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An Investigation on the Therapeutic Properties of Novel Compounds in Models of Asthma, Inflammation and Delayed Type Hypersensitivity

By

Carina Cogan

A thesis presented to the University of Dublin

for the degree of Doctor of Philosophy

Department of Pharmacology

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April 2000

To My Parents

DECLARATION

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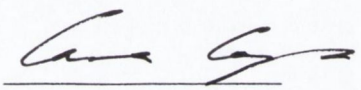

Carina Cogan

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Table of Contents

Abbreviations

AA	Arachidonic Acid
AT	Antitrypsin
ATPase	Adenosine Triphosphatase
AlOH	Aluminium Hydroxide
β_2-agonists	beta$_2$-Adrenergic Stimulants
BAL	Bronchoalveolar lavage
BN	Brown Norway
BPM	Breaths Per Minute
BSF-1	B cell stimulatory factor-1
BSS	Buffered Salt Solution
°C	Degrees Centigrade
Ca^{2+}	Calcium Ion
$CaCl_2$	Calcium Chloride
CO_2	Carbon Dioxide
Conc	Concentration
COX	Cyclo-oxygenase
cAMP	cyclic Adenosine Monophosphate
cGMP	cyclic Guanine Monophosphate
CMC	Carboxymethyl Cellulose
CRAC	Ca^{2+} release-activated Ca^{2-} channels
cLTs	cysteinyl leukotrienes
Cm	Centimetre
cyA	Cyclosporin A
DAG	Diacylglycerol
ΔP_{bc}	Change of pressure within the chamber during each breath cycle
DEPT	Distortionless Enhancement by Polarisation Topography
Dex	Dexamethasone
dH_2O	Distilled Water
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic Acid
DSCG	Di-sodium cromogylcate
DTH	Delayed Type Hypersensitivity
ECP	Eosinophil Cationic Protein
EIA	Enzyme Immunoassay
ELAM	Endothelial Leucocyte Adhesion Molecule
ET_B	Endothelin$_B$ receptor
ETOH	Ethanol
FCA	Freund's complete adjuvant
FCA(B)	Freund's complete adjuvant *(mycobacterium butyricum)*
FCA(T)	Freund's complete adjuvant *(mycobacterium tuberculosis)*
FI	Fluorescent Intensity
FIA	Freund's incomplete adjuvant
FLAP	5-Lipoxygenase activating protein
Fn	Fibronectin

FTIR	Fourier Transform Infared Spectrometry
G	Gram
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GDP	Guanine Diphosphate
GIT	Gastrointestinal Tract
GTP	Guanine Triphosphate
h	Hour
HETE	Hydroxyeicosatetraenoic Acid
HPLC	High-Performance Liquid Chromatography
ICAM	Intercellular Cell Adhesion Molecule
i.d.	Intra-dermal
IFN	Interferon
IL	Interleukin
iNOS	inducible Nitric Oxide Synthase
i.p	Intra Peritoneal
IP_3	Inositol Triphosphate
K^+	Potassium Ion
KCl	Potassium chloride
KLH	Keyhole Limpet Hemocyanin
λ	Lambda (wavelength)
λex	Excitation wavelength
λem	Emission wavelength
L	Litre
LAF	Lymphocyte Activating Factor
LFA	Lymphocyte Function-Associated Antigen
LPS	Lipopolysaccharides
LT	Leukotriene
5-LO	5-Lipoxygenase
μ	micro
M	Molar
Max	Maximum
MBP	Major Basic Protein
mg	Milligram
min	Minute
ml	Millilitre
mM	Millimolar
mm	Millimetre
mmHg	Millimeters of Mercury
6MNA	6 Methoxy-2-Naphthyl Acetic Acid
mBSA	methyl bovine serum albumin (mBSA)
Mg^{++}	Magnesium Ion
$MgCl_2$	Magnesium Chloride
MHC	Major Histocompatiblilty Complex
mRNA	messenger Ribonucleic Acid
NaCl	Sodium Chloride
NADPH	Nicotinamide Adenine Dinucleotide Phosphate, reduced form

NaH_2PO_4	Sodium Di-hydrogen Phosphate
$NaHCO_3$	Sodium Hydrogen Carbonate
NANC	Nonadrenergic Noncholinergic
NaOH	Sodium Hydroxide
NF-κB	Nuclear Factor-κB
NK	Natural Killer
NMR	Nuclear Magnetic Resonance
NO	Nitric Oxide
NO_2^-	Nitrite
NO_3^-	Nitrate
NS	Not Significant
NSAIDs	non-Steroidal Anti-Inflammatories
O_2	Oxygen
O_2^-	Superoxide Anion
OH^-	Hydroxy Free Radical
$ONOO^-$	Peroxynitrite
oPT	*O*-phthaldialdehyde
OVA	Ovalbumin
PAPase	Phosphatidate Phosphohydrolase
PDE	Phosphodiesterase
PG	prostaglandins
PI	Phosphatidylinositol
PIP_2	Phosphatidylinositol bisphosphate
PKC	Protein kinase C
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PLD	phospholipase D
p.o	*per os*
RA	Rheumatoid Arthritis
RBC	Red Blood Cells
ROCs	Receptor Operated Channels
Rpm	Revolutions Per Minute
S1P	Sphingosine-1-phosphate
s.c	Subcutaneous
Sec	Seconds
SEM	Standard Error of the Mean
SK	Sphingosine Kinase
SR	Sacroplasmic Reticulum
TBI	Total Body Irradiation
TCR	T cell receptor
TNFα	Tumour Necrosis Factor
TPA	12-*O*- tetra-decanoylphorbol 13 acetate
TV	Tidal Volume
UV	Untra Violoet
-ve Ctrl	Negative Control
+ve Ctrl	Positive Control

v/v	Volume/Volume
VCAM	Vascular Cell Adhesion Molecule
VLA	Very Late Antigen
VOC	Voltage Operated Channels
w/v	Weight/Volume

Summary

There are many drugs on the market for the treatment of asthma, inflammation and delayed type hypersensitivity (DTH), however few are disease modifying. The potential therapeutic effect of three novel compounds, 3C8, 6C6 and 7C9 was investigated for the treatment of these diseases.

The compounds 3C8, 6C6 and 7C9 are synthetic derivatives of the natural pharmacological products, pterosin Z and acetylpterosin Z originating from the fern *Pteridium aquilinium*. These natural products have previously been demonstrated to inhibit calcium induced contractions in depolarised guinea-pig ileum, and histamine intestinal contractions. These observations indicated a potential therapeutic application, particularly with respect to bronchodilation and asthma.

There are two forms of asthma intrinsic and extrinsic. Extrinsic asthma develops following exposure to an allergen resulting in T_H2 activation, mast cell degranulation, bronchoconstriction and the influx of inflammatory cells, eosinophils and neutrophils, to the site of inflammation. In contrast, intrinsic asthma results in the development of a T_H1 response and the development of chronic inflammation. This condition is thought to be as a consequence of a respiratory tract infection. Initial *in vitro* studies showed the potential bronchodilatory and anti-inflammatory effect of these compounds with particular focus on asthma. 3C8 inhibited depolarised smooth muscle contractions as induced by calcium and potently inhibited rat peritoneal mast cell degranulation as induced by compound 48/80. While 6C6 had minimal effect on smooth muscle contraction, 6C6 was like 3C8 a potent mast cell stabiliser. *In vivo* studies of asthma supported the *in vitro* findings. An *in vivo* model was set up where animals were sensitised to the antigen ovalbumin (OVA) using AlOH (T_H2 inducer) and freund's complete adjuvant, containing *mycobacterium tuberculosis* (FCA(T); T_H1 inducer), as adjuvants. 3C8 was found to prevent the late phase response to the antigen as seen by the significant reduction of breaths per minute and tidal volume as compared to untreated OVA challenged animals. 3C8 also reduced the inflammatory response seen in this asthmatic model as measured by the influx of leucocytes in the bronchoalveolar lavage (BAL) fluid. This compound however had no therapeutic effect on the inflammatory asthmatic model sensitised to OVA using either AlOH (T_H2 model of extrinsic asthma) or FCA(T) (T_H1 model of intrinsic asthma) as separate adjuvants.

Post mortem studies on the animals from the *in vivo* asthmatic study showed that severe peritonitis developed as a result of the sensitisation procedure. This was seen as severe fibrosis and increased vascularity of the liver and fibrosis of gastrointestinal tract. A slight improvement was seen with di-sodium cromogylcate (DSCG) treated animals however in contrast inhaled 3C8 appeared to act systemically and completely abolish any signs of inflammation in the abdomen. As a consequence work on the anti-inflammatory effects of the three compounds was set up and studies highlighted 3C8 and 7C9 as effective anti-inflammatories. 3C8 and 7C9 were shown to act by inhibiting the arachidonic acid (AA)-induced mouse ear oedema, where the effects of leukotrienes

dominate and rat paw oedema, where in contrast, the effects of prostaglandins dominate. 12-*O*-tetra-decanoylphorbol 13 acetate (TPA)-induced mouse ear oedema, which is known to induce prostaglandin production, was also inhibited by 3C8. Finally a chronic model of inflammation supported the acute studies where cellular infiltration and the development of fibrosis in sponge implants were prevented by 3C8.

Final studies investigated DTH, a T_H1 type disease. These were carried out to clarify the mode of action of 3C8 following its potent inhibitory action on the asthmatic model induced by OVA using both FCA(T) (T_H1 inducer) and AlOH (T_H2 inducer) as adjuvants. 3C8 prevented a delayed type hypersensitivity response to methyl bovine serum albumin (mBSA) in mice sensitised to the antigen using Freund's complete adjuvant containing *mycobacterium butyricum* (FCA(B)) (T_H1 inducer) as an adjuvant.

These studies have shown the three test compounds, particularly 3C8 to have potential activity against asthma, inflammation and DTH and autoimmune diseases. However, the exact mode of action is as yet unclear. Extensive studies at a molecular and biochemical level would prove invaluable in elucidating the exact role or point of action of these compounds and should give distinct direction to the study.

Chapter 1

Introduction

Contents **Page No.**

Contents **Page No.**

HISTORICAL OVERVIEW

Pterosin Z and acetylpterosin Z are naturally occurring pharmacological products from the fern *Pteridium aquilinum*. These products together with phenolic pterosins, onitin onotisin and otninoside possess smooth muscle relaxant activity. They have been shown to inhibit intestinal contractions to histamine and 5-hydroxy tryptamine (5-HT). Furthermore, they have been shown to inhibit calcium-induced contractions in K^+-depolarised smooth muscle guinea-pig ileum (Sheridan *et al* 1999).

In house studies revolved around the development of a synthetic route for these natural compounds. Pharmacological testing of Pterosin Z demonstrated potent inhibitory activity of the compound with an EC_{50} of $1.3 \pm 0.1 \times 10^{-6}$M. This activity was over 100 times greater than that of Onitin (EC_{50} of 1×10^{-4}M), Onotisin (EC_{50} of 2×10^{-3}M) and Otninoside (EC_{50} of 7×10^{-4}M) (Sheridan *et al* 1999). The exact mode of action of the natural products is unknown however calcium and the influx of this ion play an essential role in smooth muscle contraction. The antagonistic activity of the natural products may be due to the prevention of calcium influx or inhibition of calmodulin, which would prevent the formation of the calmodulin/calcium complex needed to induce cell contraction.

Following this, full-scale research and development of the derivatives of the natural products was launched and this led to the development of over 200 compounds. All compounds were screened for smooth muscle relaxant and mast cell stabilisation activities.

A potent smooth muscle relaxant and mast cell stabiliser would give a potential anti-asthmatic drug with bronchodilatory and anti-inflammatory properties. This line of research was chosen, as it is believed that the mechanism of degranulation of mast cells also revolves around calcium influx and the formation of the calmodulin/calcium complex. The screening of the 200 synthetic derivatives in these two tests resulted in the selection of three compounds, 3C8, 6C6 and 7C9, with either smooth muscle relaxant or mast cell stabilising properties or both (Work carried out by the Chemistry sector of the project Sheridan, H., Walsh, J.J., Jordan, M.J. & Butterly, S. Dept of Pharmacognosy, School of Pharmacy, TCD (1994-2000).

This present study was designed to test the three compounds in various models of asthma. Studies proved quite fruitful and extended into inflammation and delayed type hypersensitivity (DTH). This thesis will detail the pharmacological experimentation carried out to elucidate the therapeutic roles of these three compounds if any in the disease states outlined above and will close with a conclusion giving direction for the future research of these novel compounds.

1. INTRODUCTION

1.1 Asthma

The number of cases of bronchial asthma worldwide has escalated over the last two to three decades. It has been classified as a chronic inflammatory disease and its prevalence, severity and mortality are increasing significantly (Anderson,1992; Lazarus, 1998). Bronchial asthma can be defined as reversible bronchial constriction and inflammation that leads to hypersensitivity of the airways (Galli & Costa, 1995). There are two types of asthma clinically defined, extrinsic or allergic asthma and intrinsic asthma.

In extrinsic asthma, bronchoconstriction occurs following exposure to allergens, for example, pollen and dust mites. These allergens perturb the mast cell *via* allergen specific IgE antibodies, as well as inducing degranulation and subsequent release of preformed mediators, such as histamine, and newly synthesised mediators, namely the eicosanoids, (for example leukotrienes and prostaglandins). Release of these mediators not only induces bronchoconstriction, but also vasodilation, increased capillary permeability and chemotaxis. This process, termed the early phase in asthma, occurs within five to fifteen minutes after allergen exposure and lasts for up to 1h. A second or late phase also occurs and develops two to 6h after allergen exposure and can last from twelve to twenty four hours. This later phase is caused by the influx of inflammatory cells: T-lymphocytes, monocytes macrophages, eosinophils and neutrophils (Kay,1991; Krishna *et al* 1996; Lazarus, 1998). Airway inflammation also includes mucus hypersecretion and shedding of

the epithelial surface that leads to bronchial hyper-reactivity (Aizawa *et al* 1988; Kay, 1991). Bronchial hyper-reactivity is defined as hypersensitivity of the airways to a variety of stimuli such as histamine, eicosanoids, acetylcholine, allergens (aerosols and food), infections (bacteria and viruses), exercise, cold, humidity and environmental irritants, such as ozone (Barnes & Adcock, 1997; Denburg, 1996; Barrios *et al* 1998).

Intrinsic asthma usually begins around the age of 40 or even later on in life. It is often referred as non-allergic, non-atopic or postinfectious asthma. The exact cause is unknown, however it has been found to develop following a viral respiratory tract infection. Other possible causes include an autoimmune disease or even a hidden allergen. Total IgE levels are normal and IgE levels specific to house mites, dust, grass pollen are untraceable. Little is known about intrinsic asthma, however it has been associated with T-lymphocyte activation, eosinophilia and bronchial hyper-reactivity. Evidence of mast cell or B-cell involvement is unfounded (Walker & Virchow, 1993; Virchow *et al* 1996).

1.1.1 Extrinsic or Allergic Asthma

1.1.1.1 Early Phase Response

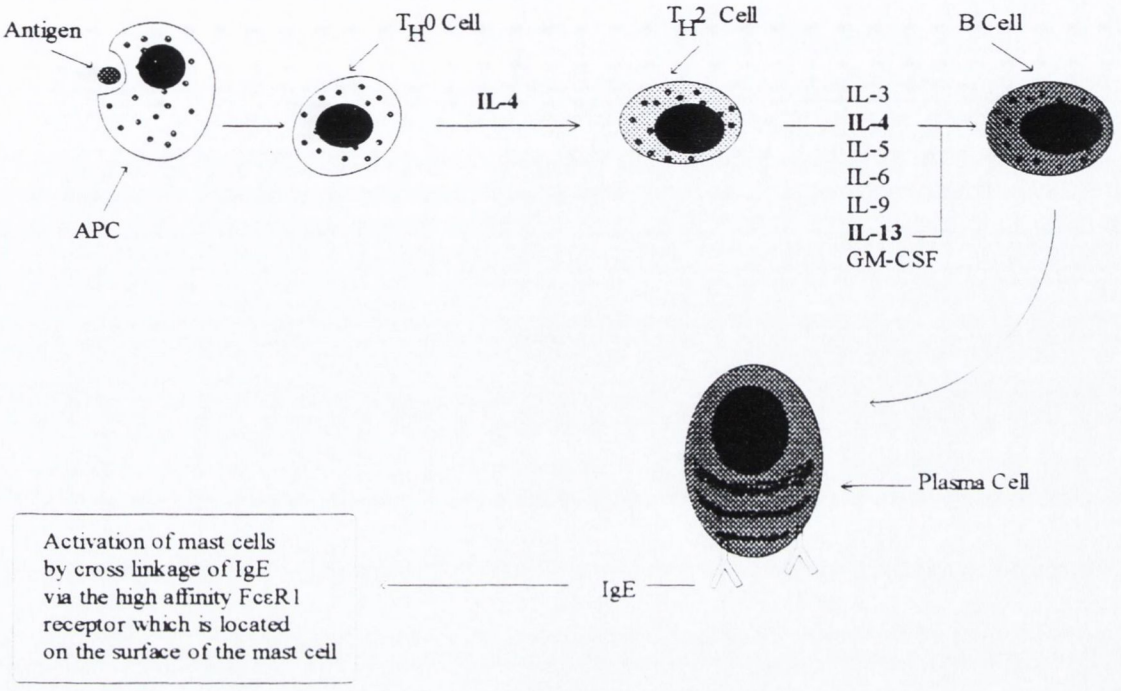
1.1.1.1(a) Isotope switching of B cells to produce IgE antibodies

Exposure of a sensitised asthmatic patient or animal to an allergen or antigen leads to an almost immediate response by the patient or animal. This response has been termed the

early phase response and occurs within five to fifteen minutes of antigen exposure and lasts for up to 1h. The initial stages of the early phase are the stimulation of B cells to produce the IgE antibody specific to the antigen. This occurs *via* a number of steps as illustrated in figure 1.1.

The antigen is firstly presented to T_H0 cells by Antigen Presenting Cells (APCs), for example macrophages. The T_H0 cells produce the cytokine interleukin (IL)-4 that specifically stimulates maturation and proliferation of T_H2 cells (Spits *et al* 1987). The T_H2 cell itself then synthesises and releases more IL-4 and also cytokines IL-3, IL-5, IL-6, IL-9, IL-13 and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Krishna *et al* 1996). Following this, IL-4 together with the appropriate cytokine milieu promotes the induction of IgE *via* isotype switching of B cells and the proliferation of these B cells (Defrance *et al* 1987; Spits *et al* 1987; Geha, 1992; Krishna *et al* 1996). IL-4 has been proven to be one of the primary most important cytokines in IgE synthesis. The ability of human T cell clones to induce or enhance IgE synthesis was found to correlate significantly with its ability to release IL-4 (Del Prete *et al* 1988). Moreover, mouse B cell stimulatory factor-1 (BSF-1), later known as IL-4, enhanced the IgE response of lipopolysaccharides (LPS)-stimulated B cells by at least 100 fold (Coffman *et al* 1986; Finkelman *et al* 1988). This finding was supported by the demonstration, in mice, both *in vitro* and *in vivo* of total inhibition of BSF-1 / IL-4 induced IgE production by a monoclonal antibody to BSF-1 (Coffman *et al* 1986; Finkelman *et al* 1986; Finkelman *et al* 1988; Zhou *et al* 1997).

Figure 1.1 The activation of B cells and the synthesis of IgE antibody to antigen



(Adapted from Krishna *et al* 1996)

Although IL-4 is important in the synthesis of IgE antibodies, it is not sufficient on its own, and requires the help of not only more cytokines but also, more importantly, a T cell-B cell interaction (Vercelli *et al* 1989; Krishna *et al* 1996). Briefly, T cell-B cell interaction is a method for T cells to deliver a second signal to B cells by interaction between the T cell receptor (TCR)/CD3 and major histocompatibility complex (MHC) class II antigens. Recently, in humans, IL-13 has in addition to IL-4 been shown to induce IgE synthesis in *in vitro* cultures of mononuclear cells derived from spleen, tonsils and blood. IL-13 has also been found to induce transcription of the germline ε messenger

ribonucleic acid (mRNA), which ultimately results in the induction of isotype switching of B cells to produce the IgE antibody. IL-13 has also been shown to induce and sustain B cell growth and differentiation with equal potency to IL-4. IL-4 and IL-13 appear to be independent from each other. Both T_H1 and T_H2 cells produce IL-13 while IL-4 is a product of T_H2 cells but not T_H1. IL-13 however, unlike IL-4, cannot activate T cells. This implies that while IL-13 can induce IgE synthesis it cannot do so without the help of IL-4, the T_H2 cell activator. IL-4 can produce much higher concentrations of IgE, at saturating concentrations of the antibody, than IL-13. However IL-13 is quite effective in the production of IgE antibodies where little or no IL-4 is present. Overall this suggests that IL-13, like IL-4 plays an important role in the induction and sustenance of B cell growth and differentiation, along with IgE synthesis. However studies have shown that IL-13 is produced earlier upon T cell activation and sustained for longer than IL-4 indicating that IL-13 may act to sustain IgE synthesis even when IL-4 production has been down regulated (Bousquet *et al* 1997; Jung *et al* 1996; Robinson, 1996).

The cytokines IL-3, IL-5, IL-6, IL-9 and GM-CSF are also released from the T_H2 cell when activated by IL-4 (Krishna *et al* 1996). Of these listed cytokines, only IL-5 and IL-6 appear to be important in isotope switching of B cells to IgE synthesising cells. IL-5 and IL-6 strongly upregulate IL-4 induced IgE synthesis on human B cells with some cell proliferation activity (Geha, 1992). IL-5 has been shown, in human peripheral blood mononuclear cells, to have an additive effect on IL-4 induced IgE production, particularly when IL-4 concentrations are suboptimal (Pene *et al* 1988). IL-6 however appears to play an obligatory role in IL-4 induced IgE synthesis. Anti IL-6 polyclonal antibody

strongly inhibited IL-4 driven IgE production in human B cells (Vercelli *et al* 1989; Lacy *et al* 1998).

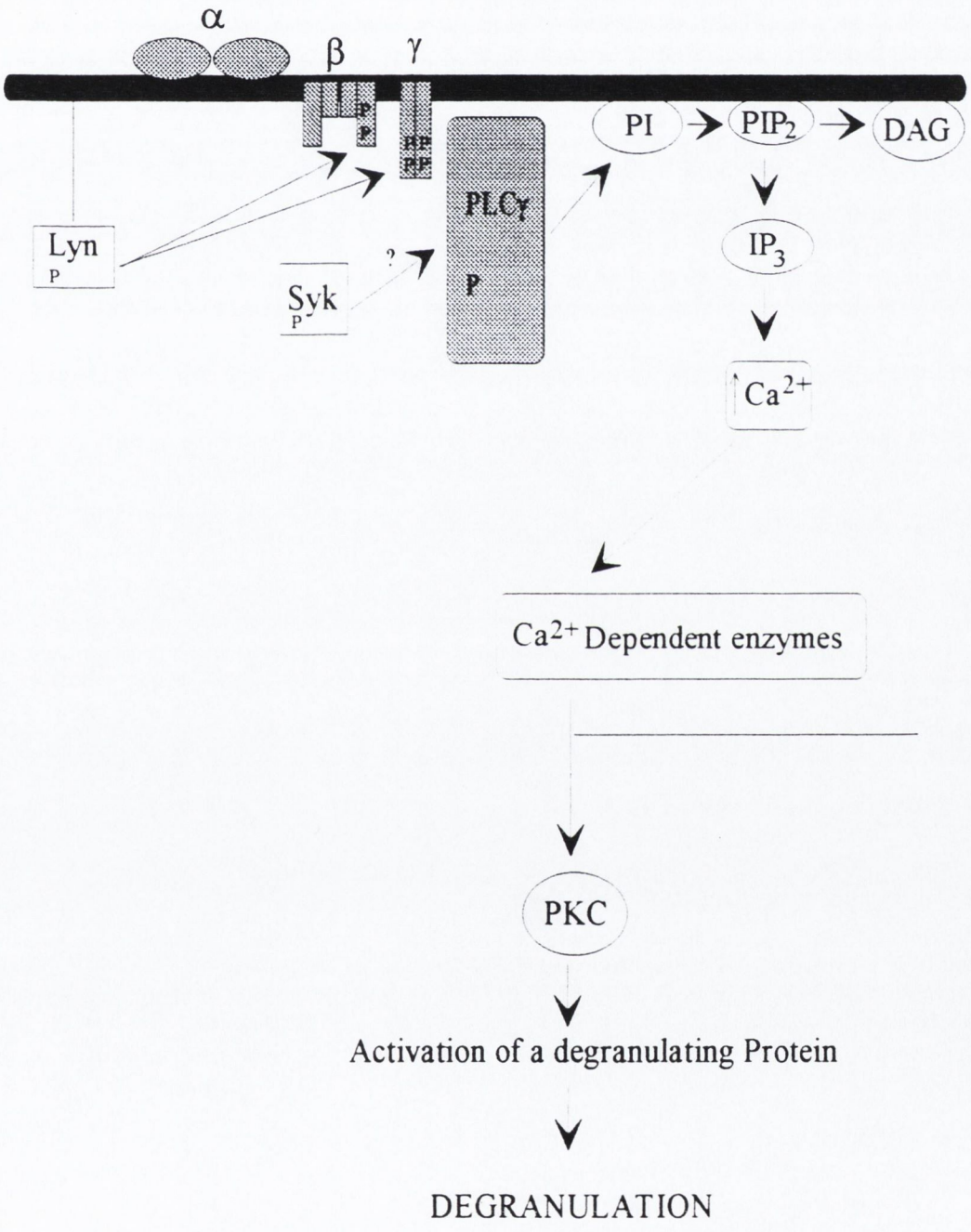
1.1.1.1(b) Mast cell Degranulation.

Mast cells are activated following significant production of the IgE antibody by the B-cells. Activation occurs *via* cross-linkage of the high affinity mast cell receptors $Fc_{\varepsilon}RI$ that occurs through binding of IgE and antigen (Figure 1.2 & Figure 1.3). Cross-linkage is dependent on diffusion and persistent aggregation of the $Fc_{\varepsilon}RI$ receptors, which consist of three subunits α, β and γ. Binding of wheat germ agglutinin, a lectin that binds to the α subunit of $Fc_{\varepsilon}RI$, resulted in immobilisation of the receptors and inhibition of cultured RBL-2H3 mast cell activation following antigen addition (McCloskey, 1993).

A number of events, leading to release of histamine-containing granules, occur, following aggregation and cross-linkage of the $Fc_{\varepsilon}RI$ receptors. Protein-tyrosine phosphorylation is the earliest detectable event that occurs after receptor aggregation and takes place within five to fifteen seconds. This process is well characterised and has been demonstrated in rodent mast cells (RBL-2H3), rat basophilic leukaemia cells (RBL-2H3) and human cultured mast cells (Benhamou *et al* 1990; Benhamou & Siraganian, 1992; Scharenberg & Kinet, 1994; Hamawy *et al* 1995; Suzuki *et al* 1997). The β and γ subunits of the cross-linked FcεRI receptor are phosphorylated at the tyrosine residue by a tyrosine kinase, lyn, which is activated on FcεRI aggregation. This leads to activation of another kinase, syk, also *via* phosphorylation, by lyn. Following this, phospholipase C ($PLC\gamma1$ and $PLC\gamma2$) is

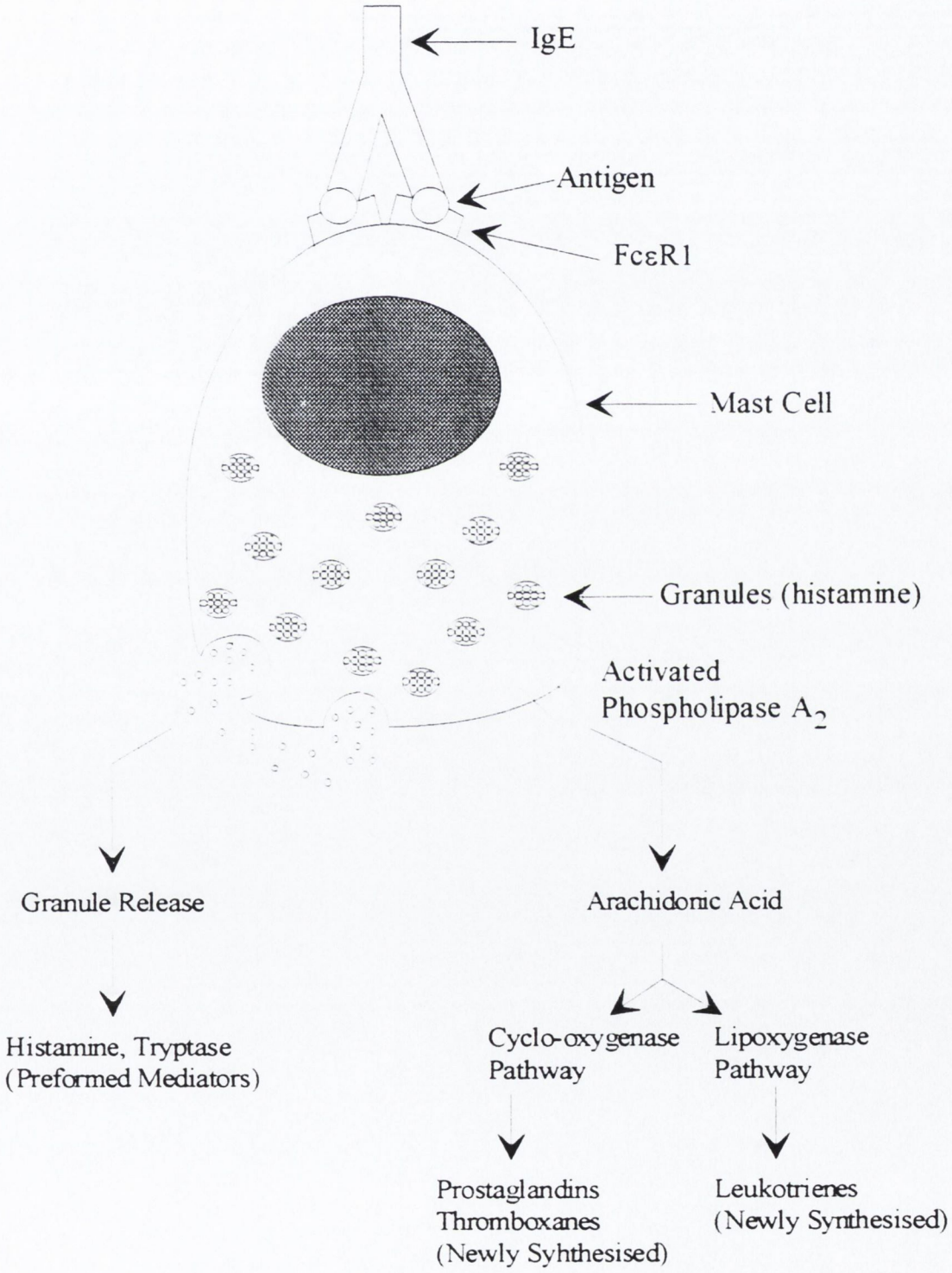
activated again *via* phosphorylation by the kinase, syk, however the exact kinase has not been demonstrated conclusively.

Figure 1.2 Mast cell degranulation



(Adapted from Hamaway *et al* 1995 & Benhamou & Siraganian 1992)

Figure 1.3 Release of preformed and newly synthesised mediators following mast cell degranulation



PLCγ when activated translocates from the cytosol to the membrane allowing co-localisation with substrates. It catalyses the hydrolysis of phosphatidylinositol (PI) through phosphatidylinositol bisphosphate (PIP_2) to inositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3, a secondary messenger, increases intracellular Ca^{2+} through its action on Ca^{2+} intracellular stores, namely the endoplasmic reticulum (SR). An alternative secondary messenger sphingosine-1-phosphate (S1P), formed by the enzyme sphingosine kinase (SK), has recently been identified in RBL-2H3 mast cells (Suzuki *et al* 1997). Results gathered by Choi *et al* (1996), suggest that FcεRI uses S1P to mobilise Ca^{2+} from intracellular stores in this particular cell line. Depletion of the Ca^{2+} intracellular stores in turn induces Ca^{2+} influx from extracellular sources *via* plasma membrane channels known as Ca^{2+} release-activated Ca^{2+} channels (CRAC). Chloride ions appear to play an important role in the influx of extracellular Ca^{2+} by hyperpolarising a depolarised cell and enabling the entry of calcium. The need for chloride ions in degranulation has been demonstrated in both rat peritoneal mast cells and the rodent mast cell line RBL-2H3 (Alton & Norris, 1996; Redrup *et al* 1997). Elevation of Ca^{2+} through both release of sequestered or stored intracellular Ca^{2+} and through extracellular influx results in the formation of the calcium/calmodulin complex and in turn the activation of a number of Ca^{2+} dependent enzymes. It is thought that the Ca^{2+} dependent enzymes together with DAG, activate protein kinase C (PKC) which, *via* phosphorylation, activates a degranulating protein and hence degranulation (Barnes, 1991; Wilson *et al* 1991; Grosman, 1992; Chakravarty, 1992).

Mast cells can also be shown to degranulate after exposure to non-immunological agents, namely, compound 48/80 and substance P (Wu *et al* 1993; Mousli *et al* 1991; Shefler *et al* 1998; Senyshyn *et al* 1998; Chakravarty, 1992). However, the mechanism of action of these secretagogues is different to that of antigen. These agents act by directly activating the heterotrimeric Guanine triphosphate (GTP)-binding proteins on peritoneal mast cells and sensitised RBL-2H3 mast cells. This in turn activates the enzymes, in a manner similar to that of the antigen challenge mechanism, resulting in the activation of PLCγ, the breakdown of PI to IP_3 and DAG, and the increase in intracellular calcium. There follows an influx of extracellular calcium. While, this is chloride ion dependent in the antigen-stimulated model of mast cell degranulation it does not appear to be the case with degranulation induced with compound 48/80 (Redrup *et al* 1997; Alton & Norris 1996). The influx of extracellular calcium in this model therefore must involve other undefined mechanisms. Once calcium levels have reached a certain threshold the final stage in mast cell degranulation is the formation of the calcium/calmodulin complex, the activation of PKC and degranulation of mast cells.

1.1.1.1(c) Preformed Mediators Released on Mast Cell Degranulation

Granules released from mast cells contain preformed mediators and heparin bound histamine and tryptase (Barnes, 1991). These components have been shown to be released in guinea pigs and in asthmatic patients (Turner & Dollery, 1988; Holgate *et al* 1986; Biggs, 1984; Mauser *et al* 1990). Histamine acts on the H_1-receptors located on the airway smooth muscle and induces bronchoconstriction. Histamine has also been shown

to be involved in stimulation of secretion of mucus, vasodilatation and the increase of pulmonary epithelial and capillary permeability. Tryptase is a proteolytic enzyme, however its actions, in tissues, upon mast cell degranulation are not as well defined as histamine. He & Walls (1997) found that the human mast cell tryptase was involved in further mast cell activation. They also demonstrated that tryptase was a stimulus to microvascular leakage. Recent evidence links the enzyme to the induction of hypersensitive airway smooth muscle by antigen in antigen-sensitised animals (Barrios *et al* 1998). This had been demonstrated previously with the supernatant of degranulated dog mastocytoma cells on isolated dog bronchial smooth muscle (Sekizawa *et al* 1989).

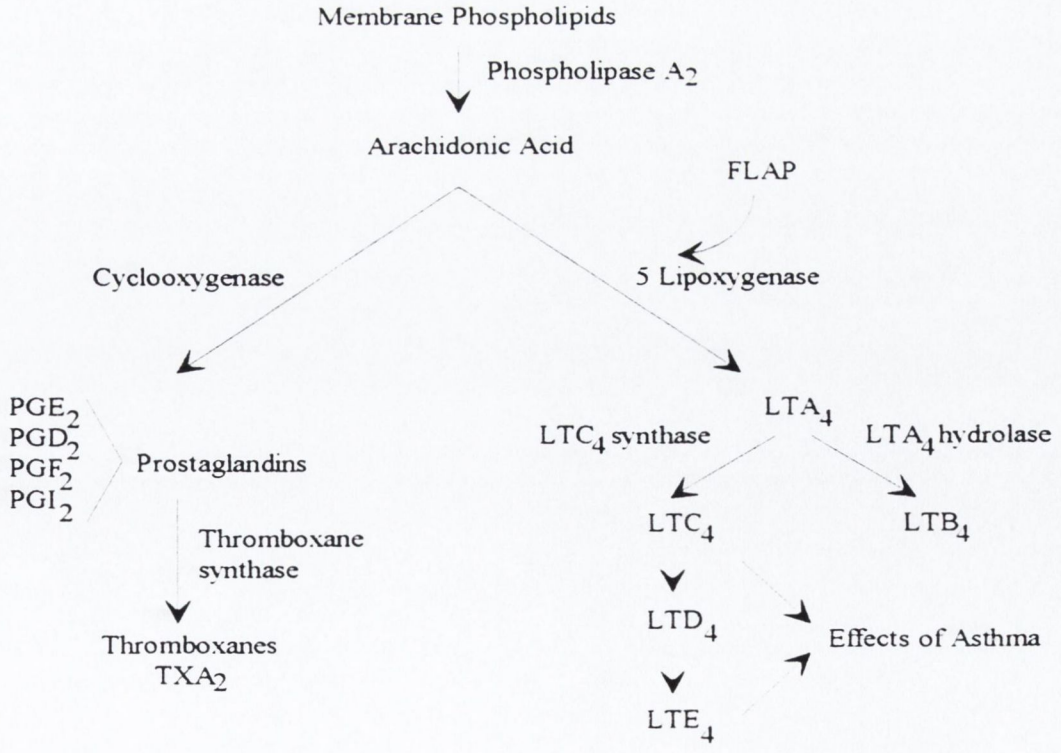
1.1.1.1(d) Newly Formed Mediators Released on Mast Cell Degranulation

Phospholipase A_2, (PLA_2) is activated by phosphorylation after aggregation of the FcεRI receptor on both peritoneal mast cells and MCII mast cells (Currie *et al* 1994; Ishimoto *et al* 1996). Cytosolic PLA_2, upon phosphorylation by a protein kinase has been thought to translocate to the membrane and liberate arachidonic acid (AA), *via* Ca^{2-} dependent methods. Recent studies have noted that, although PLA_2 is activated on mast cell degranulation, a prolonged concentration of intracellular calcium is needed for hydrolysis of membrane lipids to liberate AA release. This concentration of calcium, however, has not been demonstrated in rat peritoneal mast cells on antigen activation. There is evidence of a sequential pathway from phosphatidylcholine to DAG leading to AA release and eicosanoid production in antigen stimulated rat peritoneal mast cells. Enzymes involved in this pathway are phospholipase D (PLD) - phosphatidate phosphohydrolase (PAPase)

while DAG lipase is believed to act on DAG leading to AA release (Ishimoto *et al* 1996a; Ishimoto *et al* 1996b). These observations lead us to believe that the PLD-PAPase-DAG lipase pathway is equally important as the PLA_2 pathway in AA release and eicoisanoid production.

AA, on release from mast cells can be broken down by a number of mechanisms. These pathways are well documented in a number of human and animal models (Tan & Spector 1997, Barnes, 1991, Lazarus, 1998, Chabot-Fletcher *et al* 1995, Bell *et al* 1997). The two most important of these pathways are the, 5-lipoxygenase (5-LO) pathway and the cyclo-oxygenase (COX) pathway (Figure 1.3 & Figure 1.4).

Figure 1.4 The synthesis of the newly formed mediators in mast cells.



(Adapted from Barnes, 1991)

The enzyme, 5-LO, breaks down AA to leukotriene (LT) A_4. However before this takes place activation of 5-LO require a second enzyme, 5-Lipoxygenase activating protein (FLAP). LTA_4 is unstable and can be broken down to either LTB_4 or LTC_4 by LTA_4 hydrolase and LTC_4 synthase respectively. LTC_4 is also unstable and therefore is broken down to LTD_4 and subsequently LTE_4. LTB_4 appears to play a role as a chemoattractant, however its exact actions are as yet unclear. The cysteinyl leukotrienes (cLTs), namely LTC_4, LTD_4 and LTE_4, induce bronchoconstriction, increase vascular permeability, mucus and inflammatory cell infiltration, namely eosinophil influx. Finally, leukotrienes have been shown to be involved in remodelling of the airway smooth muscle in both cell cultures and animal studies. Interpretation of this evidence may implicate leukotrienes in the development of bronchial hyper-sensitivity.

The second pathway in the breakdown of AA is the COX pathway. The COX enzyme breaks down AA to form the prostaglandins (PG), PGE_2, PGD_2, PGF_2 and PGI_2. Thromboxanes are also formed in this pathway. There are two forms of the COX enzyme, namely, COX-I and COX-II. COX-I is a constitutive enzyme and always present in the cell, although in small quantities. On the other hand COX-II is an inducible enzyme and when induced, as in inflammatory conditions, large quantities are produced to accommodate for the increase in concentration of AA. Prostaglandins, act on receptors and can cause a bronchoconstrictory response, however they are not potent constrictors. The exact role of prostaglandins in asthma is as yet unclear as indomethacin, a COX inhibitor, provides no clinical benefit (Barnes, 1991, Benyon *et al* 1989; Skidmore & Vardey 1984). Of the mediators released from mast cells, leukotrienes appear to be the

most potent of the bronchoconstrictors with activity over a thousand times greater than histamine and prostaglandins (Tan & Spector 1997).

1.1.1.1(e) Bronchoconstriction and Contraction of the Airway Smooth Muscle

Studies on human and guinea-pig trachea have shown that binding of agonist to receptor operated Ca^{2+} channels (ROCs: calcium channels) leads to the contraction of smooth muscle. Histamine H_1-receptor and leukotriene cys-LT_1 receptor stimulation leads to contraction of the airways probably through G-protein mechanisms. G-proteins have three subunits, namely, α, β and γ, and their association with each other depends on a guanine diphosphate (GDP)/ (GTP) exchange mechanism. In the normal relaxed inactive state GDP is bound to the α subunit which in turn is bound to the other subunits β and γ. Following the binding of the agonist to the receptor, GDP dissociates from the α subunit and GTP takes its place. This leads to dissociation of the $\beta\gamma$ dimeric subunit from the α subunit. The activation of the G-protein to GTP-α gives rise to the stimulation of PLC. As described earlier in mast cell activation PLC cleaves the membrane lipid PIP_2 generating IP_3 and DAG. While IP_3 is involved in the release of calcium from the SR giving rise to a large rapid contraction, DAG appears to be involved in maintaining the tone of the contraction through PKC dependent mechanisms. Calcium released from the SR by IP_3 binds to the calcium binding protein, calmodulin. This complex in turn activates myosin light chain kinase, which phosphorylates light chain myosin. Induction of myosin adenosine triphosphatase (ATPase) and cross bridging between actin and myosin leads to contraction of the smooth muscle. IP_3 acts only to provide the initial burst of

intracellular calcium, which is needed to initiate the contractile response. The sustained contraction is thought to be due in part to the activation of PKC by DAG and in part to the decrease of membrane potential. PKC is thought to enhance the sensitivity of the cell to calcium by phosphorylating the contractile proteins. The decrease in membrane potential is due to release of calcium from the intracellular stores and this leads to further influx of calcium (Rodger, 1992, Hall *et al* 1989, Ahmed *et al* 1984; Schultz *et al* 1990).

There are other mechanisms of smooth muscle contraction other than that of ROC mediated contractions. Potassium chloride (KCl) has also been shown to induce contraction of smooth muscle trachea. Large concentrations of KCl (45mM) would decrease the membrane potential from the resting potential (-45 to -60mV). The depolarisation of the membrane leads to the opening of voltage operated channels (VOC) and influx of calcium. Calcium influx would lead to contraction of smooth muscle as described above (Rodger, 1992, Foster *et al* 1984, Ahmed *et al* 1985).

1.1.1.2 Late Phase Response

1.1.1.2(a) The Role of Mast Cells in the Onset of the Late Phase Response

Mediators released from mast cells play a pivotal role in the onset of the late phase respiratory response (Kumar *et al* 1998; Galli & Costa 1995; Barnes & Adcock, 1997; Krishna *et al* 1996). Tumour Necrosis Factor-α (TNFα), IL-3, IL-4, IL-5, IL-6 and GM-CSF are among the cytokines released from mouse, rat and human mast cells following

IgE activation (Gordon *et al* 1990; Galli & Costa, 1995; Krishna *et al* 1996; Hide *et al* 1997). Of the cytokines released TNFα is one of the most critical participants involved in the mast cell-leucocyte cytokine cascade. It is produced not only by mast cells but also by a variety of others, namely, epithelial cells, macrophages and eosinophils (Renzetti *et al* 1996). TNFα has many pro-inflammatory effects however one of particular importance is that of the recruitment of leucocytes, i.e. eosinophils, neutrophils and basophils and T-lymphocytes.

Histamine and TNFα have been shown to induce expression of adhesion molecules, involved in leucocyte recruitment, on the endothelial surface (Galli & Costa 1995; Krishna *et al* 1996). While histamine upregulates the expression of the P-selectin, TNFα, *via* activation of nuclear factor-κB (NF-κB) has been shown to upregulate E-selectin, P-selectin, intercellular cell adhesion molecule (ICAM)-1 ICAM-2 and vascular cell adhesion molecule (VCAM)-1 (Kumar *et al* 1998; Galli & Costa 1995; Barnes & Adcock, 1997; Krishna *et al* 1996). T-cells are recruited through binding of sialyl lewis X, an adhesion molecule located on the T-cell, to P-selectin and E-selectin located on the endothelial cell. A second mechanism of recruitment of T-cells is through the binding of another one of its adhesion molecules, lymphocyte function-associated antigen (LFA)-1, to ICAM-1 and ICAM-2 on the endothelial cell (Krishna *et al* 1996). IL-4 together with TNFα are associated with eosinophil recruitment through the promotion of the VCAM endothelial adhesion molecule, which binds to the ligand, very late antigen (VLA)-4, located on the surface of the eosinophil (Krishna *et al* 1996).

It is believed that TNFα can upregulate IL-5 expression in recruited T-lymphocytes and IL-5 enhances differentiation and prolongs eosinophil survival (Meng *et al* 1997; Sur *et al* 1996; Ohnishi *et al* 1993; Kumar *et al* 1998). IL-3 and GM-CSF are also understood to be involved in enhancing eosinophil differentiation, activation and survival. Of the three, IL-5 is the most important constituent and is particularly unique in acting specifically on mature eosinophils (Meng *et al* 1997; Sur *et al* 1996; Ohnishi *et al* 1993). In addition it is a chemoattractant for eosinophils and promotes degranulation of eosinophils (Meng *et al* 1997). IL-6 released from T_H2 cells, mast cells and eosinophils plays an important role in the IL-4 dependent induction of IgE antibody. IL-6, released from mast cells and eosinophils, acts as a positive feedback mechanism, amplifying the reaction (Vercelli *et al* 1989; Lacy *et al* 1998).

1.1.1.2(b) Leucocytes

Eosinophils

Eosinophils play a particularly important role in asthma and the onset of airway hyper-responsiveness and epithelial shedding (Barnes, 1991; Krishna *et al* 1996). This has been demonstrated in both human subjects and Brown Norway (BN) rats (Renzetti *et al* 1996; Barnes, 1991; Krishna *et al* 1996). Eosinophils release a mixture of basic proteins on degranulation. They include major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil-derived neurotoxin and eosinophil peroxidase (Barnes, 1991; Robinson, 1996). These proteins are highly charged and are thought to be associated with epithelial

shedding and airway hyper-responsiveness. Eosinophils synthesise, store and release approximately 18 cytokines. Some of these include IL-3, IL-4, IL-5, IL-6, GM-CSF and TNFα, and appear to act as a positive feedback loop that amplifies the asthmatic process (Lacy *et al* 1998; Galli & Costa, 1995). TNFα is also thought to upregulate cytotoxic effector functions in eosinophils and other leucocytes. It assists tissue remodelling through its effects on fibroblasts and other cells. In addition to the release of cytotoxic proteins and cytokines, eosinophil degranulation induces the production of leukotrienes particularly LTC_4 (Kay, 1991; Robinson, 1996).

Neutrophils and Basophils

Other leucocytes, namely, neutrophils and basophils are also found in inflammation (De La Puerta *et al* 1996; Taub *et al* 1996; Irani *et al* 1998). However, although neutrophils accumulate in the airways in asthma, the evidence that they play a role in asthma and bronchial hyper-responsiveness remains a controversial topic. Neutrophils are believed to induce tissue damage in many inflammatory conditions. This is carried out through the release of oxygen metabolites, proteases and cationic materials. Prostaglandins, leukotrienes, thromboxanes and platelet activating factor (PAF) have also been shown to be released on neutrophil activation. The fact that neutrophils are capable of altering airway functions suggests that they probably do play a role in asthma, however, it has been shown in some animal models and in some human cases, that neutrophils appear both in normal and asthmatic subjects (Kay, 1991; Kay & Corrigan 1992; Frew *et al* 1996). Moreover, myeloperoxidase, a neutrophil-derived mediator does not appear in the

bronchoalveolar lavage (BAL) fluid of asthmatics (DuBuske, 1995). This is in contrast to eosinophils and the release of ECP from these cells following an asthmatic attack. These finding would then indicate, that despite the fact neutrophils are present, they are not activated and do not contribute to airway hyper-responsiveness. Basophils, although they play an important role in many inflammatory diseases and appear to be present in other organs following allergic reactions, have not however been identified in bronchial inflammation (Irani *et al* 1998; Kay, 1991).

1.1.1.2(c) Epithelial Cells

Epithelial cells are also thought to be involved in the propagation of the asthmatic response. It has been shown that they are a source of cytokines, eicosanoids, nitric oxide (NO) and endothelin (Busse, 1998; Krishna *et al* 1996; Sparrow *et al* 1995). The cytokines and eicosanoids appear to amplify the asthmatic process. The constitutive expression of the cytokines, IL-1β, IL-6, IL-8 and GM-CSF has been demonstrated in human lung (Wang *et al* 1994). IL-1β, together with TNFα and interferon (IFN)γ, is a major contributor to the induction of the inducible Nitric Oxide synthase (iNOS) (Barnes & Liew 1995). As already mentioned IL-6 strongly upregulates IL-4 induced IgE synthesis in B cells and GM-CSF enhances eosinophil differentiation, activation and survival (Vercelli *et al* 1989; Lacy *et al* 1998). IL-8 has been shown recently to bind to the immunoglobin IgA and this complex increases eosinophil chemotaxis (Krishna *et al* 1996). Furthermore, Erger & Casale (1995), showed the capability of IL-8 to induce eosinophil migration in human pulmonary endothelial and epithelial cells. The release of

eicosanoids from human bronchial epithelium has also recently been demonstrated. These mediators, namely leukotrienes, prostaglandins and thromboxanes may further intensify the process through their ability to induce bronchoconstriction and chemotaxis (Busse, 1998; Krishna *et al* 1996; Sparrow *et al* 1995).

NO can be produced by the enzyme iNOS in the epithelial cells of the airways. mRNA for iNOS and NO the product of iNOS has been detected in asthmatic patients, human lung cell lines and ovalbumin (OVA) sensitised BN rats (Asano *et al* 1994; Robbins *et al* 1994, Nadaud *et al* 1994; Liu *et al* 1997). It can also be produced, again by the enzyme iNOS, in macrophages, mast cells and lymphocytes all of which are abundant in asthma (Liu *et al* 1997). The activity of iNOS however seems to be predominant in macrophages during inflammation. NO as a compound has the potential to reverse the effects of the bronchoconstriction seen in asthma. It has a nonadrenergic noncholinergic (NANC) bronchodilatory effect, which acts *via* cyclic guanine monophosphate (cGMP) (Sadeghi-Hashjin *et al* 1996; Mehta *et al* 1997). Moreover NO has been shown, in guinea pig, to shift the AA breakdown away from the potent bronchoconstrictors (leukotrienes) and towards the production of the weaker and less potent bronchoconstrictors (prostaglandins) (Folkerts *et al* 1995). From this evidence therefore, it is conceivable to think that NO produced in small quantities has the potential to alleviate the symptoms of asthma.

However the pathophysiological effects of NO in asthma and respiratory disorders have not been fully elucidated. Contrary to NO's bronchodilatory ability, it as a compound can

have detrimental effects. NO may increase blood flow in the airways, it may also induce eosinophil inflammation and it has the potential to cause cytotoxic effects that may contribute to epithelial shedding leading to hyper-reactive airways. Increased blood flow is caused by vasodilatation, which in turn is induced by NO acting *via* cGMP, which relaxes the vascular endothelium (Krishna *et al* 1996; Barnes & Liew 1995). NO may also indirectly exhibit its effect on eosinophilic inflammation. Studies on mouse have shown that NO acts through the inhibition of T_H1 lymphocytes, which in turn, would inhibit IFNγ, the cytokine that inhibits T_H2 lymphocytes, which are the cells involved in extrinsic asthma. NO therefore has the potential to indirectly promote the proliferation of the antigen-driven T_H2 cells and increased production of IL-4, and IL-5, the cytokines that activate mast cells and eosinophils respectively (Barnes & Liew 1995). Finally NO has the ability to act as a cytotoxic molecule and thereby induce epithelial damage. Inhibition of iron-containing enzymes by NO has been demonstrated in the trachea of hamster and rat hepatocytes (Radi *et al* 1991, Heiss *et al* 1994). NO itself can inhibit mitochondrial respiration *via* inactivation of the enzymes aconitase, complex II and I. The inhibition of deoxyribonucleic acid (DNA) synthesis by NO through the inactivation of ribonucleotide reductase has also been demonstrated. Inhibition of these enzymes appears to be through the binding of the free radical, NO to the iron cluster in the enzyme to form iron-dinitrosyl-dithiolate complexes. As a free radical, it can induce some damage however it is more potent following binding with a second molecule, O_2^-, which is also present during inflammation. NO and O_2^- bind to form peroxynitrite anion ($OONO^-$), a compound that can induce lipid oxidation, protein degradation and DNA damage in rat hepatocytes (Patel

& Block 1986; Wink *et al* 1991; Moriguchi *et al* 1992). The presence of $OONO^-$ in asthmatic patients has been demonstrated therefore NO may indirectly induce cytotoxic effects in the lung (Saleh *et al* 1998).

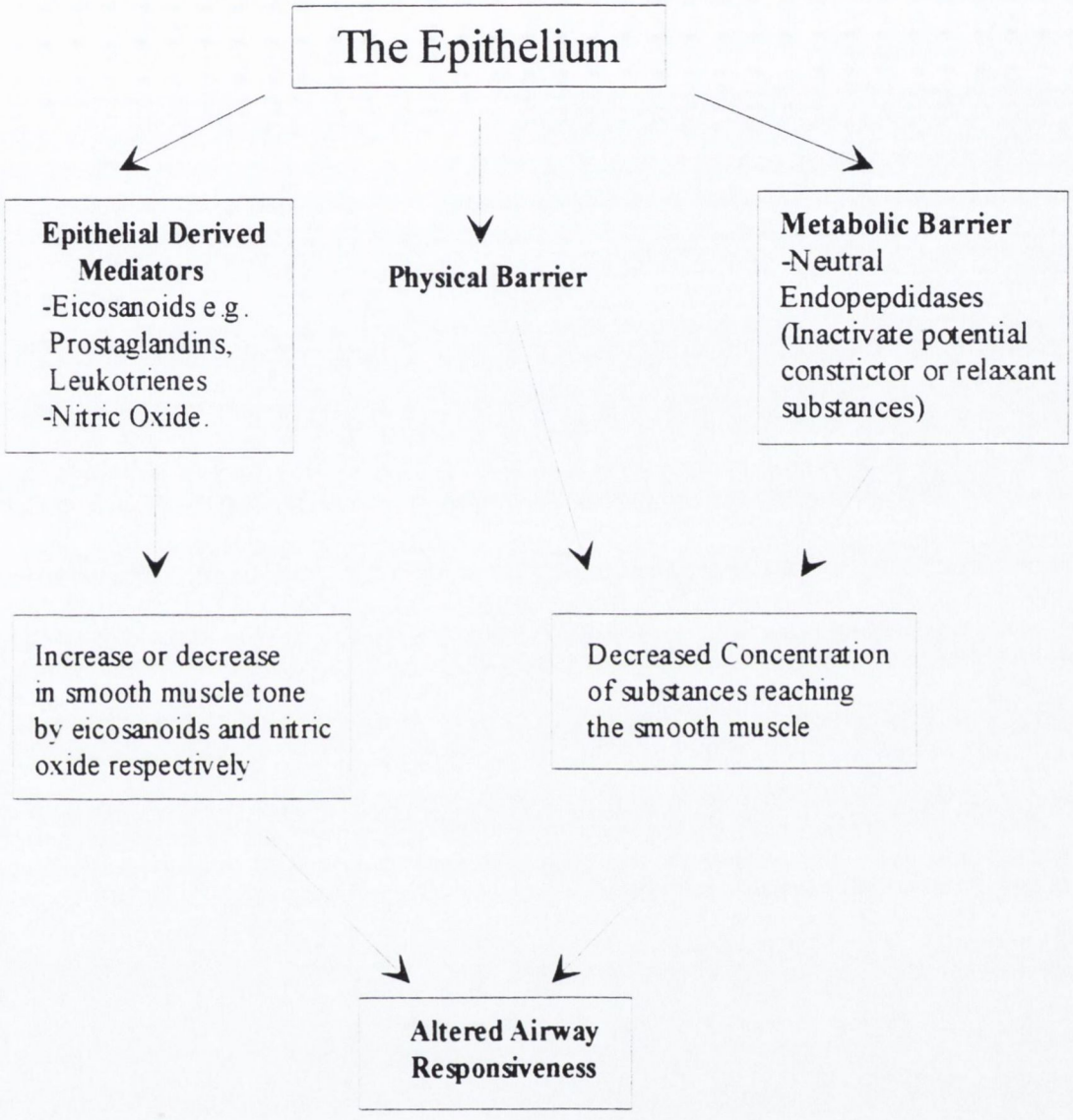
Finally, endothelin has been shown to be elevated sixfold in the airways of asthmatics (Barnes, 1996a). Release of endothelin has been detected in cultured porcine, canine and human bronchial epithelial cells and in rabbit and guinea-pig trachea epithelial cells (Goldie *et al* 1996). Endothelin-like immunoreactivity has also been detected in rat and mouse airway epithelium (Hay *et al* 1993). It is thought to be stimulated by either endotoxins or various combination of cytokines some of which include IL-1, IL-2, IL-6, IL-8, TNFα and TGFβ (Goldie *et al* 1996). These cytokines are released either at the early stages of asthma or by the epithelial cells themselves (Barnes & Liew, Wang *et al* 1994). In guinea pig trachea the effects of endothelin has been shown to be attenuated by the intact epithelium through the metabolic actions of a neutral endopeptidase. Endothelin was also shown to be metabolised by human activated neutrophils. Endothelin induces bronchoconstriction, by mobilising intracellular calcium. This is seen particularly in conditions of chronic asthma where the endothelium is damaged. It is thought that endothelin acts *via* the endothelin$_B$ (ET_B) receptor in the airways and induces the activation of PLC and release of IP_3 through a G-protein mechanism (Hay *et al* 1993). IP_3 leads to release of calcium from the intracellular stores and depolarisation of the membrane. The depolarisation of the membrane leads to further increases of intracellular calcium through voltage operated channels and bronchoconstriction. Endothelin, is also thought to be involved in airway remodelling, however the mechanisms of action are

unclear. It is thought that it acts by inducing subepithelial fibrosis (Krishna *et al* 1996; Barnes, 1996a).

1.1.1.2(d) The Epithelial Barrier and the Development of Airway Hyper-responsiveness

The hypersensitivity of the airways of asthmatics towards ozone, viruses and certain chemicals may be linked to that of epithelial shedding (Barnes, 1996a). The importance of the bronchial epithelium in regulating airway smooth muscle function has been frequently demonstrated through the observation that loss or damage to the epithelium leads to respiratory disease. The role of the epithelium is complex, as it appears to manifest its effects on modulating smooth muscle tone in several ways (Figure 1.5). Firstly, it may act as a physical barrier to diffusion preventing the actions of potential bronchoconstrictors or bronchorelaxants. This barrier therefore would prevent the penetration of allergens in mild cases of asthma. Secondly, it contains degradative enzymes, namely neutral endopeptidase which can inactivate inflammatory mediators. Thirdly, the epithelium produces and secretes substances that can themselves modulate the tone of the smooth muscle. These substances include NO, cytokines and the eicosanoids, leukotrienes and prostaglandins (Sparrow *et al* 1995). The concentrations of these substances are low in mild cases of asthma, however when the inflammatory process develops high concentrations may be produced which would induce a deleterious response as described earlier (Sparrow *et al* 1995).

Figure 1.5:- Epithelial Functions



(Adapted from Sparrow *et al* 1994)

Although the exact mechanism underlying the development of airway hyper-responsiveness is unclear, many substances have been proven to be involved in epithelial shedding and tissue remodelling. Tissue remodelling is caused by proliferation of fibroblasts leading to subepithelial fibrosis and the laying down of type III and V collagen.

Fibrosis may inhibit pulmonary functions and the contractile myofibroblasts present beneath the basement membrane may contribute to persistent hyper-responsiveness (Smith, 1992; Barnes, 1996a).

Many substances are thought to play a role in the development of airway hyperresponsiveness. Some of these include the fibrogenic cytokines, eosinophil granule proteins namely, ECP and MBP, high concentrations of NO, and endothelin (Smith, 1992; Barnes, 1996a; Kraneveld *et al* 1997; Kovacs, 1991).

Macrophages, T-cells, B cells and mast cells, have been shown to release fibrogenic cytokines, for example TNFα. TNFα appears to have the ability to directly promote tissue remodelling through its proliferating effects on fibroblasts (Galli & Costa 1995; Kovacs, 1991). TNFα, along with other pro-inflammatory cytokines, IL-1β and IFNγ has the potential to induce iNOS, leading to the production of large toxic quantities of NO. As described earlier NO may elicit its effects in many ways. It is a compound with potent vasodilatory activity, which may also increase plasma exudate in the airways (Barnes, 1996a). It has the ability to inhibit mitochondrial respiration and DNA synthesis (Radi *et al* 1991; Heiss *et al* 1994). It also may amplify the T_H2-lymphocyte mediated response. (Barnes, 1996a; Barnes & Liew 1995). Peroxynitrite, the product of NO and superoxide anion (O_2^-), has the potential to destroy the epithelium through the induction of lipid oxidation, protein degradation and DNA damage (Patel & Block 1986; Wink *et al* 1991; Moriguchi *et al* 1992).

The ability of TNFα to induce hypersensitive airways extends deeper as this cytokine has the potential to act by indirectly recruiting and activating eosinophils. This proinflammatory cytokine acts by upregulating the adhesion molecules involved in eosinophil recruitment and also by upregulating a second cytokine IL-5 involved in eosinophil maturation and activation (Galli & Costa 1995: Kovacs, 1991; Kips *et al* 1992). Eosinophils when activated release the ECP and MBP. It has been demonstrated both in *in vitro* and *in vivo* animal models that the products of eosinophils, ECP and MBP have the ability to induce airway hyperresponsiveness. Airway hyper-responsiveness, was induced by eosinophil granule proteins both in guinea pig trachea preparations and in an *in vivo* rat model (Kraneveld *et al* 1997).

The exact mechanism by which eosinophils and their proteins promote their effects is not however clearly defined, although numerous hypotheses have been described. Eosinophils can secrete fibroblast growth factors and ECP has been shown to stimulate the production of hyaluronan and proteoglycan in human fibroblasts (Du Buske, 1995; Smith, 1992). It is possible that these cationic proteins may induce changes in the epithelial layer, for example through inactivation of neutral endopeptidase. As mentioned earlier neutral endopeptidase in turn inactivates inflammatory mediators including endothelin a protein thought to be involved in bronchoconstriction and tissue remodelling (Krishna *et al* 1996; Barnes, 1996a). Furthermore, release of sensory neuropeptides, such as tachykinin, may take place following eosinophil activation. Eosinophilic mediators have been shown to induce the release of neuropeptides in human bronchi that may lead to activation of the NANC nerves. Studies on OVA-induced airway hyperresponsiveness in guinea pigs have

shown neuropeptide depletion resulted in complete inhibition of hyperresponsiveness in the guinea pigs (Kraneveld *et al* 1997).

1.1.1.3 Treatment of Extrinsic Asthma

Although, to date, many drugs have been developed for the treatment of asthma its prevalence, severity and mortality are increasing significantly (Anderson, 1992; Lazarus, 1998). The symptoms of a mild to moderate asthmatic can be alleviated effectively, with bronchodilators. However, asthmatics with severe inflammatory symptoms rely mainly on the use of corticosteroids which, although extremely effective may have severe adverse effects if used continuously.

1.1.1.3(a) Bronchodilators

There are two types of bronchodilators, beta$_2$-adrenergic stimulants (β_2-agonists) and phosphodiesterase (PDE) inhibitors. β_2-agonists stimulate receptors, which increase the synthesis of cyclic adenosine monophosphate (cAMP), which in turn relax airway smooth muscle (Nials *et al* 1997; Nials *et al* 1993; Ball *et al* 1991). The second type of bronchodilators are PDE inhibitors. These drugs act by preventing the breakdown of cAMP through the inhibition of PDE (Kleerup, 1997; Lazarus, 1998). PDE inhibitors have also been shown to possess anti-inflammatory properties. Theophylline has demonstrated adenosine antagonistic properties. This drug has been shown act by

blocking the adenosine receptor, which is known to be present on many inflammatory cells, including mast cells. As activation of adenosine receptors can trigger mast cell degranulation antagonism of this receptor would in turn prevent bronchoconstriction by the mediators released. The PDE inhibitor theophylline has been show to also act as an adenosine antagonist whereby the activation of phospholipase C and the mobilisation of calcium were prevented (Linden *et al* 1998; Feoktistov *et al* 1998).

β_2-agonist activity has been demonstrated *in vitro* and *in vivo* in guinea pigs and in the human bronchus in man (Ball *et al* 1991). They can be classified into two groups, short acting β_2-agonists and long acting β_2-agonists. Short acting β_2-agonists include, salbutamol and terbutaline and are taken when needed to relax the bronchus. The effects of these drugs last only for 4-6h and are limited in cases where extended protection is needed, particularly in cases of nocturnal asthma. Long acting β_2-agonists include salmeterol and eformoterol and have the advantage over short acting β_2-agonists in that their relaxant effects last for up to 12h. It has been suggested that the long acting action of salmeterol is due to its lipophilic N-substituted phenylalkyloxyalkyl 'tail'. This tail appears to bind adjacent to the active site placing the drug in such a position, which would enable the phenylethanolamine 'head' to repeatedly interact and activate with the β_2-adrenoreceptor (Nials *et al* 1997; Nials *et al* 1993; Ball *et al* 1991). In contrast to short acting β_2-agonists, long acting β_2-agonists are not taken for the relief of an acute attack but are taken twice daily to prevent an attack. Long acting β_2-agonists are generally used for patients requiring long-term regular bronchodilatory therapy and are taken together

with anti-inflammatories, for example corticosteroids or Di-sodium cromoglycate (DSCG) (Anderson, 1993; Nials *et al* 1993; Ball *et al* 1991).

Since the discovery of the potent bronchodilatory effect of β_2-agonists, other possible applications have been investigated with particular focus on salmeterol and its potential anti-inflammatory effect. The mechanism by which salmeterol acts as an anti-inflammatory is unclear. Some studies on guinea pig and human lung tissue would indicate that the actions are *via* the β_2-adrenergic receptor and propranolol, a β-adrenergic receptor antagonist blocks the anti-inflammatory effect of the drug (Butchers *et al* 1991; Whelan & Johnson, 1992; Gentilini *et al* 1994). In contrast to this however other studies show that the actions are independent of the β-adrenergic receptor and are resistant to the inhibitory effects of propranolol. It is thought that in these experiments the drug acts by stabilising the membrane (Nials *et al* 1997; Chong *et al* 1998). Salmeterol has been found to be a potent inhibitor of the release of histamine, leukotrienes (LTC_4/LTD4) and PGD_2 in human lung *in vitro* (Butchers *et al* 1991, Gorenne *et al* 1995; Gentilini *et al* 1994). *In vivo* studies on guinea-pig lung showed that salmeterol inhibited histamine induced plasma protein extravasation into the airway lumen. These studies also showed *in vivo* and *in vitro* inhibition of neutrophil and eosinophil accumulation and activation in lung by salmeterol (Whelan & Johnson 1992; Pedersen *et al* 1993; Anderson *et al* 1996). The bronchodilatory and anti-inflammatory properties of salmeterol give it the potential to act at both the early and late phases of asthma, preventing bronchoconstriction and bronchial hyperresponsiveness in atopic asthmatic patient. It is

possible therefore that salmeterol may be used without the use of additional therapy from other anti-inflammatories particularly steroids (Anderson *et al* 1996; Pedersen *et al* 1993).

Although, β_2-agonists are useful therapeutically, regular use of these drugs leads down-regulation and the development of tolerance towards the drug. Drug tolerance appears to occur with or without the use of concomitant corticosteroid therapy. Increase in non-specific airway responsiveness results and physiological indicators of airway inflammation in asthma appear to worsen by chronic use of β_2 agonists (Kleerup, 1997).

Theophylline or aminophylline, the modified formulation of theophylline are the main PDE inhibitors available for the treatment of asthma. Through the inhibition of the PDE enzyme these drugs prevent the breakdown of cAMP thus increasing the local concentration available to relax the airways (Kleerup, 1997). There are many types of PDE enzymes and PDE III and PDE IV are the two of importance in the airways. Although inhibition of PDE III and PDE IV may provide bronchodilation relief for asthmatics inhibition of PDE III may also result in unwanted cardiovascular side effects. Theophylline is limited in that it is a non-selective bronchodilator and treatment with this drug leads to inhibition of all PDE enzymes (I-V) (Kleerup, 1997). Because of this theophylline's therapeutic window is narrow (10-20µg/ml) as the side effects are prominent above this level. At this concentration however theophylline has only weak bronchodilatory effects, therefore it is relatively ineffective in the treatment of asthma (Page, 1999)

Further studies on theophylline have demonstrated that this drug has anti-inflammatory properties together with its bronchodilatory ability. *In vitro* and *in vivo* studies on both animal models and human beings have show that inhibition of PDE results in a reduction in the inflammatory process. Low doses of theophylline resulted in the inhibition of the late phase bronchial response to allergen inhalation and airway hyper-responsiveness. Moreover PDE inhibitors, such as theophylline, have also been shown to possess the ability to antagonise adenosine receptors present on many inflammatory cells such as mast cells. Antagonism of the adenosine receptor by theophylline prevents mast cell granulation and the release of inflammatory mediators involved in the constriction of the airways (Linden *et al* 1998; Feoktistov *et al* 1998). Studies also demonstrated a decrease in the proliferation and activation of lymphocytes and inhibition of the release of pro-inflammatory cytokines with theophylline (Jaffar *et al* 1996; Barnes & Pauwels, 1994). Furthermore, a combination of PDE inhibition and β_2-adrenoreceptor stimulation results in the inhibition of chemotaxis for and activation of eosinophils (Szefler & Nelson, 1998; Kleerup, 1997; Barnes & Pauwels, 1994). The exact mechanism of theophylline's anti-inflammatory affects remains a controversial topic. Recent evidence has shown that theophylline can alter IL-5 mediated eosinophil survival and increase the rate of apoptosis, however it is thought that its actions are *via* PDE IV-independent mechanisms as rolipram, a selective PDE IV inhibitor, has little effect on eosinophil survival (Yasui *et al* 1997). Moreover the theophylline plasma levels required to induce a significant anti-inflammatory effect are below 10μg/ml whilst 10-20μg/ml show only a very weak bronchodilatory effect (Page, 1999).

1.1.1.3(b) Prophylactics or Preventors

The drugs DSCG and nedocromil sodium can prevent an attack of asthma. Although the exact mechanism of action of DSCG and nedocromil sodium are unknown they are believed to act as an anti-inflammatories at the early phase through the prevention of mast cell degranulation and at the last phase by blocking eosinophil recruitment. Recent studies in rodent mast cells have proposed that both these drugs inhibit mast cell degranulation by inhibiting chloride influx. It is believed that following the release of Ca^{2+} from the intracellular stores, the influx of chloride ions brings the membrane potential to a more negative value, which would then allow the slow influx of more Ca^{2+} ions, through CRAC channels, resulting in sufficient levels of intracellular calcium concentration needed to induce degranulation of the mast cell (Alton & Norris, 1996; Redrup *et al* 1997). *In vitro* studies showed the inhibitory effect of nedocromil sodium on antigen or anti-IgE induced histamine and leukotriene release from human, rat and monkey sensitised lung mast cells (Napier *et al* 1990; Pearce, 1993; Moqbel *et al* 1988). Moreover, DSCG was found to be an effective inhibitor of histamine and leukotriene release from rat peritoneal and pleural mast cells *in vitro* and human mast cells recovered from BAL (Butchers *et al* 1979; Flint *et al* 1985). Both drugs inhibited the release of the TNFα from rat peritoneal mast cells and the cytotoxicity experienced with this cytokine. The inhibitory activity of these drugs on this cytokine from rat mast cells appears to differ from that of histamine release as inhibition of protein and mRNA synthesis appears to play an intricate part (Bissonnette *et al* 1995). These drugs were also found to reduce the effect of both antigen and anti-IgE induced contraction of passively sensitised human and guinea-pig bronchial lung strips

(Armour *et al* 1982; Church & Young, 1983; Napier *et al* 1990; Butchers *et al* 1979; Busse, 1998). An *in vivo* model, using antigen-sensitised guinea pigs supported previous studies. They showed that nedocromil sodium prevented antigen-induced bronchoconstriction, airway epithelial eosinophilia and the development of airway hyper-responsiveness (Schellenberg *et al* 1991; Church *et al* 1993). Similar *in vivo* studies were carried out looking at DSCG's anti-anaphylactic properties. This drug reduced bronchial anaphylaxis in guinea pigs sensitised to OVA (Andersson, 1980). Studies on the late phase of asthma showed that both drugs inhibit isolated human eosinophil and neutrophil chemotaxis *in vitro* (Bruijnzeel *et al* 1990; Warringa *et al* 1993; Moqbel *et al* 1988). Clinical studies have supported previous findings. They show that both drugs significantly improve pulmonary function, inhibit early and late phase bronchoconstriction allergen responses, inhibit BAL eosinophilia and decrease bronchial hyper-reactivity (Busse, 1998, Schellenberg *et al* 1991; Calhoun *et al* 1996; Szefler & Nelson, 1998).

1.1.1.3(c) Steroidal Anti-inflammatories

Inhaled corticosteroids, often found to be the only real effective treatment, are used for patients with chronic asthma. Some of the steroids on the market include, beclomethasone and budesonide and they appear to have their effect through their many anti-inflammatory properties (Lazarus, 1998).

These compounds may act through either inducing or inhibiting the transcription of many genes. They induce the transcription of the gene for the protein lipocortin, which in turn

inhibits PLA_2. This enzyme is involved in the release of AA, the precursor for the production of the bronchoconstrictor and inflammatory mediators, leukotrienes and prostaglandins. Therefore, although steroids do not inhibit the bronchoconstrictor action of histamine they have the potential to inhibit the synthesis of new mediators by the mast cell. Steroids also have the potential to inactivate NF-κB, by initiating the transcription of its inhibitory protein IκBα. NF-κB plays an important part in the asthmatic inflammatory process through the stimulation of cytokine production and also the production of adhesion molecules involved in the influx of T-lymphocytes and leucocytes (Kumar *et al* 1998; Galli & Costa 1995; Barnes & Adcock, 1997; Krishna *et al* 1996; Baraniuk, 1996; Schwiebert, 1996).

Other target genes are those transcribing inflammatory cytokines. In these cases steroids inhibit the transcription of the genes. Cytokines affected are IL-1β, TNFα, GM-CSF and IL-2 through to IL-6 (Barnes, 1996b; Schwiebert, 1996). As described in earlier sections many of these cytokines play a significant role in the pathogenesis of asthma. IL-1β is involved in the induction of iNOS an enzyme whose transcription is also inhibited by steroids (Barnes, 1996b). TNFα, is involved in the production of adhesion molecules through the activation of NF-κB (Kumar *et al* 1998; Galli & Costa 1995; Barnes & Adcock, 1997; Krishna *et al* 1996). It upregulates IL-5 in T-lymphocytes (Meng *et al* 1997; Sur *et al* 1996; Ohnishi *et al* 1993; Kumar *et al* 1998). It is also thought to be involved in the remodelling of airways through its effect on fibroblasts (Kay, 1991; Robinson, 1996). IL-3, IL-5 and GM-CSF are involved in the activation, differentiation and maturation of eosinophils and finally IL-4 is involved in the isotype switching of B-

cells to produce IgE antibodies. Steroids also inhibit release of IL-4 and IL-13 from lymphocytes, the cytokines involved in IgE synthesis. (Baraniuk, 1996; Schwiebert *et al* 1996).

Although steroids are extremely effective in the treatment of asthma, they have many side effects and are only used in closely monitored doses when other treatments fail to alleviate symptoms. Potential side effects of long-term use of inhaled steroids can include hypertension, glucose intolerance, adrenal suppression, growth retardation in children, osteoporosis, glaucoma and cataracts (Lazarus, 1998; Wasserfallen & Baraniuk, 1996). These effects occur at concentrations greater than 2mg/day and less than 600-800μg/day/adult and less than 400-600μg/day/child maintains low systemic levels of the particular steroid. While the use of steroids at low doses is beneficial in mild to moderate cases of asthma, higher doses are needed for the severe cases. For these patients levels greater than 2mg/day are required and at these doses the side effects can be quite serious and therefore although steroids are effective, the search for a different drug for the treatment of asthma is ongoing. It is possible that combination therapy of low dose budesonide and low dose theophylline may show some clinical relevance. Both drugs act, as anti-inflammatories however when used at high doses they exhibit unwanted side effects. Preliminary studies demonstrated that low dose theophylline and low dose budesonide had equivalent efficacy to that of high dose budesonide (Page, 1999).

1.1.1.3(d) Anti-leukotrienes

Other potential treatments, against asthma, are anti-leukotrienes. It appears that the products of 5-LO, LTB_4, and the cLTs, LTC_4, LTD_4 and LTE_4 contribute significantly in the pathogenesis of asthma. The cLTs produce bronchoconstriction, increase vascular permeability, mucus and inflammatory cell infiltration. LTC_4 and LTD_4 are 1000 times more effective as bronchoconstrictors than histamine and they are thought to cause increased airway responsiveness to histamine and methacholine. Leukotrienes are also more potent at increasing vascular permeability than histamine, prostaglandins and PAF and are also thought to play an important role in eosinophil influx. LTD_4 has been found to potentiate mitogen-induced proliferation in cell cultures and animal studies and may contribute to bronchial hyper-responsiveness (Bell *et al* 1997; Tan & Spector 1997; Lazarus, 1998; Busse, 1998).

There are three main ways of preventing the action of leukotrienes. These include inhibition of 5-LO, the enzyme involved in the synthesis of these mediators, the inhibition of the FLAP and leukotriene receptor antagonism. A number of drugs are presently available on the market. These include the receptor antagonists, zafirlukast and pranlukast, the FLAP inhibitors MK-0591 and BAYx1005 which are undergoing clinical trials and the 5 -lipoxygenase inhibitors, zileuton, which is also currently available and ABT-761 which is undergoing pre-clinical studies. *In vivo* studies in rodent models of airway diseases have shown that inhibition of the production of leukotrienes prevents bronchoconstriction and eosinophil influx. All of these drugs to date have improved

pulmonary function and clinical symptoms and reduced night time awakenings and the use of β-agonists (Bell *et al* 1997; Tan & Spector 1997; Lazarus, 1998; Busse, 1998; Szefler & Nelson, 1998).

1.1.2 Intrinsic Asthma

Intrinsic asthma differs from allergic asthma in many ways. Patients with intrinsic asthma give a negative result when exposed to skin prick tests to common allergens such as grass pollen, house dust mites, cat fur or dog hair. IgE levels specific to the common allergens cannot be demonstrated. The symptoms of intrinsic asthmatics are more severe than those of extrinsic asthmatics and require higher doses of steroids to alleviate bronchoconstriction. This may be related to the fact that eosinophilia is more prominent in intrinsic than extrinsic subjects. In general intrinsic asthma is more severe, more difficult to control and patients with this disease have a worse prognosis (Virchow *et al* 1996; Bentley *et al* 1992).

Although little is known about the development of intrinsic asthma, many possible causes have been discussed. Strong evidence points towards that of respiratory tract infection. Respiratory syncytial virus (RSV), parainfluenza, adenovirus and *Mycoplasma pneumoniae* are among the viruses and microbes thought to be implicated in the development of wheezing and the onset of intrinsic asthma. However not enough is known about the development of the disease and it is thought that other causes, for example a hidden allergen cannot be ruled out (Busse, 1990).

Viruses are antigenic and can therefore result in polyclonal antibody production. Virus specific IgE antibodies have been demonstrated in patients following viral infection and nasal histamine release was higher in patients with RSV IgE antibodies. In intrinsic asthma therefore it can be speculated, although not proven, that virus specific IgE antibodies may induce mast cell degranulation, release of bronchoconstrictive and inflammatory mediators and result in the eventual cause of bronchial hyper-reactivity (Busse, 1990).

Intrinsic asthma can be described as a type of autoimmune disease whereby exposure of a patient to a respiratory viral infection leads to the development of the T_H1-type response. Activation of T-lymphocytes, with a cytokine profile simulating that of a T_H1-type response has been demonstrated in patients with intrinsic asthma. Cytokines, namely IL-2, IFNγ, IL-5 but not IL-4 are elevated in the BAL fluid and purified peripheral blood T-cells of these patients. Peripheral blood T cells also showed elevated levels of IL-3 and GM-CSF cytokines that together with IL-5 activate, differentiate and prolong eosinophil survival. The presence of mRNA for IL-5, GM-CSF and also IL-6 has been demonstrated in T-lymphocyte preparations in patients with intrinsic asthma. mRNA for IL-2 and IFNγ have also been found in some intrinsic asthmatic patients. The presence of IL-3, IL-5 and GM-CSF, cytokines that promote the differentiation and activation of eosinophils, indicates the mechanism whereby hypersensitivity of the airways in intrinsic asthmatics develops. Eosinophilic proteins, such as ECP and MBP, are believed to result in epithelial damage and airway remodelling and hypersensitivity towards cold and humidity and

environmental irritants, such as ozone (Walker & Virchow, 1993; Virchow *et al* 1996; Barnes & Adcock, 1997; Denburg, 1996; Barrios *et al* 1998).

1.1.2.1 Treatment of Intrinsic asthma

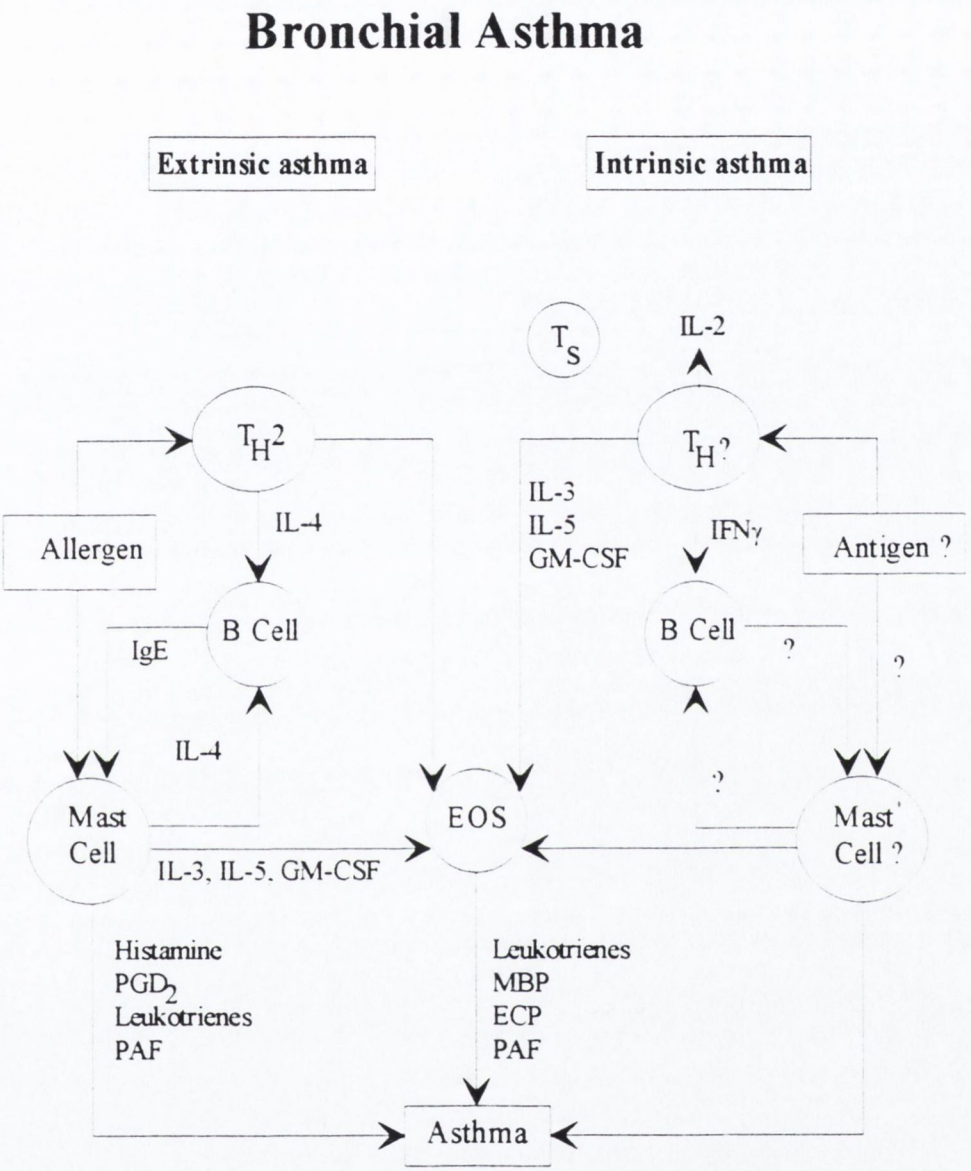
The only known treatment for this chronic disease, intrinsic asthma, is that of steroids. The steroids used are those also used for the treatment of extrinsic asthma, beclomethasone and budesonide (Lazarus, 1998). Steroids manifest their protective effects as described earlier for extrinsic asthma (section 1.1.1.3(c)) where although the initial cause of asthma is different the ultimate effect as seen in the symptoms noted are the same.

1.1.3 Extrinsic Versus Intrinsic Asthma

Figure 1.6 shows a brief outline comparing the two asthmatic diseases. Extrinsic and intrinsic asthma appear to follow a T_H2 and T_H1 type profile respectively. Extrinsic asthma follows a clearly defined path, including cytokines namely, IL-4, IL-5, IL-6, IL-10, IL-3 and GM-CSF. IL-4 has been implicated in the production of specific IgE production and B-cell activation. IL-5, IL-3 and GM-CSF are involved in eosinophil activation and differentiation. The exact profile for intrinsic asthma is not as clearly defined however it is thought to mimic a T_H1 type response. IL-2, IFNγ, IL-5, IL-3 and GM-CSF are among the cytokines produced in intrinsic asthma. IL-2 induces the T_H1 response, which in turn

produces IL-5, IL-3 and GM-CSF and leads to activation and differentiation of eosinophils. The two responses T_H1 and T_H2 are cross-regulated. IFNγ produced by T_H1 cells inhibits the T_H2 response, the proliferation of the T_H2 cells and hence IgE activation. IL-4 and IL-10 produced by T_H2 cells inhibits the T_H1 response, IL-2 production. T_H1 and T_H2 cells possess very different functions. While T_H2 cells are involved in helping B cells differentiate into Ig-producing cells, T_H1 cells participate in DTH and cytotoxic functions. Direct evidence of the existence of T_H1 and T_H2 cells in humans are as yet not well documented *in vivo*, however murine and rat studies support this idea. RNA hybridisation studies indicate two $CD4^+$ cells with two separate cytokine profiles, T_H1 and T_H2 (Walker & Virchow, 1993; Virchow *et al* 1996, Field *et al* 1993).

Figure 1.6:- Comparison between extrinsic and intrinsic asthma



(Adapted from Virchow *et al* 1996)

1.2 Inflammation

1.2.1 Acute Inflammation

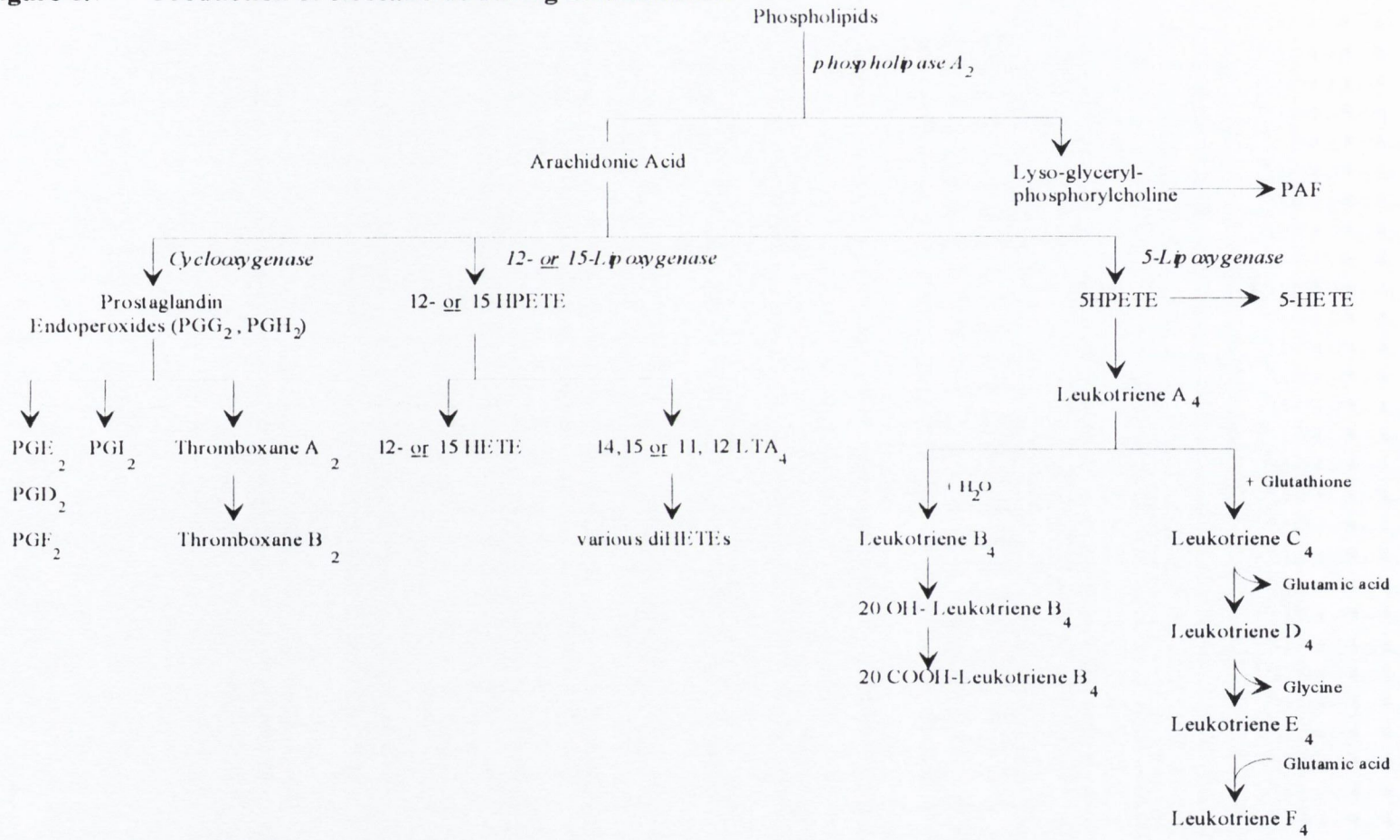
Inflammation develops after injury, trauma or bacterial infection. The pathogenesis of inflammation can be local or systemic however if not controlled properly may often lead to fibrosis and necrosis of a particular tissue or organ. Inflammatory diseases include psoriasis, septic shock, peritonitis, hepatitis and acute lung injury (Vadas *et al* 1991; Baumann & Gauldie, 1994).

The inflammatory process is a physiological response, activated to protect the host by preventing ongoing tissue damage, isolating, killing and eliminating the infective organism and repairing the damaged tissue. Following injury or a bacterial infection a series of events initiated by the host takes place (Baumann & Gauldie, 1994). Bacterial endotoxins, LPS, have been shown to induce both PLA_2 and COX-II in many cells. LPS induction of PLA_2 has been demonstrated in human monocytes and rat vascular smooth muscle cells among others (Nakano *et al* 1990; Roshak *et al* 1994). COX-II induction by LPS is also well documented and has been demonstrated in human monocytes, rat alveolar macrophages, murine macrophage cell line (J774.2) and bovine aortic endothelial cells (Roshak *et al* 1994; Lee *et al* 1992; Akarasereenont *et al* 1995; Fu *et al* 1990). From these studies it is clear that inflammation, caused by bacterial infection, leads to the activation of many cells and the upregulation of PLA_2 and COX-II enzymes (Frolich, 1997).

Activation of macrophages and monocytes following an inflammatory stimulus leads to the synthesis and release of many pro-inflammatory cytokines. These include mainly IL-1α, IL-1β, TNFα and IFNγ (Roshak *et al* 1994). These cytokines act to amplify the inflammatory process in many ways. IL-1 has also been shown to increase the activity of PLA_2 and COX-II by *de novo* protein synthesis in macrophages, monocytes, endothelial cells and fibroblasts (Arias-Negrete *et al* 1995; Raz *et al* 1988; Raz *et al* 1989; Nakazato *et al* 1991; O'Neill & Lewis, 1989; Vadas *et al* 1991). TNFα has been shown to act synergistically with IFNγ in stimulating mRNA transcription of COX-II in human macrophages (Vadas *et al* 1991; Arias-Negrete *et al* 1995; Frolich, 1997).

PLA_2 is believed to be involved in the deacylation of cellular phospholipids to AA and lyso-glycerylphosphorylcholine, the latter being the precursor to PAF. AA can be further modified or metabolised *via* a number of pathways as outlined in figure 1.7. The products of AA are the eicosanoids and while COX-II produces the prostaglandins and thromboxane (TXA_2), the lipoxygenase enzymes produce leukotrienes. Eicosanoids, promote the inflammatory process as described in asthmatic inflammation, however, their actions vary considerably depending on the site of injury. While PAF, and the leukotriene, LTB_4, induce leucocyte infiltration, LTB_4 and 12-hydroxyeicosatetraenoic acid (HETE) induce epidermal proliferation. Vascular permeability is a result of the effects of PAF, LTC_4, LTD_4 and LTE_4 while vasodilation is due to PGD_2, PGE_2 and PGI_2 (Miyake *et al* 1993; Davies *et al* 1984; Salmon & Higgs, 1987).

Figure 1.7 Production of eicosanoids during inflammation



(Adapted from Davis *et al* 1984)

1.2.2 Chronic Inflammation

1.2.2.1 Leucocyte Infiltration

Leucocytes accumulate in damaged tissue during inflammation. Vasodilation induced by the prostaglandins results in the increased concentration of leucocytes at the site of inflammation while increased vascular permeability induced by PAF and the cLTs enables migration or diapedesis of these same cells (Salmon & Higgs, 1987). Recruitment of leucocytes is a complex process and involves rolling, activation, firm adhesion and diapedesis or transendothelial migration of the cells with endothelial cells of surrounding blood vessels. Activation of macrophages in the early phase results in the release of the cytokines IL-1 and TNFα. These cytokines are imperative in activating and stimulating the endothelial cells to express adhesion molecules. Chemoattractants, namely PAF and LTB_4, also greatly influence leucocytes adherence possibly *via* transmembrane receptors located on the leucocyte (Bochner, 1997; Zimmerman *et al* 1994; Gimbrone *et al* 1984; Samuelsson, 1983). The initial rolling phase of leucocytes adhesion is mediated by selectins. While L-selectin is constitutively expressed by most leucocytes, E -selectin and P-selectin are both expressed by activated endothelial cells. L-selectin, E-selectin and P-selectin specifically interact using carbohydrate moieties with tetrasaccharide sialyl-Lewis x linked to mucin-like molecules expressed by both leucocytes and endothelial cells. Rolling of the leucocytes on the endothelial surface slows down their flow rate to a speed that will allow activation and firm adhesion. Activation of the leucocytes is carried out by chemokines which induce the shedding of the L-selectin and the activation of a second

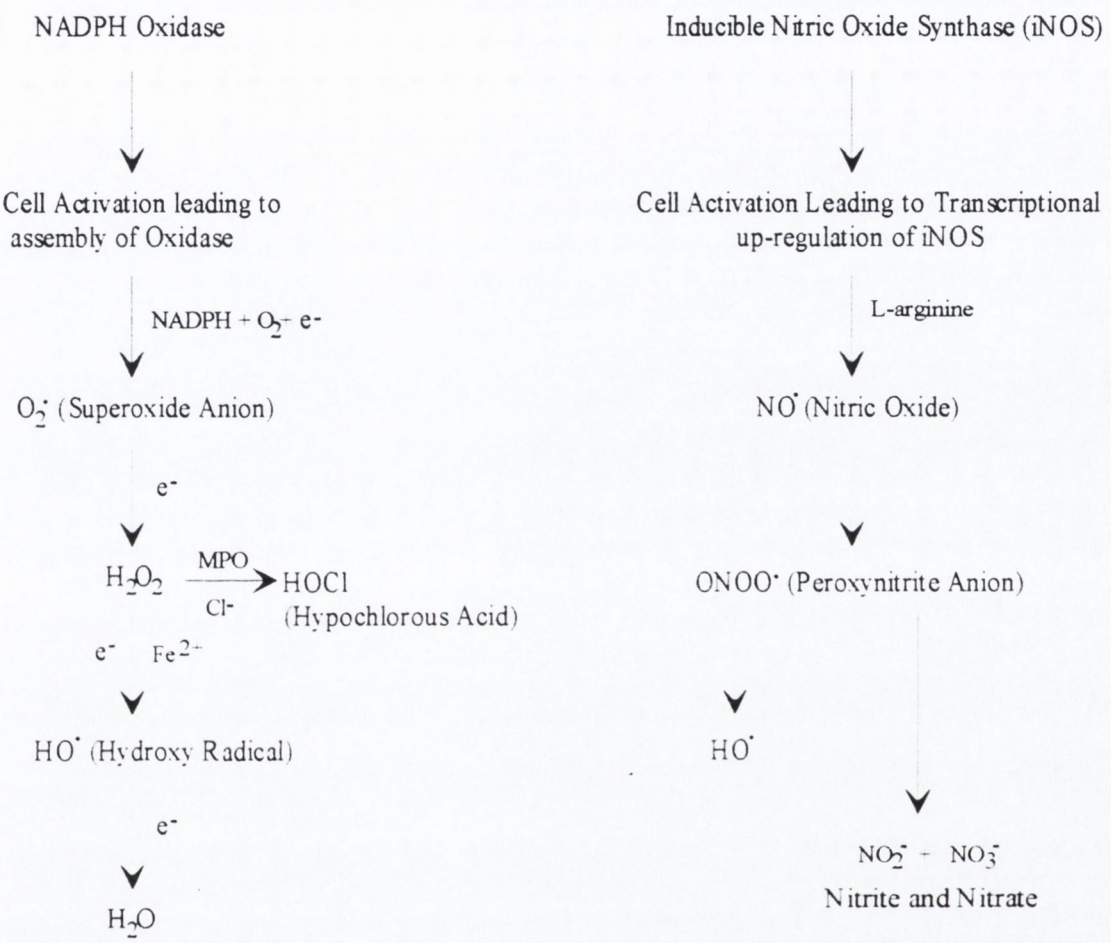
integrin adhesion molecule, VLA-4, Mac-1 (CD11b/CD18) and LFA-1 (CD11a/CD18). These molecules interact with their counter receptors on the endothelial cells, VCAM-1 for VLA-4, ICAM-1 for MAC-1 and LFA-1 to give firm adhesion onto the endothelial cell. Following this the leucocytes migrate between the endothelial cells to the site of inflammation (Diaz-Gonzalez & Sanchez-Madrid, 1998; Ward & Lentsch, 1999; Bochner, 1997; Zimmerman *et al* 1994; Gimbrone *et al* 1984; Samuelsson, 1983).

1.2.2.2 Tissue Injury

Leucocytes, in particular neutrophils, are activated following recruitment to the area of inflammation. Neutrophils contain over 40 proteolytic enzymes and have the capability to generate various oxidants (Dallegri & Ottonello, 1997). They act mainly *via* phagocytic mechanisms whereby the bacterial entity or damaged tissue is engulfed and digested. However on some occasions release of destructive granules occurs and uncontrolled destruction of healthy tissue transpires. This happens, either during the formation of a phagosome or when the cell meets with an immunocomplex or antibody coated surface too large to engulf.

There are two oxidant-generating pathways seen in inflammatory responses in rat phagocytic cells, (neutrophils, macrophages and monocytes) (Ward & Lentsch, 1999). These pathways are the nicotinamide adenine dinucleotide phosphate, reduced form (NADPH) oxidase pathway and the NO synthase pathway, figure 1.8.

Figure 1.8:- Mechanisms of Oxidant Production by activated phagocytic cells



(Adapted from Ward & Lentsch, 1999)

NADPH oxidase exists as inactive subunits located on both the cell membrane and in the cytosol. Following cell activation, the cytosolic subunit translocates to the cell membrane resulting in a multimeric active enzyme complex. The active NADPH oxidase, enables the donation of one electron from NADPH to molecular oxygen. The reduction of oxygen leads to the formation of O_2^-. Further reduction of O_2^- gives hydrogen peroxide which can be further reduced to hydroxy free radical (OH^-). This free radical is highly reactive and

damaging towards lipids, proteins and DNA. In the presence of a chloride ion, hydrogen peroxide can also be converted to hypochlorous acid, another potent oxidant, by myeloperoxidase, a neutrophil derived peptide. Hypochlorous acid not only induces oxidative tissue injury but inactivates the enzyme alpha-1-antitrypsin (AT), which in turn results in elastase-mediated digestion of the intercellular tissue matrix (Ward & Lentsch, 1999; Blake *et al* 1987; Rosen *et al* 1995; Dallegri & Ottonello, 1997).

The second oxidant-generating pathway is the NO synthase pathway (Ward & Lentsch, 1999; Rosen *et al* 1995). The enzyme NO synthase (NOS) can be induced in large quantities by bacterial endotoxins lipopoylsaccharides (LPS) and the pro-inflammatory cytokines, IL-1β, TNFα and IFNγ (Barnes & Liew 1995; Ruetten & Thiemermann, 1997). As discussed earlier, NOS reacts with L-arginine leading to the production of the free radical. NO is synthesised constitutively in small picomolar quantities and is involved in the control of blood flow, however in inflammatory conditions the NOS enzyme is induced and large nanomolar quantities are produced leading to deleterious conditions.

This free radical is capable of inhibiting a variety of enzymes involved in mitochondrial respiration and also DNA synthesis in rat hepatocytes and hamster trachea (Radi *et al* 1991; Heiss *et al* 1994). NO, an unstable molecule is readily converted to a $ONOO^{\cdot}$. This molecule is highly reactive and can induce lipid oxidation, protein degradation and DNA damage in rat hepatocytes (Patel & Block, 1986; Wink *et al* 1991; Moriguchi *et al* 1992). Peroxynitrite can be further converted to either the stable or unreactive products nitrite (NO_2^-) and nitrate (NO_3^-) or the $OH^{\cdot}$ which can go on to induce further cellular damage.

Although, the generation of these free radicals by macrophages helps to combat bacterial infection or damaged tissue, they are unfortunately not site specific and hence their presence accounts for the damage of healthy tissue (Ward & Lentsch, 1999).

Other leucocytes activated during the tissue injury process are the eosinophil and the basophil. Eosinophilia has been demonstrated mainly in asthmatic inflammation and has been found in the BAL of asthmatics. Eosinophils are similar to neutrophils however release additional proteins on activation. These include ECP, peroxidase, MBP and neurotoxin (Barnes, 1991; Robinson, 1996). The activities of these proteins have been discussed earlier. Basophils have activity similar to mast cells.

1.2.3 Treatment of Inflammation

1.2.3.1 Non Steroidal Anti-inflammatories

Anti-inflammatory drugs can be classified into two categories, non-steroidal anti-inflammatories (NSAIDs) and steroidal anti-inflammatories. NSAIDs act by inhibiting the COX enzymes and the synthesis of prostaglandins and thromboxanes. There are two isoforms of COX, COX-I and COX-II. COX-I, the constitutive form is present in small quantities, while COX-II when induced in inflammatory states is available in large quantities. COX-II differs to COX-I in that while the activity of COX-I may increase up to 2-3 fold, the activity of COX-II can increase 20 fold during an inflammatory reaction (Frolich, 1997; Mitchell *et al* 1994; Spangler, 1993; Engelhardt, 1994;).

COX produces prostaglandins and thromboxanes, which play a regulatory role under physiological conditions. COX-I has been found in blood platelets, the gastric mucosa and in the renal medulla. In blood platelets the production of thromboxane A_2 controls platelet aggregation. COX-I in the gastric mucosa leads to the production of prostaglandins, which are cytoprotective. Finally, synthesis of prostaglandins by COX-I in the renal medulla significantly influences total renal blood flow, distribution of renal blood flow and Na^+ and water reabsorption (Frolich, 1997; Engelhardt *et al* 1995). While the inhibition of COX-II would result in the reduction of inflammation, in many cases inhibition of COX-II results in the inhibition of COX-I and the development of many side effects. COX-I inhibition, would lead to the inhibition of prostaglandin synthesis in the gastric mucosa, which in turn would lead to gastric bleeding and ulceration. The inhibition of the synthesis of thromboxane by COX-I in blood platelets would lead to abnormal platelet aggregation. Finally, inhibition of the activity of COX-I in the renal medulla would lead to the reduction of glomerular filtrate rate and Na^+ and water excretion (Frolich, 1997).

The second isoenzyme, COX-II, is induced mainly during inflammatory conditions. Endotoxin, has been shown to induce COX-II in human monocytes, mouse macrophages and rat, rabbit and human alveolar macrophages. Moreover, proinflammatory cytokines, namely IL-1, TNF-α and IFN-γ induce COX-II while anti-inflammatory cytokines, such as IL-10 down-regulate COX-II expression. The large induction of COX-II leads to the

production of large quantities of prostaglandins and thromboxanes. This may have detrimental effects at the site of inflammation (Frolich, 1997).

The ideal NSAIDs therefore would be one that would effectively inhibit the inducible COX-II enzyme without affecting the constitutive enzyme COX-I. To date, many COX inhibitors have been synthesised, however, they are non-selective and have the potential to cause the side effects listed above. These drugs include, indomethacin, piroxicam, diclofenac etodolac and ibuprofen (Frolich, 1997; Mitchell *et al* 1994; Spangler, 1993; Engelhardt, 1994;). Moderately selective COX-II inhibitors have been synthesised. These include meloxicam and nabumetone (Frolich, 1997; Engelhardt, 1994; Engelhardt *et al* 1995; Melarange, *et al* 1994a; Melarange *et al* 1994b). Studies on meloxicam show that it has a very favourable COX-II : COX-I ratio (Frolich, 1997; Engelhardt, 1994; Engelhardt *et al* 1995). It showed selectivity for COX-II in whole cell assays and 75% selectivity for assays using microsomes. *In vivo* pre-clinical animal studies also demonstrated this fact. Meloxicam when used for clinical studies showed a significant anti-inflammatory effect without any effect on platelet aggregation, renal function and without development of gastric bleeding or ulceration. Nabumetone, a non-acidic drug is metabolised by the liver to the active metabolite, 6 methoxy-2-naphthyl acetic acid (6MNA). Like meloxicam, nabumetones metabolite, 6MNA, is specific for COX-II and has little effect on COX-I. *In vivo* studies on rats dosed daily for one month showed no evidence of gastrointestinal irritancy. Parameters studied included the inhibition of both gastric and ileal i6-keto-$PGF_{i\alpha}$, erosion or ulcer formation (Frolich, 1997; Melarange, *et al* 1994a; Melarange *et al* 1994b). Other more highly selective COX-II inhibitors are in early

development and show a COX-II : COX-I ratio of <0.001 in intact cells. However, at this stage it is difficult to predict whether these compounds or their derivatives would pass the now strict animal and clinical studies (Frolich, 1997, Engelhardt *et al* 1995; Melarange, *et al* 1994a; Melarange *et al* 1994b; Engelhardt, 1994; Spangler, 1993).

NSAIDs have been found to have anti-inflammatory activity alternative to that of COX inhibition. Indomethacin, diclofenac, aspirin, prioxicam and meloxicam have been found to partially inhibit the infiltration of neutrophils to the site of inflammation (Diaz-Gonzalez & Sanchez-Madird, 1998; Dallegri & Ottonello, 1997). *In vitro* studies have also demonstrated their ability to inhibit neutrophil activation by inhibiting NADPH oxidase, iNOS expression and the release of lysosomal enzymes. The inhibition of the activation of the transcription factor, NF-κB, by NSAIDS has also been suggested which would in turn inhibit the transcription of pro-inflammatory cytokines and endothelial adhesion molecules (Diaz-Gonzales & Sanchez-Madrid, 1998; Dallegri & Ottonello, 1997).

1.2.3.2 Steroidal Anti-inflammatories

Corticosteroids, namely, hydrocortisone and dexamethasone inhibit the inflammatory response in a variety of ways, however as discussed previously they also display many serious side effects. It is believed that steroids induce the *de novo* synthesis of a protein called lipocortin, which in turn inhibits action of PLA_2 on cellular phospholipids to produce AA. This has been demonstrated in a number of cells types in a number of species including human monocytes. The exact mechanism of inhibition seen with

corticosteroids is however unclear. Some believe that lipocortin directly inhibits the activity of PLA_2, in fact lipocortin has been shown to inhibit PLA_2 induced phospholipid hydrolysis in rat liver mitochondria and rat platelets. However, some believe that lipocortin binds to the enzyme's substrate, phospholipid and prevents it from acting with PLA_2 (Raz *et al* 1989; Flower, 1988; Davidson *et al* 1987; Aarsman *et al* 1987).

Other applications of corticosteroids include the down regulation of the expression of the pro-inflammatory cytokines namely, IL-1 (α,β), TNFα both of which are involved in the induction of PLA_2, COX-II and iNOS synthesis (Schwiebert *et al* 1996; Arias-Negrete *et al* 1995; Raz *et al* 1988; Raz *et al* 1989; Nakazato *et al* 1991; O'Neill & Lewis, 1989; Vadas *et al* 1991; Frolich, 1997; Barnes & Liew 1995). Corticosteroids may also exert their effects through the direct inhibition of COX-II, iNOS and PLA_2 expression from the DNA template (Barnes & Adcock, 1997). Dexamethasone acts by inducing the expression of Iκ-Bα a protein that binds and inactivates NF-κB, the potent inflammatory transcription factor needed for expression of the cytokines and the induction of enzyme synthesis (Baraniuk, 1996; Ruetten & Thiemermann, 1997; Barnes & Adcock; 1997). It has been demonstrated that dexamethasone inhibits the expression of COX-II without affecting COX-I. Treatment of rat macrophages with LPS resulted in a 20-fold increase of COX-II mRNA without any effect on the expression of COX-I mRNA. This increase was completely inhibited by dexamethasone pre-treatment (Lee *et al* 1992). Similar studies with cultured vascular smooth muscle cells showed COX-II induction by an epidermal growth factor was also inhibited by dexamethasone (Pash & Bailey, 1988). Treatment with these steroids also results in the prevention of the activation of the

endothelium and leucocyte cell surface. Steroids act through the inhibition of the expression of adhesion molecules *via* the inhibition of these cytokines (Schwiebert *et al* 1996; Diaz-Gonzalez & Sanchez-Madrid, 1998; Ward & Lentsch, 1999; Bochner, 1997; Zimmerman *et al* 1994; Gimbrone *et al* 1984; Samuelsson, 1983).

Although many drugs for the treatment of inflammation exist, the search continues for a non-steroidal effective drug that will combat not only acute inflammation, but also chronic inflammation. There are many possible therapeutic approaches. These include anti-adhesion therapy, inhibition of neutrophil activation and anti-oxidant and anti-protease therapy (Dallegri & Ottonello, 1997).

1.3 Delayed Type Hypersensitivity and autoimmune diseases

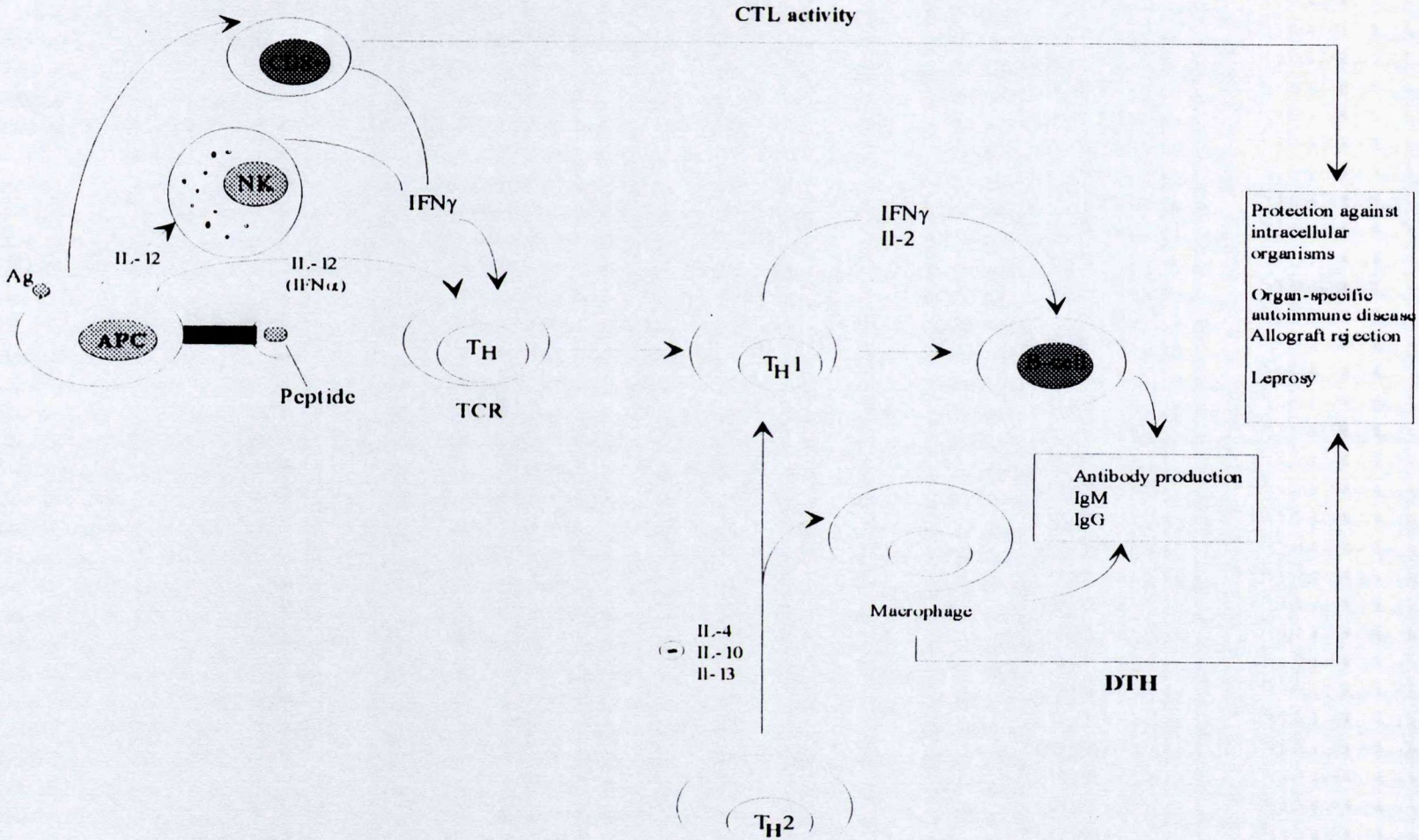
A second class of inflammatory disease is that of DTH and autoimmune disease. These include rheumatoid arthritis (RA), leprosy and allograft rejection. These diseases appear to follow a T_H1-type profile, where the T-cells produce IFNγ, IFNα, TNFβ, and IL-2 (Lucey *et al* 1996; Henderson *et al* 1987; Romagnani, 1997). .

DTH is unlike immediate hypersensitivity as it develops 2-3 days following sensitisation or exposure to a specific antigen. While the end point of both hypersensitivities is that of inflammation involving influx of T-lymphocytes, neutrophils, eosinophils and basophils they involve very different pathways, namely TH_1 and T_H2 . DTH follows the T_H1 pathway where IL-2, IFNγ, TNFβ, are produced in contrast to the synthesis of IL-4 or IL-

5 during immediate hypersensitivity. The exact mechanism for the early production of a particular group of cytokines, (which would ultimately lead to the expansion of either T_H1 or T_H2 cells), remains unclear. The factors that govern polarisation of T cells, towards T_H1 or T_H2 cells, are thought to be initiated at antigen level and are influenced by environmental and genetic factors. This has been demonstrated in mice and in human where the type of antigen and adjuvant and the dose of antigen play an important role. It is now believed that the presence of extracellular bacteria leads to polarisation of naive T cells, T_H0, into T_H2 lymphocytes and that of intracellular organisms (e.g. *Mycobacterium* species) leads to the development of T_H1 lymphocytes (Stern *et al* 1996; Romagnani, 1991; Lucey *et al* 1996; Romagnani, 1997; Murray, 1998).

DTH is an important *in vivo* observation of cell-mediated immune response. Antigens, namely, *Mycobacterium tuberculosis, butyricum* and *leprae*, are processed by APCs, e.g., dendritic cells, Langerhans' cells, B lymphocytes, macrophages and monocytes) in human and mouse tissue, cells and cell lines (Figure 1.9). The antigens are enzymatically digested, forming peptides, which are presented on MHCs to the TCR on naive T_H0 cells.

Figure 1.9 Mycobacterium infection, T_H1 response and the development of DTH



(Adapted from Romagnani, 1997)

The cytokine, IL-12 is also released from APCs. This cytokine has multiple roles and appears to have an obligatory function in the polarising of T_H0 cells towards the T_H1 pathway, the cell mediated immune response. It promotes the maturation and activation of T_H1 ($CD4^+$) cells and T_C1 ($CD8^+$), the cytolytic T lymphocyte. IL-12 is also involved in inducing the production of many cytokines including IFNγ in natural killer (NK) cells and T cells. It plays an intricate role in the proliferation of T cells and NK cells. Finally it induces the lytic activity of cytotoxic T lymphocytes ($CD8^+$), NK cells and lymphokine activated killer cells (Stern *et al* 1996; Maruo *et al* 1996; Romagnani, 1997; Gately *et al* 1996). IL-12 is responsible for the development of the cell-mediated immune response while inhibiting the humoral immune response (Stern *et al* 1996). T_H1 cells secrete IL-2, IFNγ, TNFβ and lymphotoxin, which ultimately leads to protection against intracellular parasites, organ specific autoimmune diseases and allograft rejection (Romagnani, 1997). The induction phase follows effector phase or the sensitisation to antigen and the development of the T_H1 cells. IL-2, one of the cytokines, released from T_H1 cells induces further proliferation and maturation of $CD4^-$ and $CD8^-$ T_H1 and T_C1 cells respectively. $CD8^-$ cytotoxic T cells are involved in the killing of intracellular parasites while $CD4^-$ are known for generation of the lymphokines IL-2, IFNγ and TNFβ as described earlier. A second function of IL-2 is the potent induction of IFNγ, which in turn enhances activation of macrophages, amplifying the inflammatory process (Chu *et al* 1992). Cross regulation between T_H1 and T_H2 has been demonstrated and cytokine generated by T_H1 cells can be inhibited by the cytokines of the T_H2 profile. IL-4, IL-6, IL-10 and IL-13 are the cytokines involved in regulation (Romagnani, 1997; Romagnani, 1991). IL-6 has been

found to inhibit DTH in the induction phase (Mihara *et al* 1991). IL-10 inhibits T_H1 cytokine production, vascular leakage and ultimately DTH (Li *et al* 1994).

Lymphokines, namely IFNγ and TNFα have been implicated in further lymphocyte recruitment in DTH (Buchanan & Murphy, 1997). Anti-IFNγ prevented lymphocyte recruitment in inbred rat footpads by 50-90% in a DTH reaction induced by Keyhole limpet hemocyanin (KLH) in Freund's complete adjuvant (FCA) (Issekutz, 1988). A similar experiment supported this finding where anti-IFNγ was found to inhibit 55% of the footpad swelling as induced by subcutaneous (*s.c*) injection of T_H1 clones in Balb/c and CBA/J mice (Fong & Mosmann, 1989). The actions of anti-IFNγ appear to be through inhibition of vascular leakage and recruitment. However, because complete inhibition was not seen, it is conceivable to speculate that other factors are involved. TNFα is thought to also play a significant role in tissue damage in DTH. It has been demonstrated that TNFα induces necrosis when injected in areas with DTH. TNFα is a pro-inflammatory cytokine produced by APCs namely, macrophages/monocytes and Langerhans' cells following antigen exposure (Chu *et al* 1992; Buchanan & Murphy, 1997). TNF-α, together with IL-1 a second pro-inflammatory cytokine also sourced from APCs, are thought to be involved with T cell activation and proliferation.

TNFα, IL-1 and IFNγ are involved in endothelial activation in DTH (Doukas & Pober, 1990). Activation of the endothelium leads to increased expression of adhesion molecules on its surface. As discussed previously increased expression ultimately leads to recruitment and activation of T-lymphocytes, macrophages and polymorphonuclear cells,

to the site of inflammation. Adhesion molecules involved are ICAM-1 for lymphocyte recruitment, endothelial leucocyte adhesion molecule (ELAM)-1 for neutrophil recruitment and VCAM-1 for eosinophil recruitment. TNF and IL-1 both increase endothelial expression of these adhesion molecules although it has been demonstrated that IFNγ acts synergistically with TNF to induce a more than additive increased adhesion molecule expression on endothelial cells. IFNγ has also been shown to increase MHC expression and the actions of IFNγ and TNF are more than additive in increasing MHC expression. While increased expression of adhesion molecules would induce chronic inflammation at the injury site, increased MHC expression may act to amplify the process and increase antigen processing by macrophages or as more accurately described, APCs.

The actions of neutrophils and eosinophils in inflammation have been described earlier (section 1.1.1.2(b)) . Both cells release proteolytic enzymes and oxidants, which are designed to combat infected cells or damaged tissue (Dallegri & Ottonello, 1997; Barnes, 1991; Robinson, 1996). Unfortunately, this process is not completely controlled and therefore the need for drugs to control the deleterious effects of this inflammatory process exists.

Other actions of these inflammatory cytokines are the direct induction of free radicals and oxidants. NO synthase is induced by the combination of the above cytokines, (IFNγ, TNFα and IL1-β). NO induces protein degradation, DNA deamination and lipid peroxidation (Moriguchi *et al* 1992; Wink *et al* 1991; Radi *et al* 1991). As discussed in previous sections it can covalently combine with O_2^- to form $OONO^-$ and ultimately a

$OH^{\cdot}$. This radical can further damage DNA, protein and lipids, which would ultimately, lead to necrotic conditions and the development of fibrosis (Hernandez-Pando & Rook 1994; Liew *et al* 1990).

The diseases arthritis, leprosy and allograft rejection all follow a similar profile to DTH. RA is a chronic disabling inflammatory disease with an increased global mortality rate (Wilson *et al* 1996). It is inflammation of the synovial or articulating joints and is believed to be a result of autoimmune reactivity to a component of the joint. Arthritis ultimately leads to vascularisation and to the formation of inflammatory tissue due to cell proliferation of monocytes, macrophages, endothelium, fibroblasts and T-lymphocytes (Dunn *et al* 1990; Henderson *et al* 1987). Eicosanoids appear to play an important role in the acute response in arthritis where abnormally high levels of LTB_4 have been found in the rheumatoid synovial fluid. Explants of rheumatoid synovial cell lining taken and cultured, produce significantly more PGE_2 than explants taken from normal tissue. These eicosanoids are primarily responsible for hyperalgesia or pain. However, as described earlier these acute mediators are also responsible for oedema, swelling, vascular leakage and chemotaxis of leucocytes leading to chronic inflammation (Henderson *et al* 1987).

As described for DTH, cytokines primarily involved in the development of arthritis and articular destruction are T_H1 type cytokines; IL-2, IFNγ and macrophages sourced cytokines, namely IL-1, and TNFα (Cauli *et al* 1997; Bondeson, 1997; Schultze-Koops *et al* 1995). IL-2 is involved in further T-cell activation and proliferation and cell mediated inflammation including macrophage activation and hence further IL-1 and TNFα

production (Carlson *et al* 1993; Carlson *et al* 1998; Chu *et al* 1992). It is believed that IL-1 is implicated in stimulating bone resorption, inhibiting bone formation and inducing cartilage damage while TNFα appears to induce the release of proteolytic enzymes from synovial cells, which in turn induce tissue damage (Kawai *et al* 1997). IL-1 induces the acute phase response elevating the production of fibronectin (Fn) which in turn leads to a significant increase in collagen binding, chemotaxis, adhesion molecule expression and hence neutrophil and monocyte recruitment (Henderson *et al* 1987; Connolly *et al* 1988). Other signs of the acute phase response are an increase in the plasma C-reactive protein and a decrease in plasma albumin and iron all of which have been demonstrated in both RA patients and rat adjuvant induced arthritis (Connolly *et al* 1988). Further studies have show that TNFα plays an equally significant role to IL-1 in the pathogenesis of arthritis and therefore must also be considered in the management of this disease. It appears that, like IL-1, TNFα can stimulate bone resorption, increase neutrophil adherence to the endothelial cell and induce collagenase and PGE_4 production of the synovial cells.

Leprosy is a disease recognised by hypopigmented skin lesions. There are two types of leprosy, tuberculoid leprosy and lepromatous leprosy. The host defends itself against tuberculoid leprosy through cell mediated mechanisms (T_H1 cells; IFNγ and IL-2) and has strong reactions to intracellular bacteria namely, *Mycobacterium leprae, butyricum* and *tuberculosis* (Roman & Moreno 1997; Lucey *et al* 1996; Stern *et al* 1996). *Mycobacterium leprae* has been shown to induce the production of IL-12 from monocytes. In addition to being the principal differentiator of T_H0 to T_H1 type cells, IL-12 also proliferates NK cells and T-cells and induces the lytic activity of cytotoxic T-

lymphocytes ($CD8^+$), NK cells and lymphokine activated killer cells. In contrast lepromatous leprosy follows the humoral immune response where a T_H2 cells are expressed with IL-4 and IL-10 produced (Stern *et al* 1996; Singh, 1998). It was found that TNFα is produced by macrophages following infection with intracellular bacteria. It is thought to act together with IFNγ in activating bacterial effector functions and it also has been implicated in the formation of granuloma lesions and in the inflammation developed with leprosy. Monoclonal antibodies raised against TNFα prevent granuloma formation in *Mycobacterium bovis* BCG-infected mice (Singh, 1998)

Finally allograft rejection is also mediated by cell mediated immunity where DTH and cytotoxic T lymphocytes play a significant role (Denton *et al* 1999). The graft is recognised as a foreign body and hence action in the form of $CD4^-$ and $CD8^-$ T cell is mounted against this now recognised antigen. Vascular lesions are first noted in acute rejection and cellular infiltration. This is followed by chronic rejection where arterial thickening and interstitial fibrosis develops (Pascual *et al* 1998). The antigen is presented on APCs and together with cognate ligands they activate T-cells and production of the T_H1 type cytokine IL-2 is induced. As described earlier this cytokine is the centre of the T_H1 response and induces further proliferation of $CD4^-$ and $CD8^-$ T_H1 and T_C1 cells and the production of more IL-2, IFNγ and TNFβ. The induction of IFNγ by IL-2 in turn activates macrophages, the production of TNFα and the amplification of the inflammatory response acting against the foreign allograft (Chu *et al* 1992). Immunosupressants, compounds that would depress the cell-mediated immune response have therefore been

efficacy rendering firm conclusions impossible. One *in vivo* study was carried out on LEW rats receiving MHC mismatched bone marrow transplants following treatment with a myeloablative dose of busulfan plus total body irradiation (TBI). These studies showed that post-transplantation treatment with methotrexate (0.12 and 0.25mg/kg) reduced rejection from 67% to 0% (Uharek *et al* 1991). A clinical study looked at the ability of methotrexate to prevent cardiac transplant rejection. Results, however, were inconsistent: methotrexate was found to increase rejection whilst decreasing cardiac allograft vasculopathy (Costanzo *et al* 1997).

Although methotrexate appears to demonstrate some efficacy, many drug related adverse events have been reported (Singer & McCune, 1998). It is thought that mild liver inflammation and fibrosis are related to long-term exposure to this drug. Moreover, a second side effect appears to be that of shortness of breath, cough and fever all associated with pulmonary toxicity.

1.3.1.4 Thalidomide

Thalidomide has recently been clinically tested to act as an immuno-depressant. However studies with derivatives of this drug have resulted in potentially more potent compounds presently named CC 1069 and CC-3052 (Marriott *et al* 1998; Oliver *et al* 1999). The mechanism of action appears to be through selective inhibition of TNFα synthesis by enhancing TNFα mRNA degradation (Marriott *et al* 1998; Mc Hugh *et al* 1995). As discussed previously the cytokine TNFα, released primarily in macrophages, is common to

both humoral and cell mediated inflammatory responses. It is involved in the increased expression of adhesion molecules and the chemotaxis of leucocytes to the site of inflammation. In fact thalidomide has been shown to depress the expression of the adhesion molecule β_2-integrin on the surface of lymphocytes and monocytes (Mc Hugh *et al* 1995). A second mode of action of thalidomide is through stimulation of T_H2 lymphocytes and hence the inhibition of the T_H1 cytokines (Mc Hugh *et al* 1995). The latter observation was noted in an experiment involving phytohaemagglutin (PHA) stimulated peripheral blood mononuclear cell cultures. Stimulated cells predominantly synthesise T_H1 cytokines (IFNγ), however with the addition of thalidomide T_H2 cytokines dominated and T_H1 cytokines were suppressed. Further studies looked at the production of IL-12, the cytokine involved in the activation of T_H1 cells and hence the production of IL-2 and IFNγ (Moller *et al* 1997). IL-12 production was significantly suppressed in human PBMC and primary human monocytes by thalidomide.

Thalidomide has been clinically tested, in twelve patients, to act against RA (Singer & McCune, 1998; Mariott *et al* 1998). It has been shown in previous clinical trials that anti-TNFα is beneficial to RA patients. This small trial showed a 20% improvement in nine patients. Further larger Phase III studies would be beneficial in elucidating the efficacy of this drug or its derivatives. A pre-clinical study looked at arthritis in rat paw (Oliver *et al* 1999). In this study rats were inoculated with *mycobacterium tuberculosis* (H37RA) at the base of the tail (*i.d.*). Fifteen days post inoculation all rats showed significant signs of arthritis in their paws, as measured by ankle diameter, hind limb cytokine levels and radiology. It was demonstrated that a thalidomide derivative (CC1069) reduced TNFα

and IL-2 production in the ankle joints of *mycobacterium tuberculosis* treated rat tail. Radiographical methods were also used to monitor synovial inflammation and CC1069 (50-200 mg/kg) proved to be a potent dose dependent inhibitor of the inflammation. *In vitro* studies supported this, where TNFα and IL-2 were significantly reduced in stimulated LPS and Con(A) splenocytes as compared to control. Thalidomide itself was also tested in these studies however was not as potent as the derivative.

The *in vitro* studies demonstrating thalidomides anti-TNFα activity points this drug and its derivative towards Leprosy and the treatment of this disease (Mariott *et al* 1998; Oliveira *et al* 1999). Polymorphonuclear neutrophils isolated from Lepromatous patients were stimulated with LPS, *Mycobacterium leprae* and lipoarabinomannan and secreted IL-8 and TNFα. Thalidomide inhibited the secretion of TNFα from these stimulated neutrophils. Preliminary studies have shown this drug to be a potent inhibitor of inflammation in patients with lepromatous leprosy. As described above this type of leprosy takes the profile of a T_H2 type inflammatory response (Singh, 1998; Stern *et al* 1996). Although, *in vitro* studies show that thalidomide appears to act preferentially against the cell-mediated immune responses, the fact that TNFα, released from active macrophages and monocytes, plays an imperative role in both diseases makes this study credible. Inhibition of TNFα in lepromatous leprosy would in fact reduce the inflammatory response seen. However as described earlier, thalidomide and its derivatives appear to not only act as a TNFα inhibitor but also appears to favour the T_H1 response where IL-2 and IFNγ are predominant and IL-4 and IL-10 are reduced significantly (Moller *et al* 1997; Mc Hugh *et al* 1995). It would therefore be useful to conduct clinical

studies looking also at tuberculoid leprosy, where the T_H1 profile is active (Roman & Moreno 1997; Lucey *et al* 1996; Stern *et al* 1996).

Thalidomide has also been found to potently act as an immunosuppressant in allograft or organ rejection. *In vitro* studies assessing preliminary potential effects of thalidomide showed that lymphocyte proliferation, as induced by either alloantigen (MLR) mitogens Concanavlin A or superantigen (SEB), was inhibited by thalidomide and one of its derivatives N-hydroxythalidomide (Chuong *et al* 1997). An *in vivo* study involving mongrel dogs that underwent lung transplants showed thalidomide to be a successful replacement to steroids. CyA (20mg/kg twice a day), azathioprine (2.5mg/kg twice a day) and prednisone (2mg/kg once a day) became a more potent combination when the steroid was substituted with thalidomide (50mg/kg twice a day). Another independent study with rats undergoing cardiac transplant supported the above where thalidomide successfully prevented rejection of a solid organ (Ostraat *et al* 1996). It has been shown to prevent bone marrow transplant rejection (Mc Hugh *et al* 1995; Schuler & Ehinger, 1995).

One main issue surrounding the use of this drug is that of its well-known side effects. Thalidomide has been found to exert teratogenic and neurotoxic properties and hence must be used with caution. Studies on the derivatives of thalidomide, for example the water soluble, CC-3052, have shown them to be non-toxic, non-mutagenic and non-teratogenic (Marriott *et al* 1998).

1.4 Aims

The aim of this study was to elucidate the pharmacological role of three novel compounds, 3C8, 6C6 and 7C9. The areas focused on were asthma, inflammation and DTH. To date many drugs have been developed with potential therapeutic value however most of these either are ineffective in combating these diseases or have serious side effects therefore the need for a drug which is both efficacious and safe is growing.

Specific aims of this project were to:

- set up *in vitro* models in order to look at the mast cell stabilising and smooth muscle relaxant activities of the three test compounds.

- set up and employ *in vivo* respiratory and inflammatory asthmatic rat models in order to elucidate the role of 3C8 in these models.

- set up and utilise *in vivo* acute and chronic inflammatory models to elucidate the effect of the three test compounds these models. Acute models of inflammation used were the AA mouse ear oedema, TPA mouse ear oedema and carrageenan induced rat paw oedema, while the chronic model of inflammation used was poly-vinyl sponge implant induced oedema.

- consequently set up and use an mBSA DTH model in order to elucidate the role of the three test compounds in this model.

Chapter 2

Methods and Materials

Contents **Page No.**

Contents **Page No.**

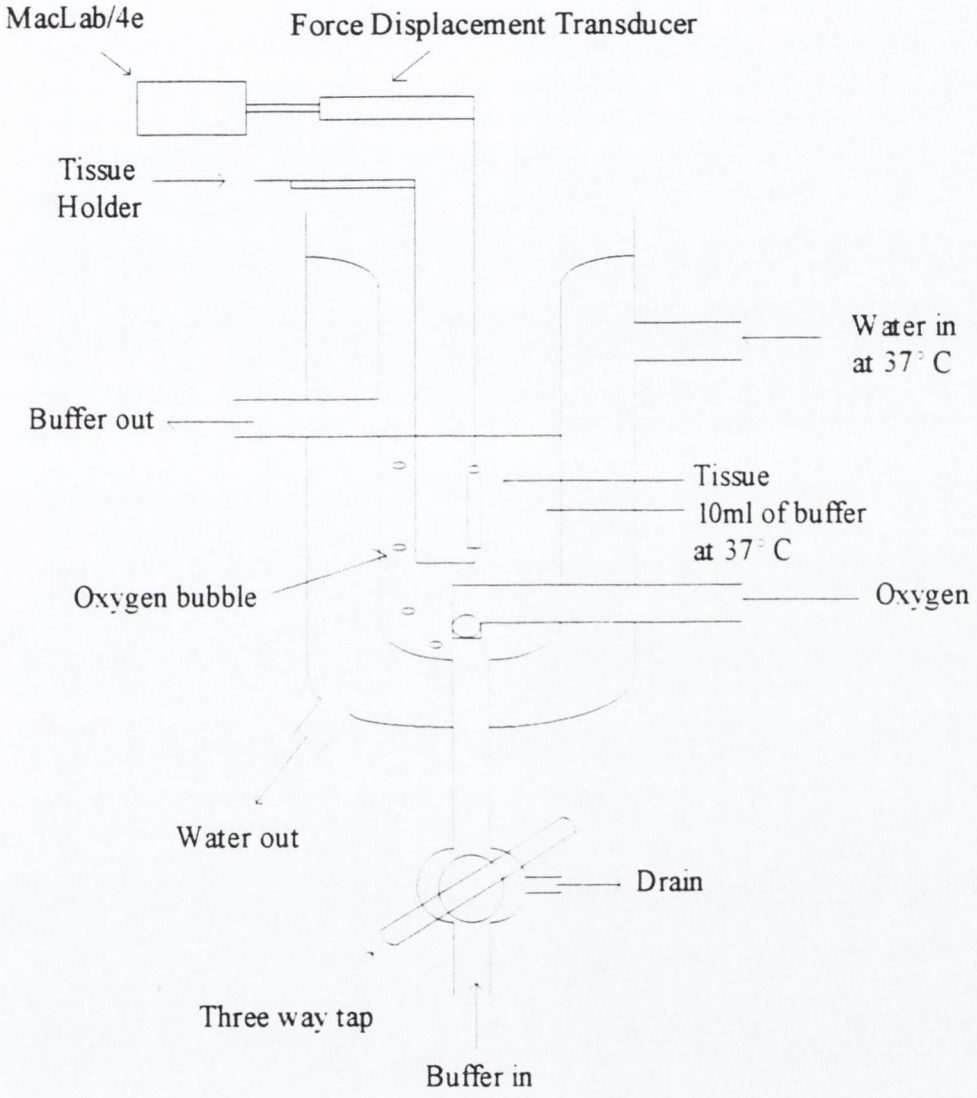
2.1 Methods

2.1.1 Contraction of Guinea Pig Ileum Smooth Muscle as Induced by $CaCl_2$

The guinea-pig ileum smooth muscle $CaCl_2$ induced contraction protocol was set up according to Sheridan *et al* 1990. Male guinea pigs (~350g) were killed in an atmosphere of saturated CO_2. The abdomen was opened by a mid-line incision and the small intestine was removed. The tissue was stored at 4°C in Krebs buffer (NaCl 118mM; KCl 4.7mM; $CaCl_2$ 2.5mM; $MgCl_2$ 1.15mM; NaH_2PO_4 1.17mM; $NaHCO_3$ 25mM; Glucose 14.4mM) either until used or up to a maximum of 48h.

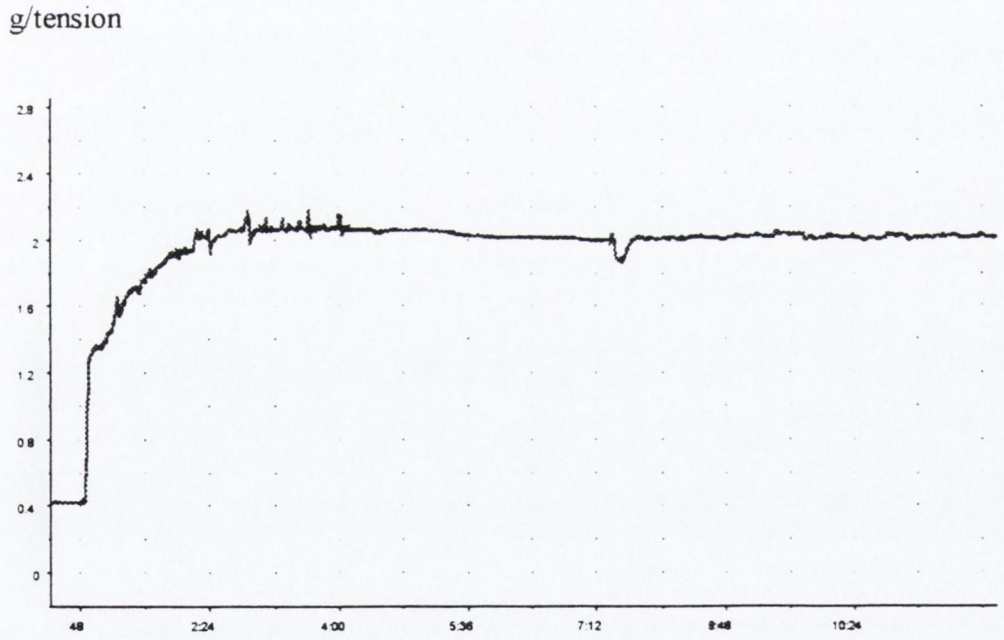
Threads were attached to either ends of a segment of ileum tissue (1-1.5cm). These threads were used to attach one end of the tissue to a tissue holder and the other end of the tissue to a force displacement transducer (Grass, FT.03; Linton instrumentation, UK). Tissues were suspended in a 10ml organ bath (Linton instrumentation, UK) containing a depolarising, high potassium, calcium free Krebs, buffer (NaCl 80mM; KCl 45mM; $MgCl_2$ 1.15mM; NaH_2PO_4 1.17mM; NaH_2CO_3 25mM; Glucose 11.1mM). The buffer was maintained at 37°C by the jacketed organ bath and gassed with 95% O_2 and 5% CO_2 (Figure 2.1). Tissues were subjected to a resting tension of approximately 1g. Isometric contractions were recorded using a MacLab/4e system in conjunction with the Chart 3.3.1 software package.

Figure 2.1:- The Jacketed organ bath containing guinea-pig ileum smooth muscle



Contractions were initiated by the addition of 25µl of 1M $CaCl_2$ (a final concentration of 2.5mM). The contractions stabilized with time (10-15 min) and could be maintained for up to 45 minutes. Figure 2.2 shows one trace of smooth muscle contraction by calcium. Up to 48 seconds the smooth muscle guinea-pig ileum is in a relaxant state with a resting tension of 0.4g. On addition of $CaCl_2$ (48 sec), the tension of the smooth muscle increases to over 2g. The contraction increased steadily for the first 2.5 minutes and stabilised over the next 7.5 minutes. Following stabilisation of contraction the smooth muscle is ready for experimentation using drugs or test compounds.

Figure 2.2:- A trace of smooth muscle contraction of Guinea-Pig ileum



The trace shows the change in tension of K^+ depolarised guinea pig ileum smooth muscle on exposure to $CaCl_2$. The ileum is suspended in High potassium no calcium buffer ($CaCl_2$ (2.5mM) added at 48 sec) and was gassed with 95% O_2 : 5% CO_2 at 37°C.

Stock solutions of test compounds and nifedipine, the positive control, were prepared at 10^{-3}M in 50% (v/v) dimethyl sulphoxide (DMSO) and distilled water (dH_20). These were diluted to give concentrations of 10^{-4}M in 5% (v/v) DMSO, 10^{-5}M in 0.5% (v/v) DMSO, 10^{-6}M in 0.05% (v/v) DMSO, 10^{-7}M in 0.005% (v/v) DMSO and 10^{-8}M in 0.0005% (v/v) DMSO. Solvent 'blank' solutions were prepared containing 50%, 5%, 0.5%, 0.05%, 0.005% and 0.0005% (v/v) DMSO. The test compound solution was added to the organ bath once a stable contraction of the tissue had been achieved. A cumulative dose-response assay was carried out for the test compound, at half log dose intervals.

The concentration ranges chosen depended on the potency of the test compound, where lower concentrations could be used for the more active compounds. A range of 10^{-10}M to 10^{-7}M was chosen for the potent positive control, nifedipine. A range of $3x10^{-9}$M to 10^{-5}M was used for 3C8 and $3x10^{-8}$M to 10^{-5}M for 6C6. Only two doses were chosen for 7C9, $3x10^{-6}$M and 10^{-5}M, because of its weak effect on the smooth muscle contraction. Because of the poor solubility of the test compounds a higher concentration than 10^{-5}M could not be achieved without increasing the DMSO concentrations to unacceptable levels. Concentrations of 10^{-5}M of test compounds required 0.5% DMSO, final concentration. This final concentration of DMSO reduced the contraction by approximately 20%. It is clear therefore that higher concentrations of DMSO would reduce the sensitivity of the assay and prevent the detection of moderately efficacious test compounds

Following the final addition of test compound the organ bath was washed out and the tissue was allowed to relax. A contraction was induced and the DMSO cumulative dose-

response assay was carried with DMSO concentrations in distilled water (dH_2O) ranging from 0.00015%-0.5% DMSO (v/v), where the percentage DMSO used corresponded to the final percentage of DMSO needed for the test compound.

Each drug was tested, in duplicate, on three different animals (n = 3). The results were expressed as percentage inhibition of the $CaCl_2$ induced contraction, for each tissue, at each concentration of drug in DMSO. The effect of DMSO, for each tissue at each concentration, was subtracted from the effect of the drug (in DMSO) to give the effect of the drug alone. A log dose vs. response curve was plotted for each drug using the mean and the standard error of the mean (SEM) for the accumulated results.

2.1.2 Histamine Release from Mast Cells as Induced by Compound 48/80

The harvesting of mast cells and the release of histamine from these mast cells was carried out according to a modified protocol described by both Loeffler *et al* 1971 and Amellal *et al* 1984. No mast cell purifying procedures by density gradient centrifugation was carried out. This was in accordance with Loeffler *et al* 1971 where it was demonstrated that consistent and uniform responses to compound 48/80 and other releasing agents were achieved without using purifying cell procedure. Modifications to the protocol outlining the harvesting of mast cells and release of mast cells are as follows. In this experiment animals were firstly sacrificed under 100% CO_2, while the procedures outlined by both Loeffler *et al* 1971 and Amellal *et al* 1984 were different. Animals were firstly anaesthesised with ether or stunned prior to exsanguination. This procedure was carried

out before the injection and massage of the krebs buffer in order to eliminate contamination of sample by the blood. It was found however that this procedure including contamination was avoided by sacrificing the animals with CO_2 and eliminating the exsanguination procedure. Using this procedure the peritoneal cavity was devoid of any contamination with blood and the mast cell preparation was clear.

2.1.2.1 The Harvest of Peritoneal Mast Cells

Female Wistar rats (250-350g) were killed in an atmosphere of saturated CO_2. A volume of 10ml of pre-warmed (37°C) buffered salt solution (BSS; NaCl 137mM; KCl 2.7mM; $MgCl_2$ 1.0mM; $CaCl_2$ 0.5mM; NaH_2PO_4 0.4mM; Glucose 5.6mM; HEPES 10mM; pH 7.2-7.4) was injected *i.p.* and the abdomen was massaged for 3 minutes. The BSS, along with suspended mast cells and other cells, were aspirated, using a 10ml syringe, following a mid-line incision. The aspirate was centrifuged for 6 minutes at 1000rpm, using a Sigma 204 centrifuge, and the supernatant removed. The cells were re-suspended in BSS, at 4°C, and centrifuged as described before. The cells were washed in this manner a total of three times. Following the final wash, the pelleted cells were stored at 4°C, for use within 2 hours. The total typical number of cells isolated were in the region of 10^8 – 10^9 cells/isolation.

2.1.2.2 Mast Cell Incubation

The BSS was dispensed into test tubes and heated to 37°C; each test tube contained 4.5ml of buffer. There was a minimum of six incubations, basal, maximum, total, (DSCG) pre-treated, DMSO pre-treated, and test compound pre-treated. The basal would show the normal release of histamine from mast cells, the maximum would show the maximum release of histamine from mast cells on addition of compound 48/80 and the total would show the total histamine content of the mast cells. The basal, maximum and the total were each pre-treated with 0.5% (v/v) dH_2O (25µl of dH_2O). The DMSO, DSCG and test compound were supplemented with 0.5% (v/v) DMSO (25µl DMSO), 0.5% (v/v) dH_2O / $2x10^{-5}$M DSCG (25µl of $4x10^{-3}$M DSCG in dH_2O) and $2x10^{-5}$M test compound / 0.5% (v/v) DMSO (25µl of $4x10^{-3}$M test compound in DMSO) respectively. This was carried out 10 minutes before the addition of compound 48/80 in order to investigate their effect on the maximum release.

The cells were re-suspended in 7ml BSS. From this, 0.5ml aliquots were transferred to each of the incubation tubes. After 10 minutes at 37°C, with gentle agitation, compound 48/80 (50µl of 0.2mg/ml) was added (final concentration of 2µg/ml) in order to stimulate histamine release in all tubes except the basal where 50µl of dH_2O was added. Cell stimulation was stopped after 2 minutes by the addition of 0.5ml BSS (4°C) and the incubation tubes were transferred immediately to an ice bath. The cell suspensions were centrifuged, using a Sigma 204 centrifuge, for 6min at 1000rpm. The 'total' tube was

placed in a water bath at 100°C for 2 minutes prior to centrifugation. Heating the 'total' tube to this temperature caused the cells to lyse and all the histamine was released. The supernatants were retained for histamine assay.

2.1.2.3 Histamine Assay

The histamine assay was carried out according to the protocol described by Shore *et al* 1959. A volume of 0.4ml of 1M NaOH and 0.1ml *O*-phthaldialdehyde (oPT) (1% (w/v) in methanol) was added to 2ml of supernatant from each tube. This was incubated at room temperature for 4 minutes. The reaction was stopped by the addition of 0.2ml of 3M HCl. The presence of the fluorescent product of the reaction was measured using a Shimadzu RF-1501 spectrofluorophotometer set at λex = 360nm and λem = 450nm. The supernatant from each incubation tube was assayed in duplicate.

Each drug was tested on at least five animals (n = 5). The basal, maximum, DMSO pre-treated, DSCG pre-treated and test compound pre-treated were all expressed as percentage total histamine release as measured by the fluorescent intensity (FI). The total quantity of histamine from compound 48/80 induced mast cells was in the region $2\mu g/1x10^6$ cells. The total quantity of histamine release from mast cells was in the region of $3\mu g/1x10^6$ cells. The standard curve was set up to measure between 50 and 1000ng/ml. In some cases this range was too low for the maximum and total histamine cells counts. Samples were therefore diluted 1 in 3 or 1 in 4 in order to fit within the range set out by the standard curve.

2.1.3 *In Vivo* Respiratory Asthma Model

Experiments were performed in male Wistar rats aged 10-12 weeks (250-350g). Rats were sensitised by the injection (1ml *s.c.*) of OVA (1mg/ml) / aluminium hydroxide (AlOH) (200mg/ml) and Freund's complete adjuvant containing *Mycobacterium tuberculosis* (FCA(T)) (1ml *i.p.*). Sensitisations were carried out according to a modified protocol described by Hessel *et al* 1998 and Renzetti *et al* 1996. The protocol was modified as follows. While Hessel *et al* utilised a mouse model this assay was set up in order to develop a rat model of asthma. The concentration antigen OVA used was similar in both studies where Hessel *et al* administered 70µg/30g mouse mouse this study administered 1mg/300g rat. In this study in order to maximise the effect AlOH and FCA(T) was also administered. These adjuvants were utilised in order to increase the asthmatic response. Renzetti *et al* 1996 described an asthmatic model whereby BN rats were sensitised to OVA using AlOH as an adjuvant. The quantity of both OVA and AlOH differed to that of this study where animals were administered 10µg OVA and 1mg AlOH in 0.5ml of saline.

Three weeks following sensitisation, each animal was sedated with sagatal (40mg/kg *i.p.*). Sedation was maintained with supplemental injections (5mg/kg *i.p.*) as required. The nose was occluded by surgical tape to prevent deposition of aerosols. The animal was placed in a respiratory chamber and allowed to settle. The chamber was connected to a differential volume transducer, which in turn was connected to a MacLab/4e system in conjunction with the Chart 3.3.1 software package. The volume transducer measured changes

pressure (mmHg) within the respirator chamber, which contained the animal (figure 2.3). A decrease in pressure was seen when the animal breathed in while an increase in pressure was seen when the animal breathed out. The maclab system plotted the pressure/mmHg over time. This enabled the measurement of respiratory parameters, namely breaths per minute (BPM) and the tidal volume (TV) or volume of each breath. The tidal volume, volume of each breath taken, was measured as a function of the change in pressure ($f\Delta$mmHg; ΔP) within the chamber (figure 2.3), which contained the rat. On set-up of this experimental apparatus a calibration demonstrating the direct comparison between known volumes of air injected into the chamber and the change of pressure as seen with the transducer was carried out (data not available). This procedure was deemed unnecessary in the experiments that followed, as the volume of air injected into the chamber was directly proportional to with the change in pressure detected by the transducer. A linear calibration was demonstrated. Throughout this work the TV will now be represented as the change in pressure within the chamber (ΔP), which directly reflects the TV. The chamber was also connected to a nebuliser, to enable drug and OVA administration (figure 2.3). The nebuliser was in turn attached to an air pump, which pumped air at a rate of 0.75L/minute. This pump aerosolised the drug/OVA contained in the nebuliser. Linton Instrumentation, UK supplied the respiratory chamber and volumetric transducer (RSP1015 respiratory measurement chamber for restrained rats and Model PT5 volumetric low pressure transducer respectively). Pari, UK supplied the nebuliser.

Animals were treated with an aerosol of ethanol (ETOH) (50% v/v as a negative control), DSCG (5 mg/ml in ETOH 50% v/v as a positive control) or 3C8 (5mg/ml in ETOH 50%

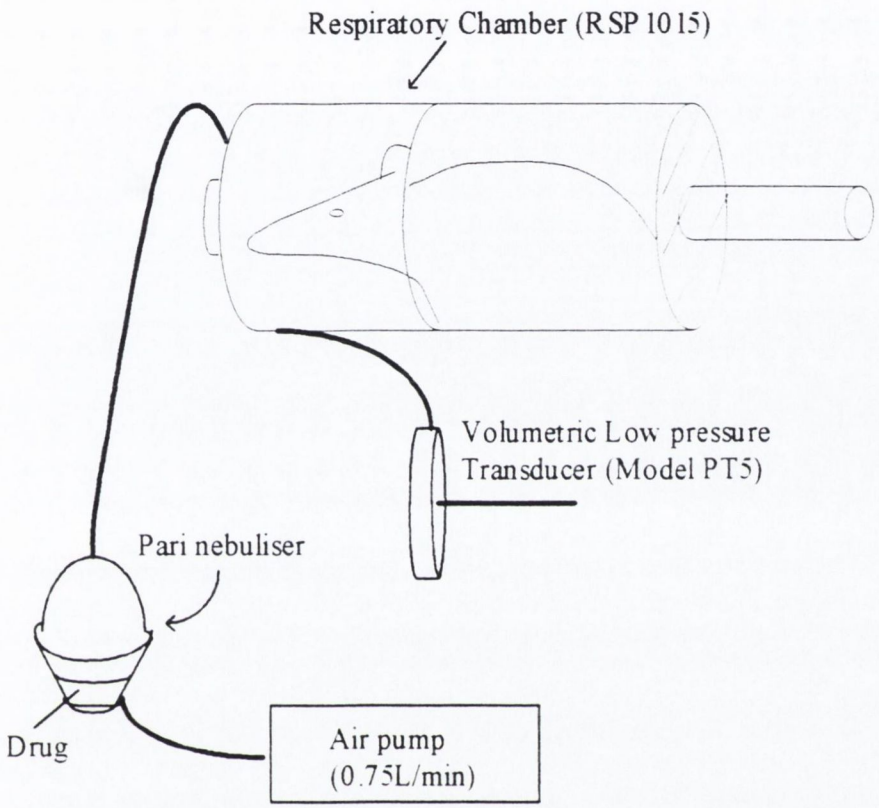
v/v) for 2 minutes. The animals were subsequently challenged with an aerosol of saline (negative control) or OVA (5% w/v) 30 minutes later, for 2 minutes. The pre-treatments and the challenging procedures of the four groups are shown in table 2.1. Changes in the respiratory parameters were monitored 30 minutes before and up to 3h after OVA challenge.

Table 2.1:- Pre-treatment and challenging procedure of all animal groups

Group	**Pre-treatment (-30mins)** (2 mins)	**Challenge (0mins)** (2 mins)
Group I (Negative control)	50% (v/v) ETOH	Saline
Group II (Positive control)	50% (v/v) ETOH	5% (w/v) OVA
Group III	DSCG (5 mg/ml in ETOH 50% v/v)	5% (w/v) OVA
Group IV	3C8 (5 mg/ml in ETOH 50% v/v)	5% (w/v) OVA

Each group was challenged and pre-treated with their corresponding vehicle/drug or test compound, as outline in table 2.1, three times over the following three weeks (once weekly). This was carried out in order to induce bronchial hyper-reactivity in group II and to investigate the effect of pre-treatment with either drug or test compound on the development of hyper-reactive airways (Hessel *et al* 1998).

Figure 2.3:- Illustration of the apparatus for the respiratory assessment of rats.



Four weeks after the initial treatment, all groups of animals were exposed to an aerosol of methacholine (acetyl-β-methylcholine chloride) for two minutes at a dose (8mg/ml), which would stimulate a significant response in hyper-reactive airways only (Wang *et al* 1986). Animals were not pre-treated with either vehicle, drug or test compound. Changes in airway responses were monitored for 1h after methacholine exposure.

2.1.4 Post Mortem of Animal Groups from the Bronchial Asthma Model

All animals in groups I - IV were examined for side effects of drug treatment. Animals were put down by CO_2 and external and internal examinations were carried out. Internal examinations of both the thoracic and the abdominal cavities were performed.

2.1.5 *In Vivo* Inflammatory Models

2.1.5.1 Acute Inflammation

2.1.5.1(a) Arachidonic Acid Induced Mouse Ear Oedema

The AA-induced mouse ear oedema inflammatory model was set up according to a modified protocol described by Lloret & Moreno, 1995. Modifications to this protocol include the mouse strain, while Lloreto & Moreno, 1995 used male Swiss Webster mice this study used male LACA mice. Other modifications include the quantity of AA applied to the ear, where 4mg rather than 2mg were required in order to induce a significant increase in oedema. Also, while Lloret & Moreno, 1995 utilised a different technique to measure ear oedema, namely leukotrienes and prostaglandin influx, this study utilised the use of a micrometer screw gauge to measure the increase in ear thickness. A final variation to the protocol was the length of time for dexamethasone application, while Loreto & Moreno, 1995 applied dexamethasone (5µg/ear) 2h before AA application, in this study dexamethasone was applied (300µg/ear) 1h prior to the application of AA. Although, a

greater increase would be expected if dexamethasone was applied 2h prior to AA, a significant although smaller increase could be achieved with a 1h pretreatment period at 300µg/ear and in such a way the pretreatment timelines and concentrations would be kept in line with the other positive control, indomethacin, and the test compounds (Inoue *et al* 1988a)

In this experiment in Mouse ear oedema was induced in male LACA mice (25-35g). The animals were sedated with Sagatal, 60mg/kg *i.p* initially and subsequently with 20mg/kg when necessary. One ear was treated by the topical application of solvent alone (10µl of acetone; positive control); dexamethasone (300µg/ear; 20µl at 15mg/ml in acetone); indomethacin (300µg/ear; 10µl at 30mg/ml in acetone) or test drug (300µg/ear; 10µl at 30mg/ml in acetone). One hour later, oedema was induced on the same ear by the topical application of AA (4mg/ear; 10µl at 0.4g/ml in acetone). The thickness of the ear was measured (mm), both before and 60 minutes after the induction of oedema, using a micrometer screw gauge. Ear oedema was calculated by comparing the ear thickness before and after induction of oedema and expressed as percentage normal.

Percentage Normal (%) =

$$\frac{[\text{Ear thickness after oedema induction}]-[\text{Ear thickness before oedema induction}]}{[\text{Ear thickness before oedema induction}]} \times 100\%$$

2.1.5.1(b) TPA-Induced Mouse Ear Oedema

The 12-*O*-tetra-decanoylphorbol 13 acetate (TPA) mouse ear oedema inflammatory model was set up according to a modified protocol described by Lloret & Moreno 1995. Modifications to this protocol were the strain of mice, the concentration of TPA, 1.5μg/ear versus 10μg/ear and the methods of oedema measurement. A significant increase in oedema was achieved in this study with a lower concentration of TPA, 1.5μg/ear, versus 10μg/ear in the study outlined by Lloret & Moreno, 1995. In contrast a higher concentration of 10μg/ear of dexamethasone was used to suppress the increase in ear thickness where Lloret & Moreno only required a concentration of 5μg/ear to reduce swelling. The final modification to this protocol was the method of oedema measurement. In this study 5mm ear punch was taken and weighted, this was compared to the untreated ear and a percentage changed was calculated. In contrast Lloret & Moreno, 1995 measured the influx of inflammatory mediators, leukotrienes and prostaglandins to the point of TPA application.

Animals used were male laca mice 30-40g in weight. The right ear of each animal was pre-treated with 20μl (10μl to both the inner and outer aspects of the ear) of either vehicle (acetone; positive control), dexamethasone (10μg/ear; 20μl at 500μg/ml in acetone) or test compound (300μg/ear; 20μl at 15mg/ml in acetone). Vehicle and test compounds were applied 1h before the induction of oedema while dexamethasone was applied 2h before the induction of oedema. Oedema was induced by the topical application of TPA (1.5μg/ear; 20μl at 75μg/ml in acetone; 10μl to both the inner and outer aspects of the ear). Six hours

after the application of TPA animals are sacrificed by cervical dislocation. 5mm diameter sections of the right and left ear were taken and weighed immediately. Oedema was calculated from the differences between the left and right ear and expressed as percentage normal.

Percentage normal (%) =

$$\frac{[\text{Weight of right ear section}] - [\text{Weight of left ear section}]}{[\text{Weight of left ear section}]} \times 100\%$$

2.1.5.1(c) Rat paw oedema

The rat paw oedema protocol was set up according to the modified protocol of Melarange *et al* 1994(a). In this study female in contrast to male Wistar rats were utilised. While the method of oedema induction was as described for Melarange *et al*, the drug dose regimen varied whereby Melarange *et al* dosed animals for 28 days up to oedema induction. In this study doses and timelines for those doses were as described in the previous acute inflammatory models, AA and TPA induced mouse ear oedema. This study also varied with regards to the apparatus employed for paw volume measurement. While Melarange *et al* had the use of the plethysmograph, paw volume was measured in this study using the method of displacement of water in a finely graduated cylinder.

Female Wistar rats (200-250g) were dosed orally with 1% Carboxy methylcellulose (CMC) (Positive control); 30mg/kg dexamethasone in 1% CMC (drug control 1); 30mg/kg; indomethacin in 1% CMC (drug control 2); 3C8, 30mg/kg in 1% CMC (test

compound 1) or 7C9, 30mg/kg in 1% CMC (test compound 2). Oral administration of positive control, drug controls and test compounds was carried out 1h before the induction of rat paw oedema. Rat paw oedema was induced by injection of 1% w/v carrageenan (100µl) below the plantar aponeurosis of the right hind paw. Paw volume was measured both before and 180 minutes after oedema induction by displacement of water in a finely graduated cylinder. Paw oedema was calculated by comparing the paw volume before and after oedema induction and expressed as percentage change.

PAW OEDEMA $_{\text{(percentage normal)}}$ =

$$\frac{\text{Volume Right Injected Hind Paw} - \text{Volume Left Uninjected Hind Paw}}{\text{Volume Left Uninjected hind Paw}} \times 100\%$$

2.1.5.2 Chronic Inflammation

2.1.5.2(a) The Induction or Chronic Inflammation with a Poly-vinyl Sponge Implant

The polyvinyl sponge implant chronic inflammatory model was set up according to a modified protocol of Boyle and Mangan, 1982. The model employed in this study is similar to that of Boyle and Mangan (1982) however with a difference in the pre-treatment of the sponge. Boyle and Mangan used either a saline-soaked or a carrageenan-impregnated saline-soaked sponge implant. Secondly, in this study we were interested in the long term-effect of the sponge implant whereby a chronic inflammatory response would develope. Because of this the length of time the sponge was implanted was 7 days, which is in

contrast to Boyle and Mangan (1982) whereby the sponge was removed within 24h and analysed for acute inflammatory mediators, leukocytes.

Four groups of female Wistar rats (200-250g) were set up. Group I was the positive control, group II the dexamethasone treated control, group III the 3C8 treated group and group IV the 7C9 treated group. All groups were treated on day 1, before the surgical procedure, and daily thereafter through to day 6 of the experiment. They were given, vehicle (Group I; 50% ETOH & 50% H_20; *i.p.*), dexamethasone (Group II; 1mg/kg in 50% ETOH & 50% H_2O; *i.p.*), indomethacin (Group III; 1mg/kg in 50% ETOH & 50% H_2O; *i.p.*) or test compound 3C8 (Group IV; 1mg/kg in 50% ETOH & 50% H_2O).

On day 1 all animals were temporarily anaesthetised with halothane. Following successful anaesthesia they were surgically implanted with a pre-weighed (~40 mg) polyvinyl sponge with dimensions of approximately $1.5cm^3$. The sponge was implanted subcutaneously in the lower back of the rat. The wound was closed using surgical staples. Seven days later the animals were sacrificed with saturated CO_2 and the sponge, together with the newly formed inflammatory capsule, was removed immediately. The sponges were placed carefully on a petri dish and left to dry for 24h at 45°C. After the 24h period the sponges were weighed and the change in weight was calculated and taken as indication of chronic inflammation.

2.1.6 *In Vivo* Inflammatory Asthma Model - BAL

A number of asthmatic models were set up to test our compounds. The models were designed according to the protocol described by Renzetti *et al* 1996 with some variations in the sensitisation procedures and timelines employed. They varied specifically in two ways. Firstly, they differed in the manner they were sensitised to OVA. Animals were sensitised to OVA using the adjuvants AlOH (T_H1 inducer) or FCA(T) (T_H2 inducer) or the combination of the two. Secondly, they differed by the time interval between the OVA sensitisation and final OVA challenge. A three-week time interval before challenge to OVA was referred to as the acute model and an eight-week time interval was referred to as the chronic model. The respiratory chamber and nebuliser utilised were used for the challenging procedure and were as described in section 2.1.3. Inflammatory asthma was measured 24h following OVA challenge, the animals were sacrificed and BAL was carried out. The differential cell count, focusing mainly on leucocytes, was carried out and an increase in leucocyte number was used as a marker for increased asthmatic inflammation.

2.1.6.1 Acute Models of Inflammatory Asthma

Male BN rats were sensitised to OVA on days 1, 2 and 3 using adjuvants, AlOH and FCA(T) in three different combinations, the mixtures of which are described below in sections 2.1.6.1(a), 2.1.6.1(b) and 2.1.6.1(c) for the three acute models. On day 21, rats from Groups I - IV were pre-treated with either vehicle (1% CMC), dexamethasone or

3C8 all administered orally (*p.o.*) 1h prior to a 30 minutes challenge with either vehicle (dH_2O) or 5% OVA by nebuliser. BAL was carried out 24h following OVA challenge.

2.1.6.1(a) Sensitisation of Animal Groups to OVA using AlOH as an Adjuvant (acute model)

The sensitisation mixture used to sensitise animals to OVA in groups I - IV are described in table 2.2, where AlOH was used as an adjuvant. The vehicle/drug pre-treatment and OVA challenge are shown in table 2.3. BAL was carried out 24h later following OVA challenge.

Table 2.2:- Sensitisation of all groups to OVA using AlOH as an adjuvant

Sensitisation mixture / administered *i.p.* (1ml)		
OVA	AlOH	FCA(T)
1mg/ml	100mg/ml	----

Table 2.3:- Pre-treatment and challenging procedure of animals sensitised to OVA using AlOH as an adjuvant

		Pre-treatment (*p.o.*) 1h prior to challenging	Challenging 30 min by nebuliser
Group I	Negative control	Vehicle (1% CMC)	dH_2O
Group II	Positive control	Vehicle (1% CMC)	5% OVA
Group III	Dexamethasone	1mg/kg (in 1% CMC)	5% OVA
Group IV	3C8	10mg/kg (in 1% CMC)	5% OVA

2.1.6.1(b) Sensitisation of Animal Groups to OVA using FCA(T) as an Adjuvant (acute model)

All groups of animals (groups I-IV) were sensitised to OVA using FCA(T) as an adjuvant, table 2.4. The vehicle/drug pre-treatment and the challenging procedure for this second model are described in table 2.5. BAL was carried out 24h later following OVA challenge.

Table 2.4:- Sensitisation of all groups to OVA using FCA(T) as an adjuvant

Sensitisation mixture / administered *i.p.* (1ml)		
OVA	AlOH	FCA(T)
1mg/ml	----	66% (v/v); 660µl/ml

Table 2.5:- Pre-treatment and challenging procedure of animals sensitised to OVA using FCA(T) as an adjuvant

		Pre-treatment (*p.o.*) 1h prior to challenging	Challenging 30 min by nebuliser
Group I	Negative control	Vehicle (1% CMC)	dH_2O
Group II	Positive control	Vehicle (1% CMC)	5% OVA
Group III	Dexamethasone	10mg/kg (in 1% CMC)	5% OVA
Group IV	3C8	10mg/kg (in 1% CMC)	5% OVA

2.1.6.1(c) Sensitisation of Animal Groups to OVA using AlOH and FCA(T) as Adjuvants (acute model)

Animals groups I - IV are sensitised to OVA using using both AlOH and FCA(T) as adjuvants, table 2.6. The vehicle/drug pre-treatment and the challenging procedure are shown in table 2.7. BAL was carried out 24h later following OVA challenge.

Table 2.6:- Sensitisation of all groups to OVA using AlOH and FCA(T) as adjuvants

Sensitisation mixture / administered *i.p.* (1ml)		
OVA	AlOH	FCA(T)
1mg/ml	100mg/ml	66% (v/v); 660µl/ml

Table 2.7:- Pre-treatment and challenging procedure of animals sensitised to OVA using AlOH and FCA(T) as adjuvants

		Pre-treatment (*p.o.*) 1h prior to challenging	Challenging 30 min by nebuliser
Group I	Negative control	Vehicle (1% CMC)	dH_2O
Group II	Positive control	Vehicle (1% CMC)	5% OVA
Group III	Dexamethasone	10mg/kg (in 1% CMC)	5% OVA
Group IV	3C8	10mg/kg (in 1% CMC)	5% OVA

2.1.6.2 Chronic Models of Inflammatory Asthma

All rats in the groups I - IV were sensitised to OVA using either a combination of the two adjuvants, AlOH and FCA(T) or AlOH alone. On week 4, rats from groups I - IV were treated with vehicle, drug or test compound. This was carried out 30 minutes prior to OVA challenge. Eight weeks later, the animal groups were pre-treated again with either vehicle, drug or test compound 30 minutes prior to OVA challenge. Bal was carried out 24h following OVA challenge and the increase in leucocyte number in the BAL cell count was taken as a reflection of inflammatory asthma. These protocols are described in more detail in the following sections.

2.1.6.2(a) Sensitisation of Animal Groups to OVA using AlOH as an Adjuvant (chronic model)

Animals in all groups were sensitised to OVA using only AlOH as an adjuvant (table 2.8). Four weeks following sensitisation, they were challenged to OVA. This was carried out after pre-treatment with vehicle, budesonide or 3C8 (table 2.9). Finally, eight weeks following sensitisation, all animal groups were pre-treated with their corresponding drug/vehicle prior to challenging with OVA (table 2.10). BAL was carried out 24h later.

Table 2.8:- Sensitisation of all groups to OVA using AlOH as an adjuvant

Sensitisation mixture /administered *i.p.* (1ml)		
OVA	AlOH	FCA(T)
1mg/ml	100mg/ml	----

Table 2.9:- The four week pre-treatment of animals with vehicle, budesonide or 3C8, 30mins prior to challenging with OVA

		Pre-treatment (-30min) 1 min by nebuliser	Challenging (0min) 1 min by nebuliser
Group I	Negative control	Vehicle (66% ETOH)	dH_2O
Group II	Positive control	Vehicle (66% ETOH)	5% OVA
Group III	Budesonide	5mg/ml (in 66% ETOH)	5% OVA
Group IV	3C8	5mg/ml (in 66% ETOH)	5% OVA

Table 2.10:- The eight week pre-treatment of animals with vehicle, budesonide or 3C8, 30min prior to challenging with OVA

		Pre-treatment (-30min) (*i.p.*)	Challenging (0min) 45 min by nebuliser
Group I	Negative control	Vehicle (66% ETOH)	dH_2O
Group II	Positive control	Vehicle (66% ETOH)	1% OVA
Group III	Budesonide	10mg/kg (in 66% ETOH)	1% OVA
Group IV	3C8	10mg/kg (in 66% ETOH)	1% OVA

2.1.6.2(b) Sensitisation of Animal Groups to OVA using AlOH and FCA(T) as Adjuvants (chronic model)

Animals in groups I - IV were sensitised to OVA as shown in table 2.11. Four weeks following sensitisation groups I - IV were challenged with OVA. This was carried out following pre-treatment with their corresponding drug or vehicle (table 2.12). Eight weeks after sensitisation, all rats from groups I - IV were pre-treated with their corresponding drug or vehicle prior to a second challenge to OVA (table 2.13). BAL was carried out 24h later.

Table 2.11:- Sensitisation of all groups to OVA using AlOH and FCA(T) as adjuvants

Sensitisation mixture / administered *i.p.* (1ml)		
OVA	AlOH	FCA(T)
1mg/ml	100mg/ml	66% (v/v); 660µl/ml

Table 2.12:- The four week pre-treatment of animals with vehicle, dexamethasone or 3C8, 30mins prior to challenging with OVA

		Pre-treatment (-30min) 1 min by nebuliser	Challenging (0min) 1 min by nebuliser
Group I	Negative control	Vehicle (66% ETOH)	dH_2O
Group II	Positive control	Vehicle (66% ETOH)	5% OVA
Group III	Dexamethasone	5mg/ml (in 66% ETOH)	5% OVA
Group IV	3C8	5mg/ml (in 66% ETOH)	5% OVA

Table 2.13:- The eight week pre-treatment of animals with vehicle, dexamethasone or 3C8, 30min prior to challenging with OVA

		Pre-treatment (-30min) (*i.p.*)	Challenging (0min) 45 min by nebuliser
Group I	Negative control	Vehicle (66% ETOH)	dH_2O
Group II	Positive control	Vehicle (66% ETOH)	1% OVA
Group III	Dexamethasone	10mg/kg (in 66% ETOH)	1% OVA
Group IV	3C8	10mg/kg (in 66% ETOH)	1% OVA

2.1.6.3 Measurement of Eosinophilia by Bronchoalveolar Lavage

Rats in all groups were killed 24h after their final challenge to OVA and a BAL was carried out. The rat was firstly anaesthetised with halothane, opened by mid-line incision and then bled by cutting the *vena cava*. This procedure would kill the animal and eliminate blood from the preparation. Following this the diaphragm was cut in order to deflate the lungs. The trachea was cannulated and 4 x 1ml (per 100g rat) of buffer (BSS without Ca^{++} and Mg^{++}; NaCl 137mM, KCl 2.7mM, $NaH_2PO_4.2H_2O$ 0.4mM, $C_6H_{12}O_6.H_2O$, 5.6mM, HEPES 10mM) was used to lavage the lungs. All aliquots were pooled together and concentrated by centrifugation at 1000rpm for 6 minutes at 25°C, using a Sigma 204 centrifuge.

Red blood cells (RBC) were lysed from the resulting pellet with dH_20, 0.5ml for 30sec, and osmolarity was restored immediately with the addition of 4.5ml BSS. The sample was centrifuged for a second time at 1000rpm for 6 minutes at 25°C. This procedure was repeated until all the RBCs were lysed. Following the removal of RBC, the supernatant was pippetted off and the pellet was resuspended in 0.5ml of BSS buffer. For differential cell count, a 50µl aliquot of the cell sample was dropped and smeared onto a slide. The slide was left to dry before staining with Leishmann's stain. The slide was stained by pippetting 1ml of the stain onto the slide and adding 2ml of dH_2O 20sec later. The 2:1 mixture of dH_2O : stain was left on the slide for 8-10 minutes before washing off with dH_2O and allowing the slide to dry. The cells on the slide were counted under a magnification of 100X with the aid of immersion oil, which was used to improve the refractive index. The percentage leucocytes, namely, eosinophils neutrophils and basophils in the BAL fluid were calculated from the total cells counted, for each group, for each protocol.

2.1.7 mBSA delayed-type hypersensitivity model

The mBSA delayed-type hypersensitivity model was set up according to a modified protocol as described by Tarayre *et al* 1990. Modifications to this protocol were the strain of mice, the quantity of mBSA used on challenge and finally the method of measurement of oedema. Tarayre *et al* 1990, used male swiss mice while this study used male CD-1 mice. Although, the quantity of mBSA used for sensitisation was the same in this study, the quantity used for challenge was less, whereby, 0.1mg /20µl rather than 0.25mg/25µl

(Tarayre *et al* 1990), was an adequate amount to induce DTH in the hind paw. Finally, Tarayre *et al* 1999, looked at changes in paw weight to measure DTH, which is in contrast to this study where changes in paw volume with the use of a plethysmometer was employed in oedema measurement.

Anaesthetised (halothane) CD-1 mice (25-35g) were immunised *i.d.* with mBSA/Freund's complete adjuvant containing *Mycobacterium butyricum* (FCA(B)) emulsion (appendix 1) at four sites (62.5µg/25µl each site) on the shaved chest on day 1. Oral administration of 1% CMC, cyA (50mg/kg in 1% CMC) and test compounds (3 and 10mg/kg in 1% CMC) to CD-1 mice began 1-day prior to challenge with mBSA. The animals were also dosed on the day of challenge to mBSA (2h prior to challenge). The challenging with mBSA (appendix) was carried out eight days following immunisation. Anaesthetised mice were injected *s.c.* in the dorsal surface of one hind foot with mBSA (appendix 1) solution in saline and *s.c.* in the contralateral foot with saline alone (20µl each injection). Swelling of each of the paws of the mice were measured at 24h with a plethysmometer (Model 7140; Ugo Basile, Comerio, Italy). Triplicate measurements were made on each paw of each animal. Mice were sacrificed by cervical dislocation just prior to the paw measurement.

The experiment was run in groups of 6-8 mice. Included was a group that was "primed" with FCA(B) (appendix 1) alone, followed by the mBSA challenge on day 8. This group of animals were dosed orally with 1% CMC. This controlled for the modest background swelling reaction seen upon injection of the mBSA solution. The paw volume

measurements (in ml) were used to calculate the increase of the mBSA-challenged paw compared to the saline-injected contralateral paw of each mouse, as follows:

$$\text{Paw Swelling (\% diff.)} = \frac{[\text{mBSA injected paw (ml) measurement}] - [\text{saline injected paw (ml) measurement}]}{\text{Saline injected paw (ml) measurement}} \times 100\%$$

2.1.8 Statistical analysis

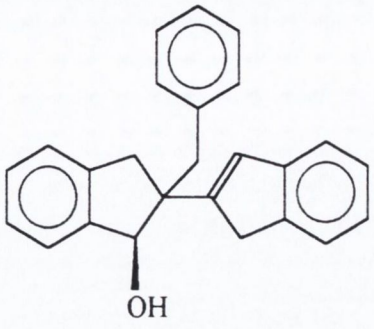
The Mann Whitney, unpaired, one tailed test was carried out on results from the histamine release assay, the *in vivo* inflammatory models and the mBSA DTH model. Mann Whitney, unpaired, two-tailed test was used for analysis of the respiratory asthma results. Data for the inflammatory asthma model was analysed using a one-way ANOVA followed by Bonferroni t-test (Bland & Altman, 1995).

2.2 MATERIALS

2.2.1 The chemistry of three novel compounds, 3C8, 6C6 and 7C9

The chemical structures of three novel compounds 3C8, 6C6 and 7C9 are shown in figures 2.4a, 2.4b and 2.4c respectively. The structures are accompanied by their compound name, their chemical name, their molecular weight and their melting point. Unimed Plc and Dept. of Pharmacognosy School of Pharmacy, Trinity College Dublin. manufactured 3C8, 6C6 & 7C9. Compounds were identified using ^{1}H NMR (Nuclear Magnetic Resonance), ^{13}C NMR, DEPT (Distortionless enhancement polarisation topography), FTIR (Fourier Transform Infared Spectroscopy), UV (Ultraviolet) and Mass Spectrometry. No batch recognition was considered necessary at this stage in the research as only mg quantity samples were synthesised. 3C8 and 7C9 were stored between 0-4°C, while 6C6 was stored at –20°C in a container wrapped in tin foil as it was a light sensitive compound. Experiments to determine shelf life were not carried out, however all compounds were found to remain stable for at least three months at the storage conditions outlined above. All compounds were tested for impurities, before use, using TLC (thin layer chromatography).

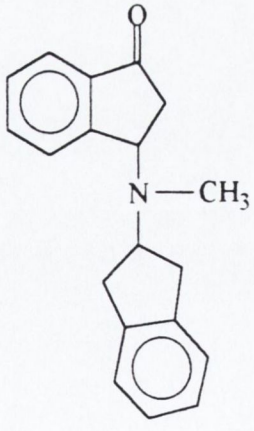
Figure 2.4a:- The chemical structure and properties of compound 3C8



3C8, 2-(2-indenyl)-2-benzylindan-1-ol.

Molecular weight:- 338. Melting point:- 132.5-133.7°C.

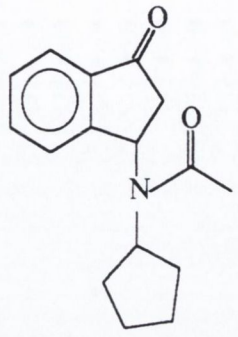
Figure 2.4b:- The chemical structure and properties of compound 6C6



6C6, 3-(N-methyl-N-2-indanylamino)-indan-1-one.

Molecular weight:- 277. Melting point:- 113.7 - 114.6°C.

Figure 2.4c:- The chemical structure and properties of compound 7C9



7C9, N-cyclopentyl-N-3-indan-1-onyl ethanamide.

Molecular weight:- 257. Melting point:- 160.0 - 161.2°C.

2.2.2 Sigma Aldrich, Airton Rd, Tallaght, Dublin, Ireland.

The following chemicals were obtained from Sigma Aldrich, budesonide, indomethacin, dexamethasone, Freund's incomplete adjuvant (FIA), oPT, cyA, TPA, OVA (chicken egg), compound 48/80, mBSA, methacholine histamine, CMC, DMSO, AlOH and FCA(T) (1ml contains 1mg of *Mycobacterium tuberculosis* (H37RA, ATCC 25177) heat killed and dried, 0.85ml mineral oil and 0.15ml mannide monooleate). Carrageenan was also obtained from Sigma Aldrich (C3799; cas no. 9062-07-1). The carrageenan was a type V iota carrageenan sourced from Eucheuma Spinosa.

2.2.3 Lennox Chemicals Ltd., John F. Kennedy Drive, Naas Rd., Dublin 12, Ireland (local suppliers).

Merck, KGaA, 64271 Darmstadt, Germany.

The following chemicals were obtained from Merck by Lennox chemicals Irl., NaCl, KCl, $CaCl_2.2H_2O$, $NaH_2PO_4.2H_2O$, $C_6H_{12}O_6.H_2O$, HEPES.

BDH, Laboratory Supplies, Poole, BH15 1TD. England.

The following chemicals were obtained from BDH by Lennox chemicals Irl., $MgCl_2.6H_2O$, Leishmann Staining solution.

2.2.4 Unitech Ltd, United drug house, Belgard Road, Dublin 24, Ireland.

Mycobacterium butyricum was obtained from Unitech Ltd., United Drug House, Belgard Rd., Dublin 24. The producers of the product were Difco Lab, Detroit, Michigan, USA.

2.2.5 Rhone Merieux Irl, Tallaght, Dublin, Ireland (Supplied by Bioresources Unit (BRU) TCD).

Sagatal (Pentobarbitone Sodium) was obtained from Rhone Merieux by the BRU TCD.

2.2.6 Bioresources Unit, TCD, Ireland.

The following animals were obtained from the BRU, TCD, Wistar rats, female (250g-350g), Wistar rats, male (250-350g), guinea pigs (male) (350g), Laca mice, male (35-45g), *BN rats, male (250g-350g), *CD-1 mice, male (35g-45g) (* indicates that the animals were supplied to the Bioresources Unit, TCD, by Harlan, UK. The animal housing conditions were 12hr/light dark cycle (6.00am to 6pm). The room temperature was 22°C. The feed was standard pollet chow supplied by Red Mills.

Chapter 3

Results

Contents **Page No.**

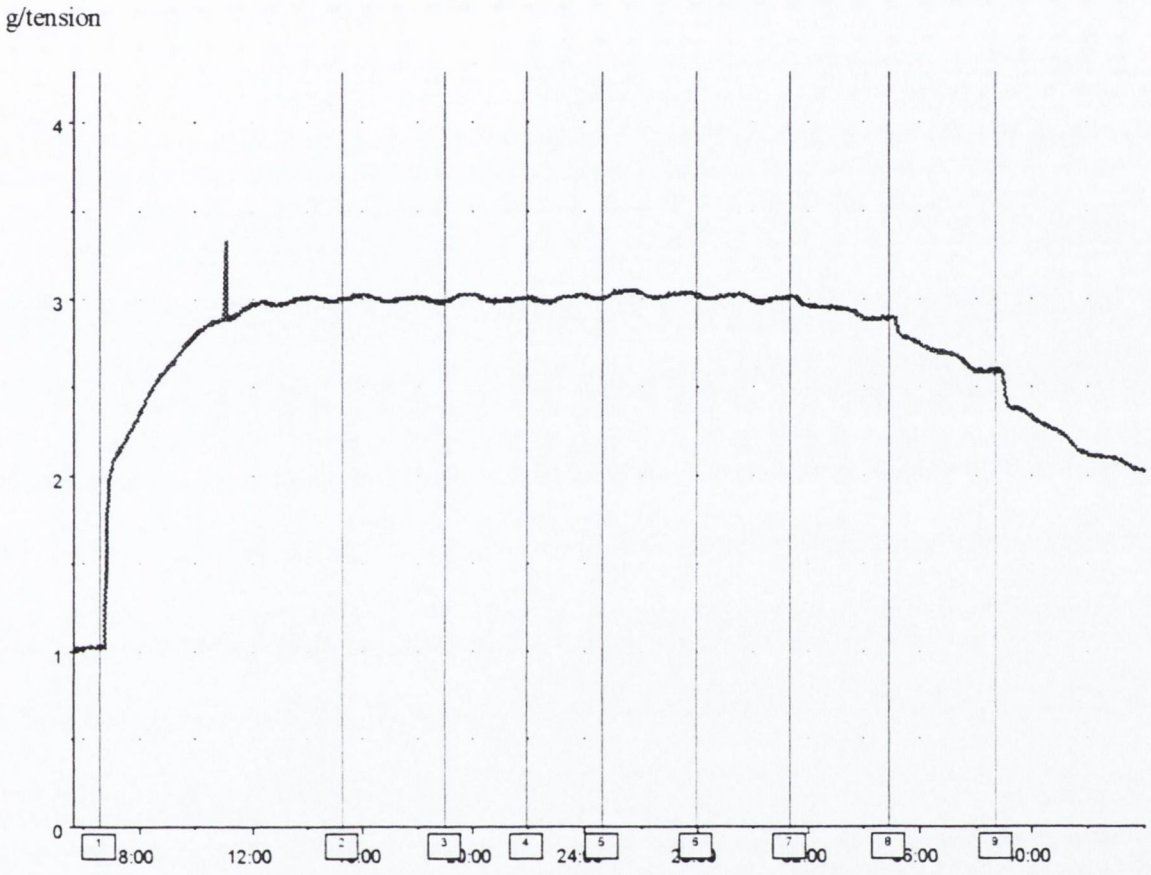
3.1 The Effect of 3C8, 6C6 and 7C9 on Calcium Induced Contraction of K^+ depolarised Guinea-Pig ileum Smooth Muscle

Studies carried out demonstrate the effect of three test compounds on $CaCl_2$ induced contraction of K^+ depolarised guinea pig ileum smooth muscle. The tension of the ileum was measured in grams. Figure 3.1a shows a trace illustrating the inhibition of a $CaCl_2$ (2.5mM) induced contraction by 3C8. The addition of $CaCl_2$ (2.5mM) induced an increase in tension of 2g. Test compounds were added cumulatively to the guinea-pig ileum preparation at half–log dose intervals, ranging from 3 $X10^{-9}$M to 10^{-5}M. 3C8 ($3x10^{-6}$M and 10^{-5}M) reduced the $CaCl_2$ induced contraction by 0.5g and 1g respectively.

A trace demonstrating the effect of 6C6 on $CaCl_2$ (2.5mM) induced contraction of guinea-pig ileum smooth muscle is shown in figure 3.1b. A 2.2g contraction was seen on addition of $CaCl_2$ to this preparation of ileum. A cumulative half log dose response of concentrations ranging from $3x10^{-8}$M to 10^{-5}M was used for 6C6. 6C6 ($3x10^{-8}$M - 10^{-5}M), however had minimal relaxant effect against the $CaCl_2$ induced contraction.

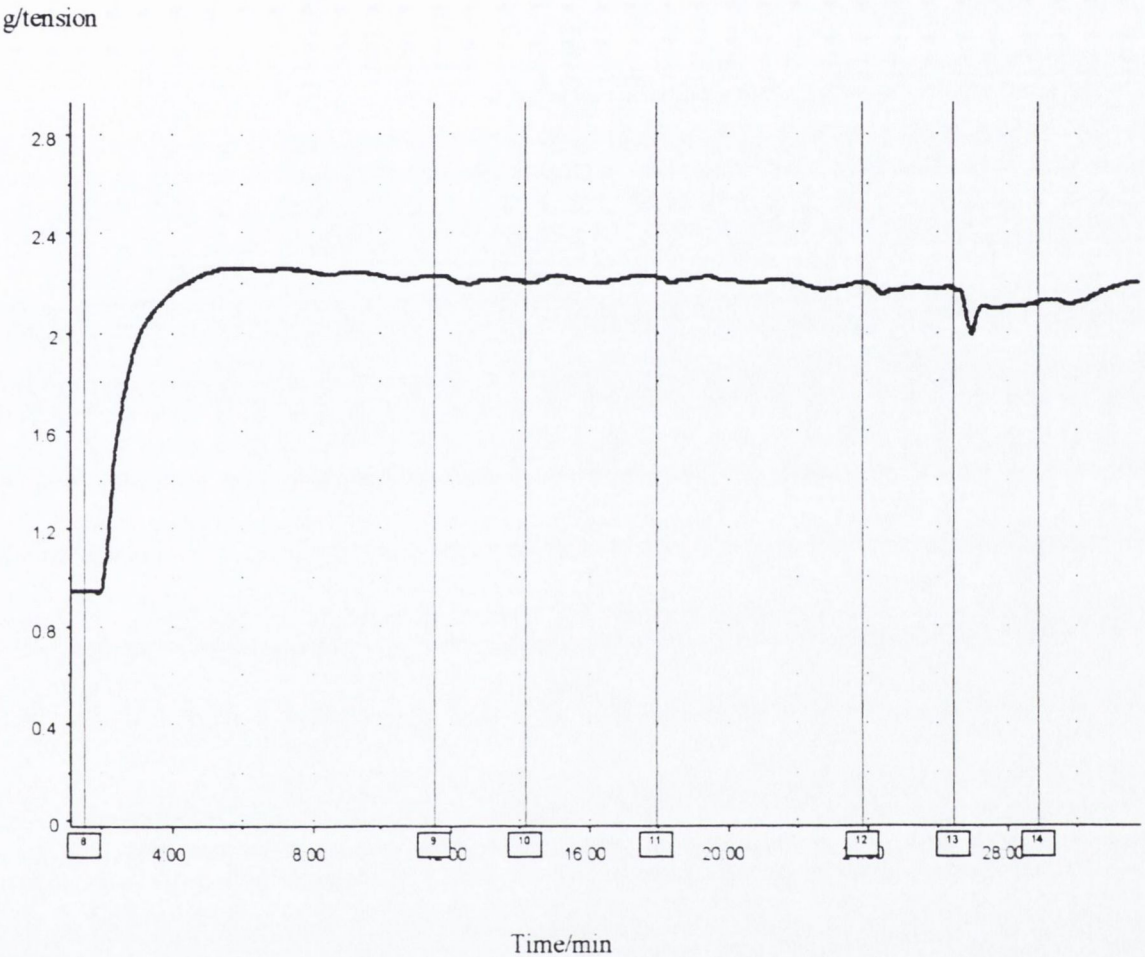
Figure 3.1c shows a trace of the effect of 7C9 on a $CaCl_2$ (2.5mM) induced contraction of guinea-pig ileum smooth muscle. A contraction of 1.5g was seen on addition of $CaCl_2$ to this ileum preparation. Two concentrations, $3x10^{-6}$M and 10^{-5}M were used for this test compound. 7C9 (10^{-5}M) was found to marginally reduce this contraction by 0.25g.

Figure 3.1a:- A trace of a $CaCl_2$ induced contraction of guinea-pig smooth muscle and the effect of 3C8 on this contraction



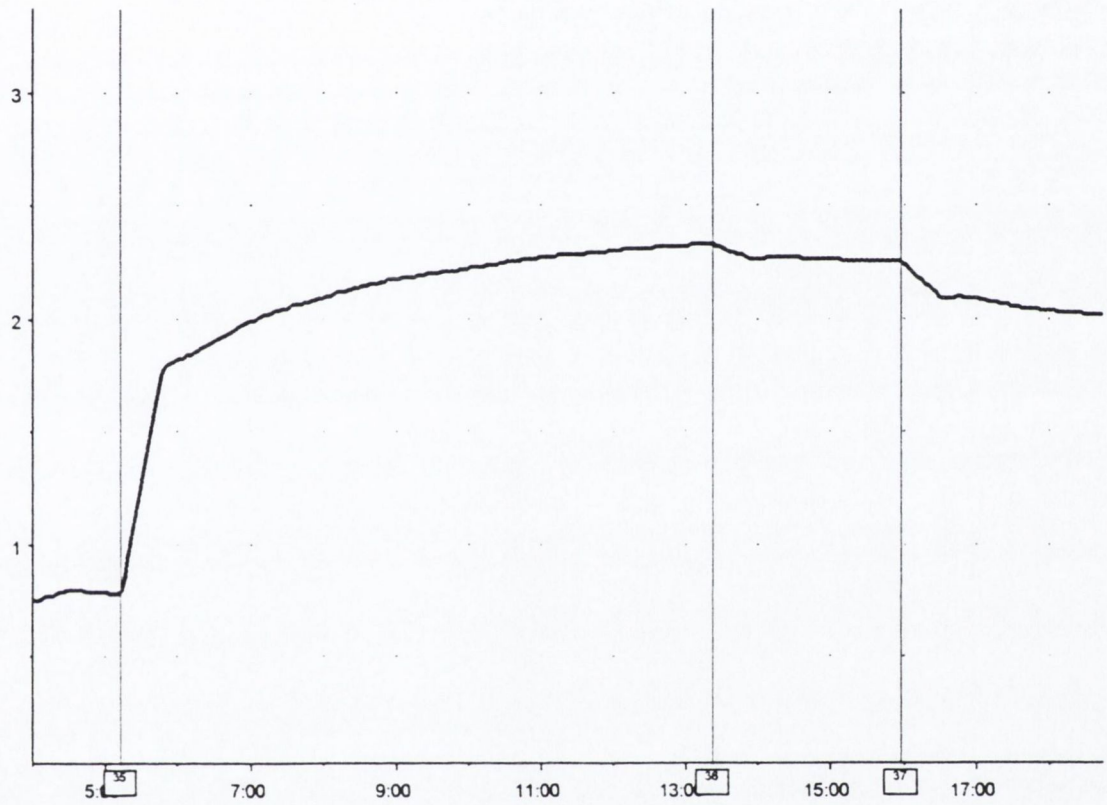
The trace shows the change in tension of guinea-pig ileum exposed firstly to $CaCl_2$ and then to cumulative ranges of test compound. The ileum is suspended in K^+ depolarising medium at 37°C and gassed at 95 % O_2 : 5% CO_2. The first dotted line marks the addition of 2.5mM $CaCl_2$. The second through to the ninth lines mark the cumulative addition of $3x10^{-9}$M, 10^{-8}M, $3x10^{-8}$M, 10^{-7}M, $3x10^{-7}$M, 10^{-6}M, $3x10^{-6}$M and 10^{-5}M 3C8 respectively.

Figure 3.1b:- A trace of a $CaCl_2$ induced contraction of guinea-pig smooth muscle and the effect of 6C6 on this contraction



The trace shows the change in tension of guinea-pig ileum exposed firstly to $CaCl_2$ and then to cumulative ranges of test compound. The ileum is suspended in K^+ depolarising medium at 37°C and gassed at 95 % O_2 : 5% CO_2. The first dotted line marks the addition of 2.5mM $CaCl_2$. The second through to the seventh lines mark the cumulative addition of $3x10^{-9}$M, 10^{-8}M, $3x10^{-8}$M, 10^{-7}M, $3x10^{-7}$M, 10^{-6}M, $3x10^{-6}$M and 10^{-5}M 6C6 respectively.

Figure 3.1c:- A trace of a $CaCl_2$ induced contraction of guinea-pig smooth muscle and the effect of 7C9 on this contraction

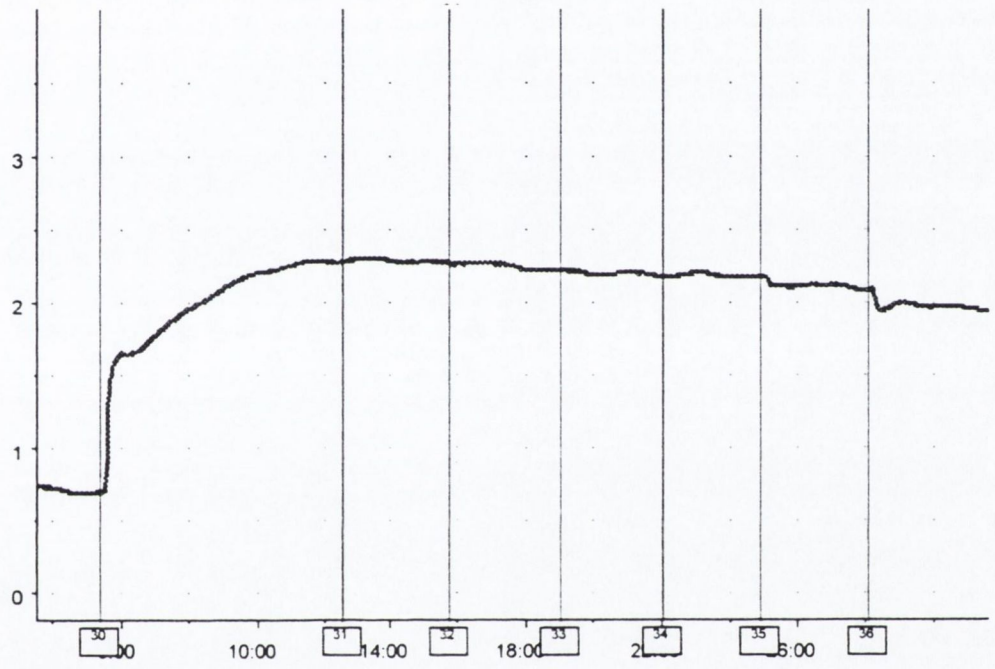


The trace shows the change in tension of guinea-pig ileum exposed firstly to $CaCl_2$ and then to cumulative ranges of test compound. The ileum is suspended in K^+ depolarising medium at 37°C and gassed at 95 % O_2 : 5% CO_2. The first dotted line marks the addition of 2.5mM $CaCl_2$. The second and third lines indicate the cumulative addition of $3x10^{-6}$M and 10^{-5}M 7C9 respectively.

Figure 3.1d shows a trace of the effect of the solvent DMSO on a $CaCl_2$ (2.5mM) induced contraction of guinea-pig ileum smooth muscle. A contraction of 1.5g was seen on addition of $CaCl_2$ to this ileum preparation. The effect of the solvent is shown in this trace, where 0.0015-0.05% DMSO had minimal effect on smooth muscle contraction, however, the higher concentrations of DMSO 0.15% and 0.5% showed a decrease in tension of 0.2g (13%) and 0.3g (20%) respectively.

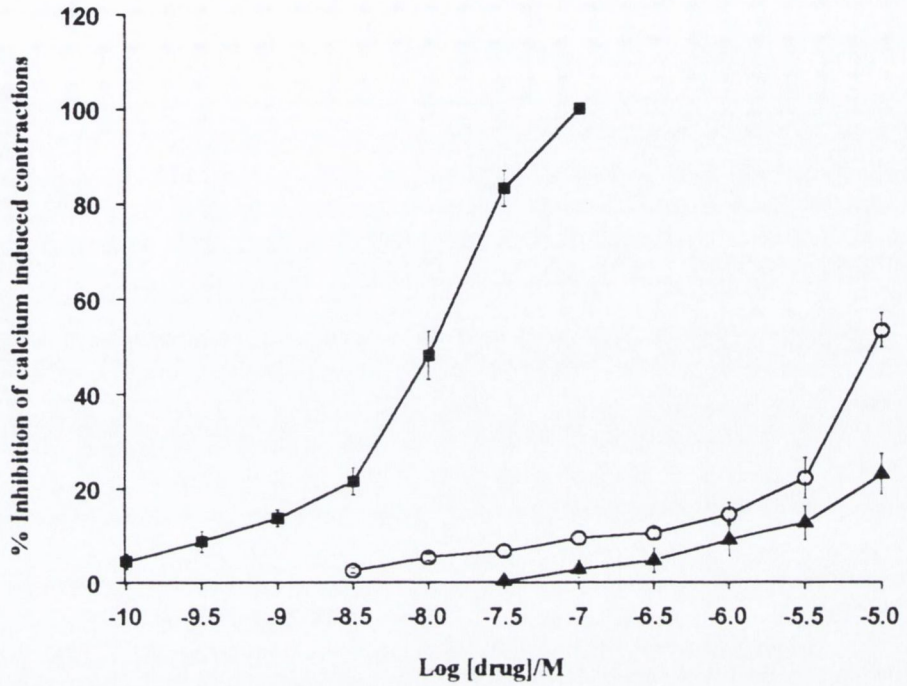
Figure 3.2 illustrates the percentage inhibition (mean ± sem) of calcium-induced contractions by 3C8, 6C6 and nifedipine (n = 3) in K^+ depolarised guinea-pig ileum smooth muscle. Nifedipine was the most potent of the drugs/compounds tested and 100% inhibition was demonstrated at 10^{-7}M. 3C8 was the most potent inhibitor of the three test compounds where a concentration of 10^{-5}M resulted in 53.4 ± 3.6% inhibition. 6C6 (10^{-5}M) showed a slight inhibitory action of 23.0 ± 4.3%. 7C9 (3 x 10^{-6}M & 10^{-5}M) was not plotted on figure 3.2 as only two concentrations were tested. This is as a result of the minimal inhibition of 11.6±4.9% and 15.4 ± 4.6% seen with 7C9, 3 x 10^{-6}M and 10^{-5}M respectively. Results are tabulated and shown on table 3.1 (Appendix 2).

Figure 3.1d:- A trace of a $CaCl_2$ induced contraction of guinea-pig smooth muscle and the effect of DMSO on this contraction



The trace shows the change in tension of guinea-pig ileum exposed firstly to $CaCl_2$ and then to cumulative ranges of solvent, DMSO. The ileum is suspended in K^+ depolarising medium at 37°C and gassed at 95 % O_2 : 5% CO_2. The first dotted line marks the addition of 2.5mM $CaCl_2$. The second through to the seventh lines mark the cumulative addition of 0.0015%, 0.005%, 0.015%, 0.05%, 0.15% and 0.5% of DMSO respectively.

Figure 3.2:- Percentage inhibition of $CaCl_2$ induced contraction by Nifedipine, 3C8 and 6C6 in K^+depolarised guinea-pig ileum smooth muscle.



This figure illustrates the percentage inhibition of $CaCl_2$ (mean ± sem; n=3) induced contraction of K^+ depolarised guinea pig ileum smooth muscle in a buffer gassed with 95% O_2 : 5% CO_2 at 37°C.
Symbols: —■— represents nifedipine; — 0 — represents 3C8; —♦— represents 6C6;
Log [Conc]/M = Log_{10} [Conc]/M

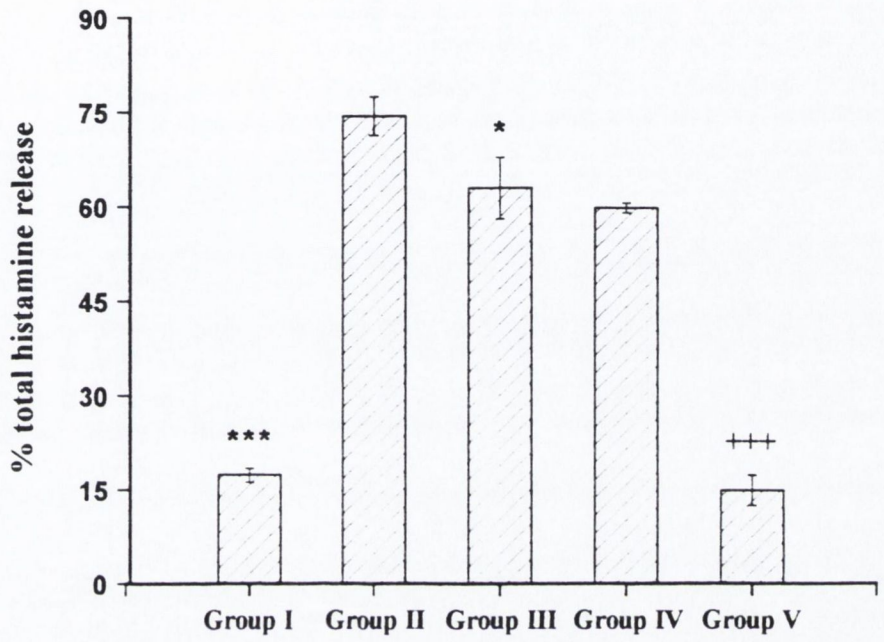
3.2 The Investigation of the Ability of 3C8, 6C6 and 7C9 to Stabilise Mast Cells

Release of histamine from isolated rat mast cells following the addition of compound 48/80 was measured. The inhibition of the release of histamine from isolated rat mast cells was used as an indicator of the stabilisation of these cells. There are five groups set up each showing the mean ± sem (3C8 (n = 5); 6C6 (n = 8); 7C9 (n = 6) of histamine release from

rat mast cells (table 3.2; Appendix 2). Group I, basal, represents the release of histamine from unstimulated mast cells, pre-treated with dH_2O. Group II, represents the maximum release of histamine, on stimulation with compound 48/80, following pre-treatment with dH_2O. Group III, DSCG, represents the histamine release from mast cells that were treated with DSCG (2×10^{-5}M) prior to stimulation with compound 48/80. Group IV represents the maximum release of histamine from compound 48/80 stimulated cells that were pre-treated with DMSO (final concentration 0.5%). Group V, Test Cpds (2×10^{-5}M), represents mast cells pre-treated with test compound, 3C8, 6C6 or 7C9 dissolved in DMSO (final concentration 0.5%) before stimulation with compound 48/80. A typical amount of histamine released per sample on stimulation with compound 48/80 was $2\mu g/10^6$ cells. The typical total amount of histamine released from the isolated mast cells was $3\mu g/10^6$ cells.

The addition of compound 48/80 induced a four-fold increase in histamine release from mast cells. The increase was found to be statistically significant ($p<0.001$) and was seen in all control groups associated with the three test compounds, 3C8, 6C6 and 7C9. The positive drug control, DSCG, was found to reduce the increase in histamine release from mast cells. This was found to be statistically significant, $P<0.05$, $P\leq0.05$, and $P<0.1$ for DSCG associated with 3C8, 6C6 and 7C9 respectively. Test compounds 3C8 and 6C6 were both found to be potent inhibitors of histamine release from mast cells when compared to the maximum which had been pre-treated only with DMSO (Figures 3.3 & 3.4). Statistical comparisons showed a difference of $P<0.001$ for both compounds. However, test compound 7C9 did not inhibit histamine release (Figure 3.5).

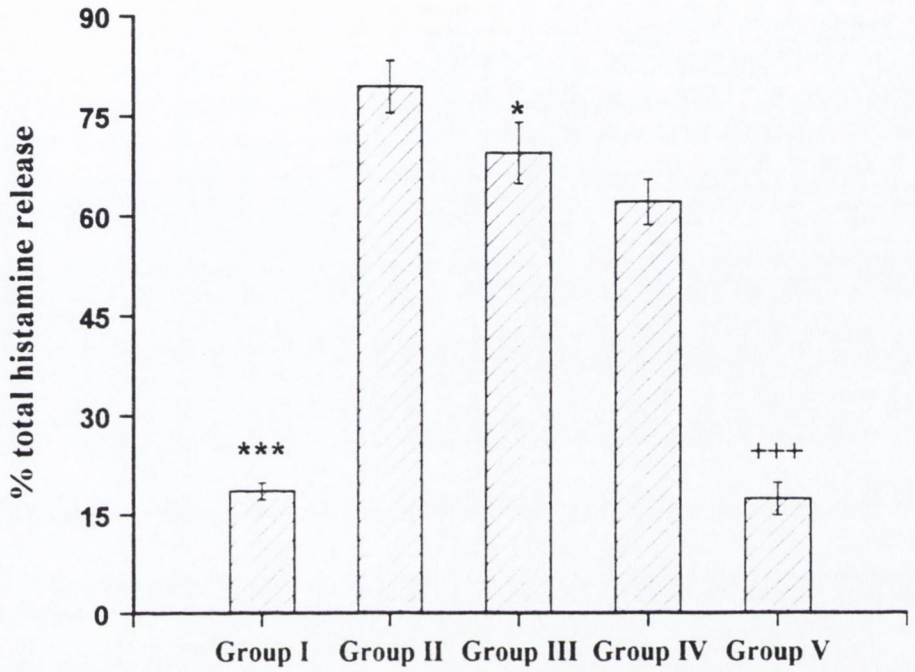
Figure 3.3:- Inhibition of histamine release by 3C8



This figure shows the percentage inhibition of histamine release from rat peritoneal mast cells, as induced by compounds 48/80 by DSCG ($2x10^{-5}$ M) and test compound 3C8 ($2x10^{-5}$ M). Group I represents the basal release of histamine. Group II represents the maximum release of histamine upon stimulation with compound 48/80. Group III represents the release of histamine upon stimulation with compound 48/80 in DSCG pre-treated mast cells. Group IV represents the maximum release of histamine upon stimulation with compound 48/80 in the 0.5% DMSO (solvent for 3C8) pre-treated mast cells and group V represents the release of histamine in 3C8 pre-treated mast cells

Statistical analysis included a Mann Whitney test, unpaired and one tailed.
*** indicates $P<0.001$ as compared to Group II.
* indicates $P<0.05$ as compared to Group II.
+++ indicates $P<0.001$ as compared to Group IV.

Figure 3.4:- Inhibition of histamine release by 6C6 from isolated rat mast cells

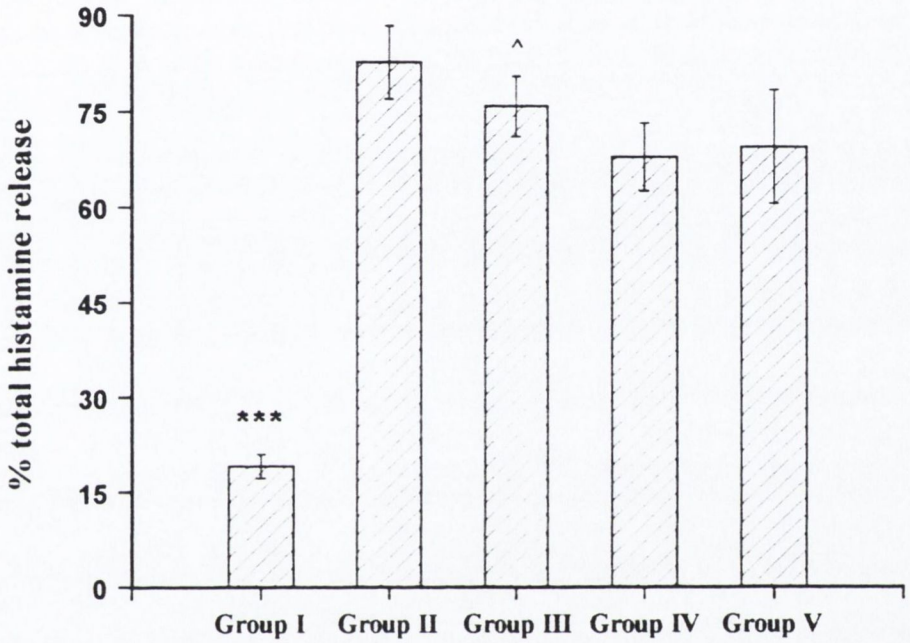


This figure shows the percentage inhibition of histamine release from rat peritoneal mast cells, as induced by compounds 48/80, by DSCG ($2x10^{-5}$ M) and test compound 6C6 ($2x10^{-5}$ M). Group I represents the basal release of histamine. Group II represents the maximum release of histamine upon stimulation with compound 48/80. Group III represents the release of histamine upon stimulation with compound 48/80 in DSCG pre-treated mast cells. Group IV represents the maximum release of histamine upon stimulation with compound 48/80 in the 0.5% DMSO (solvent for 6C6) pre-treated mast cells and group V represents the release of histamine in 6C6 pre-treated mast cells

Statistical analysis included a Mann Whitney test, unpaired and one tailed.

*** indicates $P<0.001$ as compared to Group II.
* indicates $P\leq0.05$ as compared to Group II.
+++ indicates $P<0.001$ as compared to Group IV.

Figure 3.5: Inhibition of histamine by 7C9 from isolated rat mast cells



This figure shows the percentage inhibition of histamine release from rat peritoneal mast cells, as induced by compounds 48/80, by DSCG ($2x10^{-5}$ M) and test compound 7C9 ($2x10^{-5}$ M).). Group I represents the basal release of histamine. Group II represents the maximum release of histamine upon stimulation with compound 48/80. Group III represents the release of histamine upon stimulation with compound 48/80 in DSCG pre-treated mast cells. Group IV represents the maximum release of histamine upon stimulation with compound 48/80 in the 0.5% DMSO (solvent for 7C9) pre-treated mast cells and group V represents the release of histamine in 7C9 pre-treated mast cells

Statistical analysis included a Mann Whitney test, unpaired and one tailed.

*** indicates $P<0.001$ as compared to Group II.
^ indicates $P<0.1$ as compared to Group II.

3.3 Respiratory Asthma

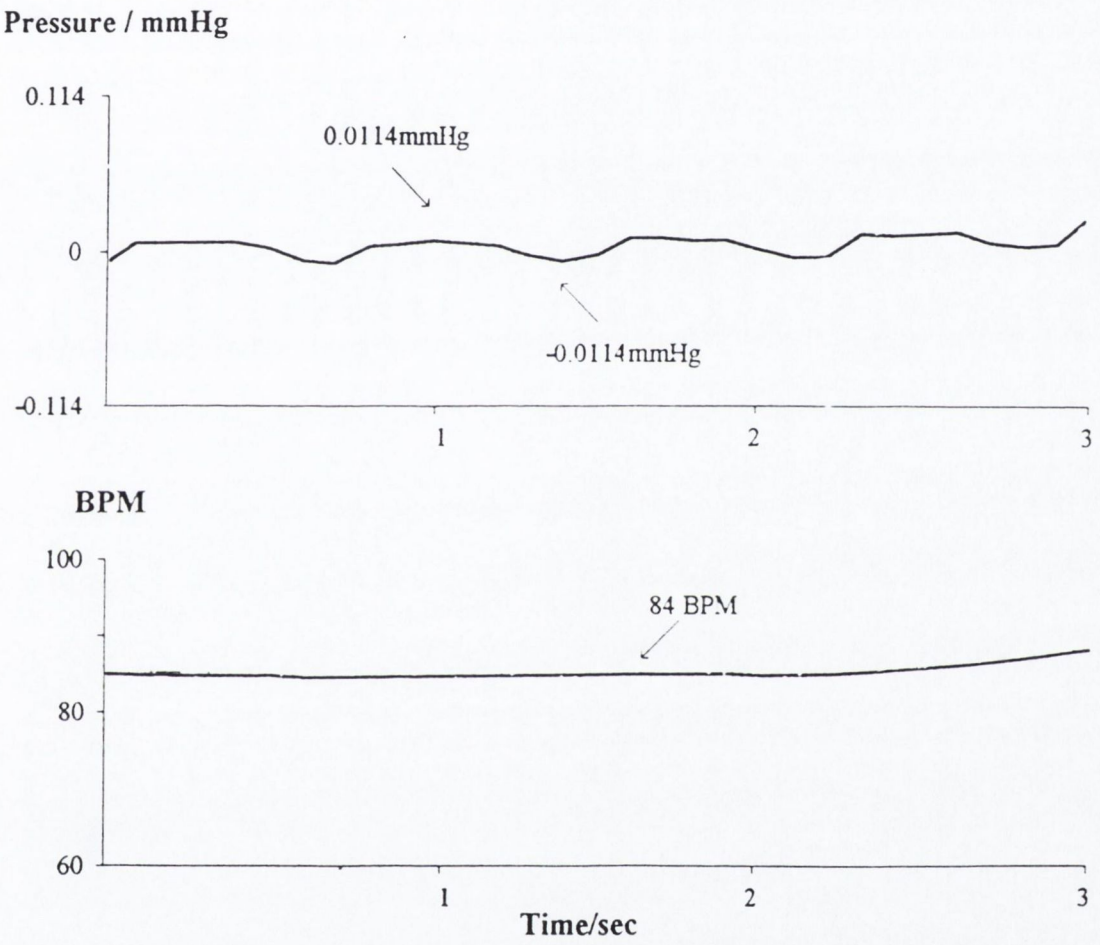
3.3.1 Respiration in Rats with Acute Asthma

The rate of respiration and change of pressure within the chamber during each breath cycle (ΔP_{bc}) of Wistar rats were monitored over a 3h period, during which time animals were challenged with OVA. Asthma was induced in all rats by sensitising them to OVA using AlOH and FCA(T) as adjuvants. Respiration was measured in BPM. Four groups of animals were set up. Group I, the negative control (-ve ctrl; n = 4) was untreated (50% ETOH, at -30mins), and unchallenged (at 0 minutes). Group II, positive control (+ve ctrl; n = 4) was untreated (50% ETOH, at –30 minutes) and OVA challenged (5% w/v, at 0mins). Group III was DSCG (n = 5) treated (5mg/ml in 50% ETOH, at -30mins) and OVA challenged (5% w/v, at 0mins). Group IV was 3C8 (n = 6) treated (5mg/ml in 50% ETOH, at -30mins) and OVA challenged (5% w/v, at 0mins). Drug/vehicle was administered by nebuliser for 2 minutes prior to challenge with OVA which was also administered, by nebuliser for a 2-minute duration.

Figures 3.6a and 3.6b show respiratory traces before OVA challenge and 135 minutes after OVA challenge, respectively. The traces were taken from one animal from the positive control group that was untreated (solvent only) and OVA challenged. These traces show the change in pressure over three seconds within the chamber containing the animal.

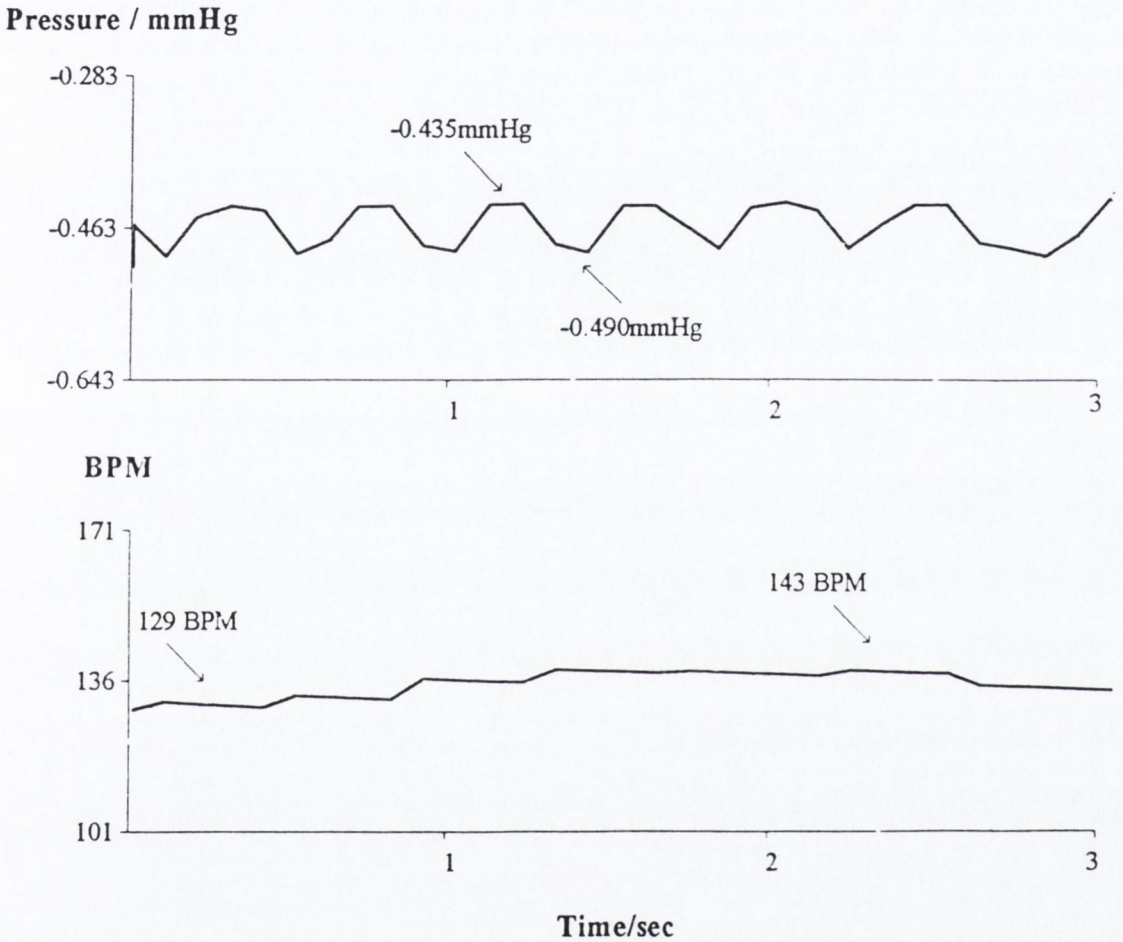
This change in pressure within the chamber was measured in mmHg and is inversely proportional to the intake and output of air, a breath. Increase of pressure represents breathing out and decrease of pressure represents breathing in. The frequency of the wave represents the rate of respiration. The respiration rate both before OVA challenge and 180 minutes afterwards was calculated and replotted by the Maclab software. This was represented as BPM. The rate was shown to be 84BPM before OVA challenge, figure 3.6a, and increased to 143BPM, 135 minutes after OVA challenge, figure 3.6b. The measurement of the change of pressure within the chamber during each breath cycle was directly proportional to the tidal volume or volume of each breath taken (data not shown). The tidal volume, volume of each breath taken, was therefore represented as a function of the change in pressure ($f\Delta$mmHg; ΔP_{bc}) within the chamber (figure 2.3), which contained the rat. This latter measurement can detect constriction of the airways whereby an increase in constriction would be represented by a decrease in the change of pressure within the chamber during the breath cycle. The change in pressure within the chamber during each breath cycle (ΔP_{bc}) was taken from the height of the wave, which was calculated from the difference in pressure between the intake and the output of a breath. The ΔP_{bc} was 0.0228mmHg before OVA challenge, figure 3.6a and increased to 0.055mmHg 135 minute after OVA challenge, figure 3.6b.

Figure 3.6a:- A trace measuring the respiratory parameters of a positive control rat prior to challenging with OVA



The first trace shows the change in pressure within the chamber that holds the rat. Pressure oscillated from 0.0114mmHg to –0.0114mmHg. The high pressure of 0.00114mmHg represents the exhaled state and the low pressure of –0.0114mmHg represents inhaled state. The rate of respiration of the animal was calculated using the MacLab software and is illustrated in the second trace. The rate of respiration was 84BPM.

Figure 3.6b:- A trace measuring the respiratory parameters of a positive control rat following challenge with OVA



The first trace shows the change in pressure within the chamber that holds the rat. Pressure oscillated from -0.435mmHg to -0.490mmHg. The high pressure of -0.435mmHg represents the exhaled state and the low pressure of -0.490mmHg represents inhaled state. The rate of respiration of the animal was calculated using the MacLab software and is illustrated in the second trace. The rate of respiration ranged from 129BPM to 143BPM.

The BPM and the change of pressure within the chamber during each breath cycle (mean ± sem) of the four groups as described above are illustrated in figure 3.7 and figure 3.8 respectively. The two respiratory parameters were monitored before, immediately after and up to 180 minutes following exposure to OVA. The BPM is represented as the percentage normal and is calculated as a percentage of the BPM taken 15 minutes prior to OVA challenge (table 3.3; appendix 2). No significant change in BPM was noted in the positive control immediately after OVA challenge, however an increase was seen at 135 minutes. This increase was a statistically significant in the BPM compared to the negative control at 135 minute ($P<0.05$), and 150 minutes ($P<0.05$).

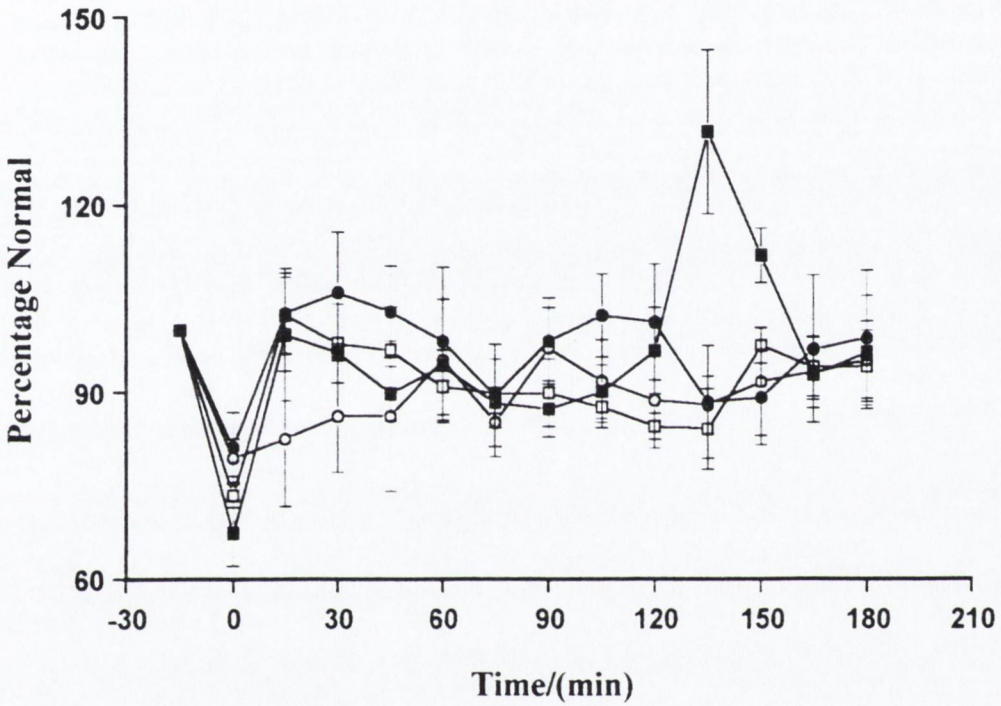
DSCG prevented the increase in respiration at 135 minutes, $P<0.01$, but not at 150 minutes, as compared the positive control. Our test compound, 3C8, prevented the increase in respiration at both time points, 135 and 150 minutes, $P<0.05$ and $P<0.1$ respectively as compared to the positive control.

The change of pressure within the chamber during each breath cycle (ΔP_{bc}) on OVA challenge between groups I-IV is illustrated in figure 3.8. The ΔP_{bc} is represented as the percentage normal and is calculated as a percentage of the ΔP_{bc} taken 15 minutes prior to OVA challenge (table 3.4; appendix 2). No significant change in ΔP_{bc} was noted immediately after OVA however like with the BPM the ΔP_{bc} increased at later time-points. The first change in ΔP_{bc} was seen between Group I and Group II and was at 60 minutes where the ΔP_{bc} in Group II started to marginally climb ($P<0.1$ as compared to Group I). However the ΔP_{bc} returned to normal soon after and was not significantly different from

Group I, the negative control, at the next time point, 75 minutes following OVA challenge. The ΔP_{bc} increased again, however, more significantly at 120 minutes ($P<0.05$ as compared to control). The ΔP_{bc} continued to climb and at 135 minutes a statistical P value of <0.01 as compared to control was calculated. The increase in ΔP_{bc} was maintained through the next time point 150 minute ($P<0.01$ as compared to control) however began to decrease at 165 minutes ($P<0.05$ as compared to control) and returned to normal at 180 minutes.

Pre-treatment with either DSCG and 3C8 showed some therapeutic effect. The increase in ΔP_{bc} was prevented somewhat by both treatments at 135 minute in both groups ($P<0.1$ as compared to control). In the DSCG group the increase in ΔP_{bc} was also prevented slightly at 165 minute ($P<0.1$ as compared to control).

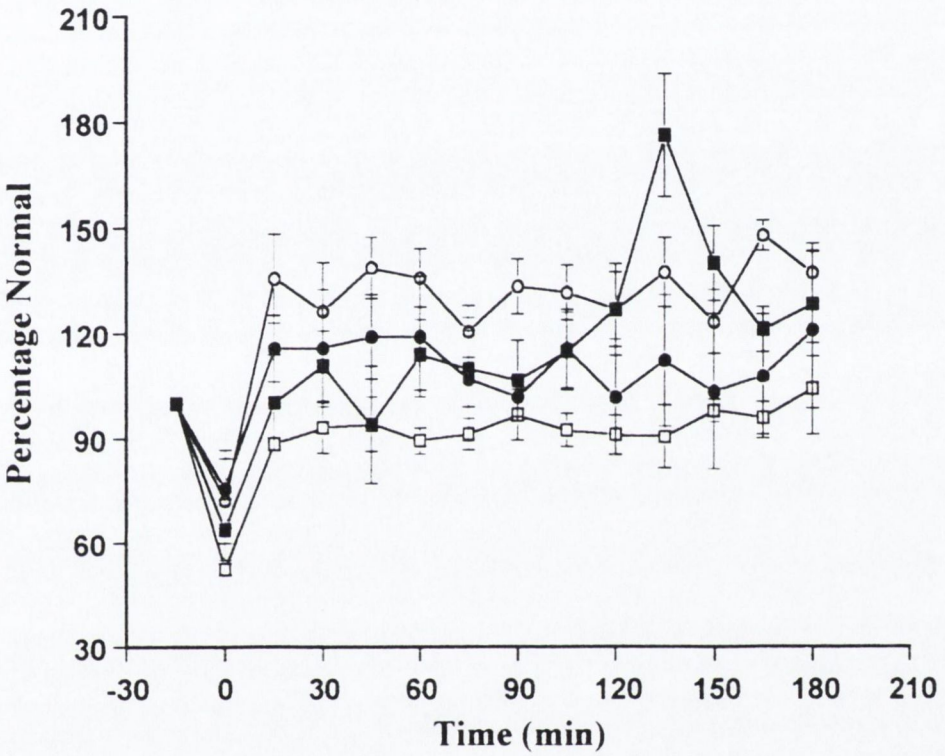
Figure 3.7:- Rate of respiration in rats with acute asthma



This figure represents the percentage change in the rate of respiration in OVA (adjuvants: ALOH + FCA(T)) sensitised rats on challenge with OVA following pre-treatment with vehicle, DSCG or 3C8. Data is represented as percentage normal and calculated as a percentage of the BPM noted 15 minutes prior to OVA challenge.

- –□– Group I (n=4) - untreated (50% ETOH), @ -30min, unchallenged (NaCl, 0.9% w/v) @ 0min.
- –●– Group II (n=4) - untreated (50% ETOH), @ -30 min, OVA challenged (5% w/v) @ 0min.
- –■– Group III (n=5) - DSCG (5mg/ml in ETOH, 50%) treated @ -30mins, OVA challenged (5%w/v) @ 0min.
- –○– Group IV (n=6) - 3C8 (5mg/ml in ETOH, 50%) treated @ -30mins, OVA challenged (5% w/v) @ 0min.

Figure 3.8:- Change in pressure within the chamber during the breath cycle (ΔP) of rats with acute asthma



This figure represents the percentage change in chamber pressure before and after a breath (ΔP) in OVA (adjuvants: ALOH + FCA(T)) sensitised rats on challenge with OVA following pre-treatment with vehicle, DSCG or 3C8. Data is represented as percentage normal and calculated as a percentage of the ΔP noted 15 minutes prior to OVA challenge.

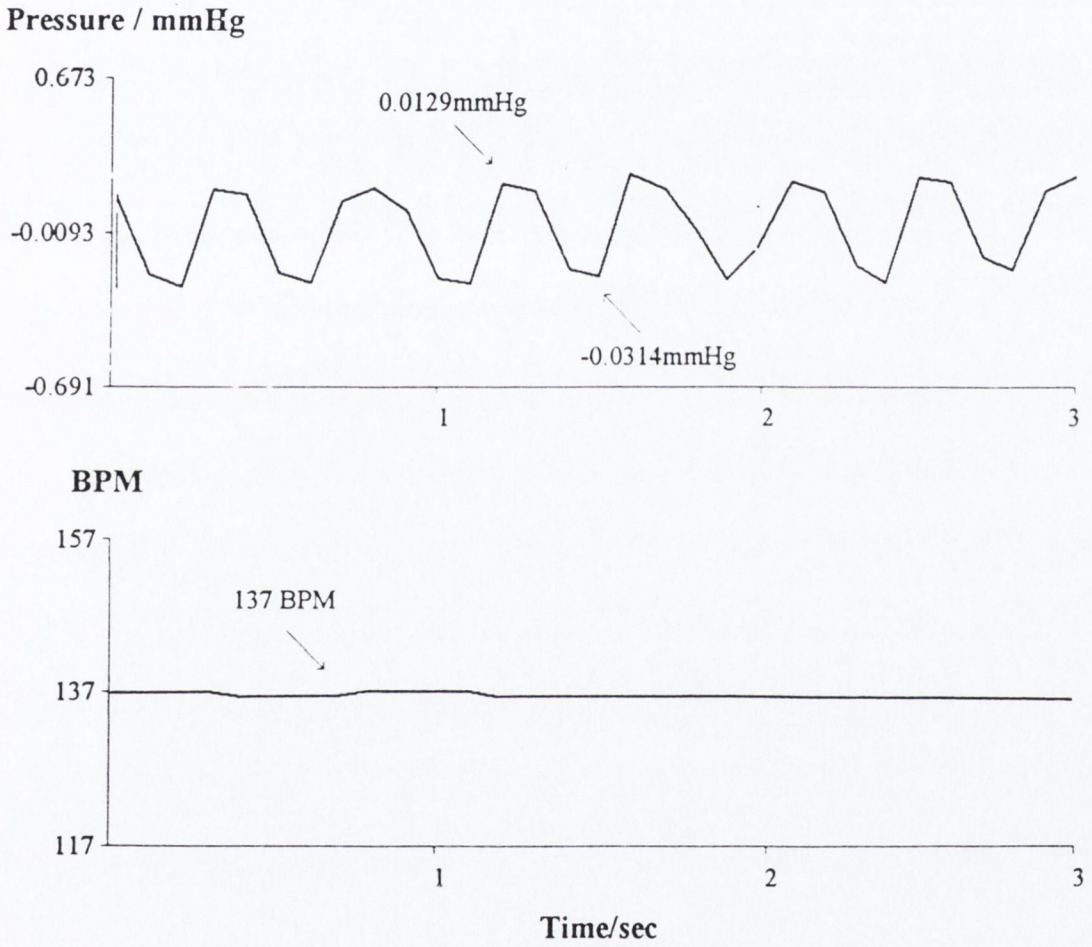
–□– Group I (n=4)- untreated (50% ETOH), @ -30min, unchallenged (NaCl, 0.9% w/v) @ 0min.
–●– Group II (n=4)- untreated (50% ETOH), @ -30 min, OVA challenged (5% w/v) @ 0min.
–■– Group III (n=5) - DSCG (5mg/ml in ETOH, 50%) treated @ -30mins, OVA challenged (5%w/v) @ 0min.
–○– Group IV (n=6) - 3C8 (5mg/ml in ETOH, 50%) treated @ -30mins, OVA challenged (5% w/v) @ 0min.

3.3.2 Respiration in Bronchiolar Hyper-reactive Rats

Following this, groups II - IV as described in section 3.3.1 were exposed repetitively to OVA in the same manner and following pre-treatment with their corresponding solvent or drug. In the case of Group I, animals were again untreated and unchallenged each time. This procedure was carried out in order to induce bronchiolar hyper-reactivity in the rats. The development of this bronchiolar hyper-reactive response was then investigated by exposing all groups to methacholine (8mg/ml).

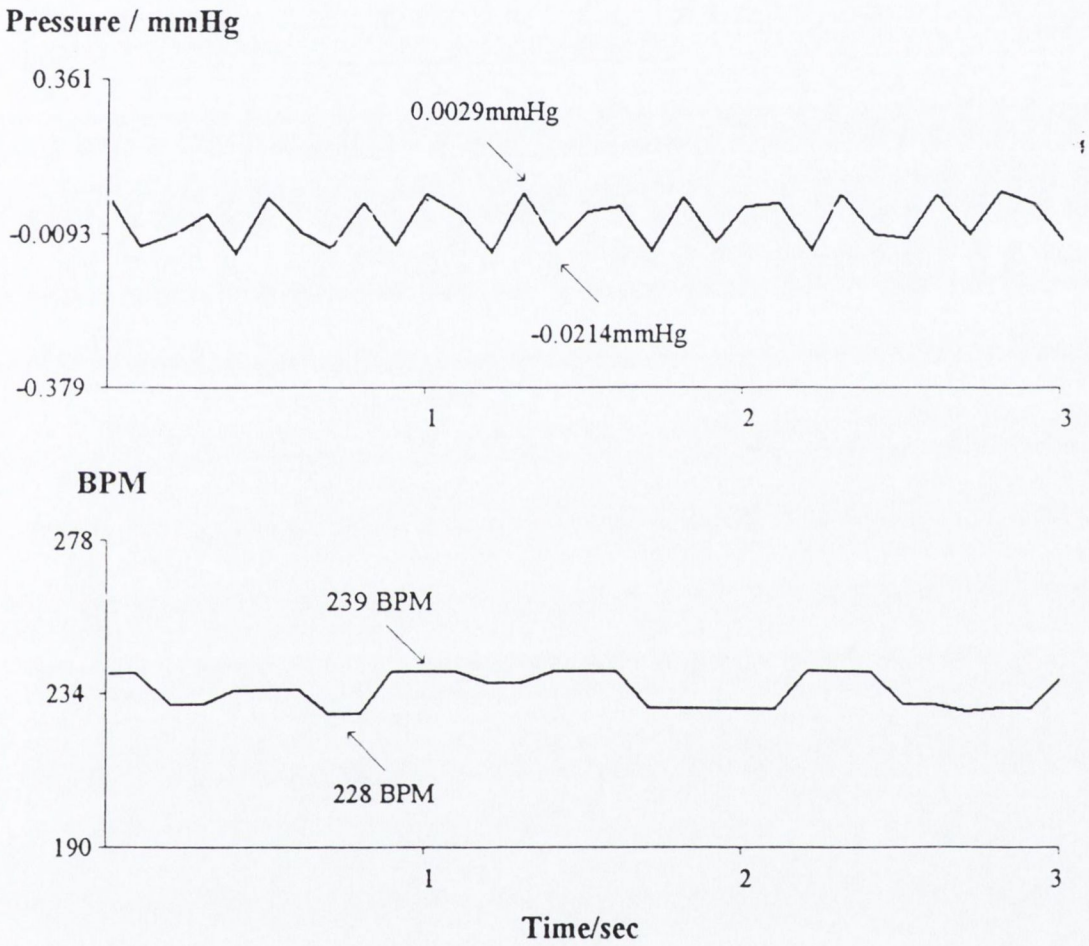
A trace of the respiration as measured in BPM and ΔP_{bc} of one animal, from the positive control group, before, (figure 3.9a) and immediately after methacholine exposure (figure 3.9b) are shown. The two traces again represent the change in pressure of the chamber containing the rat and are measured over 3 seconds only. The principal of the pressure change within the chamber is as described in section 3.3.1. Comparing the two traces directly we find that before exposure to methacholine, the animal was seen to be breathing normally at a rate of 137 BPM, while almost immediately after exposure to methacholine the respiration became erratic and increased to 279BPM. The ΔP_{bc}, measured as a function of change in pressure, before methacholine challenge was 0.0443mmHg and decreased to 0.0243mmHg following exposure. The decrease in ΔP_{bc} was however not exclusive to this group and was noted in all groups including the negative control where the animals were not hypersensitive to methacholine (figure 3.11 & Table 3.6, Appendix 2).

Figure 3.9a:-A trace monitoring the respiratory parameters of a positive control rat prior to exposure to methacholine



The first trace shows the change in pressure within the chamber that holds the rat. Pressure oscillated from 0.0129mmHg to –0.0314mmHg. The high pressure of 0.0129mmHg represents the exhaled state and the low pressure of –0.0314mmHg represents inhaled state. The rate of respiration of the animal was calculated using the MacLab software and is illustrated in the second trace. The rate of respiration was 137BPM.

Figure 3.9b:-A trace monitoring the respiratory parameters of a positive control rat following methacholine exposure



The first trace shows the change in pressure within the chamber that holds the rat. Pressure oscillated from 0.0029mmHg to –0.0214mmHg. The high pressure of 0.0029mmHg represents the exhaled state and the low pressure of –0.0214mmHg represents inhaled state. The rate of respiration of the animal was calculated using the MacLab software and is illustrated in the second trace. The rate of respiration ranged from 228BPM to 239BPM.

The rate of respiration and ΔP_{bc} of groups I-IV was monitored, before, immediately after and up to sixty minutes following the exposure to methacholine (8mg/ml). The BPM and ΔP_{bc} are represented as the percentage normal and are calculated as a percentage of the BPM and ΔP_{bc} taken 30 minutes prior to methacholine exposure, respectively. Figure 3.10, figure 3.10a and figure 3.11 illustrate the results from groups I - IV. The mean ± sem are shown on tables 3.5 and 3.6 (Appendix 2) for the change in BPM and ΔP_{bc} respectively.

The rate of respiration of group II (n = 3), the positive control increased significantly over the negative control (n = 5), $P<0.1$ immediately after methacholine exposure. It was also found that pre-treatment of DSCG (n = 4) and 3C8 (n = 6) before each OVA challenge prevented the DSCG and 3C8 groups respectively from becoming hyper-reactive and overly sensitive to methacholine, $P<0.05$ compared to the positive control. Finally, fifteen minutes after exposure to methacholine statistical comparisons showed a reduction in the 3C8 treated group as compared to the positive control group ($P<0.05$). No difference was noted between the negative control and the positive control groups at this time point. The results taken from the ΔP_{bc} monitored in each group did not reflect the results seen for the change in respiration. The ΔP_{bc} decreased following methacholine exposure in all groups. No statistical significant difference was found when comparing the groups to the positive control at the time points taken.

Figure 3.10:- Rate of respiration in broncho-hyperreactive rats

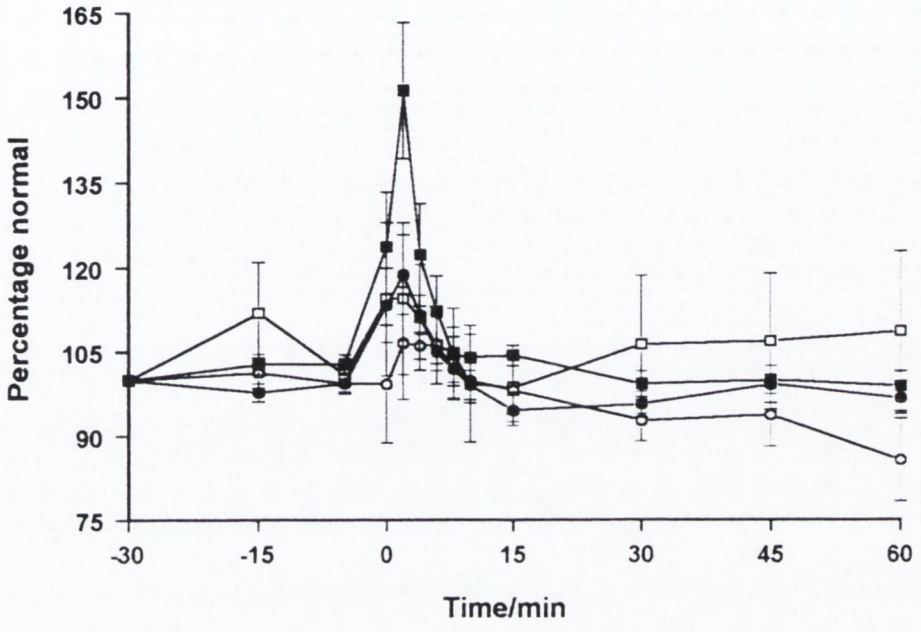
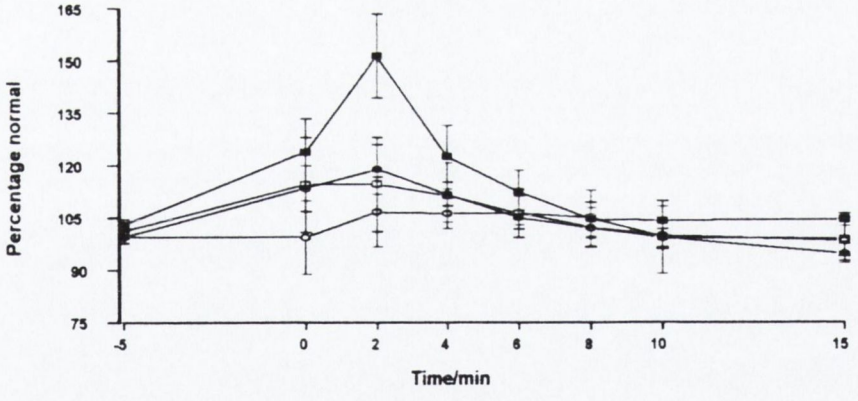


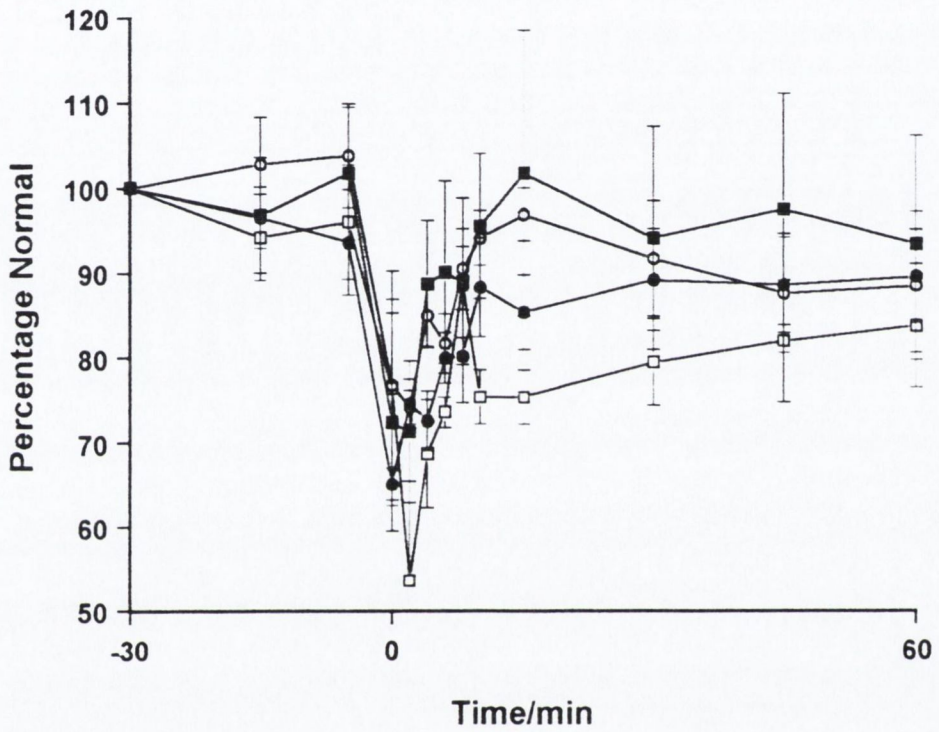
Figure 3.10a:- Rate of respiration in broncho-hyperreactive rats (Time -5 to 15min)



These figures illustrates the percentage change in BPM, on exposure to methacholine, in rats with hyper-reactive airways. Hyper-reactive airways were induced by repeated challenge to OVA prior to treatment with vehicle (group I & II) DSCG (Group III) and 3C8 (Group IV). This was carried out following sensitisation to the OVA using AlOH and FCA(T) as adjuvants. Data is represented as percentage normal and calculated as a percentage of the BPM noted 30 minutes prior to methacholine exposure. Figure 3.10a is a close up of the rate of respiration seen 5 minutes before and up to 15 minutes after OVA challenge.

—□— Group I (n=3) - untreated, unchallenged , Methacholine (8mg/ml) @ 0min.
—▶— Group II (n=5) - untreated, OVA challenged, Methacholine (8mg/ml) @ 0min.
—▶— Group III (n=4) - DSCG treated, OVA challenged, Methacholine (8mg/ml) @ 0min.
—▶— Group IV (n=6) - 3C8 treated, OVA challenged, Methacholine (8mg/ml) @ 0min.

Figure 3.11:- The change of pressure within the chamber during the breath cycle (ΔP_{bc}) for broncho-hyperreactive rats



The figure illustrates the percentage change in Pressure within the chamber during the breath cycle, on exposure to methacholine, in rats with hyper-reactive airways. Hyper-reactive airways were induced by repeated challenge to OVA prior to treatment with vehicle (group I & II) DSCG (Group III) and 3C8 (Group IV). This was done following sensitisation to the OVA using AlOH and FCA(T) as adjuvants. Data is represented as percentage normal and calculated as a percentage of the ΔP_{bc} noted 30 minutes prior to methacholine exposure

—□— Group I (n=3) - untreated, unchallenged , Methacholine (8mg/ml) @ 0min.
—►— Group II (n=5) - untreated, OVA challenged, Methacholine (8mg/ml) @ 0min.
—►— Group III (n=4) - DSCG treated, OVA challenged, Methacholine (8mg/ml) @ 0min.
—►— Group IV (n=6) - 3C8 treated, OVA challenged, Methacholine (8mg/ml) @ 0min.

3.3.3 Problems experienced with the in *vivo* respiratory model of asthma

Many problems were found with this experiment, the in vivo respiratory asthma model. It was because of these problems that the compounds 6C6 and 7C9 were not also tested. In this experiment a late phase response was detected. This response was seen approximately 2.5h following the challenging procedure. However other models designed by Wanner & Abraham (1982) and Wang et al 1986 also demonstrated an early phase response within minutes of OVA challenge. This response was believed to be as a result of mast cell degranulation and the release of histamine, tryptase and the synthesis and release of leukotrienes, prostaglandins and thromboxanes. It was not noticed in this study. It is possible that the effect of the nebuliser, which delivered OVA, masked the early phase response. At this point it cannot be clarified and can only be speculated that the animals experienced an early phase response. The masking effect of the nebuliser was also experienced in the measurement of the hyper-reactive response of the animals to metacholine. While the increase in the rate of respiration was detected no difference in ΔP was seen across the five groups. In contrast during the time of challenge the ΔP of all animals groups decreased highlighting the problems of the pressure in the chamber immediately following OVA challenge. A second problem was seen with the results. In an asthmatic attack the expected change in the breathing pattern would be an increase in BPM and a decrease in ΔP. The late phase respiratory pattern seen in this experiment was an increase in BPM however an increase in ΔP was also noticed. This would mirror rapid deep breaths in contrast to rapid shallow breaths normally seen in an attack. In the hyper-reactive response as induced by the challenging of hyper-sensitive animals to metacholine

an increase in BPM was again demonstrated however ΔP was not differentiated between groups.

3.3.4 Post Mortem of Groups I - IV

A post mortem was carried out on all of the rats in groups I - IV used in sections 3.3.1 & 3.3.2. The post mortem extended from the thoracic cavity to the abdominal cavity. The thoracic cavity appeared normal in all groups. Group I (negative control) and II (positive control) however, developed severe fibrosis of the liver and the gastrointestinal tract (GIT). The liver and the GIT had developed white adhesions. The lobes of the liver were inseparable and an increase of vascularity of this organ was also noted. Moreover, a build up of adipose tissue was found throughout the abdominal cavity. It is believed that this inflammatory response may have been caused by the sensitising procedure. As described earlier, in section 2.1.3, rats were sensitised by the injection (1ml *s.c.*) of OVA (1mg/ml) / aluminium hydroxide (AlOH) (200mg/ml) and Freund's complete adjuvant containing *Mycobacterium tuberculosis* (FCA(T)) (1ml *i.p.*).

Group III, the DSCG pre-treated group, developed fibrosis of the liver and some white adhesions appeared on the surface. However, in this case the inflammation was believed not to be as severe as all liver lobes were separable and distinguishable.

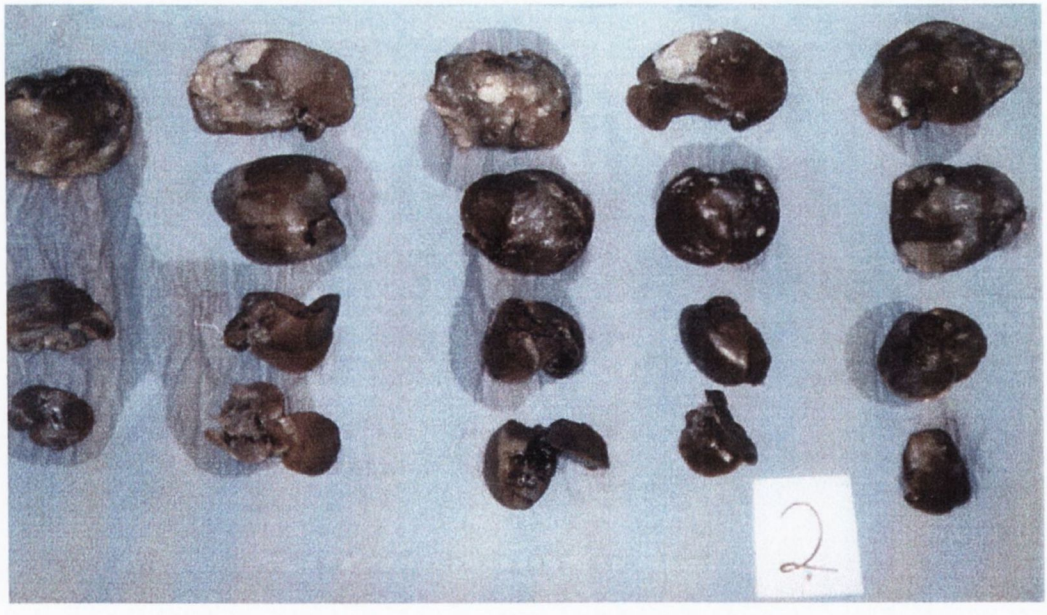
Post mortem studies on the abdominal cavities of group IV, the 3C8 pre-treated group appeared normal. Figure 3.12 a,b,c,d show the liver post mortems of groups I - IV respectively.

Figure 3.12(a)Livers excised post mortem from group I, untreated unchallenged, rats.

Treatment of animals:

1. Sensitised by the injection (1ml *s.c.*) of OVA (1mg/ml) / aluminium hydroxide (AlOH) (200mg/ml) and Freund's complete adjuvant containing *Mycobacterium tuberculosis* (FCA(T)) (1ml *i.p.*).
2. Three weeks following sensitisation animals treated with 50% ETOH (2 mins by aerosol) and challenged with the saline (2 mins by aerosol). This latter procedure was repeated weekly for a further 3 weeks.

Figure 3.12(b) Livers excised post mortem from group II, untreated OVA challenged, rats.



Treatment of animals:

1 Sensitised by the injection (1ml *s.c.*) of OVA (1mg/ml) / aluminium hydroxide (AlOH) (200mg/ml) and Freund's complete adjuvant containing *Mycobacterium tuberculosis* (FCA(T)) (1ml *i.p.*).

2 Three weeks following sensitisation animals treated with 50% ETOH (2 mins by aerosol) and challenged with the 5% OVA (2 mins by aerosol). This latter procedure was repeated weekly for a further 3 weeks.

Figure 3.12(c) Livers excised post mortem from group II, DSCG treated and OVA challenged, rats.

Treatment of animals:

1 Sensitised by the injection (1ml *s.c.*) of OVA (1mg/ml) / aluminium hydroxide (AlOH) (200mg/ml) and Freund's complete adjuvant containing *Mycobacterium tuberculosis* (FCA(T)) (1ml *i.p.*).

2 Three weeks following sensitisation animals were treated with DSCG (5mg/ml in ETOH 50% v/v; 2 mins by aerosol) and challenged with the 5% w/v OVA (2 mins by aerosol). This latter procedure was repeated weekly for a further 3 weeks.

Figure 3.12(d) Livers excised post mortem from group II, 3C8 treated and OVA challenged, rats.

Treatment of animals:

1 Sensitised by the injection (1ml *s.c.*) of OVA (1mg/ml) / aluminium hydroxide (AlOH) (200mg/ml) and Freund's complete adjuvant containing *Mycobacterium tuberculosis* (FCA(T)) (1ml *i.p.*).

2 Three weeks following sensitisation animals were treated with 3C8 (5mg/ml in ETOH 50% v/v; 2 mins by aerosol) and challenged with the 5% w/v OVA (2 mins by aerosol). This latter procedure was repeated weekly for a further 3 weeks.

3.4 Inflammation

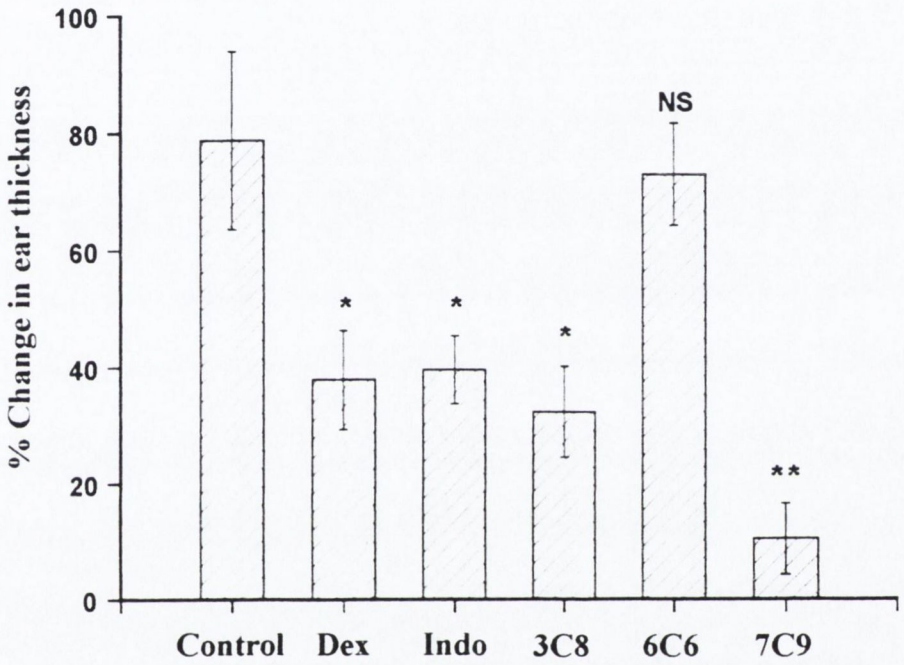
3.4.1 Acute Inflammation

3.4.1.1 The Effect of 3C8, 6C6 and 7C9 on Arachidonic Acid Induced Mouse Ear Oedema as measured by the change in ear thickness

The percentage change in ear thickness, mean ± sem, as induced by AA is shown in figure 3.13 and table 3.7 (Appendix 2). The solvent, drug or test compounds were applied to the same ear as AA 1h prior to application of AA, in order to investigate the change in inflammation. There were six groups, all of which were treated with AA (4mg / ear; applied only to one ear). The positive control was pre-treated with acetone, the solvent (n = 8). The second and third groups were pre-treated, with dexamethasone (n = 4) and indomethacin (n = 4) at 300μg/ear respectively. The other groups were pre-treated with their respective test compounds 3C8 (n = 4), 6C6 (n = 4), and 7C9 (n = 4) again at 300μg/ear.

A percentage change of 78.8 ± 15.2 in ear thickness was found on the application of AA in the positive control. This increase in ear thickness was found to be suppressed to a significant extent when ears were pretreated with either dexamethasone or indomethacin, $P \leq 0.05$. The test compounds, 3C8 and 7C9 also significantly reduced ear thickness, $P < 0.05$ and $P < 0.01$ respectively. However pretreatment with 6C6 had no effect on ear thickness induced by AA.

Figure 3.13:- The change of AA induced ear thickness by compounds 3C8, 6C6 and 7C9



The graph represents the percentage change in AA (4mg/ear)-induced mouse ear oedema as measured by ear thickness on pre-treatment with acetone (control; n=8), dexamethasone (300µg/ear; n=4). Indomethacin (300µg/ear; n=4), 3C8 (300µg/ear; n=4), 6C6 (300µg/ear; n=4) and 7C9 (300µg/ear; n=4)

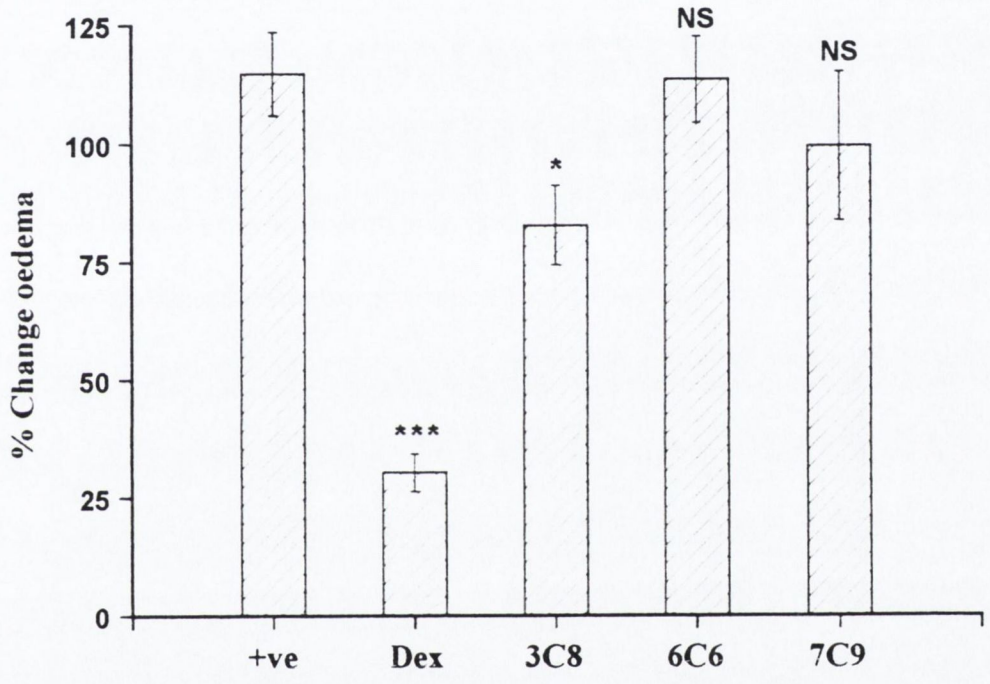
Statistical analysis included a Mann Whitney test, unpaired and one tailed.
* indicates $P \leq 0.05$ as compared to control
** indicates $P < 0.01$ as compared to control
NS indicates no significant difference as compared to control

3.4.1.2 The Effect of 3C8, 6C6 and 7C9 on TPA Induced Mouse Ear Oedema

The percentage change in oedema, mean ± sem, as induced by TPA is shown in figure 3.14 and table 3.8 (appendix 2). Five groups of animals were set up, group I, positive control, was pre-treated with acetone followed by TPA one hour later (n = 10). Group II was pre-treated with dexamethasone (10μg/ear; n = 10) followed by TPA two hours later. Group III (n = 5), Group IV (n = 5) and Group V (n = 5) were pre-treated with 3C8 (300μg/ear), 6C6 (300μg/ear) and 7C9 (300μg/ear) respectively followed by TPA one hour later. The solvent, drug or test compounds were applied by topical application to the same ear as TPA (1.5μg / ear) in order to investigate the change in inflammation. The change in oedema was determined six hours after the application of TPA.

A percentage change of 114.7 ± 8.8 ear punched weighed was found on the application of TPA in the positive control. This increase in ear weight was found to be suppressed significantly when ears were pre-treated with dexamethasone ($P<0.001$). The increase in ear weight was also found to be suppressed to a significant extent with the pre-treatment with, 3C8 ($P<0.05$). However pre-treatment with either 6C6 or 7C9 had no effect on the oedema induced by TPA

Figure 3.14:- The change of TPA induced ear oedema by compounds 3C8, 6C6 and 7C9



The graph represents the percentage change in TPA (1.5µg/ear)-induced mouse ear oedema with 1h pre-treatment with acetone (control; n=10), dexamethasone (10µg/ear; n=10), 3C8 (300µg/ear; n=5), 6C6 (300µg/ear; n=5) and 7C9 (300µg/ear; n=5).

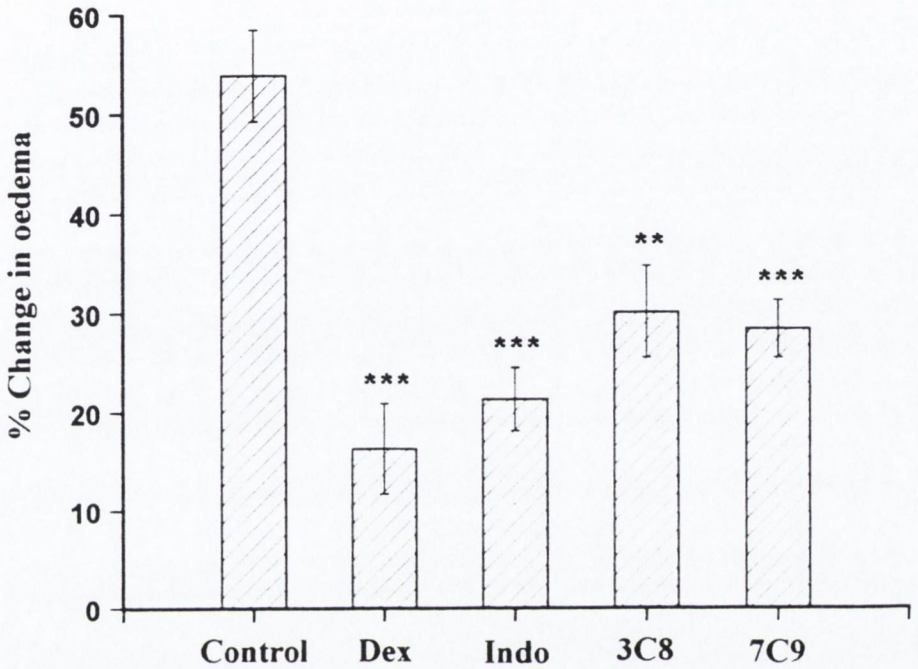
Statistical analysis included a Mann Whitney test, unpaired and one tailed.
*** indicates $P<0.001$ as compared to control
*indicates $P<0.05$ as compared to control
NS indicates no significance difference as compare to control

3.4.1.3 The Effect of 3C8 and 7C9 on Carrageenan Induced Rat Paw Oedema

The effect of 3C8 and 7C9 on a third acute inflammatory model was assessed using carrageenan induced rat paw oedema. The percentage change in rat paw oedema (mean ± sem) is shown in figure 3.15 and table 3.9 (appendix 2). There were five groups of female rats set up. Oedema was induced in all groups by injecting 100µl of carrageenan (1% w/v) in below the plantar aponeurosis of the right hind paw 1 h following treatment with vehicle, drug or test compound. Group I was the control group and was pre-treated with vehicle only (1% CMC; n = 15). Group II (30mg/kg; n = 6) and Group III (30mg/kg; n = 6) were drug control groups and were pre-treated with dexamethsone and indomethacin respectively. Group IV (30mg/kg; n = 6) and Group V (30mg/kg; n = 9) were test compound treated groups and were pre-treated with 3C8 and 7C9. Oedema was measured 180 minutes after induction.

The percentage change in paw volume as induced by carrageenan in the positive control was 53.9±4.6%. This increase was attenuated by both drug control groups dexamethasone (16.3±4.6%) and indomethacin (21.3±3.2%) $P<0.001$ & $P<0.001$ respectively. Both drugs 3C8 and 7C9 reduced oedema to 30.1±4.6% ($P<0.01$ as compared to control) and 28.4±2.9% ($P<0.001$ as compared to control) respectively.

Figure 3.15:- The effect of 3C8 and 7C9 on carrageenan induced rat paw oedema



The graph represents the percentage change in carrageenan (1% w/v) -induced rat paw oedema on pre-treatment with 1% CMC (control; n=15), dexamethasone (30mg/kg; n=6), indomethacin (30mg/kg; n=6) 3C8 (30mg/kg; n=6) and 7C9 (30mg/kg; n=9) 1h prior to the induction of oedema with carrageenan.

Statistical analysis included a Mann Whitney test, unpaired and one tailed.
*** indicates $P<0.001$ as compared to control
** indicates $P<0.01$ as compared to control

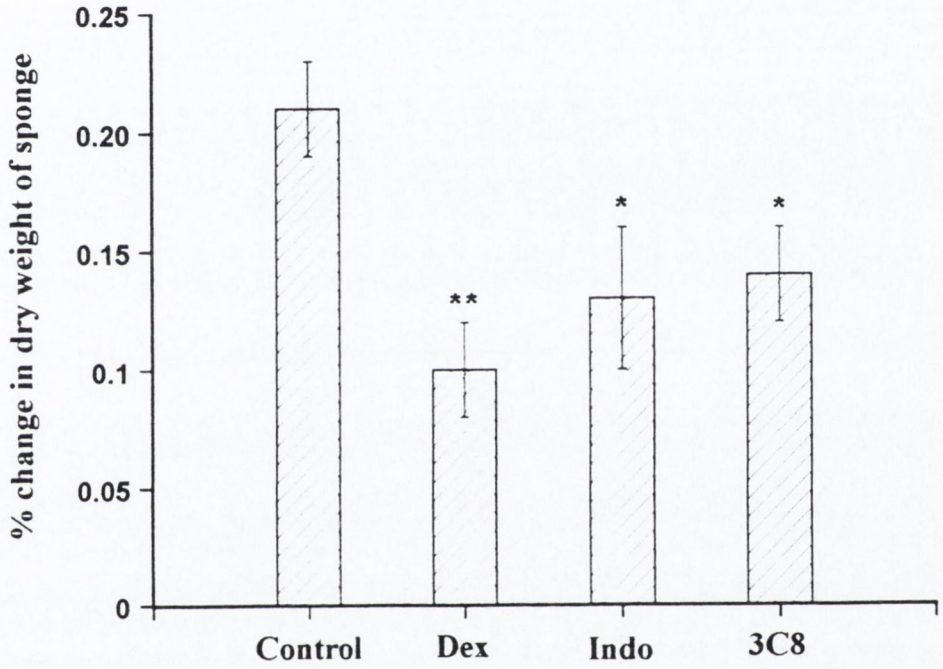
3.4.2 Chronic Inflammation

3.4.2.1 The Effect of 3C8 on Chronic Inflammation Induced by the Subcutaneous Implant of a Polyvinyl Sponge in Rat

The effect of 3C8 on a chronic model of inflammation was studied using the technique of polyvinyl sponge subcutaneous implants in the lower back of the rat. Four groups were set up and all groups were implanted with the sponge. All groups were treated with either vehicle, drug control or test compound. Group I (n = 6) was the positive control and was given the vehicle only, group II (n = 5) and III (n = 5) were the drug controls and were given dexamethasone (1mg/kg daily) and indomethacin (1mg/kg daily) and finally group IV, the test compound, was given 3C8 (1mg/kg daily).

The results are represented in grams as the mean ± sem change in dry weight of sponge before and after implantation (figure 3.16 and table 3.10 (Appendix 2)). The positive control showed an increase in weight of 0.21±0.02g. This increase was suppressed to a significant extent by dexamethasone and indomethacin, $P<0.01$ and $P<0.05$ respectively, as compared to control. The test compound 3C8 also showed a decrease in weight and was statistically significant from the positive control ($P<0.05$).

Figure 3.16:- The effect of 3C8 on a chronic model of inflammation as induced by sponge implants



The graph represents the percentage change oedema as measured by the change in sponge dry weight on daily treatment with ETOH (50%w/v; 1ml/kg) (control; n=6), dexamethasone (1mg/kg; n=5), indomethacin (1mg/kg; n=5), 3C8 (1mg/kg; n=4)

Statistical analysis included a Mann Whitney test, unpaired and one tailed.
** indicates $P<0.01$ as compared to control
* indicates $P<0.05$ as compared to control

3.5 Inflammatory Asthma - BAL

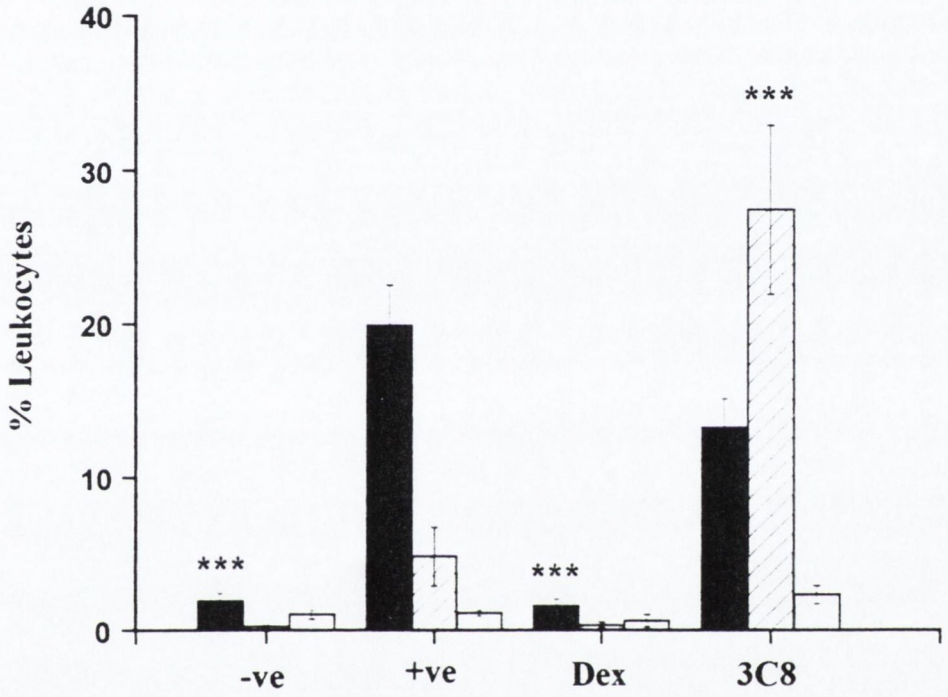
3.5.1 Acute Inflammatory Asthma

3.5.1.1 BN Rats Acutely Sensitised to OVA with AlOH as an Adjuvant

The leucocyte influx (mean ± sem of % leucocytes in total cell count) as seen in BAL fluid taken from BN rats acutely sensitised to OVA using AlOH as an adjuvant is illustrated on figure 3.17 and tabulated on Table 3.11 (Appendix 2). There are four groups, group I, the negative control (-ve ctrl; n=5) or untreated, unchallenged group, group II, the positive control (+ve ctrl; n=8) or untreated, OVA challenged group, group III, the dexamethasone (1mg/kg; n=4) treated, OVA challenged group and Group IV, the 3C8 (10mg/kg; n=5) treated, OVA challenged group.

A ten-fold increase in eosinophils was found on challenge with OVA. This increase was found to be statistically significant, $P<0.001$. Dexamethasone reduced the positive value to a value comparable to the negative control, $P<0.001$. The test compound, 3C8, however had no significant effect on the increase eosinophils seen on challenge with OVA. No statistically significant increase in neutrophil number was noted on OVA challenge. Pre-treatment with dexamethasone did not alter the neutrophil number, which was comparable to the negative and positive controls. However pretreatment with the test compound 3C8 before OVA challenge induced a significant increase in neutrophils in the BAL fluid ($P<0.001$). No change in basophil number was noted across the groups.

Figure 3.17:- Percentage of each type of leucocytes in BAL of BN rats acutely sensitised to OVA using AlOH as an adjuvant



This graph represents the percentage of each type leucocyte found in the BAL fluid of rats acutely sensitised and challenged to OVA using AlOH as an adjuvant on sensitisation.
■ = eosinophils; ▨ = neutrophils; □ = basophils
-ve ctrl:- Negative control (Group I; n=5), untreated and unchallenged.
+ve ctrl:- Positive control (Group II; n=8), untreated and OVA challenged.
Dex:- Group III, dexamethasone (1mg/kg; n=4) treated and OVA challenged.
3C8:- Group IV, 3C8 (10mg/kg; n=5) treated and OVA challenged.

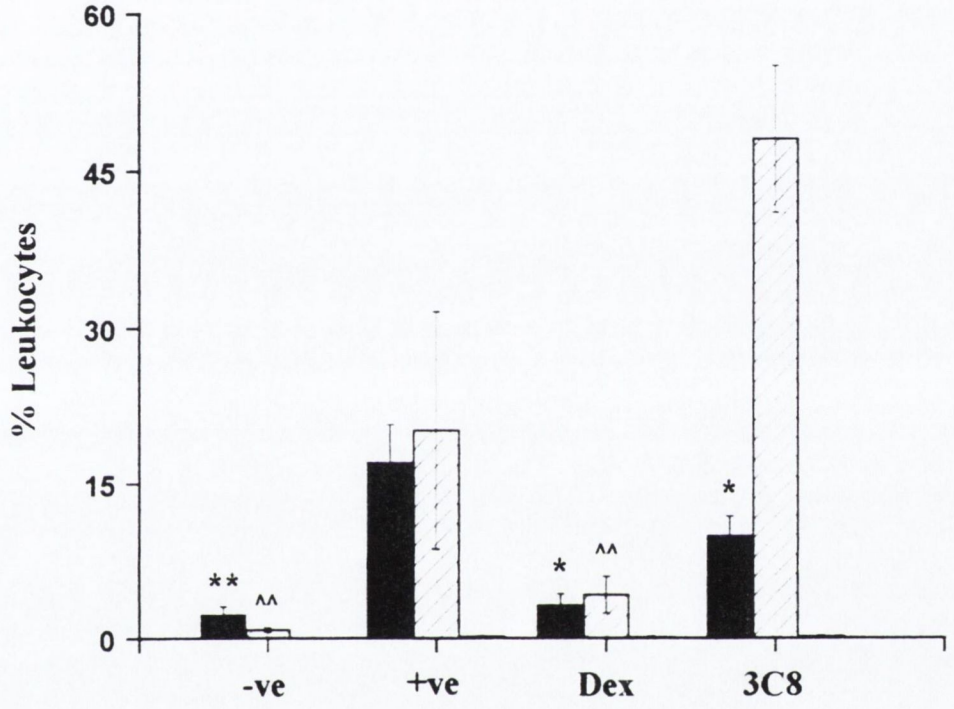
Statistical analysis included a one way ANOVA followed by Bonferroni t-test.
*** indicates $P<0.001$ as compared to the positive control for the respective leucocyte.

3.5.1.2 BN Rats Acutely Sensitised to OVA with FCA(T) as an Adjuvant

The leucocyte influx (mean ± sem of % leucocytes of total cell count) calculated from the BAL fluid taken from BN rats acutely sensitised to OVA using FCA(T) as an adjuvant is shown on figure 3.18 and tabulated on table 3.12 (Appendix 2). This experiment was essentially the same as that described in section 3.5.1.1 however the AlOH was omitted from the sensitising mixture and FCA(T) was the only adjuvant used. The four groups were Group I, the negative control (-ve ctrl; n=6) or untreated, unchallenged group, group II, the positive control (+ve ctrl; n=5) or untreated, OVA challenged group, group III, the dexamethasone (10mg/kg; n=5) treated, OVA challenged group and Group IV, the 3C8 (10mg/kg; n=5) treated, OVA challenged group.

A statistically significant increase in eosinophilia was found on challenge with OVA ($P<0.01$). An eight-fold increase was seen between negative control and positive control. Dexamethasone reduced the increase seen on OVA challenge, $P<0.05$. However, our test compound, 3C8 had no significant effect on inflammation seen in the positive control. A statistically significant increase in neutrophil number was not seen on OVA challenge in this model. However it was noted that pretreatment with 3C8 induced an increase as compared to negative control ($P<0.01$) and the dexamethasone pretreated group ($P<0.01$). It must be noted however that no statistical difference was found between 3C8 and the positive control. Basophil number did not increase on OVA challenge. Also, no increase in basophil number was found on pretreatment with either dexamethasone or test compound 3C8.

Figure 3.18:- Percentage of each type of leucocyte in BAL of BN rats acutely sensitised to OVA using FCA(T) as an adjuvant



This graph represents the percentage of each type of leucocyte found in the BAL fluid of rats acutely sensitised and challenged to OVA using FCA(T) as an adjuvant on sensitisation.
■ = eosinophils; ▨ = neutrophils; □ = basophils
-ve ctrl:- Negative control (Group I; n=6), untreated and unchallenged.
+ve ctrl:- Positive control (Group II; n=5), untreated and OVA challenged.
Dex:- Group III, dexamethasone (10mg/kg; n=5) treated and OVA challenged.
3C8:- Group IV, 3C8 (10mg/kg; n=5) treated and OVA challenged.

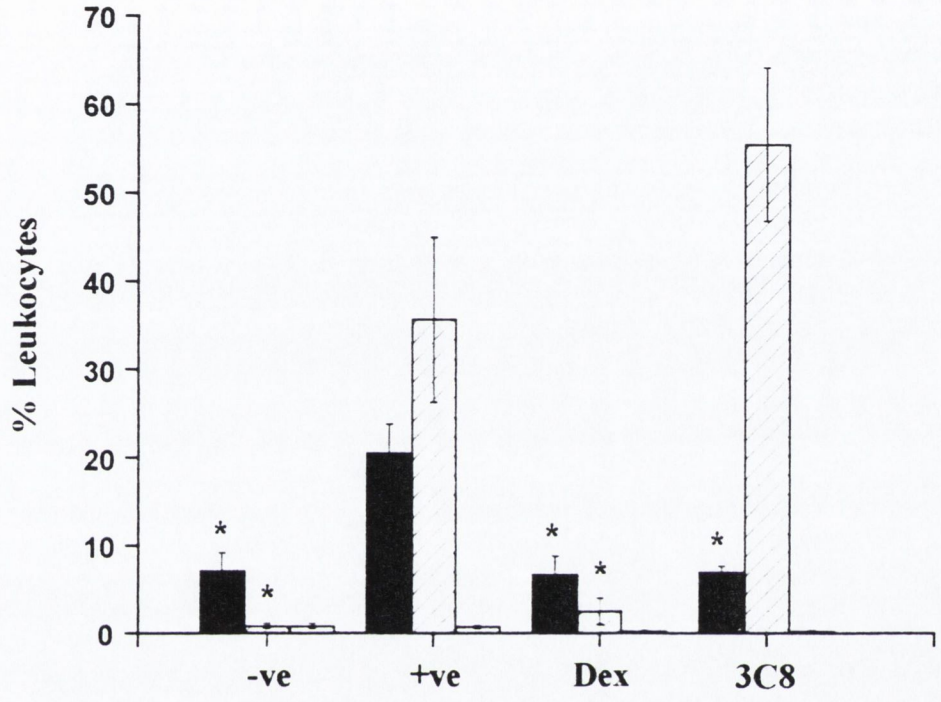
Statistical analysis included a one way ANOVA followed by Bonferroni t-test.
**indicates $P<0.01$ as compared to the positive control for the respective leucocyte.
* indicates $P<0.05$ as compared to the positive control for the respective leucocyte.
^^ indicates $P<0.01$ as compared to the 3C8 pretreated group for the respective leucocyte.

3.5.1.3 BN Rats Acutely Sensitised to OVA with AlOH and FCA(T) as Adjuvants

The leucocytes influx as seen in BAL fluid taken from BN rats acutely sensitised to OVA using both AlOH and FCA(T) as adjuvants is shown on figure 3.19 and table 3.13 (Appendix 2). These results are represented as mean ± sem. The four groups were, group I, the negative control (-ve ctrl; n=5) or untreated, unchallenged group, group II, the positive control (+ve ctrl; n=5) or untreated, OVA challenged group, group III, the dexamethasone (10mg/kg; n=5) treated, OVA challenged group and Group IV, the 3C8 (10mg/kg; n = 5) treated, OVA challenged group.

A two-fold increase in eosinophilia was seen on challenge with OVA. This increase between negative and positive controls was found to be statistically significant, $P<0.05$. Both dexamethasone and test compound, 3C8 reduced this increase to values comparable to the negative control, $P<0.05$. An increase in neutrophil number was also found on challenge with OVA. A significant P value ($P<0.05$) was calculated when comparisons were carried out between Group I and Group II. Pretreatment with dexamethasone was found to significantly reduce the increase in influx of neutrophils upon OVA challenge ($P<0.05$). 3C8 however did not show any inhibitory action on neutrophil influx. No increase in basophil number was seen on OVA challenge. This result is consistent throughout all groups where pretreatment with either dexamethasone or test compound, before OVA challenge, shows no significant change in basophil number found in BAL.

Figure 3.19:- Percentage of each type of leucocyte in BAL of BN rats acutely sensitised to OVA using AlOH and FCA(T) as adjuvants



This graph represents the percentage of each type of leucocyte found in the BAL fluid of rats acutely sensitised and challenged to OVA using AlOH and FCA(T) as adjuvants on sensitisation.
■ = eosinophils; ▨ = neutrophils; □ = basophils
-ve ctrl:- Negative control (Group I; n=5), untreated and unchallenged.
+ve ctrl:- Positive control (Group II; n=5), untreated and OVA challenged.
Dex:- Group III, dexamethasone (10mg/kg; n=5) treated and OVA challenged.
3C8:- Group IV, 3C8 (10mg/kg; n=5) treated and OVA challenged.

Statistical analysis included a one way ANOVA followed by Bonferroni t-test.
* indicates $P<0.05$ as compared to the positive control for the respective leucocyte.

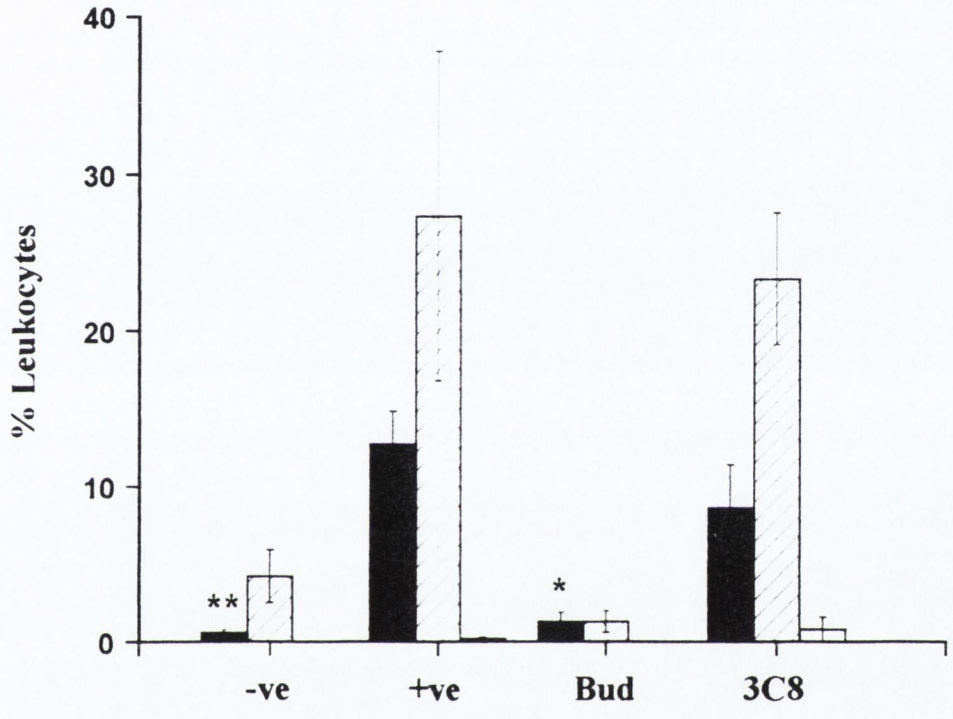
3.5.2 Chronic Inflammatory Asthma

3.5.2.1 BN Rats Chronically Sensitised to OVA with AlOH as an Adjuvant

The mean ± sem percentage leucocytes in the total cell count of BAL fluid, from OVA challenged, BN rats chronically sensitised to OVA using AlOH only as an adjuvant is shown on figure 3.20 and tabulated on table 3.14 (Appendix 2). Unlike the acutely sensitised BN rats described in sections 3.5.1.1, 3.5.1.2 and 3.5.1.3, these rats were chronically sensitised and repeatedly challenged with OVA over an eight-week period. Group I represents the negative control (-ve ctrl; n=5) untreated, unchallenged group. Group II represents the positive control (+ve ctrl; n=5) untreated, OVA challenged group. Group III represents the budesonide (10mg/kg; n=4) treated and OVA challenged group. Group IV represents the 3C8 (10mg/kg; n=4) treated and OVA challenged group.

On challenge with OVA a twenty-fold increase in eosinophilia was demonstrated. The increase in eosinophilia was found to be statistically significant, $P<0.01$. Budesonide was found to reduce this increase down to values comparable to negative values ($P<0.05$). However, 3C8 had no significant effect on OVA challenging. No significant increase in either neutrophil or basophil number was found on OVA challenge. Moreover no change was noted in neutrophils or basophils when pre-treated with budesonide or the test compound 3C8 prior to OVA challenge.

Figure 3.20:- Percentage of each type of leucocyte in BAL of BN rats chronically sensitised to OVA using AlOH as an adjuvant



This graph represents the percentage of each type of leucocyte found in the BAL fluid of rats chronically sensitised and challenged to OVA using AlOH as an adjuvant on sensitisation.
■ = eosinophils; ▨ = neutrophils; □ = basophils
-ve ctrl:- Negative control (Group I; n=5), untreated and unchallenged.
+ve ctrl:- Positive control (Group II; n=5), untreated and OVA challenged.
Bud:- Group III, budesonide (10mg/kg; n=4) treated and OVA challenged.
3C8;- Group IV, 3C8 (10mg/kg; n=4) treated and OVA challenged.

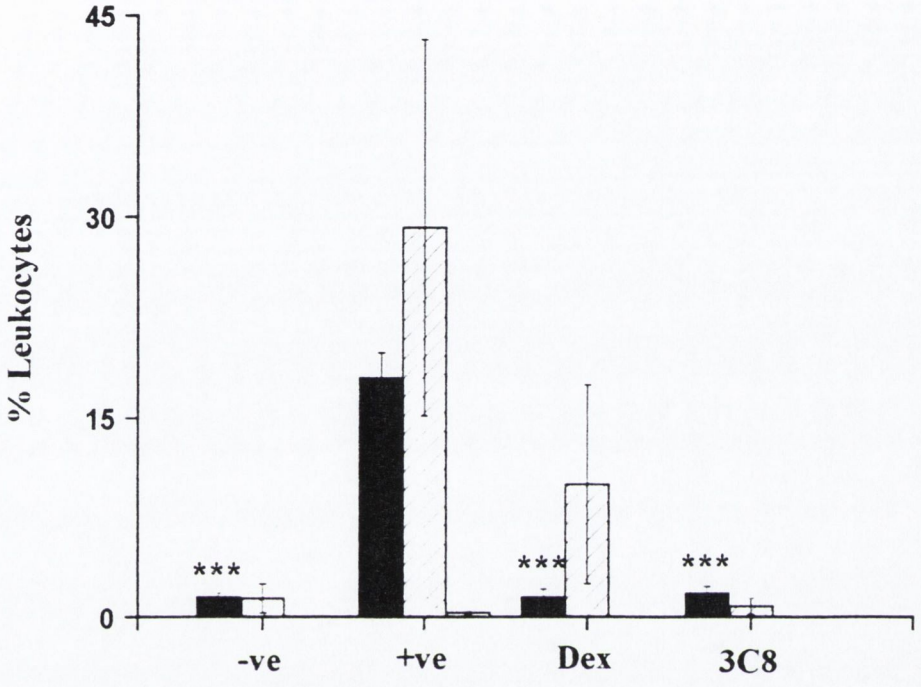
Statistical analysis included one way ANOVA with Bonferroni t-test.
** indicates $P<0.01$ as compared to the positive control for the respective leucocyte.
* indicates $P<0.05$ as compared to the positive control for the respective leucocyte.
Note:- The above figure includes the final pre-treatment and OVA challenge. A middle, 4 week vehicle/drug pre-treatment and OVA challenge was also carried out (section 2.1.6.2 (a))

3.5.2.2 BN Rats Chronically Sensitised to OVA with AlOH and FCA(T) as Adjuvants

The mean ± sem percentage leucocytes in total cell count of BAL in BN rats chronically sensitised to OVA using both AlOH and FCA(T) as adjuvants are shown in figure 3.21 and tabulated on table 3.15 (Appendix 2). The study was essentially as described in section 3.5.2.1, however AlOH and FCA(T) were used as adjuvants. Group I represents the negative control (-ve ctrl; n=5) untreated, unchallenged group. Group II represents the postitive control (+ve ctrl; n=5) untreated, OVA challenged group. Group III represents the dexamethasone (10mg/kg; n=5) treated and OVA challenged group. Group IV represents the 3C8 (10mg/kg; n=5) treated and OVA challenged group.

A ten fold increase in eosinophilia was seen on challenge with OVA. This increase was found to be statistically significant, $P<0.001$. Treatment with both dexamethasone and 3C8 reduced this increase with a statistical significance of $P<0.001$ in both cases. No significant change, in either neutrophil or basophil numbers was seen on OVA challenge. The same result was noted on pre-treatment with either dexamethasone or test compound before OVA challenge.

Figure 3.21:- Percentage of each type of leucocyte in BAL of BN rats chronically sensitised to OVA using AlOH and FCA(T) as adjuvants



This graph represents the percentage of each type of leucocyte found in the BAL fluid of rats chronically sensitised and challenged to OVA using AlOH and FCA(T) as adjuvants on sensitisation.
■ = eosinophils; ▨ = neutrophils; □ = basophils
-ve ctrl:- Negative control (Group I; n=5), untreated and unchallenged.
+ve ctrl:- Positive control (Group II; n=5), untreated and OVA challenged.
Dex:- Group III, dexamethasone (10mg/kg; n=5) treated and OVA challenged.
3C8:- Group IV, 3C8 (10mg/kg; n=5) treated and OVA challenged.

Statistical analysis included one way ANOVA with Bonferroni t-test.
*** indicates $P<0.001$ as compared to the positive control for the respective leucocyte.
Note:- The above figure includes the final pre-treatment and OVA challenge. A middle, 4 week vehicle/drug pre-treatment and OVA challenge was also carried out (section 2.1.6.2 (b))

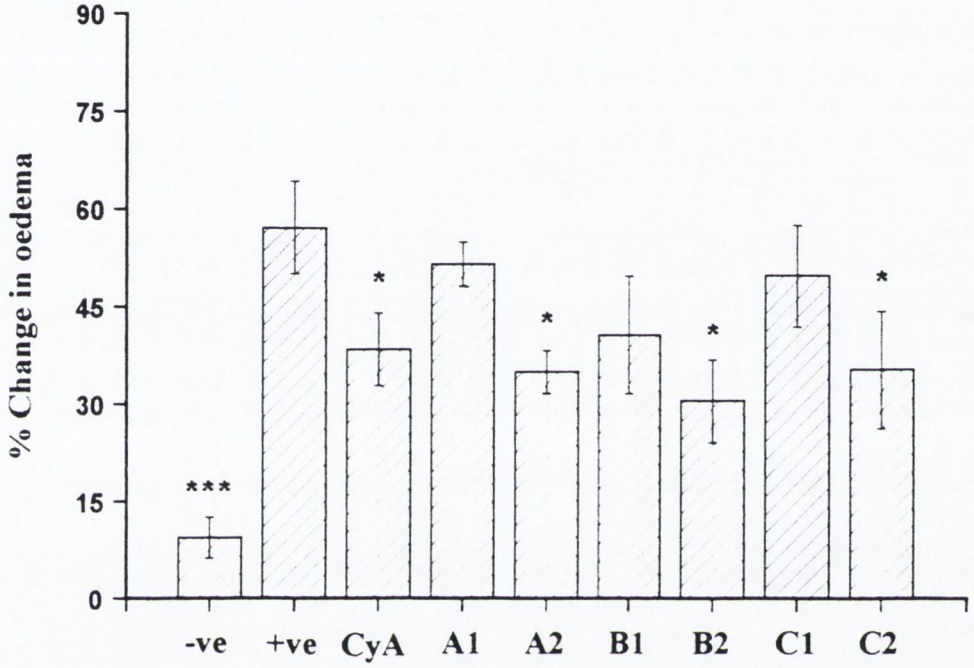
3.6 Delayed Type Hypersensitivity

3.6.1 The Effect of 3C8, 6C6 and 7C9 on a Delayed-Type Hypersensitivity Model in Mice

The percentage change in paw swelling of mice challenged with mBSA is illustrated in figure 3.22 (table 3.16 Appendix 2). All mice, excluding the negative control, were previously sensitised to mBSA using FCA(B) as an adjuvant, prior to challenge with mBSA. The negative control was sensitised with the adjuvant FCA(B) only. Both the negative control group and the positive control group were untreated (1% CMC) and mBSA challenged. The other groups were all pretreated with either drug or test compound at indicated concentrations before they were challenged with mBSA.

The negative control group (n = 12), which was not sensitised to mBSA shows the percentage inflammation caused by mBSA challenge alone. The percentage change in inflammation of the negative control was 9.4 ± 3.2%. The positive control (n = 12) was sensitised to mBSA and as a result developed a six fold increase in paw swelling on challenge with mBSA as compared to negative control, $P<0.001$. Pre-treatment with cyA (50mg/kg; n = 12) resulted in a 20% decrease of paw oedema to that observed in the positive control group of mice, $P<0.05$. Pre-treatment with test compounds 3C8, 6C6 and 7C9 (10mg/kg; n=6)) also resulted in a 20% decrease of paw oedema when compared to the positive control group of mice, $P<0.05$. Lower concentrations of each of these test compounds showed no statistical difference from the positive control group of mice.

Figure 3.22:- The change of mBSA induced paw oedema (DTH) by compounds 3C8, 6C6 and 7C9



The graph represents the percentage change in mBSA-induced mouse paw oedema (DTH) on treatment with 1% CMC (+ve control; n=12), cyA (50mg/kg; n=12), 3C8 (A1, 3mg/kg (n=5) A2, 10mg/kg (n=6)), 6C6 (B1, 3mg/kg (n=6) B2, 10mg/kg (n=6)), 7C9 (C1, 3mg/kg (n=6) C2, 10mg/kg (n=6)),). A negative control (-ve n=12) was carried out to sow background inflammation not releated to mBSA sensitisation.

Statistical analysis included a Mann Whitney test, unpaired and one tailed.
*** indicates $P<0.001$ as compared to the positive control
* indicates $P<0.05$ as compared to the positive control

Chapter 4

Discussion

Contents **Page No.**

Contents **Page No.**

4. Discussion

Pterosin Z and acetylpterosin Z are natural products isolated form the fern, *Pteridium aquilinimu*. These products have been shown to have smooth muscle relaxant properties (Sheridan *et al* 1999). It is thought that the smooth muscle relaxant activity is due to the inhibition of calcium influx or possibly the inhibition of the calmodulin, which would prevent the formation of the calcium/calmodulin complex and the contractile response.

Initial in-house studies revolved around the development of a synthetic route for the manufacture of these compounds and their derivatives, with the aim of discovering a potent smooth muscle relaxant. An *in vitro* screening program looking at over 200 compounds highlighted three, 3C8, 6C6 and 7C9, with potential smooth muscle relaxant activity and also mast cell stabilisation activity. The latter *in vitro* study was carried out because calcium mobilisation and the calcium/calmodulin complex also play a pivotal role in mast cell degranulation. The study was therefore designed to look at the effects of the compounds on compound 48/80 induced rat peritoneal mast cell degranulation. (section 1.1.1.1(b)). Compounds with activity against both smooth muscle contraction and mast cell degranulation may potentially act as bronchodilators and mast cells stabilisers in asthma. In asthmatics mast cell degranulation occurs following exposure to a particular antigen. This in turn leads to the release of preformed and newly synthesised mediators, which induce bronchoconstriction of the airway smooth muscle (sections 1.1.1.1 (b), (c), (d), & (e)). It is believed that the process of mast cell degranulation and subsequent smooth muscle contraction in the

airways play a detrimental role in an asthma attack and hence compounds that prevent both these actions would be invaluable.

The aim of this study was to carry out a pre-clinical evaluation of the three novel compounds 3C8, 6C6 and 7C9. The studies set up focused initially on the potential properties of these drugs to act against extrinsic (T_H2 response) and/or intrinsic (T_H1 response) asthma. Results from these asthmatic models were extremely informative and also directed the research to acute and chronic inflammation and DTH (T_H1 response). The aim of this chapter will be to interpret the action(s) of the compounds using the data at hand and also to give direction to the project with a view to elucidating the exact mechanism of action of the compounds in the models.

4.1 The effects of 3C8, 6C6 and 7C9 on a $CaCl_2$ induced contraction of K^+ depolarised guinea-pig ileum smooth muscle

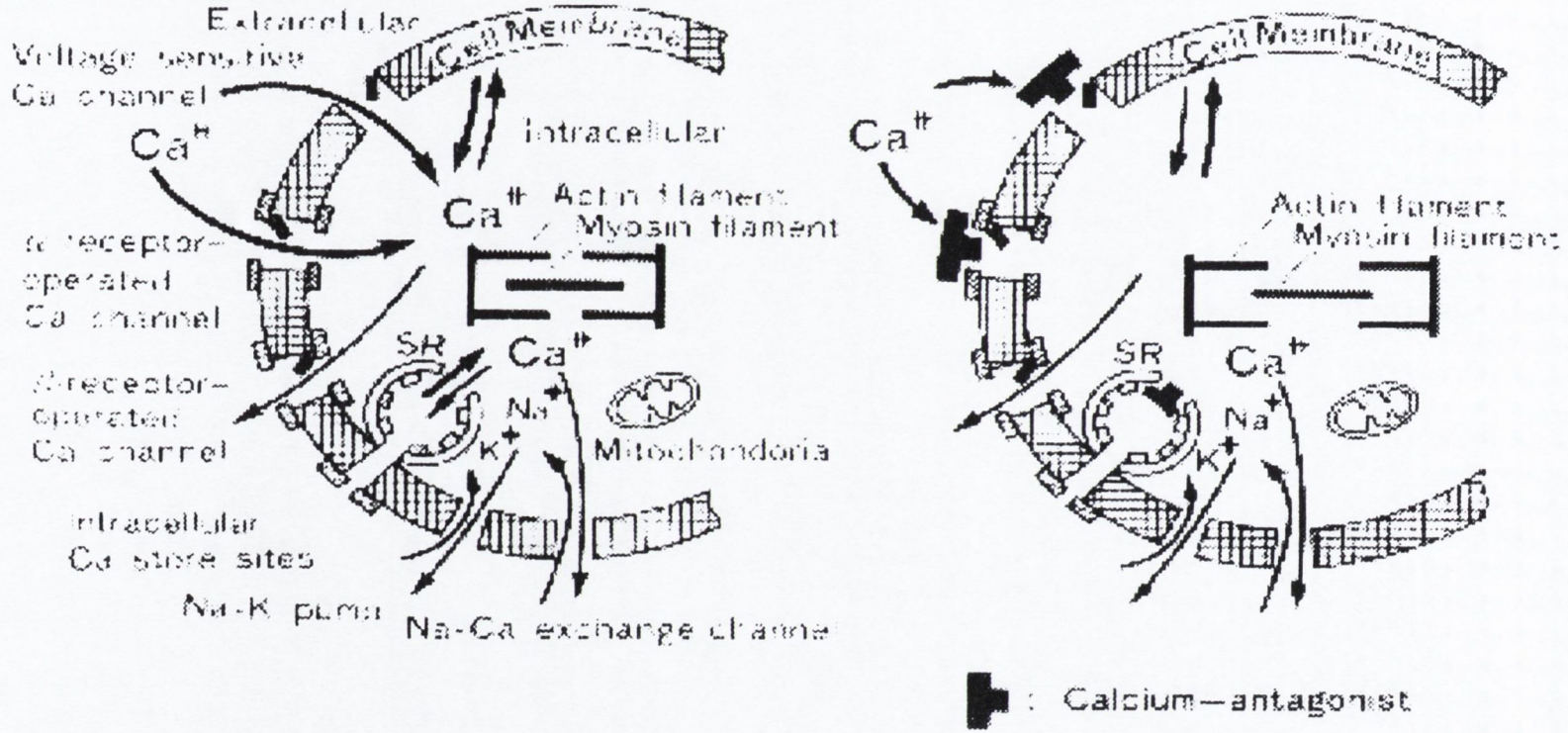
The effects of the test compounds 3C8, 6C6 and 7C9 on $CaCl_2$ induced contraction of K^+ depolarised male guinea-pig ileum smooth muscle was investigated. Smooth muscle cells contain a number of receptors each governing the physiology of the cell with regards to uptake and output of ions such as Ca^{2+}, Na^+, K^+ among others. Each of these ions contributes to the membrane potential of the cell, which under normal physiological conditions is between -45 and -60mV. The receptors involved in maintaining this membrane potential include the VOC, a Ca^{2+} channel, the α-ROC, the β-ROC, Na-K pump, the Na-Ca exchange channel and finally the calcium channel on the SR membrane. A clear diagram illustrating the physiological state of the

smooth muscle membrane is shown in figure 4.1 (Aoki & Sato, 1995; Rodger, 1992; Foster *et al* 1984; Ahmed *et al* 1985).

The potential smooth muscle relaxant properties of the test compounds were investigated. Guinea-pig ileum was suspended in high potassium no calcium buffer, a buffer, which induces depolarisation of the cell (a decrease in membrane potential). Depolarisation would immediately induce the opening of the voltage operated calcium channels and the addition of calcium (2.5mM) to the preparation would result in immediate uptake of calcium, the formation of the calcium/calmodulin complex and contraction of the smooth muscle through the activation and cross bridging of actin and myosin.

The addition of calcium chloride to guinea-pig ileum set up in a physiological environment (95% O_2 : 5% CO_2 at 37°C) in K^+ depolarising medium induced contractions of between 1.5g and 2.5g. Calcium-induced contractions have been previously demonstrated using K^+ depolarising guinea-pig ileum smooth muscle preparations (Mahmoudian *et al* 1997; Staneva-Stoytcheva & Venkova, 1992). Cumulative, half log-dose, concentrations of nifedipine (10^{-10}M to 10^{-7}M), 3C8 ($3x10^{-9}$M to 10^{-5}M), 6C6 ($3x10^{-8}$M to 10^{-5}M) and 7C9 ($3x10^{-6}$M and 10^{-5}M) were added to the calcium-contracted preparation to investigate their smooth muscle relaxant properties.

Figure 4.1:- Physiological scheme of smooth muscle



Aoki & Sato, 1995

The positive control nifedipine (10^{-7}M) completely reversed the calcium-induced contraction. 3C8 ($3x10^{-6}$M and 10^{-5}M) reduced the contraction by 22.2 ± 4.3% and 53.4 ± 3.6% respectively. 6C6 ($3x10^{-6}$M and 10^{-5}M) proved to be less effective and reduced the contraction by 12.6 ± 3.6% and 23.0 ± 4.3% respectively. The third compound 7C9 reduced the contraction by only 11.6 ±4.9% at a concentration of $3x10^{-6}$M and 15.4 ± 4.6% for 10^{-5}M.

Of the three test compounds 3C8 (10^{-5}M) appears to be the most potent inhibitor of calcium contracted in K^+ depolarised guinea-pig ileum smooth muscle. However this did not compare to the potent activity of nifedpine where 100% inhibition was noted at concentrations of 10^{-7}M. Nifedipine (EC_{50} = 10^{-8}M) was seen to be 1000 times more potent that 3C8 (EC_{50} = 10^{-5}M) (figure 3.2). Previous studies on calcium antagonists have proven verapamil and nifedipine to be potent inhibitors, of the calcium induced contraction in K^+ depolarised smooth muscle. These studies showed inhibition of calcium chloride induced contraction by both verapamil and nifedipine in both guinea-pig ileum and trachealis smooth muscle. In guinea-pig ileum the EC_{50} of verapamil was $2x10^{-6}$M with $CaCl_2$ and KCl concentrations of 2.5mM and 30mM respectively (Staneva-Stoytcheva & Venkova, 1992). Nifedipine ($2x10^{-9}$M) significantly inhibited guinea-pig ileum $CaCl_2$ (3mM) induced contraction in K^+ depolarising medium by 10% (Mahmoudian *et al* 1997). In addition, in a similar experiment, the EC_{50} of nifiedpine with 2.5mM $CaCl_2$ was shown to be 10^{-8}M (Sheridan *et al* 1999). In guinea-pig trachealis smooth muscle, maximum contractions o, K^+ depolarised tissue were seen on addition of 1mM and 10mM concentrations of $CaCl_2$. This contraction was reduced, at both calcium concentrations, by 60% with

nifedipine (10^{-9}M-10^{-6}M) and 40% with verapamil (10^{-4}M-10^{-5}M) (Ahmed *et al* 1985; Ahmed *et al* 1984).

Smooth muscle contraction has previously been shown to be reversed by verapamil and nifedipine (Mahmoudian *et al* 1997; Staneva-Stoytcheva & Venkova; Ahmed *et al* 1985; Ahmed *et al* 1984). Figure 4.1 shows the points of action of these calcium antagonists, namely, the VOC, the α-ROC and the calcium channel located on the SR. Relaxation of the smooth muscle is seen as the intracellular [Ca^{2+}] decreases. Depletion of intracellular calcium is due to both the prevention of entry of the ion and also to the loss of the ion through the β-ROC and Ca-Na exchange channels which are unaffected by the drugs.

A further mechanism of smooth muscle relaxation is *via* inhibition of calmodulin. Formation of the calcium/calmodulin complex upon increase in intracellular calcium is imperative in the steps leading to the activation and cross-linkage of actin and myosin (section 1.1.1.1(e)). Calmidazolium and trifluoperazine are calmodulin inhibitors and have been shown to decrease calcium contraction in K^+ depolarised rat vas deferens (Nahazawa *et al* 1993).

It is clear from these results that the test compounds particularly 3C8 have potential smooth muscle relaxant properties in K^+ depolarised guinea-pig ileum smooth muscle. It is possible that, like nifedipine and verapamil, the test compounds may prevent the increase of intracellular calcium by blocking the calcium channels as outlined in figure 4.1. It is also possible that the compounds act similarly to calmidazolium and trifluoperazine by inhibiting the calmodulin protein, the formation of the

calcium/calmodulin complex and the activation and cross-linkage of actin and myosin, the contractile proteins.

Although the exact mechanism of the test compounds on smooth muscle relaxation is undefined at present, from these results it is conceivable to believe that the compounds may also have mast cell stabilising activity. Mast cell degranulation has been described in detail in section 1.1.1.1(b) and occurs following antigen challenge or stimulation with compound 48/80. Like with smooth muscle contraction, calcium mobilisation and the calcium/calmodulin complex play an imperative role in mast cell degranulation. It is probable therefore that the test compounds may also act as anti-inflammatories preventing the degranulation of mast cells.

4.2 The effects of 3C8, 6C6 and 7C9 on compound 48/80-induced rat peritoneal mast cell degranulation

Mast cells play a pivotal role in asthmatic attacks. They can be found in the peritoneal cavity, skin and lung among other sites. Activation occurs following FcεRI receptor aggregation and is induced by antigen challenge or in *in vitro* studies by non-immunological stimuli such as compound 48/80 and substance P (McCloskey, 1993; Wu *et al* 1993; Mousli *et al* 1991; Shefler *et al* 1998; Senyshyn *et al* 1998). Mast cell activation by antigen or compound 48/80 results in an increase of intracellular calcium from the SR and extracellular influx of the same ion, through the CRAC channels. An increase in intracellular calcium leads to the formation of the calcium/calmodulin complex and the subsequent activation of a calcium sensitive degranulation protein and degranulation of the mast cell (Wilson *et al* 1991; Grosman, 1992; Chakravarty,

1992; Benhamou *et al* 1990; Benhamou & Siraganian, 1992; Scharenberg & Kinet, 1994; Hamawy *et al* 1995; Suzuki *et al* 1997; Wu *et al* 1993; Mousli *et al* 1991; Shefler *et al* 1998; Senyshyn *et al* 1998; Barnes *et al* 1991). Upon activation the pre-formed mediators such as histamine and tryptase are released and synthesis and release of newly formed mediators, leukotrienes and prostaglandins begin (Barnes, 1991; Turner & Dollery, 1988; Holgate *et al* 1986; Biggs, 1984; Mauser *et al* 1990; Tan & Spector 1997, Barnes, 1991, Lazarus, 1998, Chabot-Fletcher *et al* 1995, Bell *et al* 1997). Both the pre-formed and newly synthesised mediators are involved in the pathogenesis of asthma through bronchoconstriction of the airways and chemotaxis of inflammatory mediators.

An experiment to investigate the potential mast cell stabilisation properties of our three test compounds was set up using compound 48/80 (2μg/ml) as a non-immunological stimulus for mast cell activation and histamine as a marker for the degranulation. 3C8 appears to inhibit calcium-induced contraction in guinea-pig ileum therefore this compound may also potentially prevent mast cell degranulation where an increase in intracellular calcium, the formation of the calcium/calmodulin complex and the subsequent activation of the degranulating protein in mast cells play important roles.

Stimulation of female peritoneal Wistar rat mast cells resulted in a 75-80% and 59-66% release of histamine for dH_2O and DMSO pre-treated cells respectively. Previous studies on mast cells support these findings. Studies were carried out on human skin and rat Wistar and Sprague Dawley peritoneal mast cells. Concentrations of 1μg/ml and 10μg/ml of compounds 48/80 resulted in a 65% increase in histamine

release from purified rat peritoneal mast cells (Atkinson *et al* 1979). Previous studies on non-purified rat peritoneal mast cells show a similar result, where compound 48/80 (1μg/ml) gave a 52.5±1.7% increase in histamine release (Loeffler *et al* 1971). This experiment is comparable to the experiment carried out in this study and verifies the opinion that a consistent and uniform response from mast cells can be achieved without purification of the cells through a density gradient extraction method, which would, if used, significantly reduce the total number of mast cell harvested.

In this study pre-treatment with DSCG ($2x10^{-5}$M or 10μg/ml) showed a 20% inhibition of mast cell degranulation as induced by compound 48/80 and significantly reduced the release of histamine as compared to the maximum pre-treated with dH_2O. Previous studies using compound 48/80, as a mast cell stimulant, showed similar inhibition of 10-20% by DSCG (10^{-7} to 10^{-5}M) (Tanizaki *et al* 1992). In contrast, the same concentration range of DSCG resulted in 50% inhibition of antigen-induced rat peritoneal mast cell degranulation (Tanizaki *et al* 1992). Further studies support the more potent effect of DSCG in antigen-stimulated models. DSCG (0.18 to 18μg/ml) was a more effective inhibitor of mast cell degranulation when DNP-EA, was used as the antigen in previously sensitised rat peritoneal mast cells (Butchers *et al* 1979). DSCG was shown to inhibit histamine release in a dose-dependent manner (EC_{50} 4.8μg/ml).

The exact mechanism of action of DSCG upon mast cells is as yet unknown however it has been suggested that it prevents chloride influx. This may explain the variations in potency of DSCG in the antigen stimulated and compound 48/80 stimulated mast cells. Chloride influx is important in hyperpolarizing the cell to allow for further

influx of calcium in antigen stimulated mast cells, however compound 48/80 appears to have minimal effects on the chloride ion influx (Redrup *et al* 1997; Alton & Norris 1996). It should also be noted that the mechanisms outlined for degranulation with antigen and compound 48/80 vary. While antigen stimulation is due to cross linkage of receptors, compound 48/80 is thought to act through activation of the heterotrimeric GTP-binding proteins. The final outcome however is similar where PLCγ is activated and intracellular calcium concentrations increase. It has also been demonstrated, in human skin mast cells, that although the levels of histamine release is similar in the two models outlined, the concentration of leukotrienes and prostaglandins is considerably lower with compound 48/80 activation than that seen with antigen activation (Benyon *et al* 1989).

Pre-treatment of rat peritoneal mast cells with 3C8 ($2x10^{-5}$M) and 6C6 ($2x10^{-5}$M) resulted in marked inhibition of histamine release induced by compound 48/80. The concentration was comparable to basal levels of histamine release from the cells. A 75% reduction of histamine release was seen with pre-treatment with both these compounds when compared to maximum, which was pre-treated with solvent alone, DMSO (0.5%).

The exact mechanisms of these drugs are at this point is difficult to decipher. Since 3C8 has been shown to inhibit calcium-induced contraction of depolarised guinea-pig ileum smooth muscle (section 4.1) it may inhibit mast cell degranulation in the same manner. This compound may act either as a calcium antagonist preventing the influx of calcium or *via* the inhibition of calmodulin and the formation of the calcium/calmodulin complex. This however does not explain the fact that 6C6

potently inhibits mast cell degranulation while having minimal effect on smooth muscle relaxation. While the different outcome may be related to differences in species and tissues involved in the experiments we cannot assume that the mechanisms of action of the two compounds are the same. Moreover, we cannot assume that these compounds act in a similar fashion to DSCG, as it appears that chloride channel blockers do not play a significant role in mast cell degranulation when compound 48/80 is used as an activator of these cells. In contrast 7C9 failed to cause a significant reduction in histamine from mast cells when the cells were treated with this compound prior to stimulation with compound 48/80.

A second experiment was attempted to measure mast cell degranulation using LTC_4 as a marker (data not available). As discussed earlier in this section leukotrienes are synthesised and subsequently released upon mast cell degranulation. An enzyme immunoassay (EIA) specific for the detection of mouse LACA luekotrienes was attempted. The assay was based on the competition between unlabelled LTC_4 (sample/standard) and a fixed quantity of peroxidase labelled LTC_4 for a limited number of sites on the peptido leukotriene specific antibody. With fixed amounts of antibody and peroxidase labelled LTC_4 the amount of peroxidase labelled ligand bound to the antibody was inversely proportional to the concentration of added unlabelled ligand, sample/standard.

The mouse mast cell degranulation procedure was set up as described for rat mast cell degranulation with a few minor modifications. The isolation of mast cells was as described in section 2.1.2.1 with the exception of the volume (3ml) of BSS injected into the mouse abdomen and the number of animals used to isolate a single sample. In

order to obtain a large sample of mast cells, the isolated mast cells of 4 female LACA mice were pooled together and utilised in mast cell incubation with compound 48/80. The mast cell incubation was as described in section 2.1.2.2 section with three incubation tubes, basal, maximum and DSCG ($2x10^{-5}M$) treated tube, set up exactly as outlined earlier. The concentration of compound 48/80 utilised to induced mast cell degranulation was higher than described previously with 25µl of 0.5mg/ml solution added to give a final concentration of 5µg/ml. The time-plan for incubation of mast cells with compound 48/80 was also modified, where aliquots of 0.5 mls were taken 135 and 225 min following the addition of compound 48/80. All samples were placed immediately on ice in order to terminate the experiment. All samples were subsequently stored at -20°C until analysis for LTC_4 was carried out.

A pilot LTC_4 standard curve was set up using a small number of standard concentrations, 0pg/ml, 0.75pg/ml, 12pg/ml 48pg/ml (n=1). This standard curve formed a linear curve when the %tracer bound to the antibody was plotted against the Log_{10} of concentration of LTC_4. Results from the basal, maximum and the DSCG treated samples were not however as expected. While it appears that some LTC_4 was synthesised from the mast cells at both time points, no significant difference was seen between the groups. Release of LTC_4 was the same for all groups basal, maximum and DSCG pre-treated groups, at 135 min. At the second time point 225 min a higher increase in LTC_4 was demonstrated however again a similar trend was seen between groups where no significant difference was seen between basal, maximum and DSCG treated cells.

It has been reported that LTC_4 is synthesised and released within a 5-15 minutes of mast cell degranulation in anti-IgE induced IgE sensitised mast cell degranulation (Kay. 1991, Krishna et al 1996, Lazarus, 1998). At present little evidence has been reported of compound 48/80 induced synthesis of LTC_4 or any other newly synthesised mediator in human mast cells (Gibbs et al 1999, Church & Clough 1999). Benyon *et al* 1989 however did report minute prostaglandin and leukotriene release from mouse mast cell with compound 48/80 as the stimulant. While the amount of histamine released was similar to that of anti-IgE stimulated mast cell degranulation, compound 48/80 released 12 times less PGD_2 and LTC_4 than anti-IgE in this same preparation. In this experiment the time between stimulation of mast cell degranulation and the termination of the experiment was 135 minutes and 225 minutes. This timeline was chosen in order to give ample time for the synthesis and release of the leukotrienes. It is possible that this time may have in fact been too long and that a range of points from 5-60 minutes may have better differentiated the three groups. While LTC_4 was synthesised, similar amounts were however seen in all groups. It is possible that while compound 48/80 may have induced synthesis and release of LTC_4 however the time left was in fact too long and cells in all groups may have induced leukotriene synthesis due to injury and cell death in the *in vitro* incubation.

Finally, it is possible that a vital step to isolate leukotrienes may have been eliminated in the preparation of sample. This step was a high performance liquid chromatography (HPLC) step designed to separate lipoxygenase metabolites of AA, namely leukotrienes and prostaglandins (Richmind *et al* 1987; Osborne *et al* 1983). As a HPLC system was not available in the laboratory we were unable to carry out

this purification step. It is therefore possible that elimination of this step may have in fact resulted in inaccurate readings from the EIA.

4.3 *In vivo* respiratory asthma model

4.3.1 Respiration in acute asthmatic rats

Experimental models of asthma in sheep, dog, rat and guinea-pig have been developed and studied in an attempt to reproduce human bronchial asthma and to ultimately develop a greater understanding of the disease with a view to developing more potent and effective drugs (Wanner & Abraham, 1982; Wang *et al* 1986). Sheep sensitised to the antigen *Ascaris suum* developed changes in pulmonary function when challenged with the same antigen. This model appeared to follow a T_H2 type response resulting in the development of extrinsic asthma. Bronchoconstriction as seen by, increased resistance, increase in the frequency of breaths taken and a decrease in tidal volume was observed immediately following challenge. This was seen to return to normal within minutes, however a second or late phase response was noted 8h following antigen challenge.

The early phase response is believed to be a result of mast cell activation and the release of both preformed and newly synthesised mediators (Barnes, 1991 Turner & Dollery, 1988; Holgate *et al* 1986; Biggs, 1984; Mauser *et al* 1990). The actions of histamine and leukotrienes play an important role in immediate brochochonstriction, which is induced by the activation of the H_1 and cys-LT_1 receptor respectively (Tan & Spector 1997). The late phase response is believed to develop as a result of the

release of these mediators, which together with cytokines from macrophages, epithelial and mast cells lead to influx of inflammatory leucocytes particularly eosinophils (Kay,1991; Krishna *et al* 1996; Lazarus, 1998). Pre-treatment with DSCG, the mast cell stabiliser was found to completely inhibit the early and late response in the *in vivo* model of asthma in the sheep (Wanner & Abraham, 1982; Alton & Norris, 1996; Redrup *et al* 1997). DSCG has been shown to prevent antigen-stimulated mast cell degranulation *in vitro* and therefore has the potential to prevent the immediate bronchoconstrictor response caused the mast cell mediators (Butchers *et al* 1979; Tanizaki *et al* 1992). Mast cell stabilisation would also significantly reduce the late phase response, as histamine, prostaglandins, leukotrienes, and thromboxanes play an important role in the activation of endothelial and leucocyte cell surfaces and ultimately the influx of leucocytes (Tan & Spector 1997, Barnes, 1991, Lazarus, 1998, Chabot-Fletcher *et al* 1995, Bell *et al* 1997).

In this study the effect of 3C8 was also investigated on an *in vivo* model of asthma. Male Wistar rats were sensitised to OVA using both AlOH (T_H2-inducer) and FCA(T) (T_H1-inducer) as adjuvants. Subsequent challenge with OVA, the antigen, would result in a mixed T_H1/T_H2 asthmatic response, which ultimately may lead to influx of the leucocytes and the late phase bronchoconstrictor response. Respiratory parameters, BPM and the TV which was measured by the change in chamber pressure during the breath cycle (ΔP_{bc}) were monitored before, immediately after and up to 3h following OVA challenge. No significant change in BPM and ΔP_{bc} was noted between groups immediately following the challenge. This included the untreated, unchallenged negative control group, the untreated OVA challenged positive control group, the DSCG treated OVA challenged drug control group and the 3C8 treated

OVA challenged group. It appears from this result that no early phase response, as would be expected with mast cell activation, was experienced by any of the animal groups. It must be stated however that both BPM and ΔP_{bc} reduced in all groups following vehicle or OVA challenge. The fact that this effect was also seen in the unchallenged group, which was given dH_2O only, shows that the change in respiration was probably as a result of the action of the nebuliser and the effects experienced by the animals in all groups. The respiratory chamber was connected to the nebuliser, to enable vehicle (group I) or OVA (groups I-IV) administration (figure 2.3). The nebuliser was in turn attached to an air pump, which aerosolised the vehicle/OVA contained in the nebuliser and directed them into the chamber. This action was seen to disturb the pressure in the chamber as well as disturbing the breathing pattern of the animal.

A significant increase in BPM and ΔP_{bc} was seen 120-150 minutes following OVA challenge as compared to the negative unchallenged control group. BPM was significantly increased at both 135 and 150 minutes following OVA challenge. This increase was prevented with the pre-treatment of DSCG at 135mins and 3C8 at both 135 and 150 minutes. Change of pressure within the chamber or the volume of air taken with each breath for the OVA challenged group, appeared to increase also as compared to the unchallenged group. This increase in ΔP_{bc} was marginally suppressed by DSCG and 3C8. The type of late phase in this model represents that of deep and rapid breaths. This is not as seen with the sheep asthmatic model where shallow and fast breaths are seen.

Many problems were experienced in this acute model of asthma. Firstly, an early phase response was not detected. This response should occur immediately after challenge and as described earlier would be due to mast cell mediators released on mast cell degranulation. It is possible however that the early phase response (increase in BPM and decrease in ΔP_{bc}) was masked by the effect of the nebuliser as the negative control group merely exposed to dH_2O, also experienced changes in breathing during and immediately following solvent delivery. This was seen as a decrease in BPM and ΔP_{bc}. It must also be stated that this model of asthma differs to that of the sheep extrinsic (T_H2) model described earlier. The model in this study may be described as a mixed T_H1/T_H2 model and it is possible that mast cell degranulation, resulting in the early response, did not occur. This would support an intrinsic type T_H1 model of asthma where to date mast cell degranulation has not been demonstrated. In this model, TNFα released from macrophages, epithelial cells may activate the endothelial cell surface and prime it for leucocyte adhesion and influx. IL-3, Il-5 and GM-CSF have the potential to differentiate, mature and activate eosinophils. Indeed, TNFα, IL-3, IL-5 and GM-CSF have the potential to induce a late phase response without involving the mast cell. In contrast however DSCG and 3C8, mast cell stabilisers (section 4.2), appear to be effective in alleviating the symptoms seen in the late phase response in this model. The fact that these drugs inhibit the late phase may signify that they have anti-inflammatory and anti-chemotactic properties exclusive from mast cell activation. However the probability that mast cell degranulation does play a role in this model cannot be excluded which brings us back to the original point and the possibility that the actions of the nebuliser masked the early response. Secondly, the late phase response seen 120-150 minutes following OVA challenge was not as expected. Rapid deep breaths were seen and

interpreted from an increase in ΔP_{bc} and BPM. This result does not mirror that seen in other asthmatic models where shallow rapid breathing developed as interpreted from a decrease in ΔP_{bc} and increase in BPM (Wanner & Abraham, 1982; Wang *et al* 1986). An explanation into the differences between the two responses during the late phase response is unfounded at present. Further investigation and work will need to be invested in the model. This would extend from variations in the sensitisation and challenging procedures to changes in the method of detection of an asthmatic attack.

4.3.2 Respiration in broncho hyper-reactive rats

Continuously exposure of animals to antigen, results in hyper-reactive or hypersensitive airway. The hypersensitivity follows epithelial shedding and exposure of the receptors to histamine, eicosanoids and other potent bronchoconstrictors (Aizawa *et al* 1988; Kay, 1991). Epithelial shedding develops with the activation of eosinophils and the release of degradative proteins ECP and MBP, eosinophil-derived neurotoxin and eosinophil peroxidase (Barnes, 1991; Robinson *et al* 1996). Hyper-reactivity in animal models of asthma can be detected with exposure to methacholine (cholinergic stimulant). Hyper-reactive animals are sensitive to low doses of methacholine while the breathing of normal subjects remains unperturbed when exposed to the compound (Wanner & Abraham, 1982). Sensitive rats challenged with methacholine result in an increase in BPM and a decrease in ΔP_{bc} (Wang *et al* 1986).

A state of hyper-reactive airways was induced in groups I to IV in the above asthmatic model. Group I was unchallenged and hence was treated *via* aerosol with dH_2O (4 treatments; One per week). Groups II-Groups IV were challenged with OVA on a

weekly basis until a total of 4 challenges had been administered. Each group received their respective solvent, drug or test compound before each OVA challenge. The degree of hyper-reactivity was measured by exposing all animals including group I, the OVA unchallenged group, to methacholine (8mg/ml). A low dose of methacholine was chosen specifically to highlight hypersensitive airways. The BPM and ΔP_{bc} were measured before, immediately after and up to one hour following methacholine exposure. Methacholine induced a marginal increase in BPM, as compared to the OVA unchallenged negative control group, immediately following exposure to the chemical. This increase was significantly decreased by both DSCG and 3C8 to levels below the negative control. The ΔP_{bc} was not affected by methacholine. However the nebuliser appears to induce a decrease in the volume of intake. This decrease in ΔP_{bc} was seen in all groups measured.

A hyper-reactive state was induced following repeated exposure to the antigen. This was demonstrated in group II where an increase in BPM was seen following methacholine exposure. Hyper-reactivity was however weak as only a marginal increase was noted in the frequency of breaths (BPM) however the depth of the breaths as seen by ΔP_{bc} appeared to be unaffected or to be again masked by the effect of the nebuliser. Further, more frequent challenges with the antigen would be necessary to induce a greater hyper-reactive response in the animals with methacholine. Although the hyper-reactive state was weak, the state was induced by the repeated exposure to the antigen. The development of hyper-reactive airways was prevented by pre-treatment with both DSCG and 3C8 before each OVA challenge.

4.3.3 Critical evaluation of the in vivo respiratory model of asthma

As discussed in depth in sections 4.3.1 and 4.3.2 many problems occurred in both the asthmatic model and the methods of measurement of the respiratory parameters. It is clear however the most problems were related to the concomitant use of the nebuliser and the pressure transducer. In this study it was difficult to measure changes in pressure, in the respiratory chamber, immediately following aerosolisation of OVA and the delivery of the antigen into the chamber with the use of the pump. This action would effectively disturb the animal and the surrounding atmosphere and ultimately the respiratory measurement.

Indeed, the results from this study proved interesting and 3C8 was found to prevent a late type asthmatic response and also the development of hyper-reactive airways in rats. Following these findings research focused on the development of a second more informative model. This model and its results are described and discussed in section 4.5 and look at the measurement of influx of leucocytes in the lung using BAL. Because the pressure transducer was not used to monitor respiratory changes, the error experienced with concomitant use of both the nebuliser and the volume transducer was eliminated. Animals were sensitised and challenged to OVA, BAL was carried out and the differential cell count used to determine the percentage leucocytes in the fluid.

4.3.4 Post-Mortem of Groups I-Groups IV

A post-mortem was carried out on all groups (groups I-IV) to see the local and systemic effect of the repeated exposure to vehicle/DSCG or 3C8. All groups showed normal thoracic cavities, however examination of the peritoneal cavity resulted in different unexpected results. Groups I (negative control) and Groups II (positive control) had developed severe peritonitis with significant inflammation as seen by development of fibrotic tissue around the liver. The lobes on the liver were unrecognisable and impossible to dissect apart. The liver showed increased vascularity and GIT contained white adhesions. This appeared to be as a result of the sensitisation procedure where animals were given *i.p.* injections of OVA, AlOH and *s.c.* FCA(T). DSCG showed some anti-inflammatory effect and less fibrosis was seen both on the liver and the GIT, as compared to group I and group II. The lobes of the liver were somewhat recognisable and most were successfully dissected apart. In complete contrast to the negative control, positive control and DSCG treated groups the 3C8 treated group showed absolutely no inflammatory signs and the liver and peritoneal cavity appeared normal.

Inflammation induced by the OVA, AlOH and FCA(T) mixture was prevented by exposure to the test compound 3C8. This result highlights the fact that 3C8 is an effective systemic anti-inflammatory. Repeated exposure to a mere concentration of 5mg/ml 3C8, aerosolised and directed into the respiratory chamber containing the animal, was capable of potently inhibiting the development of severe peritonitis. It is difficult to decipher the exact pathway of inflammation seen here. However, it is clear that the influx and activation of inflammatory cells normally seen in a chronic state

was prevented. It is evident from this result that 3C8 has potent anti-inflammatory properties with the potential to act systemically at very low concentrations. Further investigations into the role of this drug in acute and chronic inflammatory disease should be carried out.

4.4 Inflammation

4.4.1 Acute inflammation

4.4.1.1 The effects of 3C8, 6C6 and 7C9 on arachidonic acid-induced mouse ear oedema

The effects of the three test compounds on AA-induced mouse ear oedema was investigated using male Laca mice. Topical application of AA to mouse ear has the potential to induce oedema at that site. The products of AA metabolism, leukotrienes and prostaglandins, induce inflammation. These products appear to be synthesised by the 5-LO and the COX-II pathways respectively (Miyake *et al* 1993; Davies *et al* 1984; Salmon & Higgs, 1987).

Although AA produces prostaglandins and leukotrienes, the leukotrienes appear to play a more pivitol role in AA-induced mouse ear oedema. It is believed that, although the prostaglandin, PGE_2 is present, the leukotriene, LTC_4, predominately mediates the inflammation and prostaglandin, PGE_2 merely compliments it (Inoue *et al* 1988b). Studies with NSAIDs, inhibitors of prostaglandin synthesis, demonstrate that apart from indomethacin, these compounds have little or no effect on the AA-

induced mouse ear oedema (Inoue *et al* 1988a). These NSAIDs include aspirin, ibuprofen and naproxen. Aspirin did not reduce ear swelling moreover it enhanced LTC_4 production. Moreover, to further highlight the importance of leukotrienes and the 5-LO pathway in this model, 5-LO inhibitors, such as zileuton, potently inhibit AA-induced mouse ear oedema (Inoue *et al* 1988a; Carter *et al* 1991). Finally the steroid dexamethasone was also shown to inhibit oedema seen in this model. This observation suggests that novel synthesis of enzymes or inflammatory mediators is necessary for oedema (Inoue *et al* 1988a).

The topical application of AA (4mg/ear) on mouse ear resulted in a 78.8±15.2% increase in ear oedema compared to normal. An increase in the ear thickness was used as a marker of inflammation or oedema. Treatment with dexamethasone (300μg/ear) and indomethacin (300μg/ear) reduced the inflammation to 37.9±8.5% and 39.6±5.8% of normal respectively. Attenuation of AA induced ear oedema, by both dexamethasone and indomethacin, has been demonstrated previously using LTB and PGE levels in ear tissue (Lloret & Moreno 1995). The concentration of LTB_4 increased from 0ng/ml to 17.1ng/ml and PGE_2 increased from 0ng/ml to 85.3ng/ml one hour after AA application to the ear. Pre-treatment with indomethacin (500 & 1000μg/ear) resulted in significantly reduced prostaglandin production while higher concentrations (1000μg/ear) were needed to significantly reduce leukotriene levels. Treatment with dexamethasone (1μg/ear) two hours prior to AA application potently reduced the levels of both eicosanoids. Both this study and that of Lloret and Moreno (1995) show comparable effects of indomethacin at near similar concentrations. However, the concentrations of dexamethasone used in the studies are different. Lloret and Moreno found a significant decrease in leukotriene (400%) and

prostaglandin (100%) ear content with dexamethasone (1μg/ear) applied 2 hours prior to AA application. In this study 300μg/ear of dexamathasone was applied only 1 hour before AA application and resulted in significant although only 50% reduction in oedema. The difference in potency can be explained by previous studies carried out by Inoue *et al* (1988a) who showed that the length of time of application of dexamethasone (100μg/ear) before AA application is imperative to the reduction of oedema. An inhibitory effect of only 6% was noted when the steroid was administered 30mins prior to AA application. The inhibitory effect increased to 50% ($P<0.001$) when applied three hours prior to induction of oedema.

The effects of the three test compounds on AA-induced mouse ear oedema was investigated. 3C8 (300μg/ear) and 7C9 (300μg/ear) significantly reduced oedema from positive control levels where an increase in ear thickness of 78.8±15.2% was reduced to 32.4±7.8 and 10.5±6.2% respectively. The effect of 3C8 was comparable to those of the effects of dexamethasone and indomethacin, however 7C9 showed a more potent effect and reduced inflammation to near normal levels. 6C6 however appeared to have no effect on AA-induced mouse ear oedema.

As discussed above, inhibitors of the AA-induced mouse ear oedema model appear to act by inhibiting either the synthesis or action of leukotrienes. This may occur in a number of ways (section 1.1.1.1(d) & 1.2.1). The 5-LO enzyme or even FLAP, the enzyme that activates 5-LO may be inhibited by these compounds whereby the synthesis and hence the action of the leukotrienes would be prevented. On the other hand the compounds may antagonise the actions of the leukotriene, which in turn

would prevent leucocyte infiltration, epidermal proliferation, chemotaxis and vascular permeability.

4.4.1.2 The effects of 3C8, 6C6 and 7C9 on TPA-induced mouse ear oedema

The effects of the three test compounds were also investigated in a TPA-induced mouse ear oedema model. This model specifically investigates potential COX-II inhibitors and prostaglandin antagonists. TPA has been shown to induce PGE_2 production in the ears with little or no LTC_4 and LTB_4 synthesised (Inoue *et al* 1989). Inhibitors of this model are, in contrast to the AA-induced mouse ear oedema model, the NSAIDs, indomethacin, aspirin and naproxen. Dexamethasone has also been shown to potently inhibit oedema in this model suggesting that novel synthesis of enzymes or inflammatory mediators are involved.

Topical administration of TPA, to the mouse ear, induced a 114.7±8.8% increase in oedema 6h following application. This increase was inhibited by treatment with dexamethasone (10μg/ear) 2h prior to the induction of oedema. Dexamethasone significantly decreased the inflammation from 114.7±8.8% to 30.3±4.0%. Inoue *et al* also found dexamethasone (100μg/ear) to be a potent inhibitor of TPA mouse ear oedema when dexamethasone was applied 30mins following TPA application. It is believed that new protein synthesis is involved in TPA-induced oedema as both dexamethasone (Inoue *et al* 1989; present study) and cyclohexamide (Inoue *et al* 1989) inhibit the inflammation.

3C8, 6C6 and 7C9 were tested for potential anti-inflammatory properties in the TPA-induced ear oedema model. 3C8 significantly reduced the oedema from 114.7±8.8% to 82.7±8.4%. Thus a 30% reduction was seen with 3C8 showing some potency against COX, the induction or the action of the enzyme or possible even the actions of prostaglandins. 6C6 and 7C9 however do not significantly inhibit the TPA-induced oedema.

4.4.1.3 The effects of 3C8 and 7C9 on carrageenan-induced rat paw oedema

A third acute inflammatory model of inflammation was set up using carrageenan to initiate the inflammatory response in the rat hind paw. Carrageenan has been found to induce high levels of COX-II mRNA and an increase in PGE_2 has been demonstrated (Kennedy *et al* 1993; Melarange *et al* 1994b). The injection of carrageenan (100µl of 1% w/v) induced a 53.9±4.6% increase in oedema as measured by paw volume. A study using Lewis rats also demonstrated an inflammatory reaction to the same volume and concentration of carrageenan. However, Sekut *et al* (1995) found a greater increase in oedema than that seen in this study with female Wistar rats (Sekut *et al* 1995). Sekut *et al* demonstrated a 100% increase in paw oedema in an undefined strain of rat where the thickness of the paw was measured before (4mm) and after oedema induction (8.3mm).

Pretreatment with dexamethasone (30mg/kg) and indomethacin (30mg/kg) significantly reduced inflammation to 16.3±4.6% and 21.3±3.2% respectively. Both these drugs have been shown to inhibit COX-II, but in very different manners. Dexamethasone inhibits the induction of synthesis of the enzyme while indomethacin

has been shown to inhibit the activity of the enzyme (Frolich, 1997; Mitchell *et al* 1994; Schwiebert *et al* 1996; Arias-Negrete *et al* 1995; Raz *et al* 1988; Raz *et al* 1989; Nakazato *et al* 1991; O'Neill & Lewis, 1989; Vadas *et al* 1991; Barnes & Liew 1995). Indomethacin has previously been shown to suppress carrageenan-induced rat paw oedena with an ED_{50} of 6mg/kg (Chang *et al* 1987).

The effects of 3C8 and 7C9 on this inflammatory model was investigated. The test compound 3C8 significantly reduced the inflammation from 53.9±4.6% to 30.1±4.6% increase in volume. A similar reduction was seen with 7C9 where the increase was reduced to 28.4±2.9%. This study is comparable to that of the TPA-induced mouse ear oedema where COX-II and the prostaglandins play an important role in the inflammatory process.

It appears however that the carrageenan rat inflammatory model is more sensitive to 3C8 and 7C9 while the TPA mouse model showed only 3C8 with potential anti-inflammatory properties. A second reason for these differences may be that our compounds are more effective in inhibiting rat COX-II and prostglandins synthesis and action than those of mice, however, a study looking at the molecular level of these inflammatory models would need to be carried out to clarify this point. Further studies validating these models at a molecular level would highlight the differences seen with carrageenan-induced rat paw oedema and TPA-induced mouse ear oedema.

4.4.2 Chronic inflammation

4.4.2.1 The effect of 3C8 on chronic inflammation induced by the subcutaneous implant of a poly-vinyl sponge

This model was set up to elucidate the role of 3C8, the most promising anti-inflammatory of the three, in chronic inflammation. Chronic inflammation was induced by 7-day subcutaneous implant of poly-vinyl sponge into the lower back of the rat. The model is similar to that of Boyle and Mangan (1982) however with a difference in the pre-treatment of the sponge. Boyle and Mangan used either a saline-soaked or a carrageenan-impregnated saline-soaked sponge implant. In depth evaluation of the model set up by Boyle and Mangan showed that the inflammatory response begun with leukotriene and prostaglandin production, followed by the influx of polymorphonuclear leucocytes (PMNLs), the influx of monocytes and phagocytic erythrocytes and finally fibroblasts (Boyle & Mangan, 1982). This pattern was similar to that seen with cotton pellet implants (Freeman *et al* 1979). Comparisons between the saline-soaked and the carrageenan-impregnated saline soaked-sponge showed that both models produced an inflammatory response with leucocyte infiltration seen within 24h of implantation, however the carrageenan–impregnated sponge proved to be marginally more effective in inducing inflammation

In this study, the change in dry weight of sponge implants was measured following a 7-day implantation. A vascularised capsule had developed around the sponge by day 7 and some fibrotic tissue was noticed. This is indicative of chronic inflammation (Vadas *et al* 1991; Baumann & Gauldie, 1994). The dry weight of the sponge had

increased by 0.21±0.02g over this time period. This increase in sponge weight was suppressed to a value of 0.10±0.02g with the daily treatment of dexamethasone (1mg/kg). The daily treatment with indomethacin (1mg/kg) was also effective at decreasing the inflammatory response as seen by the decrease in dry weight to 0.13±0.03g from 0.21±0.02g. The degree of vascularisation and fibrosis was less than that seen without the pre-treatment with both dexamethasone and indomethacin. These drugs also proved to be effective in the carrageenan sponge implant experiment as described by Boyle & Mangan (1982). It was found that both dexamethasone (45μg/kg) and indomethacin (3mg/kg) reduced 24h leucocyte influx in saline-soaked implants while indomethacin (2mg/kg) reduced 24h leucocyte influx in carrageenan-impregnated saline-soaked implants. The effect of dexamethasone was not investigated in the carrageenan-impregnated sponge implant study.

Daily treatment with 3C8 (1mg/kg) resulted in a marked decrease in vascularisation and fibrosis as compared to positive control. The dry weight was comparable to that seen with indomethacin treatment and reduced to 0.14±0.02g. It is difficult to describe the exact mode of action of 3C8 in this chronic inflammatory model. We have observed that it is effective in reducing AA-and TPA-induced mouse ear oedema and carrageenan-induced rat paw oedema (sections 4.4.1.1, 4.4.1.2 & 4.4.1.3). These results demonstrate the inhibitory actions of this test compound on leukotriene and prostaglandin synthesis and/or the actions of these eicosanoids once produced. While prostaglandins induce vasodilatation, PAF and cLTs induce vascular permeability and prime the leucocytes for endothelial adherence and infiltration to the site of inflammation (Salmon & Higgs, 1987; Bochner, 1997; Zimmerman *et al* 1994; Gimbrone *et al* 1984; Samuelsson, 1983). It is possible that the onset of chronic

inflammation may be impeded by the eicosanoid inhibitory actions of 3C8. However, as discussed previously (section 1.2.2.1) the process of infiltration of these compounds is complex and involves many mediators and steps, all of which need to be given attention. Cytokines, IL-1 and TNFα, released from activated macrophages are believed to be imperative in the activation of the endothelium and the expression of the adhesion molecules E-selectin and P-selectin, both of which are needed to attract the leucocytes. Moreover the effect of 3C8 may also extend to the latter stages of inflammation. This may include the activation of neutrophils and esoinophils at the site of inflammation and the development of fibrotic cells.

4.5 Inflammatory asthma models

In vivo models of acute and chronic inflammatory asthma were set up using OVA as an antigen. This was designed to follow and improve on the previous model of asthma where changes in respiratory parameters were used as a marker for the disease. In the acute model, rats were firstly sensitised to the antigen, using AlOH, FCA(T) or a combination of the two as adjuvants, and then challenged, 3 weeks later, to the same antigen. In the chronic model, rats were also sensitised to the antigen using the same adjuvant(s) however rats were challenged twice over an 8-week period before BAL was carried out. This was done to induce a more hyper-reactive or chronic state of asthma as compared to the acute model. OVA challenge, following sensitisation, resulted in the activation of the airways and influx of inflammatory mediators, namely, eosinophils and neutrophils. A differential cell count was carried out and eosinophil, neutrophil and basophil influx into the airways was used as a marker for inflammation.

The exact pathway taken, leading to airway inflammation depends on the type of asthma induced, extrinsic or intrinsic. This pathway is determined at the sensitisation stage where the antigen is presented by an APC. This cell processes and presents the antigen to naïve T_H0 cells in the form of a peptide. The T_H0 cells polarise to either T_H1 or T_H2 cells depending on the peptide presented which is governed by the adjuvant used on sensitisation. OVA mixed with AlOH is believed to induce a T_H2 response while OVA presented with FCA (T) is thought to result in a T_H1 response (Stern et al 1996; Romagnani, 1991; Lucey *et al* 1996; Romagnani, 1997; Murray, 1998; Chuang *et al* 1997; Wiedermann *et al* 1998; Sinha *et al* 1997).

4.5.1 Extrinsic inflammatory asthma model

Sensitisation of BN rats to OVA using AlOH as an adjuvant resulted in an *in vivo* model of extrinsic in *vivo* asthma (Renzetti *et al* 1996; Chuang *et al* 1997; Wiedermann *et al* 1998). Subsequent challenge with OVA leads to the production of T_H2 type inflammation of the airways and influx of more T_H2 lymphocytes, eosinophils and neutrophils.

4.5.1.1 The effect of 3C8 on Brown Norway rats acutely and chronically sensitised to OVA using AlOH as an adjuvant

The effect of 3C8 was investigated in an *in vivo* model of extrinsic inflammatory asthma and changes in eosinophil, neutrophil and basophils influx were monitored. As discussed in section 2.1.6.1(a) all animals were sensitised to the antigen using

AlOH as an adjuvant 3 weeks before challenge. Challenging the animal with OVA resulted in a significant influx of eosinophils (19.9±2.6) as compared to the negative control (1.9 ± 0.5%), which was exposed to dH_2O, the solvent for OVA. In contrast, neutrophil or basophil influx was not demonstrated. Inflammatory asthma using similar methods of induction has previously been shown in both guinea-pigs and BN rats (Renzetti *et al* 1996). In these models eosinophil cell count increased to 40% from 1% of total cell count on OVA challenge while neutrophil count increased to 20% from 2% of total cell count. The inflammatory response seen by Renzetti *et al* (1996) was stronger than that seen in the present study and contrasted with it in that a significant neutrophil influx was demonstrated. Although experimental procedures were similar, variations between laboratories must be considered. In this study the OVA was given to the animal by aerosolising the compound using an air pump with a rate of 0.75L/min. This method makes it difficult to quantify the exact concentration delivered to the animal and comparisons to that of Renzetti *et al* (1996) where methods to aerosolise the antigen were not described, are not possible.

The influx of eosinphils into the lung was reduced to levels comparable to the negative control by use of the positive drug control dexamethasone (1.6±0.4%; 1mg/kg). Renzetti *et al* (1996) also demonstrated the potent prophylactic activity of this steroid on neutrophil and eosinophil influx. Neutrophil and eosinophil cell counts were reduced to levels comparable to control by dexamethasone (0.3mg/kg). In this study, pretreatment with 3C8 (10mg/kg) had no significant effect on eosinophil influx (13.2±1.9%) into the lung of OVA challenged rats. However pre-treatment with this compound resulted in an increase in the neutrophil count (27.4±5.5%) in the BAL. This demonstrates that while 3C8 has minimal prophylactic activity on an eosinophilic

model of extrinsic asthma it appears to induce neutrophil influx in the lungs of asthmatic rats. Basophil levels remained consistently low throughout all groups. This is in agreement with Renzetti *et al,* 1996 where minimal changes in basophil number as compared to the negative unchallenged group were also noted.

Similar results were seen in the present study with the chronic model of asthma. A statistically significant increase in eosinophil influx (0.6±0.2% to 12.7±2.1%) was seen as compared to control. While this increase was reduced to 1.3±0.62%, of total cell count, by the steroid budesonide (10mg/kg), 3C8 (10mg/kg) again showed minimal effect on this extrinsic-type model of chronic inflammatory asthma and the eosinophil count (8.62±2.8%. of total cell count) was found to be statistically comparable to the positive control.

Neutrophilia was also measured but proved to be erratic. Neutrophil influx did not statistically differ between groups, however neutrophil count in the positive control (27.3±10.5%) and 3C8 (23.3±4.2%) group was raised. Huge variability in neutrophil count was found between individual animals in both the positive control and the 3C8-treated group. This resulted in the calculation of large standard error means rendering the groups statistically insignificant from the other groups, negative control and the dexamethasone-treated group. Erratic neutrophil counts are not uncommon in both non-asthmatic and asthmatic subjects (Kay, 1991; Kay & Corrigan 1992; Frew *et al* 1996). Moreover, the role of neutrophilia in asthma is unclear and activation of neutrophils has not been demonstrated in asthmatics. Myeloperoxidase, a neutrophil-derived mediator does not appear in the BAL fluid of asthmatics (DuBuske, 1995).

As with the acute model of extrinsic asthma, basophils remained low throughout all groups.

The steroids, dexamethasone and budesonide proved effective in inhibiting intrinsic asthma. These steroids may act in many ways, starting from inhibition of PLA_2, COX-II expression and COX-II synthesis, and inhibition of the production of cytokines. They have the potential to indirectly act on PLA_2 *via* the induction of its inhibitor lipocortin preventing AA and ultimately eicosanoid production. They may also act to inhibit COX-II mRNA expression, which in turn would depress prostaglandin synthesis and hence result in chemotaxis inhibition. A further action of steroids is cytokine inhibition at a transcriptional level. These steroids have the potential to inhibit cytokines such as IL-4, TNFα, IL-3, IL-5 and GM-CSF preventing influx and activation of eosinophils.

The result seen in this extrinsic model does not reflect or support the result seen in the earlier *in vitro* model of mast cell degranulation. 3C8 has been shown to inhibit 48/80 induced mast cell degranulation (section 4.2). However it was not effective at preventing leucocyte influx in the extrinsic model of asthma following OVA challenge in previously sensitised BN rats using AlOH as an adjuvant. It must be stated however that although 3C8 is proven to inhibit 48/80-induced mast cell degranulation its inhibitory action has not been demonstrated in an antigen-induced mast cell degranulation *in vitro* assay. Antigen-induced mast cell degranulation is more indicative of the *in vivo* model of extrinsic asthma described here. It is possible therefore that 3C8 is not as effective in inhibiting antigen-induced mast cell degranulation in this model of asthma. Differences in the mechanisms of activation of

mast cells when stimulated by compound 48/80 or by antigen have been described in detail in the introduction (section (1.1.1.1(b)). Furthermore, DSCG has shown differences in its inhibitory effects on the two *in vitro* models and while it can potently inhibit antigen-stimulated mast cell degranulation, it has only a slight inhibitory effect on compound 48/80-induced mast cell degranulation (Butchers *et al* 1979 Tanizaki *et al* 1992). Moreover, it must be stated that different tissues and different strains of rat are used in the two models. 3C8 has demonstrated inhibitory action on Wistar rat peritoneal mast cells while BN rat lung mast cells are involved in the asthma model. While the mediators and cytokines released from mast cells are important in leucocyte influx, other cells namely T_H2 lymphocytes and macrophages are equally important. TNFα, released from macrophages and epithelial cells and IL-4, released from T_H2 cells activate the endothelial cell wall, which induces the expression and synthesis of adhesion molecules necessary for leucocyte adherence and ultimate influx to the site of inflammation (Kumar *et al* 1998; Galli & Costa 1995; Barnes & Adcock, 1997; Krishna *et al* 1996). IL-3, IL-5 and GM-CSF, released from T_H2, cells are involved in activation, maturation and differentiation of eosinophils. It is possible therefore that although 3C8 potently inhibits mast cell degranulation it has no effect on the cytokines released from other inflammatory cells, which are ultimately capable of inducing a late phase response.

4.5.2 Intrinsic-type inflammatory asthma model

A second model of asthma was developed using OVA as the antigen, however in this experiment FCA(T) was used as the adjuvant. FCA(T) contains the bacteria *Mycobacterium tuberculosis* H37Ra which is believed to induce a T_H1 type response

(Stern et al 1996; Romagnani, 1991; Lucey *et al* 1996; Romagnani, 1997; Murray, 1998; Sinha *et al* 1997). Secretory proteins isolated from cultures of *Mycobacterium tuberculosis* H37Ra have been shown to induce cell-mediated T_H1-cell proliferation and the release of IL-2 and IFNγ (Sinha *et al* 1997). On sensitisation, OVA together with the FCA(T) is processed by the APCs and presented to T_H0 cells which in turn are polarised to T_H1 cells and T_H1 type cytokines (Romagnani, 1997).

The combination of antigen and adjuvant has the potential to induce intrinsic asthma where T_H1 lymphocytes predominate and subsequently synthesise and release IL-2 and IFNγ. IL-2 and IFNγ have been found in BAL fluid of intrinsic asthmatics and are thought to induce B cell activation and possibly the production of antibodies specific to the antigen. T_H1 lymphocytes have also been shown to release IL-3, IL-5 and GM-CSF (cytokines involved in eosinophil differentiation and activation). Activation of eosinophils results in the release of proteolytic proteins, ECP and MBP leading to epithelial damage and airway remodelling and hypersensitivity (Walker & Virchow, 1993; Virchow *et al* 1996; Barnes & Adcock, 1997; Denburg, 1996; Barrios *et al* 1998).

4.5.2.1 The effect of 3C8 on Brown Norway rats acutely sensitised to OVA using FCA(T) as an adjuvant

BN rats were sensitised to OVA using FCA(T) as the adjuvant. Three weeks later, the animals were challenged with OVA and BAL was carried out 24h later. Eosinophil influx resulted after challenge and a significant increase of 17.0±3.7% was seen as compared to the negative control group challenged with dH_2O (2.2±0.9%). Treatment

with dexamethasone (3.2±1.1%; 10mg/kg) prior to OVA challenge resulted in a significant reduction in eosinophilia when compared to the negative control. Pre-treatment with 3C8 (9.9±1.9%; 10mg/kg) did not result in a significant reduction in eosinophil influx on OVA challenge.

Neutrophils were also found to increase upon OVA challenge in this model of asthma. The increase however was not found to be statistically significant as compared to the unchallenged negative control. Treatment with dexamethasone prior to OVA challenge resulted in levels of neutrophila comparable to that of the unchallenged group. Pre-treatment with 3C8 resulted in a significant increase in the influx of the leucocyte in the lung as compared to the negative control. However it should be noted that in this experiment the positive control was not significantly different from 3C8 treated group or the negative control due to the erratic nature of neutrophilia. This makes the results difficult to interpret at this point. The percentage basophil count remained comparable to the negative control groups at $<0.2 \pm 0.2\%$.

While clear observations cannot be made on neutrophils, changes in eosinophil influx have proven to be quite informative. In this model of intrinsic asthma, dexamethasone inhibited the influx of eosinophils. This supports previous findings where dexamethasone can potentially inhibit the induction and expression of cytokines, namely IL-1β, TNFα, GM-CSF and IL-2 through to IL-6 (Barnes, 1996b; Schwiebert, 1996). These cytokines play a significant role in intrinsic asthma and the influx of eosinophils. While IL-2 released from T_H1 cells plays a significant role in further proliferation of T_H1 cells and B cells, TNFα is involved in the activation of the endothelial cell surface leading to leucocyte influx. IL-3, IL-5 and GM-CSF are also

important and have been shown to differentiate, activate eosinophils at the site of inflammation.

4.5.3 Mixed Extrinsic/Intrinsic type asthma model

A third asthma model was set up using both adjuvants, ALOH and FCA(T) when sensitising to OVA. The exact nature of the T-cell response, whether T_H1 or T_H2, is uncertain at this point. It is clear however that both T_H1 and T_H2 are inversely related to each other and IL4, IL-10 and IL-13 may potentially inhibit T_H1 cytokines and IL-2 and IFNγ may inhibit the T_H2 response (Romagnani, 1997).

4.5.3.1 The effect of 3C8 on Brown Norway rats acutely and chronically-sensitised to OVA using both AlOH and FCA(T) as an adjuvant

In the acute model of inflammatory asthma BN rats were sensitised to OVA using a mixture of both AlOH and FCA(T) as adjuvants. Subsequent challenge with OVA resulted in a significant increase in both eosinophils (20.5±3.3%) and neutrophils (35.6±9.3%) in the BAL fluid as compared to the unchallenged negative control (7.1±2.1% eosinophilia; 0.8±0.3% neutrophilia). This increase was significantly reduced with the pre-treatment of dexamethasone and the levels of both eosinophils (6.7±2.1%) and neutrophils (2.5±1.5%) were comparable to the negative control. 3C8 was found to significantly reduce the percentage increase in eosinophils (6.9±0.7%) without significantly affecting the neutrophil (55.4±8.7%) number. Basophils were not found to vary on challenge with OVA.

A chronic asthmatic model was also set up using the same combination of adjuvants, FCA(T) and AlOH. As in the acute model of inflammatory asthma, eosinophil (18±1.9%) and neutrophil (29.2±14%) count increased while basophil number remained comparable to the negative unchallenged control. Unchallenged animals showed a percentage eosinophila and neutrophilia of 1.5±0.3% and 1.4±1.1% respectively. The influx of the eosinophils (1.5±0.6%) decreased significantly upon pre-treatment with dexamethasone. However, in this chronic model of asthma, the steroid appeared to be ineffective in significantly inhibiting the neutrophil influx (10.0±7.5%). In contrast, 3C8 potently inhibited influx of both leucocytes where the percentage of eosinophils and neutrophils were 1.8±0.5% and 0.8±0.6% respectively. Basophil number remained statistically comparable in all groups.

This model of asthma is difficult to explain fully without molecular analysis, which would enable the elucidation of the exact cytokine profile. While AlOH appears to act as an adjuvant to promote the T_H2 response, FCA(T) is believed to promote the T_H1 response. As discussed previously, in section 1.1.3, both profiles are inversely controlled where one inhibits the other. The test compound, 3C8 does not inhibit the extrinsic asthma model or the intrinsic-type asthma model, however does appear to potently inhibit the mixed T_H1/T_H2 type asthma model. While the test compound appears to be active, the exact mechanism of action or its potential therapeutic benefit is unclear. Analysis of the extrinsic asthma model has been studied and reveals a T_H2 type profile (Renzetti et al 1996). In contrast, the T_H1-type (FCA(T)), and the T_H1/T_H2 mixed type (FCA(T) and AlOH) of asthma model needs to be studied and mechanism of pathogenesis validated. We cannot clearly state that intrinsic asthma is induced in the animals sensitised to OVA using FCA(T) as an adjuvant without

looking at the cytokine profile. Moreover, elucidation of the cytokine profile of the mixed-type asthma will answer questions as to the exact mode of action of our test compound.

4.6 Delayed type-hypersensitivity.

Results to date have shown that 3C8 is effective as an anti-asthmatic (mixed T_H1/T_H2) and an anti-inflammatory agent. The exact mode of action in the asthma model is however unclear and investigation of the actions of the three test compounds on a DTH model would highlight any possible anti- T_H1 inhibitory potential. The effect of the three test compounds on a T_H1 DTH was therefore investigated.

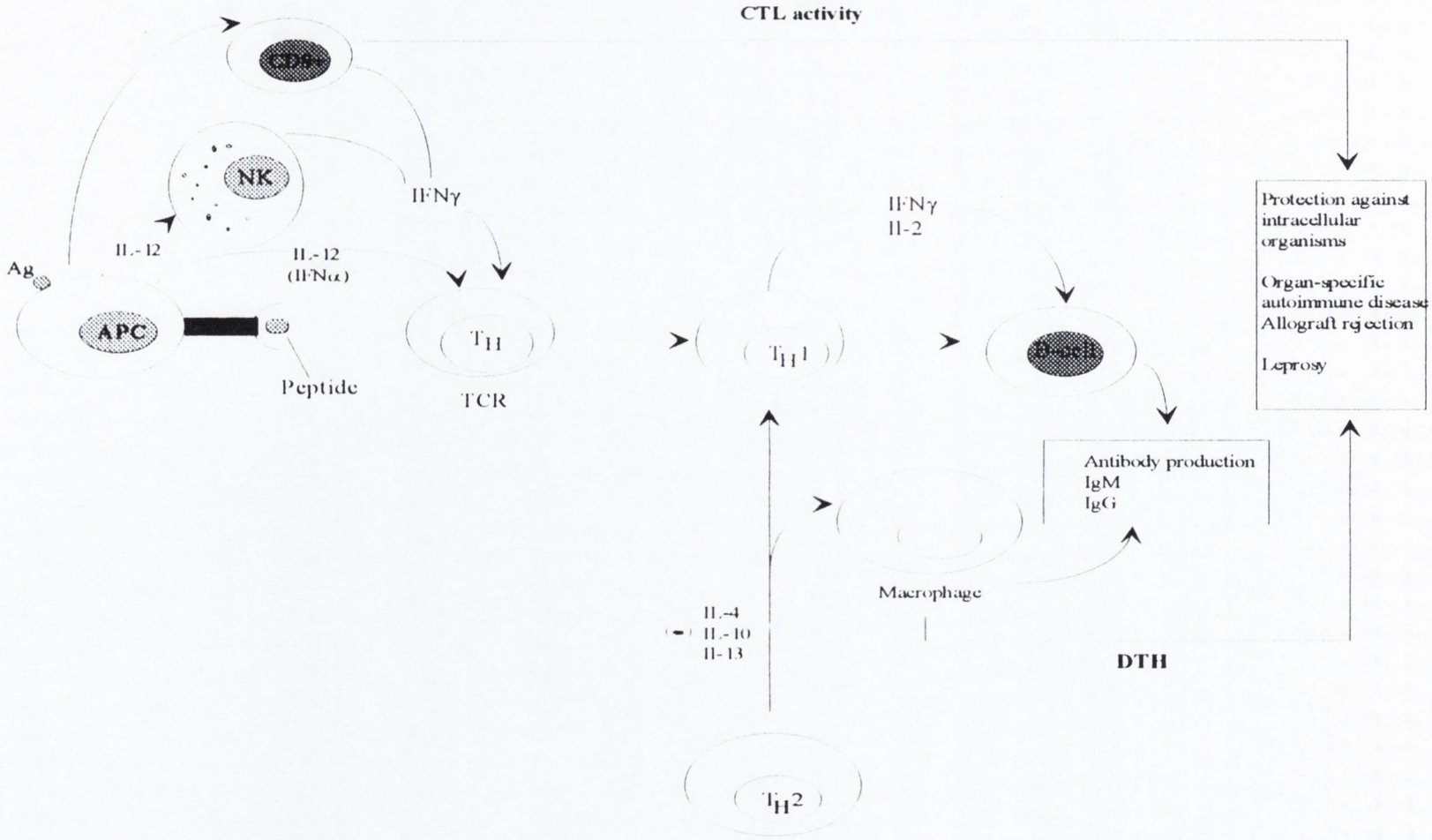
4.6.1 The effects of 3C8, 6C6 and 7C9 on mBSA induced mouse paw DTH

Intradermal injection of a mixture of mBSA and FCA to mice sensitises the animals to mBSA developing a T_H1 cytokine profile and DTH (Tarayre *et al* 1990). FCA may contain either *Mycobacterium butyricum* or *tuberculosis* and both are universally believed to induce DTH (Roman & Moreno 1997; Lucey *et al* 1996). The effect of the three test compounds on DTH to mBSA was investigated. Mice were sensitised to mBSA using a homogenised mixture of mBSA and the adjuvant FCA, containing *Mycobacterium butyricum*. Subsequent challenge of these mice to mBSA, eight days following sensitisation, resulted in a maximum increase in paw volume from 9.4 ± 3.2% to 57.1 ± 7.1% 24h later. DTH reactions have been demonstrated in earlier studies where paw weight increased by 80mg, 24h following mBSA challenge.

Treatment with the immunosuppressant, cyA (50mg/kg), 24h and 2h prior to mBSA challenge, resulted in a significant reduction of paw swelling to 38.4 ± 5.6% of normal. This gave a 33% reduction of DTH by cyA. Tarayre *et al* 1990 also showed cyA's potent inhibitory actions of 29% at 50mg/kg and 49% at 100mg/kg when dosed 24h and 2h before challenge to mBSA. 3C8, 6C6 and 7C9 were also tested, for immunosuppressive properties, in the mBSA DTH model. All compounds were investigated separately at 2 different concentrations, 3mg/kg and 10mg/kg, given both 24h and 2h before mBSA challenge. The concentration of 3mg/kg proved to be ineffective at reducing the DTH reaction however the higher concentration of 10mg/kg proved to be comparable to cyA (50mg/kg) in reducing the T_H1 type inflammation. 3C8 (10mg/kg), 6C6 (10mg/kg) and 7C9 (10mg/kg) reduced oedema to 35.0 ± 3.3%, 30.5 ± 6.4% and 35.3 ± 9.0% of normal (57.1±7.1%), respectively.

It is clear from these results that cyA (50mg/kg), 3C8 (10mg/kg), 6C6 (10mg/kg) and 7C9 (10mg/kg) can inhibit the development of mBSA induced DTH in mice. As discussed previously (section 1.3.1) the adjuvant *Mycobacterium butyricum*, has been shown to direct the polarisation of the naïve T_H0 cell towards T_H1 lymphocyte in both human and mice, figure 4.2 (Stern et al 1996; Romagnani, 1991; Lucey *et al* 1996; Romagnani, 1997; Murray, 1998).

Figure 4.2 Mycobacterium infection, T_H1 response and the development of DTH



(Adapted from Romagnani ,1997)

The *buytricum*/mBSA is processed by the APC and presented to T_H0 cells. The T_H0 cell polarises to a T_H1, IL-2 producing cell, with the help of IL-12 and IFNγ, released from APC and NK respectively. This in turn leads to activation and recruitment of more T_H1 lymphocytes, macrophages, neutrophils, basophils and eosinophils. CyA is thought to act through the inhibition of the synthesis of IL-2 in T_H1 cells which in turn would prevent further activation and proliferation of T_H1-cells and macrophages (Carlson *et al* 1993; Chaudhuri *et al* 1997; Denton *et al* 1999). Firstly cyA binds with cyclophilin to form the cyA-cyclophilin complex. This complex prevents transcriptional activation of IL-2 by inhibiting the calcium/calmodulin-dependent phosphatase calcineurin, which is imperative for the dephosphorylation of NF-AT a nuclear factor required for the transcription of IL-2. A second mechanism, that has been discussed, is the inhibition of the release of IL-1 and TNFα from macrophages (Chaudhuri *et al* 1997; Panayi & Tugwell, 1997). The test compounds may inhibit the DTH response in a number of ways, however the fact that cyA inhibits the calcium/calmodulin-dependent phosphatase, calcineurin is interesting. We have shown that 3C8 inhibits both smooth muscle relaxation and mast cell degranulation, both of which require the calcium/calmodulin complex for contraction and degranulation respectively. It would therefore be interesting to look at possible inhibitory actions of 3C8 on IL-2 production with particular focus on calcineurin inhibition. To date we are unsure of the actions of 6C6 and 7C9. While 6C6 inhibits mast cell degranulation it does not affect smooth muscle contraction. Moreover 7C9 does not affect either mast cell degranulation or smooth muscle contraction. If 3C8 did work in a manner similar to cyA then the results prior to this experiment, smooth muscle contraction and mast cell degranulation, point us again towards the fact that these latter compounds may act in a different manner to 3C8, however we must again

stress the fact that this third model is that of a mouse paw model where the species and tissue investigated are different to the above *in vitro* experiments.

4.7 Conclusion

The study was designed to investigate the effects of three test compounds 3C8, 6C6 and 7C9 on asthma, inflammation and DTH. *In vitro* and *in vivo* studies were set up to look at the anti-asthmatic (extrinsic and intrinsic) properties of these compounds with particular interest given to 3C8. The *in vitro* studies demonstrated the possible bronchodilatory and mast cell stabilisation potential of 3C8. In contrast while 6C6 and 7C9 did not show any smooth muscle relaxant properties, 6C6 did potently inhibit mast cell degranulation of rat peritoneal mast cells. Extensive *in vivo* studies of asthma showed that although 3C8 was not effective in alleviating the symptoms seen on an extrinsic or intrinsic-type asthma model it was very potent in preventing respiratory changes and influx of inflammatory cells in a mixed extrinsic/intrinsic type model of asthma. The exact cytokine profile seen in this model is at present unclear and elucidation of this profile would give clear direction to the research. Studies to clarify whether this model is T_H1, T_H2 or a mix of both types could be set up by collecting BAL at various intervals following the challenging period and detecting changes in IL-2/IFNγ and IL-4/IL-10 levels. This study would give a map of the exact cytokine profile seen upon OVA challenge, when the antigen was sensitised with both adjuvants AlOH and FCA(T).

Post mortem studies carried out on rats treated with OVA, FCA(T) and AlOH showed severe peritonitis and fibrosis of the liver. This inflammation was completely abolished by 3C8 and partially inhibited by DSCG. *In vivo* inflammatory studies revealed that 3C8 and 7C9 inhibited AA mouse ear oedema, as measured by the change in ear thickness. 3C8 was also found to potentially inhibit both carrageenan

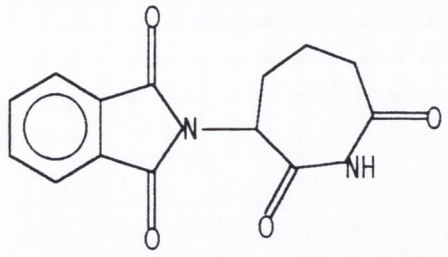
induced rat paw oedema and TPA induced mouse ear oedema. The actions of 7C9 may need further study as controversial results between carrageenan rat paw and TPA-induced mouse ear oedemas were found. These discrepancies may however merely stem from differences between species and tissue studied. 3C8 was also found to alleviate chronic inflammation where vascularisation and fibrosis was prevented on treatment with this test compound. Further studies at a molecular and biochemical level looking at inhibition of enzyme induction, inhibition of enzyme activity and antagonistic properties of these test compounds would be useful to support these anti-inflammatory findings and moreover to pin-point their exact mechanism(s) of action.

Following the positive result of 3C8 on the mixed T_H1/T_H2 type asthma model an *in vivo* DTH model was set up to clarify the mode of action of 3C8 and the other test compounds. The DTH model was validated in past studies (Tarayare *et al* 1990) and believed to follow a T_H1 type profile. The immuno-suppressant activity of 3C8, 6C6 and 7C9 was demonstrated and these test compounds were found to potently inhibit the T_H1 type response in mice. The exact point of action of these compounds is at present unclear and future studies on the effects of the test compounds on the synthesis and release of IL-2, IFNγ and also the macrophage-derived cytokine TNFα would be imperative at this stage.

3C8 (indanol), 6C6 (indanone) and particularly 7C9 (indanol) show similar structural similarity to the indole, thalidomide (Figure 4.3). The activity of thalidomide has been given extensively investigated and found to be extremely effective as an

immuno-suppressant with particular activity against TNFα. Thalidomide appears to act by inhibiting the synthesis of the cytokine *via* the induction of the degradation of the mRNA specific for TNFα (Marriott *et al* 1998; Mc Hugh *et al* 1995). Further studies on thalidomide have demonstrated other properties of this drug. These include the inhibition of T_H1 cytokines by increasing the expression of T_H2 cytokines. As discussed earlier, T_H2 cytokines, IL-4 and IL-10 directly inhibit the production of T_H1 cytokines. PHA-stimulated PMNLs show a T_H1 cytokine profile with the synthesis of IFNγ and treatment with thalidomide exhibited inhibition of IFNγ production by these cells (Mc Hugh *et al* 1995). Inhibition of IL-12 in human peripheral mononuclear cells and primary human monocytes has also been extensively investigated by thalidomide (Moller *et al* 1997). IL-12 is imperative in the polarisation of naïve T_H0 cells towards T_H1 type cells. It is released from APCs (macrophages) following antigen processing and on presentation of the processed peptide to the T-cell. IL-12 has also been shown to be involved in the release of IFNγ from NK cells and T-cells. Inhibition of this cytokine would potentially inhibit the polarisation of the T-cell towards a T_H1 type cytokine and depress the immune system.

Figure 4.3:- Thalidomide



2-(2,6-Dioxo-3-piperidinyl)-1H-iso-indole-1,3(2H)dione (Gunzler, 1992).

The findings of thalidomide and its immuno-suppressant effects support the findings in this study. There is some structural similarity between the test compounds and thalidomide. Thalidomide and our test compounds all consist of a 6-membered ring fused to a 5-membered ring with a carbonyl group. It is therefore possible that the modes of therapeutic action are comparable. Thalidomide is an effective inhibitor of TNFα, which in turn would prevent production of adhesion molecules and the subsequent influx of inflammatory cells (leucocytes, macrophages and T-cells) to the site of inflammation. This result supports the findings of 3C8 and its activity against chronic inflammation both in the asthmatic models and chronic inflammatory models. This test compound inhibited influx of the inflammatory cells and possible modes of action of 3C8 may lie with an inhibitory action of TNFα synthesis.

As discussed above, thalidomide also has the potential to induce T_H2 production and in turn inhibit T_H1 cytokine production. Moreover this drug appears also to promote T_H2 production by inhibiting IL-12 a cytokine imperative in the polarisation of T-cells towards T_H1 cells. This finding supports the inhibitory actions of our test compounds

in the model of DTH whereby the T_H1 inflammatory condition was suppressed by all three test compounds.

These findings present a very interesting future for these drugs and further evaluation of the activity of 3C8, 6C6 and 7C9 at a biochemical and molecular level would give firm direction to evaluating the exact mode of action of these compounds against asthma, inflammation and auto-immune diseases like arthritis, leprosy and transplant rejection.

Chapter 5

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Appendices

Appendix 1

Solutions for the mBSA delayed-type hypersensitivity model

Preparation of mBSA solution:

1. mBSA was dissolved in sterile dH_20 at 10mg/ml
2. The mBSA solution was diluted to 5mg/ml with 2X sterile saline.

Preparation of FCA(B):

For each 1ml adjuvant:

1. 1mg of desiccated *Mycobacterium butyricum* was ground gently with mortar and pestle for approximately 1min.
2. 1ml IFA was added and gently ground again to produce FCA(B).

Preparation of mBSA/FCA(B):

1. Equal volumes of FCA(B) and mBSA solution were emulsified.

Appendix 2

Table 3.1:- The effect of nifedipine, 3C8, 6C6 and 7C9 on calcium induced contractions in K^+ depolarised guinea-pig ileum smooth muscle

	Percentage Inhibition of $CaCl_2$ Induced Contractions							
Conc.(M)	**$3x10^{-9}$**	**10^{-8}**	**$3x10^{-8}$**	**10^{-7}**	**$3x10^{-7}$**	**10^{-6}**	**$3x10^{-6}$**	**10^{-5}**
3C8 (n=3) Mean±sem	2.8±0.5	5.3±0.7	6.7±1.3	9.4±1.3	10.4±1.3	14.5±2.4	22.2±4.3	53.4±3.6
6C6 (n=3) Mean±sem	---	---	0.2±0.4	2.8±1.9	4.6±2.6	9.0±3.5	12.6±3.6	23.0±4.3
7C9 (n=3) Mean±Sem	---	---	---	---	---	---	11.6±4.9	15.4±4.6
	10^{-10}	**$3x10^{-10}$**	**10^{-9}**	**$3x10^{-9}$**	**10^{-8}**	**$3x10^{-8}$**	**10^{-7}**	**$3x10^{-7}$**
Nifedipine (n=3) Mean±Sem	4.4±1.6	8.7±2.2	13.6±1.8	21.5±2.9	48.1±5.1	83.3±3.8	100±0	---

This table shows the percentage inhibition of $CaCl_2$ (2.5mM; mean ± sem; n=3) induced contraction of K^+ depolarised guinea pig ileum smooth muscle in a buffer gassed with 95% O_2 : 5% CO_2 at 37°C.

Table 3.2:- Histamine release from isolated rat mast cells

	Histamine Release				
	Group I Basal (% Total)	**Group II Max/dH_2O (% Total)**	**Group III DSCG (% Total) $2x10^{-5}M$**	**Group IV Max/DMSO (% Total)**	**Group V Test Cpds (% Total) $2x10^{-5}M$**
3C8 (n=5) Mean±sem	17.3±1.1	74.4±3.1	63.0±4.9	59.8±0.8	14.9±2.4
6C6 (n=8) Mean±sem	18.6±1.3	79.4±3.9	69.1±4.6	65.1±3.4	17.4±2.5
7C9 (n=6) Mean±sem	18.8±1.7	79.9±5.2	69.3±5.7	66.7±4.5	68.4±7.2

	Basal	**Maximum**	**Total**
Typical quantity of histamine released from $1x10^6$ mast cells	0.1µg/10^6 cells	2µg/10^6 cells	3µg/10^6 cells

The table represents the percentage inhibition of histamine release from rat peritoneal mast cells, as induced by, compounds 48/80 by DSCG ($2x10^{-5}$ M), 3C8 ($2x10^{-5}$ M), 6C6 ($2x10^{-5}$ M), and 7C9 ($2x10^{-5}$M). This table also represents the typical amount of histamine released per preparation. This table also represents a typical total amount of cells per assay.

Max/H is the maximum release of histamine by compound 48/80 for cells pre-treated with dH_2O.
Max/D is the maximum release of histamine by compound 48/80 for cells pre-treated with 0.5% DMSO.

Table 3.3:- Respiration of rats with acute atopic asthma

Time/ Min	Group I (-ve Ctrl) (n=4)	Group II (+ve Ctrl) (n=4*)	Group III (DSCG) (n=5)	Group IV 3C8 (n=6)
-15	100±0	100±0	100±0	100±0
0	73.4±1.9	67.4±5.3	79.5±3.0	81.2±5.6
15	102.3±6.1	99.2±10.6	82.5±10.9	102.6±6.5
30	97.8±6.3	96.4±1.4	86.2±9.1	106.0±9.7
45	96.8±6.9	89.8±4.4	86.2±12.1	102.9±7.3
60	90.9±5.8	94.3±2.5	95.2±9.7	98.2±11.8
75	89.8±5.5	88.3±1.7	85.2±5.6	89.5±8.2
90	89.9±5.4	87.3±4.5	97.1±6.4	98.2±6.9
105	87.6±3.3	90.1±4.3	91.8±6.6	102.3±6.6
120	84.5±2.1	96.6±6.9	88.7±7.7	101.2±9.3
135	84.1±6.5 *	131.7±13.1 (n=3)	88.0±4.6 **	88.4±9.1 *
150	97.5±2.8 *	111.9±4.4 (n=3)	91.7±8.7	89.2±7.7 ^
165	94.0±4.7	92.8±4.0 (n=3)	93.2±5.8	96.9±11.8
180	94.2±5.7	96.4±9.1 (n=3)	95.2±6.2	98.7±10.8

This table tabulates the percentage change in the rate of respiration in OVA (adjuvants: ALOH + FCA(T)) sensitised rats on challenge with OVA following pre-treatment with vehicle, DSCG or 3C8. Data is represented as percentage normal and calculated as a percentage of the BPM noted 15 minutes prior to OVA challenge.

Note:- Statistical analysis included Mann Whitney, unpaired and two tailed.
* indicates $P<0.05$ as compared to the positive control, at the same time point.
** indicates $P<0.01$ as compared to the positive control, at the same time point.
^ indicates $P<0.1$ as compared to the positive control, at the same time point.

Table 3.4:- Tidal volume from rats with acute atopic asthma

Time	Group I (-ve Ctrl) (n=4)	Group II (+ve Ctrl) (n=4*)	Group III (DSCG) (n=5)	Group IV (3C8) (n=5)
-15	100 ± 0	100 ± 0	100 ± 0	100 ± 0
0	52.4 ± 1.8	63.8 ± 8.8	72.1 ± 12.2	75.5 ± 11.3
15	88.4 ± 1.9	100.2 ± 13.7	135.6 ± 12.6	115.7 ± 9.5
30	93.1 ± 7.3	110.8 ± 10.0	126.3 ± 13.9	115.8 ± 16.7 (n=4)
45	94.2 ± 7.8	93.9 ± 16.9	138.7 ± 8.7	119.1 ± 12.1
60	89.4 ± 3.8 ^	114.0 ± 10.0	135.8 ± 8.3	119.1 ± 17.2
75	91.3 ± 4.5	109.9 ± 10.9	120.5 ± 7.4	106.9 ± 17.6
90	96.8 ± 7.3	106.6 ± 11.5	133.4 ± 7.8	101.9 ± 5.7 (n=4)
105	92.4 ± 4.7	114.9 ± 11.0	131.6 ± 7.8	115.6 ± 11.1 (n=4)
120	91.1 ± 5.5 *	126.8 ± 13.0	126.8 ± 10.6	101.8 ± 16.5 (n=4)
135	90.5 ± 9.0 **	176.3 ± 17.4 (n=3)	137.4 ± 9.9 ^	112.3 ± 18.7 (n=4) ^
150	98.1 ± 3.2 **	140 ± 10.6 (n=3)	123.4 ± 9.1	103.5 ± 22.2 (n=4)
165	95.9 ± 4.6 *	121.2 ± 6.4 (n=3)	147.9 ± 4.3 ^	107.8 ± 17.7 (n=4)
180	104.3 ± 13.0	128.3 ± 14.8 (n=3)	137.3 ± 8.4	121.1 ± 22.5 (n=4)

This table shows the percentage change in tidal volume in OVA (adjuvants: ALOH + FCA(T)) sensitised rats on challenge with OVA following pre-treatment with vehicle, DSCG or 3C8. Data is represented as percentage normal and calculated as a percentage of the TV noted 15 minutes prior to OVA challenge.

Note:- Statistical analysis included Mann Whitney, unpaired and two tailed.
* indicates $P<0.05$ as compared to the positive control, at the same time point.
** indicates $P<0.01$ as compared to the positive control, at the same time point.
^ indicates $P<0.1$ as compared to the positive control, at the same time point.

Table 3.5:- Respiration in broncho-hyperreactive rats

Time/ min	Group I (-ve Ctrl) (n=3)	Group II (+ve Ctrl) (n=5*)	Group III (DSCG) (n=4)	Group IV 3C8 (n=6)
-30	100±0	100±0	100±0	100±0
-15	111.9±9.1	102.8±1.8	101.3±2.7	97.8±1.6
-5	101.0±2.0	102.9±1.6	99.4±1.8	99.4±1.5
0	114.4±13.6	123.7±9.7	99.3±10.5	113.3±6.6
2	114.5±13.5 ^	151.3±12.0(n=4)	106.6±10.0 *	118.8±7.1 *
4	111.2±9.4	122.3±9.1	106.1±2.4	111.6±3.5
6	106.2±6.9	112.3±6.2	106.4±4.7	105.0±2.1
8	104.8±8.0	104.5±4.9	102.1±5.6	101.9±2.9
10	99.3±10.4	104.0±4.1	99.9±4.0	99.1±2.6
15	98.7±6.1	104.4±1.8	98.2±4.3	94.6±2.7 *
30	106.3±12.3	99.3±2.3	92.8±3.7	95.7±2.3
45	106.9±12.1	100.0±2.5	93.8±5.6	99.2±3.3
60	108.7±14.3	98.9±2.6	85.7±7.4	96.8±2.9

This table shows the percentage change in BPM. on exposure to methacholine, in rats with hyper-reactive airways. Hyper-reactive airways were induced by repeated challenge to OVA prior to treatment with vehicle (group I & II) DSCG (Group III) and 3C8 (Group IV). This was carried out following sensitisation to the OVA using AlOH and FCA(T) as adjuvants. Data is represented as percentage normal and calculated as a percentage of the BPM noted 30 minutes prior to methacholine exposure

Note:- Statistical analysis included Mann Whitney, unpaired and two tailed.
^ indicates $P<0.1$ as compared to the positive control, at the same time point.
* indicates $P<0.05$ as compared to the positive control, at the same time point.

Table 3.6:- Tidal volume in broncho-hyperreactive rats

Time/ min	Group I (-ve Ctrl) (n=3)	Group II (+ve Ctrl) (n=5)	Group III (DSCG) (n=4)	Group IV (3C8) (n=6)
-30	100 ± 0	100 ± 0	100 ± 0	100 ± 0
-15	94.2 ± 5.1	96.8 ± 6.8	102.8 ± 5.5	96.5 ± 3.6
-5	96.0 ± 6.7	101.7 ± 7.8	103.8 ± 6.1	93.6 ± 6.2
0	76.4 ± 13.8	72.4 ± 12.9	76.8 ± 10.1	65.1 ± 1.9
2	53.6 ± 11.9	71.3 ± 8.4	74.2 ± 2.0	74.6 ± 2.9
4	68.7 ± 6.4	88.7 ± 7.5	85.0 ± 3.6	72.6 ± 3.5
6	73.6 ± 1.8	90.1 ± 10.7	81.7 ± 3.5	80.0 ± 3.0
8	89.0 ± 9.8	88.8 ± 6.4	90.5 ± 2.6	80.2 ± 5.5
10	75.4 ± 3.2	95.5 ± 8.5	94.1 ± 3.2	88.3 ± 5.8
15	75.4 ± 3.2	101.7 ± 16.8	96.9 ± 3.1	85.4 ± 4.4
30	79.5 ± 5.1	94.1 ± 13.1	91.7 ± 6.8	89.2 ± 6.0
45	82.0 ± 7.2	97.5 ± 13.6	87.6 ± 7.0	88.6 ± 5.6
60	83.8 ± 7.3	93.4 ± 12.8	88.5 ± 8.7	89.6 ± 5.5

This table represents the percentage change in TV, on exposure to methacholine, in Rats with hyper-reactive airways. Hyper-reactive airways were induced by repeated challenge to OVA prior to treatment with vehicle (group I & II) DSCG (Group III) and 3C8 (Group IV). This was done following sensitisation to the OVA using AlOH and FCA(T) as adjuvants. Data is represented as percentage normal and calculated as a percentage of the TV noted 30 minutes prior to methacholine exposure

Note:- Statistical analysis included Mann Whitney, unpaired and two tailed.
No significant difference was calculated between the groups over the time points given.

Table 3.7:- The effect of 3C8, 6C6 and 7C9 on AA induced oedema

Group	Percentage change / Mean±sem	n
Group I:-Positive Control Acetone (10µl/ ear)	78.8±15.2	8
Group II:- Dexamethasone (300µg/ ear)	37.9±8.5	4
Group III:- Indomethacin (300µg/ ear)	39.6±5.8	4
Group IV:- 3C8 (300µg/ ear)	32.4±7.8	4
Group V:- 6C6 (300µg/ ear)	73.0±8.7	4
Group VI:- 7C9(300µg/ ear)	10.5±6.2	4

The table represents the percentage change in AA (4mg/ear)-induced mouse ear oedema on pre-treatment with acetone (control; n=8), dexamethasone (300µg/ear; n=4). Indomethacin (300µg/ear; n=4), 3C8 (300µg/ear; n=4), 6C6 (300µg/ear; n=4) and 7C9 (300µg/ear; n=4)

Table 3.8:- The effect of 3C8, 6C6 and 7C9 on TPA induced ear oedema

Group	Percentage change / Mean±sem	n
Group I:-Positive Control Acetone (20µl/ ear)	114.7±8.8	10
Group II:- Dexamethasone (10µg/ ear)	30.3±4.0	10
Group III:- 3C8 (300µg/ ear)	82.7±8.4	5
Group IV:- 6C6 (300µg/ ear)	113.5±9.1	5
Group V:- 7C9(300µg/ ear)	99.4±15.7	5

The table represents the percentage change in TPA (1.5µg/ear)-induced mouse ear oedema on pre-treatment with acetone (control; n=10), dexamethasone (10µg/ear; n=10), 3C8 (300µg/ear; n=5), 6C6 (300µg/ear; n=5) and 7C9 (300µg/ear; n=5) 1h prior to the induction of oedema by TPA.

Table 3.9:- The effect of 3C8 and 7C9 on carrageenan induced rat paw oedema

Group	Percentage change / Mean±sem	n
Group I:-Positive Control (1% w/v CMC; p.o)	53.9±4.6	15
Group II:- (Dexamethasone; 30mg/kg suspended in 1% w/v CMC; p.o)	16.3±4.6 ***	6
Group III (Indomethacin; 30mg/kg suspended in 1% w/v CMC; p.o.)	21.3±3.2 ***	6
Group IV:- (3C8; 30mg/kg suspended in 1% w/v CMC; p.o)	30.1±4.6 **	6
Group V:- (7C9; 30mg/kg suspended in 1% w/v CMC; p.o)	28.4±2.9 ***	9

This table represents the percentage change in carrageenan (1% w/v) -induced rat paw oedema on pre-treatment with 1% CMC (control; n=15), dexamethasone (30mg/kg; n=6), indomethacin (30mg/kg; n=6) 3C8 (30mg/kg; n=6) and 7C9 (30mg/kg; n=9) 1h prior to the induction of oedema with carrageenan.

Table 3.10:- The effect of 3C8 on a chronic model of inflammation as induced by sponge implants

Group	Change in dry weight of sponge implant Mean±sem (g)	n
Group I:-Positive Control (Vehicle; 50% ETOH 50% H_2O; i.p 1ml/kg)	0.21±0.02	6
Group II (Dexamethasone; 1mg/kg in 50% ETOH 50% H_2O; i.p)	0.10±0.02 **	5
Group III (Indomethacin; 1mg/kg in 50% ETOH 50% H_2Ol; i.p)	0.13±0.03 *	5
Group IV:- (3C8; 1mg/kg in 50% ETOH 50% H_2O; i.p)	0.14±0.02 *	4

This table represents the percentage change oedema as measured by the change in sponge dry weight on daily treatment with ETOH (50%w/v; 1ml/kg) (control; n=6), dexamethasone (1mg/kg; n=5), indomethacin (1mg/kg; n=5), 3C8 (1mg/kg; n=4)

Table 3.11:- Percentage leukocytes in BAL from BN rats acutely sensitised to OVA using AlOH as an adjuvant

	Group I -ve ctrl [Mean± SEM] (n=5)	Group II +ve ctrl [Mean± SEM] (n=8)	Group III DEX 1mg/kg [Mean± SEM] (n=4)	Group IV 3C8 (10mg/kg) [Mean± SEM] (n=5)
Eosinophils	1.9±0.5	19.9±2.6	1.6±0.4	13.2±1.9
Neutrophils	0.2±0.1	4.8±1.9	0.3±0.2	27.4±5.5
Basophils	1.0±0.3	1.1±0.2	0.6±0.4	2.3±0.6

This graph represents the percentage number of leukocytes found in the BAL fluid of rats acutely sensitised and challenged to OVA using AlOH as an adjuvant on sensitisation.

-ve ctrl:- Negative control (Group I; n=5), untreated and unchallenged.
+ve ctrl:- Positive control (Group II; n=8), untreated and OVA challenged.
Dex:- Group III, dexamethasone (1mg/kg; n=4) treated and OVA challenged.
3C8:- Group IV, 3C8 (10mg/kg; n=5) treated and OVA challenged.

Table 3.12:- Percentage leukocytes in BAL from BN rats acutely sensitised to OVA using FCA(T) as an adjuvant

	Group I -ve ctrl [Mean± SEM] (n=6)	Group II +ve ctrl [Mean± SEM] (n=5)	Group III DEX 10mg/kg [Mean± SEM] (n=5)	Group IV 3C8 (10mg/kg) [Mean± SEM] (n=5)
Eosinophils	2.2±0.9	17.0±3.7	3.2±1.1	9.9±1.9
Neutrophils	0.8±0.2	20.1±11.5	4.2±1.8	48.0±7.0
Basophils	0.03±0.03	0.2±0.1	0.1±0.1	0.2±0.1

This table represents the percentage number of leukocytes found in the BAL fluid of rats acutely sensitised and challenged to OVA using FCA(T) as an adjuvant on sensitisation.

-ve ctrl:- Negative control (Group I; n=6), untreated and unchallenged.
+ve ctrl:- Positive control (Group II; n=5), untreated and OVA challenged.
Dex:- Group III, dexamethasone (10mg/kg; n=5) treated and OVA challenged.
3C8:- Group IV, 3C8 (10mg/kg; n=5) treated and OVA challenged.

Table 3.13:- Percentage leukocytes in BAL from BN rats acutely sensitised to OVA using AlOH and FCA(T) as adjuvants

	Group I -ve ctrl [Mean± SEM] (n=5)	Group II +ve ctrl [Mean± SEM] (n=5)	Group III DEX 10mg/kg [Mean± SEM] (n=5)	Group IV 3C8 (10mg/kg) [Mean± SEM] (n=5)
Eosinophils	7.1±2.1	20.5±3.3	6.7±2.1	6.9±0.7
Neutrophils	0.8±0.3	35.6±9.3	2.5±1.5	55.4±8.7
Basophils	0.8±0.3	0.7±0.2	0.2±0.1	0.2±0.1

This table represents the percentage number of leukocytes found in the BAL fluid of rats acutely sensitised and challenged to OVA using FCA(T) as an adjuvant on sensitisation.

-ve ctrl:- Negative control (Group I; n=6), untreated and unchallenged.
+ve ctrl:- Positive control (Group II; n=5), untreated and OVA challenged.
Dex:- Group III, dexamethasone (10mg/kg; n=5) treated and OVA challenged.
3C8:- Group IV, 3C8 (10mg/kg; n=5) treated and OVA challenged.

Table 3.14 Percentage leukocytes in BAL from BN rats chronically sensitised to OVA using AlOH as an adjuvant

	Group I -ve ctrl [Mean± SEM] (n=5)	Group II +ve ctrl [Mean± SEM] (n=5)	Group III Budesonide 10mg/kg [Mean± SEM] (n=4)	Group IV 3C8 (10mg/kg) [Mean± SEM] (n=4)
Eosinophils	0.6±0.2	12.7±2.1	1.3±0.62	8.62±2.8
Neutrophils	4.2±1.7	27.3±10.5	1.3±0.7	23.3±4.2
Basophils	0±0	0.2±0.1	0.04±0.04	0.8±0.8

This table represents the percentage number of leukocytes found in the BAL fluid of rats chronically sensitised and challenged to OVA using AlOH as an adjuvant on sensitisation.

-ve ctrl:- Negative control (Group I; n=5), untreated and unchallenged.
+ve ctrl:- Positive control (Group II; n=5), untreated and OVA challenged.
Dex:- Group III, budesonide (10mg/kg; n=4) treated and OVA challenged.
3C8:- Group IV, 3C8 (10mg/kg; n=4) treated and OVA challenged.

Note:- The above figure includes the final pre-treatment and OVA challenge. A middle, 4 week vehicle/drug pre-treatment and OVA challenge was also carried out (section 2.1.6.2 (a))

Table 3.15:- Percentage leukocytes in BAL from BN rats chronically sensitised to OVA using AlOH and FCA(T) as adjuvants

	Group I -ve ctrl [Mean± SEM] (n=5)	Group II +ve ctrl [Mean± SEM] (n=4)	Group III DEX 10mg/kg [Mean± SEM] (n=5)	Group IV 3C8 (10mg/kg) [Mean± SEM] (n=6)
Eosinophils	1.5±0.3	18±1.9	1.5±0.6	1.8±0.5
Neutrophils	1.4±1.1	29.2±14	10.0±7.5	0.8±0.6
Basophils	0±0	0.3±0.1	0±0	0±0

This table represents the percentage number of leukocytes found in the BAL fluid of rats chronically sensitised and challenged to OVA using AlOH and FCA(T) as adjuvants on sensitisation.

-ve ctrl:- Negative control (Group I; n=5), untreated and unchallenged.
+ve ctrl:- Positive control (Group II; n=5), untreated and OVA challenged.
Dex:- Group III, dexamethasone (10mg/kg; n=5) treated and OVA challenged.
3C8:- Group IV, 3C8 (10mg/kg; n=5) treated and OVA challenged.

Note:- The above figure includes the final pre-treatment and OVA challenge. A middle, 4 week vehicle/drug pre-treatment and OVA challenge was also carried out (section 2.1.6.2 (a))

Table 3.16:- The effect of 3C8, 6C6 and 7C9 against mBSA induced paw oedema

Group	Percentage change (mean ± sem)	Number (n)
Negative Control	9.4 ± 3.2	12
Positive Control	57.1 ± 7.1	12
CyA (50mg/kg)	38.4 ± 5.6	12
3C8 (3mg/kg)	51.5 ± 3.4	5
3C8 (10mg/kg)	35.0 ± 3.3	6
6C6 (3mg/kg)	40.6 ± 9.0	6
6C6 (10mg/kg)	30.5 ± 6.4	6
7C9 (3mg/kg)	49.7 ± 7.8	6
7C9 (10mg/kg)	35.3 ± 9.0	6

The table represents the percentage change in mBSA-induced mouse paw oedema (DTH) on treatment with 1% CMC (+ve control; n=12), cyA (50mg/kg; n=12), 3C8 (A1, 3mg/kg (n=5) A2, 10mg/kg (n=6)), 6C6 (B1, 3mg/kg (n=6) B2, 10mg/kg (n=6)), 7C9 (C1, 3mg/kg (n=6) C2, 10mg/kg (n=6)),). A negative control (-ve n=12) was carried out to sow background inflammation not releated to mBSA sensitisation.