Peripheral blood neutrophil activation markers in severe corticosteroid-dependent asthmatics, and the effect of prednisolone.

Submitted for the degree of MD

by

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Author's declaration

The following tests on patients were carried out by nurses in the asthma clinic at the Royal Brompton Hospital: skin testing, lung function measurements, measurements of airway hyprresponsiveness, exhaled NO measurements, sputum induction, and calibration of spirometers. Patients were recruited by me with the help of the asthma nurse in the chest clinic.

The experiments carried out in this MD were done by me using the latest equipment available to me in the National Heart and Lung Institute, and the body of evidence available in the literature at that time. These include sputum processing, isolation of peripheral blood mononuclear cells and granulocytes from whole blood, direct and indirect staining of whole blood and isolated cells with monoclonal antibodies for FACS analysis, ELISA techniques, flow cytometry, and whole blood culture techniques.

The work presented in Chapter 4, on histone acetylation was done in collaboration with Dr Borja Cosio, who was also working at the National Heart and Lung Institute. I was responsible for recruiting patients for work on the peripheral blood part of the experiments, and isolated peripheral blood mononuclear cells and granulocytes for these experiments. Dr Cosio carried out the bronchoscopies, processing of bronchial alveolar cell macrophages, measurement of HDAC in alveolar macrophages and peripheral blood cells, IL-8 ELISAs in whole blood culture and the statistical analysis and graphs for this chapter.

Signed.....

Abstract

Background

Severe asthmatics have increased numbers of airway neutrophils compared to mild asthmatics, and blood neutrophils may be activated in asthmatics. Activated neutrophils may contribute to airway inflammation and tissue damage.

Hypothesis

Activation of both eosinophils and neutrophils is important in severe asthmatics, through enhancement of mediator release. The effect of corticosteroids on inhibition of neutrophils from severe asthma may be attenuated. Patients with severe allergic asthma may respond to anti-IgE monoclonal antibody treatment.

Methods

The level of expression of baseline and stimulated blood neutrophil surface markers was investigated in severe corticosteroid-dependent asthmatics and compared to normal subjects and milder asthmatics. The effect of adding or increasing maintenance oral prednisolone (30mg/day for one week), on surface molecules, IL-8 and myeloperoxidase levels, neutrophil elastase and oxidative burst in blood was also studied.

The level of expression and effect of corticosteroids on histone deacetylase (HDAC) activity from asthmatics was studied. The immediate effect of inhaled fluticasone propionate was also investigated, along with the efficacy of subcutaneous anti-IgE therapy.

Results

CD35 and CD11b expression was increased in neutrophils from severe asthmatics compared to normal subjects. Prednisolone inhibited surface marker expression, oxidative burst and IL-8 release from mild but not severe asthmatics. HDAC activity was reduced in alveolar macrophages compared to PBMCs in asthmatics. Prednisolone increased HDAC activity in PBMCs from mild, but not severe

asthmatics. HDAC activity was generally reduced in granulocytes compared with PBMCs.

Inhaled fluticasone reduced exhaled nitric oxide levels in moderate asthmatics at four hours. Subcutaneous administration of anti-IgE therapy was well tolerated and decreased high affinity IgE receptor levels on basophils and monocytes during treatment.

Conclusion

Peripheral blood neutrophils from severe asthmatics show a higher state of activation than normal subjects, and show loss of inhibitory corticosteroid response. Potential alternative treatments for severe asthma include anti-IgE antibody therapy.

Acknowledgement

I would like to thank my wife and family for their support, patience, and understanding during my MD, and indeed, throughout my medical career. I would like to dedicate this MD to the memory of my mother who passed away during the early stages of my research, and who always gave me love and encouragement. She was never far from my thoughts. Finally, I would like to thank Professor Fan Chung for giving me the opportunity to undertake research with him, and for his helpful advice, comments and support along the way.

Publications and abstracts arising from Thesis

Abstracts

1. Peripheral blood neutrophil function and activation in severe steroid-dependent asthmatics. B.S.Mann, K.F.Chung – American Thoracic Society, October 2002.

2. Effect of prednisolone on activation markers on blood neutrophils and eosinophils in mild and severe asthma. B.S.Mann, K.F.Chung – American Thoracic Society, October 2002.

3. Histone deacetylase activity is reduced in alveolar macrophages but not peripheral blood monocytes from asthmatics. Cosio B, Mann B, Jazrawi E, Adcock I, Chung KF, Barnes PJ – American Thoracic Society, October 2002

Publications

1. Blood neutrophil activation markers in severe asthma: lack of inhibition by prednisolone therapy. Mann BS, Chung KF. Respir Res. 2006; 7: 59-63.

2. Histone acetylase and deacetylase activity in alveolar macrophages and blood monocytes in asthma. Cosio BG, Mann B, Ito K, Jazrawi E, Barnes PJ, Chung KF, Adcock IM. Am J Respir Crit Care Med 2004; 170(2); 141-147.

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List of abbreviations

ADCC- antibody dependant cell mediated cytotoxicity
AFU- arbitrary fluorescence units
AM- alveolar macrophages
AP-1- apoprotein 1
APC- antigen presenting cell
ARDS-acute respiratory distress syndrome

BAL-bronchoalveolar lavage
BD- 'bid' dosing, or twice daily
BDP-beclomethasone dipropionate
BGP- basic granule protein
BPI- bacterial permeability increasing protein
BSA- bovine serum albumin

$^{\circ}$C –degrees centigrade
CBP- CREB binding protein
CCR3- chemokine receptor 3
$C0_2$-carbon dioxide
COPD- chronic obstructive pulmonary disease
COX - cyclooxygenase
CPB- cardiopulmonary bypass

DCF- dichlorofluorescein
DCFH- 2,7 dichlorofluorescein
DHR- dihydrorhodamine
DNA- deoxyribonucleic acid
DTT- dithiothretol

ECP- eosinophilic cationic protein
EDTA- ethylenediaminetetraacetic acid
EGFR-epidermal growth factor
ELISA- Enzyme-linked ImmunoSorbent Assay
eNO- exhaled nitric oxide

FACS-fluorescent activated cell sorter
FEV1-forced expiratory volume in one second (litre)
FITC- Fluorescein isothiocyanate
FL1-fluorescence 1 channel
FL2-fluorescence 2 channel
fMLP- N-formyl-methyl-leucyl-phenylalanine
FP-fluticasone propionate
FVC-forced vital capacity (litre)

GC-glucocorticoid
GCSF-granulocyte colony stimulating factor
GMCSF-granulocyte macrophage colony stimulating factor
GR-glucocorticoid receptor
GRE-glucorticoid response element

HAT- histone acetyltranferase

HBSS- Hank's balanced salt solution
HDAC- histone deacetylation activating complex
H_20_2-hydrogen peroxide
HLA-DR- Human Leukocyte Antigen DR-1
HUVEC-human umbilical vein endothelial cells

ICAM-1- Intracellular Adhesion Molecule One
ICS-inhaled corticosteroid
IFNγ-interferon gamma
IgE-immunoglobulin E
INOS-inhaled nitric oxide synthase
IL-interleukin
IU/ml- international units per millilitre
IV-intravenous

KDa- kiloDalton

LPS- lipopolysaccharide
LTB4-leucotriene B4

M-molar
mcg (μg) -microgram
MFI-mean fluorescence intensity
mg-milligrams
MHC- major histocompatibility complex
ml-millilitre
mM-millimoles
MMP-9- metallomatrix protein 9
MPO-myeloperoxidase
MRNA-messenger ribonucleic acid
MS-multiple sclerosis
MW-molecular weight

NADPH- nicotinamide dehydrogenase phosphate
NCA-neutrophil chemotactic activity
ng-nanogram
nGRE-negative glucocorticoid response element
NK cells- natural killer cells
NO-nitric oxide
NOS-nitric oxide synthase

02-oxygen
0^{2-} superoxide anion
OH- hydroxyl radical

PAF-platelet activating factor
PBMC-peripheral blood mononuclear cells
PBO-placebo
PBS- phosphate buffered saline
PBS- phosphate buffered saline with bovine serum albumin and sodium azide
PC_{20}- concentration of methacholine (mg/ml) required to produce a 20% fall in FEV1 from baseline

PCR- polymerase chain reaction
PE- phycoerythrin
PEF-peak expiratory flow
pg-picogram
PMA- phorbol myristate acetate
PMN-polymorphonuclear cell
ppb-parts per billion
PTCA-percutaneous coronary angioplasty

rpm-revolutions per minute

SEM-standard error of the mean

Th2- T-helper type 2 cell
TLC-total lung capacity (litre)
TNFα-tumour necrosis factor alpha

Chapter 1

1. Introduction

1.1 Inflammatory basis of asthma

Bronchial asthma is a common clinical condition which can affect individuals of almost any age, and is an important cause of respiratory morbidity and mortality. It is characterised by variable dyspnoea, wheeze, cough and chest tightness due to widespread narrowing of the peripheral airways in the lungs, which varies in severity over short periods of time either spontaneously or as a result of medication. Usually, asthma develops during childhood in atopic individuals (1). The prevalence of asthma is highest in the second decade of life where this disease affects 10-15% of the population. There is also geographical variation with asthma being common in, for example, New Zealand, but rarer in Far Eastern countries. Studies of occupational asthma suggest that a high percentage of the workforce, perhaps up to 20% may become asthmatic if exposed to potent sensitizers (2).

There is substantial evidence for the role of inflammation in asthma and bronchial hyperreactivity. Early autopsy studies of patients who died following attacks of fatal asthma showed epithelial denudation, mucosal oedema, glandular hypertrophy, and dense eosinophilic infiltration (3). More recently, techniques including bronchoalveolar lavage (BAL) and bronchial biopsy have been used to investigate airway inflammation in mild asthmatics. Elevated numbers of eosinophils, both in the bronchial mucosa and in BAL fluid were shown to be a constant feature of patients with mild asthma (4, 5). Similarly, increased numbers of activated lymphocytes were also seen. These were identified as either irregular, atypical lymphocytes by transmission electron microscopy (6) or as CD25+ cells as shown by immunocytochemistry, most of which were shown to be T-lymphocytes (7).

Studies in asthmatic patients challenged with aeroallergens have shown increased numbers of BAL fluid eosinophils, mast cell mediators, and products of other inflammatory cells (8, 9). In addition, transbronchial biopsies in patients with mild well controlled asthma, showed significant inflammatory changes. Asthma is thus associated with chronic inflammation of the airways, characterised by infiltration of the airway mucosa by eosinophils, mast cells, and T-lymphocytes, by subepithelial fibrosis, and almost always by intraluminal eosinophilia. The inflammation is mediated by the

release of an array of cytokines and inflammatory mediators (10, 11). Furthermore, studies have shown an association between the degree of inflammatory changes on bronchial biopsies, and measurements of non-specific bronchial hyperresponsiveness (12, 13).

Role of Neutrophils in Asthma

The majority of asthmatics can be managed with intermittent short-acting beta-2-agonists and low dose inhaled corticosteroids or combination therapy consisting of inhaled corticosteroids and long-acting bronchodilators, but a small minority of patients require high doses of inhaled corticosteroids as well as oral corticosteroids (corticosteroid-dependent asthmatics) in order to maintain control. These patients often have severe and difficult to control asthma and develop side-effects due to their medication. They also account for a large proportion of total asthma costs in terms of medication and resource utilization. The pathogenesis and mechanism(s) involved in the development of this severe asthmatic state have not been fully elucidated. Although the role of eosinophils in the pathogenesis of asthma is well established, the role of neutrophils in asthma is more controversial, but there is increasing evidence to suggest the importance of neutrophils in more severe asthma and the allergic process.

1.2.1 *Evidence for the Involvement of Neutrophils in Asthma Exacerbations*

Airway hyperresponsiveness is an exaggerated bronchoconstrictive response to physical, chemical, or pharmacological stimuli (14) and is a common and important feature of bronchial asthma. Evidence suggests that in asthma, airway hyperresponsiveness is associated with the presence of airway inflammation (15, 16), but the relative role of each inflammatory cell remains unclear. In stable mild asthmatics, long-lasting airway hyperresponsiveness significantly correlates with bronchoalveolar eosinophils, metachromatic cells, and epithelial cells (15), but not with neutrophils, suggesting that neutrophils may not be involved in long-lasting hyperresponsiveness and ongoing asthma.

The importance of neutrophils for asthma exacerbations has been examined in experimentally induced airway hyperresponsiveness in normal and asthmatic subjects. Exposure to ozone (17, 18), nitrogen dioxide (19) and inhalation of inflammatory mediators such as platelet activating factor (PAF) (20), increase the airway

responsiveness of normal subjects and cause transient airway neutrophilia. BAL studies in asthmatics exposed to ozone, show evidence of significant neutrophilia and increased levels of the cytokines interleukin-8, (IL-8) and IL-6, even at levels of ozone exposure that do not reduce pulmonary function (18). Similarly, aerosolized tumour necrosis factor-alpha, (TNF-α) causes a transient increase of airway responsiveness associated with sustained sputum neutrophilia but not eosinophilia in normal subjects (21), further suggesting that they may be involved in the transient increase of airway responsiveness induced by different inflammatory stimuli. Whether the increase in neutrophils is merely associated with such transient increases of airway responsiveness or whether neutrophils are involved in the development of such transient increase of airway responsiveness is unclear.

Studies in asthmatics of spontaneous moderate to severe asthma exacerbations by *Fahy et al* (22) and *Sur et al* (23), have shown increased numbers of neutrophils and eosinophils in sputum and BAL samples respectively. In contrast, mild asthma exacerbations that occur during tapering of inhaled glucocorticoid treatment (24) and exacerbation induced by natural allergen exposure (25) are associated with a more prominent airway eosinophilia rather than neutrophilia, suggesting that the triggers of exacerbations may be important in determining the type of cellular response.

1.2.2 *Evidence for the Involvement of Neutrophils in Stable Severe Asthma*

Although several studies have shown no difference in the numbers of neutrophils in bronchial biopsy, BAL and sputum specimens in stable asthmatic patients compared to healthy control subjects (15, 16, 25), other studies have reported an increase of sputum and BAL neutrophils in more severe asthmatics (26, 27). *Boulet et al* showed that subjects with occupational asthma induced by different sensitizing agents have a significant increase of neutrophil number in bronchial biopsy specimens even after removal of the causal agent (28). *Frangova et al* found a slight but significant increase of BAL neutrophils and eosinophils in both atopic and non-atopic moderate asthmatics (27). Similarly, *Kelly et al* showed a significant increase in the number of BAL neutrophils and also showed a correlation between the number of neutrophils and the degree of airway hyper-responsiveness in stable asthmatic subjects (25), but this correlation was not found in other studies (15, 16).

The lack of increase of neutrophils in the airways of asthmatics does not necessarily rule out their involvement in the inflammatory process, as airway

neutrophils may be activated despite a lack of increase in their number. Studies have shown increased expression of cyclooxygenases, 15-lipooxygenase, neutrophil myeloperoxidase (MPO), and lipocalin in neutrophils in the blood and sputum of patients with atopic asthma (29, 30). Also, in corticosteroid-resistant asthmatics, there is an increased production of leucotriene B4 (LTB4) by neutrophils in response to cytokines produced by peripheral blood mononuclear cells (PBMCs), (31).

Jatakanon et al showed increased numbers of neutrophils and IL-8 levels in the sputum of patients with stable severe asthma (32), and increased levels of neutrophils have been demonstrated in bronchial biopsies from severe asthmatics on high doses of maintenance oral corticosteroids (33). Thus, although neutrophils do not seem to play a significant role in mild to moderate stable asthma, they might be involved in ongoing severe asthma.

1.2.3 *Nocturnal Asthma*

The involvement of neutrophils in nocturnal asthma is controversial. Whereas *Wenzel et al* showed a significant increase of BAL neutrophils and eosinophils at night in patients with nocturnal asthma (34), this has not been confirmed in other studies. Transbronchial biopsy specimens (but not endobronchial biopsies) of nocturnal asthmatics showed an increase of eosinophils only when compared to those of non-nocturnal asthmatics (35). It is possible that only severe nocturnal asthma is associated with a significant increase of inflammatory cells in the BAL (36). Treatment with systemic corticosteroids and theophylline reduces nocturnal symptoms, airflow limitation, and the associated BAL neutrophilia and eosinophilia, suggesting the importance of bronchoalveolar inflammation, and possibly of neutrophils, in nocturnal asthma

1.2.4 *Neutrophils in severe asthma exacerbations*

Mild asthma exacerbations are typically associated with sputum eosinophilia, or sputum eosinophilia and neutrophilia (24), whilst severe asthma exacerbations have been shown to be associated with a predominant sputum neutrophilia (22). In addition, high levels of IL-8 and neutrophil elastase activity have been shown in the sputum of patients with severe exacerbations. *Slindo et al* showed that the arterial blood concentrations of the neutrophil chemotaxin LTB4, correlated with the severity of asthma exacerbations and levels were reduced by glucocorticoids, further suggesting

the importance of neutrophils in severe asthma exacerbations (37). Previous studies by *Buchanan et al* (38) also showed that severe asthma exacerbations were associated with an increase of serum neutrophil chemotactic activity (NCA). Further, *Lamblin et al* showed massive BAL neutrophilia in mechanically ventilated patients with noninfectious severe asthma exacerbations (39).

1.2.5 *Neutrophils and fatal asthma*

Death from asthma may occur either from slow progression of the disease, or by sudden death (40). Asthma death associated with a slow progression of the disease is characterized by plugging of the airways with mucus, inflammatory cells, smooth muscle hyperplasia, oedema, and massive infiltration of the airway walls with inflammatory cells (41). In particular, a dramatic increase of eosinophils in the submucosa has been demonstrated. In contrast, sudden-onset fatal asthma is characterized by more neutrophils in the airway submucosa (42) and less mucus in the airway lumen. These observations suggest that the mechanism of airway inflammation and airway narrowing in sudden-onset fatal asthma may be different from that in slow-onset fatal asthma. Thus the time course of the asthma exacerbation and possibly the nature of the trigger may be important in determining the type of cellular response (42).

1.2.6 *Neutrophils and bronchoprovocation with allergens and sensitizing agents*

BAL studies have shown that neutrophilia is present before the onset and during the first few hours of the late asthmatic reactions induced by allergens (43), and sensitizers such as toluene diisocyanate (44). Further, bronchial biopsy specimens showed a significant increase in neutrophil numbers following local allergen challenge in the airways of sensitized asthmatics (45). The increase in neutrophils has been shown to usually precede, but sometimes parallel or follow, the increase of eosinophils, metachromatic cells, and lymphocytes (43).

It has been suggested that the allergen-induced BAL neutrophilia might be due to either a nonspecific effect of the procedures involved in local bronchoprovocation or to endotoxin contamination of the allergen extracts. However, previous studies have shown BAL neutrophilia during late asthmatic reactions to allergens or isocyanates even in sites not previously lavaged (44). This suggests that the neutrophilia may be specific, and that the nonspecific increase observed after saline solution infusion is

linked to the specific maneuver of lavaging the same segment twice after a short interval. The specificity of airway neutrophilia that occurs during the first few hours after allergen challenge is also suggested by biopsy studies, which show increased neutrophil numbers in allergen-challenged airways only and not in control airways (45). In addition to neutrophil numbers, the serum heat-stable NCA has been shown to be elevated during early and late asthmatic reactions induced by allergens and toluene diisocyanate (46). Further, the percentage increase in plasma NCA induced by inhalation of toluene diisocyanate has been shown to correlate with the percentage decrease of FEV1 of the early asthmatic reaction.

1.2.7 *Animal Experimental Studies Investigating the Role of Neutrophils in Asthma*

In dogs, the transient increase in airway responsiveness induced by allergen inhalation, or by exposure to ozone and PAF, is associated with airway neutrophilia in BAL and/or biopsy specimens. The increased airway responsiveness and neutrophilia can be prevented by depletion of circulating neutrophils induced by the cytotoxic agent hydroxyurea (47). Similarly, in rabbits actively sensitized to *Alternaria,* an increased number of neutrophils in the airway mucosa are present during the late asthmatic reactions and during the transient increase of airway responsiveness induced by inhalation of *Alternaria* extracts. These effects of the inhalation challenge were prevented by neutrophil depletion and reproduced following neutrophil repletion of the animals with concentrated neutrophils (48). Other experiments in rabbits showed a neutrophilia in the airway mucosa during the transient increase of airway responsiveness induced by aerosolized complement factor 5a (C5a) des arg, (a complement fragment with marked neutrophil chemotactic activity), and this effect was prevented by neutrophil depletion (49). In a murine model of atopic asthma, infiltration of lung tissue and airways with eosinophils was associated with increased numbers of other leukocytes including neutrophils (50). Further, transgenic mice expressing IL-4 in the lung showed airway inflammation with infiltration of lung tissue and airways with both eosinophils and neutrophils (51). In general, animal studies have suggested a significant pathogenic role of neutrophils in late asthmatic reactions, and for the transient increase of airway responsiveness induced by allergen challenge.

Both human and animal studies suggest that the long-lasting hyperresponsiveness which is present between asthmatic exacerbtions is associated with mild airway inflammation due to activation of eosinophils, T-lymphocytes and mast cells but not neutrophils. Further, neutrophils do not appear to be involved in the chronic airway inflammation associated with mild and moderate stable asthma, but may have a role in severe stable asthma. There is evidence, particularly in humans, that neutrophils may be involved in exacerbations of asthma, and particularly in the early stages of sudden and severe asthma exacerbations. Also, the predominant involvement of neutrophils in some types of asthma exacerbations, such as those induced by occupational sensitizers and following severe asthma exacerbations, suggests that different triggers of asthma may induce airway inflammation through different mechanisms.

1.3 Neutrophil biology

1.3.1 *Neutrophil development*

The neutrophil is derived from a pluripotent stem cell which gives rise to the lymphoid and myeloid stem cells. From the latter, under the influence of stem cell factor (SCF), IL-3, and granulocyte/macrophage colony stimulating factor (GM-CSF), the colony forming unit-granulocyte/erythrocyte/macrophage/megakaryocyte (CFU-GEMM) is formed. This multipotent cell gives rise to the colony forming unit-granulocyte/macrophage (CFU-GM) under the continued influence of SCF, IL-3, and GM-CSF. The colony-forming unit-granulocyte (CFU-G) is the neutrophil-committed product of the CFU-GM, under the influence of IL-3, GM-CSF, and granulocyte-colony stimulating factor (G-CSF), a cytokine that is specific for neutrophil development. In the presence of G-CSF and GM-CSF, the neutrophil myeloblast develops and is the first morphologically distinct cell in neutrophil development. All subsequent stages of neutrophil development occur in the setting of G-CSF and GM-CSF (52).

It takes 4-6 days for maturation to pass through to the myelocyte stage in the bone marrow, and a further 5-7 days for the myelocyte to develop into a mature neutrophil. Following the myelocyte stage, the maturing neutrophil passes through the metamyelocyte and band stages before developing into a mature neutrophil (fig. 1.1). The mature neutrophil measures 10-12μm, and has a highly condensed, segmented, multilobulated nucleus with 3-5 lobes. Approximately $1x10^{11}$ neutrophils are generated

daily, and this number can increase by another order of magnitude in the setting of infection. The calculated circulating granulocyte pool is $0.3x10^9$ cells per kilogram of blood, and the marginated pool is $0.4x10^9$ cells per kilogram of blood, comprising 3% and 4% of the total granulocyte pool, respectively (53). The normal mechanisms by which neutrophils exit the bone marrow are unclear. However, administered cytokines such as GM-CSF and G-CSF lead to substantial increases in neutrophil production and release. Neutrophils have a circulating half-life of about 6 hours and survive in the tissue for 1-2 days.

1.3.2 *Granule content and function*

Characteristic neutrophil cytoplasmic granules are first found at the promyelocyte stage of development. The first granules that can be recognized are called primary granules or azurophilic, because they stain blue with Wright's stain in bone marrow preparations. Primary granules arise by the budding of vacuoles from the concave face of the Golgi apparatus (54). The promyelocyte develops into the myelocyte, during which stage, secondary or specific granule synthesis occurs from the convex surface of the Golgi apparatus. The secondary granules fuse with incoming phagocytic vacuoles containing ingested organisms and release their contents, which include lactoferrin (which scavenges available iron), the N-formyl-methionyl-leucyl-phenylalanine fMLP receptor (FPR), complement receptor 3 (CR3), and cytochrome 558, the membrane bound component of the nicotinamide dehydrogenase phosphate (NADPH) oxidase that is required for the generation of superoxide, hydrogen peroxide, and hypohalous acid. By 3 minutes after ingestion, the primary granules have fused to the phagolysosome and this results in a marked lowering of the intravacuolar pH due to the release of MPO, lysozyme, acid hydrolases, and defensins. MPO, a primary granule component, catalyzes the conversion of hydrogen peroxide (H_20_2) to hypohalous acid which has anti-microbial action. Lack of development of neutrophil granules and their contents can lead to serious and frequent infections in early life.

Chediak-Higashi syndrome is a rare autosomal recessive disorder with a generalized abnormality of development of the primary granules. These patients show impaired bacterial killing by phagocytes leading to recurrent infections. Natural Killer (NK) cell function is also defective and many patients die in childhood from infection. Neutrophil specific granule deficiency is a rare autosomal recessive condition clinically characterized by a profound susceptibility to bacterial infections. There is a paucity of

neutrophil specific granules and specific granule proteins and their respective messenger ribonucleic acids (mRNAs), and there are very low levels of the primary granule products defensins and their mRNAs. This is thought to be due to loss of a tissue-specific transcriptional factor (55).

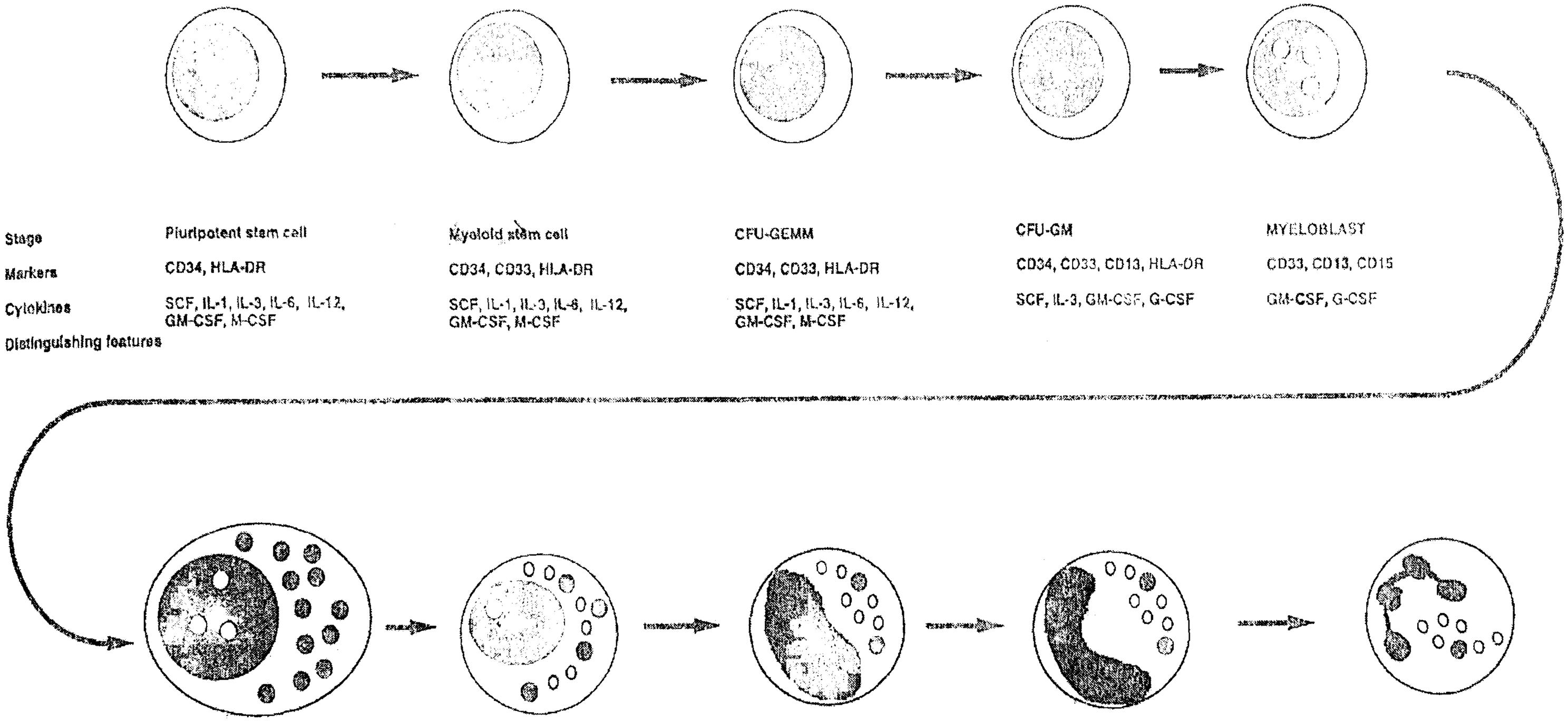


Fig 1.1 Neutrophil development from a pluripotent stem cell into a mature neutrophil (from reference 52).

1.3.3 *Chemoattractants and chemotaxis*

Neutrophils are able to move toward very slight gradients of chemical signals (56), and this process of chemotaxis involves the complex machinery of cellular redirection, orientation, and locomotion. The classical chemoattractants include N-formyl-methionyl-leucyl-phenylalanine (fMLP), C5a, LTB4, and PAF. More recently, a class of small (<10 kDa), proteins called alpha or cysteine-X-cysteine (CXC) chemokines have been shown to be potent chemoattractants for neutrophils, and are characterized by a CXC pattern in the amino terminal portion of the molecule. Among the alpha chemokines, the peptide sequence, glutamic acid-leucine-arginine (ELR) immediately proximal to the C-X-C motif confers potent neutrophil chemoattraction. IL-8 is the most extensively studied of the chemokines, and is a very potent chemoattractant and neutrophil activator. Many different cellular sources for IL-8 have been identified, including neutrophils, monocytes, T-cells, B-cells, NK cells, basophils, eosinophils, fibroblasts, endothelial cells, keratinocytes, and smooth muscle. Blockade of IL-8 by neutralizing anti-IL-8 antibody in rabbit models of pulmonary inflammation have been to be successful in preventing neutrophil accumulation and reperfusion injury, confirming the central role for this chemokine in neutrophil recruitment to sites of inflammation (57).

Neutrophil movement toward a source of stimulus involves complex changes in the viscosity of the cytoplasm, and relocation of the nucleus, centriole, and organelles. The cell projects a pseudopod in the direction of motion and trails a uropod behind. Retraction fibres may extend from the uropod that are resorbed or that break off as the cell moves forward (58). The creation of structures within the cell that can permit locomotion depends on the ability of actin to move from sol to gel to sol as shape changes. Actin monomers (G-actin) can polymerize into double-helical actin filaments (F-actin). Crosslinking of these filaments by actin-binding protein results in the formation of a rigid gel. Actin monomers are kept from polymerization in leukocytes by proteins that bind them, such as profilin. Actin polymerization is also controlled at the level of the growing filament end. Gelsolin binds to the rapidly growing end of the actin filament, preventing further elongation, and severs the existing filament. Gelsolin's binding to actin, prevention of filament elongation, and filament scission are all calcium regulated. Therefore, the rise in intracellular calcium caused by the stimulation of a chemoattractant receptor has an influence on the cell cytoskeleton through the assembly and disassembly of actin filaments.

1.3.4 *Neutrophil adhesion to endothelium*

Neutrophils are designed to roll along the endothelium, recognize sites of inflammation, adhere to inflamed sites, and traverse the endothelium in order to enter the involved tissue and fight infection. The physical interaction of neutrophils with endothelium and other leukocytes is mediated by three main sets of molecules: selectins, integrins, and members of the immunoglobulin supergene family (intercellular adhesion molecules, or ICAMs). The selectins are designated E (endothelial), P (platelet), and L (leukocyte). Endothelial cells express E-selectin (CD62E) and P-selectin (CD62P), whereas leukocytes express L-selectin (CD62L) (59). This family of molecules mediates the low-affinity rolling of neutrophils along the endothelium. Although E-selectin and P-selectin bind to the sialyl Lewis X (SLe^X) antigen on neutrophils, L-selectin on neutrophils binds to distinct antigens on endothelium, including CD34. L-selectin is shed by neutrophils on activation and is absent in exudate cells, implying that release of L-selectin and migration into sites of inflammation are linked (60). The identification of sites of inflammation is accomplished largely through chemoattractants, which trigger high-affinity binding by integrins (fig. 1.2).

The integrins are heterodimers consisting of a specific alpha (α) chain bound to a common beta (β) subunit, CD18. CD18 is a 95 kiloDalton (kDa) protein expressed on phagocytes, monocytes, macrophages, and lymphocytes and is obligatory for expression of the respective alpha chains. Integrins are required for the neutrophil's tight adherence to endothelium, and to other leukocytes, complement, and bacteria (61). The heterodimers of the integrin family include CD11a/CD18 (LFA-1), CD11b/CD18 (Mac-1, Mo-1, or CR-3), and CD11c/CD18 (p150, 95 or CR4). The CD11b/CD18 complex mediates the binding of cleavage products of C3 (62).

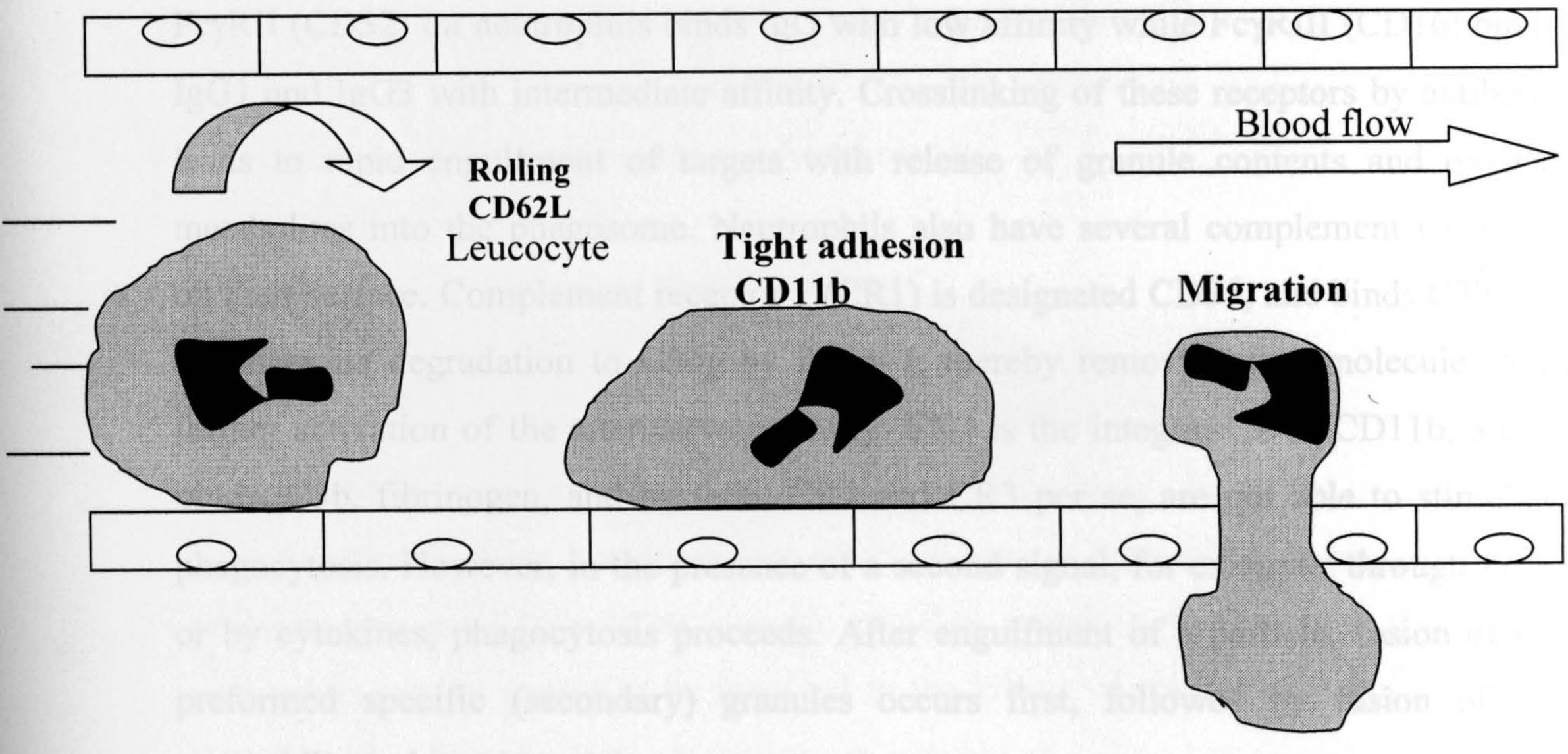


Fig. 1.2 Leukocyte trafficking. CD62L mediates the initial low affinity rolling of neutrophils along the endothelium. Integrins such as CD11b, then allow neutrophils to bind tightly to endothelium, other leukocytes, complement and bacteria.

1.3.5 *Phagocytosis*

Neutrophils bind Immunoglobulin G (IgG) antibodies via receptors for the Fc portions of IgG (FcγR). FcγRI (CD64) is a receptor for IgG1 and IgG3 on monocytes and macrophages and is expressed on neutrophils after interferon-γ stimulation (63). FcγRII (CD32) on neutrophils binds IgG with low affinity while FcγRIII (CD16) binds IgG1 and IgG3 with intermediate affinity. Crosslinking of these receptors by antibody leads to rapid engulfment of targets with release of granule contents and oxygen metabolites into the phagosome. Neutrophils also have several complement receptors on their surface. Complement receptor 1 (CR1) is designated CD35, and binds C3b and enhances its degradation to C3dg by factor I, thereby removing that molecule from further activation of the alternative pathway. CR3 is the integrin CD18/CD11b, and it binds iC3b, fibrinogen, and bacteria. CR1 and CR3 per se, are not able to stimulate phagocytosis. However, in the presence of a second signal, for example, through FcγR or by cytokines, phagocytosis proceeds. After engulfment of a particle, fusion of the preformed specific (secondary) granules occurs first, followed by fusion of the azurophilic (primary) granules.

1.3.6 *Exudative Neutrophils*

Exudative neutrophils differ from peripheral blood neutrophils and acquire a ruffled irregular membrane, extend pseudopods, assume a triangular shape, and cap certain membrane components, such as concanavilin A binding sites. These changes in exudative neutrophils can be mimicked in vitro by exposing peripheral blood neutrophils to chemotactic factors. Neutrophil exudation is also associated with shedding of L-selectin (60), and up regulation of certain plasma membrane components such as CR3, FcγRIII, and cytochrome b558. Exudative neutrophils also acquire the protein synthetic apparatus for IL-8 and are capable of producing, and storing IL-8 in a readily mobilizable, compartment distinct from primary or secondary granules in amounts comparable to mononuclear phagocytes (64)

1.3.7 *Mechanisms of killing*

These mechanisms fall into two categories: those that are dependent on the generation of oxygen metabolites, and those that are independent. In addition to mobilizing its own resources, neutrophils can produce a variety of cytokines such as IL-1β, IL-6, IL-8, and TNF-α, which may significantly affect processes such as modulation of the immune responses (65) by stimulating and attracting other neutrophils as well as monocytes and lymphocytes

1.3.7.1 *Oxygen-Independent Pathways*

Bacterial permeability increasing protein (BPI) is a highly potent antibacterial granule protein stored in the primary granules. BPI is a highly basic and is cytotoxic to gram-negative bacteria but much less effective against gram-positive organisms. The defensins are small (<4 kDa) cationic proteins found in the primary granules of neutrophils, which appear to be involved in the killing of ingested gram-positive and gram-negative bacteria (66). Defensins are capable of killing transformed mammalian cells, as well as prokaryotes and yeasts. Lactoferrin is an iron-binding protein present in specific granules of neutrophils but also in mucosal secretions. It is inhibitory to gram positive and gram-negative bacteria and some fungi. Although lactoferrin exerts much of its antimicrobial action through the depletion of iron from an organism's environment, it may possess independent antimicrobial activity as well (67).

1.3.7.2 *Oxygen-Dependent Pathways*

Neutrophils can produce toxic oxygen metabolites in response to activation by IL-8, TNF-α, C5a, or fMLP through the assembly of the reduced nicotinamide dehydrogenase phosphate (NADPH) oxidase complex. This enzyme complex catalyzes the addition of an electron to molecular oxygen, leading to the formation of superoxide anion in a reaction known as the oxidative respiratory burst. Superoxide anion is rapidly converted by the enzyme superoxide dismutase to H_20_2 and hydroxyl radicals. In the presence of MPO, H_20_2 and halide ions (usually chloride) form hypohalous acids that are intensely microbicidal (fig. 1.3). H_20_2 in the presence of iron salts leads to the production of hydroxyl radicals, which can remove single electrons from other compounds and turn them into powerful oxidants (68, 69).

1.3.8 *Neutrophils and tissue damage*

Neutrophils contain many inflammatory mediators capable of not only killing microbial pathogens, but also injuring host tissues and amplifying the inflammatory response. These inflammatory mediators have been implicated in the pathogenesis of many inflammatory diseases, including bronchial asthma.

1.3.8.1 *Neutrophil elastase*

Neutrophil elastase is a serine protease released by neutrophils on activation in response to inflammatory stimuli, and can potentially damage tissues in many ways. In vitro, neutrophil elastase induces proteolysis of elastin, fibronectin, type 3 collagen, and type 4 procollagen (70). Neutrophil elastase cleaves and inactivates α1-proteinase inhibitor, tissue inhibitor of metalloproteinases, complement fragments (C3), and CR1 (71). It can also cause airway epithelial cell damage (72), reduce ciliary beat frequency (73), and stimulate epithelial surface and airway gland cell secretion. Other actions of neutrophil elastase that can be harmful to host tissue include injuring pulmonary endothelial cells (in part through release of PAF) (74), increasing endotheliall cell permeability (75), causing degranulation of eosinophils resulting in release of inflammatory mediators such as eosinophilic cationic protein (ECP) (76), and modulation of neutrophil adhesion (77). In vivo experiments in animals have show that human neutrophil elastase induces airway constriction and hyperresponsiveness, increases lung permeability, causes secretory cell metaplasia and pulmonary emphysema (78).

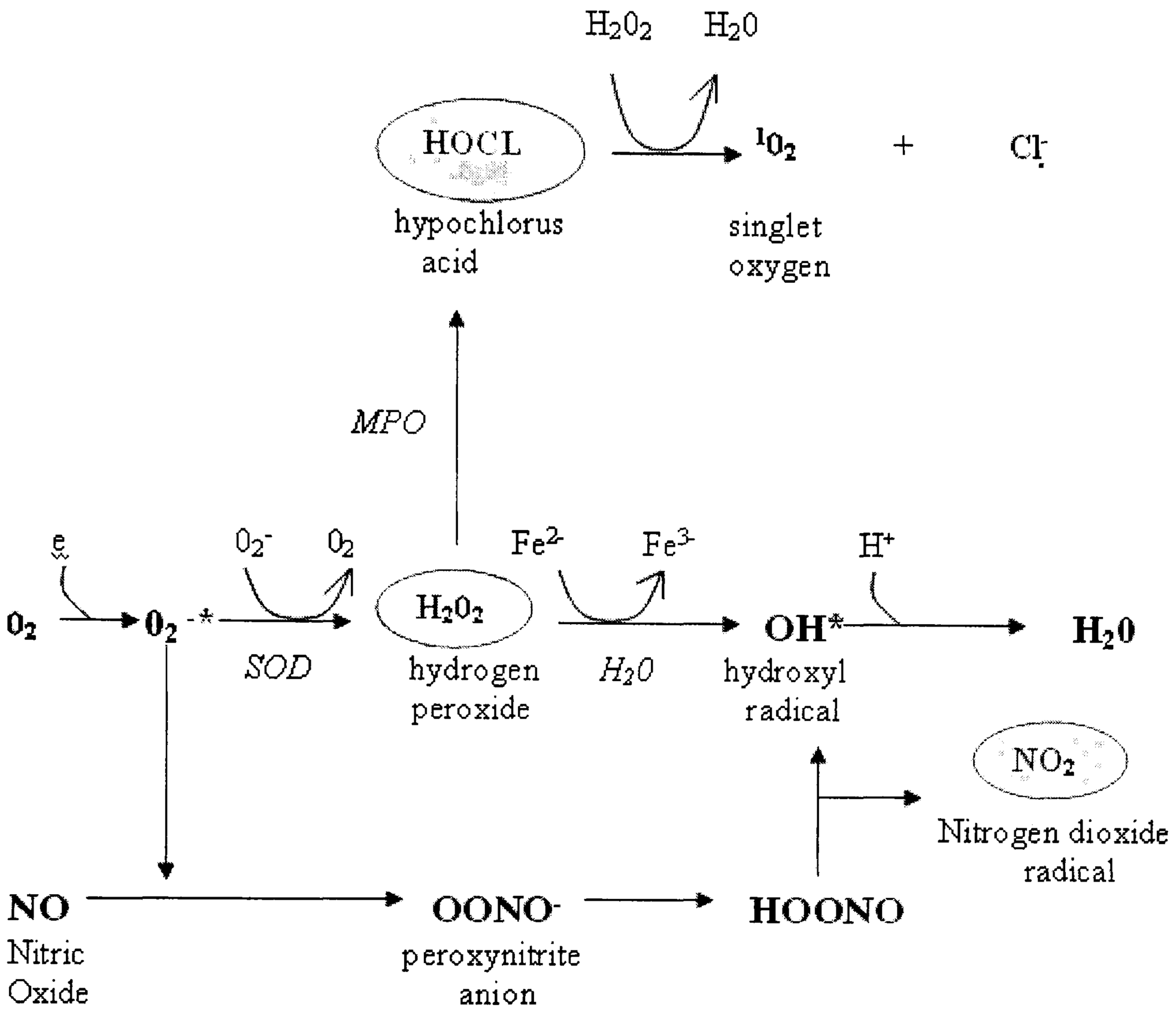


Fig. 1.3 Oxidative burst. NADPH oxidase catalyzes the addition of an electron (e) to oxygen (02), leading to the formation of superoxide anion (0_2^{-*}). This is then converted to H_20_2 and hydroxyl radicals by the enzyme superoxide dismutase (SOD). H_20_2 is then converted to hypochlorus acid, (which is microbiocidal), in the presence of MPO and chloride ions (Cl^-).

1.3.8.2 *Oxidants*

Oxidants produced as a result of the respiratory burst, can produce many of the airway lesions associated with asthma, including ciliary dysfunction and mucus hypersecretion (79), airway epithelial cell damage, bronchial smooth muscle contraction, and airway hyperresponsiveness (80). It has also been shown that oxidants may activate mast cells to release histamine (81).

1.4 Granulocyte priming in peripheral blood

Although the main site of action for neutrophils and eosinophils is probably the lung, several studies that show that these cells are already activated or primed in the circulation of asthmatic patients. For example, *Sedgwick et al* (82) showed that hypodense eosinophils isolated from the peripheral blood of asthmatic patients generated significantly more superoxide anion when activated with fMLP or opsonized zymosan (OZ),compared to eosinophils from normal controls. Furthermore, eosinophil populations and corresponding neutrophil isolates, from asthma patients generated significantly more superoxide anions (O_2-) than control granulocytes when activated by phorbol myristate acetate (PMA).

Kato et al (83) showed that the ability of neutrophils from asthmatic children to generate O_2- and other active oxygen species was significantly greater than that of neutrophils from control subjects when stimulated with OZ, PMA, or fMLP. In the same asthmatic children, the generation of O_2- and other active oxygen species was found to be significantly higher during asthma attacks. *Warringa et al* (84) showed that eosinophils from allergic asthmatics exhibited a markedly increased sensitivity in their chemotactic response toward PAF compared with eosinophils from normal donors. This in vivo-primed phenotype could be mimicked in vitro, by preincubating eosinophils from peripheral blood of healthy individuals with either GM-CSF, IL-3, or IL-5 suggesting that release of cytokines may be an important in-vivo priming mechanism in the circulation of allergic asthmatic patients. Similarly, *Hakansson et al* (85) showed that IL-3 primed the migratory function of both eosinophils and neutrophils, while IL-5 was a more potent primer of eosinophil than of neutrophil migration in response to

chemoattractants such as LTB4, PAF, fMLP, and C5a. *Leckie et al* (86) showed significant differences in the amount of light scatter by peripheral blood neutrophil populations in severe symptomatic asthmatics compared with the normal controls, using the Bayer haematology analyser. Further, when whole blood was activated in vitro with fMLP, similar changes were seen in neutrophil light scatter, suggesting that neutrophil activation is present in the peripheral blood of symptomatic asthmatics.

1.5 Expression of activation molecules on neutrophils and eosinophils in allergic asthma

Using flow cytometry techniques, *Berends et al* (87) studied the expression of CD35 and CD11b on neutrophils and eosinophils from asthmatic and non-asthmatic children using whole blood. CD35 mediates the binding and phagocytosis of C3b coated particles and immune complexes. CD11b not only mediates the phagocytosis of opsonized particles, but is also involved in cell adhesion. Results showed that on resting cells, only CD35 on neutrophils was significantly elevated in asthmatic children. After stimulation with fMLP, the CD11b expression on neutrophils and eosinophils was significantly higher in asthmatic children than in controls, suggesting that the inducible expression of CD11b on neutrophils and eosinophils in allergic asthmatic children is primed in vivo.

In contrast, *Int Veen et al* (88) showed that in stable disease, CD11b and CD62L expression on eosinophils or neutrophils from blood and sputum did not differ between normal, mild, or moderate-severe asthmatics that were on high dose inhaled corticosteroids (ICS). However, sputum eosinophils showed a higher expression of CD11b and lower expression of CD62L compared with peripheral blood eosinophils. The level of bronchial hyperreactivity as measured by PC_{20}, in the patients with moderate to severe asthma was shown to be related to CD11b expression on blood and sputum eosinophils. One explanation for these findings was that the modulation of CD11b and CD62L on airway granulocytes is not specific for asthmatic airway inflammation, but probably the result of tissue migration per se. Further, *Maestrelli et al* showed no difference between CD11a, CD11b, or CD11c in blood mononuclear cells between controls and asthmatics (89). However, in contrast to the findings of *Int Veen et al*, sputum CD11a and CD11b expression on mononuclear cells in asthmatics were expressed at a lower level compared with blood, but not in normal subjects. In both

control and asthmatics, sputum neutrophils tended to decrease their expression of integrin alpha chains compared to circulating neutrophils, suggesting that there is a downregulation of integrins in asthmatic airways after cell recruitment from the circulation.

1.6 Effect of corticosteroids on neutrophil function

1.6.1 Inhibitory effect of systemic corticosteroids on neutrophil function

Most studies of the effect of glucocorticoids on circulating neutrophils have been done in-vitro, using high doses of synthetic steroids such as dexamethasone. Most of these studies show that the effects of glucocorticoids on neutrophils are inhibitory and therapeutically this can be couterproductive as one of the most notable side effects of high dose steroids is decreased resistance to infection due to impaired neutrophil bactericidal activity. Corticosteroids have been shown to modulate neutrophil-endothelial cell interactions. *Yoshida et al* (90) showed that treatment of human umbilical vein endothelial cells (HUVEC) with methylprednisolone inhibited adherence and migration of neutrophils. Methylprednisolone also inhibited the IL-1β induced upregulation of E selectin and ICAM1 on HUVEC. Studies of neutrophil adhesion molecule expression in rheumatology patients (91) showed that methylprednisolone in doses of 1g decreased radiolabelled neutrophil ingress into inflamed joints. *Burton et al* (92) showed that 3 days treatment with dexamethasone in cows reduced CD62L and CD18 expression on bovine neutrophils. Glucocorticoids can also inhibit expression of the adhesion molecules ELAM-1 and ICAM-1 on endothelial cells (93).

The effect of dexamethasone on leucocyte extravasation in the post capillary venules of the hamster cheek pouch was studied by *Mancuso et al* (94) using intravital microscopy technique. Pretreatment with dexamethasone was shown to reduce neutrophil transmigration. Corticosteroids have been shown to have an inhibitory effect on cytokine production. *Pang et al* (95) looked at sputum and blood in patients with chronic bronchial sepsis and showed that in sputum, increased neutrophil numbers correlated with increased concentrations of IL-8, IL-1β, and TNF-α. Cytokine secretion was shown to be inhibited by dexamethasone. Dexamethasone also inhibited lipopolysaccharide (LPS) induced production of IL-6 (96) and IL-8 (97) by human

neutrophils. *Petroni et al* (98) showed that dexamethasone decreased interferon gamma (IFN-γ) stimulated phagocytosis by neutrophils, as well as reducing the IFN-γ induced increase in FcγRI receptor sites per cell by 39-73%.

Pretreatment with methylprednisolone inhibited the LPS induced superoxide production by pulmonary and circulating blood neutrophils in rats (99). The inhibitory effect of methylprednisolone on pulmonary neutrophils was greater than that on circulating blood neutrophils. Neutrophils pre-treated with hydrocortisone or dexamethasone showed a reduced percentage of Aspergillus fumigatus hyphal damage (100). Pre-treatment of neutrophils with GCSF and/or IFN-γ prevented the suppression of hyphal damage that occurred in the presence of hydrocortisone or dexamethasone. Similarly, *Abe et al* (101) showed that prednisolone inhibited neutrophil activity versus candida albicans.

1.6.2 Effect of glucocorticoids on adhesion molecules on resting neutrophils

The mechanisms by which glucocorticoids reduce leukocyte influx and inflammation are not fully understood. Some of the mechanisms of anti-inflammatory action of glucocorticoids that have been put forward include inhibition of phopholipases, inhibition of the transcription of various cytokines, and stabilization of lysosomal and other cell membranes. However, none of these hypotheses is sufficient to account for their effects on leucocyte traffic into inflamed tissues.

Several animal studies have looked at the effect of in-vivo glucocorticoids on surface molecule expression on neutrophils. *Burton et al* (102) treated dairy cows for 3 days with placebo (PBO), cortisol or dexamethasone using whole blood that was unstimulated or treated with PAF. Dexamethasone almost completely down-regulated CD62L and and reduced expression of CD18 by 50%. Dexamethasone also caused a reduction in the proportion of cells staining positive for CD62L, but did not alter the number of cells staining for CD18. Cortisol had a similar effect. This suggests that one mechanism of the anti-inflammatory action of glucocorticoids is to induce dramatic down-regulation of CD62L and CD18 adhesion molecules on blood neutrophils. *Burton et al* also showed that in-vivo glucocorticoid treatment induced significant downregulation of both CD62L and CD18 expression on resting bovine neutrophils. However these effects of glucocorticoids were detectable only 8 to 16 hours and 2 to 3

days after treatment respectively. As neutrophils reside in the peripheral blood for only a few hours, it would suggest that in this situation, glucocorticoids are affecting neutrophil precursors in the bone marrow rather than circulating neutrophils.

Studies in rabbits show that inflammatory stimuli cause the release of polymorphonuclear cells (PMNs) from the bone-marrow which show an increase in the expression of CD62L. This is in contrast to glucocorticoid induced granulocytosis which is associated with decreased CD62L expression on peripheral neutrophils (103). Intravenous (i.v.) dexamethasone given to rabbits showed that dexamethasone decreased CD62L expression on segmented neutrophils in the bone marrow but not on neutrophils already in the circulation.

In contrast to the studies showing downregulation of surface molecule expression with glucocorticoids, *Trowald et al* (104) investigated the in-vivo effect of prednisolone on canine neutrophil function in whole blood and found no change in the expression of CD11b/CD18 expression. However, their results showed that neutrophil adherence was reduced, whilst chemiluminescence and chemotaxis were increased. *O'Learry et al* showed that neutrophils in BAL fluid in rats showed increased CD11b and reduced CD62L compared to blood neutrophils (105). This upregulation of CD11b was insensitive to dexamethasone pretreatment, and may in part explain the lack of efficacy of steroids in the management of acute respiratory distress syndrome (ARDS). In human studies of in-vivo glucocorticoid treatment, *Jilma et al* (106) showed that repeated administration of i.v. dexamethasone over a 48 hour period downregulated basal CD62L expression on human neutrophils. *Crockard et al* (107) studied 15 patients with multiple sclerosis (MS) receiving i.v. methylprednisolone prior to and at 6 and 24 hrs following the initial 500mg dose. A follow up sample was obtained 48 hrs after a 5 day course. Neutrophil counts were elevated at 6 hrs (3 fold) and 24 hrs (2 fold) and this was associated with a 40% reduction in CD62L expression at 6 and 24 hrs and a 35% reduction in CD11b expression at 6 hrs. Short term in-vitro cultures at 6 and 24 hrs of blood samples from untreated MS patients and controls showed minimal reductions in neutrophil CD62L and CD11b expression.

Cardiopulmonary bypass (CPB) is known to upregulate CD11b in humans. In a randomized trial, patients were given 1g i.v. methylprednisolone 5 minutes prior to CPB compared to another group which received no steroid. (108). In the non-treatment group, CD11b was significantly upregulated, whilst in the treatment group, no significant changes were seen. During CPB, IL-1β, TNF-α, and endotoxin are released in humans and it is known that glucocorticoids suppress the release of these cytokines.

Therefore it has been hypothesised that the blunting effect by glucocorticoids on CD11b expression on neutrophils during and after CPB in humans is due to suppression of cytokine release by steroids.

Youssef et al (109) looked at the effect of 1g i.v. methylprednisolone on the expression of cell adhesion molecules on peripheral blood and synovial fluid neutrophils in rheumatoid arthritis. A significant decrease was seen in CD11b and CD18 expression on synovial fluid neutrophils at both 4 and 24 hours post methylprednisolone and an increase in CD62L expression. There was less effect on peripheral blood neutrophils although results approached statistical significance. In a study of oral prednisolone in humans, *Laroche et al* (110) looked at the effect of ketoprofen and prednisolone on CR1 and CR3 and FcγRIII receptor expression on PMN in an ex-vivo study using a randomised, single blind PBO controlled parallel design. Twenty four healthy males were given prednisolone 5mg bd or PBO for 7.5 days. No significant difference in CR1, CR3 and FcγRIII expression on unstimulated and fMLP stimulated neutrophils using monoclonal antibodies and flow cytometry.

1.6.3 Effect of glucocorticoids on adhesion molecules on stimulated neutrophils

Filep et al (111) showed that dexamethasone at clinically relevant concentrations attenuated the PAF induced downregulation of CD62L and upregulation of CD11b/18 adhesion molecules on human neutrophils. There was no detectable effect on the expression of adhesion molecules on resting neutrophils .The effects of dexamethasone were aborted by RU486 which blocks transcriptional activation of the glucocorticoid receptor and by the protein synthesis inhibitor cycloheximide. In addition to the effects of glucocorticoids, *Spoelstra et al* showed that theophylline at a pharmacological dose also inhibited the CD11b upregulation and L-selectin shedding induced by PAF on both eosinophils and neutrophils (112). In animal experiments, *Fraile et al* (113) showed that dexamethasone caused a dose-dependant inhibition of LPS induced CD11b expression, while *Za zhi et al* showed that dexamethasone inhibited the increase in CD18 on rabbit neutrophils caused by TNF-α and this inhibitory effect was reversed by RU486 suggesting that it was mediated through the glucocorticoid receptor (114).

1.6.4 Effect of inhaled corticosteroids in asthma

Inhaled corticosteroids are the most effective treatment available for the treatment of chronic asthma. Regular treatment with ICS reduces airway inflammation and can improve lung function in asthmatics. In addition, there is improvement in airway hyperresponsiveness and improved asthma control. *Jatakanon et al* (115) showed significant improvement in the forced expiratory volume in 1 second (FEV1) following treatment with inhaled fluticasone propionate (FP) 400μg/day or 1600μg/day budesonide for 4 weeks compared to PBO, and this was accompanied by significant reductions in eosinophil numbers in induced sputum. Levels of exhaled nitric oxide (NO) , which is an indirect marker of airway inflammation, were reduced following doses of budesonide as low as 100μg/day, whilst PC_{20} was improved only after the high dose was used. Similarly, *van Rensen et al* (116) showed significant increase in PC_{20} with a decrease in sputum eosinophil and decreased exhaled NO compared to baseline at 2 and 4 weeks of treatment with inhaled FP 500μg/day for 4 weeks compared to PBO. Treatment with inhaled beclomethasone dipropionate (BDP) for 3 weeks at a dose of 1000μg/day produced no significant change in FEV1 or forced vital capacity (FVC), but PC_{20} methacholine rose significantly. Exhaled NO fell after 1 week of ICS therapy, and then increased at 1 week after treatment was stopped (117).

The relatively short lived effects of ICS was confirmed by *Gershman et al* (118) who showed significant increase in FEV1 with high (1000μg/day) and low (100μg/day) FP therapy for 6 weeks, and reduction of sputum eosinophil counts. In addition there were improved symptoms and morning peak expiratory flow rates (PEFR), PC_{20}, and sputum ECP and tryptase measurements. After stopping therapy, only improvements in PEFR and tryptase activity persisted in both groups, with improvement in sputum eosinophilia persisting only in the high dose group. This study suggests that low dose inhaled FP is effective but the effects seen are short lived after treatment is stopped. Similarly, in a dose response study of inhaled FP, *O'Sullivan et al* (119) showed that optimal improvement in lung function and markers of airway inflammation occurred at a dose of 500μg/day with no evidence of a dose response relationship at the higher dose of 2000μg/day. This provides evidence for the importance of add on therapy over increasing doses of ICS.

That exhaled NO may be useful in monitoring asthma control with ICS therapy, was suggested by the study by *Kharitonov et al* (120) who showed that exhaled NO was

increased significantly compared to baseline after the dose of budesonide in asthmatics was reduced by 200μg/day but this was not accompanied by a significant decrease in spirometry or PEF variability. An increase in nocturnal symptoms however was reported. The levels of exhaled NO were decreased when the dose of inhaled budesonide was subsequently increased by 200μg/day, and this was accompanied by a decrease in diurnal variability and nocturnal symptoms. *Khartinov et al* also showed that a significant and progressive fall in exhaled NO occurred from week 1 to week 3 following treatment with budesonide 800μg/day for 3 weeks in mild steroid naïve asthmatics with no significant change in FEV1 but an improvement in methacholine PC_{20} (121). *Carra et al* also showed a reduction of exhaled NO following 6 weeks treatment with budesonide in stable asthmatic children in the absence of changes in lung function (122). The reduction in NO is thought to be due to an inhibitory effect of ICS on the induction of the enzyme NO synthase in the respiratory tract (121).

Inoue et al (123) showed that 4 weeks treatment with budesonide 800μg/day resulted in improved lung function along with a mean reduction in the percentage of eosinophils in induced sputum but not in the percentage of neutrophils. In addition, mean levels of sputum supernatant IL-8 and GM-CSF were also decreased significantly after treatment. The improvement in lung function and airway inflammation may thus be, in part, due to the inhibition of the synthesis of inflammatory cytokines such as IL-8 and GM-CSF.

Systemic corticosteroids are used in the management of acute severe asthma as opposed to ICS, as aerosol penetration is poor in the presence of bronchoconstriction. Time course studies have shown that i.v. administered hydrocortisone can cause bronchodilation within 3-4 hours of administration, and also improve the bronchodilator response of an inhaled beta-adrenergic agonist (124). More recently, studies have shown that inhalation of large doses of corticosteroids can also be beneficial in patients presenting with acute severe asthma. In one double blind study (125), 94 patients with an acute exacerbation of asthma admitted to the emergency room were randomised to receive inhaled salbutamol and PBO (n=47) or salbutamol and inhaled flunisolide (n=47) through metered dose inhaler (MDI) and volumatic. Combined therapy produced significantly better results at all time points up to 180 minutes, suggesting that ICS added to salbutamol are an effective therapy for patients with acute asthma. *Levy et al* (126) showed that high dose FP (2mg) was as effective as a short course of prednisolone (40mg per day, decreasing by 5mg every other day) in treating an exacerbation of asthma not severe enough to need hospital treatment. In total, 413 patients were

recruited from 47 GP practices in the UK. No difference was seen in the efficacy between reducing dose of oral prednisolone and high dose inhaled FP in mild exacerbations of asthma that did not need hospital admission.

Studies with inhaled budesonide (800μg bd) indicate that improvements in FEV1 and PC_{20} occurred after the first dose, and this was maximal at 6 hours. Further, these parameters returned to baseline within 36 hours of stopping the last dose following 6 week regular treatment (127). Large dose of budesonide (dose up to 1600μg) have been shown to cause maximal bronchodilation at 5-6 hours following administration in patients with asthma, with significant effects already observed by 2 hours of administration. There were also falls in blood eosinophils and serum ECP occuring 3-5 hours after budesonide inhalation (128). These studies indicate that there is a rapid component to the effect of ICS, starting as early as 2 hours. In a recent study, *Gibson et al* showed that high dose inhaled budesonide (2400μg) via turbohaler was associated with a 2.2 fold improvement in airway responsiveness compared to PBO, 6 hours after inhalation of inhaled budesonide. No significant effect was seen on mast cells, apoptotic eosinophils, symptoms or lung function (129).

The mechanisms of these early rapid changes are not entirely clear, since it is thought that many of the mechanisms of corticosteroids may involve interference with transcriptional mechanisms. However, it is possible that acute non-transciptional mechanisms may be important. Corticosteroids may, for instance, interfere with mechanisms of oedema formation or of cytokine release. In addition, it is possible that transcriptional mechanisms may occur more rapidly in-vivo than in-vitro.

Interestingly, *Green et al* (130) demonstrated a distinct subgroup of patients with mild to moderate asthma who showed a predominantly neutrophilic airway inflammation in their sputum and responded less well to ICS in terms of improvement in symptoms, FEV1, and PC_{20}. The presence of increased neutrophils in asthmatic airways may thus account for less improvement following ICS therapy in such patients. *Gauvveau et al* (131) showed that ICS attenuated allergen induced airways inflammation but were less effective at inhibiting allergen induced sputum eosinophils after challenge in asthmatics that had increased levels of sputum neutrophils at baseline. This suggests that ICS are less effective in attenuating allergen induced airway inflammation in subjects with increased levels of neutrophils and that the heterogeneity of airway inflammation in some asthmatics may influence response to steroid treatment. This lends support to the fact that alternative therapy should be considered in addition to steroids for treating patients with increased neutrophilic inflammation in their airways.

1.6.5 Histone deactylation and effect of corticosteroids in asthma

Glucocorticoids are effective at repressing inflammation in asthma. One possible mechanism of action involves the binding of activated glucocorticoid receptor (GR) to several transcription co-repressor molecules that associate with proteins with histone deacetylase activity (HDAC), resulting in increased coiling of deoxyribonucleic acid (DNA) around core histone proteins, and thus preventing binding of transcription factors, leading to gene repression (132).

In resting cells, DNA is tightly packaged into chromatin which is a highly organized protein-DNA complex. Chromatin is made up of subunits called nucleosomes which are composed of core histone proteins, surrounded by 146 base pair DNA (132). In this state, DNA is tightly compacted to prevent accessibility of transcription factors which are capable of switching on inflammatory genes. When the cell is activated, local unwinding of nucleosomal DNA occurs so that the DNA is accessible to transcription factors and to RNA polymerase 2, thus allowing gene transcription to occur. This unwinding is regulated by acetylation of specific lysine residues in the N-terminal tails of the core histone proteins. This histone acetylation is carried out by a group of enzymes with histone acetlytransferase (HAT) and HDAC activity that regulate the chromatin structure, and through this effect, the inflammatory gene expression. (Fig. 1.4)

Transcription factors such as STATs, AP-1 and NF-κB, bind to co-activator molecules such as CREB binding protein (CBP) or p300, which have intrinsic HAT activity, resulting in acetylation of histone residues. This leads to unwinding of DNA and allows increased binding of transcription factors resulting in increased gene transcription. (133). The process of deacetylation involves the binding of hormone or vitamin receptors to co-repressor molecules such as nuclear receptor co-repressor (N-CoR), which forms a complex with another repressor molecule, Sin3, and a histone deacetylase (134,135). Deacetylation of histone increases the winding of DNA round histone residues, resulting in dense chromatin structure and reduced access of transcription factors to their binding sites, thereby leading to repressed transcription of inflammatory genes (136).

Ito et al (137) have shown an increased HAT activity in bronchial biopsies from asthmatics, which returned to normal following treatment with ICS. This suggests that

the use of steroids can lead to histone deacetylation, which represents one mechanism of their anti-inflammatory action.

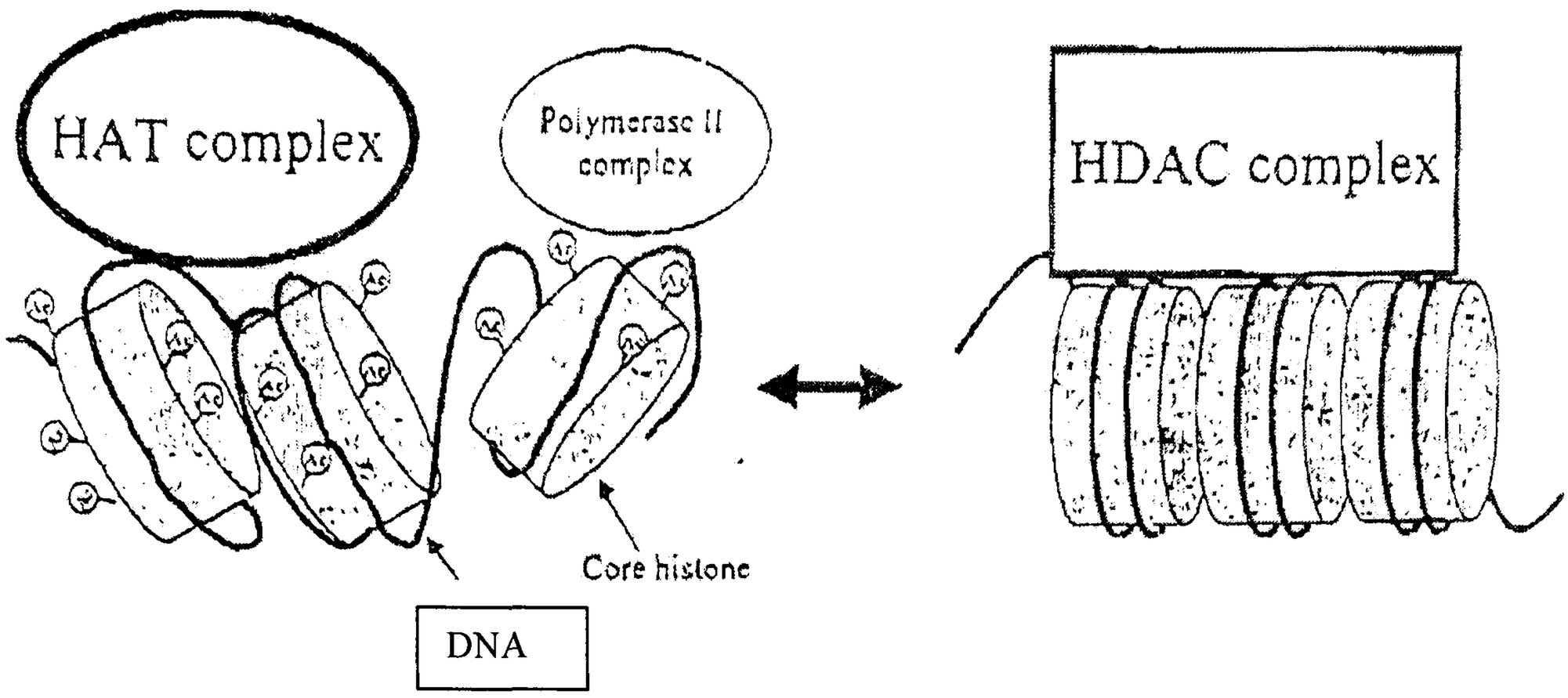


Fig. 1.4 Model of histone acetylation and histone deacetylation. HAT complex induces acetylation of lysines in the core histone. This reduces affinity of histone N-terminal domains for DNA, resulting in unwrapping of DNA from core histone. This allows large molecules, such as RNA polymerase II, to gain access to DNA, allowing gene transcription. In contrast, HDAC causes deacetylation of histones, resulting in tightening of the DNA coiling to core histone proteins. (From reference 137).

1.7 Allergic basis of asthma

1.7.1 Asthma and atopy

The onset of asthma commonly begins in early life, and childhood asthma is frequently associated with atopy to common environmental allergens, such as dust mites, tree and grass pollens, and animal dander (138). Atopy is the genetic predisposition for the development of an IgE-mediated response to common aeroallergens. Allergens play an important role in the development of adult-onset asthma, but not all adults who develop asthma have IgE antibodies to allergens or a family history of asthma (139). Asthma has been shown to be associated with increased levels of total IgE, even in subjects negative for specific IgE to common allergens (140). It is known that allergen exposure can result in increased airway hyperresponsiveness in sensitized subjects. In one survey of 875 young adults aged between 20-41 years, elevated serum IgE levels were associated with a 3-fold increase in bronchial hyperresponsiveness, independently of the presence of asthma symptoms or specific IgE (141). These results suggest that asthma is frequently associated with some type of IgE-related reaction and therefore has an allergic basis (140).

A minority of asthmatic patients have late onset of asthma associated with negative skin tests to common allergens and a normal circulating IgE. These patients who have non-atopic, or 'intrinsic', asthma tend to have more severe disease. Recently, patients with 'intrinsic' asthma have been shown to produce IgE widely in the airways (142) suggesting that IgE also plays a role in these patients.

1.7.2 Mechanism of allergic response

During an allergic immune response, allergen-specific B cells are stimulated to produce IgE antibodies following stimulation by IL-4 and IL-13 cytokines provided by T-helper type 2 (Th2) cells. IgE antibodies bind to the surface of effector cells (mast cells and basophils) bearing high-affinity (FcεRI) or low-affinity (FcεRII or CD23) receptors which subsequently become sensitized. The subsequent interaction of allergen with IgE-FcεRI complexes on mast cells and basophils causes crosslinking and aggregation of IgE receptors leading to effector cell degranulation and release of inflammatory mediators such as histamine (143). These mediators are thought to produce the airway smooth muscle contraction, mucosal hypersecretion and mucosal

edema representative of the immediate bronchoconstriction of the early asthmatic response (fig.1.5).

The bronchoconstriction associated with the late phase asthmatic response occurs two or more hours after allergen inhalation and is associated with increased airway reactivity to non-specific stimuli. The role of IgE and its high and low-affinity receptors in the release of inflammatory mediators in the late asthmatic response to inhaled allergen is not well established. However, human lung mast cells can generate and release, via IgE-dependent signalling, a wide variety of cytokines (144,145) suggesting that IgE has an important role in the ongoing airway inflammation associated with the late asthmatic response.

1.7.3 Difficult/severe asthma- possible alternative therapy in severe asthma

The majority of asthmatic patients can be managed with short acting β2-agonists, along with maintenance low dose ICS, or combination therapy with a long acting bronchodilator. A minority of asthmatics however, show persistent symptoms and experience frequent exacerbations despite high dose ICS and sometimes oral corticosteroid therapy (146). Although this group constitutes only 1% of asthmatics (147), they account for a larger share of total asthma costs. Difficult asthma represents a real challenge for physicians, patients, and the community. Apart from the economic impact of this condition, other implications include an increase in mortality and psychological problems, with an increased prevalence of depression in severe asthmatics. The pathogenesis and mechanism(s) involved in the development of this severe asthmatic state have not been fully elucidated. Patients may present with different clinical patterns including intermittent severe exacerbations, persistent chronic symptoms or with rapid catastrophic attacks. Many of these patients have corticosteroid-dependent asthma (146).

Treatment of severe asthma is currently limited since corticosteroids are not entirely effective and long-term treatment with corticosteroids is associated with a variety of side-effects. Current evidence suggests that methotrexate, cyclosporin, and gold salts may be considered for their 'steroid-sparing' effects in severe asthmatics, but the possibility of short and long-term side effects should be borne in mind. On the other hand, the anti-leukotriene agents may provide safe and effective alternatives in the treatment of certain groups of patients, such as exercise-induced, aspirin-sensitive and possibly corticosteroid-dependent asthmatics. The efficacy and place of these agents the

treatment of asthma is still being determined. Anti-IgE therapy represents a new therapeutic approach for the treatment of allergic disease and has been shown to be effective in treating patients with mild and severe asthma, with very few side-effects.

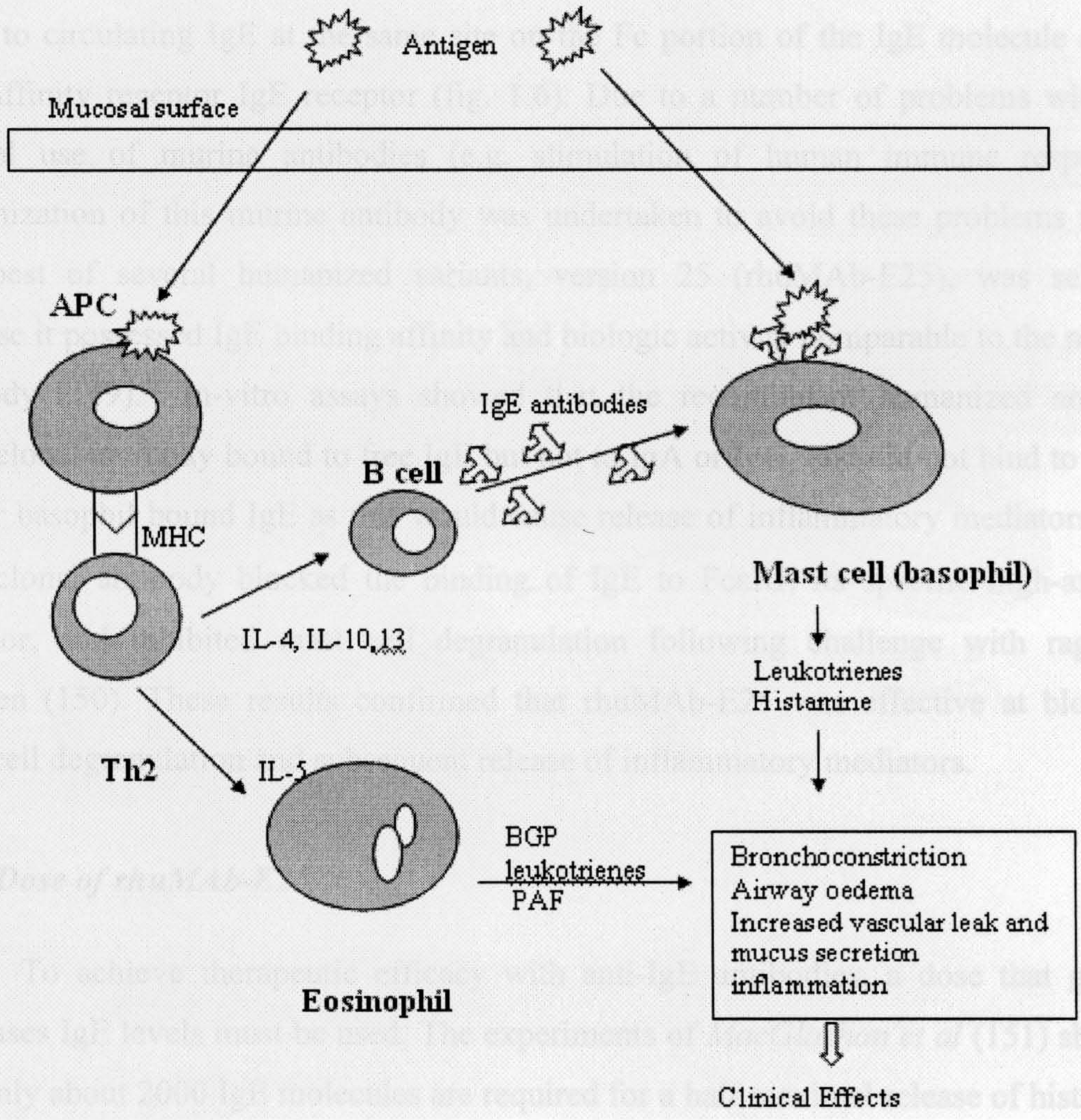


Fig. 1.5 Overview of the allergic immune response. Specific antigens are processed and presented to Th2 cells by antigen presenting cells (APC). Allergen-specific B cells are stimulated to produce IgE antibodies following stimulation by IL-4 and IL-13 cytokines provided by Th2 cells. IgE antibodies then bind to the surface of effector cells (mast cells) bearing high-affinity or low-affinity receptors which subsequently become sensitized. The subsequent interaction of allergen with IgE-FcεRI complexes on effector cells causes crosslinking and aggregation of IgE receptors leading to effector cell degranulation and release of inflammatory mediators such as histamine, which mediate various clinical effects. (BGP-basic granule proteins. PAF-platelet activating factor.)

1.7.4 Overview of anti-IgE therapy

Genentech and Novartis have developed a murine monoclonal antibody which binds to circulating IgE at the same site on the Fc portion of the IgE molecule as the high-affinity receptor IgE receptor (fig. 1.6). Due to a number of problems with the clinical use of murine antibodies (e.g. stimulation of human immune response), humanization of this murine antibody was undertaken to avoid these problems (148). The best of several humanized variants, version 25 (rhuMAb-E25), was selected because it possessed IgE binding affinity and biologic activity comparable to the murine antibody (149). In-vitro assays showed that the recombinant humanized anti-lgE monoclonal antibody bound to free IgE but not to IgA or IgG, and did not bind to mast-cell or basophil bound IgE as this would cause release of inflammatory mediators. The monoclonal antibody blocked the binding of IgE to FcεRI, its specific high-affinity receptor, and inhibited mast cell degranulation following challenge with ragweed allergen (150). These results confirmed that rhuMAb-E25 was effective at blocking mast cell degranulation and subsequent release of inflammatory mediators.

1.7.5 Dose of rhuMAb-E25

To achieve therapeutic efficacy with anti-IgE antibodies, a dose that greatly decreases IgE levels must be used. The experiments of *MacGlashan et al* (151) showed that only about 2000 IgE molecules are required for a half maximal release of histamine from basophils exposed to specific allergen. Their study also showed that FcεR1 receptor density on IgE effector cells is regulated by circulating levels of IgE. They found that treatment with rhuMAb-E25 decreased free IgE levels to 1% of pretreatment levels and resulted in marked down-regulation of FcεR1 on basophils. Median receptor densities were approximately 220,000 receptors per basophil at baseline and decreased to approximately 8,300 receptors per basophil after 3 months of treatment. Ideally, serum IgE levels should be suppressed to below 25ng/ml. This explains why anything less than near-complete suppression of IgE levels allows sufficient IgE binding to FcεR1 receptors to occur resulting in full basophil activation. From a therapeutic standpoint, this means that anti-IgE dosing needs to be individualized and depends on the patient's total IgE level, and body weight. RhuMAb-E25 can be given by i.v. or subcutaneous routes, but nebulized rhuMAb-E25 has not been shown to cause reduction in circulating IgE levels.

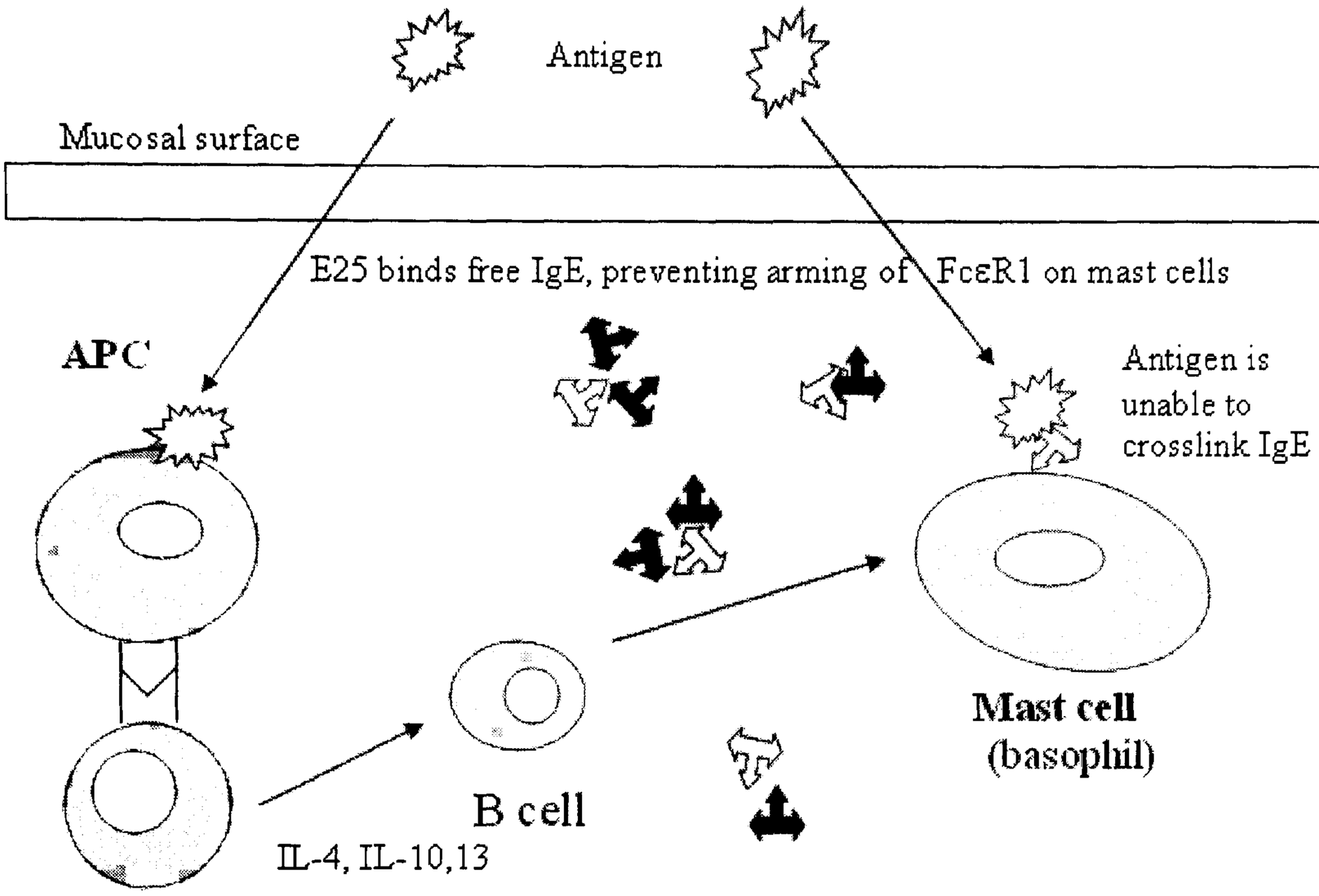


Fig. 1.6 RhuMab-E25 binds to circulating IgE at the same site on the Fc portion of the IgE molecule as the high-affinity receptor IgE receptor. This blocks the binding of IgE to the high affinity IgE receptor on effector cells and thus inhibits mast cell degranulation and subsequent release of inflammatory mediators.

1.7.6 Safety of anti-IgE therapy

The 3 main safety concerns surrounding the anti-IgE treatment strategy were, firstly, that it may cause anaphylaxis; secondly it could predispose the patient to parasitic infections; and finally it may provoke immune responses, including complement fixation or antibody formation. The first concern was eliminated by the development of non-anaphylactogenic antibodies, as described above.

The second concern arises from data suggesting an important role for IgE in eliminating parasites (152). However, studies of the role of IgE in host defence against parasitic infections, such as Schistosoma mansoni, have produced conflicting reports. *Amiri et al* (153) treated mice with a rabbit polyclonal antimouse anti-IgE or with an isotype control antibody and then infected them with S. mansoni. Results showed that reduction of the IgE response by anti-IgE antibody decreased the worm burden, the number of eggs per worm, and the number of hepatic granulomas. *Amiri et al* concluded that IgE plays a detrimental, rather than beneficial, role for the host in schistosomiasis. In contrast, *King et al* (154) infected normal mice and IgE-deficient mice (animals with a null mutation of the relevant gene and thus incapable of making IgE) with S. mansoni and found that the IgE-deficient mice were significantly more susceptible to primary infection, developing worm burdens twofold greater than those of wild-type mice, suggesting that IgE participates in parasite elimination in primary infection with S.mansoni. Overall, however, there is evidence for a potentially important role for IgE in host defense against parasites and clinical trials of anti-IgE treatment will need to monitor the incidence of parasitic infections closely.

The third concern was that anti-IgE may provoke an immune response. This is generic to all protein therapeutics but is considerably reduced by the "humanization step" in the engineering of anti-IgE molecules (155). Thus the development of antibodies against rhuMAb-E25 has not been detected in studies of more than 1000 patients in which the drug has been administered parenterally (156-158). Interestingly, the aerosol route of administration may be more immunogenic than the i.v. or subcutaneous route. One study of aerosolized rhuMAb-E25 treatment in 20 patients showed the presence of an antibody to rhuMAb-E25 in 1 patient (159).

Although rhuMAb-E25 has an IgG1-kappa structural framework, it does not fix complement. In addition, rhuMAb-E25: IgE complexes are of limited size (1 million MW or smaller) (160,161) and no precipitating immune complexes were detected.

These complexes cannot bind IgE receptors and thus lack the biological activity of IgE. Studies in cynomolgus monkeys (which have naturally high levels of IgE) indicate that these relatively small complexes do not accumulate in any body organs, including the kidney (161). In human studies, treatment with rhuMAb-E25 has been well tolerated in all studies to date, with no specific adverse effects apart from an urticarial rash after the first dose in a small number of subjects (3%) in a trial of 300 patients (162).

1.7.7 Clinical Studies

The efficacy of rhuMAb-E25 in mild atopic asthma was shown in two studies which looked at the effect of rhuMAb-E25 on bronchial challenge with allergen and on allergen-induced skin tests (158,164). The effect of rhuMAb-E25 on the provocation concentration of allergen and methacholine causing a 15 and 20% fall (PC_{15} and PC_{20}, respectively) in FEV1 during the allergen-induced early asthmatic response was assessed in 20 atopic patients with mild asthma (158). RhuMAb-E25 produced significant inhibitory effects on allergen-induced bronchospasm relative to PBO. The methacholine PC_{20} improved slightly but significantly after rhuMAb-E25 treatment while no change was observed with PBO. RhuMAb-E25 treatment reduced mean serum free IgE levels by 89% while no changes were observed with PBO. Free IgE levels fell to below the threshold for detection in 7 of 10 patients. Overall, these results showed that rhuMAb-E25, administered at regular intervals over 10 weeks, effectively suppressed allergen-induced early asthmatic response in patients with asthma.

A further randomized double-blind study showed that rhuMAb-E25 (0.5 mg/kg or placebo weekly for 9 weeks) suppressed both the early- and late-phase responses to inhaled allergen in 19 atopic patients with asthma (163). RhuMAb-E25 significantly reduced maximal bronchoconstriction to airway challenge during the early and late phases by 40% and 63% as evidenced by the reduction in magnitude of fall in FEV1. The PC_{20} improved slightly but not significantly, in the rhuMAb-E25 group relative to the PBO group. At 24 hours after allergen challenge, eosinophilia in induced sputum was reduced 11 fold, while free serum IgE levels fell by about 90% in the rhuMAb-E25 group and were unchanged in the PBO group. The reduction in the number of eosinophils in the sputum and improved airway hyperresponsiveness (158,164), suggest that rhuMAb-E25 has a long-term anti-inflammatory effect.

The clinical efficacy and safety of rhuMAb-E25 has recently been assessed in a large multicentre, randomized, double-blind, PBO-controlled study in 317 patients with

moderate to severe allergic asthma requiring ICS and/or oral corticosterolds (164). Following a 4-week run-in period, patients received either PBO, or low dose or high dose rhuMAb-E25 i.v. biweekly for 20 weeks. During the last 8 weeks of treatment, the dosages of corticosteroids were tapered in an attempt to discontinue this therapy. Both high and low dose rhuMAb-E25 produced a greater than 95% mean decrease in serum IgE levels (164). The proportion of patients in the high-dose and low dose rhuMab-E25 groups experiencing a ≥ 50 % reduction in asthma symptom scores (the primary outcome measure) was significantly higher than in the PBO group. A significantly higher proportion of patients in the high-dose group than in the low dose and PBO groups were able to achieve a ≥ 50% reduction in oral corticosteroids. For high dose, low dose and PBO groups, respectively, oral corticosteroids were discontinued in 33, 43, and 17% of patients and ICS were discontinued in 18% (p=0.27), 23% (p=0.048) and 12% of patients.

Compared with PBO, rhuMAb-E25 significantly reduced the symptoms of asthma and improved the subjects' quality of life, with a moderate increase in airway function and a reduced need for the use of bronchodilators as rescue medication. The proportions of patients experiencing asthma exacerbation during the 20-week treatment period were 30% (p=0.03) in the high dose, 28% (p=0.01) in the low dose as compared with 45% in the PBO group. There were no significant differences in adverse events between the active-treatment and PBO groups, and no serious drug-related adverse events were reported. This suggests that rhuMAb-E25 may be a useful steroid-sparing therapy in patients with severe asthma (165). Subcutaneous rhuMab-E25 has also been shown to produce improvements in daily nasal symptoms (sneezing, itchy nose, runny nose and stuffy nose), concomitant antihistamine rescue medication use, and rhinitis-specific quality of life in patients with seasonal allergic rhinitis (166,167). The improvement in seasonal allergic rhinitis and reduction in rescue medication use was directly related to reduction in free IgE levels (166).

Interestingly, *Yamaoka et al* showed that neutrophils can synthesize CD23 molecules following stimulation with GM-CSF, and this CD23 expression might be of pathophysiological relevance in IgE dependent activation during allergic processes (168). In addition, *Gounni et al* showed that peripheral blood and BAL neutrophils from asthmatics expressed FcεR1, which is the main receptor involved in binding IgE molecules to the surface of mediator cells. Further, activation of these FcεRI receptors induced the release of IL-8 by neutrophils (169). These studies suggest that neutrophils may play a role in allergic inflammation through an IgE-dependent activation

mechanism. In the context of severe allergic asthma, this is important as increased numbers of neutrophils have been demonstrated in sputum and bronchial biopsy samples in patients with severe corticosteroid-dependent asthma.

1.8 Aim of study

Neutrophils have been shown to have an important role in the pathogenesis of asthma. Recent studies have shown that patients with severe asthma have increased numbers of activated neutrophils in their airways, both in BAL samples and in sputum. Activated neutrophils can lead to increased airway inflammation and damage and consequently airway wall remodelling through the release of toxic enzymes and chemicals. In vitro, neutrophils can also activate eosinophils through the release of stored enzymes and cytokines. Further, it has been shown that corticosteroids may lead to increased neutrophil survival and hence activation by inhibiting their apoptosis. The hypothesis of this thesis is that concomitant activation of both eosinophils and neutrophils may be important in patients with severe asthma through the enhancement of mediator release and subsequent remodelling of the airways. Further, as corticosteroids may lead to continued activation of neutrophils, this form of treatment may allow the inflammatory process to persist in severe asthmatics.

Little is known about the level of activation of neutrophils in the peripheral blood of asthmatic patients. The aim of this research was to investigate neutrophil activation and function in severe corticosteroid-dependent asthmatics, and to compare this with mild, and moderate asthmatics and normal control subjects. The relevance of this is important as neutrophils may lead to airway remodelling and fixed airway disease in more severe asthmatics, requiring further treatment with corticosteroids and the development of potential side-effects of treatment. Using a fluorescent activated cell sorter (FACS) scan and peripheral whole blood, several aspects of neutrophil function were analysed including surface marker expression, measurement of neutrophil elastase and oxidative burst, and measurement of IL-8 levels in cultured whole blood. Further, the effect of a standard therapeutic dose of prednisolone (30mg for 1 week) on these functional markers on neutrophils was studied.

The level of expression of HDAC in isolated blood neutrophils from asthmatics was studied and compared to levels in isolated peripheral blood mononuclear cells and BAL alveolar macrophages, and the effect of corticosteroids on HDAC activity in these cell populations investigated. The immediate effect of ICS on various parameters

including neutrophil numbers, cytokine production and clinical parameters were studied. Finally, the efficacy and tolerability of subcutaneous administration of anti-IgE therapy over a one year period was studied in patients with severe atopic asthma.

Chapter 2

2.1 Clinical methods

2.1.1 *Ethics*

Written informed consent to participate in the studies was obtained and all studies were approved by the Ethics Committee of The Royal Brompton NHS Trust. Clinical studies were done in accordance with the guidelines on 'Good Clinical Practice'. Each subject was allocated a hospital number and a set of medical notes that were kept in the asthma laboratory at the Royal Brompton Hospital.

2.1.2 *Subjects*

Asthmatic patients were recruited from the outpatients department at The Royal Brompton Hospital as well as from advertisements. Asthma was clinically diagnosed by a history of recurrent wheezing and chest tightness and by a previous physician diagnosis. The diagnosis was confirmed either by measuring methacholine airway hyperresponsiveness (PC_{20}<4mg/ml) when the FEV1 was >70%, or by demonstrating >15% improvement in baseline FEV1 or >10% predicted FEV1 after inhaled salbutamol 200μg, when the FEV1 was < 70%.

Normal subjects had no history of chest symptoms and an FEV1 >90% predicted. All subjects were either lifelong non-smokers, or had not smoked for at least one year, and had a total smoking history of less than 10 pack years. Asthmatics were stable with no changes in asthma symptoms and medication for at least one month, except for the use of short acting inhaled beta-2 agonists. No subjects had a history of an upper respiratory tract infection within the previous 6 weeks. Asthma severity was documented by [1] asthma questionnaire and previous medical records involving asthma history and asthma medication required to maintain asthma control, [2] symptom diary card, and [3] spirometry.

Asthma severity was classified according to guidelines produced by the National Heart and Lung Institiute (170) into 3 groups depending on level of symptoms, spirometry, and medication required in order to maintain control:

Group 1: mild intermittent, if symptoms less than or equal to 2 times a week, with normal lung function. These patients required only a short acting inhaled beta-2 agonist as required.

Group 2: moderate persistent asthmatics had daily asthma symptoms requiring daily use of inhaled short acting beta-2 agonists and medium dose ICS (BDP 400 to 1000μg daily or equivalent) via an MDI.

Group 3: severe persistent asthmatics had continuous symptoms, frequent exacerbations, limited physical activity, frequent night-time symptoms with FEV1 or PEF<60 % predicted. These patients were all treated with high dose ICS (BDP 2000μg/day or equivalent), and were taking maintenance oral prednisolone tablets, along with any other asthma medication in order to control symptoms.

2.1.3 *Skin prick testing*

These were performed on the anterior aspect of one forearm. The test site was marked with a ball point pen. A lancet was used to breach the surface epidermis through a drop of allergen extract on the skin, and inserted at a 90 degree angle. It is important that blood is not drawn. Any surplus fluid was blotted with a tissue. After 10 minutes, the skin was examined visually and the reaction to individual allergens measured by placing a transparent ruler over the wheal matching one of a number of drawn circles of known diameters to the wheal size. The measurements were recorded in the notes. The allergens tested were *Dermatophagoides pteronyssimus*, mixed grass pollen, cat hair, and aspergillus fumigatus, with histamine (10mg/ml) and normal saline serving as positive and negative controls respectively. Each solution was placed about 3 centimetres apart. A reaction is positive reaction if 3 millimetres or more in diameter.

2.1.4 *Lung function measurements*

Baseline lung function was measured in all subjects. FEV1 and FVC were measured with calibrated, dry wedge spirometer (Vitalograph, Buckingham, UK). For all experiments, baseline lung function was measured following a standardised protocol. On arrival in the laboratory, each subject rested for 15 minutes prior to three measurements of FEV1 and FVC. The procedure was performed with the subject standing. The subject was asked to take a full inspiration, seal his/her lips around the

cardboard mouthpiece tube, and perform an expiratory manoeuvre as rapidly and forcefully as possible. The subject was then encouraged to breathe out for a full 6 seconds or until a 2 second plateau was achieved as indicated by the stylus on the Vitalograph chart. The manoeuvre was repeated a further 2 times with a one minute rest between each manoeuvre, and the best of three attempts recorded.

2.1.5 *Measurement of airway hyperresponsiveness*

A standard procedure was employed for bronchial challenge with methacholine. On arrival in the laboratory, subjects were required to rest for 15 minutes before assessment of lung function. The best of 3 technically acceptable recordings of FEV1 taken at 1 minute intervals, was taken and had to be 70% of predicted value before histamine challenge. Following this baseline assessment, subjects inhaled five breaths of 0.9% saline nebulised from a breath activated dosimeter by inspiring slowly from functional residual capacity (FRC) to total lung capacity (TLC) over 3 seconds and then breath holding for 6 seconds. The subject then exhales from TLC, as hard and as fast as possible for one second and the FEV1 noted. This measurement is performed at 30 seconds, 90 seconds and 180 seconds post nebulization. At each time period (30, 90, and 180 seconds) at least 3 measurements of FEV1 are made to confirm reproducibility, and the highest of each three taken. Unless a fall in FEV1 of greater than 10% from baseline is seen, subjects then inhale doubling increments of methacholine until a $\geq$ 20% fall in FEV1 from the post saline value is achieved. This concentration of methacholine is known as the PC_{20}. FEV1 is recorded 1.5 and 2.5 minutes after each concentration of methacholine. The starting concentration for methacholine was 0.031mg/ml. In the event of a post saline fall in FEV1 of > 10% from baseline, subjects are rested once only for 30 minutes before the procedure is repeated. Patients were given beta-2 agonist if required

2.1.6 *Exhaled NO measurement*

Exhaled NO was measured using a chemiluminescence analyzer (Model LR2000; Logan Research, Rochester, UK) sensitive to NO from 1 to 5000 parts per billion (ppb, by volume), and a resolution of 0.3 ppb. In addition to NO, the analyzer also measured carbon dioxide (CO_2), (resolution 0.1% CO_2) and sample pressure and

volume in real time. The sampling rate of the analyzer was 250ml/min for all measurements. The analyzer was calibrated daily by the asthma clinic staff using certified NO mixtures (90ppb and 500ppb) in nitrogen and certified CO_2 (BOC special gases, Surrey Research Park, Guildford UK). Ambient air NO was recorded before and after each subject was studied.

Measurements of exhaled NO–obstructed, were made by exhalation from TLC via a wide bore Teflon tube into the analyzer, with a flow of approximately 1L/minute, and therefore provided resistance to flow (exhaled NO-obstructed). Peak exhaled NO values were measured during the obstructed maneuver. Subjects then performed a similar maneuver with slow exhalation for 30 seconds from TLC with a flow of 5L/min, bypassing the analyzer and thus with minimal resistance to flow; NO was sampled from a side-arm (end-exhaled NO-unobstructed). For unobstructed end-exhaled NO concentrations, the value corresponding to the plateau of the end-exhaled CO_2 reading (5% CO_2) was taken as representative of an alveolar sample. This measurement using free flow was repeated following a 20 second breath hold (end-exhaled NO breath-hold). In all these maneuvers subjects wore a nose clip, and the pressure during expiration was kept constant (3 +/- 0.4mm Hg) by using a visual display of expiratory flow measured by pressure and volume sensors in the analyzer.

2.2 Laboratory methods

2.2.1 *Sputum induction and processing*

Sputum induction was performed using the method described by *Keatings et al* (172). Inhaled salbutamol was given 15 minutes before sputum induction. Hypertonic saline solution was prepared by adding 57mls of water for injection to 43mls of 7% sodium chloride. Twenty mls of this solution was placed into the cup of the ultrasonic nebuliser. After baseline spirometry was recorded, patients were instructed to wash their mouths thoroughly with water, and then inhale (breathing normally) 3% nebulized saline using an ultrasonic nebulizer (DeVilbiss Co, Heston, UK) at the maximal saline output (4 ml/min), for 5 minutes. The subject must wear a nose clip for the procedure, as is monitored closely for adverse effects such as dyspnoea and wheezing. The total period of sputum induction was 15 minutes and subjects were encouraged to cough deeply at 3 minute intervals until the 15 minute induction time had elapsed. Mouthwashing before each cough was encouraged in order to minimize salivary

contamination. Following sputum induction, spirometry was repeated and if there was >15% fall in FEV1, the patients were required to stay for obervation until it returned to baseline values, and given a salbutamol nebuliser if required. Sputum was collected into a 50ml polypropylene tube, and processed within 2 hours.

Sputum was processed by adding 1ml Hank's balanced salt solution (HBSS, Sigma Aldridge, Irvine, UK) containing 1% dithiothreitol (DTT) (Sigma chemicals, Poole, UK). The mixture was vortexed and aspirated repeatedly at room temperature until the sputum was homogenized. Sputum volume was then recorded and further diluted with HBSS to 5 ml, vortexed briefly and centrifuged at 400g for 10 minutes at 4°C. Sputum supernatants were removed and kept at –70° C until needed for subsequent cytokine assays. Total cell counts were performed by resuspending the cell pellets and then counting using a haemocytometer after staining with Kimura stain. Cytopinslides were then prepared (Shandon, Runcorn, UK) and stained with May-Grunwald-Giemsa for differential cell counts. At least 400 inflammatory cells were counted in each sample

2.2.2 *Isolation of PBMC and granulocytes by Ficoll-Hypaque gradient centrifugation*

Twenty millilitres of heparinised blood were diluted in an equal volume of RPMI 1640 (Gibco, Invitrogen, UK) in a 50mls polypropylene tube and carefully layered on top of 10ml Ficoll-Hypaque (Amersham Biosciences, UK) taking care not to disturb the interface. The tube was then centrifuged at 2000 rpm at 20°C for 20 minutes, following which the PBMC layer was withdrawn with a pipette and transferred to a new polyproplene tube and washed by adding 5mls phosphate-buffered saline (PBS, Gibco, Invitrogen, UK) and centrifuging at 1800rpm for 5 minutes. The supernatant was discarded and the cell pellet resuspended in 1 ml PBS. The cells were counted using a haemocytometer and Kimura stain and the cell concentration adjusted to $1x10^6$ in PBS. Cells were placed on ice until required.

To obtain granulocytes, all the layers above the red cell layer were removed and 10ml PBS and 3ml 3 % Dextran T-500 (Pharmacia Biotech, Uppsala, Sweden) were added and left at room temperature for 40 minutes. The supernatant was carefully removed and centrifuged at 200g for 10 minutes. The red cells were lysed by adding 2ml 10% HBSS in distilled water for 20 seconds, following which excess HBSS (minimum 12 ml) was added and the contents of the tube mixed and centrifuged at 1800rpm for 5 minutes. The cells were washed twice in HBSS (Ca and Mg free)

containing 15% fetal calf serum. Cells were counted with Kimura stain on a haemocytometer and resuspended at 1 x 10^6 /ml, and viability checked using trypan blue.

2.2.3 *Use of flow cytometry for measurement of neutrophil activation*

Several aspects of neutrophil activation can be evaluated by flow cytometry including phagocytosis, respiratory burst (superoxide anion generation, intracellular H_20_2 production), intracellular pH, actin polymerisation, degranulation and surface marker expression. Flow cytometry also has the advantage in that it allows the identification of neutrophils from anticoagulated blood, without any isolation procedure. Some neutrophil functions, such as phagocytosis, respiratory burst, and surface marker expression can be studied using whole blood, whilst other aspects of neutrophil physiology such as actin polymerization and cytotoxicity require the use of purified neutrophils (173).

For operation of a flow cytometer, cells must be kept in suspension so that they can flow in single file and this is accomplished by injecting the sample into a sheath fluid as it passes through a small (50-300μm) orifice. The cells then pass through a light source focused on the same point where cells have been focused and the amount of light scattered in the forward direction (along the same axis that the laser light is travelling) is detected in the forward scatter channel. The intensity of the forward scatter is proportional to the size, shape and optical homogeneity of the cells (or other particles). The amount of light scattered to the side (perpendicular to the axis that the laser light is travelling) is detected in the side or 90° scatter channel. The intensity of side scatter is proportional to the size, shape and optical homogeneity of the cells (or other particles). Forward scatter tends to be more sensitive to surface properties of particles (eg. cell ruffling) than side scatter, and can be used to distinguish live from dead cells. Side scatter tends to be more sensitive to inclusions within cells than forward scatter and can be used to distinguish granulated from non granulated cells.

The fluorescence emitted by each flurochrome is usually detected in a unique fluorescence channel. The specificity of detection is controlled by the wavelength selectivity of optical filters and mirrors. Optical filters are constructed from materials that absorb certain wavelengths while transmitting others. Long pass filters transmit wavelengths above a certain wavelength, whilst short pass filters transmit wavelengths below a cut off wavelength. To measure more than one scatter or fluorescence from

each cell, multiple channel/detectors are used. The fluoresence is collected, filtered and electronics converted to digital values that are stored on a computer for analysis (fig 2.1).

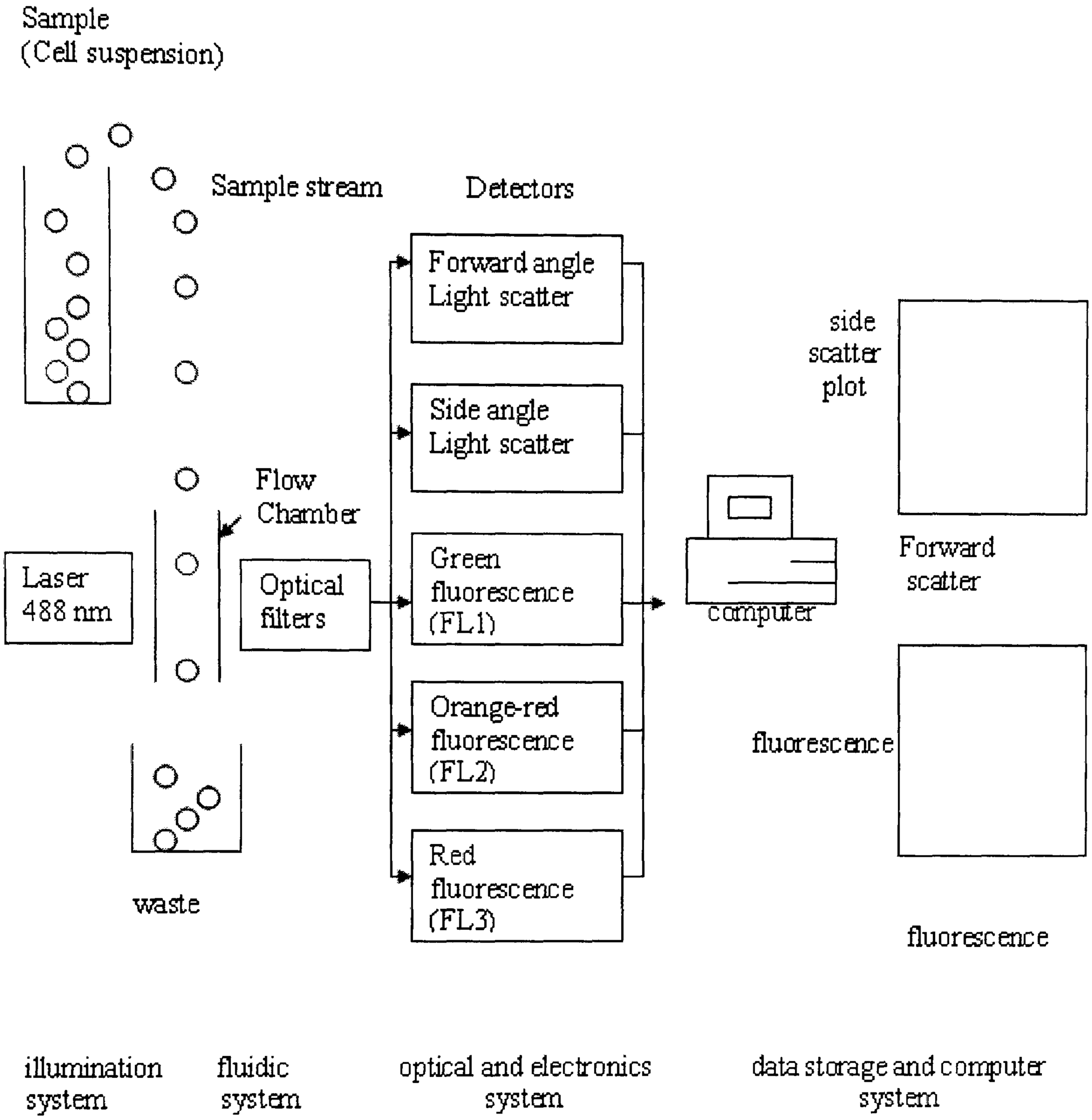


Fig. 2.1 A schematic of the primary components that comprise a flow cytometer. Dark arrows indicate the flow of particles and information. A fluidic system transports particles or cells from a prepared suspension past a focused laser beam that is generated by an illuminated system. Particle interrogation takes place, one cell at a time, in a flow chamber. The resulting scattered light and fluorescence is gathered by an optical and electronics system that translates the light signals into information that is saved by the data storage and comport system. After data has been stored, retrospective graphical data analysis can be performed with the aid of software.

2.2.3.1 *Use of whole blood for neutrophil activation studies and flow cytometry*

The use of whole blood methods to measure neutrophil function has been shown to be very specific and sensitive and avoids the artificial changes resulting from collection and isolation procedures. For example, the functional behaviour of neutrophils as well as the number and the affinity of their surface receptors can be affected by the type of anticoagulant used to collect the blood. *Repo et al* (174) showed that calcium chelating agents such as EDTA should be avoided when using monoclonal antibodies whose binding are cation dependent or when measuring functions that are calcium dependent.

Artificial upregulation of sensitive adhesion receptors such as CD11b ex-vivo can be minimized by cooling blood samples on ice. However, whether cells are kept at room temperature or cooled, further procedures such as cell labelling and fixation should be completed at the same temperaure as fluctuations in temperature have a significant effect on the expression of receptors such as CD11b and CD62L (174). *Haslett et al* (175) pointed out the importance of ensuring that reagents were pyrogen free and of tissue culture grade and the importance of avoiding endotoxin contamination of reagents or buffers as bacterial components can lead to artificial upregulation of certain activation markers. Certain procedures such as density centrifugation (176) and hypotonic lysis of red cells (177) can artificially increase expression of certain adhesion molecules. If purified neutrophils are used, then the interpretation of sensitive surface markers such as CD11b should be reserved (176).

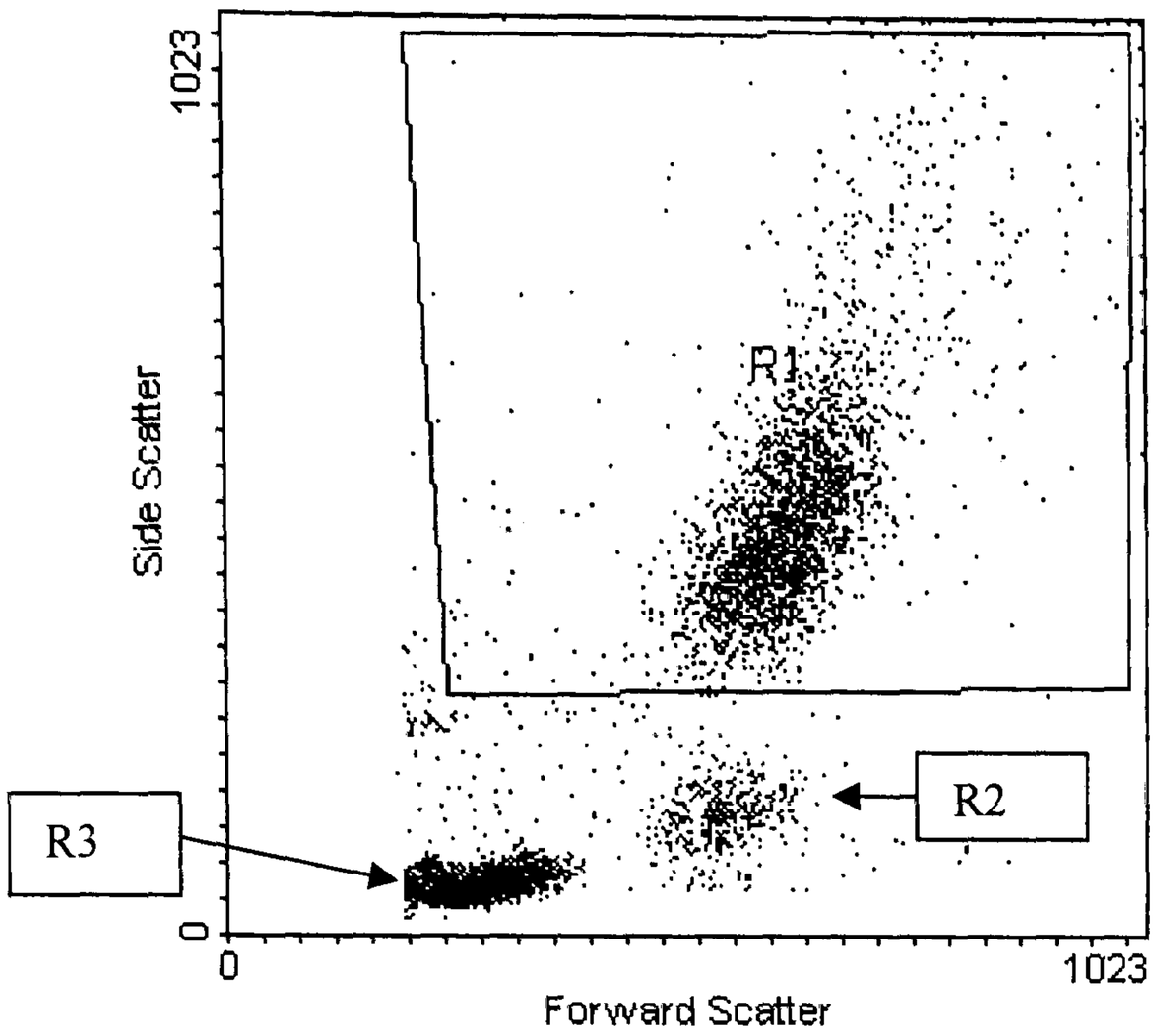
2.2.3.2 *FACS analysis*

Flow cytometry was performed using a FACScan analyser (Becton Dickinson, Oxford, UK) with Argon-ion laser. Fluorescence gain was adjusted using a gate drawn around the granulocyte population on an unstained lysed whole blood sample, and then placing this population in the first log decade. Compensation was set by using blood incubated with either fluorescein isothiocyanate (FITC) or phycoerythrin (PE) monoclonal antibodies in order to correct for spectral overlap. As an instrumental control measure, CaliBRITE beads (Becton Dickinson, Oxford, UK) were used to ensure the correct alignment and calibration of the flow cytometer. Ten thousand events were collected from each sample. Granulocytes were discriminated from other cell populations in lysed whole blood by their characteristic high side scatter and forward

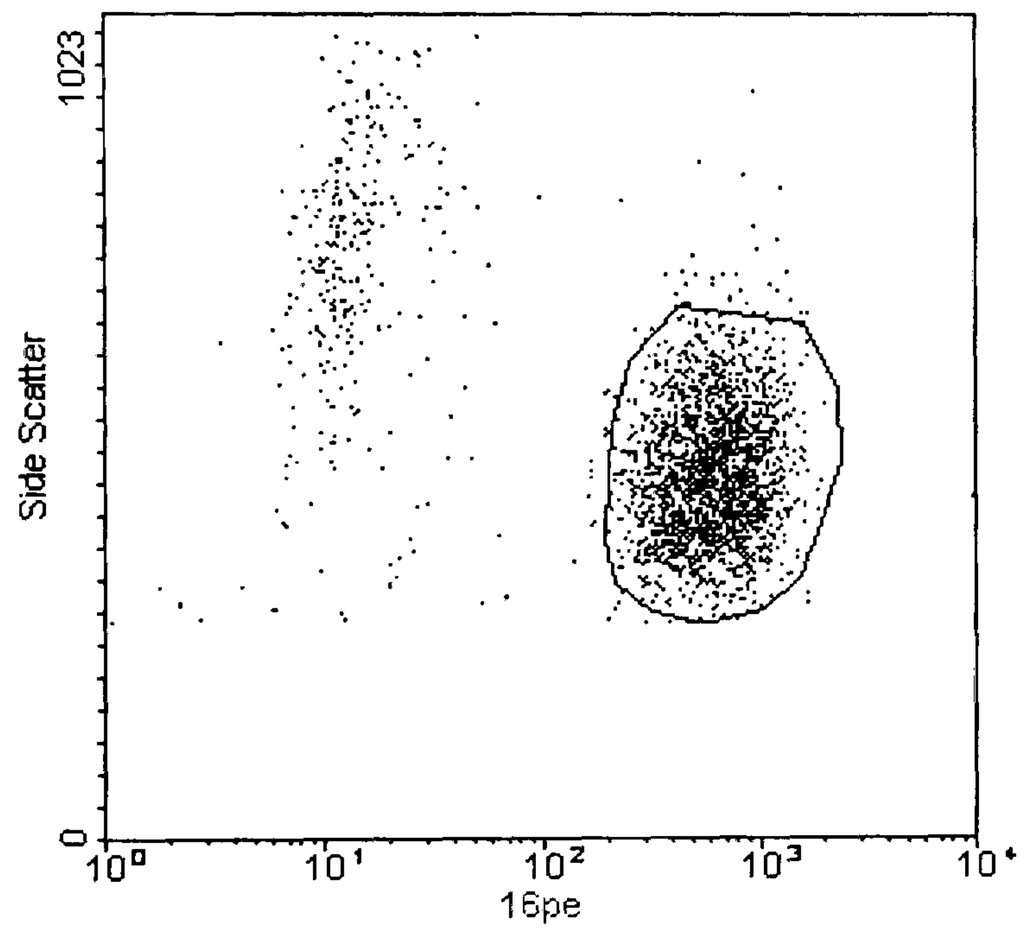
light scatter characteristics. A gate was drawn around the granulocytes (R1 or region 1) and this population was reflected into a dot plot of side-scatter and CD16. Two populations are then produced. Neutrophils are CD16 positive cells whilst cell sorting studies (176) have shown that CD16 negative cells with high side scatter properties represent eosinophils. A further gate can then be drawn around the neutrophil (CD16+) or eosinophils (CD16-) populations and reflected into a histogram so that cells labelled with a further fluorochrome (eg. CD11b or CD18) are produced (fig. 2.2). Results were recorded as mean fluorescence intensity (MFI) which represents the receptor density on the cell surface.

2.2.3.3 *Discrimination of cell populations in lysed whole blood using flow cytometry*

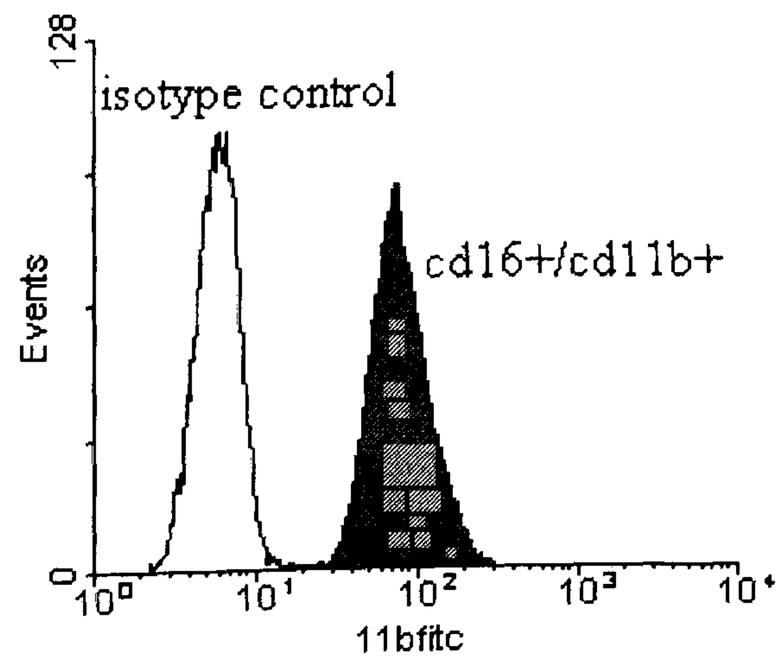
A typical dot plot pattern of lysed whole blood showing three main cell populations can be seen (fig 2.3). Lymphocytes are small and relatively agranular, and therefore display low forward and side scatter. Granulocytes have a high forward scatter corresponding to increased cell size and high side scatter corresponding to increased granularity and therefore appear in the upper right hand of the dot plot. Monocytes have intermediate scattering properties. As previously mentioned, a gate can be drawn around the granulocyte population (R1 or region 1) and this population can be reflected into a dot plot of side scatter and CD16. CD16 is also known as the FcγIII receptor and is found on neutrophils, and is involved in phagocytosis, antibody dependent cell mediated cytotoxicity (ADCC), and cytokine production. CD16 is expressed initially at the metamyelocyte stage and is almost uniformly expressed on neutrophil band forms and segmented neutrophils. Two populations are then produced. Neutrophils are CD16 positive cells whilst cell sorting studies done by Gopinath et al (178) have shown that CD16 negative cells with a high side scatter represent eosinophils. A further gate can then be drawn around the neutrophil (CD16+) or eosinophils (CD16-) populations and reflected into a histogram so that cells labelled with a further fluorochrome (eg. CD11b or CD18) are produced. The appropriate isotype control is also shown and this is a marker of background staining. Eosinophils, in contrast, are for the most part CD16 negative, although they can express a low level of this marker if stimulated in vitro by IFNγ.



Dot plot of lysed whole blood showing different cell populations based on forward and side scatter properties. R1-gated cells represent granulocytes, R2 cells are monocytes, and R3 cells are lymphocytes.



CD16 positive cells are neutrophils (gated), whilst CD negative cells with high side scatter represent eosinophils.



CD 16 postive cells are then reflected into a histogram so that cells labelled with another flourochrome, eg. CD11b can be studied.

Fig. 2.2 Discrimination of cell populations using flow cytometry.

2.2.3.4 *Direct immunofluorescence staining for flow cytometry*

Venous blood was drawn into a heparinised syringe and processed within 2 hours. Ten microlitres (µl) of conjugated antibody (FITC or PE labelled) were added to 100µl of whole blood and incubated at 4°C for 30 minutes in the dark. The appropriate isotype-matched control samples were included to detect any background staining. After the incubation period, the cells were washed by adding 2mls PBS, (Gibco, Invitrogen, UK) containing 0.5% bovine serum albumin (Sigma Aldridge, Poole, UK) and 0.5% sodium azide (Sigma Aldridge, Poole, UK), (PBA-BA) and centrifuged for 5 minutes at 400g. The supernatant was discarded and 2mls of freshly prepared Erythrolyse (Serotec Ltd, Oxford, UK) added and followed by incubation for 10 minutes at room temperature. After further centrifugation the supernatant was discarded. Cells were then washed with 2ml PBS-BA and centrifuged as above. The supernatant was discarded and the cells were resuspended in 0.5mls of 0.5% paraformaldehyde (PFA, Sigma Chemical Co, Poole, UK) prior to analysis by flow cytometry. Duplicate samples prepared and analysed.

2.2.3.5 *Indirect immunofluorescence staining for flow cytometry*

Heparinized whole blood (100µl) or PBMC at a concentration of $1x10^6$ were added to round bottom glass tubes (Marathon Ltd, UK) and the appropriate volume of primary antibody added at the recommended dilution. The cells were mixed and incubated at 4°C for 30 minutes in the dark. The cells were washed by adding 2 mls of PBS-BA, followed by centrifugation at 400g for 5 minutes. The supernatant was discarded and the appropriate volume of secondary conjugated antibody was added at the recommended dilution, and incubated as above. If using PBMC, the cells were washed with PBS-BA, centrifuged, the supernatant discarded and the cells resuspended with 0.5% PFA prior to aquiring data by flow cytometry. If using whole blood, red blood cells were lysed by adding 2 mls of freshly prepared Erythrolyse, mixing well and incubating for 10 minutes at room temperature. The tubes were then centrifuged and the supernatant discarded, followed by washing and further centrifugation as described above. Finally cells were resuspended in 0.5% PFA prior to flow cytometry. Duplicate samples were prepared and analysed.

2.2.3.6 *Indirect immunofluorescence staining for measurement of FcεR1 receptors on basophils*

30mls of venous blood were venesected into a heparinised syringe and diluted in an equal volume of RPMI 1640 (Gibco, Invitrogen UK) in a conical tube. The diluted blood was then carefully layered onto Ficoll Paque medium (Amersham Biosciences, Uppsala, Sweden). The blood was then centrifuged at 2000 revolutions per minute (rpm) for 20 minutes at 20°C, following which, the PBMC layer was carefully drawn off with a pipette and transferred to another conical tube. Phosphate buffered saline (Gibco, Invitrogen, UK) was added and the PBMC fraction was washed and centrifuged again for 5 minutes as above. Any remaining red blood cells were removed by adding 5 mls of distilled water to the cell pellet and slowly resuspending the cell pellet several times. After a further wash with PBS, the cell concentration was adjusted to 1×10^5 to 1×10^6 with PBS. The cells were then stained with 10µl rabbit anti-human FcεR1 antibody (Upstate biotechnology, Lake Placid, USA) for 30 minutes in the dark at 4°C. After washing with PBS, the cells were stained with FITC conjugated swine anti-rabbit secondary antibody (1 in 40 dilution, Dako, Denmark) again for 30 minutes at 4°C in the dark. The cells were washed in PBS and then stained with 10µl CD123 PE (Pharmingen International, Europe) and HLA-DR RPE-Cy5 (Serotec Ltd, Oxford, UK) conjugated monoclonal antibodies for 30 minutes at 4°C in the dark. Appropriate isotype controls were purchased from the companies mentioned above. After this final incubation, the cells were washed and resuspended in 0.5ml 0.5% PFA (Sigma Chemical Co, Poole, UK) prior to analysis by flow cytometry. Duplicate samples were prepared and analysed.

2.2.3.7 *Monocytes and FcεR1 receptor expression*

PBMCs were isolated from venesected venous blood as outlined in section 2.2.1. Indirect staining for FcεR1 subunit was performed as described in section 2.2.3.6. After washing and centrifugation, the cells were resuspended in PBS (Gibco, Invitrogen, UK) working solution and incubated with mouse anti-human CD14 PE monoclonal antibody along with appropriate isotype controls (Serotec Ltd, Oxford, UK) for 30 minutes at 4°C in the dark in order to identify monocytes in the PBMC population. After further

washing, the cells were resuspended in 0.5ml of 0.5% PFA (Sigma Chemical Co, Poole, UK) before analysis by flow cytometry.

2.2.3.8 *Lysed whole blood cell surface marker analysis- rhuMab E25study*

100µl of whole blood were added to round bottom tubes (BD Falcon, Oxon, UK) following which 10µl FITC or PE conjugated mouse anti-human monoclonal antibodies were added and the cells incubated for 30 minutes at 4°C in the dark. FITC conjugated CD95, CD23, and PE conjugated CD9, CD14 and CD4 along with the appropriate isotype controls were purchased from Serotec Ltd, Oxford, UK. FITC conjugated rat anti-human CCR3 and appropriate isotype control was purchased from R&D systems Europe, Oxon, UK. After incubation, the red blood cells were lysed by adding 2 mls Erythrolyse (Serotec Ltd, Oxford, UK) for 10 minutes at room temperature. After further centrifugation, the supernatant was discarded, and cells washed with 2ml PBS and centrifuged again. Cells were then resuspended in 0.5 mls of 0.5% PFA (Sigma Chemical Co, Poole, UK) before analysis by flow cytometry.

2.2.4 *Enzyme-linked immunosorbent assay (ELISA) measurements*

IL-8 (duoset, R&D systems, Oxon, UK), MPO (Calbiochem, San Diego, USA), IL-5 (duoset R&D systems, Oxon, UK) concentrations were measured in the culture supernatants with specific immunoassay kits according to the manufacturer's instructions. The detection limit was 31.2pg/ml for IL8, 1.6ng/ml for MPO, and 15.6pg/ml for IL-5. Samples were prepared and analysed in duplicate.

2.2.5 *Stimulation of whole blood*

Prior to incubation with conjugated monoclonal antibodies, blood was incubated with fMLP, (Sigma Aldridge, Poole, UK) dissolved in dimethyl sulfoxide (Sigma Aldridge, Poole, UK) at a concentration of 10^{-7}M, or PAF at a concentration of 10^{-6}M (Sigma Aldridge, Poole,UK) dissolved in PBS (Gibco, Invitrogen, UK) for 10 mins at 37°C. The reaction was stopped by adding cold PBS solution. The tubes were then centrifuged for 5 minutes at 1800rpm and the supernatant discarded. The blood was then incubated with monoclonal antibodies as previously described.

2.2.6 *Incubation of whole blood or isolated granulocytes with dexamethasone*

100µl whole blood or $1x10^6$ isolated granulocytes were preincubated at 37°C, 5% CO_2 with 10µl dexamethasone at concentrations ranging from 10^{-12} to 10^{-6} for up to 24 hours. After each incubation period, the blood was stimulated with fMLP at a final concentration of 10^{-7}M at 37°C for a further 10 minutes. The reaction was stopped with cold PBS, following which the supernatant was decanted, washed and centrifuged and finally resuspended in 100µl PBS working solution. Staining was then carried using a direct method as outlined previously.

2.2.7 *Oxidative burst*

The most widely used standard methods to assess the oxidative burst in neutrophils measure the extracellular release of 0^{2-} or H_20_2 by their ability to reduce or oxidise substrates to coloured or fluorescent products (179). The major disadvantages of these assay methods are that cells must be separated from red cells and plasma and that relatively large numbers of neutrophils are needed. In these assays, the measured response is of a population and individual response cannot be assessed.

In 1983, *Bass et al* (180) developed a method to measure 'oxidant product formation' by flow cytometry using 2'7'-dichlorofluorescein (DCF). In this method, neutrophils are incubated with the diacetate form of 2'7'-dichlorofluorescein (DCFH), which is transported across the plasma membrane into the cytoplasm where the acetate groups are removed by esterases concentrating the polar non-fluorescent DCFH in the cytoplasm. During the oxidative burst, DCFH is converted to DCF through mainly by H_20_2. Dihydrorhodamine 123 (DHR123) is used in a similar way, resulting in red fluorescence (181,182). DHR 123 is the uncharged and nonfluorescent reduction product of the mitochondrion selective dye rhodamine 123. This leuco dye passively diffuses across most cell membranes and is oxidized to cationic rhodamine 123, which localises in mitochondria. Strong non-physiological stimuli such as PMA and calcium ionophores cause a sustained respiratory burst resulting in a high fluorescence that can be detected by flow cytometry. DHR 123 has been reported to be more sensitive than DCFH for detecting granulocyte respiratory burst activity (183). The use of flow cytometry to measure the products of the oxidative burst offers an easy semi-quantitative technique that is applicable to a few thousand unpurified cells, and can be performed in mixed cell samples including whole blood where neutrophil responses can

be determined using gating techniques. The small number of unpurified cells necessary for the flow cytometry assay allows studies on small sample volumes such as neonatal blood.

Studies of granulocyte oxidative burst in asthmatics have shown that eosinophils from atopic asthmatics stimulated with PMA and fMLP showed increased free radical production versus normal patients (184). No significant difference was seen in neutrophil chemiluminescence activated by PMA and fMLP between asthmatics and normal patients. *Teramoto et al* however (185), showed that PMA and zymosan stimulated blood in asthma and/or COPD showed increased 0^{2-} generation versus normals controls, but increased resting neutrophil 0^{2-} production in asthmatic patients only.

In animal experiments, *Carey et al* (186) showed that neutrophils are primed as shown by the 2.7 times increase in 0^{2-} production above baseline from neutrophils isolated from sheep given a low dose endotoxin infusion compared to control animals. The effect of glucocorticoids on oxidative burst in bovine granulocytes was investigated by *Hoeben et al* (187) who showed that prednisolone resulted in inhibition of respiratory burst at therapeutic doses, but no effect was seen on neutrophil myeloperoxidase activity. In normal subjects, i.v. hydrocortisone decreases reactive oxygen species production by neutrophils and monocytes as measured by chemiluminometry (188). *Bekesi et al* showed that human neutrophils incubated with various steroids such as oestradiol, progesterone, testosterone and hydrocortisone, showed decreased 0^{2-} production, but this effect was not seen with the mineralocorticoid aldosterone (189). In a negative study of the effect of corticosteroids, *Toft et al* showed that pretreatment of patients undergoing cardiopulmonary bypass surgery with methylprednisolone had no effect on oxidative burst as measured with DHR123 versus control patients (190).

2.2.7.1 *Oxidative burst measurement*

Respiratory burst was measured according to the method of Rothe and Valet (191). 3ml of heparinised blood were layered onto 3ml of Ficoll Hypaque medium (Amersham Biosciences, UK) and left at room temperature for 45 minutes. 800µl of the supernatant was then removed and transferred to an Eppendorf tube and stored on ice.

1 ml of HBSS, (Sigma Aldridge, Irvine,UK) was added to 20µl of cell suspension and 10µl of DHR123, $1x10^{-3}$M, (Molecular Probes, Upsaala, Sweden), and the cells incubated for 5 minutes at 37°C. Following this, PMA, (Sigma Aldridge, Poole, UK) was added to a final concentration of 1µM and the samples incubated for 20 minutes at 37°C. Reagent blank tubes were prepared by adding only PBS to the cell suspension, while resting cells were initially incubated with PBS, followed by the addition of DHR123.

After the final incubation, 250µl of the sample was analysed immediately on the FACS scanner. The FACS machine was set up so that unstimulated cells were placed in the first log decade. DHR 123 reacts with H_20_2 as a result of the respiratory burst to give rhodamine, which emits a green fluorescence which can be measured on the FL1 channel of the FACS machine (wavelength 530 nm). Samples were prepared and analysed in duplicate.

2.2.8 *Neutrophil elastase measurement*

Neutrophil elastase was also measured according to the method described by Rothe and Valet (192). 3 mls of heparinised blood was layered on top of 3 ml Ficoll Hypaque medium (Amersham Biosciences, UK), and the erythrocytes allowed to sediment for 40 minutes at room temperature. The plasma supernatant (800µl) was withdrawn and stored on ice. One ml of HBSS supplemented with 10mM HEPES (Sigma Aldridge, Poole, UK) was added, along with 20µl cell suspension, and 1µl (N-benzyloxycarbonyl-Ala-Ala)$_2$-rhodamine 110 (Molecular probes, Upsaala, Sweden) at a final concentration of 4µM. The samples were incubated at 37°C and analysed on the flow cytometer after 20 minutes.

2.2.9 *Whole blood culture for serum cytokine measurement*

Much of the work carried out using Elisa quantitation of cytokines has been carried out on supernatants from isolated cell culture systems. The whole blood culture method is thought to be more representative of an in-vivo environment than other methods which involve isolated cell populations and may give better elucidation of the actual events occuring in-vivo, as the natural cell to cell interactions are preserved and

circulating stimulatory and inhibitory mediators are also present in their normal concentrations. This method does not, however, allow determination of which cell type is involved in cytokine production. With undiluted whole blood only early produced cytokines can be studied due to the poor stability of long term undiluted whole blood (193, 179). Samples were prepared and analysed in duplicate.

2.2.9.1 *Whole blood culture-methodology*

Venous blood was collected in heparinized tubes and diluted 1:10 with RPMI 1640 (Gibco, Invitrogen, UK) containing 10% fetal calf serum (FCS), 100u/ml penicillin, 100mcg/ml streptomycin and 2mM L-glutamine. The blood was distributed in 1 ml wells and the plates were incubated at 37°C with 5%CO_2 for 24hours. In addition, in duplicate wells, diluted blood was stimulated with IL-1β (10ng/ml final concentration), LPS (10ng/ml), and GMCSF (1ng/ml) prior to incubation. The plates were then centrifuged at 1800 rpm for 5minutes, following which the supernatants were removed and frozen at minus 70°C until analysed. Samples were prepared and analysed in duplicate.

2.2.10 *Neutrophil Myeloperoxidase measurement*

Granulocytes were isolated as described in section 2.2.2. One million granulocytes per ml were then added to 1ml wells of a 48 well plate and incubated with IL-1β (10ng/ml), or PBS for 0, and 30 minutes and incubated at 37°C and 5% CO_2. After each time point, the plates were centrifuged at 1800rpm for 5 minutes following which the supernatant was withdrawn and stored at –70°C until analysed by ELISA. Samples were prepared and analysed in duplicate.

2.2.11 *Fiberoptic bronchoscopy, BAL collection and processing of BAL macrophages.*

Bronchoscopies were performed by Dr. B. Cosio. Volunteer subjects attended the bronchoscopy suite at 8.30 a.m. after having fasted overnight. They were sedated with midazolam (5-7.5 mg i.v.) and alfentanyl (0.5 mg i.v.). Local anaesthesia was provided by linocaine gel to each nostril, and lidocaine 2% to the upper airways and larynx. After passing the fiberoptic bronchoscope (Olympus BF30 Key-Med, Southall,

UK) through the nasal passages into the trachea, warmed saline (4 x 60 ml) was instilled and aspirated from the right middle lobe. BAL cells were spun down (500g for 10 min) and washed twice with HBSS. Cytospins were prepared and stained with May-Grunwald stain for differential cell count. Cell viability was assessed by using the trypan-blue exclusion method.

2.2.12 *Measurement of HDAC activity.*

HDAC activity of nuclear extracts was measured with a non-isotopic assay using a fluorescent derivative of epsilon-acetyl lysine (HDAC fluorescent Activity Assay Kit, BIOMOL, Plymouth, PA, USA) by Dr B. Cosio. This assay was based on the Fluor de Lys^{TM} (Fluorogenic Histone deAcetylase Lysyl Substrate/Developer) Substrate and Developer combination. The assay was performed as recommended by the manufacturer and emitted light detected at 460nm in a fluorometric plate reader. Activity was measured in AFU (arbitrary fluorescence units).

2.3 Statistical methods

Unless otherwise stated, data are expressed as mean ± standard error of the mean (SEM). Differences in surface marker expression between two groups were analysed using the Unpaired-t test. Differences in surface marker expression between more than two groups were evaluated using one way analysis of variance (ANOVA). Where there was one independent treatment variable, one way ANOVA with Bonferroni post testing was carried out to take into account the effect of multiple comparison. If there were 2 independent treatment variables, 2 way ANOVA with Boferroni correction was carried out. The effect of a treatment within a single group was analysed using a Paired-t test. Where a PBO group was included in the study design, any positive results were compared with the PBO group. A p value of 0.05 or less was considered to be statistically significant.

Chapter 3

Peripheral blood neutrophil activation in mild, moderate and severe asthmatics and the effect of corticosteroids on neutrophil function in mild and severe asthmatics

3.1 Introduction

Techniques such as flow cytometry allow investigation of the state of activation of peripheral blood cells by looking at the expression of specific surface activation markers. This allows comparison of neutrophil activation in peripheral blood samples between severe asthmatics and milder asthmatics, and avoids the need to do more invasive procedures which have potential complications such as bronchoscopy and biopsies. The use of blood samples also allows the effect of any treatment to be assessed (in this case, oral prednisolone), without having to repeat investigations such as bronchoscopy. Recruitment for studies using more invasive investigations can be particularly difficult, especially in the more severe group of patients.

Although the effect of corticosteroids on neutrophil function has been investigated, there is no data on the effect of a standard course of prednisolone on neutrophil activation markers in asthmatics, which more closely resembles the situation when dealing with asthmatics in a clinical setting. The findings of other workers that neutrophils are important mediator cells in severe asthmatics, and that corticosteroids may prolong survival of neutrophils by inhibiting their apoptosis, would allow these cells to potentially cause more tissue damage and airway remodelling through the release of their chemical mediators. This suggests that corticosteroids may in fact be causing more harm in certain asthmatics and lends weight to the argument that alternative therapies are needed, rather than persisting with a form of treatment which can be potentially harmful and which is associated with significant long term side effects.

Use of a whole blood system enables the usual interaction between various cellular components, cytokines and specific messengers in the inflammatory cascade to proceed as far as possible as in the real life situation, whereas this vital interaction may be lost in situations where cells are isolated from whole blood.

3.2 Methods and materials

3.2.1 *Study design*

Subjects attended the laboratory on two occasions. During the screening visit a standard questionnaire was completed and clinical examination was performed, followed by skin prick testing, spirometry, methacholine challenge test, or reversibility testing as described in section 2.1 in order to assess asthma severity and inclusion and exlusion criteria. Atopic status was defined as having a positive skin prick test to at least one of four common aeroallergens (grass pollen, cat dander, Dermatophagoides pteronyssinus, and Aspergillus fumigatus and skin prick testing was performed as outlined in section 2.1.3. Sputum induction was performed and blood was taken for investigation of surface activation marker expression, oxidative burst and neutrophil elastase measurement using FACS analysis, and cytokine and mediator release measurements using ELISA, as described in section 2.2 on laboratory methods.

To investigate the effect of corticosteroid therapy, mild asthmatics were given 30mg of prednisolone for 1 week, whilst corticosteroid-dependent asthmatics took an extra 30 mg of prednisolone for 1 week on top of their usual maintenance prednisolone dose. Patients were asked to return after 1 week for repeat spirometry and to provide blood and sputum samples. Blood was also taken for prednisolone and cortisol measurements to ensure compliance with prednisolone treatment. Patients with the following conditions were not given prednisolone: diabetes, hypertension, high risk of osteoporosis, and active peptic ulceration. Compliance with asthma medication was reinforced and inhaler technique was checked at all clinic visits by asthma nurses.

3.2.2 *Flow cytometry*

Direct flow cytometry was performed on whole blood as described in section 2.2.3.4. FITC–conjugated monoclonal antibodies against CD11b, CD62L, CD35 and CD18, along with the appropriate isotype controls were purchased from Serotec Ltd, Oxford, UK. PE-conjugated CD16 monoclonal antibody was purchased from Becton Dickinson, Oxford, UK.

3.3 Results

3.3.1 Patient characteristics

In order to study the baseline expression of neutrophil activation in asthmatic patients, 10 patients with mild asthma, 10 patients with moderate asthma, 10 severe corticosteroid-dependent asthmatics, and 10 healthy normal subjects were included in the initial part of the study. The characteristics of normal and asthmatic subjects are shown in table 3.1, and the individual characteristics of mild and severe asthmatics taking oral prednisolone are shown in table 3.2 and table 3.3 respectively. Characteristics of the moderate patients are shown in appendix 5.1 (page 262).

Mild asthmatics had symptoms less than once weekly, requiring only a short acting inhaled β2-agonist as needed; moderate asthmatics had asthma symptoms requiring daily use of ICS (BDP 1000-2000μg/day or equivalent), but not requiring chronic oral prednisolone therapy; severe steroid-dependent asthmatics had persistent symptoms with frequent exacerbations, limited physical activity and frequent night-time symptoms requiring high dose ICS (BDP 2000μg/day or equivalent), with maintenance oral prednisolone (> 5mg per day for at least one year). The mean maintenance dose of oral prednisolone was 14mg/day (range 10-20mg/day).

3.3.2 *Pilot study*

Initial experiments involving the stimulation of lysed whole blood with fMLP were carried out to look at effect of fMLP on surface marker expression on neutrophils and eosinophils. These consistently showed that CD11b expression on neutrophils was significantly upregulated with a concentration of fMLP of between 10^{-8} M and 10^{-5} M, and in addition, CD62L was significantly down regulated in a dose dependent manner at doses of fMLP of 10^{-7} M and 10^{-5} M. This characteristic pattern of change in CD11b and CD62L surface marker expression was demonstrated initially in 10 subjects who included normal volunteers and asthmatics of varying severity. CD63 was not significantly changed in neutrophils or eosinophils following stimulation with fMLP, and therefore this marker was not used for later studies. This marker was replaced by CD18 and CD35, which showed similar up regulation to CD11b following stimulation with fMLP. The dose of fMLP chosen for future studies was 10^{-7}M (fig 3.1), following a time course study done on 3 normal subjects. The effect of PAF on peripheral blood neutrophils in whole blood produced similar results to fMLP, again in a dose dependent manner. However the effect of PAF on CD62L was less marked than with fMLP (fig 3.2). In contrast to the effect on neutrophils, fMLP did not cause a significant increase

in CD11b or reduction in CD62L on eosinophils (fig. 3.3). Time course studies on 3 normal subjects showed that fMLP caused upregulation of CD11b and downregulation of CD62L within two minutes of stimulation, with submaximal changes occurring at approximately 10 minutes (fig. 3.4). After these initial experiments, the aim was to see if there was any difference in surface marker expression between normal subjects and asthmatics of different severities.

Table 3.1 Clinical characteristics of asthmatic patients and non asthma controls

Variable	Mild asthma N=10	Moderate asthma N=10	Severe asthma N=10	Non asthma control. n=10.
Sex	2M/8F	4M/6F	4M/6F	7M/3F
Age (years)	33.4+/- 3.2	47.7+/- 4.0	51.3+/- 2.7	36.0+/- 0.7
Mean FEV1 (L)	95.8+/- 3.3	66.7+/- 5.0	51.4+/- 3.7	95.5+/- 1.8
Inhaled corticosteroid: µg/day BDP (or equivalent)	1 patient on 400 µg/day only	1500+/- 166.7	All patients taking 2000 µg/day	0
Atopy	7Y/3N	10Y	5Y/5N	0
NO (ppb)	27.1+/-8.9	18.7+/-3.4	30.9+/-6.0	ND

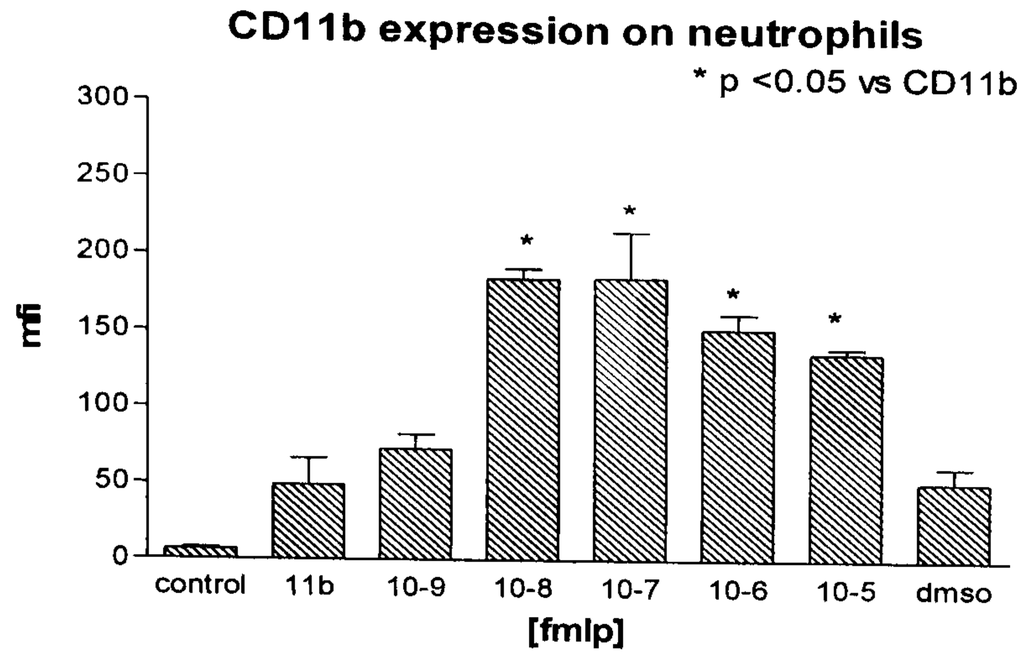
M: male; F: female: NO: nitric oxide; ND: not determined
Patients were non-smokers or had a total smoking history of less than 10 pack years and had not smoked for at least one year.
Results expressed as mean +/- SEM.

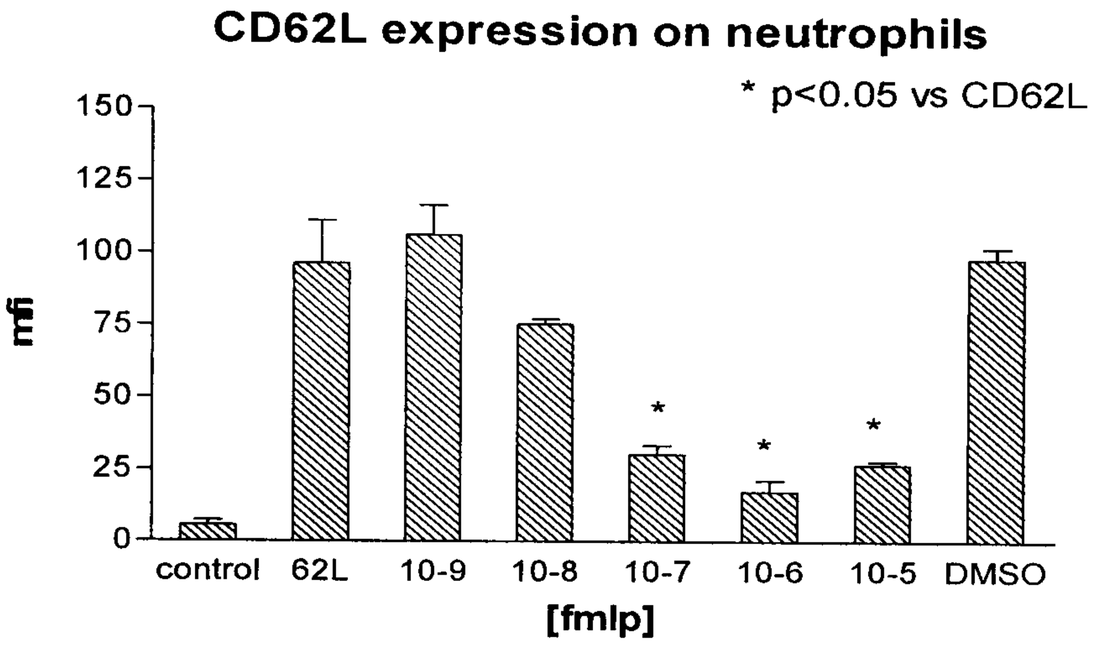
Table 3.2 Characteristics of mild patients taking oral prednisolone (30mg/day) for 1 week. All patients took salbutamol as required only, except [gj], who took BDP 400 mcg/day as maintenance therapy.

patient	Age-years	Neutrophil count ($x10^9$/l)	Eosinophil count ($x10^9$/l)	FEV (L) (% predicted)	Prednisolone (nmol/l)	Cortisol (nmol/l)	NO (ppb)
ss	49	2.5	0.3	1.8 (98%)	116	52	7.1
sm	45	3.2	0.1	1.65 (95%)	1057	<20	14
mm	22	1.8	0.2	4.64 (104%)	1540	<20	39.7
ap	45	5.6	0.2	2.95 (77%)	961	34	13
bp	24	3.9	0.1	2.56 (88%)	2143	124	53.4
st	36	4.7	0.7	4.04 (106%)	914	37	19.2
jh	36	3.2	0.4	4.26 (92%)	823	53	8.9
gm	25	6.2	0.2	3.53 (100%)	N/A	N/A	22.7
lm	23	5.4	0.3	3.17 (111.9%)	749	<20	7.6
gj	29	5.1	0.1	2.78 (91%)	1582	<20	6.2

Table 3.3 Characteristics of severe patients taking oral prednisolone (30mg/day) for 1 week. All patients took salbutamol and atrovent MDI/nebs.

patient	Age-years	Neutrophil Count ($x10^9$/l)	Eosinophil Count ($x10^9$/l)	FEV (L) (% predicted)	Treatment	Prednisol-one (nmol/l)	Cortisol (nmol/l)	NO (ppb)
ah	60	6.5	1.21	1.62 (70.7%)	BDP 2000µg/day Formoterol 12 µg bd PNL 20mg/day	<20	<20	53.5
ir	50	13.7	0.3	1.24 (48.4%)	BDP 2000 µg /day PNL 15mg/day	1555	<20	27.1
mg	52	9.7	0.6	1.5 (49.8%)	Seretide 500 µg bd PNL 11mg/day	580	<20	65.2
jm	49	15.5	0.2	1.65 (64%)	Seretide 500 µg bd Theophylline 225mg bd; PNL 20mg/day	1381	47	33.4
mmm	49	14.4	0.4	1.52 (42%)	Seretide 500 µg bd PNL 5mg/day	983	<20	20.1
rs	51	5.2	1.2	0.95 (30%)	BDP 2000 µg /day Formoterol 12 µg bd PNL 5mg/day	798	35	30.9
ls	53	7.1	2.1	1.76 (56%)	BDP 2000 µg /day Formoterol 12 µg bd PNL 10mg/day Aminophylline 225mg	408	<20	13.2
jr	53	8.9	0.1	0.96 (41.7%)	Seretide 500 µg bd PNL 5mg/day	701	<20	ND
vb	65	12.9	0.2	1.7 (61%)	BDP 2000 µg /day Formoterol 12 µg bd PNL 5mg/day	1207	<20	53.5
tj	31	13.2	1.4	1.4 (51%)	Seretide 500 µg bd PNL 20mg/day	N/A	N/A	27.1





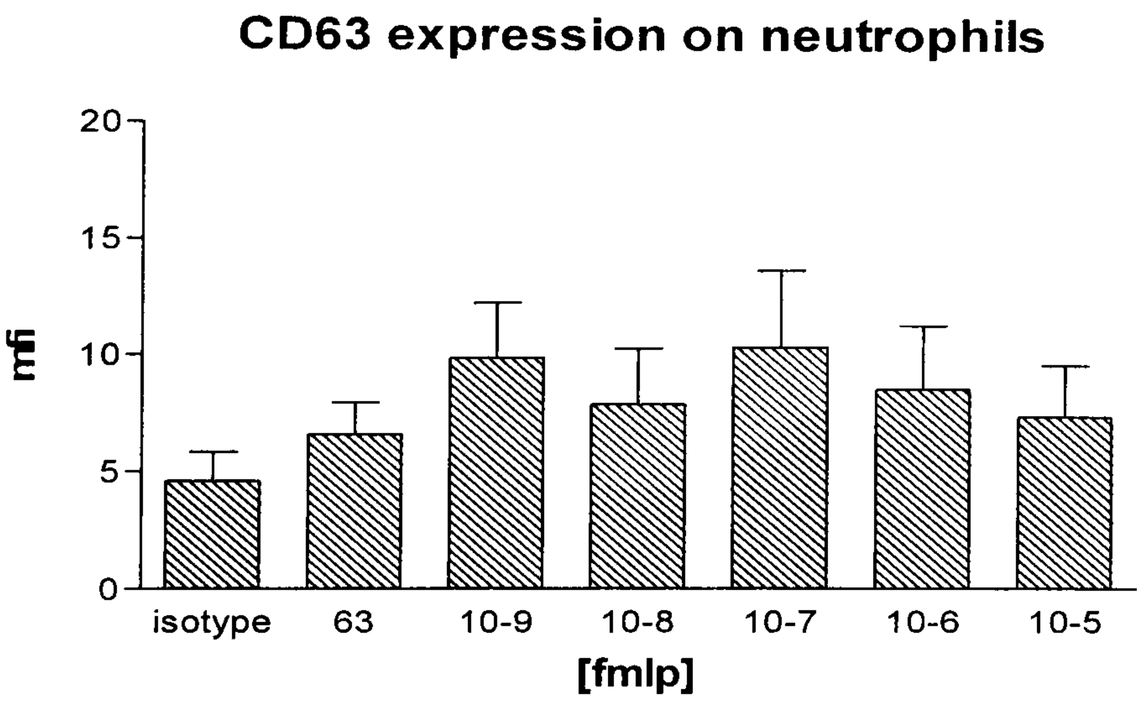
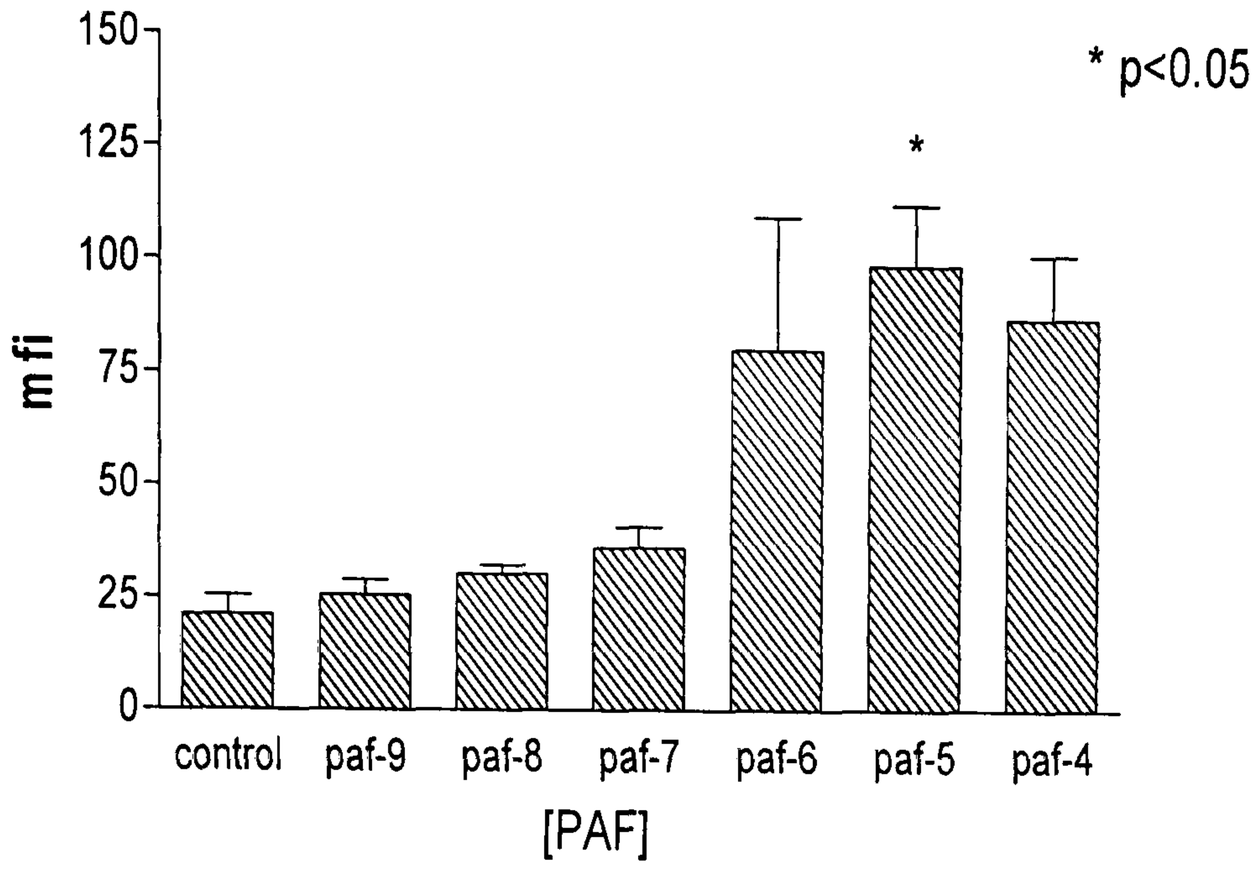


Fig. 3.1 Dose response of surface molecules CD11b, CD62L, and CD63 on peripheral blood neutrophils in lysed whole blood to fMLP, (10^{-5}-10^{-9}M). DMSO- diluent for fMLP. control =isotype control. mfi = mean fluorescence intensity. fMLP upregulated CD11b expression and reduced CD62L expression on neutrophils in lysed whole blood. There was no effect on CD63 expression.(* p <0.01 compared to markers in unstimulated whole blood.) n=3, normal subjects.



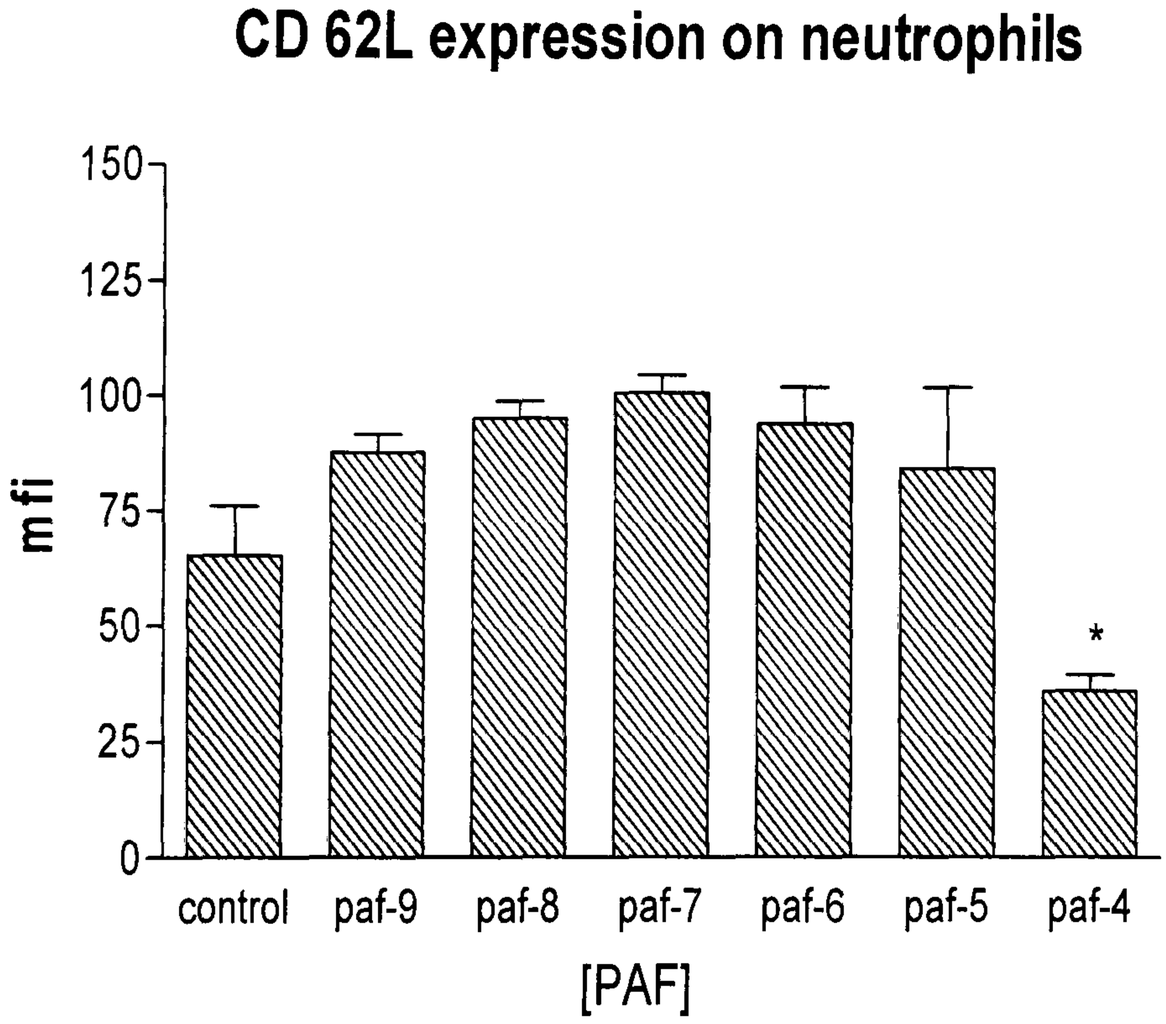
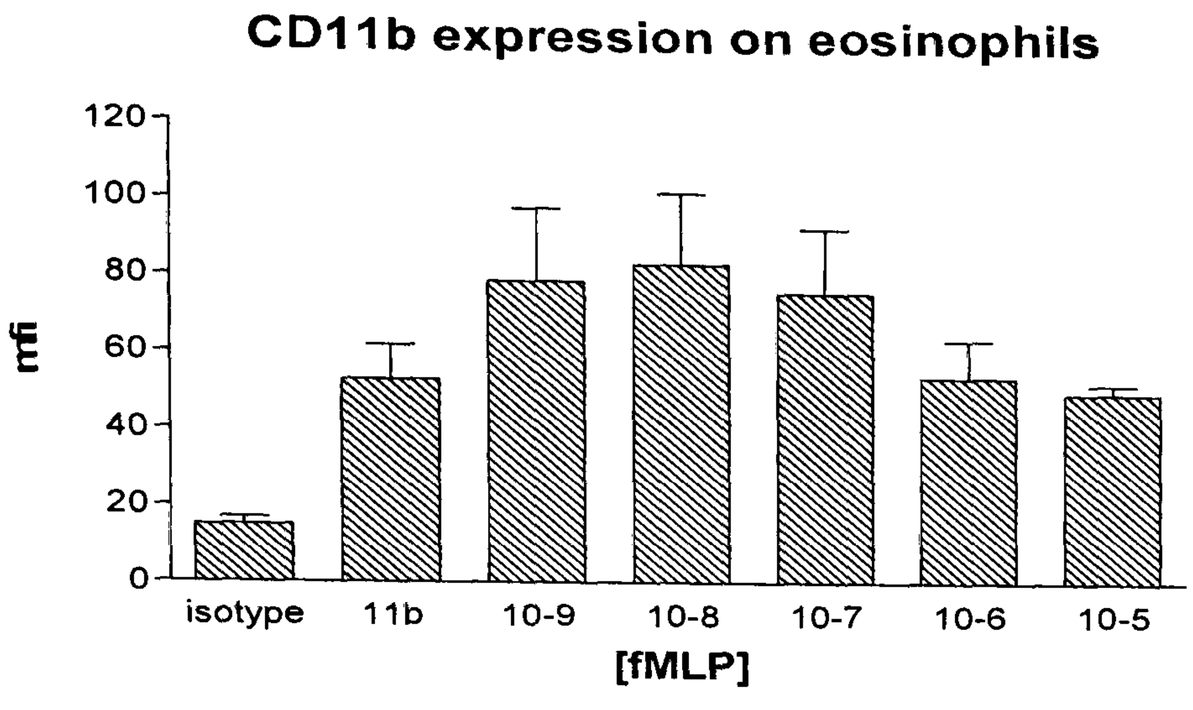
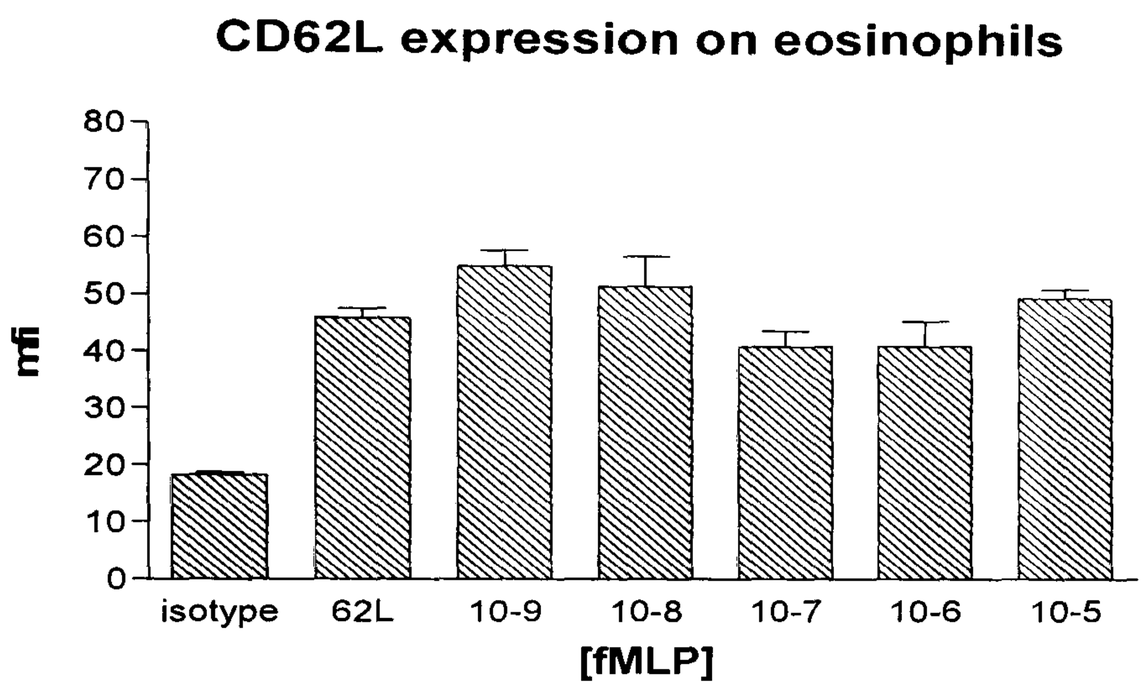


Fig. 3.2 Dose response of CD11b and CD62L to PAF (10^{-4}-10^{-9}M) on neutrophils in lysed whole blood. For CD11b, expression is increased with PAF (10^{-5}M) compared to unstimulated sample (control, *p<0.05) . For CD62L, expression is reduced with PAF (10^{-4}M) compared to PAF (10^{-6}, 10^{-7}, 10^{-8}M), (*p<0.05). n=3, normal subjects.





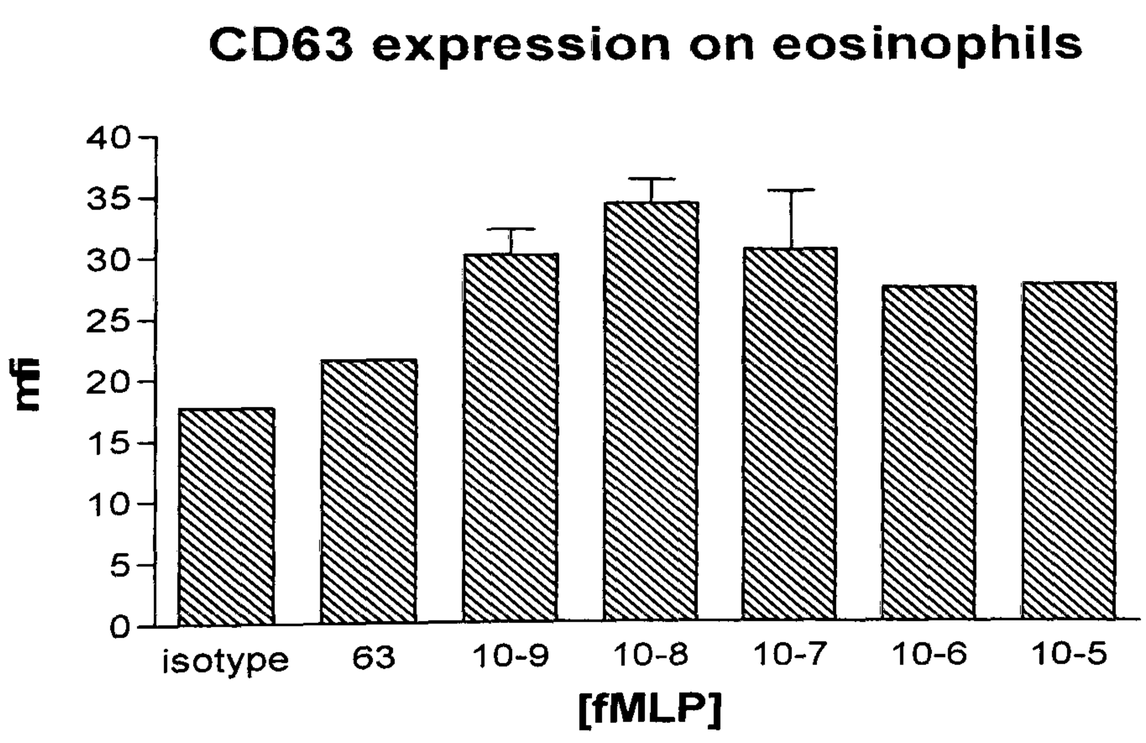
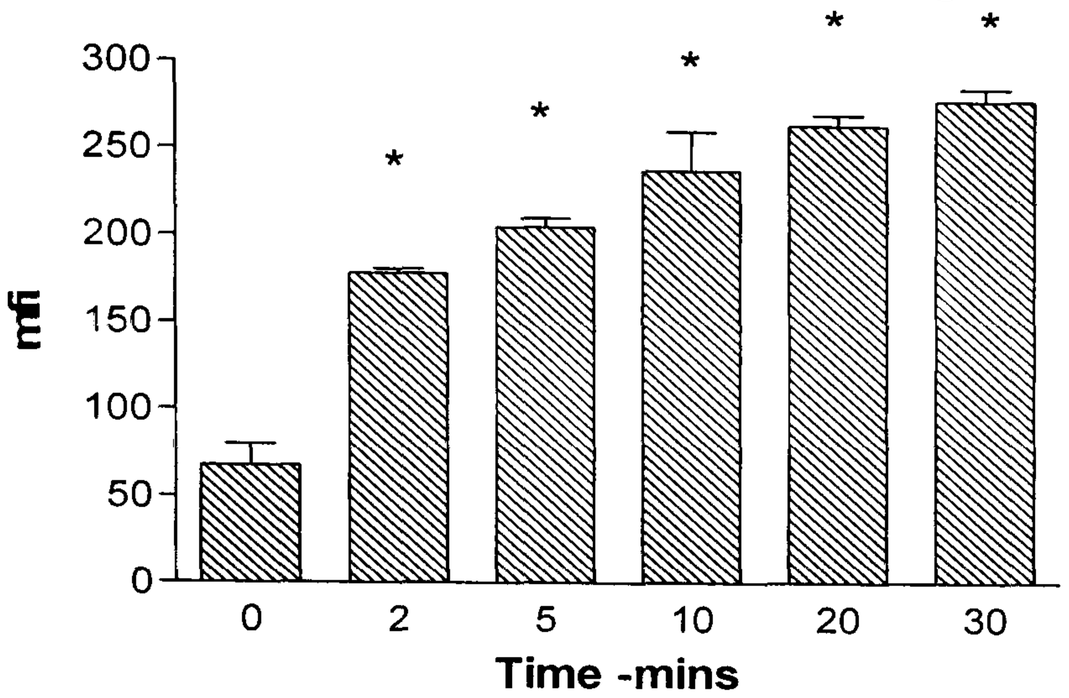


Fig. 3.3 Dose response of CD11b, CD62L, and CD63 on eosinophils in lysed whole blood to fMLP, (10^{-5}-10^{-9}M). Isotype = isotype control. No significant effect of fMLP on surface marker expression on eosinophils. n=3, normal subjects.



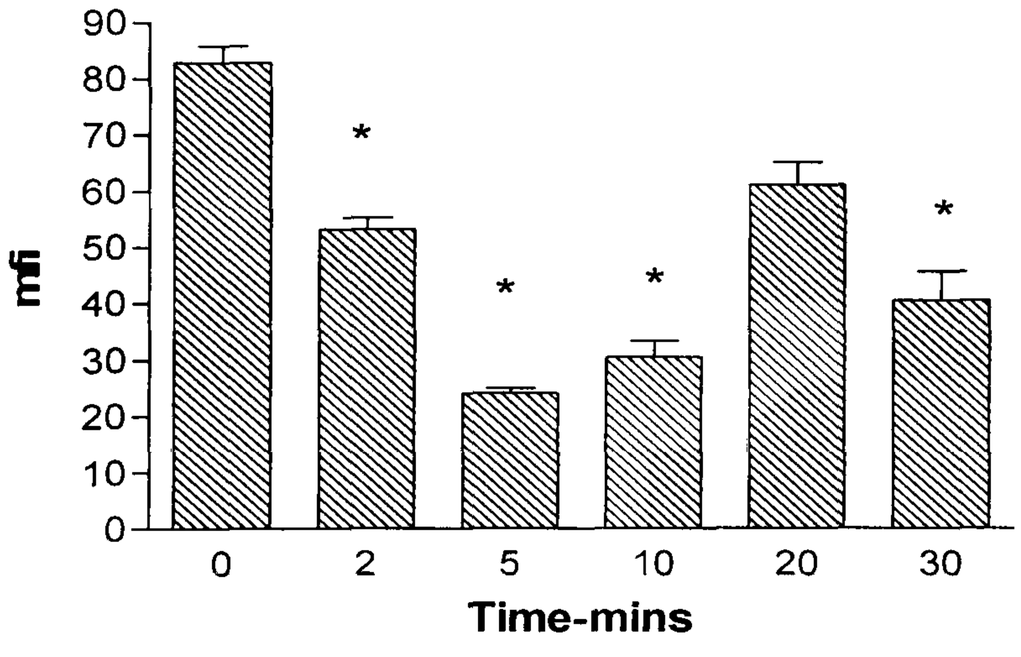


Fig. 3.4 CD11b and CD62L expression on neutrophils and fMLP (10^{-7}M) stimulation-time course study. Upregulation of CD11b with fMLP, and down regulation of CD62L over time (*p<0.05 compared to time- 0 minutes). n=3, normal subjects.

3.3.3 *Surface molecule expression on unstimulated lysed whole blood*

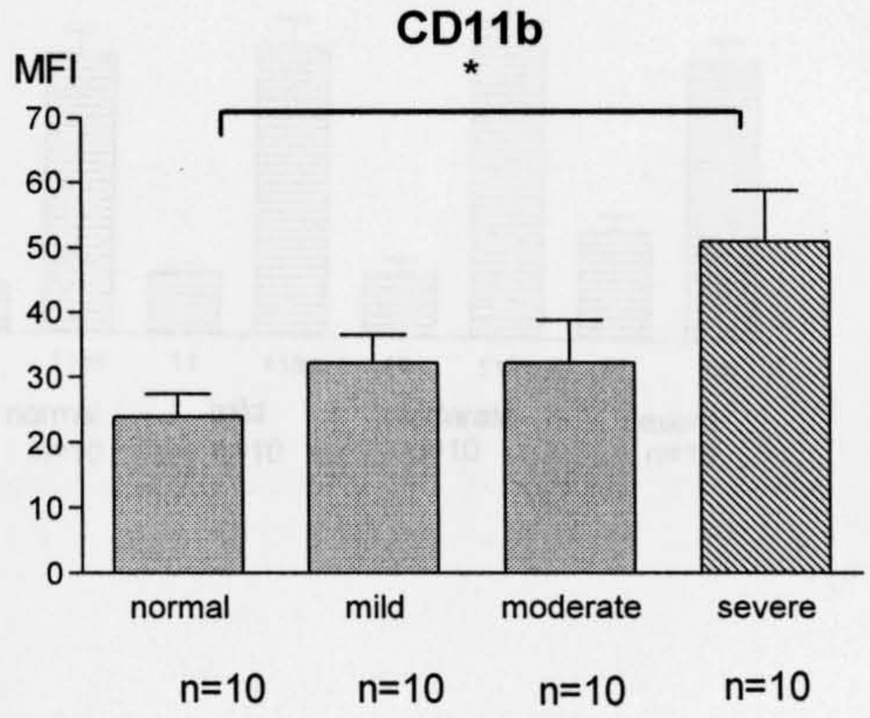
Study of the baseline expression of activation molecules in unstimulated whole blood from normal subjects and in asthmatics showed that there was increased expression of CD35 and CD11b on neutrophils in patients with severe corticosteroid - dependent asthma compared to normal subjects ($p<0.05$), but not compared to mild and moderate asthmatics. There was no difference in the expression of CD62L or CD18, (fig 3.5).

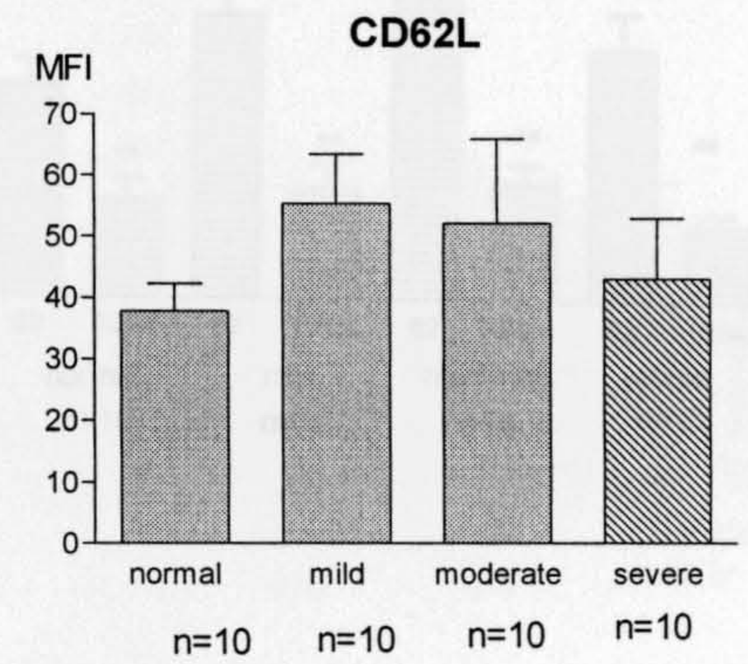
3.3.4 *Surface molecule expression on stimulated lysed whole blood*

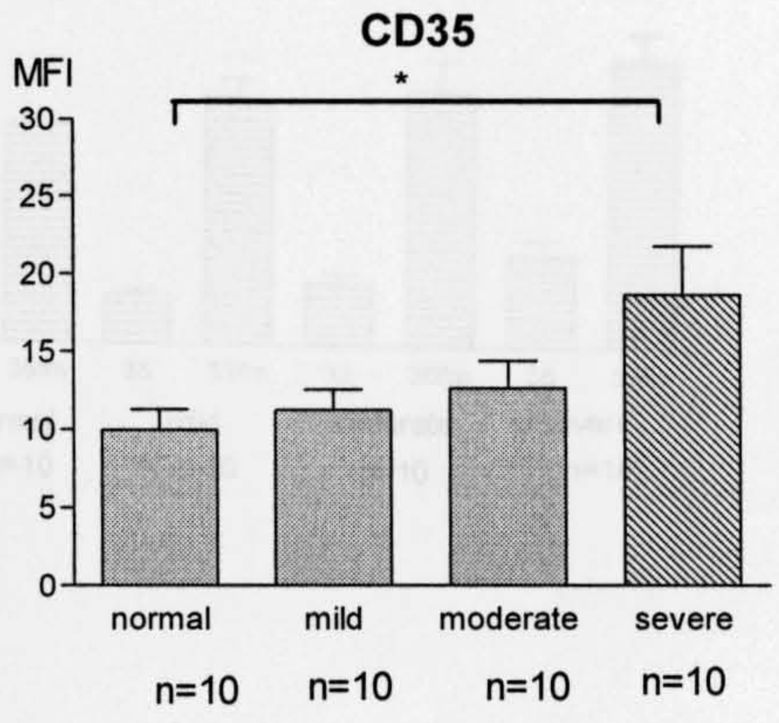
Stimulation of neutrophils in lysed whole blood by fMLP resulted in a significant increase in CD11b, CD35, and CD18 surface molecules on neutrophils in normal subjects and all patient groups as well as a significant decrease in CD62L (fig 3.6). Stimulation of peripheral blood neutrophils with PAF also resulted in significant upregulation of CD11b, CD18, and CD35 in all groups, with downregulation of CD62L only in normal subjects, and not in asthmatics (fig 3.7). In eosinophils, there was no difference in the expression of surface molecules on unstimulated eosinophils in lysed whole blood between the patient groups or normal subjects. CD11b and CD62L levels were increased on eosinophils after stimulation with fMLP in normal subjects (CD11b only), and in mild and moderate asthmatics (CD11b and CD62L), but not in severe asthmatics (figs 3.8 and 3.9).

3.3.5 *Effect of prednisolone on surface molecule expression on neutrophils in mild and severe asthmatics*

Prednisolone at a dose of 30 mg for one week had no effect on the baseline level of expression of CD11b, CD62L, CD35 or CD18 in severe patients, but in mild asthmatics there was a significant reduction in the level of CD62L expression ($p< 0.05$), (fig. 3.10). In contrast, there was significant reduction in the expression of surface molecules CD11b and CD62L on eosinophils in both mild and severe patients (fig. 3.11), and reduction in CD18 levels in mild asthmatics, with no effect on CD35 expression in either group. The mean prednisolone level in mild patients was 1098+/- 195 nmol/l, compared with 848.1+/- 162.7 nmol/l in the severe group. Levels were not available for 1 patient in each group, and at least 1 severe patient had not taken prednisolone according to levels or else was not absorbing it. Otherwise patients were compliantwithsteroids.







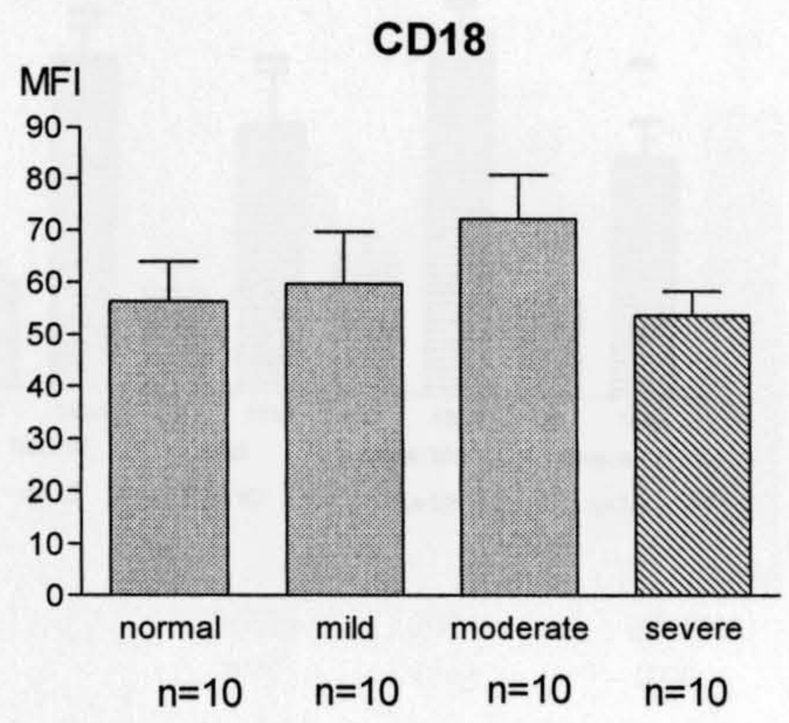
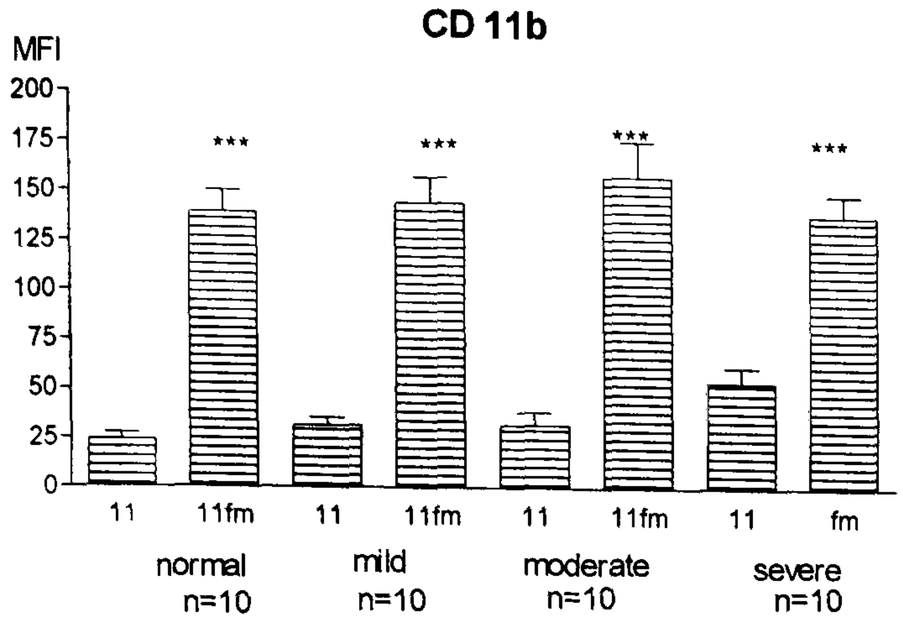
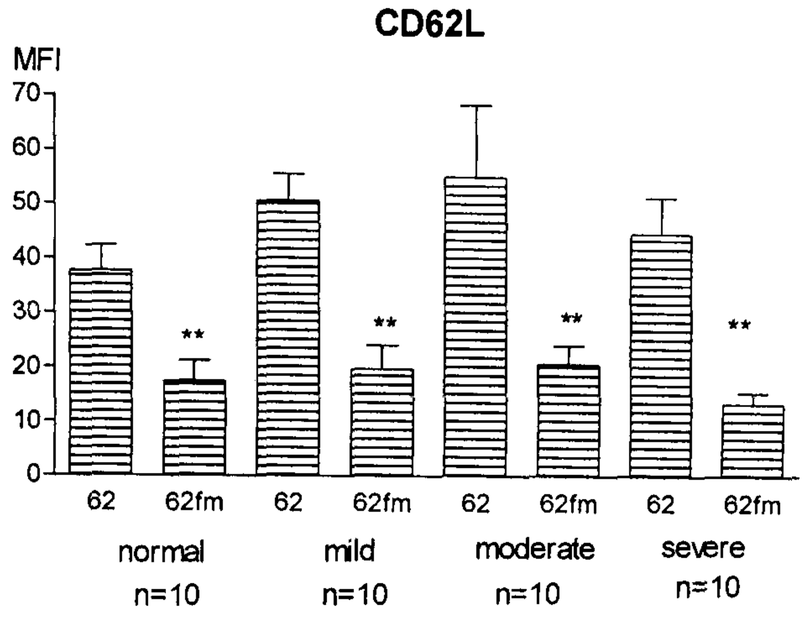
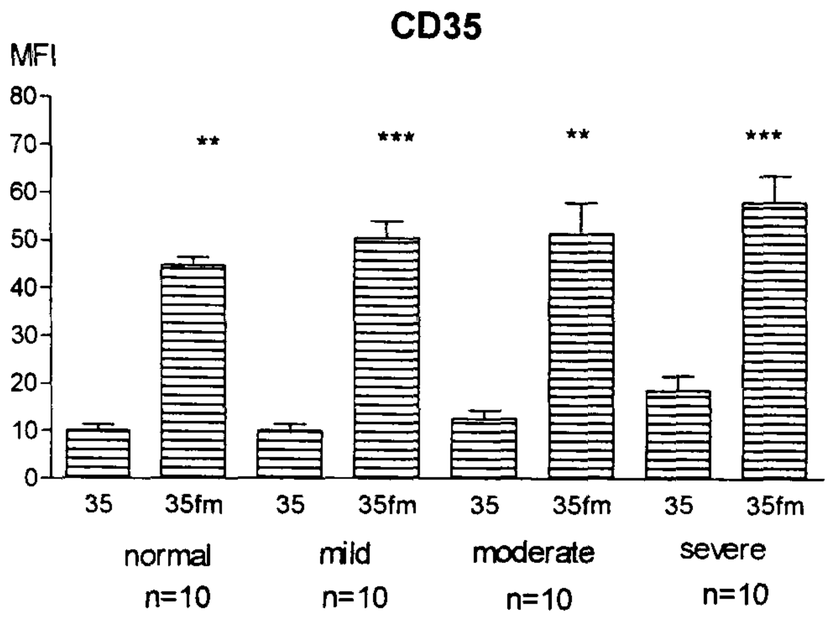


Fig. 3.5 Expression of CD11b, CD62L, CD35, and CD18 in normal subjects, and mild, moderate and severe patients in neutrophils in lysed whole blood showing increased expression of CD11b and CD35 in corticosteroid-dependent asthmatics compared with normal subjects. (*P<0.05).







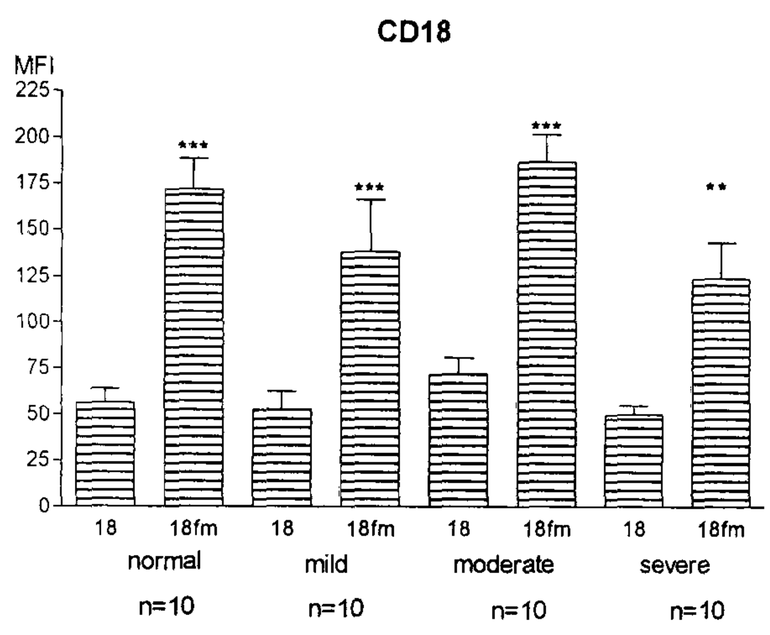
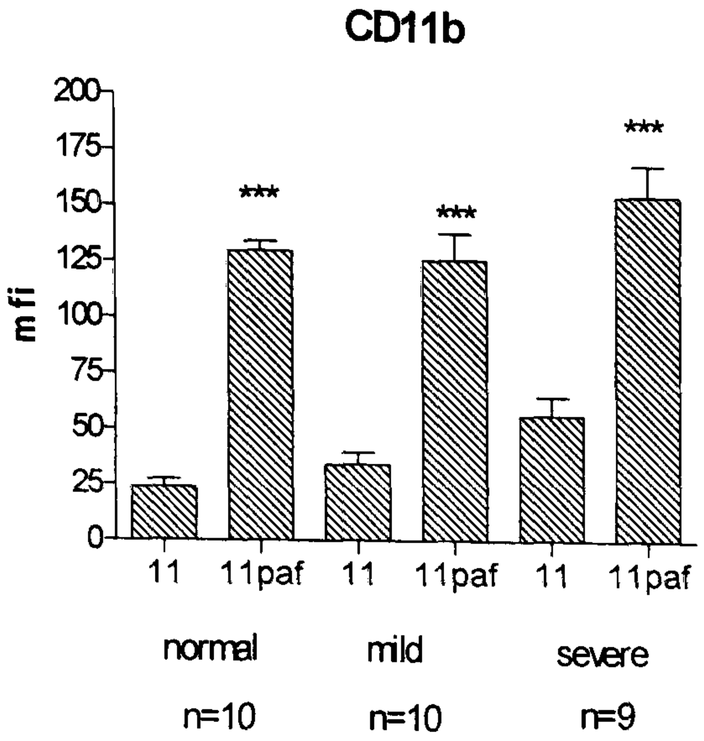
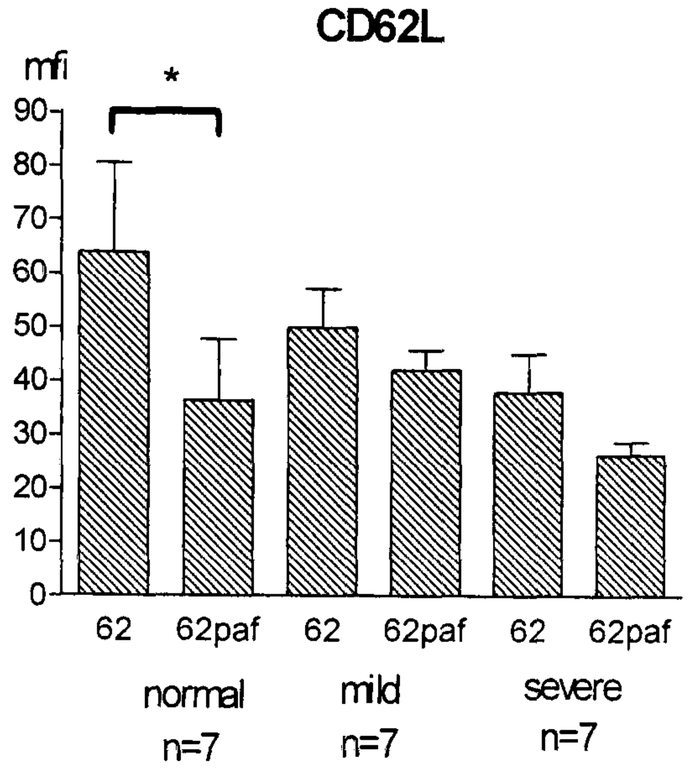
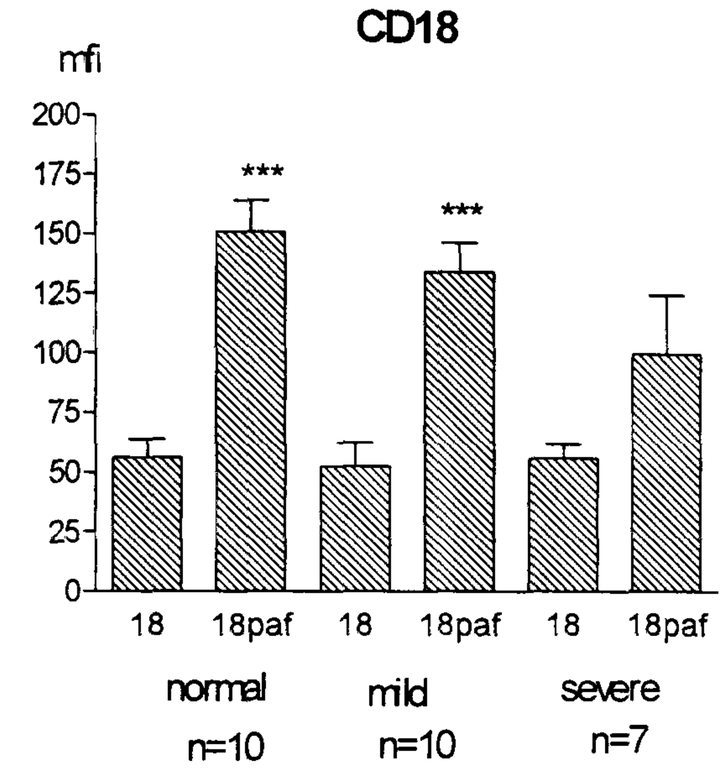


Fig.3.6. Stimulation of lysed whole blood with fMLP in normal subjects and asthmatics of different severity showing significant upregulation of CD11b, CD18, CD35 and downregulation of CD62L on neutrophils compared to unstimulated neutrophils. Asterisks indicate a significant difference compared to resting levels. No difference was seen between stimulated samples between the four groups with any of the surface markers. (P<0.01, ***P<0.0001)**







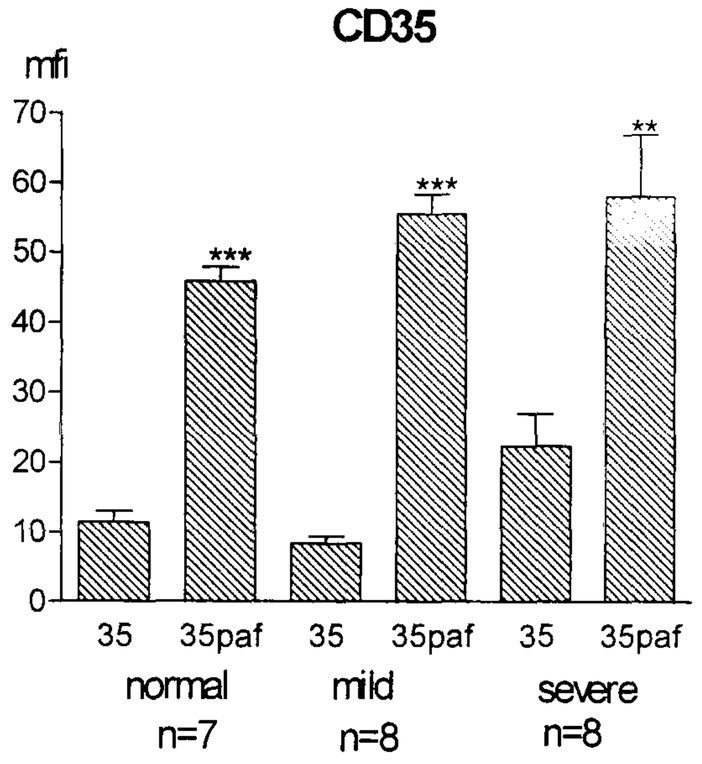
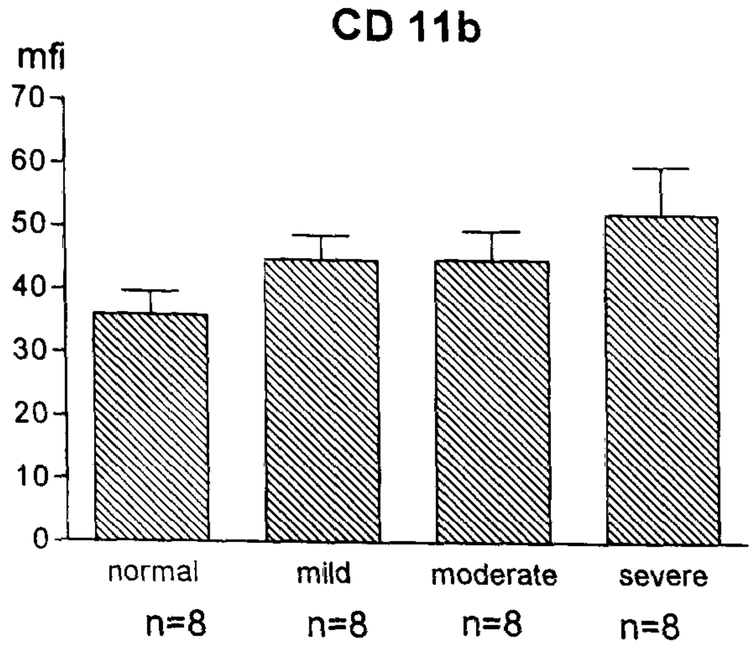
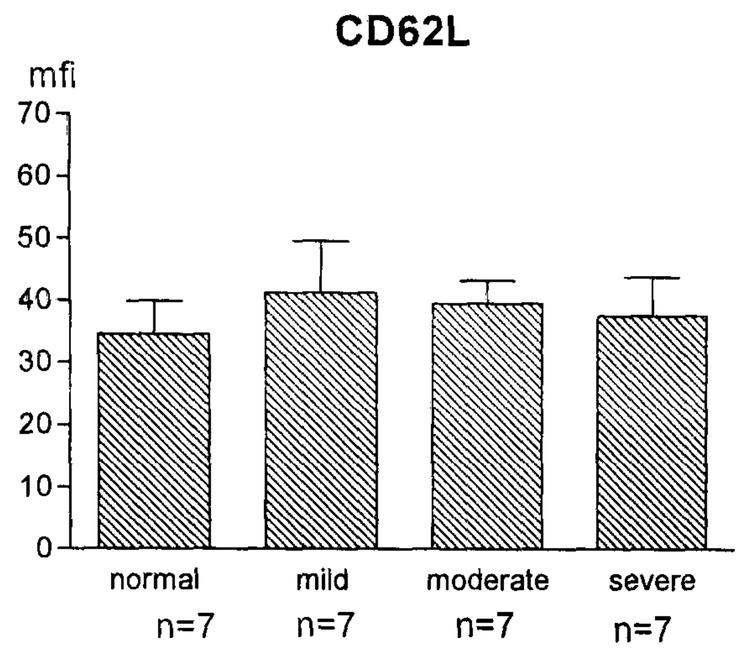
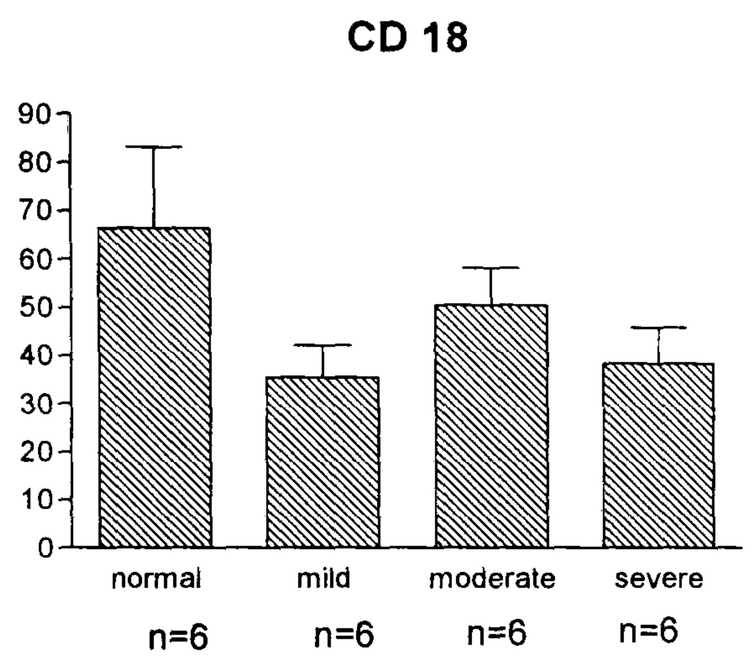


Fig. 3.7 Effect of PAF (10^{-6}M) stimulation on CD11b, CD62L, CD18, and CD35 expression on neutrophils in lysed whole blood. in normal subjects and asthmatics of different severity. Upregualtion of CD11b, CD18, and CD35 in normal subjects and asthmatics, and down regulation of CD62L in normal subjects only (*p <0.0001, **p <0.002, * P<0.05).**







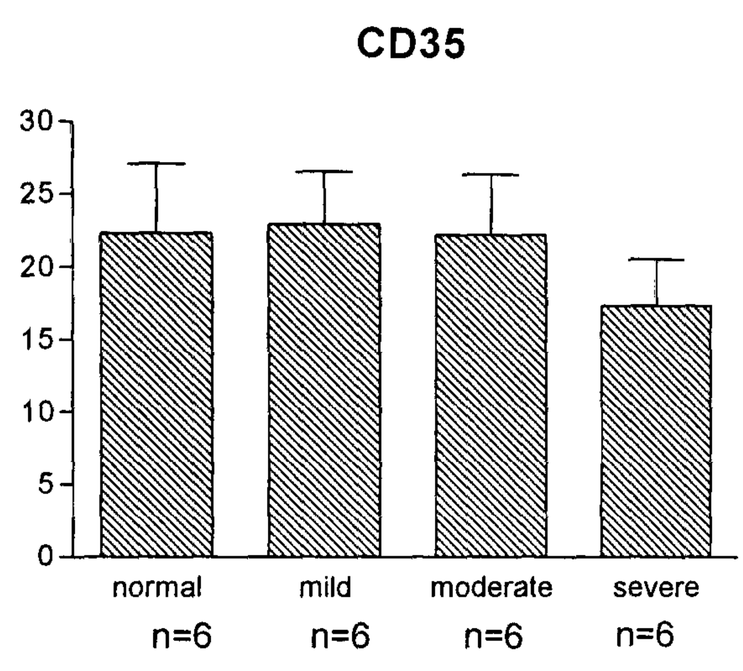
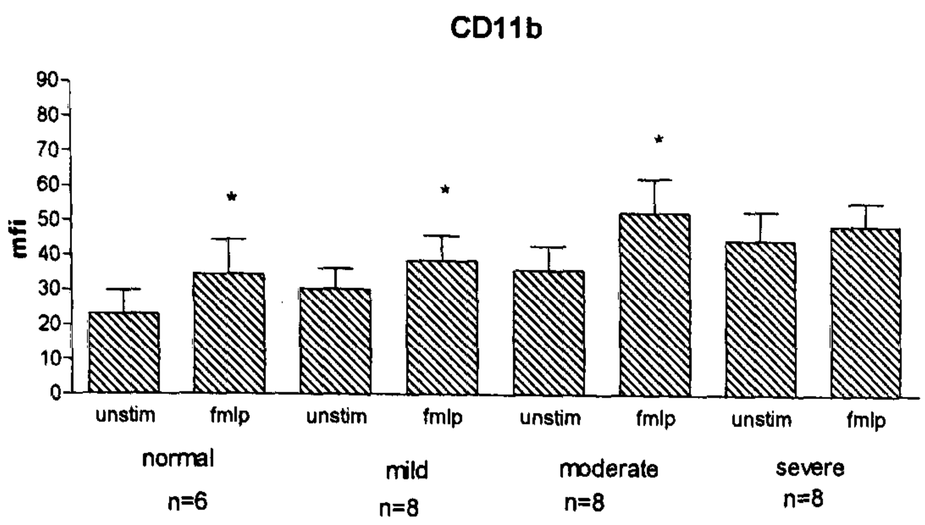
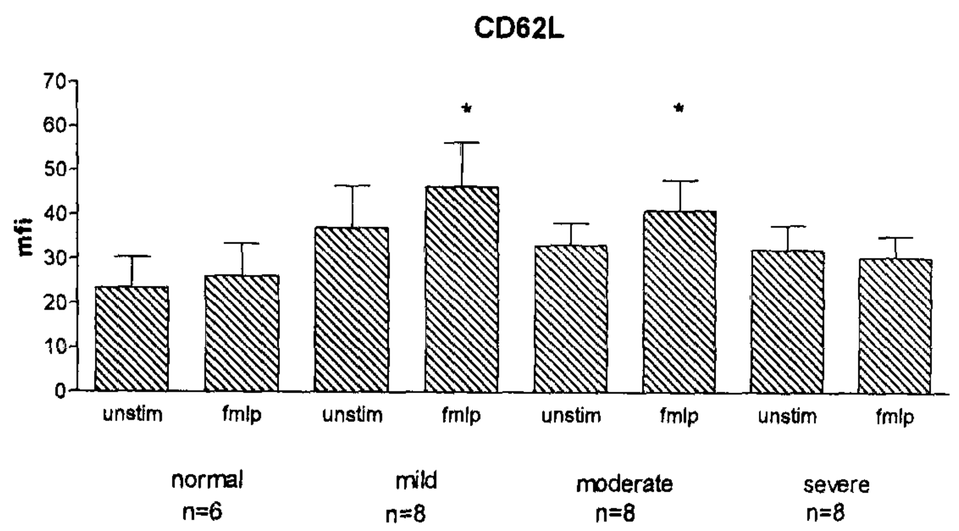
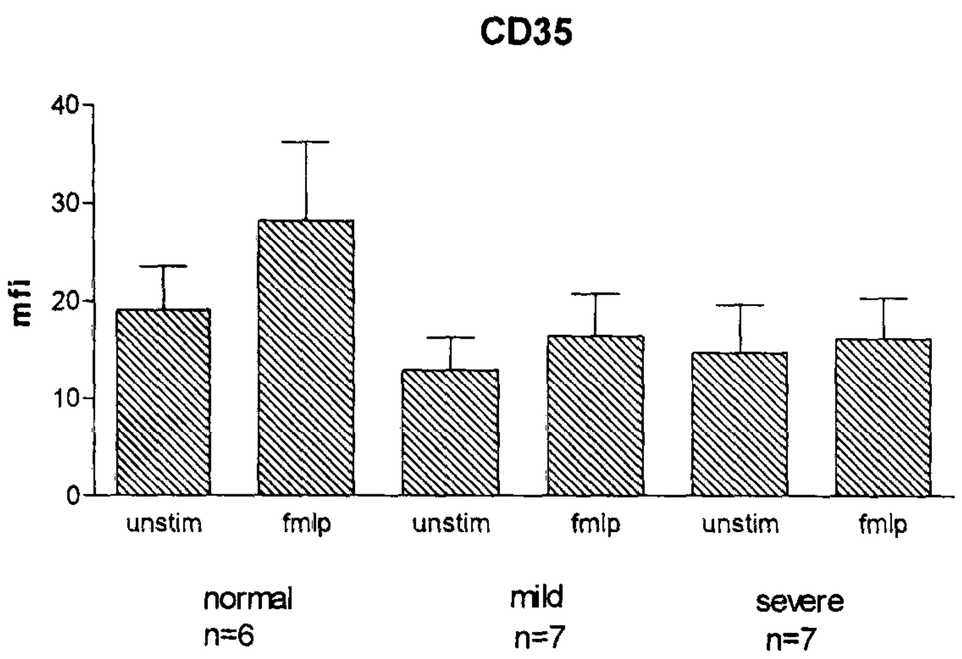


Fig. 3.8 Expression of CD11b, CD62L, CD18, and CD35 on resting eosinophils in lysed whole blood in normal subjects and asthmatics of different severity. No significant difference was seen in surface marker expression between the groups.







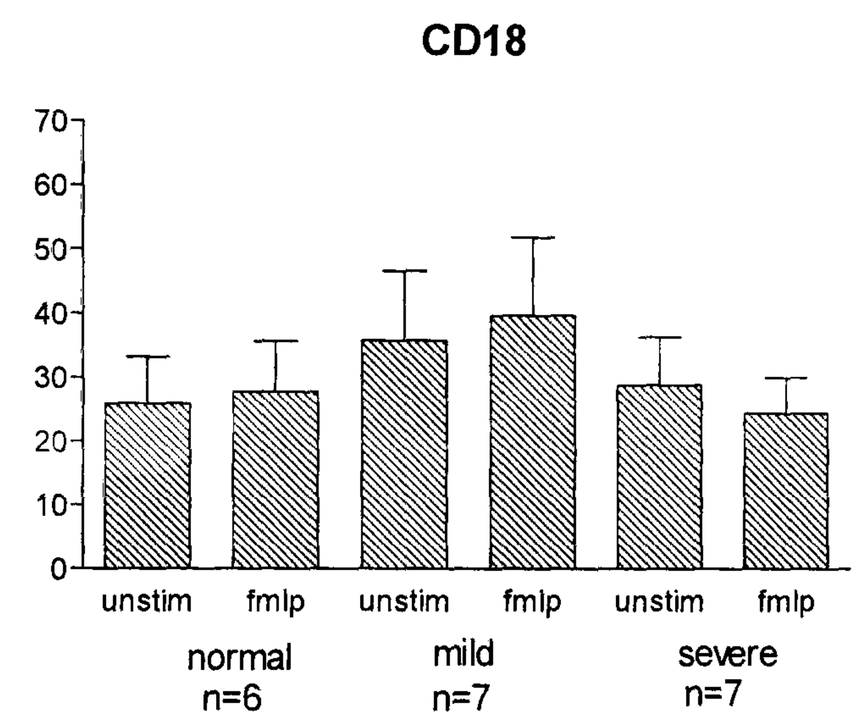
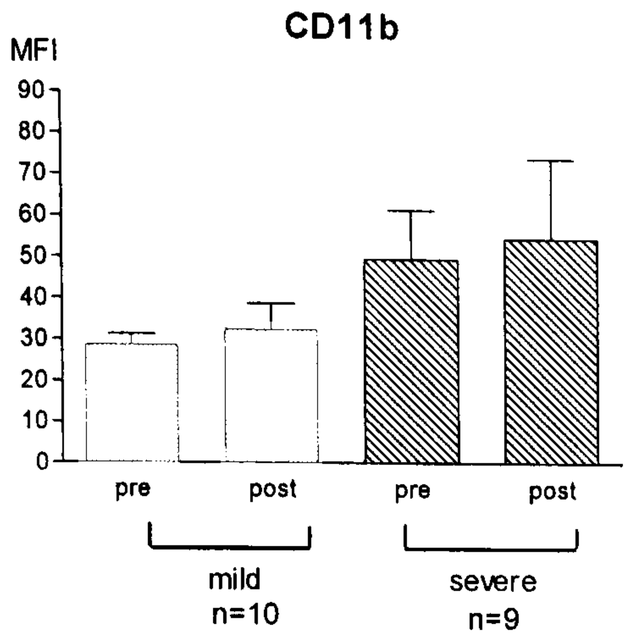
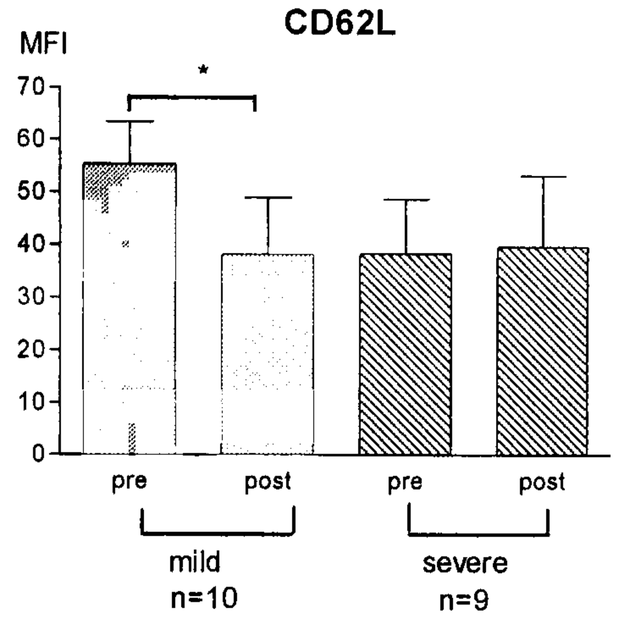
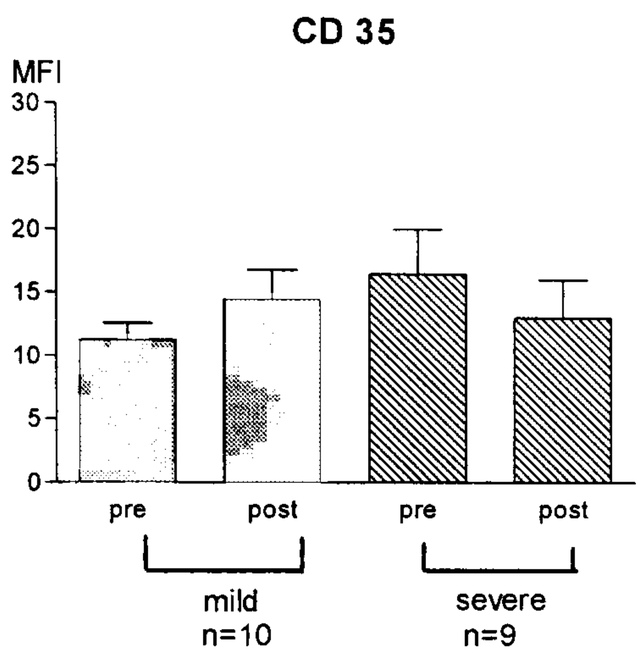


Fig. 3.9 Effect of stimulation of whole blood with fMLP (10^{-7}M) on expression of CD11b, CD62L, CD35, and CD18 on eosinophils in normal subjects and asthmatics of different severity. CD11b expression is increased in normal subjects and mild and moderate asthmatics after fMLP compared to unstimulated blood (*p<0.05). CD62L expression is increased in mild and moderate asthmatics with fMLP compared with unstimulated blood (* p<0.05).







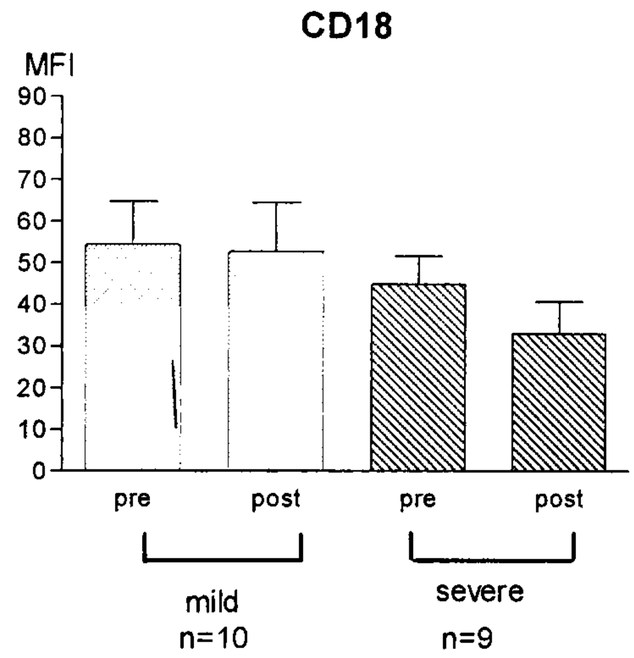
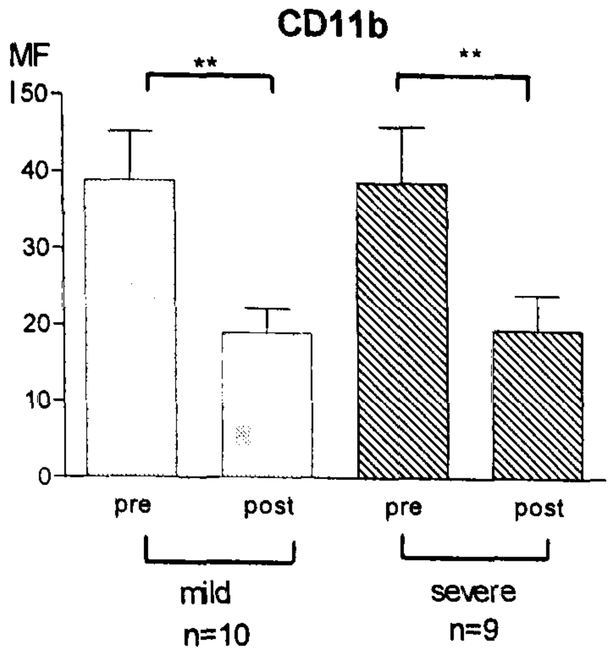
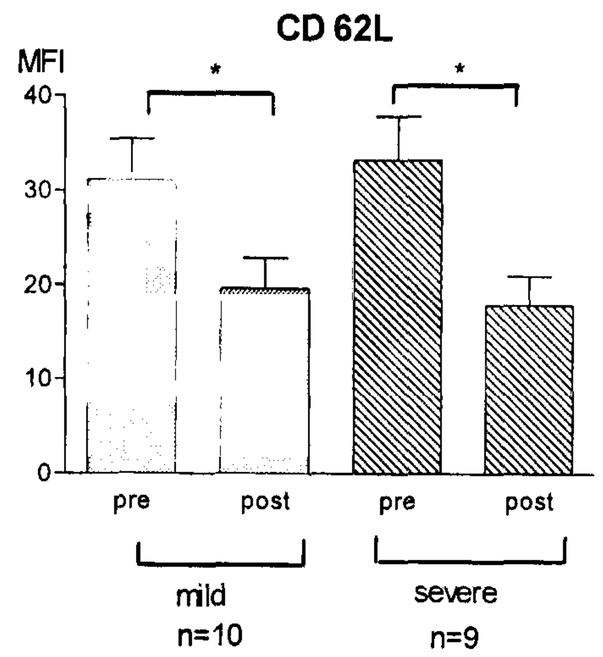
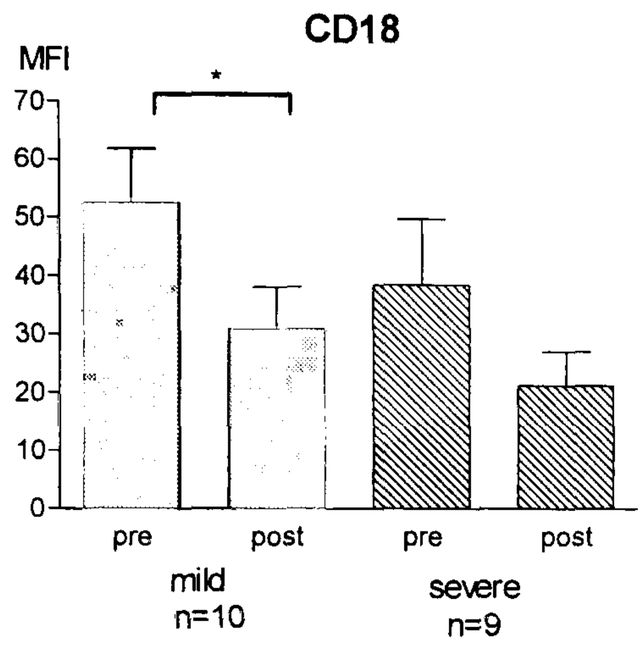


Fig. 3.10 Effect of a 1 week course of prednisolone 30mg/day on surface activation molecules on peripheral blood neutrophils in mild and severe asthmatics. CD62L expression was decreased on neutrophils in mild asthmatics following prednisolone, but no effect was seen on neutrophils from patients with corticosteroid-dependent asthma.
(* P<0.05)







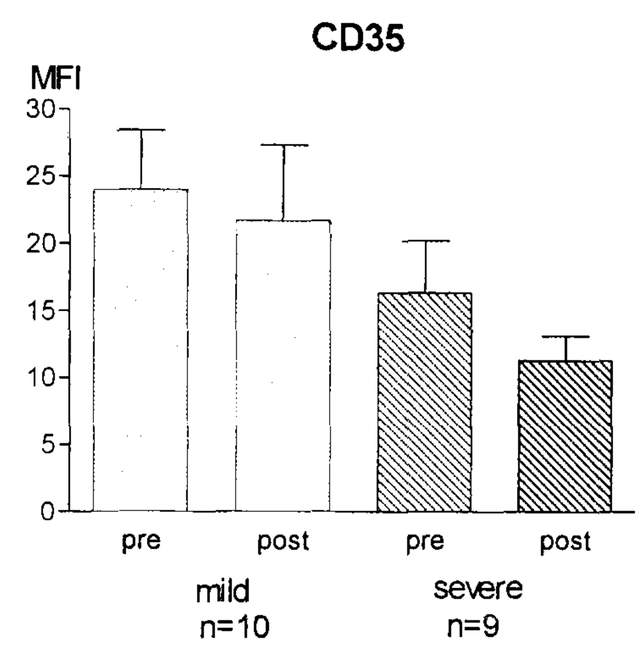


Fig. 3.11 Effect of prednisolone 30mg for 1 week on surface activation molecules on peripheral blood eosinophils, showing reduced expression of surface molecules on eosinophils from both mild and severe asthmatics. No effect was seen on CD 35 expression.(P=<0.007,*P<0.05).**

3.3.6 *Effect of dexamethasone on activation molecule expression in peripheral whole blood*

Whole blood pre-incubated ex-vivo with dexamethasone (10^{-6} to 10^{-8} M) for 1 hour or 3 hours, produced no difference in the expression of CD11b or CD62L on neutrophils or eosinophils in unstimulated or fMLP stimulated blood (figs. 3.12-3.14).

3.3.7 *Surface molecule expression on isolated granulocytes*

In order to see if the lack of effect of dexamethasone on surface molecule expression in peripheral blood neutrophils and eosinophils was due to an inhibitor of dexamethasone function present in whole blood, the above studies were carried out on isolated granulocytes (1×10^{5} cells per well).

Baseline expression of surface molecules was higher in isolated neutrophils and eosinophils compared to granulocytes in lysed whole blood and this may represent activation of granulocytes by the separation techniques involved, such as Ficoll separation and centrifugation. In the severe group, there was significant increase in CD11b expression and decrease in CD62L on isolated neutrophils after stimulation with fMLP and PAF but not in the mild group (fig. 3.15). Increased CD11b expression was seen on unstimulated isolated eosinophils in the mild group compared to the severe group, and there was no significant change following stimulation with fMLP and PAF (fig 3.16). Similar to the effect of prednisolone on peripheral blood neutrophils in mild asthmatics, isolated neutrophils from mild asthmatics showed reduction in the expression of CD62L and CD18 after prednisolone, with no effect of prednisolone on isolated neutrophils from severe asthmatics (fig 3.17). Isolated eosinophils from both mild and severe asthmatics showed a reduction in CD11b and CD62L following prednisolone, in addition to a decrease in CD18 in mild asthmatics (fig 3.17). Interestingly, in severe asthmatic group, fMLP stimulated isolated neutrophils showed an increase in CD11b expression after additional prednisolone suggesting that prednisolone may have resulted in further neutrophil activation in this situation. However, prednisolone caused a significant downregulation in CD11b, CD62L, and CD18 on isolated eosinophils in mild asthmatics, with a corresponding reduction in CD11b approaching significance on isolated eosinophils in the severe group ($p=0.06$) (fig. 3.18). Preincubation of isolated granulocytes with dexamethasone again appeared to have no effect on the expression of surface molecules on either unstimulated or

stimulated cells on neutrophils or eosinophils, confirming the results seen in whole blood pre-incubated ex-vivo with dexamethasone (figs. 3.19 and 3.20).

3.3.8 *Neutrophil oxidative burst in normal subjects, and mild and severe asthmatics, and effect of prednisolone*

Dose response studies of oxidative burst measurement in neutrophils using flow cytometry showed that PMA stimulated neutrophil oxidative burst in a dose dependent manner, and maximal stimulation was achieved after 20 minutes as seen on a time course study (n=3, normal subjects, fig.3.21). The results showed that the oxidative burst in mild asthmatics was significantly greater than in normal subjects and severe patients whilst similar levels were seen between normal subjects and severe patients (fig.3.22). Further, the oxidative burst was reduced in mild patients after prednisolone but not in the severe patients.

3.3.9 *Neutrophil elastase in peripheral blood neutrophils*

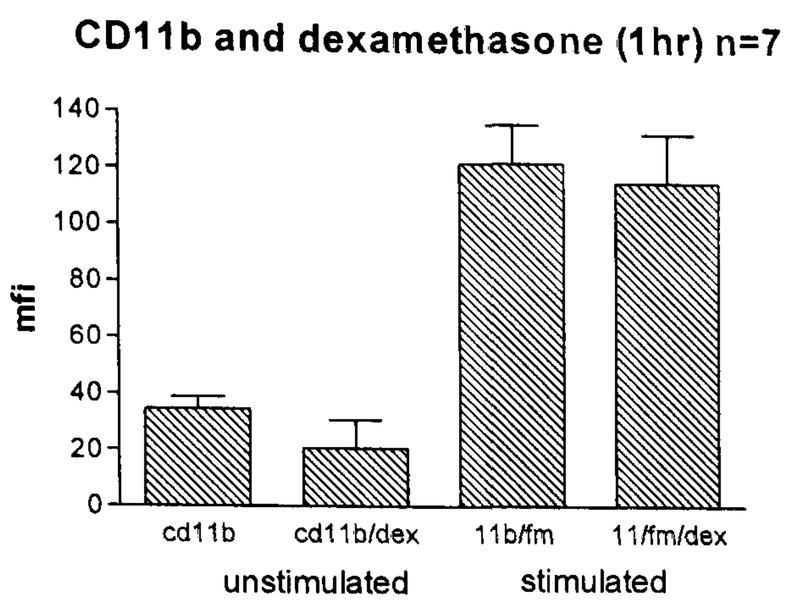
Following the recommended protocol for measurement of neutrophil elastase, a dose of 1µl of DHR110 resulted in an MFI similar to that of 5µl and 10µl, and time course study suggested 20 minutes incubation was optimal (n=3, normal subjects, fig 3.23). No significant difference was seen in the levels of neutrophil elastase in peripheral blood neutrophils between normal subjects, mild, and severe asthmatics. Although there appeared to be reduction in neutrophil elastase levels after prednisolone, this was not statically significant (fig 3.24).

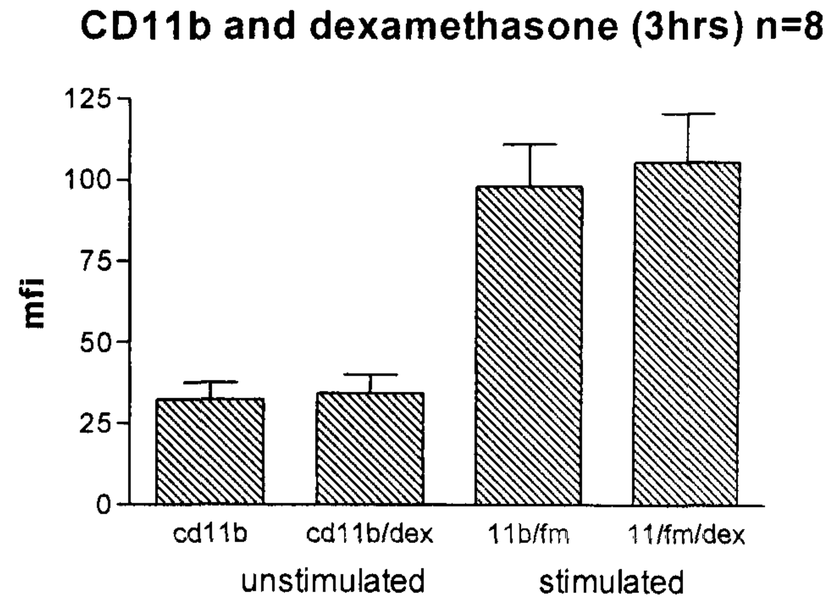
3.3.10 *Serum cytokine levels and the effect of prednisolone*

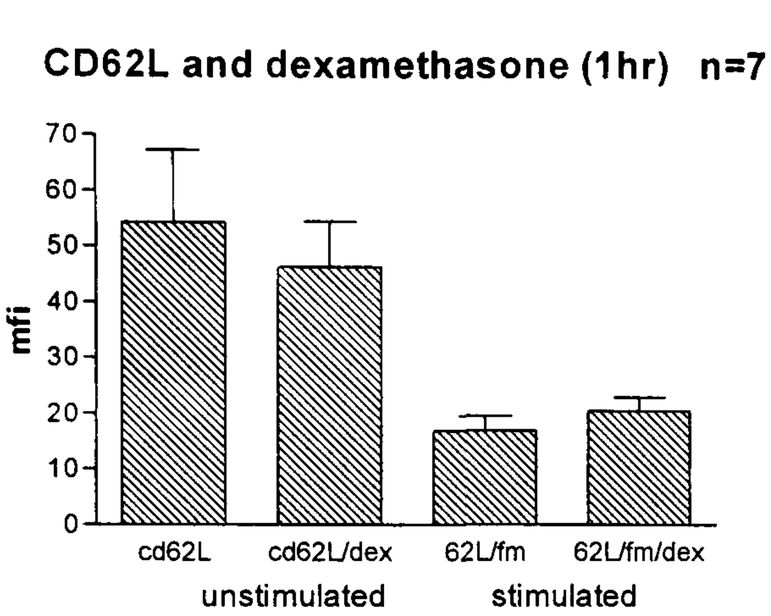
IL-8 was not detectable in serum in mild or severe asthmatics at baseline (visit 1), in unstimulated blood. After 24 hours blood culture however, IL-8 levels were detectable in both groups. IL-8 levels in mild asthmatics appeared elevated after stimulation with IL-1β, LPS, and GM-CSF compared to severe asthmatics, but this was not statisitically significant. In the mild group however, there was a significant decrease in LPS and GM-CSF induced IL-8 production after prednisolone therapy, and this effect of prednisolone was not seen in the severe group (fig 3.25).

3.3.11 *Neutrophil myeloperoxidase release*

No difference was seen in the basal or IL-1β stimulated MPO levels in isolated granulocytes between mild and severe asthmatics, and there was no obvious effect of prednisolone therapy (fig 3.26).







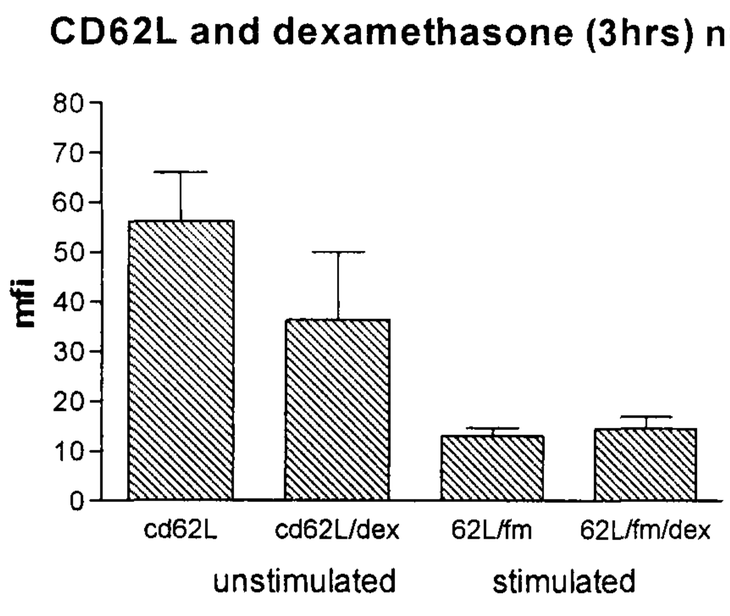
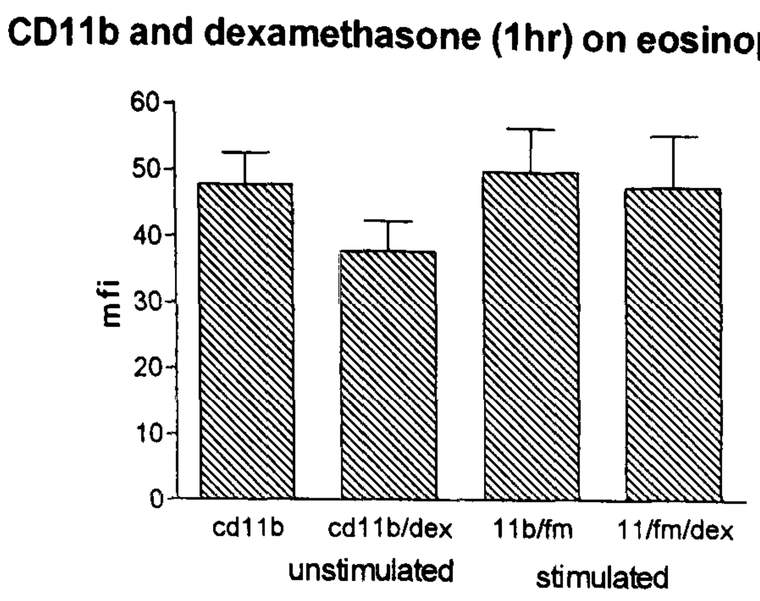
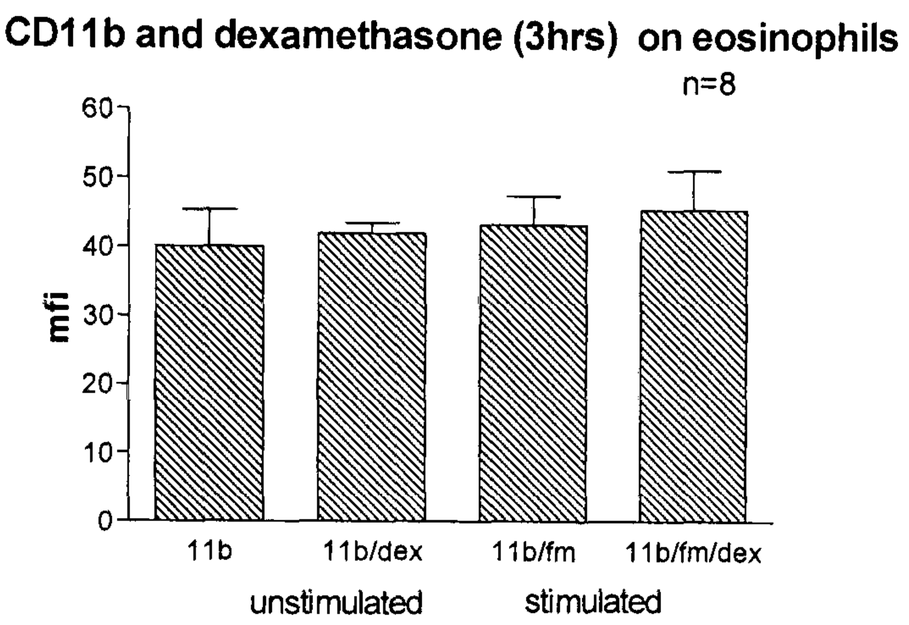
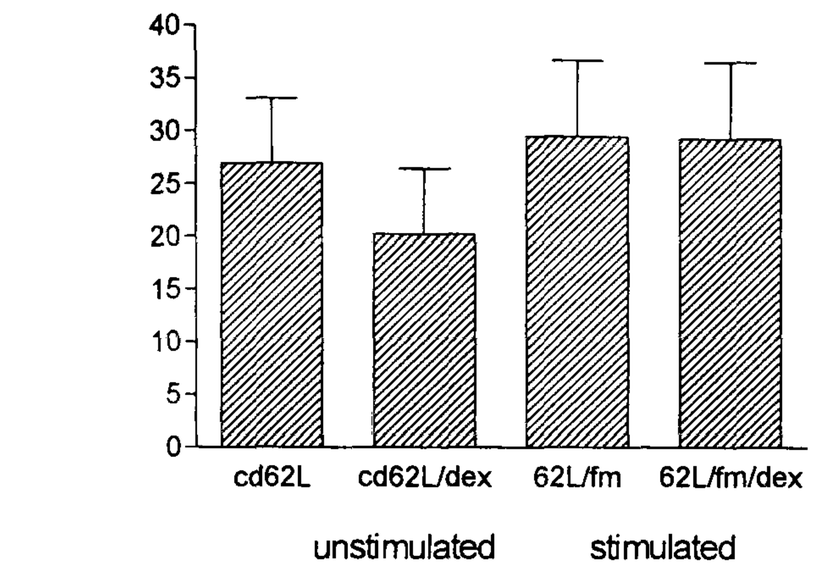


Fig. 3.12 Effect of dexamethasone (10^{-6}M) on CD11b and CD62L expression on peripheral blood neutrophils, in unstimulated and fMLP (10^{-7}M) stimulated whole blood. No significant effect of dexamethasone was seen in unstimulated or stimulated neutrophils.







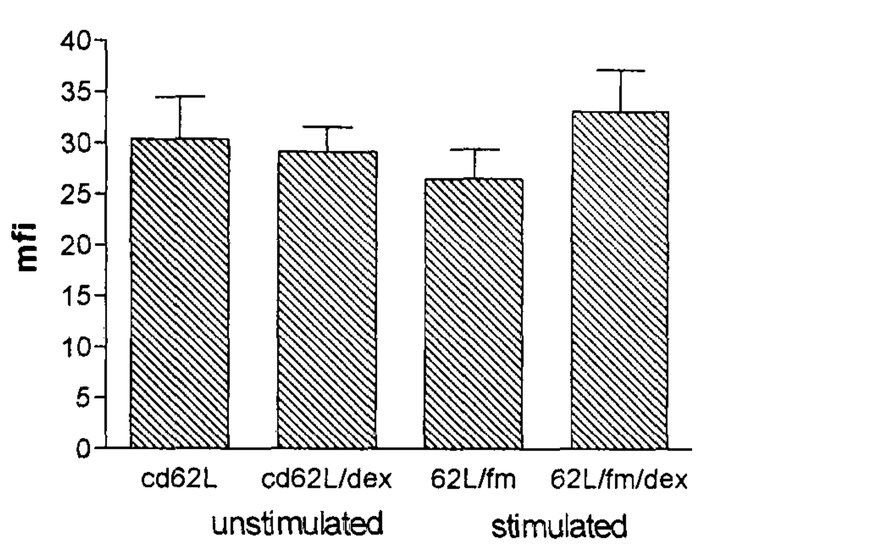
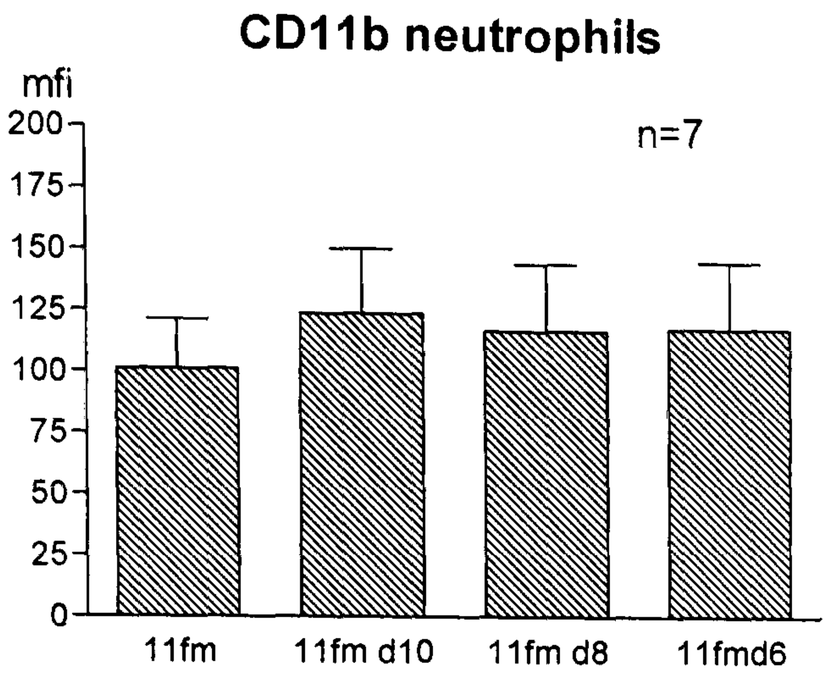
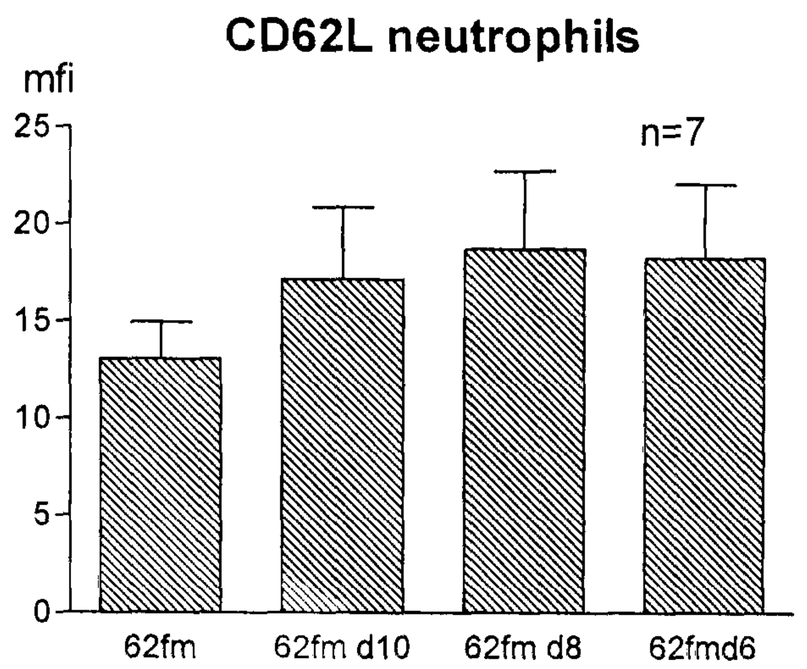
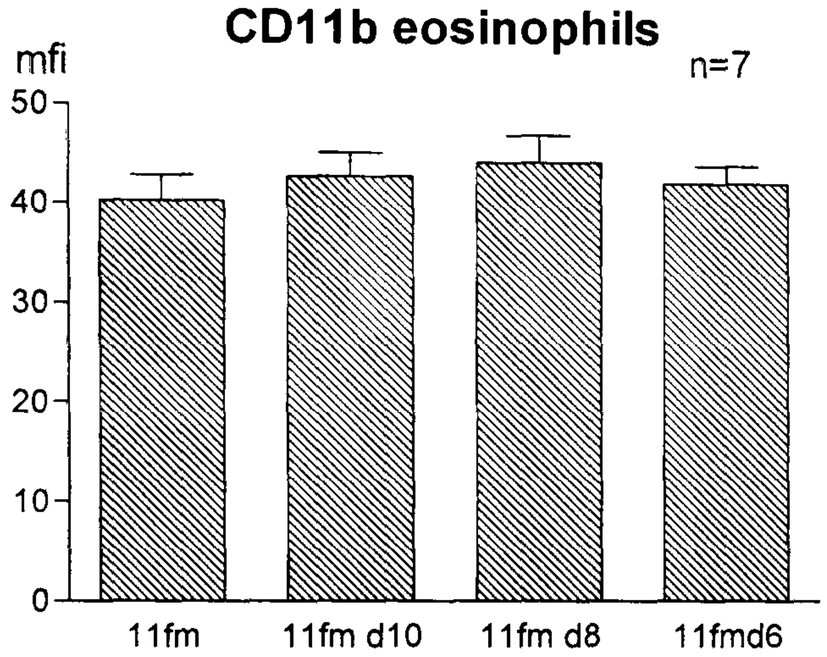


Fig. 3.13 Effect of dexamethasone (10^{-6}M) on CD11b and CD62L expression on peripheral blood eosinophils, in unstimulated and fMLP (10^{-7}M) stimulated whole blood. No effect of dexamethasone was seen in stimulated or unstimulated whole blood.







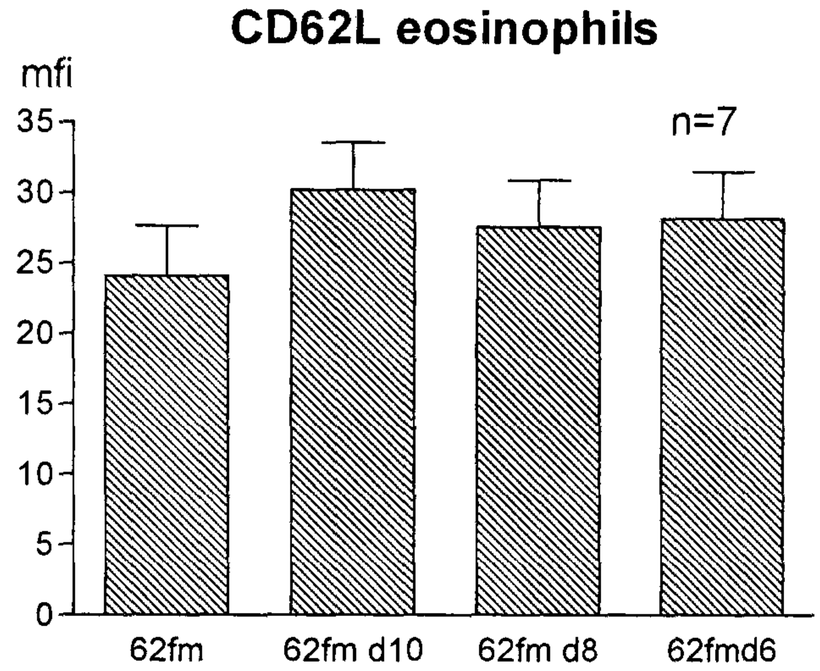
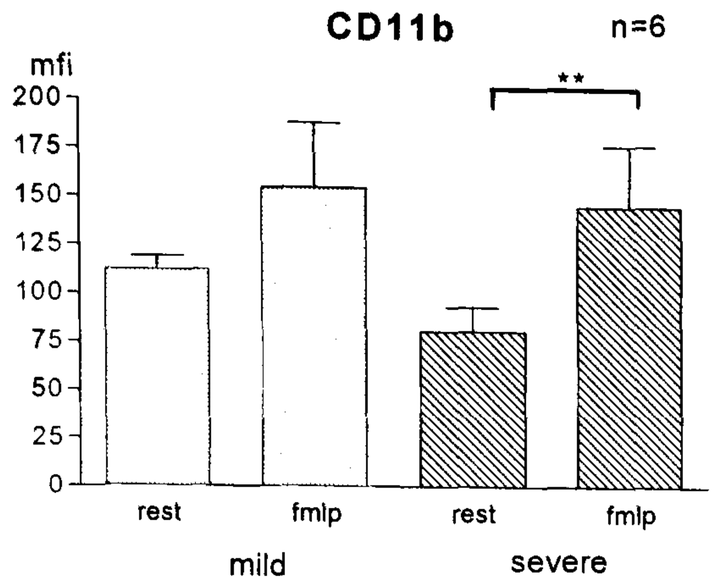
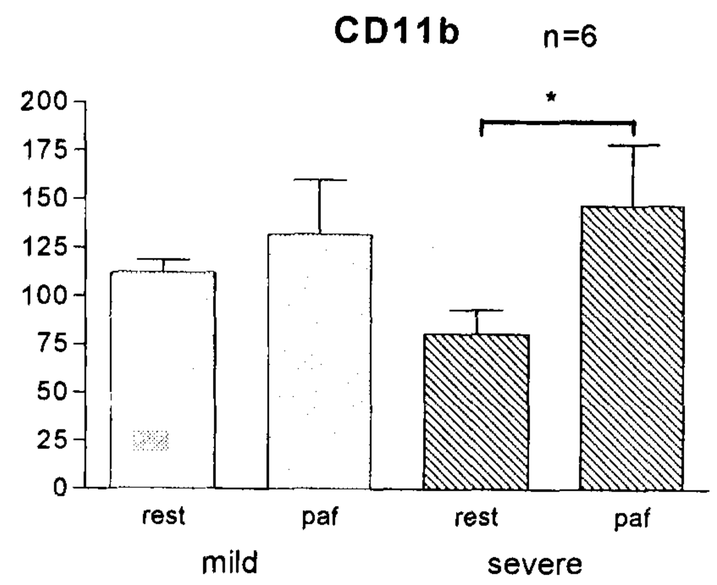
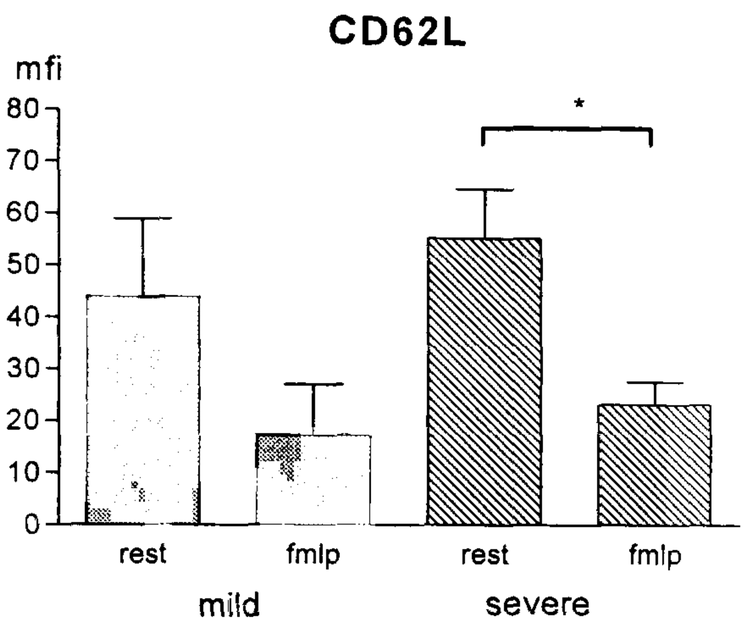
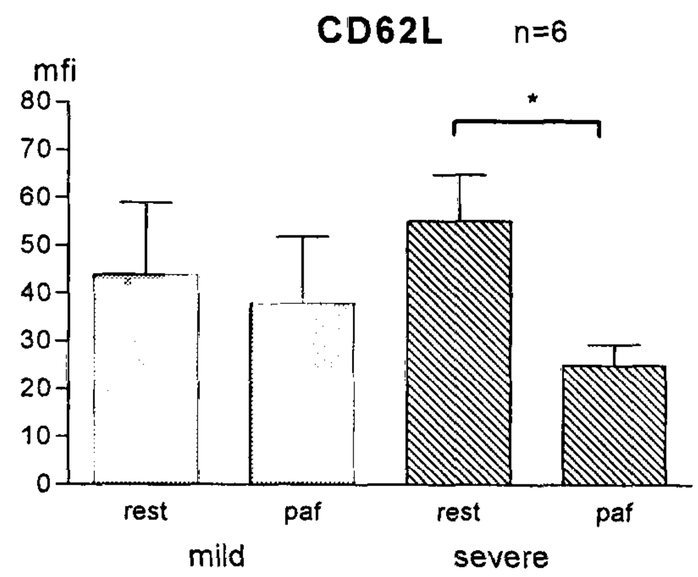


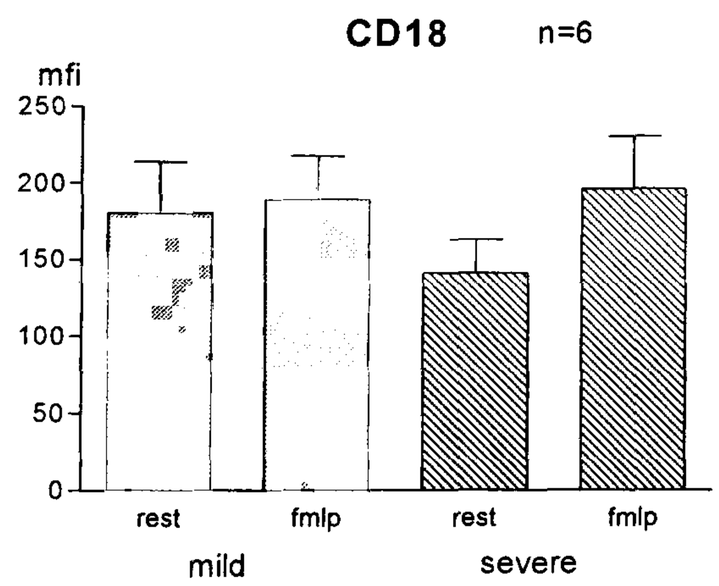
Fig. 3.14 Effect of dexamethasone (10^{-6}- 10^{-8} M) on whole blood neutrophils and eosinophils stimulated with fMLP (10^{-7}M) and incubated for 1 hour. No effect of dexamethasone was seen.











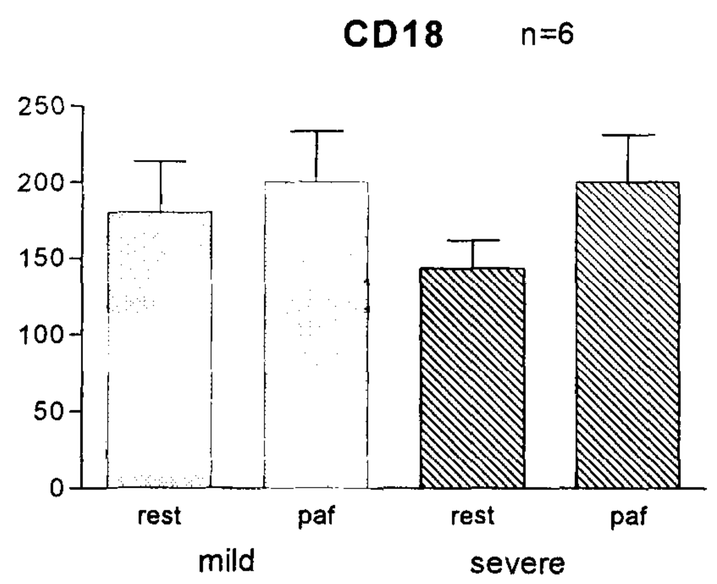
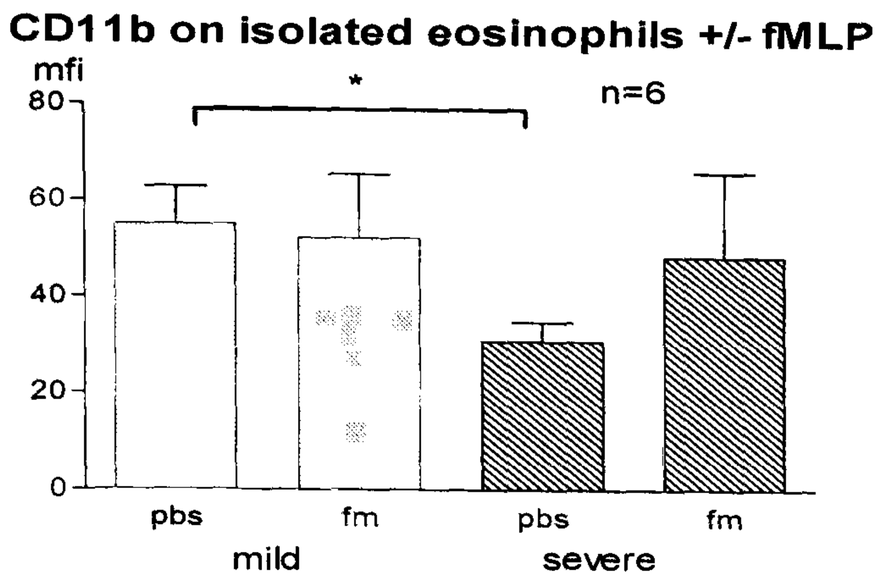
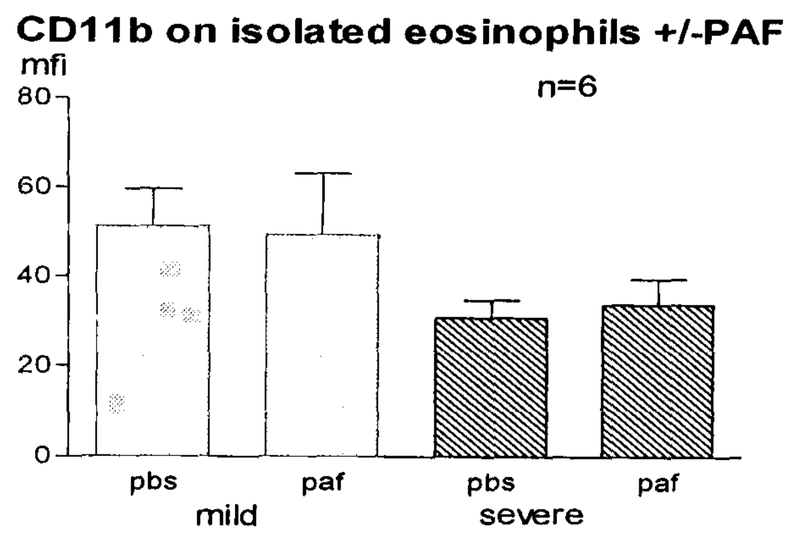
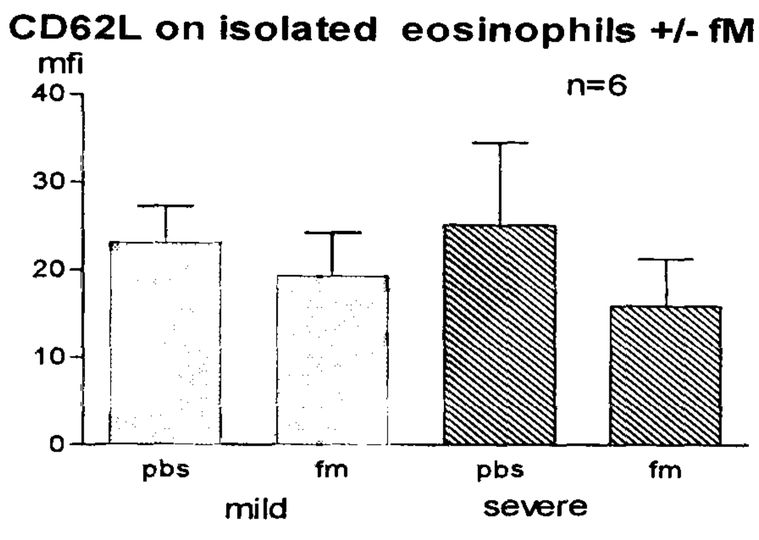
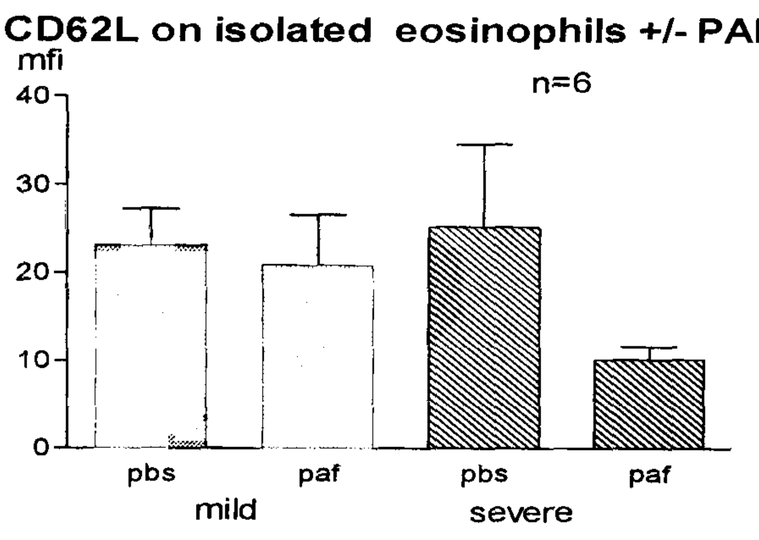


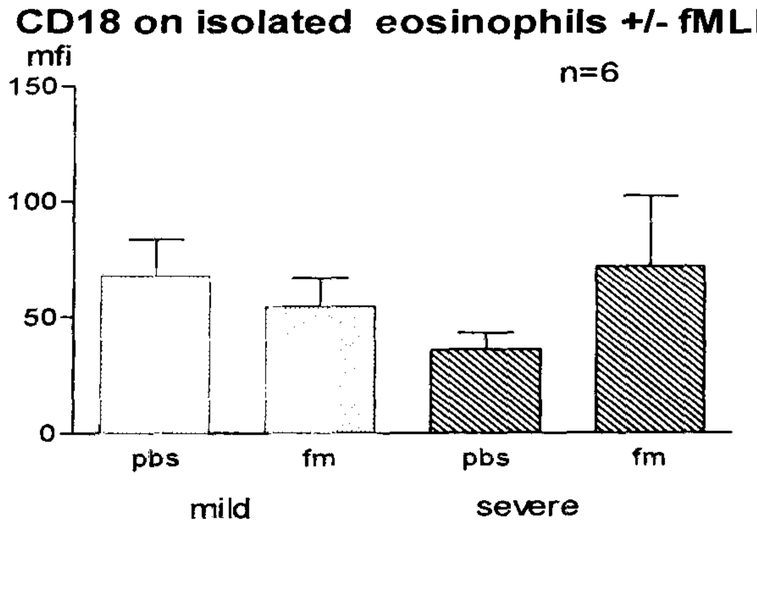
Fig. 3.15 Expression of CD11b, CD62L, and CD18 on resting and fMLP (10^{-7}M) and PAF (10^{-6}M) stimulated isolated neutrophils in mild and severe asthmatics. CD11b was increased, and CD62L reduced after stimulation with fMLP and PAF in severe asthmatics only. No effect was seen on CD18 expression in either group (p <0.001, * p <0.03).**











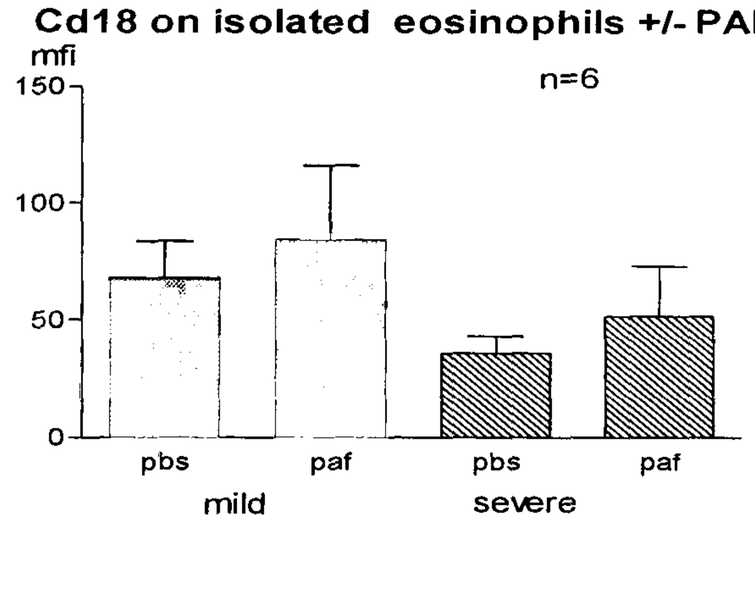
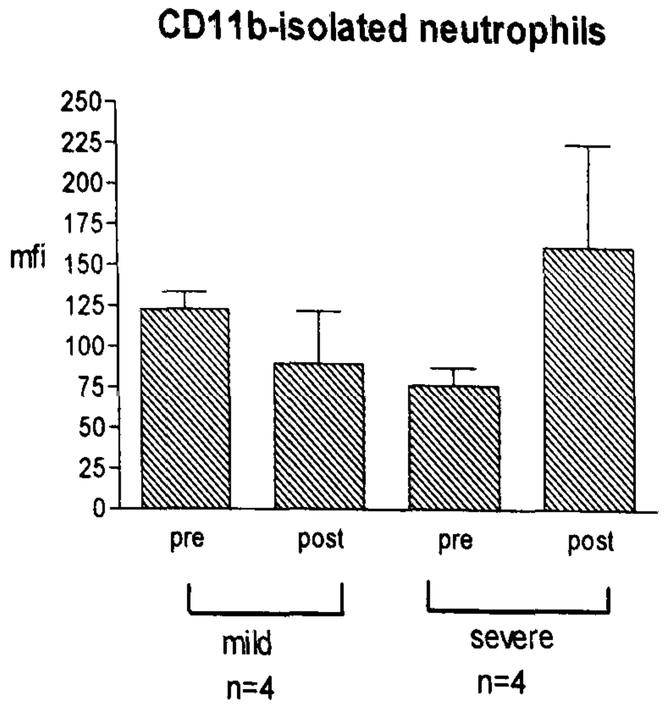
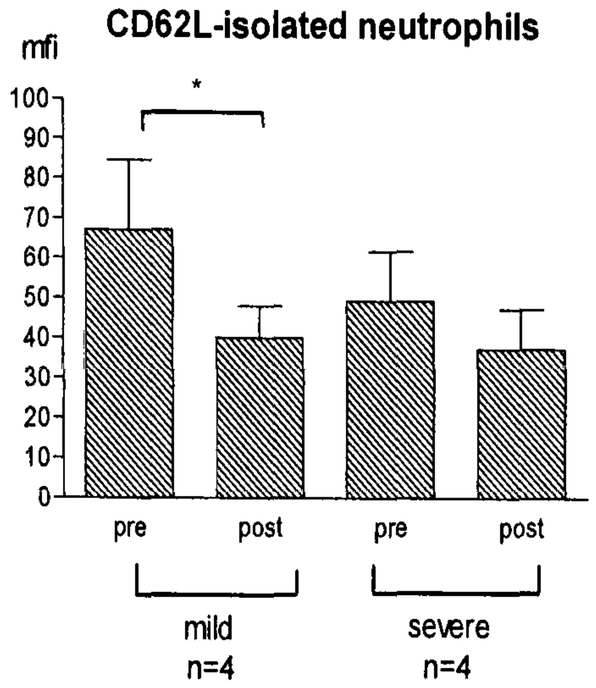
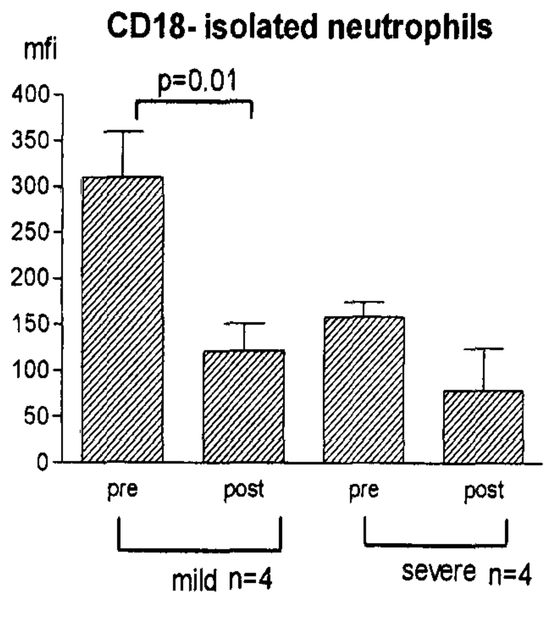
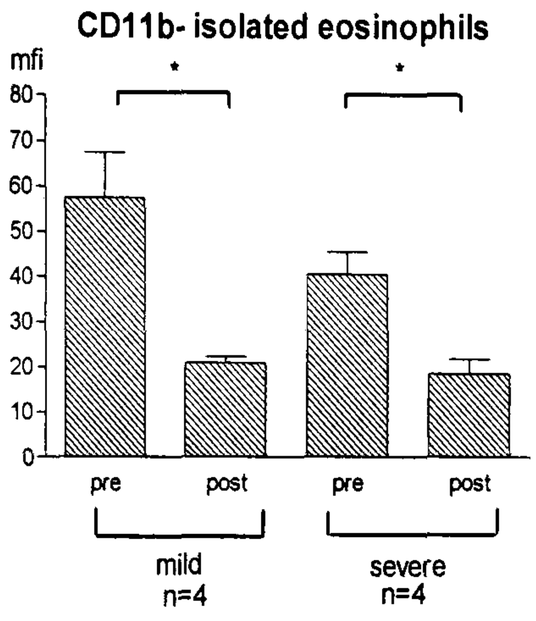


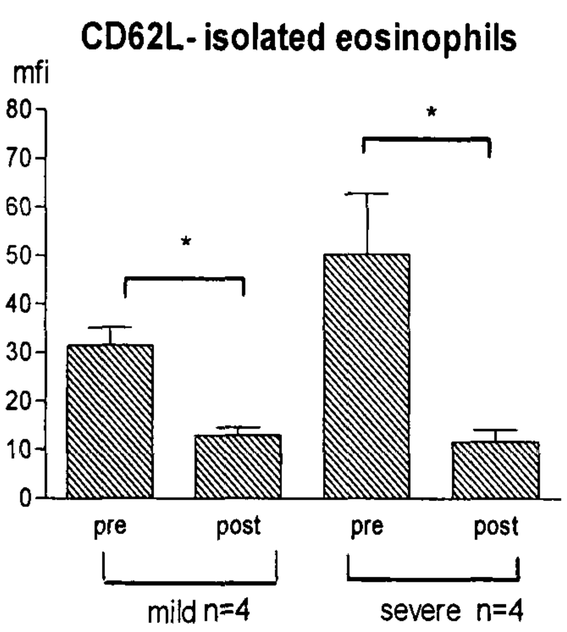
Fig. 3.16 Expression of CD11b, CD62L, and CD18 on unstimulated and fMLP (10^{-7}M) and PAF (10^{-6}M) stimulated isolated eosinophils in mild and severe asthmatics. n=6 mild, and 6 severe asthmatic patients. Stimulation with fMLP or PAF did not significantly increase surface marker expression compared with unstimulated samples. (* p =0.03).











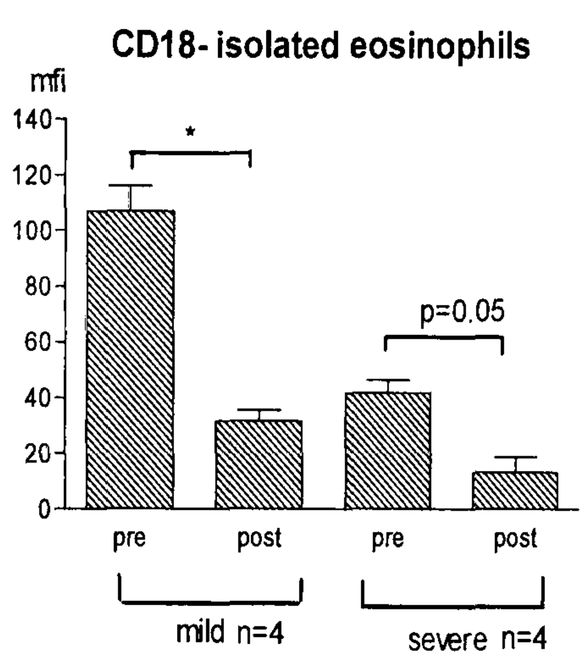
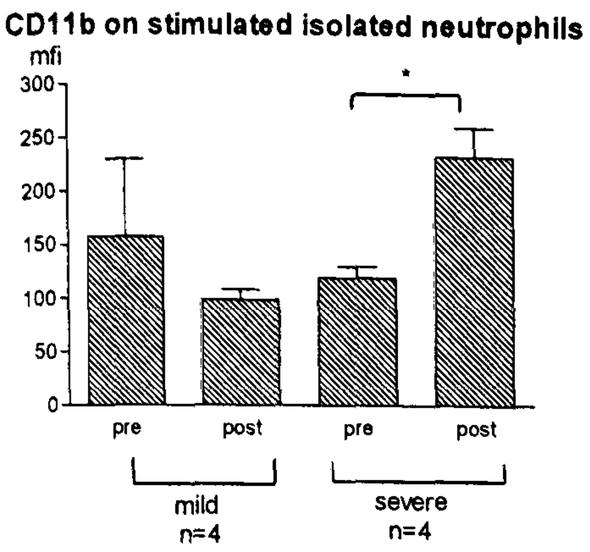
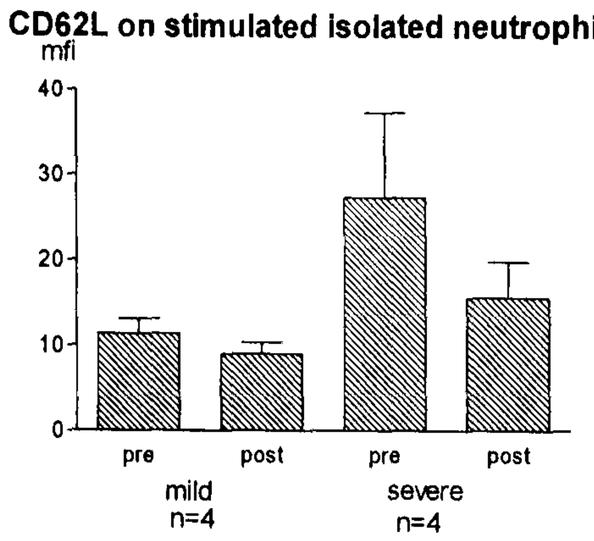
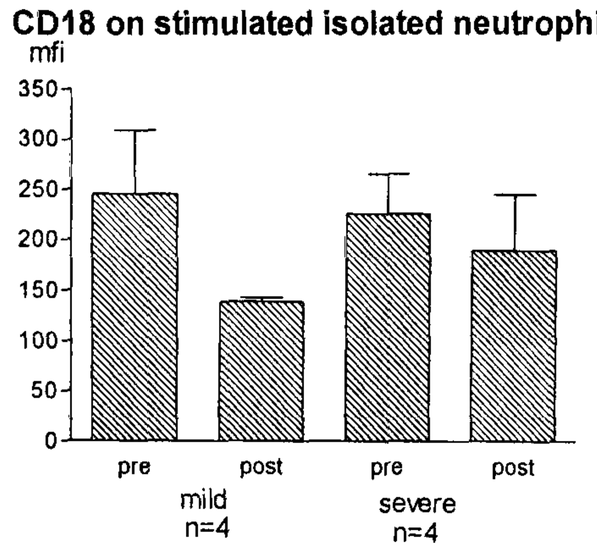
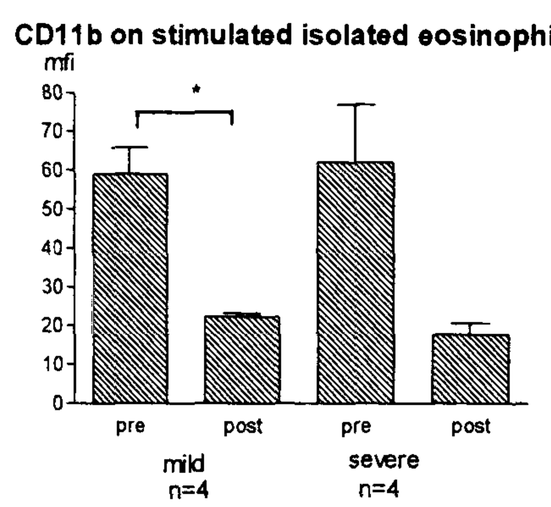


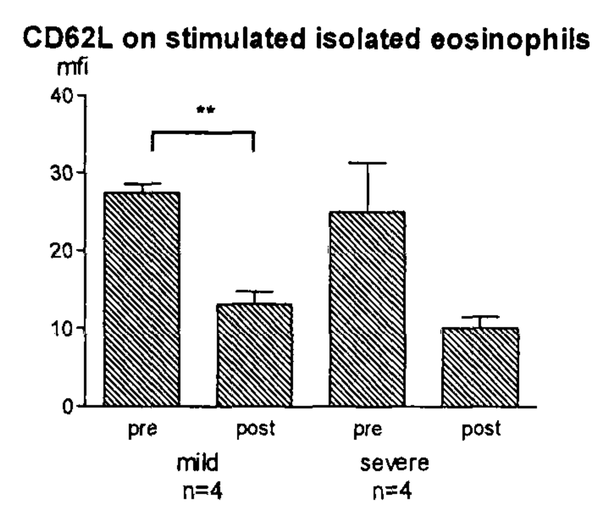
Fig. 3.17 Effect of prednisolone 30mg PO for 1 week on CD11b, CD62L, and CD18 expression on unstimulated isolated neutrophils and eosinophils in mild and severe asthmatics. Prednisolone reduced CD62L and CD18 expression on isolated neutrophils in mild asthmatics only, whereas isolated eosinophils in mild and severe asthmatics were more responsive to the inhibitory effect of prednisolone (* $p < 0.04$).











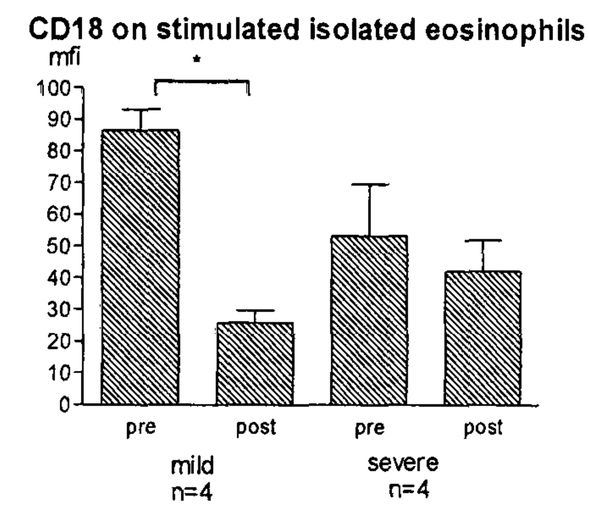
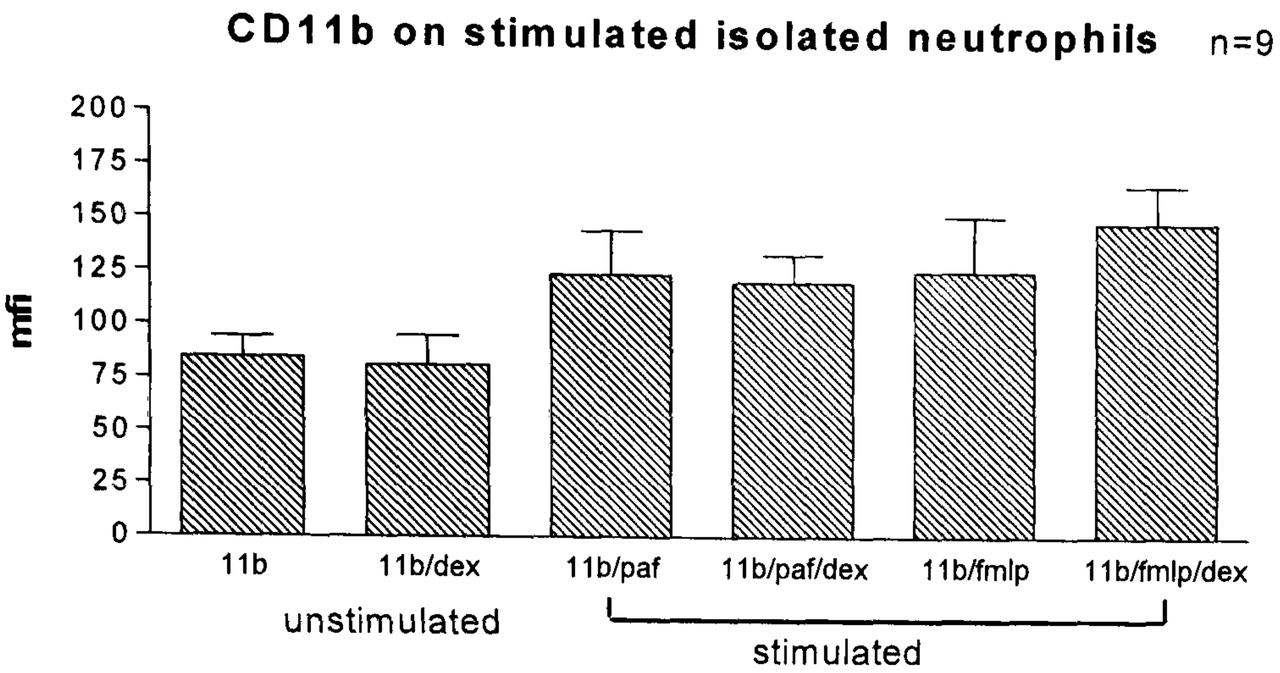
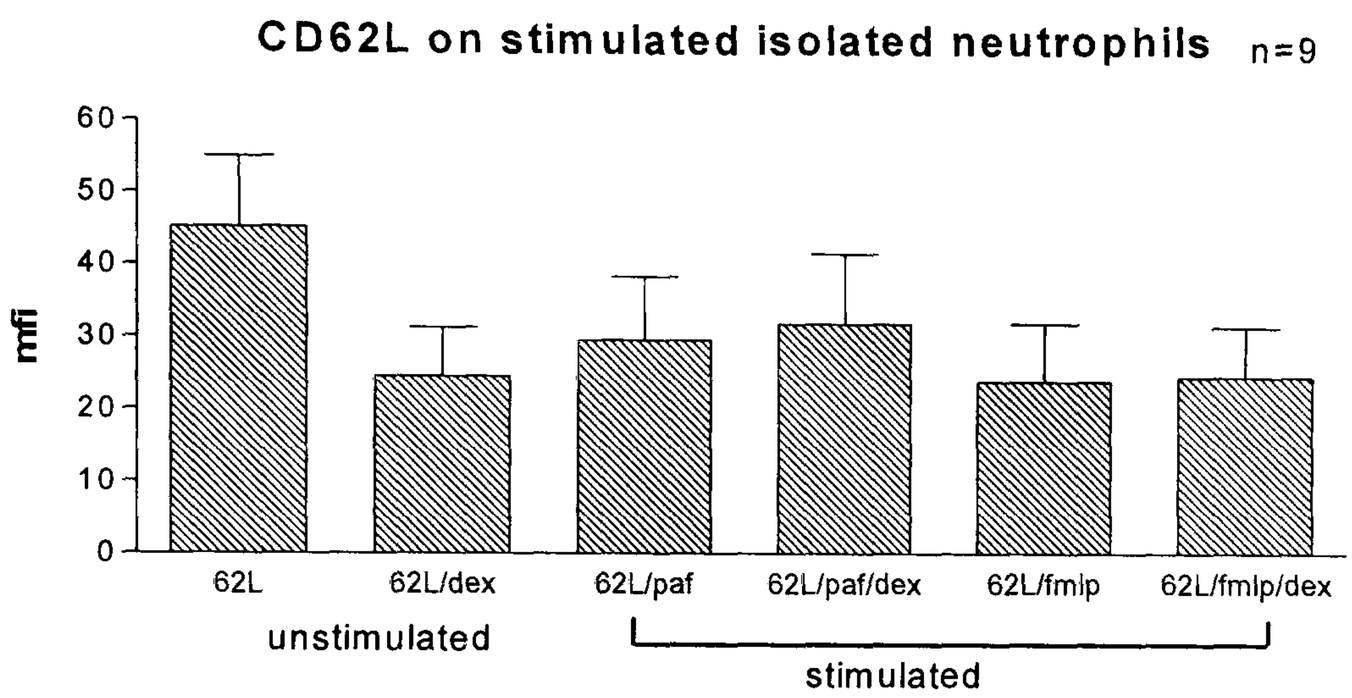


Fig. 3.18 Effect of prednisolone 30mg PO for 1 week on CD11b, CD62L, and CD18 expression on fMLP (10^{-7}M) stimulated isolated neutrophils and eosinophils in mild and severe asthmatics. (p< 0.006, *p<0.01). Prednisolone decreased surface marker expression on isolated stimulated eosinophils in mild asthmatics only, and increased CD11b expression on isolated stimulated neutrophils in severe asthmatics.**





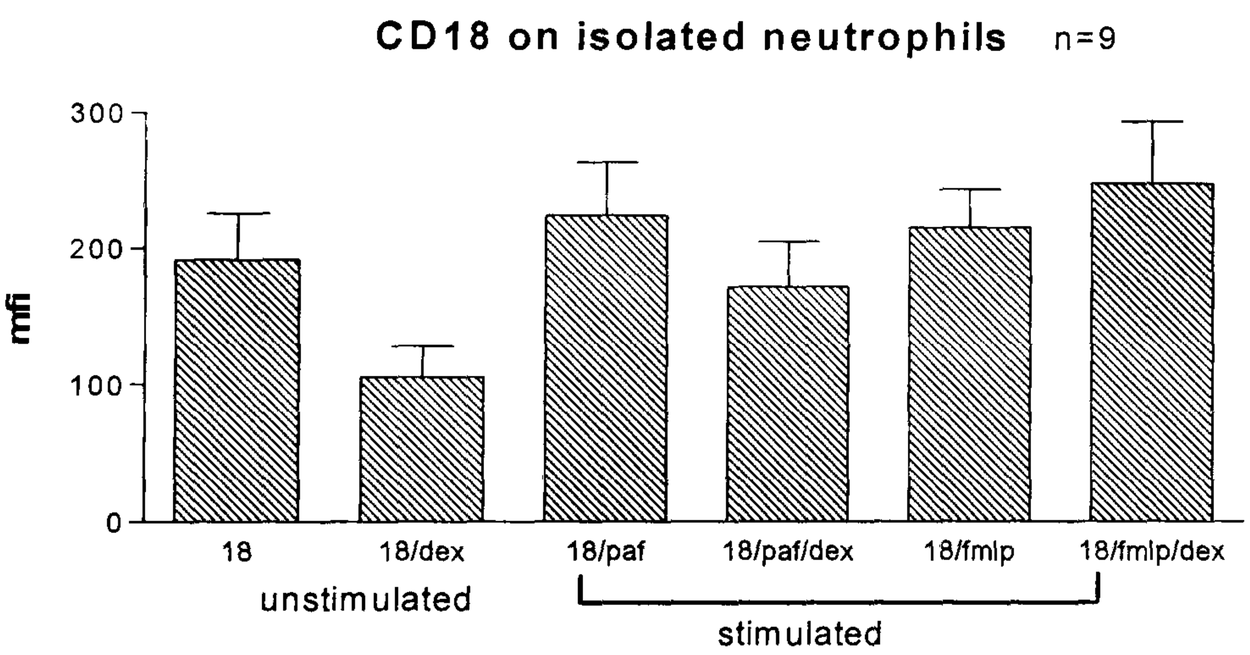
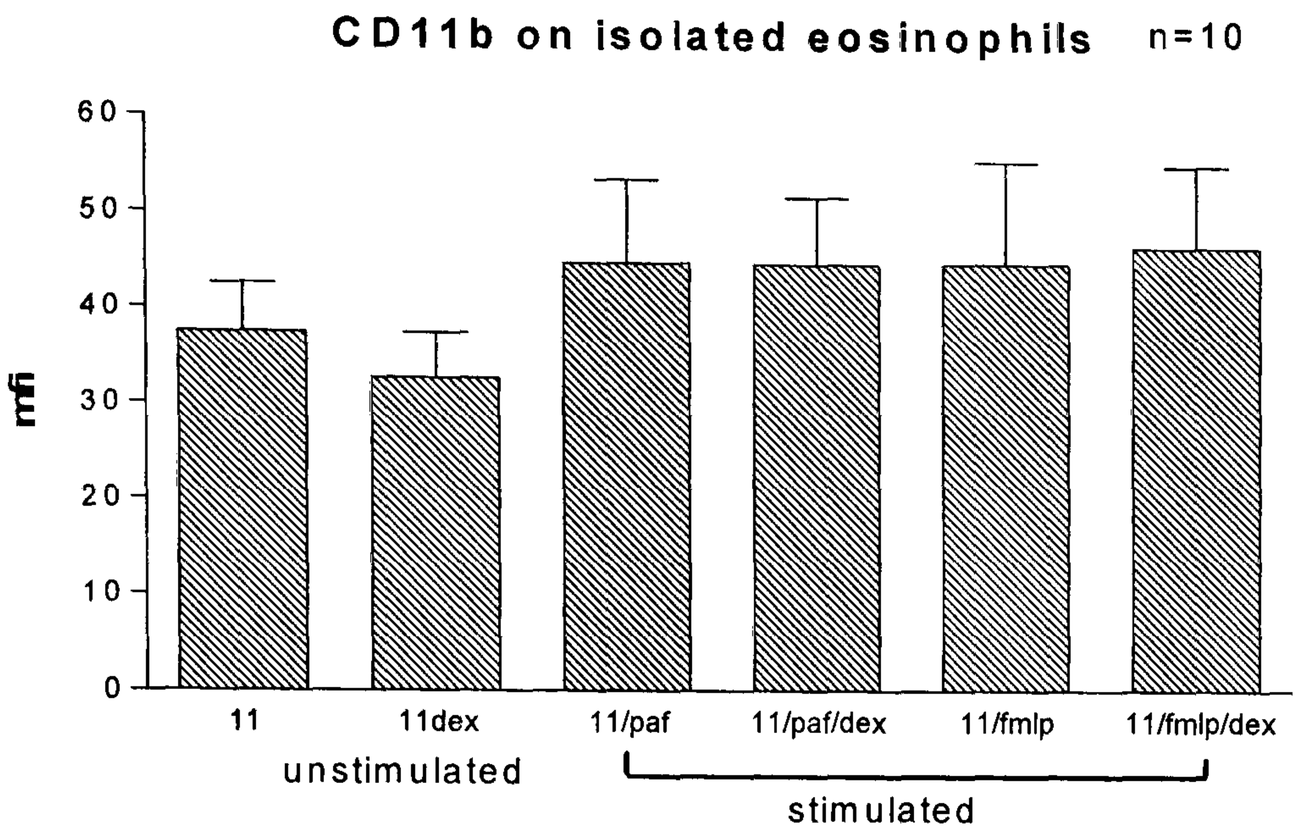
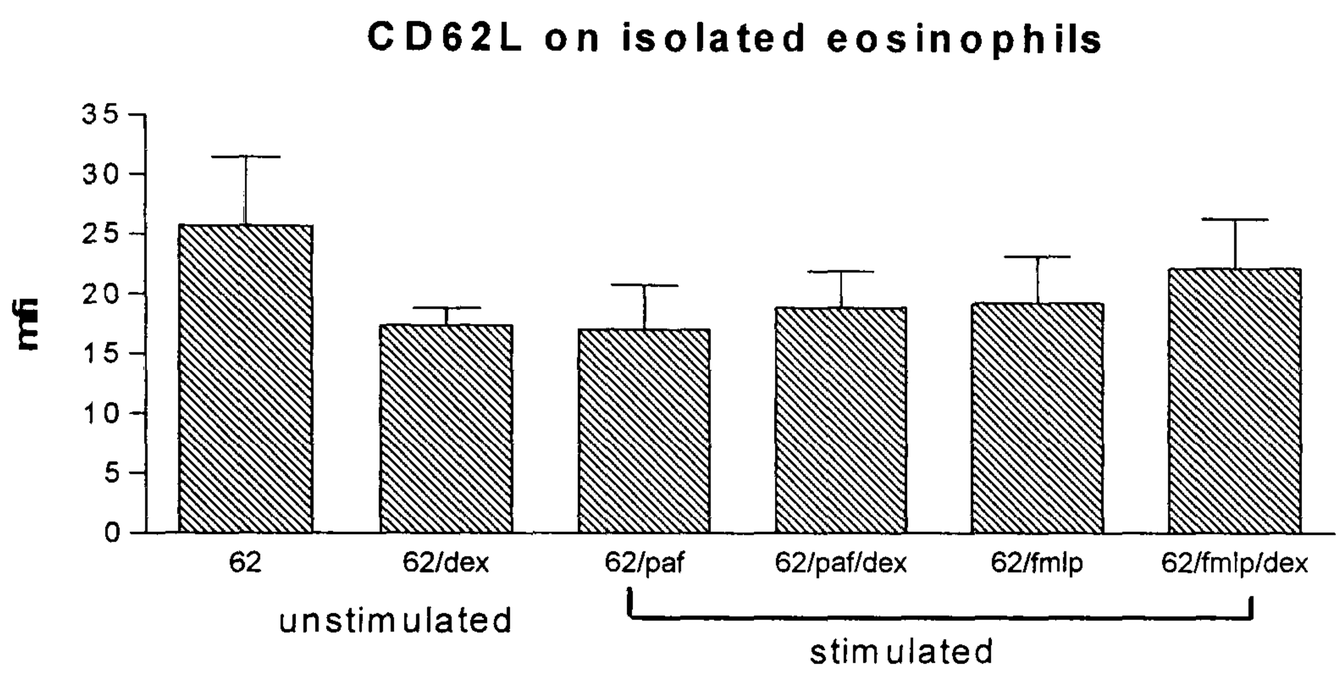


Fig. 3.19 Effect of dexamethasone (10^{-6}M) on CD11b, CD62L, CD18 expression on unstimulated or fMLP (10^{-7}M) and PAF (10^{-6}M) stimulated isolated neutrophils. dex = pretreated with dexamethasone. No significant effect of dexamethasone was seen unstimulated or stimulated isolated neutrophils.





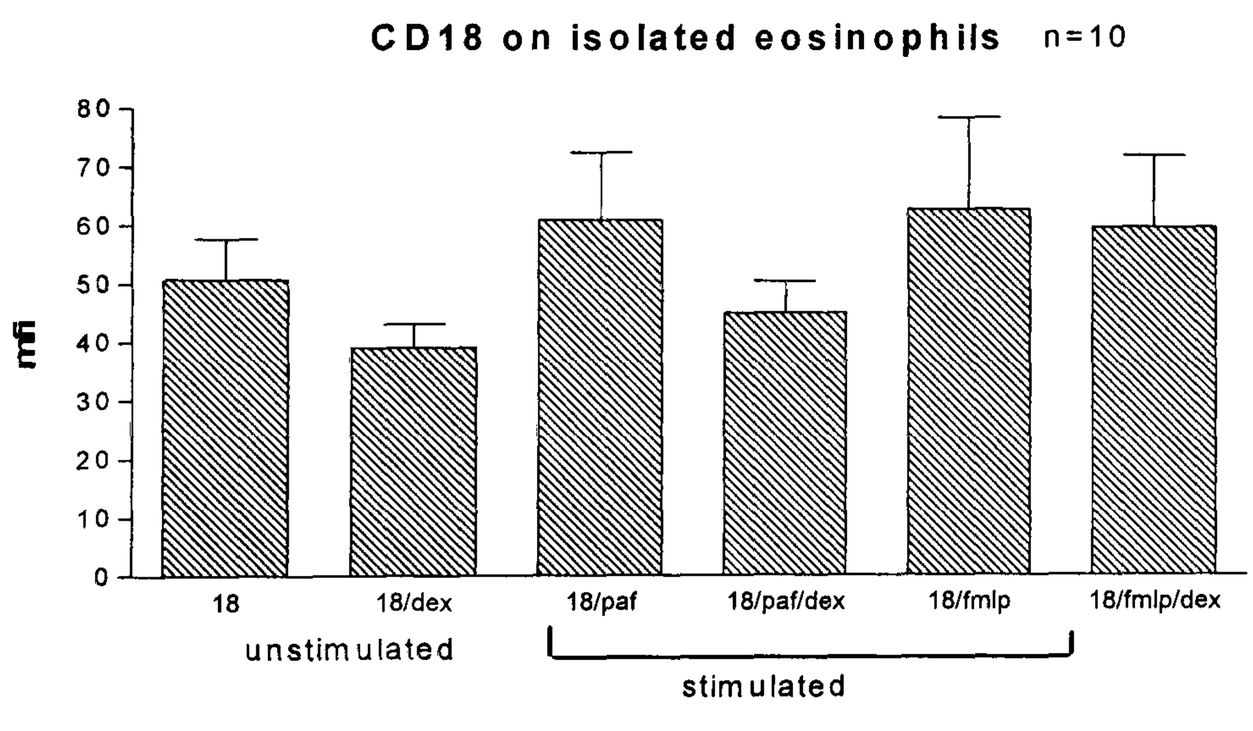
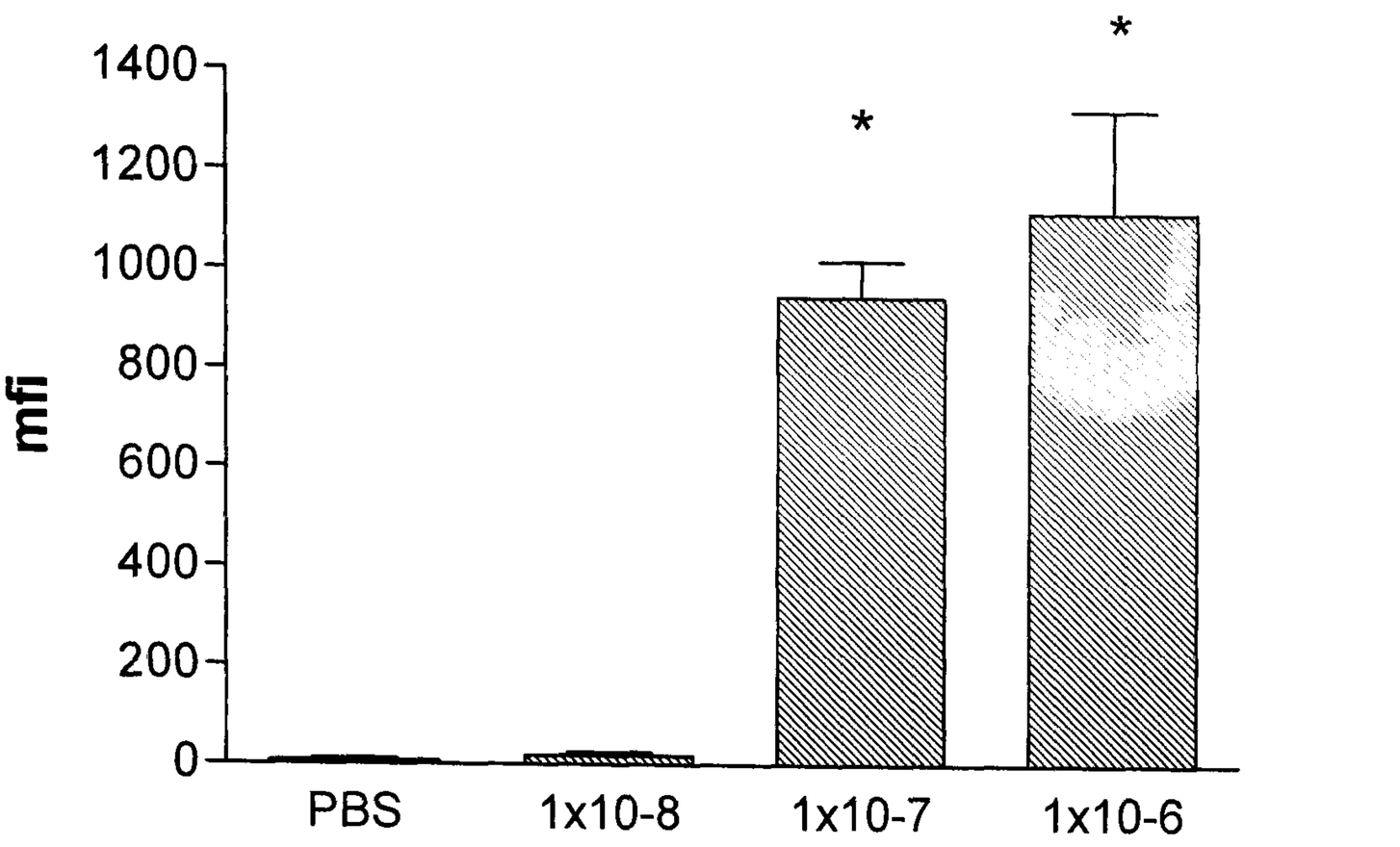


Fig. 3.20 Effect of dexamethasone (10^{-6}M) on unstimulated or fMLP (10^{-7}M) and PAF (10^{-6}M) stimulated isolated eosinophils. dex= preincubated with dexamethasone. Dexamethsone had no significant effect on unstimulated or stimulated isolated eosinophils.



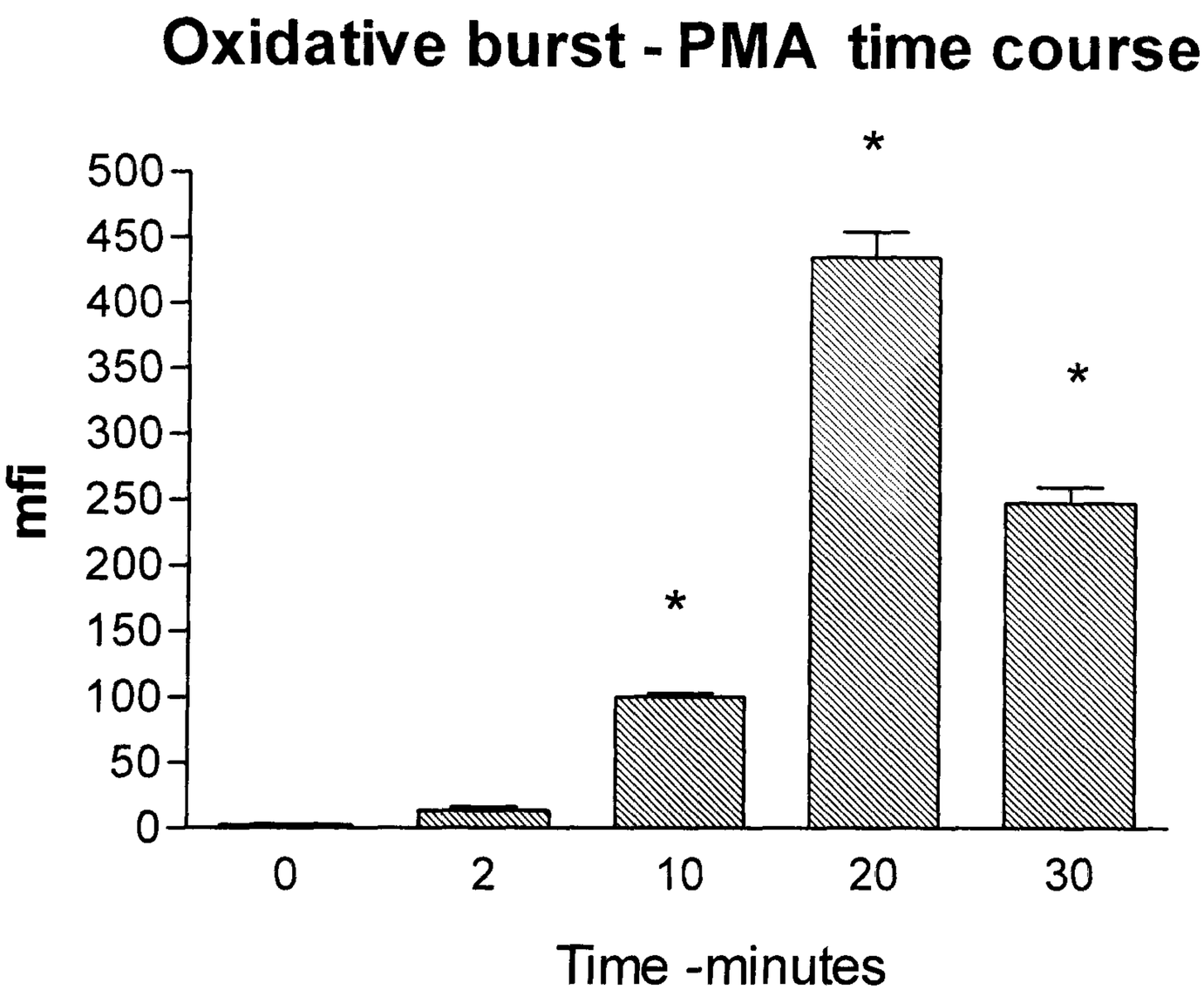


Fig. 3.21 Dose response of neutrophil oxidative burst following stimulation with PMA, (10^{-6}M- 10^{-8}M), and time course study following stimulation with PMA (10^{-6}M). (*p< 0.05 compared to pbs and [1x10^{-8}M], and time-0 and 2 minutes). n= 3, normal subjects.

Oxidative burst in peripheral blood using rhodamine 125 and flow cytometry

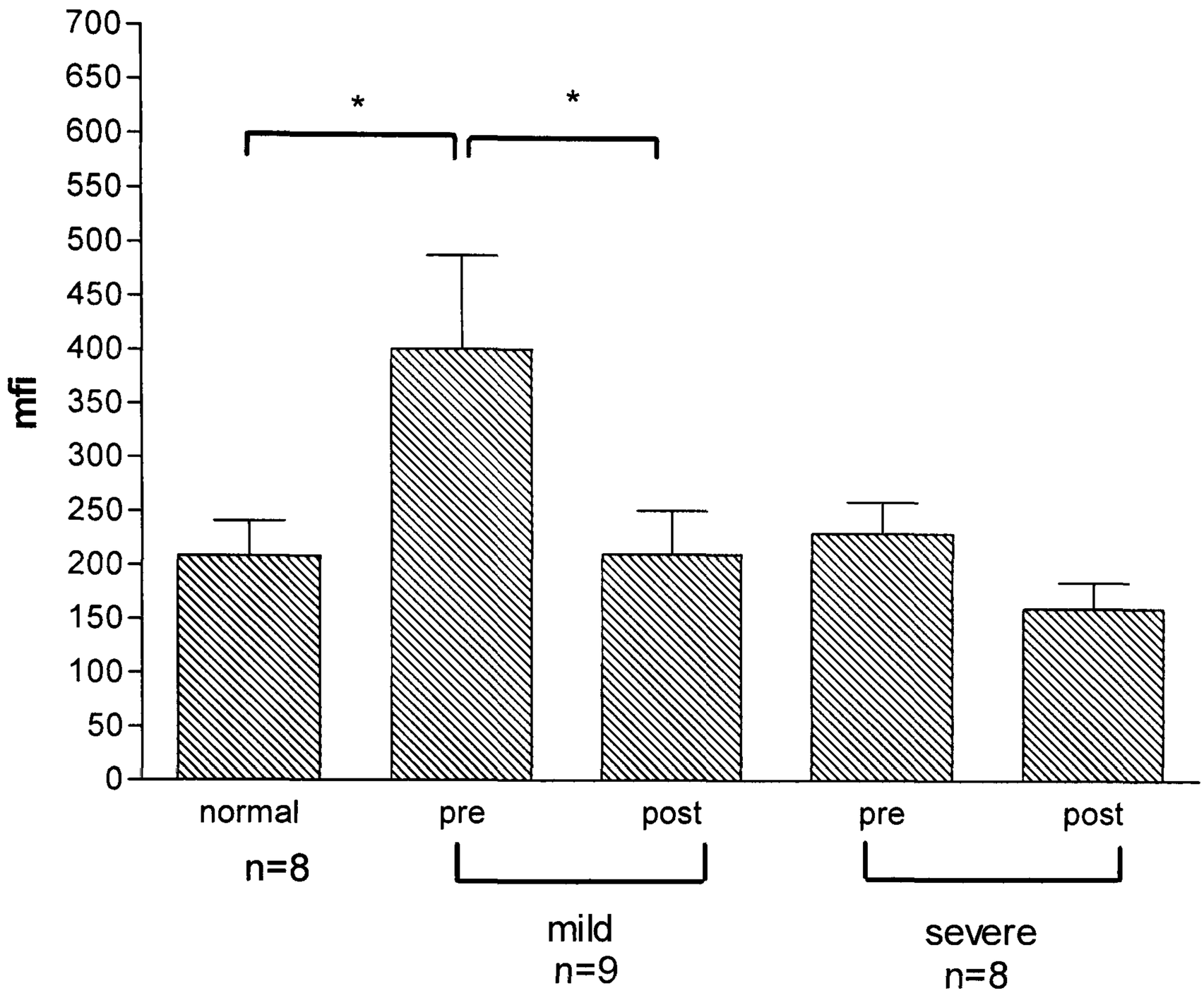
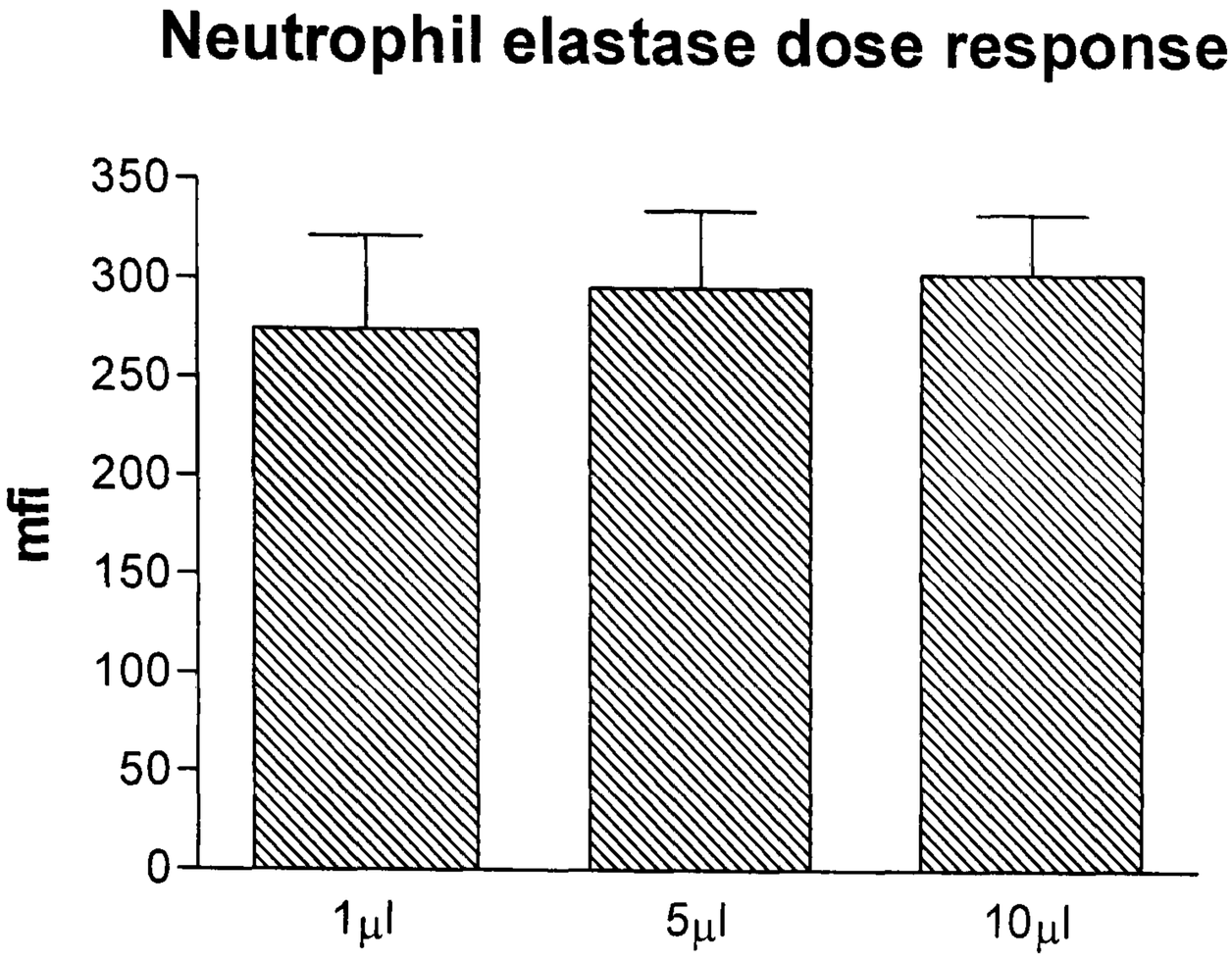


Fig. 3.22 Effect of prednisolone (30mg/day PO) on oxidative burst in peripheral blood in mild and severe asthmatics using rhodamine 125 and flow cytometry. Baseline oxidative burst was higher in mild asthmatics compared with normal subjects and severe asthmatics. Prednisolone reduced oxidative burst in mild, but not severe asthmatics.
pre= before prednisolone, post =after prednisolone (* p= <0.03).



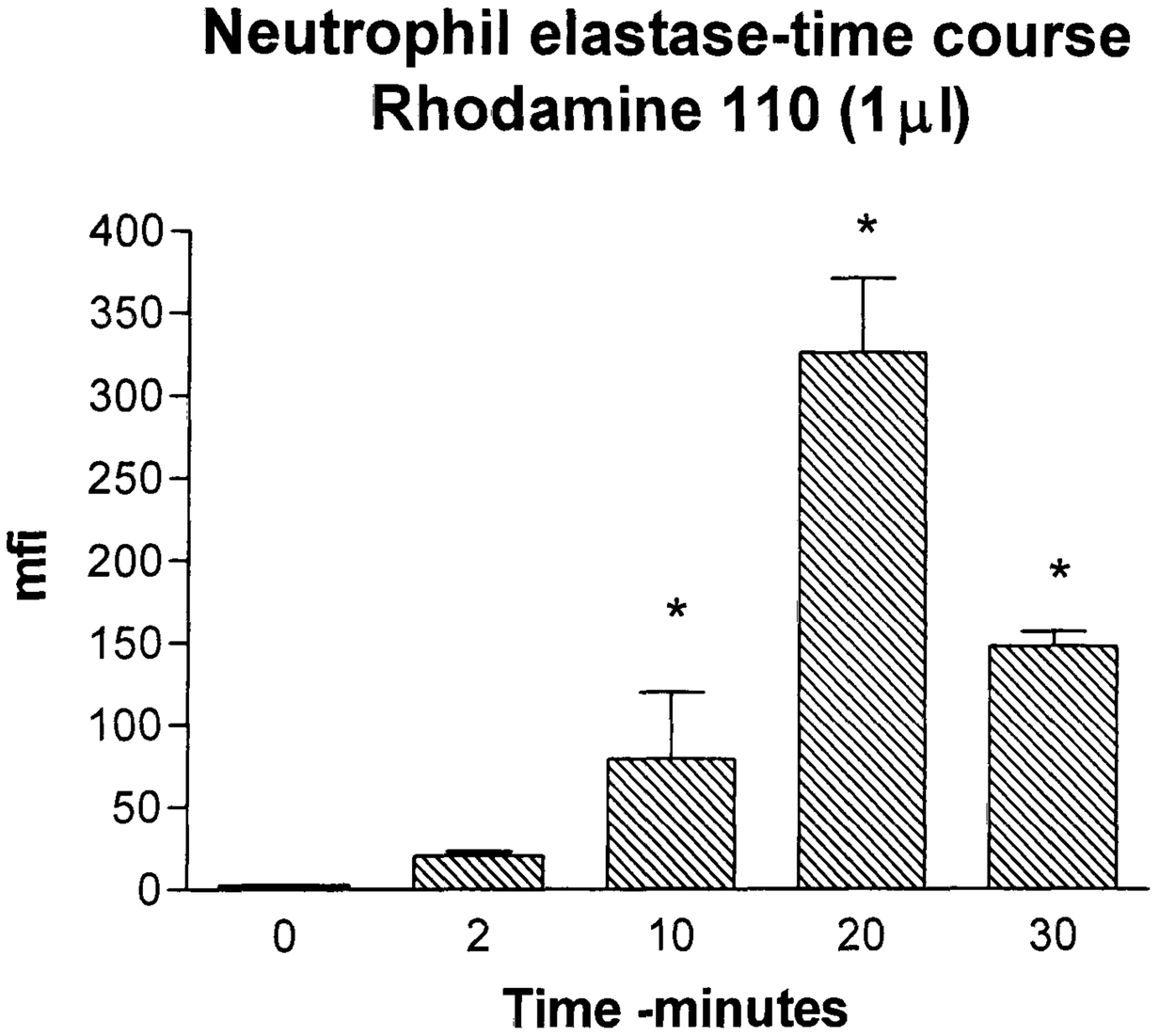


Fig. 3.23 Dose and time response studies of neutrophil elastase using Rhodamine 110 (1μl). * $p<0.05$ compared with time=0, and 2 minutes. 1μl Rhodamine 110 equivalent to other doses. n=3, normal subjects.

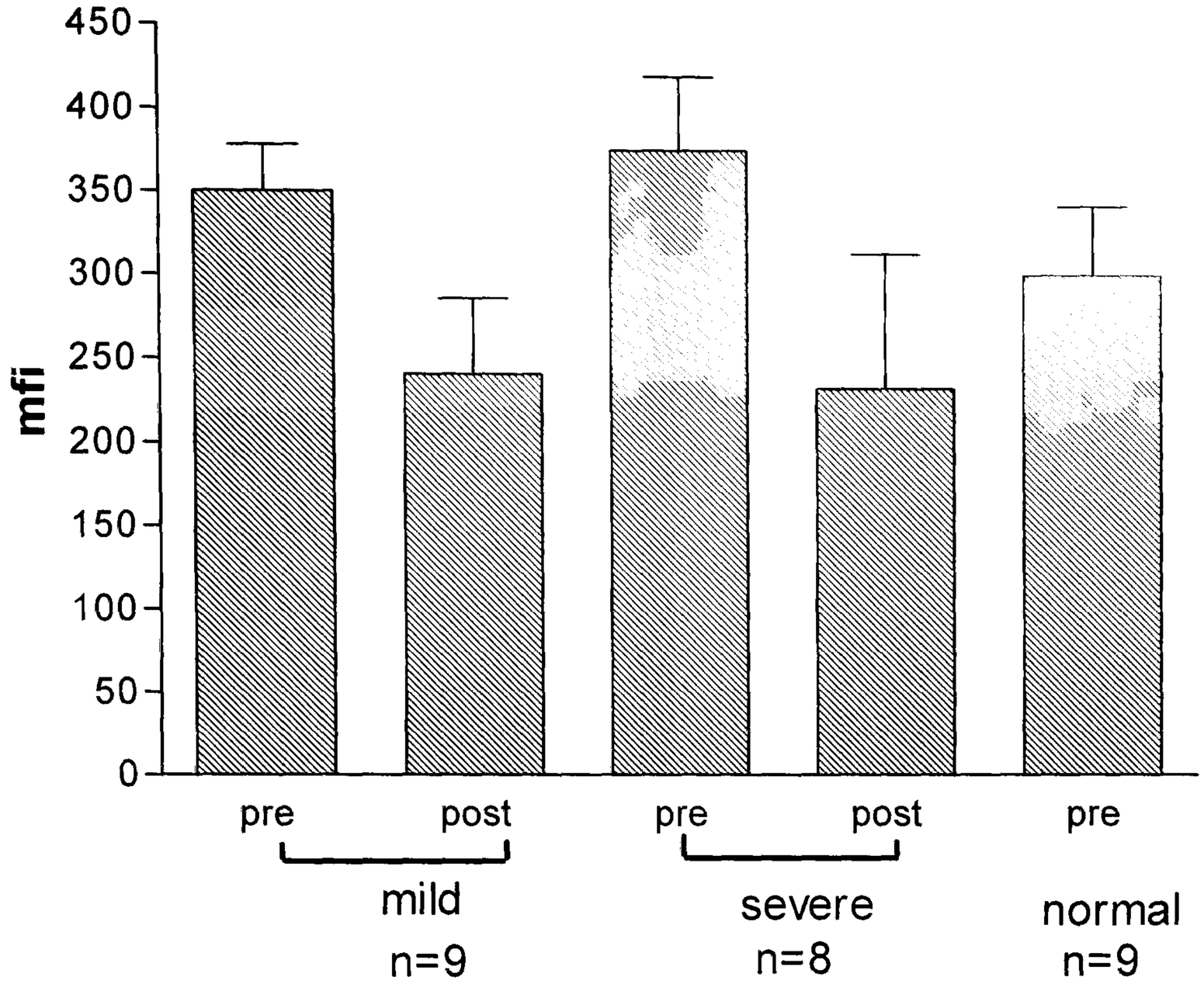
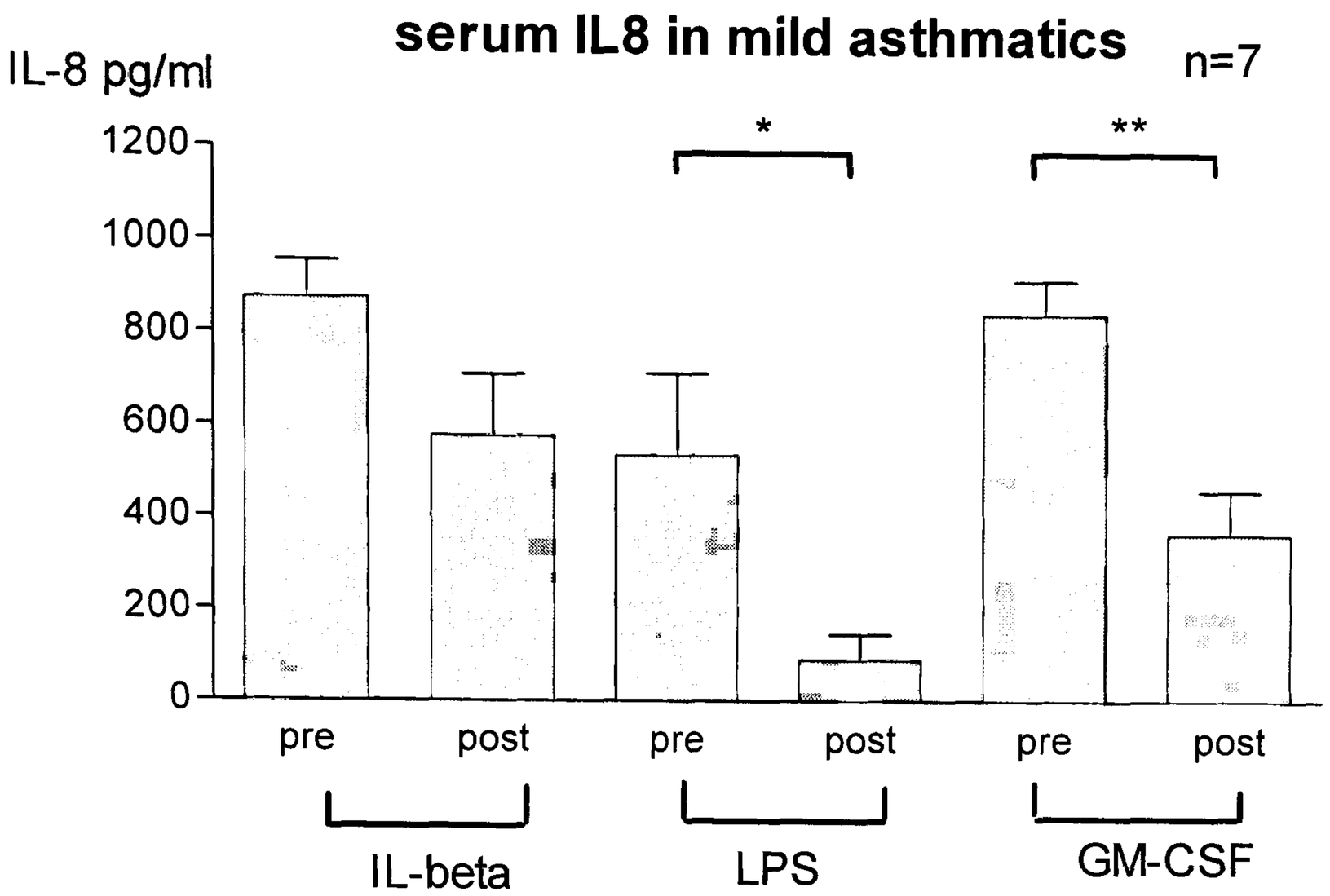


Fig. 3.24 Neutrophil elastase in normal subjects and mild and severe asthmatics (pre = baseline), and effect of prednisolone 30mg PO for 1 week (post), using flow cytometry and rhodamine 110. No significant difference was seen at baseline between the groups, and no effect of prednisolone.



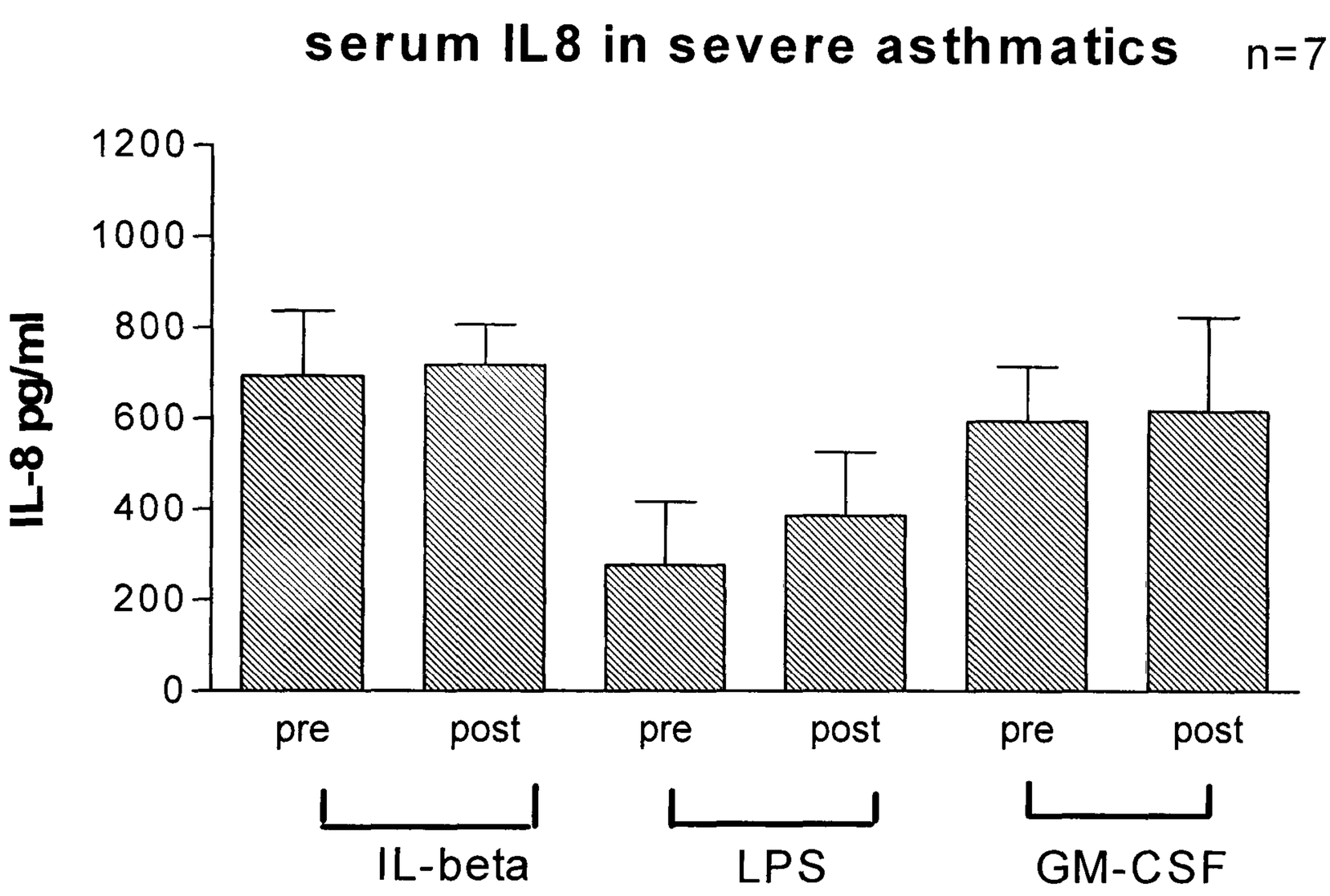
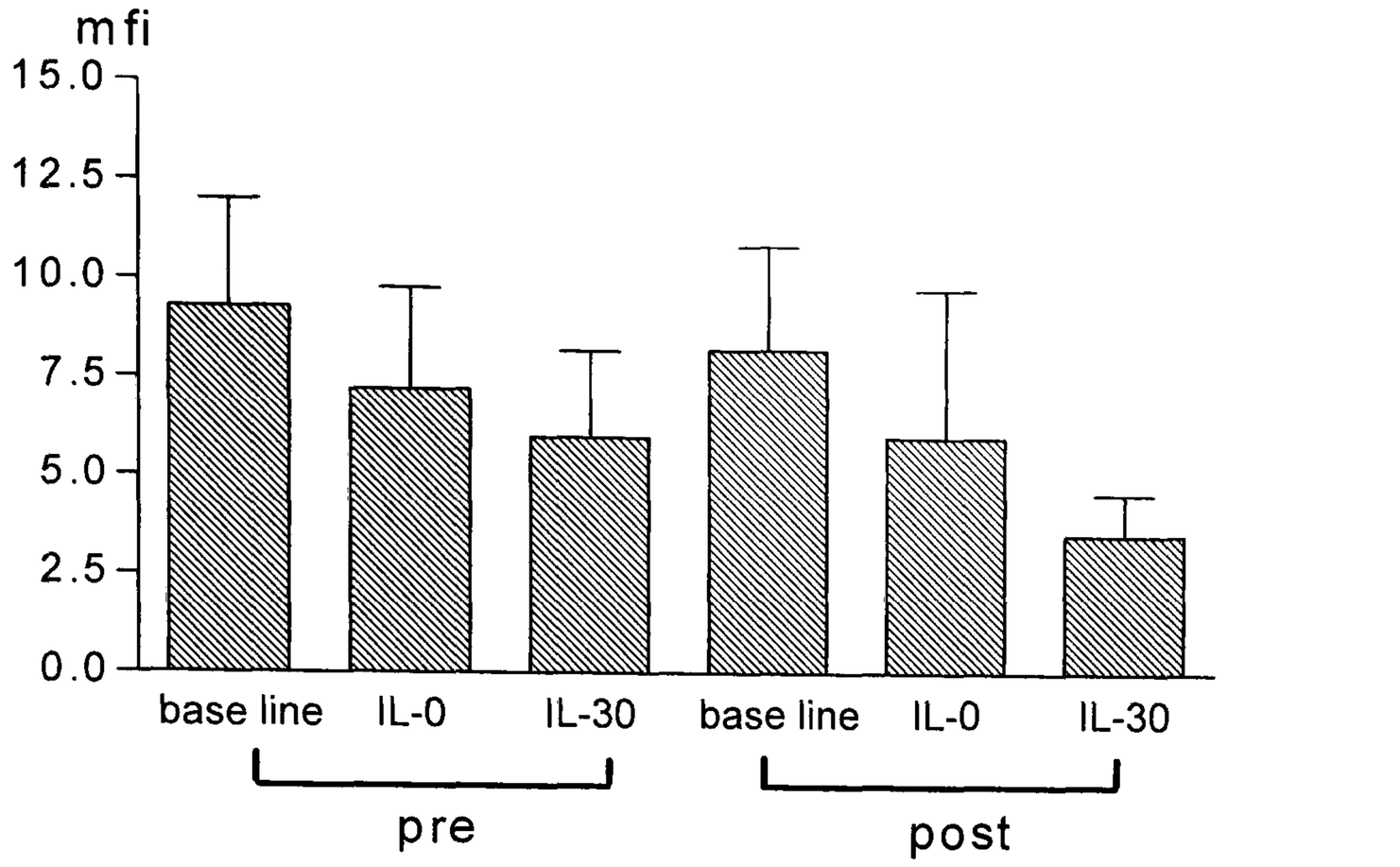


Fig. 3.25 IL-8 levels in mild and severe asthmatics, after 24 hour diluted whole blood (1:10) culture and stimulation with IL-1β (10ng/ml final concentration), LPS (10ng/ml), and GM-CSF (1ng/ml). Levels are shown before (pre) and after (post) prednisolone, 30mg/day for 1 week. Prednisolone significantly reduced the LPS and GMCSF stimulated production of IL-8 in mild patients. (*P=0.03, **P=0.004).



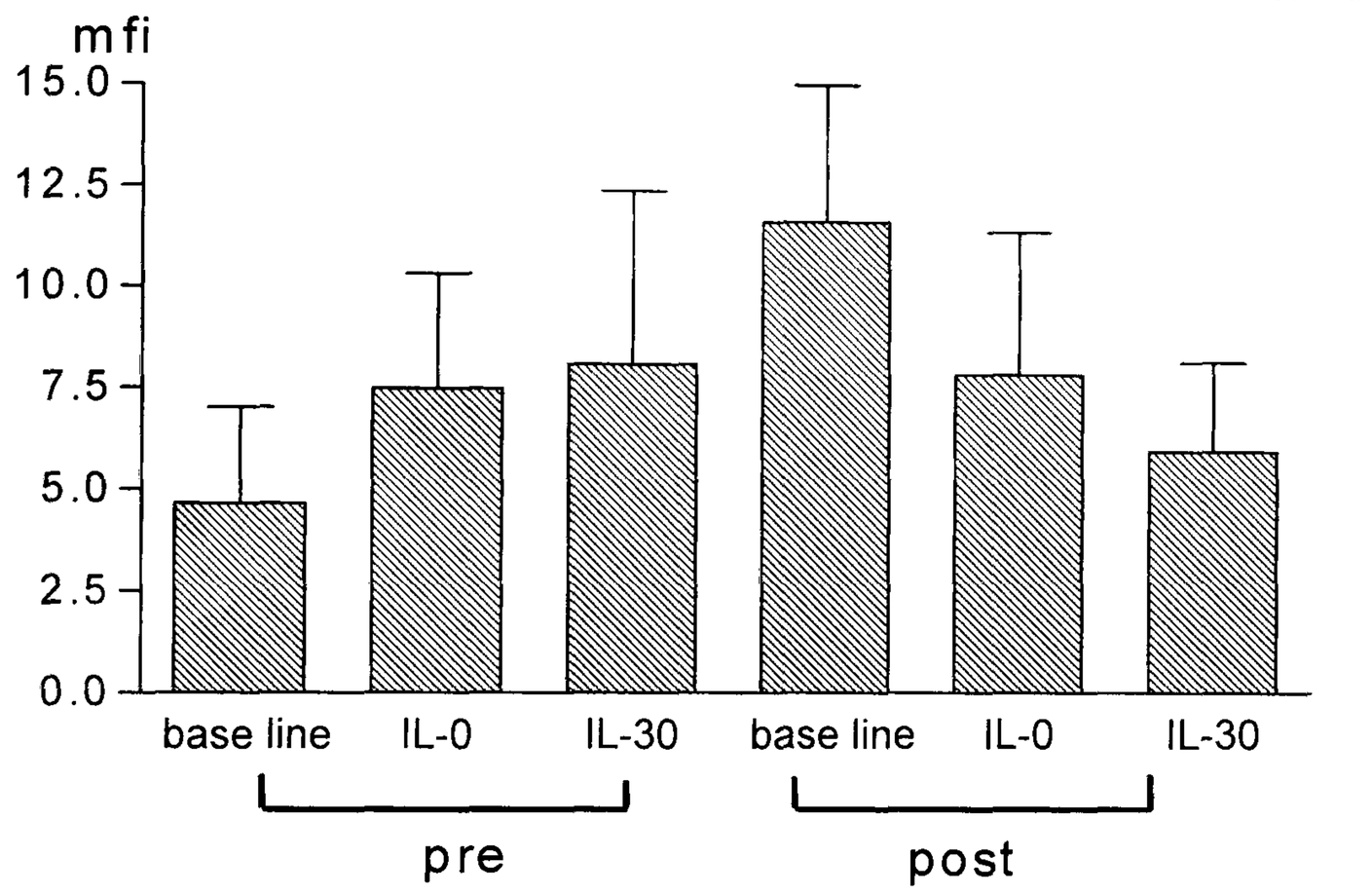


Fig. 3.26 Effect of prednisolone 30mg/day PO for 1 week on MPO release from resting and Il-1β isolated granulocytes, in mild and severe asthmatics. Time intervals: baseline, IL-0= immediately after adding IL-1β; IL-30= 30 minutes after adding IL-1β. Pre= before prednisolone, post= after prednisolone. No significant difference was seen between mild and severe asthmatics, and there was no effect of prednisolone.

3.4 Discussion

The results from this study showed that CD35 and CD11b expression was higher on resting peripheral blood neutrophils in severe corticosteroid-dependent asthmatics compared to normal subjects, suggesting that neutrophils may be in a state of higher activation in the circulation of severe asthmatics, compared to normal subjects. No difference was seen between severe corticosteroid-dependent asthmatics and mild or moderate asthmatics. One explanation for this difference may be related to the effect of chronic oral corticosteroid therapy on surface molecule expression in the more severe group. It would be difficult to completely exclude the effect of oral corticosteroids on surface molecule expression, as by definition, corticosteroid-dependent asthmatics require maintenance oral steroids for control of their symptoms, and thus stopping their steroids to exclude a possible steroid treatment effect would not be possible. The moderate patients in this study were of similar age to the severe group, and taking high doses of ICS, suggesting that high dose ICS do not affect surface marker expression. Many of the subjects in this study were non smokers, or else had not smoked for at least one year prior to the start of the study, and thus it was not felt that this had a direct bearing on granulocyte surface molecule expression.

The power of the study may have been insufficient to detect whether there was a true difference between severe asthmatics and other asthmatic groups, and a repeat study with larger numbers of patients in each group would help to answer this question. As it was only possible to recruit 10 severe patients who agreed to take additional prednisolone on top of their usual maintenance prednisolone dose, it was decided to recruit 10 patients in the other groups to keep patient numbers equal. In addition, the normal subjects were generally younger and non-atopic compared to the more severe group, and this may have some influence on results. A better control group including more age matched controls containing more atopic individuals, and more equal gender mix (most of the the normal subjects were male, and most of the mild asthmatics were female) may have allowed better comparison between groups.

No difference in the expression of the surface molecules was seen on resting eosinophils in any of the groups. Stimulation of neutrophils in lysed whole blood with fMLP and PAF led to activation of neutrophils in all groups as demonstrated by an increase in expression of CD11b, CD18 and CD35 surface markers, and down regulation of CD62L levels. PAF was less potent in reducing CD62L expression than fMLP, and a significant reduction was seen only in the normal subjects and not in the asthmatic

groups. Stimulation of eosinophils with fMLP led to increased levels of CD11b and CD62L in mild and moderate asthmatics but not in severe asthmatics, suggesting that further inducible expression of these markers is not possible in severe corticosteroid-dependent asthmatics, or else, modulation of expression is prevented by prednisolone therapy. These results are similar to a study done in asthmatic children by *Berends et al* (87) who showed that baseline CD35 expression on peripheral blood neutrophils was significantly elevated in mild allergic asthmatic children (inhaled beta-2 agonist only) and stimulation with fMLP increased expression of CD11b on neutrophils and eosinophils in asthmatics versus control subjects, suggesting that inducible expression of CD11b on granulocytes in allergic children is primed in-vivo. In this study however, no increase in surface molecules was seen in mild adult asthmatics compared to normal subjects. In contrast, *Int Veen et al* (88) showed that in stable disease, CD11b and CD62L on eosinophils and neutrophils from blood did not differ between normal, mild or moderately severe asthmatics. However, the most severe group in their study was on high dose ICS and not on maintenance oral steroids. It is interesting that the enhanced expression of CD11b was not accompanied by an associated increase in CD18 expression, since CD11b and CD18 constitute the Mac-1 integrin. One explanation is that there may be enhanced shedding of CD18 or redistribution of CD18 complexes within the CD11 family upon activation of neutrophils.

As previously mentioned, the increased expression of surface molecules on resting neutrophils seen in the severe group in this study could have been due to the effect of chronic steroid therapy which results in persistent activation of circulating neutrophils. In general however, both in-vitro and in-vivo studies have shown that corticosteroids have an inhibitory effect on neutrophil function and result in down regulation of surface molecules such as integrins. In this study, additional 30 mg prednisolone per day for 1 week had no effect on surface molecule expression in the severe group. It is possible that higher doses of prednisolone given for a longer duration may have been required to see an effect on surface molecule expression in this group. Prednisolone did however, significantly decrease CD62L expression on neutrophils in mild asthmatic patients. This finding in humans is similar to in-vivo experiments in cows (102) which showed that i.v. dexamethasone induced down regulation of the adhesion molecules CD62L and CD18 on peripheral blood neutrophils. *Jilma et al* (106) have also shown down regulation of CD62L expression on neutrophils in healthy men following administration of i.v. dexamethasone. This reduction of surface molecule expression by glucocorticoids is thought to occur on segmented PMNs in the maturation

pool of the bone marrow rather than on PMNs already in the circulation (103). This may be one mechanism of the anti-inflammatory action of glucocorticoids in mild asthma, whereby the initial weak binding of leucocytes to the endothelium which is mediated by L-selectin molecules, is reduced. The adhesion molecules on neutrophils in severe asthmatics may be more resistant to the effects of steroids, thus allowing granulocyte recruitment into inflammatory sites to continue. In contrast, the results show that surface molecule expression on eosinophils from both mild and severe patients were reduced by prednisolone suggesting that eosinophils from both groups are more sensitive to the effects of prednisolone than neutrophils, implying relative steroid resistance in the neutrophil population.

Previous human studies on the effects of corticosteroids on granulocyte surface molecules have used high i.v. doses in patients. For example, i.v. methylprednisolone at a dose of 500mg for 5 days in multiple sclerosis patients resulted in a 40% decrease in CD62L and a 35% decrease in CD11b at 6hours (107). *Hill et al* showed that 1g of methylprednisolone inhibited the upregulation of CD11b usually induced by CPB, probably by suppressing cytokine release such as IL-1β, TNF-α, and endotoxin during CPB and which are known to be suppressed by steroids (108). *Laroche et al* (110) showed that in a randomised single blind PBO controlled parallel study in 24 healthy males, prednisolone 5mg bd for 7.5 days showed no significant effect on CD35, CD11b or FcγIII expression on unstimulated and fMLP stimulated neutrophils. However, this dose is lower than that which is usually given during an asthma exacerbation and in a clinical setting.

There appeared to be no effect of dexamethasone (10^{-5} to 10^{-9}M) on either resting or stimulated neutrophil or eosinophil surface molecule expression in peripheral whole blood in-vitro. This discrepancy between in-vivo and in-vitro results has been confirmed by other workers in respect to glucocorticoid induced shedding of L-selectin, and suggests that glucocorticoids do not directly affect neutrophil L-selectin expression but instead act via an additional second messenger in vivo, thought to be annexin 1 (111).

Studies on isolated granulocytes showed similar results to peripheral blood neutrophils and eosinophils. Baseline expression of surface molecules (as demonstrated by the mfi), was higher in isolated granulocytes and this may reflect activation of granulocytes through separation and isolation procedures such as Ficoll separation and density centrifugation. No difference was seen in the baseline expression of surface markers on isolated neutrophils between mild and severe asthmatics and this may be

due to the fact that the number of patients analysed was too small. Isolated neutrophils from severe asthmatics did however show significant activation of neutrophils after stimulation with fMLP and PAF but this was not seen in the mild group. This suggests that neutrophils from severe patients may be more activated after an exacerbation, with the possibility of causing tissue damage from release of stored mediators.

Isolated eosinophils from mild patients did show increased CD11b expression compared to severe patients. As with peripheral blood eosinophils, isolated eosinophils did not show significant activation after stimulation with fMLP or PAF. It has been shown that eosinophils are more sensitive to the effects of steroids than neutrophils, and the maintenance steroids in the severe patient group may be important in modulating surface marker expression on eosinophils in this group.

As with peripheral blood neutrophils, prednisolone reduced levels of CD62L and CD18 on isolated neutrophils in mild patients only, suggesting that the reduction in adhesion molecules in mild asthmatics may be one mechanism for the anti-inflammatory action of corticosteroids in these patients. Again prednisolone did not appear to have any effect on neutrophils from severe patients, suggesting that these neutrophils from steroid-dependent patients are resistant to this action of corticosteroids. Isolated eosinophils from both groups again demonstrated sensitivity to prednisolone, and this was also the case for fMLP stimulated isolated eosinophils in mild asthmatics. Interestingly, fMLP stimulated isolated neutrophils in severe patients showed further activation with increased CD11b expression after prednisolone therapy. Dexamethasone showed no reduction in surface molecule expression in resting or stimulated isolated neutrophils or eosinophils in either group, giving weight to the hypothesis that a second messenger in-vivo is required for modulation of adhesion molecule expression.

It is known that cytokines such as GM-CSF may be able to increase the in-vitro inducible expression of adhesion molecules such as CD11b (106). Further, administration of i.v. methylprednisolone or dexamethasone reduces serum cytokine secretion (107,108). In this study, the levels of IL-8 after whole blood culture were not significantly different between mild and severe steroid-dependent asthmatics, either before or after prednisolone. In mild patients however, there was a significant reduction in LPS and GM-CSF induced IL-8 production following prednisolone therapy, which was not seen in severe patients. This would suggest that induceable cytokine levels can be suppressed by corticosteroids in mild asthmatics, thus dampening the inflammatory response. This effect is not seen in severe asthmatics and it is therefore possible that this may allow the inflammatory cascade to continue in these severe patients allowing on

going tissue damage and remodelling to occur. It must be noted that although neutrophils stimulated with IL-1β, LPS and GM-CSF may be an important source of IL-8, other cells such as monocytes may also be contributory.

Results from this study showed that the oxidative burst activity of granulocytes was greater in mild asthmatics compared to normal subjects. Further, prednisolone decreased oxidative burst activity in mild asthmatics but there was no significant effect in patients on chronic steroid therapy. This is in agreement with studies that have shown PMA and zymozan stimulated blood in asthmatics patients showed increased superoxide anion generation compared to normal subjects (184). *Hoeben et al* showed that prednisolone inhibited respiratory burst activity in bovine granulocytes at therapeutic doses (187). The reduced oxidative burst seen in corticosteroid-dependent asthmatics is partly explained by a study by *Fukushima et al* (194) which showed that prolonged corticosteroid therapy in patients receiving steroids for a variety of reasons such as neurological, collagen vascular diseases, and respiratory conditions showed inhibition of PMN superoxide production as measured by cytochrome C and spectometry following fMLP and PMA stimulation. One possible mechanism for this is thought to be inhibition of NADPH oxidation or impairment of transduction reactions in the respiratory burst. In addition, the loss of suppressibility of the oxidative burst to prednisolone in severe asthma could be due to the reduced basal release possibly induced by chronic prednisolone therapy. Examination of the effect of prednisolone in normal volunteers, in whom the baseline respiratory burst is similar to that of severe asthmatics, would resolve the issue as to whether further suppression is possible.

In comparison to whole blood culture results, no difference was seen in the basal or IL-1β stimulated MPO release in isolated granulocytes between normal subjects, mild, or severe asthmatics. This may be explained by the fact that the isolation procedure itself may be responsible for causing cellular degranulation and release of MPO from granulocytes from all samples. This study showed no difference in peripheral blood neutrophil elastase levels between normal subjects, mild and severe asthmatics using a flow cytometric method of analysis. Increased levels of neutrophil elastase have been reported in sputum from asthmatics with an exacerbation due to a respiratory tract infection (22), and in patients with status asthmaticus undergoing mechanical ventilation in the absence of infection (195). The negative result seen in peripheral blood suggests that a severe insult, either inflammatory or infectious is required before any increase in neutrophil elastase is seen and even then, perhaps only in the lung compartment rather than in the peripheral blood.

Conclusion and future studies

This study shows that the constitutive expression of surface activation markers CD35 and CD11b on peripheral blood neutrophils from severe corticosteroid-dependent asthmatics is elevated compared to normal subjects. Further, stimulation with fMLP and PAF results in further activation of certain adhesion molecules in isolated neutrophils from severe asthmatics. Adhesion molecule expression on peripheral neutrophils from the severe asthmatic group remains unchanged following an additional dose of corticosteroids, whilst CD62L and CD18 levels are reduced in mild asthmatics, both in peripheral and isolated neutrophils using a clinically relevant dose of prednisolone. This suggests that circulating neutrophils may be in a more activated state in steroid-dependent asthmatics compared with normal subjects, and that steroids may be less able to reduce recruitment of these cells into areas of inflammation in the more severe group, possibly as a result of their inability to directly reduce surface molecule expression, or indirectly, through failure of inhibition of cytokines that may be important in recruitment and activation of these cells. This study showed that inducible levels of IL-8 are significantly reduced by prednisolone in mild asthmatics, but the lack of this effect in severe asthmatics may allow continuing activation and uncontrolled inflammatory response to continue in the more severe patients, with further scope for tissue damage to occur.

Corticosteroids may have a differential effect on the activation and function of neutrophils in severe steroid-dependent asthmatics, as oxidative burst capacity in steroid dependent-asthmatics is reduced by their long term steroid therapy. This immunosuppresive effect may play a role in the pathogenesis of the severe phenotype by allowing recurrent episodes of infection to occur in these patients. Eosinophils from both mild and severe groups appear to be sensitive to the effects of corticosteroids and this has been shown to be the case in peripheral blood eosinophils and eosinophils from isolated granulocytes.

The dual nature of corticosteroids in the treatment of asthma was confirmed in a study by *Fukakusa et al* (196) who looked at the effect of a 2 week course of oral methylprednisolone (40mg/day) on the expression of chemokines in bronchial biopsy samples from 13 patients with moderate-to severe asthma using immunocytochemistry techniques. Corticosteroids reduced the number of CD3-positive T cells and major basic protein-positive eosinophils, whereas the number of neutrophils were increased. Corticosteroids also reduced the number of eotaxin, and monocyte chemotactic protein

(MCP) 3 and 4 mRNA positive cells. However, corticosteroids caused a significant increase in the epithelial expression of IL-8, and MCP-2 mRNAs. The failure of oral corticosteroids to inhibit IL-8 mRNA may contribute to the persistent airway neutrophilia in patients with severe asthma, despite treatment with corticosteroids.

Apart from the possible damage to airway tissue in asthma, animal experiments have shown that neutrophils are important in the ischaemia and subsequent reperfusion injury in other organs. Upon reperfusion, neutrophils accumulate in an ischaemic area through activation of their surface adhesion molecules, and produce an inflammatory response through the production of free radicals, proteases and leukotrienes, with deleterious effects on cell membranes and endothelial cells. *Tajra et al* (197) showed that renal ischaemia could be decreased in rats by administering anti-CD11b antibodies prior to reperfusion, resulting in less tubular necrosis, neutrophil infiltration, and decreased CD11b expression on neutrophils as seen by flow cytometry. *Jaeschke et al* (198) showed that in rats treated with anti-CD11b antibodies, hepatic ischaemia followed by reperfusion resulted in decreased neutrophil numbers in post-ischaemic liver by 59% and decreased superoxide formation by neutrophils by 56%.

In dogs, a positive correlation was seen between neutrophil count and severity of coronary artery disease (199). Using anti-CD11b/CD18 monoclonal antibodies it was possible to limit myocardial infarct size. Neutrophil activation during angioplasty (PTCA) resulted in release of oxygen free radicals which may aggravate the endothelial damage induced by PTCA, and further stimulate platelets, with important implications for restenosis. Neutrophils also release LTC4 which causes vasoconstriction and a reduction in coronary blood flow.

Antibodies to the surface adhesion molecules CD62L, CD18, and ICAM-1 in a cat model of myocardial ischaemic reperfusion injury, have shown attenuated coronary endothelial dysfunction, lower MPO activity in areas of ischaemic tissue, and attenuation of neutrophil accumulation (200), as well as lower plasma creatinine kinase levels, and a reduced area of cardiac necrosis (201,202), suggesting that inhibition of neutrophil adherence to the endothelium exerts protective effects in reperfusion injury. Further, monoclonal antibodies to sialyl Lewis antigen, which is a ligand to the selectin molecules involved in slow rolling of leucocytes along the endothelium, reduced the extent of myocardial infarction after ischaemia followed by reperfusion in a rat model of myocardial reperfusion injury (203).

In humans, *Kamimura et al* (204) showed that anti-CD11b and anti-CD18 monoclonal-antibodies inhibited migration of neutrophils into middle ear effusion in an

in-vitro study of acute otitis media in children, suggesting this maybe useful in the future treatment of human otitis media. In man, increased production of oxygen radicals and neutrophil elastase, have been noted in patients with coronary artery disease, in chronic lower limb arteriopathy, and in association with vascular risk factors such as diabetes (205). The use of pentoxifylline has been shown to decrease neutrophil adherence and free radical production and decrease TNF-α cytokine levels. Further, other cytokines such as IL-4 and IL-10 have been shown to decrease the formation of tissue factor. Therefore, multiple pharmacological targets are possible but their use in vascular disease is at preliminary stage.

In studies involving the NF-kappa B inhibitor, tepoxalin, suppression of the cell surface adhesion molecules CD11b/CD18, inhibited adhesion of neutrophils to IL-1β activated HUVEC (206). Tepoxalin also inhibited the secretion of IL-8, which is a known inducer of CD11b/CD18 expression. Tepaxolin may therefore be useful in treating adhesion molecule mediated events such as leukocyte migration. Neutrophils can therefore contribute to tissue injury by release of oxygen derived free radicals, proteases and leukotrienes, and interventions aimed at neutrophil inhibition may exert protective effects. Corticosteroids alone may not be able to suppress these actions and a specific targeted approach to therapy may be more beneficial when these are developed in the future, especially in the more severe asthmatic group.

The experiments in this chapter could have improved in many respects. Recruiting more age, gender and atopy matched normal control subjects as well as asthmatic subjects would have removed the effects of these confounding variables on the results. There may have been selection bias in the study as the normal subjects were selected from doctors working in the asthma laboratory at the Royal Brompton hospital, at the time of this study. The study could have been better powered by consulting a statistician before the start of the study. There was no statistician in place at the Brompton at the start of the study. It would have been interesting to look at the effect of oral corticosteroids on neutrophil activation parameters in the moderate asthmatic group on high dose ICS. This was attempted, but difficult to achieve because the moderate asthmatic patients were not keen to take oral corticosteroids in view of their potential side-effects. In addition, the prednisolone study would have been strengthened if there was PBO group. The design could have been improved by using a double blind, PBO controlled crossover trial with mild and severe asthmatics having placebo or prednisolone after a wash out period of a week. This might be difficult however, as ingestion of prednisolone tablets, even for a short period may be associated with some

adverse effects (e.g. insomnia, euphoria, minor weight gain) in some patients, compared with PBO. Looking at the effect of prednisolone on granulocyte surface markers in normal subjects was attempted, but not possible due to the reluctance of normal subjects to take prednisolone tablets. The dose of prednisolone used and duration of treatment was felt to closely resemble the clinical response to treatment during an exacerbation, but using higher doses of prednisolone, such as 40-50mg for a longer period of time, for example 2 weeks, may have provided different results, especially in the severe group. The effect of corticosteroids on the expression of surface markers is difficult to exclude, but investigation of a subgroup of severe asthmatics who are felt to be severe enough to require long term maintenance corticosteroid therapy, can be undertaken to look at peripheral blood neutrophil surface markers before and 2-4 weeks after starting corticosteroids, to see if there is any difference as a result of steroid therapy. However, recruitment is likely to be difficult and require some time to achieve.

Neutrophil elastase in the blood was measured by a relatively new method using FACS scanning techniques. Perhaps using an ELISA technique may have been better. Neutrophil elastase could also have been measured in sputum supernatant before and after a course of steroids.The method of defining a neutrophil population in lysed whole blood using a FACS scanner according to the literature was correct, although this could have been refined by using a FACS machine incorporating a cell sorter, although this type of FACS scanner was not available for use. Further, CD49 staining on a CD16 negative cell population may help to further exclude eosinophils from a neutrophil population. It would have been interesting to look at surface marker expression in sputum/BAL samples in addition to peripheral blood in the various asthmatic groups compared to control subjects. However, this can be technically difficult given the background 'noise' in these samples, and perhaps due to the lack of cellular material obtained.

Performing ELISA on blood samples for IL-8, MPO, and neutrophil elastase before and after fMLP stimulation, would be useful to see if the level of neutrophil surface marker expression correlated with mediator release. Newer FACS scanning techniques now allow measurement of cytokines using specific monoclonal antibodies and only a small peripheral blood sample, thus allowing an exciting and rapid means of analysing neutrophil activation markers in patients. Despite the advantages of whole blood culture techniques, it is important to remember that other cells in the blood are a potential source of IL-8, and thus isolating and culturing neutrophils specificily from the

blood to look at release of IL-8 before and after prednisolone therapy, rather than using whole blood culture, would be interesting for comparison.

Chapter 4

Investigation of histone deacetylase activity in peripheral blood neutrophils in mild and moderate asthmatics compared to peripheral blood mononuclear cells and alveolar macrophages, and the effect of corticosteroids on histone deacetylase activity.

4.1 Introduction

Asthma is a chronic inflammatory disease that is characterized by increased expression of multiple inflammatory genes. Acetylation of histones by histone acetyltransferases (HATS) is associated with increased gene transcription, whereas hypoacetylation induced by histone deacetylases (HDACs) is associated with suppression of gene expression. Studies on bronchial biopsies from patients with asthma and normal subjects, have shown reduced HDAC enzymatic activity in asthmatics, with greater HDAC activity seen in those asthmatics treated with ICS compared to untreated asthmatics, although still lower than control subjects. Further, the asthmatic group demonstrated increased HAT activity compared to controls, and this was reduced to control levels in asthmatics that were treated with ICS (137). Thus the increase in HAT activity and reduced HDAC activity in asthma may underlie the increased expression of multiple inflammatory genes. Corticosteroids have been shown to switch off inflammatory gene transcription in asthma through the inhibition of HAT activity and by the recruitment of histone deacetylase 2, (HDAC2) to the actively transcribing gene. HDAC activity is thus increased by treatment with corticosteroids, leading to repressed transcription of inflammatory genes.

In the last chapter, it was shown that the expression of certain surface activation markers on neutrophils was increased in severe asthmatics, and oral prednisolone had little effect on the level of expression of these markers. The aim of this study was to investigate the activity of HDAC activity in peripheral blood neutrophils from normal, mild, and severe asthmatic subjects, and to compare this with HDAC activity in alveolar macrophages (AM) and peripheral blood monocytes (PBMCs). The effect of oral prednisolone on HDAC activity in these cell populations was also investigated. Measurement of HDAC activity in neutrophils allows assessment of the state of activation of these cells in the peripheral circulation of patients with varying severity of asthma.

Macrophages and monocytes play an important role in the regulation of the immune and inflammatory responses. AM and PBMCs from subjects with asthma have been reported to display an activated phenotype, with upregulated expression of surface markers such as HLA-DR, CD35 and CD23 receptors (207,208). AM play a critical part in orchestrating the chronic inflammatory cascade through the release of proteases such as MMP-9, inflammatory cytokines such as TNFα, and chemokines such as IL-8 that attract neutrophils into the airways. *Vicksman et al* (209) reported evidence of activation of AM but not PBMC in subjects with allergic rhinitis and asthma.

4.2 Methods

4.2.1 *Subjects*

The contribution of Dr B Cosio to this chapter is acknowledged in the author's declaration. Eighteen stable patients with asthma were recruited for bronchoscopy. Ten were mild asthmatics receiving only intermittent treatment with the inhaled β-adrenergic agonist, salbutamol. The other eight were moderate persistent asthmatics who were taking regular ICS (500 μg/day of FP or equivalent). All patients demonstrated greater than 15% improvement in FEV1 after 200μg of salbutamol and airway responsiveness to a provocative concentration of methacholine producing a 20% fall in FEV1 (PC_{20}). All patients were atopic, as defined by two or more positive skin prick tests to common allergens. Current smokers or ex-smokers of more than 5 pack-years were excluded. Ten healthy non-atopic non-smoking volunteers were also recruited (Table 4.1).

In a separate experiment, blood was venesected from 6 patients with mild persistent asthma and 6 patients with severe asthma receiving ICS (> 1000μg/day FP or equivalent), long acting inhaled β-adrenergic agonist, and oral corticosteroids if required (n=3), for control of asthma. Blood was taken before, and after a one week course of oral prednisolone 30 mg/day (in mild asthmatics) or an additional 30 mg/day of prednisolone for one week in severe asthmatics already on maintenance prednisolone. Prednisolone blood levels and cortisol were checked to ensure compliance and absorption of the treatment. Exhaled NO was also measured before and after treatment as described in section 2.1.6.

BAL was performed according to guidelines at the Royal Brompton Hospital. All patients gave written informed consent, and the study was given approval by the Royal Brompton Hospital's Ethics committee.

TABLE 4.1. Characteristics of subjects undergoing bronchoscopy

	Control (*n = 10*)	Mild Asthma (*n = 10*)	Moderate Asthma (*n = 8*)
Age, yr	30.6 ± 3	31 ± 3	31.1 ± 2.5
Sex, M:F	7:3	5:5	5:3
FEV_1, (L)	4.5 ± 0.2	3.5 ± 0.3	3.5 ± 0.2
FEV_1, % predicted	104 ± 3	88.1 ± 5	91.8 ± 2
PC_{20}, mg/ml	ND	1 ± 0.2	1.3 ± 0.4
BAL fluid recovered, ml	99.4 ± 8	105.7 ± 5	105.6 ± 6
Macrophage number, cells x 10^3/ml	92.9 ± 11	102.4 ± 14	120.1 ± 17
Eosinophils, %	0.2 ± 0.03	1.8 ± 0.4	1.6 ± 0.3
Neutrophils, %	3.3 ± 0.1	4.1 ± 0.2	2.2 ± 0.1

Definition of abbreviations: BAL = bronchoalveolar lavage; ND = not determined; PC_{20} = provocative concentration of methacholine producing a 20% fall in FEV_1. Results are presented as mean ± SEM.

TABLE 4.2. Characteristics of subjects in whole blood culture and prednisolone study

	Mild Asthma (*n = 6*)	Severe Asthma (*n = 6*)
Age; yr	36.3 ± 5	46.6 ± 4
Sex; M:F	3:3	2:4
FEV_1; % predicted	93.6 ± 3.6	43.75 ± 8.2
FEV_1; l, pre	3.13 ± 0.2	1.29 ± 0.3
FEV_1; l, post	3.06 ± 0.2	1.74 ± 0.3
eNO; ppb, pre	42.5 ± 20	30.4 ± 5
eNO; ppb, post	23.5 ± 9*	18.3 ± 4
Prednisolone; nmol/L	1,588.6 ± 225**	983.6 ± 127**

Definition of abbreviations: eNO = exhaled nitric oxide; pre = before treatment with prednisolone; post = after treatment with prednisolone. * $p < 0.05$ compared with pretreatment levels. Results are presented as mean ± SEM.

** Incomplete data available-see discussion.

TABLE 4.3. Bronchoalveolar lavage characteristics in subjects with asthma and control subjects

Subject	TCC	Macro (%)	Neut (%)	Lym (%)	Eos (%)	CV (%)
Control	109.5 ± 5.2	85.1 ± 0.9	3.3 ± 0.1	9.9 ± 0.9	0.2 ± 0.03	93.2 ± 1.3
Mild asthma	126.4 ± 2.1	79.9 ± 1.3	4.1 ± 0.2	14 ± 1.1	1.8 ± 0.4†	89 ± 1.1
Moderate asthma	136.5 ± 4.7	87.8 ± 0.9*	2.2 ± 0.1	7.8 ± 0.3	1.6 ± 0.3†	96.3 ± 1.3

Definition of abbreviations: CV = cell viability; Eos = eosinophil; Lym = lymphocyte; Macro = macrophage; Neut = neutrophil; TCC = total cell count (cells x 10^3/ml).

* $p < 0.05$ versus subjects with mild asthma.

† $p < 0.05$ versus control subjects.

4.2.2 *Whole blood culture for IL-8 measurement*

Whole blood from normal, mild and moderate asthmatics was cultured as described in section 2.2.9.1. The supernatant was removed and stored at -70°C until analysed.

4.2.3 *ELISA*

IL-8 levels in cultured whole blood were measured by duoset ELISA kit (R&D systems Europe, Oxon, UK). The lower limit of detection was 31.2 pg/ml.

4.2.4 *Isolation of peripheral blood mononuclear cells (PBMCs) and granulocytes*

PBMCs and granulocytes were isolated from venesected whole blood as described in section 2.2.2. Cells were stained with Kimura stain for determination of total cell number, and the cell concentration was adjusted to $5x10^6$ with PBS. Cell viability, as determined by trypan blue exclusion was uniformly greater than or equal to 97%.

4.2.5 *Fiberoptic Bronchoscopy, BAL collection and processing of BAL macrophages.*

The methods used for bronchoscopy and sample collection are described in section 2.2.11.

4.2.6 *Measurement of HDAC activity.*

HDAC was measured as decribed in section 2.2.12.

4.2.7 *Statistics*

Results are expressed as mean ± standard error of the mean (SEM). All statistical testing was performed by using a two-sided 5% level of significance.

4.3 Results

4.3.1 *Patient characterization*

Baseline characteristics and investigation results of the patients undergoing bronchoscopy are summarized in Table 4.1. No differences were found in BAL total cell counts or BAL neutrophils between normal subjects and those with asthma, but the number of BAL macrophages was significantly higher in the group with moderate asthma than in the group with mild asthma (Table 4.3). The numbers of eosinophils were higher in subjects with asthma than in normal subjects, but there was no difference between the mild and moderate asthma groups. The number of macrophages per ml of recovered BAL fluid was significantly higher in moderate to severe patients receiving ICS (Table 4.3). Characteristics of the mild and severe asthmatics in whom blood studies were performed, are summarized in table 4.2, and are presented in more detail in appendix 2.1 (page 239), and 2.2 (page 240) . Three of the patients in the moderately-severe group were receiving maintenance prednisolone (5, 10 and 15 mg/day). There was a trend toward an improvement in FEV_1 (from 1.29 ± 0.3 to 1.74 ± 0.2 L, p = 0.32), and a decrease in eNO (30.4 ± 5 to 18.3 ± 4 ppb, p = 0.09) in the group with severe asthma after receiving oral prednisolone, although this did not reach significance.

4.3.2 *HDAC activity in alveolar macrophages in mild and moderate asthmatics*

AM nuclear extracts from mild asthmatics (n=10) showed a reduced HDAC activity compared to healthy age-matched controls (n=10) (3017.9 vs. 4944 AFU/10μg, $p<0.01$). However, the group of patients with moderate persistent symptoms receiving ICS as a regular treatment (n=8), showed an increased HDAC activity (3540.2 AFU/10μg) although did not reach the levels of normal controls (fig. 4.1). There was no correlation between the levels of HDAC activity and post-bronchodilator FEV_1 or PC_{20}.

4.3.3 *HDAC activity of PBMCs and granulocytes in mild and severe asthmatics and control subjects*

PBMCs did not show a significant difference in baseline HDAC activity when comparing mild or moderate asthmatics to normal controls. There was also no difference in granulocyte HDAC activity between normal subjects, and mild asthmatics and severe asthmatics (fig. 4.2). Granulocytes from whole blood showed a reduced

HDAC activity, when compared to PBMCs (1869.4±100 vs. 4788.7±1056 AFU/10μg, $p<0.001$) in blood taken from asthmatics and normal subjects suggesting a different regulation or a predominant pro-inflammatory role of these cells. (Fig. 4.3)

4.3.4 *Effect of treatment with prednisolone increased HDAC activity in PBMCs*

Prednisolone 30mg/day for a week significantly increased HDAC activity in mild asthmatics (4303.2±16.22 vs. 5333.3±21.32 AFU/10μg, $p<0.05$) but this increase was not significant in the more severe group (fig. 4.4). An improvement in lung function was observed after treatment in the moderately severe group, and in both mild and moderate-severe group, a reduction in eNO was observed.

4.3.5 *Effect of treatment with prednisolone on HDAC activity in neutrophils*

No change in HDAC activity in granulocytes was seen after prednisolone treatment.

4.3.6 *Effect of treatment with prednisolone on IL-8 release in whole blood*

Whole blood IL-8 release was decreased after a week of prednisolone therapy in mild asthmatics but not in severe asthmatics (fig 4.5, $p<0.05$).

HDAC activity in alveolar macrophages in normal subjects and mild and moderate asthmatics

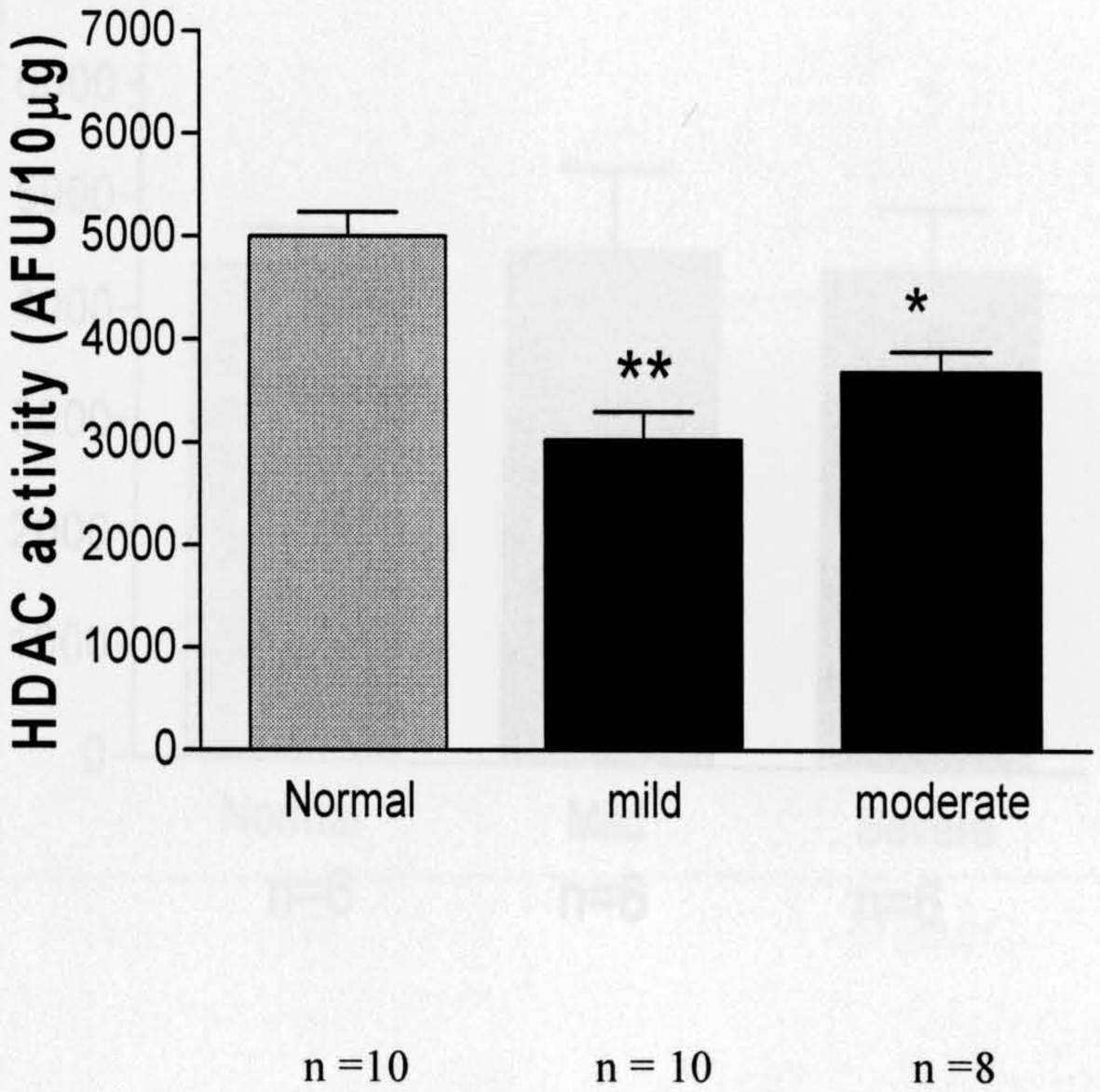


Fig. 4.1 HDAC activity in AM in BAL from normal subjects, and mild and moderate asthmatics, showing reduced HDAC activity in AM from mild asthmatics. Increased HDAC activity was seen in moderate group on ICS. (* p = 0.05, ** p< 0.01).

Basal HDAC activity of PBMC in asthmatics with different severity

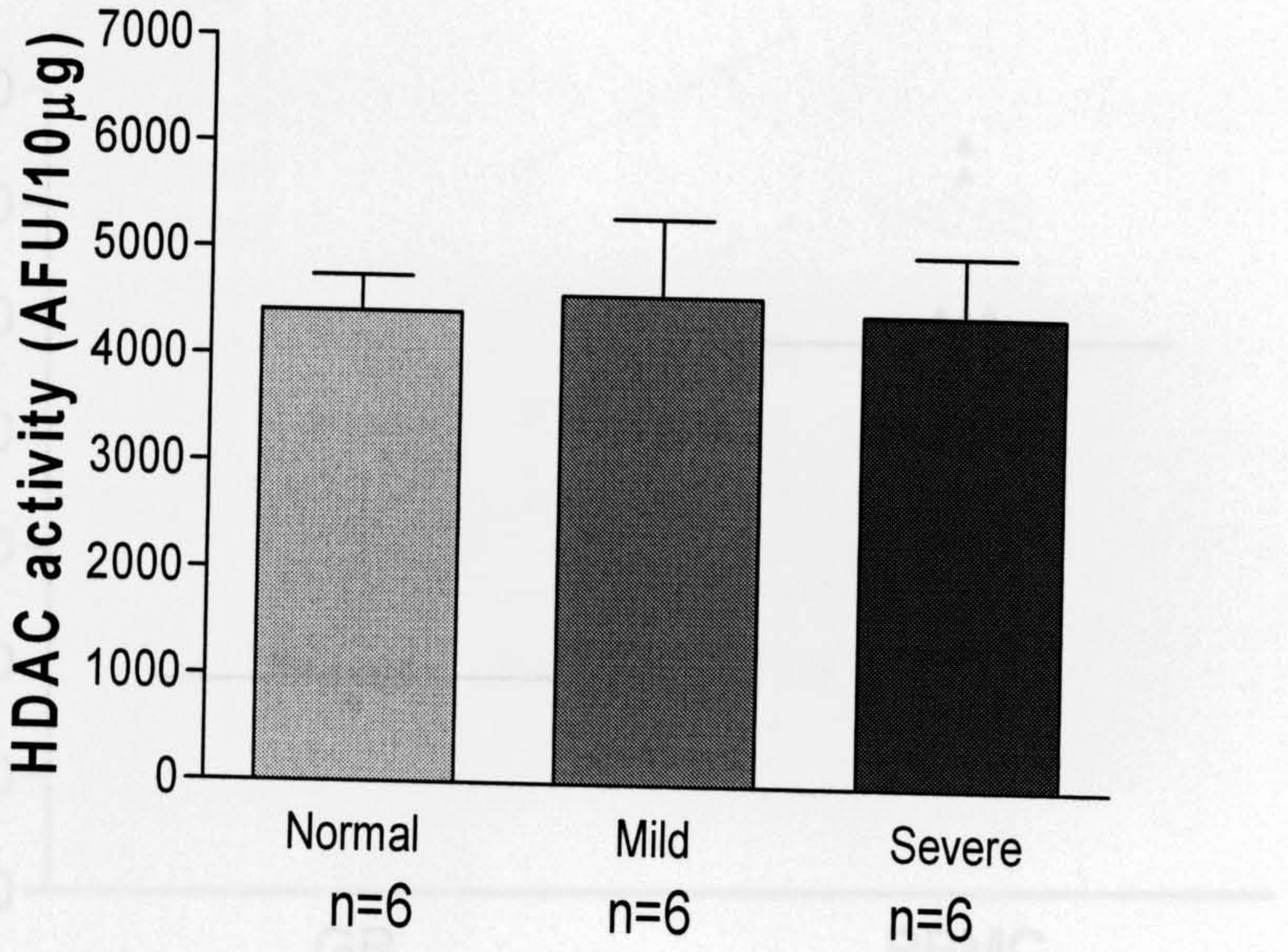


Fig. 4.2 Basal HDAC activity in PBMCs in control, mild, and severe asthmatics. No difference in HDAC activity was seen between the groups.

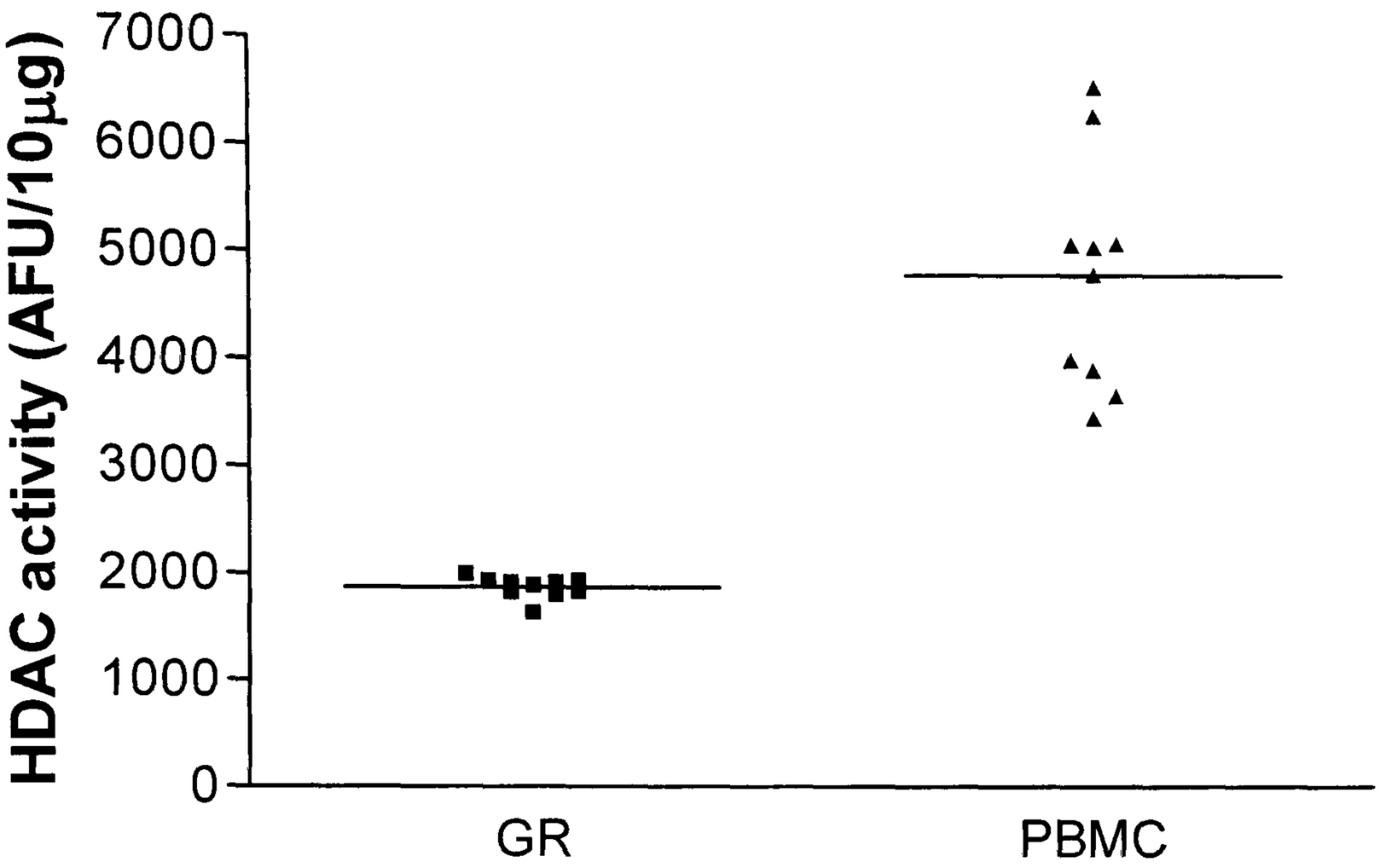
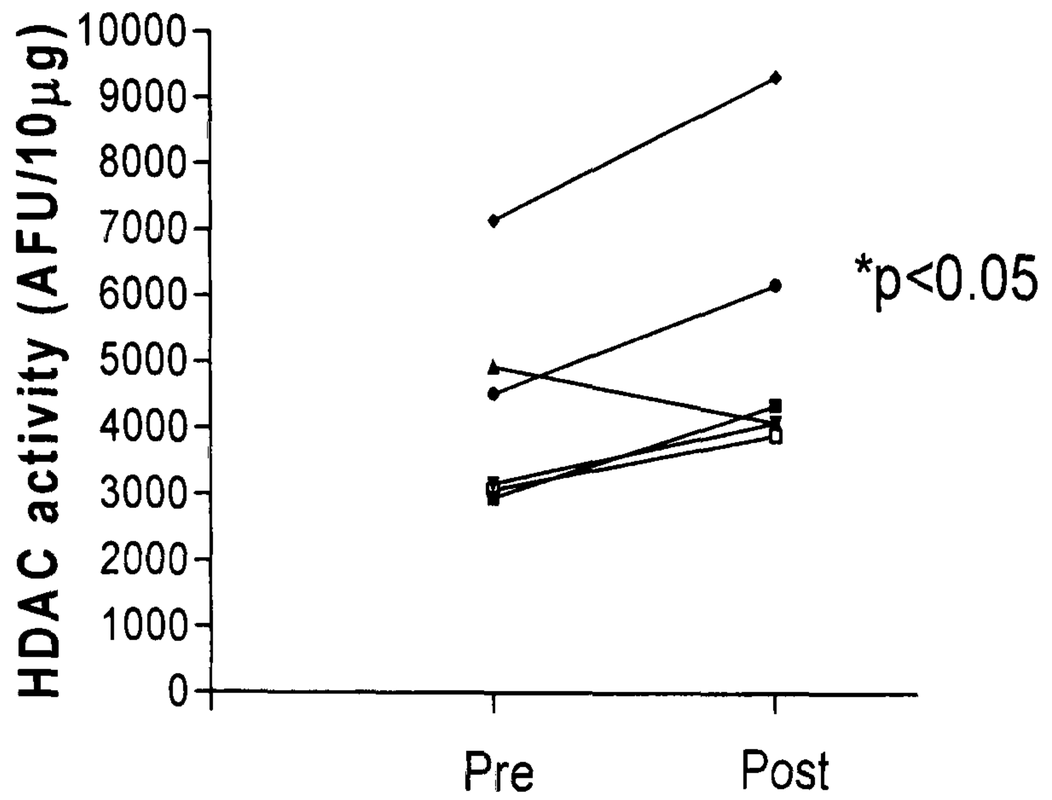


Fig. 4.3 HDAC activity in peripheral blood granulocytes and PBMCs. Granulocytes from whole blood expressed less HDAC activity than did PBMCs (1869 +/- 40 vs. 4789 +/- 431 AFU/10 µg, respectively; $p<0.001$) from peripheral blood from all groups.



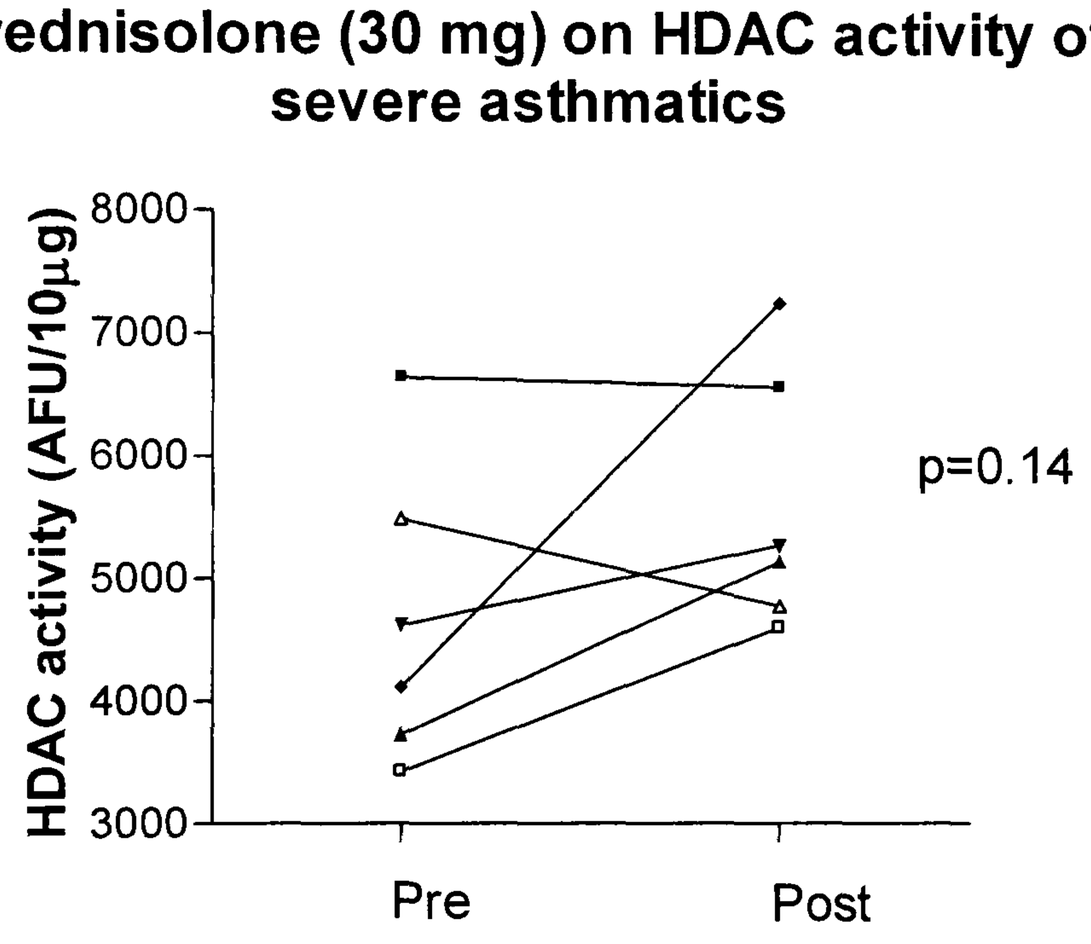


Fig. 4.4 Effect of a 1 week course of prednisolone 30mg/day on HDAC activity on PBMCs in mild and severe asthmatics. Prednisolone increased HDAC activity in PBMCs from mild but not severe asthmatics.

IL-8 in whole blood culture in mild and severe asthmatics

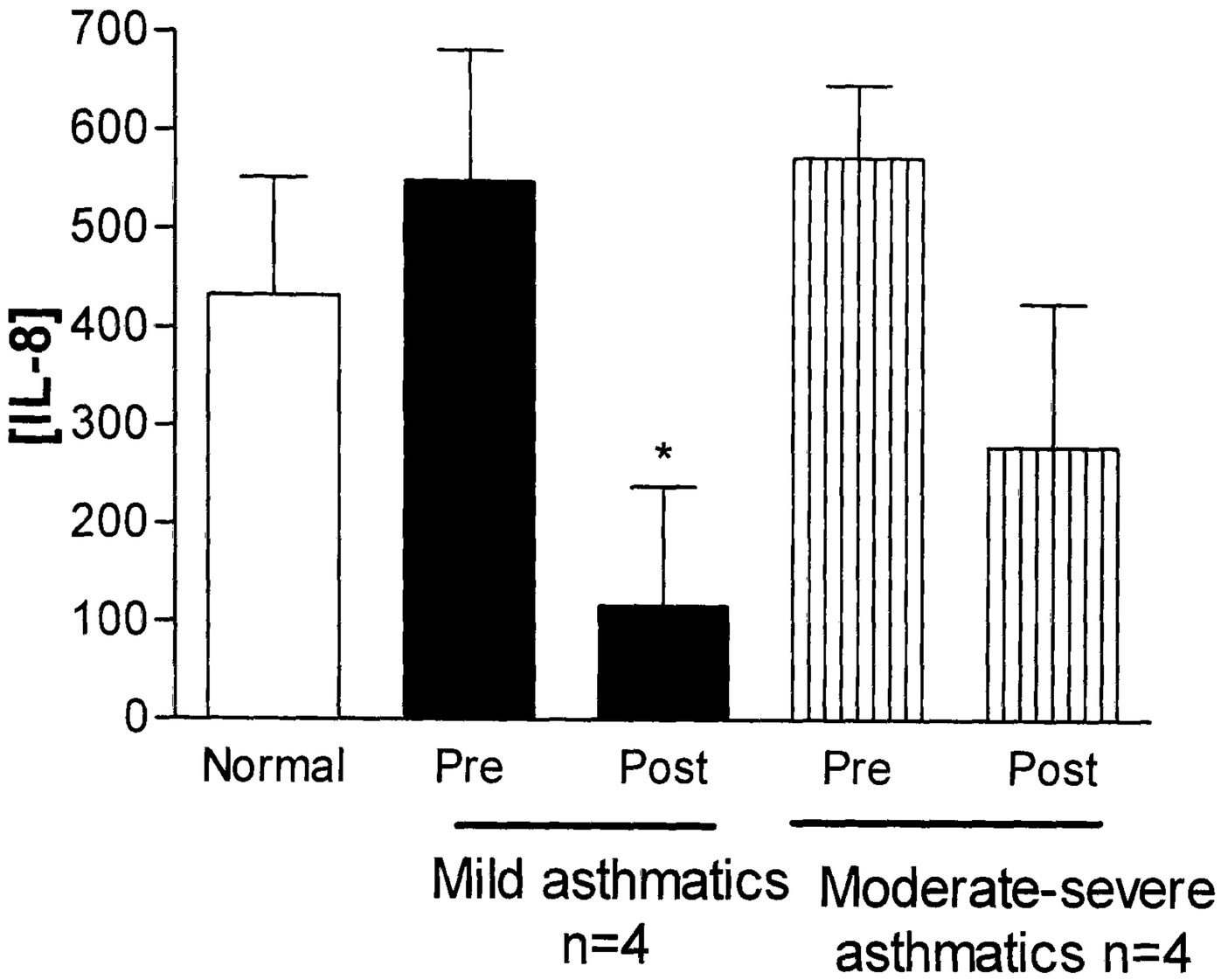


Fig. 4.5 IL-8 levels in cultured whole blood in normal subjects, and mild and severe asthmatics, before and after 1 week course of prednisolone 30mg/day. IL-8 release was decreased in mild asthmatics but not in severe asthmatics. (* $p < 0.05$).

4.4 *Discussion*

Glucocorticoids are the most effective therapy for the treatment of chronic inflammatory diseases such as asthma, rheumatoid arthritis, inflammatory bowel disease and autoimmune diseases, all of which are associated with increased expression of inflammatory genes. Most glucocorticoid actions on cells are mediated via interaction with specific, intracellular type 2 glucocorticoid receptors (GR). The human type 2 GR is a member of the steroid receptor super-family with characterisitc 'zinc fingers' motifs for DNA binding. These receptors are expressed in monocytes, macrophages, granulocytes and all lymphocyte subpopulations, with the density and affinity of glucocorticoid receptors varying between leukocyte types. The number of GR depends on the ambient glucocorticoid concentration. For example, experimental adrenalectomy results in increased GR expression in most tissues. Conversely, prolonged glucocorticoid administration results in a fall in GR numbers (210).

Glucocorticoids bind to GRs in the cytoplasm of target cells which then dimerize and translocate to the nucleus, where they bind to glucocorticoid response elements (GRE) on glucocorticoid-responsive genes, resulting in increased transcription of genes coding for anti-inflammatory proteins, including lipocortin-1, IL-10, IL-1 receptor antagonist and neutral endopeptidase. Glucocorticoids inhibit the expression of multiple inflammatory genes through a direct inhibitory interaction between activated glucocorticoid receptors and activated transcription factors, such as nuclear factor-kappa B and activator protein-1, which regulate the inflammatory gene expression (210), (fig 4.6).

There has recently been increasing evidence that glucocorticoids may have effects on the chromatin structure. GR also interact with CBP, which acts as a co-activator of transcription, binding several other transcription factors that compete for binding sites on this molecule. Increased transcription is associated with uncoiling of DNA wound around core histone proteins and this is secondary to acetylation of the histone residues by the enzymatic action of CBP. Glucocorticoids may lead to deacetylation of histone proteins, resulting in tighter coiling of DNA and reduced access of transcription factors to their binding sites, thereby suppressing gene expression (211).

This study showed that AM from asthmatics have a reduced HDAC activity compared to normal subjects, but there was no difference in HDAC activity in PBMCs between asthmatics and controls. The reduction in HDAC activity is associated with an

increased transcription of inflammatory proteins and thus an increase in inflammation (137). This suggests that AM from asthmatics show a higher level of activation in the lungs (the main site of inflammation), than PBMCs in the systemic circulation.

Ito et al (137) found an increased HAT activity in bronchial biopsies from mild asthmatics and a reduced HDAC activity. Their study also showed that treatment with ICS reduced inflammation by increasing HDAC activity and suppressing NF-κB activation, which causes a reduction in HAT activity. It has previously been shown that glucocorticoids require recruitment of HDAC2 to the p65-HAT complex for their repressive actions (212). The reduced HDAC activity found in AM confirms previous studies (213) while the increase in HDAC activity observed in the ICS-treated group would be consistent with the findings of *Ito et al* (137).

An interesting finding in this study was the very low HDAC activity in granulocytes found generally in normal subjects and mild and severe asthmatics, suggesting a higher inflammatory potential of these cells in general compared to PBMCs and AM. No difference in HDAC activity in neutrophils was seen between normal subjects and mild or severe groups, and treatment with prednisolone did not increase HDAC activity in these cells, implying a reduced capacity of glucocorticoids to regulate neutrophil inflammatory potential. In contrast, prednisolone did increase HDAC activity in PBMCs in the mild group, suggesting that they can reduce the activation of these cells. In the severe group however, the increase in HDAC following prednisolone was not statistically significant possibly due to the effect of baseline steroid therapy and the sample size. In addition, prednisolone reduced IL-8 levels in whole blood culture in mild asthmatics but not in more severe asthmatics. It is not clear why it was possible to measure serum IL-8 levels in asthmatics in this study, whereas it was not possible to measure baseline serum IL-8 in the prednisolone study (chapter 3). The IL-8 ELISA experiments in this chapter were performed by Dr B Cosio, and thus a difference in operator technique may have been a factor.

In both groups, there was a reduction of exhaled NO after treatment, which is an indirect estimation of the effect on the bronchial inflammation. The mean prednisolone level after one week of treatment was higher in mild asthmatics than in the more severe asthmatics. However, in the final analysis, only 3 patients in each group had prednisolone and cortisol levels available, and thus this is not an accurate reflection of compliance in these patients with prednisolone therapy.

In conclusion, analysis of the HAT and HDAC status can give a reflection on the activation status of a cell population. Neutrophils in general have significantly reduced

HDAC activity suggesting increased inflammatory potential, and this was not altered by prednisolone, in contrast to the effect on PBMCs. This lack of modulation of neutrophil HDAC activity and prolonged survival of neutrophils by inhibition of their apoptosis, suggests that activated neutrophils at sites of inflammation can continue to release intracellular mediators potentially resulting in airway wall damage and remodelling. This pattern of activity in neutrophils is in direct contrast to HDAC activity in AM, as is the inhibitory effect of corticosteroids on PBMCs in mild asthmatics.

The pattern of reduced HDAC activity and poor response to the anti-inflammatory actions of corticosteroids seen in neutrophils from patients with severe asthma is mirrored in chronic obstructive pulmonary disease (COPD) which also shows multiple activation of inflammatory genes. Interestingly, *Ito et al* (214) showed that patients with COPD have a progressive reduction in total HDAC activity in specimens of lung tissue obtained from patients with increasing severity of the disease. It is known that oxidative stress, which represents an imbalance between production of reactive oxygen species and antioxidant defences, and cigarette smoke increase histone acetylation and activate inflammatory gene transcription. Exhaled markers of oxidative stress, such as 8-isoprostane and ethane, are increased in healthy smokers but are raised to a much greater extent in patients with COPD (215,216). It has been proposed that oxidative stress impairs the function of HDAC2 in COPD resulting in resistance to corticosteroids, and that this is due in part, to the greater numbers of neutrophils and macrophages (215). In asthma, oxidative stress is low (217), but severe asthma is characterised by an increase in neutrophils resulting in greater oxidative stress, which could account for the reduced responsiveness to corticosteroids in these patients.

The mechanisms whereby oxidative stress results in impaired HDAC activity are unclear, but one possibility is that the increased formation of NO results in the production of peroxynitrite which impairs the activity of HDAC2 by nitration of tyrosine residues on HDAC or associated proteins (218). The HDAC2 complex is now unable to reverse histone acetylation, and this may be a factor in the relative resistance to corticosteroids seen in severe asthmatics. The use of effective anti-oxidants that can neutralise oxidative stress may help to increase the response to corticosteroids.An alternative approach may be through blocking the production of peroxyntrite from NO, by inhibition of inducible NO synthase (iNOS). Initial studies have shown that the prodrug L-N6 is rapidly converted to

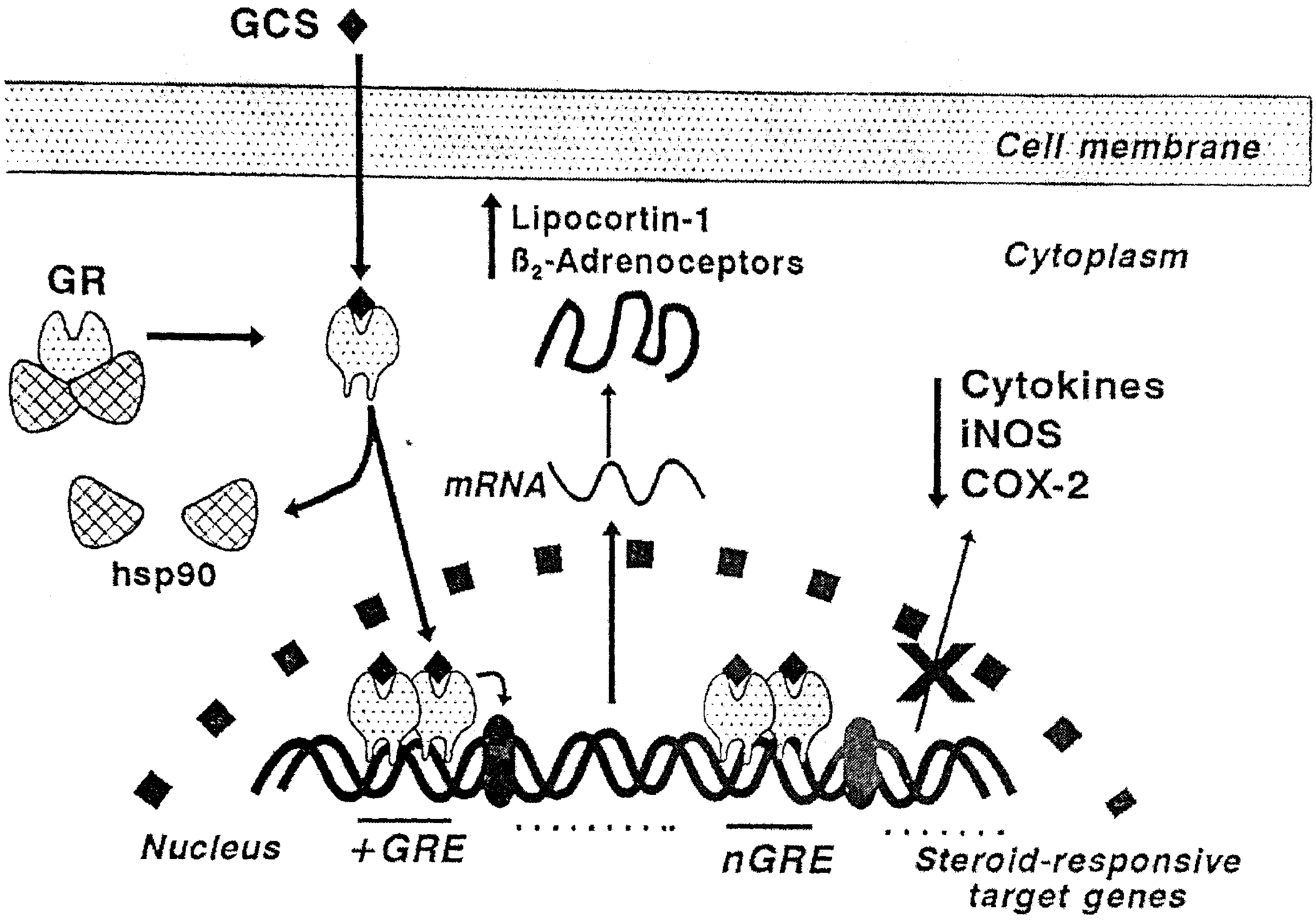


Fig 4.6 Mechanism of action of glucocorticoids. The glucocorticoid (GCS) enters the cellular cytoplasm and binds to a glucocorticoid receptor (GR) which is complexed with 2 molecules of a 90 kDa heat shock protein (hsp 90). GR then translocates to the nucleus, where, as a dimer, it binds to glucocorticoid response elements (GRE) on glucocorticoid-responsive genes, resulting in increased transcription of genes coding for anti-inflammatory proteins. In contrast, nGREs may decrease transcription, resulting in decreased mRNA and protein synthesis. Glucocorticoids inhibit the expression of multiple inflammatory genes through a direct inhibitory interaction between activated GRs and activated transcription factors, such as nuclear factor-kappa B and activator protein-1, which regulate the inflammatory gene expression. (From reference 136). COX-2, inducible cyclooxygenase; iNOS, inducible nitric oxide synthase.

an active metabolite which causes inhibition of iNOS in in-vitro experiments in human primary airway epithelial cells. A randomised, double blind, PBO crossover trial involving an oral form of this drug, decreased exhaled breathe NO levels to less than 2 ppb in both groups within 15 minutes, representing greater than 90% inhibition of baseline levels of NO in asthmatics, with no effects on lung function and no significant side-effects. This suggests the potential use of iNOS inhibitors to treat a range of inflammatory clinical disorders including severe asthma (219). Further, new classes of drugs that can activate HDACs may be identified in the future.

In this study, the level of HDAC activity was measured in isolated granulocytes. Specifically isolating neutrophils further from eosinophils would have allowed the difference in HDAC activity in these individual cell populations to be studied. It would be interesting to look at HDAC levels in asthmatic patients having exacerbations. This would be easier to do in blood samples rather than performing bronchoscopy. In addition, the level of oxidative stress in patients with severe steroid dependent asthma compared with milder asthmatics and normal subjects would be interesting to study. Repeating HDAC measurements in the blood study with more patient numbers in each of the mild and severe groups would increase the reliability of the results. In addition, the results would be more meaningful by ensuring that all patients were compliant with prednisolone therapy, by ensuring that serum prednisolone levels were available for all patients.

Chapter 5

A study of the immediate effects of inhaled Fluticasone Propionate on lung function and neutrophil and eosinophil activation in patients with stable mild and moderately severe asthma.

5.1 Introduction

Inhaled corticosteroids are the most effective treatment available for the management of chronic asthma, and have been shown to improve airway hyperresponsiveness and lead to improved asthma control. Several studies have shown an improvement in asthma symptom scores, FEV1 and PC_{20} measurements, reduction in sputum eosinophil counts and NO levels following treatment with ICS.

Lemerre et al (128) demonstrated a reduction in blood eosinophils and serum ECP occuring after budesonide inhalation and in addition, *Inoue et al* (123) showed that ICS resulted in a significant decrease in the mean levels of sputum supernatant IL-8 and GM-CSF, suggesting that the improvement in lung function and airway inflammation may, in part, be due to the inhibition of the synthesis of inflammatory cytokines such as IL-8 and GM-CSF. ICS however, have not been shown in studies to reduce the number of sputum neutrophils, and it is known that there is a subgroup of asthmatics with predominantly airway neutrophilia who repond less well to ICS (130). ICS have been shown to produce early improvements in lung function and reduction in eosinophil counts within a few hours of their administration, indicating a rapid onset of effect, possibly through non-transcriptional mechanisms such as interference with cytokine release.

5.2 Aims of study

The objectives of this study were to examine the early effects of a single dose of inhaled fluticasone propionate (FP, Flixotide Accuhaler) in patients with mild asthma and moderately severe asthma, and to monitor changes in (1) lung function-FEV1 (L), (2) bronchial responsiveness: PC_{20} methacholine, (3) inflammatory markers in exhaled NO, (4) eosinophil and neutrophil counts, (5) serum IL-8 levels, and (6) IL-8 and IL-5 levels in induced sputum.

In particular, the early effects of ICS on peripheral blood neutrophil and eosinophil numbers, as well as any early effects on cytokine production were studied.

5.3 Methods

5.3.1 *Study design*

This was a single centre study supported in part by Glaxo Wellcome Pharmaceutical Company. The study was approved by the Ethics committee of the Royal Brompton and Harefield NHS Trust, and the National Heart and Lung Institute. The study was a double-blind, randomised, PBO controlled, cross-over trial involving 3 separate days, separated by at least 1 week.

5.3.2 *Patients*

The study required 12 mild and 12 moderate patients to be recruited. However, 12 patients with mild asthma and 9 patients with moderately severe asthma were recruited from outpatient clinics and by advertisement, in the time available. Patients (age 18-60 years) with mild asthma were on Step 1 or 2 of the asthma guidelines with normal lung function (FEV1>75%), while those with moderately-severe asthma required treatment equivalent to steps 3 and 4 of the asthma guidelines in order to maintain control of their symptoms, (Table 5.1). All patients showed evidence of reversibility following beta-2 agonist administration, and had stable asthma for 6 weeks prior to entering the trial. Patients on maintenance oral corticosteroid therapy or those requiring oral corticosteroids within the previous 6 weeks were excluded, as well as patients with significant other medical disease. Characteristics of the individual mild and moderate asthmatics are shown in tables 5.2 and 5.3 respectively.

Table 5.1 Patient characteristics for fluticasone study

Mild asthma (n=12)	Moderate asthma (n=9)
Age mean 35.8 +/- 2.9 years (range 23-52)	Age mean 43.7 +/- 5.2 years (range 19-61)
Sex: 3 male/ 9 female	5 male/4 female
FEV1(L)- mean 3.03 +/- 0.1	FEV1(L)- mean 3.05 +/- 0.32
FVC (L)- mean 3.79 +/- 0.2	FVC (L) - 4.03 +/- 0.35
PC_{20} (mg/ml)- mean 1.31+/- 0.27	PC_{20} (mg/ml)- mean 1.32 +/- 0.31
NO (nitric oxide [ppb])- mean 26.6	NO (nitric oxide [ppb])- mean 26.3
Mean dose BDP or equivalent 471.8 +/- 56.5µg/day	Mean dose BDP or equivalent 1000 +/- 145.3µg/day
Short acting β2 agonist alone, n=5	Long acting β2 agonist, n=4

BDP-beclomethasone dipropionate

ppb-parts per billion

Results-mean+/-SEM

Table 5.2 Characteristics of mild patients in fluticasone study. N=12

patient	gender	Age-years	atopic	Treatment step	FEV1(L)	FVC(L)	Predicted FEV1 (%)	PC20 (mg/ml)	medication
bb	f	42	y	2	2.7	4.01	81.8	0.53	BDP 400µg/day
zc	f	33	y	2	2.84	3.41	90.4	2.31	BDP 400 µg /day
hl	f	26	y	2	3.79	4.87	78.1	0.37	BDP 400 µg /day
vl	f	44	y	2	2.8	3.59	89.4	0.55	BDP 400 µg /day
jn	m	37	y	1	2.94	4.02	81.8	n/a	Salbutamol As needed
cdb	f	29	n	2	2.65	2.82	78.8	0.67	BDP 800 µg /day
ss	m	52	y	2	3.37	4.91	83.0	1.08	BDP 500 µg /day
lmcr	f	23	y	2	3.18	3.71	99.3	2.19	BDP 400 µg /day
jp	m	52	n	1	3.72	4.6	96.3	0.7	Salbutamol as needed
bp	f	24	n	1	2.6	3.0	78.7	2.72	Salbutamol as needed
gj	f	28	y	1	2.65	2.86	78.7	0.84	Salbutamol as needed
sg	f	40	y	1	3.12	3.8	99.0	2.52	Salbutamol as needed

Table 5.3 Characteristics of moderate patients in fluticasone study. N=9

patient	gender	Age-years	atopic	Treatment step	FEV1(L)	FVC(L)	Predicted FEV1 (%)	PC20 (mg/ml)	Drugs
cam	m	60	y	4	2.67	3.65	71.9	0.39	BDP 2000 µg /day formoterol 12 µg bd
jj	f	61	y	3	1.43	2.55	70.0	1.15	BDP 400 µg /day Salmeterol 25 µg bd
rp	f	52	y	3	2.0	3.7	79.0	1.15	BDP 800 µg /day
lm	m	48	y	3	3.7	4.52	95.6	0.16	BDP 1000 µg /day Salmeterol 25 µg bd
nd	m	28	y	3	4.0	5.6	87.3	0.73	BDP 800 µg /day
gb	f	52	n	3	2.96	3.4	97.5	2.79	BDP 1200 µg /day
gc	m	25	y	3	4.52	5.77	99.5	1.09	BDP 1000 µg /day
ep	m	19	y	3	3.45	4.07	71.7	2.35	Seretide 500/50 µg bd
rf	m	49	y	3	3.4	4.85	90.9	2.14	BDP 800 µg /day

5.3.2 *Fluticasone administration*

Two doses of FP and PBO were administered on separate days: 500μg and 2000μg FP from an accuhaler. A single inhalation of each drug was given in the morning (8am) and the patients were observed hourly for 8 hours and then again at 24 hours. Patients already taking ICS were asked not take these for 48 hours prior to arrival at the laboratory. The use of rescue medication was prohibited within 6-8 hours of the study. The use of long-acting beta-agonists was prohibited for 48 hours prior to the start of the study. Leukotriene receptor antagonists were withheld for 24 hours.

5.3.4 *Protocol and measurements*

Lung function was measured regularly during the day using a vitalograph as described in section 2.1.4, and the best of 3 successive measurements recorded.

PC_{20} methacholine was measured at baseline, at 6 hours and at 24 hours at each of the 3 visits. After initial lung function measurement (FEV1), patients were asked to inhale 5 breathes of 0.9% sodium chloride solution (control diluent), followed by repeat measurements of FEV1 (section 2.1.5). Then increasing doubling concentrations of methacholine were inhaled every 5 minutes, and FEV1 measured after each dose. The test was stopped when more than 20% fall in FEV1 occurred. The PC_{20} is the concentration of methacholine needed to cause a 20% fall in FEV1 measured after initial saline inhalation. The study protocol required measurement of PC_{20} at baseline at initial screening for entry into the trial, but did not include measuring PC_{20} at time '0' at the start of the trial. Thus the PC_{20} measurements in the graphs at baseline were the same in each group (figs 5.5 and 5.6), but PC_{20} measurements were different at 6 and 24 hours in each group.

NO was measured in exhaled air using a chemiluminescence machine as previously described in section 2.1.6.

Blood was sampled through an indwelling cannula in a forearm vein of each subject at 0,1,2,3,5,6,8 hours after inhalation of FP. Blood eosinophil count and neutrophil counts were measured in the laboratory at the Royal Brompton hospital using a haemocytometer.

In addition, whole blood was cultured at 1, 4 and 24 hours to measure spontaneous and IL-1β induced release of the proinflammatory cytokine IL-8.

Induced sputum was performed before and at 6 hours after inhaled therapy. Supernatant was assayed for IL-5, IL-8. The method for collecting and processing induced sputum was as described in section 2.2.

ELISA: IL-8 and IL-5 levels were measured using Duoset ELISA kits purchased from R&D systems, Abingdon, Oxon, UK. The lower limit for detection was 31.25pg/ml for IL-8, and 15.65pg/ml for IL-5. The standards for IL-8 and IL-5 were prepared as recommended by the manufacturer, but when assaying sputum supernatant, 0.05% DTT was added to the reagent diluent in order to allow for the possible effect of the DTT on cytokine levels during processing of the sputum.

5.4 Results

5.4.1 *Lung function*

No significant difference was seen in FEV1 or FVC in mild patients over 24 hours following high or low dose inhaled FP, compared to PBO (figs. 5.1 and 5.2). In the moderate group however, an increase in FEV1 was seen at 8 and 24 hours compared with time point '0' with high dose FP ($p< 0.01$, fig. 5.3), and an increase in FEV1 at 8 hours compared with time point '0' with low dose FP ($p< 0.05$, fig 5.3). However, these were not significant when compared with PBO results at each time point. An increase in FVC ($p= 0.01$) was seen in moderate asthmatics at 24 hours with low dose inhaled FP compared to baseline (fig. 5.4), but this was not significant when compared to PBO at the same time point.

5.4.2 *Bronchial hyperreactivity*

In mild and moderate patients, PC_{20} was not significantly increased over 24 hours with high or low dose FP compared to baseline levels, or when compared with the PBO group (figs. 5.5 and 5.6). In the moderate group, all patients had PC_{20} measured at baseline, but during the trial, the FEV1 of 3 patients fell below 65% predicted, and therefore these patients did not have further measurements of bronchial hyperreactivity. The number of moderate patients who completed PC_{20} measurements was therefore six.

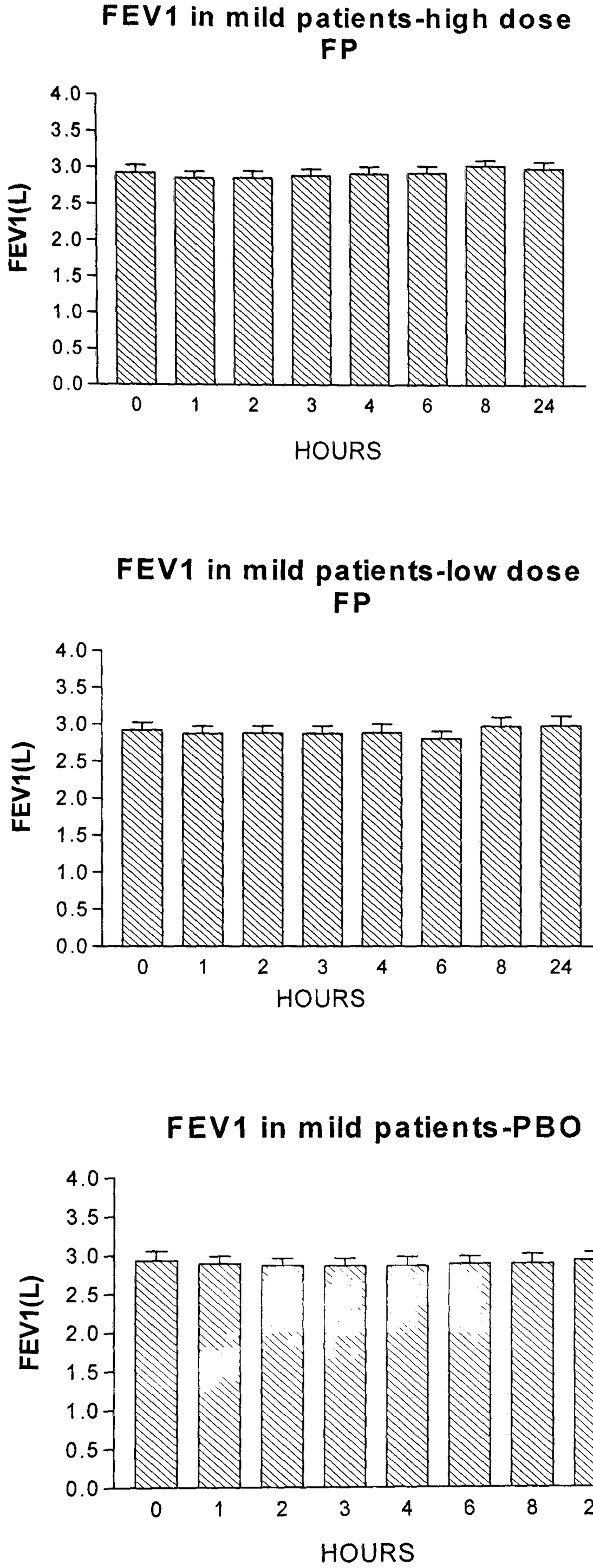


Fig. 5.1 Effect of high and low dose inhaled FP and PBO on FEV1 in mild asthmatics. No significant effect of ICS was seen on FEV1 in mild asthmatics, n=12.

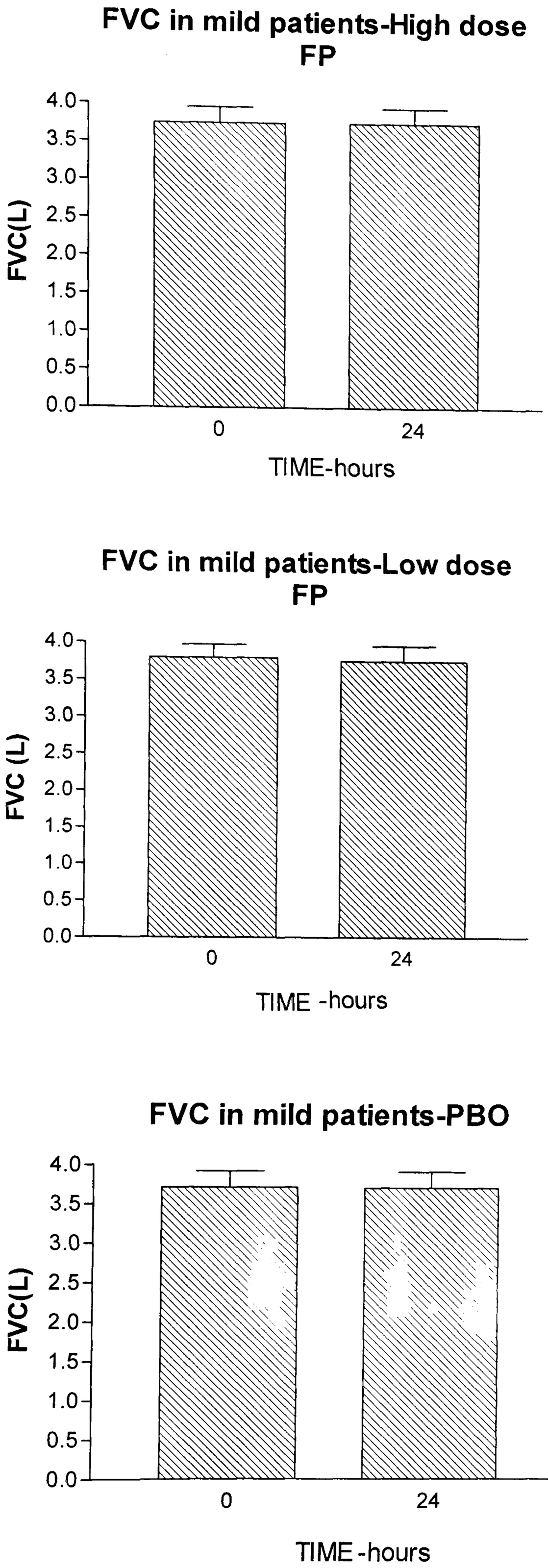
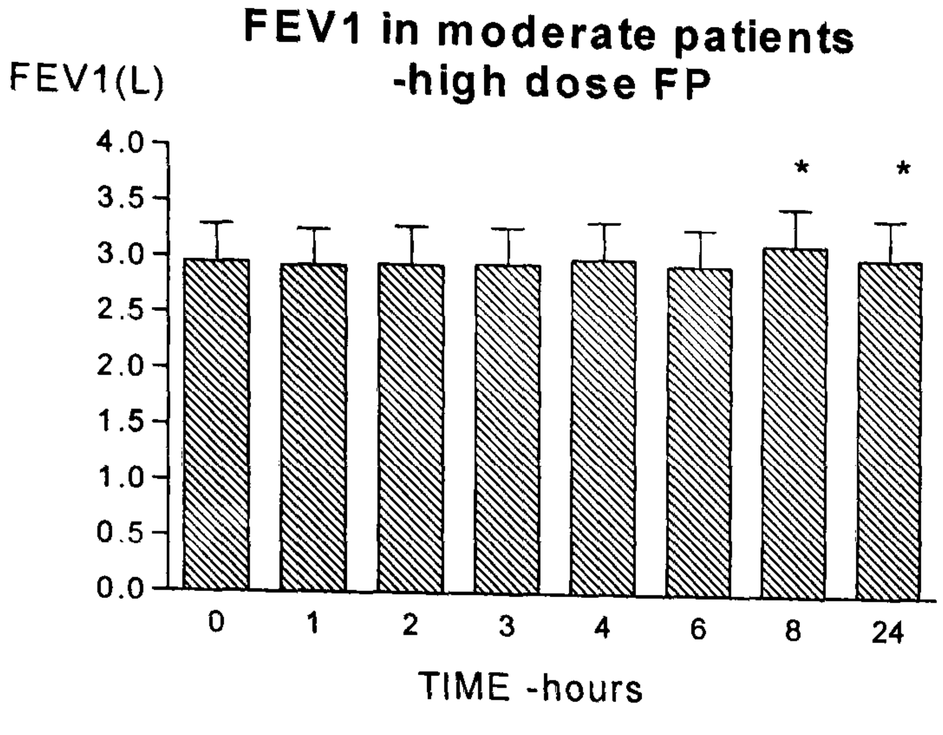
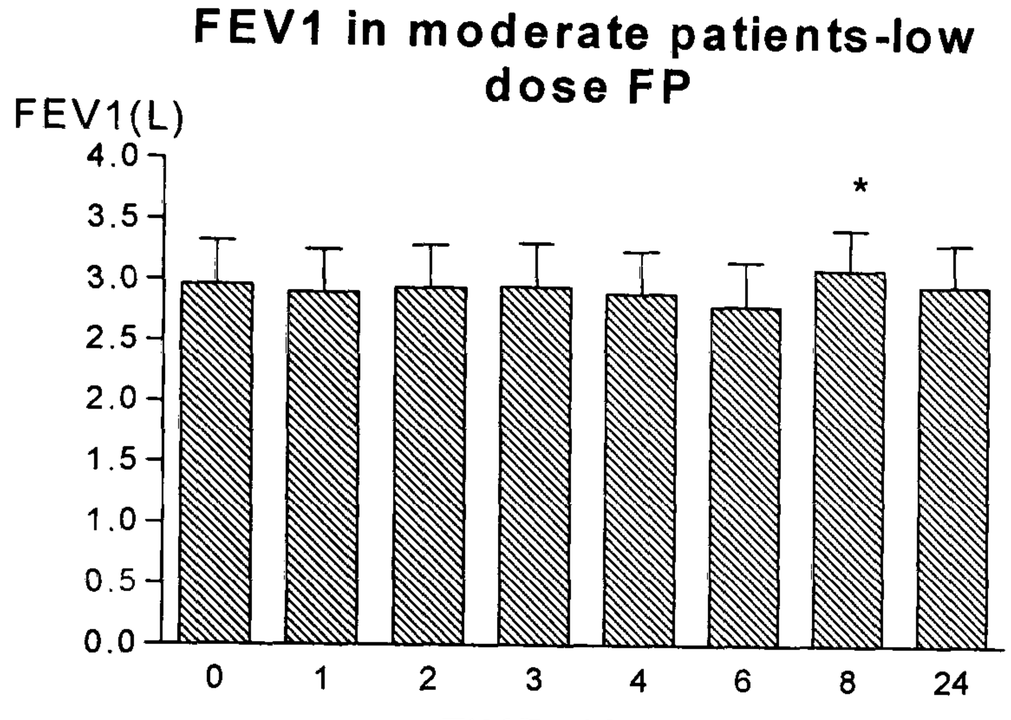


Fig. 5.2 Effect of high and low dose FP and PBO on FVC in mild asthmatics. No significant effect of ICS was seen. N=12.





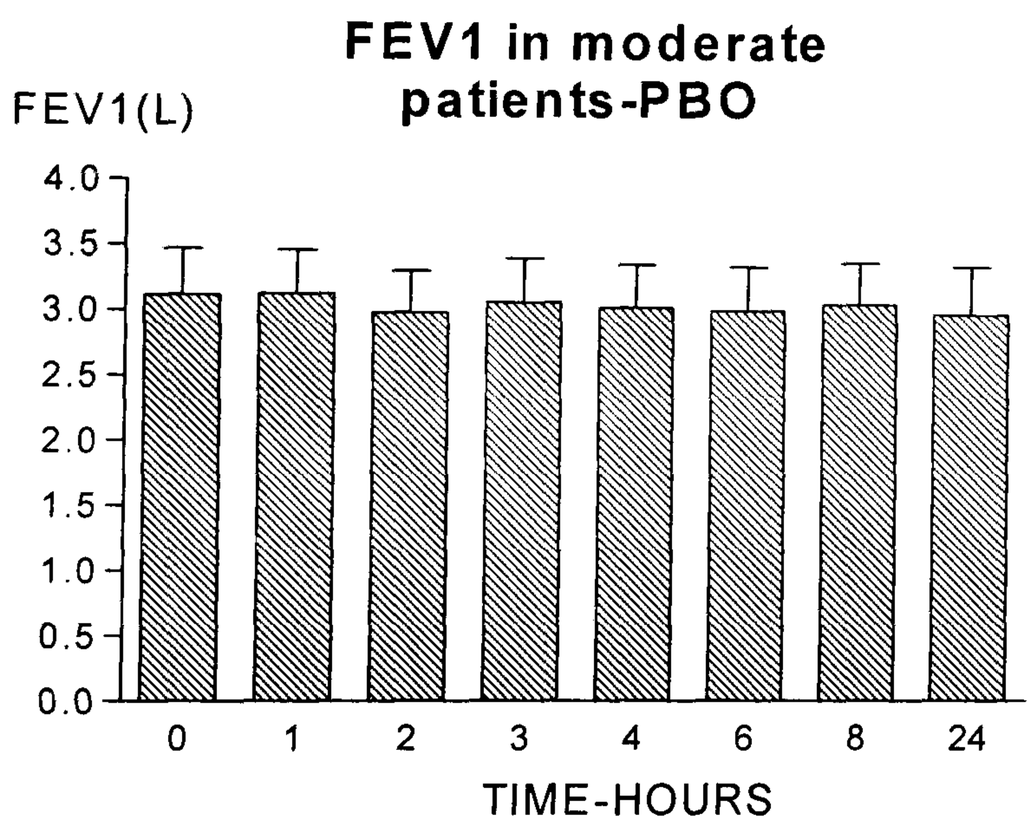
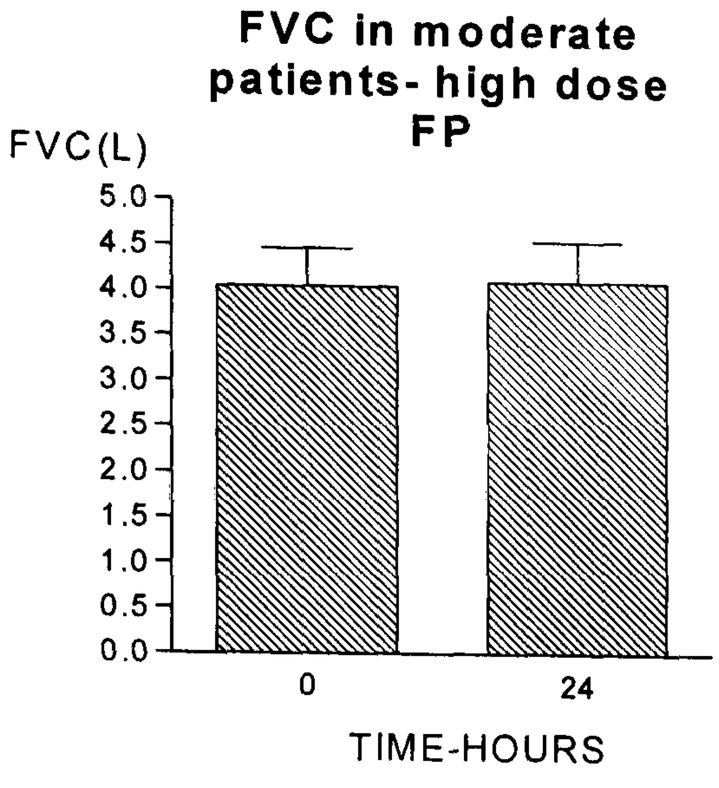
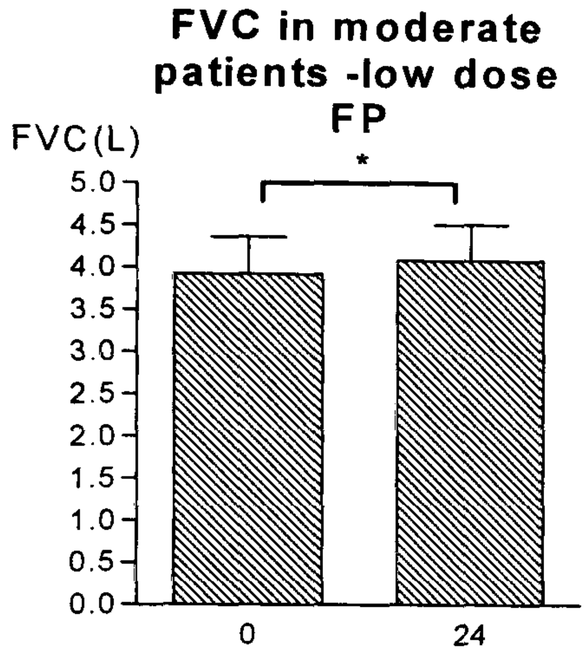


Fig. 5.3 Effect of high and low dose FP and PBO on FEV1 in moderate patients. Both doses increased FEV1 at 8 hours (and at 24 hours in the high dose FP group) compared to time=0, but this was not significant compared to PBO. n=9.





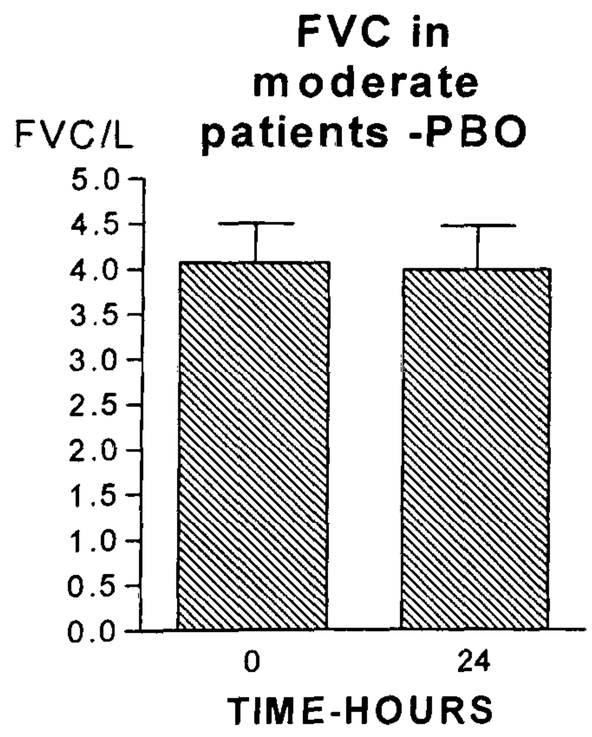


Fig. 5.4 Effect of high and low dose FP and PBO on FVC in moderate patients. Low dose FP increased FVC in moderate patients at 24 hours, (*p = 0.01), but this was not significant when compared to PBO.

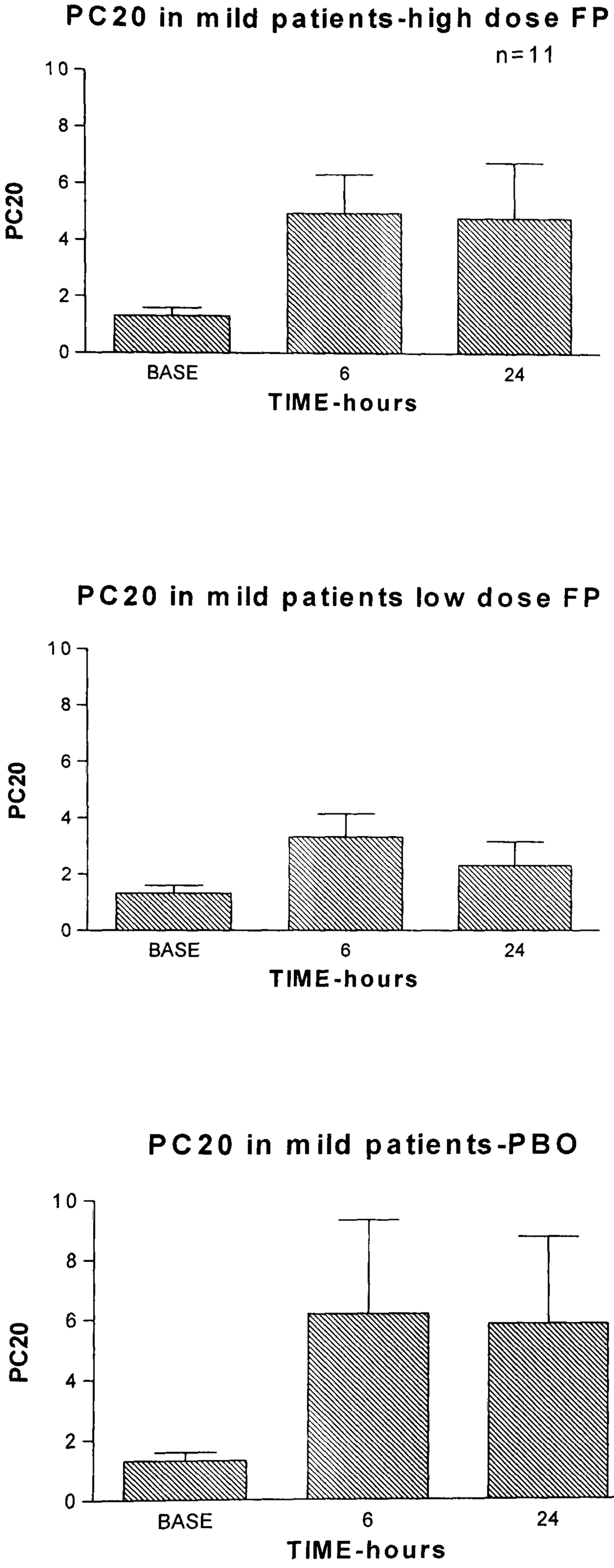
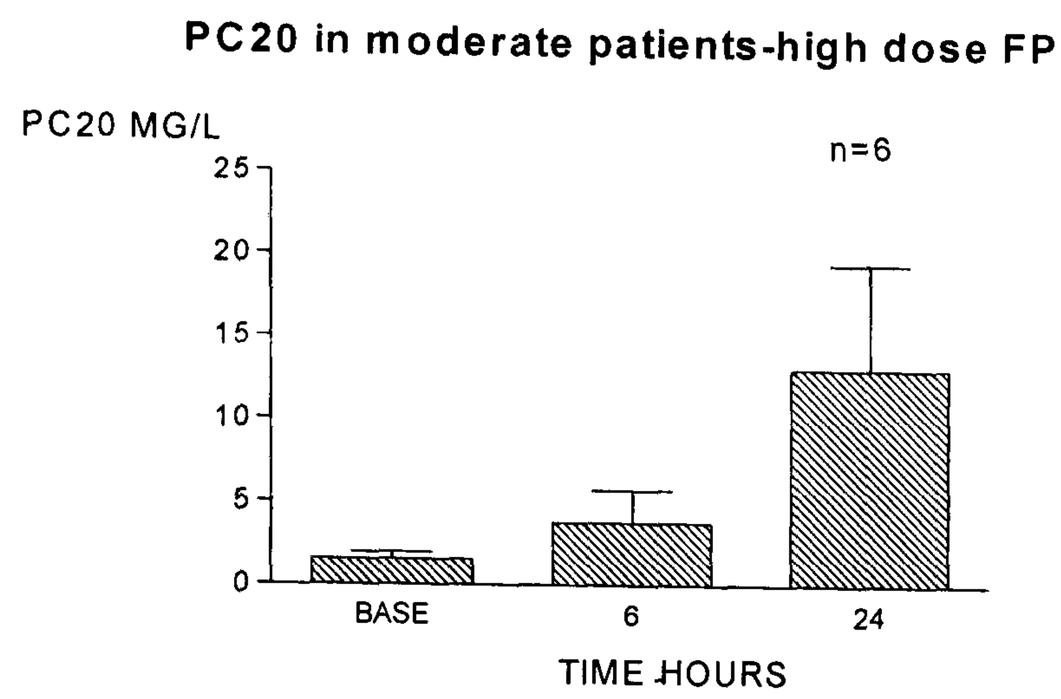
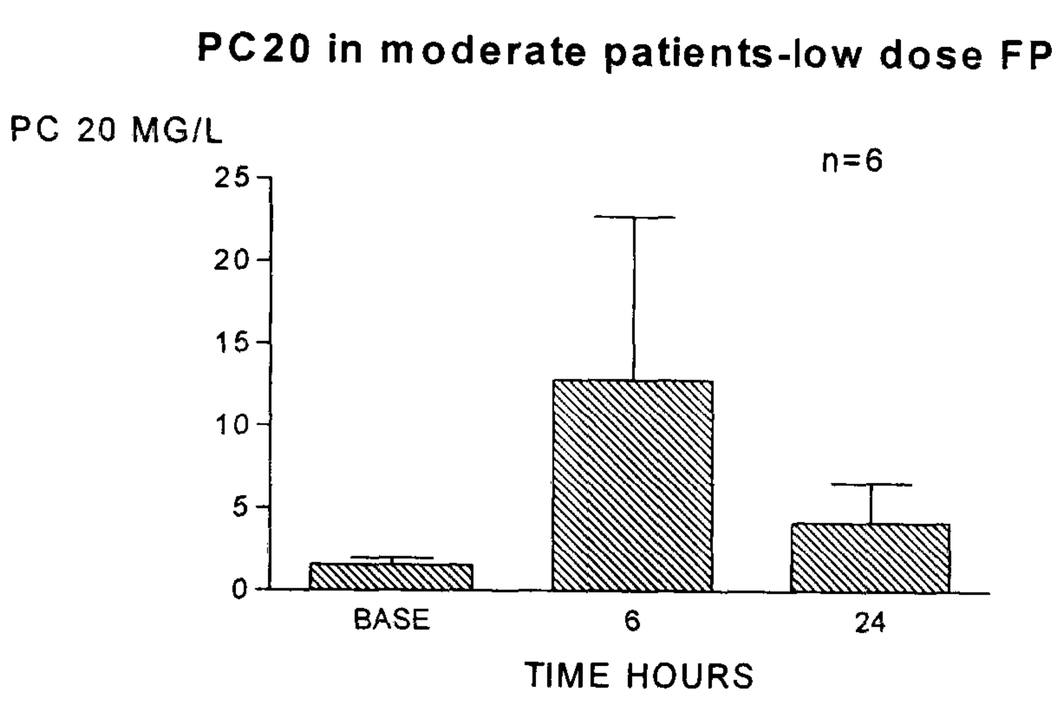


Fig. 5.5 Effect of high and low dose inhaled FP and PBO on PC_{20} (mg/ml) in mild patients. No significant effect was seen. Baseline was done at screening visit and not at start of the study (see methodology). N=11.





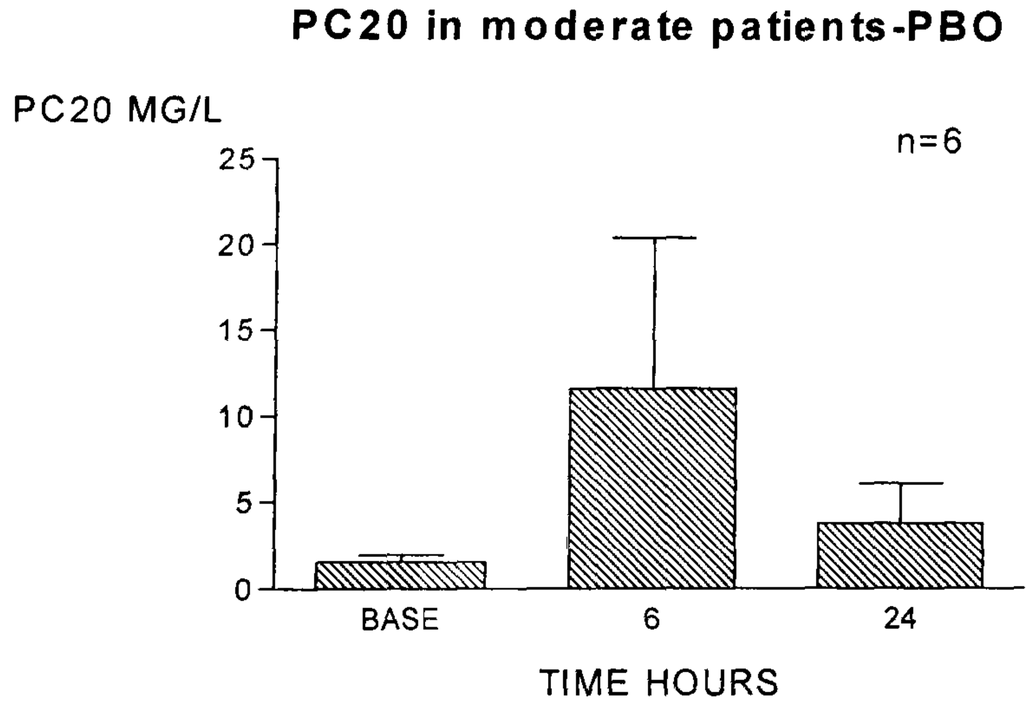


Fig. 5.6 Effect of high and low dose inhaled FP and PBO on PC_{20} (mg/ml) in moderate patients. No significant effect was seen. Baseline was done at screening-see methodology.

5.4.3 *Exhaled Nitric oxide*

In the mild asthma group, high dose FP consistently reduced exhaled NO levels after 3 hours onwards, compared to the first 3 time points. Low dose FP reduced NO levels at 24 hours, compared to preceding time points. However, NO levels were also reduced in the PBO group at 3, 6 and 8 hours compared to baseline. Comparisons between the treatment groups showed that NO levels were significantly higher at 2 hours with low dose FP than high dose FP but not PBO. At 24 hours however, exhaled NO levels were significantly lower in mild asthmatics taking high dose FP group than compared to PBO (figs. 5.7 and 5.8). In the moderate group, high dose FP decreased exhaled NO levels at 8 hours but this was not significant when compared to placebo. Low dose FP reduced NO consistently after the first hour compared with baseline, but this was significant against PBO only at 4 hours. PBO did not reduce NO levels. Both high and low dose FP reduced NO levels significantly when compared to PBO, at 4 hours.

5.4.4 *Peripheral blood eosinophil and neutrophil counts*

High dose FP had no effect on eosinophil counts in mild or severe asthmatics. In mild asthmatics, only low dose FP resulted in a significant decrease in eosinophil count four hours post inhalation ($p=0.01$) compared with baseline, but this was not significant compared with PBO at the same time point. No reduction in eosinophil count was seen in moderate asthmatics with high or low FP (figs. 5.9, 5.10). In the mild group, there was no significant effect on neutrophil count with high dose FP, but this dose did increase neutrophil counts at 8 hours in moderate asthmatics, though this was not significant when compared to PBO. Low dose FP led to a non-significant increase in neutrophil count after 3 hours in mild asthmatics, and this dose had no significant effect in moderate asthmatics (fig.5.11, 5.12). Low dose FP increased neutrophil counts in mild asthmatics at 8 hours compared to PBO. Neutrophil counts were higher in moderate asthmatics receiving high dose FP in compared with PBO at 1 hour ($p<0.05$).

5.4.5 *Serum IL-8 levels*

Serum IL-8 levels were increased after whole blood incubation with IL-1β in mild patients receiving PBO at 4 hours, in moderate patients receiving PBO at 1 hour, and moderate asthmatics receiving high dose FP at 4 hours. A significant difference was seen

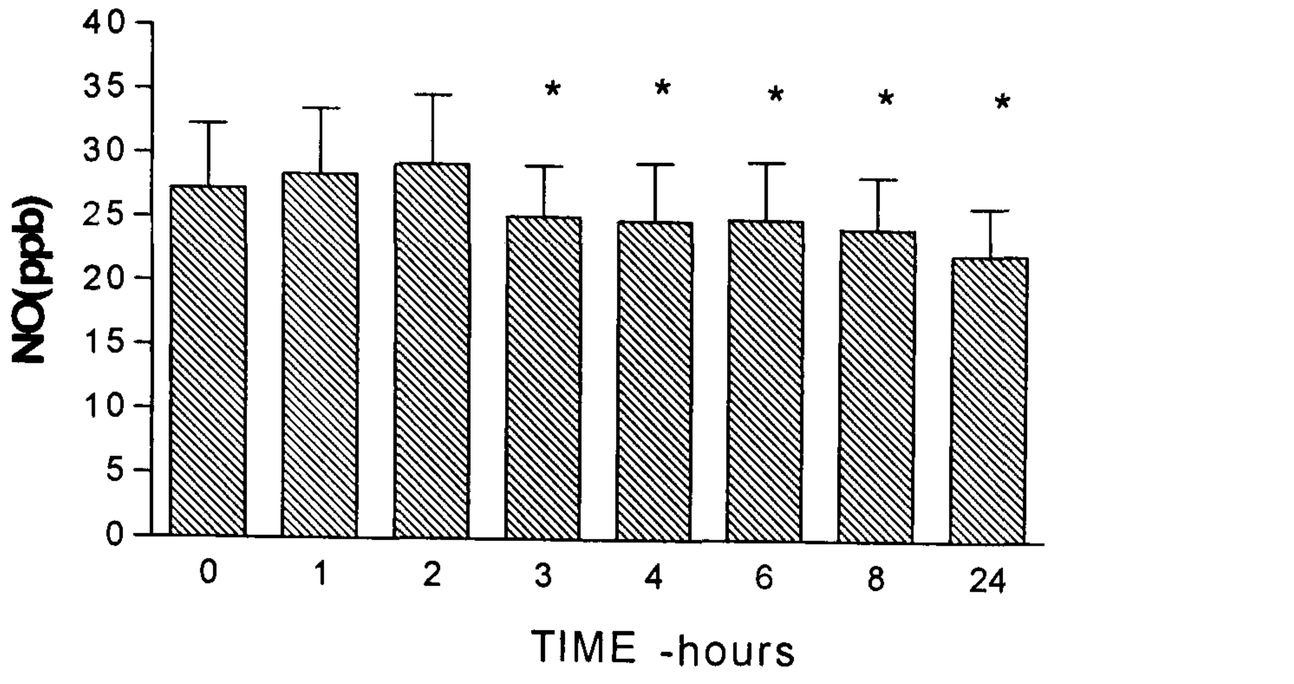
in baseline serum IL-8 levels in mild asthmatics receiving high dose FP, with lower levels of IL-8 seen in the blood cultured with IL-1β compared with serum IL-8 levels in blood which had not been cultured with IL-1β. The levels of baseline IL-8 seen in this group however were not statistically significant compared with PBO, and the significance of this effect is unclear. There was no significant difference in the effect of high or low dose FP treatment groups on IL-8 levels in mild asthmatics compared with PBO (figs. 5.13 and 5.14). FP, at either dose, had little effect on reducing IL-8 levels in moderate patients, except at 1 hour, when IL-8 levels were significantly reduced with high dose FP compared with PBO.

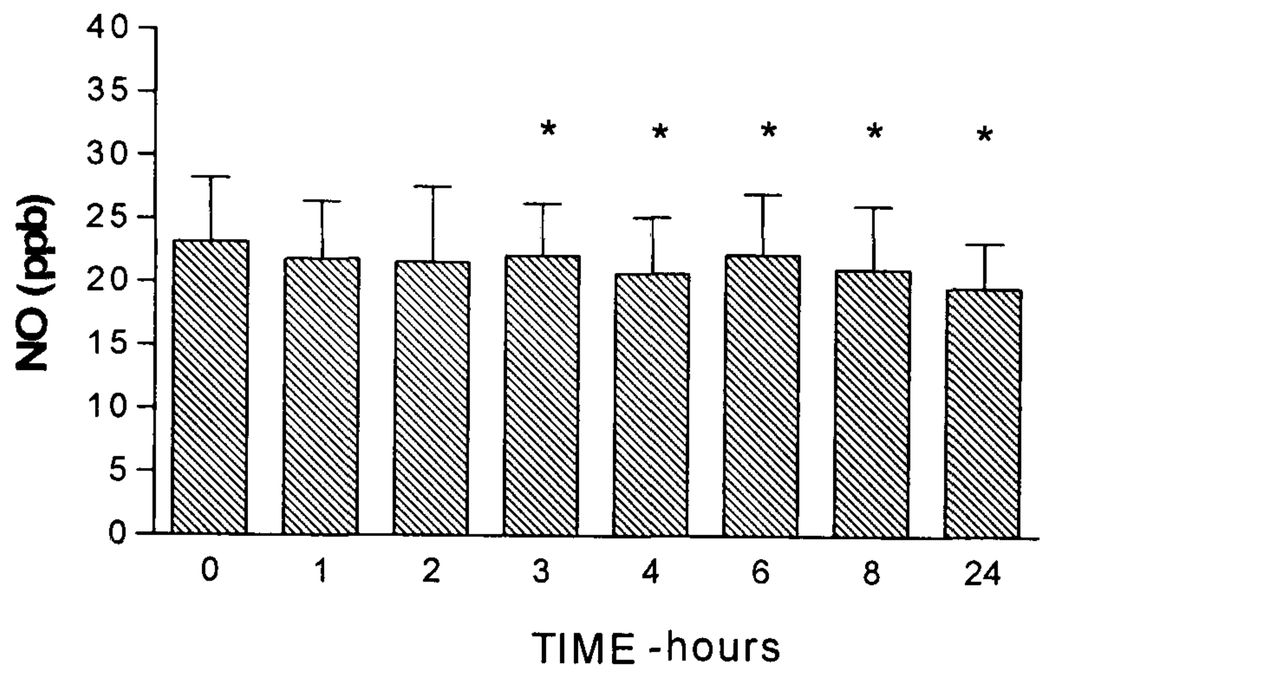
5.4.6 *Sputum cytokine levels*

At baseline, sputum IL-8 levels in moderate asthmatics were higher than in mild asthmatics (fig. 5.15, p=0.01). There was no effect of high or low dose FP on IL-8 levels at 6 or 24 hours in either group compared with PBO (figs. 5.16 and 5.17). IL-5 levels were not detectable in sputum supernatants from mild or moderate asthmatics.

5.4.7 *Sputum neutrophil and eosinophil counts*

It was not possible to arrive at any meaningful conclusion on the effect of ICS on neutrophil and eosinophil counts as many patients were unable to produce adequate sputum samples for sputum slide analysis.





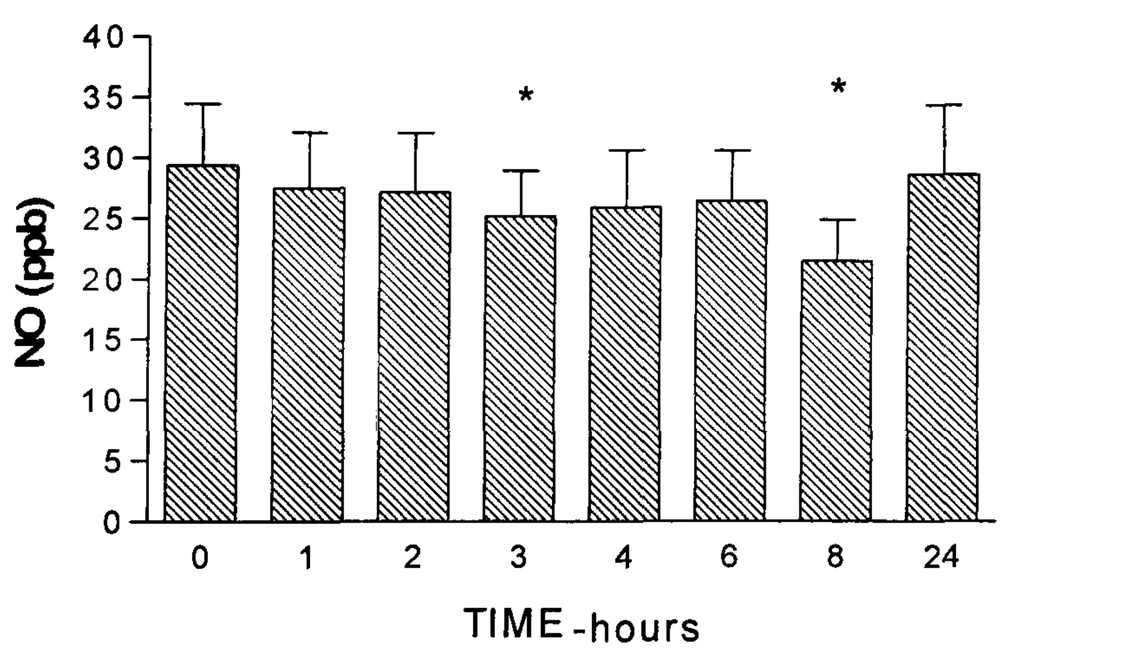
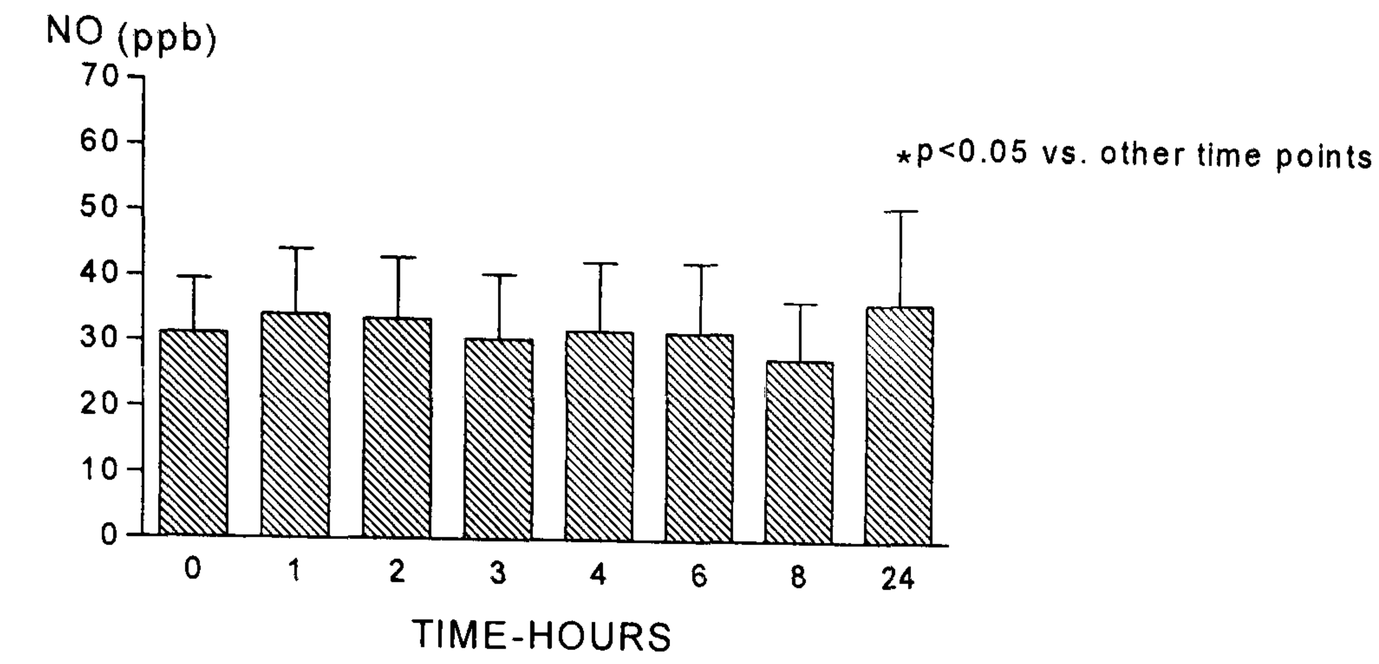
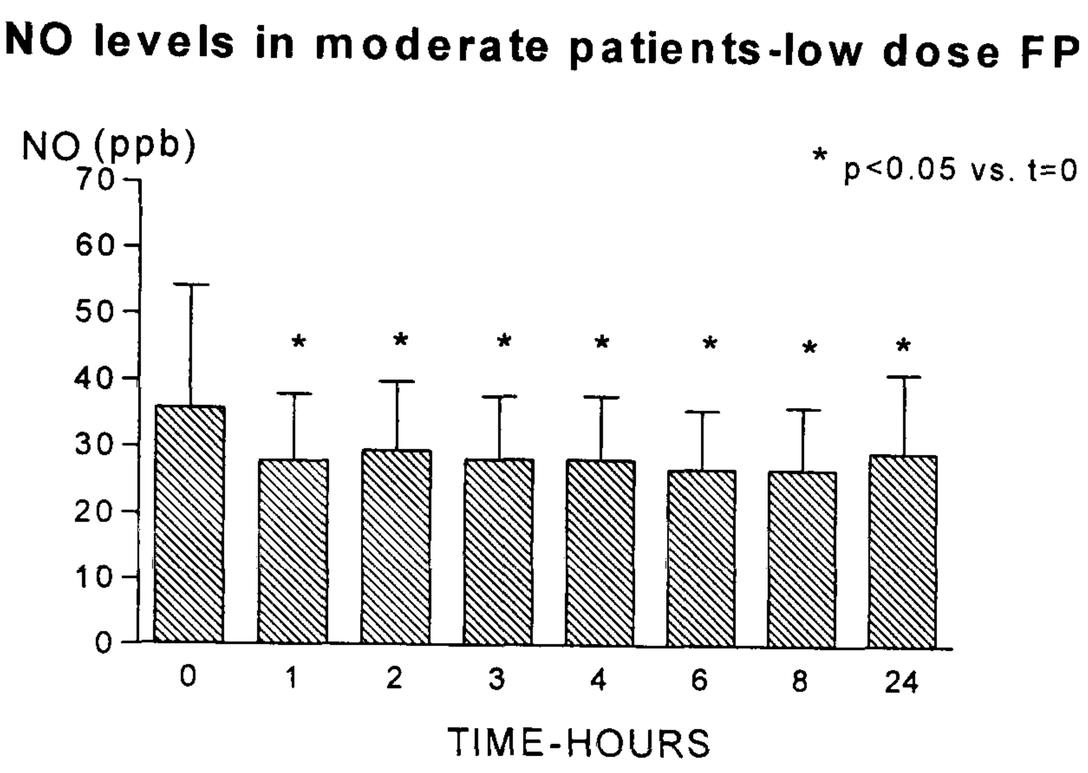


Fig. 5.7 Effect of high and low dose FP and PBO on NO levels in mild patients. High dose FP significantly reduced NO against PBO at 24 hours. (*p<0.05 as shown).





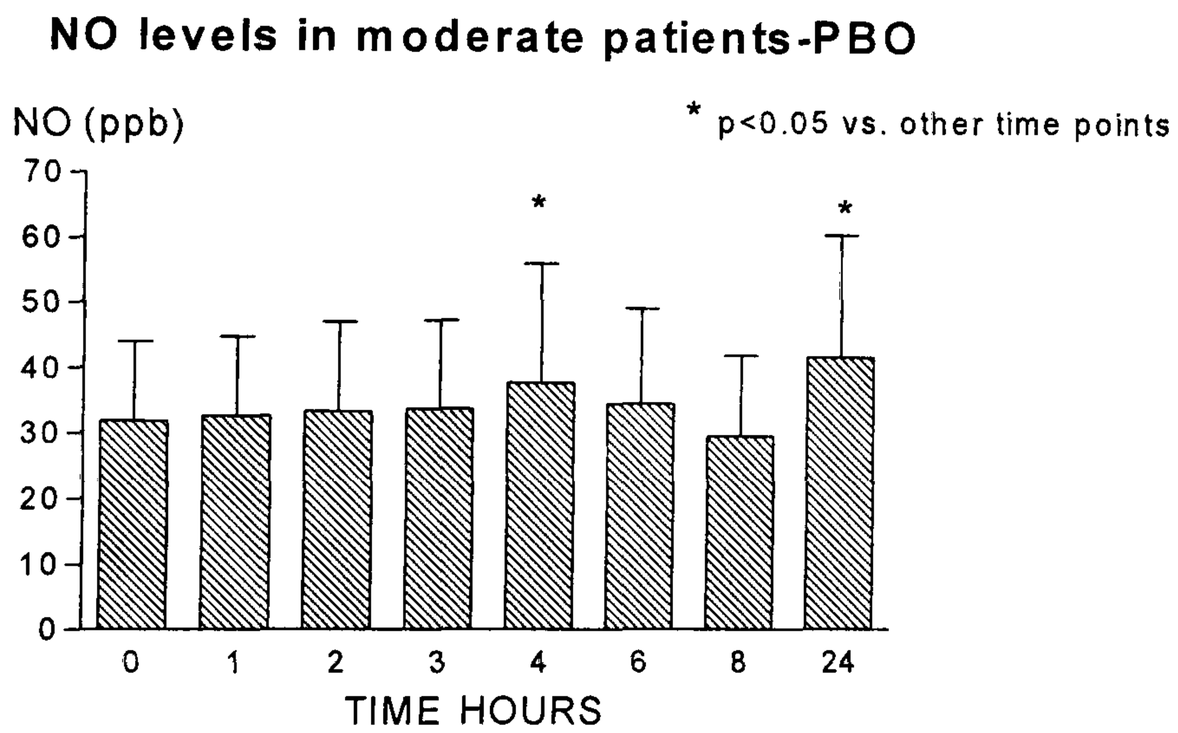


Fig. 5.8 Effect of high and low dose inhaled FP and PBO on exhaled NO in moderate patients. High and low dose FP reduced NO levels compared to PBO at 4 hours only.

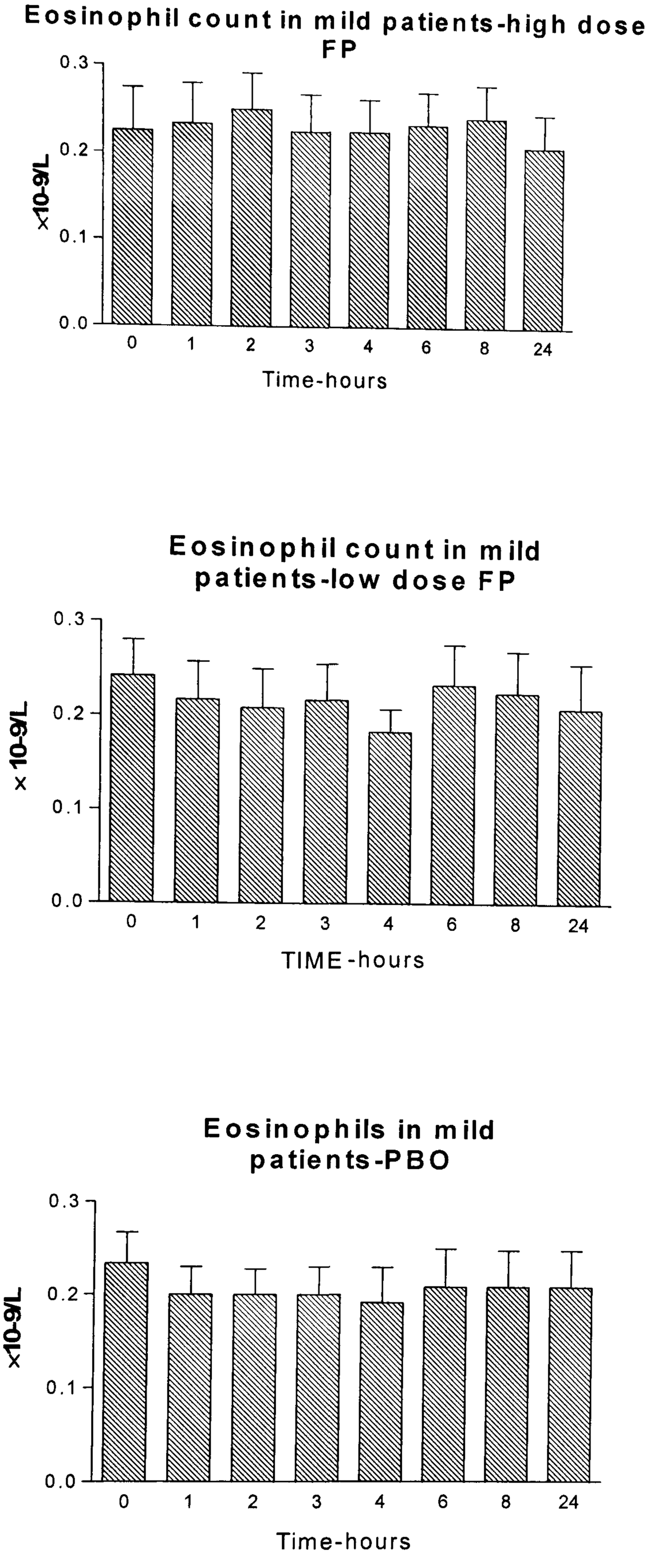
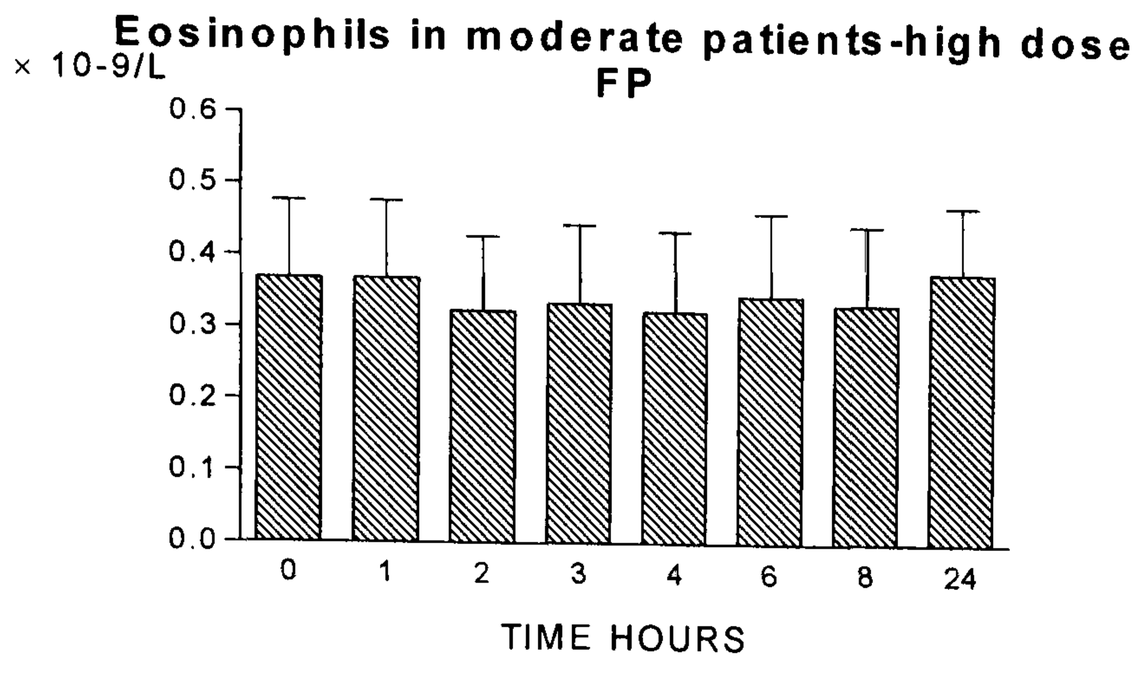
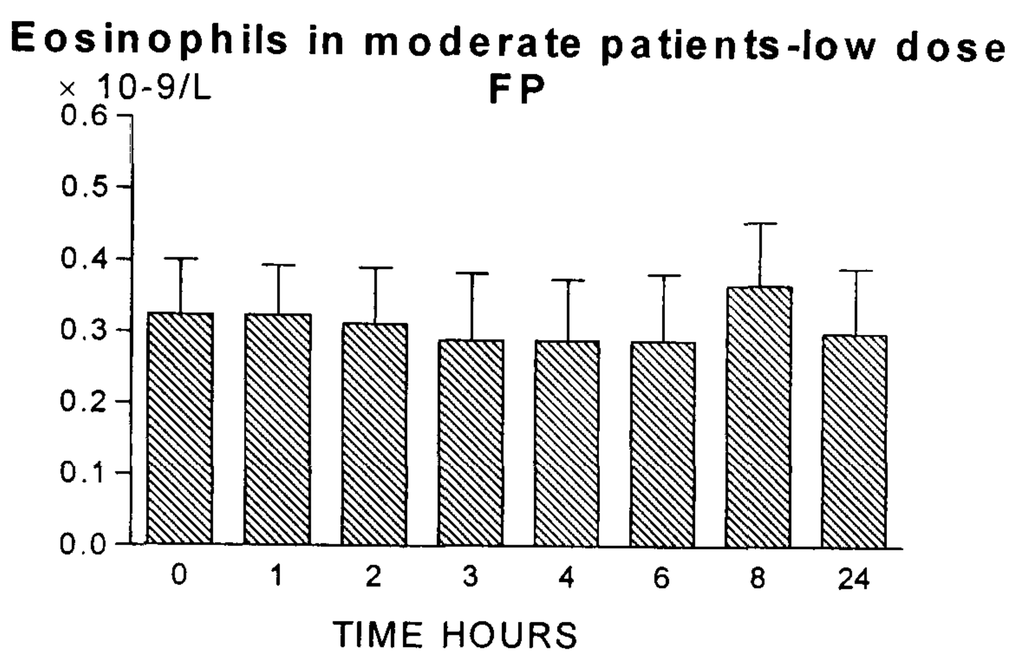


Fig. 5.9 Effect of high and low dose inhaled FP and PBO on eosinophil count in mild asthmatics. No effect of either dose of FP on eosinophil count compared with PBO, in mild asthma.





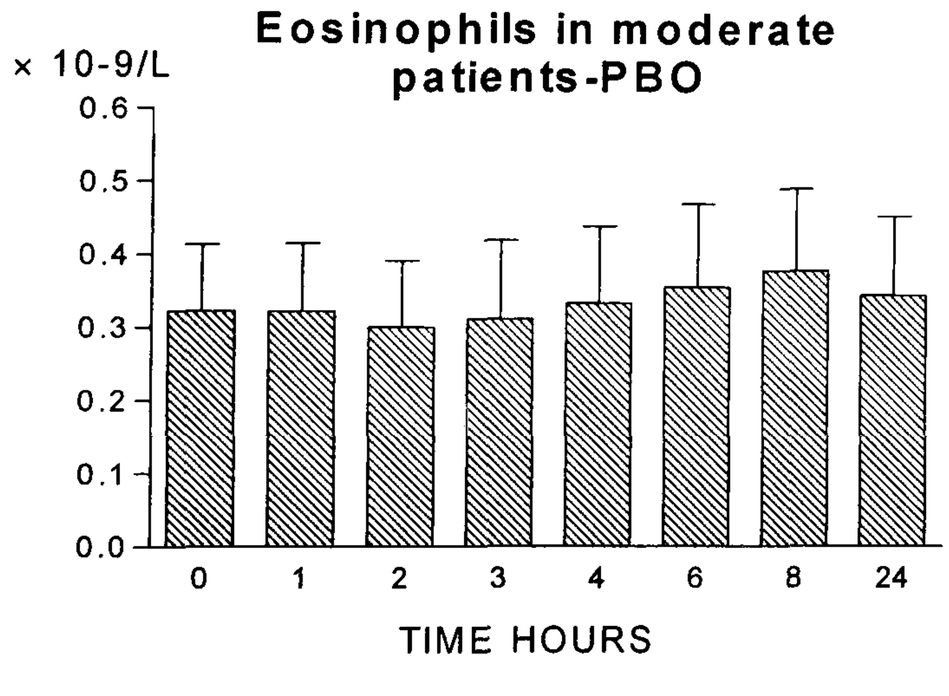


Fig. 5.10 Effect of high and low dose inhaled FP and PBO on eosinophil counts in moderate asthmatics. No significant effect on eosinophil count compared with PBO.

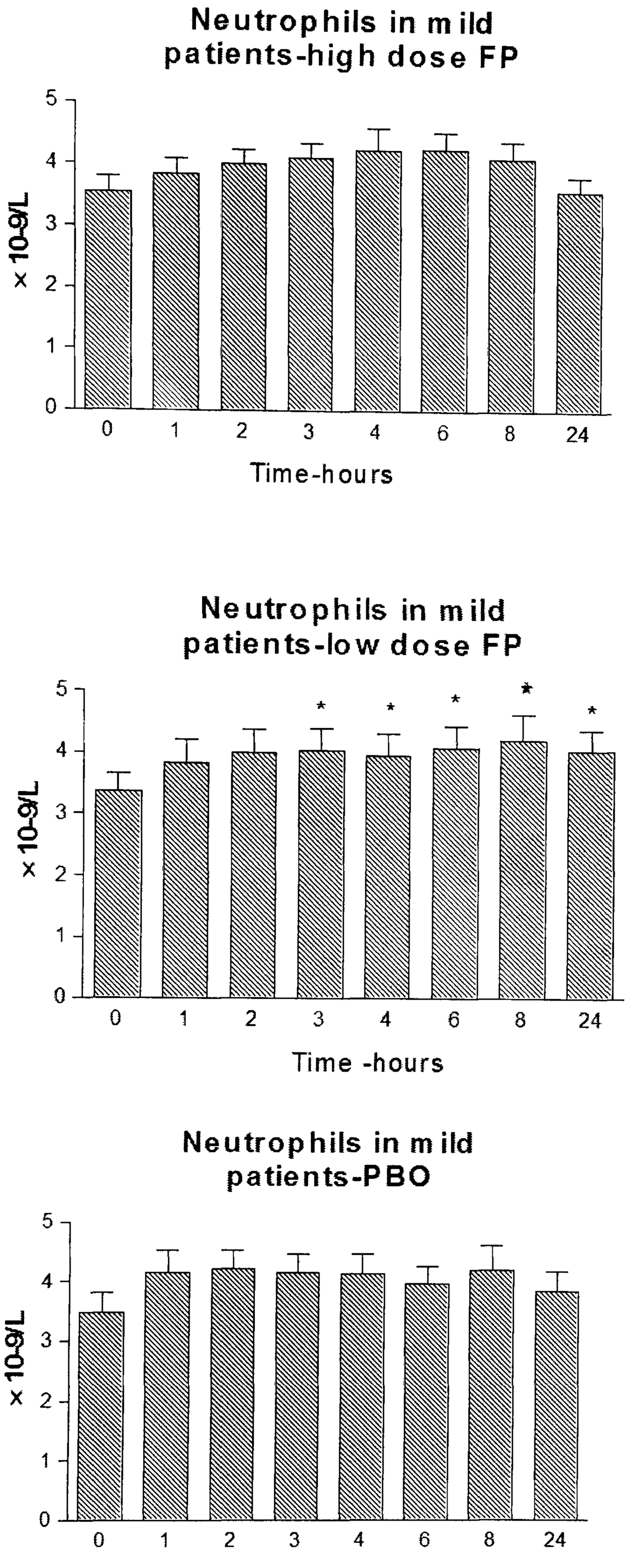
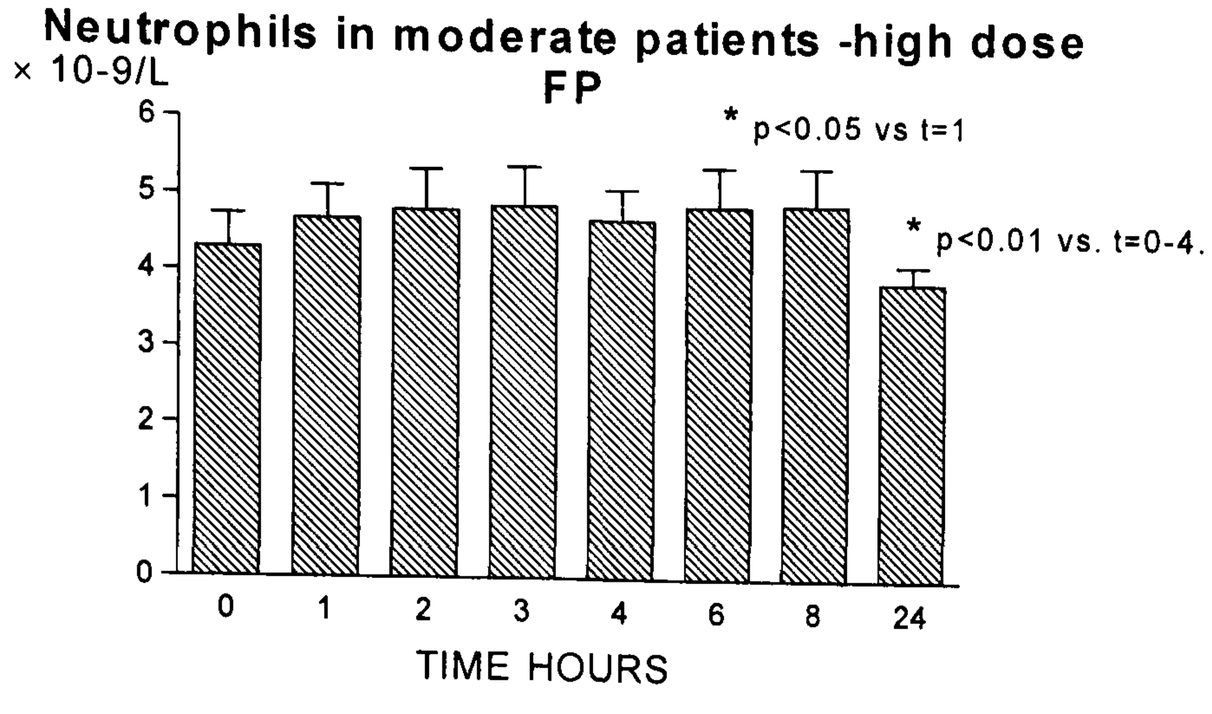
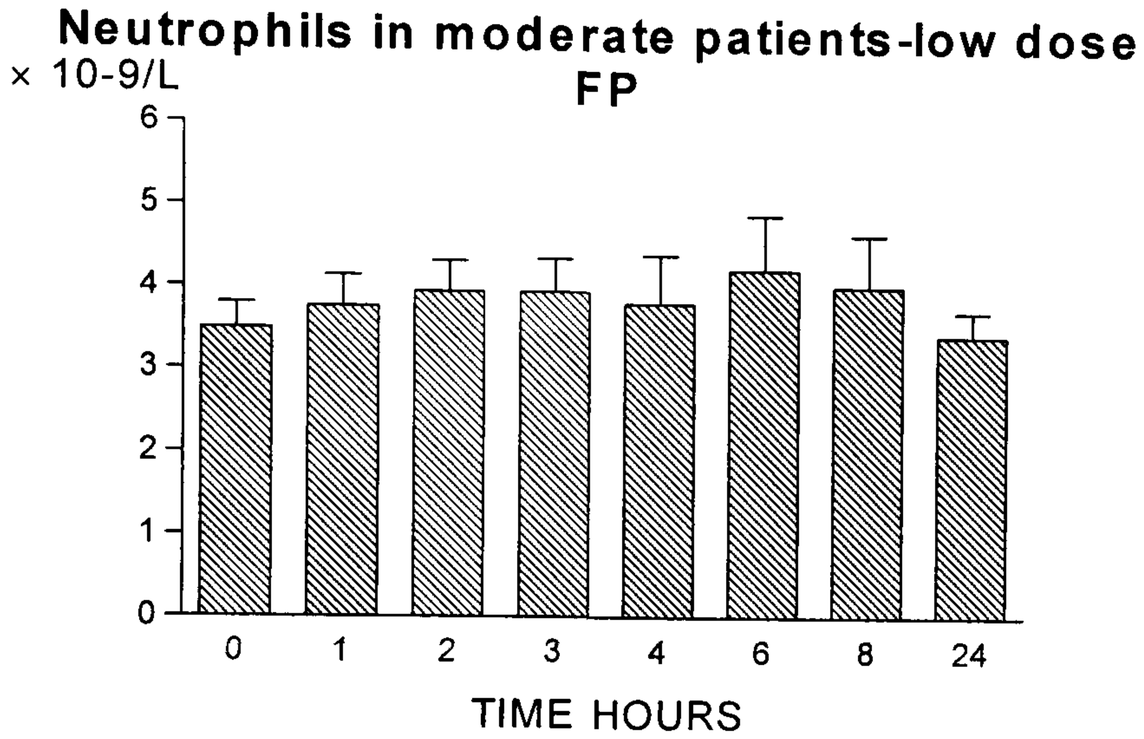


Fig. 5.11 Effect of high and low dose inhaled FP on neutrophil count in mild asthmatics. * $p<0.04$ compared with time 0. Low dose FP increased neutrophil counts at 8 hours compared to PBO.





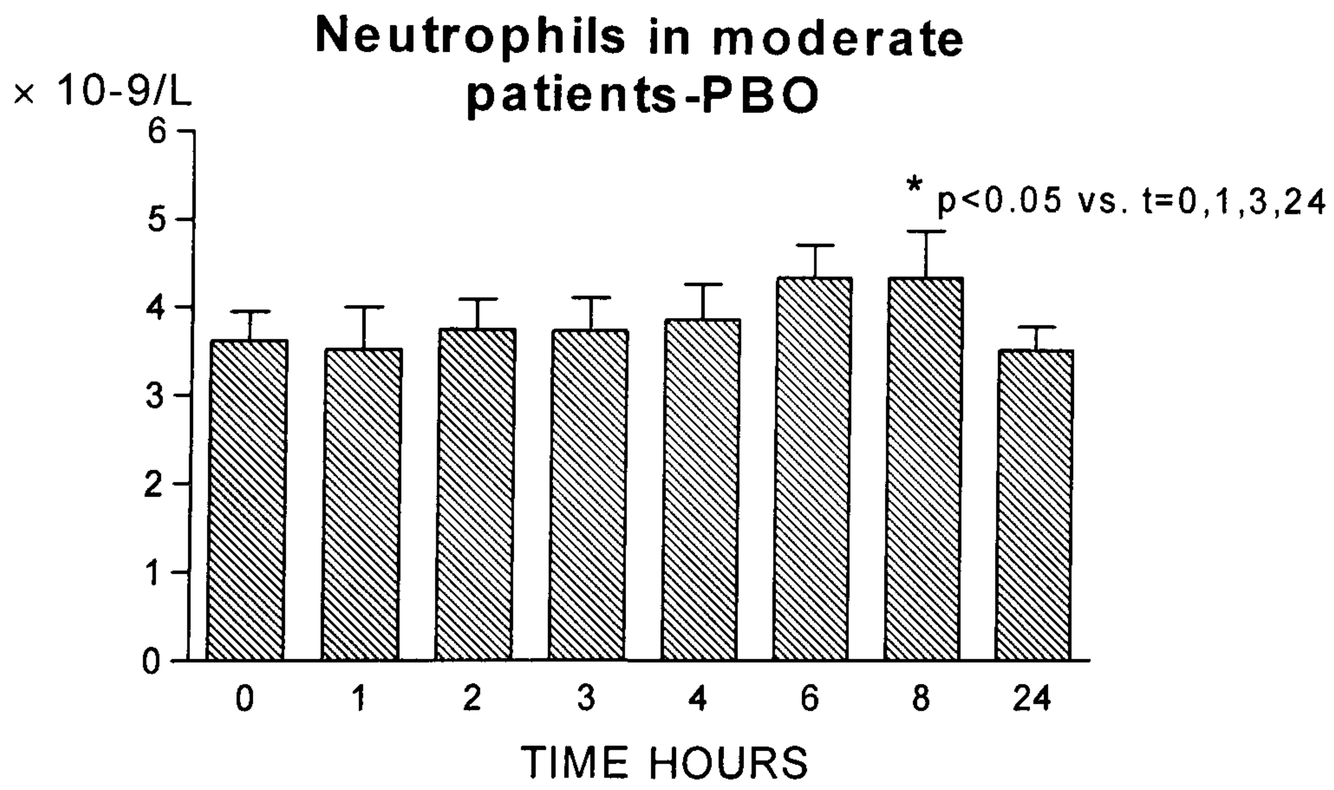
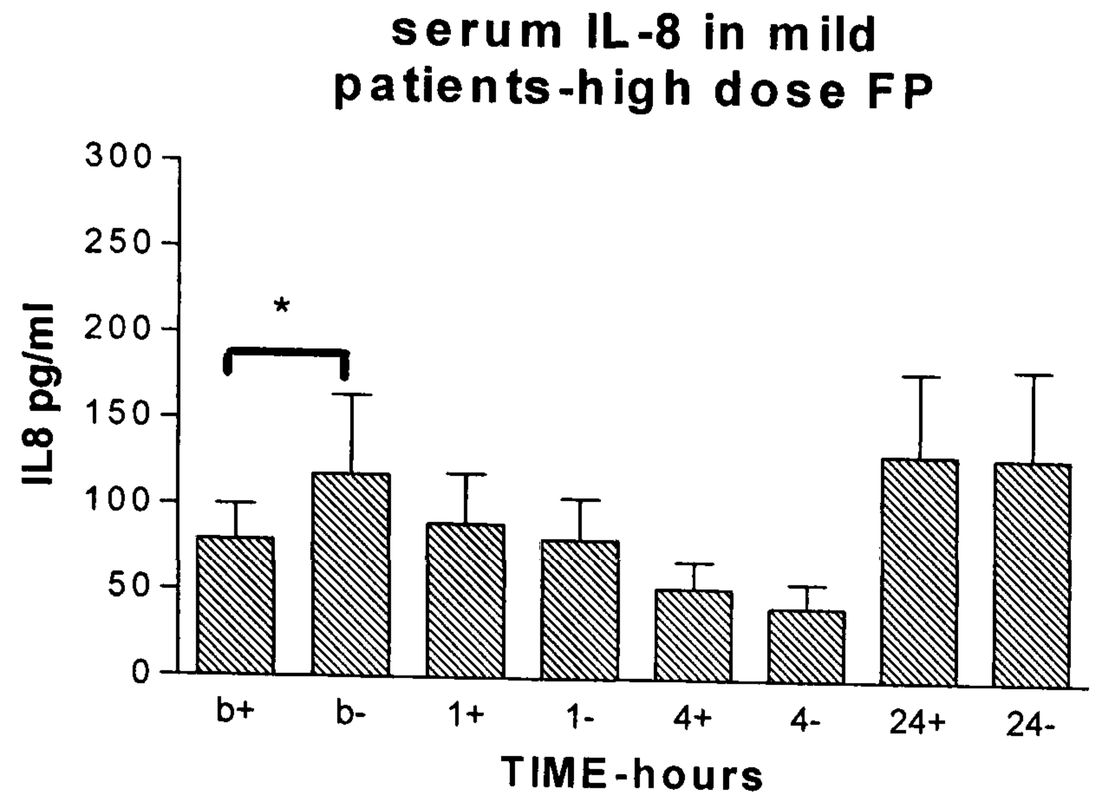
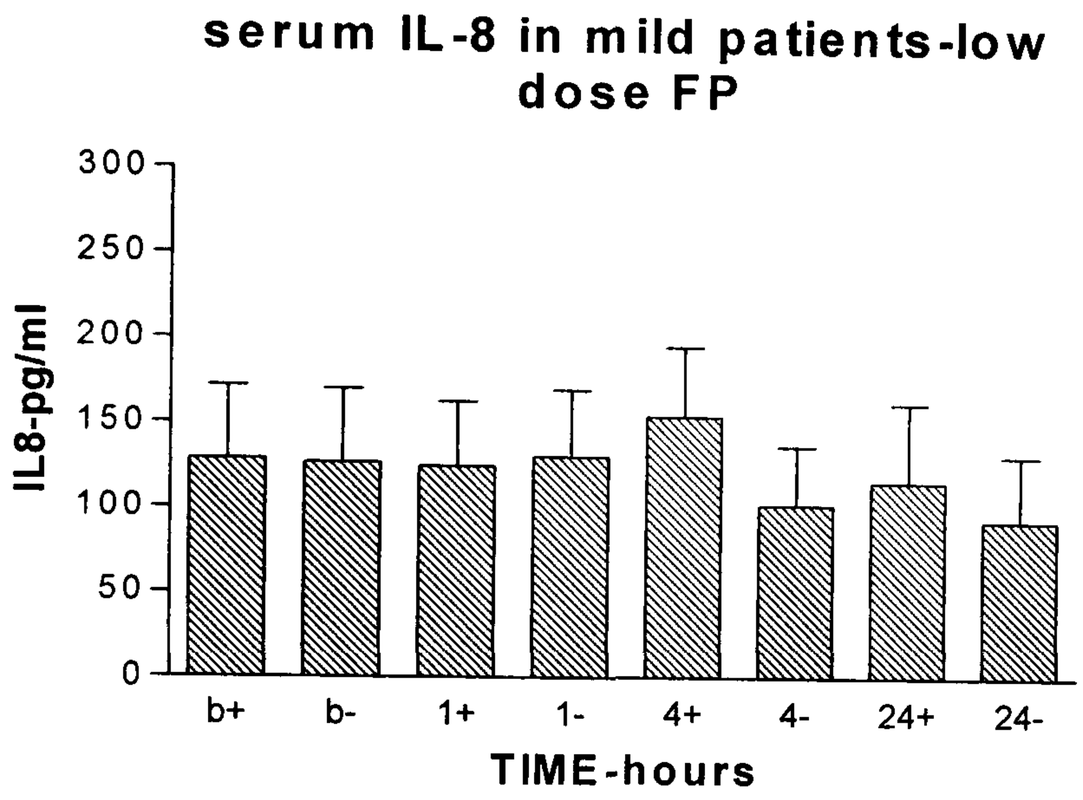


Fig. 5.12 Effect of high and low dose inhaled FP and PBO on neutrophil count in moderate asthmatics. High dose FP increased neutrophil counts compared to PBO at t=1 hour (* p <0.05)





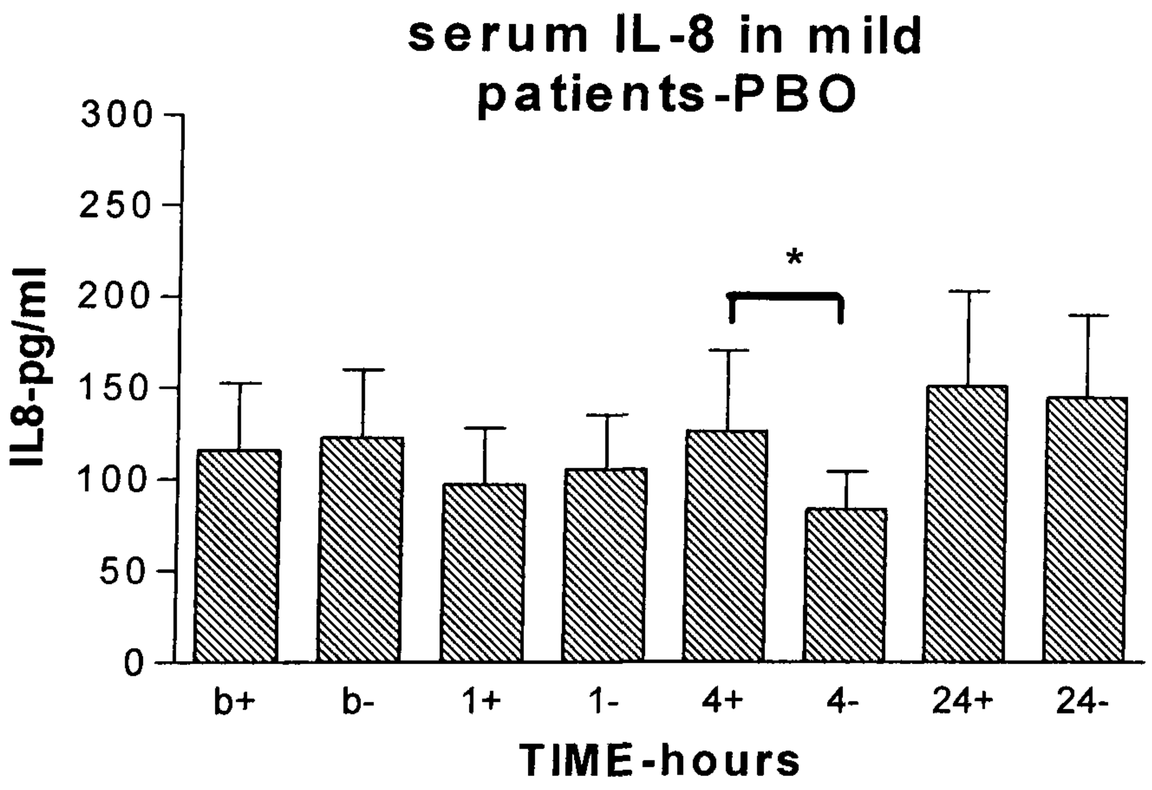


Fig. 5.13 Effect of high and low dose inhaled FP and PBO on serum IL-8 levels in mild patients. Time: b=baseline, 1, 4, 24 hrs whole blood incubation, + = with IL-1β, - = without IL-1β. No significant effect of FP compared with PBO. N=8.

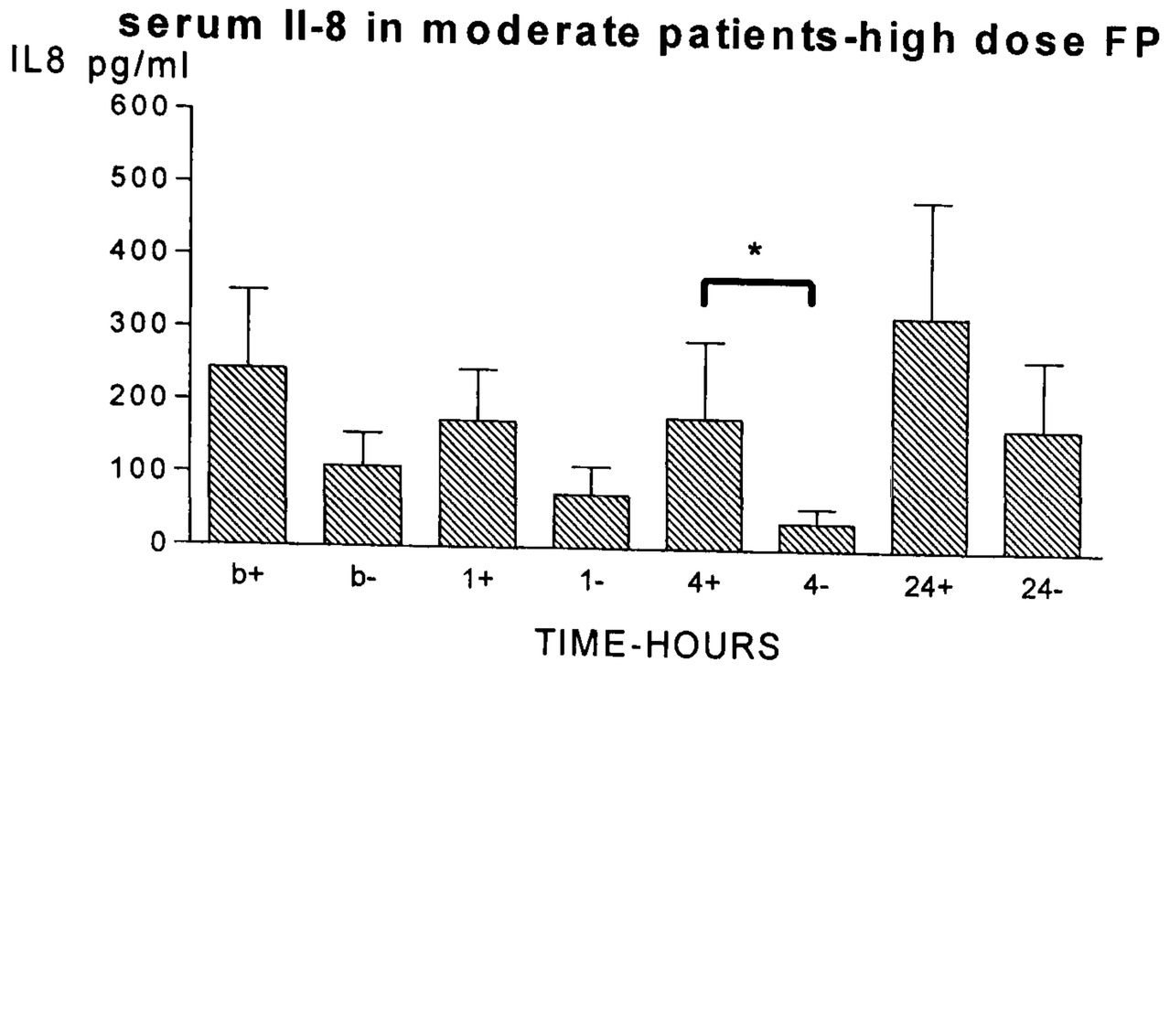


Fig. 5.14 Effect of high and low dose inhaled FP and PBO on serum IL-8 levels. Time: b=baseline, 1, 4, 24 hrs whole blood incubation, + = with IL-1β, - = without IL-1β. High dose FP reduced 1L-8 levels at 1 hour compared with PBO (*p<0.05). n=7.

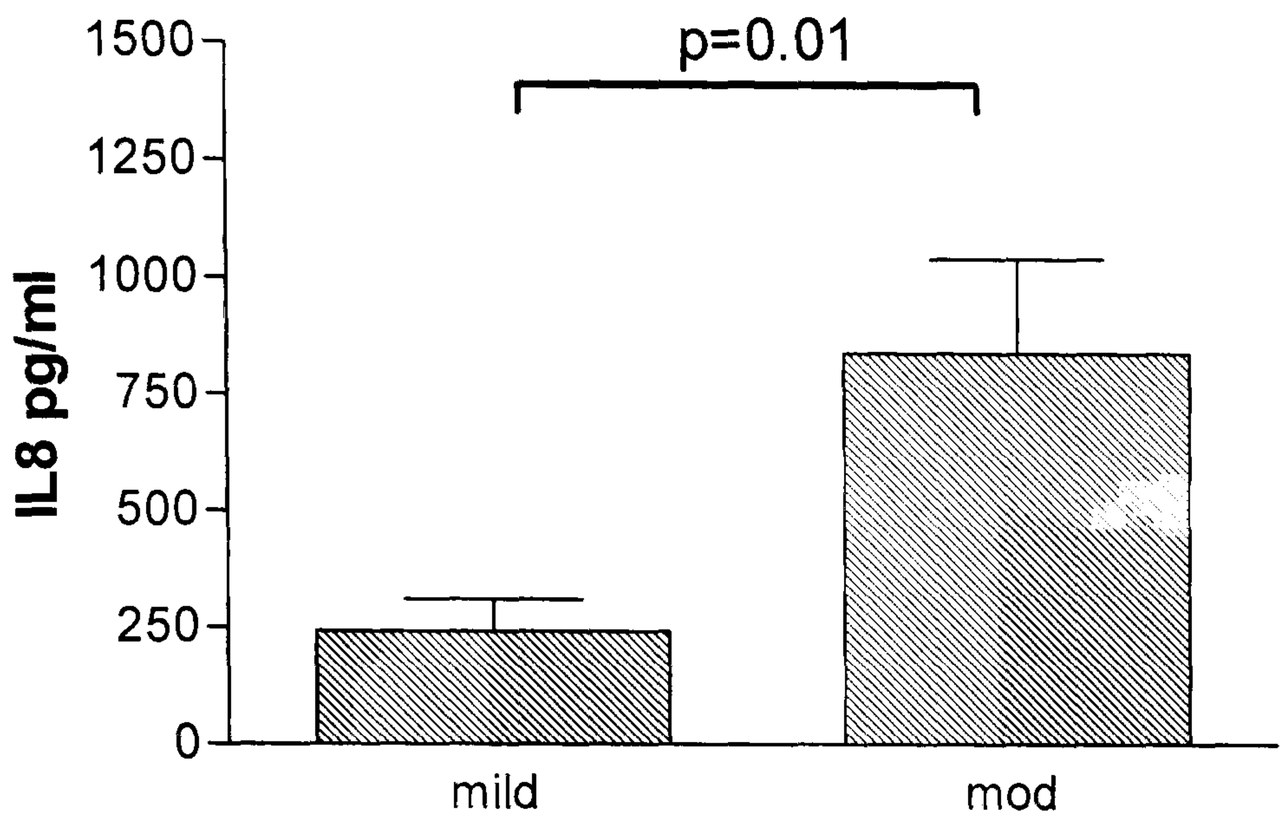
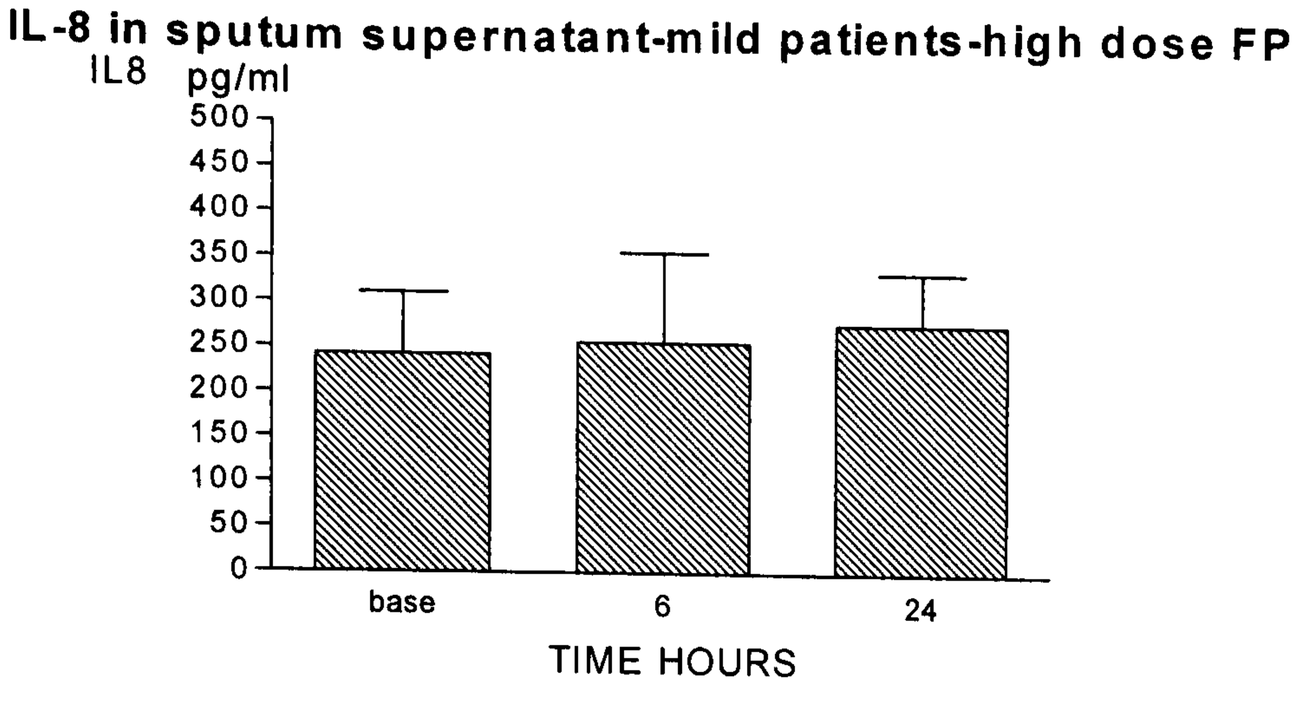
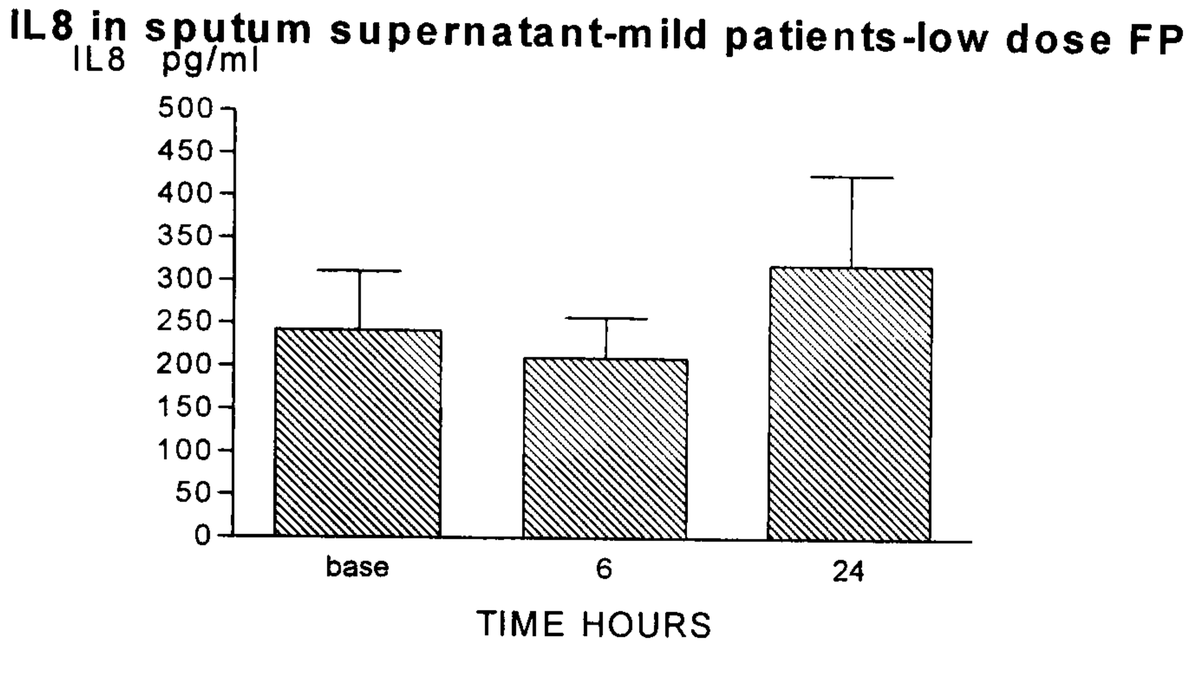


Fig. 5.15 Baseline expression of IL-8 in sputum supernatant in mild and moderate asthmatics. IL-8 levels were higher in moderate asthmatics compared to mild asthmatics. N=9 mild, and 6 moderate patients.





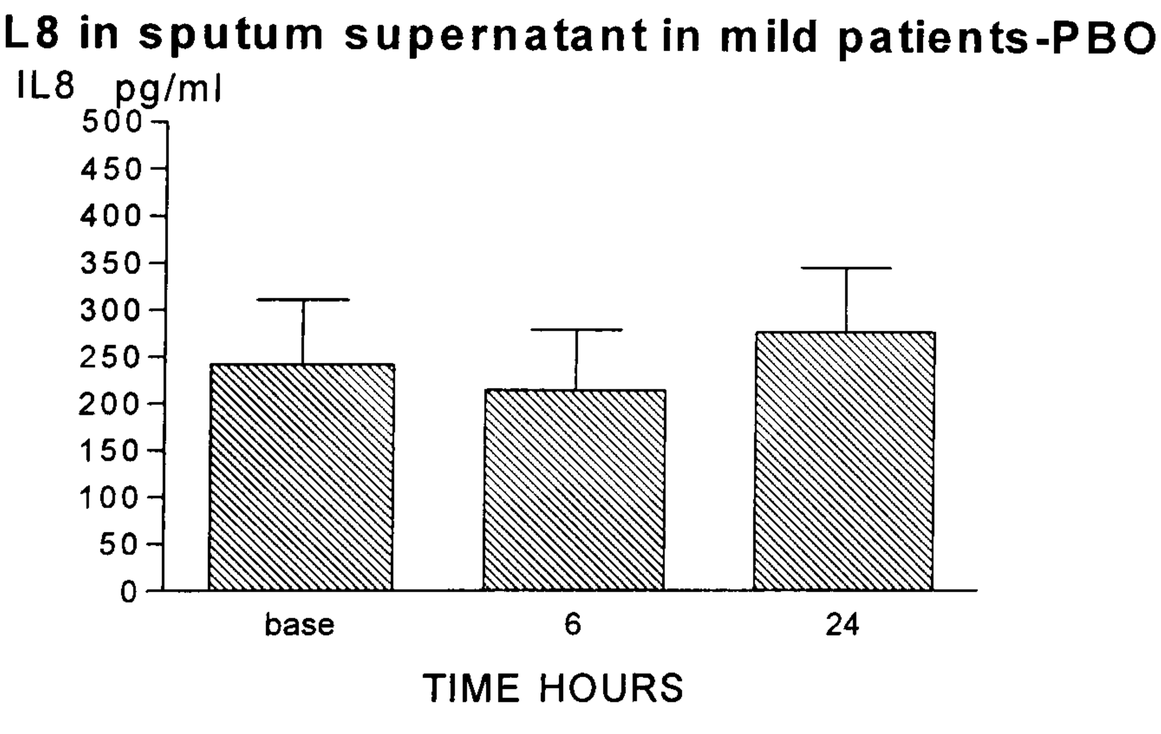
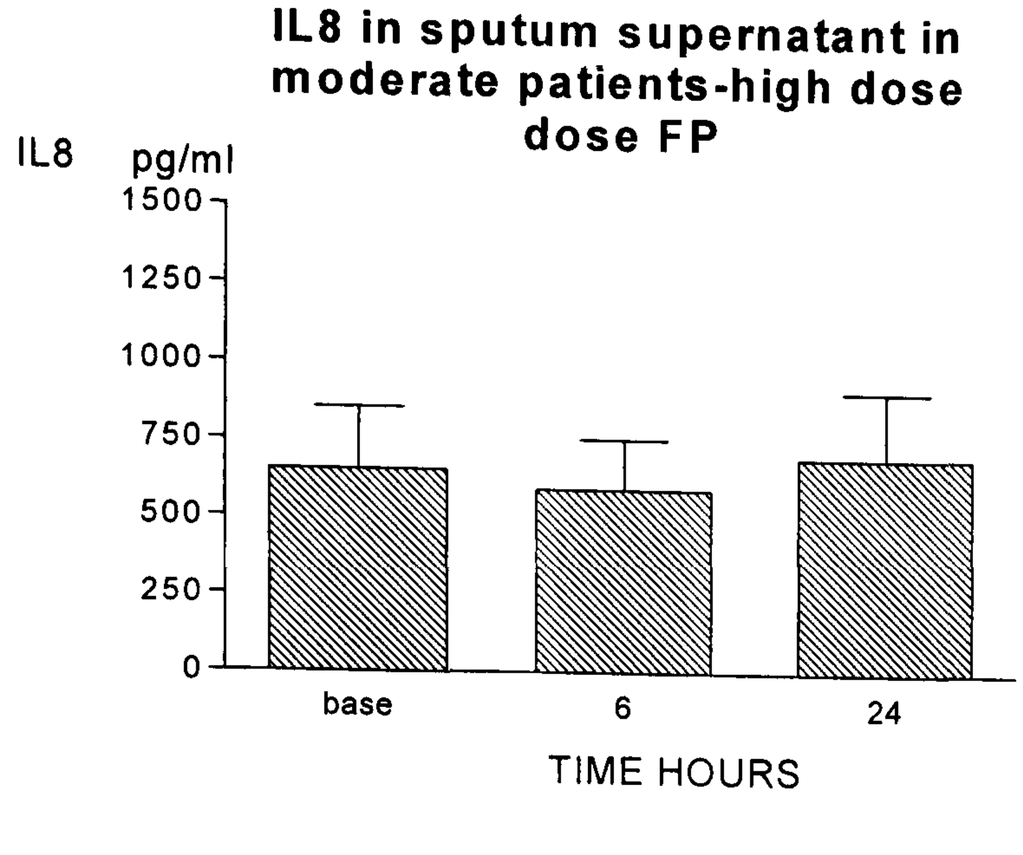
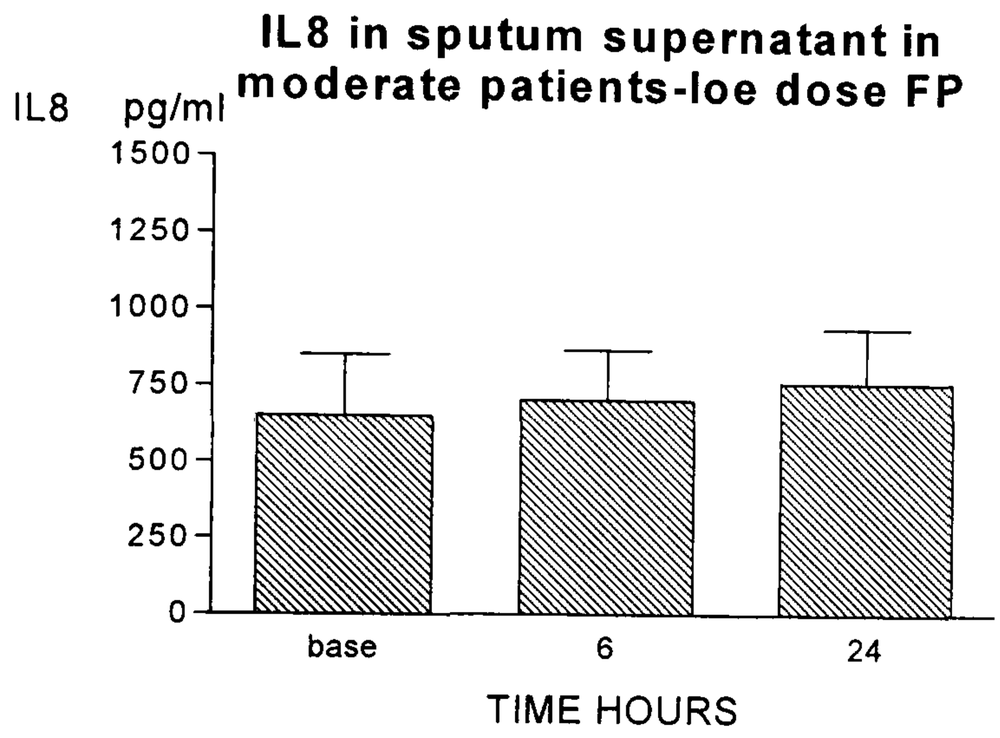


Fig. 5.16 Effect of high and low dose inhaled FP and PBO on IL-8 levels in sputum supernatant in mild asthmatics. No significant effect of FP was seen at either dose compared with PBO. N=9.





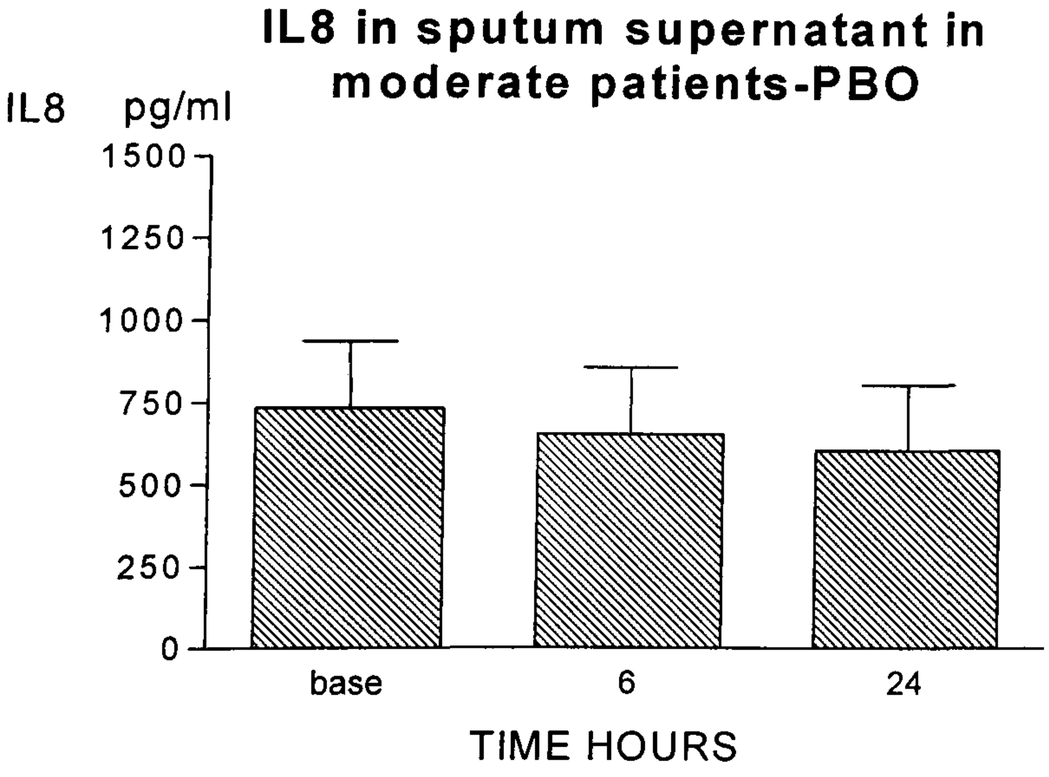


Fig. 5.17 Effect of high and low dose inhaled FP and PBO on IL-8 levels in sputum supernatant in moderate asthmatics. No significant effect of FP was seen at either dose compared with PBO. N=7.

5.5 Discussion

Previous studies have shown that regular use of ICS is effective in improving lung function and markers of airway inflammation in patients with mild asthma. However, although bronchial hyperreactivity was shown to decrease in several studies, many studies did not show an improvement in lung function (117,121). No effect of high or low dose FP was seen in mild asthmatics, and although a trend towards an increase in FEV1 was seen in moderate asthmatics with both high and low dose FP at 8 hours, this was not significant when compared with PBO. There was a significant increase in FVC in moderate asthmatics at 24 hours with low dose FP only, but this was not significant when compared with PBO. That this occurred with a low dose of FP suggests that low dose FP can result in improvements in airway inflammation and lung function, and no additional benefit is gained by increasing the dose (118). There was no effect on bronchial hyperreactivity with either dose of FP in mild or moderate asthmatics.

There was a trend for both high and low dose FP to reduce exhaled NO levels in mild asthmatics from baseline values, but at most time points, this was not statistically significant when compared with PBO. Exhaled NO was significantly reduced however at 24 hours in mild asthmatics with high dose FP compared with PBO ($p<0.05$). In moderate asthmatics, both high and low dose FP significantly reduced NO levels at 4 hours only, compared with PBO. Although regular use of ICS has been shown in studies to reduce NO levels at reasonably high levels (for example, budesonide 800μg bd) after a few weeks therapy, consistent reduction is not seen in this study after a single inhalation of low or high dose inhaled FP, suggesting that regular maintenance treatment is capable of inhibiting the induction of NO synthase in the respiratory tract, rather than a single dose.

Low and high dose inhaled FP had no significant effect on eosinophil counts in mild or moderate patients. In mild asthmatics, high dose FP had no effect on neutrophil counts, but low dose FP resulted in a higher neutrophil counts after 3 hours compared to baseline values, but this was only significant compared to PBO at 8 hours. It is not clear why low dose, but not high dose FP had this effect on neutrophil counts. In the moderate group, a trend towards a higher neutrophil count was seen at 8 hours after high dose FP, although this was not statitistically significant when compared with PBO. The increase in neutrophil numbers seen in this study after a single dose of low or high dose inhaled FP is difficult to explain, unless in terms of rapid mobilisation of cells from the bone marrow

or demargination of neutrophils, or response to an unknown antigen in the inhaler powder vehicle.

This effect of inhaled FP in causing a peripheral blood neutrophilia is interesting in that *Gizycki et al* demonstrated that endobronchial biopsies in patients with mild to moderate COPD receiving high dose inhaled FP unexpectedly showed significantly more neutrophils than in the PBO group (220). Further, inhaled FP and budesonide have been shown to prolong human neutrophil survival by inhibiting apoptosis at clinically relevant drug concentrations (221). This effect of ICS to mobilise neutrophils into bronchial airways and to prolong their survival may be important in maintaining both beneficial and harmful effects of neutrophils in airway tissue through the continued release of inflammatory mediators. In contrast, *Vagaggini et al* (222) showed that in induced sputum, moderate doses of inhaled budesonide given for 4 weeks was able to reduce the increase in the percentage of sputum neutrophils and IL-8 concentrations in sputum supernatants in response to ozone exposure.

Serum IL-8 levels were increased after whole blood incubation with IL-1β in mild patients receiving PBO at 4 hours, and in moderate patients receiving PBO at 1 hour. Serum IL-8 levels in mild and moderate patients were otherwise not significantly increased after incubation of whole blood with IL-1β, 10ng/ml. Serum IL-8 levels in mild and moderate asthmatics in each treatment group were higher after 24 hour incubation, with or without IL-1β. No effect of high or low dose FP was seen on serum IL-8 levels in mild patients over 24 hours, compared with PBO. In moderate asthmatics, whole blood culture with IL-1β resulted in higher levels of IL-8 at 4 hours compared to levels without IL-1β incubation, in the group receiving high dose FP. In moderate asthmatics, high dose FP significantly reduced serum IL-8 levels (after incubation with IL-1β) at one hour compared with PBO. The number of mild patients for whom IL-8 results were available was 8, compared with 7 moderate patients. The curves for the diluted IL-8 standards were satisfactory in all IL-8 ELISA experiments, but no results were recorded for some patients, suggesting that the sample preparation or technique may have been suboptimal. Repeating the experiment may have given more reproducible results.

The relative lack of effect of high and low dose FP on serum IL-8 levels may be due to the fact that a single dose of FP may be insufficient to significantly alter serum cytokine levels. However, it is difficult to determine if either dose is having an effect on serum IL-8 levels, as culture with IL-1β may have some bearing on the final IL-8 levels.

That ICS may not have any effect on circulating cytokines was shown in a study carried out in asthmatic children by *Visser et al* showed that no significant effect of prolonged treatment with high dose inhaled FP was seen on the release of IL-4, IL-5, IL-10 and IFN-γ by Con A stimulated PBMCs (223). Also in that study, serum IL-4 and IL-5 levels were undetectable in the majority of children. In contrast, *Morali et al* were able to show that inhaled budesonide (800μg/day) given for 4 weeks in adult mild to moderate asthmatics significantly reduced serum IL-5 levels, suggesting that this may be one possible mechanism of the anti-inflammatory action of ICS (224).

In sputum supernatant, IL-8 levels were significantly higher in the moderate group compared to mild asthmatics, but in addition, measuring IL-8 levels in a non asthmatic control group would have been interesting for comparison. There was no effect of high or low dose FP on sputum IL-8 levels in mild or moderate asthmatics over 24 hours, compared with PBO. It has been shown however, that a prolonged course of ICS can reduce IL-8 and GM-CSF concentrations in sputum supernatant (123). In the current study, IL-5 levels were undetectable in sputum supernatant samples. The measurement of IL-5 in sputum in several studies has produced variable results, and one explanation is that there may be substances in the sputum that interfere with the recognition of IL-5 epitopes by immunoassay, such as sputum proteases, or autoantibodies. The use of DTT for sputum homogenisation is known to cause a significant fall in the amount of sputum eosinophil peroxidase and MPO (225), but this is not thought to be the case with sputum IL-5 levels (226). *Simpson et al* (227) showed that the addition of a protease inhibitor to sputum processed with DTT significantly increased the amount of IL-5 recovery from sputum samples spiked with recombinant human IL-5. This has been confirmed by other workers (228), and thus suggests that a protease inhibitor should be added to processed sputum before regarding a sample as being negative for IL-5.

Problems encountered with this study included recruitment of patients, as each subject had to be available for approximately one and a half days per week for 3 consecutive weeks, which is not always convenient. The study protocol required that 12 asthmatics in each group were recruited, but only 9 moderate asthmatics were recruited. Increased patient numbers in each group would increase the power of the study, and a more prolonged course of inhaled FP (for example, twice daily for a week) may have resulted in a more marked change in the measured parameters. *Wood et al* (229) showed that an eight day course of inhaled budesonide was sufficient to demonstrate a systemic

effect in inhibiting the turnover of bone marrow derived eosinophil progenitors, although this inhibitory effect was overcome by inhalation of allergen. Although patients in the moderate group required a higher dose of ICS, and in some cases a long acting beta-2 agonist to maintain symptom control, the baseline spirometry and NO values were not significantly different compared to milder asthmatics. Sputum slides were, in many cases, difficult to read as patients were unable to produce adequate sputum samples and therefore it was not possible to look at effects of FP on sputum eosinophil or neutrophil counts. Taking into account recent advances in sputum processing techniques, the addition of a protease inhibitor may provide different results with respect to IL-5 levels.

A more useful study may have been to look at markers of neutrophil function after one week of inhaled high dose and low dose FP, as the present study was designed to look at the immediate effects of a single dose of inhaled FP. FACS analysis of neutrophil surface markers in blood samples would also be interesting to look at the effects of ICS on neutrophil activation markers. Using a longer course of inhaled FP may have detected any effect of inhaled FP on sputum supernatant IL-8 levels. The study protocol required that PC_{20} was measured at the initial screening visit, and thus the baseline levels of PC_{20} were the same in each group at the start of the study. It would have been better to measure PC_{20} in each group at time '0' to allow better comparison of treatment effect on PC_{20} at 6 and 24 hours against this value. Further, the trial was not designed to see if PC_{20} was improved before 6 hours. However, further measurements of bronchial hyperresponsiveness within each 24 hour period would have been too demanding for patients. Repeat sputum analysis for IL-5 using the current knowledge of the effect of DTT on IL-5 recovery may provide positive results.

The effect of ICS on neutrophil number and function in severe corticosteroid-dependent asthmatics would be difficult to investigate given the need for high dose inhaled steroids in this population, and also the possible confounding contribution of concomitant oral steroid therapy on peripheral neutrophil numbers. Further, ICS may not be beneficial in patients with severe asthma, especially those who who go on to develop relatively fixed airflow obstruction. Tsoumakidou *et al* (230) showed that patients with COPD and severe persistent asthma with fixed airflow limitation had similar numbers of neutrophils in their sputum, suggesting that a similar lack of response to ICS in severe asthmatics, as occurs in COPD, may be expected.

Chapter 6

Role of anti-IgE therapy in poorly controlled moderate to severe allergic asthma

6.1 Introduction

In atopic asthmatics, exposure to specific allergen can result in increased airway responsiveness with subsequent exacerbation of asthma symptoms through IgE mediated pathways. It has also been shown that non atopic or 'intrinsic' asthmatics also produce IgE in their airways, suggesting that IgE may also play a role in these patients. IgE therapy represents a new therapeutic approach for the treatment of allergic disease, and has been shown in initial studies to reduce asthma symptom scores and improve quality of life, as well as reduce oral and ICS requirements and the need for rescue inhaled bronchodilators. They have also been shown to reduce the number of exacerbations and have been shown to be safe with no reports of any significant side effects. IgE therapy is therefore useful as a steroid sparing agent. IgE therapy can be given intravenously or by subcutaneous injection, although frequent i.v. injections may not be acceptable to some patients. The efficacy of a long term subcutaneous form of therapy is unknown.

More recently, *Gounni et al* showed that peripheral blood and BAL neutrophils from asthmatics expressed the high affinity IgE receptor, FcεR1, which is the main receptor involved in binding IgE molecules to the surface of mediator cells. Further, activation of these FcεRI receptors induced the release of IL-8 by neutrophils (169). These studies suggest that neutrophils may play a role in allergic inflammation through an IgE-dependent activation mechanism. In the context of severe allergic asthma, this is important as increased numbers of neutrophils have been demonstrated in sputum and bronchial biopsy samples in patients with severe corticosteroid-dependent asthma.

6.1.1 Aim of study

The aim of this study was to evaluate the efficacy and tolerability of subcutaneous administration of rhuMab-E25 in subjects with moderate persistent to severe persistent allergic asthma over period of 1 year. All previous studies had involved administering this therapy through intravenous injections. In addition, the effect of rhuMab-E25 on

surface activation molecules on cells thought to be important in the allergic inflammatory cascade was carried out.

6.2 Methods and study protocols

6.2.1 *Protocol IA04*

Protocol IA04 at the Brompton hospital was part of an international multicenter, randomized, open label, controlled study to evaluate efficacy, tolerability, and effectiveness of subcutaneous administration of rhuMab-E25 (Omalizumab) in subjects with moderate persistent to severe persistent allergic asthma over a 1 year period. The trial was sponsored by Novartis Pharmaceuticals Corporation. Patients were randomized to receive rhuMAb-E25 on top of their current asthma treatment (according to the best medical practice), compared to patients receiving their current asthma treatment alone. Its aim was to see if there was benefit of Omalizumab in a setting more representative of real life clinical practice. This was a multicentre study in 5 European countries: France (10 centres), Spain (7 centres), Germany (9 centres), Switzerland (3 centres), and the United Kingdom (20 centres). Although the quality and access to healthcare may vary from one country to another, entry criteria to the IA04 study required all randomized patients to have had their asthma therapy optimized according to U.S. NHLBI treatment guidelines irrespective of country.

Inclusion criteria were as follows:

- Males and females between 12 and 75 years of age
- Asthma for ≥ 2 years
- 1 emergency visit and 1 course of oral corticosteroids in the last year
- Positive skin test to common aero-allergens
- Total serum IgE between 30-700 IU/ml
- Reversibility of ≥ 12% improvement in FEV1
- Inhaled corticosteroid dose of at least 800μg beclomethasone (BDP) equivalent for adults and 400μg BDP equivalent for children.

Exclusion criteria were as follows:

- Pregnant, nursing mothers or planning to become pregnant
- Previous treatment with E25 or known to be hypersensitive to any of its components or other drugs used in this trial
- Active lung disease other than asthma
- Elevated IgE levels for reasons other than atopy
- Smoking history ≥ 10 pack years
- Clinically significant condition that may compromise the subject's safety
- Treatment with depot corticosteroids or investigational product within 30 days of starting the trial
- Ongoing desensitization immunotherapy
- Any factor that may limit the subjects ability to co-operate

6.2.1.1 *Methods*

Patients were recruited by advertisement and from outpatient clinics from the Royal Brompton Hospital. All patients gave written informed consent (or parents/legal guardians if subject < 18 years of age) and the study had approval from the Hospital's Ethics committee. All patients underwent a screening period in which all inclusion/exclusion criteria were met.

Skin prick test was carried out as described in section 2.1.3.

Total/free IgE was done at screening (Visit 1).

Spirometry was carried out at every 3 monthly visits and at the end of the study, as described in section 2.1.4.

Asthma reversibility testing was carried out at screening (Visit 1) as described in section 2.1.5. The reversibilty of bronchoconstriction was demonstrated by an increase in FEV1 of more than 12% from baseline, within 30 minutes after inhalation of up to 4 puffs (100μg/puff) or nebulization (2.5mg) of salbutamol.

Clinical symptom score was recorded at each scheduled physician visit during the trial core period using asthma and rhinoconjuntivitis symptom score questionnaire, developed and validated by Wasserfallen. A quality of life questionnaire was completed at each visit during the trial core period using the self-administered Mini Asthma Quality of Life Questionnaire was completed at each visit. Review of concomitant medication and adverse experiences was carried out at each scheduled visit. The dose of rhuMAb-E25 was determined according to patient's body weight and total serum IgE level from a dosing table.

Characteristics of patients who took part in this trial are given in Table 6.1. Details of the control patients and asthmatics who received anti-IgE therapy are given in appendix 4.2 (page 258), and appendix 4.3 (page 259) respectively.

6.2.2 *Protocol 11 extension substudy*

11 extension substudy was a separate open label study for patients who had previously completed the rhuMab E25 01 011 study. It was conducted in 11 of the 15 countries which had previously participated in the rhuMab E25 01 011 trial. All patients received subcutaneous rhuMab E25 treatment every 2 or 4 weeks based on their baseline IgE level and current body weight for 2 years. Assessments included safety, spirometry, steroid use, assessment of asthma control and rescue utilization, and were carried out approximately every 3 months, with some additional assessments in the first month of the study and a 6 and 12 week follow up following the last treatment.

The primary objective was to assess safety and tolerability of long term subcutaneous rhuMab E25 treatment in a more natural clinical setting. The secondary objective of the study was to investigate the effects of long term rhuMab E25 treatment on bronchial inflammation in adult patients with severe asthma, by analysis of cell surface markers in blood samples over the course of the year. This was compared with a control asthmatic population who did not receive trial treatment. Blood was analyzed with flow cytometry for changes in cell profile and surface markers, including the low-affinity IgE receptor, and the Fas receptor, CD95. CD95 is a cell surface glycoprotein (MW 40-50 KDa) that was initially identified by 2 apoptosis inducing antibodies, anti-APO-1, and anti-FAS. It is expressed in a minority of resting T and B-cells and monocytes, and appears to be a cell surface apoptosis signalling molecule. It has been

postulated that CD95 plays a role in T-cell mediated cytotoxicity and possibly in the normal apoptotic process that occurs during T-cell and monocyte development. CCR3 is a chemokine receptor, and is a member of the G-protein coupled, seven-transmembrane receptor family. It is expressed on eosinophils, basophils, and monocyte derived dendritic cells, and acts as a receptor for several different chemokines including eotaxin and RANTES, which are involved in the allergic response and in stimulating migration of eosinophils.

Inclusion criteria

- Males and females between 12 and 75 years of age
- Chronic severe asthma for ≥ 1 year
- Positive skin test to at least 1 common aeroallergen, or positive RAST test if borderline
- Total serum IgE between 30-700 IU/ml
- Reversibility of ≥ 12% improvement in FEV1
- Inhaled corticosteroid dose of at least ≥ 1000μg inhaled FP or equivalent for adults and ≥ 500μg FP or equivalent for children.
- Use of maintenance oral corticosteroids allowed, up to a maximum dose of 20mg/day, and maximum of 5 years duration.
- No change in concomitant medication for 4 weeks prior to entry into the study.

Exclusion criteria

- Pregnant, nursing mothers or planning to become pregnant
- Patients treated with an experimental or investigational drug within 30 days prior to starting the study.
- Patients with a history of noncompliance or who are considered potentially unreliable.

In total, 10 patients were recruited into the 11 extension substudy and received rhuMAb-E25, whilst a control population of adults with severe allergic asthma who did not participate in the original protocol 01 11 study but who met the eligibility criteria were recruited from outpatient clinics at the Royal Brompton Hospital (n=5). The

characteristics of these patients are shown in appendix 4.4 (page 260). All patients gave written informed consent and the study received ethics approval from the Royal Brompton Hospital's Ethics committee.

As previously mentioned, patients with severe allergic asthma who completed the original phase 3 trial of rhuMAb-E25 study protocol 01 11 were included in this study. The protocol 01 11 study looked at the safety and corticosteroid sparing effect of Omalizumab in 246 patients with severe allergic asthma requiring daily treatment with high dose ICS (FP $\geq$ 1000μg/day) and in whom long acting beta agonists were allowed (275). This was a randomized, double blind, PBO controlled trial, and included a 16 week steroid stable phase with study medication added, followed by a 16 week steroid reduction phase. The percent reduction in ICS dose at the end of the treatment phase was significantly greater in Omalizumab treated patients compared to PBO patients (p=0.003). The proportion of Omalizumab patients who were able to reduce their FP dose to $\leq$ 500μg/day was 60.3%, compared to 45.8% in the PBO group (p=0.026). The Omalizumab group also showed less rescue medication use, improved asthma symptoms, and asthma related quality of life compared to PBO.

6.2.2.1 *General Methods*

Spirometry, skin testing and reversibilty testing were carried out as described in section 2.1.

6.2.2.2 *FACS analysis*

Indirect immunofluorescence staining for measurement of FcεR1 receptors on basophils and monocytes, and direct immunofluorescence on lysed whole blood samples were carried out as described in 2.2.3.6, 2.2.3.7, and 2.2.3.8.

6.2.2.3 *Sputum analysis*

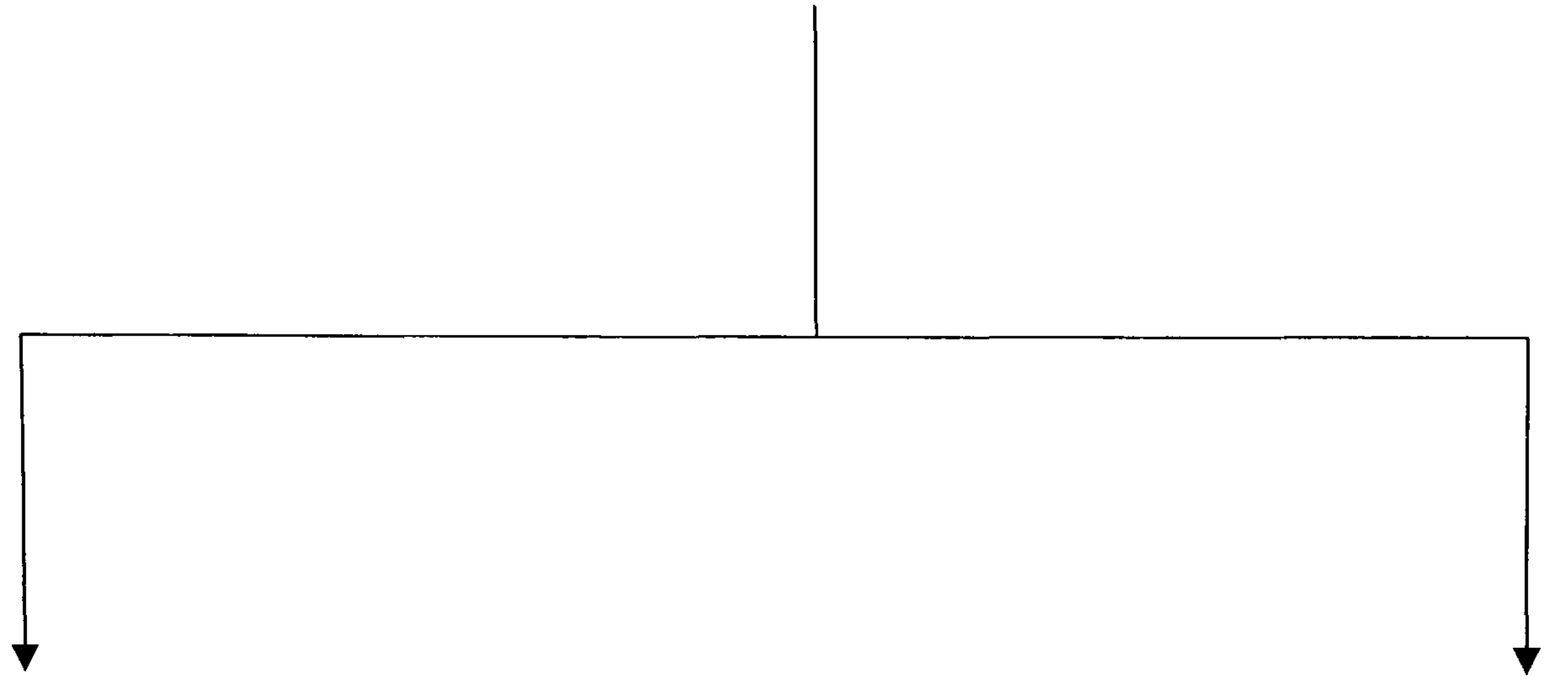
Sputum from patients was processed at baseline, and at 6 months and 12 months, as described in section 2.2.1.

Table 6.1 Characteristics of patients in IA04 study.

	RhuMAb-E25 group	Control group
N=	5	4
Male/female	1M/4F	1M/3F
Age years (mean)	59.4 +/- 3.17	35.5 +/- 10.2
Age (range)	52-68 years	18-65 years
FEV1 (L) (mean)	1.42+/- 0.17	2.0 +/- 0.57

Results expressed as mean +/- SEM.

Table 6.2 Summary of studies involving anti-IgE therapy in this chapter



Protocol IA04	Protocol 11 extension substudy
N= 5 patients receiving rhuMab E25	n=10 patients receiving rhuMab E25
N= 4 control patients	n=5 control patients
Measurements:	Measurements:
FEV1	FCεR1 on basophils: 6 patients on rhuMab E25; 5 control patients
QOL scores	FCεR1 on monocytes: 7 patients on rhuMab E25; 5 control patients
Clinical symptom scores	CD23 marker on CD14 (+) and CD4 (+) cells; 10 patients on rhuMab E25
Blood eosinophil counts	CD95 marker on CD14 (+) cells; 10 patients on rhuMab E25
Blood neutrophil counts	CCR3 marker on eosinophils; 10 patients on rhuMab E25
Dose of ICS	
Number of oral steroid courses used	

6.3 Results

6.3.1 *IA04 study*

6.3.1.1 *Lung function*

There was a significant increase in FEV1 in patients treated with rhuMAb-E25, at visits 3 and 5 compared to visit 2 (fig. 6.1), but the FEV1 readings were not significant when compared with the control group. No increase in FEV1 was seen in the control group from visit 1 through to visit 7.

6.3.1.2 *Quality of life*

There was no significant effect on quality of life scores in the rhuMAb-E25 treated asthmatics, during the course of the treatment compared to the start of the trial. The asthmatics who received rhuMAb-E25 however, showed a significant improvement in quality of life scores at visit 6 compared with the control group ($p=0.03$, fig. 6.2).

6.3.1.3 *Clinical symptom score*

At the start of the trial (visit 2), clinical symptom scores were significantly higher in treatment group compared to the control group ($p=0.03$), suggesting that the patients in the treatment group were more symptomatic. Treatment with anti-IgE therapy resulted in lower symptom scores at visit 5 compared to the end of the trial (visit 7, $p=<0.05$, fig. 6.3). There was no significant change in symptom scores in the control group, and scores in the rhuMAb-E25 treated group were not significantly different compared to corresponding scores in the control group, except at visit 2.

6.3.1.4 *Peripheral blood eosinophils and neutrophils*

No significant effect of anti-IgE therapy was seen on eosinophils or neutrophil counts during the trial, compared to the control group (fig. 6.4).

6.3.1.5 *Inhaled corticosteroid dose*

No significant decrease in the dose of ICS was seen over 1 year in rhuMAb-E25 treated patients compared with the control group (fig. 6.5), but there was a reduction in the number of courses of prednisolone required during the year in patients receiving rhuMAb-E25, and this approached significance ($p=0.06$, fig. 6.6).

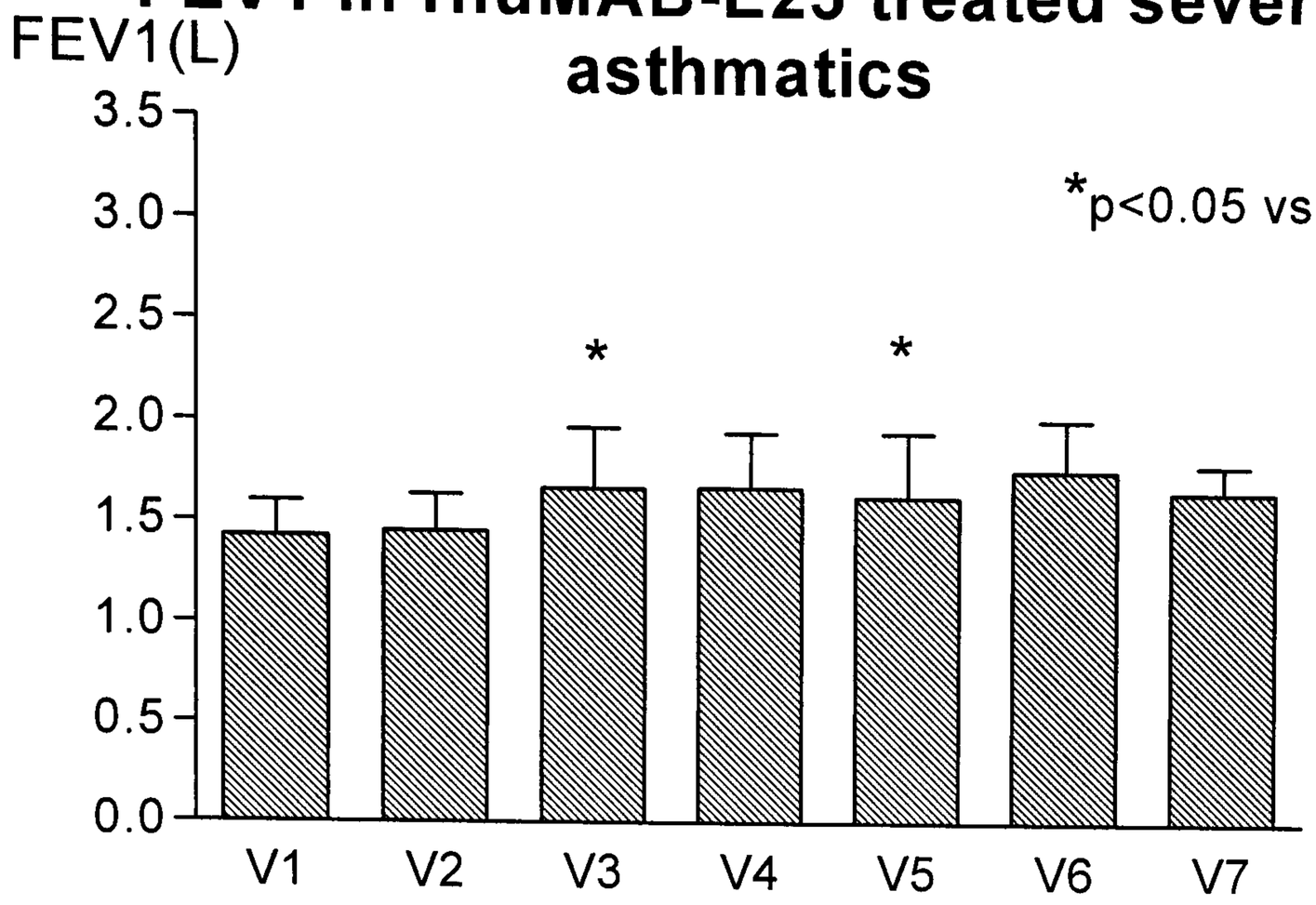
6.3.2 *Results - Protocol 11 extension*

6.3.2.1 *FcεR1 expression on basophils and monocytes and whole blood analysis*

Anti-IgE therapy significantly reduced FcεR1 expression on basophils and monocytes over a 3 month period compared to baseline levels, but not when compared to control subjects (fig. 6.7). No effect was seen on CD23 (low affinity IgE receptor), on CD14+ monocytes or CD4+ T-helper cells. No significant difference was seen in CD95 or CCR3 expression on monocytes and eosinophils respectively after anti-IgE therapy (fig. 6.8).

6.3.2.2 *Sputum counts*

No significant difference was seen in the sputum differential counts in patients receiving rhu-Mab E-25 over the course of treatment.



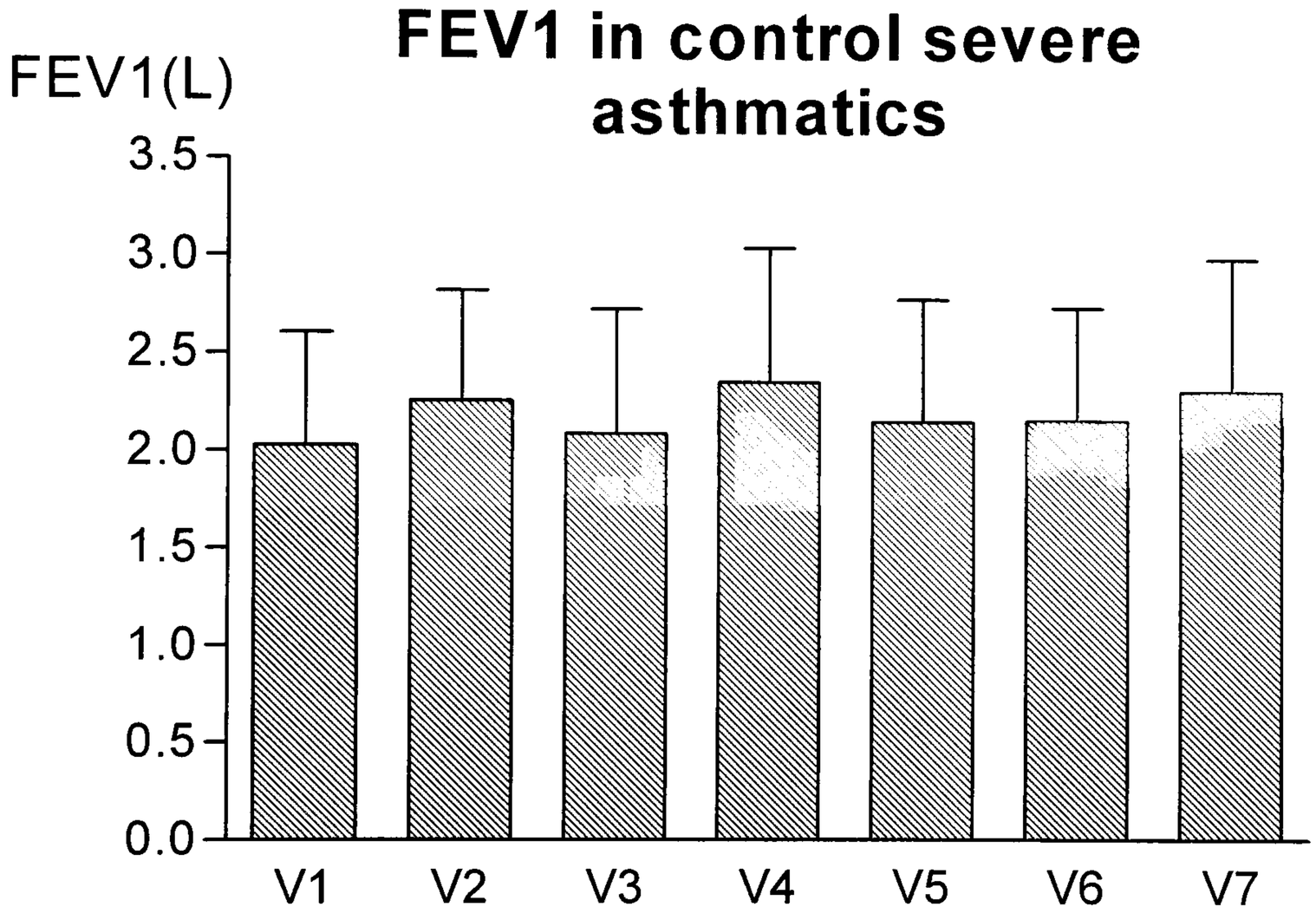
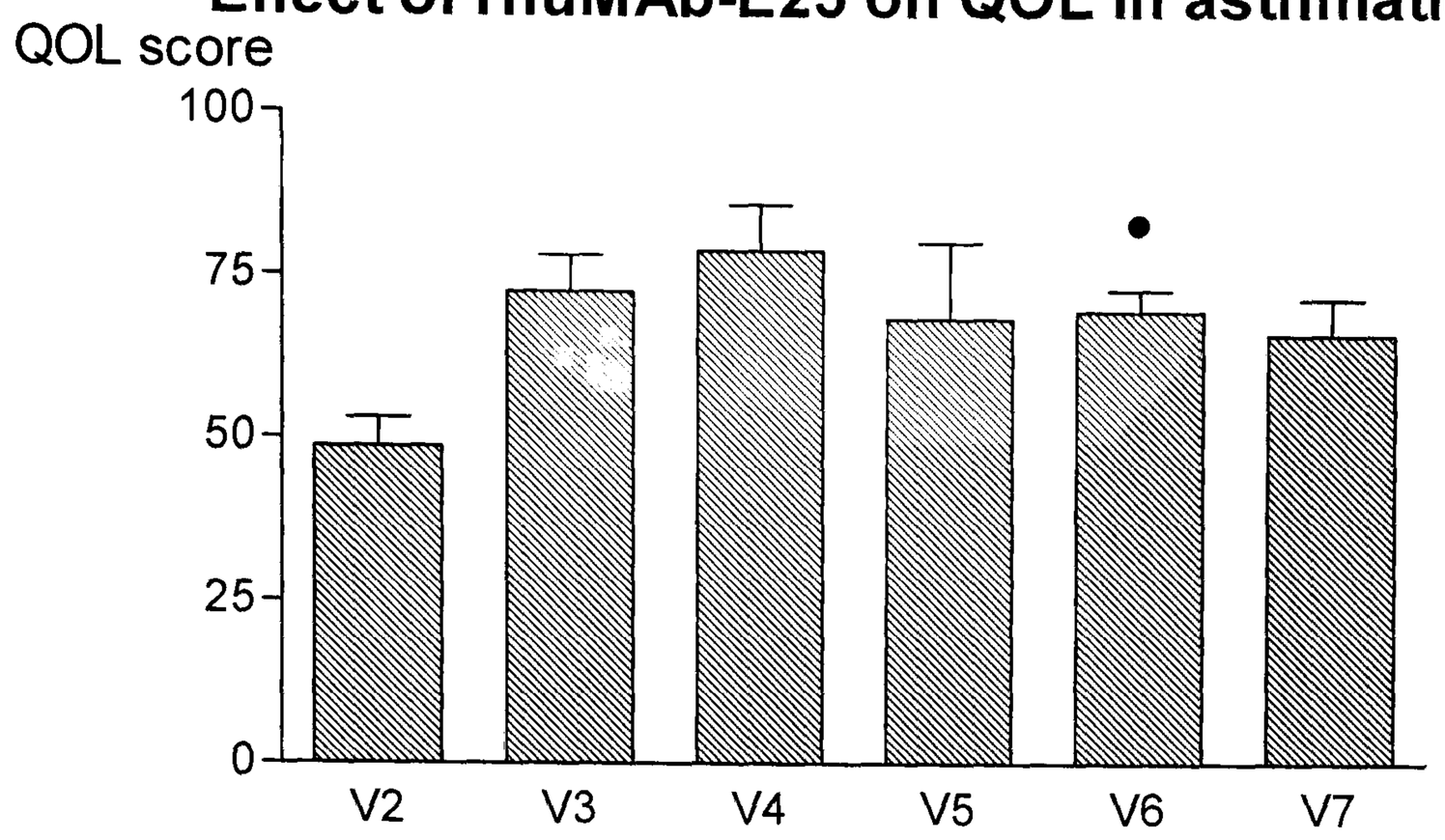


Fig. 6.1 FEV1 in rhuMAb–E25 treated severe allergic asthmatics (n=5) and controls. (n=4). V1=screening visit. V2=randomization. V6=end of study visit. V7=final follow up visit. No significant effect on FEV1 in the treatment group compared to controls.



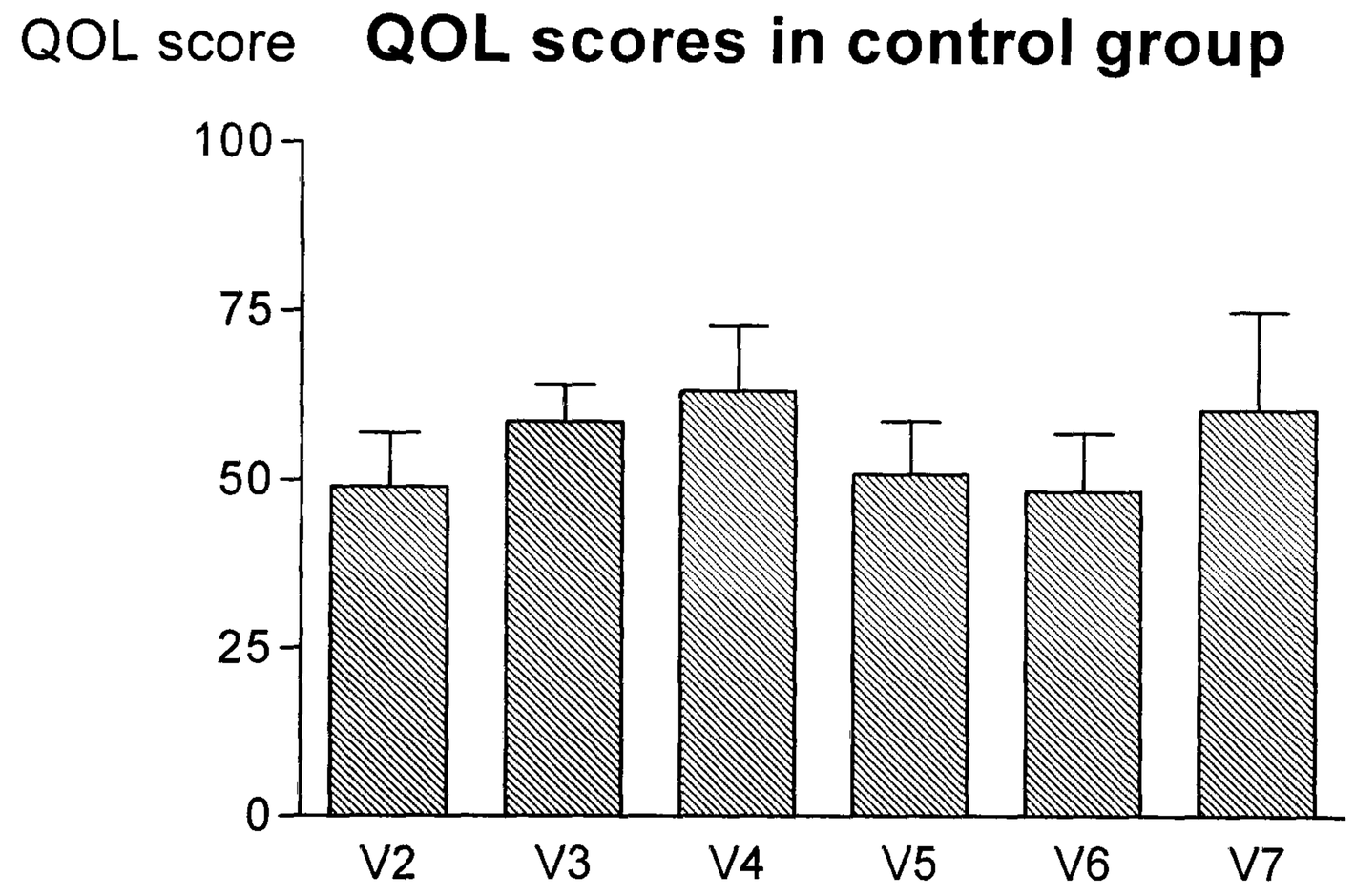
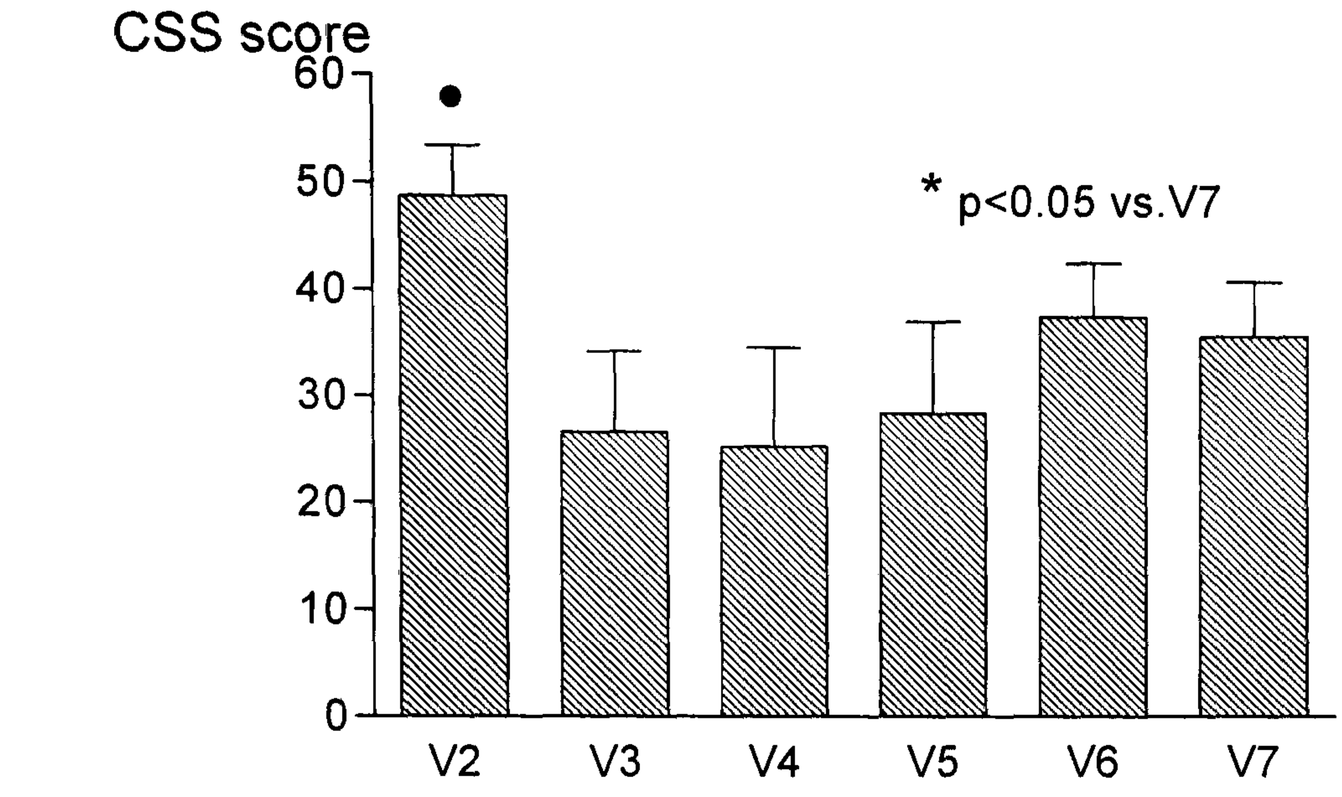


Fig. 6.2 Quality of life scores (Juniper–Mini asthma quality of life questionnaire) in asthmatics treated with rhuMAb-E25 (n=5) and in control subjects (n=4). Quality of life score was higher in the rhuMAb-E25 treated group at visit 6 compared with controls (• p< 0.03).



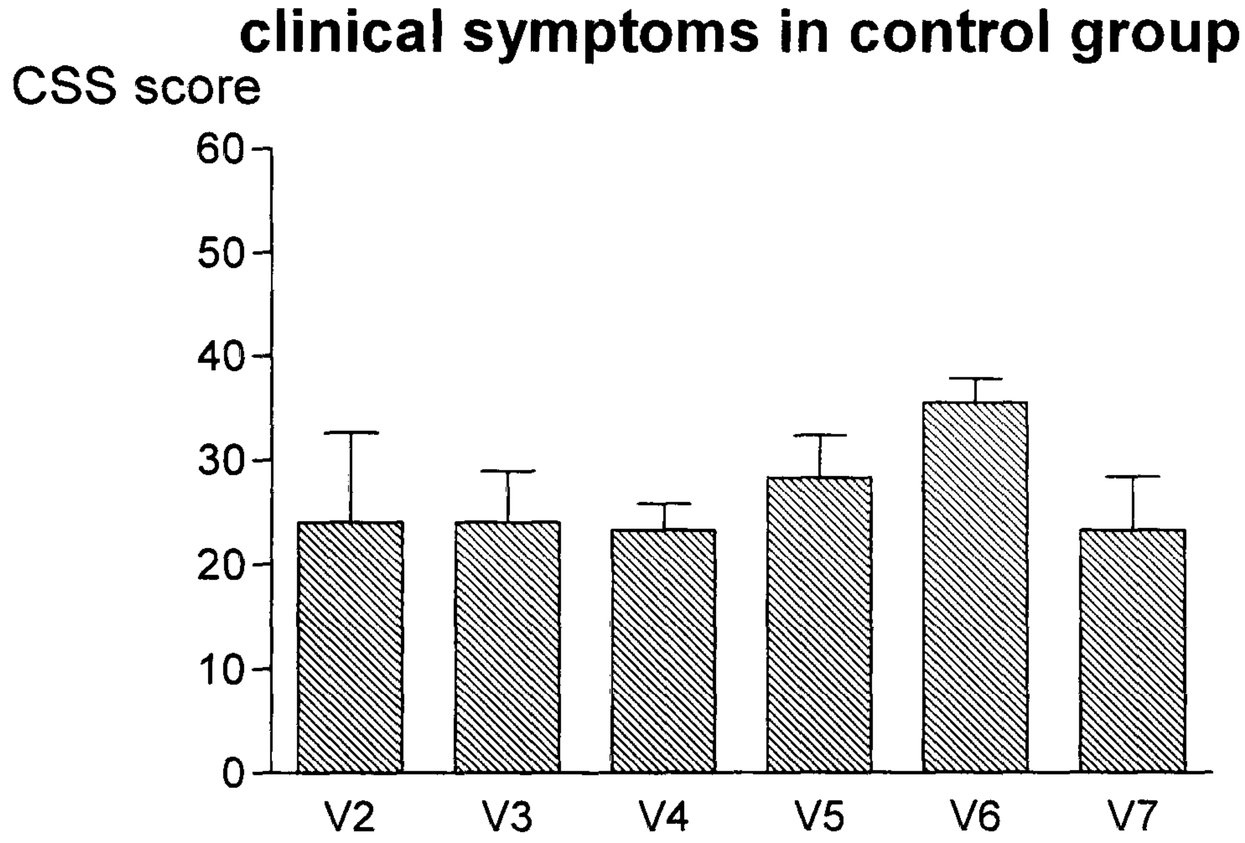
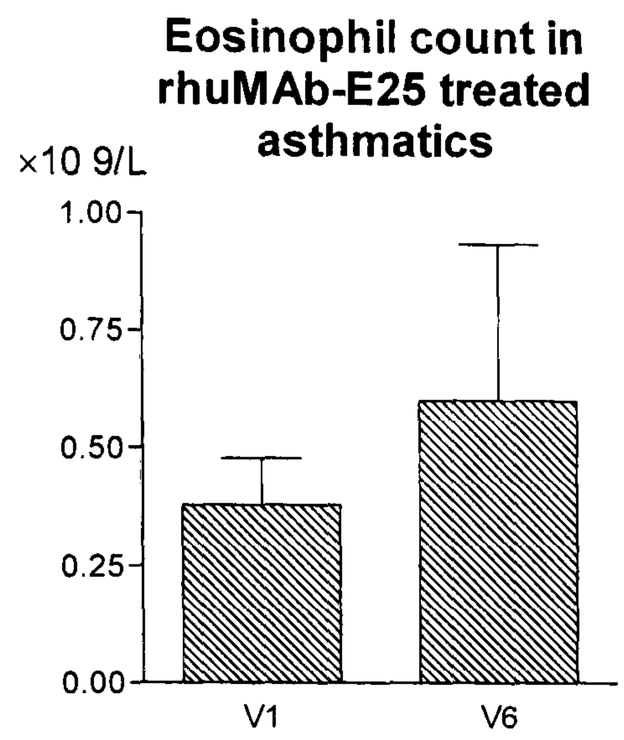
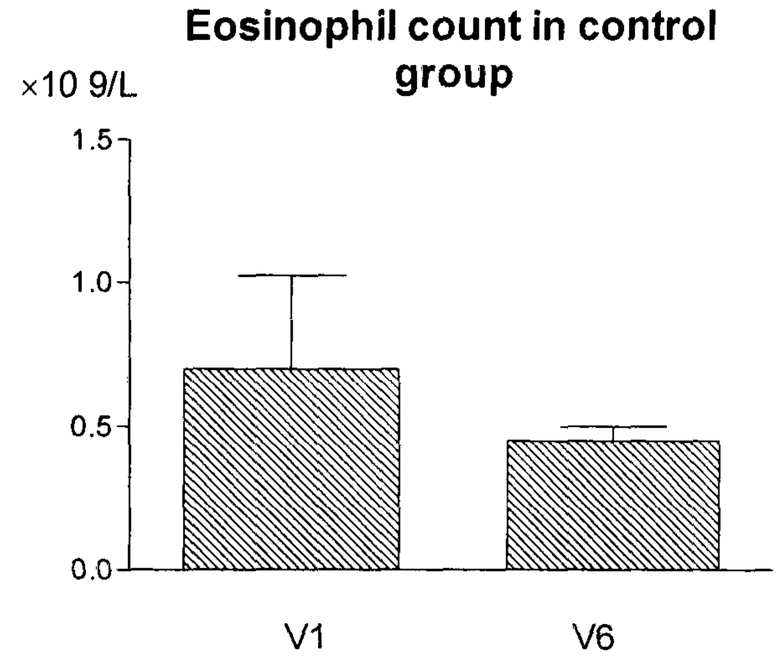
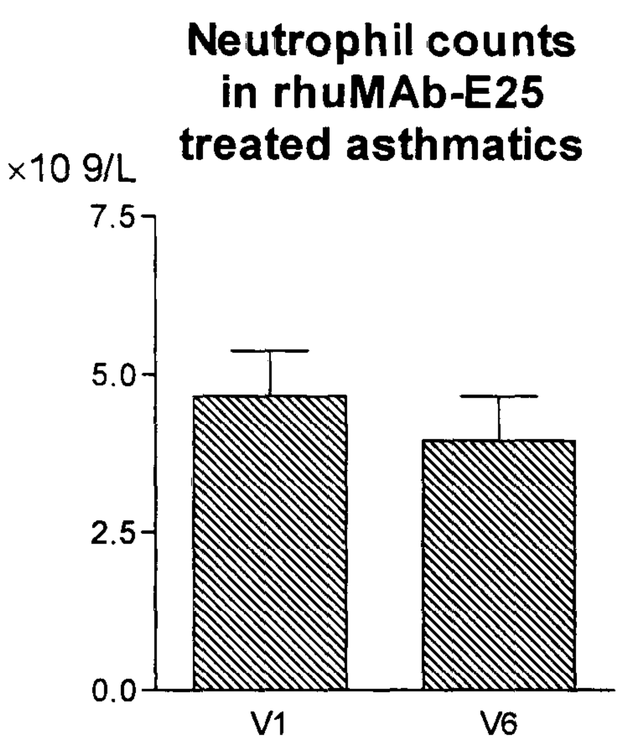


Fig. 6.3 Clinical symptom scores (Wasserfallen-combined clinical symptom score) in rhuMAb-E25 treated asthmatics (n=5) and control subjects (n=4). Clinical symptom scores were higher at visit 2 compared with the control group. (• p =0.03).







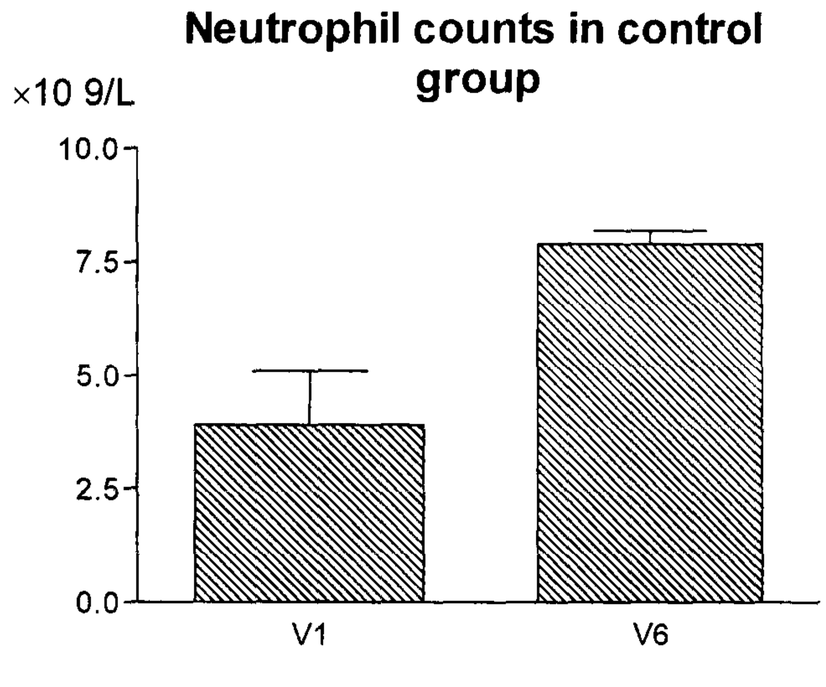
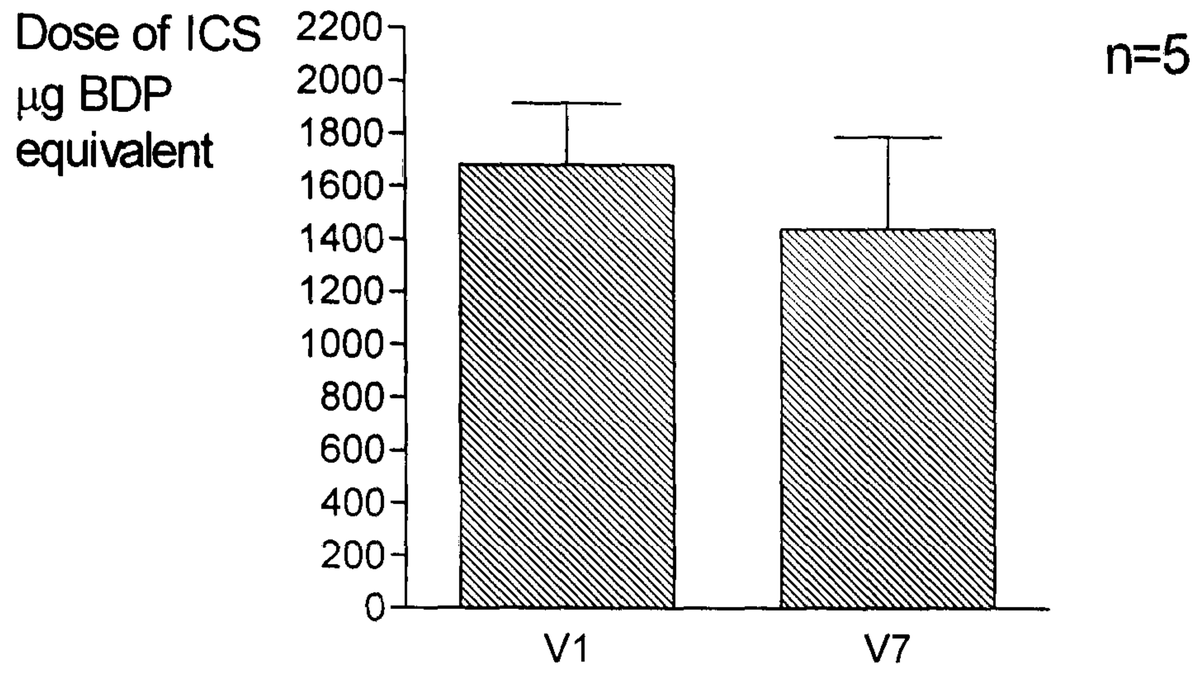


Fig. 6.4 Peripheral blood eosinophil and neutrophil counts in rhuMAb treated asthmatics (n=5) and control subjects (n=4).V1=screening visit. V6=end of study visit. No significant difference was seen in neutrophil or eosinphil counts in either group.



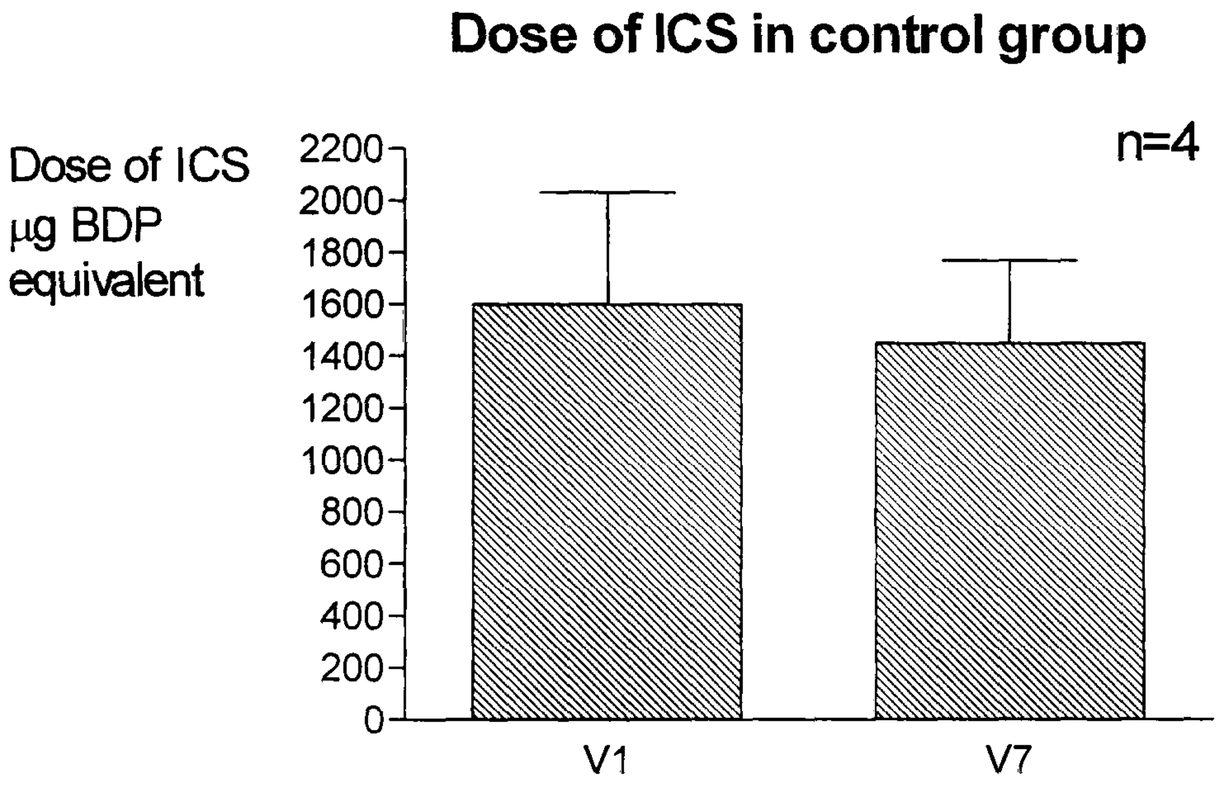


Fig. 6.5 Dose of ICS, (BDP µg/day or equivalent) in rhuMAb-E25 treated asthmatics and in control subjects. No significant reduction in dose of ICS was seen at the end of the trial in rhuMAb-E25 treated asthmatics comapered with control subjects.

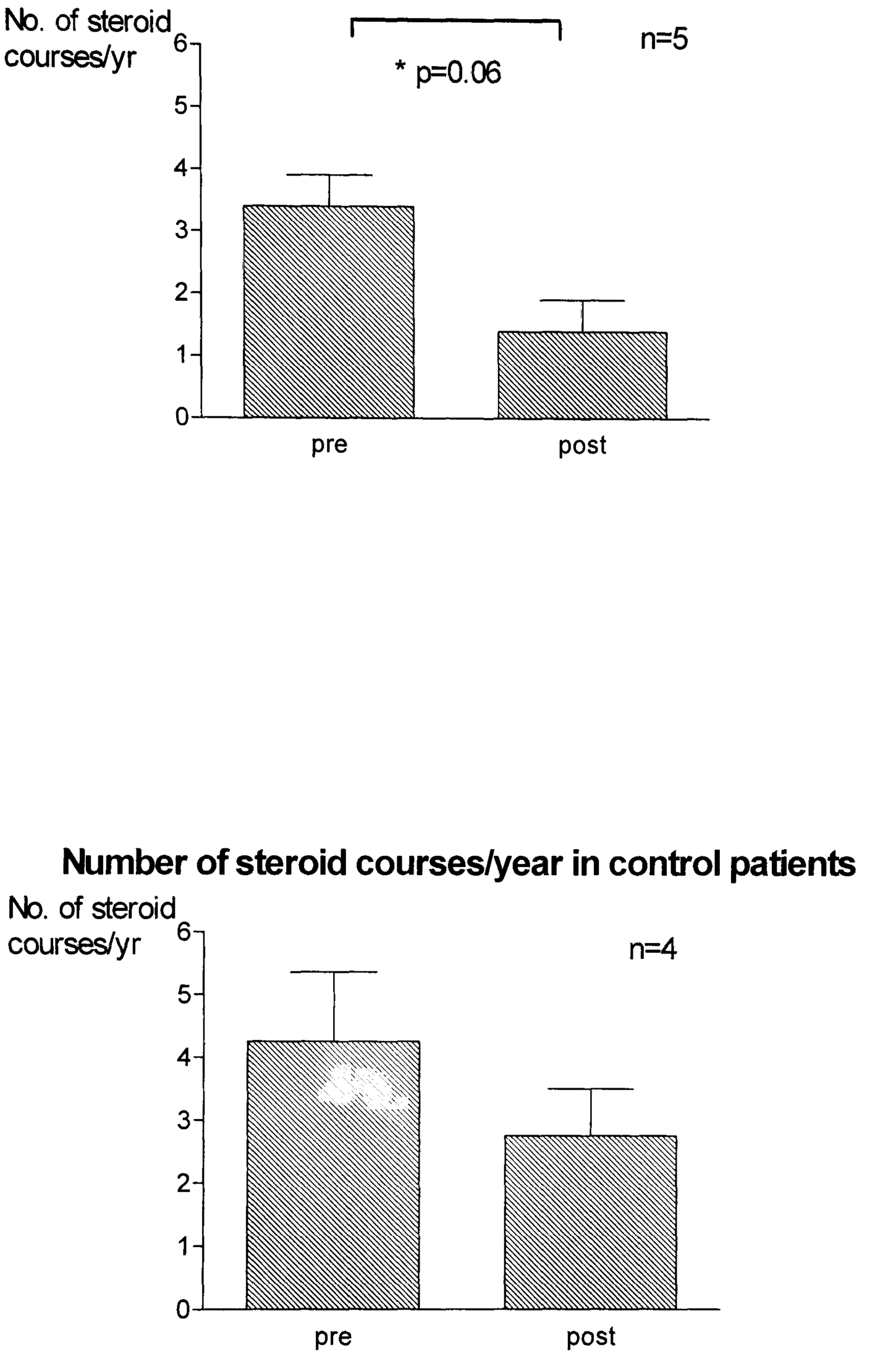
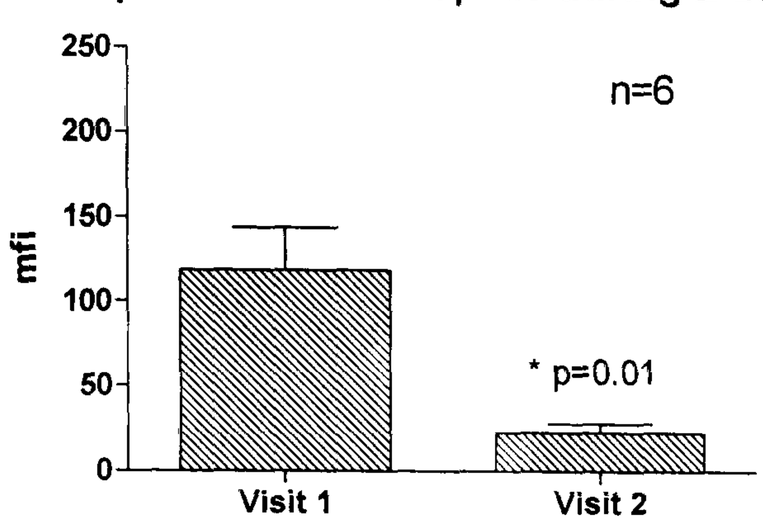
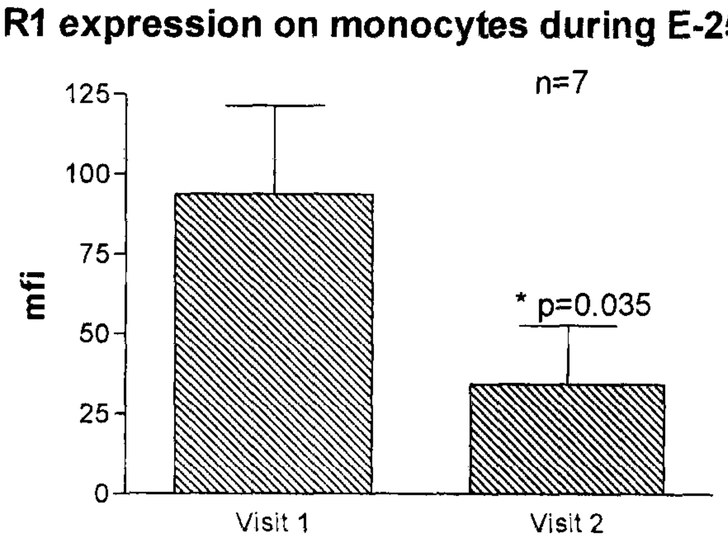
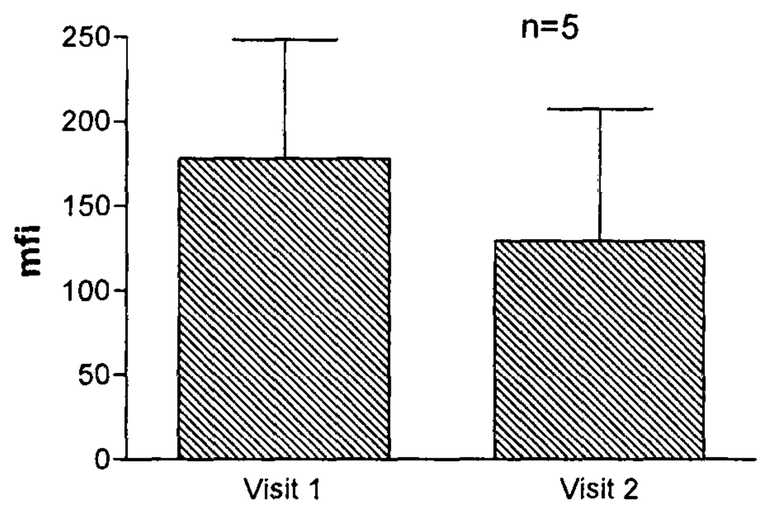


Fig. 6.6 Number of courses of prednisolone required per year, during the year prior to treatment and during treatment in rhuMAb-E25 treated asthmatics and in control subjects. Reduction in steroid courses approached significance in rhuMAb-E25 treated asthmatics, but this was not significant compared with the control group.







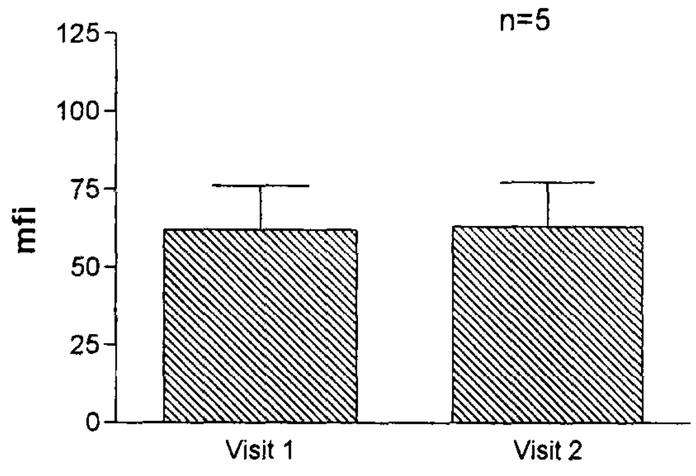
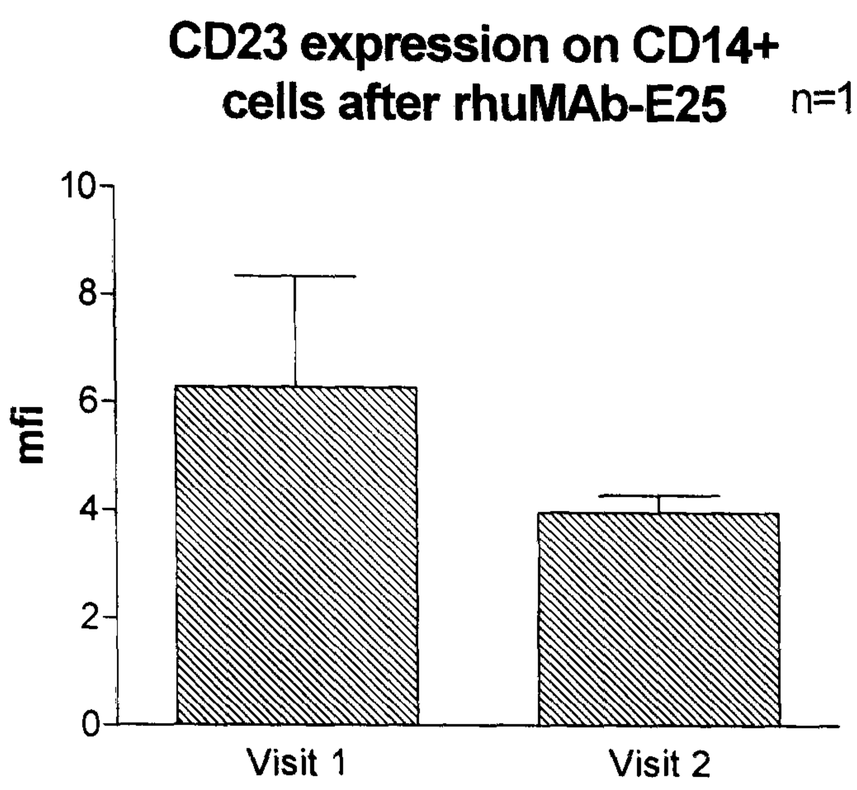
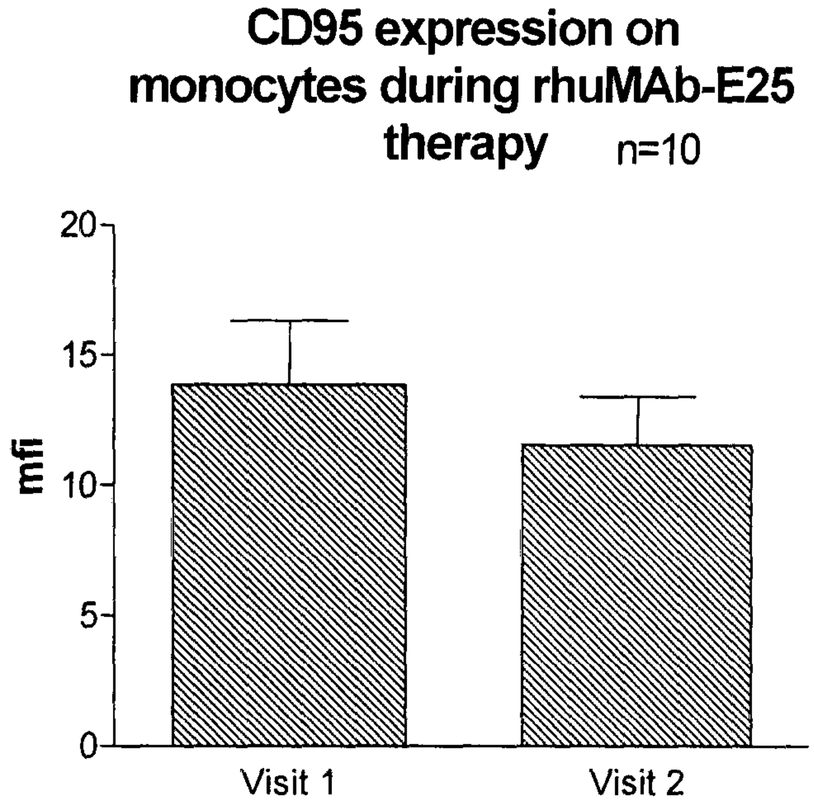
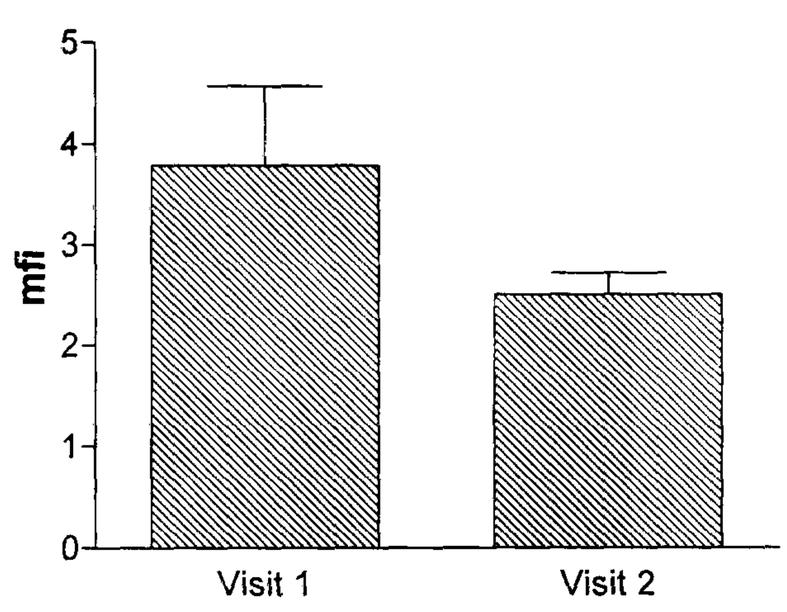


Fig. 6.7 FcεR1 expression on basophils and monocytes in rhuMAb-E25 treated asthmatics and in control subjects (n =5) after 3 month period. RhuMAb-E25 significantly reduced FcεR1 expression on basophils and monocytes at visit 2 during treatment, compared with baseline. This was not significant compared with the control group.







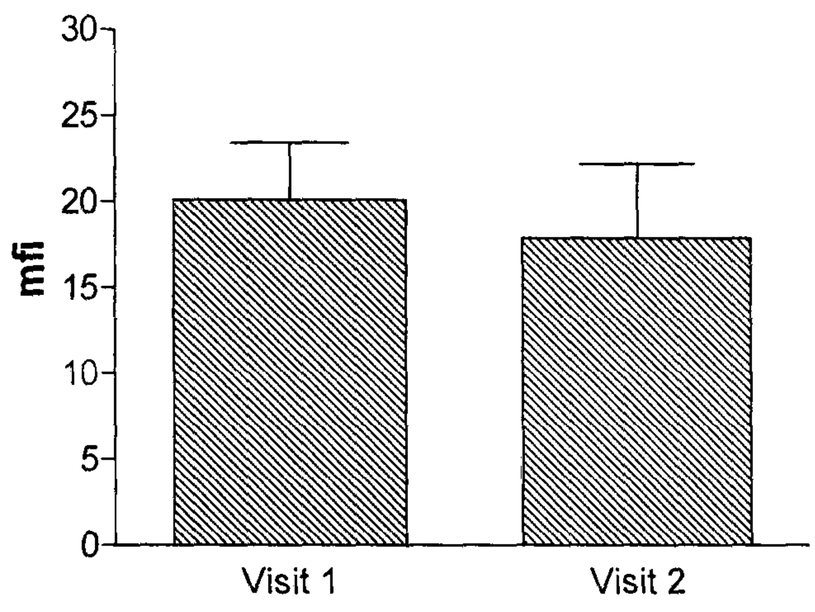


Fig. 6.8. Protocol 11 extension substudy. Expression of CD23, CD95, and CCR3 on monocytes (CD14+), lymphocytes (CD4+) and eosinophils (CD9+) in rhuMAb-E25 treated asthmatics over a 3 month period. Total number of patients in Protocol 11 extension substudy =10. No effect of rhuMAb-E25 treatment on surface markers.

6.4 Discussion

Despite recent advances in the understanding of the role of inflammation in asthma, and improved drug treatments, asthma morbidity and mortality continues to rise worldwide (231). Asthma is a multifactorial disease in which interaction between allergic factors and non-allergic triggers result in bronchial obstruction and inflammation. Most patients with asthma have atopy, with specific IgE antibodies to inhaled indoor and outdoor allergens (e.g. house dust mite allergen, cockroach allergen, pollen, and mould) which drive the inflammatory process in the airways. Although allergen avoidance is recommended in the management of allergic asthma, it is rarely completely effective. Immunotherapy directed against common allergens in patients with asthma is not typically used in the UK, but US studies have shown that this treatment is disappointing in such patients and carries a small risk of serious adverse effects.

Corticosteroids are the mainstay of anti-inflammatory treatment for asthma and produce their effects on various cells by binding to intracellular glucocorticoid receptors, which in turn inhibit transcription factors that are normally activated by inflammation. Many patients can be maintained on a low dosage of ICS with minimal risk of adverse effects, but systemic effects can occur with longterm oral corticosteroids. These include hypothalamic-pituitary-adrenal axis suppression, osteoporosis, cataracts, hyperglycaemia, dermal thinning and growth retardation in children.

Because of the central role it plays in atopic disease, inhibition of IgE with IgE antibodies represents a novel approach to improve IgE-mediated allergic reactions and to reduce the severity of asthma. RhuMAb-E25, a humanized murine monoclonal antibody directed to the FcεRI binding domain of human IgE, inhibits the binding of IgE to mast cells without provoking mast cell activation.

In clinical studies, rhuMAb-E25 exhibited a prolonged pharmacological effect without inducing anaphylaxis, blunted the early and late-phase allergic response to inhaled allergen, reduced the symptoms of seasonal allergic rhinitis, reduced the symptoms of asthma (and improved some physiological measures of asthma) and reduced corticosteroid use. RhuMAb-E25 has been well tolerated in clinical trials to date. One of the limitations of rhuMAb-E25 is that it is a protein that must be given by injection. Initial studies with rhuMAb-E25 involved giving twice weekly i.v. injections.

Results from the Protocol IA04 study did not show a significant increase in FEV1 in rhuMAb-E25 treated asthmatics during the course of the treatment compared to the

control group. There was no effect on quality of life scores in the treated group, although scores were significantly higher in the rhuMAb-E25 treated group at visit 6 compared with PBO (p=0.03). Clinical symptom scores were improved at visit 5 in the treatment group, but this was not significant when compared with PBO. There was no effect of rhu-MAb-E25 on the peripheral eosinophil or neutrophil counts, and although there was no significant reduction in the final dose of ICS at the end of the study in patients receiving anti-IgE therapy, the total number of courses of prednisolone required for treatment of asthma exacerbations fell from 17 (mean 3.4 courses per patient) over the course of the previous year to 7 (mean-1.4 courses per patient) over the year in patients receiving rhuMAb-E25 (p=0.06). Although not statistically significant, this compares to 15 courses of prednisolone over the previous year in control patients (mean of 3.75 courses per patient) and 11 courses (mean of 2.75 courses per patient) during the course of the study. The final dose of ICS may have been affected by the timing of any exacerbations which may have necessitated an increase in ICS dose in order to maintain symptom control.

The study showed that rhuMAb could be given safely by subcutaneous injections over a one year period, and that the subcutaneous route of treatment was well tolerated with no significant adverse effects being reported. Only one patient reported mild influenza like symptoms after rhuMAb-E25 injections which persisted for 4 months although symptoms responded to paracetmol and were not severe enough to stop treatment.

The main problem with the IA04 trial was the short recruitment time available before the commencement of the trial period. This meant that patient numbers in the active and control groups were low, and therefore the trial at this centre was not adequately powered to detect any significant changes in the parameters being measured. Apart from one patient, FEV1 measurements in the control group were generally higher than in the treatment group, although there was not a statistically significant difference. Patients in the control group were also much younger than the rhuMAb-E25 treated asthmatics, and thus not well age matched.

The IA04 study, also known as the ETOPA study, has since been published in 2004 by Ayres et al (276). In total, 312 patients aged between 12 to 73 years, receiving 400μg/day or more (if adolescent) or 800μg/day (adults) inhaled BDP or equivalent were included. Although long acting beta agonists were not an entry criteria, 78% of patients were using them at baseline. Patients received best standard care with asthma medication

with or without Omalizumab (with appropriate dosing) every 4 weeks for 12 months. Results showed that the mean number of asthma deterioration related incidents was from 9.76 with best standard care alone in 106 patients, to 4.92 in 206 patients who received Omalizumab. Patients who received omalizumab experienced 49.6% fewer asthma deterioration related related incidents per patient-year compared to best standard of care alone. The Omalizumab group showed a 61% reduction in clinically significant asthma exacerbation rate ($p<0.001$) than the best asthma treatment alone.

Basophils are a constituent of the cellular infiltrate in bronchial tissue of atopic asthmatic patients and are further increased after allergen challenge. They are also a major constituent of the cellular infiltrate in the cutaneous late-phase reaction to allergens. Basophils can be identified as a single population of cells that stain positively for CD123 (anti-IL-3Rα which stains predominantly basophils and monocyte derived dendritic cells, and which plays an important role in haematopoietic progenitor cell growth and differentiation), and negatively for HLA-DR (which labels the dendritic cells and distinguishes them from basophils). Anti-CD14 labelled monocytes were detected by their increased fluorescence on the FL1 channel. The experiments performed on blood samples in protocol 11 extension substudy showed that rhuMAb-E-25 significantly reduced FcεR1 expression on basophils and monocytes after 3 months treatment compared to baseline, as a consequence of reducing serum IgE levels which can regulate the expression of high affinity IgE receptors. In contrast, no significant difference was seen at 3 months in the levels of FcεR1 receptor on basophils or monocytes in the control patients. The reduction in FcεR1 expression on basophils and monocytes was not significant compared to control subjects, and this is likely due to the fact that there were insufficient patients in the treatment group to reach significance.

No significant difference was seen in the expression of the low affinity IgE receptor (CD23) on monocytes or CD4+ lymphocytes, or of CD95 on monocytes following anti-IgE therapy. Studies in mice have suggested that rhuMAb can affect cells other than mast cells and basophils, and may inhibit CD23 on other cells such as macrophages and T-cells (232). Pre-treatment of allergen challenged mice with anti-IgE treatment has been shown to reduce serum IgE levels and eosinophil infiltration into the lungs, and also reduce IL-4 and IL-5 production from Th2 lymphocytes. *Coyle et al* (232) showed that anti-IgE antibodies achieve this by inhibiting IgE-CD23 facilitated antigen presentation to T-cells.

Also no effect of anti-IgE therapy was seen on CCR3 expression on eosinophils, which can be identified by expression of surface CD9 molecules and high side scatter or granularity. Survival of eosinophils has been shown to be reduced by treatment of cells with anti-CCR3 antibody (233). The chemokine eotaxin, which is a highly specific chemoattractant for eosinophils, appears to be a principal ligand for CCR3. Thus although anti-IgE therapy can reduce sputum eosinophil numbers (163), it does not appear to reduce peripheral blood eosinophil numbers or the expression of CCR3 receptors on eosinophils, suggesting an alternative mechanism of its anti-inflammatory action.

Although rhuMAb-E25 is likely to be expensive, as are other antibody treatments such as anti-TNF-α, such a treatment may be justified in patients with severe asthma who are only controlled on doses of oral corticosteroids that cause side effects (165). Patients with severe atopic disease, in addition to asthma may also benefit.

The role of neutrophils in the allergic inflammatory cascade is now being realized. *Gounni et al* showed that peripheral blood and BAL neutrophils from asthmatics expressed FcεR1, which is the main receptor involved in binding IgE molecules to the surface of mediator cells. Further, activation of these FcεRI receptors induced the release of IL-8 by neutrophils (169). Further, *Yamaoka et al* showed that neutrophils can synthesize CD23 molecules following stimulation with GM-CSF, and this CD23 expression might be of physiopathological relevance in IgE dependent activation during allergic processes (168). The presence of three forms of IgE receptor has been demonstrated on neutrophils, as well the observation that in vitro challenge of neutrophils with allergens to which the patients were sensitive, elicited a release of elastase by these cells, suggesting that an IgE dependent mechanism may promote elastase release by neutrophils at allergic sites (234). Taken together, these studies suggest that neutrophils may play a role in allergic inflammation through an IgE-dependent activation mechanism. In the context of severe allergic asthma, this is important as increased numbers of neutrophils have been demonstrated in sputum and bronchial biopsy samples in patients with severe corticosteroid-dependent asthma.

It has been shown that corticosteroids may prolong the life of neutrophils by inhibiting their apoptosis and, in some instances, can lead to activation of neutrophil function, both of which can result in possible tissue damage through the inappropriate release of intracellular mediators and enzymes. Corticosteroid therapy could also, by

prolonging the life of neutrophils at the sites of inflammation, allow them to participate in IgE mediated allergic responses through their IgE receptors which would further drive the inflammatory cascade and lead to worsening symptoms in asthmatics. Anti-IgE therapy may thus prove to be a useful steroid sparing agent in these more severe asthmatics who show many troublesome side-effects of their current medication, and in whom an alternative safe and effective treatment is required. Further, the recognition that IgE mediated mechanisms may occur in asthma irrespective of the atopic status of the patient, suggests that despite the distinct clinical and biological features, there is a common immunopathological mechanism, suggesting a possible role for anti-IgE therapy in severe non-atopic asthmatics.

The studies in this chaper could have been improved by including larger numbers of patients in each of the groups, to increase the power of the studies. The IA04 trial protocol only required measurement of serum total IgE levels at the initial screening visit, but it would have been interesting to measure IgE levels during the 3 monthly scheduled visits to look at the effect of Omalizumab on IgE levels. The reduced numbers of patients in the 11 extension substudy, in whom expression of FcεR1 on basophils and monocytes was measured by FACS, was due to the fact that the staining antibody required was not available for use in all patients at the time of commencement of the study. FACS analysis was thus not possible in some of the early entrants into the IA04 study. It would have useful to look at the level of neutrophil surface molecule expression in peripheral blood using FACS during treatment with anti-IgE therapy in severe asthmatics, to see if this form of treatment has any effect on neutrophil activation markers. A study of anti-IgE therapy in non atopic severe asthmatics to see if there is any beneficial effect in improving symptom control, may result in an increase in the number of patients who can be treated with form of therapy, if benefits were shown.

Chapter 7

Overall conclusion

Eosinophils have long been thought to be the main cell causing bronchial mucosal injury in asthma, and it has been shown that eosinophil numbers and eosinophil mediator levels in blood, BAL and bronchial biopsies correlate with asthma severity. However, several studies suggest that airway eosinophilia is not a universal finding and that the neutrophil may be the more important effector cell in certain circumstances. *Douwes et al* (235) for example, showed that eosinophilic inflammation is seen in the airway lumen of only 50% of asthmatic subjects, whilst intense eosinophilic inflammation, as occurs in eosinophilic bronchitis, fails to induce asthma (236). Many asthma exacerbations show increased levels of neutrophils rather than eosinophils in the airways, and studies have shown that the use of specific anti-eosinophil cytokine agents such as anti-IL-5 and anti-IL-12 are poorly efficacious in-vivo (237-238). *Frangova et al* (239) showed increased neutrophils, eosinophils, and MPO in asthmatics versus controls, but found similar levels of neutrophils, eosinophils, and epithelial cells numbers and MPO in allergic versus non-allergic asthmatics, suggesting that airway recruitment and activation of neutrophils occurs in parallel with eosinophils and that airway neutrophils do not contribute significantly to epithelial cell injury or to airway hyperresponsiveness in the steady state, in mild asthma.

The role of neutrophils in more severe asthmatics however, has been highlighted in a number of studies. *Wenzel et al* (240) showed that in severe disease, eosinophils and neutrophils are usually found together but that neutrophils may gradually replace eosinophils in proportion to the severity and/or duration of the disease. Further characterization of severe corticosteroid-dependent asthmatics showed two distict pathological subtypes based on the presence or absence of airway eosinophils. In both types however, neutrophil numbers were elevated. That neutrophils may have a role in the development of asthma was shown by *Yasui et al* (256), who showed a low incidence of asthma in a group of Japanese children with autoimmune neutropenia, with the development of asthma after the neutropenia reolved. Evidence of airway neutrophil activation leading to more severe disease was demonstrated by *Little et al* (241), who showed that the FEV1 was inversely proportional to neutrophil numbers in sputum

samples, and that neutrophil numbers correlated well with markers of neutrophil degranulation which implies that these cells are also activated. *Kikuchi et al* (242) showed that, in severe persistent asthmatics treated with oral and high dose ICS, there was a correlation between the percentages of neutrophils and eosinphils in induced sputum, which may be responsible for the eventual manifestation of airway inflammation in severe asthma.

Silvestri et al (257) showed that in severe asthmatics, elevated levels of the neutrophil chemoattractant TNFα, released in IgE mediated responses by mast cells and eosinophils, correlated with increased numbers of airway neutrophils. TNFα has been shown to contribute to bronchial hyperesponsiveness in asthma. *Halasz et al* (277) showed that increased levels of TNFα and its soluble receptors 55kDa (sTNF-R1) and 75kDa (sTNF-R2) in patients with hyperreactive airways. There was a positive correlation between cytokine levels and cytokine receptor levels and negative correlation between serum TNFα and concentrations of sTNF-R2, and PC_{20} in hyperreactive patients. Inhaled TNFα induces airway neutrophilia and increases airway reactivity in normal subjects. *Thomas et al* (278) showed reduction in PC_{20} and thus increased bronchial hyperresponsiveness after inhaled recombinant human TNFα compared to PBO and an increase in sputum neutrophilia and eosinophilia at 24-48 hours versus the control group. The increase in airway responsiveness and sputum inflammatory cell influx suggests that TNFα may contribute to the airway inflammation in asthma.

Patients with refractory asthma have been shown to have evidence of up regulation of TNFα. *Berry et al* (279) showed increased TNFα activity in PBMCs with increased expression of membrane bound TNFα, TNFα receptor 1, and TNFα converting enzyme by PBMCs in patients with refractory asthma compared to mild and moderate asthmatics and control subjects. Following on from this, the same authors conducted a PBO controlled, double blind, crossover pilot study lasting 10 weeks, aimed at looking at the effect of etanercept, a soluble TNF-α receptor antagonist in patients with refractory asthma, mild to moderate asthmatics and control subjects. Compared with PBO, treatment with etanercept was associated with an improvement in airway hyperresponsiveness ($p=0.05$), an increase in asthma related quality of life scores ($p=0.02$), and an increase in post bronchodilator FEV1 ($p=0.01$).

Rouhani et al (280) looked at the effect of treatment with etanercept on the early and late phase response to inhaled allergen challenge in 26 patients with mild to moderate asthma, who were receiving only β-2 agonists. Anti-TNFα therapy with etanercept did

not attenuate pulmonary eosinophilia, and was associated with an increase in IL-4 at 24 hours following segmental allergen challenge. Treatment with etanercept was not associated with a change in airway hyperresponsiveness to methacholine, suggesting that this therapy may not be effective for preventing allergen mediated eosinophilic airway inflammation in mild to moderate asthmatics. In addition, one patient in the trial developed a transient hemiplegia, thought to be a potential toxicity of anti-TNFα therapy, and this resulted in suspension of the study.

Morjaria et al recently conducted a recent 12 week study of the effect of etanercept in 39 patients with severe corticosteroid refractory asthma (281). Significant reductions in sputum macrophages and serum C-reactive protein levels were seen, along with an increase in serum TNFα and albumin levels following therapy. There was a small but significant difference in reduction of asthma control questionnaire scores between treatment and PBO. However, there was no significant difference in asthma related quality of life scores, lung function, PEFR, bronchial hyperresponsiveness, or exacerbation rates between the groups. There was an increase in minor adverse effects such as injection site pain and skin rashes with etanercept. Larger trials are thus needed to clarify the role of treatment with TNFα antagonists in patients with severe corticosteroid refractory asthma.

Neutrophils have also been shown to be involved in the acute inflammatory reaction of the airways that occur after bronchoprovocation with allergens and sensitising agents during spontaneous asthma exacerbations, during nocturnal asthma, and in the dramatic bronchopulmonary inflammation associated with asthma death. *Wark et al* (243) showed that patients with proven virus induced exacerbation of asthma (by sputum immunofluorescence antigen detection, sputum viral culture, and sputum PCR for respiratory viruses) had evidence of increased sputum neutrophilia and increased levels of neutrophil elastase compared to patients with non infective asthma. One possible reason for this may be due to the increased expression of epithelial growth factor receptor (EGFR) levels. Expression of EGFR is increased in proportion to asthma disease severity and is a reflection of the extent of epithelial injury. *Hamilton et al* (244) showed that epithelial damage in bronchial biopsies of severe asthmatics showed increased levels of epithelial IL-8 and submucosal neutrophils and increased EGFR levels compared to milder disease. There was a strong correlation between EGFR levels and IL-8 expression, suggesting that epithelial damage may contribute to neutrophilic

inflammation through the enhanced production of IL-8 via an EGFR dependent mechanism.

Neutrophils contain several mediators which are capable of killing bacteria, but they also release enzymes and cytokines and oxygen radicals which play an important part in tissue damage, tissue remodelling and which are capable of activating other cells such as eosinophils resulting in degranulation and release of mediators which are potentially capable of causing further tissue damage. Studies on sputum, BAL and endobronchial biopsy specimens have shown that neutrophils and its chemoattractant, IL-8 and MPO, are significantly elevated in more severe asthmatics in the absence of infection. *Little et al* (241) showed that some patients with chronic asthma develop irreversible airflow obstruction and disease duration was shown to be a predisposing factor. Sputum MPO levels in patients was shown to be proportional to numbers of sputum neutrophils and inversely associated with FEV1 suggesting that neutrophils may be involved in the pathophysiology of irreversible airflow obstruction in chronic asthma. Neutrophils can also release matrix-metalloproteinases (MMP) such as MMP-9 following stimulation with IL-8 and GM-CSF (245), and have been shown to express MMP-9 in bronchial biopsies from asthmatics (246). MMP-9 can lead to airway wall remodelling which may be an important factor in the development of severe asthma.

The consistency of the finding of airway neutrophilia in more severe chronic asthmatics cannot be solely explained by the effects of smoking and concomitant acute infection. One possible explanation for the airway neutrophilia in this subgroup of asthmatics is that these patients may have had chronic airway infection with viruses or atypical bacteria such as mycoplasma or Chlamydia. There is serological evidence for infection with Chlamydia pneumonia in asthma, and evidence linking infection with more severe disease (258). In addition, *Kraft et al* (259) showed evidence of airway infection with mycoplasma pneumonia in asthmatics using PCR techniques. However, treatment with macrolide antibiotics failed to show sustained improvement in pulmonary function in asthmatics with serological evidence of Chlamydia pneumoniae infection (260). Another possible mechanism in the development of airway neutrophilia in chronic severe asthma is that airway epithelial cells are chronically activated and hypersecrete neutrophil chemoattractants including IL-8 in response to environmental pollutants such as diesel exhaust particles (261). This suggests that lower airway epithelial cells may have an intrinsic functional abnormality that is important in the pathogenesis of asthma.

Linden et al (247) showed that IL-17 may be responsible for mobilising neutrophils into airways of patients in acute severe asthma exacerbations. IL-17 is produced by T-lymphocytes of the memory (CD45 RO+) subset, and causes human bronchial epithelial cells in vitro to produce and release IL-8 and IL-6 which can lead to neutrophil recruitment and activation in the airways. Further, *Hellings et al* (248) looked at the IL-17 expression in the airways of ovalbumin sensitized mice following inhalation of nebulised ovalbumin. Increased levels of IL-17 mRNA expression were seen in the inflamed lung tissue, and this was associated with a prominent bronchial neutrophilic influx. As neutrophils may be important in airway remodelling in chronic severe asthma, targeting IL-17 may be a useful therapeutic strategy in human asthma.

The regular use of ICS improves lung function, bronchial hyperreactivity and markers of airway inflammation in sputum and exhaled NO in asthma. Some studies also show a decrease in sputum cytokine levels, which can influence recruitment of inflammatory cells into airways. Further, use of high dose ICS have been shown to be effective in treating acute asthma exacerbations and regular high dose inhaled FP, has been shown to be better than oral prednisolone at improving PC_{20} to methacholine whilst producing similar trends in FEV1, and reduction in sputum eosinophils and ECP as prednisolone (249). However, in a study by *Green et al* (130), a distinct subgroup of patients with mild to moderate asthma showed a predominantly neutrophilic airway inflammation in their sputum and responded less well to ICS in terms of improvements in symptoms, FEV1, or PC_{20}. The presence of increased neutrophils in asthmatic airways may thus account for less improvement following ICS therapy in such patients. *Gauvreau et al* (250) showed that ICS attenuated allergen induced airways inflammation but were less effective at inhibiting allergen induced sputum eosinophils after challenge in asthmatics that had increased levels of sputum neutrophils at baseline. This suggests that ICS are less effective in attenuating allergen induced airway inflammation in subjects with increased levels of neutrophils and that the heterogeneity of airway inflammation in some asthmatics may influence response to steroid treatment.

The beneficial effects of ICS on airway inflammation may also be attenuated by continued smoking in asthmatics. Compared to never smokers, smokers with asthma have been shown to have worse median total asthma control scores using the Juniper asthma control questionnaire, and this was independent of the FEV1 (282). Further, in a study of 104 uncontrolled asthmatics, of who 33 were ex-smokers, asthma was more severe in former smokers both before and after treatment (283). Severe asthma and cigarette

smoking were associated with difficulty in asthma control. Quality of life (as measured by the St George Hospital respiratory questionnaire) was improved in non-smokers but not in former smokers, suggesting that smoking has a persistent negative impact on the response to treatment in patients with uncontrolled asthma, even after smoking cessation. This highlights the importance of smoking cessation as an important part of the treatment of asthmatics.

Tomlinson et al (284), showed that compared with non smokers, smokers with mild persistent asthma are insensitive to the therapeutic effect of low dose ICS therapy given for 12 weeks. In addition, morning peak expiratory flow values were shown to be significantly lower after treatment with FP (1000μg/day) in asthmatics who smoked, compared to non-smoking asthmatics, suggesting that smoking impairs the efficacy of short term ICS therapy in mild asthma (285). This has important implications in mild asthmatics who smoke.

The use of induced sputum and bronchoscopy studies has supported the idea of a 'non-eosinophilic' asthmatic phenotype. *Simpson et al* (262) confirmed the heterogeneity of airway inflammation pattern seen in astmatics. In their study, 41% had predominantly eosinophilic inflammation in induced sputum, whilst 28% had predominantly sputum neutrophilia. Eight percent had both eosinophils and neutrophils in their sputum, whilst 31% demonstrated a 'pauci-immune' phenotype with neither eosinophils nor neutrophils in airway secretions. The eosinophil phenotype had increased levels of MMP-9, whilst those with predominantly neutrophilic inflammation had increased levels of neutrophil elastase. Whether the distinct inflammatory phenotypes occur as a result of different aetiological pathways is unclear, although recent studies suggest that the innate immune system is more activated in neutrophilic asthma, with increased expression of Toll like receptors (TLR), TLR2 and TLR4 and CD14 in induced sputum (263) in these patients. Both innate and adaptive mechanisms are important in the initiation and maintenance of asthma. The hygiene hypothesis suggests that respiratory infections should protect individuals from allergic disease, but epidemiological data in asthma is contradictory. It is thought that Toll receptors, which are present on dendtritic cells and mast cells, may control Th1 and Th2 cell differentiation, with signalling through TLRs leading to decreased Th2 allergic inflammatory response through induction of Th1 responses.

There are several definitions of severe asthma in the literature. The term 'refractory' asthma was coined by the American Thoracic Society in 2000 (264), to describe those asthmatics whose asthma proved troublesome and difficult to control. It is

an umbrella term and includes, among other patients, those with fatal or near fatal asthma, corticosteroid dependent or resistant asthmatics, brittle asthmatics and those with fixed irreversible asthma. Diagnosis depends on the presence of one or both major criteria and 2 minor criteria and requires the exclusion of other conditions and exacerbating factors, and demonstration of good compliance with therapy. Refractory asthma may represent a different inflammatory process than mild asthma, with more neutrophilic inflammation which is poorly responsive to corticosteroids, or else, the poor response to therapy can be explained by airway remodelling, which differs in amount, type, and distribution of airway smooth muscle proliferation and fibrosis, in the more severe patient group. Evidence suggests, for example, an increased proportion of cells that stain positive for TGFβ, a cytokine with fibrogenic properties, are found in the airway submucosa, in patients with refractory asthma. Possible molecular mechanisms that have been put forward to explain the more severe asthmatic phenotype includes down regulation of the binding affinity of glucocorticoid receptors, or up regulation of the less active glucocorticoid-beta receptor which prevents transcription. In addition, there may be reduced ability of corticosteroids to interfere with nuclear transcription factor binding, and failure to suppress c-jun-N-terminal phosphorylation.

The European Network for Understanding the Mechanism of severe asthma (ENFUMOSA) study also showed increased sputum neutrophilia in patients with severe asthma compared to those with well controlled asthma (265), with no difference in the number of other inflammatory cells, and this was independent of regular use of corticosteroids. Patients with severe asthma were also more likely to be female and non atopic. More recently, the severe asthma research program (SARP) showed that risk factors for severe asthma included a low initial FEV1, a history of pneumonia, and less atopic disease (266). Interestingly, *Nguen et al* (267) suggested that ICS alone do not contribute to elevated neutrophil numbers in asthmatics, whereas oral corticosteroids appeared to do so. It is unclear whether corticosteroid use results in, or is the reason for, the increase in airway neutrophilia.

The use of anti-IgE treatment represents a novel treatment for patients with allergic asthma and has been shown to be useful in patients with mild and severe asthma. The fact that it can be administered by subcutaneous injection twice weekly and is relatively free of side effects, means it will be of benefit in severe corticosteroid-dependent asthmatics in whom other treatments, including the use of corticosteroids, fail

to provide symptom control. Neutrophils from asthmatics express the FCεR1, whereas neutrophils from non-asthmatics do not. Activation of these FCεR1 receptors leads to release of IL-8, which is a potent neutrophil chemoattractant and activator. The fact that neutrophils have been shown to express high and low affinity receptors for IgE antibodies, suggest that they may play a role in allergic inflammation through an IgE dependant activation mechanism. *Monteserin et al* (251) showed that neutrophils challenged in vitro with allergens to which atopic asthmatics were known to be sensitive, resulted in increased release of MPO. This appears to be an allergen specific response in that no degranulation occurred in response to allergens to which patients were not known to be sensitive. Allergen exposure did not result in MPO release from neutrophils in healthy donors. Therefore, an IgE dependant mechanism may be important in promoting MPO release by neutrophils at allergic sites.

To date, seven large phase 3 trials of Omalizumab (Xolair Novartis Pharmaceuticals S.A.S. Huningue, France), a humanized monoclonal antibody that targets IgE, have been completed in adults and adolescents with allergic asthma. These studies consist of 5 double-blind, PBO-controlled studies and 2 randomized open-label standard therapy controlled studies. Omalizumab was given as add-on therapy to at least ICS in all studies and in some patients, to long acting beta agonists. Ninety three percent of the enrolled patients met the Global Initiative for Asthma (GINA) 2002 step 4 criteria for severe persistent asthma. Of these studies, the INNOVATE study included 419 patients aged between 12-75 years with allergic asthma. Patients treated with Omalizumab in addition to their existing therapy had a 26.2% lower rate of clinically significant asthma exacerbations. In addition, emergency room visits were reduced by 44% and hospital visits reduced by 51% (268).

Omalizumab was launched in the UK in 2005, and has been approved in 53 countries world wide. The National Institute for Health and Clinical excellence (NICE) recommended omalizumab for severe persistent allergic asthma in August 2007 as add-on therapy for patients with clinically confirmed severe persistent IgE mediated asthma. Specifically, patients should have experienced either 2 or more severe exacerbations of asthma requiring hospital admissions within the previous year, at least one of which required hospital admission and a further 2 which required treatment or monitoring in excess of the patient's usual regimen, in an emergency department. Patients must have a positive skin or blood test for a perennial aero-allergen, an FEV1 of less than 80%, as

well as frequent day time symptoms and/or night time awakenings, and FEV1 less than 60% and PEF variability of more than 30%.

Studies in the United States have looked at using anti-IgE therapy in patients with allergic bronchopulmonary aspergillosis (ABPA) who have IgE levels outside the recommended dosing limits. It is likely however, that a large component of non-specific IgE is present within the total IgE, and routine treatment with anti-IgE therapy at present cannot be recommended for patients outside the approved dosing range (the upper limit being 700 IU/ml). There are reports however, of Omalizumab being used as a corticosteroid sparing agent in cystic fibrosis patients with ABPA, with a reduction in respiratory symptoms and improved lung function (269).

At present, a study looking at the benefit of anti-IgE treatment after an acute exacerbation of asthma requiring hospitalisation is being conducted at the Royal Brompton Hospital, to look at its effect on the improvement in lung function and use of anti-asthma medication. It would be useful to look at activation markers such as CD11b, CD62L, CD18, CD35 and CD23 and FcεR1 on neutrophils before and during treatment with anti-IgE treatment to look at the effect of this form of treatment on neutrophil activation and receptor levels. Indeed, in a study involving anti-IgE antibodies, *Monteserin et al* (252) challenged blood lymphocytes in vitro from normal patients and adult allergic asthmatics some of whom had received immunotherapy. CD62L was shown to be reduced in a dose dependant manner with anti-IgE antibody, more so in non-immunotherapy patients than in the normal or immunotherapy groups, suggesting that CD62L can be modulated by an IgE dependant mechanism and that it can be affected by immunotherapy. Further, *Monteserin et al* (253) also showed that asthmatics showed a greater fMLP induced MPO release from neutrophils compared to healthy controls. MPO release was reduced in asthmatics receiving immunotherapy, suggesting that MPO release is modified in vitro by neutrophils from allergic asthmatic patients. The effect of treatment with anti-IgE treatment on MPO release or IL-8 release in cultured whole blood would also be useful to study.

This thesis adds to the hypothesis that neutrophils may be activated in the circulation of severe asthmatics. This may be a result of the chronic steroid therapy that is used to treat these patients, suggesting that steroids may be having an adverse effect in these patients by prolonging neutrophil survival and allowing them to release tissue damaging mediators. Alternative pharmacological therapy can, in theory, target various

stages in neutrophil recruitment, transmigration, and interaction to prevent tissue injury. Possible mechanisms of action include inhibiting the release or accumulation of proinflammatory mediators, altering neutrophils or endothelial cell activation, and attenuating adhesion molecule expression on neutrophils, endothelium and target cells such as myocytes or airway epithelial cells. *Martin et al* (254) for example, showed that neutrophils have a short half life in the asthmatic airways using BAL fluid levels (<8 hours) suggesting a high state of flux of neutrophils in the airway wall. Stratergies designed to block influx may be efficacious. *Cadwallader et al* (255) have recently shown that the enzyme phosphoinositide 3-kinase is important in controlling neutrophil migration, activation and survival, and the discovery of neutrophil specific isoforms makes selective therapeutic targeting a possibility.

Other potential new targets for treatment include antagonists of neutrophil chemoattractants including GM-CSF and IL-8, and blockade of neutrophil products such as neutrophil elastase. LTB4 antagonists may be particularly important as LTB4 has a central role in enhancing neutrophil survival in response to cytokines and corticosteroids. LTB4 antagonists may promote neutrophil apoptosis in the airway, leading to resolution and repair. The finding of 'non-eosinophilic' asthma in induced sputum samples may provide a case for the regular use of this technique in identifying asthma subtypes, but although relatively safe, sputum induction can produce respiratory symptoms in some patients. The finding of increased levels of urinary LTE4 and LTB4 in severe asthmatics, suggests that 5-lipoxygenase inhibitors, such as zileuton, may be efficacious in neutrophil predominant asthma, as it inhibits LTB4 along with the cysteinyl leukotrienes. This suggests that in future, targeted therapy against both eosinophils and neutrophils may be the best form of treatment in severe steroid-dependent asthmatics.

Current available therapy, in the form of long acting beta-2 agonists, has been shown to have some anti-neutrophil activity. Salmeterol given at a dose of 50μg twice daily, significantly reduced neutrophil numbers in bronchial biopsy samples, and the concentrations of MPO compared to PBO. Compared to FP, salmeterol also reduced neutrophil number and human neutrophil lipocalin level in BAL fluid (270). Further, *Maneechotesuwan et al* (271) showed that treatment with formoterol significantly reduced neutrophil numbers and sputum IL-8 levels compared to PBO. There was a significant correlation between reduction in sputum IL-8 levels and the number of neutrophils, indicating that formoterol may attenuate neutrophil airway inflammation by inhibiting IL-8 production.

Macrolides have also been shown to reduce neutrophilic inflammation. A study by *Simpson et al* (272) showed that a group of patients with severe refractory asthma treated with oral clarithromycin (500mg bd) for 8 weeks, had reduced sputum concentrations of IL-8 and neutrophil numbers, and showed improved quality of life scores compared with PBO. These reductions were most marked in those with refractory non-eosinophilic asthma. *Johnston et al* (273) studied the effect of Telithromycin in 278 asthmatics within 24 hours of an acute asthma exacerbation, versus PBO. Although a reduction in asthma symptoms was demonstrated, no effect of on PEF was demonstrated. In-vitro studies have shown that theophylline at therapeutic concentrations can restore the the reduced HDAC activity that is induced by oxidative stress, and can return steroid responsiveness to normal. Low dose theophylline can therefore lead to increased HDAC activity, thus reducing the inflammatory potential of effector cells in COPD and severe asthma (274). New classes of drug that activate HDACs might be identified in future.

In conclusion, asthma can no longer be considered a chronic eosinophilic airway disorder triggered by various allergens and stimuli. Mounting evidence suggests it is characterized by heterogenous airway inflammation, with neutrophils predominating in certain asthma phenotypes, and after certain trigger factors. The increase in neutrophil numbers is associated with evidence of neutrophil activation and mediator release, and correlates with asthma disease severity. Patients with severe asthma demonstrate different pathology on biopsy of their airways compared to milder asthmatics, and a number of molecular mechanisms have been put forward to explain the state of relative corticosteroid resistance in this group. Both biopsy studies and large observational studies point to the relative increase in airway neutrophil numbers as a possible explanation for the more severe asthma phenotype. Whether severe asthmatics represent a small number of patients mild disease who are unresponsive to corticosteroids, or whether it is a separate entity characterized predominantly by airway neutrophilia is unclear. It is also unclear whether the airway neutrophilia is a result of corticosteroid useage, or whether the persistent symptoms in severe corticosteroid dependent asthmatics are the result of ongoing neutrophilic inflammation which is unresponsive to corticosteroid therapy. Some evidence does suggest however, that sputum neutrophilia can be seen in mild steroid niave asthmatics.

This thesis shows that, in addition to the airways, neutrophils may also be activated in the peripheral circulation of patients with severe asthma. The use of corticosteroids is associated with unacceptable side effects in a number of corticosteroid-

dependent asthmatics, and may in fact lead to further airway and tissue damage through the promotion of neutrophil survival and mediator release as a result of reduced neutrophil apoptosis. The development of alternative forms of therapy to target either eosinophilic or neutrophilic inflammation, or perhaps both, seems an attractive way in treating this condition. Although several current therapies have been shown to have anti-neutrophil effect as outlined above, the use of these therapies is not the answer in more severe asthmatics as many patients are already on these treatments and demonstrate ongoing symptoms. A theoretical disadvantage of targeting neutrophils however is that a state of neutropenia may be induced with the risk of developing infections as a result. Anti-IgE therapy is an exciting new development in the field of asthma, and is associated with considerable clinical benefit and significantly less adverse effects than other immunosuppressant drugs that have been used in the treatment of severe asthmatics. However, it is likely that only a small proportion of severe asthmatics will benefit from this form of therapy as less than 50% of severe asthmatics are atopic, and in this atopic group, issues regarding body weight (increased through the use of oral corticosteroids), cost of treatment, and IgE levels above the recommended dosing range means that in practice, only between 10-15% of these patients are likely to benefit. New pharmaceutical developments are thus urgently required in the field of non-eosinophilic asthma, and this may provide a significant break through in the management of severe asthmatics.

The shortcomings of the experiments in this Thesis are presented in the discussions at the end of each chapter. A major problem was the lack of patient recruitment into various studies such as the IA04 and 11 extension substudy (chapter 6), which were due to commence shortly after the research period started. There was thus insufficient time to recruit patients into these trials and to become familiar with FACS analysis techniques for the 11 extension substudy. Unfortunately, it was not possible to get any help with the experiments at the time, and I had to attend a course on FACS analysis in Oxford to become familiar with FACS analysis techniques. The protocol for the fluticasone study (chapter 5) was difficult to recruit to, as it meant that patients had to be available for one and a half days per week for three consecutive weeks, which is demanding for patients. Recruiting subjects well in advance would help to increase numbers and the power of the studies. It would have been beneficial to consult a statistician from the outset. The lack of a non asthmatic control group taking prednisolone (chapter 3) meant that it is difficult to analyse fully the effect of prednisolone on cell surface activation markers. Trial designs could also have been improved to give more

meaningful results. For example, bronchial hyperreactivity could have been measured at the start of the fluticasone study, so that comparisons with later time points could have been carried out. In addition, the trial design for the IA04 study did not allow the routine measurement of serum IgE levels at each scheduled 3 monthly hospital visit. The control population in some of the studies were not well matched in terms of age, sex, and severity of disease and this may have had some effect on the results, and again highlights the importance of timely and careful patient selection. Most of the mild and severe patients who took prednisolone in chapter 3 were compliant with treatment as can be judged by the prednisolone and cortisol levels. However this may not necessarily have been the case for asthmatics who took prednisolone in the HDAC study (chapter 4), as not all prednisolone levels were available to ensure patient compliance with therapy. Finally, the IL-8 ELISA experiements in blood and sputum failed to produce results in some patients and thus repeating these experiments would be useful to demonstrate reproducibility and accuracy of results.

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Appendix 1- List of equipment/reagents

Spirometer: Dry Bellows vitalograph spirometer (Vitalograph Business Park, Maids Moreton, Buckinghamshire, MK18 TSW).

Methacholine: Stockport Pharmaceuticals, (Pharmacy department, Stepping Hill hospital, Poplar Grove, Stockport).

Allergens for skin prick testing: Soluprick SQ, (Alk abello Ltd. Hungerford, Berkshire RG17 OYT).

Sputum induction: Ultra-Neb 2000 Ultrasonic nebuliser (De Vilbiss Co, Heston, UK).

Nitric oxide chemiluminescence analyzer (ModelLR2000; Logan Research Ltd, Rochester, UK, ME2 4NP).

FACScan flow cytometer, with MacIntosh G4 FACStation and CellQuest software. ((Becton Dickinson, 21 Between Towns road, Oxford, Oxfordshire OX4 3LY, UK).

CaliBrite Beads (no.349502, unlabelled, FITC, PE, 25 tests, BectonDickinson, 21 Between Towns road, Oxford, Oxfordshire OX4 3LY, UK).

Solutions/reagents

Hank's balanced salt solution (HBSS, H6648, Sigma Aldridge, Irvine, UK)

Dithiothreitol (DTT) (Sigma chemicals, Fancy road, Poole, Dorset, UK).

RPMI 1640 (no.21875-091, 10x500ml, Gibco, Invitrogen, UK)

Ficoll-Hypaque (no.17-14440-03, Amersham Biosciences, UK)

Dulbecco's Phosphate-buffered saline (no.14190-185, 20x100ml, Gibco, Invitrogen, UK)

3% Dextran T-500 (Pharmacia Biotech, Uppsala, Sweden)

Bovine serum albumin (A2153-50g, Sigma Aldridge, Fancy road, Poole, UK)

Sodium azide (no.58032-100g, Sigma Aldridge, Poole, UK)

Erythrolyse (BUF04A, 20 ml of 10x concentration, Serotec Ltd, Oxford, UK)

Paraformaldehyde (no.16005-1KG-R, Sigma Chemical Co, Poole, UK)

DMSO, dimethyl sulfoxide (D1435, 500ml, Sigma Aldridge, Poole, UK)

PAF, platelet activating factor, (p7568, 1mg, Sigma Aldridge, Poole, UK)

PMA, phorbol myristate acetate, (P8139, 5mg, Sigma Aldridge, Poole, UK)

HEPES solution (H4034, Sigma Aldridge, Poole, UK)

fMLP, N-formyl-methyl-leucyl-phenylalanine (F3506, 10mg, Sigma Aldridge, Poole, UK)

Dexamethsone (D8893, 1mg, Sigma Aldrich, Poole, UK)

Lipopolysaccharide, LPS (L7770, 1mg, Sigma Aldrich, Poole, UK)

IL-1 beta (no. 19401, 5µg/vial, Sigma Aldrich, Poole, UK)

GMCSF (RDI-303, 1mg, R&D systems, Oxon, UK)

Cytopinslides (Shandon, Runcorn, Cheshire, UK)

Round bottom glass tubes (no.352085, 1000/case, BD Falcon, Marathon Ltd, UK)

Monoclonal antibodies

Rabbit anti-human FcεR1 antibody (no.06-725, Upstate biotechnology, Lake Placid, USA)

FITC conjugated swine anti-rabbit secondary antibody (F020502, 2mls, Dako, Via Real Carpinteria, Denmark)

CD123 PE (no.33335B, mouse IgG1, κ, 0.2mg, Pharmingen International, Europe, Heidelberg, Germany).

RPE-Cy5 conjugated HLA-DR (MCA1721C, mouse igG2b, 100 tests/0.5ml, Serotec Ltd, 22 Bankside Station Approach, Kidlington, Oxford, OX5 1JE, UK)

PE conjugated CD14 PE (MCA596PE, IgG2a, 100 tests/0.1ml, Serotec Ltd, Oxford, UK)

FITC conjugated CD95 (MCA 1539F, IgG, 1ml/0.1 mg, (Serotec Ltd, Oxford, UK)

FITC conjugated CD23 (MCA 1316F, IgG, 0.1mg/1ml, Serotec Ltd, Oxford, UK).

PE conjugated CD9 (MCA469PE, IgG2b, 100 tests/ml, Serotec Ltd, Oxford, UK).

PE conjugated CD14 (MCA596PE, IgG2a, 100 tests/ml, Serotec Ltd, Oxford, UK).

PE conjugated CD4 (MCA1267PE, IgG1, 100 tests/ml -Serotec Ltd, Oxford, UK).

FITC conjugated rat anti-human CCR3 (FAB 155F, 50µg/ml)- R&D systems Europe, Oxon, UK.

PE conjugated CD 16 (MCA1193PE, IgG1, 100 tests/ml, Serotec Ltd, Oxford, UK).

FITC conjugated CD 62L (MCA1076F, IgG2b, 100 tests/ml, Serotec Ltd, Oxford, UK).

FITC conjugated CD 11b (MCA551F, IgG1, 100 tests/ml, Serotec Ltd, Oxford, UK).

FITC conjugated CD 18 (MCA5, IgG2b, 100 tests/ml, Serotec Ltd, Oxford, UK).

FITC conjugated CD 35 (MCA554F, IgG1, 100 tests/ml, Serotec Ltd, Oxford, UK).

RPE-Cy5 conjugated igG2b negative control, (MCA691C, 0.5ml/100 tests, Serotec Ltd, Oxford, UK).

PE conjugated IgG1 negative control (MCA928PE, IgG1, 100 tests/ml, Serotec Ltd, Oxford, UK).

FITC conjugated IgG2b negative control, (MCA691F, IgG2b, 100 tests/ml, Serotec Ltd, Oxford, UK).

PE conjugated IgG2a negative control (MCA929PE, IgG2a, 100 tests/ml, Serotec Ltd, Oxford, UK).

FITC conjugated IgG1 negative control, (MCA928F, IgG1, 100 tests/ml, Serotec Ltd, Oxford, UK).

FITC conjugated IgG2a negative control, (IC006F, IgG2a, 25μ/ml, R&D systems Europe, Barton Lane, Abingdon, Oxon, OX14 3YS, UK).

N-benzyloxycarbonyl-Ala-Ala)$_2$-rhodamine 110 (R-6504, 5mg, Molecular probes, Upsaala, Sweden)

Dihydrorhodamine 123 (D-632, 10mg, Molecular Probes, Upsaala, Sweden),

ELISA

Human IL-8 Duoset ELISA (no. DY208, R&D systems, Oxon, UK).

MPO ELISA kit (no.475918, Calbiochem, San Diego, USA).

Human IL-5 Duoset ELISA (no. D5000, R&D systems, Oxon, UK).

HDAC

HDAC fluorometric cellular activity assay kit, (AK-503, 1 kit, BIOMOL International Inc., Plymouth Meeting, PA, USA).

Graphpad Prism

Version 4.0, Graphpad software, Inc. La Jolla, CA 92037 USA.

Appendix 2.1 Characteristics of mild asthmatics taking prednisolone 30mg/day for one week in HDAC study (Chapter 4). n=6. N/A= not available

Patient	Age (years)	FEV1 (L) Visit 1	FEV1 (L) Visit 2	NO (ppb) Visit 1	NO (ppb) Visit 2	PC20 (mg/ml)	Treatment	Prednisolone Level (nmol/L)	Cortisol Level (nmol/L)	Eosinophil Count (x109/l)	Neutrophil count (x109/l)
GM	27	3.57 (104%)	3.51 (99%)	8.0 7.2	6.9 6.2	8.49	Salbutamol prn	N/A	N/A	Visit1-0.2 Visit 2-0.1	Visit1-6.2 Visit 2-7.0
SG	41	3.05 (99%)	3.14 (102%)	114 104.4	52.8 50.7	2.92	Salbutamol prn	N/A	N/A	Visit1-0.2 Visit 2-0.1	Visit1-5.3 Visit 2-6.9
GJ	29	2.78 (91%)	2.65 (86.3%)	5.30 7.10	15.9 14.3	0.84	Salbutamol BDP-400µg/day	1582	<20	Visit1-0.1 Visit 2-0.0	Visit1-5.1 Visit 2-11.7
DP	49	3.5 (94%)	3.4 (92%)	100 104.6	41.5 46.2	1.0	Salbutamol prn	1041	<20	Visit1-0.3 Visit 2-0.1	Visit1-4.9 Visit 2-8.8
JP	52	3.35 (96%)	3.25 (85%)	22.1 16.0	N/A	0.03	Salbutamol prn	N/A	N/A	Visit1-0.4 Visit 2-0.2	Visit1-4.0 Visit 2-N/A
BP	25	2.56 (78%)	2.45 (74%)	4.1 3.7	2.4 2.6	6.97	Salbutamol prn	2143	124	Visit1-0.1 Visit 2-0.1	Visit1-3.9 Visit 2-5.6

Appendix 2.2 Characteristics of severe asthmatics taking additional prednisolone 30mg/day for one week in HDAC study (Chapter 4). n=6.

	Age (years)	FEV1 (L) Visit 1	FEV1 (L) Visit 2	NO (ppb) Visit 1	NO (ppb) Visit 2	PC20 (mg/ml)	Treatment	Prednisolone Level (nmol/L)	Cortisol Level (nmol/L)	Eosinophil Count (x109/l)	Neutrophil count (x109/l)
JK	52	0.9 (36%)	0.87 (34%)	19.9 18.9	19.1 22.7	Not Done (N/D)	Serevent 50 µg bd Flixotide 250 µg bd Prednisolone 5mg/day	N/A	N/A	Visit1-0. Visit 2-0.	Visit1- N/A Visit 2-N/A
DA	48	0.9 (32%)	1.5 (35%)	10.5 14.6	14.1 11.7	N/D	Seretide 500 µg bd	N/A	N/A	Visit1-0.4 Visit 2-0.1	Visit1-1.6 Visit 2-3.2
CH	47	0.9 (33%)	2.7 (41.1%)	41.1 38.8	28.4 35.8	N/D	Flixotide 500 µg bd Serevent 50 µg bd	N/A	N/A	Visit1-. N/A Visit 2- N/A .	Visit1- N/A Visit 2-N/A
RS	52	0.95 (41%)	1.15 (51%)	N/D	N/D	N/D	Flixotide 1mg bd Formoterol 24 µg bd Aminophylline 450mg bd	793	<20	Visit1-1.2 Visit 2-0.1	Visit1-5.2 Visit 2-7.5
TJ	32	1.4 (51%)	1.85 (66%)	N/D	N/D	N/D	Seretide 1000 µg bd Uniphylline 400mg bd Salbutamol & atrovent nebs Prednisolone 20mg/day	2147	<20	Visit1-0.5 Visit 2-0.2	Visit1-1.0 Visit 2-1.8
CA	59	2.46 (74.3%)	2.37 (71.6%)	14.3 14.9	3.1 4.0	0.34mg/ml	Seretide 500 µg bd Uniphylline 400mg bd	814	26	Visit1-0.3 Visit 2-0.1	Visit1-2.7 Visit 2-4.7

All patients used salbutamol either regularly or as required. N/A=not available
N/D-PC_{20} not done in severe asthmatics

Appendix 3-Data for chapter 5

Data for Fig. 5.1 (page 152). FEV1 (L) in mild patients taking high dose FP

	0	1	2	3	4	6	8	24hours
bb	2.30	2.53	2.46	2.74	2.76	2.91	2.87	2.76
zc	2.94	2.85	2.92	2.98	2.93	2.93	2.83	2.83
hl	3.65	3.50	3.61	3.56	3.72	3.75	3.62	3.78
vl	2.82	2.80	2.68	2.73	2.75	2.80	2.96	2.94
jn	2.94	2.85	2.80	2.96	3.00	3.15	3.20	3.05
cdb	2.43	2.35	2.50	2.34	2.34	2.42	2.67	2.60
ss	3.13	3.12	2.95	2.95	2.85	2.68	3.20	3.28
lmcr	2.90	2.66	2.76	2.67	2.83	2.93	3.11	3.06
jp	3.35	3.25	3.36	3.32	3.42	3.22	3.36	3.22
bp	2.75	2.63	2.58	2.68	2.64	2.73	2.80	2.75
gj	2.85	2.76	2.70	2.73	2.82	2.72	2.76	2.71
sg	3.08	3.00	3.00	3.08	3.02	3.00	3.05	3.00

FEV1 (L) in mild patients taking low dose FP

	0	1	2	3	4	6	8	24hours
bb	2.72	2.67	2.82	2.90	2.99	2.94	2.91	2.50
zc	2.68	2.78	2.65	2.76	2.67	2.67	2.81	2.62
hl	3.71	3.55	3.65	3.47	3.75	3.65	3.90	3.93
vl	2.76	2.79	2.77	2.80	2.78	2.87	2.92	2.85
jn	2.92	2.85	2.77	2.99	2.76	2.77	2.84	3.08
cdb	2.32	2.21	2.37	2.23	2.24	2.35	2.45	2.51
ss	3.35	3.07	3.07	3.05	3.27	2.82	3.07	3.25
lmcr	2.85	2.93	2.98	2.95	3.03	2.83	3.10	3.10
jp	3.15	3.34	3.25	3.35	3.15	3.05	3.55	3.58
bp	2.77	2.57	2.52	2.35	2.30	2.37	2.51	2.68
gj	2.78	2.75	2.74	2.75	2.80	2.52	2.56	2.72
sg	3.04	3.00	3.03	3.00	2.98	2.92	3.15	3.10

FEV1 (L) in mild patients taking PBO

	0	1	2	3	4	6	8	24hours
bb	2.43	2.74	2.70	3.12	3.02	3.01	2.92	2.63
zc	2.86	2.79	2.46	2.38	2.95	2.90	2.80	2.98
hl	3.85	3.66	3.60	3.45	3.71	3.56	3.30	3.72
vl	2.62	2.72	2.82	2.66	2.85	2.83	2.95	2.78
jn	2.67	2.78	2.95	2.66	2.58	2.57	2.97	2.91
cdb	2.49	2.41	2.38	2.56	2.29	2.31	2.00	2.43
ss	3.25	3.10	2.93	2.93	2.43	2.61	2.89	2.99
lmcr	3.08	2.91	3.01	2.95	2.88	3.00	3.12	3.05
jp	3.55	3.30	3.23	3.45	3.43	3.35	3.68	3.48
bp	2.70	2.58	2.70	2.61	2.55	2.77	2.95	2.67
gj	2.78	2.76	2.70	2.64	2.75	2.75	2.25	2.65
sg	2.95	2.96	2.95	2.97	3.00	3.02	2.92	3.01

Data for Figure 5. 2 (page 153). FVC (L) in mild patients-high dose FP

	0	24 hours
bb	3.83	3.83
zc	3.39	3.38
hl	4.69	4.90
vl	3.50	3.57
jn	4.00	4.16
cdb	-	2.63
ss	4.71	4.84
lmcr	3.25	3.60
jp	4.40	4.39
bp	3.17	3.05
gj	3.00	2.90
sg	3.80	3.80

FVC (L) in mild patients taking low dose FP

	0	24 hours
bb	3.83	3.83
zc	3.39	3.38
hl	4.69	4.90
vl	3.50	3.57
jn	4.00	4.16
cdb	-	2.63
ss	4.71	4.84
lmcr	3.25	3.60
jp	4.40	4.39
bp	3.17	3.05
gj	3.00	2.90
sg	3.80	3.80

FVC (L) in mild patients taking PBO

	0	24 hours
bb	3.67	3.86
zc	3.55	3.49
hl	4.78	4.64
vl	3.46	3.55
jn	3.95	3.86
cdb	2.55	2.46
ss	4.82	4.68
lmcr	3.60	3.70
jp	4.59	4.60
bp	3.10	3.10
gj	2.95	2.80
sg	3.65	3.80

Data for Figure 5.3 (page 154).FEV1 (L) in moderate patients taking high dose FP

	0	1	2	3	4	6	8	24 hours
rf	3.35	3.20	3.25	3.22	3.41	3.40	3.63	3.42
gb	2.90	2.73	2.70	2.65	2.71	2.62	2.80	2.76
rp	1.65	1.60	1.85	1.70	1.74	1.81	1.77	1.55
ep	3.38	3.56	3.65	3.50	3.75	3.52	3.72	3.55
gc	4.57	4.50	4.52	4.45	4.48	4.33	4.45	4.44
jj	1.38	1.48	1.38	1.50	1.47	1.45	1.55	1.47
LM	3.53	3.35	3.23	3.20	3.40	3.39	3.45	3.75
nd	3.65	3.65	3.81	3.95	3.85	3.90	4.30	4.12
ca	2.21	2.31	2.19	2.45	2.26	2.20	2.65	2.30

FEV1 (L) in moderate patients taking low dose FP

patient	0	1	2	3	4	6	8	24 hours
rf	3.15	3.10	3.36	3.35	3.20	3.20	3.35	3.40
gb	3.73	2.70	2.80	2.79	2.51	2.00	2.64	2.87
rp	1.41	1.73	1.63	1.62	1.60	1.56	1.80	1.56
ep	3.30	3.62	3.49	3.55	3.50	3.42	3.70	3.45
gc	4.38	4.56	4.51	4.55	4.55	4.45	4.55	4.60
jj	1.35	1.38	1.60	1.71	1.54	1.35	1.63	1.32
LM	3.55	3.25	3.43	3.39	3.33	3.35	3.56	3.41
nd	3.70	3.90	3.85	4.00	3.93	3.85	4.13	3.72
ca	2.10	1.88	1.83	1.66	1.96	2.08	2.60	2.42

FEV1 (L) in moderate patients taking PBO

patient	0	1	2	3	4	6	8	24 hours
rf	3.26	3.40	3.30	3.37	3.50	3.38	3.40	3.33
gb	2.85	2.65	2.72	2.85	2.61	2.74	2.75	2.75
rp	1.46	1.69	1.54	1.53	1.50	1.40	1.75	1.36
ep	3.65	3.65	3.54	3.73	3.70	3.75	3.65	3.60
gc	4.51	4.40	4.35	4.45	4.37	4.45	4.40	4.50
jj	1.36	1.41	1.75	1.77	1.75	1.55	1.68	1.41
LM	3.48	3.60	3.48	3.55	3.35	3.41	3.47	3.68
nd	4.00	3.85	3.88	3.99	3.91	3.67	3.95	3.86
ca	3.46	3.48	2.28	2.35	2.51	2.65	2.37	2.29

Data for Figure 5. 4 (page 155). FVC (L) in moderate patients taking high dose FP

	0	24 hours
rf	4.70	4.80
gb	3.45	3.15
rp	2.20	2.10
ep	4.85	4.75
gc	5.65	5.67
jj	2.55	2.62
LM	4.26	4.55
nd	5.55	5.93
ca	3.23	3.33

FVC (L) in moderate patients taking low dose FP

	0	24 hours
rf	4.62	4.87
gb	3.00	3.40
rp	1.99	2.13
ep	4.68	4.80
gc	5.54	5.72
jj	2.46	2.45
LM	4.25	4.20
nd	5.60	5.63
ca	3.16	3.59

FVC (L) in moderate patients taking PBO

	0	24 hours
rf	4.75	4.80
gb	3.30	3.25
rp	2.06	2.01
ep	4.85	4.75
gc	5.62	5.56
jj	2.58	2.58
LM	4.20	-
nd	5.74	5.73
ca	3.61	3.29

Data for fig 5.5. PC_{20} (mg/ml) in mild patients, taking high dose FP.

Patient	BASE	6 hours	24 hours
bb	0.53	2.53	0.50
zc	2.31	2.50	12.67
hl	0.37	1.80	1.20
vl	0.55	1.20	0.94
jn	-	2.90	1.79
cdb	0.67	13.12	21.09
ss	1.08	-	-
lmcr	2.19	2.30	0.24
jp	0.70	13.60	4.16
bp	2.72	4.80	4.61
gj	0.84	8.00	4.76
sg	2.52	1.84	0.61

PC_{20} in mild patients, taking low dose FP.

Patient	BASE	6 hours	24 hours
bb	0.53	1.940	1.17
zc	2.31	7.900	1.28
hl	0.40	1.290	0.73
vl	0.60	3.300	1.71
jn	-	9.100	1.37
cdb	0.70	4.700	11.51
ss	1.08	0.810	2.10
lmcr	2.19	1.980	0.68
jp	0.70	2.950	2.56
bp	2.80	4.980	1.61
gj	0.84	0.250	1.63
sg	2.52	0.810	1.36

PC_{20} in mild patients, taking PBO.

Patient	BASE	6 hours	24 hours
bb	0.53	2.24	0.53
zc	2.31	3.07	7.85
hl	0.37	0.54	0.59
vl	0.55	0.34	0.40
jn	-	3.64	4.98
cdb	0.67	16.00	8.00
ss	1.08	0.50	2.79
lmcr	2.19	2.00	1.47
jp	0.70	0.98	0.72
bp	2.72	7.13	5.79
gj	0.84	37.70	36.49
sg	2.52	0.25	0.686

Data for fig 5.6. (page 157) PC_{20} (mg/ml) in moderate patients, taking high dose FP.

Patient	BASE	6 hours	24 hours
rf	2.140	2.350	1.660
gb	2.790	4.000	24.800
ep	2.350	0.870	9.290
jj	1.150	2.030	2.750
LM	0.160	13.100	39.000
nd	0.730	0.230	0.850

PC_{20} in moderate patients, taking low dose FP.

Patient	BASE	6 hours	24 hours
rf	2.1400	0.670	0.710
gb	2.7900	52.100	15.500
ep	2.3500	7.600	5.740
gc	1.0900	-	0.710
LM	0.1600	3.480	0.070
nd	0.7300	0.500	2.320

PC_{20} in moderate patients, taking PBO.

Patient	BASE	6 hours	24 hours
rf	2.14000	0.830	0.830
gb	2.79000	-	2.220
ep	2.35000	9.590	15.270
gc	1.09000	1.140	2.230
LM	0.16000	46.100	1.590
nd	0.73000	0.500	0.500

Data for fig. 5.7 (page 160). NO levels (ppb) in mild patients taking high dose FP.

	0	1	2	3	4	6	8	24 hours
bb	32.45	33.95	44.35	40.50	28.25	33.70	36.90	43.70
zc	26.75	26.45	27.30	22.75	27.65	23.80	21.55	19.75
hl	43.75	45.85	48.45	41.10	48.15	45.50	39.90	34.60
vl	28.20	30.20	25.65	22.95	16.15	18.40	25.10	26.40
jn	59.65	70.05	70.30	49.90	58.05	59.20	53.30	38.40
cdb	11.10	10.75	10.60	8.60	8.800	8.50	8.50	7.65
ss	51.00	33.85	36.70	29.30	27.65	27.35	24.00	32.25
lmcr	17.75	17.05	16.85	19.40	20.45	21.60	27.35	16.20
jp	26.30	33.10	33.00	29.60	29.90	28.80	21.80	20.90
bp	2.55	7.75	7.45	6.00	6.00	7.90	5.80	4.50
gj	6.00	7.95	8.05	8.20	8.20	9.50	7.20	9.15
sg	21.55	23.55	21.35	23.15	18.80	15.95	20.70	14.70

NO levels in mild patients taking low dose inhaled FP.

	0	1	2	3	4	6	8	24 hours
bb	24.70	24.20	26.10	24.30	27.550	22.30	21.250	24.10
zc	17.40	22.35	0.00	18.75	17.300	18.35	17.950	17.65
hl	45.90	36.60	44.80	44.00	41.800	48.45	42.300	39.95
vl	21.60	19.10	18.70	21.70	16.500	13.95	16.350	16.80
jn	7.85	7.35	7.75	9.15	6.900	8.45	8.500	11.80
cdb	7.70	5.80	6.20	7.10	6.850	7.75	7.600	6.60
ss	33.30	22.00	20.30	20.85	17.900	16.95	17.650	19.05
lmcr	21.60	19.50	21.60	23.85	19.650	21.65	22.750	20.80
jp	23.30	28.00	23.80	24.35	23.100	31.15	21.750	23.75
bp	3.20	8.10	8.95	11.75	9.450	11.10	6.600	4.85
gj	8.10	6.50	6.60	6.25	5.450	6.65	4.250	6.60
sg	63.55	62.30	74.80	54.00	57.450	60.70	66.700	44.70

NO levels in mild patients taking PBO.

	0	1	2	3	4	6	8	24 hours
bb	67.20	56.350	51.80	40.0	60.050	51.60	39.60	48.30
zc	25.05	24.550	22.90	23.7	24.800	19.05	19.60	24.55
hl	42.65	44.700	42.70	37.55	33.750	41.40	29.15	42.60
vl	19.95	18.700	16.75	18.7	16.150	21.95	16.85	19.00
jn	30.75	29.650	35.70	33.15	25.850	31.50	29.45	27.65
cdb	29.80	8.500	8.90	9.75	8.800	7.90	6.50	11.85
ss	31.10	35.550	32.50	34.2	30.500	32.85	31.35	59.20
lmcr	23.40	25.600	22.95	24.65	28.550	25.75	23.60	22.65
jp	18.75	18.750	20.60	18.8	19.800	19.60	15.80	12.80
bp	7.95	11.450	8.20	9.4	7.550	9.85	5.70	6.40
gj	4.30	7.150	5.90	7.55	6.200	10.35	5.55	6.95
sg	51.75	49.200	57.50	46.15	49.700	46.40	35.90	63.50

Data for fig. 5.8 (page 161). NO (ppb) levels in moderate patients taking high dose FP.

	0	1	2	3	4	6	8	24 hours
rf	72.150	75.30	69.80	66.30	75.350	62.30	57.70	93.50
gb	15.400	13.35	15.30	14.00	14.500	16.55	12.65	11.50
rp	8.100	13.50	14.40	11.00	8.500	10.80	10.45	9.20
ep	28.500	29.80	32.60	28.20	30.700	27.90	24.60	16.80
gc	69.500	92.10	88.70	91.50	90.400	101.20	80.30	130.10
jj	11.650	18.05	19.05	6.00	6.050	5.55	6.40	6.25
LM	13.000	11.05	12.05	12.85	8.900	10.25	8.75	15.30
nd	44.950	40.60	38.85	37.85	43.600	42.10	41.10	30.30
ca	16.700	13.00	11.10	7.10	8.700	8.60	8.40	14.10

NO (ppb) in moderate patients taking low dose FP.

	0	1	2	3	4	6	8	24 hours
rf	177.40	96.60	98.50	87.20	83.70	82.20	80.50	114.10
gb	5.30	8.10	10.10	12.35	12.20	12.20	7.55	5.45
rp	13.95	14.45	15.20	11.90	10.25	11.10	12.90	11.50
ep	14.90	16.30	18.00	20.50	16.70	15.70	13.40	16.90
gc	51.10	55.40	60.10	64.40	65.90	55.60	69.60	57.30
jj	6.15	5.20	5.10	7.65	6.55	4.10	5.50	6.45
LM	12.30	15.80	13.00	11.35	9.75	11.50	11.00	11.75
nd	32.80	30.70	36.95	31.00	42.70	39.20	30.20	31.60
ca	7.60	8.70	7.70	8.00	6.60	9.60	10.70	9.10

NO levels (ppb) in moderate patients taking PBO.

	0	1	2	3	4	6	8	24 hours
rf	116.950	115.90	131.0	127.90	175.40	137.50	116.0	178.60
gb	15.000	13.60	12.15	13.40	12.20	14.70	10.1	13.30
rp	10.750	12.90	10.7	11.10	9.50	9.70	8.4	10.35
ep	23.700	20.60	19.2	19.20	19.50	19.40	18.6	14.20
gc	46.400	46.20	45.7	52.50	56.10	51.90	45.1	41.50
jj	9.300	8.80	8.75	6.25	7.70	7.30	6.35	8.45
LM	11.500	18.85	14.35	16.25	11.00	10.80	8.4	11.95
nd	53.500	57.60	58.4	57.70	48.90	60.80	53.9	81.00
ca	13.10	8.13	7.90	12.1	12.9	8.7	14.2	17.5

Data for fig. 5.9 (page 162). Eosinophil counts (x10^9/L) in mild asthmatics taking high dose FP.

	0	1	2	3	4	6	8	24 hours
bb	0.50	0.50	0.50	0.50	0.40	0.40	0.40	0.30
zc	0.50	0.40	0.40	0.40	0.40	0.30	0.40	0.30
hl	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.00
vl	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.30
jn	0.30	0.40	0.40	0.30	0.30	0.30	0.30	0.30
cdb	0.10	0.20	0.20	0.10	0.10	0.20	0.20	0.20
ss	0.00	0.00	0.20	0.10	0.20	0.20	0.20	0.20
lmcr	0.30	0.30	0.30	0.30	0.30	0.30	0.40	0.30
jp	0.40	0.40	0.40	0.40	0.40	0.50	0.40	0.40
bp	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
gj	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
sg	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.00

Eosinophil counts (x10^9/L) in mild asthmatics taking low dose FP.

	0	1	2	3	4	6	8	24 hours
bb	0.50	0.40	0.40	0.40	0.30	0.50	0.40	0.60
zc	0.30	0.30	0.30	0.30	0.30	0.30	0.40	0.30
hl	0.20	0.10	0.10	0.10	0.10	0.20	0.20	0.10
vl	0.30	0.20	0.20	0.20	0.20	0.20	0.20	0.20
jn	0.20	0.30	0.30	0.30	0.20	0.30	0.30	0.30
cdb	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.00
ss	0.30	0.30	0.20	0.20	0.20	0.30	0.30	0.20
lmcr	0.30	0.10	0.20	0.20	0.20	0.10	0.10	0.10
jp	0.40	0.50	0.50	0.50	0.30	0.50	0.50	0.40
bp	0.10	0.10	0.00	0.10	0.10	0.10	0.00	0.10
gj	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
sg	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10

Eosinophil counts (x10^9/L) in mild asthmatics taking PBO.

	0	1	2	3	4	6	8	24 hours
bb	0.30	0.20	0.20	0.20	0.10	0.10	0.10	0.40
zc	0.40	0.30	0.30	0.20	0.30	0.30	0.30	0.30
hl	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.00
vl	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.10
jn	0.30	0.30	0.30	0.30	0.40	0.40	0.30	0.30
cdb	0.20	0.10	0.20	0.10	0.00	0.20	0.20	0.10
ss	0.30	0.20	0.20	0.30	0.20	0.20	0.30	0.30
lmcr	0.30	0.30	0.20	0.30	0.30	0.30	0.30	0.30
jp	0.40	0.40	0.40	0.40	0.40	0.50	0.50	0.40
bp	0.10	0.10	0.10	0.10	0.10	0.00	0.00	0.10
gj	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
sg	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10

Data for fig. 5.10 (page 163). Eosinophil counts (x10^9/L) in moderate asthmatics taking high dose FP.

	0	1	2	3	4	6	8	24 hours
rf	1.10	1.10	1.0	1.0	1.0	1.0	1.0	0.90
gb	0.20	0.20	0.2	0.1	0.2	0.2	0.2	0.20
rp	0.20	0.30	0.2	0.2	0.2	0.2	0.2	0.20
ep	0.30	0.20	0.2	0.2	0.1	0.1	0.1	0.30
gc	0.70	0.70	0.7	0.8	0.8	0.9	0.8	0.80
jj	0.10	0.10	0.1	0.1	0.1	0.1	0.1	0.10
LM	0.20	0.30	0.2	0.2	0.1	0.2	0.2	0.30
nd	0.30	0.20	0.1	0.2	0.2	0.2	0.2	0.30
ca	0.10	0.2	0.3	0.2	0.4	0.2	0.3	0.10

Eosinophil counts (x10^9/L) in moderate asthmatics taking low dose FP.

	0	1	2	3	4	6	8	24 hours
rf	0.800	0.800	0.80	0.80	0.80	0.80	0.80	0.80
gb	0.100	0.200	0.20	0.20	0.20	0.10	0.10	0.10
rp	0.300	0.300	0.30	0.30	0.30	0.30	0.40	0.20
ep	0.200	0.200	0.10	0.10	0.10	0.10	0.20	0.20
gc	0.600	0.500	0.60	0.70	0.60	0.70	0.80	0.70
jj	0.100	0.100	0.10	0.10	0.10	0.10	0.10	0.10
LM	0.300	0.300	0.30	0.30	0.30	0.30	0.40	0.30
nd	0.200	0.200	0.20	0.10	0.20	0.20	0.20	0.30

Eosinophil counts (x10^9/L) in moderate asthmatics taking PBO.

	0	1	2	3	4	6	8	24 hours
rf	0.90	0.90	0.80	1.0	1.0	1.10	1.10	1.10
gb	0.20	0.10	0.10	0.2	0.2	0.20	0.20	0.20
rp	0.40	0.40	0.30	0.3	0.3	0.30	0.40	0.30
ep	0.20	0.20	0.20	0.2	0.2	0.20	0.20	0.20
gc	0.60	0.60	0.70	0.7	0.7	0.70	0.70	0.60
jj	0.20	0.20	0.10	0.1	0.1	0.10	0.10	0.20
LM	0.20	0.30	0.30	0.2	0.3	0.40	0.40	0.20
nd	0.00	0.00	0.00	0.0	0.0	0.00	0.00	0.00
ca	0.20	0.20	0.20	0.1	0.2	0.20	0.30	0.30

Data for fig. 5.12 (page 165). Neutrophil counts (x10^9/L) in moderate asthmatics taking high dose FP.

	0	1	2	3	4	6	8	24 hours
rf	4.10	4.10	4.90	4.80	5.10	6.10	6.50	3.80
gb	4.30	4.10	4.40	4.20	5.00	4.20	4.40	4.20
rp	3.70	4.20	3.70	3.70	3.80	4.30	4.50	4.20
ep	5.40	5.60	7.10	7.10	6.10	7.70	7.30	4.80
gc	4.60	5.70	5.90	6.40	6.00	6.40	6.30	4.40
jj	3.20	3.90	3.70	3.80	3.70	3.50	3.90	3.00
LM	3.40	4.10	3.70	3.70	3.30	3.30	4.10	4.20
nd	2.80	3.10	2.70	3.10	3.10	3.40	2.80	3.60
ca	7.20	7.40	7.20	7.00	6.00	4.80	4.10	2.70

Neutrophil counts (x10^9/L) in moderate asthmatics taking low dose FP.

	0	1	2	3	4	6	8	24 hours
rf	5.0	5.70	5.0	5.20	6.0	6.50	6.30	4.10
gb	4.1	5.20	5.2	5.20	5.3	6.30	5.70	4.50
rp	3.5	2.90	3.3	3.50	3.7	3.80	3.90	3.30
ep	2.4	2.70	3.0	3.30	3.8	4.70	4.80	2.50
gc	4.6	4.60	5.7	5.90	5.4	5.80	5.20	4.70
jj	3.0	3.50	3.5	3.60	3.5	3.60	3.30	3.30
LM	3.6	3.30	3.7	3.30	3.3	4.10	4.20	3.50
nd	2.7	3.20	3.8	3.20	3.2	3.10	2.70	2.50
ca	2.5	2.70	2.3	2.30	0.0	0.00	0.00	2.30

Neutrophil counts (x10^9/L) in moderate asthmatics taking PBO.

	0	1	2	3	4	6	8	24 hours
rf	4.20	4.80	4.60	4.5	4.60	5.30	7.10	4.3
gb	4.40	4.40	4.10	5.0	5.50	5.50	5.70	4.0
rp	3.60	3.40	3.30	3.1	3.30	4.30	4.40	3.0
ep	3.20	3.70	4.40	4.7	4.70	5.30	4.80	4.8
gc	5.60	4.80	5.60	4.9	5.30	5.60	5.60	4.1
jj	2.30	3.60	2.10	1.9	1.90	3.80	2.00	2.9
LM	3.50	3.90	3.30	4.0	3.70	3.90	3.90	3.4
nd	2.90	3.10	3.30	3.0	3.00	3.00	2.80	2.5
ca	2.90	0.00	3.10	2.6	2.90	2.50	2.90	2.9

Data for Fig 15.3 (page 166). Serum IL-8 levels (pg/ml) in mild asthmatics taking high dose FP.

	b+	**b-**	**1+**	**1-**	**4+**	**4-**	**24+**	**24-hours**
bb	69.3	46.1	32.6	52.8	112.8	35.6	221.1.0	179.8
zc	24.5	27.3	35.4	26.4	18.8	34.9	187.6	177.6
hl	132.6	210.8	124.5	153.0	92.3	135.4	153.0	190.9
vl	0.0	0.0	1.8	0.0	103.8	18.5	0.0	0.0
jn	145.4	161.6	210.0	170.1	70.4	69.6	271.4	211.9
cdb	24.1	18.2	6.3	20.2	31.2	14.3	132.5	189.2
lmcr	49.2	25.2	34.9	41.1	18.6	21.7	11.3	31.7
bp	145.4	161.6	210.0	170.1	70.4	69.6	271.4	211.9
gj	196.9	542.9	249.6	140.3	151.3	124.0	534.1	620.6
sg	160.8	236.7	202.6	223.8	0.0	0.0	155.1	123.7

Serum IL-8 levels (pg/ml) in mild asthmatics taking low dose FP.

	b+	**b-**	**1+**	**1-**	**4+**	**4-**	**24+**	**24-hours**
bb	0.0	0.0	97.0	88.8	153.3	43.1	55.8	43.8
zc	55.8	77.1	56.7	112.7	31.1	35.8	0.0	0.0
hl	116.9	119.8	116.5	135.9	195.2	152.0	146.8	111.4
vl	0.0	0.0	28.1	7.1	295.0	14.6	69.4	67.7
jn	283.2	281.7	104.7	172.7	69.3	34.2	185.3	205.2
cdb	421.6	426.0	394.6	442.8	411.7	342.9	0.0	0.0
lmcr	110.2	113.8	34.7	26.8	32.7	35.4	0.0	0.0
gj	175.6	179.0	159.3	165.0	152.1	152.5	446.5	380.9
sg	122.5	66.7	254.2	130.5	201.8	210.1	253.4	118.7

Serum IL-8 levels (pg/ml) in mild asthmatics taking PBO.

	b+	**b-**	**1+**	**1-**	**4+**	**4-**	**24+**	**24-hours**
bb	18.3	63.3	0.0	0.0	44.6	66.3	28.8	1.1
hl	190.0	238.0	220.0	178.8	184.0	168.3	239.5	293.5
vl	54.3	33.9	65.2	42.0	382.0	52.9	107.0	93.2
jn	212.5	243.7	179.5	215.2	103.2	73.3	133.0	217.0
cdb	64.0	5.0	3.5	58.7	15.5	6.7	40.8	30.7
lmcr	30.8	49.0	48.4	41.8	30.9	35.4	34.0	19.1
gj	56.4	89.5	73.3	95.8	41.5	100.0	163.3	179.2
sg	300.1	259.8	191.2	213.9	213.9	169.2	471.2	332.3

b=baseline
(+) =cultured with IL-1β, (-) =without IL-1β

Data for Fig 15.4 (page 167). Serum IL-8 levels (pg/ml) in mild asthmatics taking high dose FP.

	b+	**b-**	**1+**	**1-**	**4+**	**4-**	**24+**	**24-hours**
rf	325.6	199.8	181.3	226.1	276.5	129.5	899.5	617.3
gb	342.8	168.3	322.1	17.8	62.3	44.7	238.5	21.3
rp	32.9	25.9	0.00	0.00	3.8	0.00	33.8	62.4
ep	71.9	8.0	93.0	0.00	33.4	0.00	0.00	55.0
gc	686.5	257.3	429.9	160.2	670.9	23.5	730.8	246.5
jj	0.00	0.00	15.7	35.1	36.4	29.1	35.1	16.3

Serum IL-8 levels (pg/ml) in mild asthmatics taking low dose FP.

	b+	**b-**	**1+**	**1-**	**4+**	**4-**	**24+**	**24-hours**
rf	552.2	460.3	419.1	374.6	396.6	371.7	0.00	0.00
gb	221.6	157.8	180.8	148.6	129.6	70.7	370.4	256.2
LM	0.00	0.00	32.3	44.5	16.1	10.9	86.3	83.7
rp	5.3	2.9	28.6	25.7	16.1	21.5	0.00	0.00
ep	1054.5	740.4	1028.7	949.0	1009.0	962.2	650.5	175.2
gc	590.0	253.7	502.5	87.1	286.1	78.1	694.3	477.9
nd	48.5	16.8	34.0	13.2	47.9	5.8	0.00	0.00
jj	27.8	21.1	-2.0	16.3	34.8	10.6	107.9	66.3

Serum IL-8 levels (pg/ml) in mild asthmatics taking PBO.

	b+	**b-**	**1+**	**1-**	**4+**	**4-**	**24+**	**24-hours**
rf	341.0	309.3	196.2	171.8	275.7	321.2	612.7	538.9
gb	70.1	47.5	154.5	144.9	145.1	203.4	134.4	36.0
LM	33.1	33.4	6.5	20.5	7.2	0.9	10.2	29.7
rp	0.00	3.6	43.4	47.2	14.7	49.4	43.7	54.
ep	147.0	48.7	417.9	114.	197.4	60.7	286.7	201.6
gc	1263.1	813.0	1178.0	699.1	991.6	624.1	1233.7	810.6
nd	5.0	0.00	4.1	0.00	4.7	5.3	34.43	54.2
jj	94.6	166.1	220.5	257.1	7.4	31.3	22.7	25.6

Data for Fig. 5.15. Baseline IL-8 (pg/ml) levels in sputum supernatant in mild and moderate asthmatics.

Mild (n=9)	Moderate (n=6)
285.30	1372.53
139.40	313.25
201.34	1342.74
74.24	247.13
417.91	790.59
211.76	1429.03
65.44	
574.30	
683.30	

Data for Fig 5.16, (page 169). IL-8 levels (pg/ml) in sputum supernatant, in mild asthmatics taking high dose FP.

	base	6 hours	24 hours
lmc	285.3	92.6	173.3
sg	139.4	58.1	99.9
jn	201.3	-	66.4
zc	12.3	-	74.7
cduB	74.2	-	309.1
vl	417.9	718.8	410.2
bp	211.7	414.9	419.1
gj	65.4	183.3	225.3
jp	574.3	968.2	387.7
ss	683.3	377.6	686.2

IL-8 levels (pg/ml) in sputum supernatant, in mild asthmatics taking low dose FP.

	base	6 hours	24 hours
lmc	285.3	82.5	236.6
sg	139.4	88.9	122.8
jn	201.3	144.9	-
zc	12.3	24.0.	-
cduB	74.2	260.5	-
vl	417.9	334.3	946.3
bp	211.7	423.8	917.8
gj	65.4	275.1	329.0
jp	574.3	307.1	386.5
ss	683.3	407.2	586.7

IL-8 levels (pg/ml) in sputum supernatant, in mild asthmatics taking PBO.

	base	6 hours	24 hours
lmc	285.3	72.8	70.1
sg	139.4	225.2	223.3
jn	201.3	-	-
zc	12.3	50.8	1.7
hl	-	-	84.
cduB	74.2	-	395.4
vl	417.9	491.3	411.9
bp	211.7	611.0	489.0
gj	65.4	217.6	257.3
jp	574.3	310.6	727.1
ss	683.3	387.7	392.4

Individual data for Fig 5.17, (page 170). IL-8 levels (pg/ml) in sputum supernatant, in moderate asthmatics taking high dose FP.

	base	6 hours	24 hours
rf	1372.5	1002.7	1808.1
gb	313.2	-	104.6
rp	1342.7	687.6	1268.1
ep	386.5	683.3	407.2
gc	247.1	-	219.6
LM	790.5	790.5	410.2
nd	1429.0	1372.5	1377.6
ca	-	763.3	650.3

IL-8 levels (pg/ml) in sputum supernatant, in moderate asthmatics taking low dose FP.

	base	6 hours	24 hours
rf	1372.5	1404.3	1458.8
gb	313.2	343.3	126.0
rp	1342.7	643.7	1311.7
ep	386.5	214.5	359.6
gc	247.1	-	-
jj	-	912.3	597.9
LM	790.5	643.7	1311.7
nd	1429.0	1418.7	989.3
ca	-	773.6	720.1

IL-8 levels (pg/ml) in sputum supernatant, in moderate asthmatics taking PBO.

	base	6 hours	24 hours
rf	1372.5	-	-
gb	313.2	99.4	-
rp	1342.7	890.7	1117.8
ep	386.5	586.7	387.7
gc	247.1	-	71.6
LM	790.5	945.9	727.0
nd	1429.0	1420.8	1284.1
ca	-	1308.8	1268.7

Appendix 4.1 - Data for chapter 6

Data for Fig 6.1 (p.185). Effect of rhuMab E25 on FEV1 (L) in asthmatics. (V=visit number)

	V1	V2	V3	V4	V5	V6	V7
cmd	2.03	1.80	2.85	2.66	2.89	2.75	2.13
os	1.41	1.20	1.45	1.32	1.22	1.50	1.55
sem	1.17	1.75	1.25	1.72	1.41	1.59	1.62
wh	1.01	0.85	1.26	1.05	1.25	1.37	1.33
ma	1.51	1.65	1.52	1.60	1.38	1.59	1.65

FEV1 (L) in control group.

	V1	V2	V3	V4	V5	V6	V7
jae	0.60	0.71	0.61	0.63	0.45	0.60	0.60
vc	1.67	2.33	1.45	1.85	2.01	2.03	1.85
cc	3.23	3.37	3.15	3.45	3.34	3.26	3.34
mb	2.62	2.62	3.15	3.48	2.80	2.75	3.45

Data for Fig 6.2. (p.186). Effect of rhuMab E25 on QOL scores in asthmatics. (V=visit number)

	V2	V3	V4	V5	V6	V7
cmd	53.0	93.0	96.0	99.0	81.0	57.0
os	34.0	75.0	63.0	66.0	67.0	76.0
sem	52.0	67.0	73.0	77.0	65.0	70.0
wh	44.0	63.0	66.0	72.0	63.0	49.0
ma	60.0	64.0	95.0	27.0	71.0	77.0

QOL scores in control group.

	V2	V3	V4	V5	V6	V7
jae	58.0	53.0	40.0	36.0	35.0	34.0
vc	25.0	49.0	59.0	39.0	33.0	38.0
cc	56.0	74.0	67.0	63.0	68.0	76.0
mb	57.0	59.0	87.0	66.0	58.0	94.0

Data for Fig 6.3. (p.187). Effect of rhuMab E25 on clinical symptom scores in asthmatics. (V=visit number)

	V2	V3	V4	V5	V6	V7
cmd	46.0	9.0	7.0	2.0	26.0	47.0
os	59.0	49.0	61.0	55.0	44.0	49.0
sem	32.0	19.0	23.0	22.0	26.0	26.0
wh	54.0	16.0	21.0	28.0	39.0	30.0
ma	53.0	40.0	14.0	35.0	52.0	26.0

Clinical symptom scores in control group.

	V2	V3	V4	V5	V6	V7
jae	40.00	37.00	28.00	40.00	42.00	38.00
vc	0.00	22.00	16.00	21.00	34.00	22.00
cc	24.00	24.00	25.00	27.00	31.00	18.00
mb	32.00	13.00	24.00	25.00	35.00	15.00

Data for Fig 6.5 (p.189). Effect of rhuMab E25 on dose of ICS (BDP equivalent µg/day) in asthmatics. (V=visit number)

	V1	V7
cmd	1600	800
os	2000	2000
sem	2000	2000
wh	2000	2000
ma	800	400

Dose of inhaled corticosteroid (BDP equivalent µg/day) in control group.

	V1	V7
jae	1600	2000
vc	2000	2000
cc	2400	1000
mb	400	800

Data for Fig 6.6 (p. 190). Effect of rhuMab E25 on number of corticosteroid courses per year in asthmatics.

	Pre-trial period	post-trial period
ma	2	1
os	3	2
cmd	3	1
wh	5	0
sem	4	3

Number of oral corticosteroid courses per year in control group.

	Pre-trial period	post-trial period
jae	2.0	2.0
vc	3.0	2.0
cc	7.0	5.0
mb	5.0	2.0

Data for Fig 6.7 (page 191)

FCεR1 expression on basophils during E25 therapy (3 months)

	Visit 1		Visit 2	
Patient	mean mfi	SEM	mean mfi	SEM
vt	195.150	11.05	29.850	3.55
sj	129.450	20.25	4.440	0.94
bh	182.800	18.60	24.350	2.65
gg	49.450	10.95	39.750	6.04
jh	59.350	4.35	25.700	0.30
ms	95.600	12.00	13.850	2.95

FCεR1 expression on monocytes during E25 therapy (3 months)

	Visit 1		Visit 2	
Patient	mean mfi	SEM	mean mfi	SEM
vt	47.80	9.40	11.50	1.90
sj	55.40	0.30	4.96	4.74
bh	35.93	0.765	20.58	1.61
gg	90.00	2.50	78.05	3.14
jh	197.90	11.50	125.60	1.09
ms	195.90	12.80	0.20	0.10
pg	33.40	1.40	0.30	0.30

FCεR1 expression on basophils-control patients

	Visit 1		Visit 2	
Patient	mean mfi	SEM	mean mfi	SEM
at	145.05	3.65	61.95	17.15
lc	435.95	22.34	423.90	66.89
pk	5.15	2.45	8.45	0.15
ca	142.15	3.04	150.35	17.65
eh	166.05	29.65	3.650	1.960

FCεR1 expression on monocytes-control patients

	Visit 1		Visit 2	
Patient	mean mfi	SEM	mean mfi	SEM
at	78.95	3.95	76.60	19.20
lc	43.45	1.64	76.45	43.75
pk	59.85	4.95	34.20	1.29
ca	104.80	3.20	101.90	33.70
eh	22.70	1.40	25.60	1.80

mfi-mean fluorescence intensity
SEM-standard error of the mean

Appendix 4.2 Patient characteristics IA04 study (chapter 6). N= 4, control asthmatics.

Patient	measurements	Visit 1 (screening)	Visit 2	Visit 3	Visit 4	Visit 5	Visit 6	Visit 7	Medication at start of trial→ end of trial
JAE	FEV1(L) QOL score CSS Neutrophils Eosinophils	0.6 7.3 0.7	0.71 58 40	0.61 53 37	0.63 40 28	0.45 36 40	0.6 35 42 8.2 0.5	0.6 34 38	Budesonide 800µg/day→2000 µg /day
MB	FEV1(L) QOL CSS Neutrophils Eosinophils	2.62 2.1 0.4	2.62 57 32	3.15 59 13	3.48 87 24	2.8 66 25	2.75 58 35 3.2 0.5	3.45 94 15	Budesonide 400 µg /day→ 1600 µg /day
CC	FEV1(L) QOL CSS Neutrophils Eosinophils	3.23 3.8 0.1	3.37 56 24	3.15 74 24	3.45 67 25	3.34 63 27	3.26 68 31 7.6 0.4	3.34 76 18	Budesonide 2400 µg /day→ 1000 µg /day
VC	FEV1(L) QOL CSS Neutrophils Eosinophils	1.67 2.4 0.0	2.33 25 24	1.45 49 22	1.85 59 16	2.01 39 21	2.03 33 34	1.85 38 22	Budesonide 1000 µg /day→ 2000 µg /day

FEV1- forced expiratory volume in 1 second (L); QOL- quality of life score; CSS- clinical symptom score; neutrophil/eosinophil numbers ($x10^9$/L).

Appendix 4.3 Patient characteristics IA04 study (chapter 6). Asthmatic patients receiving rhuMab E25, N= 5.

Patient	measurements	Visit 1 (screening)	Visit 2	Visit 3	Visit 4	Visit 5	Visit 6	Visit 7	Medication at start of trial→ end of trial
SEM	FEV1	1.17	1.75	1.25	1.72	1.41	1.42	1.56	Budesonide 1000 µg /day→ 1000 µg /day
	QOL		52	67	73	77	65	68	
	CSS		32	19	23	22	26	25	
	Neutrophils	2.7					2.6		
	Eosinophils	0.2					0.1		
WH	FEV1	1.01	0.85	1.26	1.05	1.25	1.22	1.33	Budesonide 1000 µg /day→ 1000 µg /day
	QOL		44	63	66	72	63	49	
	CSS		54	16	25	28	39	30	
	Neutrophils	5.9					6.5		
	Eosinophils	0.5					1.9		
CMD	FEV1	2.03	1.8	2.85	2.66	2.89	2.75	2.13	Budesonide 1600 µg /day→ 800 µg /day
	QOL		53	93	96	99	81	57	
	CSS		46	9	7	2	26	47	
	Neutrophils	3.1					3.5		
	Eosinophils	0.6					0.4		
OS	FEV1	1.41	1.2	1.45	1.32	1.22	1.5	1.55	Budesonide 1000 µg /day→ 2000 µg /day
	QOL		34	75	63	66	67	76	
	CSS		59	49	61	55	44	49	
	Neutrophils	5.7					4.3		
	Eosinophils	0.5					0.5		
MA	FEV1	1.51	1.65	1.52	1.60	1.38	1.59	1.65	Budesonide 800 µg /day→ 400 µg /day
	QOL		60	64	95	27	71	77	
	CSS		53	40	14	35	52	26	
	Neutrophils	5.9					2.9		
	Eosinophils	0.1					0.1		

FEV1- forced expiratory volume in 1 second (L); QOL- quality of life score; CSS- clinical symptom score; neutrophil/eosinophil numbers ($x10^9$/L)

Appendix 4.4 Patient characteristics: 11 extension substudy (chapter 6). Asthmatic patients on anti-IgE treatment. n=10,

Patient	Age- years	Male/female	FEV1 (L)	FEV%	Dose Fluticasone propionate	IgE level (IU/l)
VAT	50	F	2.27	89%	500 µg/day	102
GCG	45	F	1.33	43.9%	750 µg /day	159
AFG	59	M	1.56	42.5%	3000 µg /day	585
LMH	48	F	2.07	92.2%	1250 µg /day	267
MCS	69	F	1.27	64.9%	2000 µg /day	330
SJJ	43	F	2.48	93.2%	1000 µg /day	397
JEH	26	F	3.18	101%	1500 µg /day	149
BMH	36	M	2.87	70.4%	250 µg /day	123
AMB	47	F	2.07	76.6%	1000 µg /day	235
PG	50	M	3.45	86.9%	2000 µg /day	155

Patient characteristics: 11 extension substudy. Control subjects, n=5.

Patient	Age	Male/female	FEV1 (L)	FEV%	Dose Fluticasone propionate	IgE level (IU/l)
AT	63	F	1.07	54%	1000 µg /day	300
PK	63	F	1.22	55.7%	1000 µg /day	76
CGA	58	M	1.72	46.9%	800 µg /day	88
HE	51	M	2.40	69.2%	1000 µg /day	125
LSC	58	F	1.10	52.1%	3200 µg /day	290

Appendix 5.1. Characteristics of moderate asthmatic patients in prednisolone study (chapter 3). N=10.

Patient	age	FEV1 (L)	FEV1 %	PC_{20} (mg/ml)	Atopic Y/N	NO (ppb)	Treatment
ca	59	1.92	74.6	0.34	Y	13.5	Seretide 500µg bd Formoterol 12µg bd Uniphyllin 400 mg bd
jj	48	1.49	67	1.15	Y	19.4	Pulmicort 2mg/day nebs. Formoterol 24 µg bd.
at	54	2.0	54	N/D	Y	9.2	Flixotide 250 µg bd Formoterol 12 µg prn Iprtropium prn
tg	73	1.9	70.6	N/A	Y	N/A	Seretide 500 µg bd
cc	31	3.67	84.6	N/A	Y	19	Pulmicort 800 µg tds Formoterol 24 µg bd Montelukast 10mg
lc	50	0.9	45	N/D	Y	23.1	Pulmicort 1600 µg bd Iptratropium 2 puffs tds MDI
cha	50	2.5	78	N/A	Y	N/A	Seretide 250 µg bd
ip	41	1.0	38.5	N/A	Y	13.9	Flixotide 250 µg bd Formoterol 24 µg bd.
db	38	2.45	70	0.8	Y	12.3	Flixotide 250 µg bd Formoterol 12 µg bd.
chi	33	2.6	85	2.19	Y	39.9	Flixotide 500 µg bd Formoterol 24 µg bd.

N/D- not done as low FEV1
N/A-not available
ppb-parts per billion