CYTOKINE EXPRESSION IN MAST CELLS AND RAT LUNG TISSUE

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by

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To Steve and Aidan

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Abstract

Although mast cells are known to initiate early phase allergic reactions it is not known whether they contribute to the progression of late phase reactions. Therefore, the potential of mast cells to act as a source of cytokines was studied. Immunological activation of rat PMC induced expression of mRNA encoding IL-5, IL-6, TNF-α, MIP-2 and IFN-γ and release of biologically active TNF-α. Additionally, Cl.MC/C57.1 murine mast cells released TNF-α and IL-6 protein and release of TNF-α was enhanced after activation of the cells for 1 h with IgE/antigen. Unstimulated HMC-1 human mast cells expressed mRNA encoding IL-3, IL-4, IL-8 and TNF-α; mRNA levels encoding IL-3, IL-4 and IL-8 but not TNF-α were enhanced after stimulation of the cells for 4h with ionomycin.

Culture of rat PMC with Dex or CsA (10^{-7}M, 24h) inhibited the anti-IgE induced release of 5-HT. Dex also inhibited the release of arachidonate from these cells. However, BTS 71321 had no effect on the release of these mediators. Culture of rat PMC with Dex or CsA (10^{-6}M, 24h) also inhibited the anti-IgE-induced expression of mRNA encoding IL-5, IL-6, TNF-α, MIP-2 and IFN-γ and the release of TNF-α protein. CsA (10^{-6}M) inhibited ionomycin-induced gene expression of IL-3 by HMC-1 cells and both CsA and BTS 71321 inhibited ionophore-induced release of TNF-α from Cl.MC/C57.1 cells.

Culture of Cl.MC/C57.1 mast cells with SCF (100ng/ml, 48h) enhanced the IgE/antigen-induced release of TNF-α and culture of HMC-1 cells with IL-4 (100ng/ml, 24h) enhanced ionomycin-induced gene expression of IL-3 and IL-8.

At various times after a single *i.v.* injection of Sephadex particles into rats, mRNA encoding the cytokines IL-5, IL-6, TNF-α, MIP-2 and IFN-γ was induced transiently in BAL cells. These changes occurred alongside transient increases in mononuclear cell, neutrophil, eosinophil and mast cell numbers in BAL cell populations. A temporal relationship appeared to exist between bronchoalveolar neutrophilia and induced expression of MIP-2 both of which peaked at 24-48h. Induced expression of IL-5 mRNA was also related to the increase in eosinophil numbers both of which peaked 72h after Sephadex administration. A similar pattern of events was observed in pleural lavage fluid after ovalbumin challenge of ovalbumin-sensitised rats, thus demonstrating similarities between antigen-induced inflammation and that induced by a physical stimulus (Sephadex). Pre-treatment of rats with Dex (0.1mg/kg or 0.3mg/kg), CsA (10mg/kg or 30mg/kg) or BTS 71321 (10 mg/kg) prior to administration of Sephadex inhibited BAL cell cytokine gene transcription. Dex and CsA also inhibited cell mobilisation although BTS 71321 did not. However, BTS 71321 may have some therapeutic potential through its inhibition of TNF-α expression. Additionally, in view of the fact that activated mast cells can express mRNA encoding IL-5, IL-6, TNF-α, MIP-2 and IFN-γ, each of which were up-regulated at various times during the progression of Sephadex-induced lung inflammation and antigen-induced pleurisy, mast cells may be considered as candidate sources for the production of these cytokines. However, cytokine mRNA expression by BAL cells particularly up to 72h appeared in the absence of detectable mast cells.

Publications

Buckley M.G., Williams C.M.M., Thompson J., Pryor P., Ray K., Butterfield J.H. and Coleman J.W. 1995. IL-4 enhances IL-3 and IL-8 gene expression in a human leukemic mast cell line. *Immunology* **84**: 410-415.

Williams C.M.M. and Coleman J.W. 1995. Induced expression of mRNA for IL-5, IL-6, TNF-α, MIP-2 and IFN-γ in immunologically activated rat peritoneal mast cells: inhibition by dexamethasone and cyclosporin A. *Immunology* **86**: 244-249.

ABBREVIATIONS

A	Adenosine
AIDS	Acquired Immune Deficiency Syndrome
AMV	Avian myoblastosis virus (reverse transcriptase)
Anti-IgE	IgE-crosslinking antibody
ATP	Adenosine triphosphate
BAL	Bronchoalveolar lavage
BMMC	Bone marrow-derived cultured mast cells (mouse)
B9	Murine IL-6 dependent cell line
bp	Base pairs
BSA	Bovine serum albumin
BTS 713121	N-[1-(4-chlorophenyl)-1-methylethyl]-3-(imidazol-1-yl)propylamine
C	Cytosine
CD	Cluster differentiation
cDNA	Complimentary DNA
C5a	Complement component 5a
Ci	Curies
c-kit	Stem cell factor receptor
Cl.MC/C57.1	Growth factor-independent mouse mast cell line derived from bone marrow cells
cpm	Counts per minute
CsA	Cyclosporin A

DAG	Diacylglycerol
DEPC	Diethyl pyrocarbonate
Dex	Dexamethasone
dig 11 UTP	Digoxigenin labelled UTP
DMEM	Dulbecco's modified Eagle's medium
DNAse	Deoxyribonuclease I
DNP-HSA	Dinitrophenyl-human serum albumin conjugate
dNTP	Dinucleotide triphosphate
DTT	Dithiothreitol
EAR	Early asthmatic reaction
ECP	Eosinophil cationic protein
EDTA	Ethylene diamine tetraacetic acid
ELAM-1	Endothelial leucocyte adhesion molecule-1
ELISA	Enzyme-linked immunosorbent assay
EPO	Eosinophil peroxidase
FcϵRI	High affinity IgE receptor
FCS	Foetal calf serum
5-HT	5-Hydroxytryptamine (serotonin)
G	Guanosine
GM-CSF	Granulocyte-macrophage colony stimulating factor
GTP	Guanosine triphosphate
HMC-1	Growth factor-independent mast cell line derived from a patient with a mast cell leukemia
h	Hour(s)

ICAM-1	Intercellular adhesion molecule-1
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin
IMDM	Iscove's modified Dulbecco's medium
IP_3	Inositol 1, 4, 5-trisphosphate
kDa	Kilodalton (a molecular weight of 1000)
LAR	Late asthmatic reaction
LB	Liquid broth
L929	Murine cell line sensitive to TNF-α
LT	Leukotriene
MBP	Major basic protein
MC^T	Human mast cells containing tryptase but not chymase
MC^{TC}	Human mast cells containing both tryptase and chymase
min	Minute(s)
MIP	Macrophage inflammatory protein
mRNA	Messenger RNA
MTT	3 - [4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
p	Probability
PAF	Platelet activating factor
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
PG	Prostaglandin
PMC	Peritoneal mast cells
RNAsin	Recombinant inhibitor of RNAse
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute culture medium
RT	Reverse transcription
SCF	Stem cell factor
SDS	Sodium dodecyl sulphate
SSC	Sodium citrate / sodium chloride buffer
T	Thymidine
TAE	Tris-acetate-EDTA buffer
TBE	Tris-borate-EDTA buffer
TBS	Tris buffered saline
TE	Tris-EDTA buffer
Th	Helper T lymphocyte
TMA	Tetramethyl ammonium chloride
TNF-α	Tumor necrosis factor-alpha
U	Units
VCAM-1	Vascular cell adhesion molecule-1
VLA-4	Very late antigen-4 (the ligand for VCAM-1)

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CHAPTER 1

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1.1 GENERAL INTRODUCTION

1.1.1 The immune system

The immune system has developed as an efficient means of eliminating pathogenic viruses, microorganisms and parasites from the body. The response involves two phases; a recognition phase mediated by lymphocytes and an effector phase which involves a variety of cell types and is directed towards the elimination of the initiating antigen. The recognition phase must identify antigens of potential pathogens whilst tolerating the body's own tissues. Any failure to do so will result in autoimmune disease. The effector phase involves the activation of effector systems including antibody production, macrophage activation and generation of cytotoxic cells. An inappropriate response will result in insufficient elimination of the invading pathogen and an overpowerful response may result in hypersensitivity.

Re-exposure to an antigen results in a more rapid and effective immune response. The readily available and expanded population of memory cells generated in the recognition phase produce antigen-specific antibodies which precipitate the antigen and induce opsonization by macrophages. These adaptive responses develop in parallel with the non-adaptive or innate immune system which involves the physical barriers of the skin and mucus membranes and other non-immunologically specific cells such as phagocytes and natural killer cells.

Occasional failures within the immune system such as immunodeficiency, autoimmune disease and hypersensitivity can lead to

serious and even life threatening problems. An inadequate response to an invading pathogen resulting from immunodeficiency may be inherited as in thymic aplasia or acquired as in Acquired Immune Deficiency Syndrome (AIDS). Immunodeficiency can effect all aspects of the immune system but an impairment of T and B cell responses is particularly serious. A breakdown in tolerance to self tissues can result in autoimmune disease whereas an overpowerful immune response leads to hypersensitivity-type reactions such as allergy.

1.1.2 Allergy

The original definition of allergy by Von Pirquet in 1906 was a "specifically changed reactivity of the host to an agent on a second or subsequent occasion". Allergic reactions are in essence, hypersensitivity-type reactions involving damage to normal tissues via interactions of the allergen/antigen with specific antibodies. Many allergic reactions are mediated by Immunoglobulin (Ig) E antibodies bound predominantly to mast cells and basophils. Immunoglobulin E-mediated allergic reactions include rhinitis, sinusitis, asthma, hypersensitivity pneumonitis, extrinsic allergic alveolitis, conjunctivitis, urticaria, eczema, atopic dermatitis, anaphylaxis and angioedema. Other disorders such as serum sickness are mediated by IgG or IgM antibodies and complement, whereas allergic contact dermatitis is caused by T lymphocytes. Atopy, the enhanced ability of an individual to generate IgE antibodies in response to common allergens is frequently a feature of allergy. Immunoglobulin E is the primary antibody involved in

inducing an immediate allergic (type I hypersensitivity) reaction (Gould, 1993). It binds with high affinity to receptors on mast cells and basophils termed FcϵRI, sensitizing these cells for subsequent release of preformed and *de novo* synthesised mediators which initiate an immediate allergic reaction. Other low affinity IgE receptors (FcϵRII) are found on macrophages, eosinophils, platelets, B and T cells, Langerhans cells, follicular dendritic cells and natural killer cells (Gould, 1993). In addition to its ability to bind IgE with low affinity, FcϵRII induces the growth and differentiation of activated B cells and acts as a co-factor for the IL-4 induced heavy chain switching to IgE expression. Additionally, the binding of IgE/antigen complexes to the receptor on B cells, and subsequent internalization, contributes to antigen presentation (Gould, 1993).

Immediate allergic reactions often lead to secondary or late phase reactions which occur three to six hours after the initial attack. These more serious late phase reactions are frequently observed in patients suffering form asthma and are not confined to mast cell and basophil release of histamine and eicosanoids. Late phase reactions and allergic inflammation appear to involve other cell types including T cells macrophages and eosinophils with increased expression of several pro-inflammatory cytokines.

1.2 CYTOKINES AND ALLERGIC DISEASE

1.2.1 An introduction to cytokines

Cytokines are soluble proteins or glycoproteins which act as chemical communicators between cells. They are produced by a variety of cell types and are usually secreted, but in some instances may be expressed on cell membranes or stored within reservoirs in the extracellular matrix. They bind to specific receptors on surfaces of target cells which are coupled to intracellular signal transduction and second messenger pathways and are involved in the differentiation of pluripotent and oligopotent stem cells, tissue growth and repair, mediation of nervous stimulation and chemotaxis. Although cytokine nomenclature owes little to any systematic relationships between molecules a number of families exist based on similar structures. These are shown in Table 1.2.1. Haematopoietic growth factors, peptide growth factors, interleukins, interferons, neuropeptides and chemokines all have potentially important roles in the development and maintenance of allergic disease.

1.2.2 Cytokines in immune responses

Through the regulation of immunoglobulin isotype switching and clonal amplification of B cell responses, cytokines can contribute extensively to antigen specific immunity. For example IL-4 specifically induces IgG4 and IgE production by human B cells thus assuming a central role in the development of IgE-dependent allergic reactions (Pène *et al.*, 1988a;

Finkleman *et al.*, 1988). These effects are modulated by many other cytokines. Interferon-γ and interferon-α inhibit IgE synthesis *in vitro* (Pène *et al.*, 1988a, 1989) and *in vivo* (King *et al.*, 1989; Souillet *et al.*, 1989) whereas IL-5, IL-6 and TNF-α enhance IL-4-induced IgE synthesis (Pène *et al.*, 1988b, 1989; Vercelli *et al.*, 1989). Additionally, IL-5 promotes the immunoglobulin switch to IgA and IFN-γ induces the switch to IgG2a. Additionally, a number of cytokines including IL-4, IL-10 and IFN-γ regulate major histocompatibility complex (MHC) class II expression on a variety of cells thus influencing their antigen presenting capacity.

1.2.3 Cytokines in inflammation

Cytokines initiate a cascade of pro-inflammatory responses following tissue damage leading to acute and chronic inflammation. For example, IL-1, IL-6 and TNF-α, the principle pro-inflammatory cytokines, promote the stimulation of gene expression for acute phase proteins, the activation of inflammatory cells through the secondary release of haematopoietic growth factors and chronic tissue reparative or damaging processes such as fibrosis which in turn involve other cytokines (Denburg, 1993). Other haematopoietic growth factors initiate the influx, activation and prolonged survival of inflammatory cells. For example, IL-5 specifically increases the production and prolongs the survival of eosinophils. Nerve release of neuropeptides such as substance P initiate the release of cytokines such as G-CSF, GM-CSF and M-CSF from stromal cells which can activate neutrophils, eosinophils, basophils and macrophages (Denburg, 1993). Additionally, cytokines can increase the

expression of cell surface molecules which is important in adherence to tissues.

Family	Members	Receptor type
Haematopoietins (4 α-helical bundles)	IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-13, G-CSF, GM-CSF, CNTF, OSM, LIF, Epo	Cytokine receptor class I
	IL-10, IFN-α, IFN-β, IFN-γ	Cytokine receptor class II
	M-CSF	Tyrosine kinase
EGF (β-sheet)	EGF, TGF-α	Tyrosine kinase
β-Trefoil	FGF-α, FGF-β,	split tyrosine kinase
	IL-1α, IL-1β, IL-1Rα	IL-1 receptor
TNF (jelly roll motif)	TNF-α, TNF-β, LT-β	NGF receptor
Cysteine knot	NGF	NGF receptor
	TGF-β1, TGF-β2, TGF-β3	Serine/threonine kinase
	PDGF, VEGF	Tyrosine kinase
Chemokines (triple-stranded, anti parallel β-sheet in Grey key motif)	IL-8, MIP-1α, MIP-1β, MIP-2 PF-4, PBP, MCP-1, MCP-2 MCP-3	Rhodopsin superfamily

Table 1.2.1 Structural families of cytokines (Callard and Gearing, 1994)

(Abbreviations: IL, interleukin; G-CSF, granulocyte colony stimulating factor; GM-CSF, granulocyte/macrophage colony stimulating factor, CNTF, ciliary neutrophic factor; OSM, oncostatin M; LIF, leukemia inhibitory factor; Epo, erythropoietin; IFN, interferon; M-CSF, macrophage colony stimulating factor; EGF, epidermal growth factor; TGF, transforming growth factor; FGF, heparin binding growth factor; TNF, tumour necrosis factor; NGF, nerve growth factor; PDGF, platelet derived growth factor; VEGF, vascular endothelial derived growth factor; MIP, macrophage inflammatory protein; PF, platelet factor; PBP, platelet basic protein; MCP, monocyte chemoattractant protein.

1.2.4 Cytokines in allergic reactions

Allergic reactions combine inflammatory and immune responses and it has recently become apparent that the orchestration of these events is directed by an extensive variety of cytokines. For example cytokines are produced by immune and inflammatory cells that are activated in asthma, resulting in cell recruitment, survival and activation. Interleukin-1, one of the first cytokines to be discovered and released predominantly by monocytes and macrophages, is up-regulated in asthmatic airways (Mattoli *et al.*, 1991). It activates T cells to express IL-2 and airway epithelial cells to produce IL-8 and GM-CSF (Cromwell *et al.*, 1992). Interleukin-2 is produced by activated Th1 cells and has been reported to induce bronchial hyperresponsiveness in Brown-Norway rats (Renzi *et al.*, 1991) whereas IL-3, produced primarily by T cells, but also mast cells, promotes the development of mast cells and

basophils and also the survival of eosinophils (Galli, 1990; Clutterbuck *et al.*, 1989). Interleukin-4 plays a critical role in immunoglobulin isotype switching to IgE synthesis and is up-regulated in mast cells from asthmatic airways (Bradding *et al.*, 1994). It also increases the expression of vascular cell adhesion molecule (VCAM)-1 which promotes eosinophil adhesion in the bronchial circulation (Schleimer *et al.*, 1992). Interleukin-5 is produced by Th2 lymphocytes and other cells and is associated with eosinophilic inflammation (Sanderson, 1992). Elevated IL-5 concentrations have been reported in bronchoalveolar lavage fluid and increased IL-5 mRNA expression has been detected in bronchial biopsies from asthmatic patients (Hamid *et al.*, 1991; Walker *et al.*, 1992; Bentley *et al.*, 1993). There is also evidence of increased release of IL-6 from alveolar macrophages from asthmatic patients and increased basal release compared to non-asthmatic patients (Gosset *et al.*, 1991, 1992). IL-6 acts as a co-stimulatory factor with a number of cytokines on a variety of inflammatory and immune cells and contributes extensively to the acute phase response (van Snick, 1990). Chemokines also play an important role in the development of allergic inflammation. Interleukin-8, the major neutrophil chemoattractant in the human lung is released by a variety of airway cells (Kunkel *et al.*, 1991) including macrophages (Cromwell *et al.*, 1992), eosinophils, epithelial cells (Nakamura *et al.*, 1991) and fibroblasts (Rolff *et al.*, 1991). Other members of the chemokine family such as RANTES act as chemoattractants for eosinophils (Rot *et al.*, 1992; Kameyoshi *et al.*, 1992). Enhanced expression of GM-CSF has been detected in epithelial cells of asthmatic patients (Sousa

et al., 1993) and in T lymphocytes and eosinophils after endobronchial allergen challenge (Broide and Firestein, 1991; Broide *et al.*, 1992). GM-CSF is known to activate neutrophils, eosinophils and macrophages (Denburg, 1993; Bittleman and Casale, 1994) and is important for the survival of eosinophils in culture (Hallsworth *et al.*, 1992). Tumour necrosis factor-α is also present in BAL fluid from asthmatic patients (Broide *et al.*, 1992) and increased gene expression has been detected after IgE triggering of blood monocytes and alveolar macrophages *in vitro.* This effect is further enhanced by IFN-γ (Gosset *et al.*, 1992). TNF-α enhances the expression of intracellular adhesion molecule-1 (ICAM-1) on airway epithelial cells (Tosi *et al.*, 1992) and synergizes with IL-4 and IFN-γ to increase VCAM-1 expression on endothelial cells (Thornhill *et al.*, 1991) thus leading to increased adhesion of leucocytes to airway surfaces. Furthermore, infusion of TNF-α causes increased bronchial hyperresponsiveness in Brown Norway rats (Kips *et al.*, 1992) and inhalation by normal human subjects results in an increase in sputum neutrophils and increased airway responsiveness at 24 h (Yates *et al.*, 1993). Interferon-γ expression is increased in culture supernatants of peripheral blood mononuclear cells from patients with mite sensitive asthma (Okubo *et al.*, 1994) and is known to amplify the release of TNF-α from alveolar macrophages induced by IgE triggering or by endotoxin (Gosset *et al.*, 1992; Gifford and Lohmann-Matthess, 1987). Additionally, airway epithelial cells, eosinophils and airway smooth muscle secrete a number of peptide growth factors including PDGF and TGF-β (Kelley, 1990) which may activate fibroblasts to proliferate and secrete

collagen (Rose *et al.*, 1986).

It is clear that cytokines are critical to the development of allergic responses such as asthma. In this thesis five cytokines, namely IL-5, IL-6, TNF-α, MIP-2 and IFN-γ, were selected for study on the basis of their potential involvement in inflammation and also for technical reasons. The structures, functions and receptors for these cytokines are further discussed in section 1.2.5.

1.2.5.1 Interleukin-5

Interleukin-5 is a glycoprotein produced by T cells, mast cells and eosinophils. It is also known as eosinophil differentiation factor, eosinophil colony stimulating factor, B cell growth factor II, B cell differentiation factor for IgM, IgA enhancing factor or T cell replacing factor. Three dimensionally it exists as an antiparallel disulphide-linked homodimer. Its unique structure is made up of two left handed bundles of four α-helices with two short β-sheets on opposite sides of the molecule (Callard and Gearing, 1994). It has a novel two domain structure each domain sharing structural homology with the cytokine fold in GM-CSF, M-CSF, IL-2, IL-4 and growth hormone (Callard and Gearing, 1994).

Interleukin-5 selectively induces eosinophil differentiation and stimulates the production and maturation of eosinophils at terminal stages (Clutterbuck *et al.*, 1987 1989; Clutterbuck and Sanderson, 1988; Saito *et al.*, 1988). It is also a potent eosinophil chemoattractant and activator (Lopez *et al.*, 1988; Wang *et al.*, 1989). Murine IL-5 also stimulates B cell

activation, growth and differentiation although whether human IL-5 has activity on B cells remains controversial (Sanderson *et al.*, 1988). Although the gene in mouse and human is situated on the long arm of chromosome 5 in close vicinity to IL-3, IL-4 and GM-CSF (van Leeuwen *et al.*, 1989) its DNA promoter region is more diverse than the other cytokines. The phorbol ester-responsive element, TGACTCA which is present in the IL-2 promoter and is common for many PMA-inducible genes, differs by two nucleotides in the IL-5 promoter (TGACT<u>GG</u>) as too does the ten-base-pair lymphokine consensus sequence which is common to mouse and human IL-2, IL-4, GM-CSF and human IFN-γ. However, the IL-5 gene does contain a number of conserved sequences between mouse and human, one of which differs by only one nucleotide from the -34 to -52 base pair sequence in the GM-CSF responsive element, suggesting that in some tissues or in some instances the two genes may be expressed together (Staynov and Lee, 1992). Staynov and Lee (1992) have also suggested that the time course of activation of IL-5 gene transcription is tissue specific.

The IL-5 receptor (Fig. 1.2.5.1) consists of a low affinity binding α-chain (CD125) and a non-binding β-chain (KH97). In humans the non-binding β-chain converts the low affinity receptor to a high affinity receptor but it does not bind ligand itself. However it has a much longer intracytoplasmic portion and is able to transduce a signal. The mouse IL-5 receptor has two β subunits AIC2A and AIC2B. The AIC2B subunit has similar activity to the human KH97 form. Signal transduction mechanisms are less well characterized although tyrosine phosphorylation takes place.

A soluble secreted form of the IL-5 receptor has also been described (Murata *et al.*, 1993) although its function is not clear.

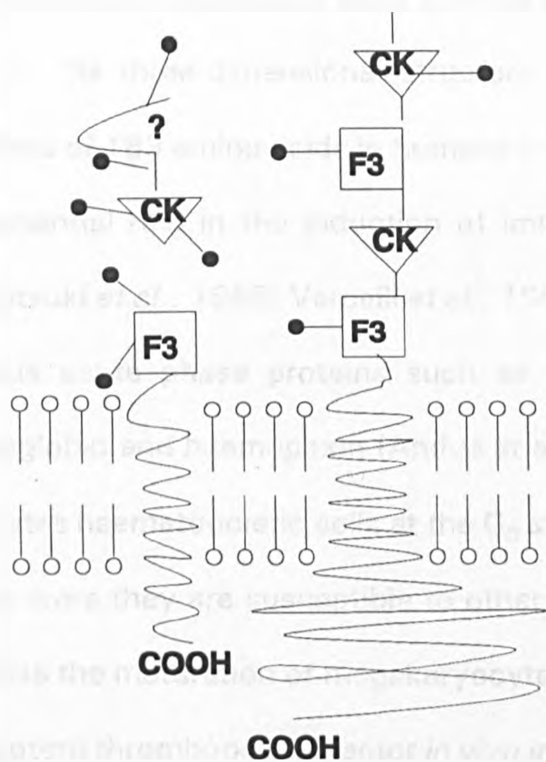

Fig.. 1.2.5.1 The IL-5 receptor. CK Cytokine receptor, F3 Fibronectin type III domain, —● N-glycosylation sites.

1.2.5.2 Interleukin-6

Interleukin-6 is also known as IFN-β2, 26-kDa protein, B cell stimulatory factor, hybridoma/plasmacytoma growth factor, hepatocyte stimulating factor, monocyte granulocyte inducer type 2, cytotoxic T cell differentiation

factor and thrombopoietin. It was originally identified as a T cell derived cytokine but is also produced by non-lymphoid cells including macrophages, bone marrow stromal cells, fibroblasts, keratinocytes, mesangium cells, astrocytes and endothelial cells (Callard and Gearing, 1994; Sehgal *et al.*, 1987). Its three-dimensional structure is unknown but its mature form consists of 183 amino acids in humans and 187 amino acids in mice. It has an essential role in the induction of immunoglobulin synthesis in B cells (Takatsuki *et al.*, 1988; Vercelli *et al.*, 1989) and acts on liver cells inducing various acute phase proteins such as C-reactive protein, β_2-fibrinogen, haptoglobin and haemopexin (Andus *et al.*, 1987; Gauldie *et al.*, 1987). It activates haematopoietic cells at the G_0 stage inducing them to enter the G_1 stage were they are susceptible to other cytokines such as IL-3. IL-6 also induces the maturation of megakaryocytes *in vitro* (Ishibashi *et al.*, 1989a), is a potent thrombopoietic factor *in vivo* in mice (Ishibashi *et al.*, 1989b) and acts on nerve cells (Satoh *et al.*, 1988), epidermal keratinocytes (Grossmann *et al.*, 1989; Yoshizaki *et al.*, 1990) and T cells (Lotz *et al.*, 1988) inducing their growth or differentiation.

The IL-6 gene is situated on chromosome 7 whereas the IL-6 receptor α chain is found on chromosome 1 and the signal transducer gp130, is located on chromosomes 5 and 17 in primates (Callard and Gearing, 1994). The IL-6 receptor (Fig. 1.2.5.2) is expressed on a variety of cells including activated B cells, T cells, hepatocytes, epidermal keratinocytes, epithelial cells, fibroblasts and neural cells (Kishimoto, 1992; Callard and Gearing, 1994).

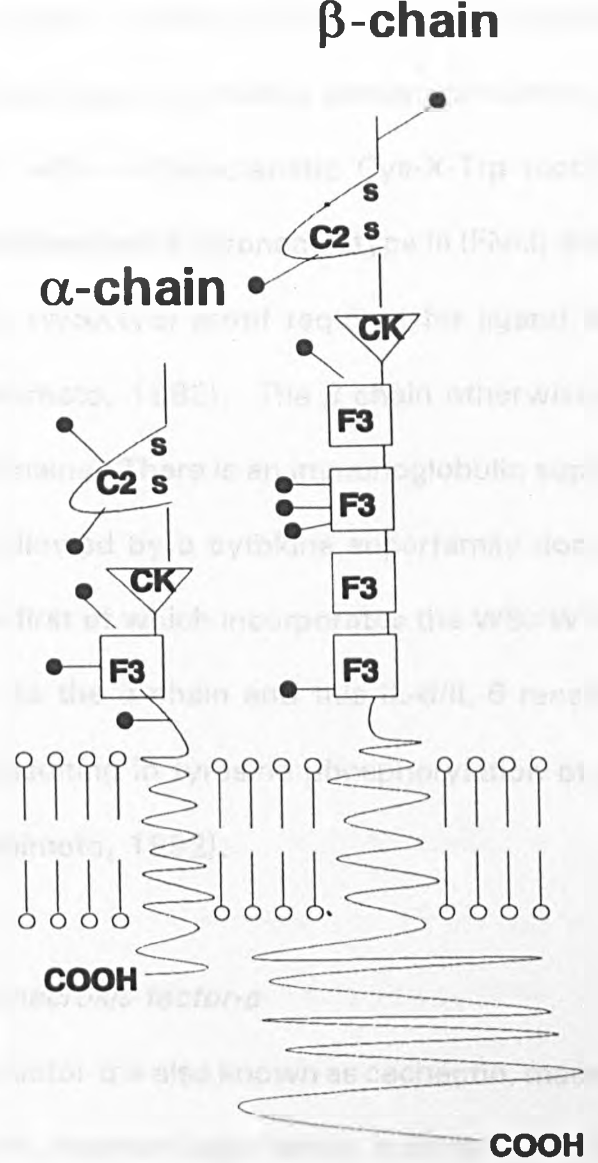

Fig. 1.2.5.2. The IL-6 receptor. CK Cytokine receptor, F3 Fibronectin type III domain, C2 Immunoglobulin superfamily domain, —● N-glycosylation sites.

The IL-6 receptor is formed by non-covalent binding of the *α* chain (CD 126) with the *β* chain (CD 130) (Kishimoto *et al.*, 1992). The extracellular region

of the α chain consists of three domains, an immunoglobulin superfamily domain at the N terminus, a cytokine domain consisting of approximately 100 amino acids with a characteristic Cys-X-Trp motif and three other conserved Cys residues and a fibronectin type III (FNIII) domain including the Trp-Ser-X-Trp-Ser (WSXWS) motif required for ligand binding and signal transduction (Kishimoto, 1992). The β chain otherwise known as gp130 consists of six domains. There is an immunoglobulin superfamily domain at the N terminus followed by a cytokine superfamily domain and then four FNIII domains the first of which incorporates the WSXWS motif. IL-6 binds with low affinity to the α chain and this IL-6/IL-6 receptor complex then binds to gp130 resulting in tyrosine phosphorylation of gp130 and signal transduction (Kishimoto, 1992).

1.2.5.3 Tumour necrosis factor-α

Tumour necrosis factor-α is also known as cachectin, macrophage cytotoxin, necrosin, cytotoxin, haemorrhagic factor, macrophage cytotoxic factor and differentiation-inducing factor. It is mainly a monocyte derived cytokine (Vilcek and Lee, 1991) but its production can also be induced in other cell types such as T cells (Turner *et al.*, 1987) and endothelial cells (Warner and Libby, 1989). Its tertiary structure is very similar to the jelly-roll motif of some plant and animal virus capsids (Jones *et al.*, 1989) and it is normally secreted as a homotrimer. Tumour necrosis factor-α mediates both apoptotic and necrotic forms of cell death (Laster *et al.*, 1988) as well as the growth of haematopoietic progenitor cells. However, its effects on growth

are less clear as TNF-α has been reported to inhibit proliferation in some circumstances (Broxmeyer *et al.*, 1986; Murase *et al.*, 1987) whereas in other cases it has been found to act as a stimulator (Caux *et al.*, 1990, 1991). Jacobsen *et al.* (1992) have further demonstrated that TNF-α directly inhibits M-CSF-stimulated proliferation of murine bone marrow progenitor cells which directly correlates with CSF receptor inhibition and induces their formation through at least the induction of G-CSF which may mediate the response through up-regulation of the CSF receptor. TNF-α is also known to induce the transcription of a variety of other haematopoietic growth factor cytokines including IL-1, IL-6, IL-8 and GM-CSF (Nawroth *et al.*, 1986; Munker *et al.*, 1986; Brouckaert *et al.*, 1989; Cromwell *et al.*, 1992) and to enhance endothelial cell adhesiveness for T cells (Cavender *et al.*, 1987) and neutrophils (Gamble *et al.*, 1985; Schleimer and Rutledge, 1986) via up-regulation of adhesion molecules such as intercellular adhesion molecule (ICAM)-1 and endothelial adhesion molecule (ELAM)-1. It also acts as a stimulator for human skin mast cells to secrete histamine and tryptase (van Overveld *et al.*, 1992). Many other effects occur in combination with other cytokines as part of the "cytokine network".

There are two receptors for TNF-α (Fig. 1.2.5.3). The type I receptor with a molecular weight of 55 000 is found on most cell types whereas type II with a molecular weight of 75 000 appears to be more restricted to haematopoietic cells. Both receptors have four Cys-rich repeats in the extracellular domain but share very little homology. Soluble forms of each receptor which are thought to act as TNF-α antagonists (Nophar *et al.*,

1990) have also been detected in the serum of cancer patients and in urine. Following receptor cross-linkage with the TNF-*α* trimer, diacyl-glycerol (DAG) and inositol 1,4,5, triphosphate (IP_3) are generated from the phospholipase C (PLC) mediated hydrolysis of membrane-bound inositol phosphate. DAG acts to stimulate protein kinase C (PKC) whereas IP_3 regulates intracellular calcium by mobilizing calcium form internal stores and possibly stimulating calcium entry.

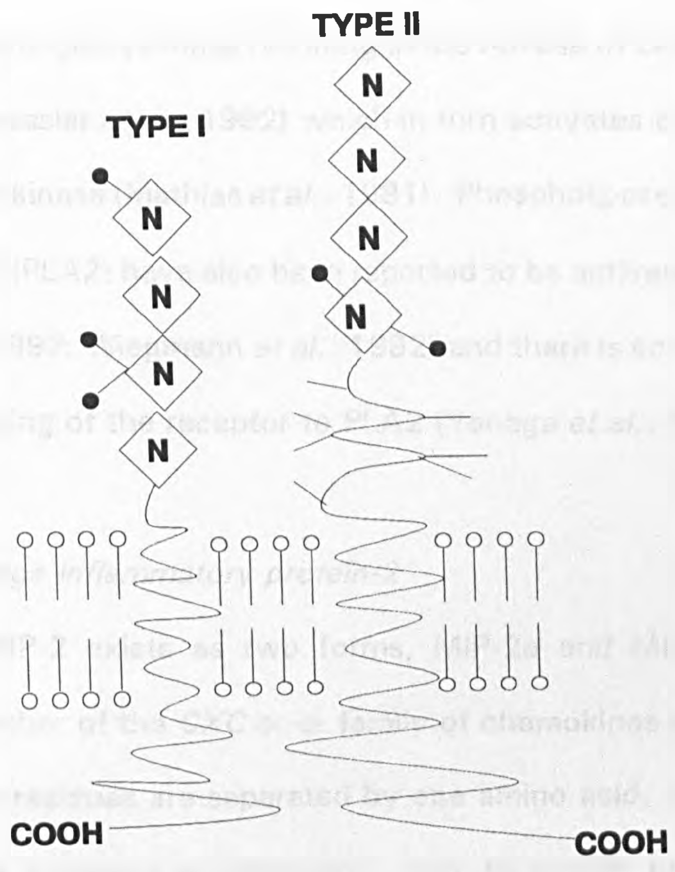

Fig. 1.2.5.3. The TNF-*α* receptors. ⟨N⟩ Nerve growth factor receptor, —● N-glycosylation sites, —— O-linked glycosylation.

Inhibitors of PLC such as neomycin and RHC 80267, an inhibitor of diacylglycerol lipase, do not prevent TNF-mediated cytotoxicity of L929 cells (Suffys *et al.*, 1987) whereas lithium which is known to inhibit some of the enzymes that hydrolyse inositol phosphates, which in effect reduce the supply of precursor inositol lipids, potentiates the cytotoxicity of TNF-α (Beyaert *et al.*, 1989) supporting the hypothesis that the phosphatidylinositol pathway mediates the protective actions of TNF-α. Receptor crosslinkage also activates a sphingomyelinase resulting in the release of ceramide from sphingomyelin (Dressler *et al.*, 1992) which in turn activates a magnesium dependent protein kinase (Mathias *et al.*, 1991). Phospholipase D (PLD) and phospholipase A2 (PLA2) have also been reported to be activated by TNF-α (De-Valck *et al.*, 1993; Wiegmann *et al.*, 1992) and there is some evidence of G protein coupling of the receptor to PLA2 (Yanaga *et al.*, 1992).

1.2.5.4 Macrophage inflammatory protein-2

In the human, MIP-2 exists as two forms, MIP-2α and MIP-2β. Each molecule is a member of the *CXC* or α- family of chemokines in which the first two cysteine residues are separated by one amino acid. Although its three dimensional structure is unknown it may be similar to IL-8 which consists of a triple-stranded antiparallel β-sheet. In both mice and humans MIP-2 consists of 73 amino acids and has a molecular weight of 7.9 kD. Macrophage inflammatory protein-2 is chemotactic for neutrophils *in vitro* and elicits a localized neutrophil response when administered sub-cutaneously to mice or intracisternally to rabbits (Wolpe *et al.*, 1989,

Saukkonen *et al.*, 1990). Messenger RNA expression for MIP-2 has been detected in rat alveolar macrophages and expression is increased with TNF-*α*, adherence to plastic and mineral dust exposure (Driscoll *et al.*, 1993). Furthermore, studies with a rat fibroblast and epithelial cell line have demonstrated that MIP-2 expression can be detected by these cells after stimulation with TNF-*α* (Driscoll *et al.*, 1993). These observations are consistent with recent findings for the structurally and functionally related cytokine IL-8, which in response to various stimuli such as TNF-*α* can be induced in epithelial cells, fibroblasts and endothelial cells as well as in macrophages and neutrophils (Larsen *et al.*, 1989; Strieter *et al.*, 1989; 1990; Elner *et al.*, 1991; Bazzoni *et al.*, 1991). Although the structure of the MIP-2 receptor is unknown it has been shown to compete with IL-8 for binding to the low affinity IL-8 receptors on neutrophils (LaRosa *et al.*, 1992).

1.2.5.5. Interferon-γ

Interferon-*γ* is also known as immune interferon, type II interferon or T cell interferon. It has a globular structure and exists as a homodimer formed by antiparallel association of two subunits each consisting of six *α*-helices held together by short non-helical sequences (Callard and Gearing, 1994). It is produced by CD8+ and CD4+ T cells and has a principle role in the regulation of IgE synthesis. High levels of IL-4 induce lipopolysaccharide (LPS)-stimulated B cells to produce IgE *in vitro* and these effects are antagonized by IFN-*γ* (Snapper and Paul, 1987). Additionally, IFN-*γ* has

been shown to suppress IgE production induced by soluble antigen and *Nippostrongylus braziliensis* and *Heligmosomoides polygyrus in vivo* in mice (Finkelman *et al.*, 1990). Furthermore, an association between reduced IFN-γ production and increased IgE has been reported in patients with hyper-elevated IgE levels whose peripheral blood lymphocytes have a reduced capacity to produce IFN-γ (Paganelli *et al.*, 1991). Interferon-γ also promotes the development of T helper 1 (Th1) cells (Gajewski *et al.*, 1989) inhibits the growth of Th2 cells (Fernandez-Botran *et al.*, 1988), enhances TNF-α cytotoxicity (Trinchieri *et al.*, 1985), and induces MHC class I and II expression in target tissues (Wong *et al.*, 1983, 1994; Skoskiewicz *et al.*, 1985; Giacomini *et al.*, 1988; Gerrard *et al.*, 1988). It also combines with TNF-α to enhance endothelial cell adhesiveness for T cells (Thornhill *et al.*, 1991) and up-regulates the IgE- or endotoxin-induced release of TNF-α from alveolar macrophages (Gosset *et al.*, 1992; Gifford and Lohmann-Matthess, 1987). Additionally, IFN-γ is the major molecule recognized as macrophage-activating factor (Adams and Hamilton, 1987).

The IFN-γ receptor (Fig. 1.2.5.5) consists of a high affinity binding chain and a secondary accessory protein required for signal transduction (Soh *et al.*, 1994; Hemmi *et al.*, 1994). The binding chain is a glycoprotein, the extracellular region consisting of approximately 200 amino acids with two FNIII domains. Signal transduction involves phosphorylation of the receptor and is mediated by phosphorylation and activation of JAK1 and JAK2 protein tyrosine kinases (Hunter, 1993; Watling *et al.*, 1993; Muller *et al.*, 1993).

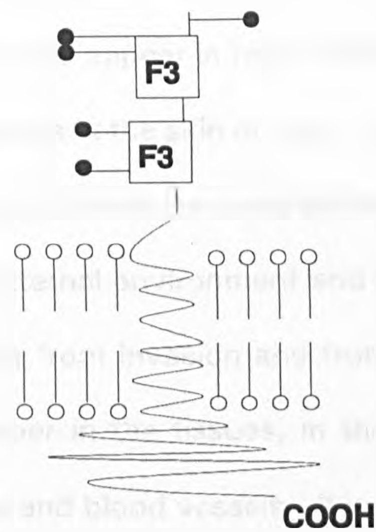

Fig. 1.2.5.5 The IFN-γ receptor. F3 Fibronectin type III domain, —● N-glycosylation sites

1.3 MAST CELLS

1.3.1 Mast cell distribution

Mast cells are distributed throughout the vascular tissue, epithelia, and serosal cavities in rodents and appear in high numbers at mucosal surfaces and within connective tissues in the skin or lung. In the lung, mast cells are found predominantly at the luminal mucosal surface of the bronchi where they interface with the external environment and have a suspected role in the protection of the lung from invasion and from foreign proteins. Skin mast cells are found deeper in the tissues, in the dermis rather than the epidermis close to nerves and blood vessels. It is thought that cells at this sight are involved in angiogenesis, nerve regeneration and tissue repair rather than defence.

1.3.2 Mast cell heterogeneity

Visualisation of mast cells by electron microscopy has demonstrated that they contain electron dense granules. Stored within the granules are the preformed mediators such as heparin proteoglycan and neutral proteases. Human mast cells are categorised into sub-types on the basis of their protease content. Those that contain tryptase alone are designated as the MC^{T} subtype whereas mast cells that contain chymase, carboxypeptidase and tryptase are known as the MC^{TC} subtype. The two subtypes are easily distinguishable from each other using immunohistochemical techniques (Craig *et al.*, 1989) and by electron microscopy (Craig *et al.*, 1989; Dvorak

et al., 1991). Mast cells containing tryptase alone, the MC^T phenotype are generally found at luminal mucosal surfaces in the lung and appear to be dependent on T cells for their generation as they predominate at sites of inflammation where there is a massive T cell infiltration (Church and Caufield, 1993). Irani *et al.* (1987) have also observed that numbers are reduced in patients with reduced T cell activity. The MC^{TC} phenotype are more prevalent in the skin and the small intestine submucosa and appear to be unaffected by reduced T cell activity (Irani *et al.*, 1987). However, both mast cell phenotypes have been located at each anatomical site preventing categorisation upon the basis of their location alone (Church and Caufield, 1993). Other differences in the two subtypes involve their release of mediators upon activation; human lung mast cells, the MC^T subtype, produce more leukotriene C4 (LTC_4) than the MC^{TC} subtype (Lawrence *et al.*, 1987) and unlike the MC^{TC} subtype do not release histamine upon challenge with the neuropeptide substance P (Pearce *et al.*, 1991).

Rodent mast cells can also be subdivided into two distinct groups which share a number of similarities with their human counterparts. Those mast cells which reside in the intestinal lamina have been designated as the mucosal mast cell subtype (MMC) and have similar properties to those of MC^T subtype in humans. They do not release histamine upon challenge with substance P (Pearce *et al.*, 1989) and they are highly dependent on T cells for growth and function (Galli, 1990). Rodent mast cells found in the skin and peritoneal cavity are known as the connective tissue-type mast cells (CTMC) (Galli, 1990). They are bigger, contain more histamine and 5-HT

than the mucosal mast cells and release histamine upon substance P activation (Pearce *et al.*, 1989). However, they do not produce LTC_4 upon activation and in that sense are unlike their MC^{TC} counterparts in humans (Gurish and Austin, 1989). Activation of CTMC is inhibited by sodium cromoglycate whereas activation of MMC is unaffected by this drug (Church and Caufield, 1993). Additionally, mucosal mast cells contain the proteoglycan, chondroitin sulphate whereas CTMC contain heparin. The two subtypes are therefore easily distinguishable by differential staining techniques; MMC stain with the alcain blue dye whereas the heparin binding safranin stain is used to identify mast cells of the CTMC subtype.

The effects of cytokines on mast cell phenotype are important. Mouse bone marrow cells cultured with IL-3 and cells of the 3T3 murine fibroblast cell line develop into mast cells that contain heparin or chondroitin sulphate in their granules and thus appear to resemble both the MMC and CTMC rodent mast cell phenotypes (Levi-Schaffer *et al.*, 1986). Nerve growth factor on the other hand appears to promote the generation of rodent mast cells containing heparin only (Matsuda *et al.*, 1991) and culture of CD34+ pluripotent human bone marrow progenitor cells with IL-3 and stem cell factor (SCF) results in the generation of heparin containing mast cells (Kirshenbaum *et al.*, 1992). Further work has demonstrated that culture of human umbilical cord blood cells with SCF promotes the generation of mast cells with a phenotype resembling that of human lung mast cells (MC^{T}) whereas co-culture with 3T3 fibroblasts is required for the generation of mast cells resembling the MC^{TC} phenotype (Mitsui *et al.*, 1993).

Furthermore, Irani *et al.* (1992) have demonstrated that co-culture of human foetal liver cells with 3T3 fibroblasts results in the generation of immature mast cells with an MC^{T} phenotype whereas under the same conditions co-culture of human umbilical cord blood mononuclear cells gives rise to cells of the MC^{TC} phenotype. Nilsson *et al.* (1993) have also indicated that foetal liver cells cultured with SCF generate mast cells that do not bear high affinity IgE receptors on their cell surfaces and express only low levels of mRNA encoding this receptor. However, other research has shown that mast cells derived *in vitro* from human bone marrow progenitors and human umbilical cord blood cells express high affinity IgE receptors (Rottem *et al.*, 1991; Ishizaka *et al.*, 1991; Mitsui *et al.*, 1993).

1.3.3 Mast cell growth and differentiation

Mast cells are derived from pluripotent bone marrow stem cells (Kirshenbaum *et al.*, 1991) that complete their differentiation and maturation in vascularised tissues, epithelia and serosal cavities (Church and Caulfield, 1993). The evidence that they can arise from CD34+ bone marrow cells depleted of T lymphocyte, B lymphocyte, macrophage and eosinophil committed progenitors indicates that they do not arise from cells of these types (Kirshenbaum *et al.*, 1991). Additionally, mast cells do not appear to share a common ancestor with basophils as the generation of either basophils or mast cells form human umbilical cord blood cells *in vitro* requires different culture conditions (Furitsu *et al.*, 1989).

Stem cell factor is required for the generation of mast cells from

haematopoietic stem cells. Mice with mutations at either the *W/c-kit* locus on chromosome 5 or the *Sl* locus on chromosome 10 which encodes the ligand (SCF) for the c-*kit* receptor are virtually devoid of mature mast cells (Galli, 1990). Recombinant forms of SCF induce proliferation, differentiation and survival of mast cells both *in vitro* and *in vivo* and effects have been reported in the mouse (Tsai *et al.*, 1991a, 1991b; Gurish *et al.*, 1992; Iemura *et al.*, 1994), rat (Ulich *et al.*, 1991; Haig *et al.*, 1994), experimental primates (Galli *et al.*, 1993) and humans (Irani *et al.*, 1992; Valent *et al.*, 1992; Mitsui *et al.*, 1993; Costa *et al.*, 1994). Furthermore, both IL-3 and IL-4 promote proliferation of mast cells. In mice IL-3 appears to be important for the expansion of the MMC phenotype (Abe and Nawa, 1988). Interleukin-4 appears to augment these effects and also favours the *in vitro* growth of murine mast cells with the CTMC phenotype (Hamaguchi *et al.*, 1987).

1.3.4 IgE-mediated activation of mast cells

The mast cell has been associated with the initiation of allergic reactions through its ability to be activated by an IgE-dependent mechanism (Fig. 1.3.1). Upon their cell surface are high affinity IgE receptors (FcεR1) which bind IgE secreted by B cells (Nissim *et al.*, 1991). The FcεR1 consists of four subunits, one α, one β, and two identical γ subunits (Metzger, 1992) but it is through its α subunit that it binds to the constant region of an IgE molecule (Hakimi *et al.*, 1990). However, it is only when bound IgE molecules become themselves cross-linked by antigen that mast cell

degranulation occurs (Metzger, 1992). Each IgE molecule has two antigen binding sites and mast cells can become sensitised to a particular antigen by binding of antigen specific IgE molecules, a process known as "sensitisation". Upon crosslinking of two or more IgE molecules which are bound to FcεR1, a number of phosphorylation take place which lead to mast cell degranulation. Benhamou *et al.* (1990) have demonstrated that early phosphorylation of tyrosine residues on cellular proteins leads to the release of histamine from rat basophilic leukemia cells (RBL-2H3, cells of a cloned mast cell line). These events are easily reversible by displacement of the multivalent antigen with excess monovalent antigen but are not prevented by prolonged incubation with phorbol ester to down-regulate protein kinase C and do not require the presence of extracelluar calcium (Benhamou *et al.*, 1990). This suggests that tyrosine phosphorylation is an early event in the mast cell signal transduction pathway which occurs prior to calcium mobilisation or the activation of protein kinase C. Further studies have indicated that FcεRI phosphorylation of tyrosine and serine residues on the β chain and tyrosine and threonine residues on the γ chain also represents an important signal in the stimulus-secretion pathway of immunologically-activated mast cells (Paolini *et al.*, 1991; Metzger, 1992; Benhamou and Siraganian, 1992) and although these studies have been performed using cloned rodent mast cells of the RBL-2H3 cell line, studies using human mast cells have also shown that tyrosine kinase is involved in the signal transduction pathway of IgE-dependent mast cell stimulation (Warner *et al.*, 1992; Lavens *et al.*, 1992). Increases in levels of intracellular free calcium

are also important for the activation of mast cells. Church *et al.* (1989) have demonstrated that challenge of mast cells with specific antigen in the absence of extracelluar calcium fails to induce degranulation although further work by von zur Muhlen *et al.* (1991) using a GTP analogue which induces the release of calcium from intracellular stores but does not induce mast cell degranulation, has indicated that an increase in intracellular calcium ions is an important component of mast cell activation by immunological stimuli but is not sufficient by itself to induce exocytosis. Stimulation of mast cells by IgE receptor aggregation leads to the hydrolysis of phosphatidylinositol 4, 5-bis phosphate by phospholipase C, generating inositol 1, 4, 5-trisphosphate (IP_3) and diacylglycerol (DAG) as its products (Ishizaka *et al.*, 1987). IP_3 can bind to high affinity receptors on the intracellular calcium storage sites inducing calcium ion release whereas DAG can stimulate protein kinase C which is thought to play an important role in IgE dependent mast cell activation. Ishizaka *et al.* (1987) have reported that IgE-dependent stimulation of mast cells enhances protein kinase C activity and further studies have revealed that inhibition of protein kinase C diminishes the release of histamine by human mast cells (Massey *et al.*, 1991). It has also been suggested that two GTP binding proteins regulate mast cell granule secretion; one form is involved in the regulation of the inositide-specific phospholipase C (which generates IP_3 and DAG) and another form appears to control an unknown event immediately preceding exocytosis (Gomperts, 1990).

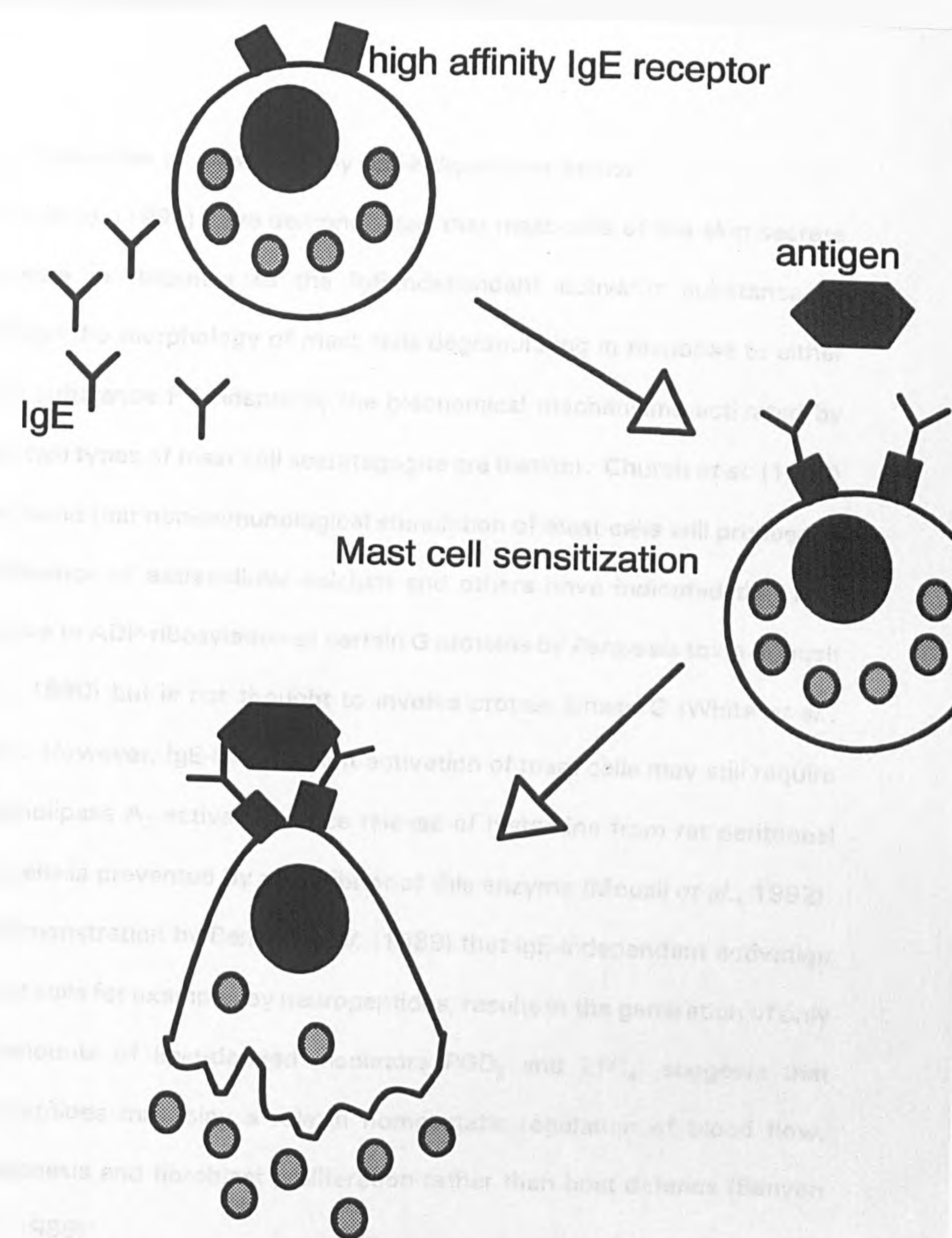

Fig. 1.3.1. The release of mediators from mast cells following activation of the cells via an IgE-dependent process.

1.3.5 Activation of mast cells by IgE-independent stimuli

Pearce *et al.* (1991) have demonstrated that mast cells of the skin secrete histamine in response to the IgE-independent activator substance P. Although the morphology of mast cells degranulating in response to either IgE or substance P is identical, the biochemical mechanisms activated by these two types of mast cell secretagogue are distinct. Church *et al.* (1989) have found that non-immunological stimulation of mast cells will proceed in the absence of extracellular calcium and others have indicated that it is sensitive to ADP-ribosylation of certain G proteins by *Pertussis* toxin (Mousli *et al.*, 1990) but is not thought to involve protein kinase C (White *et al.*, 1990). However, IgE-independent activation of mast cells may still require phospholipase A_2 activation since release of histamine from rat peritoneal mast cells is prevented by an inhibitor of this enzyme (Mousli *et al.*, 1992). The demonstration by Benyon *et al.* (1989) that IgE-independent activation of mast cells for example, by neuropeptides, results in the generation of only low amounts of lipid-derived mediators PGD_2 and LTC_4, suggests that neuropeptides may play a role in homeostatic regulation of blood flow, angiogenesis and fibroblast proliferation rather than host defence (Benyon *et al.*, 1989)

1.3.6 Mast cell mediators

Mast cells contain a number of preformed mediators which are stored in their secretory granules. Upon stimulation these are rapidly released into the extracellular fluid. Histamine, which is generated in the Golgi apparatus by

decarboxylation of the amino acid histidine (Barnes *et al.*, 1988), causes bronchoconstriction and leakage of plasma proteins from the post capillary venules in the large airways leading to oedema. It also has the ability to increase mucus production and has some ability in acting as a neutrophil and eosinophil chemoattractant (Church and Caufield, 1993; Barnes *et al.*, 1988). Serotonin or 5-hydroxytryptamine (5-HT) which is involved in bronchoconstriction is only found in the granules of rodent mast cells. They acquire the amine by actively uptaking it from the extracellular environment. However, human mast cells do not possess this uptake mechanism and so do not contain 5-HT within their granules (Church and Caulfield, 1993).

Proteoglycans consist of long protein cores to which glycosaminoglycans are attached (Ruoslathi, 1989). The nature and extent of sulphation of the glycosaminoglycans determines the species of proteoglycan, i.e. heparin or chondroitin sulphate. Proteoglycans are the main structural unit of mast cells and form the matrix with which other granule mediators are associated. They have a number of functions within mast cells. Heparin, an anticoagulant, is thought to stabilize the neutral protease tryptase (Schwartz *et al.*, 1990) and to be involved in the regulation of cell proliferation (Ruoslathi, 1989) whereas secretory proteoglycans may prevent the autolysis of the ionically bound mast cell neutral proteases (Gurish and Austin, 1989).

The proteases found within human mast cells are tryptase, chymase, and carboxypeptidase A. Tryptase is the most abundant human mast cell protease. It is stored in association with the proteoglycans heparin and

chondroitin sulphate and the active tetramer is stabilised by heparin (Schwartz *et al.*, 1990). Although no clear role for this enzyme has been determined it does possess kininogenase activity (Walls *et al.*, 1992) and has been shown to degrade the vasodilator calcitonin gene related peptide to an inactive form. The MC^T human mast cell subtype found predominantly in the lung, contains only tryptase whereas MC^{TC} mast cell subtype contains tryptase, chymase and carboxpeptidase A within its granules (Church and Caufield, 1993). Although there is little information regarding the effects of chymase and carboxypeptidase, chymase may be involved in the control of local blood flow by converting angiotensin I to angiotensin II (Church and Caufield, 1993) and in the conversion of an IL-1ß precursor to an active IL-1 species (Mizutani *et al.*, 1991) whereas carboxypeptidase A may be involved in the cleavage of leu^5 - enkephalin to an inactive form (Goldstein *et al.*, 1991).

Mast cells also release a number of *de novo* synthesised mediators upon stimulation. These include the lipid mediators of arachidonic acid metabolism. Arachidonic acid is metabolised along two distinct pathways which generate either the prostaglandins or leukotrienes. Mast cells make PGD_2 and LTC_4. After secretion these lipids can be further metabolized to PGE_2 or LTD_4 and LTE_4 respectively. PGD_2 has marked bronchoconstrictor properties through its interaction with TP_1 thromboxane receptors on the surface of airway smooth muscle (Church and Caufield, 1993). It also causes increases in mucus production (Barnes *et al.*, 1988). LTC_4 and LTD_4 also induce bronchoconstriction (Church and Caufield, 1993) and stimulate

mucus secretion (Barnes *et al.*, 1988).

Another lipid mediator known as platelet activating factor (PAF) is also produced by mast cells. Cleavage of membrane phospholipids at the sn-2 position of glycerol by the enzyme phospholipase A_2 generates arachidonic acid and 1-alkyl, 2-phosphatidylcholine and it is the acetylation of this phosphatidylcholine product that generates platelet activating factor (Kennerly and Duffy, 1993). Platelet activating factor is a potent bronchoconstrictor in man acting via platelets and causes long lasting bronchial hyperresponsiveness to a number of constrictors (Barnes *et al.*, 1988). It also acts as a neutrophil and eosinophil chemoattractant and enhances their activity (Johnston and Holgate, 1990).

1.3.7 Mast cells as a source of cytokines

The increasing information regarding mast cells as a source of several multifunctional cytokines suggests an important mechanism whereby these cells may influence many physiological, immunological and pathological processes including late phase hypersensitivity reactions, host responses to parasites and neoplasms, immunologically non-specific inflammatory and fibrotic conditions, angiogenesis and tissue remodelling and wound healing. A wide variety of cytokines are known to be produced by rodent and human mast cell lines although relatively less is known about mature tissue mast cells. However, rat and mouse tissue mast cells express mRNA and biologically active product for TNF-α (Gordon and Galli, 1990, 1991; Bissonnette *et al.*, 1991) and more recently rat peritoneal connective tissue-

type mast cells (PMC) have been reported to express IL-6 (Leal-Berumen *et al.*, 1994) and LIF (Marshall *et al.*, 1993). Human skin mast cells are known to provide a source of TNF-α (Walsh *et al.*, 1991; Benyon *et al.*, 1991) and IL-8 (Möller *et al.*, 1993) and human lung mast cells express IL-4, IL-5, IL-6, and TNF-α (Bradding *et al.*, 1994).

The expression of individual cytokines by mast cells appears to be differentially regulated. For example, IgE-dependent stimulation of mast cell lines or bone marrow derived mast cells (BMMC) results in rapid increase in mRNA encoding TNF-α, TCA3, JE, MIP-1α, MIP-1β and IL-6 but not IL-1, IL-3, IL-5 or GM-CSF which does not appear at substantially elevated levels until 90-120 minutes after stimulation (Gordon *et al.*, 1990). Additionally, stimulation of BMMC by agents that specifically activate either protein kinase C or phospholipase C can produce distinct consequences for mast cell cytokine expression. Stimulation of BMMC by activation of protein kinase C with PMA results in an increase in mRNA encoding TNF-α, TCA3, MIP-1α, MIP-1β, IL-1 and IL-6 but not IL-2, IL-3, IL-4, IL-5, GM-CSF or IFN-γ whereas treatment of BMMC with both PMA and calcium ionophore A23187 is required for optimal induction of IL-2, IL-3, IL-5, and GM-CSF (Gordon *et al.*, 1990). IgE-dependent or calcium ionophore-induced mast cell stimulation results in a more rapid augmentation of cytokine mRNA levels and significant histamine release than stimulation by PMA (Gordon *et al.*, 1990). These results suggest that different regulatory mechanisms exists in mast cells for the production of different groups of cytokines. The possibility therefore exists that different *in vivo* mechanisms of stimulation may result in the

generation of different patterns of mast cell cytokine expression (Gordon *et al.*, 1990) which result in distinctive effects on physiological, immunological and pathological processes.

1.4 ASTHMA

1.4.1 The characteristics of asthma

Asthma is characterised by obstruction of the airways with spontaneous and pharmacological reversibility, increased responsiveness (bronchial hyperresponsiveness) to exogenous and endogenous stimuli and inflammation of the airways (Djukanović *et al.*, 1990). Atopy, "a propensity to produce IgE antibodies" is a common feature of asthma in children and young adults and occupational sensitizing agents are often the cause of asthma in older patients. However, other forms of asthma known as intrinsic or cryptogenic asthma appear to show no underlying stimuli. Asthma usually appears in children in Western societies around the age of two years, it increases in prevalence up to the age of nine or ten and then begins to decrease (Woolcock and Jenkins, 1993). However, moderate and severe forms of childhood asthma are likely to continue into adult life. In adults, asthma remission is uncommon. It is less likely in patients with frequent symptoms, lung function abnormalities and co-existing smoking related lung disease (Woolcock and Jenkins, 1993).

Inhalation of an allergen results in a decrease in lung function in asthmatic patients. This is measured as a decrease of 20% or more in the forced expiratory volume in one second (FEV_1). Typically the FEV_1 declines sharply and recovers to near baseline values within one hour. The initial decrease in airway volume which is referred to as the early asthmatic reaction (EAR) is then often followed by a second more prolonged reduction

in lung function which lasts up to several hours (Fig. 1.4.1). This more serious phase is known as the late asthmatic reaction (LAR). The EAR is thought to be caused by bronchial smooth muscle contraction following release of mediators from mast cells whereas other pro-inflammatory cell mediators are believed to contribute to the development of the LAR.

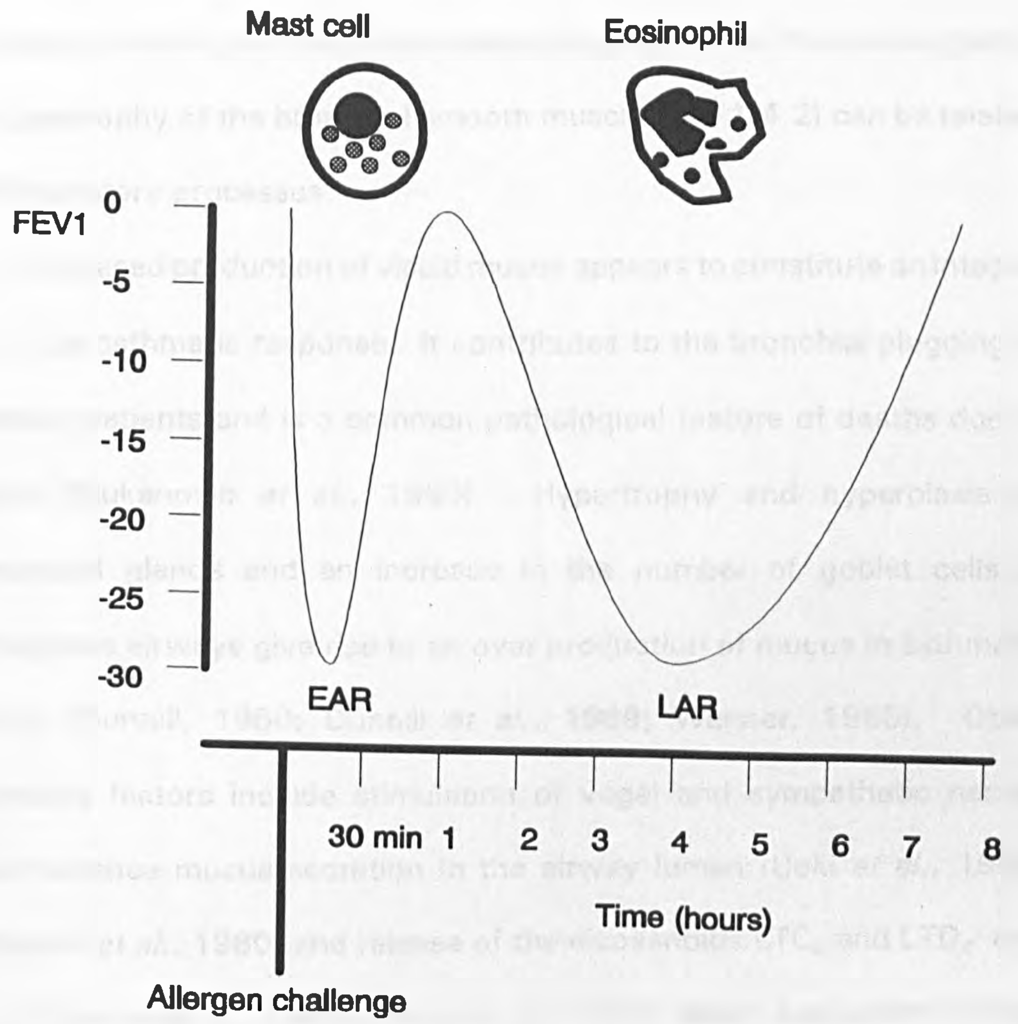

Fig. 1.4.1. The kinetics of the decrease in lung function in an asthmatic patient as measured by the forced expiratory volume in one second (FEV1). The EAR is caused by the release of mediators from mast cells whereas the

LAR involves other cells such as eosinophils.

1.4.2 Inflammation in asthma

In most forms of asthma many of the pathological changes including occlusion of the bronchi with a mixture of serum proteins and cellular debris, epithelial sloughing, apparent thickening of the epithelial basement membrane, oedema, leucocyte infiltration, hyperplasia of the mucus glands and hypertrophy of the bronchial smooth muscle (Fig. 1.4.2) can be related to inflammatory processes.

Increased production of viscid mucus appears to constitute an integral part of the asthmatic response. It contributes to the bronchial plugging in asthmatic patients and is a common pathological feature of deaths due to asthma (Djukanovic *et al.*, 1990). Hypertrophy and hyperplasia of submucosal glands and an increase in the number of goblet cells in cartilaginous airways give rise to an over production of mucus in asthmatic patients (Dunnill, 1960; Dunnill *et al.*, 1969; Wanner, 1988). Other influencing factors include stimulation of vagal and sympathetic nerves which enhance mucus secretion in the airway lumen (Ueki *et al.*, 1980; Shelhamer *et al.*, 1980) and release of the eicosanoids LTC_4 and LTD_4 and $PGF_{2\alpha}$ (Marom *et al.*, 1981; Coles *et al.*, 1983) which are potent mucus secretagogues in human airway tissue. The neuropeptides substance P and neurokinins A and B may also induce mucus secretion (Baker *et al.*, 1977; Peatfield *et al.*, 1983; Borson *et al.*, 1984). Mucus retention is also enhanced in asthma due to a reduction in mucociliary clearance caused by

structural and functional damages to the mucociliary apparatus (Laitinen *et al.*, 1985) and an increase in microvascular leakage in asthma can lead to an increase in the amount of albumin in the lung tissue which can increase the viscosity of mucus by the formation of viscous glycoprotein complexes.

In healthy individuals the bronchial vasculature serves a homeostatic function to enable warming and humidification of inspired air. However, in asthmatic patients endothelial cell permeability is enhanced due to the presence of large gaps between the endothelial cells of the submucosal venules (Laitinen and Laitinen, 1988). The vascular bed therefore serves as a source for leucocyte recruitment and conveyance of locally acting mediators. Components of the complement, kinin and fibrinolytic systems can also leak into the extravascular spaces to enhance and perpetuate the inflammatory response. The anaphylatoxin complement peptides C3a and C5a and bradykinin are potent smooth muscle spasmogens. C3a and C5a also act as chemotactic factors for neutrophils and eosinophils and fibrin may contribute to the increase in mucus viscosity (Djukanovic *et al.*, 1990).

Epithelial desquamation and sloughing are common pathological features of death from asthma as too are the apparent thickening and hyalinization of the epithelial basement membrane (Dunnill, 1960; Callerame *et al.*, 1971). However, further studies have revealed that dense deposition of subepithelial collagen fibrils, of which fibroblasts may be the source, is the abnormality that has previously been mistaken for thickening of the epithelial basement membrane (Roche *et al.*, 1989). Other pathological features of asthmatic inflammation include cellular infiltration of the bronchial

wall and an increase in immunoglobulins, particularly IgE, in the bronchial mucosa.

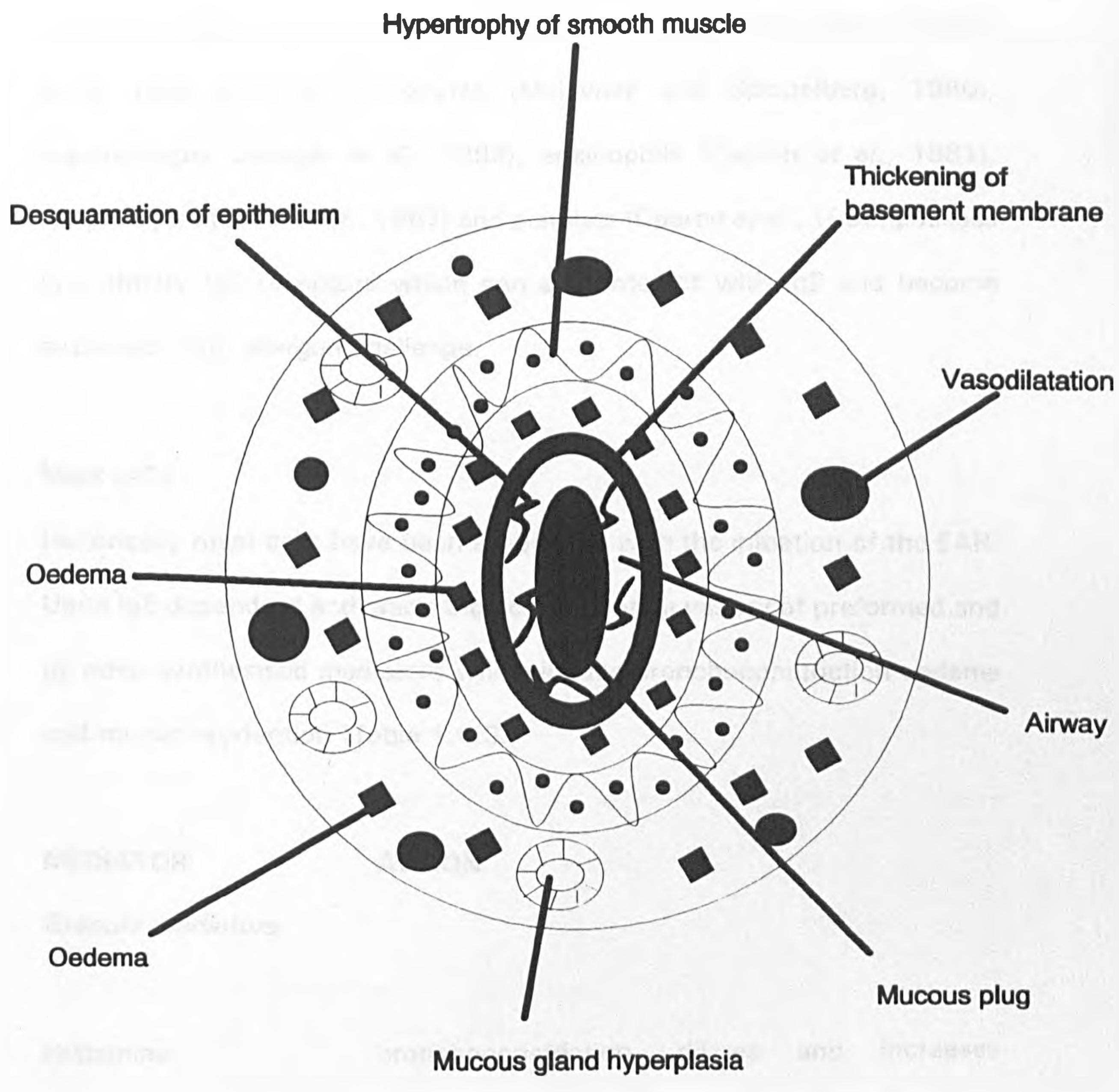

Fig. 1.4.2 Diagramatic representation of the pathology of asthma showing changes in tissues surrounding an asthmatic airway.

1.4.3 Inflammatory cell effector mechanisms in bronchial asthma

Many different cell types are known to be involved in the asthmatic reaction. Mast cells possess high affinity IgE receptors on their cell surfaces and upon activation can release a variety of mediators which can initiate an EAR. Other cells such as monocytes (Melewicz and Spiegelberg, 1980), macrophages (Joseph *et al.*, 1983), eosinophils (Capron *et al.*, 1981), lymphocytes (Ludin *et al.*, 1987) and platelets (Capron *et al.*, 1985) possess low affinity IgE receptors which can also interact with IgE and become activated upon allergen challenge.

Mast cells

Historically mast cells have been associated with the initiation of the EAR. Upon IgE-dependent activation they can secrete a variety of preformed and *de novo* synthesised mediators which induce bronchoconstriction oedema and mucus production (Table 1.4.3.)

MEDIATOR	**ACTION**
Granule mediators	
Histamine	bronchoconstriction, dilates and increases permeability of bronchial vascular bed, stimulates mucus secretion and modulates its viscosity.

Tryptase	augments airway smooth muscle responsiveness in dogs, degrades bronchodilator neuropeptides such as VIP, cleaves complement components to yield anaphylatoxins such as C3a and C5a and kininogen to yield bradykinin and lysylbradykinin which are potent bronchoconstrictors

De novo synthesised mediators

PGD_2	direct smooth muscle spasmogen 30 times more potent than histamine, a potent vasodilator and increases vascular permeability.
$PGF_{2\alpha}$	a metabolite of PGD_2 which is a potent mucus secretagogue, vasodilator, increases vascular permeability
15-hydroxyeicosatetraenoic acid (15-HETE)	potent mucus secretagogue
LTC_4	bronchoconstriction, potent mucus secretagogue, vasodilation and increases vascular permeability

LTD_4	bronchoconstriction, potent mucus secretagogue, vasodilation and increases vascular permeability, decrease in mucociliary clearance
PAF	vasodilation and an increase in vascular permeability, neutrophil and eosinophil chemoattractant

Table 1.4.3. Activities of mast cell mediators (Djukanovic *et al.*, 1990) in the asthmatic lung.

A role for mast cells in the late asthmatic reaction

Until recently mast cells were considered to be primarily involved in the EAR. However due to the discovery that mast cells provide a source of several pro-inflammatory cytokines which can be stored preformed (Bradding *et al.*, 1994) the possibility exists that upon activation they can rapidly release a wide variety of cytokines which can in turn promote the entry of eosinophils into the lung thereby initiating the LAR.

Neutrophils

Neutrophils are the predominant circulating white blood cell and are usually the most numerous inflammatory cells occurring in acute inflammatory reactions. Their migration from the blood begins within minutes of an

inflammatory stimulus and usually subsides after 4-24 hours. In some types of reaction the decrease in the numbers of neutrophils coincides with an increase in the numbers of other cell types including monocytes, T-lymphocytes, and eosinophils. These cells persist in the tissues for a much longer period than neutrophils and as a result of this are strongly associated with the chronic effects of allergy. Neutrophils, however, occur in the phase between the immediate response and the late phase response and so may have an important role as intermediary cells in the transition between the two.

Although there is some controversy over the importance of neutrophils in human asthma, neutrophil cytotoxic function has been linked to the magnitude of the fall in FEV_1 in experimental exercise-induced asthma and enhanced neutrophil complement expression has been shown to accompany allergen-induced EAR and LAR (Papageorgiou *et al.*, 1983; Durham *et al.*, 1984; Carroll *et al.*, 1985; Moqbel *et al.*, 1986). Neutrophil numbers are also increased in the LAR in humans after toluene diisocyanate challenge (Fabbri *et al.*, 1987) and Metzger *et al.* (1986) have shown that four hours after allergen challenge both neutrophil and eosinophil numbers are elevated with neutrophil numbers subsiding rapidly and eosinophils remaining elevated at twenty-four hours.

A number of agents including C5a, LTB_4 and chemotactic cytokines such as IL-8 induce neutrophil migration whereas other factors such as TNF-α, IL-1 and PAF can exert neutrophil-attracting effects *in vivo* by inducing the release of specific chemotaxins from other cells in the microenvironment

and by inducing the expression on local microvascular endothelial cells of adhesion molecules which influence neutrophil adhesion and transmigration (Haslett, 1993). Chemotaxins bind to specific G protein-receptor complexes on the surfaces of neutrophils and lead to activation of phospholipase C which hydrolyzes inositol (4,5)-bisphosphate into IP_3 and DAG. The IP_3 causes a rapid release of calcium from intracellular stores followed by a slower influx of calcium from the extracellular environment. DAG activates protein kinase C to promote protein phosphorylation processes critical to cell locomotion and degranulation. It is also linked to the membrane-associated NADPH oxidase which converts O_2 to superoxide (Haslett, 1993). Neutrophil adhesion and transmigration are influenced by a variety of adhesion molecules and ligands including ICAM-1, ICAM-2, ICAM-3 and ELAM-1. Chemotactic factors such as C5a can induce the up-regulation of adhesion molecules on the surfaces of neutrophils whereas cytokines such as IL-1 can induce the expression of ELAM-1 on endothelial cells (Haslett, 1993). In the lung, neutrophils adhere to capillary surfaces through the interactions of neutrophil and endothelial cell surface adhesion proteins. They then squeeze through endothelial cells by a process known as diapedesis, traverse the basement membrane and cross the epithelial cell layer into an inflammatory focus in a lung airspace. Although these events are rather obscure many inflammatory diseases in which neutrophils are recruited, are associated with excessive endothelial and epithelial injury and persistent microvascular leakage. Neutrophils generate a variety of oxygen intermediates which are highly toxic to cells in the close vicinity. Neutrophil

elastase may also mediate neutrophil-associated tissue injury. It is a cationic molecule which is capable of digesting a variety of proteins and has been shown to be toxic to endothelial cells *in vitro* (Haslett, 1993). Neutrophils also release a variety of cytokines including TNF-α (Djeu *et al.*, 1990), IL-1 (Tiku *et al.*, 1986), IL-6 (Cicco *et al.*, 1990) and IL-8 (Bazzoni *et al.*, 1991) which may be important in mediating further cellular recruitment and tissue damage. The fact that neutrophils appear in the period between early and late phase reactions and the evidence that they provide a source of several mediators which are highly toxic to endothelial cells and other cells in the microenvironment, suggests that they may play a prominent role in the maintenance of allergic inflammation. For instance, neutrophils may be important in tissue destruction in asthma and through their release of cytokines may play a role in the recruitment of other cell types into the lung.

Eosinophils

Eosinophils are derived form the bone marrow and are distributed to the tissues via the bloodstream. Under normal conditions they are dormant but can become activated to release a wide variety of toxic mediators which can cause cellular injury in the host. Eosinophilic inflammation accompanies both atopic and non-atopic asthma (Bousquet *et al.*, 1990, 1994) and is also a feature of occupational forms of asthma (Saetta *et al.*, 1992). Increased numbers have been reported in bronchoalveolar lavage (BAL) fluid and sputum of asthmatic patients compared to controls (Bousquet *et al.*, 1990; Gibson *et al.*, 1989) and significant correlations have been found between

BAL fluid eosinophilia and bronchial hyperresponsiveness or the severity of asthma (Walker *et al.*, 1991a; Kelly *et al.*, 1988). After allergen challenge the LAR is associated with increased BAL fluid eosinophils (De Monchy *et al.*, 1985) and increased levels of eosinophil cationic protein (Metzger *et al.*, 1987). Human lung biopsies also revealed that allergen challenge induces eosinophil recruitment (Bentley *et al.*, 1993) and cytokine mRNA expression for IL-5 and GM-CSF.

Recruitment from the blood to tissues initially involves adherence of eosinophils to endothelial cells and subsequent migration into the inflamed tissue. There are a number of chemotactic factors for eosinophils (Fig. 1.4.3), many of which also induce the influx of neutrophils. These bind to specific cell surface receptors and initiate a cascade of events leading to transendothelial migration and activation. The adhesion of eosinophils to the vascular endothelium is the result of the interaction between various receptors on both cell types which can be induced and up-regulated by a variety of cytokines. Eosinophils express the adhesion molecules very late antigen-4 (CD49d) (VLA-4), lymphocyte function associated antigen-1 (CD11a) (LFA-1), macrophage antigen-1 (Mac-1) and gp150,95. These molecules interact with the vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (CD54) (ICAM-1), ICAM-2 and as yet unidentified molecules on the endothelial cells. As neutrophils do not express VLA-4 the interaction of this molecule with VCAM-1 on endothelial cells may explain the selective accumulation of eosinophils in the LAR.

Eosinophilic inflammation is regulated by cytokines acting at different

levels, from the production of cells by the bone marrow, their migration into tissues to the differentiation and activation of fully mature eosinophils present in inflamed tissues. A number of cell types including mast cells and T cells are known to release cytokines such as IL-3, IL-5 and GM-CSF which are known to promote the development and chemotaxis of eosinophils. These cells may therefore participate in the regulation of eosinophil inflammation and possibly the generation of a chronic allergic reaction.

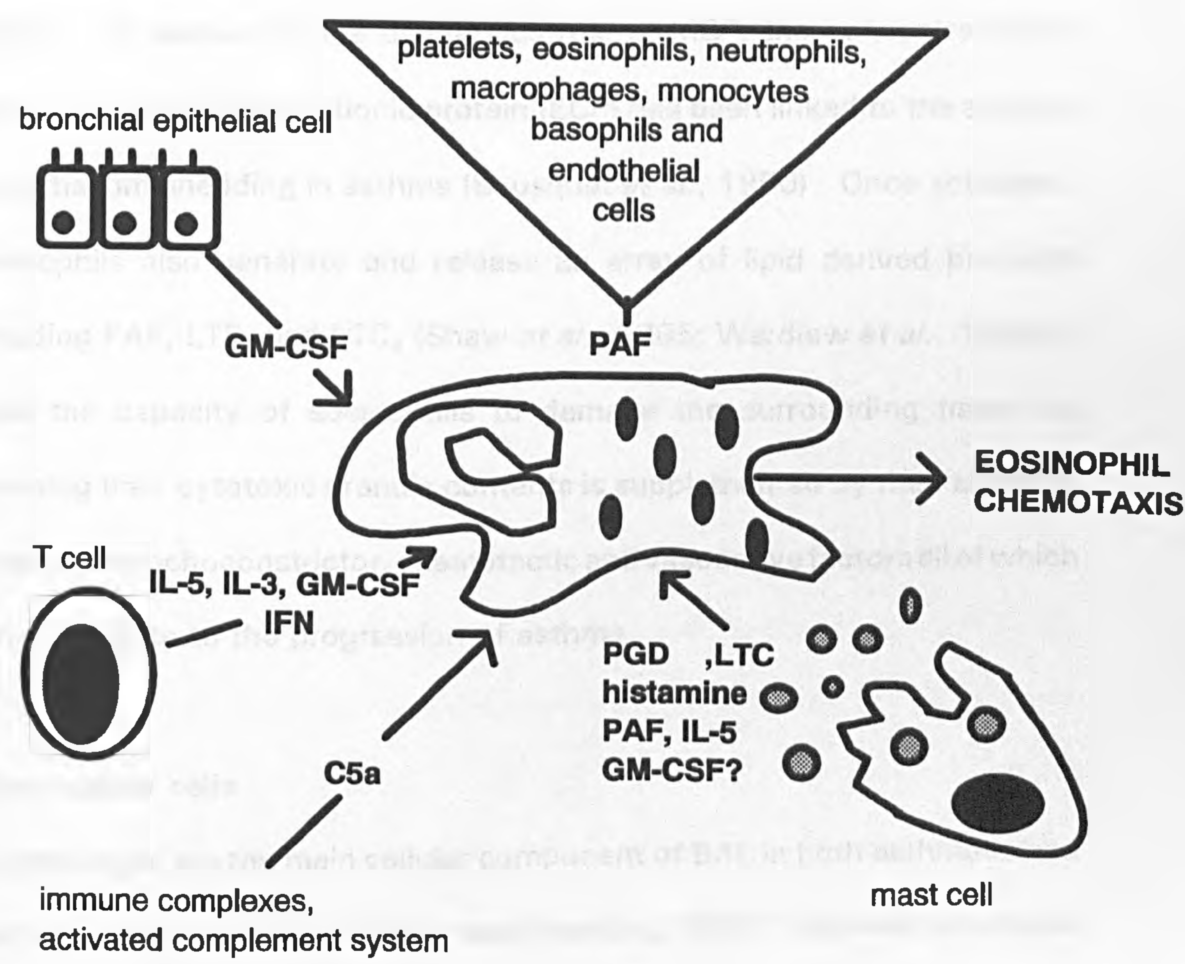

Fig. 1.4.3. Chemoattractants for eosinophils

Eosinophils contain a number of preformed mediators which are stored in granules within the cells. Of these, major basic protein (MBP) and eosinophil cationic protein (ECP) are the most toxic. Localised to the crystalline core of the granule (Lewis *et al.*, 1978) MBP has been studied most extensively. It is toxic to guinea pig tracheal epithelial cells (Gleich *et al.*, 1979) and human bronchial tissue explants (Frigas *et al.*, 1980) and inhibits ciliary function (Hastie *et al.*, 1987). In one study the concentration of BAL fluid MBP was also shown to correlate with indices of airway responsiveness and the number of ciliated epithelial cells recovered by BAL (Wardlaw *et al.*, 1988a). In addition to the deleterious role of MBP, the concentration of extracelluar eosinophil cationic protein (ECP) has been linked to the amount of epithelium shedding in asthma (Bousquet *et al.*, 1990). Once activated, eosinophils also generate and release an array of lipid derived products including PAF, LTB_4 and LTC_4 (Shaw *et al.*, 1985; Wardlaw *et al.*, 1988b). Thus the capacity of eosinophils to damage the surrounding tissue by releasing their cytotoxic granule contents is supplemented by their ability to generate bronchoconstrictor, chemotactic and vasoactive factors all of which can contribute to the progression of asthma.

Mononuclear cells

Macrophages are the main cellular component of BAL in both asthmatic and normal individuals (Eschenbacher and Gravelyn, 1987). However, increased numbers of macrophages have been reported in BAL fluid after allergen challenge (Metzger *et al.*, 1987). Once activated they can release a variety

of potent chemotactic substance including PAF, LTB_4 a number of peptides and a variety of pro-inflammatory cytokines (Nathan, 1987; Denburg, 1993). Macrophages have therefore been implicated in the promotion of leucocyte recruitment in the LAR and an increase in airway responsiveness observed after allergen challenge (Woolcock, 1988).

T Lymphocytes

T lymphocytes play a central role in any antigen-driven inflammatory process as they are the only cells capable of recognizing foreign antigenic material after processing by antigen presenting cells (Corrigan and Kay, 1993). Furthermore, upon activation by antigen, T cells release a wide variety of cytokines which can lead to selective accumulation and activation of pro-inflammatory cells into inflammed tissues. For example, T cells provide a major source of IL-5 which is a potent eosinophil chemoattractant and activator and Walker *et al.* (1991b) have demonstrated that cultured T lymphocytes from patients with asthma, secrete cytokines which can prolong eosinophil survival. Additionally, mast cells also appear to be dependent on T cells as a deficiency of mucosal mast cells is seen in the gastrointestinal tract of humans with defective T lymphocyte function (Irani *et al.*, 1987).

Some studies have revealed similar numbers of T lymphocytes in BAL fluid of controls and asthmatic patients (Wardlaw *et al.*, 1988a) whereas others have demonstrated an increase in T cell numbers in the BAL fluid of asthmatics (Graham *et al.*, 1985). Further work by Azzawi *et al.* (1990) and

Bradley *et al.* (1991), in which numbers and activation status of mucosal T lymphocytes were assessed by immunostaining with monoclonal antibodies directed against T lymphocyte phenotypic and activation markers, has indicated that total numbers of CD4+ and CD8+ T cells are not significantly elevated in the bronchial mucosa of mild asthmatics, as compared to normal controls and CD4+ cells predominate over CD8+ cells in both cases. Nevertheless, only T cells in biopsies from asthmatics showed evidence of IL-2 receptor expression suggesting activation and numbers of activated T cells correlated with both total numbers of eosinophils and numbers of activated eosinophils. Additionally, the degree of activation of T cells could be correlated with disease severity as assessed by bronchial hyperresponsiveness. Immunostaining and flow cytometry have also revealed a proportion of activated CD4+ but not CD8+ T cells in the peripheral blood of patients with acute severe asthma (Corrigan *et al.*, 1988) and the degree of T cell activation has been shown to decrease after therapy to an extent that could correlate with the degree of clinical improvement (Corrigan and Kay, 1990).

Results therefore suggest that T cells may provide an important link between the induction of an asthmatic response and its subsequent propagation. In particular, activated T cells may control the numbers and activation status of eosinophils in asthmatic bronchial inflammation and the degree of activation of T cells may be one factor which determines disease severity.

1.5 THE PREVENTION OF ALLERGY

The prevention of asthma and other allergic reactions without pharmacological intervention is preferable but not always effective. It is known that once an atopic individual has become sensitized to an allergen, the most effective means of treatment is that of prolonged and if possible permanent avoidance of that allergen. However removal of allergens and in particular the aeroallergens, is not always possible. Suitable symptom relieving and anti-inflammatory drugs must therefore be administered.

1.5.1 Corticosteroids

Corticosteroids have been used in the treatment of chronic severe asthma as early as 1950 (Church, 1993). More recently it has been established that corticosteroids are also effective in the treatment of milder forms of asthma as they reduce bronchial inflammation and control the progression of the disease. The naturally occuring glucocorticoid hydrocortisone has both anti-inflammatory, glucose metabolism and mineral absorption effects and has therefore been unsuccessful in the treatment of allergic disease. Although some glucocorticoid activity still exists in synthetic molecules chemical modulation of hydrocortisone has removed many of these unwanted mineralocorticoid effects. Chemical manipulations have also increased potency and duration of action which are a requirement for systemic administration whereas other manipulations confering slow absorption from mucosal surfaces and rapid metabolism on entry into the systemic circulation

have been developed for local corticosteroid administration to the lung in aerosol form.

At the cellular level corticosteroids suppress both acute and chronic inflammation by acting at a number of steps in the inflammatory response. The molecular basis of their activity has been extensively studied. Corticosteroids are highly lipophilic compounds that bind to either of two plasma proteins: transcortin which binds hydrocortisone and prednisolone with high affinity or albumin which binds all steroids with low affinity. Free steroid molecules diffuse across the cell membrane and bind to an inactive cytosolic glucocorticoid receptor. Glucocorticoid receptors are maintained in an inactive state by being bound to a 90 kDa heat shock protein which is shed upon interaction with the glucocorticoid molecule to reveal the active site of the receptor (Church, 1993). The active glucocorticoid receptor-drug complex then rapidly translocates to the nucleus. Within the nucleus the complex binds to specific sites on DNA termed glucocorticoid response elements (GRE) upstream of the promoter region in steroid responsive genes. Binding of the steroid-GR complex to the GRE enhances or represses the transcription of the target gene. An example of a protein whose transcription is up-regulated is lipocortin which exerts anti-inflammatory activity by inhibiting the activity of phospholipase A2. Alternatively the drug-receptor complex can bind to active transcription factors by a direct protein-protein interaction. These transcription factors such as activating protein-1 (AP-1) and nuclear factor κB (NF-κB) themselves stimulate transcription but can be inactivated by binding of the drug-receptor complex.

For example IL-5 expression may be inhibited by the binding of the drug-receptor complex to AP-1 thereby inhibiting the interaction of AP-1 with the AP-1 response element sequence upstream of the 5′ region of the human IL-5 gene (Rolfe *et al.*, 1992).

Unfortunately a small proportion of asthmatic patients are resistant to the therapeutic effects of glucocorticoids possibility because of a reduced ability of the active drug-glucocorticoid receptor complex to bind to GRE. This reduction may be a result of an abnormal interaction with AP-1 or NF-κB which would decrease the amount of drug-receptor complexes available to bind to GRE within the nucleus (Adcock *et al.*, 1995). Patients within this steroid resistant group must therefore be treated with a variety of other anti-inflammatory drugs.

1.5.2 Cyclosporin A

Cyclosporin A (CsA) has been used in the treatment of a number of allergic diseases including allergic conjunctival disorders (Lightman, 1993) and atopic dermatitis (MacKie, 1993). It has also recently been administered to patients with chronic severe asthma and was found to improve lung function (Alexander *et al.*, 1992).

Cyclosporin A appears to act at a step distal to the cell membrane receptors and known second messengers, but proximal to late signalling events such as transcriptional activation of early genes or the degranulation of mast cells. It diffuses across the cell membrane and binds to an intracellular protein known as cyclophilin which acts as an intracellular

receptor for the drug. Calcineurin, a calcium and calmodulin dependent protein phosphatase then binds with high affinity to the biologically active immunophilin-drug complex (Harding *et al.*, 1986). Calcineurin may play a central role in the signalling pathway carrying information from the cell membrane to the nucleus and it has been suggested that binding of the immunophilin-drug complex to calcineurin can inhibit nuclear translocation of various cytosolic subunits required for activation of transcription factors (Schreiber and Crabtree, 1992). Studies on the IL-2 enhancer have shown that transcription factor proteins act cooperatively to activate the IL-2 gene and the transcription factors NF-AT and OAP (octamer associated protein) are highly sensitive to the drug (Grannelli-Piperno, 1990). Transcription controlled by these sites is reduced by over 100-fold at concentrations of drug which block T cell activity but have no inhibitory effect on most other cell types. In contrast the AP-1 and NF-κB sites which are present in most tissues are unaffected by CsA (Granelli- Piperno, 1990). Studies on the mode of action of CsA in blocking the activity of NF-AT have shown that it does not inhibit the synthesis of the transcription factor protein but rather that it inhibits its assembly (Flanagan *et al.*, 1991). NF-AT is a complex protein consisting of two or more subunits. One of these is predominantly nuclear, is found in most cells and is induced by activators of protein kinase C whilst the other appears to be T cell specific and is contained within the cell cytoplasm. Intracellular increases in calcium can lead to translocation of the cytosolic protein component into the nucleus were it combines with the nuclear subunit to form a functional transcription factor (Flanagan *et al.*,

1991). It has been suggested that calcineurin is involved in the translocation of the cytosolic component into the nucleus. Upon antigen presentation signalling mechanisms are activated leading to a generation of inositol tris phosphate (IP_3) and diacyl-glycerol. IP_3 leads to an influx in calcium ions which cause the activation of the phosphatase activity of calcineurin which could then be involved in the dephosphorylation of the cytosolic subunit of NF-AT thus inducing nuclear translocation (Schreiber and Crabtree, 1992).

1.5.3 Drugs in development

Even with the present range of anti-inflammatory drugs such as corticosteroids and the wide variety of symptom relieving drugs, the prevalence and severity of asthma is rapidly increasing within the Western world. There is therefore a need to develop more effective and reliable therapies. A number of approaches include calcium antagonists which aim to reduce the stimulus induced entry of calcium into smooth muscle and thus decrease its contractility, potassium channel opening drugs which lead to hyperpolarization and consequently a reduction in contractile sensitivity of bronchial smooth muscle and leukotriene and PAF antagonists which reduce the bronchoconstrictor and pro-inflammatory properties of asthma (Church, 1993). An example of a new potential oral anti-inflammatory drug for asthma has been developed by Knoll Pharmaceuticals (formerly Boots Pharmaceuticals). It is known as N-[1-(4-chlorophenyl)-1-methylethyl]-3-(imidazol-1-yl)propylamine (BTS 71321) and is a novel 1-substituted

imidazole which has recently been found to inhibit the late phase response to antigen challenge in guinea pigs and the release of arachidonic acid, superoxide and cytokines from leucocytes stimulated *in vitro* (Steele *et al.*, 1995). Although its mechanism of action is not characterised it has been developed for asthma prophylaxis and in healthy male volunteers it is well tolerated in single doses up to 800 mg (Garratt *et al.*, 1995) suggesting its suitability for further clinical investigation.

1.6 AIMS AND OBJECTIVES

As cytokines are involved in many cell to cell signalling events and have the capacity to influence the function of target cells, they may play important roles in the development and progression of allergic disease. A central theme of study in this thesis is the involvement of these mediators in the progression of allergic inflammation. Furthermore, the possibility that mast cells may play a key role in the regulation of allergic disease such as asthma by the production of cytokines is considered. A number of different drugs are used in the treatment of asthma. These include corticosteroids and more recently CsA has also been found to be effective in the treatment of chronic severe asthma. Thus another central theme of study throughout this thesis has centred on the efficacy of drugs such as these at inhibiting cytokine expression by mast cell *in vitro* and by rat BAL cells *in vivo*. As the novel 1-substituted imidazole compound, BTS 71321, has been developed as a potential drug for asthma prophylaxis its effects on mast cell mediator release and cytokine gene expression by mast cells and BAL cells are also investigated.

The effects of BTS 71321 on mast cell mediator release are unknown. Therefore, chapter 3 considers the effects of BTS 71321 compared to Dex and CsA, on the inhibition of 5-HT and arachidonate from immunologically activated rat PMC.

The release of preformed and *de novo* synthesised mast cell mediators are not only known to be regulated by drugs but also cytokines such as IL-3,

IL-4, SCF and IFN-γ. Therefore, in chapter 4 the effects of cytokines on the expression of cytokines by Cl.MC/C57.1 murine mast cells, rat PMC and HMC-1 human mast cells are investigated.

In chapter 5 immunologically activated rat PMC have been studied more closely as a source of several pro-inflammatory cytokines. Originally a panel of eight cytokines, namely IL-3, IL-4, IL-5, IL-6, TNF-α, MIP-2, IFN-γ and GM-CSF, were chosen for investigation. However, only five PCR primer pairs were found to work consistently and so studies have centred on the mRNA expression of IL-5, IL-6, TNF-α, MIP-2 and IFN-γ. Chapter 5 also considers the effects of Dex, CsA and BTS 71321 on mast cell cytokine expression *in vitro*.

Chapter 6 investigates whether the cytokines IL-5, IL-6, TNF-α, MIP-2 and IFN-γ are induced in a Sephadex-induced model of lung inflammation in the rat. In addition to assessing changes in cell numbers in BAL fluid, chapter 6 includes studies of the mRNA expression of these cytokines by BAL cells at various times after a single *i.v.* injection of Sephadex particles. Furthermore, in an attempt to determine specific cellular sources of some of these cytokines, expression of mRNA encoding IL-6 and TNF-α were investigated in lung tissues of controls and diseased rats by *in situ* hybridization.

Chapter 7 considers the effectiveness *in vivo*, of Dex, CsA and BTS 71321 on the inhibition of cellular infiltration in the bronchoalveolar cavity and BAL cell cytokine mRNA expression in the rat Sephadex-induced model of lung inflammation. In this chapter the effects of these drugs on BAL cell

cytokine gene expression and numbers of bronchoalveolar cells are investigated 24 h and 72 h after administration of Sephadex particles.

Chapter 8 describes an investigation of an antigen-driven model of pleurisy in the rat and of rat pleural cells as a source of induced expression of IL-5, IL-6, TNF-α, MIP-2 and IFN-γ.

Each experimental chapter (chapters 3-8) includes a short introduction including background information and rationale for the experimental work followed by a results section and a discussion of results. Chapter 2 describes the materials and methods used within the project and to conclude, chapter 9 discusses results in general and points of interest arising during the course of this work.

CHAPTER 2

MATERIALS AND METHODS

2.1 CELL CULTURE

2.1.1 Rat Peritoneal Mast Cells

2.1.2 Mast cell-depleted peritoneal cells

2.1.3 Cl.MC/C57.1 mouse mast cells

2.1.4 HMC-1 human mast cells

2.1.5 L929 Cells

2.1.6 B9 Cells

2.1.7 L929 Cytotoxicity Assay to measure TNF-α bioactivity

2.1.8 The B9 proliferation assay for IL-6

2.1.9 The Enzyme Linked Immunosorbent Assay (ELISA) for IL-5

2.1.10 Investigation of the effects of IL-3, IL-4, SCF or IFN-γ on the release of cytokines by murine Cl.MC/C57.1 mast cells

2.1.11 Investigation of the effects of IL-4 or SCF on the release of TNF-α by rat peritoneal mast cells

2.1.12 Investigation of the effects of IL-4 on cytokine gene expression in the HMC-1 human leukemic mast cell line

2.1.13 Investigation of the effects of cyclosporin A, dexamethasone or BTS 71321 on TNF-α release from the Cl.MC/C57.1 murine mast cell line

2.1.14 Investigation of the effects of cyclosporin A or dexamethasone on TNF-α release from rat peritoneal mast

cells

2.1.15 Investigation of the effects of cyclosporin A, dexamethasone or BTS 71321 on cytokine mRNA expression in HMC-1 cells

2.1.16 Detection of mRNA for IL-5, IL-6, TNF-α, MIP-2 and IFN-γ in activated rat peritoneal cells

2.1.17 Investigation of the effects of cyclosporin A or dexamethasone on cytokine mRNA expression in activated rat peritoneal cells

2.1.18 Investigation into the effects of cyclosporin A, dexamethasone or BTS 71321 on serotonin and arachidonate release from activated rat peritoneal mast cells

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2.2.1.2 Lung Tissue

2.2.1.3 Lung Histology: Staining for mast cells and neutrophils

2.2.1.4 Lung Histology: Staining for eosinophils

2.2.1.5 Investigation of the effects of cyclosporin A, dexamethasone or BTS 71321 on bronchoalveolar cell infiltration and BAL cell cytokine mRNA levels in the rat Sephadex model of lung inflammation

2.2.2 Antigen-induced pleurisy in the rat

2.2.2.1 Pleural lavage cells

2.3 IMMUNOHISTOCHEMISTRY

2.3.1 Staining for IL-6 or TNF-α in bronchoalveolar cell cytospin preparations

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2.4.2 Reverse Transcription

2.4.3 PCR of rat cDNA

2.4.4 PCR of human cDNA

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2.4.6 Restriction Digests of PCR products

2.4.7 Preparation of RNA in situ probes.

2.4.7.1 Cloning of PCR products

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2.4.7.3 Restriction Digests of plasmid DNA

2.4.7.4 Large scale preparations of plasmid DNA

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2.4.8.3 Hybridisation

2.4.8.4 Post Hybridisation Washes

2.4.8.5 Visualisation of Probe

2.1 CELL CULTURE

2.1.1 Rat peritoneal mast cells (PMC)

Mast cells were obtained by peritoneal lavage of male or female Wistar rats (Liverpool outbred stock). Rats were killed by asphyxiation in CO_2 followed by cervical dislocation. The skin was then removed from the abdomens of the animals and 60 ml of sterile Hanks' balanced salt solution (GIBCO, Uxbridge, U.K.) was injected into the peritoneal cavity. Cavities were massaged vigorously and the fluid was removed using a sterile needle and syringe. This procedure was repeated twice more. Peritoneal cell suspensions were then sedimented by centrifugation at 150 *g* for 8 min and cells were washed once in complete DMEM (Dulbecco's modified Eagle's Medium (GIBCO) supplemented with 5% heat inactivated foetal calf serum (FCS) and 40 μg/ml gentamycin; see Appendix 1 for preparation and composition of a 5L stock solution of DMEM). To purify mast cells the peritoneal cells were suspended in 72.5% isotonic Percoll (Pharmacia, Uppsala, Sweden) and centrifuged at 200 *g* for 10 min. Mast cells were recovered as the pelleted fraction. Mast cells comprised approximately 1% of unfractionated cells and ≥ 99% of cells after density gradient fractionation as determined by metachromatic staining in 0.01% toluidine blue. Cells were stained for 10 min at 37°C and then counted on an improved Neubauer haemocytometer (Weber Scientific Instruments Ltd, Teddington).

2.1.2 Mast cell-depleted peritoneal cells

After density gradient fractionation of peritoneal cells through Percoll, the upper cell layer (containing < 1% mast cells) was removed and pelleted at 150 *g* for 5 min, washed twice, resuspended and cultured in 1.0 ml of complete DMEM for 24 h (37° and 5% CO_2) at a concentration of 10^4 cells/ml, corresponding to the number of non-mast cells in purified mast cell preparations.

2.1.3 Cl.MC/C57.1 mouse mast cells

The Cl.MC/C57.1 growth factor independent mouse mast cell clone (Young *et al.*, 1987) was kindly provided by Dr. S.J. Galli (Beth Israel Hospital, Boston MA, USA). Cells were seeded at a density of $5x10^5$/ml in cell culture medium (DMEM supplemented with 10% FCS, 2 mM L-glutamine and 40 μg/ml gentamycin). Cells were cultured in 75 cm^2 culture flasks (37°C, 5% CO_2) and were fed twice weekly by replenishment of the medium. Cell viability was assessed by staining in 0.02% trypan blue dye which stains non viable cells blue. These cells were excluded from cell counts.

2.1.4 HMC-1 human mast cells

The HMC-1 growth factor-independent human leukemic mast cell line (Butterfield *et al.*, 1988) was kindly provided by Dr. Butterfield (Mayo Clinic, Rochester, MN, USA). Cells were grown in complete IMDM (Iscove's modified Dulbecco's medium (Gibco) supplemented with 10% FCS, 2 mM L-glutamine, 1.2 mM monothioglycerol and 40 μg/ml gentamycin) and

maintained as described above (section 2.1.3).

2.1.5 L929 Cells

The L929, TNF-α sensitive cell line was kindly supplied by Dr. S. J. Galli, (Beth Israel Hospital, Boston MA, USA). The cells adhere to flask surfaces so culture flasks were placed horizontally in the incubator (37°C / 5%CO_2). Cells were seeded at a concentration of 5×10^5/ml in cell culture medium. Cells that had reached confluence were removed from flask surfaces by the addition of trypsin (GIBCO). Culture medium was removed from flasks, adherent cells were washed in phosphate buffered saline (PBS; see Appendix 1 for preparation and composition) and 2 ml of 10 x trypsin (25 mg/ml) were added to each flask so that the cell layer was completely covered. Flasks were incubated at 37°C for 2 min before the addition of 10 ml of cell culture medium. Dislodged cells were pipetted into sterile 30 ml universal tubes, sedimented by centrifugation at 100 *g* for 3 min and washed in cell culture medium prior to resuspension in fresh culture medium. Cell viability was assessed as described above (section 2.1.3) and cells were reseeded at the appropriate concentration.

2.1.6 B9 Cells

The murine B9 hybridoma cell line (Aarden *et al.*, 1987) was kindly provided by Dr. I. Hashim, Royal Liverpool University Hospital, Liverpool. Cells were maintained in complete B9 cell culture medium (RPMI 1640 (GIBCO) supplemented with 10% FCS, 2 mM L glutamine, 860 IU/ml crude IL-6

(Cambio, Cambridge UK) and 40 μg/ml gentamycin). Cells were seeded at a density of $5x10^4$/ml and maintained as described above (section 2.1.3). The cells were used up to the 14th passage after which time the cells were discarded due to the loss of their IL-6 dependency. A fresh vial of cryopreserved cells was taken from stocks stored under liquid nitrogen (see Appendix 1 for preparation and composition of freezing solution).

2.1.7 L929 Cytotoxicity Assay to measure TNF-α bioactivity

TNF-*α* bioactivity was determined by the L929 cytotoxicity assay as described by Gordon and Galli (1990). Cells were diluted to $3x10^5$/ml in cell culture medium and 120 μl of the cell suspension was pipetted into each well of a 96 well plate. Plates were incubated overnight at 37°C in 5% CO_2 in air to enable the L929 cells to adhere to the well surfaces. On the following day spent medium was gently flicked out and 100 μl of samples (in duplicate) or standards (in triplicate) were added to the wells. Actinomycin D (Sigma, Poole, Dorset) was added to the 100 μl culture volumes to give a final concentration of 5 μg/ml in each well. Plates were then incubated for 18-24 h at 37°C in 5% CO_2 in air. Biologically active TNF-*α* in culture supernatants was then detected by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma), colorimetric assay (Green *et al.*, 1984). Twenty microlitres of MTT (5 mg/ml in PBS) were added to each microculture well and samples were incubated for a further four hours (37°C /5% CO_2). The blue formazan crystals were solubilized with 20% Triton X in 0.5 M HCl with vigorous shaking on a plate

shaker. Absorbances were read at 570 nm on an automated plate reader (Dynatech MR600, Dynatech Laboratories Ltd. Surrey, England). The specificity of the cytotoxic activity was demonstrated by neutralisation with a monospecific rabbit anti-mouse TNF-α antibody (final concentration of 50 μg/ml; Serotec Ltd. Oxford, England).

2.1.8 The B9 proliferation assay for IL-6

IL-6 bioactivity was determined by the B9 murine hybridoma proliferation assay as described by Aarden *et al.* (1987). B9 cells were washed and diluted to a concentration of 1×10^5 cells/ml in IL-6 free cell culture medium. Standards (50 pg/ml), heat treated samples (56°C for 30 min) or IL- 6 free culture medium alone were added to the top row of wells of a 96 well plate and serially diluted two-fold down the plate. Fifty microlitres of B9 cells (1×10^5 cells/ml) were then added to each well and plates were incubated for 4 days at 37°C in 5% CO_2. MTT was added to each well as described above, formazan crystals were solubilised in the usual way and absorbances read at 570 nm as before (section 2.1.7).

2.1.9 The Enzyme Linked Immunosorbent Assay (ELISA) for IL-5

Samples were assayed with pairs of rat anti-mouse IL-5 murine antibody (AMS Biotechnology, Witney, Oxon) in a two-site sandwich ELISA (Schaumer *et al.,* 1988). Flexible polyvinyl chloride microtitre plates were coated with 50 μl of 1 μg/ml coating antibody in PBS and incubated at either 4°C overnight or at 37°C for 2 h. Wells were then blocked by the addition

of 150 μl of 10% FCS in PBS, with incubation at 37 °C for 30 min. Sample and standard dilutions were made in RPMI-1640 medium plus 10% FCS in a separate 96 well flat bottom microtitre plate and 50 μl volumes were transferred to the ELISA plate which had been washed three times in PBS containing 0.05% Tween 20 (PBS-Tween, pH 7.2). Samples were incubated in the assay plates for either 1 h at 37 °C or 2 h at room temperature. After washing three times in PBS-Tween each well received 50 μl of antibody-biotin conjugate at 0.5 to 4 μg/ml in 1% bovine serum albumin (BSA)/PBS-Tween. After a 1 h incubation period at room temperature streptavidin - horseradish peroxidase conjugate (75 μl/well) was added at between 1 and 2 μg/ml in 1% BSA/PBS-Tween. Plates were incubated at room temperature for 1 h, washed three times in fresh PBS-Tween and 100 μl of freshly prepared substrate solution, containing 0.1% H_2O_2 (30% w/v) and 400 μg/ml of *o*-phenyldiamine dihydrochloride (Sigma) in 0.15 M citrate phosphate buffer (see Appendix 1 for preparation and composition of citrate phosphate buffer) were added to each well. The enzyme-substrate reaction was terminated after 5 min by addition of 25% H_2SO_4 (25 μl/well). Absorbances were read at 450 nm on a dual wavelength automated plate reader (Dynatech) with the reference wavelength set at 630 nm.

2.1.10 Investigation of the effects of IL-3, IL-4, SCF or IFN-γ on the release of cytokines by murine Cl.MC/C57.1 mast cells.

Cl.MC/C57.1 mast cells (1 x 10^6 cells in 1.0 ml of cell culture medium) were cultured for 48 h in the presence or absence of either recombinant mouse

IL-3, recombinant mouse IL-4, (Genzyme, New Brunswick Scientific, Suffolk, U.K.) recombinant rat SCF^{164} (Amgen, Thousand Oaks, CA) or recombinant mouse IFN-γ (provided by Dr. A.G. Morris, University of Warwick, U.K.) each at a concentration of 100 ng/ml. One hour before challenge, mouse monoclonal IgE anti-dinitrophenyl (anti-DNP) antibody (ICN-Flow, High Wycombe, U.K.) was added at a concentration of 0.2 μg/ml (Holliday *et al.*, 1994). Prior to challenge, cells were washed twice in DMEM to remove unbound IgE. Cells were resuspended in 200 μl of cell culture medium. Cell suspensions (200 μl) were then added to 200 μl of prewarmed cell culture medium containing DNP-human serum albumin antigen (DNP_{30-40}-HSA, Sigma) to give a final concentration of DNP-HSA in the tubes of 50 ng/ml (Holliday *et al.*, 1994). Control cells were resuspended in 400 μl of cell culture medium alone. Cells were challenged for 1 h (37°C, 5% CO_2) and then sedimented at 150 *g* for 5 min. Supernatant fractions were removed and assayed for cytokines.

2.1.11 Investigation of the effects of IL-4 or SCF on the release of TNF-α by rat peritoneal mast cells.

Purified rat PMC (1×10^6 cells/ml) were cultured in complete DMEM for 48 h (37°C, 5%CO_2) in the presence of recombinant mouse IL-4 (25 ng/ml or 100 ng/ml) or recombinant rat SCF (50 ng/ml). Cells were then pelleted at 150 *g* for 5 min, the supernatant fraction was discarded and the cell pellet was washed once in complete DMEM. Cells (200 μl) were challenged with sheep anti-rat IgE antibody (anti-IgE; ICN Biomedicals, Thame, Oxfordshire,

UK). Anti-IgE was used at a final dilution of 1/100. Pilot experiments had revealed that this concentration gave optimal release of TNF-*α* after a 1 h challenge. Cells (10^6) were suspended in 100 μl of complete DMEM and 100 μl of anti-IgE (1/50) were added. Cells were challenged for 1 h at 37°C in 5% CO_2. Cells were then pelleted at 150 *g* for 5 min and supernatant fractions tested for biologically active TNF-*α*.

2.1.12 Investigation of the effects of IL-4 on cytokine gene expression in the HMC-1 human leukemic mast cell line

HMC-1 cells (10^6/ml) were cultured with or without recombinant human IL-4 (100 ng/ml, R&D Systems, Minneapolis, USA) for 24 h. Cells were then sedimented (150 *g*, 5 min) resuspended in complete IMDM and challenged for 4 h with 10 μM ionomycin (Buckley *et al.*, 1995). RNA was extracted from cells and cytokine mRNA levels analyzed by RT-PCR (sections 2.4.1, 2.4.2, 2.4.4).

2.1.13 Investigation of the effects of cyclosporin A, dexamethasone or BT 71321 on TNF-α release from the Cl.MC/C57.1 murine mast cell line

Cl.MC/C57.1 mast cells (10^6/ml) were cultured in cell culture medium (37°C, 5% CO_2) for 24 h in the presence or absence of cyclosporin A (CsA; Sandoz, Feltham, Middlesex U.K.), dexamethasone (Dex; Sigma), or BTS 71321 (Knoll Pharmaceuticals; not commercially available) at either 10^{-5}M or 10^{-6}M. Stock solutions of CsA and Dex were made up in absolute ethanol and stored at -70°C prior to use. Stock solutions of BTS 71321 were made

up fresh in cell culture medium. All drugs were diluted in cell culture medium before use and final concentrations of ethanol with which cells were exposed to did not exceed 1 %. After the 24 h culture period cells were pelleted by centrifugation (150 *g*, 5 min), washed once, resuspended in 300 μl of cell culture medium and challenged for 1 h with calcium ionophore A23187 (final concentration 10^{-7}M). Pilot experiments had revealed that cell challenges with 10^{-7}M calcium ionophore A23187 for 1 h were optimal for TNF-α release. Supernatant fractions were removed after centrifugation and assayed for biologically active TNF-α (section 2.1.7).

2.1.14 Investigation of the effects of cyclosporin A or dexamethasone on TNF-α release from rat peritoneal mast cells

Rat PMC (2 x 10^6/ml) were cultured with or without CsA or Dex both at a concentration of 10^{-6}M for 24 h. One hour before challenge IgE anti-DNP (0.2 μg/ml) was added. After the 24 h culture period cells were washed once, resuspended in complete DMEM and challenged for 1 h with DNP-HSA antigen (final concentration 50 ng/ml) in a final volume of 250 μl. Supernatant fractions were assayed for TNF-α bioactivity as described above (section 2.1.7).

2.1.15 Investigation of the effects of cyclosporin A, dexamethasone or BTS 71321 on cytokine mRNA expression in HMC-1 cells.

HMC-1 mast cells were cultured (10^6/ml) in complete IMDM for 24 h in the presence or absence of either CsA, Dex or BTS 71321 (each at a

concentration of 10^{-6}M). Cells were then washed once, resuspended in complete IMDM and challenged for 4 h with 10 μM ionomycin in a final volume of 0.5 ml. RNA was extracted and cytokine mRNAs were analyzed by RT-PCR (sections 2.4.1, 2.4.2, 2.4.4).

2.1.16 Detection of mRNA for IL-5, IL-6, TNF-α, MIP-2 and IFN-γ in activated rat peritoneal mast cells

Purified rat PMC (10^6/ml) were cultured for 24 h (37°C, 5%CO_2) in complete DMEM. Cells were then washed, resuspended in complete DMEM and challenged for 4 h with anti-IgE (final concentration of 1:100) in a final volume of 0.5 ml at 37°C in 5% CO_2 in air. RNA was extracted and cytokine mRNA levels analyzed by RT-PCR (sections 2.4.1-2.4.3).

2.1.17 Investigation of the effects of cyclosporin A or dexamethasone on cytokine mRNA expression in activated rat peritoneal mast cells

Purified rat PMC (10^6/ml) were cultured in complete DMEM for 24 h (37°C, 5%CO_2) in the presence of CsA or Dex each at 10^{-6}M. After 24 h cells were washed, resuspended in complete DMEM and challenged for 4 h with anti-IgE as described above. RNA was extracted and cytokine mRNA levels analyzed by RT-PCR (sections 2.4.1-2.4.3).

2.1.18 Investigation into the effects of cyclosporin A, dexamethasone or BTS 71321 on serotonin and arachidonate release from activated rat peritoneal mast cells.

Purified rat PMC (5×10^4/ml) were cultured in 1.0 ml aliquots of complete DMEM for 24 h (37°C, 5% CO_2) in the presence or absence of CsA, Dex (each at 10^{-7}M) or BTS 71321 (10^{-4}M - 10^{-7}M). Radiolabelled arachidonic acid ([5,6,8,9,11,12,14,15-^{3}H(*N*)]Arachidonic acid, 2.0 μCi/ml; ARC, St Louis, MO; sp. act. 200 Ci/mmol) was added to cells at the beginning of the 24 h culture period. 5-[1,2-^{3}H(*N*)]-hydroxytryptamine creatinine sulphate (1.0 μCi/ml; NEN, Dreiech, Germany, sp. act. 27 Ci/mmol) was added to other cell cultures 1 h before termination of cultures. Cells were sedimented (150 *g*, 5 min) washed twice and resuspended in 1.0 ml of challenge medium (complete DMEM supplemented with 10 mM HEPES to give pH 7.0 at 37°C). The cells (150 μl) were added to 150 μl of anti-IgE (final concentration of 1:100) in challenge medium, challenge medium alone, or 0.05% Triton X-100 (Sigma), to lyse cells for determination of total incorporated [^{3}H]5-HT or [^{3}H]arachidonate. Cells were challenged for 30 min for assay of 5-HT release or for 1 h for assay of arachidonate release. Pilot experiments had shown that these times were optimal for release of these two mediators. All cell challenges were performed in duplicate and released radioactivity in supernatant fractions (150 μl) from challenged cells was measured by scintillation spectrometry. Percentage specific stimulated release was measured as: $[(a-b)/c] \times 100$, where a is the radioactivity (c.p.m) released from stimulated cells, b is that released from unstimulated cells, and c is total incorporated cellular radioactivity determined by detergent lysis. Background release of 5-HT from unstimulated cells was 5-10% and background release of arachidonate was 6-10%.

2.2 ANIMAL MODELS OF INFLAMMATION

2.2.1 The rat Sephadex model of lung inflammation

2.2.1.1 Bronchoalveolar lavage cells.

Male Wistar rats, weight 150 - 180 g, were purchased from Charles River, housed two per cage and fed *ad libitum* on a standard diet. Animals were anaesthetized by intraperitoneal injection of Brietal (5 mg per 100 g bodyweight) prior to receiving 1.0 ml of isotonic saline (controls) or 1.0 ml of Sephadex suspension (0.5 mg/ml) intravenously via the tail vein (Sephadex G200 (Pharmacia) was suspended in isotonic saline, autoclaved for 20 min and stored at 4°C for a minimum of 48 h prior to use). At various times after Sephadex administration (6 h, 24 h, 48 h, 72 h and 7 days) rats were sacrificed, the trachea cannulated and 5 ml of heparinised saline (5 U/ml) was injected into the airway. After 2 min, the lavage fluid was recovered by gentle aspiration. Three more lavages were performed and the four fluid collections were combined, and stored on ice prior to centrifugation. Bronchoalveolar lavage (BAL) cells were pelleted at 150 *g* at 4°C for 10 min, the supernatant fraction discarded and the cell pellet resuspended in 1.0 ml of heparinised saline. Total white cell counts were made with a Coulter Counter (Coulter Electronics). Differential leucocyte counts were made on cytocentrifuged preparations (Shandon Cytospin II; Shandon Southern Instruments) that had been stained with 'Diff-Quik' (Merz and Dade AG). A minimum of 100 cells were counted on each slide. To

eliminate bias, cytospin slides were coded by an independent observer and cells were counted blindly. Statistical analysis of differences in inflammatory cells in bronchoalveolar lavage fluid compared with control levels was determined using the two-tailed Mann Whitney *U* test at the 95% confidence interval. Remaining BAL cells were sedimented at 150 *g* for 5 min, RNA was extracted and cytokine mRNA levels investigated by RT-PCR (sections 2.4.1-2.4.3).

2.2.1.2 Lung Tissue

At each time point a small piece of lung tissue taken from the lower right lung was removed from the sacrificed rats, fixed in 10% buffered formalin for 24 h and processed to wax (Lamb Wax, Lamb, London, U.K.) for histological staining or analysis of mRNA by *in situ* hybridisation (sections 2.2.1.3, 2.2.1.4, 2.4.7).

2.2.1.3 Lung Histology: Staining for mast cells and neutrophils

Paraffin embedded sections (4-5 μM) of rat lung tissue were mounted on microscope slides, dewaxed in Xylene (2 x 5 min), rehydrated through alcohol (100% ethanol (2 x 1 min), 90% ethanol (2 x 1 min), 70% ethanol (2 x 1 min), distilled water (5 min)) and stained for mast cells and neutrophils using a Naphthol AS-D Chloroacetate Esterase staining kit (Sigma). Sodium nitrite solution (50 μl) was added to 50 μl of Fast Red Violet LB Base Solution, mixed gently by inversion and allowed to stand for 2 min. The solution was then mixed with 2 ml of 37°C prewarmed distilled

water. TRIZMAL™ 6.3 Buffer Concentrate (250 μl) and Naphthol AS-D Chloroacetate Solution (50 μl) were then added and the resulting red solution was gently pipetted onto each tissue section. Slides were incubated for 30 min at 37°C in the dark, washed in water for 5 min and mounted with aqueous mountant (Vince *et al.*, 1991).

2.2.1.4 Lung Histology: Staining for eosinophils

Paraffin embedded sections (4-5 μM) of rat lung tissue were mounted on microscope slides, dewaxed in Xylene (2 x 5 min), rehydrated through alcohol (100% ethanol (2 x 1 min), 90% ethanol (2 x 1 min), 70% ethanol (2 x 1 min), distilled water (5 min)) and stained in haematoxylin for 5 min. Slides were then dipped in 1% acid/alcohol solution (see Appendix 2) approximately 12 times then dipped in Scotts solution (see Appendix 2) for 1 min and stained in Eosin (see Appendix 2) for 2.5 min before washing quickly, dehydrating through increasing concentrations of alcohol and mounting in DPX resin.

2.2.1.5 Investigation of the effects of cyclosporin A, dexamethasone or BTS 71321 on bronchoalveolar cell infiltration and BAL cell cytokine mRNA levels in the rat Sephadex model of lung inflammation

Twenty four hours prior to the injection of Sephadex, rats were administered vehicle alone (olive oil for CsA treated rats or cellosize for Dex or BTS 71321 treated rats) or either CsA (10 mg/kg or 30 mg/kg), Dex (0.1 mg/kg or 0.3 mg/kg) or BTS 71321 (10 mg/kg) by *i.p.* injection. Ninety minutes prior to

the Sephadex injection they received a repeat dose of drug. Rats were sacrificed 24 h or 72 h post-Sephadex injection, BAL cells were extracted, stained and counted as described above (section 2.2.1.1) and BAL cell cytokine mRNA levels investigated by RT-PCR (sections 2.4.1-2.4.3). A number of cell cytospin slides were also fixed in 10% buffered formalin for 10 min, dehydrated through alcohol, air dried and stored at -20°C for immunohistochemical analysis (section 2.3).

2.2.2 Antigen-induced pleurisy in the rat

2.2.2.1 Pleural lavage cells

Male Wistar rats (150-180 g, Charles River) were sensitized by intraperitoneal (ip) injection of a mixture containing 10 μg of ovalbumin with 100 mg of $Al(OH)_3$ as an adjuvant on day 0. On day 7, rats were anaesthetized with a 50% CO_2/50% O_2 gas mixture, and received an intrathoracic (it) injection of vehicle (isotonic saline) or 12 μg of ovalbumin, in a final volume of 0.5 ml. At various times after challenge (2 h, 4 h, 24 h and 48 h) rats were sacrificed. A 1", 20 gauge disposable hypodermic needle was inserted through the left hand chest wall and 2 ml of air injected using a sterile 5 ml disposable syringe to collapse the lungs. This was followed by injection through the same needle of 4 ml of heparinised physiological saline (5 U/ml). After removing the needle the rat was inverted several times to wash any adherent pleural cells free. The chest wall was then carefully opened via the diaphragm and using a specifically adapted

needle, the pleural exudate and wash were withdrawn. Cell counts were made as described above (section 2.2.1.1), RNA was extracted and pleural cell cytokine mRNA levels investigated by RT-PCR (sections 2.4.1-2.4.3).

2.3 IMMUNOHISTOCHEMISTRY

All immunohistochemical analyses were carried out using ZYMED Antibody Staining Kits (ZYMED, Carlton Court South, San Francisco USA)

2.3.1 Staining for IL-6 or TNF-α in bronchoalveolar cell cytospin preparations

Cell cytospin slides were stained for IL-6 or TNF-*α* using an alkaline phosphatase detection system. Slides were incubated with 10% non immune goat serum for 30 min at room temperature. The serum was then tipped off and the sections incubated with primary antibody (monoclonal mouse anti-human IL-6 (Genzyme) at a final concentration of 50 μg/ml which is known to cross react with rat IL-6 (Hancock *et al.,* 1993a, 1993b) or polyclonal rabbit anti-mouse TNF-*α* (Serotec) which is known to cross react with rat TNF-*α* at a final concentration of 10 μg/ml. Both antibodies were diluted in Tris buffered saline (TBS, see Appendix 3 for preparation and composition of buffer). Sections were incubated with primary antibodies for at least 1 h. Slides were then washed in TBS for 10 min before the addition of secondary antibodies. A biotinylated goat anti-mouse secondary antibody was used for the detection of IL-6 whereas a biotinylated goat anti-rabbit

secondary antibody was used for the detection of TNF-α. Sections were incubated with secondary antibodies for 1 h. Slides were then washed in TBS for 5 min prior to the addition of streptavidin alkaline phosphatase conjugate for 15 min. Slides were washed in TBS for 5 min and incubated with Fast Red substrate solution for 5-10 min which stains positive cells red. (See Appendix 3 for preparation and composition of Fast Red Substrate Solution).

2.4 MOLECULAR BIOLOGY PROTOCOLS

2.4.1 Extraction of cellular RNA

Cells were sedimented at 150 *g* for 5 min and 1.0 ml of TRIzol Reagent, (GIBCO) a monophasic solution of phenol and guanidine isothiocyanate, was added to the cell pellets and samples were incubated at room temperature for 5 min. Chloroform (0.2 ml per 1.0 ml of TRIzol Reagent) was then added and samples were capped and shaken by hand for 15 seconds. Following a further incubation of 2-3 min at room temperature samples were centrifuged at 12,000 *g* for 15 min at 4°C. and the upper aqueous phase, containing the RNA was removed into a clean tube and precipitated in 0.5 ml isopropyl alcohol for 10 min at room temperature. The RNA was then pelleted at 12,000 *g* for 10 min at 4°C, the supernatant fraction was removed and the RNA pellet washed once in 1.0 ml of 70% ethanol. The RNA was centrifuged at 7,500 *g* for 5 min at 4°C, the pellet was air dried, dissolved in 20 μl of RNase-free water and incubated for 10 min at 60°C.

RNA yields were measured as the absorbance at 260 nm.

2.4.2 *Reverse Transcription*

All reverse transcription reactions were carried out using an RT kit obtained from Promega Southampton. Three micrograms of RNA from each sample were reversed transcribed using a deoxythimidine primer of 20 nucleotides (oligo dT) and 45 U of AMV reverse transcriptase. Each tube contained 6 μl of 10 X RT buffer, 3 μl of RNAse inhibitor, 6 μl of 10 mM dinucleotide triphosphate mix (dNTP), 6 μl of oligo dT, $MgCl_2$ (a final concentration of 5 mM) and 3 μl of AMV reverse transcriptase (15 U/μl). Reaction mixtures were made up to a final volume of 60 μl with diethyl pyrocarbonate (DEPC) treated water (see Appendix 5.6 for preparation of DEPC water) and incubated at 45°C for 1 h. Tubes were then heated to 94°C for 3 min to denature double stranded cDNA and inactivate AMV reverse transcriptase.

2.4.3 *PCR of rat cDNA*

Preliminary RT-PCR experiments using starting RNA amounts from 100 ng to 1 μg revealed that within this range the product band intensity was linearly related to the amount of starting RNA. Consequently PCR analysis was performed using 8 μl aliquots of cDNA equivalent to 400 ng of starting RNA in 50 μl reaction volumes containing 5 μl of 10 X PCR buffer (50 mM Tris-HCl pH 8; 50 mM KCl: Roche Molecular Systems, Branchburg, New Jersey USA) 5 μg/ml each primer (see Appendix 4 for sequences of primer pairs), 1 μl of 10 mM dNTPs, 1.2 U of Taq DNA polymerase (Roche) and

$MgCl_2$ (final concentrations; 1.5 mM for IL-6, TNF-α, IFN-γ and G3PDH; 3 mM for MIP-2; 5 mM for IL-5). For PCR of TNF-α, IL-6 and G3PDH, the PCR enhancer, tetramethylammonium chloride (TMA; Sigma) was added at a final concentration of 10^{-5}M. For amplification of MIP-2, TMA was added at a final concentration of 3 X 10^{-5}M. TMA was replaced by Perfect Match (0.35 U; Stratagene Ltd. Cambridge, U.K.) in reactions amplifying mRNA for IL-5; for PCR of IFN-γ neither TMA nor Perfect Match were necessary. Reaction mixtures were overlaid with mineral oil and 38 PCR cycles were performed. For the cytokines IL-6, TNF-α, MIP-2 and the housekeeping gene G3PDH, reactants were cycled at 94°C for 1 min, 60°C for 1 min and 72°C for 2 min for 37 cycles followed by a final cycle of 94°C for 1 min, 60°C for 1 min and 72°C for 5 min. For IL-5 amplification, reactants were cycled at 94°C for 1 min, 60°C for 15 min and 72°C for 2 min for the first 4 cycles followed by 34 cycles of 94°C for 1 min, 60°C for 2 min and 72°C for 2 min (Noble *et al.*,1993). For IFN-γ amplification, reactants were cycled at 94°C for 1 min, 65°C for 1 min, and 72°C for 2 min for 37 cycles followed by a final cycle of 94° for 1 min, 65°C for 1 min and 72°C for 5 min.

2.4.4 PCR of human cDNA

PCR analyses were performed using 4 μl of cDNA equivalent to 200 ng starting RNA in 50 μl volumes as described above. For amplification of all human cytokines (see Appendix 4 for sequences of primer pairs) a final concentration of 1.5 mM $MgCl_2$ was used and no PCR enhancers were added. Reaction mixtures were overlaid with mineral oil and 35 PCR cycles

were performed. Reactants were cycled at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min for 34 cycles followed by a final cycle of 94°C for 1 min, 55°C for 1 min and 72°C for 5 min.

2.4.5 Visualisation of PCR products

Aliquots (10 μl) of reactions were mixed with 1 μl of gel loading dye (Promega) and run on 2% agarose gels containing ethidium bromide (0.5 μg/ml) in Tris-acetate-EDTA-buffer (TAE, see Appendix 6.2 for preparation and composition of buffer). DNA molecular weight markers (1 μg, Promega) were run in parallel. The gels were visualized and photographed under U.V. light. Negatives were scanned by laser densitometry (LKB Ultroscan XL Pharmacia, Uppsala, Sweden) and the area under the curve of band peaks calculated (GelScan XL 2.1 software, Pharmacia). Statistically significant differences between band intensities were then determined by the two-tailed Mann Whitney *U* Test at the 95% confidence interval. The technique of measuring band intensities as an indicator of the amount of cDNA substrate had previously been validated by calculating the area under the curve of band peaks of known amounts of DNA (twofold dilutions). These values were then plotted and curve linearity and results were found to be consistent between experiments.

2.4.6 Restriction Digests of PCR products

The identities of the PCR products, all of which migrated according to their predicted size, were confirmed by restriction enzyme digestion (all enzymes

obtained from Boehringer Mannheim, Mannheim, Germany) giving predicted fragment sizes (see Appendix 5 for restriction enzymes and fragment sizes of digested PCR products). Five microlitre aliquots of PCR products were digested with an appropriate restriction enzyme (1 U/tube) in a 20 μl reaction volume for 1 h at 37°C. Digested products were then run on 2% agarose gels and visualized under U.V light as described above.

2.4.7 Preparation of RNA probes for in situ hybridisation

To prepare sufficient quantities of high quality RNA probes for *in situ* hybridization, PCR products were cloned, small scale preparations of plasmid DNA made and restriction digests performed to verify the presence of PCR inserts. Large scale preparations of plasmid DNA were then prepared and yields were measured by absorbance at 260 nm (an OD of 1 corresponds to approximately 50 μg/ml for double stranded DNA). PCR inserts were sequenced to confirm their authenticity and orientation within the plasmid and upon confirmation were converted to digoxigenin labelled RNA probes using the enzyme RNA polymerase and digoxigenin-11-UTP (dig 11 UTP). See Fig. 2.1 for a schematic diagram of the procedures involved in the preparation of digoxigenin labelled RNA *in situ* hybridisation probes.

2.4.7.1 Cloning of PCR products.

PCR products were inserted into the TA Cloning™ vector using the TA Cloning™ System (Invitrogen, R+D Systems, Abingdon, Oxon, UK).

TA Cloning™ Ligation

Concentrations of PCR products were estimated by running them on a 2% agarose gel against known amounts of DNA. The ligations with the pCR™ vector (3.9 Kb) were set up as 1:1 to 1:3 molar ratio of vector:PCR insert. To each ligation reaction was added -:

6μl sterile water

1μl 10x ligation buffer

2μl pCR™ vector (25/ng/μl)

1μl diluted PCR product

1μl T4 DNA Ligase

Reactions were incubated at 12°C for 4 h or overnight.

TA Cloning™ Transformation

Ligation reactions were centrifuged at 7500 *g* for 1 min prior to placement on ice. To each 50 μl vial of competent *E. coli* cells (1 vial for each ligation/transformation) was added 2 μl of 0.5 M β-mercaptoethanol. After mixing by gentle tapping 1 μl aliquots of each TA Cloning™ ligation reaction were added directly into the competent cells and mixed by tapping gently. Vials were incubated on ice for 30 min followed by a 45 second incubation in a 42°C water bath. Vials were then placed back on ice for 2 min prior to the addition of 450 μl of prewarmed SOC medium (provided in kit) to each vial. Vials were then placed in a microcentrifuge rack, secured with tape and shaken at 37°C for 1 h at 225 rpm in a gyratory shaker-incubator. Following the incubation period 25 μl and 100 μl aliquots from each

transformation vial were spread onto separate LB agar plates (see Appendix 6.1 for preparation) containing ampicillin (50 μg/ml) and 25 μl X-Gal (40 mg/ml stock solution). Plates were inverted and placed in a 37°C incubator overnight. Single white colonies were picked off the plates and cultured overnight (37°C in a gyratory shaker incubator) in 5 ml of LB medium (Appendix 6.1) containing 50 μg/ml ampicillin.

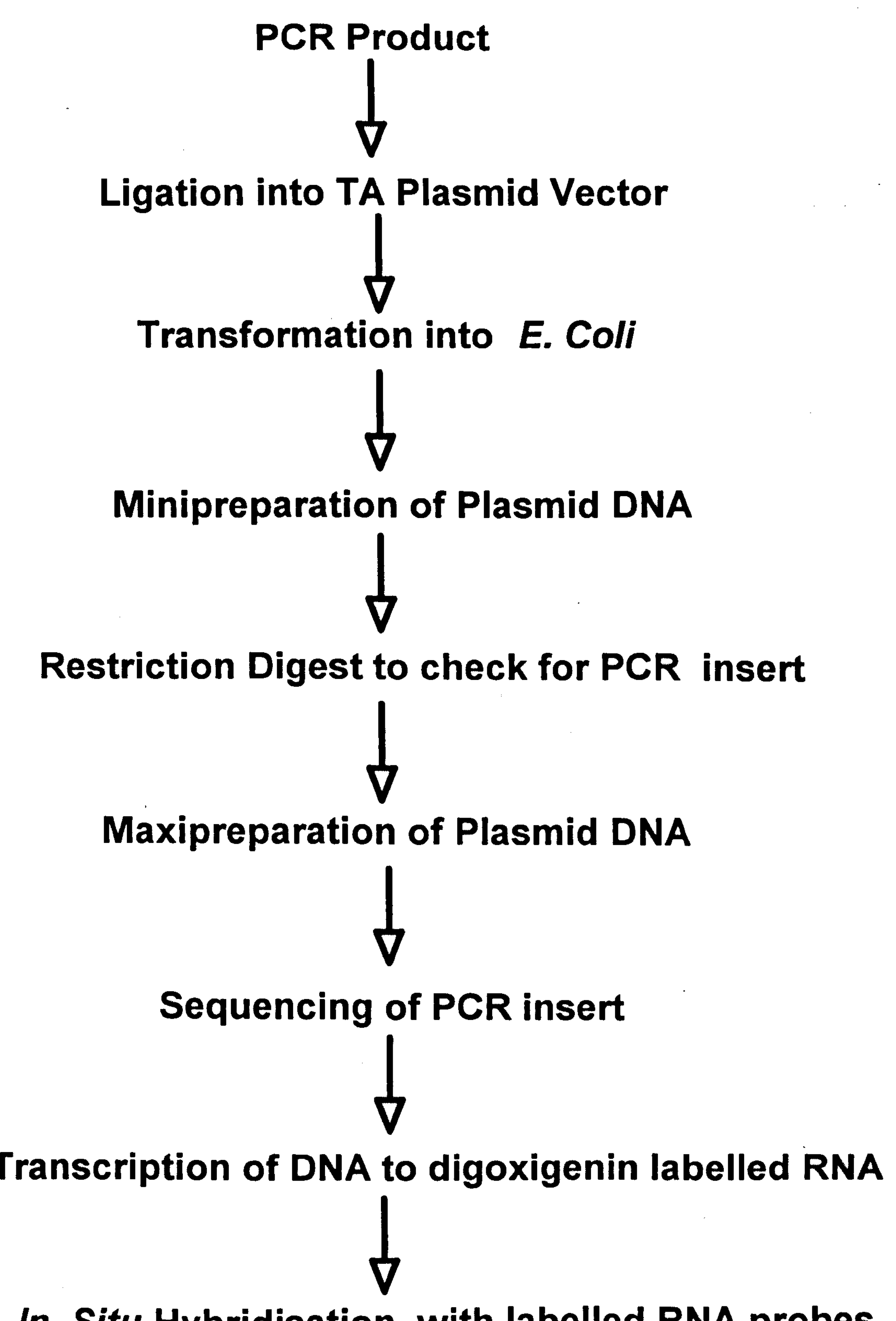


Fig 2.1. Schematic diagram of procedures involved in the preparation of digoxigenin labelled RNA *in situ* hybridisation probes.

2.4.7.2 Small scale preparations of plasmid DNA

Prior to small scale preparation (minipreparation) of plasmid DNA using a method described by Sambrook *et al.* (1989), 750 μl of transformed bacterial suspension were added to 200 μl of glycerol and samples were frozen at -70°C for future use. The remainder of the transformed *E. coli* bacteria were pelleted at 2000 rpm for 10 min. Supernatant fractions were discarded into bleach leaving bacterial pellets as dry as possible. The pellets were resuspended in 200 μl of ice cold Solution 1 (see Appendix 6.3 for preparation and composition of Solutions 1, 2, and 3) by vigorous vortexing and pipetted into microcentrifuge tubes containing 200 μl of freshly prepared Solution 2. The contents of the tubes were mixed by pipetting. Two hundred microlitres of Solution 3 were then added to each tube and contents were vortexed and placed on ice for 3 min prior to centrifugation at 12000 *g* for 5 min at 4°C. The supernatant fractions were then transferred to clean tubes and an equal volume of phenol:chloroform:isoamyl alcohol in the ratio of 25:24:1 (Sigma) was added to each tube. The solutions were vortexed, centrifuged at 12000 *g* for 5 min and the upper layer containing the DNA removed into a clean tube. The doubled stranded DNA was precipitated with 2 volumes of ethanol. Solutions were vortexed and the DNA pelleted by centrifugation (12000 g, 5 min). Supernatant fractions were discarded and pellets were dried in a vacuum line for 3 min prior to resuspension in 50 μl of water containing DNAase free pancreatic RNAase (20 μg/ml, Boehringer Mannheim). DNA was stored at -20°C.

2.4.7.3 Restriction Digests of plasmid DNA

To each tube was added -:

5μl minipreparation of plasmid DNA

2μl 10 x restriction enzyme reaction buffer

1U Sac I

1U Eco RI

The reaction mixture was made up to a final volume of 20 μl with distilled water. Tubes were incubated at 37°C for 1-2 h prior to analysis by gel electrophoresis. Plasmids containing PCR inserts should, on restriction digest, reveal 2 DNA bands, one of extremely high molecular weight (plasmid DNA) and one of a much lower molecular weight (corresponding to the weight of the inserted PCR product) or alternatively, a number of small bands due to the fact that the PCR insert itself contains Sac I and/or Eco RI restriction sites.

2.4.7.4 Large scale preparations of plasmid DNA.

Plasmids containing correct PCR inserts were grown from frozen glycerol colonies in 500 ml of LB medium containing 50 μg/ml ampicillin (37°C for 24 h in a gyratory shaker incubator). Cultures were centrifuged for 10 min at 8000rpm and supernatant fractions discarded into chlorous. Bacterial pellets were resuspended in 20 ml of Solution I (see Appendix 6.4 for preparation and composition of Solutions I, II and III) and 40 ml of freshly prepared Solution II was added to each tube. Tubes were inverted to mix contents and 30ml of Solution III was then added. Cell DNA and bacterial debris were

pelleted at 10000 rpm for 15 min. Supernatant fractions were decanted into clean tubes and plasmid DNA precipitated with 60 ml of isopropanol. Tubes were shaken vigorously and centrifuged for 15 min at 10000 rpm. Supernatant fractions were discarded and DNA was washed once in 70% ethanol, dried in a vacuum line and resuspended in 8 ml of Tris-EDTA buffer (TE buffer, see Appendix 6.1 for preparation and composition). Resuspended DNA was transferred to a fresh tube and further TE buffer was added to give a weight of 9 g. Cesium chloride (10 g), ethidium bromide (100 μl) and water (400 μl) were added to each tube, mixed well and the resulting solution transferred to ultracentrifuge tubes for ultracentrifugation at 45000 rpm for 24 h. Following ultracentrifugation the lower red band (closed circular plasmid DNA) was removed into a clean tube. To the solution of DNA was added an equal volume of 1-butanol saturated with water. The two phases were vortexed and centrifuged at 1500 rpm for 3 min. The upper pink layer was discarded and the process repeated until the solution turned clear. To precipitate the DNA an equal volume of water and 2 volumes of ethanol were added to each tube. If at this stage DNA did not come out of solution 1/10 of the original volume of 3 M sodium acetate was added and tubes were stored at -20°C for several hours. Tubes were then centrifuged at 5000 rpm for 10 min and the precipitated DNA was spooned out into a clean tube. DNA was washed in 70% ethanol to remove salt, dried in a vacuum line and resuspended in 1.0 ml of TE buffer. Concentrations were determined by measuring optical densities at 260 nm as described above (section 2.4.7).

2.4.7.5 Sequencing of PCR inserts

DNA sequencing was determined by the dideoxynucleotide chain termination method (Sanger and Coulson, 1977) using the Sequenase version 2.0 kit (United States Biochemical Co. Cleveland, Ohio, USA). Plasmid DNA had been previously purified by equilibrium centrifugation in a cesium chloride-ethidium bromide gradient as described above (section 2.4.7.4) and therefore only required denaturation of the double-stranded template by alkali treatment, prior to carrying out the sequencing reactions.

Denaturation of double-stranded DNA

To 2.5 μg of plasmid DNA was added 4 μl of 1 M NaOH. The solution was made up to 16 μl with TE buffer and incubated at room temperature for 5 min. Two microlitres of 5 M ammonium acetate (adjusted to pH 4.6 with acetic acid) was added to each tube and solutions were vortexed briefly. Ice cold ethanol (50 μl) was added, solutions were mixed by vortexing and stored on ice for 15 min. The denatured plasmid DNA was recovered by centrifugation at 12000 *g* for 15 min at 4°C. Supernatant layers were removed by gentle aspiration, denatured DNA was washed once in 70% ethanol and air dried until the last traces of ethanol had evaporated. DNA was dissolved in 10 μl of TE and stored on ice for no longer than 30 min before use.

Annealing reaction

For each set of 4 sequencing lanes, a single (and subsequent labelling)

reaction was used. To each 0.5ml microcentrifuge was added -:

1μl primer (5'-GTTTTCCCAGTCACGAC-3')

2μl reaction buffer

7μl denatured DNA

The capped tubes were warmed to 65°C for 2 min and then allowed to cool slowly to room temperature over a period of 30 min. Tubes were then placed on ice prior to use in the labelling reaction.

Labelling Reactions

To each template-primer mix (as described above) were added the following (on ice, in the order listed below) -:

1μl 0.1M dithiothreitol (DTT)

2μl labelling mix (diluted 1:5 with distilled water)

0.5μl [α-^{35}S]dATP (10μCi/μl and 10μM dATP)

2μl Sequenase (diluted 1:8 in enzyme dilution buffer)

Solutions were mixed thoroughly and incubated for 2-5 min at room temperature.

Termination Reactions

To each of the 4 prewarmed terminations tubes containing 2.5 μl of either dideoxy ATP, dideoxy TTP, dideoxy GTP, or dideoxy CTP were added 3.5μl of labelling reactions. Upon addition of the labelling mix, tubes were mixed immediately and returned to the 37°C water bath. Incubations were

continued for a total of 5 min prior to the addition of 4 μl of Stop Solution (provided in kit). Tubes were mixed thoroughly and stored on ice until ready to be loaded on the sequencing gel.

Sequencing Gel Electrophoresis

Samples were heated to 75-80°C for 2 min and loaded immediately onto 16% polyacrylamide gels (see Appendix 6.5 for preparation and composition of gels). Gels were run in Tris-borate-EDTA buffer (TBE buffer, see Appendix 6.2 for preparation and composition) at 50°C for 3-4 h. On completion of electrophoresis, gels were fixed in 10% methanol, 10% acetic acid for 15 min, dried and exposed to X-ray film (Kodak XAR; Sigma) at room temperature for 2-3 days. Sequences were read and compared to known sequences of PCR inserts.

2.4.7.6 Preparation of digoxigenin labelled RNA probes

TA cloning vectors with correct PCR inserts were linearised in 50 μl reaction volumes containing 5 μg of TA vector and 20 U of restriction enzyme. (Either Bam HI or Hind III when T7 RNA polymerase was used in the transcription step or Xba-I or Xho-I when SP6 RNA polymerase was used in the transcription step; all enzymes and other molecular biology reagents were obtained from Boehringer Mannheim). From the sequencing data it was determined which linearised products were equivalent to the sense and antisense PCR primers. To ensure complete linearisation 5 μl of each sample was run on a 0.8% agarose gel. A single 4 Kb band should be visible under

U.V illumination. An equal volume of phenol:chloroform:isoamyl alcohol was added to each DNA sample, tubes were vortexed, centrifuged at 12000 *g* for 10 min and the upper layer removed into a clean tube. DNA was precipitated with two volumes of absolute ethanol and 1/10 of the original volume of 3 M sodium acetate. DNA was pelleted by centrifugation (12000 *g*, 5 min), washed once in 70% ethanol and dried in a vacuum line for 3 min prior to resuspension in 20 μl of DEPC treated water.

Transcription Reactions

To each sterile 0.5 ml microfuge tube were added -:

2μl RNA polymerase buffer (either T7 or SP6)

1μl RNase inhibitor

5μl DNA linearised with appropriate enzyme

1μl 100mM DTT

2μl 1mM ATP/GTP/CTP mix

2μl UTP/digoxigenin 11 UTP (final concentration in each reaction tube of 0.66mM UTP and 0.33mM dig 11 UTP)

1μl RNA polymerase (either T7 or SP6)

6μl DEPC treated water

Tubes were incubated at 37°C for 30 min. Completed transcription reactions were confirmed by running 1/10 of each sample on a 2% agarose gel. Under U.V illumination 2 bands should be visible; a high molecular weight band of plasmid DNA and a very much lower molecular weight RNA band. Reactions were incubated for a further 30 min period with 1.0 μl of RNase free DNase

I to digest contaminating plasmid DNA. Concentrations of RNA were then estimated by running 1/10 of each sample on a 2% agarose gel against known amounts of RNA. Samples were aliquoted into 1.5 ml microfuge tubes to give 400-500 ng of RNA per tube. Two volumes of ethanol and 1/10 of the original volume of sodium acetate were added to each tube and samples were stored at -70°C until use.

2.4.8 In Situ Hybridisation

Throughout the *in situ* hybridisation procedure sterile techniques were employed and all solutions were made with DEPC treated water to minimize the risk of RNase contamination.

Paraffin embedded sections (4-5 μm) were mounted on 3-aminopropyl Triethoxy-Silane (APES, Sigma) coated slides. To coat the glass slides, they were washed thoroughly in detergent, rinsed in running water for 30 min, dipped in 95% ethanol and air dried before coating with freshly prepared 2% APES in dry acetone for 5 seconds. Slides were then rinsed twice (2 x 1 min) in DEPC water and dried overnight at 37°C prior to use.

2.4.8.1 Dewaxing

Wax was removed from paraffin sections by soaking in Xylene (2 x 5 min). Slides were then rinsed with 4 changes of 100% ethanol over a 5 min period, washed in DEPC water for 5 min and incubated in 2 x Sodium citrate-NaCl buffer (SSC, see Appendix 6.6 for preparation and composition) for 10 min at 60°C prior to a final rinse in DEPC water.

2.4.8.2 Proteinase K Digestion

Slides were washed in 50mM Tris (pH 7.6) for 5 min at room temperature prior to incubation with Proteinase K (Sigma). Preliminary experiments revealed that 25 μg/ml Proteinase K was the optimal concentration. Three hundred microlitres of Proteinase K solution diluted in 50 mM Tris (pH 7.6) was applied to each section and slides were incubated for 30 min at 37°C. Slides were then transferred to a wash sleeve containing PBS for 1 min prior to refixation in 0.4% paraformaldehyde in PBS for 20 min at 4°C. Slides were drained onto tissue paper and transferred to PBS to rinse.

2.4.8.3 Hybridisation

RNA probes were centrifuged at 7500 *g* for 10 min, ethanol was removed and the probes were dried in air before reconstitution in 1.0 ml of hybridisation buffer (see Appendix 6.6 for preparation and composition). Two hundred microlitres of either sense or antisense probe were applied to each section. Sections were covered with coverslips that had previously been soaked in a 3% H_2O_2 solution for at least 10 min, washed and dried at 37°C before use. The slides were incubated overnight at 42°C.

2.4.8.4 Post-Hybridisation Washes

Slides were transferred to a wash sleeve containing 2 x SSC for 10 min at room temperature to remove coverslips and excess hybridisation solution. Slides were then incubated in a solution of 0.1 x SSC/50% formamide at 42°C for 1 h.

2.4.8.5 Visualisation of Probe

Slides were washed in modified Tris (see Appendix 5.6 for preparation and composition) for 15 min at room temperature. Excess buffer was removed carefully and 300 μl of sheep anti-digoxigenin/alkaline phosphate Fab fragment (Boehringer Mannheim) at a dilution of 1:500 in modified Tris was applied to each section. Slides were incubated at room temperature for 30 min, washed in fresh modified Tris (2 x 5 min at room temperature) and rinsed in DEPC water (1 min at room temperature) before the application of 300μl of revealing agent (see Appendix 6.6 for preparation and composition). Clean coverslips were applied to each section, slides were incubated at room temperature in the dark for 6 h before washing in water for 5 min and mounting with aqueous mountant.

CHAPTER 3

The effects of cyclosporin A, dexamethasone and BTS 71321 on the release of serotonin and arachidonate from immunologically activated rat peritoneal mast cells

3.1 Introduction

3.2 Results

3.2.1. The effects of CsA, Dex and BTS 71321 on mast cell viability

3.2.2. The effects of BTS 71321 on total incorporated [^{3}H]5-HT

3.2.3. The effects of BTS 71321 on total incorporated arachidonic acid

3.2.4. The effects of CsA, Dex and BTS 71321 on the release of serotonin from anti-IgE-activated PMC

3.2.5. The effects of CsA, Dex and BTS 71321 on the

release of arachidonate from anti-IgE-activated PMC

3.3. Discussion

3.1 INTRODUCTION

Whereas CsA and Dex differ considerably in their chemical structures they share a number of pharmacological characteristics relating to their anti-inflammatory activities. Their precise mechanisms of action are as yet incompletely characterised, although a number of functional targets have been suggested. These are reviewed in chapter 1 section 1.5. CsA, a cyclic undecapeptide extracted from *Tolypocladium inflatum Gams,* is a powerful immunosuppressant and is widely used in the prevention of graft rejection following organ transplantation (Calne *et al.*, 1978). It is also effective in the treatment of some patients with various forms of autoimmune disease (Weinblatt *et al.*, 1987; Brynskov *et al.*, 1989), psoriasis (Ellis *et al.*, 1991), atopic dermatitis (van Joost *et al.*, 1987; de Prost *et al.*, 1989) and severe chronic asthma (Szczeklik *et al.*, 1990; Finnerty and Sullivan, 1991; Alexander *et al.*, 1992). Dex, a potent glucocorticoid, is not used extensively in the clinic, although other members of this class of drug are amongst the most powerful and effective agents in the treatment of chronic allergic disorders such as allergic asthma and atopic dermatitis (Schleimer 1990; Church, 1993).

Both CsA and Dex are known to inhibit mediator release from mast cells and basophils. Cirillo *et al.* (1990) have demonstrated that CsA inhibits histamine release from human peripheral blood basophils challenged with anti-IgE. CsA was shown to be more potent when the basophils were activated by the calcium ionophore A23187. Studies on rat peritoneal mast

cells revealed a similar inhibition following incubation of the cells with CsA prior to stimulation with either compound 48/80, A23187 or concanavalin A (con A) plus phosphatidylserine in the presence of extracellular calcium (Draberova, 1990). CsA was also shown to inhibit uptake of radiolabelled Ca^{2+} by secretagogue-treated cells. Others have shown that CsA inhibits secretion of serotonin from rat basophilic leukemia cells after stimulation by either IgE receptor aggregation or A23187. Interestingly, A23187-stimulated cells were more resistant to the inhibitory effects of CsA (Hultsh *et al.*, 1990). Further work on human skin mast cells and human basophils (Stellato *et al.*, 1992) revealed that CsA had the ability to inhibit in a dose-dependent manner, the release of histamine from human skin mast cells challenged with anti-IgE but had little or no effect on the release of histamine induced by activation of the cells with A23187. It completely suppressed A23187-induced release form human basophils in agreement with Cirillo *et al.* (1990). CsA was also extremely effective in the inhibition of the *de novo* synthesis of prostaglandin D_2 (PGD_2) from human skin mast cells challenged with anti-IgE (Stellato *et al.*, 1992). *In vivo* administration of Dex to rats and the subsequent removal after four days of serosal mast cells, revealed that there was a significant decrease, after antigen or A23187 activation, of release of β-hexosaminidase, serotonin and arachidonic acid (Marquardt and Wasserman, 1983). Further work on mouse bone marrow-derived mast cells (BMMC) revealed that preincubation of the cells with Dex at concentrations between 0.01 to 0.5μM for periods of 6 to 96 h, inhibited the subsequent IgE-dependent release of β-hexosaminidase in a concentration-dependent

manner, and at a concentration of 1μM inhibited granule secretion in a time-dependent manner. Furthermore, Dex was shown to inhibit IgE biosynthesis and release of the immunoreactive leukotrienes (LT)C_4 and LTB_4 and PGD_2 (Robin *et al.*, 1985). Interestingly, further work on the effects of Dex indicated that 1μM Dex induced a time-dependent increase in cell histamine content which reached a plateau after 11 days in culture. Ultrastructural analysis of Dex-treated BMMC revealed numerous cytoplasmic granules filled with abundant and uniform electron-dense matrix and following a 14 day culture period with Dex the antigen-induced IgE-mediated release of histamine, β-hexosaminidase, platelet-activating factor-acether, LTB_4, and LTC_4 was inhibited (Pitton *et al.*, 1988). Other studies on the rat basophilic leukemia 2H3 cell line, which is a mucosal mast cell-like cell, have shown that long term culture in the presence of high doses of corticosteroid can induce an increase in the cellular histamine content (Brantschen *et al.*, 1989) but that short term incubation with Dex reduces the response to antigen-mediated challenge (Collado-Escobar *et al.*, 1990). Others have demonstrated that Dex induces a down-regulation of the mast cell specific protease RMCPII content in this cell line (Saunders and Marshall, 1992) and further work on rat lung mast cells has revealed that single and repeated administrations of Dex did not influence the RMCPI concentration, but decreased the concentration of RMPCII with a parallel decrease in [^{3}H] histamine synthesis (Rouleau *et al.*, 1994).

CsA and corticosteroids are amongst the most potent anti-inflammatory agents but unfortunately they can produce a number of

undesired effects. Consequently, there is a need for the development of novel drugs which mimic the beneficial effects of these well established compounds but which do not exhibit their unwanted side effects. BTS 71321, {N-[1-(4-chlorophenyl)-1-methylethyl]-3-(imidazol-1-yl)propylamine dihydrochloride}, is a putative tyrosine kinase inhibitor. Its mechanism of action is as yet poorly understood, but it was originally selected for further development from a series of benzylaminoalkylimidazoles which were shown to inhibit the release of arachidonic acid from mouse peritoneal macrophages. However, the effects of BTS 71321 on mast cell mediator release have not yet been investigated. It is thought that inhibition of release of prestored and *de novo* synthesised chemical mediators from mast cells may be of therapeutic benefit in inflammation and allergic processes. Therefore, the aim of this study was to evaluate the role of BTS 71321 in the inhibition of release of serotonin and arachidonate from immunologically activated rat PMC. The inhibitory effects of CsA and Dex on mast cell mediator release are well documented so these compounds were included in the study as a means of comparing the activity and potency of the novel benzylaminoalkylimidizole compound to well established anti-inflammatory agents.

3.2 RESULTS

3.2.1 The effects of CsA, Dex or BTS71321 on mast cell viability

Culture of PMC in the presence of CsA or Dex for 24 h, each at a concentration of 10^{-7}M, had no effect on cell viability as determined by staining in 0.02% trypan blue dye. In addition, cell viability was unaffected after culture of the cells in the presence of BTS 71321 for 24 h at concentrations in the range of 10^{-4}M - 10^{-7}M.

3.2.2 The effects of BTS 71321 on total incorporated [^{3}H]5-HT

There was no significant difference in levels of total incorporated [^{3}H]5-HT, in cells that had been cultured for 24 h in the presence of CsA (10^{-7}M), Dex (10^{-7}M) or BTS 71321 (10^{-6}M - 10^{-7}M) compared to [^{3}H]5-HT uptake by control cells. However, levels of uptake were decreased in cells cultured in the presence of BTS 71321 at either 10^{-5}M or 10^{-4}M. Statistically, these effects were highly significant over seven independent experiments (Fig. 3.2.1).

3.2.3 The effects of BTS 71321 on total incorporated arachidonic acid

CsA (10^{-7}M), Dex (10^{-7}M) and BTS 71321 (10^{-4}M-10^{-7}M) were without effect on incorporation of [^{3}H]arachidonate (Fig. 3.2.2).

3.2.4 The effects of CsA, Dex or BTS 71321 on the release of serotonin from anti-IgE-activated PMC.

Culture of PMC in the presence of CsA or Dex, each at a concentration of 10^{-7}M for 24 h, led to a substantial and significant inhibition of anti-IgE-induced release of 5-HT (Fig. 3.2.3). In seven independent experiments CsA inhibited release of 5-HT on average by 66% whereas Dex inhibited 5-HT release by 75%. In the same experiments, BTS 71321 at concentrations in the range of 10^{-5}M-10^{-7}M, did not effect anti-IgE-induced release of 5-HT. However, at a concentration of 10^{-4}M, BTS 71321 significantly inhibited the release of 5-HT on average by 40% (Fig. 3.2.3).

3.2.5 The effects of CsA, Dex or BTS 71321 on the release of arachidonate from anti-IgE-activated PMC

Culture of PMC in the presence of CsA at a concentration of 10^{-7}M for 24 h, had no effect on the anti-IgE-induced release of arachidonate whereas 24 h culture in the presence of Dex (10^{-7}M) led to substantial and significant inhibition of release. In seven independent experiments Dex inhibited release of arachidonate on average by 62% (Fig. 3.2.4). In the same experiments BTS 71321 (10^{-4}M-10^{-7}M) was without effect on anti-IgE-induced release of arachidonate (Fig. 3.2.4).

3.3 DISCUSSION

The findings presented in the present chapter support earlier evidence that CsA and Dex are extremely effective in the inhibition of preformed mediator release from mast cells. Twenty-four hour culture of PMC in the presence of CsA or Dex, each at a concentration of 10^{-7}M which was shown to be non-toxic to cells, substantially inhibited anti-IgE-induced serotonin release. In addition, Dex was shown to inhibit arachidonate release whereas CsA was without effect. However, other research has indicated that CsA markedly inhibits *de novo* synthesis of PGD_2 from human skin mast cells challenged with anti-IgE (Stellato *et al.*, 1992) although at 10^{-7}M, the concentration of CsA used in this study, its inhibitory effects were not substantial. In the study of Stellato *et al.* (1992), maximal effects were not reached until incubation with the drug at a concentration of 8 x 10^{-7}M suggesting that, had higher concentrations of CsA been investigated in the present study, inhibition of arachidonate may have been observed. Nevertheless, the primary objective of the study was to examine the effectiveness of a novel benzylaminoalkyimidazole, BTS 71321, on the inhibition of mast cell mediator release, using CsA and Dex as reference compounds. BTS 71321 was non-toxic to cells at concentrations between 10^{-4}M-10^{-7}M as determined by trypan blue exclusion. However, at concentrations of 10^{-4}M and 10^{-5}M the uptake of 5-HT was inhibited by BTS 71321 suggesting that, at these concentrations, BTS 71321 was toxic to the 5-HT uptake mechanism. BTS 71321 had no effect on rat mast cell

arachidonate release although inhibition of serotonin release was observed at the highest concentration investigated (10^{-4}M). However, it is difficult to determine the relevance of this finding as inhibition may be due, at least in part, to toxicity of the drug during the 24 h culture period prior to activation. Therefore, the effects of BTS 71321 on rat mast cells *in vitro* do not compare favourably to those observed with either CsA or Dex. Interestingly, previous studies have indicated that BTS 71321 has the ability to inhibit the release of arachidonate from mouse peritoneal macrophages cultured *in vitro* with BTS 71321 for 24 h prior to stimulation with zymosan for 5 h (Steele *et al.*, 1995). However, in direct assays, cyclooxygenase, 5-lipoxygenase or phospholipase A_2 was not inhibited. It has therefore been suggested that BTS 71321 exerts inhibitory effects through inhibition of tyrosine kinases (Smith, 1995, Knoll Pharmaceuticals, personal communication). Zymosan, a carbohydrate rich cell wall preparation derived from *Saccharomyces cerevisiae,* binds to mononuclear cells through a β2 integrin (Ross *et al.*, 1985), is phagocytosed without opsonisation and elicits the secretion of reactive oxygen intermediates, arachidonic acid derivatives and cytokines (Suzuki *et al.*, 1985; Sanguedolce *et al.*, 1992; Elstad *et al.*, 1994). Zaffran *et al.* (1995) have also indicated that some components of the *src* tyrosine kinase family, namely p53-56^{lyn} provide the molecular link between zymosan receptors and the cytoskeleton-directed signal transduction. The possibility therefore exists that BTS 71321 may inhibit the release of arachidonic acid derivatives through the inhibition of p53-56^{lyn} or indeed other kinases involved in this pathway. Eiseman and Bolen (1992) have also shown that

engagement of the high affinity IgE receptor (FcϵR1), which is expressed on the surface of mast cells and basophils, activates *src* protein-related tyrosine kinases and upon receptor crosslinking a number of different *src* protein-related tyrosine kinases can become activated. In a rat basophilic leukemia cell line, $p56^{lyn}$ and $pp60^{c\text{-}src}$ become activated after FcϵR1 crosslinking whereas $p62^{c\text{-}yes}$ is activated in PT-18 cells after receptor engagement (Eiseman and Bolen, 1992). Nevertheless, these experiments were carried out on mast cell lines which, although important as model systems for the analysis of FcϵR1-mediated signal transduction, are not fully representative of mature connective tissue-type mast cells. Those protein-related tyrosine kinases involved in signal transduction after activation of rat PMC via the high affinity IgE receptor have not yet been elucidated and although it is highly probable that a member of the *src* tyrosine kinase family is involved it is possible that it is not $p53\text{-}56^{lyn}$ which is critical in the activation of macrophages by zymosan. The possible differences in signal transduction may therefore account for the lack of inhibition of BTS 71321 on anti-IgE-activated mast cell mediator release but its effectiveness in the down-regulation of release of arachidonate from zymosan stimulated murine peritoneal macrophages. Alternatively, BTS 71321 may inhibit other protein tyrosine kinases involved in the cytoskeleton-associated signal transduction pathway which is favoured by engagement of β2 integrins by particles such as zymosan, whereas it may not be involved in the inhibition of the cytoplasmic signal transduction pathway induced by FcϵR1 engagement. However, a better knowledge of these transductional alternates and their

activation by different stimuli is required before specific modulation of intracellular signals can be exploited using novel therapeutic agents. As yet these variations in signal transduction have not been well documented and one can only speculate as to the apparent unresponsiveness of anti-IgE-activated rat PMC to BTS 71321. However, if indeed BTS 71321 has no effect on the release of human mast cell mediators such as histamine and arachidonic acid metabolites, it would suggest that BTS 71321 would not be effective in acute allergic disease where the release of these mediators contributes substantially to the allergic response.

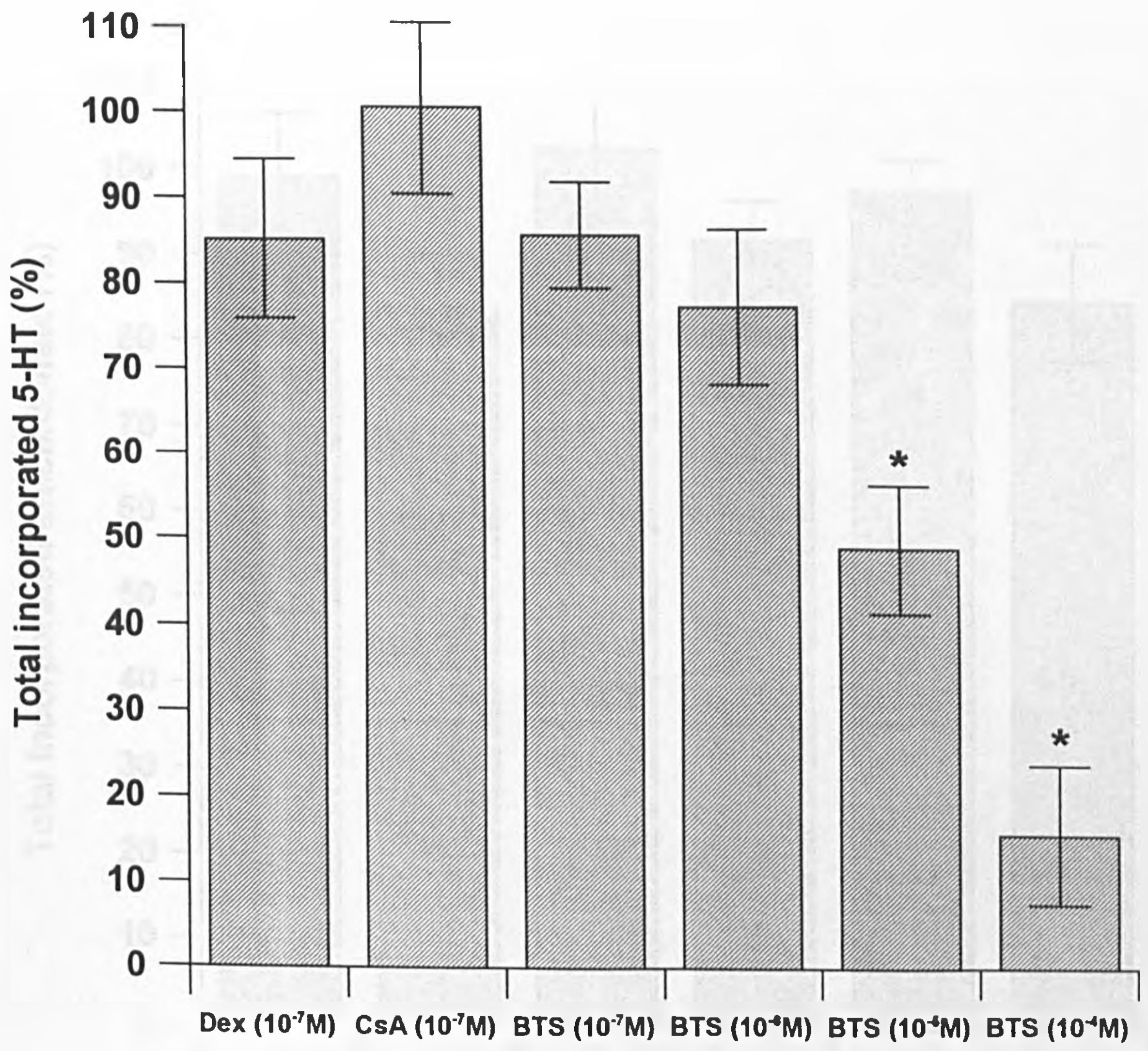


Fig. 3.2.1. The effects of culture of rat PMC with Dex, CsA or BTS 71321 for 24 h on the incorporation of [^{3}H]5-HT. Total incorporated [^{3}]5-HT is expressed as a percentage of control (no drug) levels. Results are means ± SEM for seven experiments. (* $p < 0.05$ for comparison of BTS 71321 treated cells with control cells). Statistical analyses were by the two-tailed Mann Whitney *U* test.

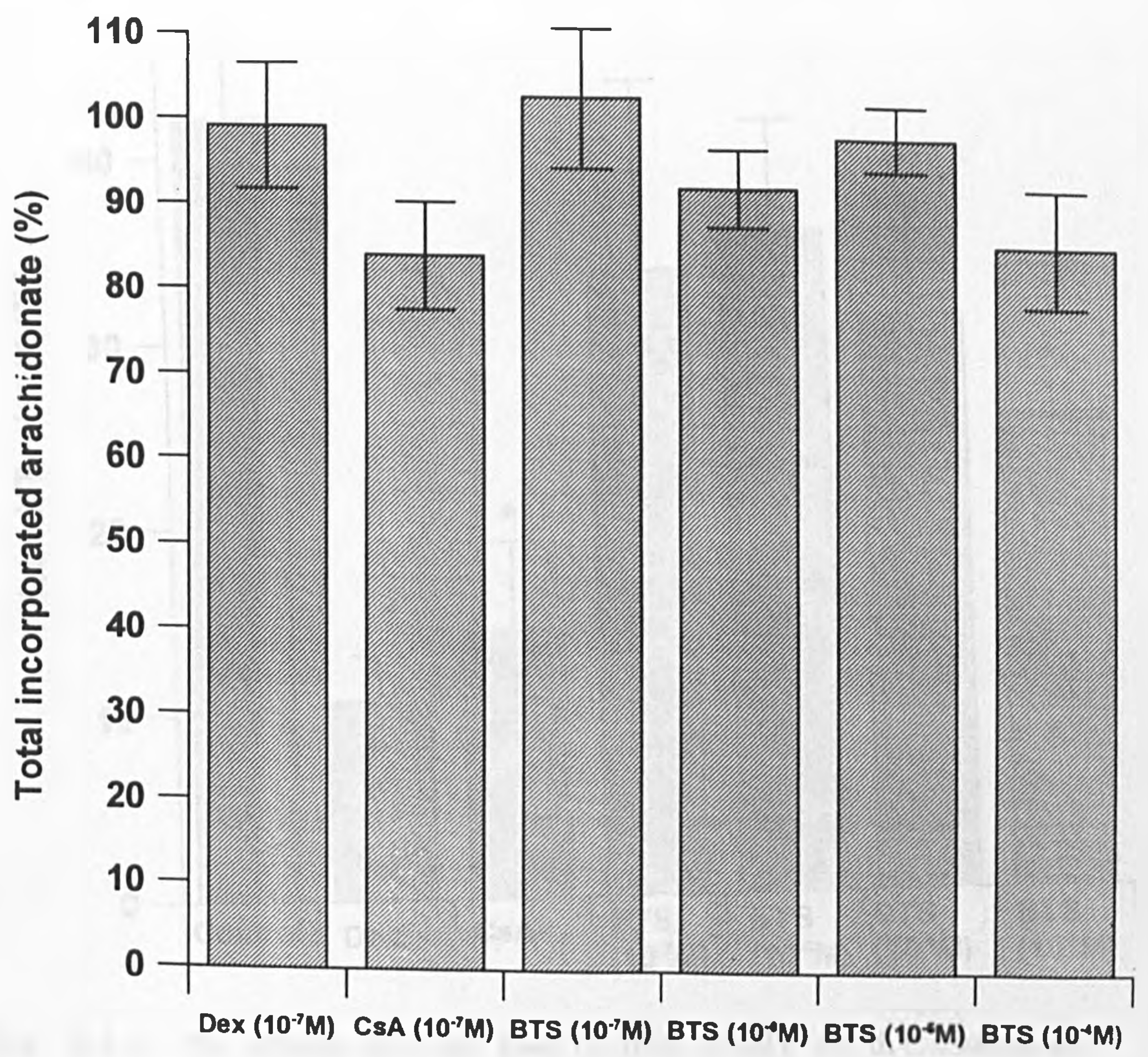


Fig. 3.2.2. The effects of culture of rat PMC with Dex, CsA or BTS 71321 for 24 h on the incorporation of arachidonic acid. Total incorporated arachidonate is expressed as percentage of control levels. Results are means ± SEM for seven experiments.

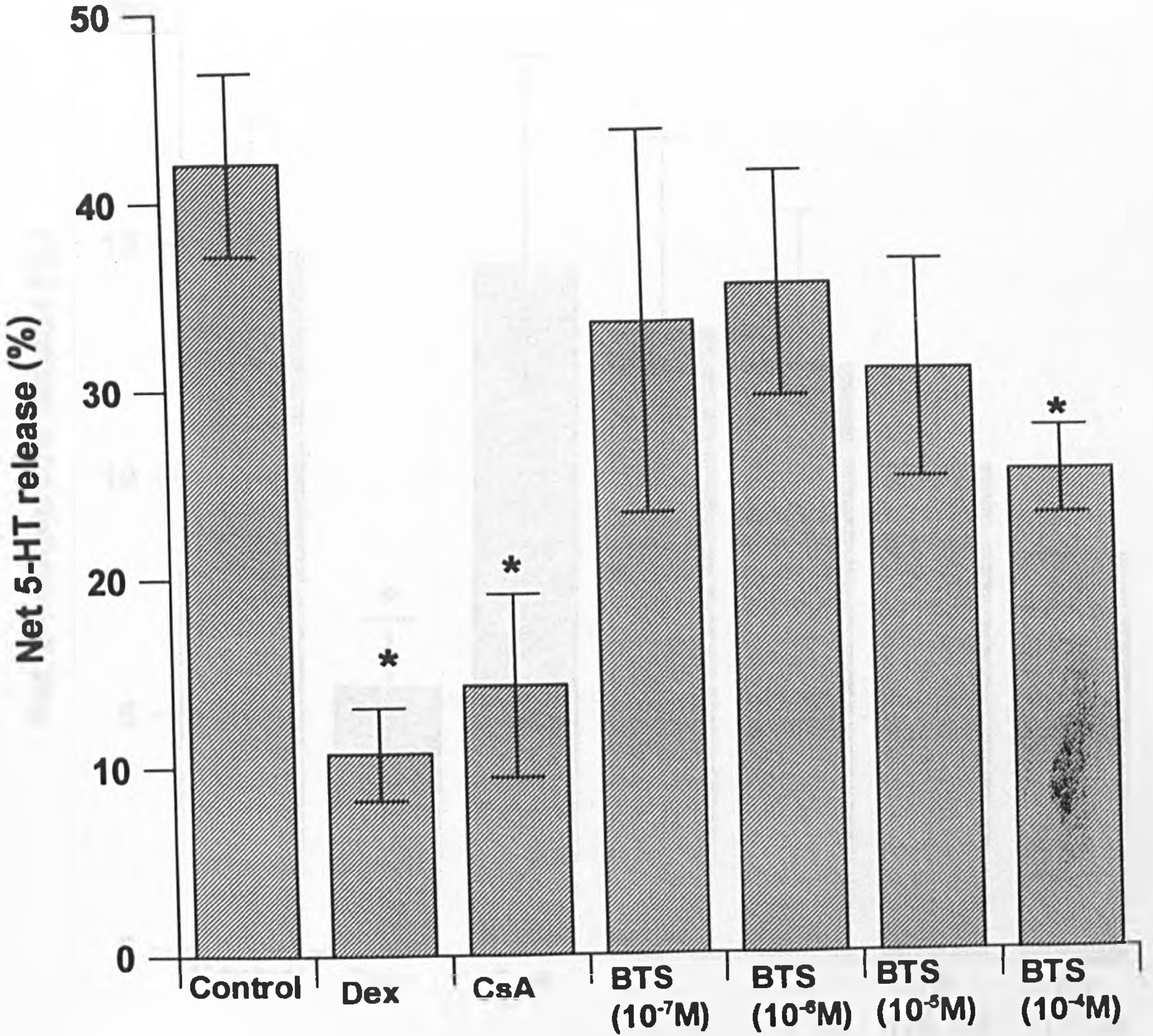


Fig. 3.2.3. The effects of CsA, Dex or BTS 71321 on the release of serotonin from anti-IgE activated rat PMC. Cells were cultured in the presence of CsA (10^{-7}M), Dex (10^{-7}M) or BTS 71321 (10^{-4}M-10^{-7}M) for 24 h washed and activated for 30 min with anti-IgE. Results are means ± SEM for seven independent experiments. (* $p < 0.03$ for comparison of drug treated cells with control). Statistical analyses were by the two-tailed Mann Whitney *U* test.

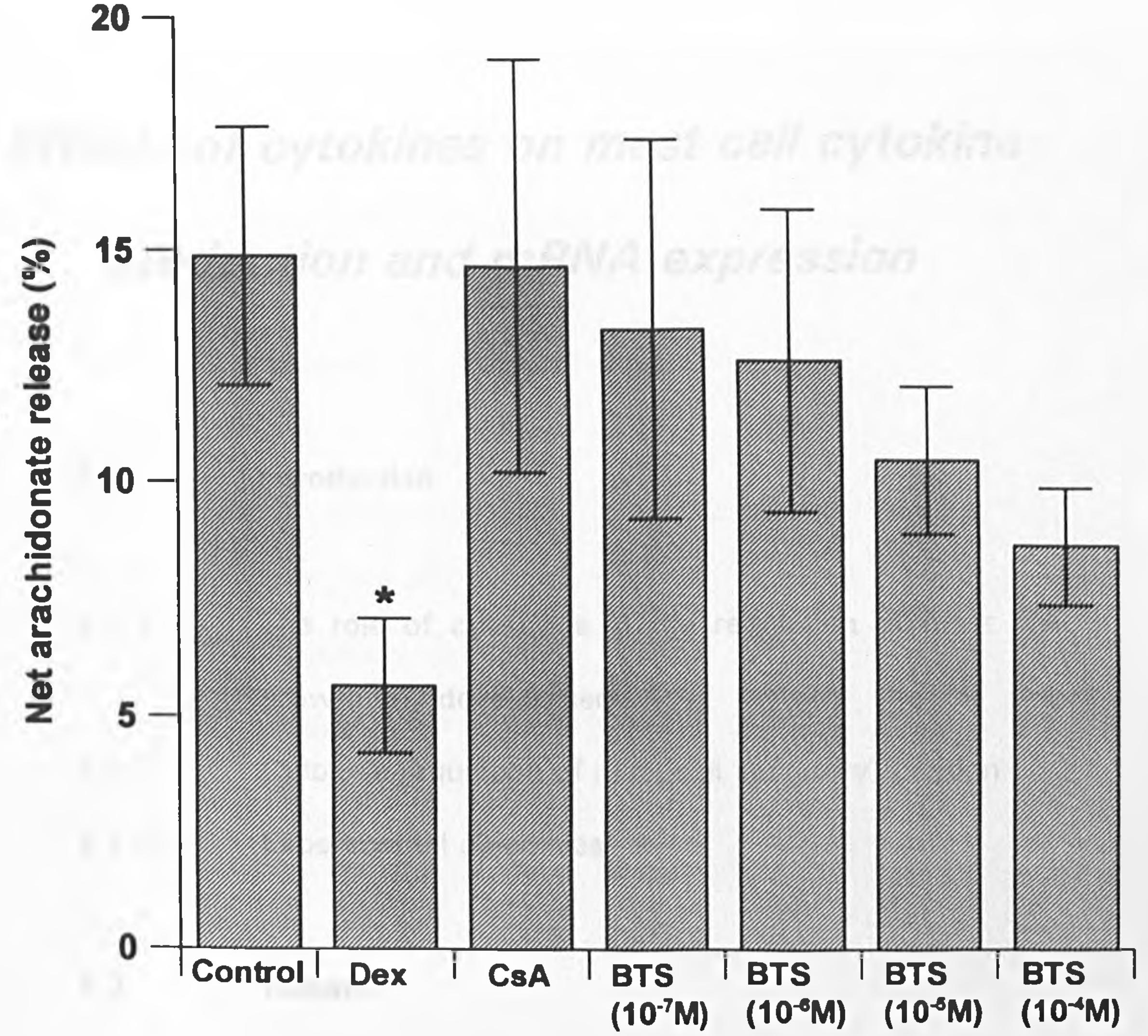


Fig. 3.2.4. The effects of CsA, Dex or BTS 71321 on the release of arachidonate from anti-IgE activated rat PMC. Cells were cultured in the presence of CsA (10^{-7}M), Dex (10^{-7}M) or BTS 71321 (10^{-4}M-10^{-7}M) for 24 h washed and activated for 1 h with anti-IgE. Results are means ± SEM for seven independent experiments. (* p = 0.0063 for comparison of Dex treated cells with control). Statistical analyses were by the two-tailed Mann Whitney *U* test.

Chapter 4

Effects of cytokines on mast cell cytokine production and mRNA expression

4.1. Introduction

4.1.1. The role of cytokines in the regulation of mast cell growth and development

4.1.2. Cytokine regulation of mast cell secretory function

4.1.3. Experimental objectives

4.2. Results

4.2.1. Cytokine production by Cl.MC/C57.1 mast cells

4.2.2. Investigation of the effects of IL-3, IL-4, SCF and IFN-γ on the release of TNF-α by Cl.MC/C57.1 mast cells

4.2.3. Investigation of the effects of IL-3, IL-4, SCF and IFN-γ on the release of IL-6 by Cl.MC/C57.1 mast cells

4.2.4. Investigation of the effects of IL-4 on the release of TNF-

α by rat peritoneal mast cells

4.2.5. Investigation of the effects of SCF on the release of TNF-*α* by rat peritoneal mast cells

4.2.6. Expression of mRNA for IL-3, IL-4, IL-8 and TNF-*α* in unstimulated and activated HMC-1 mast cells after culture with or without IL-4

4.3. Discussion

4.3.1. The production of TNF-*α* and IL-6 by mast cells

4.3.2. Effects of cytokines on mast cell cytokine production and mRNA expression

4.3.3. Concluding remarks

4.1 INTRODUCTION

4.1.1 The role of cytokines in the regulation of mast cell growth and development

It is well established that cytokines are crucial to the development, differentiation and function of many cell types of both haematopoietic and non haematopoietic origin. This situation is also apparent in the regulation of mast cell development and phenotypic heterogeneity. Mast cells, having originated from precursors in the bone marrow, migrate to tissues via the blood where they complete their differentiation process to become mature sedentary secretory cells. Stem cell factor (SCF), the product of the *Sl* gene in the mouse (Copeland *et al.*, 1990; Zsebo *et al.*, 1990) and the ligand of the tyrosine kinase-type receptor encoded by the c-*kit* proto-oncogene, (Williams *et al.*, 1992; Galli *et al.*, 1994) is critical to the survival, differentiation and proliferation of mast cells. Some of its many properties include induction of proliferation, maturation and heparin synthesis in cultured immature murine BMMC (Tsai *et al.*, 1991a), differentiation of human mast cells from bone marrow cells, peripheral blood mononuclear cells (Valent *et al.*, 1992), dispersed human fetal liver cells (Irani *et al.*, 1992) and umbilical-cord blood cells (Mitsui *et al.*, 1993). SCF also promotes mast cell survival by suppression of apoptosis (Iemura *et al.*, 1994).

In addition to SCF, a number of other cytokines have been implicated in the proliferation, differentiation and survival of mast cells. The

glycoprotein IL-3, a cytokine produced by T helper cells, has been shown to promote the development of immature mast cells from mouse bone marrow (Ihle *et al.*, 1983; Rennick *et al.*, 1985) and to induce the *in vivo* proliferation of mucosal mast cells in athymic nude mice in response to the parasite *Strongyloides ratti* (Abe and Nawa, 1988) or even in the absence of parasitic infection (Abe *et al.*, 1988). Subsequent studies have revealed that IL-4, another T helper cell-derived cytokine, which acts as a B cell growth and differentiation factor (Paul, 1991) and plays a critical role in the production of IgE class antibodies (Finkelman *et al.*, 1988), augments the IL-3-dependent proliferation of BMMC (Lee *et al.*, 1986) and acts as a co-factor with IL-3 on the *in vitro* growth of connective tissue-type mouse mast cells (Hamaguchi *et al.*, 1987, Tsuji *et al.*, 1990). Hultner *et al.* (1990) have also reported that IL-9 has mast cell growth promoting activity and others have indicated that IL-10, which is derived from both T and B cells, can enhance IL-3/IL-4 -induced proliferation of mast cell lines or immature mast cells derived from lymphoid tissue (Thompson-Snipes *et al.*, 1991). However, combinations of IL-3, IL-4, IL-9 and IL-10 are not sufficient to induce a further evolutionary change from immature mast cells into mature heparin-containing cells. This final stage in development requires the co-culture of BMMC with SCF or indeed cells that can provide this factor (Levi-Schaffer *et al.*, 1986; Tsai *et al.*, 1991a,b) or alternatively, IL-3 in combination with nerve growth factor (Matsuda *et al.*, 1991). IFN-γ is a member of the family of anti-viral proteins that exert an array of immunoregulatory activities including enhancement of natural killer cell toxicity (Mannering and Deloria,

1986), enhancement of the phagocytic and cytotoxic functions of macrophages (Mannering and Deloria, 1986), regulation of B-cell proliferation and Ig synthesis (Siegel *et al.*, 1986) and control of the expression of histocompatibility antigens on the surfaces of a range of cell types including IL-3-dependent murine mast cell lines (Wong *et al.*, 1983, 1984), rat pleural mast cells (Banovac *et al.*, 1989) and rat connective tissue-type mast cells (Warbrick *et al.*, 1995). IFN-γ has been shown to antagonize the clonal proliferation of mature tissue-type mouse peritoneal mast cells stimulated with IL-3 in conjunction with either IL-4 or IgE/antigen (Takagi *et al.*, 1990).

4.1.2 Cytokine regulation of mast cell secretory function

There is now a substantial amount of evidence that certain cytokines that regulate mast cell growth and differentiation also influence their secretory function. The effects of SCF on mast cell secretory function are varied and complex but include enhancement of IgE-mediated degranulation of mast cells isolated from the mouse peritoneal cavity (Coleman *et al.*, 1993), human skin (Columbo *et al.*, 1992) and human lung (Bischoff and Dahinden, 1992). In addition, culture of mouse PMC for 48 h in the presence of IL-3 or IL-4 ($<$ 100 ng/ml) results in a concentration-dependent increase in IgE/antigen-induced serotonin release (Coleman *et al.*, 1993) and further studies have indicated that IFN-γ at comparable concentrations, strongly inhibits serotonin release regardless of whether the cells have been maintained in the absence or presence of IL-3 and /or IL-4 (Coleman *et al.*, 1992; Holliday *et al.*, 1994).

In recent years there has been a growing interest in the mast cell as a source of multifunctional cytokines (Gordon *et al.*, 1990). The most prominent and indeed the first cytokine to be clearly associated with normal mast cells was TNF-α. However, there is a considerable lack of information regarding the effects of cytokines on the regulation of release of mast cell TNF-α and indeed other mast cell associated cytokines. Bissonnette and Befus (1990) have reported that at high concentrations IFN-α/β and IFN-γ inhibit the release of TNF-α from rat PMC. Others have demonstrated that murine BMMC cultured in the presence of either IL-3 or SCF differentially expressed cytokine genes. BMMC cultured with IL-3 took on a mucosal mast cell-like phenotype and expressed the IL-4 gene whereas cells cultured with SCF took on a connective tissue mast cell-like phenotype and possessed transcripts for both of the subunits of the IL-12 cytokine. Moreover, BMMC cultured in the presence of both IL-3 and SCF expressed the IL-4 gene at lower levels than that seen after culture with IL-3 alone but did not possess IL-12 gene transcripts (Smith *et al.*, 1994). These findings thus suggest that mast cell growth promoting cytokines, in addition to their effects on the release of mast cell histamine and serotonin, have the ability to influence the regulation of synthesis and release of mast cell-associated cytokines.

4.1.4 Experimental objectives

Considering the properties of the cytokines IL-3, IL-4, SCF and IFN-γ on mast cell growth, differentiation and secretory function it was pertinent to

evaluate their effects on cytokine expression in mast cells. As has been discussed above, there is a shortage of information regarding these effects. Experiments were therefore set up to establish the importance of these cytokines on the release of TNF-*α* and IL-6 by the IL-3 independent murine Cl.MC/C57.1 mast cell line (Young *et al.*, 1987). The effects of SCF and IL-4 on the anti-IgE-induced release of TNF-*α* from rat PMC were also investigated. In addition to these studies, a human mast cell line, HMC-1, derived from a patient with a mast cell leukemia (Butterfield *et al.*, 1988), was used to explore the effects of IL-4 on cytokine gene expression.

4.2 RESULTS

4.2.1. Cytokine production by murine Cl.MC/C57.1 mast cells

Supernatant fractions from unstimulated or IgE/antigen-stimulated Cl.MC/C57.1 mast cells were examined for the presence of biologically active TNF-α by the L929 cytotoxicity assay, for IL-6 by the B9 proliferation assay and for IL-5 by an IL-5-specific ELISA. Low levels of TNF-α were detected in supernatant fractions from unstimulated mast cells and levels increased approximately 5-fold after activation with IgE/antigen (Fig. 4.2.1). As determined by the two-tailed Mann Whitney U test, this increase was statistically significant over four separate experiments. IL-6 was also detectable in supernatant fractions from unstimulated mast cells and levels increased approximately 3-fold after activation. However, statistical analysis revealed that these changes were not significant (Fig. 4.2.2). IL-5 was not detectable in culture supernatants from either unstimulated or activated cells. Assay sensitivity was low and IL-5 at concentrations no lower than approximately 100 pg/ml could be detected.

4.2.2. Investigation of the effects of IL-3, IL-4, SCF and IFN-γ on the release of TNF-α by Cl.MC/C57.1 mast cells

Culture of Cl.MC/C57.1 mast cells with SCF but not IL-3, IL-4 or IFN-γ (100 ng/ml, 48 h) produced a significant increase in the release of TNF-α from IgE/antigen-activated cells (Fig. 4.2.1).

4.2.3. Investigation of the effects of IL-3, IL-4, SCF and IFN-γ on the release of IL-6 by Cl.MC/C57.1 mast cells

Culture of Cl.MC/C57.1 mast cells in the presence of IL-3, IL-4, SCF or IFN-γ for 48 h, each at a concentration of 100 ng/ml, had no significant effect on the release of IL-6 from unstimulated cells or cells that were subsequently stimulated with IgE/antigen for 1 h (Fig. 4.2.2).

4.2.4. Investigation of the effects of IL-4 on the release of TNF-α by rat peritoneal mast cells

In ten independent experiments culture of rat PMC in the presence of recombinant mouse IL-4 (25 ng/ml, 48 h) did not influence the release of TNF-*α* from cells subsequently challenged with anti-IgE for 1 h (Fig. 4.2.3a). Similar results were obtained when the concentration of IL-4 was increased to 100 ng/ml (Fig. 4.2.3b).

4.2.5. Investigation of the effects of SCF on the release of TNF-α by rat peritoneal mast cells

Culture of rat PMC in the presence of SCF at 50 ng/ml for 48 h had no significant effect, over three independent experiments, on the release of TNF-*α* from anti-IgE-activated cells (Fig. 4.2.4).

4.2.6. Expression of mRNA for IL-3, IL-4, IL-8 and TNF-α in unstimulated and activated HMC-1 mast cells after culture with or without IL-4

Messenger RNA encoding the cytokines IL-3, IL-4, IL-8 and TNF-*α* was

detected by RT-PCR in cytoplasmic lysates from unchallenged HMC-1 mast cells although the intensity of the PCR product bands were often weak after 30 PCR cycles. Stimulation of the cells with ionomycin (10 μM) for 4 h induced expression for IL-3 (3/3 experiments), IL-8 (3/3) and IL-4 (3/3) but rarely TNF-α (1/3). In all experiments, levels of expression of mRNA for the "housekeeping gene" β-actin, were not influenced by ionomycin (Fig. 4.2.5). Addition of IL-4 (100 ng/ml, 24 h) to the cell cultures led to a small but detectable increase in mRNA for IL-3 and IL-8 but not IL-4, TNF-α or β-actin in unstimulated cells, but enhanced mRNA expression for IL-3 and IL-8 by cells challenged with ionomycin for 4 h (Fig. 4.2.5.). IL-4 produced a smaller effect on ionomycin-induced expression of IL-4 mRNA, and was without effect on induced expression of mRNA for TNF-α and β-actin (Fig. 4.2.5). Similar results shown to those in figure 4.2.5 were seen in at least six independent experiments.

DISCUSSION

4.3.1. Production of TNF-α and IL-6 by mast cells

Biologically active TNF-*α* was detected in supernatant fractions from unstimulated and immunologically activated Cl.MC/C57.1 mast cells and rat PMC. IL-6 was detected in culture supernatants from unstimulated and IgE/antigen-activated Cl.MC/C57.1 mast cells whereas IL-5 was not detected. TNF-*α* mRNA and biologically active product have previously been detected in a number of mast cell lines including Cl.MC/C57.1 mast cells (Young *et al.*, 1987), freshly purified rodent peritoneal mast cells (Gordon and Galli 1990, 1991; Bissonnette *et al.*, 1991) and human skin and lung mast cells (Benyon *et al.*, 1991; Bradding *et al.*, 1994). In addition, peritoneal mast cells have been reported to contain extensive preformed stores of TNF-*α* which are released immediately upon appropriate cell stimulation (Gordon and Galli, 1990, 1991). Others have reported that Cl.MC/C57.1 mast cells express mRNA for a wide variety of cytokines including IL-5 and IL-6 following activation with IgE/antigen (Burd *et al.*, 1989). The lack of detectable levels of IL-5 in the present experiments may therefore be explained by the insensitivity of the ELISA detection system, or alternatively that mRNA expression for IL-5 is not translated to protein product in these cells. The release of TNF-*α* was significantly enhanced in both Cl.MC/C57.1 mast cells and rat PMC after immunological stimulation for 1 h although for the same stimulation period IL-6 production by Cl.MC/C57.1 mast cells was unaffected. This finding may therefore suggest

that although Cl.MC/C57.1 mast cells may release some IL-6 while in a quiescent state they require a longer activation period before releasing stimulated levels of this cytokine. Indeed Leal-Berumen *et al.* (1994) have reported that in rat PMC, IL-6 is not detected in cell supernatants until activation of the cells with anti-IgE for at least 3 h, suggesting that at least in mature connective tissue-type mast cells IL-6 is synthesised *de novo*.

4.3.2. Effects of cytokines on mast cell cytokine production and mRNA expression

Although IL-3, IL-4, SCF and IFN-γ have previously been reported to regulate mouse PMC secretory function *in vitro* (Coleman *et al.*, 1991, 1992, 1993; Takaishi *et al.*, 1992), their effects on the release and regulation of mast cell associated cytokines have been relatively neglected. However, the findings observed in these studies suggest that their effects on mast cell serotonin and histamine release are not reflected in the release and regulation of TNF-α at least in the Cl.MC/C57.1 mast cell line, rat PMC and HMC-1 cells. Neither IL-3, IL-4 or IFN-γ had any significant effect on the release of TNF-α from IgE/antigen-activated Cl.MC/C57.1 mast cells although others have reported that IFN-γ does inhibit TNF-α from rat PMC (Bissonnette and Befus, 1990). The difference in effect of IFN-γ observed here compared to that described by Bissonnette and Befus may possibly be explained by the fact that Cl.MC/C57.1 cells represent a source of immature mast cells, whereas rat PMC are functionally mature connective tissue-type cells with fully operational cytokine receptors. Indeed this factor must always be

considered when using mast cell lines in experiments and may further explain the relative ineffectiveness of other cytokines on Cl.MC/C57.1 mast cell TNF-α release. Only SCF was shown to enhance the release of TNF-α from immunologically activated Cl.MC/C57.1 mast cells although at 50 ng/ml it had no effect on TNF-α release from rat PMC.

Additionally, IL-3, IL-4, SCF or IFN-γ had no effect on the release of IL-6 from Cl.MC/C57.1 mast cells. Although the ineffectiveness of these cytokines on IL-6 production may be explained by the fact that Cl.MC/C57.1 cells are relatively immature mast cells with non functional cytokine receptors therefore rendering them unresponsive to the regulatory effects of cytokines, SCF were shown to enhance IgE/antigen-induced TNF-α release. Therefore, Cl.MC/C57.1 mast cells are responsive to SCF regulation and although TNF-α production is regulated by SCF, production of IL-6 is not influenced by this cytokine.

Recombinant human IL-4 was shown to enhance ionomycin-induced gene expression for IL-3, and IL-8 in HMC-1 cells. Further studies by Buckley *et al.* (1995) using a competitive PCR technique to quantify starting levels of cDNA, have revealed that IL-4 enhanced mRNA expression for IL-3 and IL-8 in ionomycin-stimulated cells by 6-fold and 4-fold respectively. The enhancing effect of IL-4 was not generalized but appeared to be targeted at certain cytokine genes only; it had no effect on IL-4 mRNA in unstimulated cells, and only weakly elevated IL-4 mRNA in activated cells and was totally without effect on TNF-α expression in unstimulated and activated cells. Buckley *et al.* also reported that this 6-fold increase in IL-3 mRNA expression

led to a disproportionate increase in secreted IL-3 product (15 to 20-fold) whereas for IL-8, both mRNA and protein product were up-regulated to a similar extent. HMC-1 cells, which are derived from a human mast cell leukemia, resemble phenotypically relatively immature mast cells. They express several characteristic mast cell molecules including the product of the protooncogene c-*kit* and the granule enzyme tryptase, but not a functional high affinity IgE receptor. They also contain histamine at relatively low levels (Butterfield *et al.*, 1988; Sillaber *et al.* 1991). However, cells of this line have been reported to express high affinity receptors for IL-4 (Valent *et al.*, 1991). IL-4 has been shown to inhibit IL-1β in these cells (Sillaber *et al.*, 1993) and to enhance cell surface expression of the intercellular adhesion molecule ICAM-1 (Valent *et al.*, 1991) but to inhibit the expression of the c-*kit* product (Sillaber *et al.*, 1991). In view of the above evidence, it is clear that IL-4 exerts diverse and complex regulatory effects on gene expression in mast cells and by doing so may influence the functional role of mast cells in immune and inflammatory reactions.

However, IL-4 had no effect on the release of TNF-α from IgE/antigen-activated Cl.MC/C57.1 mast cells and did not influence TNF-α release from immunologically activated rat PMC. Although one possible explanation may be due to the fact that cells were cultured in the presence of recombinant mouse IL-4 and not recombinant rat IL-4 which as yet is not commercially available, IL-4 was without effect on TNF-α gene expression in HMC-1 mast cells, a point also noted by Sillaber *et al.* (1993). Therefore IL-4 may be relatively ineffective in the regulation of TNF-α expression by mast cells.

TNF-*α* is known to be stored preformed in rodent peritoneal mast cells (Gordon and Galli, 1990, 1991), human skin mast cells (Walsh *et al.*, 1991) and in HMC-1 cells (Sillaber *et al.*, 1993); in mouse mast cells the stored pool of TNF-*α* is released rapidly after cell activation while TNF-*α* released subsequently is *de novo* synthesised (Gordon and Galli, 1991). Therefore, the effects of IL-4, if any, on mast cell TNF-*α* regulation may be undetectable during the initial stimulatory period when preformed stores are released, and effects may only be observed after culture of the cells with IL-4 during a regranulation period prior to a subsequent second stimulation. However, at this stage there is no clearly defined role for IL-4 on the regulation of TNF-*α* expression by mast cells.

4.3.7. Concluding remarks

In conclusion, the results presented in this chapter demonstrate that SCF can enhance IgE/antigen-induced TNF-*α* release from Cl.MC/C57.1 mast cells although at a lower concentration SCF was found to have no effect on the release of TNF-*α* from mature rat connective tissue-type peritoneal mast cells. Although IL-4 had no effect on the release of TNF-*α* or IL-6 by IgE/antigen activated Cl.MC/C57.1 mast cells and did not enhance immunologically induced release of TNF-*α* from rat PMC it was found to enhance ionomycin-induced gene expression of IL-3 and IL-8 by HMC-1 mast cells. Therefore, results suggest that IL-4 and SCF may influence mast cell function; for example by promoting expression of IL-3, a cytokine important in the activation of eosinophils (Lopez *et al.*, 1987; Fabian *et al.*, 1992), and

IL-8, a member of the *C-X-C* chemokine family important in neutrophil activation (Baggiolini *et al.*, 1989), IL-4 may promote mast cell-dependent inflammatory reactions in which eosinophils and neutrophils are recruited and activated. Additionally, SCF may enhance the release of TNF-α from immunologically activated mast cells therefore promoting and maintaining allergic inflammation.

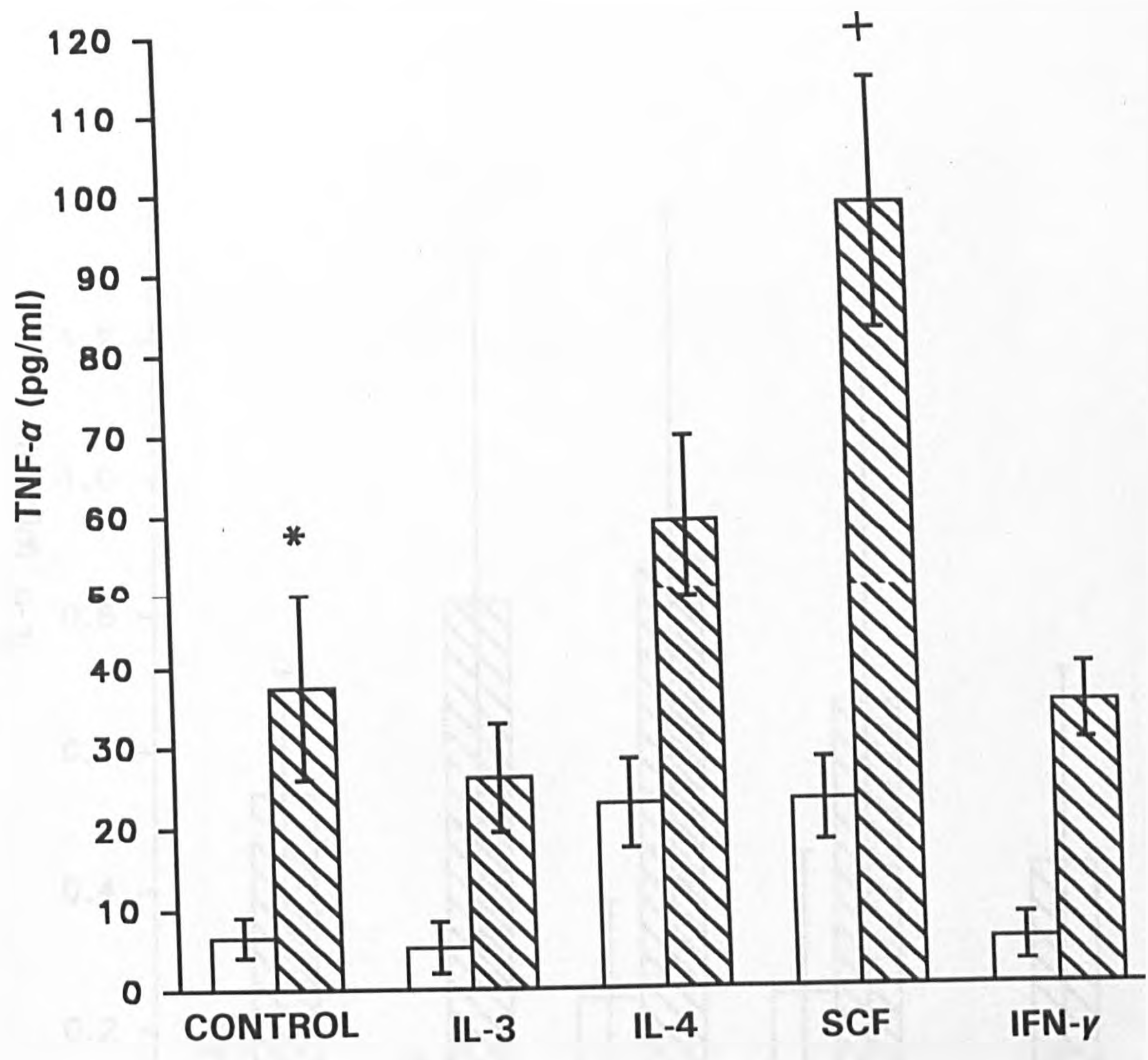


Fig. 4.2.1. Release of TNF-α from Cl.MC/C57.1 mast cells cultured with IL-3, IL-4, SCF, IFN-γ or medium alone (control) for 48 h and then washed and challenged with medium (unstimulated cells; open bars) or IgE/antigen (stimulated cells; filled bars) for 1 h. Results are means ± SEM for four experiments (* $p < 0.03$ for comparison of unstimulated control cells to IgE/antigen-stimulated control cells; + $p < 0.05$ for comparison of IgE/antigen-stimulated SCF-treated cells with IgE/antigen-stimulated non-cytokine-treated control cells. Statistical analyses were by the two-tailed Mann Whitney *U* test).

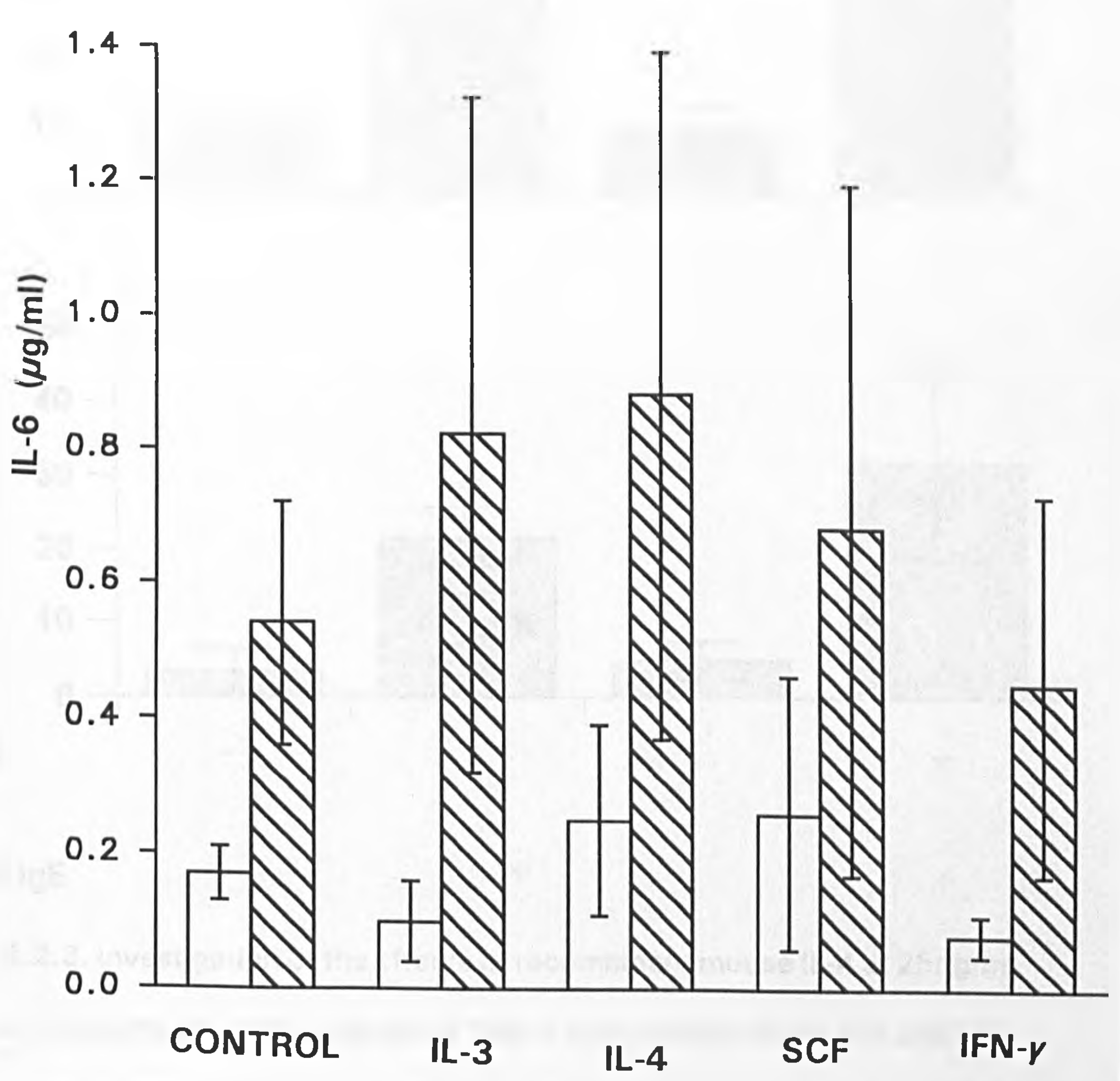


Fig. 4.2.2. Release of IL-6 from Cl.MC/C57.1 mast cells cultured with IL-3, IL-4, SCF, IFN-γ or medium alone (control) for 48 h and then washed and challenged with medium (open bars) or IgE/antigen (filled bars) for 1 h. Results are means ± SEM for three experiments

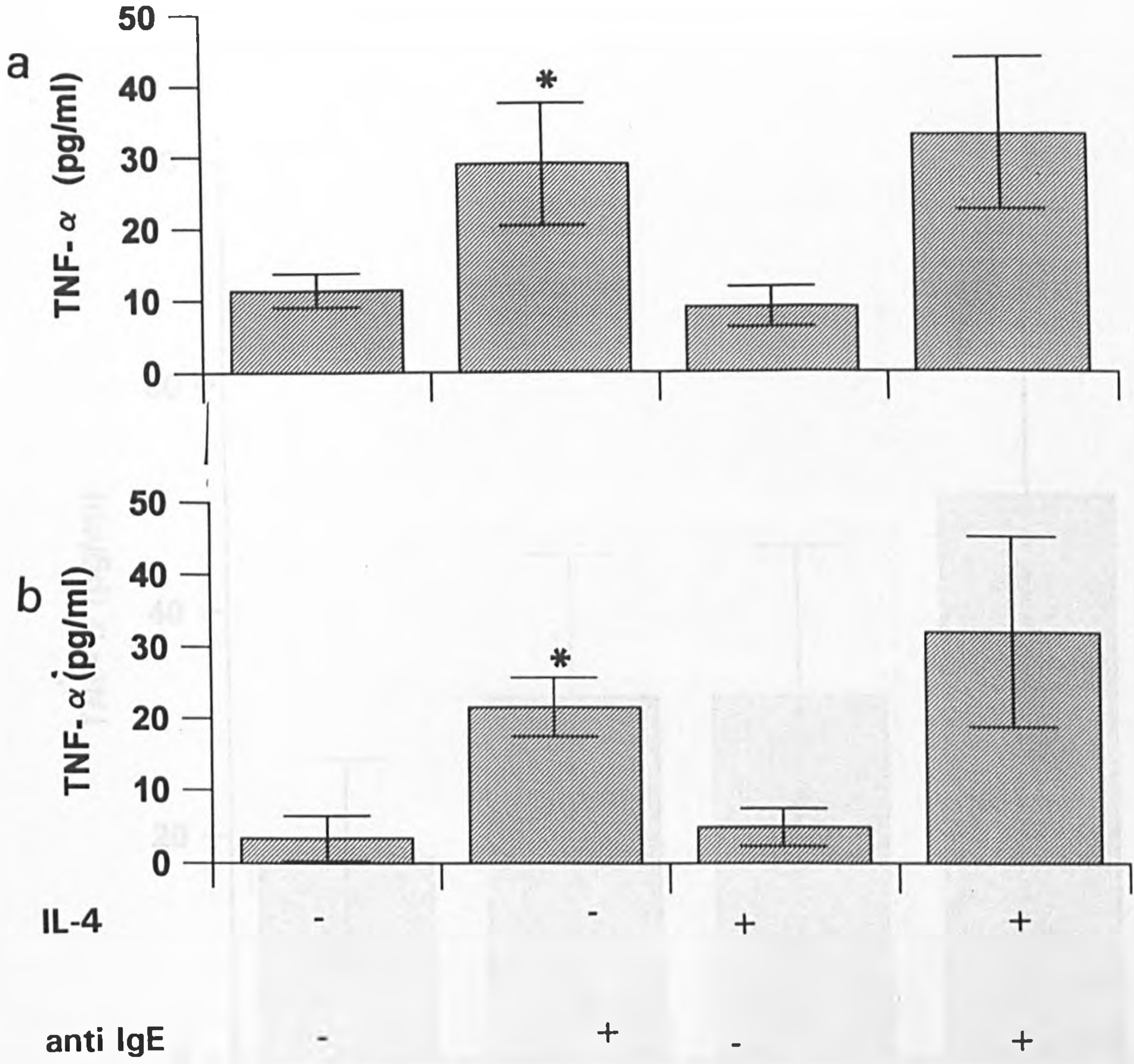


Fig. 4.2.3. Investigation of the effects of recombinant mouse IL-4 at 25ng/ml (a) or 100ng/ml (b) on the release of TNF-*α* from unstimulated and anti-IgE-activated rat PMC. Cells were cultured with (+) or without (-) IL-4 for 48 h. Cells were then washed and challenged with anti-IgE (+) or medium (-) for 1 h. Cells were then pelleted and supernatant fractions removed and assayed for TNF-*α* bioactivity using the L929 cytotoxicity assay. Results are means ± SEM for ten experiments (a) or four experiments (b). (* $p < 0.05$ for comparison of TNF-*α* released from unstimulated control cells to TNF-*α* released form anti-IgE-activated control cells. Statistical analysis was by the two-tailed Mann Whitney *U* test).

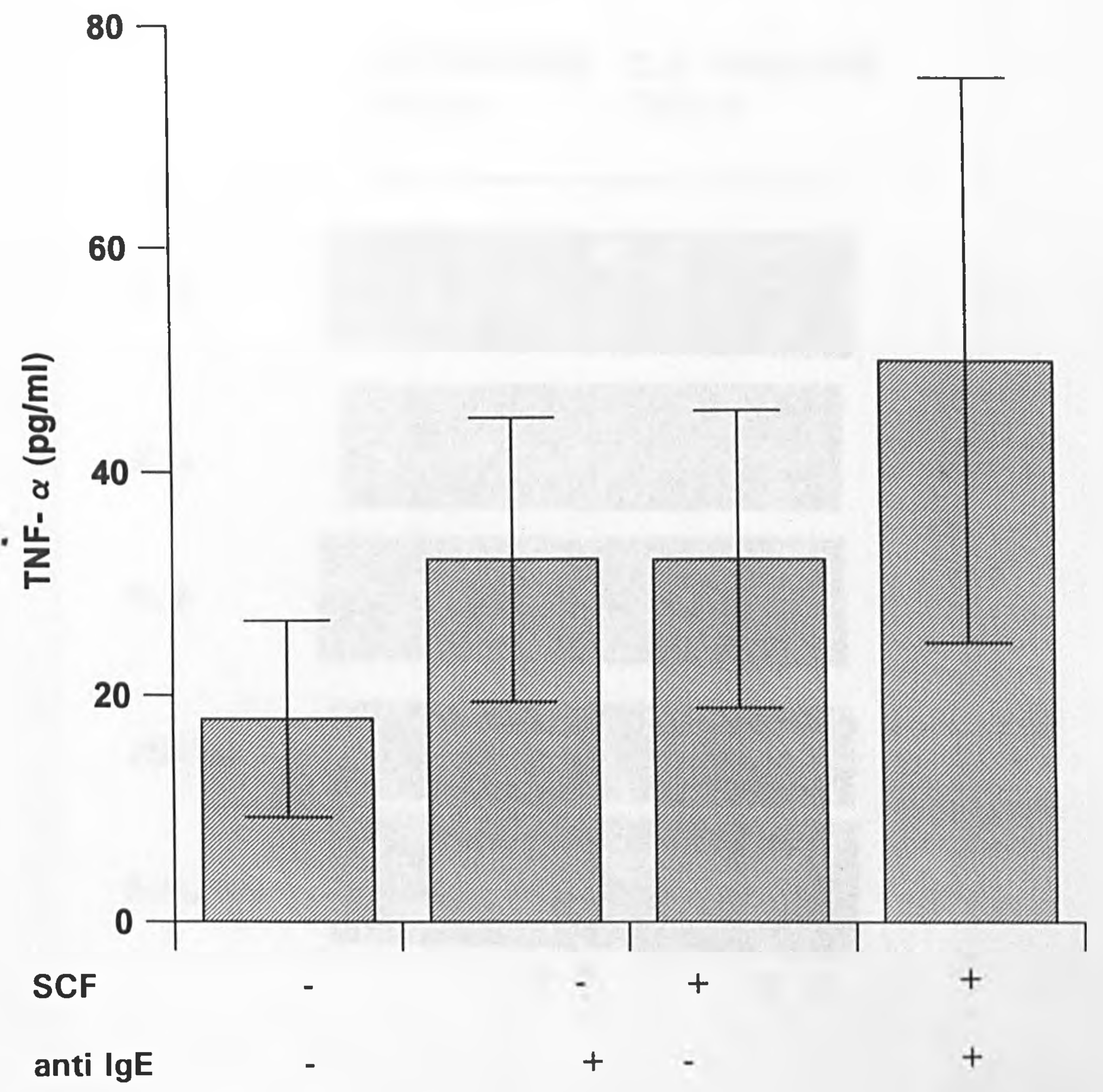


Fig. 4.2.4. Investigation of the effects of recombinant rat SCF at 50ng/ml on the release of TNF-*α* from unstimulated and anti-IgE-activated rat PMC. Cells were cultured with (+) or without (-) SCF for 48 h. Cells were then washed and challenged with anti-IgE (+) or medium (-) for 1 h. Cells were then pelleted and supernatant fractions removed and assayed for TNF-*α* bioactivity using the L929 cytotoxicity assay. Results are means ± SEM for three experiments.

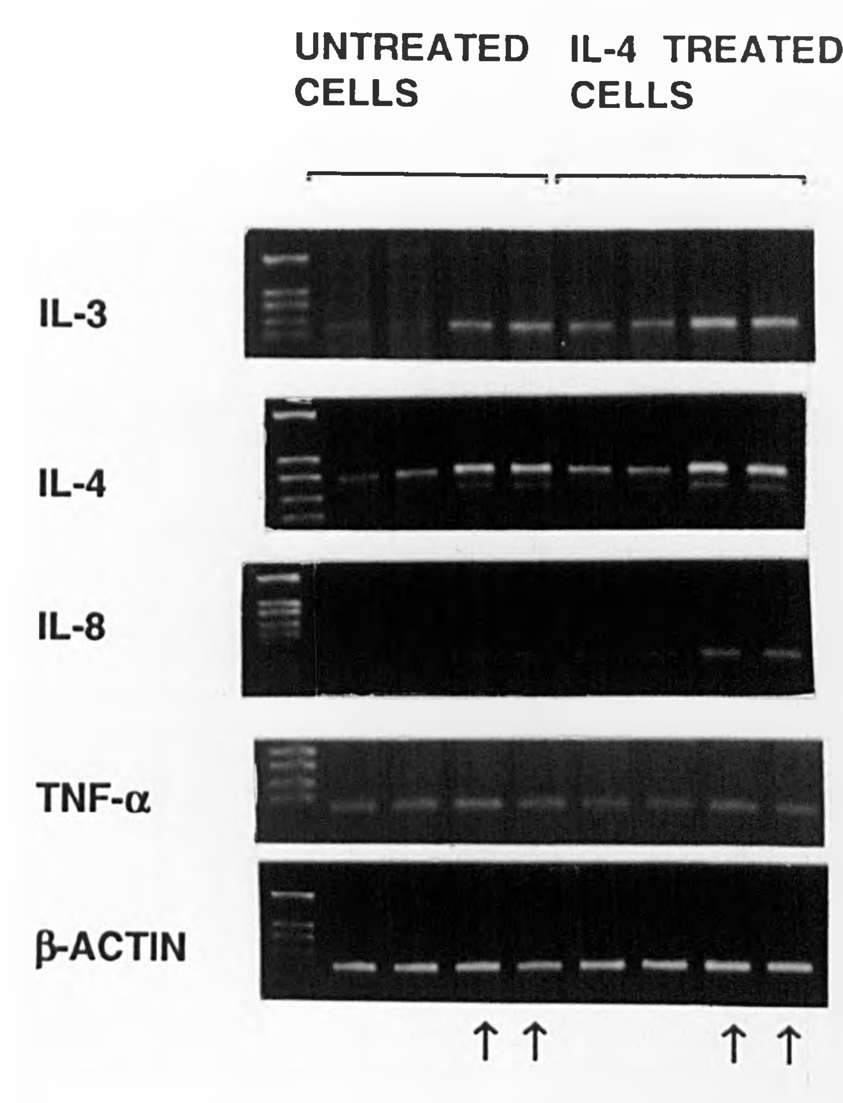


Fig. 4.2.5. RT-PCR analysis of mRNA for IL-3, IL-4, IL-8, TNF-α and β-actin in HMC-1 mast cells. Cells were cultured with or without IL-4 (100ng/ml, 24 h) and then challenged with ionomycin (10μM) or medium alone for 4 h. Arrows indicate ionomycin stimulation (lanes 4, 5, 8, 9,). Results of duplicate cell cultures from a representative experiment are shown. DNA molecular weight markers were run in the left hand lane.

Chapter 5

Effects of drugs on cytokine expression by rat peritoneal mast cells and mast cell lines

5.1 Introduction

5.1.1. Mast cells as a source of multifunctional cytokines

5.1.2. Inhibition of mast cell cytokine production by CsA or Dex

5.2 Results

5.2.1. Cytokine expression by purified rat peritoneal mast cells

5.2.2. Studies of cytokine expression in mast cell-depleted peritoneal cell populations

5.2.3 Effects of CsA or Dex on anti-IgE-induced cytokine gene expression in purified rat peritoneal mast cells

5.2.4 Effects of Dex or CsA on release of TNF-*α* from purified rat peritoneal mast cells

5.2.5 Effects of CsA, Dex or BTS 71321 on the release of TN

α from Cl.MC/C57.1 mast cells

5.2.6. Effects of CsA, Dex or BTS 71321 on cytokine mRNA expression by human HMC-1 mast cells

5.3 Discussion

5.3.1. Expression of mRNA for multiple cytokines by purified rat peritoneal mast cells

5.3.2 Inhibition of mast cell cytokine mRNA expression and release of TNF-α by anti-inflammatory drugs

5.3.3. Concluding remarks

5.1 INTRODUCTION

5.1.1 Mast cells as a source of multifunctional cytokines

It is well established that mast cells are primary instigators of the immediate allergic reaction by releasing inflammatory mediators such as histamine, neutral proteases and *de novo* synthesized products of the cyclo-oxygenase and lipoxygenase pathways of arachidonic acid metabolism (Barnes *et al.*, 1988; Church and Caulfield, 1993). Additionally, mast cells of certain types provide a source of several multifunctional cytokines, suggesting that mast cells also have the potential to contribute to chronic inflammation and regulation of the immune response. Much of the work to date on cytokine production by mast cells has been carried out on mouse or human mast cell lines or BMMC. Preliminary studies revealed that BMMC were cytotoxic towards tumour cells (Ghiara *et al.*, 1985) and Abelson murine leukemia virus (A-MuIV)-transformed tumorigenic mast cell lines expressed mRNA and biologically active product for GM-CSF (Chung *et al.*, 1986). Further investigations indicated that mRNA and biologically active product for GM-CSF were induced after IgE/antigen-activation of mouse BMMC (Wodnar-Filipowicz *et al.*, 1989). Others have identified murine mast cell lines and clones as a source of IL-1, IL-3, IL-4, IL-5, IL-6, IL-13, IFN-γ, TCA3, MIP1α and MIP1β (Brown *et al.*, 1987; Burd *et al.*, 1989, 1995; Plaut *et al.*, 1989). Interleukin-1β, IL-3, IL-4, IL-8 and TNF-α are also known to be expressed by the HMC-1 human mast cell line (Möller *et al.*, 1993; Sillaber *et al.*, 1993; Buckley *et al.*, 1995). However, information regarding cytokine expression

by tissue mast cells is less complete, although it is known that mouse and rat PMC express mRNA and biologically active product for TNF-α (Young *et al.*, 1987; Gordon and Galli, 1990, 1991) and more recently rat PMC have been reported to produce biologically active IL-6 and leukemia inhibitory factor (Leal-Berumen *et al.*, 1994; Marshall *et al.*, 1993). In addition, human skin mast cells express TNF-α (Walsh *et al.*, 1991; Benyon *et al.*, 1991) and human lung mast cells are known to provide a source of IL-4, IL-5, IL-6 and TNF-α (Bradding *et al.*, 1992, 1994). Nevertheless, examination of mature mast cells as a source of multifunctional cytokines demands further attention. Research by Burd *et al.* (1995) has indicated that when mast cell derived IL-13 is added to B lymphocytes it induces transcripts encoding the constant region of the IgE heavy chain, DNA recombination characteristic of the isotype switch to IgE and the secretion of IgE protein. Therefore, these results suggest that mast cells could play a role in the induction of IgE synthesis.

The recent interest in the role of cytokines in chronic allergic reactions such as asthma and the evidence that mRNA for a number of cytokines is up-regulated in bronchial cells of asthmatic patients (Bentley *et al.*, 1993; Hamid *et al.*, 1991; Gosset *et al.*, 1991, 1992) suggests the possibility that activation of these cells may be due in part to mast cell-derived cytokines which are released following IgE/antigen-dependent activation during the acute reaction. The possibility also exists that mast cells may play a part in the recruitment of other pro-inflammatory cells such as eosinophils into the asthmatic lung, thereby initiating the chronic allergic response. In this

chapter, rat PMC, that are representative of mature connective tissue-type mast cells, have been used to investigate whether cross-linkage of IgE leads to induction of expression of the cytokines IL-5, IL-6, TNF-α, MIP-2 and IFN-γ at the level of mRNA and TNF-α at the level of biologically active protein product.

5.1.2 Inhibition of mast cell cytokines by CsA and Dex

It has become apparent that not only do CsA and Dex inhibit the release of preformed and *de novo* synthesised mast cell mediators but they inhibit expression of a number of mast cell associated cytokines. Hatfield and Roehm (1991) have shown that CsA can inhibit the release of IL-2, IL-3, IL-4 and GM-CSF in a number of mast cell lines and Kaye *et al.* (1992) have indicated that CsA is able to inhibit IgE/antigen-induced increases in mRNA for IL-1β, TNF-α and IL-6 in murine BMMC. Others have reported that Dex inhibits production of IL-6 by rat PMC (Leal-Berumen *et al.*, 1994) and TNF-α by murine Cl.MC/C57.1 cells (Wershil *et al.*, 1995). The finding that CsA and Dex inhibit the expression of mast cell-derived cytokines is potentially of clinical importance and has revealed new possibilities whereby mast cell function may be modulated in a number of disease states. In this chapter the effects of CsA and Dex on anti-IgE-induced expression levels of mRNA for IL-5, IL-6, TNF-α, MIP-2 and IFN-γ and release of TNF-α protein product from rat PMC were examined. Additionally, the effects of CsA, Dex and the novel benzoaminoalkylimidazole compound, BTS 71321, on release of TNF-α by Cl.MC/C57.1 mast cells were investigated. The effects of CsA, Dex and

BTS 71321 on mast cell cytokine gene expression at the level of mRNA were also studied using cells of the HMC-1 human leukemic mast cell line.

5.2 RESULTS

5.2.1 Cytokine expression by purified rat peritoneal mast cells

Messenger RNA encoding the cytokines IL-5, IL-6, MIP-2 and IFN-γ was not detectable in cytoplasmic lysates from unstimulated PMC, whereas mRNA for TNF-α was detected weakly in unstimulated PMC in 3/5 experiments (Fig. 5.2.1). Following stimulation of the cells for 4 h with anti-IgE antibody, levels of mRNA encoding each of the five cytokines were elevated markedly in each of five independent experiments (Fig. 5.2.1). Analysis of band intensities by laser densitometry revealed that the anti-IgE-induced increase in mRNA expression for each of the cytokines was highly significant statistically over the five experiments each using PMC pooled from two rats ($p < 0.009$ by Mann-Whitney U test). In all experiments, the level of expression of mRNA for the housekeeping gene G3PDH, was not influenced by activation of the cells with anti-IgE (Fig. 5.2.1).

5.2.2 Studies of cytokine expression in mast cell-depleted peritoneal cell populations

Messenger RNA for each of the five cytokines was not detected in RNA extracted from 10^4 mast cell-depleted peritoneal cells activated with anti-IgE. These populations contained <1% mast cells and corresponded to the

number of non-mast cells that routinely contaminated purified mast cell preparations.

5.2.3 Effects of CsA or Dex on anti-IgE-induced cytokine gene expression in purified rat peritoneal mast cells

Treatment of rat PMC with CsA (10^{-6}M) for 24 h, prior to challenge with anti-IgE for 4 h, led to a highly significant decrease in induced levels of mRNA encoding the cytokines IL-5, IL-6, TNF-α, MIP-2 and IFN-γ. However, CsA had no effect on mRNA expression of the housekeeping gene G3PDH (Fig. 5.2.2). Expression of IL-5, IL-6 and MIP-2 mRNA was completely abrogated by the drug treatment in each of five experiments, while expression of TNF-α and IFN-γ mRNA was completely abolished in 1/5 and 2/5 experiments respectively and inhibited to a lesser degree in the remainder of the experiments (Fig. 5.2.2). Statistical evaluation of data obtained by laser densitometry of the PCR product bands, revealed that the inhibition by CsA, of mRNA levels for all five cytokines was highly significant (Table 5.2.1).

Culture of rat PMC with Dex (10^{-6}M) for 24 h inhibited the anti-IgE-induced expression of mRNA for each of the five cytokines but did not inhibit expression of mRNA for the housekeeping gene G3PDH (Fig. 5.2.3). In fact, after treatment with Dex, mRNA for IL-5, IL-6, TNF-α and MIP-2 was undetectable in anti-IgE-activated PMC, while product bands for IFN-γ were extremely weak (Fig. 5.2.3). In each of five independent experiments, anti-IgE-induced mRNA expression for each cytokine was readily detected and

a clear inhibition of expression by Dex was observed. Statistical analysis of data obtained from laser densitometric measurements of band intensities revealed a highly significant effect of the drug treatment on expression of mRNA (Table 5.2.1).

5.2.4 Effects of Dex or CsA on release of TNF-α from purified rat peritoneal mast cells

TNF-*α* bioactivity was detectable in supernatant fractions from IgE/antigen-stimulated PMC; culture of the mast cells for 24 h in the presence of Dex or CsA (each at 10^{-6}M) led to a substantial and significant reduction of induced TNF-*α* release (Fig. 5.2.4). In four independent experiments, Dex inhibited release of TNF-*α* on average by 95% whereas CsA inhibited release by an average of 85%.

5.2.5 Effects of CsA, Dex or BTS 71321 on the release of TNF-α from Cl.MC/C57.1 mast cells

Culture of Cl.MC/C57.1 mast cells with CsA (10^{-5}M or 10^{-6}M) for 24 h led to a substantial inhibition of TNF-*α* release after subsequent activation with calcium ionophore. In four independent experiments, calcium ionophore-induced TNF-*α* release was inhibited on average by 95% at 10^{-6}M and by 97% at 10^{-5}M (Fig. 5.2.5). BTS 71321 (10^{-6}M) also inhibited ionophore-induced TNF-*α* release from Cl.MC/C57.1 mast cells on average by 28% and inhibition increased to 42% after culture of the cells with BTS 71321 at a concentration of 10^{-5}M (Fig. 5.2.5). However, Dex did not inhibit the release

of TNF-α by Cl.MC/C57.1 mast cells at either 10^{-6}M or 10^{-5}M (Fig. 5.2.5).

5.2.5. Effects of CsA, Dex or BTS 71321 on cytokine mRNA expression in human HMC-1 mast cells

Incubation of HMC-1 cells with CsA (10^{-6}M) for 24 h prior to stimulation with ionomycin (10 μM) for 4 h, led to an inhibition of mRNA expression for IL-3 and IL-8 and to a lesser extent inhibition of mRNA expression for IL-4. Results from five representative experiments are shown in Fig. 5.2.6. Statistical analysis of data obtained from laser densitometric measurements of band intensities from ten independent experiments revealed a highly significant effect of the drug treatment on expression of mRNA for IL-3 and IL-8 but not IL-4 (Fig. 5.2.7). Incubation of the cells with Dex (Fig 5.2.8) or BTS 71321 (Fig. 5.2.9), each at a concentration of 10^{-6}M, prior to ionomycin stimulation, had no significant effect on mRNA expression for IL-3, IL-4 or IL-8 over ten independent experiments. In all experiments levels of the "housekeeping gene" β-actin, were not affected by culture of the cells in the presence of CsA, Dex or BTS 71321 or by activation with ionomycin.

5.3 DISCUSSION

5.3.1 Expression of mRNA for multiple cytokines by purified rat peritoneal mast cells

This chapter clearly demonstrates that immunological activation of rat peritoneal mast cells induces expression of mRNA encoding the cytokines IL-5, IL-6, TNF-*α*, MIP-2 and IFN-*γ*. Unstimulated PMC expressed low levels of TNF-*α* mRNA, but no detectable mRNA for each of the other cytokines. There can be no doubt that the induced cytokine mRNA is of mast cell origin since the purity of all PMC preparations was high (≥99%), and induction of expression was by cross-linkage of IgE that binds selectively to the Fcε receptor on mast cells. Furthermore, activation by anti-IgE of mast cell-depleted peritoneal cells, at numbers corresponding to those that routinely contaminated mast cell preparations, failed to generate any detectable cytokine mRNA. The increased expression of cytokine mRNA observed after PMC activation was seen consistently and was statistically significant over several experiments, whereas expression of mRNA for the housekeeping gene G3PDH was unaltered. Although the release of biologically active product was not measured for all of the five cytokines investigated, in the case of TNF-*α*, induction of mRNA corresponded to induced release of biological activity.

Of the five types of cytokine mRNA that were studied, IL-5, IL-6, TNF-*α* and IFN-*γ* mRNA have been reported to be expressed by activated mouse BMMC and mast cell clones (Ghiara *et al.*, 1985; Humphries *et al.*, 1988;

Burd *et al.*, 1989; Plaut *et al.*, 1989). TNF-α and IFN-γ mRNA have been reported in human skin mast cells (Walsh *et al.*, 1991; Benyon *et al.*, 1991; Horsmanheimo *et al.*, 1994) and IL-6 and TNF-α mRNA have been detected in activated rodent PMC (Young *et al.*, 1987; Gordon and Galli, 1990, 1991; Bissonnette *et al.*, 1991; Leal-Berumen *et al.*, 1994). However, IL-5 and IFN-γ mRNA have not previously been reported in rodent mast cells and mRNA for MIP-2, a neutrophil chemoattractant of the *C-X-C* family of chemokines, has not previously been reported in mast cells of any phenotype. From an inflammatory point of view these cytokines are of potential importance. IL-5 has been shown to induce eosinophil proliferation and chemotaxis (Lopez *et al.*, 1988; Clutterbuck *et al.*, 1989; Wang *et al.*, 1989; Dent *et al.*, 1990), a characteristic feature of chronic allergic reactions. IL-6 is important in stimulating the production of acute phase response proteins in hepatocytes (Andus *et al.*, 1987; Gauldie *et al.*, 1987) and has been shown to induce the proliferation and stimulation of T cells in the presence of mitogen, suggesting an important role in the physiological activation of T cells (Tosato *et al.*, 1988). Furthermore, IL-6 can strongly up-regulate the IL-4-dependent synthesis of IgE (Vercelli *et al.*, 1989). TNF-α and IFN-γ can increase endothelial cell adhesiveness for T cells and these effects are further enhanced with combinations of the two cytokines (Thornhill *et al.*, 1991). TNF-α is also known to directly and indirectly regulate haematopoietic progenitor cell proliferation (Jacobsen *et al.*, 1992) and induce the expression of MHC class I and class II (Israel *et al.*, 1989; Kingston *et al.*, 1989). IFN-γ also enhances MHC class I and class II expression (Gerrard

et al., 1988) and it up-regulates T cell activating proteins (Tap, Ly-6, Sca-1) in resting and activated murine T cells (Dumont and Boltz 1987; Dumont *et al.*, 1987, 1988). MIP-2 is a potent neutrophil chemoattractant (Wolpe *et al.*, 1988).

Inflammatory cell influx is a common feature of late phase reactions and chronic asthma is characterised by infiltration of the bronchial mucosa with large numbers of activated eosinophils and increased mRNA expression for cytokines with a predominant Th2-like profile. The results presented in this chapter, in conjunction with evidence provided by other groups, indicates that mast cells have the potential to produce cytokines which may be important in initiating these processes in the late phase inflammatory response.

Recent evidence suggests that mast cells of different phenotypes express different cytokines with the potential to influence the differentiation of T cells into either Th1 or Th2 cells (Smith *et al.*, 1994). For example, mouse BMMC cultured in SCF and resembling mast cells of the connective tissue phenotype expressed transcripts for IL-12, a Th1 cell promoting cytokine, whereas mast cells cultured in IL-3 and resembling the mucosal phenotype expressed mRNA for IL-4, a Th2 promoting cytokine, but not IL-12 (Smith *et al.*, 1994). In the present study it was found that rat PMC express mRNA for IFN-γ, a Th1 promoting cytokine (Seder *et al.*, 1994), suggesting that mast cells of connective tissue phenotype have the potential to promote Th1 cell development not only by producing IL-12 but also IFN-γ. However, HMC-1 mast cells and human lung mast cells express IL-4

(Bradding *et al.*, 1994; Buckley *et al.*, 1995), a Th2 cell promoting cytokine. It therefore appears that in terms of influencing Th1 or Th2 responses the role of mast cells may be a complex one with the possibility that mast cells may release cytokines which preferentially promote either Th1 or Th2 responses. The observation by Horsmanheimo *et al.* (1994) that IFN-γ is up-regulated in psoriatic but not atopic human skin mast cells suggests that disease state may influence the type of cytokines produced by mast cells. For example, in type I allergic reactions mast cells may release substantial amounts of cytokines such as IL-4 and IL-5 that may contribute to, or reflect a Th2 response, whereas in disorders such as psoriasis there may be a preferential release of cytokines such as IFN-γ and IL-12, by mast cells resulting in, or reflecting a typical Th1 response.

5.3.2 Inhibition of mast cell cytokine mRNA expression and release of TNF-α by drugs

The immunosuppressive agent CsA was extremely effective in inhibiting calcium ionophore A23187-induced release of TNF-α from Cl.MC/C57.1 mast cells. BTS 71321 also inhibited TNF-α release although to a much lesser extent than CsA whereas Dex was completely without effect even at a concentration of 10^{-5}M. CsA and Dex are potent inhibitors of anti-IgE-induced serotonin release from rat PMC although BTS 71321 had no effect on the release of serotonin from these cells (chapter 3). Previous studies have demonstrated that Dex does not inhibit the release of LTC_4 and LTB_4 from calcium ionophore-activated BMMC but does inhibit their release after

stimulation of the cells with IgE/antigen (Robin *et al.*, 1985). It therefore appears that Dex exerts its inhibitory effects on mast cell mediator release via regulation of components of FcεR1-dependent transduction events that occur prior to calcium mobilisation, hence explaining its ineffectiveness in inhibiting calcium ionophore-induced TNF-*α* release from Cl.MC/C57.1 mast cells and ionomycin-induced cytokine gene expression by HMC-1 cells. Mechanistically, the action of BTS 71321 is poorly understood, but is thought to act as a tyrosine kinase inhibitor (Smith, Knoll Pharmaceuticals, personal communication, 1995). Although it was shown to have no effect on anti-IgE-induced release of serotonin and arachidonate from rat PMC (chapter 3), it is known to inhibit arachidonate release from zymosan-stimulated mouse peritoneal macrophages (Steele *et al.,* 1995) and ionophore-induced TNF-*α* release from Cl.MC/C57.1 mast cells (this chapter). It therefore appears that BTS 71321 has a complex mechanism of action having the ability to inhibit both zymosan-induced macrophage activation and ionophore-induced mast cell activation.

CsA inhibited cytokine mRNA expression in HMC-1 cells whereas Dex and BTS 71321 were without effect. However, both CsA and Dex significantly inhibited immunologically-induced expression of mRNA for IL-5, IL-6, TNF-*α*, MIP-2 and IFN-*γ* in rat PMC and inhibited the release of TNF-*α* protein from these cells. CsA has previously been found to inhibit the production of mast cell IL-2, IL-3, IL-4 and GM-CSF (Hatfield and Roehm, 1991) and to down-regulate IgE-mediated gene induction of a number of cytokines in several murine mast cell lines and in BMMC (Burd *et al.*, 1989;

Kaye *et al.*, 1992). Recently, Dex has also been reported to down-regulate mRNA and inhibit the production of TNF-α protein in immunologically activated cloned mouse mast cells and examination of the effects of Dex or CsA in murine mast cell/IgE dependent cutaneous reactions has revealed a significant inhibition of leucocyte recruitment, tissue swelling and TNF-α mRNA at reaction sites (Wershil *et al.*, 1995). Dex can also inhibit IL-6 protein production by rat PMC after anti-IgE stimulation (Leal-Berumen *et al.*, 1994). The results presented in this chapter extend these findings to show that Dex or CsA inhibit induced expression of several cytokines genes, namely IL-5, IL-6, TNF-α, MIP-2 and IFN-γ, by mature tissue mast cells. In particular it has been shown that they act at the transcriptional level, and in the case of TNF-α there is a clear relationship between inhibition of transcription and inhibition of release of biologically active protein product. Considering that these cytokines have potent pro-inflammatory activities including activation of leucocytes, it appears that some of the clinical efficacy associated with corticosteroids and CsA may relate to their ability to inhibit mast cell cytokine mRNA expression and release of biologically active protein products. BTS 71321 does not appear to share these characteristics, at least on HMC-1 mast cells, although it was found to significantly inhibit the release of TNF-α from activated Cl.MC/C57.1 mast cells.

5.3.3 Concluding remarks

The findings presented in this chapter clearly demonstrate that mature

connective tissue mast cells from the rat are a potential source of IL-5, IL-6, TNF-α, MIP-2 and IFN-γ. In view of the potential role of these cytokines in the initiation and maintenance of late phase allergic reactions, it appears that mast cells may play a role in not only contributing to the early reaction through the release of their preformed mediators and *de novo* synthesised products of arachidonic acid metabolism but possibly also in initiating and maintaining the late phase reaction. In addition, the results indicate that CsA can inhibit cytokine mRNA expression by HMC-1 cells and that both CsA and Dex are potent inhibitors of cytokine mRNA expression by mature connective tissue-type mast cells. Furthermore, CsA and BTS 71321 can inhibit the release of TNF-α from ionophore-stimulated Cl.MC/C57.1 mast cells. In view of the putative role of cytokines in allergy these observations may bare particular significance in the development of novel tools for pharmaceutical intervention and may further explain the effectiveness of CsA and Dex in the treatment of serious allergic disease.

Fig. 5.2.1. RT-PCR analysis of mRNA for IL-5, IL-6, TNF-α, MIP-2, IFN-γ and G3PDH in rat PMC. After a 24 h pre-culture period, the cells were incubated with (stimulated) or without (resting) anti-IgE (1:100) for 4 h and then the RNA extracted. Results of five independent experiments are shown (lanes 2 and 7 are experiment 1, lanes 3 and 8 are experiment 2, etc). Each band represents DNA amplified from cDNA reverse transcribed from RNA extracted from purified PMC pooled from two rats. DNA molecular weight markers were run in the left hand lane (lane 1).

Resting Stimulated

IL-5

IL-6

TNF-α

MIP-2

IFN-γ

G3PDH

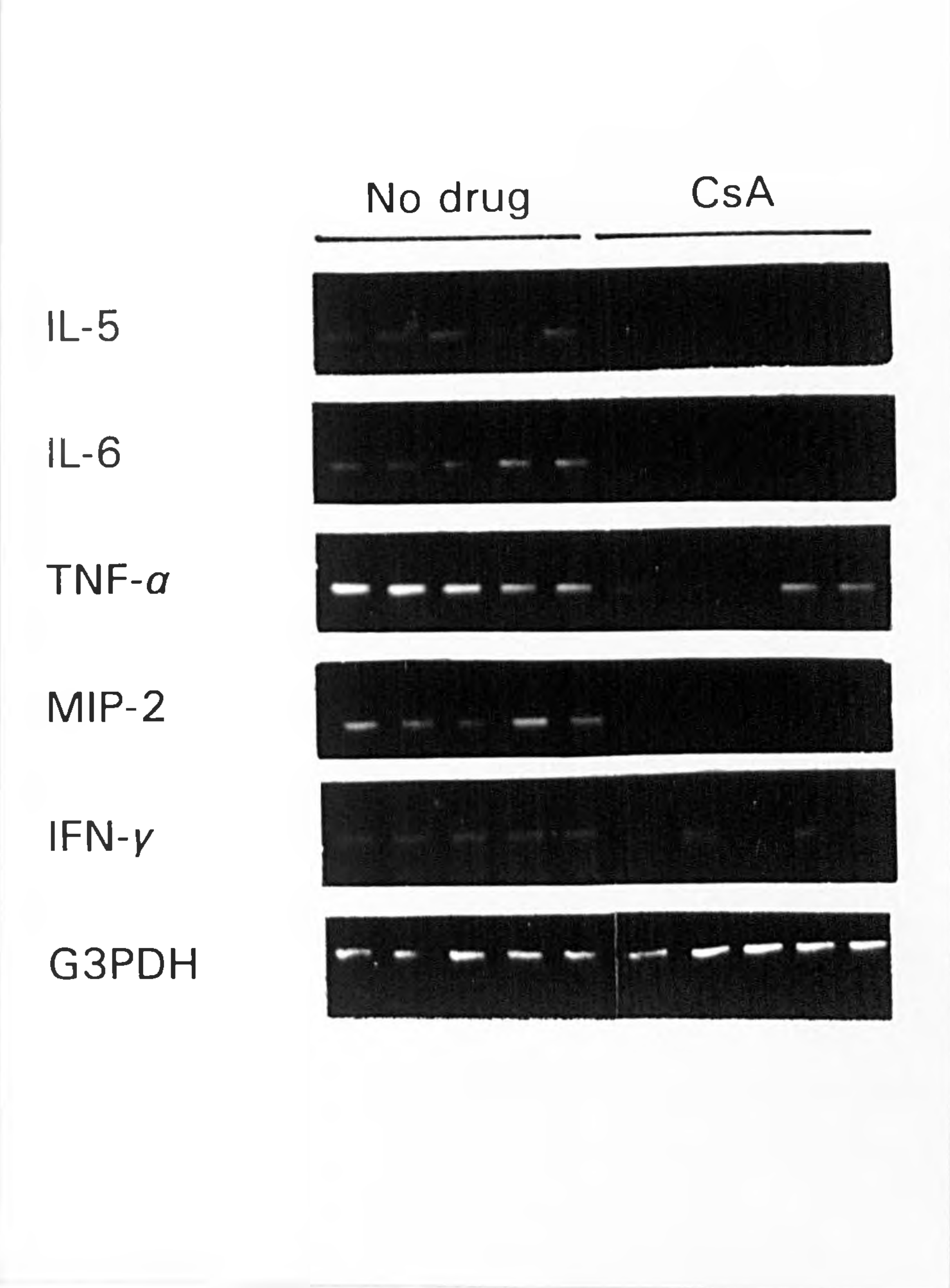


Fig. 5.2.2. RT-PCR analysis of mRNA for IL-5, IL-6, TNF-α, MIP-2, IFN-γ and G3PDH from rat PMC that had been cultured with or without CsA at 10^{-6} M for 24 h prior to stimulation with anti-IgE for 4 h. Results shown are from five independent experiments. Further details as for Fig. 5.2.1.

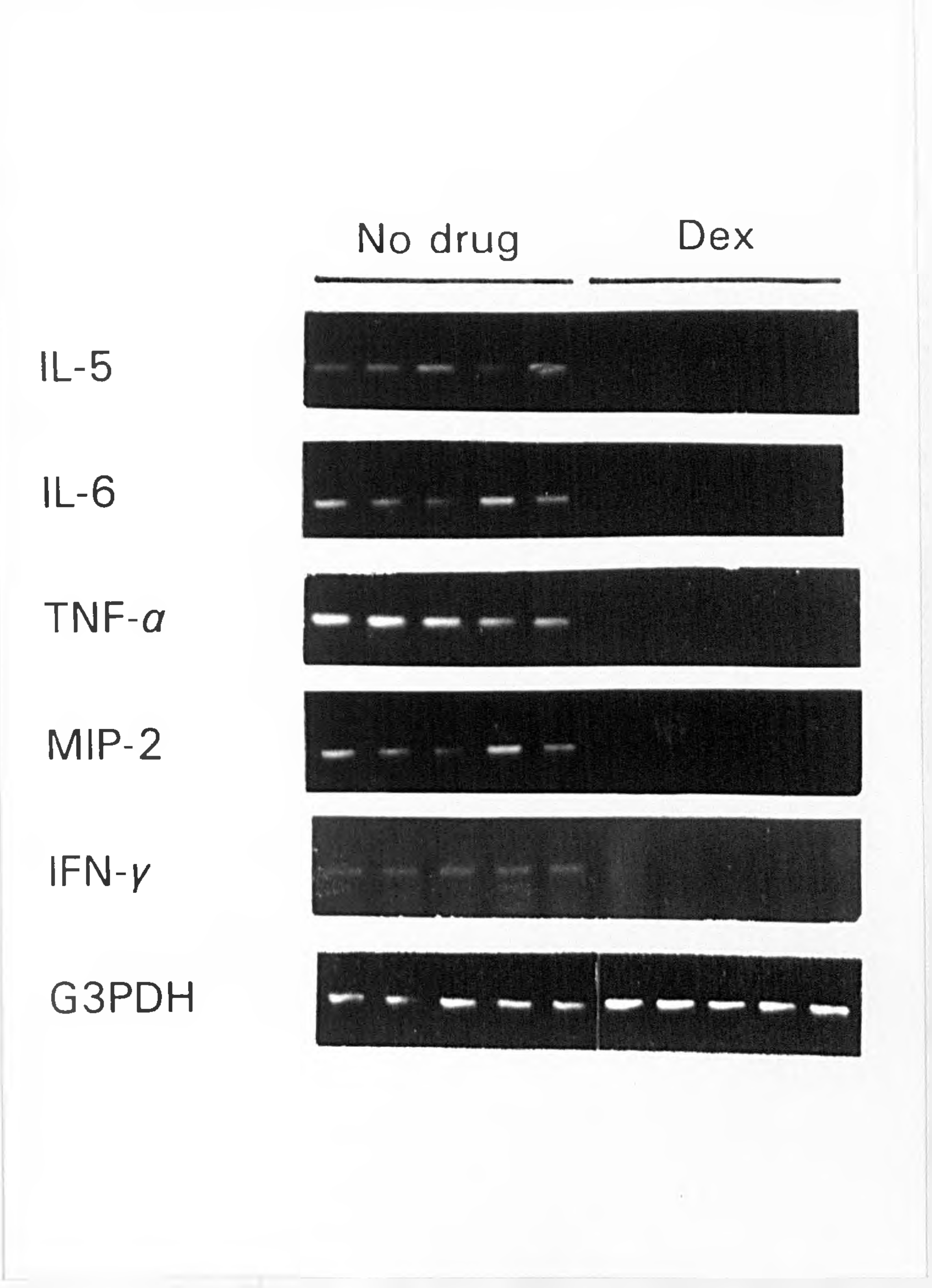


Fig. 5.2.3. RT-PCR analysis of mRNA for IL-5, IL-6, TNF-α, MIP-2, IFN-γ and G3PDH from rat PMC that had been cultured with or without Dex at 10^{-6} M for 24 h prior to stimulation with anti-IgE for 4 h. Results shown are from five independent experiments. Further details as for Fig. 5.2.1.

Table 5.2.1. Laser densitometric measurements of the area-under-the-curve of mRNA product bands generated from RNA extracted from PMC cultured with or without Dex or CsA (10^{-6} M) for 24 h and then stimulated with anti-IgE for 4 h.

	CONTROL	DEX	CONTROL	CSA
IL-5	0.22 ± 0.0	0.00 ± 0.00	0.23 ± 0.02	0.00 ± 0.00
IL-6	0.34 ± 0.07	0.00 ± 0.00	0.27 ± 0.04	0.00 ± 0.00
TNF-α	0.82 ± 0.11	0.00 ± 0.00	0.83 ± 0.12	0.22 ± 0.08
MIP-2	0.30 ± 0.07	0.00 ± 0.00	0.32 ± 0.07	0.00 ± 0.00
IFN-γ	0.11 ± 0.03	0.004 ± 0.002	0.10 ± 0.01	0.03 ± 0.02
G3PDH	0.87 ± 0.01	0.89 ± 0.01	0.80 ± 0.10	0.79 ± 0.04

Results are means ± SEM of data from five experiments. For each cytokine mRNA both drugs produced a significant decline in product intensity ($p < 0.035$ as determined by the two-tailed Mann-Whitney U test). Both drugs were without effect on levels of G3PDH mRNA.

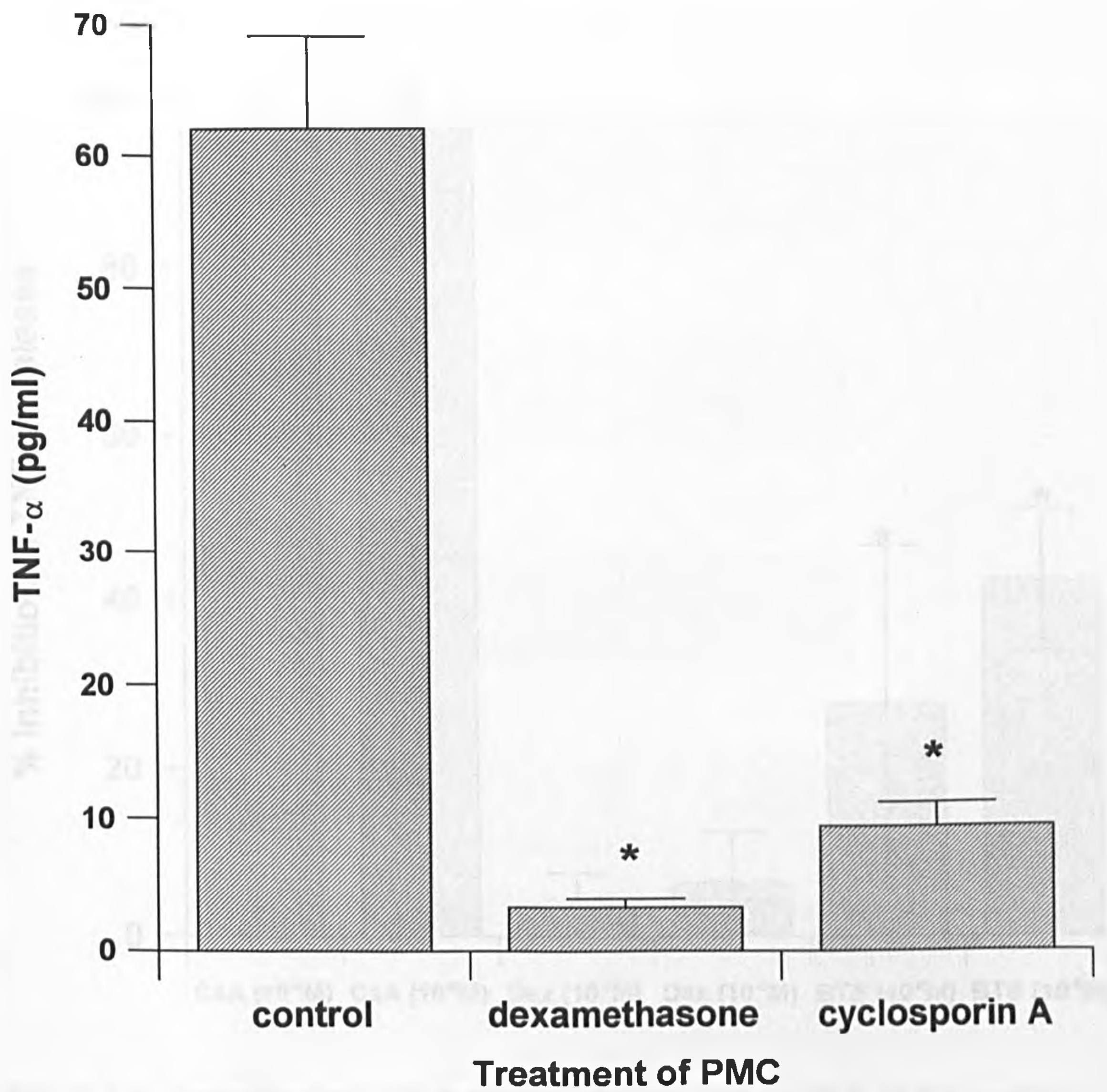


Fig. 5.2.4. The effects of Dex or CsA on antigen/IgE-induced TNF-*α* release from rat PMC. The cells were cultured with or without Dex or CsA (each at 10^{-6}M) for 24 h in the presence of IgE anti-DNP prior to stimulation with DNP-HSA for 1 h. Results are means ± SEM for four experiments each assayed in duplicate. (* $p < 0.05$ by comparison of release of TNF-*α* by drug treated cells to controls as determined by the two-tailed Mann-Whitney *U* test).

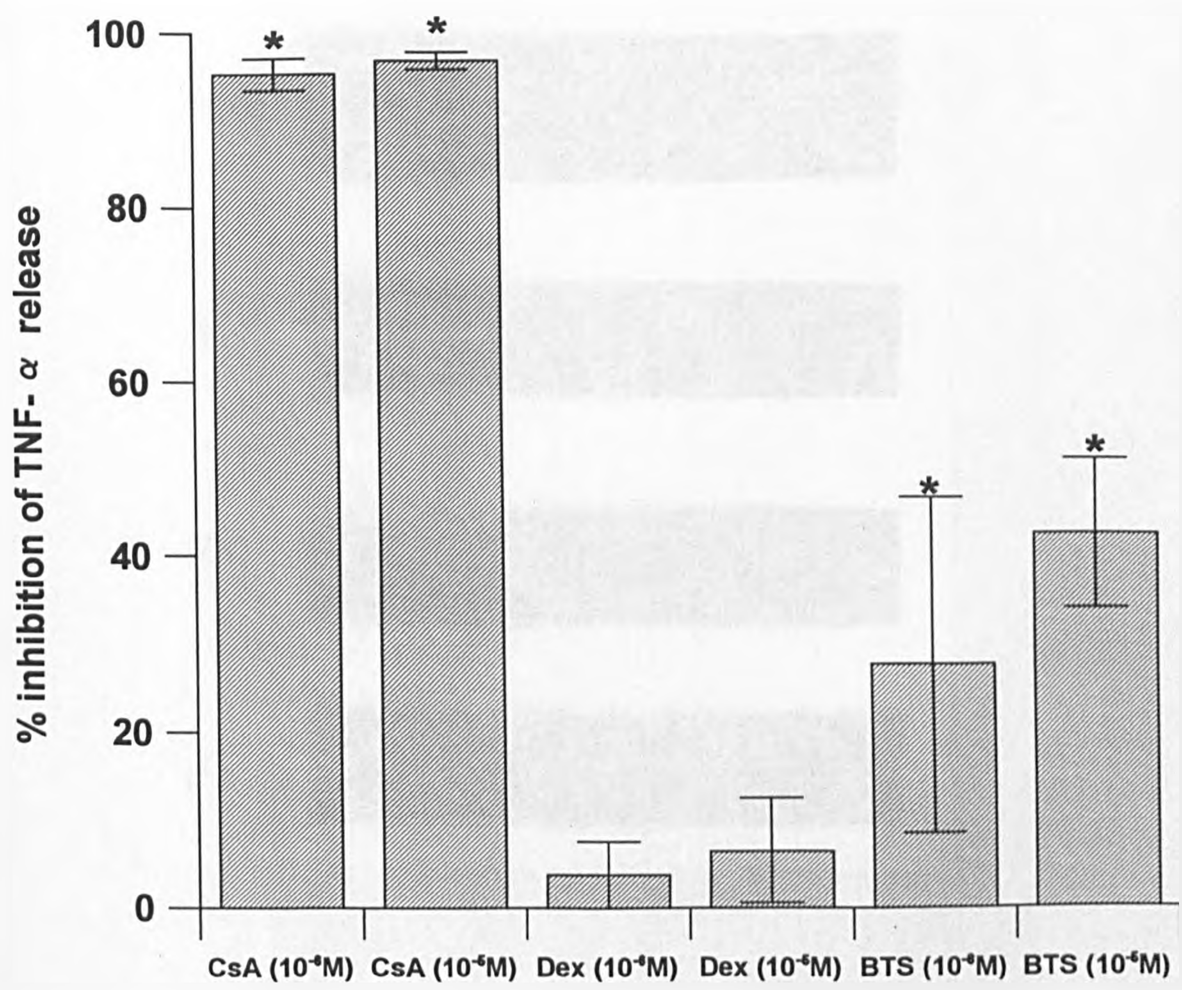


Fig. 5.2.5. Investigation of the effects of CsA, Dex or BTS 71321 on the release of TNF-*α* from Cl.MC/C57.1 mast cells. Cells were cultured with or without drugs at either 10^{-5}M or 10^{-6}M for 24 h. The cells were then pelleted and supernatant fractions removed and assayed for TNF-*α* bioactivity. Results are expressed as percentage inhibition of TNF-*α* release from non-drug treated control cells and are shown as means ± SEM for four experiments (* $p < 0.05$ for comparison of drug-treated cells with control cells as determined by the two-tailed Mann Whitney *U* test).

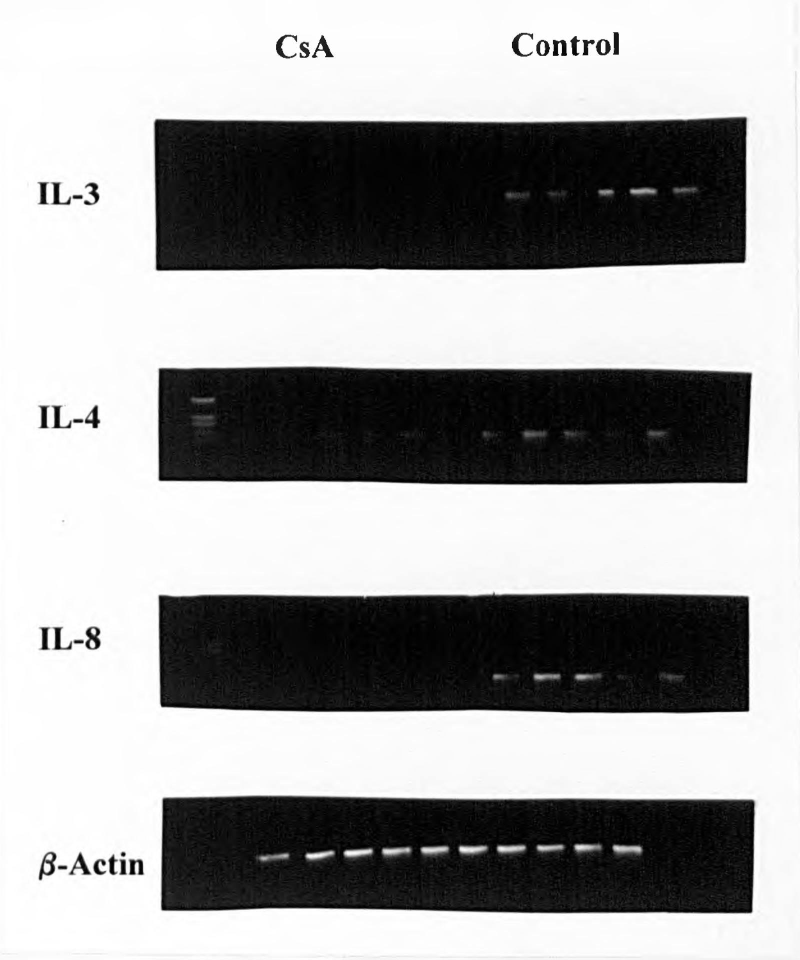


Fig. 5.2.6. RT-PCR analyses of mRNA expression for IL-3, IL-4, IL-8 and β-actin from HMC-1 cells that had been cultured with or without CsA (10^{-6}M) for 24 h prior to stimulation with ionomycin for 4 h. Results shown are from five independent experiments. For IL-4 and IL-8 the left hand lane shows molecular weight markers. For each cytokine the first five samples are from CsA treated cells; the next five samples are from control non drug-treated cells.

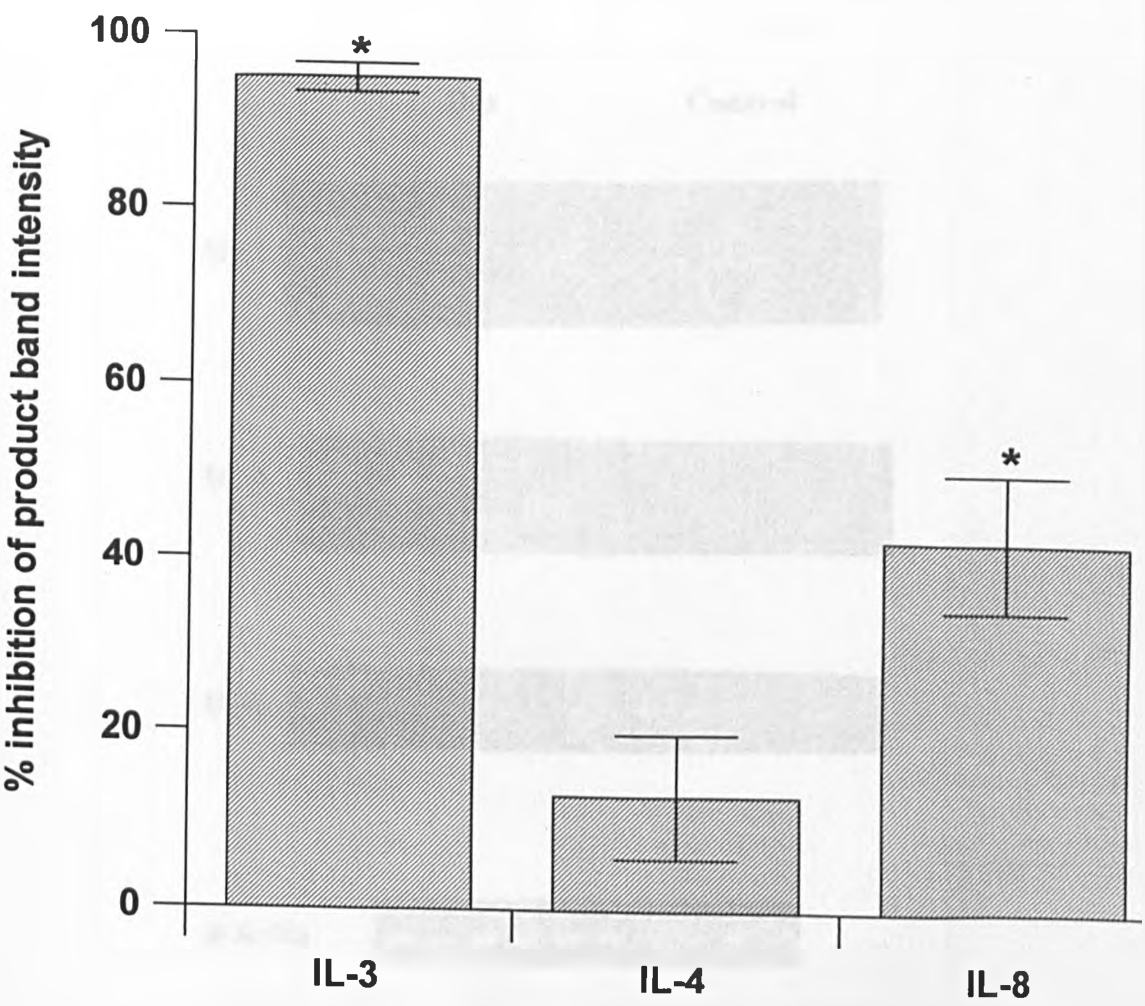


Fig. 5.2.7. Evaluation of the activity of CsA (10^{-6}M) on mRNA expression for IL-3, IL-4 and IL-8 by HMC-1 cells. Cells were cultured with or without CsA for 24 h prior to activation with ionomycin for 4 h. RT-PCR product band intensities were assessed by densitometric analysis as areas under the curve of band peaks (mm^2). Results are expressed as percentage inhibition of cytokine expression from non-drug-treated control cells and are shown as means ± SEM for ten experiments (* $p < 0.03$ for comparison of drug treated levels with control levels as determined by the two-tailed Mann Whitney *U* test).

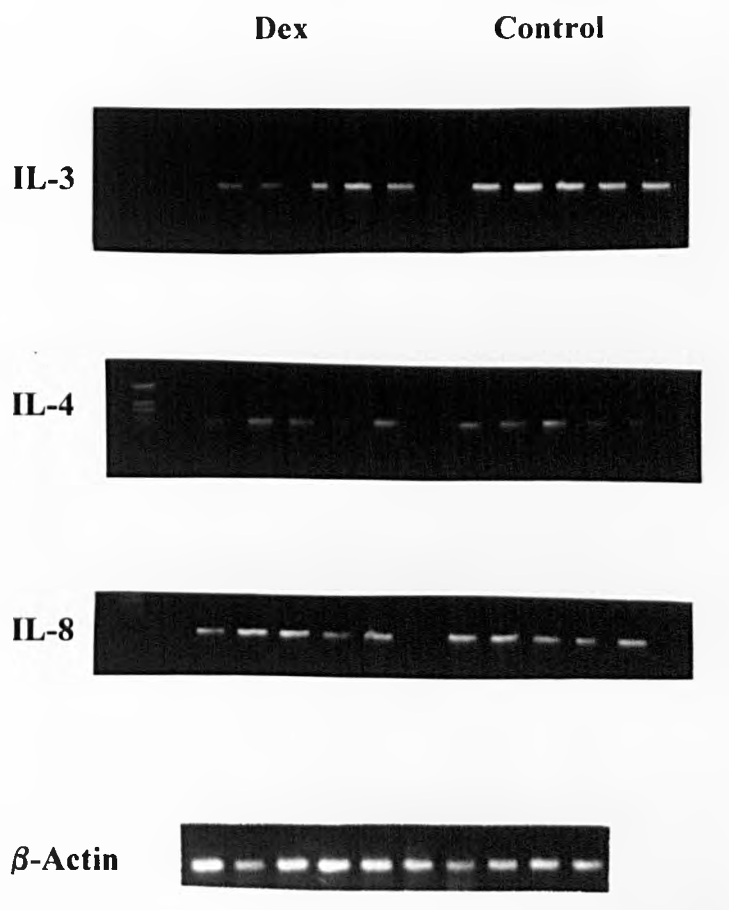


Fig. 5.2.8. RT-PCR analyses of mRNA expression for IL-3, IL-4, IL-8 and β-actin from HMC-1 cells that had been cultured with or without Dex (10^{-6}M) for 24 h prior to stimulation with ionomycin for 4 h. Results shown are from five independent experiments. For IL-4 and IL-8 the left hand lane shows molecular weight markers. For each cytokine the first five samples are from Dex treated cells; the next five samples are from control non drug-treated cells.

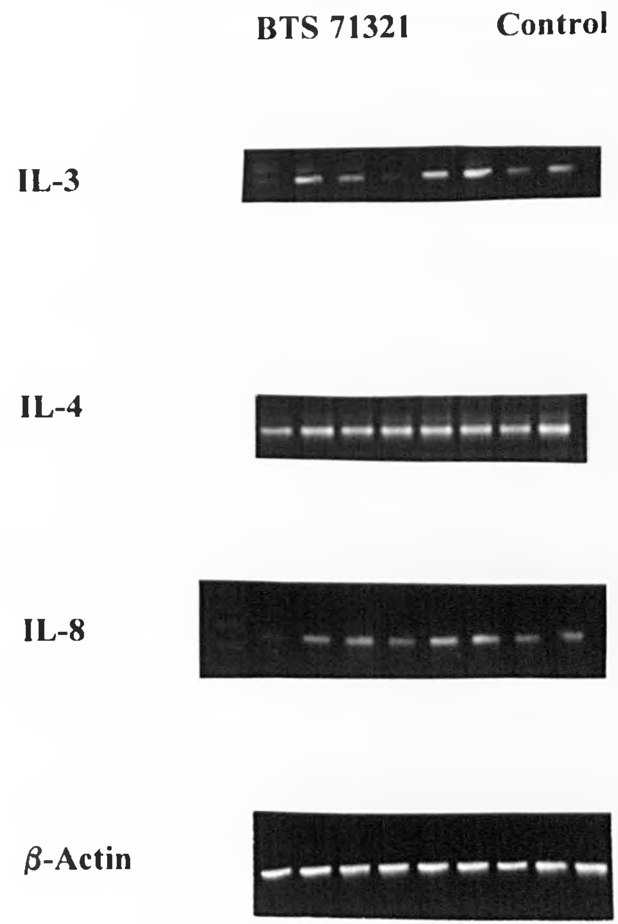


Fig. 5.2.9. RT-PCR analyses of mRNA expression for IL-3, IL-4, IL-8 and β-actin form HMC-1 cells that had been cultured with or without BTS 71321 (10^{-6}M) for 24 h prior to stimulation with ionomycin for 4 h. Results shown are from five independent experiments. For IL-3 and IL-4 lanes 1-4 are with drug, 5-8 no drug. For IL-8, a molecular weight marker is run in the left hand lane. Lanes 2-5 are with drug, 6-9 no drug. For β-Actin lanes 1-5 are with drug, 6-9 no drug.

CHAPTER 6

Cytokine expression and patterns of cell infiltration in the rat Sephadex model of lung inflammation

6.1 INTRODUCTION

6.2 RESULTS

6.2.1. Histological changes in rat lung tissue

6.2.2. Cellular content of bronchoalveolar lavage fluid

6.2.3. Expression of mRNA for IL-5, IL-6, TNF-*α*, MIP-2 and IFN-*γ* in bronchoalveolar lavage cells

6.2.4. Messenger RNA expression of IL-6 and TNF-*α* in rat lung tissue

6.2.5. Cellular sources of IL-6 and TNF-*α* mRNA in rat lung tissue

6.3 DISCUSSION

6.3.1. Cell mobilisation in the rat Sephadex model of lung inflammation

6.3.2. Cytokine expression in the rat Sephadex model of lung inflammation

6.3.3. Concluding remarks

6.1 INTRODUCTION

Asthma is characterized by episodes of acute and chronic narrowing of the airways associated with increased responsiveness to exogenous and endogenous stimuli and chronic airways inflammation. Constriction of the airways, causing many of the symptoms of asthma including wheeze and breathlessness, is a result of smooth muscle contraction following mediator release from inflammatory cells. The late phase reaction, which is clinically defined as bronchoconstriction (a fall of 20% or more in forced expiratory volume in 1 s (FEV_1) or a 50% fall in airway conductance) occurring 3 - 12 h after the early phase reaction to inhalation allergen challenge, is observed in approximately 50% of allergic asthmatic patients and in about 30% of the population with exercise induced asthma. A number of features, including occlusion of the bronchial lumina with a mixture of mucus, serum proteins and cellular debris, apparent thickening of the epithelial basement membrane and oedema and leucocyte infiltration, can be attributed to chronic inflammatory processes. These changes in the pathology of the lung can reach life threatening proportions if the disease is not monitored.

Traditionally mast cells have been associated with the initial allergen provoked bronchoconstriction. Cross-linkage of high affinity IgE receptors on their surfaces leads to degranulation and release of preformed and *de novo* synthesised chemical mediators which induce bronchoconstriction, mucus production and oedema. Nevertheless, there has been a recent shift of interest towards the involvement of other cells in the pathology of

asthma. The infiltration of eosinophils into the lung is believed to be a major pathogenic event. These cells express the low affinity IgE receptor (FcεRII) (Capron *et al.*, 1981) providing them with the capacity to interact directly with inhaled allergen and to degranulate, releasing preformed and newly synthesised mediators, some of which are known to be toxic to the human respiratory epithelium. Thus, eosinophils may be key cells in the development of inflammation of the airways and important contributors to the late asthmatic reaction. The role of neutrophils in human asthma is less clearly defined. Studies comparing levels of neutrophils in BAL fluid from asthmatic patients to controls report widely differing values, although these differences may be due to differences in the timing of lavage. Bronchoalveolar lavage performed 4 h post allergen challenge reveals elevated numbers of both neutrophils and eosinophils. Subsequently, neutrophil numbers decline rapidly whereas eosinophil counts remain elevated at 24 h post challenge (Metzger *et al.*, 1986). In occupational forms of asthma neutrophils appear to have a more dominant role (Fabbri *et al.*, 1987). Also, in sudden-onset fatal asthma, as opposed to slow-onset fatal asthma, there is a relative paucity of eosinophils in the face of an excess of neutrophils in the airway submucosa (Sur *et al.*, 1993). Macrophages constitute the major cells in the BAL fluid of both asthmatic and normal subjects (Eschenbacher and Gravelyn, 1987) and it has been reported that they become activated and increase in number after allergen challenge (Carroll *et al.*, 1985; Durham *et al.*, 1984; Metzger *et al.*, 1987). Activated macrophages are known to secrete a variety of chemotactic

factors including platelet activating factor (PAF), LTB_4 and an array of peptides (Nathan, 1987) and they have been implicated in the promotion of leucocyte recruitment during the late phase reaction and the associated increase in airway responsiveness observed with allergen challenge (Woolcock, 1988).

It is still unclear as to how eosinophils and other inflammatory cells initially accumulate and become activated in the bronchial epithelium. Although they are known to express low affinity IgE receptors on their cell surfaces, this cannot fully account for their substantial accumulation and activation observed after allergen challenge. The growing awareness of the role of cytokines in allergic disease does however point to several candidates which may be important in the initiation and maintenance of the asthmatic reaction (Arai *et al.*, 1990; Gordon *et al.*, 1990; Barnes, 1994). For example, studies on bronchial biopsies and BAL cells from patients with asthma have revealed increased mRNA expression for IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, GM-CSF and TNF-α (Hamid *et al.*, 1991; Gosset *et al.*, 1991, 1992; Ying *et al.*, 1991; Marini *et al.*, 1992; Gelder *et al.*, 1993; Broide *et al.*, 1992; Robinson *et al.*, 1992, 1993; Walker *et al.*, 1992; Okubo *et al.*, 1994; Bradding *et al.*, 1994) suggesting that at least some of these cytokines may be involved in inflammatory cell recruitment and activation.

To understand more precisely the role of cytokines in the progression of inflammatory lung disease the development of appropriate animal models is essential. One such model is that of Sephadex-induced lung injury in the rat. In this model, which is used quite extensively within the pharmaceutical

industry to assess potential anti-asthmatic compounds (Spicer *et al.*, 1990; Asano *et al.*, 1992), Sephadex particles become lodged in the capillaries of the lung, and this leads to lung eosinophilia and airway hyperresponsiveness (Laycock *et al.*, 1986; Cook, 1990). However, the molecular mechanisms underlying Sephadex-induced lung inflammation and the relevance of those mechanisms to clinical asthma have not been fully elucidated. The aim of the present chapter was to study patterns of cell mobilisation into the bronchoalveolar lumen and expression of mRNA by BAL cells in the rat Sephadex model. For this purpose a panel of eight cytokines, namely IL-3, IL-4, IL-5, IL-6, TNF-α, MIP-2, GM-CSF and IFN-γ were selected for investigation. RT-PCR primer pairs were designed and tested on positive rat cDNA. Five primer pairs, namely IL-5, IL-6, TNF-α, MIP-2 and IFN-γ were found to work consistently. Additionally, RNA *in situ* hybridization probes were prepared but only TNF-α and IL-6 were found to provide consistant results. Therefore, in this chapter investigations have centred on the changes in mRNA levels for IL-5, IL-6, TNF-α, MIP-2 and IFN-γ in BAL cells and associated changes in numbers of inflammatory cells in the bronchoalveolar lumen during the progression of lung inflammation in this model. Changes in levels of mRNA expression for IL-6 and TNF-α and corresponding changes in numbers of inflammatory cells in lung tissue following Sephadex injection have also been examined.

6.2 RESULTS

6.2.1 Histological changes in rat lung tissue

Twenty-four hours after a single *i.v.* injection of Sephadex via the tail vein, granuloma formation was observed in alveolar tissue and around bronchioles (Fig. 6.2.1b, 6.2.1c, compared to control tissue in Fig. 6.2.1a). Granulomas increased in size up to 72 h but by 7 days had declined markedly in size although were still evident. At all times mononuclear cells were the major cell type in granulomas. Eosinophils were observed in granulomas at 24 h and numbers increased up to 72 h (Fig. 6.2.1d), but thereafter declined to negligible levels by day 7. Neutrophils were also observed in granulomas (Fig. 6.2.1c): their intensity peaked between 24 h (Fig. 6.2.1c) and 48 h but declined markedly by 72 h. Mast cells did not appear to increase in number around the large airways but numbers did increase around smaller airways (Fig. 6.2.2b compared to control tissue in Fig. 6.2.2a).

6.2.2 Cellular content of bronchoalveolar lavage fluid

BAL fluid of control rats contained an average of approximately 6 million cells, comprising almost exclusively mononuclear cells. Sephadex treatment of the rats produced a substantial increase (approximately 3-fold) in numbers of total and mononuclear cells in BAL fluid. Total and mononuclear cell numbers peaked after 72 h and declined to baseline levels by 7 days (Fig. 6.2.3). A significant infiltrate of neutrophils into BAL fluid was observed 24 h to 72 h after Sephadex treatment but by 7 days neutrophils were

undetectable (Fig. 6.2.4). Eosinophils appeared in BAL fluid at significant levels 24 h after treatment and continued to rise to peak at 72 h, thereafter declining substantially although still present at significant levels after 7 days (Fig. 6.2.4). Numbers of eosinophils and neutrophils in BAL fluid peaked at an average of approximately 2 million per rat. Interestingly, a highly significant infiltration of mast cells was also observed 72 h post Sephadex injection but not at other time points, whereas no mast cells were detected in BAL fluid from control rats (Fig. 6.2.5.).

6.2.3 Expression of mRNA for IL-5, IL-6, TNF-α, MIP-2 and IFN-γ in bronchoalveolar lavage cells

Messenger RNA encoding IL-5, IL-6, MIP-2 or IFN-*γ* was either undetectable or was detected very weakly in BAL cells from saline-treated control rats in five separate experiments, each utilising cells pooled from two rats (Fig. 6.2.6). By contrast, messenger RNA encoding TNF-*α* mRNA was readily detectable in BAL cells from saline-treated rats in each of the five experiments (Fig. 6.2.6). Following a single *i.v.* injection of Sephadex particles into the rats, levels of mRNA encoding each of the five cytokines were elevated markedly at various stages during the inflammatory response (Fig. 6.2.6). Six hours after treatment, IL-6 mRNA was detected in 4/5 exeriments, TNF-*α* mRNA was elevated in 5/5 experiments, whereas IL-5, MIP-2 and IFN-*γ* mRNA were not yet detectable in any experiment (Fig. 6.2.6). After 24 h, induction of mRNA for each cytokine except IL-5 was seen (IL-6 in 3/5, TNF-*α* in 5/5, MIP-2 in 5/5 and IFN-*γ* in 4/5 experiments).

At 48 h, IL-5 mRNA appeared weakly whereas IL-6 mRNA was strongly induced in each experiment, MIP-2 mRNA had started to decline, and TNF-α and IFN-γ were maintained at high levels. At 72 h, IL-5 mRNA was detected in each experiment at maximal levels, whereas IL-6 and MIP-2 mRNA had declined. TNF-α and IFN-γ mRNA did not decline until 7 days (Fig. 6.2.6). Throughout these experiments mRNA expression for the housekeeping gene G3PDH, was not altered by injection of the Sephadex particles and levels remained constant at each time point (Fig. 6.2.6).

Analysis of band intensities by laser densitometry revealed that the Sephadex-induced increase in IL-5 mRNA was statistically significant after 48 h and 72 h, with peak values at 72 h (Fig. 6.2.7); IL-6 mRNA levels were significantly elevated only at 48 h (Fig. 6.2.8); TNF-α mRNA levels were significantly elevated from 6 h to 72 h (Fig. 6.2.9); MIP-2 mRNA was significantly elevated from 6 to 72 h with peak values at 24 h (Fig. 6.2.10); IFN-γ mRNA was significantly elevated from 24 h to 7 days (Fig. 6.2.11). Messenger RNA levels of all cytokines, except IFN-γ, had declined to baseline by 7 days. No changes were seen in the intensity of G3PDH RT-PCR products (Fig. 6.2.12).

6.2.4 Messenger RNA expression for IL-6 and TNF-α in rat lung tissue

In only one out of five control rats was mRNA expression for IL-6 detected weakly in epithelial cells surrounding large airways in lung tissue. Typical staining for IL-6, by *in situ* hybridization, of lung tissue from a control rat is shown in Fig. 6.2.13. Following injection of Sephadex particles there was

a dramatic increase in IL-6 expression in cells lining airways and in the infiltrating inflammatory cells. Messenger RNA expression for IL-6 was most intense at the 6 h (Fig. 6.2.14) and 24 h (Fig. 6.2.15) time points. After 24 h, IL-6 mRNA levels appeared to decline in the inflammatory cell granulomas particularly at the 72 h time point (Fig. 6.2.16 - Fig. 6.2.17), but continued to remain intensely elevated in cells lining airways (Fig. 6.2.16 - Fig. 6.2.17). TNF-α mRNA expression was detected in cells lining large airways from 4/5 control rats (Fig. 6.2.18). Six hours after Sephadex injection, levels in epithelial cells had increased dramatically (Fig. 6.2.19). There was also increased expression of TNF-α by infiltrating inflammatory cells. Expression of TNF-α continued to increase up to the 24 h time point (Fig. 6.2.20) but thereafter levels decreased in inflammatory cell granulomas although at 48 h (Fig. 6.2.21) and 72 h (Fig. 6.2.22), the last time point investigated, TNF-α mRNA was still markedly elevated in cells lining airways. In each experiment, controls stained with mRNA sense *in situ* hybridization probes were negative.

6.2.5 Cellular sources of IL-6 and TNF-α mRNA in rat lung tissue

In situ hybridization studies did not conclusively determine which inflammatory cells were responsible for the increased mRNA expression of IL-6 and TNF-α in rat alveolar lung tissue. However, results clearly demonstrated that mRNA for IL-6 and TNF-α was expressed by epithelial cells lining airways (Fig. 6.2.14 - Fig. 6.2.22). Sequential sections that had been stained for neutrophils or either TNF-α or IL-6 indicated that TNF-α or

IL-6 mRNA was localised in areas were neutrophils were seen (Fig. 6.2.20), but it was difficult to be certain of the precise cellular location of gene transcripts.

6.3 DISCUSSION

6.3.1 Cell mobilisation in the rat Sephadex model of lung inflammation

Inflammation of the lung leading to asthma-like disease can either be antigen/IgE-driven or non-antigen-driven. The study reported in this chapter examined a non-antigen-driven model of lung inflammation with regard to patterns of cell mobilisation and cytokine gene expression. In many respects the changes observed resembled those associated with the antigen-driven form of lung disease. A single tail vein injection of Sephadex particles into rats led to lung granuloma formation, particularly around the small airways, mobilisation of mononuclear cells, neutrophils, eosinophils and mast cells, and transient expression of several cytokine genes that are known to be important in immune and inflammatory reactions.

The patterns of cell infiltration into granulomas and BAL fluid resembled those seen in asthma. Mononuclear cells were the major component of granulomas and BAL cells; their numbers increased to reach peak levels 72 h after the Sephadex injection. Neutrophils and eosinophils appeared transiently in granulomas and BAL cells; in both sites neutrophil numbers peaked at 24 to 48 h and eosinophils at 48 to 72 h. This sequential mobilisation of neutrophils and eosinophils, against a background

of mononuclear cell mobilisation, is a well established pattern in antigen-driven asthma in animals and man (Djukanovic *et al.*, 1990; Elwood *et al.*, 1991). Mobilisation of neutrophils has not previously been reported in the Sephadex model of rat lung inflammation (Laycock *et al.*, 1986; Cook, 1990), perhaps because earlier studies focused on the influx of cells at later time points (3 days onwards) and therefore would have missed the early (24 to 48 h) appearance of these cells. The observation that neutrophils appear transiently at this early stage raises several questions regarding their role in the progression of the inflammation, and also the nature of the signals required for their activation. Almost certainly their mobilisation is not dependent on antigen/IgE-dependent activation of mast cells, since it is induced by a physical stimulus and is too early to involve an IgE response. Conceivably, non-antigen-dependent activation of mast cells could lead to rapid generation of mast cell-derived cytokines. Interestingly, mast cells did increase in number around small airways following injection of Sephadex particles, and a noticeable and highly significant mast cell influx was detected in BAL fluid 72 h post Sephadex injection. The increases in mast cells observed within this model are reminiscent of increases in mast cell numbers reported in bronchial epithelium of both allergic and non-allergic asthmatic subjects (Gibson *et al.*, 1993) and this mast cell mobilisation could be responsible for driving further airway inflammation.

6.3.2 Cytokine expression in the rat Sephadex model of lung inflammation

At a molecular level, Sephadex-induced lung inflammation was associated

with a transient expression of mRNA for IL-5, IL-6, TNF-*α*, MIP-2 and IFN-*γ* in BAL cells. Of the five cytokine genes, only TNF-*α* was constitutively expressed. MIP-2 and TNF-*α* were the earliest induced genes, with mRNA levels for both significantly elevated 6 h after the Sephadex treatment. Therefore, the question arises as to whether MIP-2 or TNF-*α* may be responsible for the early mobilisation of neutrophils. Both MIP-2 (Feng *et al.*, 1995; Appelberg, 1992) and TNF-*α* (Yates *et al.*, 1993) are known to induce neutrophil influx. However, because MIP-2 expression peaked sharply at 24 h (at or slightly before the peak neutrophil response) whereas TNF-*α* expression was sustained for 72 h (some time beyond the peak neutrophil response), MIP-2 is probably the most likely candidate in this regard. MIP-2 is an important neutrophil chemoattractant in rodents: neutralisation of MIP-2 reduced substantially the influx of neutrophils in a mouse model of peritonitis (Appelberg, 1992) and in a rat model of glomerulonephritis (Feng *et al.*, 1995) and it is likely that in rodents MIP-2 plays a similar role to that of IL-8 in man, as a neutrophil chemoattractant and activating agent (Appelberg, 1992; Feng *et al.*, 1995). Although one cannot say whether the neutrophil contributes to further inflammatory changes in the rat Sephadex model, there is accumulating evidence to implicate the neutrophil in certain forms of asthma (Tanizaki *et al.*, 1993; Basha *et al.*, 1994) and studies on patients who died from sudden-onset fatal asthma have revealed a significant increase in neutrophils as opposed to eosinophils in the airway mucosa and submucosa (Sur *et al.*, 1993).

Bronchoalveolar eosinophilia is a feature of the late asthmatic reaction

(De Monchy *et al.*, 1985; Gibson *et al.*, 1989; Adelroth *et al.*, 1990; Djukanovic *et al.*, 1990; Rossi *et al.*, 1991; Bentley *et al.*, 1993; Bousquet *et al.*, 1994) and recent reports have indicated that infiltrating cells in the late asthmatic reaction transcribe increased mRNA for IL-5 (Bentley *et al.*, 1993; Hamid *et al.*, 1991). A similar pattern of events is described here in the Sephadex model of lung inflammation in the rat: eosinophilia and IL-5 mRNA expression peaked at 72 h post-treatment. Although IL-5 is a known chemoattractant for eosinophils (Wang *et al.*, 1989), as yet a causal relationship between IL-5 induction and eosinophilia cannot be confirmed in this model. Equally, one cannot comment on the cellular source of IL-5 in rat lung although T cells (Bentley *et al.*, 1993; Robinson *et al.*, 1992; Walker *et al.*, 1991a, 1991b, 1992), mast cells (Bradding *et al.*, 1994; chapter 5) and eosinophils (Bradding *et al.*, 1994) are candidates.

Results also demonstrate a significant increase in mRNA expression for IL-6, TNF-α and IFN-γ in BAL cells following Sephadex injection. *In situ* hybridisation studies on rat lung tissue also revealed a dramatic increase in mRNA expression for IL-6 and TNF-α in cells surrounding airways and in inflammatory granulomas. IL-6, TNF-α and IFN-γ have multiple pro-inflammatory and immune regulatory activities (Barnes, 1994). IL-6 up-regulates IL-4-dependent synthesis of IgE (Vercelli *et al.*, 1989) and acts as a co-stimulatory factor with other cytokines on a variety of inflammatory and immune cells including T cells (Tosato *et al.*, 1988), and keratinocytes (Grossmann *et al.*, 1989; Yoshizaki *et al.*, 1990). IFN-γ amplifies the release of TNF-α from alveolar macrophages induced by IgE triggering or by

endotoxin (Gosset *et al.*, 1992; Gifford and Lohmann-Matthess, 1987) and increases the expression of MHC class I and class II on macrophages, epithelial cells and mast cells (Barnes, 1994; Warbrick *et al.*, 1995). TNF-*α* is a potent chemoattractant and activator of neutrophils and eosinophils and causes bronchial hyperresponsiveness and airway inflammation in the rat (Kips *et al.*, 1992). Although the results indicate that these cytokines are induced in BAL cells following injection of Sephadex particles, as yet the cellular source of IL-6, TNF-*α* and IFN-*γ* within the lung is unknown. Equally, their role within the Sephadex model of lung inflammation is as yet unidentified, although they may contribute to the later phases of airway inflammation. *In situ* hybridisation studies on the expression of IL-6 and TNF-*α* within rat lung tissue were not entirely successful in that they did not conclusively determine which cells were responsible for the induced expression of these cytokines. In hindsight, it appears that double labelling techniques are necessary to provide evidence of the cellular sources of these cytokines and results discussed within this chapter can only tentatively suggest possible candidates. It does appear that epithelial cells are a major source of both IL-6 and TNF-*α* and indeed there is evidence of increased expression of IL-6 in airway epithelium of asthmatic patients (Marini *et al.*, 1992). Neutrophils may also provide a source of both IL-6 and TNF-*α* since histological staining and *in situ* hybridisation revealed mRNA expression for IL-6 and TNF-*α* in the vicinity of neutrophils. However, mononuclear cells and eosinophils are also likely candidates. Haemotoxylin and eosin staining of lung tissue sections revealed increased numbers of mononuclear cells and

eosinophils surrounding airways at various time points after injection of Sephadex particles. Equally, inflammatory granulomas consisted of mainly mononuclear cells with appreciable numbers of neutrophils and eosinophils at various time points. Results suggest that each of these cell types is involved in the induced expression of both IL-6 and TNF-α and indeed macrophages, T cells, eosinophils and neutrophils are known to provide a source of pro-inflammatory cytokines (Kasama *et al.*, 1993; McCain *et al.*, 1993; Tiku *et al.*, 1986; Bazzoni *et al.*, 1991; Costa *et al.*, 1993; Bauer *et al.*, 1988; Denburg, 1993).

Mast cells may also contribute to the increased mRNA expression for cytokines during the latter stages of the Sephadex-induced response, particularly at the 72 h time point when mast cells appeared in BAL fluid. In chapter 5, immunologically activated mature connective tissue-type mast cells were found to express mRNA for IL-5, IL-6, TNF-α, MIP-2 and IFN-γ and to secrete TNF-α protein. Murine mast cell lines and clones are also known to provide a source of IL-1, IL-3, IL-4, IL-5, IL-6, IL-13, IFN-γ, TCA3, MIP-1α, MIP-1β and JE (Brown *et al.*, 1987; Burd *et al.*, 1989, 1995; Plaut *et al.*, 1989) and IL-1β, IL-3, IL-4, IL-8 and TNF-α are known to be expressed by the HMC-1 human mast cell line (Möller *et al.*, 1993; Sillaber *et al.*, 1993; Buckley *et al.*, 1995). Additionally, a seven-fold increase was detected in the number of mast cells staining for TNF-α in human asthmatic biopsies as well as mast cell localisation of IL-4, IL-5 and IL-6 (Bradding *et al.*, 1994) . Mast cells also accounted for 90% of the IL-4 immunoreactive cells from biopsies taken from patients with perennial allergic rhinitis and $>$ 90% of IL-

6 and > 50% of IL-5 immunoreactive cells (Bradding *et al.*, 1993). Mast cells have the capacity for mediator storage and are known to contain stores of preformed TNF-α protein. Furthermore, IL-4, IL-5 and IL-6, as well as localising to mast cells in asthmatic airways, may be contained in prestored forms (Bradding *et al.*, 1994). In contrast to T cells and monocytes/macrophages, the presence of IL-4, IL-5, IL-6 and TNF-α in stored forms within mast cells may provide a ready source of these cytokines in response to appropriate stimulation by either IgE/antigen or, as is the case in the rat Sephadex model of lung inflammation, by a non-antigenic stimulus.

Of the five cytokines that were shown to be induced in BAL cells, previously mRNA expression for IL-5, IL-6 and TNF-α has been reported to be up-regulated in asthma, whereas increased mRNA expression for IFN-γ has not been reported (Bentley *et al.*, 1993; Hamid *et al.*, 1991; Gosset *et al.*, 1991; Ying *et al.*, 1991; Marini *et al.*, 1992; Gelder *et al.*, 1993; Broide *et al.*, 1992; Robinson *et al.*, 1992; Walker *et al.*, 1992; Bradding *et al.*, 1994). However, Okubo *et al.* (1994) have reported an increase in IFN-γ in peripheral blood mononuclear cell culture supernatants obtained from patients with mite sensitive asthma, suggesting it may be induced during certain forms of human asthma. The induction of MIP-2 has not previously been reported in asthma although IL-8, the major neutrophil chemotactic factor in the human lung, is known to be up-regulated in bronchial epithelial cells of asthmatic patients (Marini *et al.*, 1992).

6.3.3 Concluding remarks

In conclusion, this study has demonstrated that Sephadex-induced lung inflammation in the rat is associated with transient increases in lung mononuclear cells, neutrophils and eosinophils, and these changes occur alongside transient induced expression of genes encoding the cytokines IL-5, IL-6, TNF-α, MIP-2 and IFN-γ. More so than was previously thought, the pathological processes and molecular events observed in this model resemble closely those seen in antigen-driven asthma, suggesting common pathoaetiological mechanisms in asthma driven by either a physical or immunological stimulus. Although at this stage these findings are largely descriptive, the similarities between what is now known about Sephadex-induced rat lung inflammation and human asthma suggest that this model could be exploited extensively for elucidation of the relationships between induction of certain cytokines and the progression of asthma-like disease.

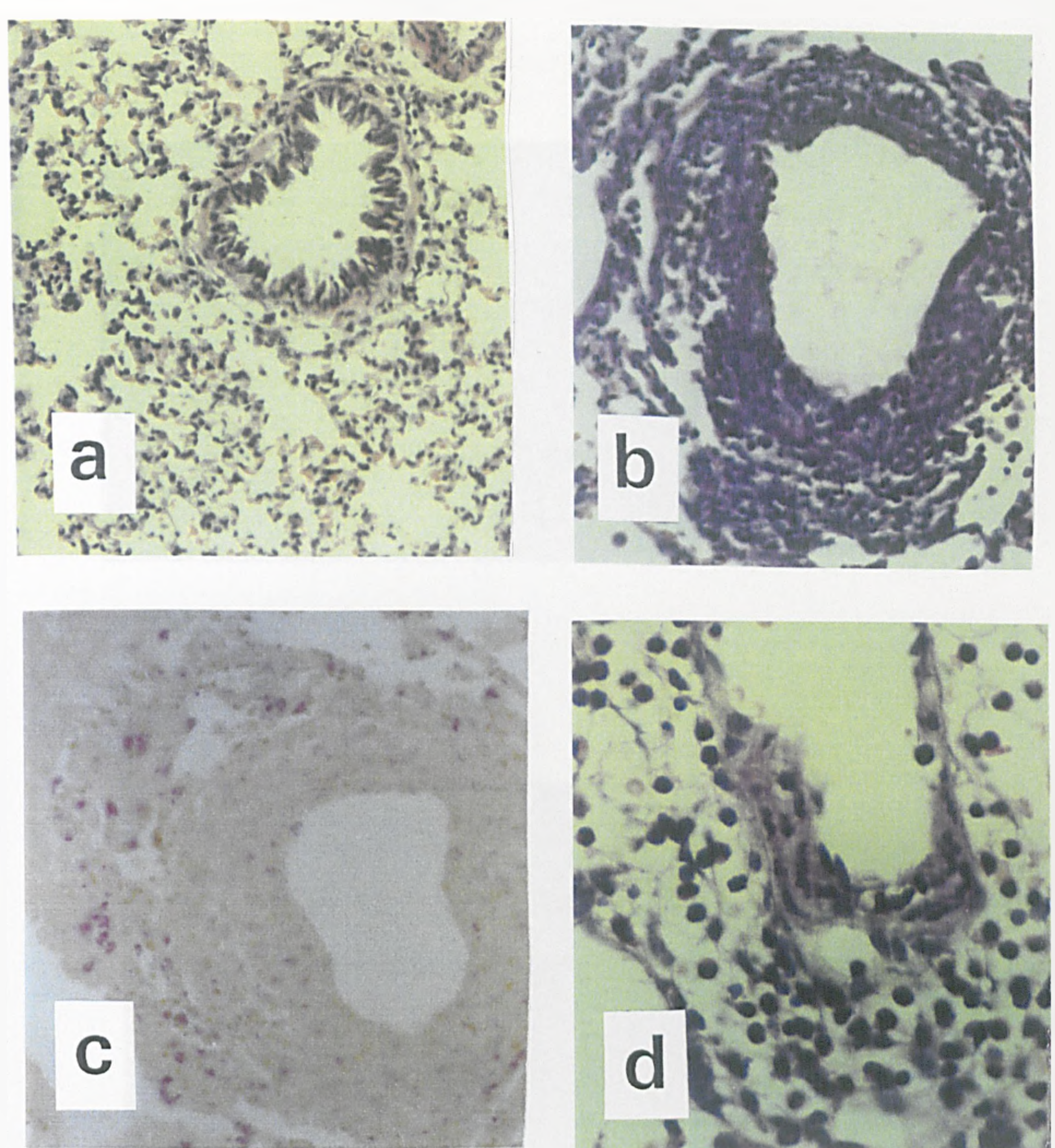
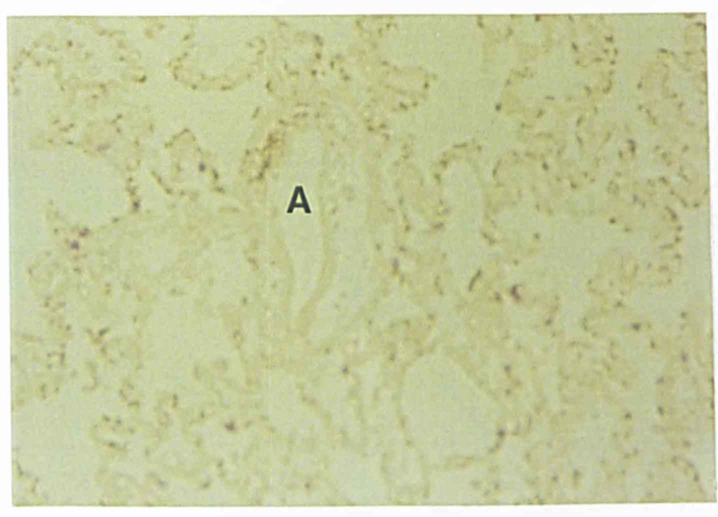


Fig. 6.2.1. Histological analysis of rat lung tissue sections. a (x200), haemotoxylin and eosin staining of a section of lung tissue from a saline treated control rat. b (x200), haemotoxylin and eosin staining of a section of lung tissue from a Sephadex-treated rat 24 h post-injection. c (x200), chloroesterase staining of a section of lung tissue from a Sephadex-treated rat 24 h post-injection (neutrophils stained pink). d (x400), haemotoxylin and eosin staining of lung tissue from a Sephadex-treated rat 72 h post-injection. b and c show granulomatous inflammation around airways following injection of Sephadex particles. c shows accumulation of eosinophils around an airway 72 h after Sephadex treatment.



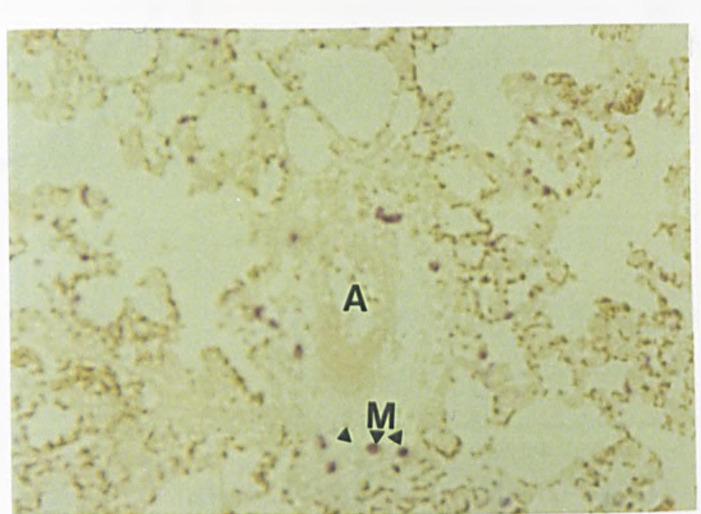


Fig. 6.2.2. Histological analysis of rat lung tissue. a (x200), chloroesterase staining of a section of lung tissue from a saline treated control rat. b (x200), chloroesterase staining of a section of lung tissue from a Sephadex-treated rat 24 h post-injection (mast cells stained red). b shows increased numbers of mast cells around a small airway following injection of Sephadex particles.

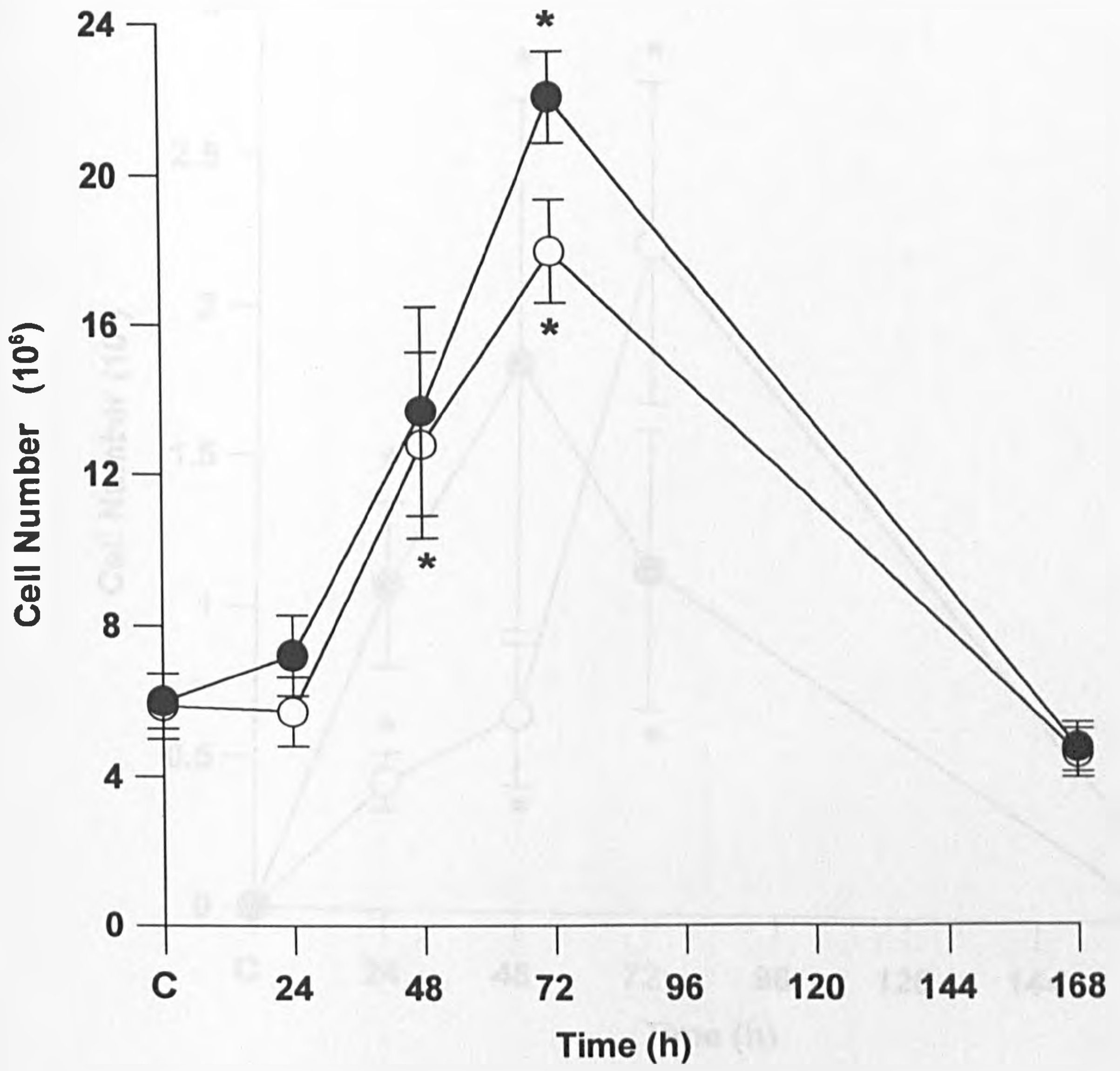


Fig. 6.2.3. Numbers of total cells (●) and mononuclear cells (○) in BAL fluid of rats following i.v. injection of Sephadex. Results are means ± SEM for 10 rats. (* $p < 0.05$, by comparison of Sephadex-treated rats to controls as determined by the two-tailed Mann Whitney *U* test). C = cell counts from saline-injected control rats.

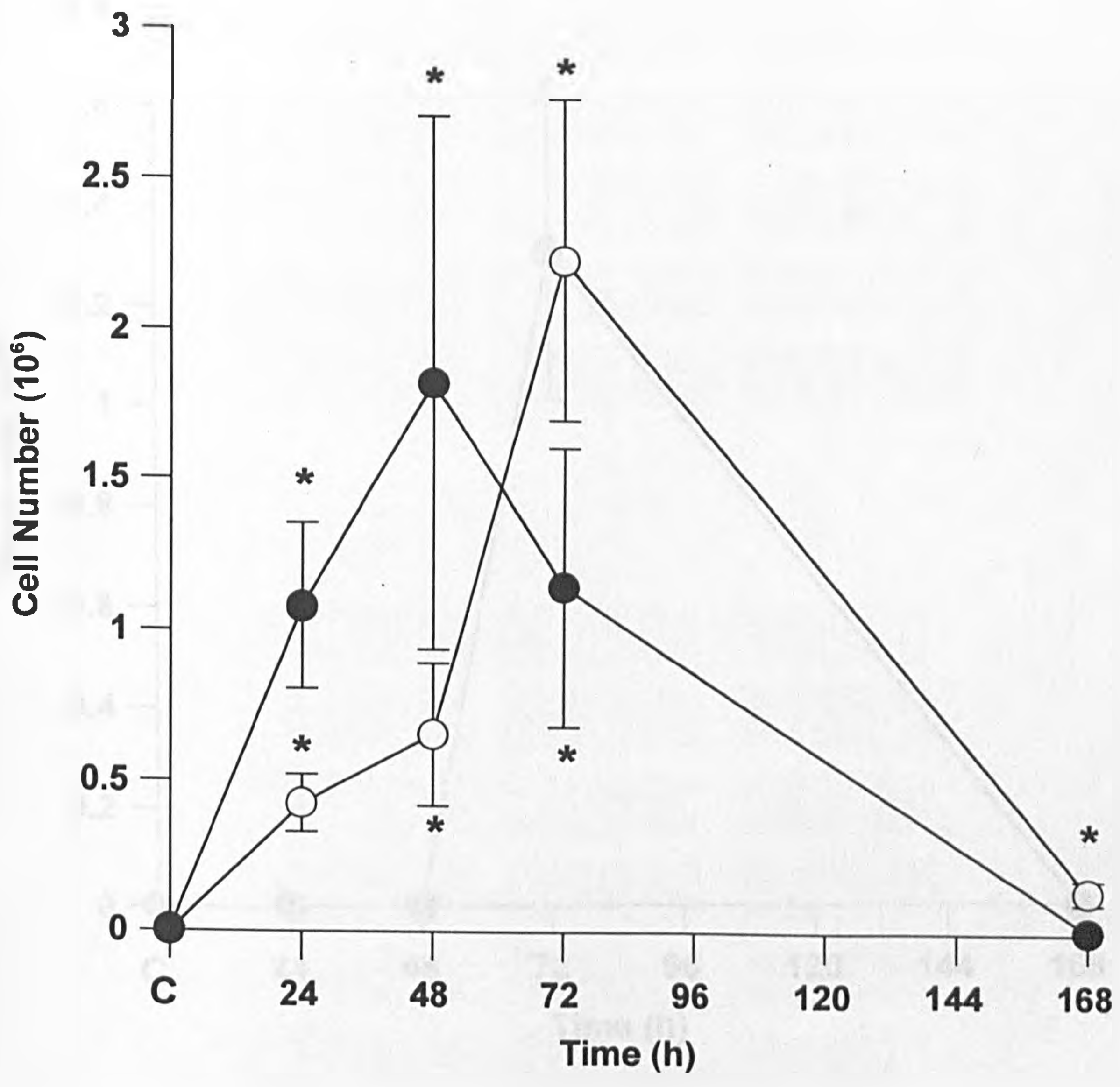


Fig. 6.2.4. Numbers of neutrophils (●) and eosinophils (○) in BAL fluid of rats following i.v. injection of Sephadex. Results are means ± SEM for 10 rats. (* $p < 0.05$ by comparison of Sephadex-treated rats to controls as determined by the two-tailed Mann Whitney *U* test). C = cell counts from saline-injected control rats.

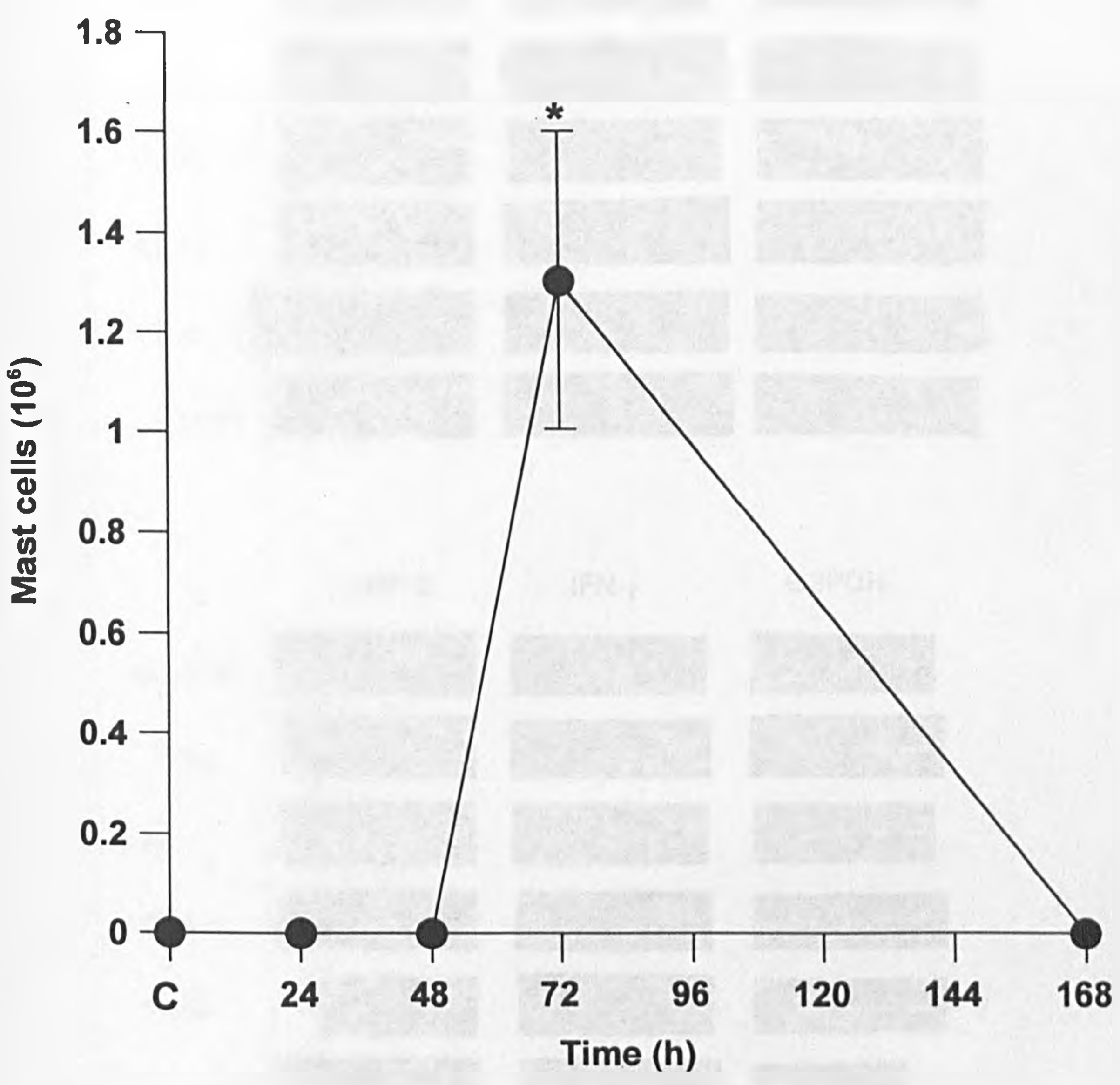


Fig. 6.2.5. Numbers of mast cells (●) in BAL fluid of rats following i.v. injection of Sephadex. Results are means ± SEM for 10 rats. (* $p < 0.05$ by comparison of Sephadex-treated rats to controls as determined by the two-tailed Mann Whitney *U* test). C = cell counts from saline-injected control rats

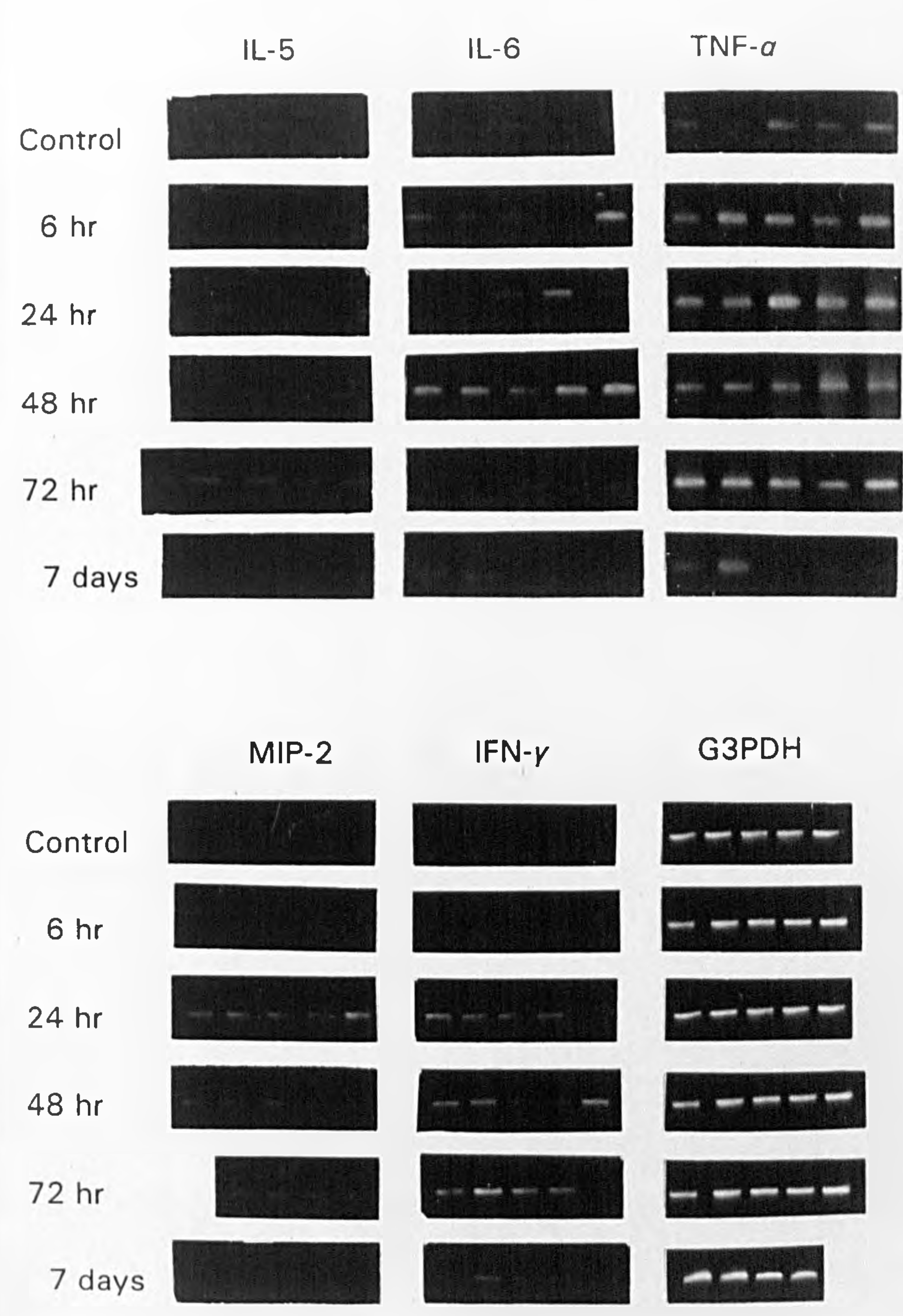


Fig. 6.2.6. RT-PCR analysis of mRNA for IL-5, IL-6, TNF-α, MIP-2, IFN-γ and G3PDH in rat BAL cells. Sephadex was injected i.v. and BAL cells obtained at the times indicated. Control refers to saline-injected rats. Each band represents DNA amplified from cDNA reverse transcribed from RNA extracted from BAL cells pooled from two rats.

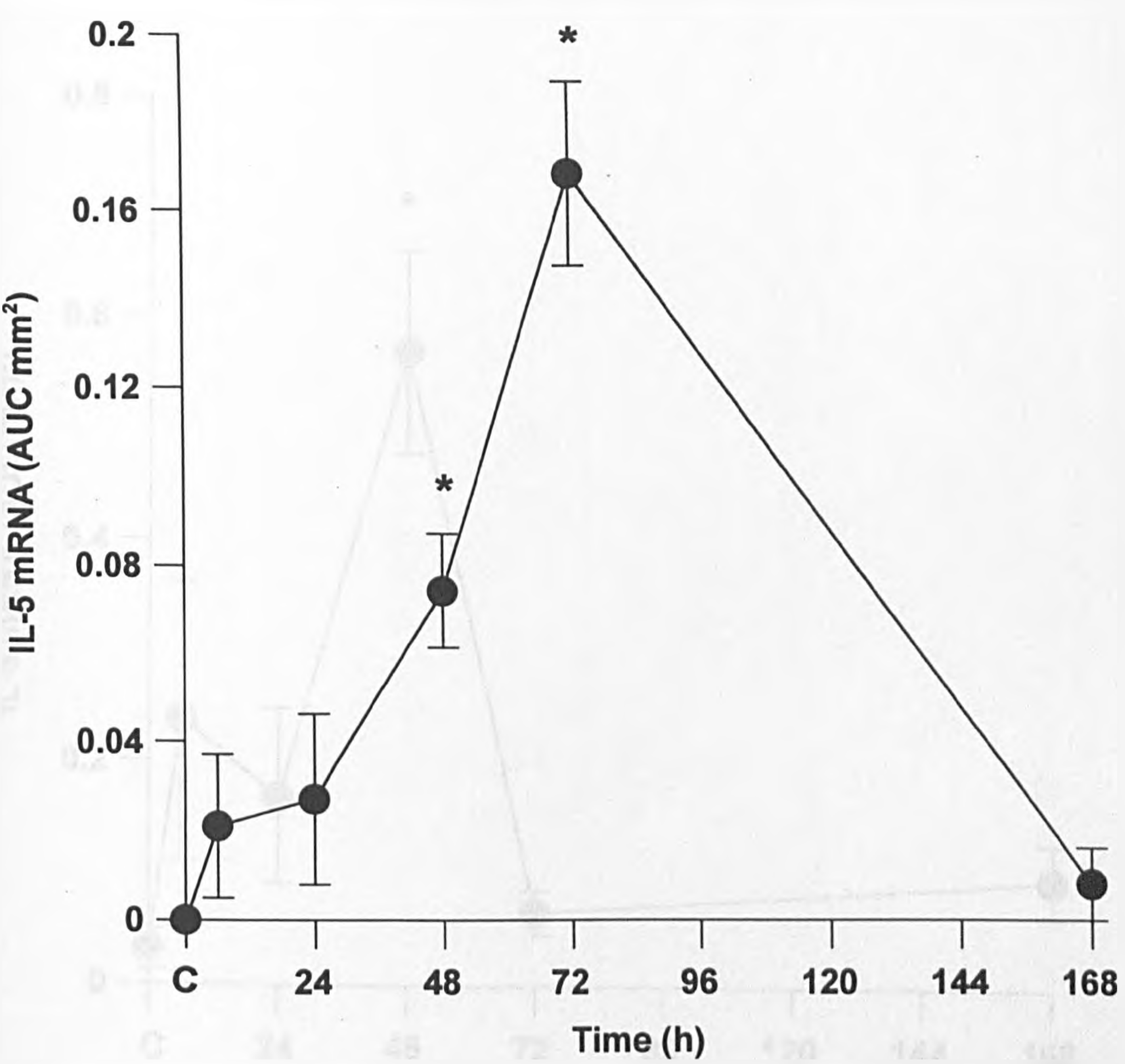


Fig. 6.2.7. Densitometric analysis of the time course of mRNA expression for IL-5 in BAL cells after treatment of rats with Sephadex. Gels were scanned by laser densitometry and the intensity of RT-PCR products calculated as area under the curve (AUC) of densitometric traces. Results are means ± SEM for five experiments, each with BAL cells pooled from two rats. (* $p < 0.05$ by comparison of Sephadex-treated rats to controls as determined by the two-tailed Mann Whitney *U* test). C = IL-5 mRNA expression from saline-injected control rats.

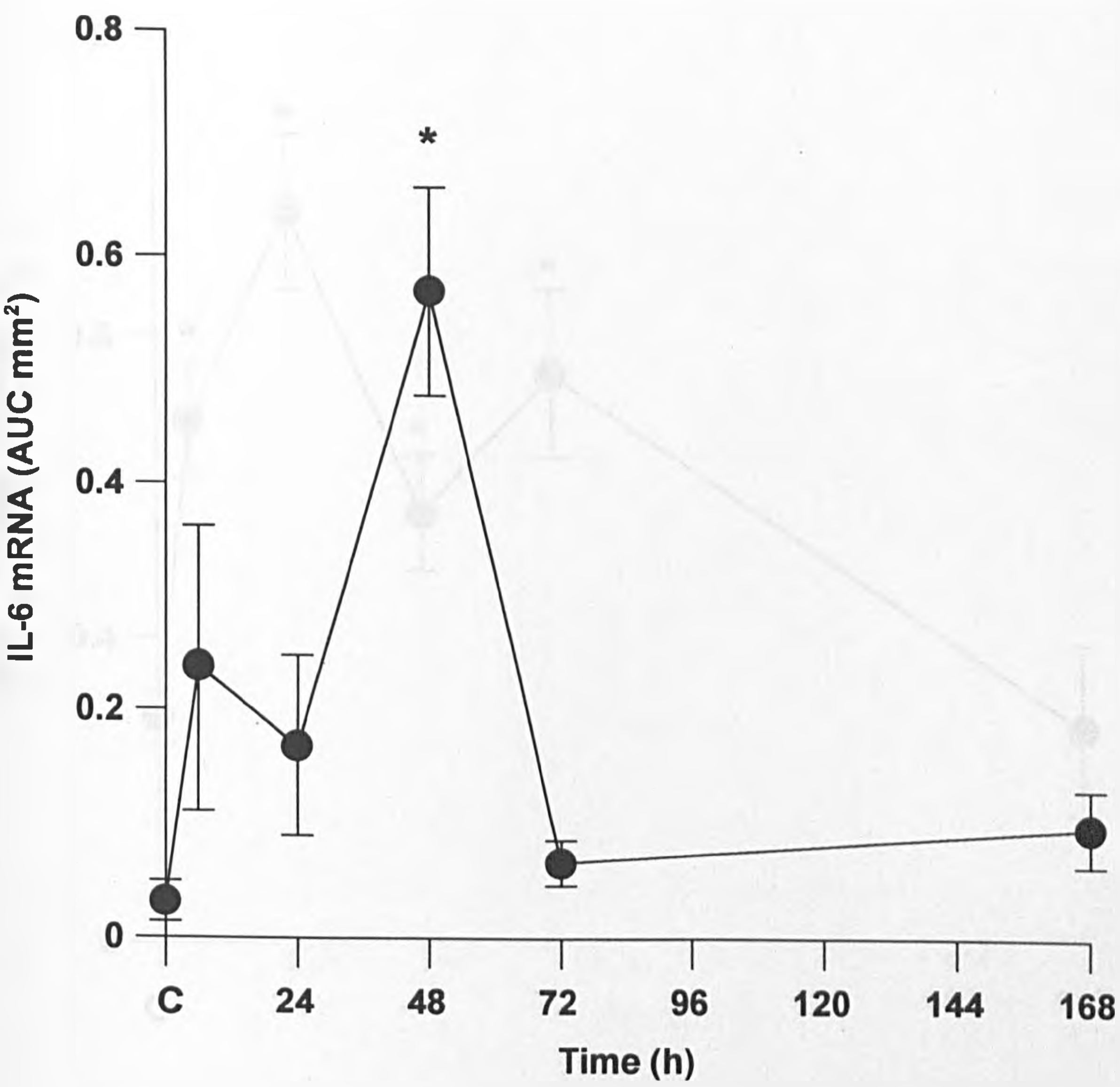


Fig. 6.2.8. Densitometric analysis of the time course of mRNA expression for IL-6 in BAL cells after treatment of rats with Sephadex. Gels were scanned by laser densitometry and the intensity of RT-PCR products calculated as area under the curve (AUC) of densitometric traces. Results are means ± SEM for five experiments, each with BAL cells pooled from two rats. (* $p < 0.05$ by comparison of Sephadex-treated rats to controls as determined by the two-tailed Mann Whitney *U* test). C = IL-6 mRNA expression from saline-injected control rats.

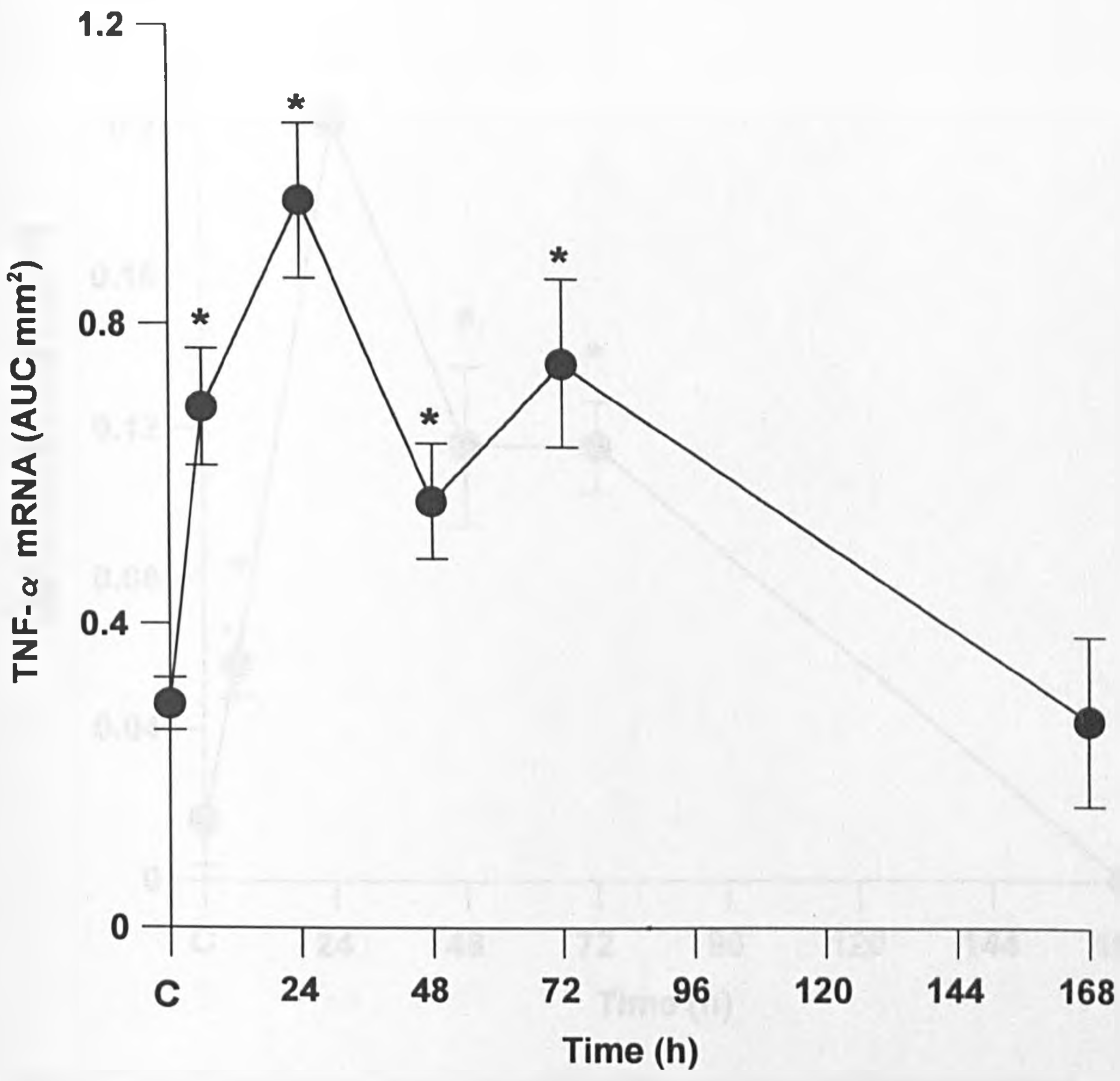


Fig. 6.2.9. Densitometric analysis of the time course of mRNA expression for TNF-*α* in BAL cells after treatment of rats with Sephadex. Gels were scanned by laser densitometry and the intensity of RT-PCR products calculated as area under the curve (AUC) of densitometric traces. Results are means ± SEM for five experiments, each with BAL cells pooled from two rats. (* $p < 0.05$ by comparison of Sephadex treated rats to controls as determined by the two-tailed Mann Whitney *U* test). C = TNF-*α* mRNA expression from saline-injected control rats.

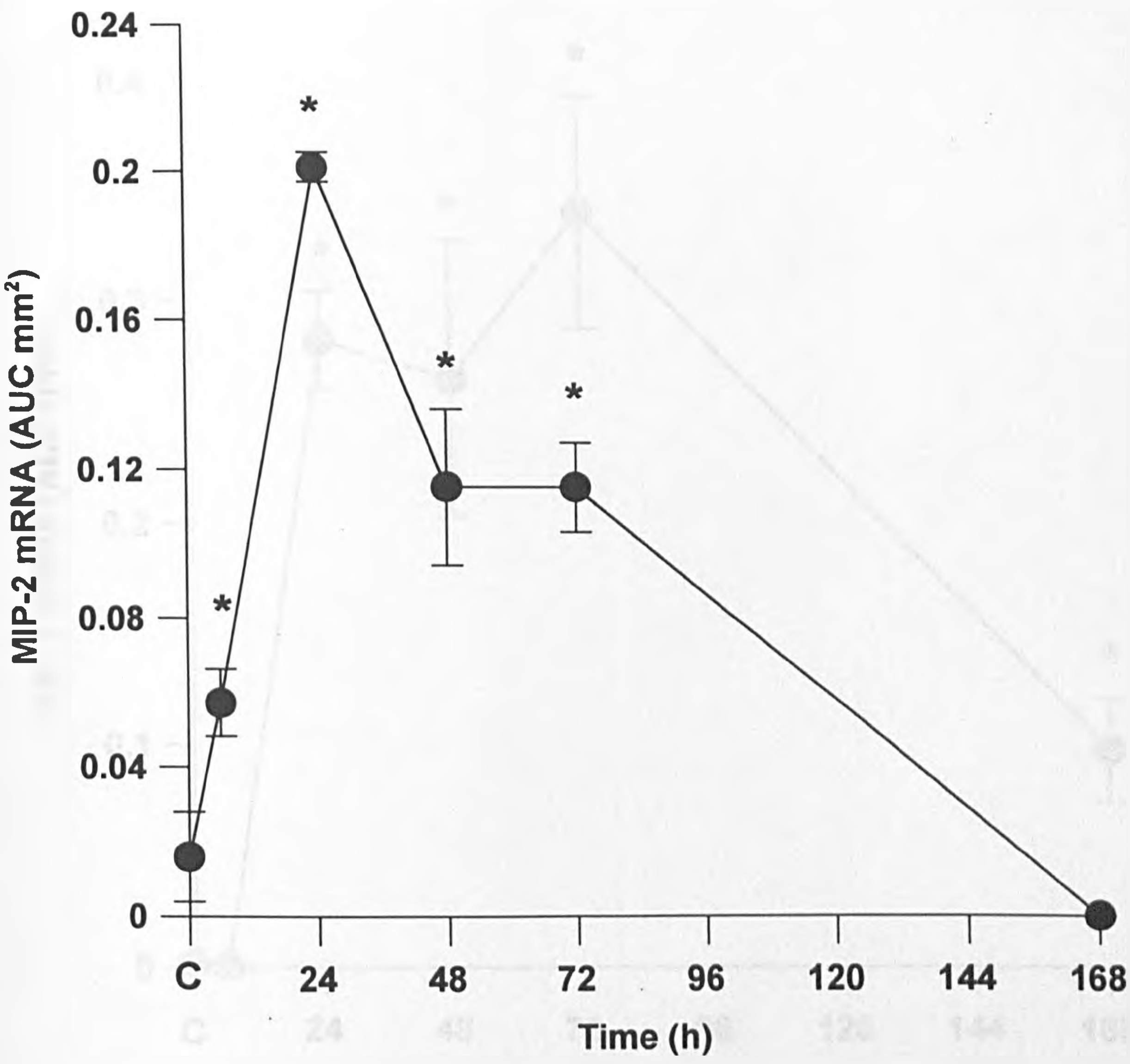


Fig. 6.2.10. Densitometric analysis of the time course of mRNA expression for MIP-2 in BAL cells after treatment of rats with Sephadex. Gels were scanned by laser densitometry and the intensity of RT-PCR products calculated as area under the curve (AUC) of densitometric traces. Results are means ± SEM for five experiments, each with BAL cells pooled from two rats. (* $p < 0.05$ by comparison of Sephadex-treated rats to controls as determined by the two-tailed Mann Whitney *U* test). C = MIP-2 mRNA expression from saline-injected control rats.

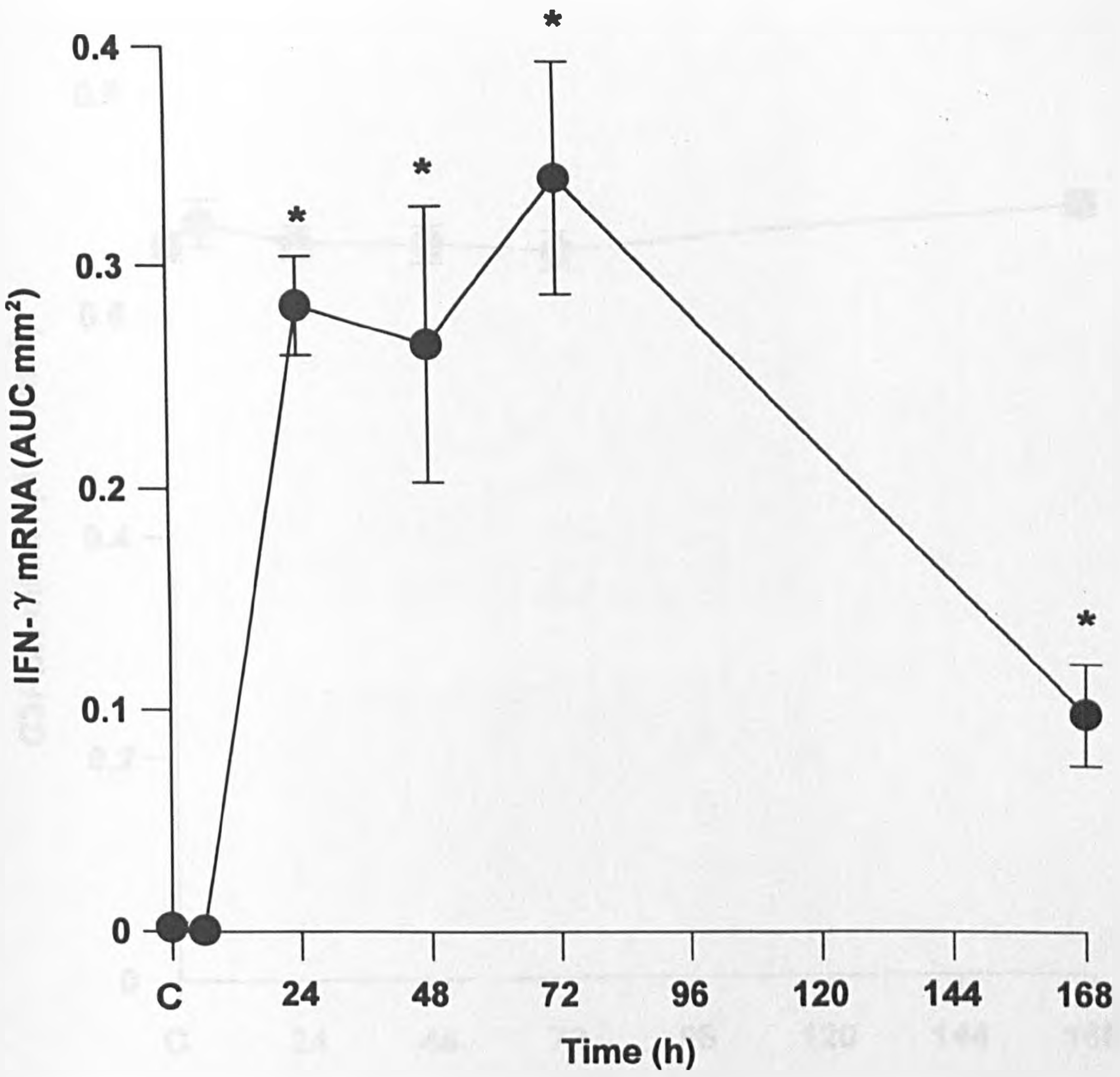


Fig. 6.2.11. Densitometric analysis of the time course of mRNA expression for IFN-γ in BAL cells after treatment of rats with Sephadex. Gels were scanned by laser densitometry and the intensity of RT-PCR products calculated as area under the curve (AUC) of densitometric traces. Results are means ± SEM for five experiments, each with BAL cells pooled from two rats. (* $p < 0.05$ by comparison of Sephadex-treated rats to controls as determined by the two-tailed Mann Whitney *U* test). C = IFN-γ mRNA expression from saline-injected control rats.

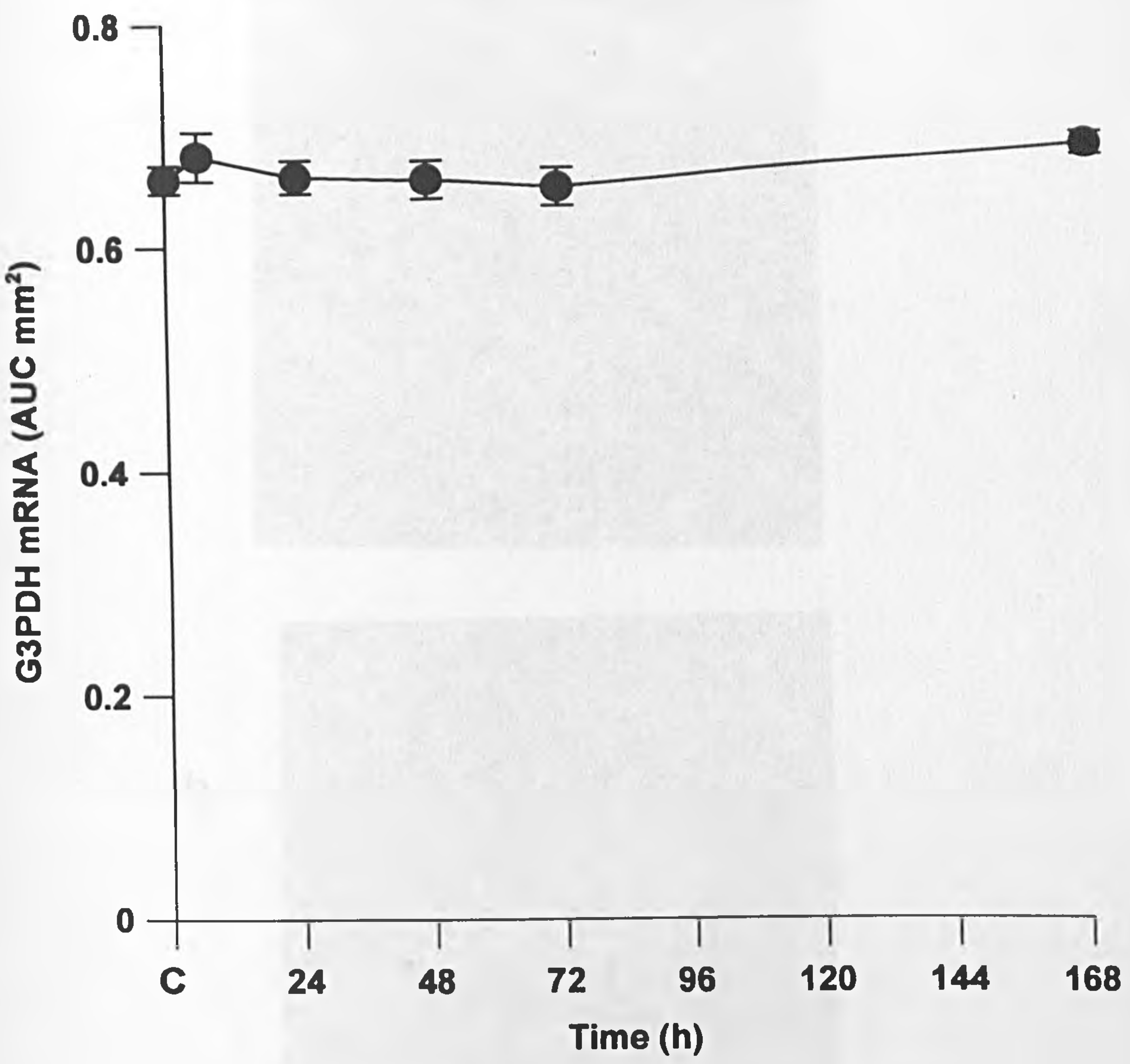


Fig. 6.2.12. Densitometric analysis of the time course of mRNA expression for G3PDH in BAL cells after treatment of rats with Sephadex. Gels were scanned by laser densitometry and the intensity of RT-PCR products calculated as area under the curve (AUC) of densitometric traces. Results are means ± SEM for five experiments, each with BAL cells pooled from two rats. C = G3PDH mRNA expression from saline-injected control rats.

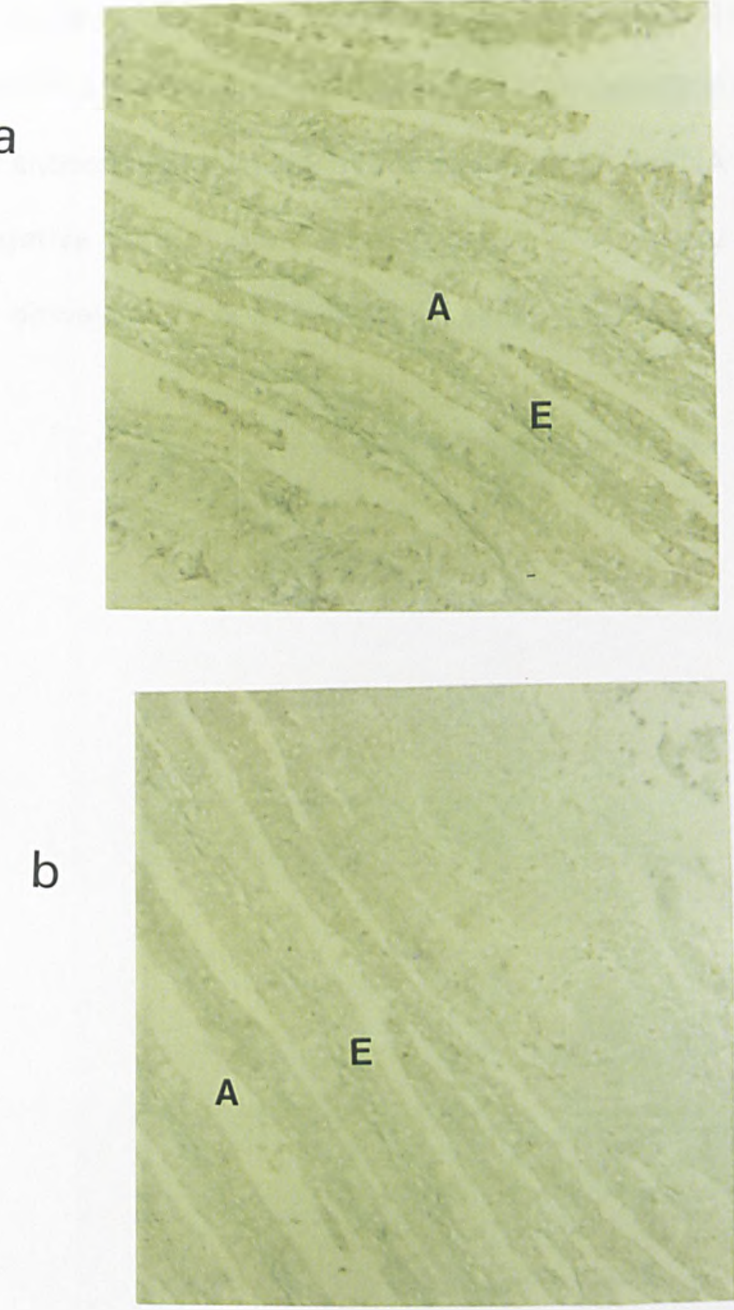
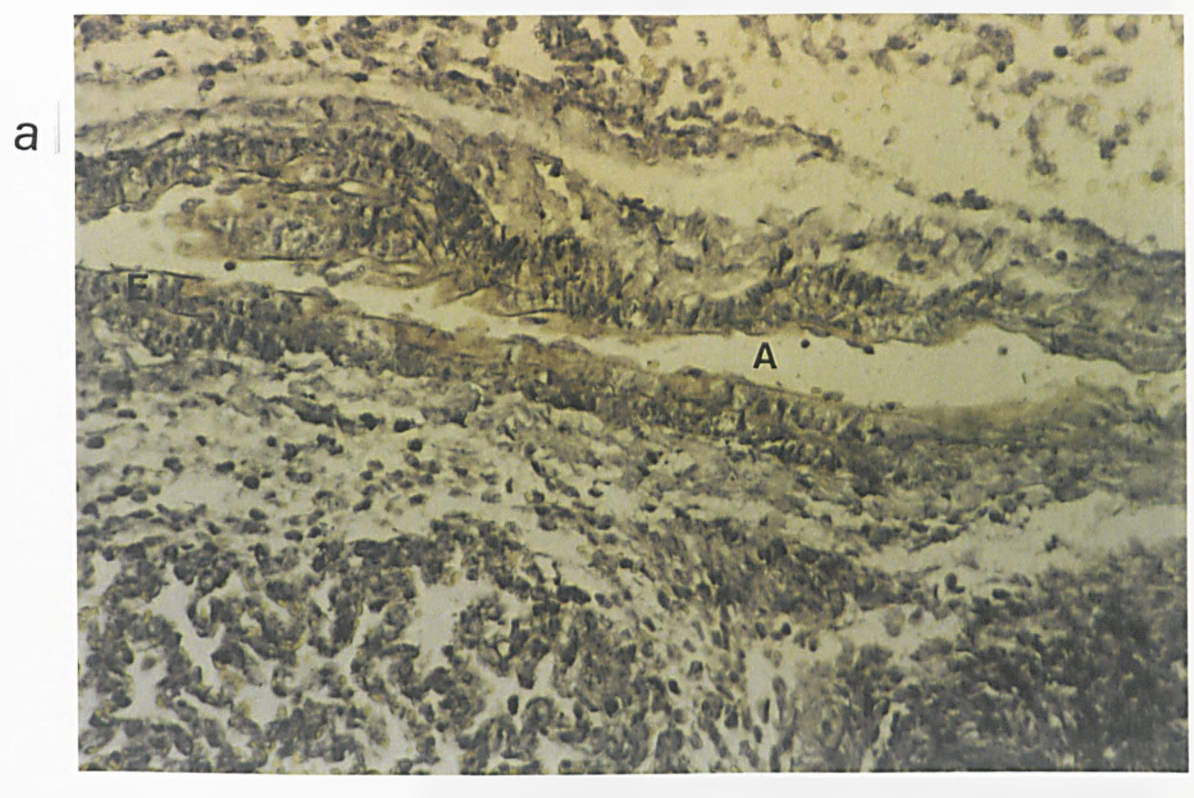


Fig. 6.2.13. *In situ* hybridization analysis of mRNA expression for IL-6 in a lung tissue section from a control (saline injected) rat. a (x200), detection of IL-6 mRNA using an IL-6 antisense RNA *in situ* hybridization probe; no IL-6 mRNA was detected. b (x200), negative control using an IL-6 sense RNA *in situ* hybridization probe. (A = airway; E = airway epithelial cells).

Fig. 6.2.14. *In situ* hybridization analysis of the expression of IL-6 mRNA in rat lung tissue 6 h after Sephadex injection. a (x200) detection of IL-6 mRNA using an IL-6 antisense RNA *in situ* hybridization probe (mRNA stains black). b (x200) negative control using an IL-6 sense RNA *in situ* hybridization probe. (A = airway; E = airway epithelial cells).

a



b

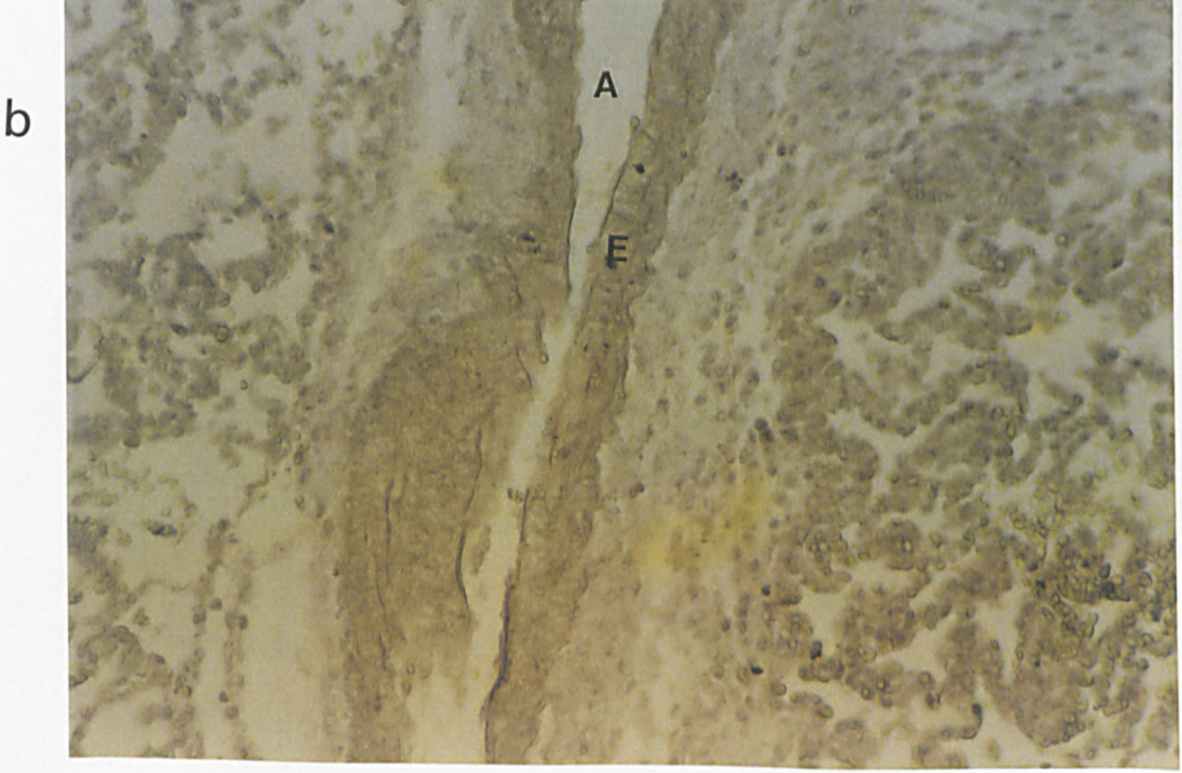


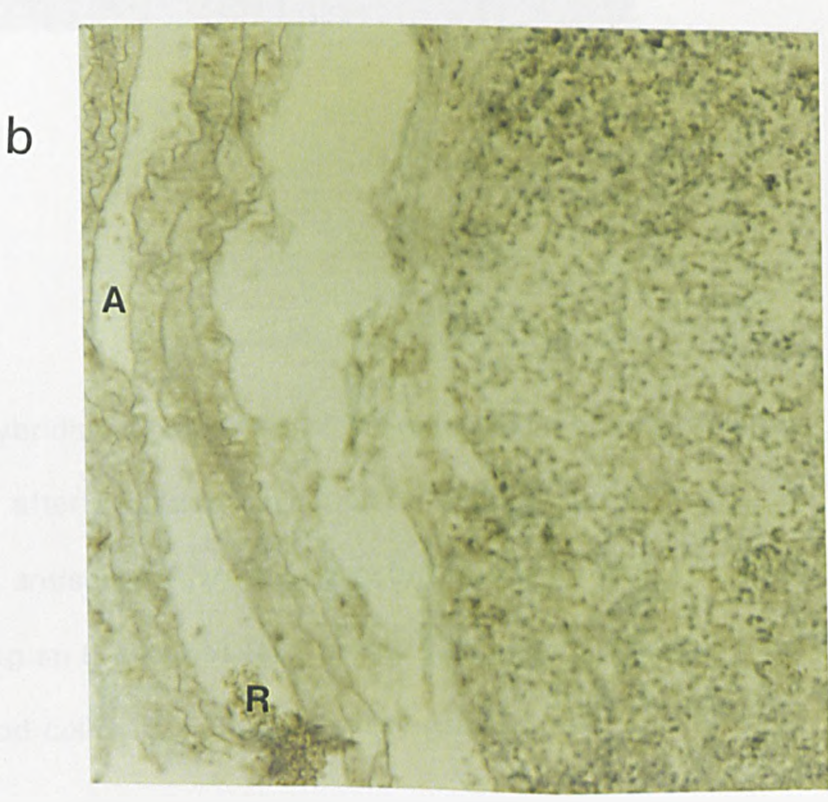
Fig. 6.2.15

a

A

R

b



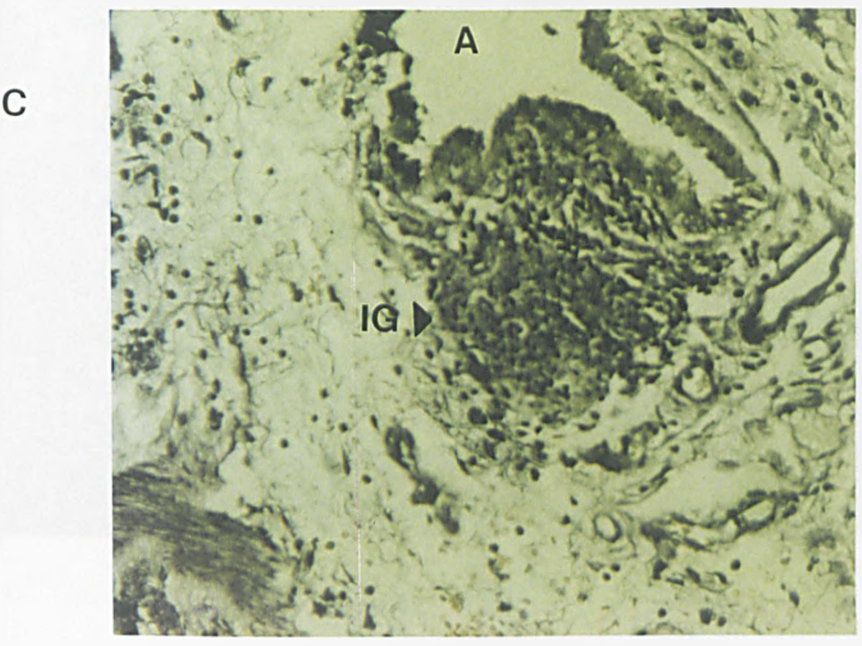
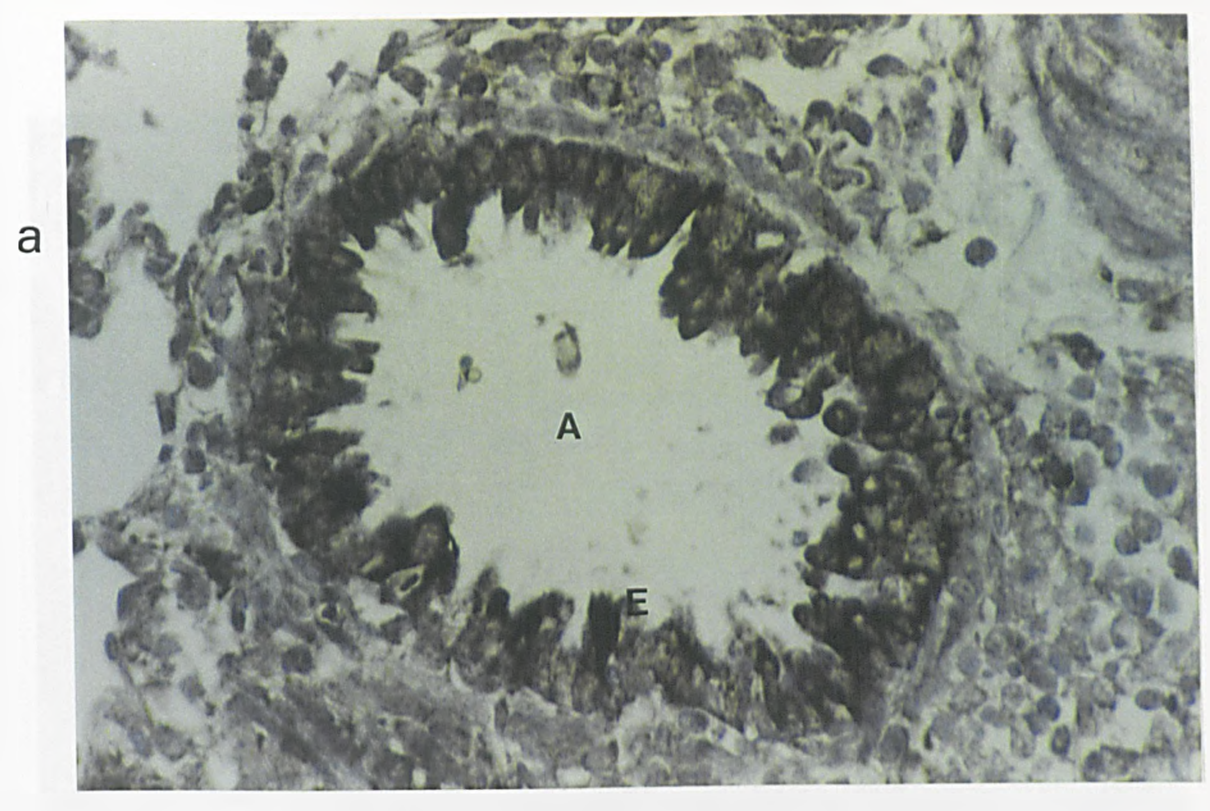


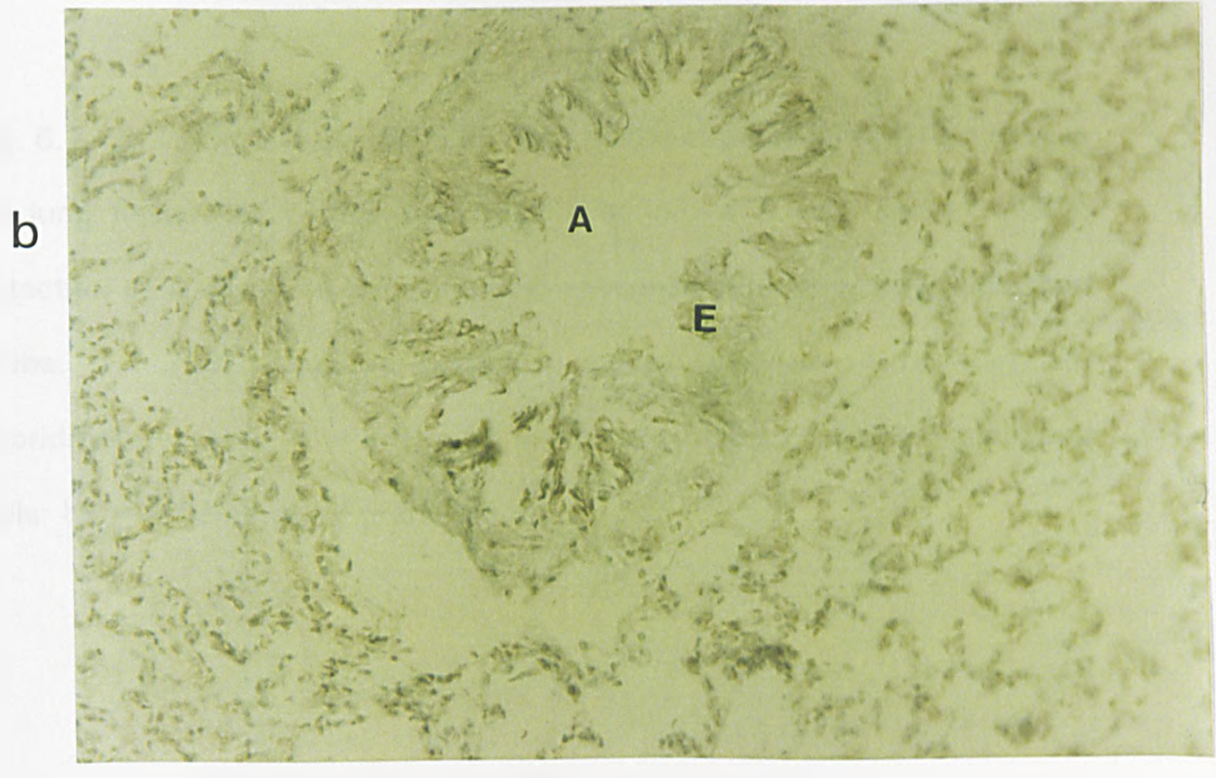
Fig. 6.2.15. *In situ* hybridization analysis of the expression of IL-6 mRNA in rat lung tissue 24 h after Sephadex injection. a (x200) detection of IL-6 mRNA using an IL-6 antisense RNA *in situ* hybridization probe. b (x200), negative control using an IL-6 sense RNA *in situ* hybridization probe. (A = airway; R = red blood cells; IG = inflammatory granuloma).

Fig. 6.2.16

a



b



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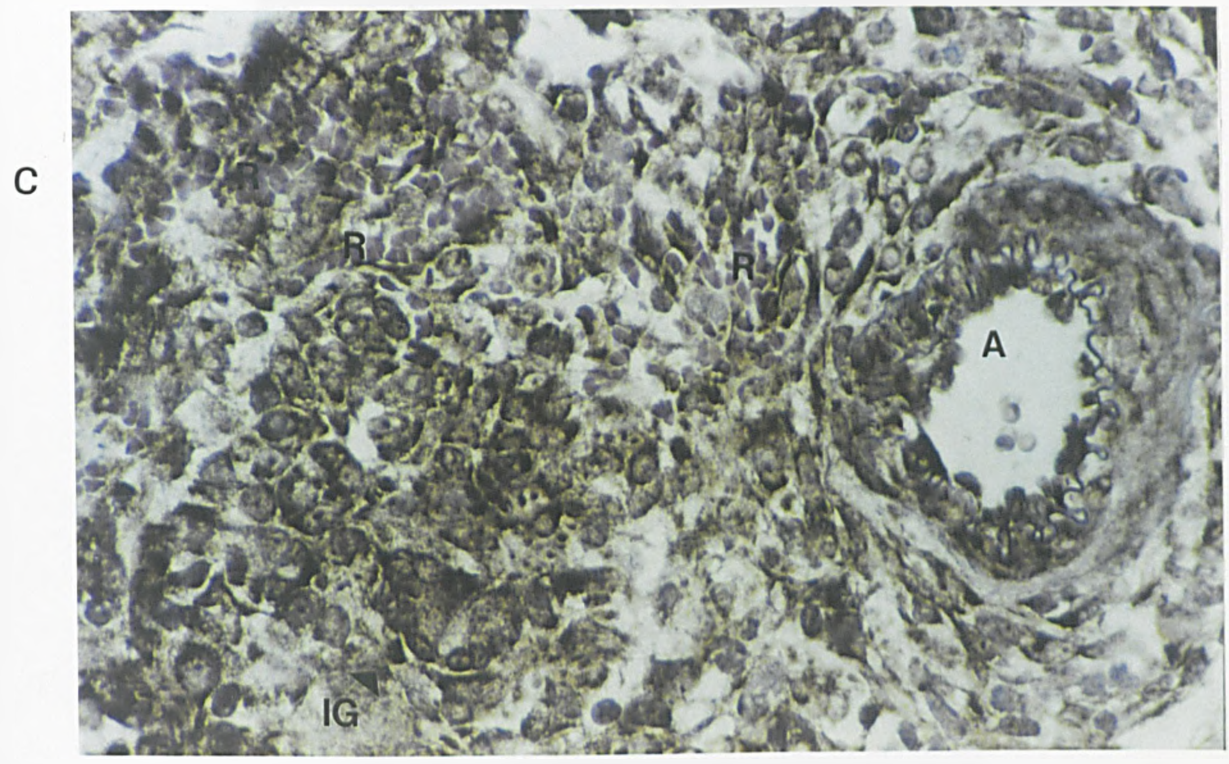
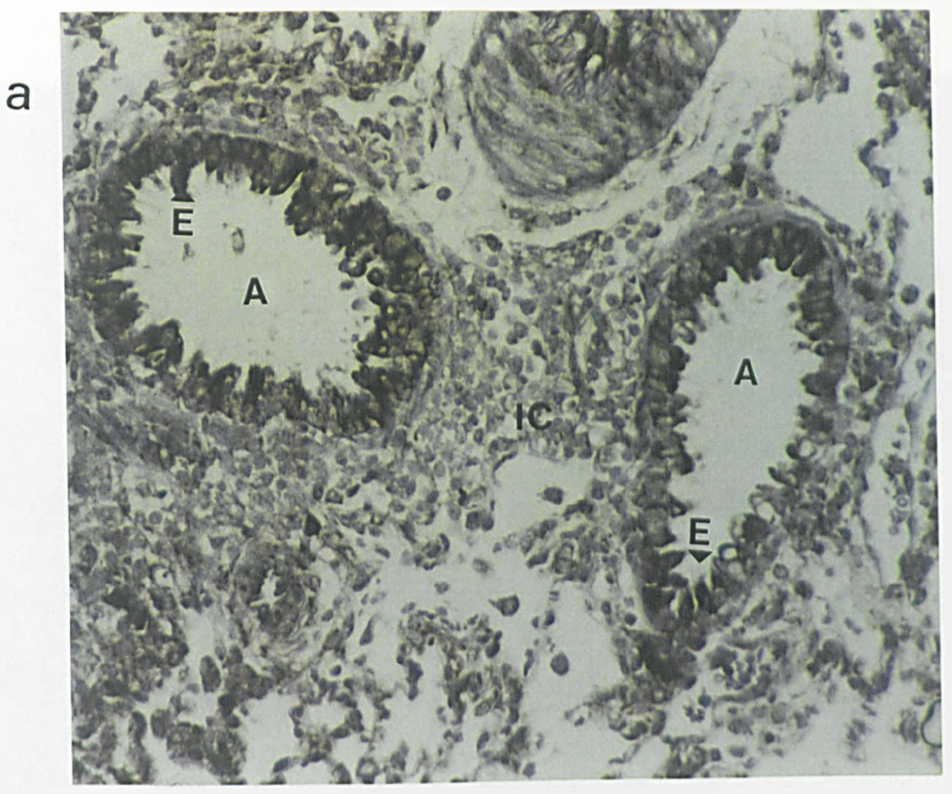


Fig. 6.2.16. *In situ* hybridization analysis of the expression of IL-6 mRNA in rat lung tissue 48 h after Sephadex injection. a (x400) and c (x400), detection of IL-6 mRNA using an IL-6 antisense RNA *in situ* hybridization probe. b (x200), negative control using an IL-6 sense RNA *in situ* hybridization probe. (A = airway; E = airway epithelial cells; R = red blood cells; IG = inflammatory granuloma).

Fig. 6.2.17

a



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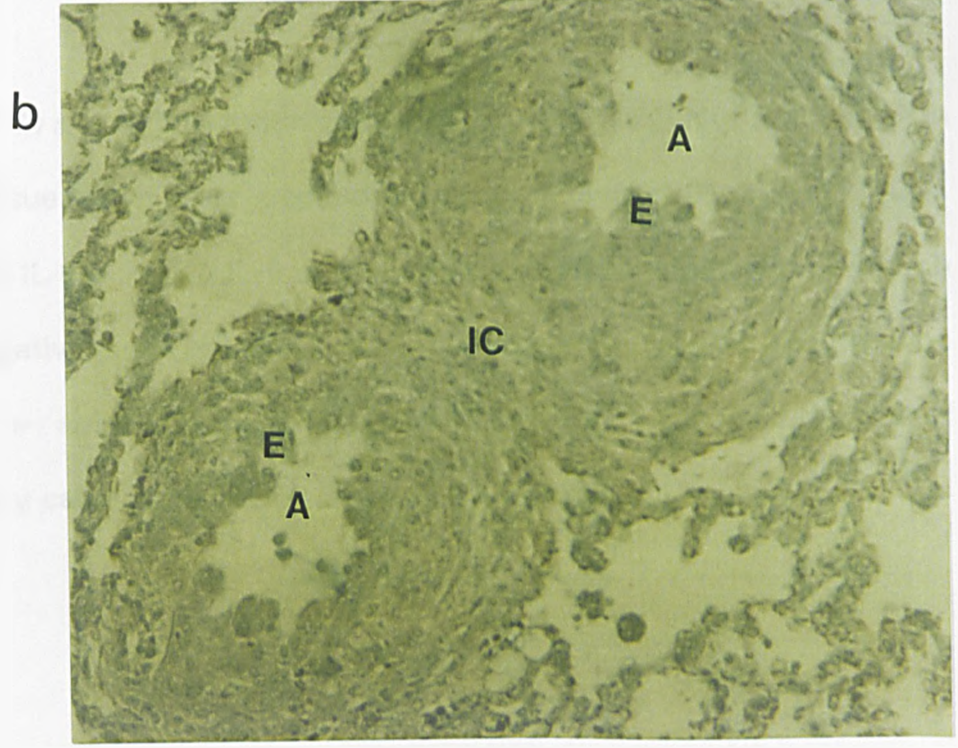


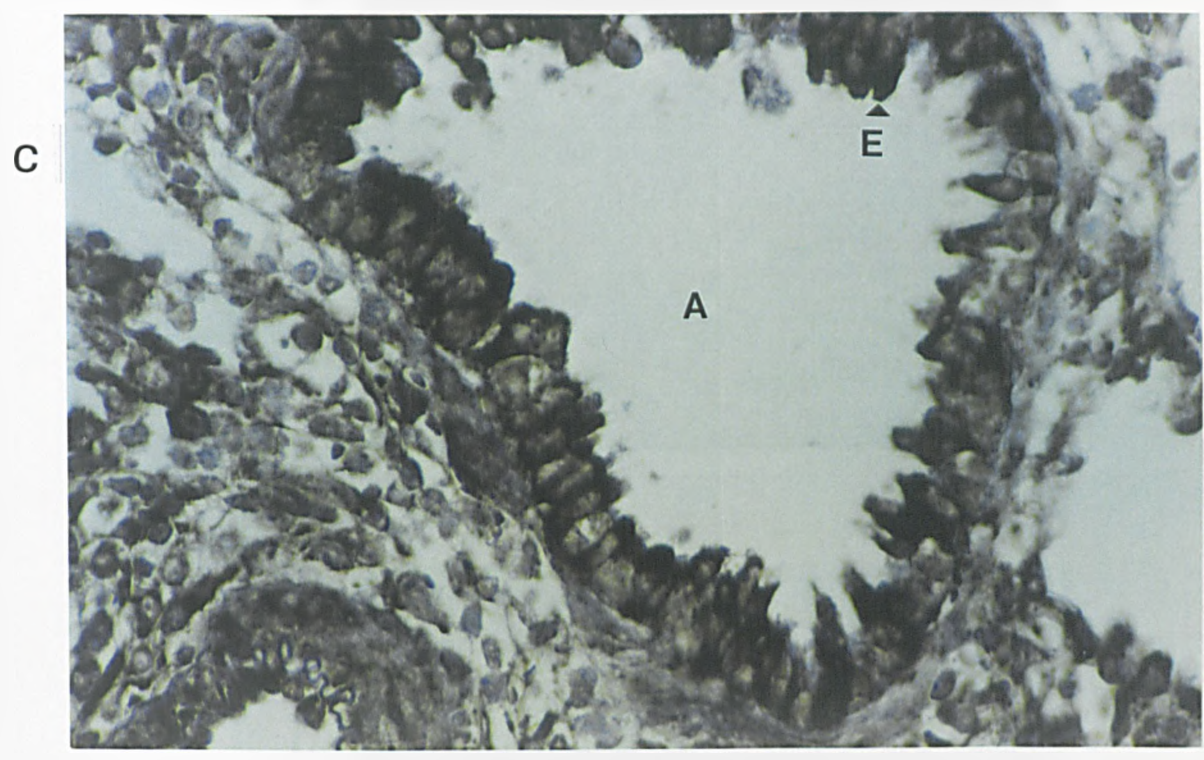
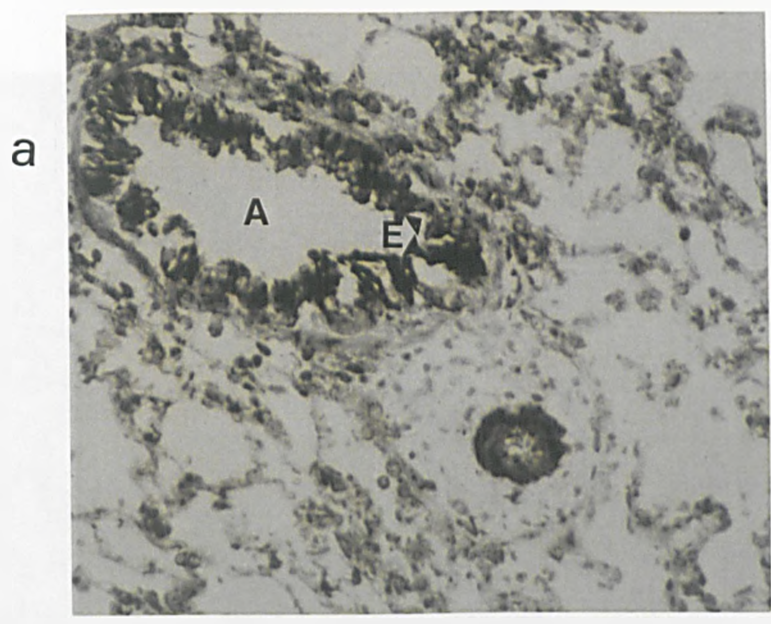


Fig. 6.2.17. *In situ* hybridization analysis of the expression of IL-6 mRNA in rat lung tissue 72 h after Sephadex injection. a (x200) and c (x400), detection of IL-6 using an IL-6 antisense RNA *in situ* hybridization probe. b (x200), negative controls using an IL-6 sense RNA *in situ* hybridization probe. (A = airway; E = airway epithelial cells; IC = infiltration of inflammatory cells).

a



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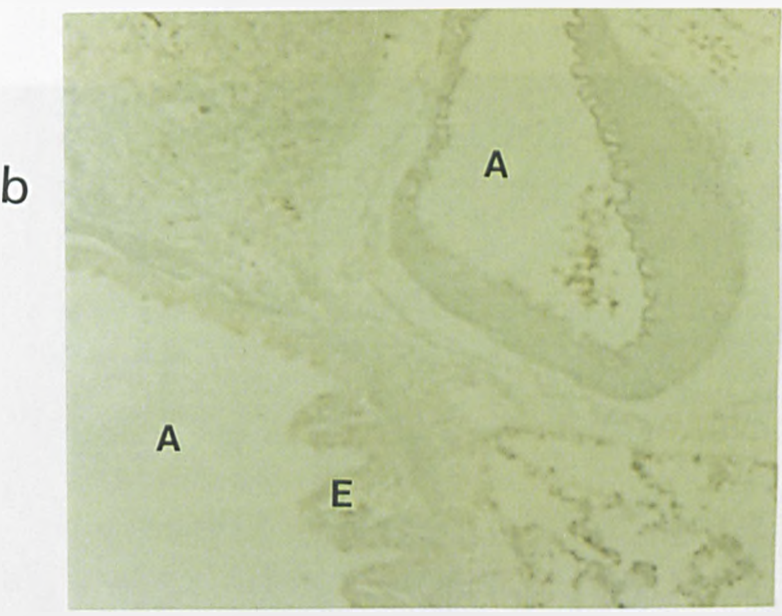


Fig. 6.2.18. *In situ* hybridization analysis of mRNA expression for TNF-*α* in lung tissue of control (saline injected) rats. a (x200), detection of TNF-*α* mRNA using a TNF-*α* antisense RNA *in situ* hybridization probe. b (x200), negative control using a TNF-*α* sense RNA *in situ* hybridization probe. (A = airway; E = airway epithelial cells).

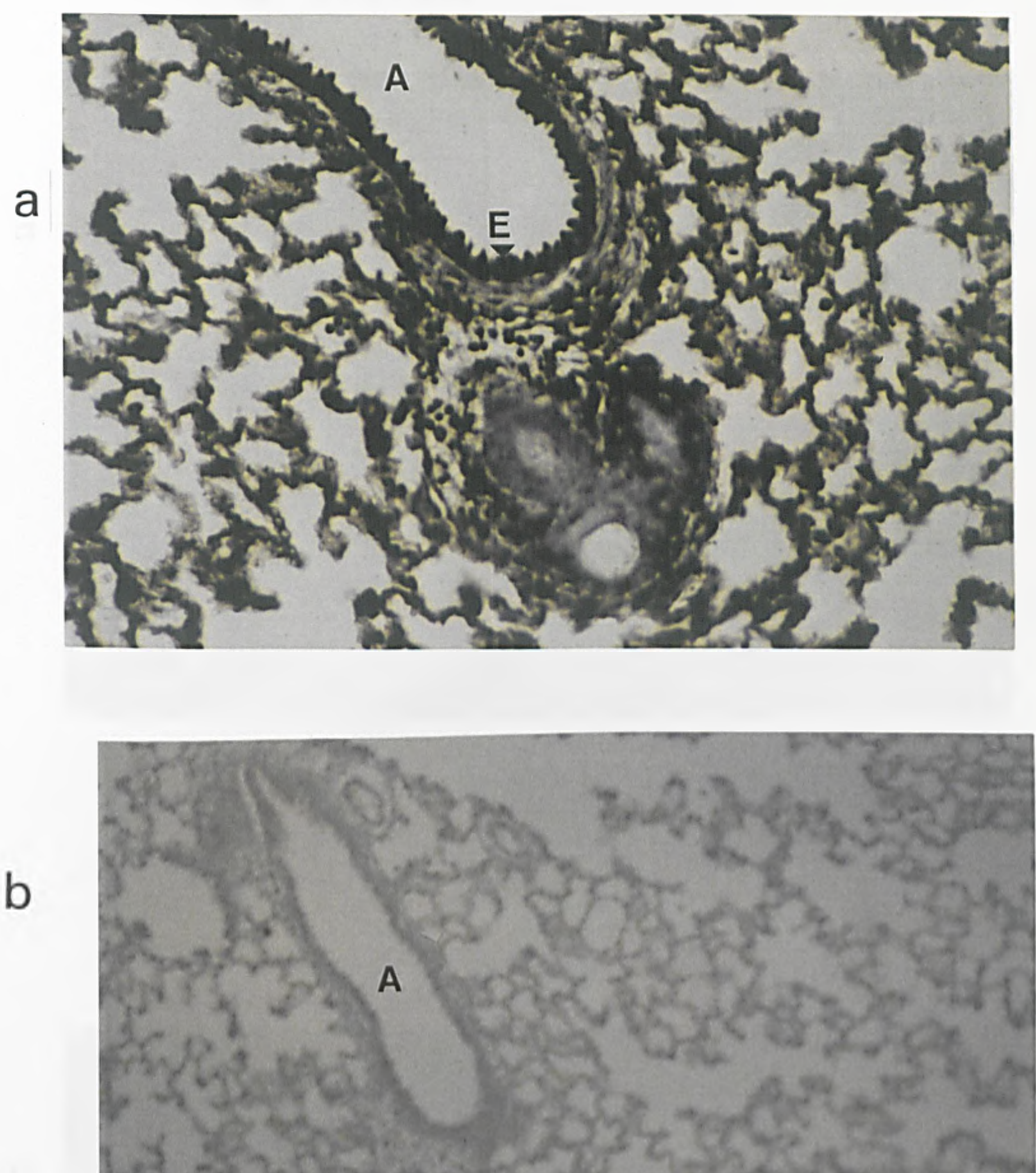
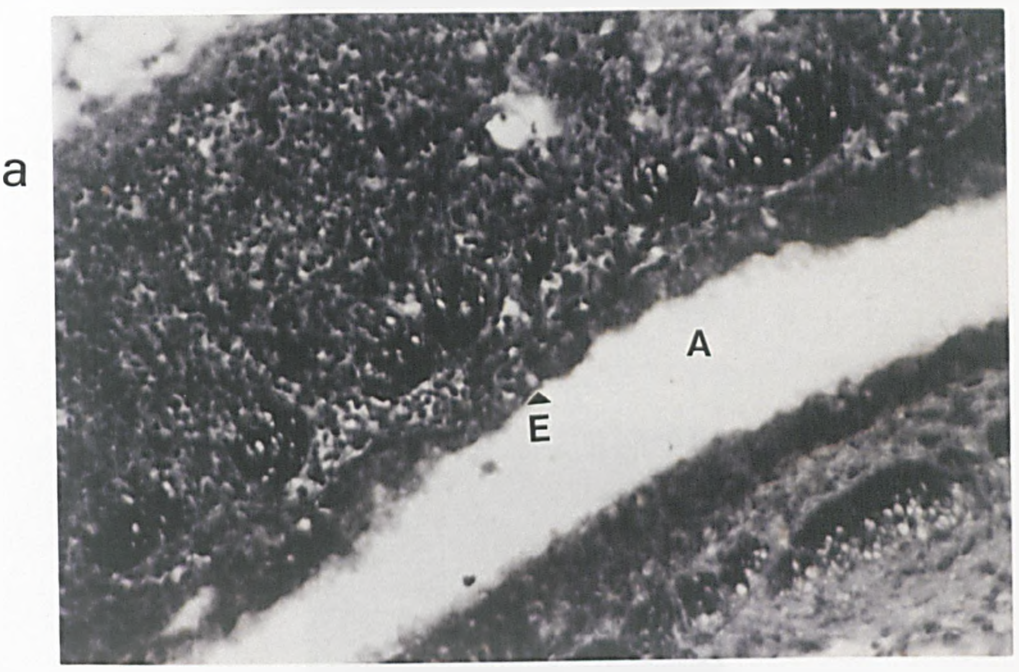


Fig. 6.2.19. *In situ* hybridization analysis of mRNA expression for TNF-α in rat lung tissue 6 h after injection of Sephadex. a (x200), detection of TNF-α mRNA using a TNF-α antisense RNA *in situ* hybridization probe. b (x100), negative control using a TNF-α sense RNA *in situ* hybridization probe. (A = airway; E = airway epithelial cells).

Fig. 6.2.20

a



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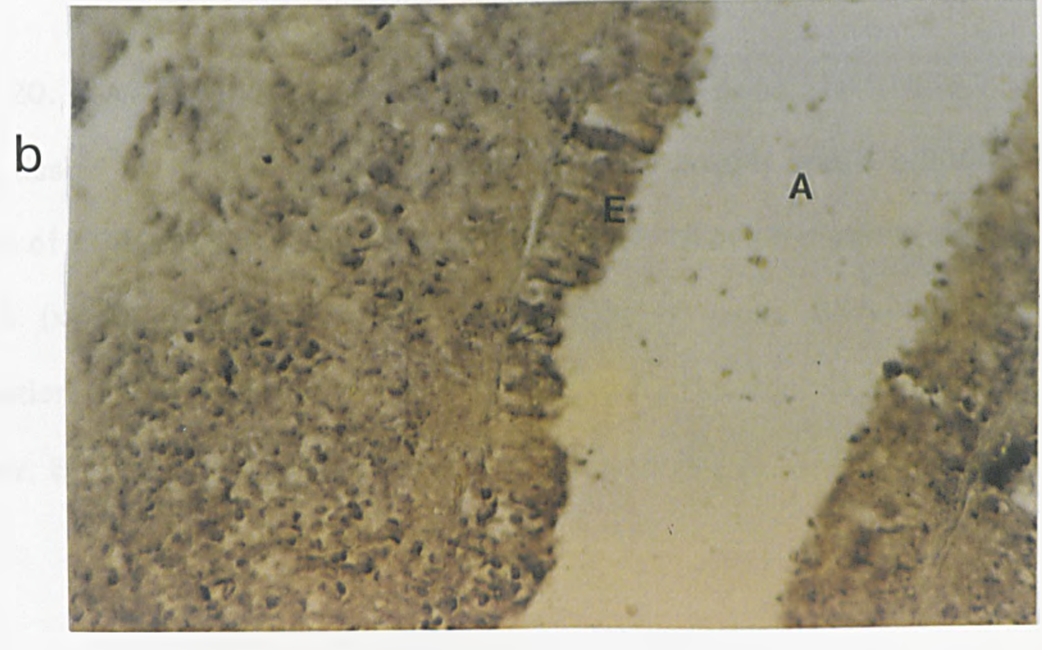


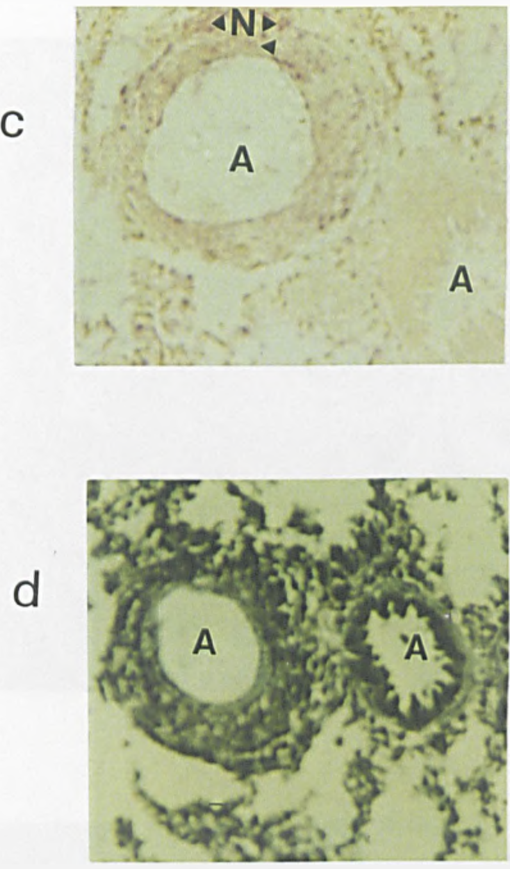
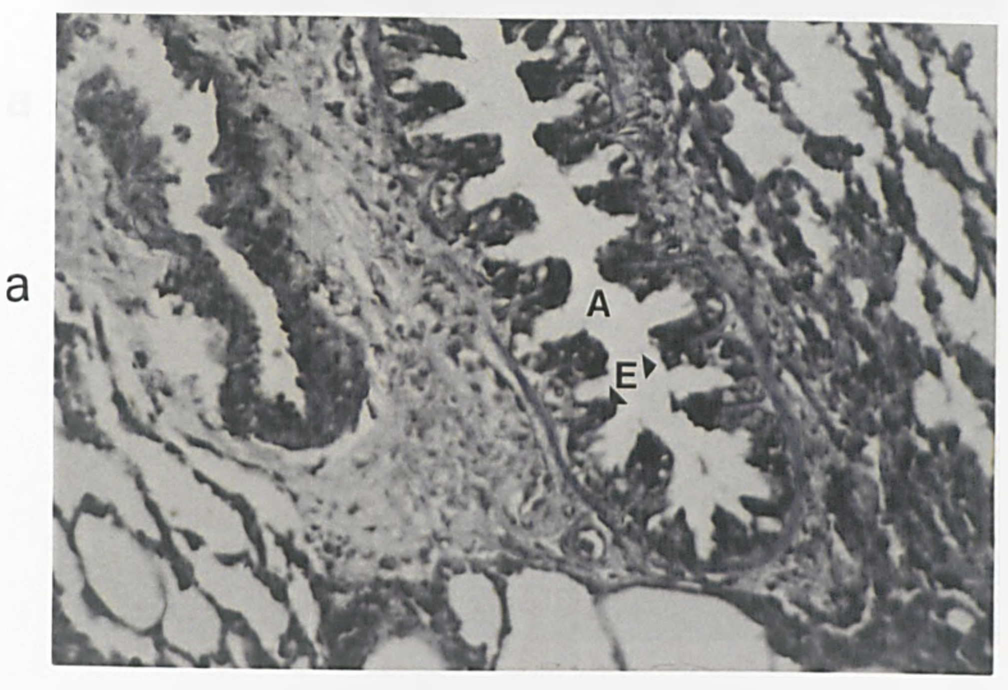


Fig. 6.2.20. *In situ* hybridization analysis of mRNA expression for TNF-α in rat lung tissue 24 h after injection of Sephadex. a (x400) and d (x200), detection of TNF-α mRNA using a TNF-α antisense RNA *in situ* hybridization probe. b (x400), negative control using a TNF-α sense RNA *in situ* hybridization probe. c, sequential section stained for neutrophils (pink). (A = airway; E = airway epithelial cells; N = neutrophils).

a



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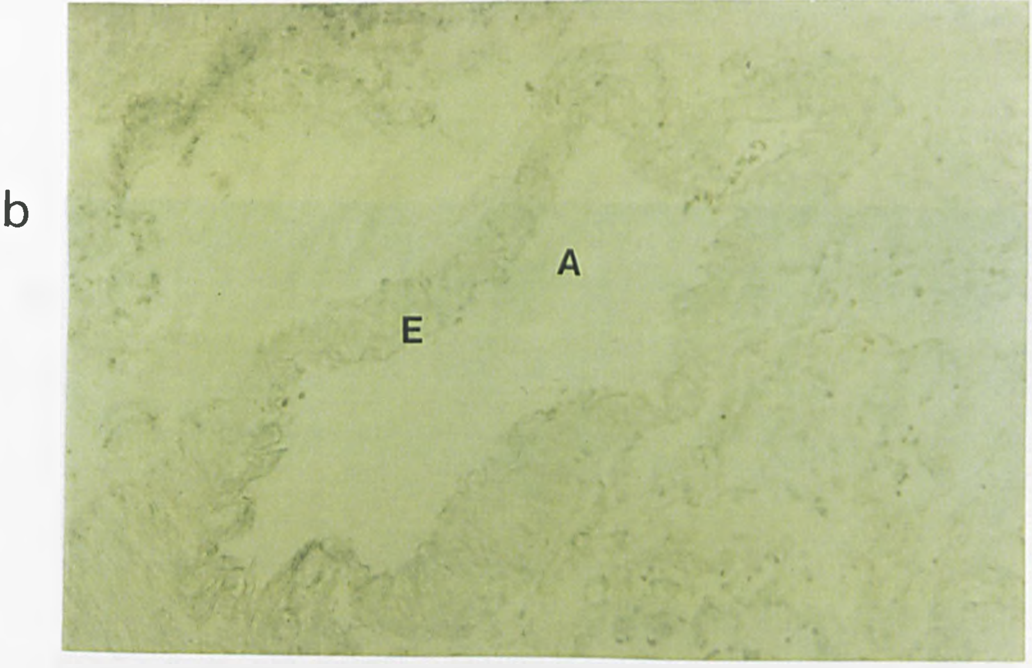
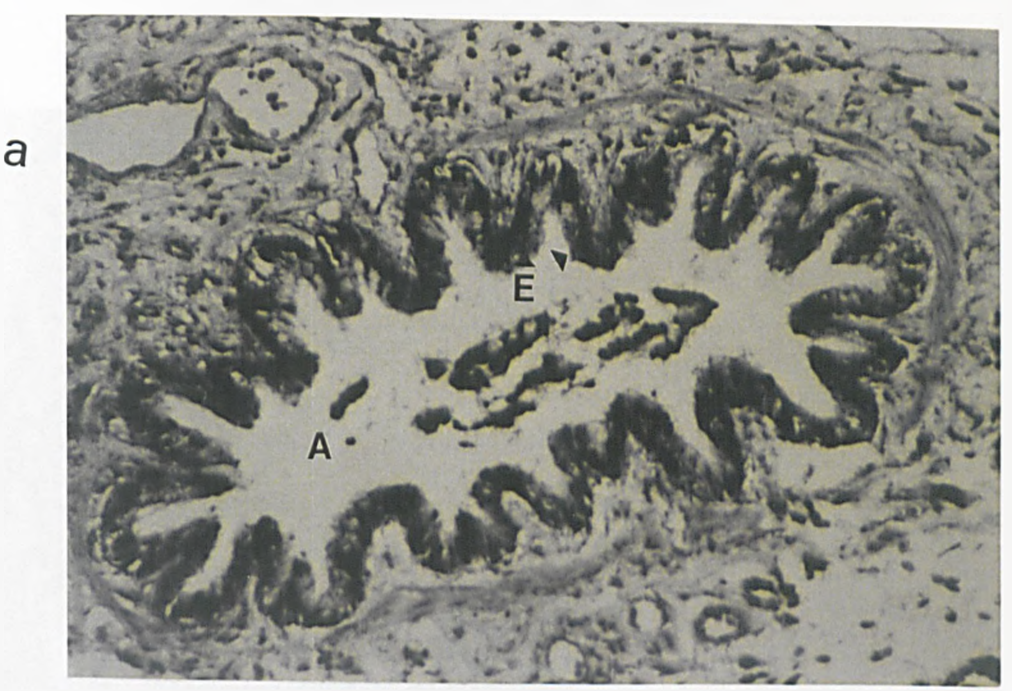


Fig. 6.2.21. *In situ* hybridization analysis of mRNA expression for TNF-α in rat lung tissue 48 h after injection of Sephadex. a (x200), detection of TNF-α mRNA using a TNF-α antisense RNA *in situ* hybridization probe. b (x200), negative control using a TNF-α sense RNA *in situ* hybridization probe. (A = airway; E = airway epithelial cells).

Fig. 6.2.22

a



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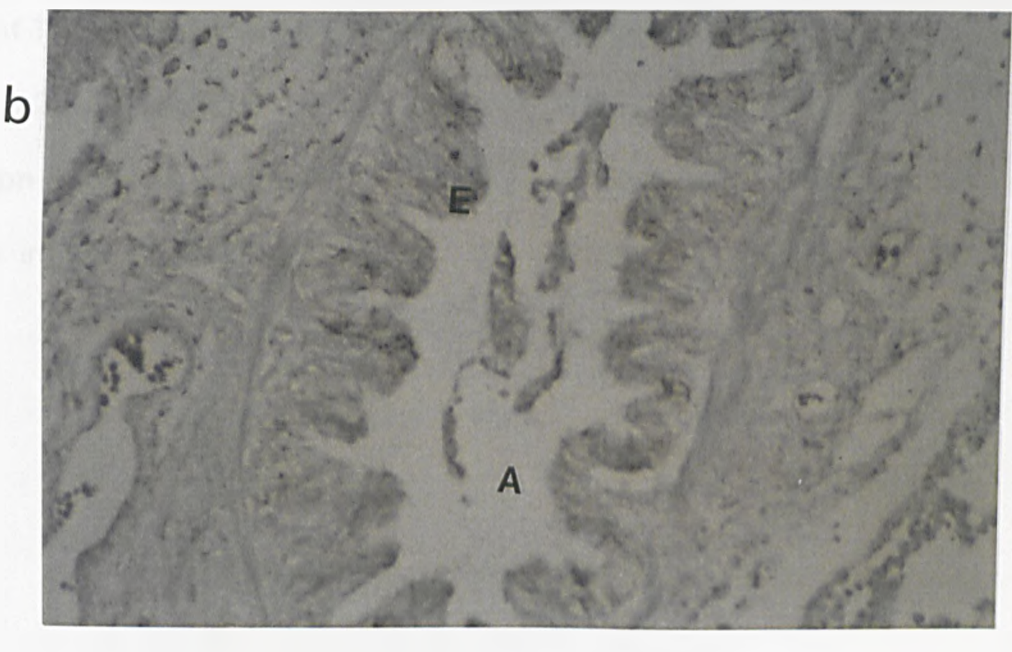

C

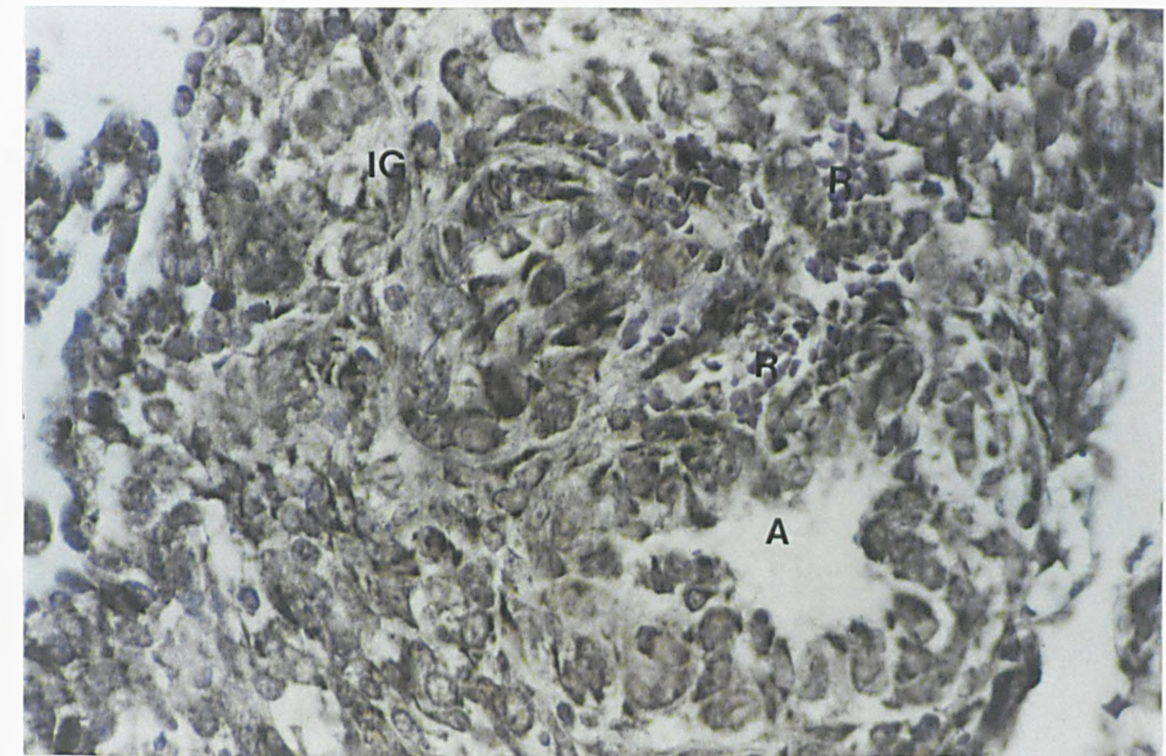

Fig. 6.2.22. *In situ* hybridization analysis of mRNA expression for TNF-α in rat lung tissue 72 h after injection of Sephadex. a (x200) and c (x400), detection of TNF-α mRNA using a TNF-α antisense RNA *in situ* hybridization probe. b (x200), negative control using a TNF-α sense RNA *in situ* hybridization probe. (A = airway; E = airway epithelial cells; R = red blood cells; IG = inflammatory granuloma).

CHAPTER 7

The effects of drugs on cytokine expression and patterns of cell infiltration in the rat Sephadex model of lung inflammation

7.1 **Introduction**

7.2 **Results**

7.2.1 The effects of Dex on bronchoalveolar cell infiltration

7.2.2 The effects of CsA on bronchoalveolar cell infiltration

7.2.3 The effects of BTS 71321 on bronchoalveolar cell infiltration

7.2.4 The effects of Dex on cytokine mRNA expression

7.2.5 The effects of CsA on cytokine mRNA expression

7.2.6 The effects of BTS 71321 on cytokine mRNA expression

7.2.7 Immunohistochemical analysis of bronchoalveolar lavage cells

7.3 Discussion

7.1 INTRODUCTION

The recent increase in the prevalence and severity of asthma in many developed countries has emphasised the need for more effective and reliable therapies. Glucocorticoids are established anti-inflammatory agents with a proven record in the treatment of chronic airways inflammation in asthma. Their precise mode of action in asthma remains unclear although they are known to reduce mast cell proliferation and mediator release (chapter 3), to reduce eosinophil adhesion, chemotaxis and activation and to decrease blood vessel permeability. In addition, they reduce macrophage eicosanoid production and enzyme release, they reduce bronchial smooth muscle β-adrenoceptor coupling and they reduce the number of CD4+ cells (Church, 1993). It has also become apparent that glucocorticoids can inhibit the synthesis and release of a number of cytokines from a variety of inflammatory cells which may explain their effectiveness in the treatment of a number of inflammatory disorders.

Cyclosporin A is a cyclic undecapeptide that has traditionally been thought of as a drug used in the prevention of organ-graft rejection. However, due to the fact that a subpopulation of asthmatic patients are resistant to the beneficial effects of glucocorticoid therapy, the anti-inflammatory effects of CsA have recently been investigated in asthmatic patients. In a trial of CsA in corticosteroid-dependent chronic severe asthma, patients had fewer disease exacerbations during CsA treatment and lung function improved throughout the twelve week treatment period

(Alexander *et al.*, 1992). However, as for the therapeutic effects of glucocorticoids, the mechanism of action of CsA is again poorly characterized. It has generally been regarded as a T cell specific drug and has been shown to be effective in the treatment of various diseases thought to be mediated by activated T cells (Thomson and Neild, 1991) including psoriasis (Ellis *et al.*, 1991), atopic dermatitis (MacKie, 1993) and Crohn's disease (Brynskov *et al.*, 1989). However, it is also known to inhibit mast cell degranulation and the transcription of a number of mast cell associated cytokine genes (chapters 3 and 5).

The increasing awareness that cytokines can contribute extensively to the aetiology of asthma suggests mechanisms whereby drugs may directly antagonize cytokines thereby instigating an improvement in lung function and the pathology associated with asthma. Indeed, as has been suggested above, the inhibition of cytokine synthesis associated with Dex and CsA therapy may account for many of their anti-inflammatory effects.

The Sephadex model of lung inflammation is often used within the pharmaceutical industry to test the effectiveness of novel anti-inflammatory drugs, but as yet these reports concentrate on the efficacy of compounds in down-regulating the inflammatory cell influx and decreasing the broncho-hyperreactivity associated with this model. Their effects on the inhibition of corresponding increases in cytokine mRNA levels and release of biologically active cytokine products have been neglected and in view of the growing evidence that cytokines are important in the initiation and maintenance of the asthmatic reaction, it seems appropriate to investigate the effects of

known and potential anti-asthmatic compounds on the expression of pro-inflammatory cell associated cytokines. Additionally, although there is an abundance of information regarding the effects of these drugs *in vitro*, there are relatively few observations on the regulation of cytokine gene expression *in vivo*. Consequently, in this chapter the rat Sephadex model of lung inflammation has been used to investigate the effects of Dex, CsA and the novel benzylaminoalkylimidazole, BTS 71321, on the changes in mRNA levels for IL-5, IL-6, TNF-α, MIP-2 and IFN-γ in BAL cells and associated changes in numbers of inflammatory cells in the bronchoalveolar lumen during the progression of lung inflammation.

7.2 RESULTS

In all experiments reported in this chapter all rats were treated by *i.v.* administration of Sephadex, and were pre-treated 24 h and 90 min before Sephadex, with either drug or appropriate vehicle. Throughout this chapter non-drug (vehicle)-treated, Sephadex-treated rats will be referred to as control rats. Drug doses, as advised by Knoll Pharmaceuticals, were chosen on the basis of previous studies that had investigated their effectiveness in reducing lung hyper-reactivity in a guinea pig model of lung inflammation.

7.2.1 The effects of Dex on bronchoalveolar cell infiltration

Injection of Dex (0.1 mg/kg or 0.3 mg/kg) into rats prior to a single *i.v.* injection of Sephadex particles, had no effect on the total number of cells lavaged from the bronchoalveolar cavity 24 h later compared to control rats (Fig. 7.2.1A). However, 72 h after Sephadex treatment there were fewer total BAL cells (approximately one third of control numbers) in rats that had been treated with Dex at either 0.1 mg/kg or 0.3 mg/kg prior to Sephadex injection compared to controls (Fig. 7.2.1B). These effects were highly significant over ten rats.

Dex had no significant effect on levels of mononuclear cells 24 h after Sephadex treatment (Fig. 7.2.2A) but did reduce mononuclear cell numbers 72 h after Sephadex injection at both doses, compared to control rats (Fig. 7.2.2B). Again these effects were highly significant over ten rats.

Twenty-four hours after Sephadex treatment a significant inhibition of

neutrophil influx was observed in rats that had been pre-treated with Dex at 0.1 mg/kg but not 0.3 mg/kg compared to control rats (Fig. 7.2.3A). However, 72 h after induction of lung inflammation both concentrations of drug had significantly inhibited the influx of neutrophils compared to control rats (Fig. 7.2.3B).

Pretreatment of rats with Dex at either 0.1 mg/kg or 0.3 mg/kg significantly inhibited the influx of eosinophils into the brochoalveolar cavity 24 h and 72 h after Sephadex injection compared to control rats (Fig. 7.2.4).

7.2.2 The effects of Cyclosporin A on bronchoalveolar cell infiltration in the rat Sephadex model of lung inflammation

Injection of CsA at 10 mg/kg into rats prior to a single *i.v.* injection of Sephadex particles had no significant effect on the total number of cells within the bronchoalveolar cavity 24 h after the Sephadex injection compared to control rats, whereas pretreatment of rats with CsA at a higher dose of 30 mg/kg prior to Sephadex injection caused a significant fall in total numbers of BAL cells in five rats (Fig. 7.2.5A). However, 72 h after Sephadex treatment no significant inhibitory effects of CsA were seen on total numbers of BAL cells (Fig. 7.2.5B).

Pretreatment of rats with CsA at either 10 mg/kg or 30 mg/kg prior to induction of lung inflammation, had no significant effect on mononuclear cell numbers 24 h or 72 h after Sephadex treatment compared to controls (Fig. 7.2.6).

Twenty-four hours after Sephadex treatment a significant inhibition of

neutrophil influx was observed in rats that had been pre-treated with CsA at 10 mg/kg and 30 mg/kg compared to control rats (Fig. 7.2.7A). CsA at 10 mg/kg inhibited neutrophil influx on average to a third of control values, whereas CsA at 30 mg/kg reduced the number of neutrophils to one seventh of control values. These effects were highly significant over five rats. However, 72 h after Sephadex injection the inhibitory effects of CsA on neutrophil infiltration were no longer observed (Fig 7.2.7B).

Pretreatment of rats with CsA at 10 mg/kg had no effect on levels of infiltrating eosinophils 24 h or 72 h post Sephadex injection compared to vehicle-treated/control rats, whereas CsA at 30 mg/kg significantly down-regulated the influx of eosinophils into the brochoalveolar cavity at both time points (Fig. 7.2.8 A, B). On average, CsA at 30 mg/kg inhibited eosinophil infiltration to a third of control levels after 24 h and to a quarter of control levels after 72 h.

7.2.3 The effects of BTS 71321 on bronchoalveolar cell infiltration in the rat Sephadex model of lung inflammation

Pretreatment of rats with BTS 71321 at 10 mg/kg, a concentration known to be effective in reducing lung-hyperreactivity in a guinea-pig model of lung inflammation, had no significant effect on total numbers of cells or mononuclear cells 24 h or 72 h post Sephadex injection compared to control rats (Fig. 7.2.9). Additionally, BTS 71321 had no effect on the infiltration of neutrophils or eosinophils at either time point following induction of lung inflammation (Fig. 7.2.10).

7.2.4 The effects of Dex on cytokine mRNA expression in the rat Sephadex model of lung inflammation

Unfortunately mRNA encoding MIP-2 was not investigated in this chapter due to a shortage of primers. However, mRNA encoding IL-6, TNF-α, and IFN-γ was readily detectable in BAL cells from Sephadex-treated control rats 24 h after Sephadex injection (Fig. 7.2.11) whereas IL-5 mRNA was detected weakly in 3/5 experiments (Fig. 7.2.11). Treatment of the rats with Dex at 0.1 mg/kg completely abolished the mRNA expression of IL-5, IL-6 and IFN-γ in 4/4 experiments 24 h after Sephadex treatment but had no effect on mRNA expression of TNF-α or G3PDH (Fig. 7.2.11). Similar effects were observed when rats were treated with Dex at a concentration of 0.3 mg/kg prior to Sephadex injection although mRNA expression of TNF-α had appeared to decline in 2/4 experiments and IFN-γ mRNA was detected very weakly in 2 experiments (Fig. 7.2.11). Densitometric analysis of the band intensities revealed that Dex (0.1 mg/kg and 0.3 mg/kg) significantly inhibited mRNA expression of IL-6 and IFN-γ 24 h after injection of Sephadex particles compared to control rats, but that it did not significantly affect mRNA expression of IL-5, TNF-α or the housekeeping gene G3PDH (Table 7.2.1).

Seventy-two hours after Sephadex treatment, mRNA expression of IL-5, IL-6, TNF-α, IFN-γ and G3PDH was detectable in BAL cells from Sephadex-treated control rats although IL-5 signals were weak (Fig. 7.2.12). Treatment of the rats with Dex at 0.1 mg/kg significantly inhibited or abolished the expression of IL-5 mRNA in 4 experiments (Fig. 7.2.12) and

similar effects were observed at a higher dose of 0.3 mg/kg (Fig. 7.2.12). Dex (0.1 mg/kg) also abolished the expression of IL-6 mRNA (Fig. 7.2.12) and similar effects were observed when rats were pre-treated with Dex at 0.3 mg/kg (Fig. 7.2.12). Dex at 0.1 mg/kg inhibited the expression of TNF-α mRNA in 4 experiments and more noticeably in 4 experiments in which the dose was raised to 0.3 mg/kg prior to Sephadex injection (Fig. 7.2.12). Pretreatment of rats with Dex at 0.1 mg/kg had no effect on mRNA expression of IFN-γ 72 h after injection of Sephadex particles whereas Dex at a concentration of 0.3 mg/kg inhibited IFN-γ mRNA in 4/4 experiments (Fig. 7.2.12). Densitometric analysis of RT-PCR band intensities revealed that Dex at both 0.1 mg/kg and 0.3 mg/kg significantly inhibited the mRNA expression of IL-5, IL-6 and TNF-α (Table 7.2.2) and at 0.3 mg/kg it significantly inhibited IFN-γ mRNA expression whereas at both concentrations it had no effect on expression levels of G3PDH compared to control levels (Table 7.2.2).

7.2.5 The effects of CsA on cytokine mRNA expression in the rat Sephadex model of lung inflammation

Messenger RNA encoding IL-6, TNF-α, and IFN-γ was detectable in BAL cells from 5/5 control rats 24 h after injection of Sephadex particles whereas IL-5 mRNA was readily detectable in BAL cells from 3 Sephadex-treated control rats and less intensely in a further 2 rats (Fig. 7.2.13). Pretreatment of the rats with CsA at 10 mg/kg had no effect on IL-5 mRNA in 3/5 experiments but totally abolished the mRNA expression of IL-5 in 1 experiment and

appeared to inhibit its expression in a further experiment (Fig. 7.2.13). Pretreatment of rats with CsA at a higher concentration of 30 mg/kg totally abolished the mRNA expression of IL-5 in 4/5 experiments (Fig. 7.2.13). Twenty-four hours after injection of Sephadex particles, CsA at 10 mg/kg had inhibited or totally abolished IL-6 mRNA expression in 4/5 experiments (Fig. 7.2.13) and at 30 mg/kg, CsA had abolished the IL-6 mRNA expression in 5/5 experiments (Fig. 7.2.13). Twenty-four hours after Sephadex injection, expression of TNF-α mRNA appeared to be unaffected by pre-treatment of the rats with CsA at 10 mg/kg (Fig. 7.2.13). Similar effects were observed when rats were pre-treated with CsA at 30 mg/kg although in 1/5 experiments TNF-α mRNA was inhibited (Fig. 7.2.13). Messenger RNA encoding IFN-γ was unaffected by CsA at 10 mg/kg although at 30 mg/kg IFN-γ mRNA appeared to be inhibited slightly in 2/4 experiments (Fig. 7.2.13). CsA had no effects on the mRNA expression of the housekeeping gene G3PDH (Fig. 7.2.13). Densitometric analysis of RT-PCR band intensities 24 h after injection of Sephadex particles revealed that pre-treatment of the rats with CsA at 10 mg/kg or 30 mg/kg significantly inhibited the mRNA expression of IL-6 (Table 7.2.3) and at 30 mg/kg it significantly inhibited IL-5 mRNA expression compared to control levels (Table 7.2.3). However, it had no effect at either 10 mg/kg or 30 mg/kg on mRNA expression levels of TNF-α, IFN-γ or G3PDH (Table 7.2.3).

Seventy-two hours after injection of Sephadex particles mRNA encoding IL-5, TNF-α, IFN-γ and G3PDH was readily detectable in BAL cells from 5/5 control rats whereas IL-6 mRNA was detected in 4/5 control rats

(Fig. 7.2.14). Pre-treatment of the rats with CsA at 10 mg/kg had no effect on mRNA expression of IL-5 whereas pre-treatment with CsA at 30 mg/kg inhibited IL-5 mRNA expression in at least 3/4 experiments (Fig. 7.2.14). CsA at 10 mg/kg inhibited the mRNA expression of IL-6 in 4/5 experiments and similar effects were observed when the dose was raised to 30 mg/kg (Fig. 7.2.14). In 2 experiments expression of IL-6 mRNA was very intense after CsA treatment, perhaps reflecting failure of correct drug administration (Fig. 7.2.14). No apparent effects of CsA at either 10 mg/kg or 30 mg/kg, on mRNA expression of TNF-α were observed (Fig. 7.2.14). Similarly, pre-treatment of rats with CsA at 10 mg/kg appeared to produce no pronounced effects on mRNA expression levels of IFN-γ although at 30 mg/kg IFN-γ mRNA appeared to be slightly inhibited (Fig. 7.2.14). G3PDH mRNA was unaffected by pre-treatment of the rats with CsA at either 10 mg/kg or 30 mg/kg (Fig. 7.2.14). Densitometric analysis of RT-PCR band intensities revealed that CsA at 30 mg/kg but not 10 mg/kg significantly inhibited the mRNA expression of IL-6 (Table 7.2.4). At 30 mg/kg CsA significantly inhibited IL-5 mRNA levels (Table 7.2.4). However, CsA at either concentration had no effect on mRNA expression levels of TNF-α, IFN-γ or G3PDH compared to controls (Table 7.2.4).

7.2.6 Effects of BTS 71321 on cytokine mRNA expression in the rat Sephadex model of lung inflammation

In these experiments because of a shortage of reagents including primers only mRNA for IL-6, TNF-α and IFN-γ was assayed.

Messenger RNA encoding IL-6 and TNF-α was readily detectable in BAL cells from Sephadex-treated control rats 24 h after injection of Sephadex particles whereas IFN-γ mRNA was detected less intensely in 3 experiments, very weakly in 2 experiments and not at all in one further experiment (Fig. 7.2.15). Pre-treatment of the rats with BTS 71321 at a concentration of 10 mg/kg totally abolished mRNA expression levels of IL-6, TNF-α and IFN-γ whereas it had no effect on G3PDH mRNA (Fig. 7.2.15). Densitometric analysis of RT-PCR band intensities revealed that BTS 71321 significantly inhibited mRNA expression of each of the 3 cytokines but that it had no effect on mRNA expression of G3PDH compared to controls (Table 7.2.5).

Seventy-two hours after injection of Sephadex particles mRNA encoding IL-6 and TNF-α was readily detectable in BAL cells from 6/6 control rats whereas IFN-γ mRNA was detected in BAL cells from 5/6 control rats (Fig. 7.2.16). Pre-treatment of the rats with BTS 71321 at a concentration of 10 mg/kg totally abolished the expression of IL-6 mRNA but had no effect on mRNA encoding TNF-α, IFN-γ or G3PDH (Fig. 7.2.16). Densitometric analysis of RT-PCR band intensities revealed that the inhibitory effect of BTS 71321 on IL-6 mRNA expression was highly significant over 6 independent experiments compared to control levels (Table 7.2.6).

7.2.7 Immunohistochemical analysis of BAL cells

BAL cell cytospin slides that had been stained with antibodies for IL-6 or TNF-α revealed that IL-6 and TNF-α were produced by BAL cells 24 h after

Sephadex administration. In the figures shown, the cells appear to be mononuclear monocyte/macrophage-like cells (Fig. 7.2.17 A and C). Figures B and D show respective negative stained controls. Pre-treatment of rats with Dex at 0.1 mg/kg completely abolished the production of IL-6 by BAL cells 24 h after Sephadex injection (Fig. 7.2.17E) but not production of TNF-α (Fig. 7.2.17F).

7.3 DISCUSSION

This study clearly demonstrates that Dex and CsA have potent anti-inflammatory effects with regard to cell mobilisation and cytokine mRNA expression within the rat Sephadex model of lung inflammation, whereas BTS 71321 does not influence cell mobilisation but does inhibit the induced expression of IL-6, TNF-α and IFN-γ. Twenty-four hours after induction of lung inflammation, Dex significantly inhibited the infiltration of neutrophils and eosinophils into the bronchoalveolar cavity, whereas after 72 h it had reduced numbers of total BAL cells, mononuclear cells neutrophils and eosinophils. Twenty-four hours after induction of lung inflammation, CsA had inhibited total numbers of infiltrating BAL cells, neutrophils and eosinophils, but by 72 h most of these effects had been overcome, although eosinophil numbers were still significantly inhibited. However, BTS 71321 had no effect, at 10 mg/kg, on numbers of any type of bronchoalveolar cells 24 h or 72 h after Sephadex administration.

Concurrent with inhibition of bronchoalveolar eosinophilia, a significant inhibition of mRNA encoding IL-5 was observed in many experiments. Twenty-four hours after induction of lung inflammation, CsA at 30 mg/kg, had inhibited the infiltration of eosinophils and had totally abolished IL-5 mRNA expression in most experiments. Similar effects were observed after 72 h. Although Dex did not significantly inhibit IL-5 mRNA expression 24 h post Sephadex-treatment, it had inhibited the infiltration of bronchoalveolar eosinophils. However, expression of mRNA encoding IL-5

was not detected in BAL cells from two control rats and only weakly in BAL cells from three further rats. Thus, analyses of changes in PCR band intensities did not reveal significant differences after 24 h. However, 72 h post Sephadex-treatment, Dex had significantly inhibited the expression of IL-5 mRNA and the infiltration of eosinophils into the bronchoalveolar cavity. IL-5 is a known eosinophil chemoattractant (Wang *et al.*, 1989) and in chapter 6, eosinophilia and IL-5 mRNA expression were found to peak together at 72 h post Sephadex-treatment. The results of the present chapter provide further circumstantial evidence for a relationship between these events.

Unfortunately, due to a shortage of primers, mRNA encoding MIP-2 was not measured in this chapter. However, previous results have suggested that neutrophil influx and expression of MIP-2 are closely related in the rat Sephadex model of lung inflammation (chapter 6). Messenger RNA encoding MIP-2, a known neutrophil chemoattractant in rodents, was found to peak at, or slightly before the peak neutrophil response, providing circumstantial evidence for a connection between these two events. Therefore, investigations of the effects of Dex and CsA, on mRNA expression of MIP-2 would prove interesting, and if inhibition of MIP-2 mRNA concurrent with inhibition of neutrophilia was observed, this would lend support to the hypothesis that MIP-2 is an important neutrophil chemoattractant in the rat.

BTS 71321 was found to have no effect on levels of infiltrating eosinophils or neutrophils. Unfortunately, due to a shortage of reagents,

including primers IL-5 and MIP-2 mRNA were not measured in these experiments. However, because of the possible relationships between IL-5 and eosinophil migration and MIP-2 and neutrophil migration, it would certainly have been of interest to measure mRNA for these two cytokines, and it might be predicted from the above, that they would not be influenced by BTS 71321.

Messenger RNA encoding IL-6 was inhibited by each of the three drugs at both 24 h and 72 h post Sephadex injection and in the case of Dex, there was a clear relationship between inhibition of transcription and inhibition of expression of immunohistochemically detected product. Previous research has demonstrated that Dex can inhibit the production of IL-6 by monocytes, endothelial cells and fibroblasts and these effects are via a receptor-mediated mechanism directed towards IL-6 transcription (Waage *et al.*, 1990). Additionally, Dex can inhibit the mRNA transcription and release of biologically active IL-6 from rat PMC (chapter 5; Leal-Berumen *et al.*, 1994) and CsA is known to inhibit the transcription of rat mast cell IL-6 (chapter 5) and its expression in murine BMMC (Kaye *et al.*, 1992). The observations presented in this chapter indicate that Dex and CsA also have pronounced effects on IL-6 mRNA expression *in vivo.* Clearly the marked suppressive effect on IL-6 transcription by rat BAL cells and other cell types, provides an additional mechanism by which these drugs exert their immunosuppressive and anti-inflammatory effects. In the case of CsA and Dex this inhibition of IL-6 mRNA may relate to inhibition of inflammatory cell recruitment that was observed after injection of Sephadex particles.

However, no such inhibition was observed with BTS 71321, although IL-6 transcription was inhibited at both 24 h and 72 h post-Sephadex-injection. It therefore appears that selective inhibition of IL-6 mRNA is not sufficient to down-regulate an inflammatory response measured as cell infiltration, as other cytokines appear to compensate for the lack of IL-6 product.

The effects of Dex, CsA or BTS 71321 on the inhibition of TNF-α mRNA differed widely. BTS 71321 inhibited the expression of TNF-α mRNA after 24 h, whereas 72 h later this effect had subsided. Dex did not inhibit TNF-α mRNA until 72 h post-Sephadex-injection, whilst CsA had no effect on transcriptional levels at either 24 h or 72 h. Previous results have indicated that expression of mast cell TNF-α is inhibited *in vitro* by CsA and Dex (chapter 5) and that these agents can also significantly suppress the tissue swelling and leucocyte infiltration associated with TNF-α-associated inflammation *in vivo* (Wershil *et al.*, 1995). However, other research has indicated that CsA does not inhibit TNF-α mRNA or protein expression in lipopolysaccharide-treated mice (Cockfield *et al.*, 1993). Additionally, TNF-α mRNA expression by the monocyte/macrophage population, is resistant to the inhibitory effects of CsA (Granelli-Piperno *et al.*, 1988), suggesting that cells of disparate origin have differing pathways for the activation of the TNF-α gene. The present observations that TNF-α transcription is resistant to inhibition by CsA suggests that the monocyte/macrophage cell population is the major cellular source of TNF-α in this model. Indeed, immunohistochemical analysis of BAL cell cytospin preparations, demonstrated that TNF-α was produced principally by mononuclear cells.

The results also demonstrate that both Dex and BTS 71321 are effective inhibitors of TNF-*α* transcription although they exhibit different kinetic profiles. BTS 71321 was found to act more rapidly than Dex with noticeable and significant inhibition after 24 h, whereas the inhibitory effects of Dex were not observed until 72 h post-Sephadex-injection. Similarly, BTS 71321 inhibited the expression of IFN-*γ* mRNA after 24 h, but these effects were not sustained and by 72 h IFN-*γ* mRNA expression had recovered. However, Dex inhibited IFN-*γ* mRNA levels 24 h and 72 h after induction of lung inflammation, whereas CsA had no effect at either time point. Although TNF-*α* and IFN-*γ* share similar profiles of mRNA expression from 24 h to 7 days after injection of Sephadex particles (chapter 6), the delayed appearance of IFN-*γ* mRNA (later than 6 h in contrast to TNF-*α* mRNA which was induced by 6 h post-injection) and the present observations that IFN-*γ* but not TNF-*γ* mRNA is inhibited by Dex 24 h after induction of lung inflammation, argues against a major role for IFN-*γ* in inducing the rapid TNF-*α* response in this model.

Previous reports have indicated that Dex can reduce bronchial hyperresponsiveness to 5-HT and inhibit blood and lung eosinophilia in the rat Sephadex model of lung inflammation (Spicer *et al.*, 1990; Piercy *et al.*, 1993). In view of the fact that TNF-*α* causes bronchial hyperresponsiveness in rats (Kips *et al.*, 1992) it is possible that TNF-*α* mediates the bronchial hyperresponsiveness associated with this model. It would therefore have been of interest to determine whether inhibition of TNF-*α* expression by Dex, CsA or BTS 71321 would have resulted in a decrease in bronchial

hyperreactivity. Such a result would support a causal link between TNF-α-mediated inflammation and hyperresponsiveness and provide a therapeutic mechanism for the effects of BTS 71321.

In conclusion, this study has demonstrated that Dex, CsA and BTS 71321 inhibit cytokine genes at a transcriptional level *in vivo*. With respect to Dex and CsA, inhibition of cytokine gene transcription appears to relate to inhibition of pro-inflammatory cell recruitment. However, similar results were not observed with BTS 71321. Although IL-6, TNF-α and IFN-γ mRNA expression were inhibited by BTS 71321 24 h after induction of lung inflammation, no significant inhibition of mononuclear cell, neutrophil or eosinophil numbers was observed. Dex and CsA are well established anti-inflammatory agents that can inhibit the transcription of a number of cytokine genes other than those investigated here. However, besides those discovered within this project, the effects of BTS 71321 on cytokine gene transcription are unknown. Results presented in this chapter imply that cell infiltration into the bronchial lumen is not fully dependent on IL-6, TNF-α and IFN-γ, since BTS 71321 inhibits the expression of these cytokines, without influencing cell numbers. Therefore, the present observations suggest that inhibition of migration of these cells by Dex and CsA is due not only to inhibition of IL-6, TNF-α and IFN-γ but also to inhibition of other cytokine genes which may be unaffected by BTS 71321.

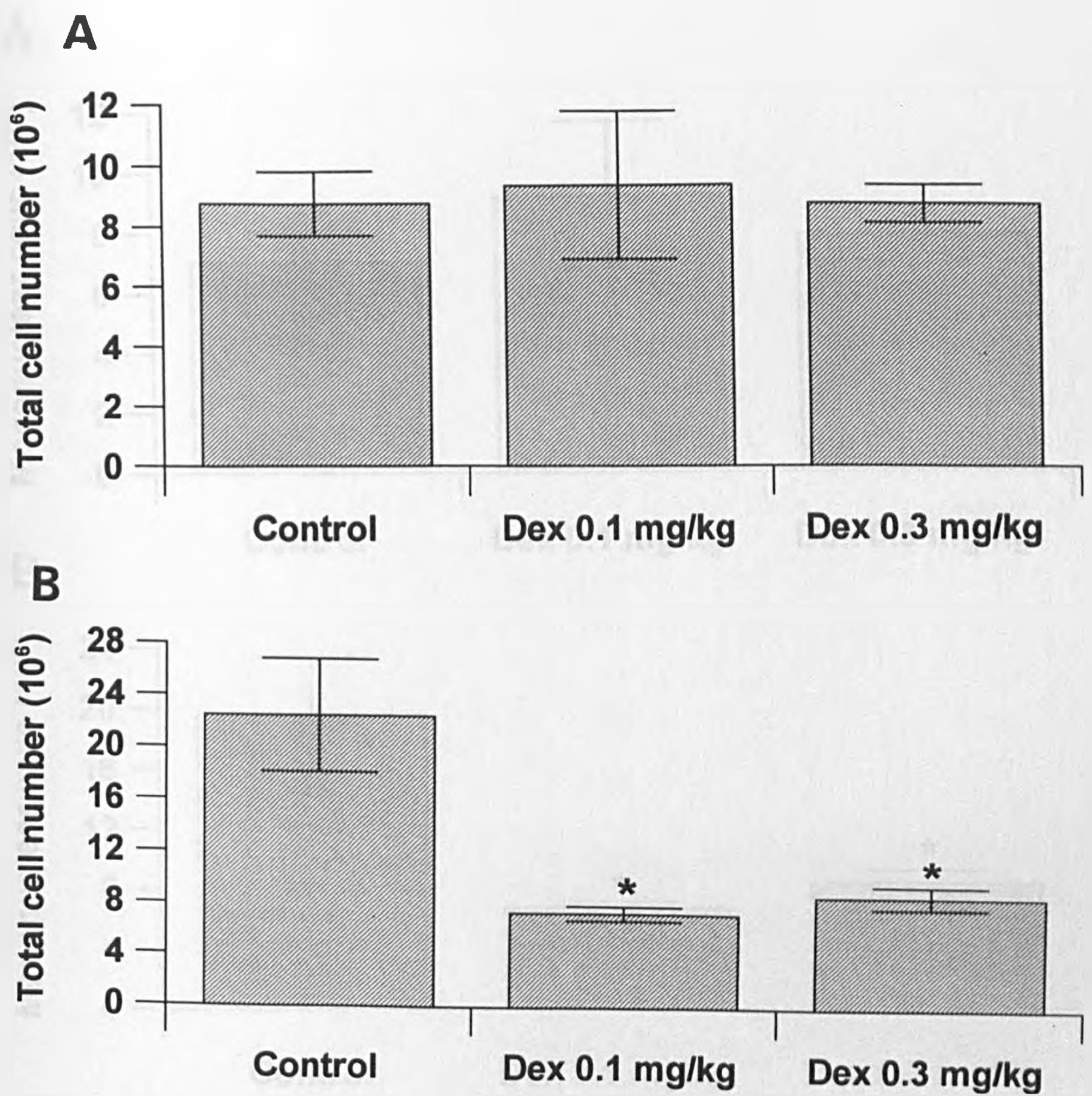

Fig. 7.2.1 The effects of Dex (0.1 mg/kg and 0.3 mg/kg) on total cell numbers in BAL fluid 24 h (A) and 72 h (B) after induction of lung inflammation. Results are means ± SEM for 10 rats. $p < 0.05$ by comparison of drug-treated/Sephadex-injected rats with vehicle-treated/Sephadex-injected control rats as determined by the two-tailed Mann Whitney *U* test.

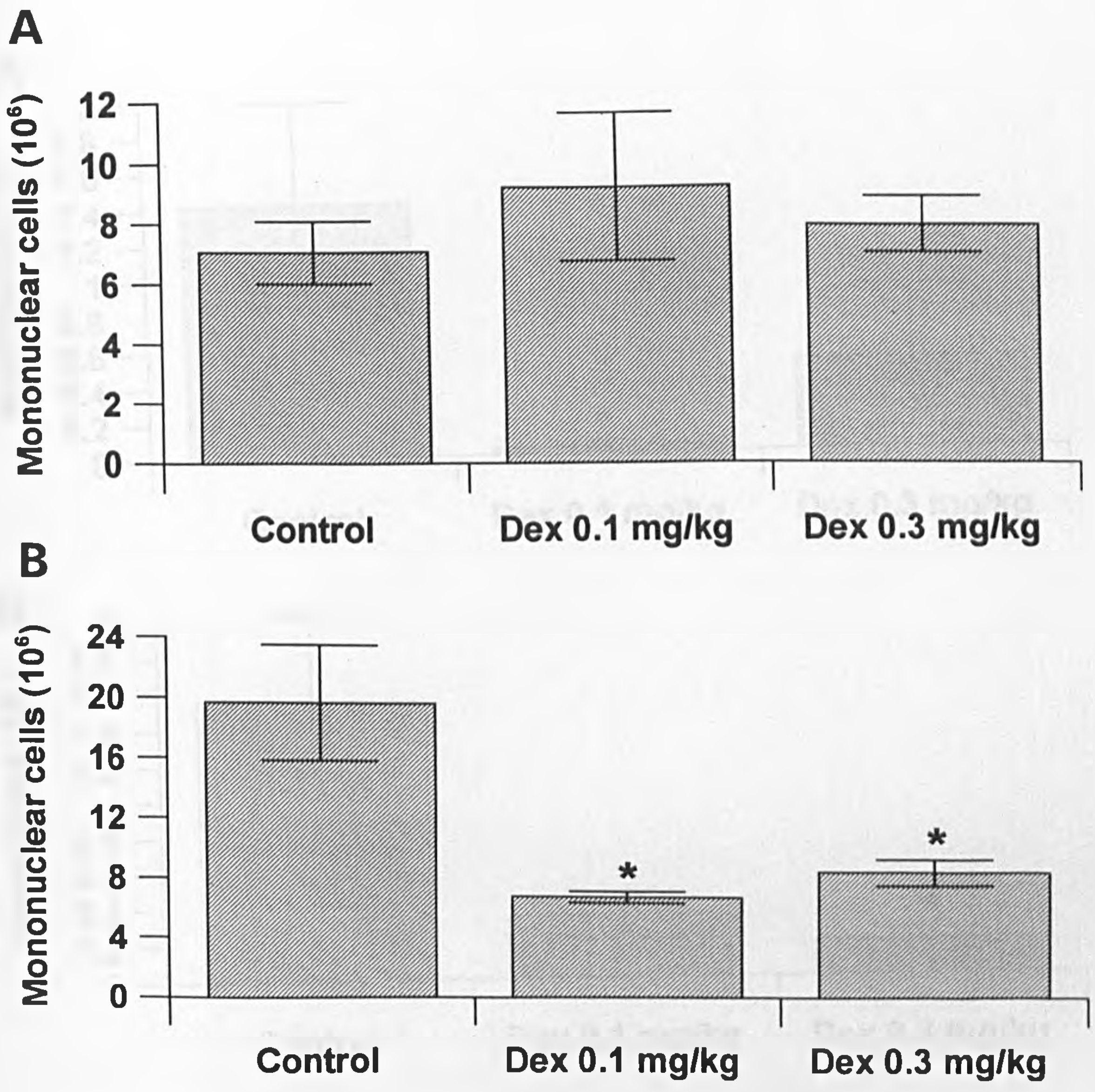

Fig. 7.2.2 The effects of Dex (0.1 mg/kg and 0.3 mg/kg) on numbers of mononuclear cells in BAL fluid 24 h (A) and 72 h (B) after induction of lung inflammation. Results are means ± SEM for 10 rats. $p < 0.05$ by comparison of drug-treated/Sephadex-injected rats with vehicle-treated/Sephadex-injected control rats, as determined by the two-tailed Mann Whitney *U* test.

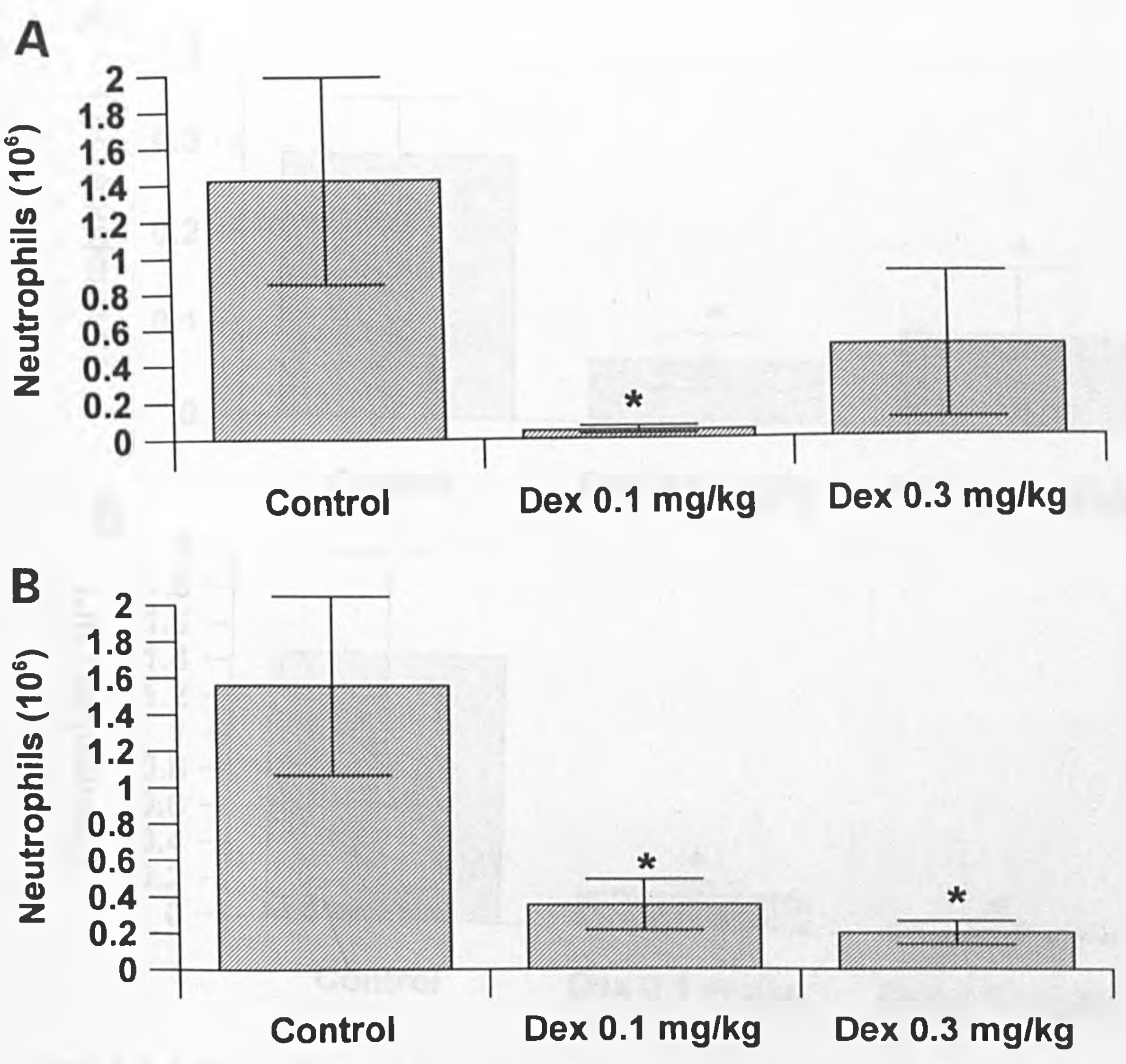

Fig. 7.2.3 The effects of Dex (0.1 mg/kg and 0.3 mg/kg) on numbers of neutrophils in BAL fluid 24 h (A) and 72 h (B) after induction of lung inflammation. Results are means ± SEM for 10 rats. $p < 0.05$ by comparison of drug-treated/Sephadex-injected rats with vehicle-treated/Sephadex-injected control rats as determined by the two-tailed Mann Whitney *U* test.

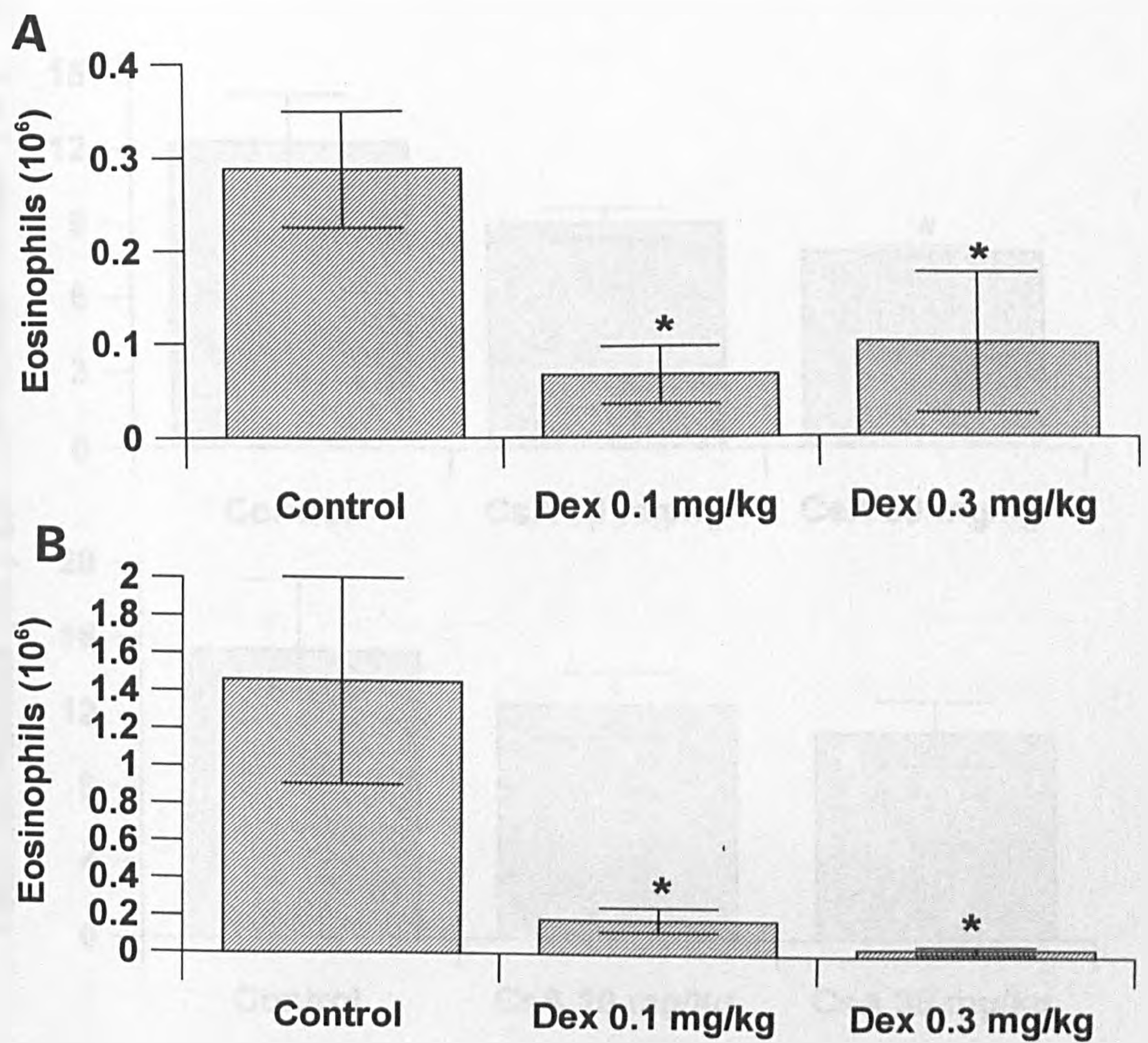

Fig. 7.2.4 The effects of Dex (0.1 mg/kg and 0.3 mg/kg) on numbers of eosinophils in BAL fluid 24 h (A) and 72 h (B) after induction of lung inflammation. Results are means ± SEM for 10 rats. $p < 0.05$ by comparison of drug-treated/Sephadex-injected rats with vehicle-treated/Sephadex-injected control rats as determined by the two-tailed Mann Whitney *U* test.

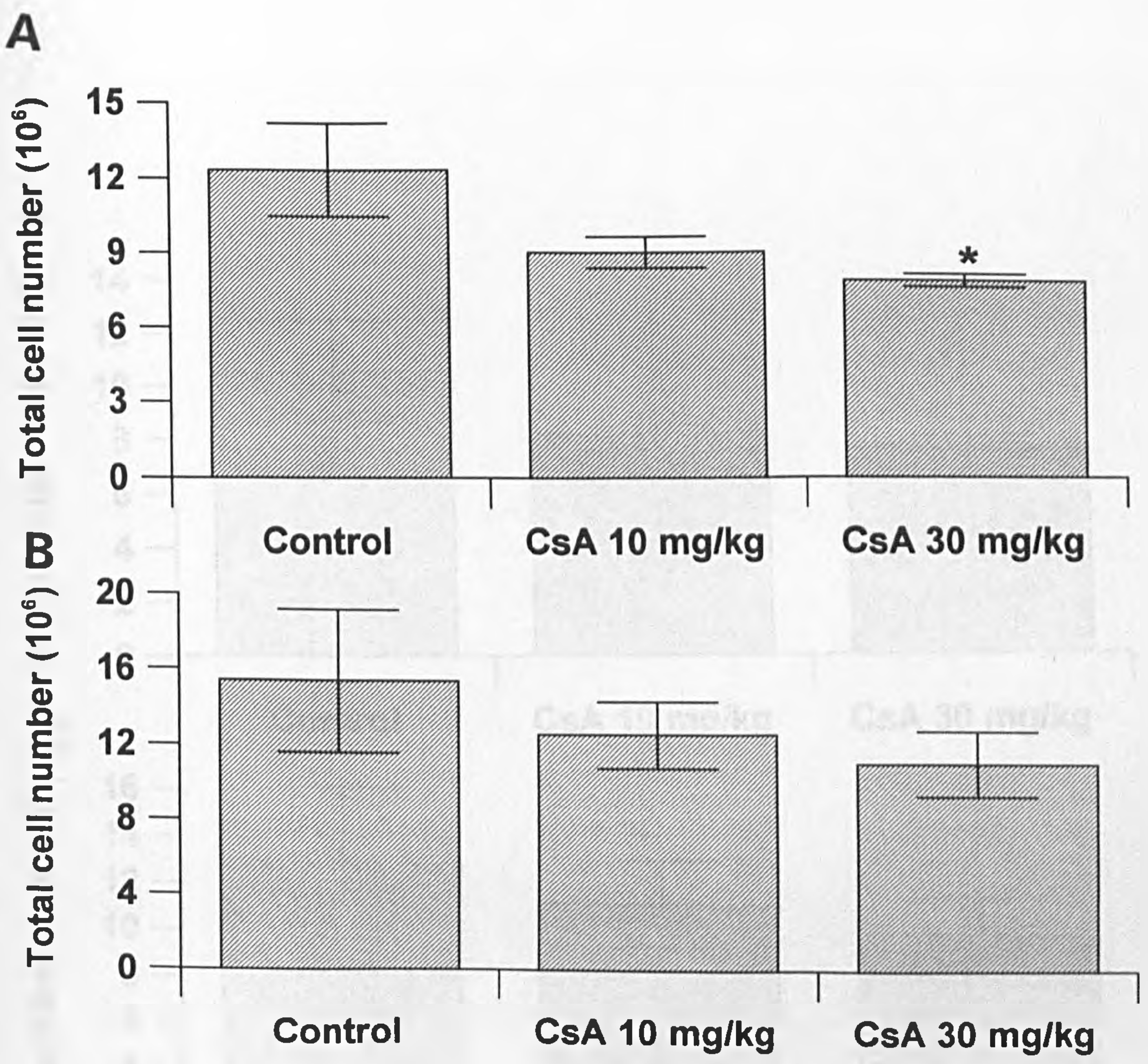

Fig. 7.2.5 The effects of CsA (10 mg/kg and 30 mg/kg) on total cell numbers in BAL fluid 24 h (A) and 72 h (B) after induction of lung inflammation. Results are means ± SEM for 5 rats. $p < 0.05$ by comparison of drug-treated/Sephadex-injected rats with vehicle- treated/Sephadex-injected control rats as determined by the two-tailed Mann Whitney *U* test.

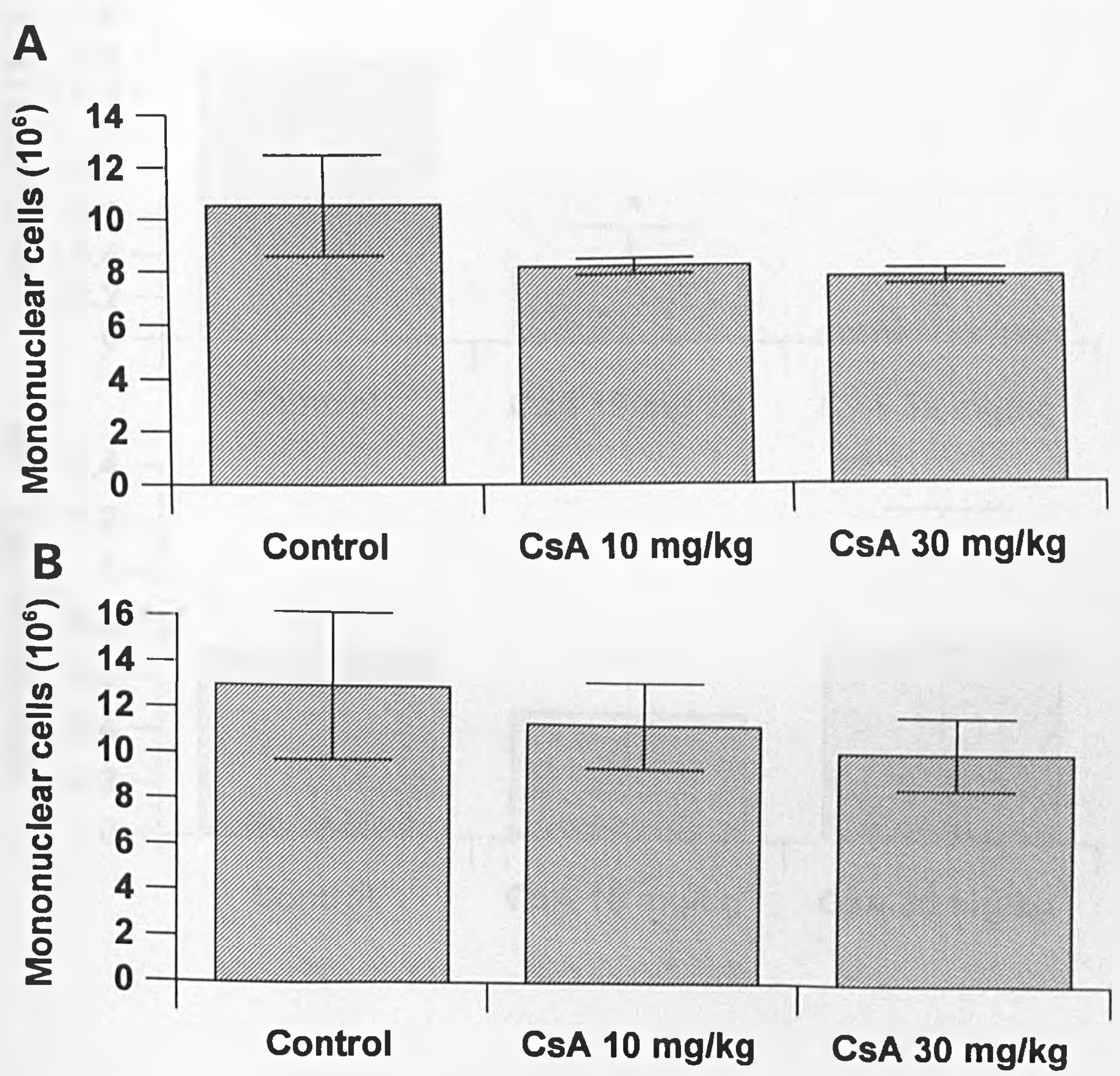

Fig. 7.2.6 The effects of CsA (10 mg/kg and 30 mg/kg) on numbers of mononuclear cells in BAL fluid 24 h (A) and 72 h (B) after induction of lung inflammation. Results are means ± SEM for 5 rats.

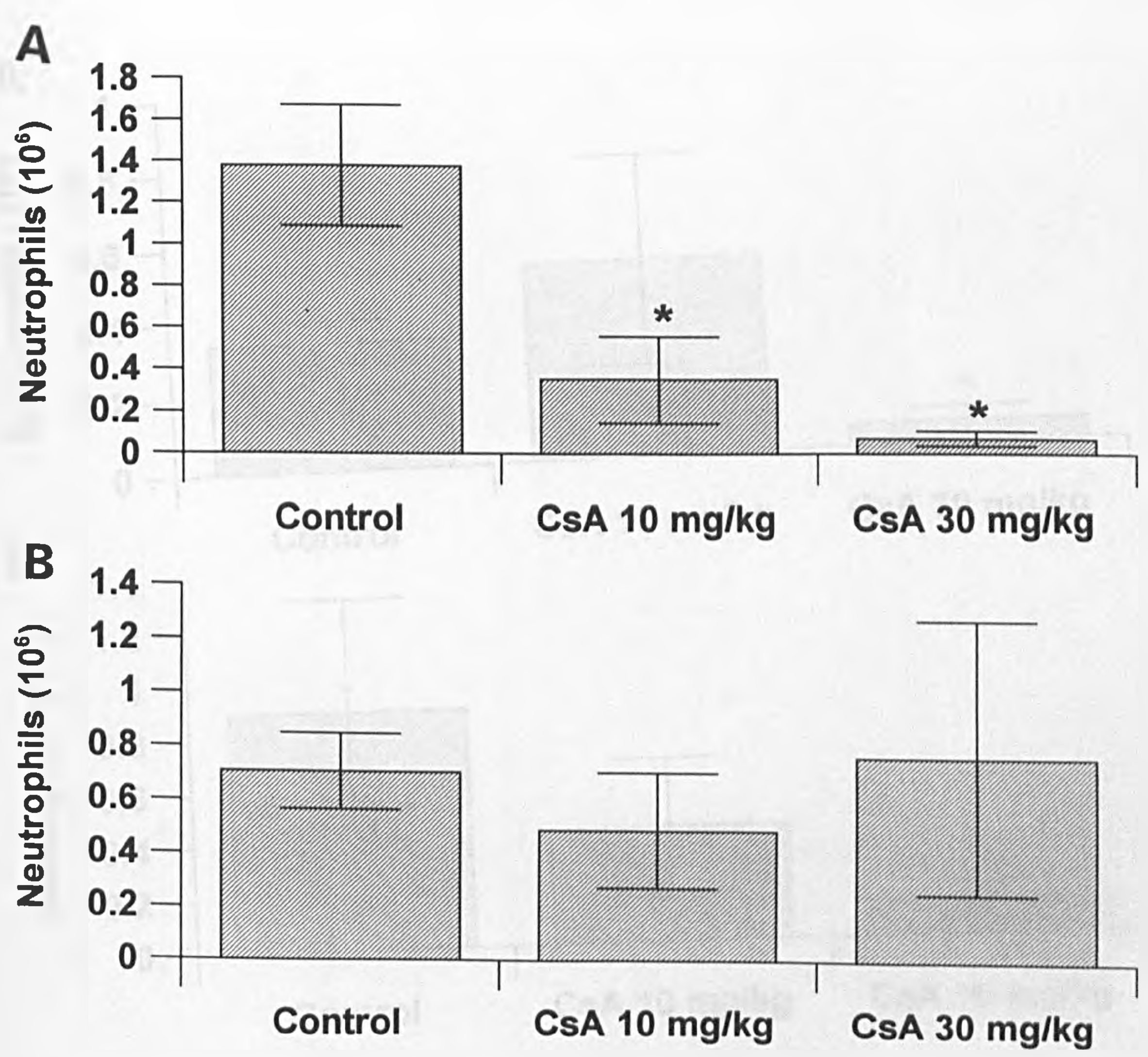

Fig. 7.2.7 The effects of CsA (10 mg/kg and 30 mg/kg) on numbers of neutrophils in BAL fluid 24 h (A) and 72 h (B) after induction of lung inflammation. Results are means ± SEM for 5 rats. $p < 0.05$ by comparison of drug-treated/Sephadex-injected rats with vehicle-treated/Sephadex-injected control rats as determined by the two-tailed Mann Whitney *U* test.

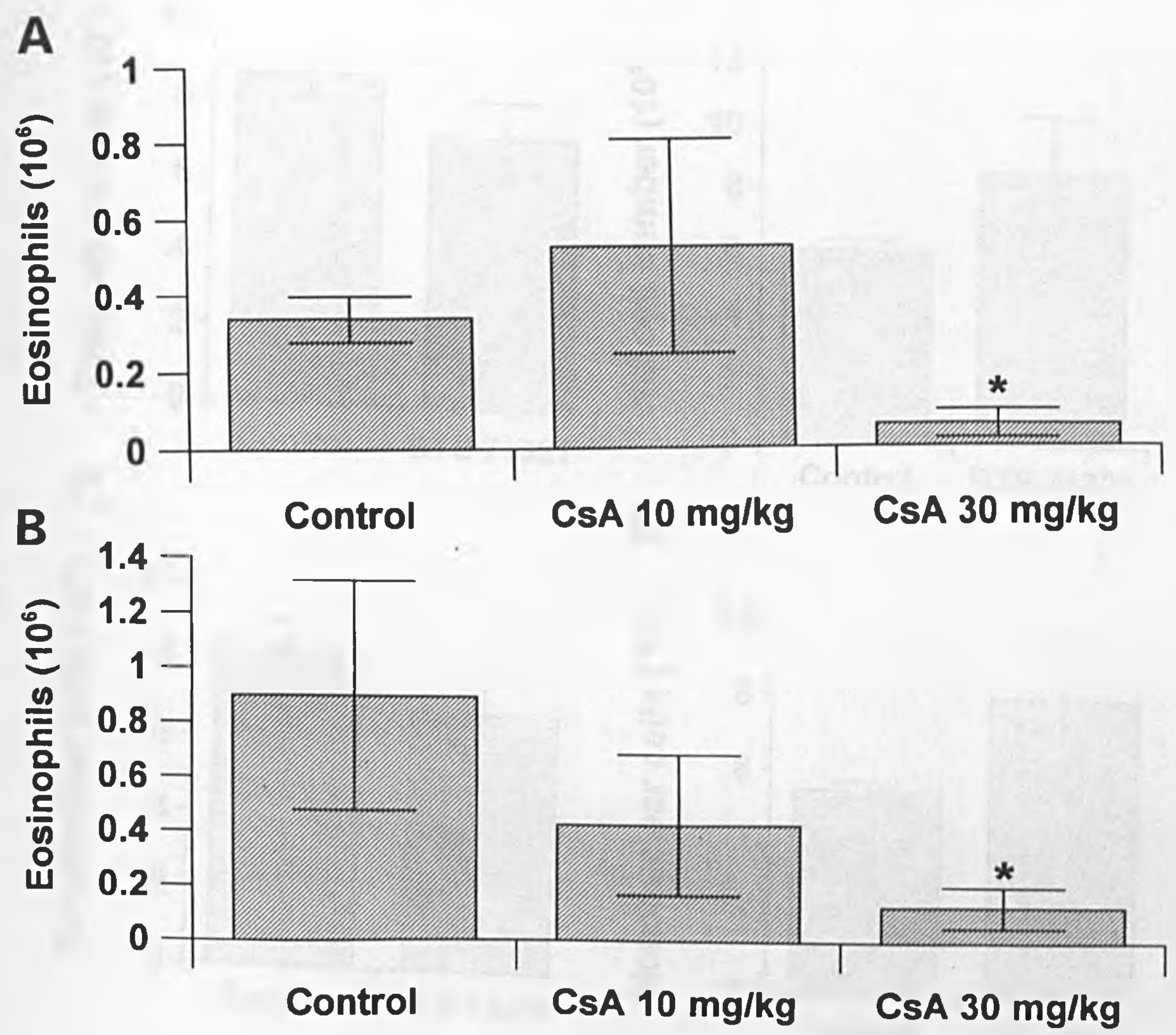

Fig. 7.2.8 The effects of CsA (10 mg/kg and 30 mg/kg) on numbers of eosinophils in BAL fluid 24 h (A) and 72 h (B) after induction of lung inflammation. Results are means ± SEM for 5 rats. $p < 0.05$ by comparison of drug-treated/Sephadex-injected rats with vehicle-treated/Sephadex-injected control rats as determined by the two-tailed Mann Whitney *U* test.

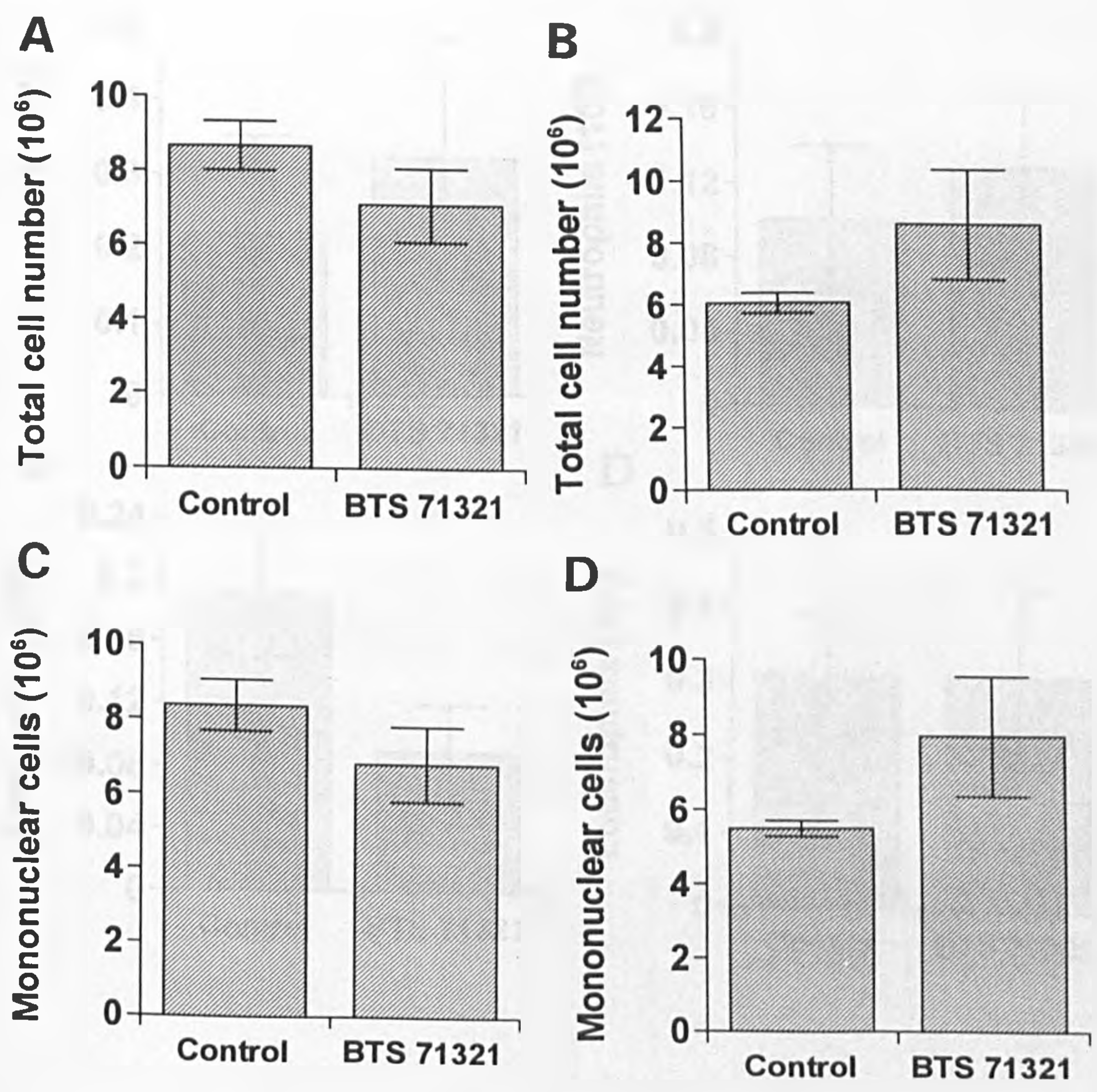

Fig. 7.2.9 The effects of BTS 71321 (10 mg/kg) on total cell numbers (A and B) and mononuclear cell numbers (C and D) in BAL fluid 24 h (A and C) and 72 h (B and D) after induction of lung inflammation. Results are means ± SEM for 6 rats.

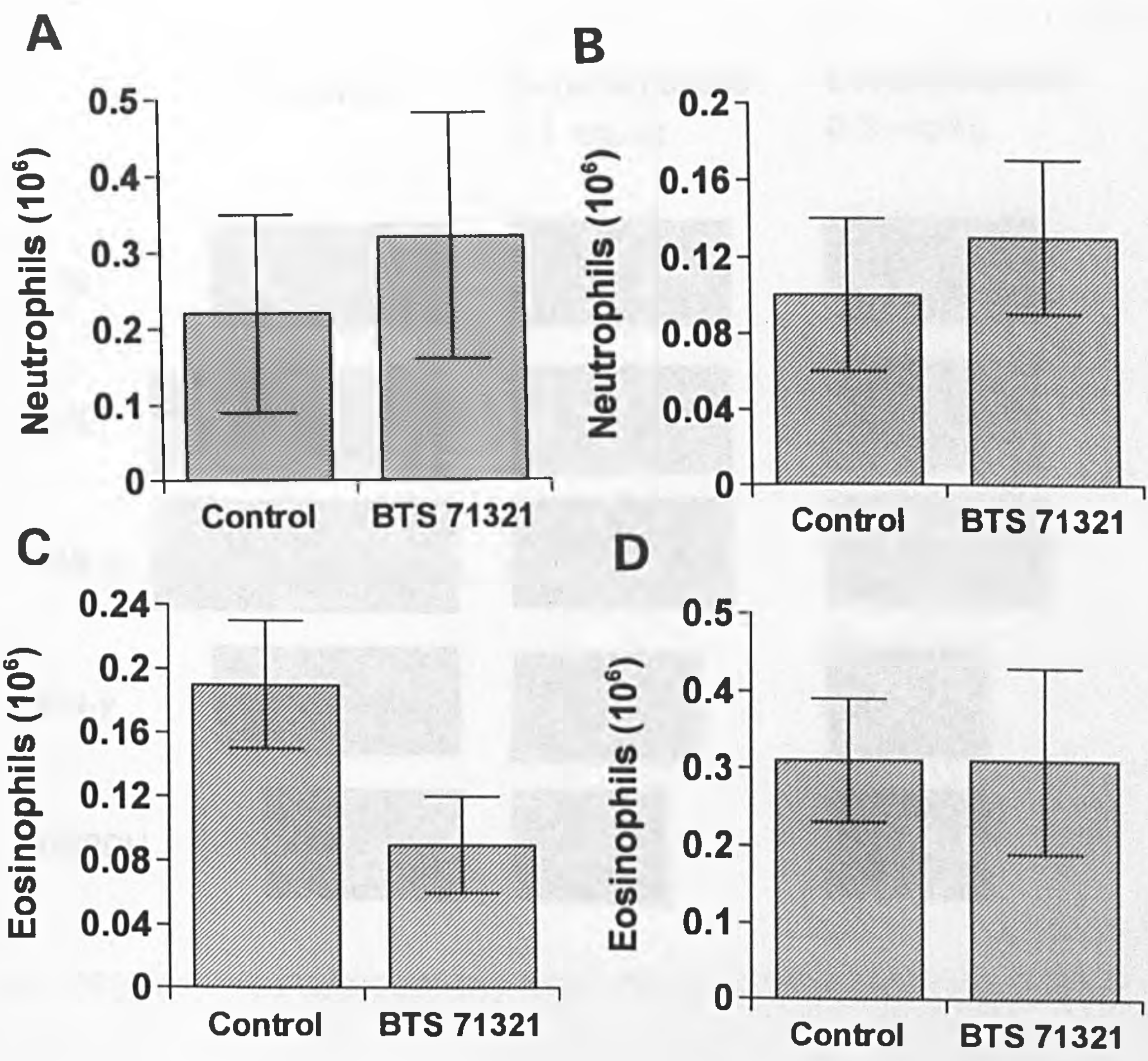

Fig. 7.2.10 The effects of BTS 71321 (10 mg/kg) on numbers on infiltrating neutrophils (A and B) and eosinophils (C and D) in BAL fluid 24 h (A and C) and 72 h (B and D) after induction of lung inflammation. Results are means ± SEM for 6 rats.

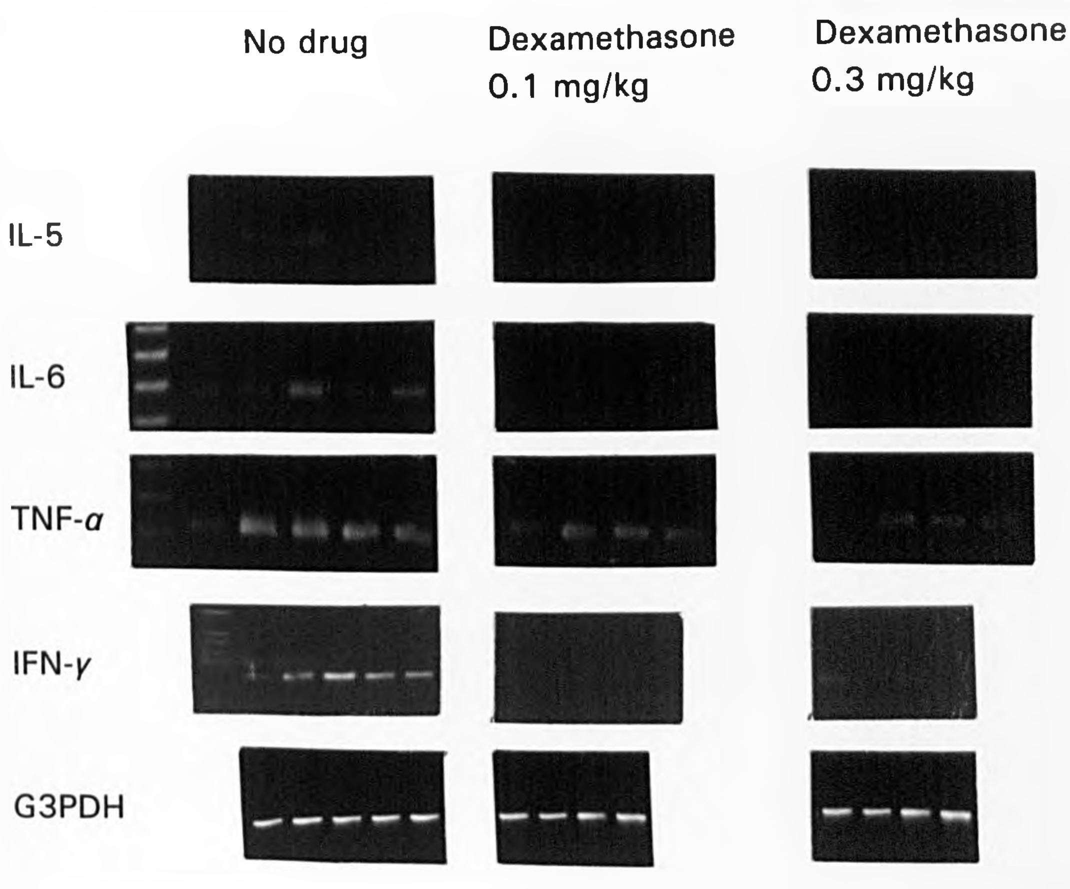

Fig. 7.2.11 RT-PCR analysis of mRNA for IL-5, IL-6, TNF-α IFN-γ and G3PDH from RNA extracted from rat BAL cells 24 h after injection of Sephadex particles. Rats were treated with or without (no drug) Dex (0.1 mg/kg or 0.3mg/kg) prior to injection of Sephadex particles. Each RNA sample was obtained from BAL cells pooled from two rats. The left hand lane for IL-6, TNF-α and IFN-γ shows molecular weight markers.

	Control	Dex (0.1 mg/kg)	Dex (0.3 mg/kg)
IL-5	0.06 ± 0.03	0.00 ± 0.00	0.00 ± 0.00
IL-6	0.30 ± 0.05	0.00 ± 0.00 *	0.00 ± 0.00 *
TNF-α	0.43 ± 0.05	0.40 ± 0.04	0.22 ± 0.05
IFN-γ	0.35 ± 0.04	0.00 ± 0.00 *	0.007 ± 0.005 *
G3PDH	0.46 ± 0.007	0.46 ± 0.01	0.44 ± 0.02

Table 7.2.1 Laser densitometric measurements of the area-under-the-curve of mRNA product bands generated from RNA extracted from BAL cells from rats that had been treated with Sephadex for 24 h. Rats were treated with or without Dex (0.1 mg/kg or 0.3 mg/kg) prior to Sephadex injection. Results are means of PCR product band intensities ± SEM for at least 4 experiments. * $p < 0.05$ by comparison of drug-treated cells with controls as determined by the two-tailed Mann Whitney *U* test.

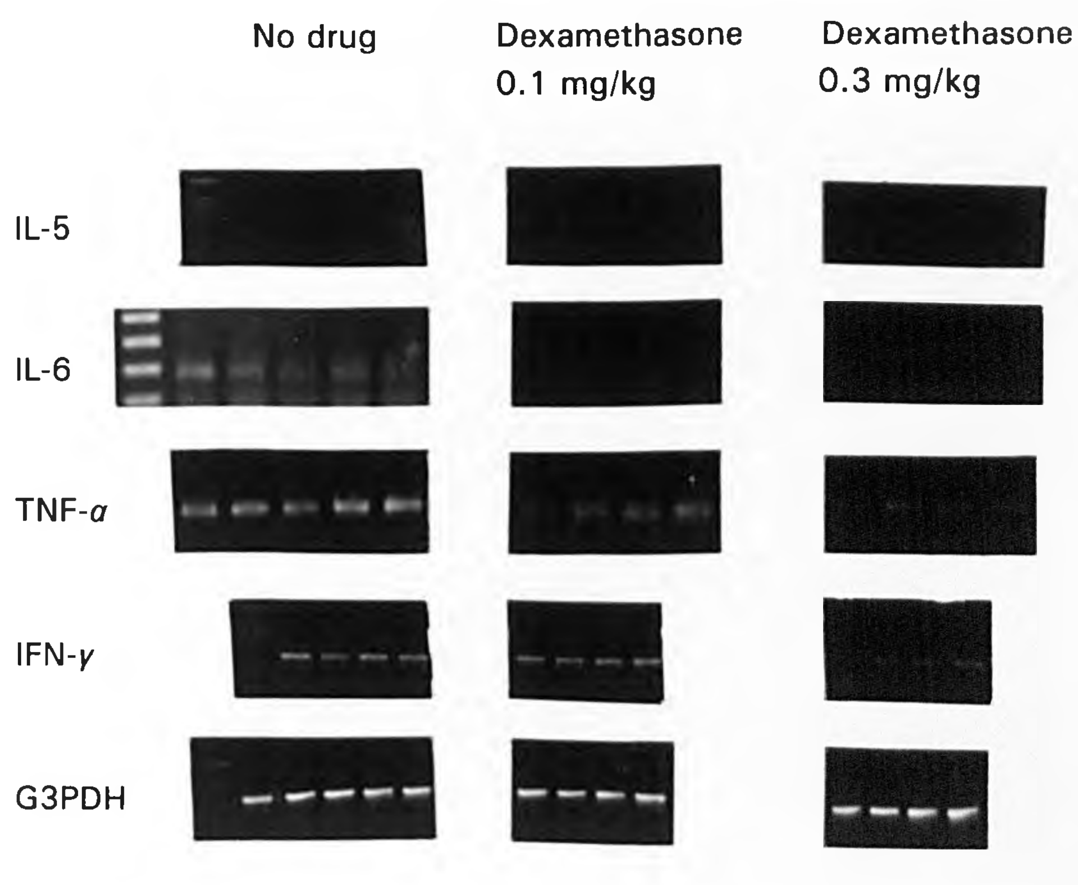

Fig. 7.2.12 RT-PCR analysis of mRNA for IL-5, IL-6, TNF-α IFN-γ and G3PDH from RNA extracted from rat BAL cells 72 h after injection of Sephadex particles. Rats were treated with or without (no drug) Dex (0.1 mg/kg or 0.3mg/kg) prior to injection of Sephadex particles. Each RNA sample was obtained from BAL cells pooled from two rats. The left hand lane for IL-5, IL-6, IFN-γ and G3PDH shows molecular weight markers.

	Control	Dex (0.1 mg/kg)	Dex (0.3 mg/kg)
IL-5	0.08 ± 0.01	0.02 ± 0.006 *	0.00 ± 0.00 *
IL-6	0.28 ± 0.03	0.00 ± 0.00 *	0.00 ± 0.00 *
TNF-α	0.66 ± 0.02	0.48 ± 0.05 *	0.17 ± 0.03 *
IFN-γ	0.26 ± 0.01	0.27 ± 0.02	0.09 ± 0.03 *
G3PDH	0.47 ± 0.02	0.46 ± 0.02	0.47 ± 0.02

Table 7.2.2 Laser densitometric measurements of the area-under-the-curve of mRNA product bands generated from RNA extracted from BAL cells from rats that had been treated with Sephadex for 72 h. Rats were treated with or without Dex (0.1 mg/kg or 0.3 mg/kg) prior to Sephadex injection. Results are means of PCR product band intensities ± SEM for at least 4 experiments. * $p < 0.05$ by comparison of drug-treated cells with controls as determined by the two-tailed Mann Whitney U test.

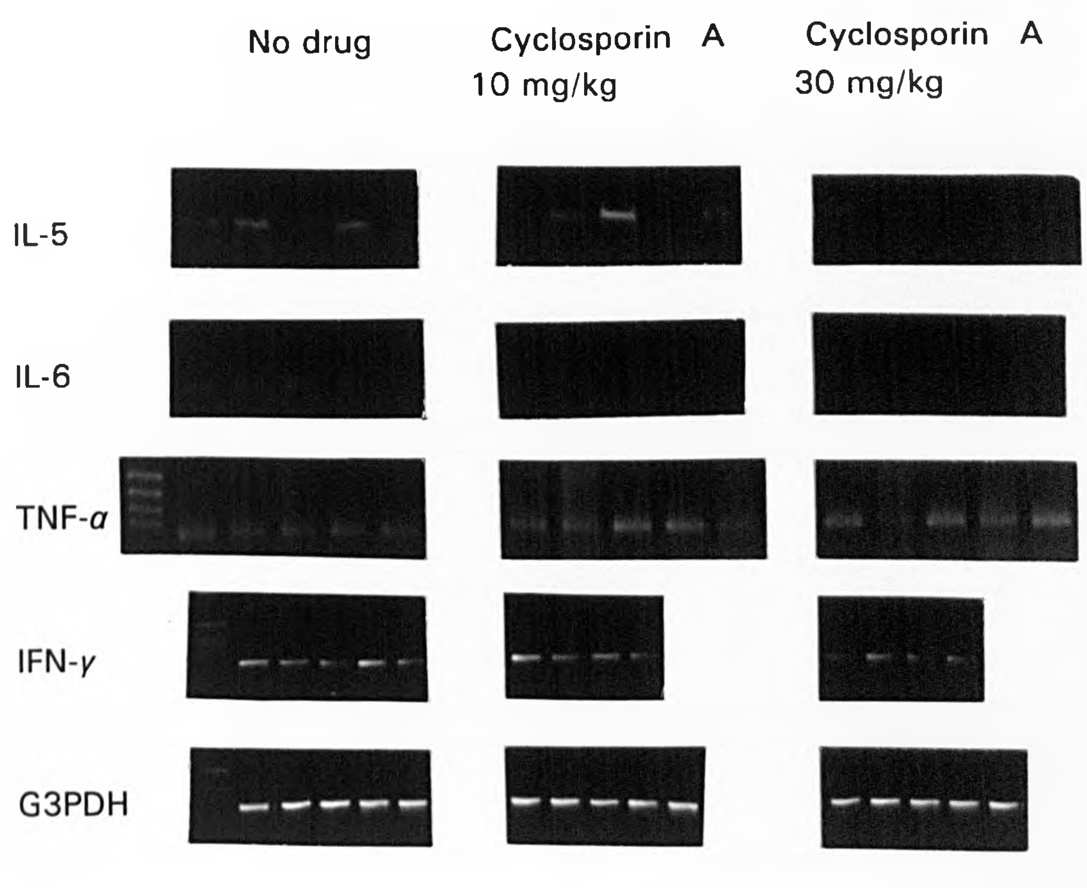

Fig. 7.2.13 RT-PCR analysis of mRNA for IL-5, IL-6, TNF-*α* IFN-*γ* and G3PDH from RNA extracted from rat BAL cells 24 h after injection of Sephadex particles. Rats were treated with or without (no drug) CsA (10 mg/kg or 30mg/kg) prior to injection of Sephadex particles. Each RNA sample was extracted from BAL cells pooled from two rats. The left hand lane for TNF-*α*, IFN-*γ* and G3PDH shows molecular weight markers.

	Control	CsA (10 mg/kg)	CsA (30 mg/kg)
IL-5	0.14 ± 0.04	0.14 ± 0.08	0.02 ± 0.02 *
IL-6	0.12 ± 0.02	0.02 ± 0.01 *	0.002 ± 0.002 *
TNF-α	0.15 ± 0.01	0.18 ± 0.03	0.19 ± 0.04
IFN-γ	0.22 ± 0.04	0.23 ± 0.05	0.12 ± 0.02
G3PDH	0.46 ± 0.02	0.48 ± 0.01	0.45 ± 0.02

Table 7.2.3 Laser densitometric measurements of the area-under-the-curve of mRNA product bands generated from RNA extracted from BAL cells from rats that had been treated with Sephadex for 24 h. Rats were treated with or without CsA (10 mg/kg or 30 mg/kg) prior to Sephadex injection. Results are means of PCR product band intensities ± SEM for at least 4 experiments. * $p < 0.05$ by comparison of drug-treated cells with controls as determined by the two-tailed Mann Whitney *U* test.

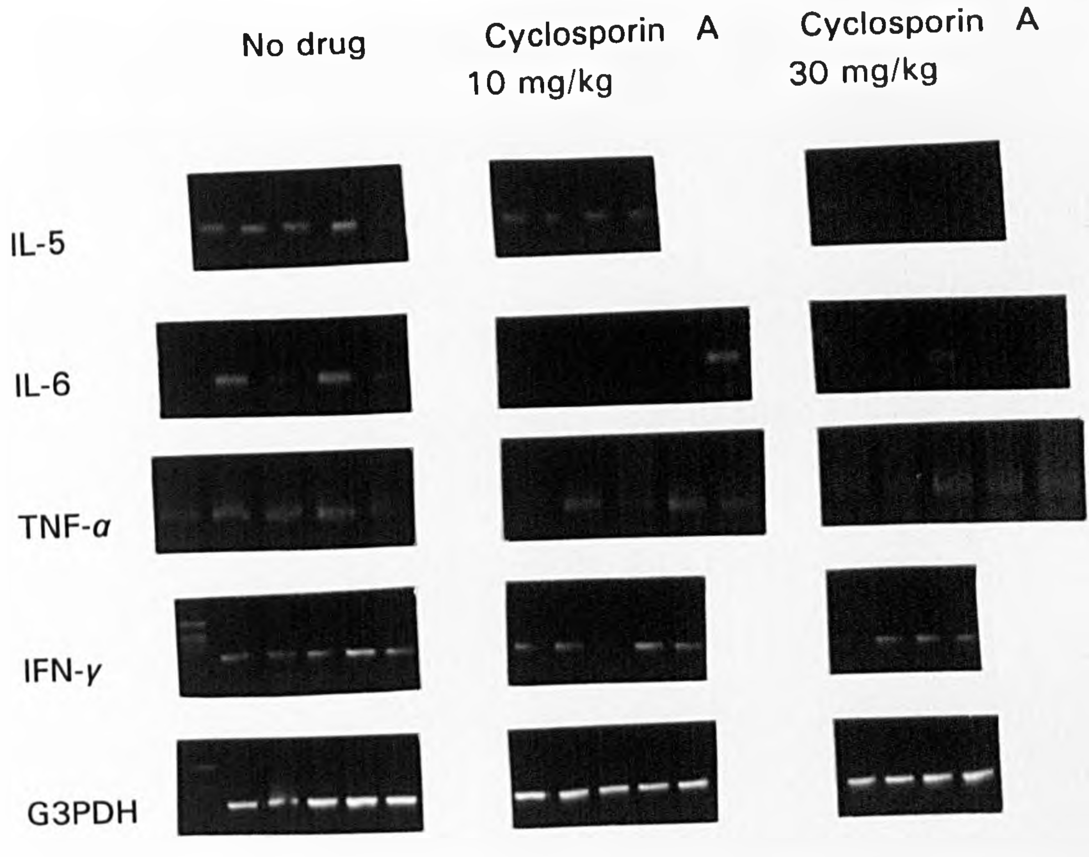

Fig. 7.2.14 RT-PCR analysis of mRNA for IL-5, IL-6, TNF-α IFN-γ and G3PDH from RNA extracted from rat BAL cells 72 h after injection of Sephadex particles. Rats were treated with or without (no drug) CsA (10 mg/kg or 30mg/kg) prior to injection of Sephadex particles. Each RNA sample was extracted from BAL cells pooled rom two rats. The left hand lane for IFN-γ and G3PDH shows molecular weight markers.

	Control	CsA (10 mg/kg)	CsA (30 mg/kg)
IL-5	0.32 ± 0.08	0.21 ± 0.02	0.08 ± 0.02 *
IL-6	0.33 ± 0.09	0.1 ± 0.09	0.09 ± 0.07 *
TNF-α	0.36 ± 0.03	0.32 ± 0.04	0.27 ± 0.04
IFN-γ	0.26 ± 0.05	0.18 ± 0.03	0.12 ± 0.02
G3PDH	0.47 ± 0.02	0.46 ± 0.02	0.48 ± 0.01

Table 7.2.4 Laser densitometric measurements of the area-under-the-curve of mRNA product bands generated from RNA extracted from BAL cells from rats that had been treated with Sephadex for 72 h. Rats were treated with or without CsA (10 mg/kg or 30 mg/kg) prior to Sephadex injection. Results are means of PCR product band intensities ± SEM for at least 4 experiments. * $p < 0.05$ by comparison of drug-treated cells with controls as determined by the two-tailed Mann Whitney *U* test.

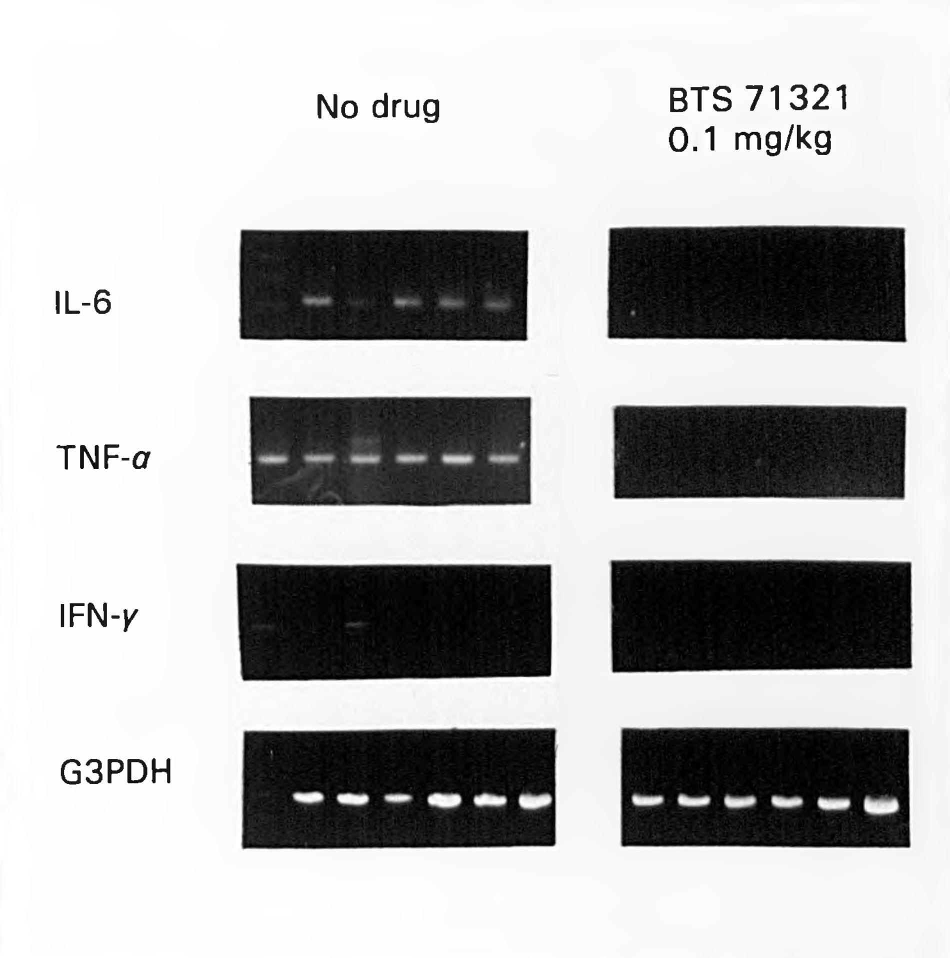

Fig. 7.2.15 RT-PCR analysis of mRNA for IL-6, TNF-α IFN-γ and G3PDH from RNA extracted from rat BAL cells 24 h after injection of Sephadex particles. Rats were treated with or without (no drug) BTS 71321 (10 mg/kg) prior to injection of Sephadex particles. Each RNA sample was extracted from BAL cells from one rat. The left hand lane for IL-6 and G3PDH shows molecular weight markers.

	Control	BTS 71321 (10 mg/kg)
IL-6	0.30 ± 0.04	0.00 ± 0.00 *
TNF-α	0.46 ± 0.01	0.00 ± 0.00 *
IFN-γ	0.14 ± 0.05	0.00 ± 0.00 *
G3PDH	0.61 ± 0.01	0.57 ± 0.02

Table 7.2.5 Laser densitometric measurements of the area-under-the-curve of mRNA product bands generated from RNA extracted from BAL cells from rats that had been treated with Sephadex for 24 h. Rats were treated with or without BTS 71321 (10 mg/kg) prior to Sephadex injection. Results are means of PCR product band intensities ± SEM for 6 experiments. * $p < 0.05$ by comparison of drug-treated cells with controls as determined by the two-tailed Mann Whitney *U* test.

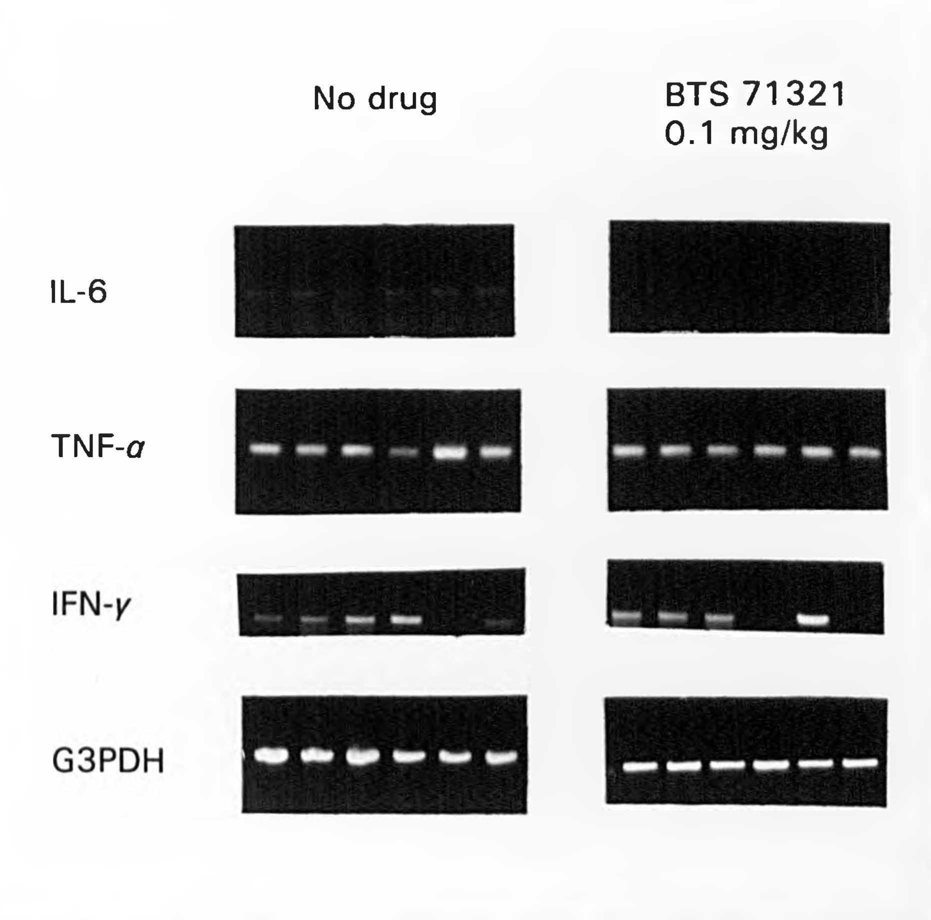

Fig. 7.2.16 RT-PCR analysis of mRNA for IL-6, TNF-α IFN-γ and G3PDH from RNA extracted from rat BAL cells 72 h after injection of Sephadex particles. Rats were treated with or without (no drug) BTS 71321 (10 mg/kg) prior to injection of Sephadex particles. Each RNA sample was extracted from BAL cells from one rat.

	Control	BTS 71321 (10 mg/kg)
IL-6	0.21 ± 0.02	0.00 ± 0.00 *
TNF-α	0.48 ± 0.04	0.47 ± 0.02
IFN-γ	0.21 ± 0.06	0.32 ± 0.01
G3PDH	0.60 ± 0.02	0.55 ± 0.01

Table 7.2.6 Laser densitometric measurements of the area-under-the-curve of mRNA product bands generated from RNA extracted from BAL cells from rats that had been treated with Sephadex for 72 h. Rats were treated with or without BTS 71321 (10 mg/kg) prior to Sephadex injection. Results are means of PCR product band intensities ± SEM for 6 experiments. * $p < 0.05$ by comparison of drug-treated cells with controls as determined by the two-tailed Mann Whitney *U* test.

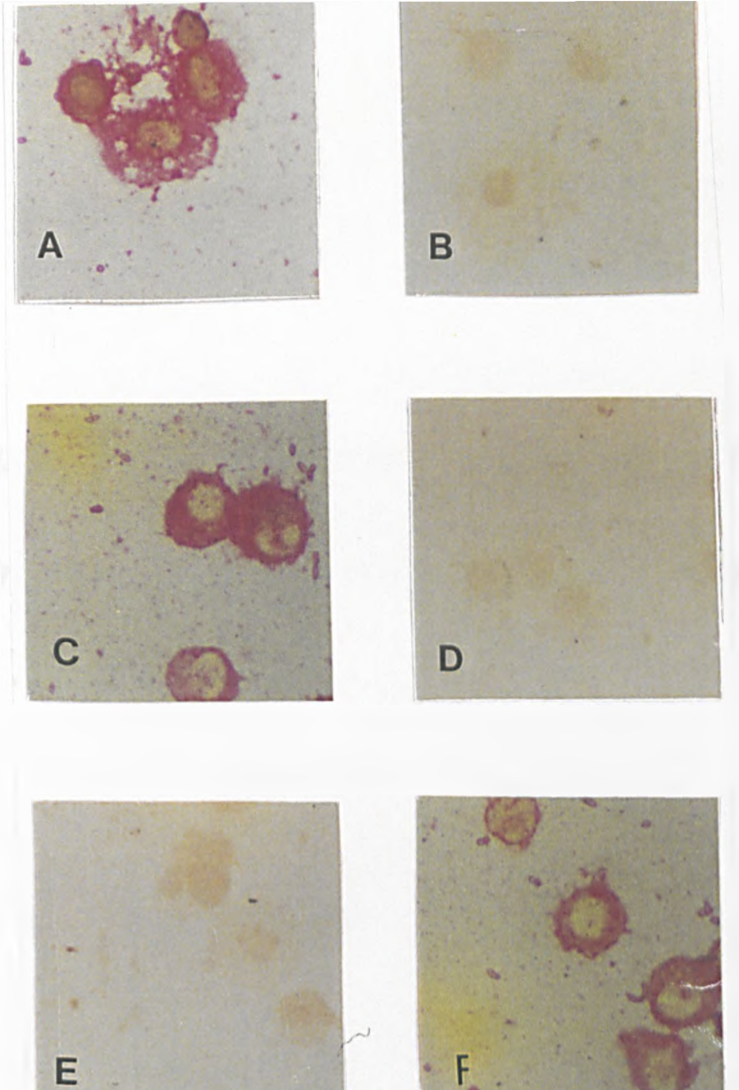

Fig. 7.2.17 Immunohistochemical analysis of BAL cells isolated 24 h after Sephadex administration to rats that had been treated with or without Dex (0.1 mg/kg) prior to injection of Sephadex particles. Cell cytospin sections were stained with antibodies for IL-6 (A and E) or TNF-α (C and F). A, BAL cells from Sephadex-treated control rats stained for IL-6; B, negative control staining (no antibody); C, BAL cells from Sephadex-treated control rats stained for TNF-α; D, negative control staining (no antibody); E, BAL cells from rats treated with Dex before Sephadex administration, stained for IL-6; F, BAL cells from rats treated with Dex before Sephadex administration, stained for TNF-α;

CHAPTER 8

Expression of cytokines and patterns of cell infiltration in an antigen driven model of pleurisy in the rat

8.1 INTRODUCTION

8.2 RESULTS

8.2.1. Cellular content of pleural lavage fluid

8.2.2. Expression of mRNA for IL-5, IL-6, TNF-α, MIP-2 and IFN-γ by pleural lavage cells

8.3 DISCUSSION

INTRODUCTION

Immunoglobulin E-mediated allergic reactions are characterized by an initial sensitization to allergen and are provoked by a later allergen challenge leading to inflammation. Most allergens are either proteins or haptens which possess the ability to breach physical defence mechanisms and gain access to the immune system. They are soluble and foreign and include plant pollens, fungal spores, animal dander, epithelium, fur, urine and saliva, bee venoms, foods, drugs and chemicals. Upon entering the body, allergens are processed by antigen presenting cells into small polypeptide fragments which are recognized, in association with major histocompatibility molecules, by T lymphocytes. These in turn secrete a wide variety of cytokines which enable B cell proliferation and secretion of allergen-specific IgE antibodies, to take place. Allergen-specific IgE antibodies can then bind to high affinity IgE receptors on mast cells or basophils. Upon re-exposure to the same allergen, these cells degranulate and release both preformed and newly synthesised chemical mediators which initiate and mediate the early phase of allergic inflammation. In the lungs these mediators induce bronchoconstriction, oedema in the airway walls and the secretion of mucus which are all characteristic of an acute asthmatic episode, whereas in the nose, mast cell mediators induce rhinorrhoea, blockage and sneezing by actions on both the vasculature and sensory nerve endings (Church and Caulfield, 1993). In the skin, local vasodilation and oedema are responsible for the weal that follows allergen injection while the flare is propagated by

axon reflex-mediated vasodilation stimulated primarily by histamine (Church and Caulfield, 1993).

The early phase of allergic inflammation is often followed by chronic inflammation which is characterized by a marked increase in eosinophils with the concomitant release of their granule mediators. Cytokines are thought to play an important role in the initiation and maintenance of the chronic allergic reaction and mRNA expression and biologically active product for a number of cytokines have been detected in an up-regulated state in allergic disease. Mast cells have also recently been implicated in the late phase allergic reaction as they are known to be an important source of TNF-α and other pro-inflammatory cytokines (chapter 4 and chapter 5)

In this chapter, an antigen driven-model of pleurisy in the rat has been used to investigate the involvement of cytokines in the progression of allergic disease. As has been discussed in chapter 6, animal models of inflammation are used extensively within the pharmaceutical industry to asses the efficacy of novel anti-inflammatory agents. However, the molecular mechanisms underlying inflammatory cell influx, oedema, mucus production and other features of inflammation have been relatively neglected. In the study reported in this chapter, rats were sensitized with a subcutaneous injection of a mixture of ovalbumin and aluminium hydroxide seven days prior to challenge with an intrathoracic injection of ovalbumin. The rats were sacrificed at various times after challenge, pleural cavities were lavaged and cellular contents were assessed histologically. In addition, RNA was extracted from the lavaged pleural cells and mRNA expression for

five cytokines, namely IL-5, IL-6, TNF-α, MIP-2 and IFN-γ was examined by RT-PCR.

8.2 RESULTS

In all the experiments reported in this chapter rats were sensitized by a subcutaneous injection of ovalbumin and challenged intrathoracically seven days later with either ovalbumin or saline as control.

8.2.1 Cellular content of pleural lavage fluid

Ovalbumin challenge produced a significant increase in total cell numbers 2 h after the injection compared to levels in control rats at the 2 h time point. Total cell numbers continued to rise until 24 h after the challenging injection but declined thereafter, although they remained significantly elevated, compared to control rats, at the 48 h time point (Fig. 8.2.1). Although there was an increase in levels of mononuclear cells after ovalbumin challenge compared to levels in control rats, this did not reach significance in at least 5 independent experiments (Fig. 8.2.2). Neutrophils increased significantly 2 h after ovalbumin challenge and peaked at 4 h with an average 7-fold increase in number compared to numbers from saline-challenged controls (Fig. 8.2.3). Neutrophil numbers then declined, although remained significantly elevated compared to controls at both the 24 h and 48 h time points (Fig. 8.2.3). Although eosinophils did increase in number compared to controls, particularly at the latest time point investigated (48 h), these changes were not significant over five independent experiments (Fig. 8.2.4). A significant mast cell infiltrate into the pleural cavity was observed 2 h after ovalbumin challenge with an approximate 6-fold increase in mast cell number

compared to control rats. However, numbers declined to baseline levels by 24 h (Fig. 8.2.5).

8.2.2 Expression of mRNA for IL-5, IL-6, TNF-α, MIP-2, and IFN-γ in pleural lavage cells

Messenger RNA encoding all of the five cytokines was detected in pleural lavage cells from ovalbumin-sensitized, saline-challenged control rats. For each experiment RNA was pooled from two rats. Messenger RNA encoding IL-5 and IL-6 was detectable in control rats at the 2 h time point in 5/5 experiments (Fig. 8.2.6), for TNF-*α* in 5/5 experiments (Fig. 8.2.7) for MIP-2 in 5/5 experiments (Fig. 8.2.7) and IFN-*γ* in 5/5 experiments (Fig. 8.2.8). At the 4 h time point mRNA encoding IL-5 and MIP-2 was reduced (Fig. 8.2.6 - Fig. 8.2.7). After 24 h neither IL-5 nor IL-6 mRNA were detectable (Fig. 8.2.6), whereas mRNA encoding MIP-2 and IFN-*γ* were detected extremely weakly in 2/5 experiments (Fig. 8.2.7 - Fig. 8.2.8). At the 24 h time point TNF-*α* mRNA was detected in control rats in 4/5 experiments although at reduced levels compared to levels detected at the 2 h and 4 h time points (Fig. 8.2.7).

Following a challenging injection of ovalbumin into rats, mRNA encoding IL-6, TNF-*α*, MIP-2 and IFN-*γ* increased noticeably compared to levels in ovalbumin-sensitized saline-challenged rats (Fig. 8.2.6 - Fig. 8.2.8). Two hours after treatment, IL-6 mRNA was detected strongly in 5/5 experiments. Increased levels persisted until 4 h after challenge and were still detectable, although weakly, in 3 experiments at the 24 h time point

(Fig. 8.2.6). After ovalbumin challenge, TNF-α mRNA was also detected intensely in 5/5 experiments at the 2 h time point and levels continued to remain elevated, compared to controls, even after 24 h (Fig. 8.2.7). Two hours after ovalbumin challenge, MIP-2 mRNA had increased in intensity in 3/4 experiments compared to controls (Fig. 8.2.7). Four hours after ovalbumin challenge MIP-2 mRNA was detected strongly in 5/5 experiments and levels were still weakly detectable in 3 experiments after 24 h (Fig. 8.2.7). IFN-γ mRNA was detected strongly in 4/4 experiments 2 h after ovalbumin challenge (Fig. 8.2.8). Levels remained elevated compared to controls at the 4 h time point in 4/4 experiments, but by 24 h IFN-γ mRNA expression had fallen away (Fig. 8.2.8). Messenger RNA encoding IL-5 was elevated at 2 h post-ovalbumin challenge, compared to later time points, but levels were no different from those seen after initial exposure to ovalbumin (sensitization). Compared to control levels, IL-5 mRNA did not increase dramatically 2 h after ovalbumin challenge (Fig. 8.2.6). Both control levels and challenged levels of IL-5 mRNA had decreased after 4 h and were not detectable at the 24 h time point (Fig. 8.2.6).

Analysis of band intensities by laser densitometry revealed that ovalbumin challenge did not significantly increase the expression of IL-5 mRNA at either 2 h, 4 h or 24 h compared to levels expressed by control saline-challenged rats (Fig. 8.2.9). However, after 2 h, IL-5 mRNA expression was extremely high in both the saline-challenged and ovalbumin-challenged groups, but by 4 h levels in both groups had decreased significantly. IL-6 mRNA expression was significantly increased at 4 h and

24 h compared to levels expressed by control rats, with peak values at 4 h (Fig. 8.2.10). TNF-α mRNA was significantly elevated at 2 h, 4 h and 24 h after ovalbumin challenge compared to controls with peak values at 4 h (Fig. 8.2.11). Expression of MIP-2 mRNA had increased significantly 4 h after ovalbumin challenge compared to control levels and although mRNA expression had declined to near baseline by 24 h, levels continued to remain significantly elevated compared to controls (Fig. 8.2.12). IFN-γ mRNA expression was significantly increased 2 h and 4 h after ovalbumin challenge compared to controls with peak values at 2 h (Fig. 8.2.13). Throughout the *experiments no changes in* the intensity of G3PDH RT-PCR products were seen (Fig. 8.2.14).

8.3 DISCUSSION

This study clearly demonstrates that antigen-induced pleurisy in the rat is associated with increased production of cytokines by pleural cells. An early and noticeably significant infiltration of mast cells and neutrophils was observed after ovalbumin challenge of ovalbumin-sensitized rats, suggesting that these cells may be important in the initiation and maintenance of the allergic reaction. Indeed others have previously reported a marked mast cell degranulation, plasma exudation and neutrophil influx in this model (Silva *et al.*, 1992). Interestingly, a significant infiltration of eosinophils into the pleural cavity was not observed under these experimental conditions whereas Silva *et al.* (1992) have reported a noticeable eosinophilia 24 h after challenge. The fact that in the study of Silva *et al.* (1992) rats were sensitized fourteen days prior to antigen challenge rather than a seven day period as here, may however explain these differences and suggests that the generation of a chronic allergic reaction, characterized by an infiltration of eosinophils, requires a longer sensitization period. It is possible that the inflammation observed here after a seven day sensitization period is not IgE-driven but rather T cell- mediated. A fourteen day sensitization period may be a requirement for the generation of IgE-mediated inflammation within this model. Furthermore, the observation that IFN-γ mRNA expression levels were elevated after ovalbumin challenge, whereas increases in IL-5 mRNA expression were not significant, may suggest that the inflammatory response is mediated by Th1 cells. No significant increase in the expression of IL-5

mRNA was observed at any time point investigated compared to control levels, although IL-5 mRNA was elevated in controls and ovalbumin-challenged rats two hours after challenge compared to later time points. This is a rather curious result which is difficult to interpret but one possible explanation may be due to the intrathoracic challenging injection which could have invoked trauma leading to induced expression of IL-5 in both groups. Although IL-5 mRNA was elevated in pleural lavage cells from saline-challenged and ovalbumin-challenged rats after 2 h, eosinophil numbers did not increase within the pleural cavity, suggesting that IL-5, a known eosinophil chemoattractant (Wang *et al.*, 1989), does not play a significant role in an early accumulation of eosinophils. Rothenberg *et al.* (1995) have recently reported that eotaxin, a member of the C-C family of chemokines, is released during antigen challenge in a guinea pig model of allergic airway inflammation. Eotaxin was found to induce the selective infiltration of eosinophils when injected into the lung and skin and compared with lungs of saline-challenged animals, eotaxin mRNA levels increased sixfold within three hours and returned to baseline by six hours. It has therefore been suggested, that although IL-5 is important in the long term accumulation of eosinophils as it promotes their growth and differentiation (Clutterbuck *et al.*, 1987, 1989) and causes a marked rise in blood eosinophilia (Dent *et al.*, 1990), it is eotaxin rather than IL-5 which promotes early and rapid tissue eosinophilia. Although the cellular source of eotaxin is unknown, it may be stored in mast cell granules (Rothenberg *et al.*, 1995). However, results presented in this chapter have indicated that an early accumulation of

eosinophils is not a feature of antigen induced pleurisy in the rat following a seven day sensitization period, implying that pleural mast cells do not provide an early and significant source of eotaxin. Nevertheless, if, as has been suggested above, the inflammation is T cell- driven rather than IgE-mediated, it is possible that the majority of pleural mast cells are not activated and hence are unable to release their stores of eotaxin or similar analogue which could initiate eosinophil accumulation.

A highly significant increase in neutrophils was observed after antigen challenge of sensitized rats with peak values at 4 h. MIP-2 expression also peaked at 4 h providing circumstantial evidence that these two events are closely related. IL-8, a major neutrophil chemoattractant in humans, has not been identified with certainty in the rat and it is possible that MIP-2 may provide this function. MIP-2 is a known neutrophil chemoattractant (Appleberg, 1992) and Feng *et al.* (1995) have reported that neutrophil influx can be inhibited significantly in rat glomerulonephritis with anti-MIP-2 antibody. Additionally, MIP-2 mRNA was found to peak at, or slightly before the peak neutrophil response observed in the rat Sephadex model of lung inflammation (chapter 6). Taken together these results thus suggest that MIP-2 may be important in initiating the influx of neutrophils in rat models of disease.

A significant increase in the mRNA expression of IL-6, TNF-α and IFN-γ was also observed following antigen challenge. IL-6 strongly up-regulates the IL-4-dependent IgE synthesis in humans (Vercelli *et al.*, 1989) indicating an important role in the initiation and maintenance of allergic reactions.

TNF-α can activate and induce the influx of neutrophils (Shalaby *et al.*, 1985; Yates *et al.*, 1993) and enhance endothelial cell adhesiveness for lymphocytes (Cavender *et al.*, 1987) and polymorphonuclear leucocytes (Gamble *et al.*, 1985) and IFN-γ, in addition to a number of other pro-inflammatory characteristics, can up-regulate the expression of MHC class II on mast cells (Warbrick *et al.*, 1995), suggesting that this cytokine may be important in the antigen - presenting function of these cells (Fox *et al.*, 1994). A similar pattern of induced expression of IL-6, TNF-α and IFN-γ was also observed at various stages during the progression of rat Sephadex-induced lung inflammation (chapter 6). The results presented in this chapter suggest that these cytokines also have the potential to contribute to the progression of antigen-driven pleurisy in the rat and provided further confirmation that there are close similarities between antigen-driven inflammation and that induced by a physical stimulus.

In conclusion, this study clearly demonstrates that intrathoracic injection of ovalbumin into sensitized rats induces a significant influx of mast cells and neutrophils into pleural cavities and these changes occur alongside transient induced expression of IL-6, TNF-α, MIP-2 and IFN-γ. Furthermore, the results presented in this chapter have demonstrated a potential relationship between induced expression of MIP-2 and the mobilisation of neutrophils. Although the importance of neutrophils in the progression of inflammation has received little formal study these cells may contribute to the inflammatory response. They are known to contain a number of agents with the capacity to injure human tissues and a study on one particular type

of fatal allergic reaction "sudden-onset fatal asthma" has indicated that neutrophils rather than eosinophils predominate (Sur *et al.*, 1993). Additionally, neutrophils are known to provide a source of several pro-inflammatory cytokines. In view of these facts, the role of neutrophils in allergy certainly merits further investigation and this model may prove useful in exploring their importance in the development and maintenance of allergic disease.

The observation that mast cell numbers increased significantly after ovalbumin challenge also leads to several interesting questions. For example, the accumulation of mast cells may indicate the presence of an as yet unknown mast cell chemotactic factor. Therefore, this model may have potential benefits in validating this suggestion.

Finally, the results presented in this chapter have suggested that there are a number of similarities, in terms of cell mobilisation and cytokine gene expression, between antigen-induced pleurisy in the rat and rat Sephadex-induced lung inflammation.

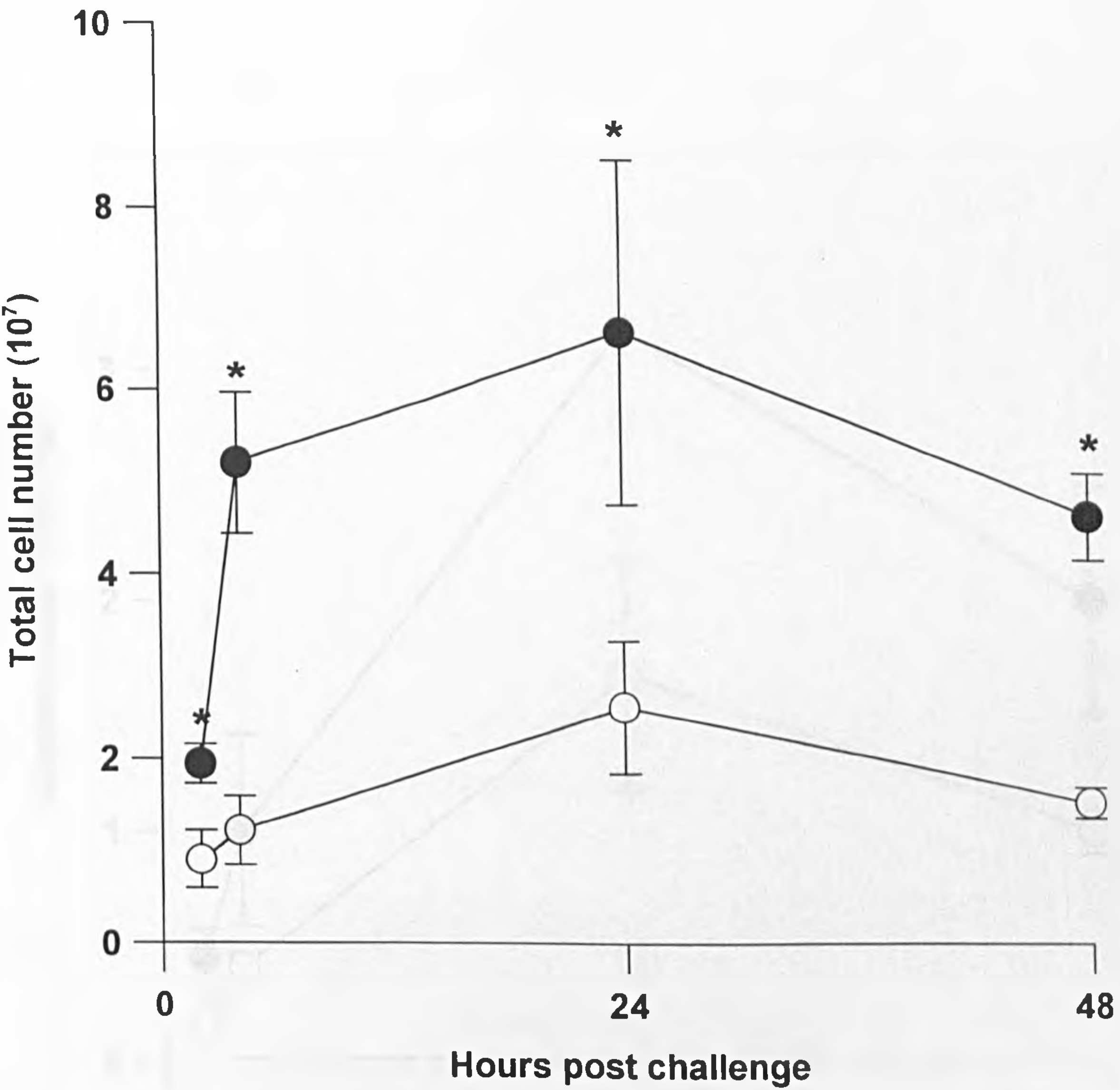

Fig. 8.2.1. Numbers of total pleural cells from control (ovalbumin-sensitized/saline-challenged) rats (o) and ovalbumin-sensitized/ovalbumin-challenged rats (●). Results are means ± SEM for at least five independent experiments (* $p < 0.035$ by comparison of ovalbumin-challenged rats with controls as determined by the two-tailed Mann Whitney *U* test).

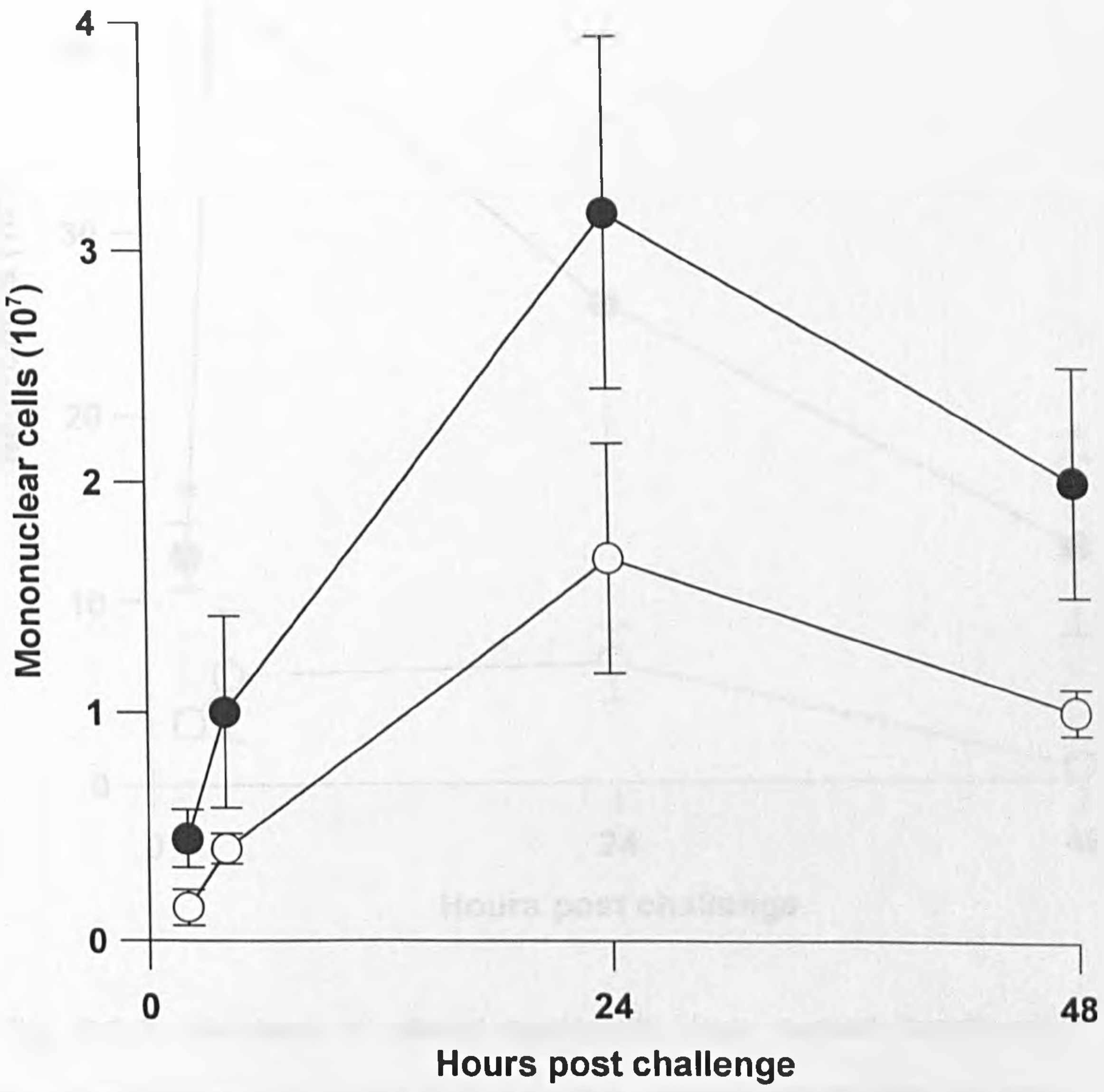

Fig. 8.2.2. Numbers of pleural mononuclear cells from control (ovalbumin-sensitized/saline-challenged) rats (o) and ovalbumin-sensitized/ovalbumin-challenged rats (●). Results are means ± SEM for at least five independent experiments.

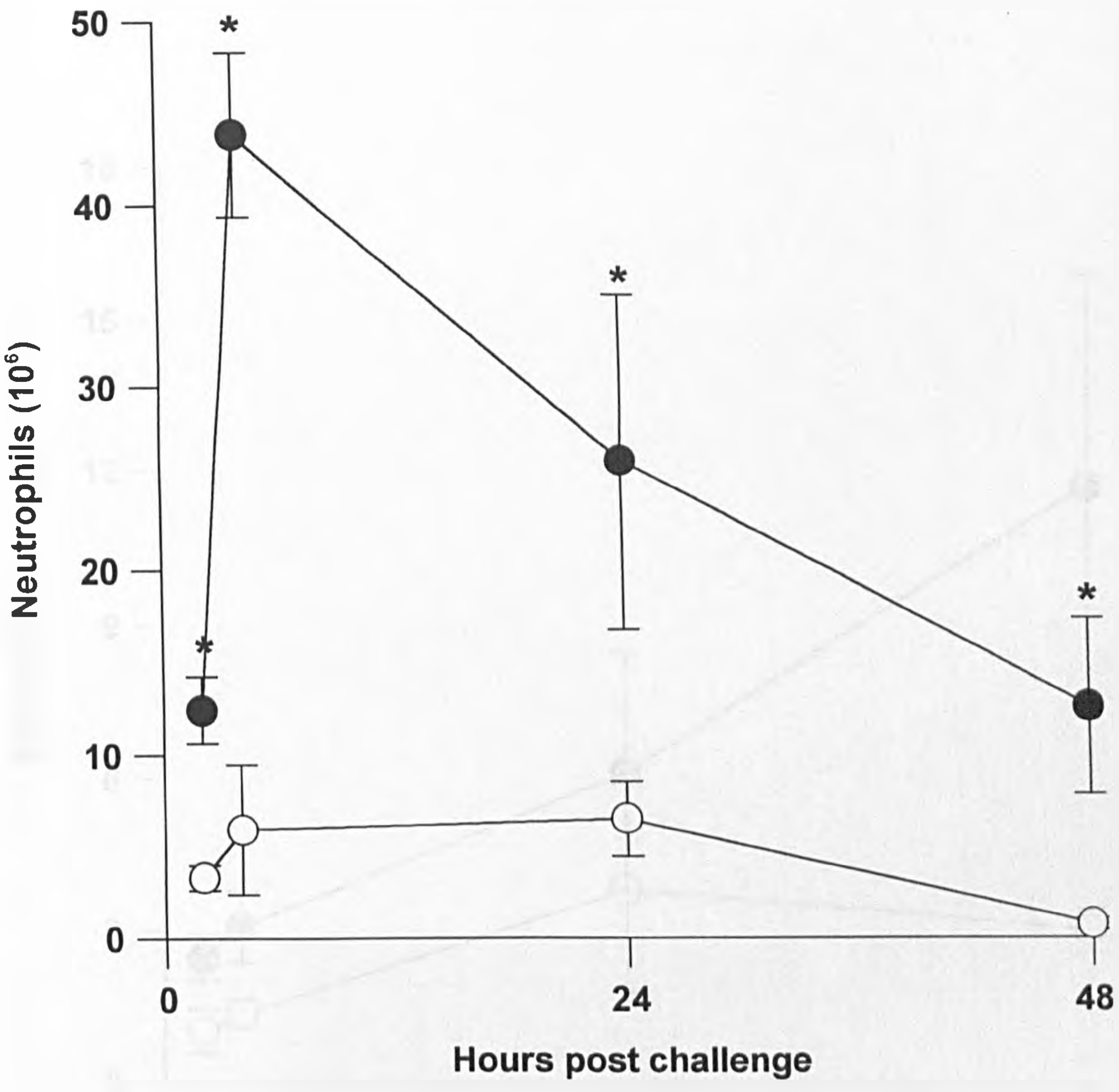

Fig. 8.2.3. Numbers of pleural neutrophils from control (ovalbumin-sensitized/saline-challenged) rats (o) and ovalbumin-sensitized/ovalbumin-challenged rats (●). Results are means ± SEM for at least five independent experiments (* $p < 0.05$ by comparison of ovalbumin challenged rats with controls as determined by the two-tailed Mann-Whitney *U* test).

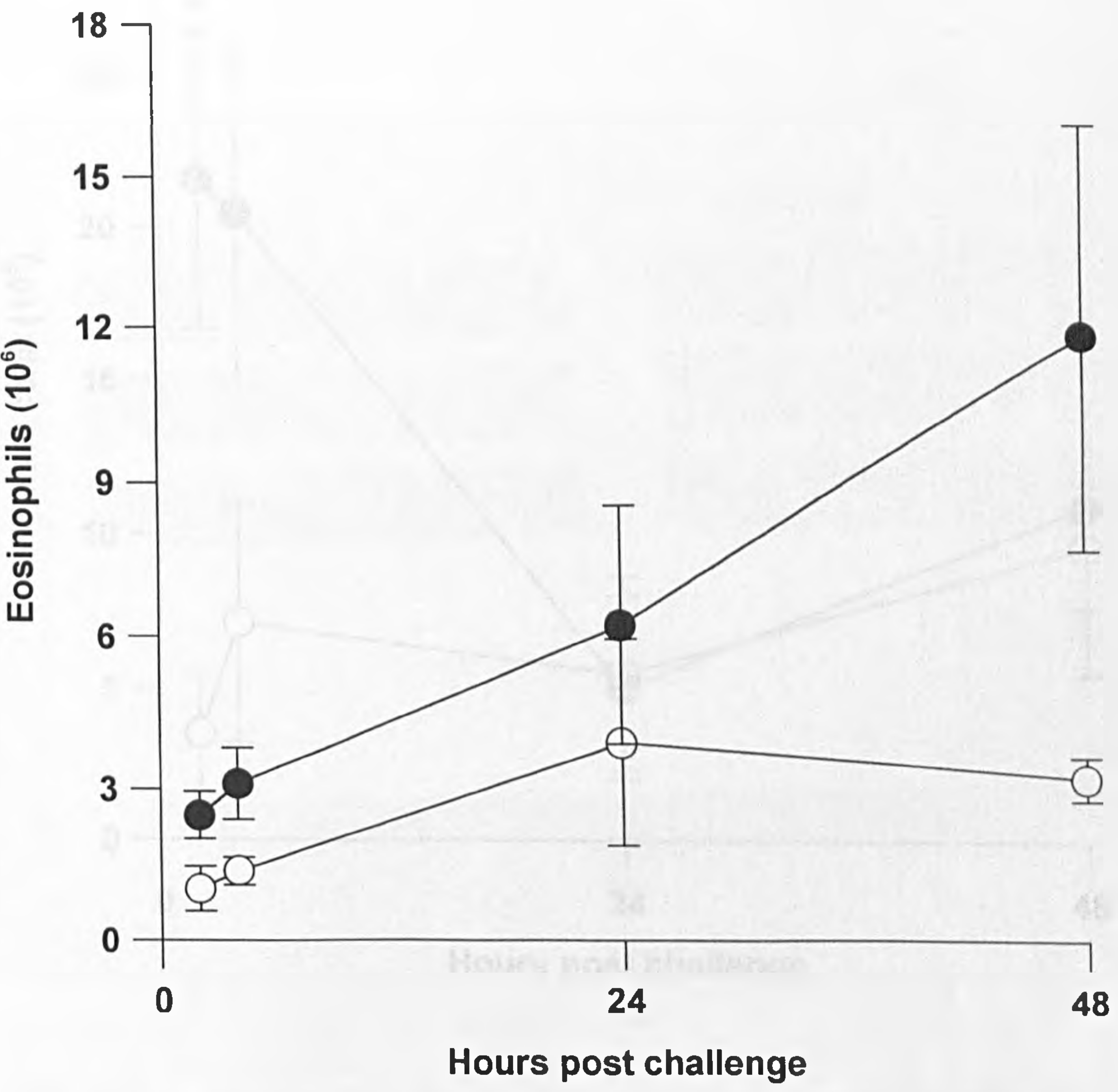

Fig. 8.2.4. Numbers of pleural eosinophils from control (ovalbumin-sensitized/saline-challenged) rats (o) and ovalbumin-sensitized/ovalbumin-challenged rats (●). Results are means ± SEM for at least five independent experiments.

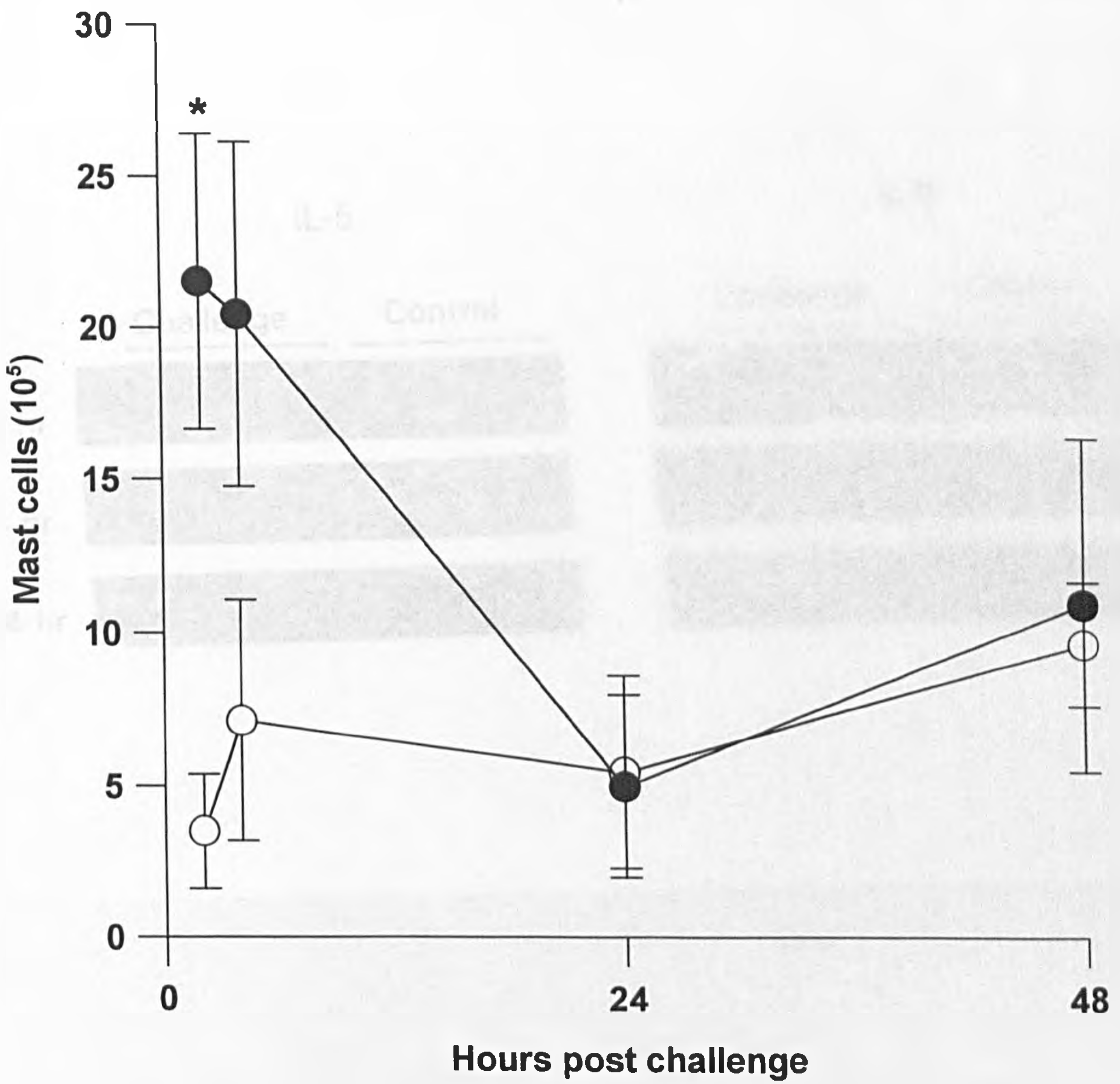

Fig. 8.2.5. Numbers of pleural mast cells from control (ovalbumin-sensitized/saline-challenged) rats (o) and ovalbumin-sensitized/ovalbumin-challenged rats (●). Results are means ± SEM for at least 5 independent experiments (* $p < 0.008$ by comparison of ovalbumin challenged rats to controls as determined by the two-tailed Mann-Whitney *U* test).

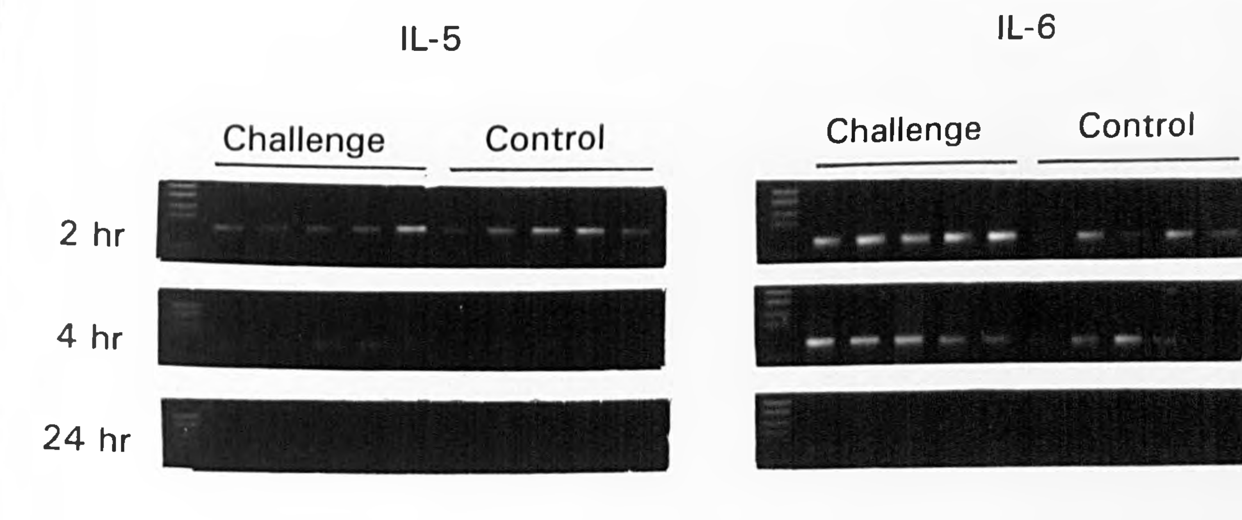

Fig. 8.2.6. RT-PCR analysis of mRNA for IL-5 and IL-6 in rat pleural cells. Ovalbumin (challenged) or saline (control) was injected intrathoracically into ovalbumin-sensitized rats and pleural cells obtained at the times indicated. Each band represents DNA amplified from cDNA reverse transcribed from RNA pooled from two rats.

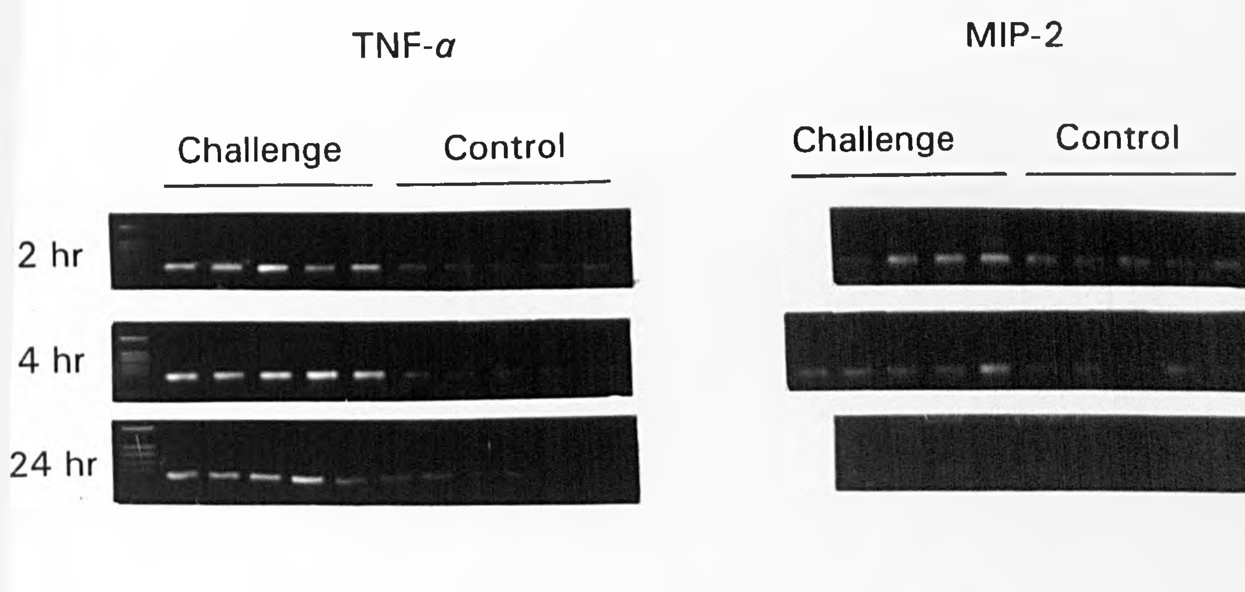

Fig. 8.2.7. RT-PCR analysis of mRNA for TNF-α and MIP-2 in rat pleural cells. Ovalbumin (challenged) or saline (control) was injected intrathoracically into ovalbumin-sensitized rats and pleural cells obtained at the times indicated. Each band represents DNA amplified from cDNA reverse transcribed from RNA pooled from two rats.

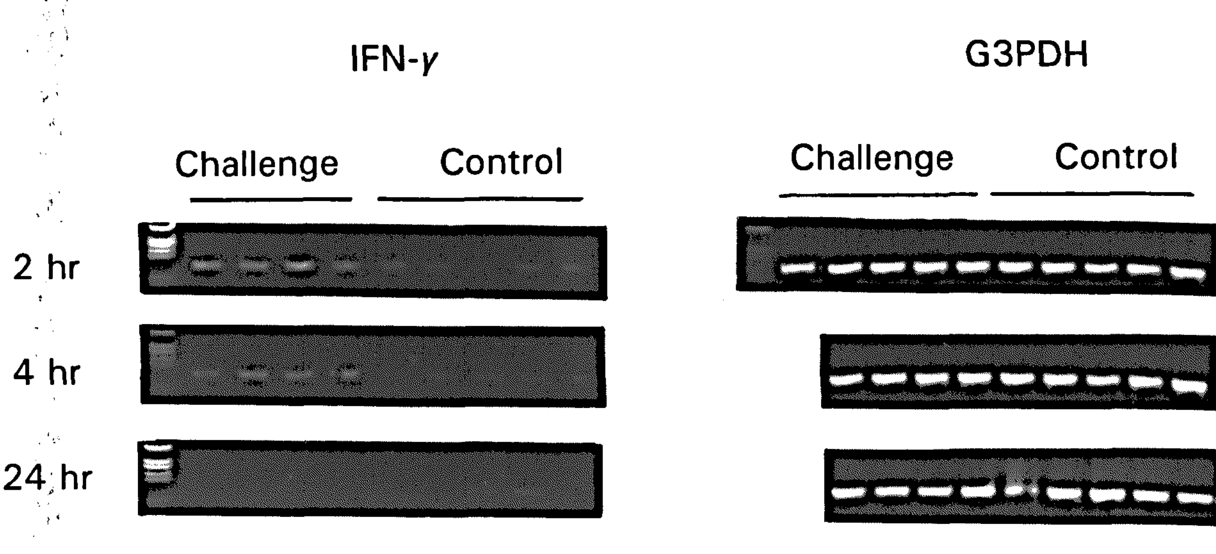

Fig. 8.2.8. RT-PCR analysis of mRNA for IFN-γ and G3PDH in rat pleural cells. Ovalbumin (challenged) or saline (control) was injected intrathoracically into ovalbumin-sensitized rats and pleural cells obtained at the times indicated. Each band represents DNA amplified from cDNA reverse transcribed from RNA pooled from two rats.

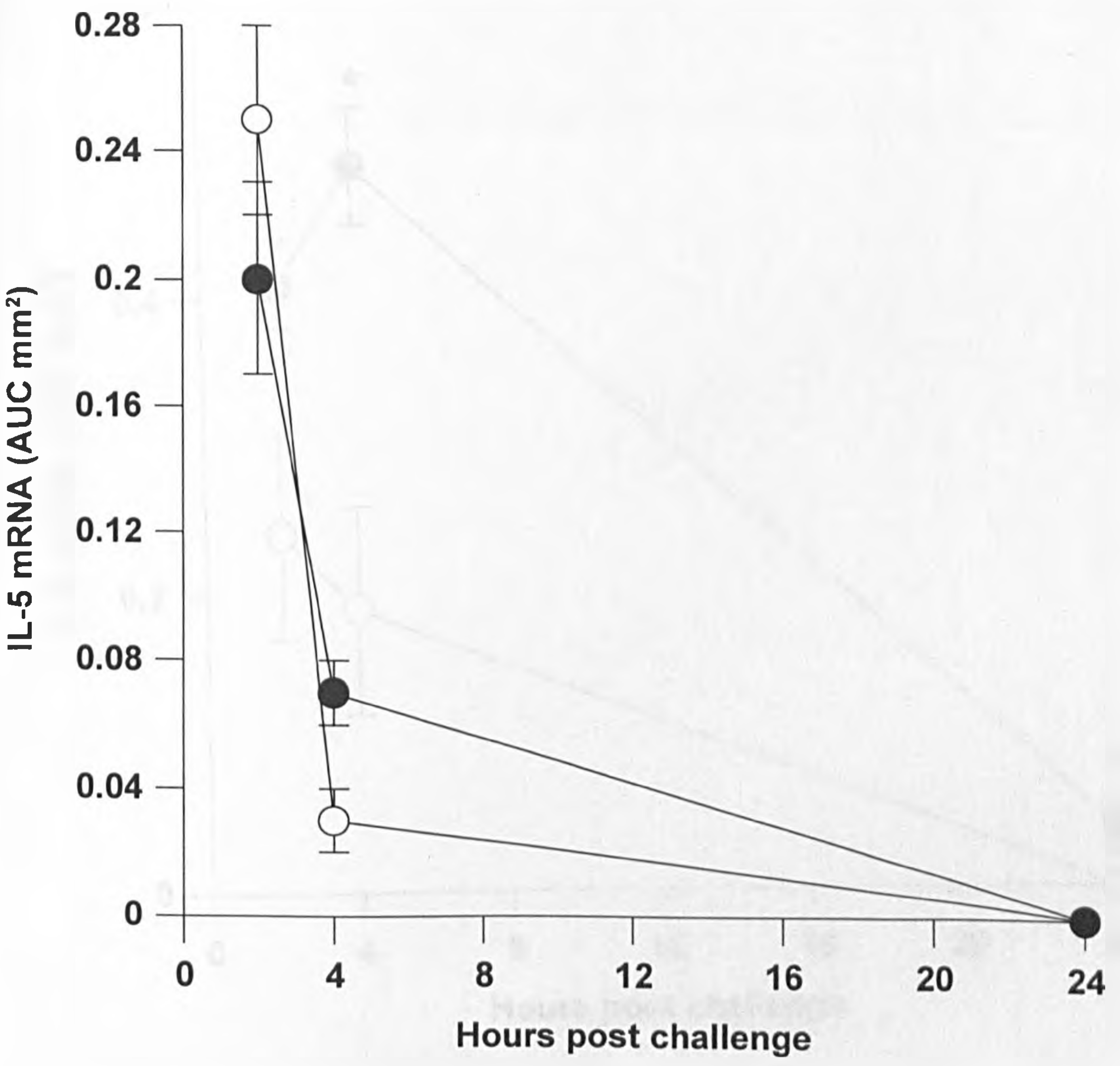

Fig. 8.2.9. Densitometric analysis of mRNA for IL-5 in pleural cells after challenge of ovalbumin-sensitized rats with either saline (○) or ovalbumin (●). Gels were scanned by laser densitometry and the intensity of RT-PCR products calculated as area under the curve (AUC) of densitometric traces. Results are means ± SEM for five experiments, each with pleural cells pooled from two rats.

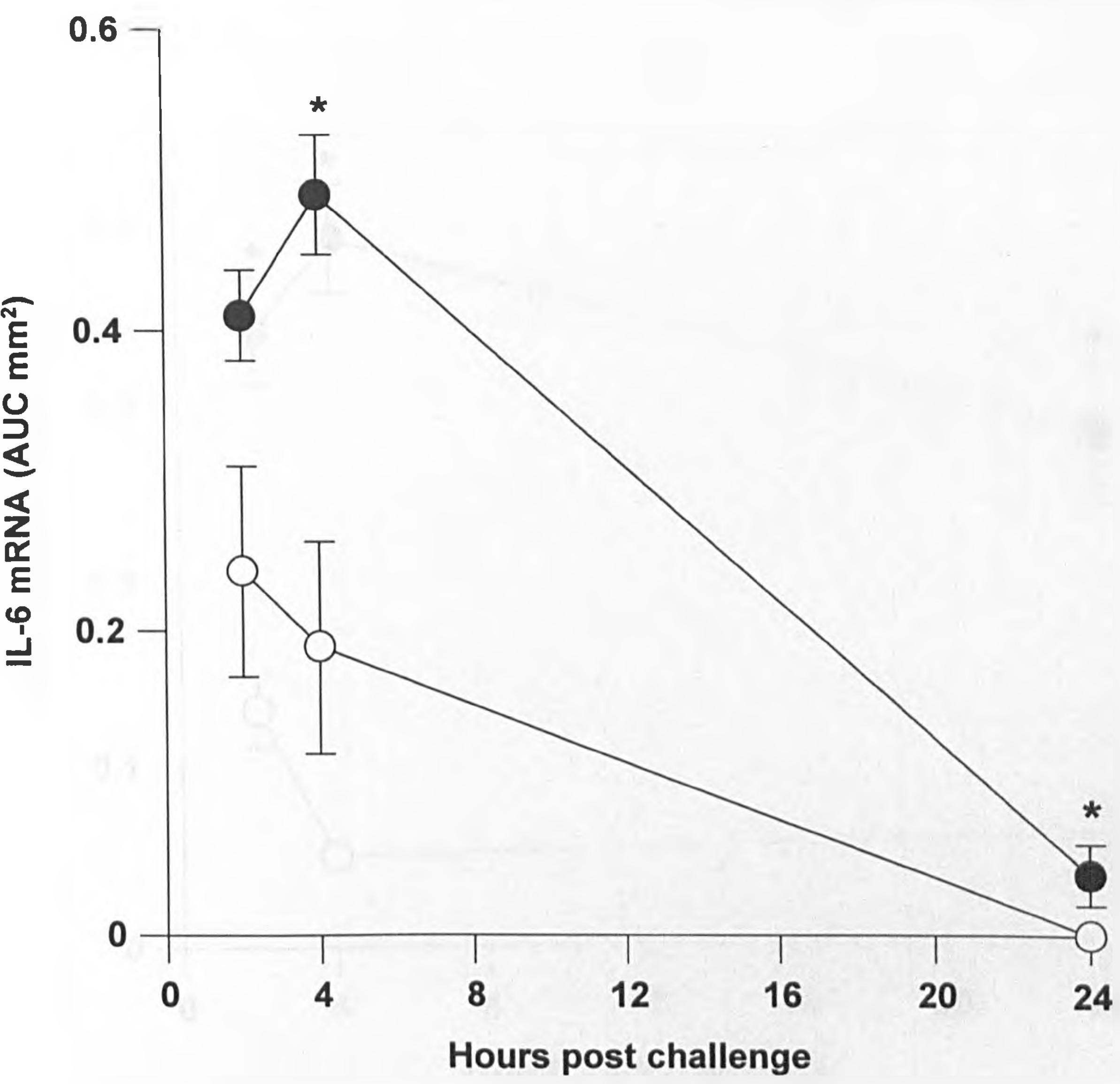

Fig. 8.2.10. Densitometric analysis of mRNA for IL-6 in pleural cells after challenge of ovalbumin-sensitized rats with either saline (○) or ovalbumin (●). Gels were scanned by laser densitometry and the intensity of RT-PCR products calculated as area under the curve (AUC) of densitometric traces. Results are means ± SEM for five experiments, each with pleural cells pooled from two rats. (* $p < 0.01$ by comparison of ovalbumin-challenged rats with controls as determined by the two-tailed Mann Whitney *U* test).

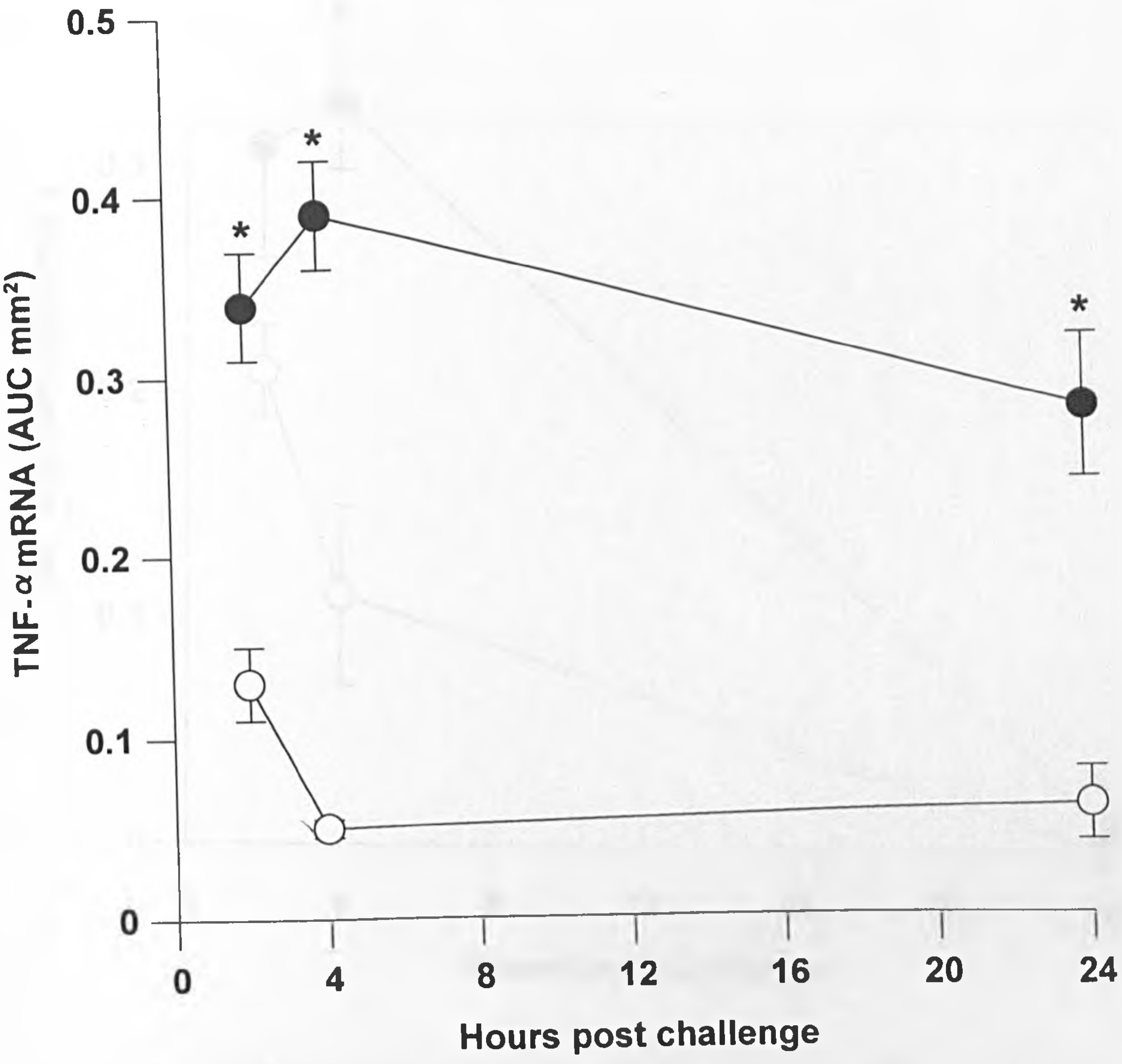

Fig. 8.2.11. Densitometric analysis of mRNA for TNF-α in pleural cells after challenge of ovalbumin-sensitized rats with either saline (○) or ovalbumin (●). Gels were scanned by laser densitometry and the intensity of RT-PCR products calculated as area under the curve (AUC) of densitometric traces. Results are means ± SEM for five experiments, each with pleural cells pooled from two rats. (* $p < 0.01$ by comparison of ovalbumin-challenged rats with controls as determined by the two-tailed Mann-Whitney U test).

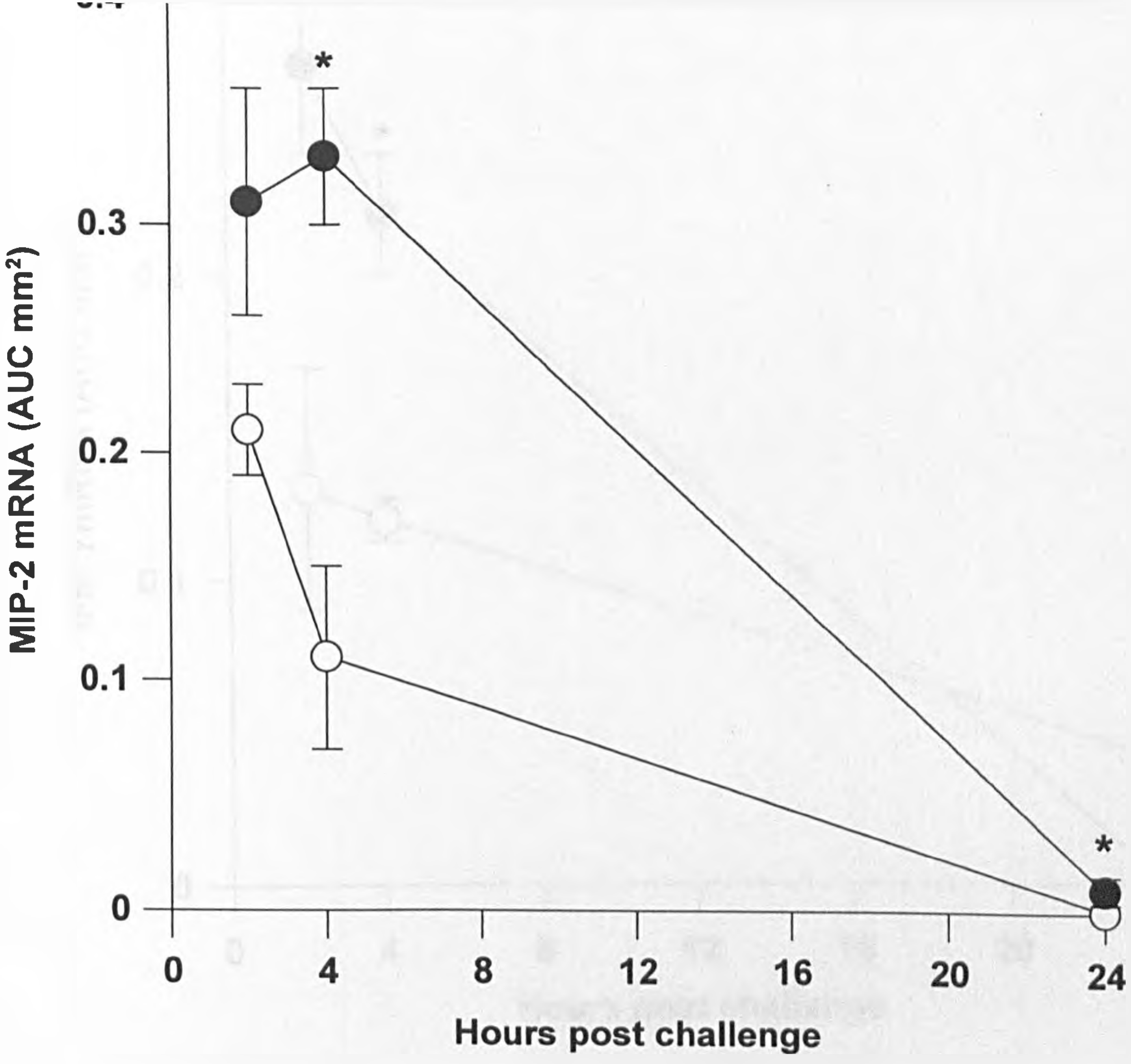

Fig. 8.2.12. Densitometric analysis of mRNA for MIP-2 in pleural cells after challenge of ovalbumin-sensitized rats with either saline (○) or ovalbumin (●). Gels were scanned by laser densitometry and the intensity of RT-PCR products calculated as area under the curve (AUC) of densitometric traces. Results are means ± SEM for at least four independent experiments, each with pleural cells pooled from two rats. (* $p < 0.01$ by comparison of ovalbumin-challenged rats with controls as determined by the two-tailed Mann Whitney *U* test).

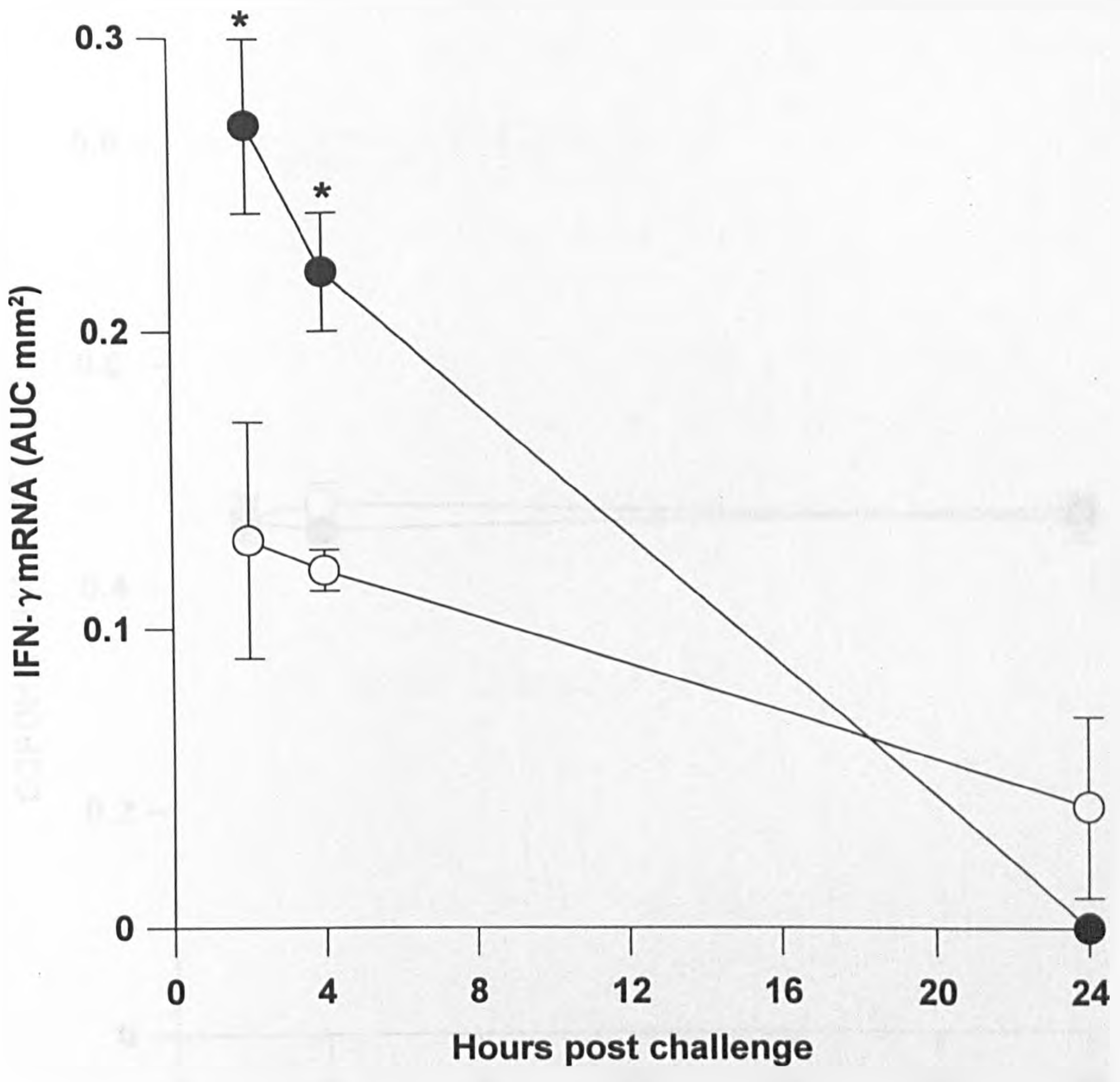

Fig. 8.2.13. Densitometric analysis of mRNA for IFN-γ in pleural cells after challenge of ovalbumin-sensitized rats with either saline (○) or ovalbumin (●). Gels were scanned by laser densitometry and the intensity of RT-PCR products calculated as area under the curve (AUC) of densitometric traces. Results are means ± SEM for at least four independent experiments, each with pleural cells pooled from two rats. (* $p = 0.0143$ by comparison of ovalbumin-challenged rats with controls as determined by the two-tailed Mann Whitney *U* test).

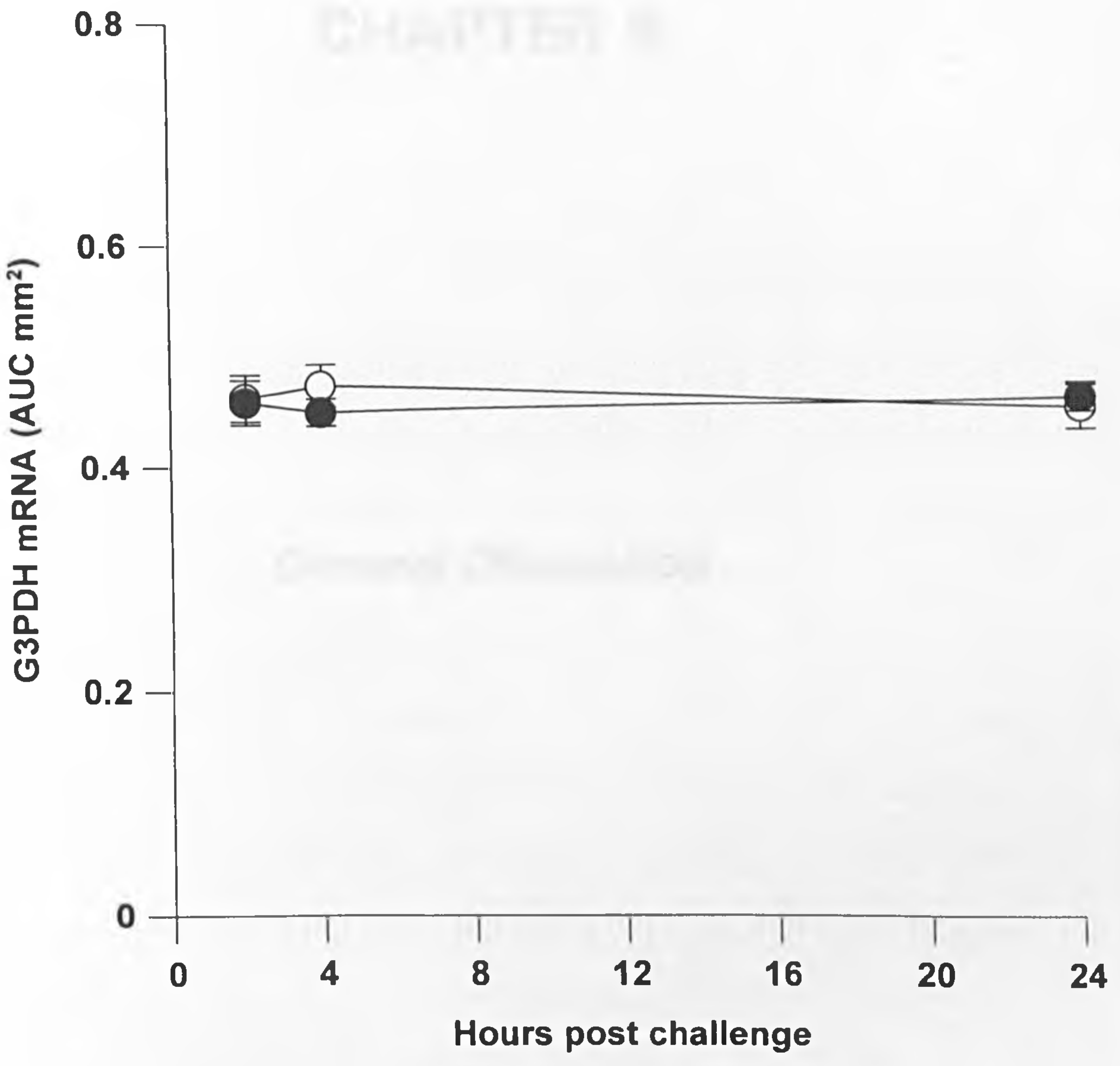

Fig. 8.2.14. Densitometric analysis of mRNA for G3PDH in pleural cells after challenge of ovalbumin-sensitized rats with either saline (○) or ovalbumin (●). Gels were scanned by laser densitometry and the intensity of RT-PCR products calculated as area under the curve (AUC) of densitometric traces. Results are means ± SEM for five experiments, each with pleural cells pooled from two rats.

CHAPTER 9

General Discussion

General Discussion

Allergic reactions are generally characterised by the appearance of an early phase reaction occurring five to thirty minutes after allergen exposure. In many individuals this phase is then followed by a later reaction which has often more serious pathological effects on surrounding cells and tissues. Cytokines, which are small protein molecules involved in many aspects of the immune system including the differentiation and maturation of haematopoietic progenitor cells, immunoglobulin synthesis and isotype switching, priming and activation of pro-inflammatory cell mediator release and pro-inflammatory cell chemotaxis, may be important to the propagation of allergic reactions. In actual fact, a new concept for the pathogenesis of late phase reactions in IgE-mediated allergy is emerging. According to this concept, cytokines, which may be produced during the early immediate phase of the IgE-mediated reaction, could play a major role in the induction of late phase cell infiltration which involves particularly eosinophils.

The cellular sources of cytokines that may contribute to allergic reactions is not entirely clear at present. On the one hand it is well known that cytokines such as IL-4, IL-5 and IL-6 are produced by activated T lymphocytes, but on the other hand activated mast cells may also produce a similar profile of cytokines. Considering that mast cells become activated almost immediately after allergen exposure, are the main contributors to early phase reactions and contain ample storage capacity for a variety of mediators including cytokines it seems appropriate to also consider their

involvement in the initiation of late phase reactions.

One of the principal themes of this thesis was to investigate whether activated mast cells are capable of producing cytokines which could be involved in the initiation of a late phase response. Previous studies on murine mast cell lines and clones have revealed that they provide a source of several cytokines and more recently mature tissue mast cells have also been found to produce some of these. In this thesis Cl.MC/C57.1 murine mast cells were found to produce TNF-α and IL-6 (chapters 4 and 5) and HMC-1 human mast cells were found to express mRNA encoding IL-3, IL-4, IL-8 and TNF-α (chapters 4 and 5). Furthermore, immunologically activated rat PMC, which are an example of mature connective tissue-type mast cells, were found to express mRNA encoding the cytokines IL-5, IL-6, TNF-α, MIP-2 and IFN-γ and to produce TNF-α protein (chapter 5). These cytokines have important immunological and pro-inflammatory functions. For example, IL-3 and IL-5 are important in the activation of eosinophils and in the regulation of eosinophil production from human bone marrow (Fabian *et al.*, 1992; Lopez *et al.*, 1987, 1988; Sanderson, 1992; Clutterbuck and Sanderson, 1988; Clutterbuck *et al.*, 1989). IL-5 is also a selective eosinophil chemoattractant (Wang *et al.*, 1989). IL-4 is a B-cell growth and differentiation factor and in T-cells promotes production of selected cytokines such as IL-4, IL-5, GM-CSF and IL-10 that together define the Th2 subset (Paul, 1991). IL-4 also exerts effects on tissue mast cells *in vitro*: it acts as a co-factor (with IL-3) for their growth (Hamaguchi *et al.*, 1987) and alone enhances their exocytotic secretory responsiveness to IgE-mediated

stimulation (Coleman *et al.*, 1993). IL-6 has an essential role in the induction of IL-4-dependent human IgE synthesis (Vercelli *et al.*, 1989) and acts as a co-stimulatory factor with other cytokines on a variety of inflammatory and immune cells. IL-8 and MIP-2 are important neutrophil chemoattractants and activating agents (Baggiolini *et al.*, 1989; Appleberg, 1992; Feng *et al.*, 1992) and TNF-α enhances endothelial cell adhesiveness for T-cells (Cavender *et al.*, 1987) and neutrophils (Gamble *et al.*, 1985; Schleimer and Rutledge, 1986). TNF-α also acts as a stimulator for human skin mast cells to secrete histamine and tryptase (van Overveld *et al.*, 1992) and causes bronchial hyperresponsiveness and airway inflammation in the rat (Kips *et al.*, 1992). IFN-γ amplifies the release of TNF-α from alveolar macrophages induced by IgE triggering or by endotoxin (Gosset *et al.*, 1992; Gifford and Lohmann-Matthess, 1987) and increases the MHC class I and class II expression on a variety of cell types (Barnes, 1994) including mast cells (Warbrick *et al.*, 1995).

Kinetic studies revealed that a four hour activation period was a requirement for optimal cytokine mRNA expression by rat PMC. Rodent and human mast cells are known to contain preformed stores of TNF-α (Gordon and Galli, 1991) and Bradding *et al.* (1994) have also recently shown that human lung mast cells store IL-4, IL-5 and IL-6. This would suggest that mast cells may provide the initial source of cytokines during an early phase reaction. Upon appropriate activation of the cells they could release their preformed mediators such as histamine and tryptase but also cytokines which may contribute to the initiation of a late phase reaction. Additionally,

the results presented in chapter 5 show that mature connective tissue-type mast cells express mRNA encoding IL-5, IL-6, TNF-*α*, MIP-2 and IFN-*γ* after a four hour activation period suggesting that mast cells could contribute to inflammatory events, such as cell mobilisation, initiated at this time corresponding to *de novo* synthesis of cytokines. Furthermore, the fact that mature mast cells are a source of IL-5, an important cytokine involved in eosinophil chemotaxis (Wang *et al.*, 1989), suggests that mast cells may provide a significant stimulus in the recruitment of eosinophils into sites of allergic inflammation during the late phase reaction.

An interesting aspect of the concept that mast cells provide a source of several cytokines which may be involved in the induction of late phase reactions is that they are known to express IFN-*γ* (chapter 5) a typical Th1 promoting cytokine as well as IL-4 (Bradding *et al.*, 1994) a typical Th2 promoting cytokine. This poses the theory that mast cells may contribute to the promotion of either Th1-like or Th2-like responses. For instance, during allergic reactions mast cells may secrete greater amounts of IL-4 but little IFN-*γ* thus promoting a typical Th2 response whereas in other diseases they may produce more IFN-*γ* and little IL-4 thereby contributing to a Th1 response. Furthermore, in view of the fact that IFN-*γ* up-regulates the expression of MHC class II on the surfaces of rat PMC (Warbrick *et al.*, 1995) the possibility exists that mast cell derived IFN-*γ* may influence the antigen presenting capacity of these cells. In Th1 type reactions in which IFN-*γ* is produced, mast cells may therefore have a greater potential to act as antigen presenting cells thus demonstrating a two pronged approach in

the regulation of T lymphocytes: firstly they could provide a source of T cell promoting cytokines and secondly they could present antigen to T cells thereby initiating a cascade of events resulting in the release of T cell derived cytokines and T cell-dependent responses.

Cytokines have the capacity to prepare pro-inflammatory cells for more efficient mediator release, a phenomenon known as "priming". For example Dahinden *et al.* (1988) have reported that *in vitro* prior contact of neutrophils with GM-CSF enhances the cells capacity to produce mediators such as LTB_4 and PAF upon challenge with the complement component C5a. Without prior contact and treatment with GM-CSF, challenge with C5a remains ineffective. Similarly, blood basophils will markedly increase their capacity to release preformed histamine or to form *de novo* sulphidoleukotrienes following a short incubation *in vitro* with IL-3, IL-5, or GM-CSF (Kurimoto *et al.*, 1989). Furthermore, *in vitro* culture of mouse PMC with IL-3, IL-4 or SCF for 48 hours results in a concentration-related increase in the IgE/antigen-induced release of 5-HT (Coleman *et al.*, 1993) and exposure of the cells to IFN-γ has been shown to inhibit the IgE/antigen-induced release of 5-HT (Coleman *et al.*, 1991, 1992) and the IL-4 enhanced secretion of both 5-HT and arachidonate from mouse PMC (Holliday *et al.*, 1994). Therefore, the effects of IL-3, IL-4, SCF and IFN-γ on the priming of mast cell cytokine release were also investigated in this thesis (chapter 4). IL-3, IL-4 and IFN-γ had no effect on the release of TNF-α from IgE/antigen activated Cl.MC/C57.1 mast cells, whereas 48 h culture with SCF significantly enhanced the IgE/antigen-induced release of TNF-α from

Cl.MC/C57.1 mast cells (chapter 4). However, similar effects were not observed when mature connective-tissue type mast cells were cultured with IL-4 or SCF for 48 hours prior to activation (chapter 4) although IL-4 was found to enhance the ionomycin-induced gene expression of IL-3 and IL-8 in HMC-1 mast cells (chapter 4). Nevertheless, if indeed mast cells do provide the initial source of cytokines in allergic reactions it is possible that the release of these cytokines by fully differentiated mature mast cells is not subject to cytokine regulation. However, it is possible that mast cell cytokine expression may be enhanced during late phase reactions when mast cells may release *de novo* synthesised cytokines. The fact that IL-4 exerted its effects on mast cell cytokine gene expression in HMC-1 mast cells after a four hour activation period (chapter 4) suggests this.

Corticosteroids and more recently CsA have been used in the treatment of allergic reactions including asthma. These drugs have a number of actions including effects on mast cell mediator release. Recent information has also indicated that they can inhibit cytokine gene transcription in a variety of different cell types. In this thesis their effects on the expression of mast cell mediators were investigated. BTS 71321, a novel 1-substituted imidazole compound which has recently been found to inhibit the late phase response in guinea pigs and the release of arachidonic acid, superoxide and TNF-α from leucocytes stimulated *in vitro* (Steele *et al.*, 1995) has also been included in many of these studies. The inhibitory effects of the three drugs on the expression of mast cell mediators are summarised in Table 9.1. Both CsA and Dex inhibited mRNA encoding IL-5,

IL-6, TNF-α, MIP-2 and IFN-γ and the release of TNF-α protein and 5-HT from immunologically activated rat PMC. Additionally, Dex inhibited anti-IgE-induced arachidonate release from these cells. However, Dex had no effect on ionomycin-induced gene expression of IL-3, IL-4 or IL-8 by HMC-1 mast cells and did not inhibit the calcium ionophore A23187-induced release of TNF-α by Cl.MC/C57.1 mast cells. Although this lack of effectiveness may be due to differences in mast cell phenotype it is also possible that immunological activation of mast cells is a requirement for the inhibition of mast cell mediators by Dex.

		CsA	**Dex**	**BTS 71321**
	Cytokine gene expression			
*	*Rat PMC*	+ + +	+ + +	ND
♣	*HMC-1*	+ + +	-	-
	TNF-α release			
♦	*Rat PMC*	+ + +	+ + +	ND
♥	*Cl.MC/C57.1*	+ + +	-	+ +
	5-HT release			
*	*Rat PMC*	+ + +	+ + +	-
	arachidonate release			
*	*Rat PMC*	-	+ + +	-

Table 9.1 The inhibitory effects of CsA, Dex and BTS 71321 on the expression of mast cell mediators. * These cells were stimulated with anti-IgE; ♣ these cells were stimulated with ionomycin; ♦ these cells were stimulated with IgE/antigen; ♥ these cells were stimulated with calcium ionophore A23187. (Abbreviations; ND, not done; -no effect; + + +, a marked inhibitory effect; + +, a moderate inhibitory effect).

BTS 71321 had no effect on mast cell cytokine gene expression but did inhibit the release of TNF-α from calcium ionophore activated Cl.MC/C57.1 mast cells. However, it did not inhibit the release of 5-HT or arachidonate from immunologically activated rat PMC. These results may therefore suggest that although 5-HT and TNF-α are stored preformed within mast cells they do not share similar regulatory and activation mechanisms. This view is further supported by the fact that although the anti-IgE-induced release of TNF-α from rat PMC is unaffected by prior culture of the cells with IL-4 (chapter 4), IgE/antigen-induced release of 5-HT from mouse PMC is enhanced (Coleman *et al.*, 1993).

As has been mentioned above, cytokines are thought to contribute to the initiation and progression of late phase allergic reactions. Therefore, in terms of cytokine expression and pro-inflammatory cell mobilisation, the Sephadex-induced model of lung inflammation in the rat was investigated as a potential model of asthma (chapter 6). Messenger RNA encoding each of the five cytokines found to be expressed by rat PMC was up-regulated in BAL cells at various times after Sephadex administration and mRNA

encoding IL-6 and TNF-α was induced in lung tissue. A significant early increase in bronchoalveolar neutrophils (24-48 h) followed by a later eosinophil influx which peaked 72 h after Sephadex injection, was detected in BAL fluid. In view of the fact that enhanced expression of IL-5, IL-6, TNF-α and in some cases IFN-γ have been detected in the lung during an asthmatic reaction (Barnes, 1994; Okubo *et al.*, 1994) and the LAR has been characterised by a significant increase in eosinophils, results presented in chapter 6 suggest that the Sephadex-induced model of lung inflammation in the rat has many similarities to human asthma.

A close relationship was found to exist between movement of eosinophils into the bronchoalveolar lumen and increased mRNA expression encoding IL-5. Both eosinophil numbers and enhanced expression of IL-5 peaked together 72 h after Sephadex administration (chapter 6). Additionally, induced expression of IL-5 mRNA was not seen in the antigen driven model of pleurisy in the rat and no significant increase in pleural eosinophil numbers was observed. Considering that IL-5 is a selective eosinophil activator (Lopez *et al.*, 1988) and chemoattractant (Wang *et al.*, 1989), results suggest IL-5 may be an important cytokine in eosinophil mobilisation within these models. Inhibition of cytokine synthesis is an important approach to asthma treatment and IL-5 is a particularly attractive target. Thus, the Sephadex-induced model of lung inflammation could be exploited within the pharmaceutical industry as a tool to investigate the effects of IL-5 antagonists and other novel cytokine inhibitors.

The appearance of increased numbers of neutrophils within the

bronchoalveolar lumen alongside enhanced expression of MIP-2 suggests that these two events may be related. As IL-8, the major neutrophil chemoattractant in the human lung (Kunkel *et al.*, 1991), has not been found with certainty in the rat, it is possible that MIP-2 may provide this function. MIP-2 is a known neutrophil chemoattractant and Feng *et al.* (1995) have reported a significant decrease in neutrophil influx in a rat model of glomerulonephritis after neutralisation of MIP-2.

Although Sephadex-induced lung inflammation in the rat is driven by a physical stimulus many similarities exist between this and allergic inflammation such as that seen in asthma. This merges again the pathophysiological concepts of allergic asthma and other forms of asthma that are non-immunologically induced. Indeed, an investigation into the involvement of cytokines in an antigen driven model of pleurisy in the rat (chapter 8) demonstrated a similar profile of cytokine expression to that of Sephadex-induced lung inflammation. However, Sephadex-induced lung inflammation occurred over a longer period of time than that of antigen-induced pleurisy, thus kinetics of cytokine mRNA expression differed. Messenger RNA encoding IL-6, TNF-α, MIP-2 and IFN-γ was induced much more rapidly in the antigen driven model of pleurisy (between 2-4 h) and levels of IL-6, MIP-2 and IFN-γ had fallen away by 24 h. Furthermore, no induction of IL-5 mRNA was observed after antigen challenge. Nevertheless, in both models induced expression of TNF-α was detected early in the inflammatory response and a close temporal relationship was demonstrated between the expression of MIP-2 mRNA and the mobilisation of neutrophils.

In both types of inflammation there was a noticeable mobilisation of mast cells into either the bronchoalveolar or pleural cavity. An interesting aspect of this finding is that a specific mast cell chemotactic factor may exist. Furthermore, the fact that significant numbers of mast cells were detected in the bronchoalveolar and pleural cavities may suggest that these cells are involved in the inflammatory responses and may possibly contribute to the induced expression of various cytokines. For example, in the Sephadex-induced model of lung inflammation mast cell numbers peaked at 72 h as too did eosinophils and IL-5 mRNA (chapter 6). As mast cells are known to express mRNA encoding IL-5 (chapter 5), they may provide a potential source of IL-5 within this model at the 72 h time point. However, many other cell types may also contribute to the cellular sources of these cytokines. For example, in the Sephadex-induced model of lung inflammation TNF-α appears to be produced primarily by BAL monocyte/macrophage mononuclear cells as immunohistochemical analysis of BAL cells revealed that only BAL monocyte/macrophage mononuclear cells stained positively for this cytokine (chapter 7). Immunohistochemistry also indicated that these cells stained positively for IL-6. Almost certainly mast cells did not contribute to the induced expression of mRNA encoding IL-5, IL-6, TNF-α, MIP-2 and IFN-γ in BAL cells from 6 h to 48 h after Sephadex administration as mast cells were not detected in BAL fluid at these times (chapter 6). However, mast cell numbers were significantly elevated 2 h after ovalbumin challenge of ovalbumin-sensitized rats (chapter 8). Therefore, mast cells may contribute to the early induced expression of

mRNA encoding various cytokines in the antigen-induced model of pleurisy in the rat.

In addition to the inhibitory effects of Dex, CsA and BTS 71321 on mast cells *in vitro* (chapters 3 and 5) these drugs have also been found to inhibit Sephadex-induced cytokine gene expression *in vivo* (chapter 7). As has been discussed above, BTS 71321 inhibits ionophore-induced TNF-α release from Cl.MC/C57.1 mast cells (chapter 5). In chapter 7 results indicate that BTS 71321 also inhibits the transcription of TNF-α in BAL cells and has additional inhibitory effects on IL-6 and IFN-γ mRNA expression in BAL cells. However, it does not inhibit cell mobilisation whereas pre-treatment of rats with Dex or CsA prior to Sephadex administration inhibits both cytokine gene expression and cell mobilisation (chapter 7). The degree of inhibition of IL-5 mRNA expression by Dex or CsA appeared to relate to the degree of inhibition of eosinophil migration into the BAL fluid, supporting the concept that a link may exist between these two events.

Incidentally, TNF-α, which has been shown to induce the expression of MIP-2 in a rat fibroblast and epithelial cell line (Driscoll *et al.*, 1993), was found to increase before the appearance of MIP-2 mRNA in the Sephadex-induced model of lung inflammation (chapter 6) suggesting that a similar pattern of events may take place *in vivo*. However, the fact that mRNA encoding TNF-α was inhibited by BTS 71321 but the mobilisation of neutrophils was not, argues against a stimulatory role of TNF-α on MIP-2 expression.

In conclusion, the work presented in this thesis has indicated that

mast cell lines and rat PMC express several cytokines, some of which may be involved in the coordination and perpetuation of an asthmatic response. As the inhibition of cytokines may be an important approach to asthma treatment this thesis has also studied the effects of Dex and CsA on mast cell cytokine expression. The results presented have suggested that the production of cytokines by mast cells may be a suitable target for pharmaceutical intervention in asthma. Furthermore, BTS 71321, the novel 1-substituted imidazole compound, may have some potential therapeutic benefit in acute reactions through its regulation of mast cell TNF-α release.

It has also been shown that the Sephadex-induced model of lung inflammation bares many similarities, in terms of cell mobilisation and cytokine gene expression, to that of antigen-induced inflammation and asthma. Although it has not been determined which cells regulate the LAR this thesis has suggested that mast cells may play an important role in the regulation of this phase. The potential contributions of mast cells to the initiation of the LAR are summarised in Fig. 9.1.

Dex and CsA were also found to inhibit cytokine gene expression and cell mobilisation *in vivo* in the Sephadex-induced model of lung inflammation. However, although BTS 71321 inhibited BAL cell cytokine gene expression it had no effect on cell mobilisation. Nevertheless, BTS 71321 may have some potential therapeutic benefit through its regulation of cytokine expression by a variety of cell types including mast cells and may possibly be effective in the treatment of bronchohyper-reactivity through its inhibition of mast cell TNF-α release and BAL cell mRNA expression. However,

although BTS 71321 appears to affect a number of different cell types it is possible that inhibition of cytokine expression by one specific cell type may provide a more effective therapy for asthma. If, as has been suggested, mast cell cytokines are important and crucial to the induction of an LAR, identification of agents which may specifically modulate mast cell cytokine release may be of greater use in the search for improved therapies for asthma.

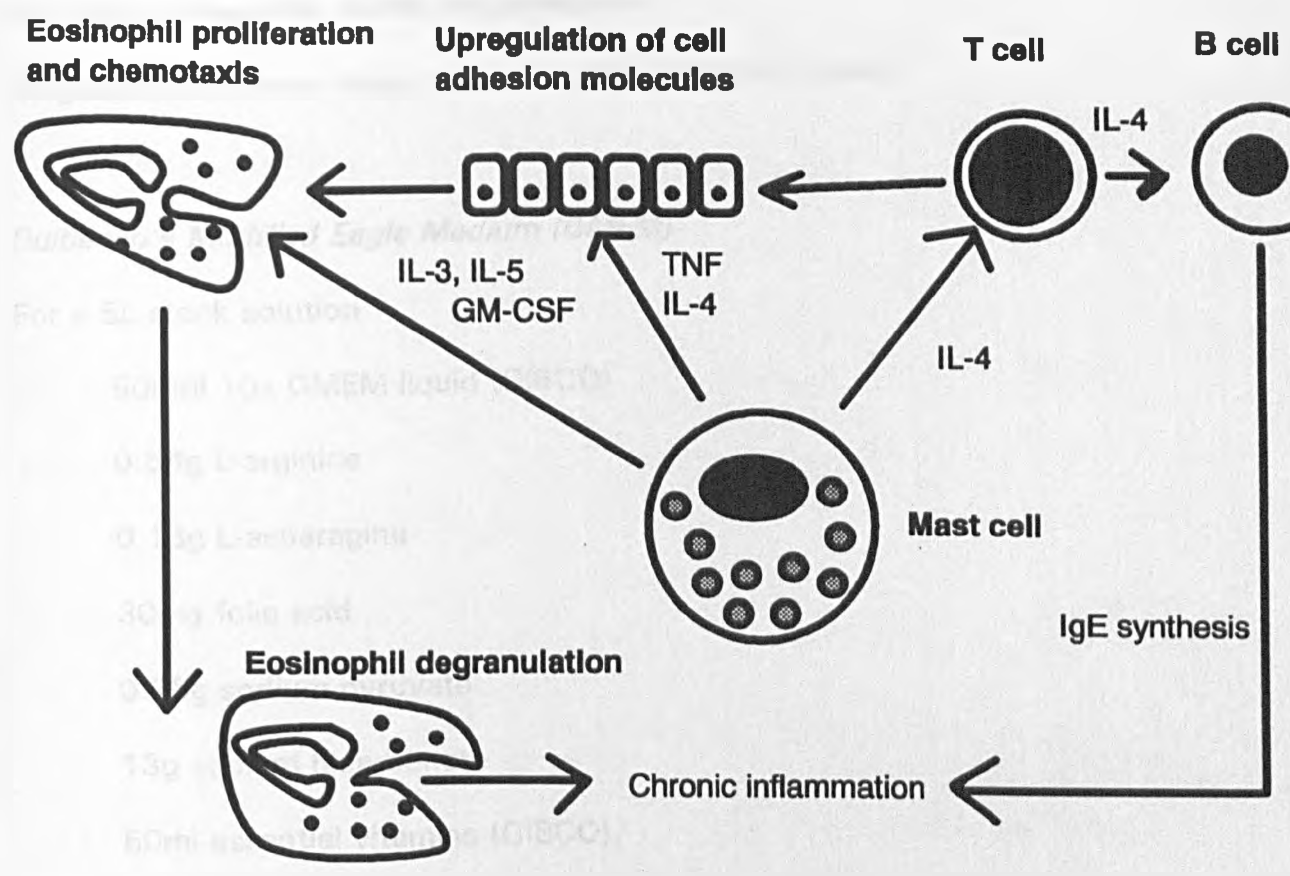

Fig. 9.1. Proposed actions of mast cells in the progression of chronic inflammation

Appendix 1

Cell culture reagents, media and solutions

All general laboratory reagents were obtained from Sigma

Dulbecco's Modified Eagle Medium (DMEM)

For a 5L stock solution

- 500ml 10x DMEM liquid (GIBCO)
- 0.58g L-arginine
- 0.18g L-asparagine
- 30mg folic acid
- 0.55g sodium pyruvate
- 13g sodium bicarbonate
- 50ml essential vitamins (GIBCO),
- 50ml non-essential vitamins (GIBCO)
- 5ml 2 mercaptoethanol.

The solution was made up to 5 L with distilled water and filter sterilized.

Cryopreservation Freezing Solution

- 1.5ml saturated hydroxyethyl starch solution
- 0.5ml dimethyl sulphoxide (DMSO)
- 3ml FCS

Cells (5 x 10^6) were resuspended in 0.5 ml of FCS in cryopreservation freezing vials and 0.5 ml of freezing solution were added to each vial. Cells were frozen in liquid nitrogen.

Phosphate buffered saline (PBS)

8g NaCl

0.2g KCl

1.44g Na_2HPO_4

0.24g KH_2PO_4

800ml water

The solution was adjusted to pH 7.4 made up to 1 L with water and autoclaved.

Citrate phosphate buffer

21g citric acid in 1L of water

35.6g Na_2HPO_4 in 1L of water

Citric acid solution (49 ml) was mixed with 51 ml of sodium phosphate solution and the pH adjusted to pH 5.0.

Appendix 2

Histology

Acid/Alcohol wash for Haematoxylin and Eosin staining

10ml concentrated HCl

990ml ethanol (70%)

Scott's Solution

2g potassium bicarbonate

20g $MgSo_4$

1L water

Eosin Stain

20g Eosin Yellowish

10g Potassium dichromate

200ml absolute alcohol

1600ml water

200ml saturated aqueous Picric acid

1 thymol crystal

Appendix 3

Immunohistochemistry

All general laboratory reagents were obtained from Sigma

Tris buffered saline (TBS)

980ml NaCl (0.9%)

20ml 1M Tris (pH 8.2)

Fast Red Substrate Solution

2mg naphthol AS-MX phosphate ($C_{19}H_{18}NO_5P$)

200μl dimethyl formamide

9.8ml 0.1M Tris buffer (pH 8.2)

10μl 1M levamisole

Just before applying to tissue sections, 10 mg of 4-chloro-2-methylbenzene-diazonium hemi [zinc chloride] salt (Fast Red TR) was added and the resulting solution was filter sterilized through a 0.2 μM syringe filter directly onto slides.

Appendix 4.

PCR Primers

Unless otherwise stated, primers were obtained from ImmunoGen International Ltd, Blaewaun Farm, Llandysul, Dafyd, Wales.

Rat Primers

IL-5 primers used were those described by Noble *et al*, (1993) which amplify a 298 bp fragment.

Sense primer $^{5'}$ TGC-TTC-TGT-GCT-TGA-ACG-TTC-TAA $^{3'}$

Antisense primer $^{5'}$ TTC-TCT-TTT-TGT-CCG-TCA-ATG-TAT-TTC $^{3'}$.

IL-6 primers were designed from the rat cDNA sequence (Northemann *et al.*, 1989) amplifying a 275 bp fragment

Sense primer $^{5'}$ GGA-GTT-CCG-TTT-CTA-CCT-GG $^{3'}$

Antisense primer $^{5'}$ GCC-GAG-TAG-ACC-TCA-TAG-TG $^{3'}$

TNF-α primers were designed from the rat TNF-α cDNA sequence (Shirai *et al.*, 1989) amplifying a 316 bp fragment

Sense primer $^{5'}$ CCA-CGT-CGT-AGC-AAA-CCA-CCA-AG $^{3'}$

Antisense primer $^{5'}$ CAG-GTA-CAT-GGG-CTC-ATA-CC $^{3'}$

MIP-2 primers used were those described by Huang *et al* (1992) which amplify a fragment of 210bp.

Sense primer $^{5'}$ GCC-AGT-GAG-CTG-CGC-TGT-CAA-TGC $^{3'}$

Antisense primer $^{5'}$ GTT-AGC-CTT-GCC-TTT-GTT-CAG-TAT-G $^{3'}$.

IFN-γ primers were obtained from Clontech Laboratories (Palo Alto CA, USA) which amplify a 288 bp fragment

Sense primer $^{5'}$ ATC-TGG-AGG-AAC-TGG-CAA-AAG-GAC-G $^{3'}$

Antisense primer $^{5'}$ CCT-TAG-GCT-AGA-TTC-TGG-TGA-CAG-C $^{3'}$.

G3PDH primers were designed from the rat cDNA sequence (Fort *et al.*, 1985) amplifying a 404 bp fragment.

Sense primer $^{5'}$ CTC-AAG-ATT-GTC-AGC-AAT-GC $^{3'}$

Antisense primer $^{5'}$ CAG-GAT-GCC-CTT-TAG-TGG-GC $^{3'}$

Human Primers

IL-3 primers were those described by Buckley *et al.* (1995) which amplify a 378 bp fragment.

Sense primer $^{5'}$ CTG-CTC-TAA-CAT-GAT-CGA-TG $^{3'}$

Antisense primer $^{5'}$ GAG-AAC-GAG-CTG-GAC-GTT-GG $^{3'}$

IL-4 primers were those described by Buckley *et al.* (1995) which amplify a 449 bp fragment.

Sense primer $^{5'}$ CTG-CAA-ATC-GAC-ACC-TAT-TA $^{3'}$

Antisense primer $^{5'}$ GAT-CGT-CTT-TAG-CCT-TTC $^{3'}$

IL-8 primers were those described by Buckley *et al.* (1995) which amplify a 248 bp fragment.

Sense primer $^{5'}$ GCA-GCT-CTG-TGT-GAA-GGT-GCA $^{3'}$

Antisense primer $^{5'}$ GAA-TTC-TCA-GCC-CTC-TTC-AA $^{3'}$

TNF-α primers were those described by Buckley *et al.* (1995) which amplify a 254 bp fragment.

Sense primer $^{5'}$ CGA-GTG-ACA-AGC-CTG-TAG-CC $^{3'}$

Antisense primer $^{5'}$ CTA-CCA-GAC-CAA-GGT-CAA-C $^{3'}$

β - Actin primers were those described by Buckley *et al.* (1995) which amplify a 362 bp fragment.

Sense primer $^{5'}$ CTG-GCA-CCC-AGC-ACA-ATG-AAG $^{3'}$

Antisense primer $^{3'}$ ACC-GAC-TGC-TGT-CAC-CTT-CA $^{3'}$

Appendix 5.

PCR Restriction Digests

PCR PRODUCT	PRODUCT SIZE	RESTRICTION ENZYME	FRAGMENT SIZES
RAT IL-5	298bp	MboII	221bp + 77bp
RAT IL-6	275bp	DdeI	200bp + 75bp
RAT TNF-α	316bp	Sau3A	84bp + 232bp
RAT MIP-2	210bp	AluI	65bp + 145bp
RAT IFN-γ	288bp	HaeIII	100bp + 188bp
HUMAN IL-3	378bp	EcoRI	107bp + 271bp
HUMAN IL-4	449bp	EcoRI	70bp + 379bp
HUMAN IL-8	248bp	HindIII	91bp + 157bp
HUMAN TNF-α	254bp	MboII	96bp + 158bp

Appendix 6.

Molecular Biology Media and Solutions

All general laboratory reagents were obtained from Sigma

6.1 Bacterial Culture Media

SOB Medium (per 1L)

20g tryptone

5g yeast extract

0.5g NaCl

Reagents were dissolved in 1 L of water, autoclaved and just prior to use 10 ml of 1 M $MgCl_2$ and 10 ml of 1 M $MgSO_4$ were added and the solution filter sterilised.

SOC Medium (per 100ml)

To 100 ml SOB medium was added 1.0 ml of a 2 M filter sterilised glucose solution prior to use.

LB Medium (per 2L)

10g NaCl

20g bactotryptone

10g yeast extract

Water was added to a final volume of 2 L, the pH adjusted to 7.0 with 5M NaOH and the medium autoclaved

LB (liquid broth) Agar Plates

400ml LB Medium

6g bactoagar

The solution was autoclaved and poured into 10 ml sterile petri dishes. For LB-Ampicillin plates, 400 μl of a 50 mg/ml solution of Ampicillin was added before pouring.

Tris-EDTA (TE) Buffer

10mM Tris-HCl (pH 7.5)

1mM EDTA

6.2 Electrophoresis Buffers

Tris-acetate-EDTA (TAE). Use working solution as 1 x TAE.

50 X : 242g Tris base

57.1ml glacial acetic acid

100ml 0.5M EDTA (pH 8.0)

The solution was made up to 1 L with distilled water

Tris-borate-EDTA (TBE). Use working solution as 1 x TBE.

10 X : 216g Tris base

110g boric acid

80ml 0.5M EDTA (pH 8.0)

The solution was made up to 1 L with distilled water

6.3 Alkaline Lysis Buffers for Minipreparations of Plasmid DNA

Solution 1

50mM glucose

25mM Tris-Cl (pH 8.0)

10mM EDTA (pH 8.0)

Solution 1 can be prepared in batches of 100 ml, autoclaved for 15 min and stored at 4°C.

Solution 2

0.2M NaOH (freshly diluted from 10M stock)

1% SDS

Solution 3

60ml 5M potassium acetate

11.5ml glacial acetic acid

28.5ml water

The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate.

6.4 Alkaline Lysis Buffers for Maxipreparations of Plasmid DNA

Solution I

To make 1L of Solution I

9g glucose

25ml 1M Tris (pH 8.0)

20ml 0.5M EDTA (pH 8.0)

955ml water

Solution II

To make 100ml of Solution II

4ml 5M NaOH

10ml 10% SDS

86ml water

Solution III

To make 1L of Solution III

600ml 5M potassium acetate

115ml glacial acetic acid

285ml water

6.5 Sequencing Gels

Polyacrylamide (16%)

288g Urea

34.2g acrylamide

1.8g bisacrylamide

Reagents were dissolved in 20 ml of water with slight heat. The solution was stirred gently and 20 g of amberlite resin were added. The solution was then left to stand for 30 min before filtering through sintered glass. Sixty millilitres of 10 X TBE was added and the solution was made up to 600 ml

with water.

Gel for bottom tray

40ml 16% polyacrylamide

100μl N,N,N',N'-tetramethyl ethylenediamine (TEMED)

300μl 10% ammonium persulphate

Main gel

70ml 16% polyacrylamide

70μl TEMED

200μl 10% ammonium persulphate

6.6 In Situ Hybridisation Buffers and Solutions

Diethyl Pyrocarbonate (DEPC) treated Water

One millilitre of DEPC was added to 1 L of water. Bottles were shaken vigorously for 2 min and left for at least 1 h with regular shaking before autoclaving.

20 X Sodium Chloride/Sodium Citrate Solution (20 X SSC)

175.3g NaCl

88.2g sodium citrate

800ml DEPC water

The pH was adjusted to 7.0 with a few drops of 10 M NaOH, made up to 1 L with DEPC water and autoclaved.

Hybridisation Buffer

5ml formamide

2ml 50% dextran sulphate

3ml 20 x SSC

Modified Tris

50mM Tris (pH 7.6)

150mM NaCl

2mM $MgCl_2$

0.1% bovine serum albumin

0.1% Triton X-100

Revealing Reagent

2.4ml 0.125M Tris (pH 9.5)

300μl 1M NaCl

300μl 0.5M $MgCl_2$

The solution was mixed by inversion. Thirteen microlitres of 4 Nitro blue tetrazolium chloride (Boehringer Mannheim), 10 μl of 5-Bromo-4 chloro-3-indoyl-phosphate 4-toluidine salt (Boehringer Mannheim) and 60 μl of 1 M levamisole were then added and the resulting solution pipetted onto tissue sections.

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