

THE EFFECT OF TREATMENT WITH LOW DOSE ORAL THEOPHYLLINE ON
THE INFLAMMATORY RESPONSE TO ALLERGEN IN ATOPIC ASTHMA.

Paul Joseph Sullivan MB BS MRCP(UK)

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The scientific work was carried out at:

The Department of Thoracic Medicine,
King's College Hospital School of Medicine and Dentistry,
London.

The Department of Lung Pathology,
The Royal Brompton National Heart and Lung Institute,
London.

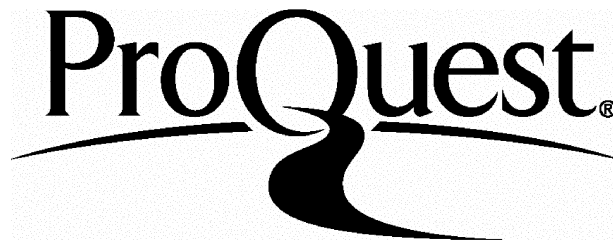
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Abstract

Theophylline inhibits a number of immunocompetent cells in vitro at clinically relevant concentrations and reduces the inflammatory response in animal models of asthma. The current study is a of double blind, parallel group design, comparing the effect of theophylline and placebo upon the inflammatory response to allergen in man. Subjects were mild asthmatics who had previously demonstrated an early and late asthmatic response. Allergen inhalation was performed before and after six weeks of treatment with slow release theophylline, 200mg 12 hourly or identical placebo. Blood samples were taken before challenge, during the early and late responses and at 24 hours and bronchoscopy with biopsy and bronchoalveolar lavage (BAL) was performed at 24 hours. Primary outcome parameters were biopsy eosinophil and T lymphocyte counts. A confidence level of 95% was regarded as representing statistical significance. This corresponded to a p value of 0.025 after correction for multiple comparisons. T cell subsets were also enumerated in blood and BAL, cell counts and inflammatory mediator concentrations were measured in BAL and mast cells and activated T cells and eosinophils were counted in biopsy specimens.

There was a reduction in the number of eosinophils but not T lymphocytes in biopsies of the airway after theophylline, and a significant treatment effect was shown. Changes were also seen in some secondary parameters. There was a reduction in the number of activated eosinophils in tissue. In the blood there was an increase in the number of activated T helper lymphocytes (CD4+, HLA DR+) following theophylline both before and after allergen. BAL revealed a reduction in the number of lymphocytes after theophylline, and a reduction in CD3+, ICAM+ and CD4+, VLA+ activated T cells. It is concluded that low dose slow release oral theophylline attenuates the airway eosinophilic response to allergen. The results also suggest that theophylline increases circulating activated lymphocytes and reduces the number of lymphocytes in the airway lumen after allergen exposure.

Aim

The aim of the current study is to investigate whether the effects of theophylline upon inflammatory cells which are described *in vitro* in animal models of asthma is translated into an anti-inflammatory effect in human disease at a dose that is tolerated. The study examines the effect of treatment with low dose oral slow release theophylline on the inflammatory infiltrate in bronchial biopsies. Primary outcome parameters are biopsy eosinophil and T lymphocyte counts. Effects on mast cells and T cell subsets in biopsies, bronchoalveolar lavage (BAL) cells and on peripheral blood and BAL lymphocyte subset activation in relation to allergen inhalation will also be examined as secondary parameters.

Ethical Approval

This study was approved by the ethical committee of King's College Hospital.

Acknowledgement

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Author's Declaration

All of the clinical procedures and measurements were carried out by the author. These include administration of inhaled allergen and subsequent supervision, spirometry, bronchoscopy, broncho-alveolar lavage and biopsy. Finger prick blood tests for theophylline determination were carried out by Mrs Sally Sampson (in order to avoid un-blinding).

Biopsy samples were mounted in cutting medium, snap frozen, sectioned and stained by immuno-histochemical techniques by the author. All cell counts and computer aided image analyses were performed by the author.

FACS analysis of peripheral blood and bronchoalveolar lavage fluid was performed by Ms Zeina Jaffar, a post doctoral research fellow using a technique devised in collaboration with the author.

Determination of bronchoalveolar lavage mediator concentrations were carried out jointly by Ms Zeina Jaffar and by the author.

All statistical analyses were carried out by the author

CHAPTER 1: INTRODUCTION

PART I: ASTHMA

Whilst asthma continues to evade precise definition (CBI Foundation, 1971) its clinical features make up a distinct recognisable syndrome. The central characteristic is a variable narrowing of the airway lumen (McFadden, 1992). The primary pathology is, however, likely to be inflammation of the bronchi.

1.1 EVALUATION OF THE EVIDENCE FOR INFLAMMATION IN ASTHMATIC BRONCHI

The earliest studies of the airway wall in asthma examined post mortem material from patients who had died during a severe exacerbation (Houston, 1953; Dummil, 1960). Findings included the presence of an inflammatory infiltrate within the bronchial wall, mucosal oedema, loss of epithelial cells and occlusion of the lumen by tenacious plugs consisting of mucus, inflammatory exudate and leukocytes. More recently, Azzawi has compared post mortem material from 15 asthmatics who died from the disease with 10 normals who died of other causes and, as a second control group, 6 patients who died from cystic fibrosis (Azzawi, 1992). In the normal subjects there were some immunocompetent cells but eosinophils were rare. In the asthmatics there was a significant increase in CD45+ cells (leukocytes), eosinophils, EG2+ activated eosinophils, and T lymphocytes. No EG2+ cell were seen in normals but the proportion of EG2+ activated divided by total eosinophils was 44% in asthmatics. There was the appearance of basement membrane thickening in the asthmatics and mucus plugging was present in most cases of asthma. Saetta and colleagues examined post mortem lung tissue from 6 asthmatics and found, in addition, an increase in the number of eosinophils in the airway wall and in the walls of arteries, even where the artery is not close to the bronchial lumen, suggesting diffuse eosinophilic inflammation within the

lung (Saetta 1991). Examination of tissue from asthmatics who died as a result of causes other than asthma have revealed similar changes within the bronchial mucosa (Jeffery, 1993).

The increasing acceptance of fibre-optic bronchoscopy as a safe technique in asthma (Djukanovic, 1991) has allowed investigation of the morphology of the bronchial mucosa in stable disease. Jeffery et al reported a study which bronchial biopsies of 11 asthmatics, 5 atopic non-asthmatics and 5 normal non-atopic subjects and showed complete loss of epithelial cells in most asthmatics but in only 1 normal subject and the difference between the two groups for degree of epithelial integrity was statistically significant. (Jeffery, 1989). The highest proportion of epithelial cover in all groups was 56%, suggesting a degree of artefactual epithelial damage; however, epithelial damage correlated with bronchial responsiveness which suggests that the finding in the asthmatic group is related in part to the disease process. A second finding in the same study was apparent thickening of the epithelial basement membrane in subjects who were hyper-responsive to methacholine compared with subjects with a normal response. However, 2 of the original asthmatic group had normal bronchial responsiveness and this finding is therefore the result of retrospective sub-grouping (in so far as the original groupings were clinical asthma versus clinical normality) which increases the chance of type I statistical error. There was no difference in airway eosinophil count between asthmatic and non asthmatic subjects; this is contrary to the increased BAL eosinophilia seen in a subgroup of the asthmatic patients in this study. The paper suggests that the reason for the discrepancy may be that eosinophils in tissue degranulate to such an extent that they are no longer stainable *or* that there is a steady state, with increased traffic of cells from blood to airway lumen but a normal tissue cell concentration along the way *or* that the BAL eosinophilia may reflect changes exclusive to the smaller distal airways which were not sampled. Another explanation, not addressed by the authors, is that the power of the study was low for biopsy cell counts, with only 5 control subjects and standard deviations for cell counts ranging

from 60-83% of the respective sample mean: the absence of a statistically significant difference between samples in this situation does not imply that no difference exists between populations.

Azzawi compared biopsies from 11 asthmatics, 9 atopic non asthmatics and 10 normal subjects (Azzawi, 1990a). Again, there was loss of epithelium in normal subjects, suggesting artefact. There was thickening of the sub-epithelial collagen layer in the asthmatic group, compared with normals. Biopsies from two airway levels were examined. In this study there was a significant eosinophilia in asthmatics compared to normals and a higher number of EG2+ activated eosinophils in central airways. An interesting finding was that the atopic, non asthmatic subjects had an activated eosinophil count between the asthmatic and normal state which was statistically different from both. In sub-segmental bronchi findings were similar. In asthma there was a significant eosinophilia, with increased EG2+ cells and eosinophil granules were seen within the extra-cellular space in asthmatics but not normals. Additionally there was an increase in the number of cells expressing the interleukin-2 (IL2) receptor CD25, the first demonstration of possible T cell activation in asthmatic bronchi. There was no significant difference in T cell counts, although again the power of the study was poor and the standard deviation (SD) of CD3 and CD4 counts were 89% and 85% of the means in the asthmatic group. There was also a wide variation in EG2 counts (SD=96% of mean). The mean activated eosinophil count was greater than the total eosinophil count which suggests a degree of imprecision (i.e. individual cell counts which vary widely around the true mean value), and such imprecision may be partly responsible for the large within-group variability observed.

Bousquet performed a larger and therefore more powerful study which included 43 asthmatics, although only 17 were biopsied, and 10 normals who underwent blood tests and bronchoalveolar lavage (BAL) (Bousquet,1990). Controls for biopsy specimens were taken post mortem from 8 non asthmatics. There was a significant

peripheral blood eosinophilia in the asthmatic group which correlated with the clinical symptom score and an increase in BAL eosinophilia and ECP concentration. In biopsies there was eosinophilic infiltration in asthma, even in mild disease, and EG2+ activated eosinophils were seen in all asthmatics with extra-cellular EG2 denoting eosinophil degranulation in 14 of 17 asthmatics. In contrast EG2 positive cells were rare in biopsies of normal subjects. These findings point to the importance of the eosinophil in asthma, although the biopsy comparisons might be criticised because the control specimens could have been subject to post mortem artefact.

The presence of eosinophilia is supported by a study with better design which used biopsy material obtained at bronchoscopy from asthmatics and normal control subjects (Foresi 1990). 13 mild asthmatics were compared with 6 normals. All of the asthmatics displayed bronchial hyper-responsiveness but all were well enough to be able to stop medication for 24 hours for the study. There was an increase in eosinophils in the submucosa ($p=0.024$) and, in addition, an increase in mast cell count in the epithelium in the asthmatics. In keeping with studies showing correlation of hyper-responsiveness with epithelial integrity, the number of epithelial cells in BAL correlated with bronchial hyper-responsiveness. BAL eosinophilia was also present compared to normals ($p=0.012$).

Ollernshaw compared 5 asthmatics on inhaled steroids, 5 asthmatics on beta -agonists alone and 4 non asthmatic patients (Ollernshaw, 1992). There was an increased lymphocyte, eosinophil and mast cell count in the lamina propria in asthmatics requiring inhaled steroids compared with normals and there was a decrease in the proportion of intact epithelium. The study also looked at 5 subjects with chronic airflow limitation (CAL). There is no consensus on the clinical difference between CAL and asthma and the selection criteria are therefore, to some extent, arbitrary and should be stated. In this study the definition of these groups was not given. The CAL group included a non smoker with bronchial hyper-responsiveness and the asthmatic

group contained 2 smokers. Furthermore, the two groups were not well matched for age. The mean age of the asthmatics was 41 years and that of the CAL group was 58 years. The lymphocyte, eosinophil and mast cell count was higher in asthmatics than the CAL group and CAL lymphocytes were found within smooth muscle, although without a clear definition of the CAL group it is difficult to interpret this finding.

Many of the studies that have examined asthmatic inflammation in the bronchi have used relatively small groups of subjects and controls and the degree of statistical significance associated with positive findings has not been great. Many of these studies have simultaneously compared numerous different cell counts between the asthmatic and normal groups and the risk of a type I statistical error, the demonstration of a difference between samples which is not actually present between corresponding populations, is increased. Bradley and colleagues have carried out a study in which a Bonferroni correction was applied to the results of statistical analyses (Bradley 1991). This mathematical technique reduces the risk of type I error by allowing for the number of comparisons used to test a single hypothesis. 21 allergic asthmatics were compared with 10 allergic non-asthmatic and 12 non allergic normal subjects. Significant differences were found between the asthmatic and non-allergic normal subjects in terms of EG2 positive activated eosinophils and IL-2 receptor (CD25) positive cells, with greater numbers of both in the asthmatics. The authors suggested that the CD25+ cells were activated T lymphocytes; this was based upon the fact that the concentration of anti-CD25 antibody used in the study was titrated in separated cells so that only lymphocytes stained. Without double marker staining is not possible to be certain that the antibody titration in separated cells will translate for use in tissue samples. It is therefore not necessarily true that there was an increase in the number of activated T lymphocytes in the asthmatics. Instead, the conclusion should be that an increased number of cells overall expressed the receptor for IL-2. There were no statistically significant differences between the groups in terms of CD3, CD4 or CD8 positive cells or mast cells, although there was a large degree of variance within

groups for lymphocyte markers which increases the risk of a type II statistical error (failing to demonstrate a true difference between groups). The number of EG2+ cells correlated with the number of T lymphocytes, suggesting some link between the two cell types in asthma, although an alternative explanation is that variations in tissue quality affected both cell types simultaneously. An important observation of this study was that some T lymphocytes were present within the airway of normal, non-asthmatic, non-allergic subjects.

Djukanovic compared 13 asthmatics and 10 non allergic normals (Djukanovic, 1992a). There was a higher light microscope eosinophil count in asthmatic than in normal subjects and the difference was highly significant ($p < 0.0005$). Such a level of significance holds true even if correction is made for the number of comparisons used to test a single hypothesis. Electron microscopy demonstrated mast cell degranulation in asthmatics but not normals and the presence of eosinophils in bronchial submucosa only in the asthmatics. This study also included 10 allergic but non asthmatic subjects. It was found that there was an increased eosinophil count in these subjects, although the statistical significance ($p < 0.05$) would have been lost if corrected for the number of 'p' values generated. Subjects in the asthmatic group had symptoms and diurnal peak flow variability, whereas the non asthmatic allergic subjects simply had no symptoms: they were not shown to have normal peak flow records and there may have been a degree of crossover, with very mild (or uncomplaining!) asthmatics included in the group.

Poulter's group have examined biopsies and BAL from 5 asthmatics with biopsies from 5 controls who were investigated for cough or respiratory tract infection (all had normal airway responsiveness) and BAL from 10 normal controls (Poulter, 1992). In the asthmatics there was an inflammatory infiltrate consisting mostly of mononuclear cells. More than 80% of T cells expressed CD45RO, a marker of memory cells and the number of CD45RO cells was increased significantly in asthmatic tissue. There was

also increased expression of HLA-DR, an activation marker found on a number of cells, in asthma. In BAL there was no increase in the expression of HLA-DR and there was no significant correlation between BAL and biopsy counts for any cells other than CD45RO+ cells. However, the lack of correlation may be the result of the small number of subjects studied, since the correlation coefficient between BAL and biopsy was 0.77 for T cells and 0.87 for monocytes, revealing a relatively strong association but the p values, which are determined partly by the size of the sample, were large, so it is not possible to be confident that the findings could possibly be due to chance. An important finding was that less than 1% of BAL lymphocytes expressed B cell markers and more than 95% were T cells, suggesting important role of the T lymphocyte in the lung. In this respect, however, it is important to distinguish between the compartments studied by BAL and by biopsy. The absence of B cells in BAL does not mean that they are not present within the tissue of the airway wall.

Poston compared 16 asthmatics, of whom 15 were atopic, with normals and used immuno-histochemical staining with a number of antibodies (Poston, 1992). There were significantly more monocytes ($p=0.002$) and more HLA class II staining ($p=0.009$) in the submucosa of asthmatics. There were also more EG2+, activated eosinophils, CD45RO+ memory T cells and macrophages (stained with the pan macrophage marker HAM56), although the level of statistical significance for the latter three cell types was not as great ($p=0.02$, 0.01 and 0.02 respectively) and might not be considered to demonstrate a statistically significant difference if corrected for the number of tests performed. In the epithelium it was observed that class II HLA was localised to basal epithelial cells in asthmatics but not normal subjects. Class II HLA is involved in antigen presentation to CD4+, helper T lymphocytes suggesting that basal epithelial cells may act as antigen presenting cells in asthma and that they are different in this respect from basal cells of non-asthmatics. There were more CD45RO+ memory T lymphocytes in the epithelium of asthmatics ($p=0.001$) and more eosinophils ($p=0.01$) although, again, the difference in eosinophil counts might be regarded as non-

significant if corrected for the number of comparisons used to test a single hypothesis. There were more macrophages in the epithelium of asthmatics than normals, but the level of significance was not great ($p=0.04$).

Tissue eosinophilia, with an increased number of activated eosinophils has been demonstrated in virtually all histological studies of the bronchi in asthma, regardless of disease severity. The level of probability accepted as statistically significant could be criticised for most of the studies because numerous comparisons were made to test a single hypothesis (i.e. that asthmatic airways differ from normal in terms of inflammation). However, in one study eosinophilia was found after Bonferroni correction (Bradley, 1991) for multiple comparisons and in another the level of significance was great (Djukanovic, 1992a), leaving little doubt that eosinophilia is a feature of the airways in asthma. An increased CD25 count has also been shown after Bonferroni correction, suggesting that cellular activation, probably T cell activation, occurs in the bronchi. There are a number of findings that have been shown with a relatively low level of significance, particularly when the number of comparisons in a study is taken into account, but which are seen in a number of different studies and which are therefore likely to represent actual differences between asthmatic and normal bronchi. These include the loss of epithelial cells, the appearance of thickening of the basement membrane and an increase in the number of CD45RO+ memory T cells. A less constant finding has been an increase in the number of mast cells in asthma.

There is, then, little doubt that bronchial inflammation is a feature of asthma and that it is present even in mild disease. The inflammatory infiltrate consists largely of eosinophils, which show features of activation, and memory T lymphocytes and is accompanied by loss of epithelial cells and sub-epithelial fibrosis. There may also be an increase in the number of mast cells, monocytes and macrophages within the bronchial wall.

1.2 BRONCHIAL RESPONSIVENESS

The airways of asthmatic subjects are hyper-responsive to a number of constrictor stimuli *in vivo*. In asthma there is a leftward shift in the dose-response curve to inhaled histamine and methacholine and the degree of sensitivity correlates with the clinical severity of asthma (Cockcroft, 1985, Josephs, 1990, Djukanovic, 1990). Isolated smooth muscle from asthmatics does not exhibit increased sensitivity to histamine *in vitro* and there is no correlation between the constrictor response *in vitro* and the degree of bronchial hyper-responsiveness *in vivo* in asthma (Cerrina, 1986). This suggests that it is some abnormal component of the bronchial environment that induces the altered responsiveness in smooth muscle cells in asthma. The inflammatory process may be the underlying stimulus for smooth muscle hyper-responsiveness. Two strands of evidence support this hypothesis. One derives from the finding that there is a positive association between the degree of bronchial hyper-responsiveness and the magnitude of cellular infiltration in the airway wall. The number of EG2+ activated eosinophils in the airway wall correlates with disease severity and bronchial hyper-responsiveness. Furthermore, in patients who experience increased symptoms of asthma at night there is a significant increase in the total BAL cell count and in the number of eosinophils and neutrophils at 0400 hr when compared with 1600 hr: this is not seen in patients who do not suffer nocturnal symptoms, suggesting a relationship between disease activity and the number of cells retrieved by BAL (Martin, 1991). The second strand derives from the fact that inhalation of pro-inflammatory products similar to those released by infiltrating cells in asthma can induce bronchial hyper-responsiveness *in vivo*. In asthmatic subjects inhalation of leukotriene E₄ causes acute bronchoconstriction which is followed by a period of enhanced bronchial reactivity to inhaled histamine (Arm, 1988). Similarly, leukotriene C₄ may cause bronchial hyper-responsiveness (Henderson, 1991). In normal subjects inhalation of platelet activating factor (PAF), a mediator that is released by mast cells and a variety

of leukocytes produces a prolonged state of hyper-responsiveness to methacholine which peaks at 6h and which may persist for weeks (Page, 1988).

1.3 THE MECHANISMS OF INFLAMMATION IN ASTHMA

1.3.1 ANTIGEN PRESENTATION - DENDRITIC CELLS

T-lymphocyte/antigen interaction requires that the antigen is presented to lymphocytes in an appropriate form. The antigen must be partly degraded and bound to a special region on a major histocompatibility complex (MHC) molecule (Davis, 1988). MHC exists as class I which is widely distributed and class II which is found on the surface of a limited group of cells which internalise antigen, process it and then present it on their surface with the class II MHC. These “antigen presenting cells” include B lymphocytes, macrophages, epithelial cells and dendritic. The latter have interdigitating projections which are seen to form an extensive network (Schon-Hegrad, 1991). In the lung dendritic cells have been differentiated into Langerhans cells with intracellular Birbeck granules, and simple dendritic cells without Birbeck granules (Hance, 1993). T helper cells have CD4 cell surface markers which bind to MHC II enabling interaction with antigen presenting cells (Agostini, 1993). Antigen presenting cells act as accessory cells and stimulate T lymphocytes by virtue of cell surface proteins. King found that monoclonal antibodies directed against cell surface markers ICAM 1 and LFA 3 on dendritic cells and CD4 on T cells reduced T cell mitogenesis resulting from interaction with dendritic cells in vitro (King, 1989). Similarly, Nicod has shown that dendritic cells from mouse lung have high concentration of the adhesion molecules ICAM 1 and LFA 3 and antibodies to these molecules attenuate T cell activation (Nicod, 1992). In vitro work has also shown that these cells emit signals which stimulate naive T cells to convert to memory cells (Inaba, 1986).

Dendritic cells seem to be the most important antigen presenting cells in the airway since depletion of macrophages, the other main group of antigen presenting cells, does

not diminish the response to antigen in animals (Hance, 1993) and dendritic cells represent the majority of MCH II staining cells in the airway. Furthermore, the number of dendritic cells is increased after exposure to acute inflammatory stimuli and in chronic inflammation (Schon-Hegrad, 1991). These cells can migrate to secondary lymphoid tissue, where T cell interaction occurs (Bujduso, 1989). In view of their interaction with T cells, their ability to migrate to secondary lymphoid tissues and the fact that asthmatic airways have an excess of dendritic cells compared with normals (Sattoli, 1992), it seems likely that these cells have an important role in the pathology of asthma.

1.3.2 LYMPHOCYTES

Lymphocytes detect specific antigen and direct the immune response. These cells are divided into B cells which produce immunoglobulin and T cells which respond to antigen on the surface of other host cells (Agostini, 1993). T cells may be further divided; the subgroup that is most studied in asthma is the T helper/inducer group. These cells can release inflammatory mediators which induce differentiation and proliferation in a number of cell types including lymphocytes themselves (Dinarelo, 1987). Lymphocytes are present in the airway wall and epithelium and in defined clusters termed bronchus associated lymphoid tissue (BALT) (Pabst, 1992). T helper cells which possess CD4 markers interact with antigen presented in association with MHC II on antigen presenting cells. The T cells have a T cell receptor (TCR) which includes a variable region that confers antigen specificity. Antigen recognition is followed by T cell activation and a number of markers are up-regulated on the cell surface, including the interleukin 2 receptor (CD25) soon after activation, HLA-DR, a class II MHC molecule, and VLA-1 which appears after 2 weeks of stimulation in vitro (Corrigan, 1990).

In the mouse the CD4 T helper subset has been divided into TH1 and TH2 groups on the basis of the pattern of cytokine release. TH2 cells seem to be most likely to

support the type of inflammation seen with asthma. They release interleukin (IL)4 which stimulates IgE production and IL5 which promotes eosinophil differentiation and survival. TH2 cells, when injected, induce a local inflammatory response in mice which is blocked by antibodies to IL4. In man, similar groups have been described. BAL from allergic asthmatics has elevated IL4 and IL5 but normal IL2 (a TH1 product). Patients with non allergic diseases such as sarcoidosis have a TH1 pattern. However, some patients show a mixed pattern with increased IL5 and IL2 suggesting that there may be cells which produce a broad range of cytokines (Walker, 1994). Messenger RNA (mRNA) for IL5 is especially prominent in asthmatic lymphocytes (Hamid, 1991) and BAL and blood lymphocytes in asthma produce more IL4 and IL5 ex vivo (Walker, 1992) and more cells express mRNA for IL4 and IL5 in asthmatic lung: most of these are activated T cells (Kay, 1995). T cells therefore appear to have an important role in the immunology of asthma. Further evidence for this role comes from experiments in which Garssen was able to transfer sensitivity to picryl chloride from exposed to naive mice by injection of lymphocytes. Selective depletion of T cells abolished the effect (Garssen, 1991).

1.3.3 MAST CELLS

Mast cells are found in and below the airway epithelium (Bradley, 1991). These cells have high affinity IgE receptors which bind to soluble IgE (Stevens, 1989). Mast cells store products such as histamine, tryptase and heparin which are released by degranulation (Warner, 1994). In addition, immuno-staining has shown that mast cells contain IL4 and IL5 within granules (Brading, 1994) and that mast cells contain mRNA for these cytokines (Kay, 1995). This suggests a more sophisticated role in asthma. Mast cells also release newly formed products such as prostaglandin D2, and leukotriene B4 and C4 (Warner, 1994).

1.3.4 EOSINOPHILS

Eosinophils are granulocytes which appear to have a role in asthma as end effectors of the inflammatory process. They have granules which contain pre-formed cationic proteins which are highly cytotoxic to helminth and to host cells (Kroegel, 1994). Eosinophils, like mast cells, also release newly formed mediators PGD₂ and LTC₄ as well as toxic oxygen species. Eosinophils therefore have the potential to cause many of the pathological features in asthma including bronchoconstriction and bronchial hyper-responsiveness and mucus hypersecretion (LTC₄), vascular permeability (LTC₄, oxygen radicals and cationic proteins) and epithelial damage (oxygen radicals and cationic proteins).

1.3.4 EPITHELIUM

Cultured epithelial cells produce neutrophil chemotactic activity in vitro and these cells can produce leukotrienes and interleukin 8 which are chemotactic signals (Shoji, 1987; Von Essen, 1988; Elling, 1986). RANTES (Regulated upon Activation in Normal T cells Expressed and Secreted) is also produced by human airway epithelial cells (Kwon, 1994); it is a small peptide which is chemo-attractant for monocytes, eosinophils and T cells (Schall, 1991). Supernatant from epithelial cell cultures increases neutrophil survival and G-CSF, which is released by these cells, prolongs eosinophil survival (Thompson, 1995). Epithelial cells may also present antigen to lymphocytes in association with MHC II (Suda, 1995).

Epithelial cells may also have anti-inflammatory effects. They produce PGE₂ which attenuates inflammation and transforming growth factor beta which inhibits lymphocyte proliferation and macrophage function (Sacco, 1992; Kehri, 1986). Epithelial cells may also release nitric oxide which relaxes smooth muscle (Robbins, 1993).

Epithelial shedding, which occurs in asthma, may have an important pro-inflammatory or anti-inflammatory role, depending upon which epithelial cell functions predominate in vivo. It is interesting that epithelial removal in rabbits leads to bronchial hyper-responsiveness although this has not been reproduced in guinea pigs (Butler, 1987; James, 1993).

1.3.5 THE ROLE OF AIRWAY NERVES

The bronchi have autonomic nerves which affect bronchial tone and possibly airway inflammation. Branches from the vagus nerve send efferent parasympathetic pre-ganglionic fibres to the airways. These fibres synapse within the lung and send post-ganglionic fibres to airway smooth muscle and their stimulation leads to contraction by the action of acetylcholine upon smooth muscle muscarinic receptors (De Jongste, 1991). There is a background vagal tone and anticholinergic drugs abolish this and cause bronchodilation in normal lungs, although the picture is complicated by the fact that complete denervation of the lung leads to a state of bronchial hyper-responsiveness without clinical asthma after lung transplantation. The sympathetic nervous system sends few nerves to airway smooth muscle (Laitinen, 1985) and β -2 receptors on airway smooth muscle must therefore respond physiologically mainly to catecholamines which circulate at low concentration. The bronchoconstriction which follows exposure to β -2 receptor antagonists (Tattersfield, 1973) may be due to blockade of pre-synaptic β -2 receptors on cholinergic parasympathetic nerves (Ind, 1989) as it is inhibited by anticholinergic drugs.

There is evidence for another type of autonomic neuronal response in the airway which is neither cholinergic or adrenergic, the non-cholinergic, non-adrenergic (NANC) nerves. Capsaicin, a substance which stimulates sensory nerves, induces bronchodilation after pharmacological blockade of adrenergic and cholinergic

transmission (Lammers, 1992). NANC nerves also affect vascular permeability and they can cause bronchoconstriction as well as dilation. Vasoactive intestinal peptide was thought to be the likely neurotransmitter for human NANC relaxation but alpha-chymotrypsin which degrades vasoactive peptide (VIP) has no effect on NANC bronchodilation in man. There is evidence NANC bronchodilation is mediated by nitric oxide (NO) since it is attenuated by the addition of NO synthetase inhibitors in vitro and this effect is partially reversed by addition of L-arginine, the precursor of NO (Belvisi, 1992). NANC bronchoconstriction may be mediated by substance P (SP) which is located in afferent axons of unmyelinated sensory nerves: SP inhalation causes bronchoconstriction in guinea pigs (Lotvall, 1990) and SP receptors are found on airway smooth muscle (Joos, 1989). It has been postulated that the physiological mechanism for transmitter release from *sensory* nerve endings might be a form of reflex retrograde impulse transmission along the axon tree.

1.3.6 SUMMARY

Modern research is beginning to unravel atopic asthma. Airborne antigen which enters the lung is processed by antigen presenting cells and recognised by T-lymphocytes which become activated and produce mediators promote inflammation by attracting and upgrading the activity of a number of different cell types. Allergen may also bind to specific IgE on the surface of mast cells which then release stored and newly formed inflammatory mediators. An inflammatory infiltrate accumulates, which includes numerous eosinophils and lymphocytes. Eosinophils release toxic substances, leading to tissue damage and oedema, bronchial hyper-responsiveness and bronchoconstriction. Mast cell products have similar effects. In addition, there appear

processes are associated with, and may be the cause of, the syndrome of recurrent symptomatic airway narrowing that we know as asthma.

1.4 PART II: HISTORY OF THEOPHYLLINE IN RELATION TO ASTHMA

In 1860 Henry Hyde Salter described the efficacious use of strong coffee taken on an empty stomach as a treatment for asthma (Salter, 1860): the recommended quantity, two breakfast cups, probably contained enough caffeine to produce significant bronchodilation (Becker, 1984). Theophylline and caffeine are similar compounds but theophylline is more potent a bronchodilator than caffeine (by approximately two and a half times)(Gong, 1986)).

Theophylline was first isolated from tea in 1888 and it came into clinical use initially as a diuretic. In vitro and animal work early in this century revealed an ability to relax airway smooth muscle and in 1922 theophylline suppositories were found to be effective in asthmatic subjects (Persson, 1986a). In 1931 Herrmen and Aynesworth successfully treated a single patient with intravenous aminophylline and 6 years later they reported a prompt and successful outcome after intravenous treatment in 14 out of 16 patients who had failed to respond to adrenaline. In the same year, Green, Paul and Feller published results showing an increase in vital capacity after intravenous aminophylline in 16 patients (Green, 1937). The ability of the drug to reverse severe bronchoconstriction was soon recognised (Brown, 1938) as was the rapid onset of relief (Carr, 1940). Further work in the 1950s and 1960s showed that oral preparations produce an improvement in symptoms associated with an increased timed vital capacity during stable chronic disease (Jackson, 1964) and during acute exacerbations (Turner-Warwick, 1957).

1.5 STRUCTURE

Theophylline is a di-methyl xanthine compound with two methyl groups attached to a basic xanthine structure. Caffeine, a tri-methyl xanthine, is a similar compound with an additional xanthine group [Figure 1].

1.6 RESPIRATORY ACTIONS OF THEOPHYLLINE

1.7 BRONCHODILATION.

Oral sustained release theophylline causes a significant increase in FEV₁ and in peak flow readings throughout the day in stable asthmatics (Alanko, 1983; Klein, 1983; Barlow, 1988) and the improvement persists during long term treatment (Grassi, 1987). When given as a single intravenous dose, aminophylline causes bronchodilation in stable asthmatics (Magnussen, 1986) even if the patient is already taking inhaled beta agonists and steroids. 1.7.2 Protection against bronchoconstriction.

Relatively low serum concentrations of theophylline (mean 6.4 µg/ml) have been shown to reduce the response of asthmatic airways to inhaled histamine and methacholine (Magnussen, 1987) and theophylline has been shown to protect against the pulmonary effects of *intravenous* histamine and methacholine challenges (Segal, 1949) and against bronchoconstriction after cold air inhalation (Magnussen, 1988). In normal subjects sensitivity to inhaled methacholine is also decreased by theophylline (Seppala, 1990).

1.8 EFFECTS ON SMOOTH MUSCLE TONE IN VITRO.

At therapeutic concentrations aminophylline induces a dose dependent relaxation of the spontaneous tone of resting guinea pig trachealis muscle. Theophylline has also been reported to relax both large and small human airways in vitro (Guillot, 1984; Finney, 1985). Results of experiments investigating the effect of theophylline on histamine induced smooth muscle contraction are conflicting. Allen found that theophylline antagonised the smooth muscle response to histamine in vitro and Supinski abolished the contractile response to histamine by adding theophylline at a concentration of 18µg/ml which is within the "therapeutic range" (Allen, 1986; Supinski, 1986). Other investigators however, have failed to show any effect on the histamine response, even at a concentration of 80µg/ml, although the effect on resting tone was demonstrated (Gustaffson, 1991).

1.9 MECHANISM OF BROCHODILATION

1.9.1 PHOSPHODIESTERASE INHIBITION

It has been suggested that theophylline causes bronchodilation by inhibition of the enzyme phosphodiesterase (PDE) which breaks down cyclic AMP, thereby increasing intracellular cyclic AMP concentration. Cyclic AMP is the second messenger which mediates the action of a number of cell surface receptor agonists including beta agonists and PGE₂ which relax bronchial muscle and cyclic AMP analogues relax bronchial smooth muscle. This mechanism is therefore plausible and is supported by the fact that there is correlation between the potency of theophylline and similar compounds in terms of tracheal relaxation and the inhibition of PDE in lung homogenates; this relationship has also been reported in uterine smooth muscle (Berg, 1987); however, there are a number of experimental findings which argue against PDE

inhibition as the chief mechanism of bronchodilation of theophylline. PDE is inhibited to a small degree at concentrations of theophylline which are therapeutically relevant (Bergstrand 1980) and dipyridamole which also inhibits PDE has no bronchodilator effect (Ruffin, 1981). Theophylline has been shown to relax airway in vitro without any significant change in cyclic nucleotide concentration (Kolbeck, 1979). A further criticism is that synergy between theophylline and beta agonists has not been demonstrated in terms of reduction of in vitro human bronchial smooth muscle tone (Handslip, 1981). If PDE inhibition were the major mode of action of theophylline there should be synergy with beta-agonists, which increase intracellular cyclic AMP production.

1.9.2 ADENOSINE RECEPTOR ANTAGONISM

Theophylline, at therapeutic concentrations, is a potent antagonist of adenosine receptors, suggesting a possible mechanism for its therapeutic effects. Adenosine is produced by several cell types, and its plasma concentration increases after inhaled antigen challenge in asthmatic subjects (Mann, 1986). Adenosine has little effect on human airway smooth muscle in vitro (Finnerly, 1985) and in isolated guinea pig trachea preparations it produces relaxation (Farmer, 1988), but when given by inhalation causes bronchoconstriction in asthmatic subjects (Cushley, 1983), suggesting that it may have an indirect effect which may be due to release mediators, possibly from airway mast cells. Theophylline inhibits adenosine-induced bronchoconstriction (Cushley, 1984). However, enprofylline, a related compound which is 3.8 times more potent than theophylline as a bronchodilator, has no significant effect on adenosine receptors (Persson, 1982; Persson, 1986b) suggesting that adenosine receptor antagonism is not important in the bronchodilator action of theophylline.

1.9.3 ENDOGENOUS CATECHOLAMINE RELEASE

Endogenous catecholamines, particularly adrenaline, may affect bronchomotor tone in asthma (Barnes, 1986) and theophylline increases the secretion of catecholamines from the adrenal medulla (Higbee, 1982). In normal subjects the bronchodilator response to aminophylline infusion is attenuated by propranolol, implying that endogenous stimulation of airway beta-receptors, presumably by circulating adrenaline, may contribute to the bronchodilator effect in normal subjects (Mackay, 1983).

1.9.4 PROSTAGLANDIN INHIBITION

Theophylline antagonises some of the effects of prostaglandins on vascular smooth muscle *in vitro* (Horrobin, 1977). However, these effects are seen at concentrations of theophylline which are much higher than those used therapeutically; Napier examined the effect of theophylline on PGF₂ and PGD₂ induced constriction in human bronchial smooth muscle and found no effect at the upper end of the therapeutic range for theophylline concentration (Napier, 1989), and are likely to be a reflection of functional antagonism, since theophylline inhibits smooth muscle contractions induced by a range of spasmogens.

1.9.5 EFFECTS ON CALCIUM MOBILISATION

Another explanation which has been suggested for the bronchodilator effect of theophylline is that there is some interference with calcium mobilisation in the target cell, since smooth muscle contraction depends on intracellular calcium (Middleton, 1984). Verapamil, which blocks cell surface calcium channels, has been found to inhibit isometric tension in guinea pig trachealis smooth muscle *in vitro* in experimental

anaphylaxis (Weiss, 1982) and nifedipine, with a similar action, relaxes Guinea pig and rat tracheal smooth muscle (Cheng, 1983). In vivo experiments have, however, shown that while nifedipine protects against exercise induced bronchoconstriction it has no bronchodilator action in mild asthmatics so prevention of calcium entry into cells is not likely to explain theophylline's bronchodilator action. Calcium might be important for other actions. For example, theophylline reduces mast cell histamine release in vitro, but this effect is not seen in calcium free media (Saeki, 1983).

1.10 EFFECTS OF THEOPHYLLINE ON THE IMMUNE SYSTEM

Recent attention has focused on evidence that theophylline may have potent anti-inflammatory activity (Pauwels, 1987). The model of airway inflammation in asthma that is most commonly used for the evaluation of the anti-inflammatory response is the late asthmatic reaction (Ward, 1993). When a susceptible asthmatic inhales an antigen to which he or she is sensitive, there is a fall in lung function within a few minutes called the "early asthmatic response" (EAR). The EAR is thought to be due in the main to the release of pre formed mediators, such as histamine, from mast cells. This recovers but may be followed 4-6 hours later by a delayed fall in lung function through recruitment and activation of inflammatory cells such as eosinophils and basophils and further release of pre-formed and newly formed mediators, termed the "late asthmatic reaction" (LAR). This LAR is therefore considered a useful model of airway inflammation in asthma and has been used to measure the putative "anti-inflammatory" effects of a wide variety of potential anti-asthmatic agents. Several studies of the effect of theophylline on the LAR have been reported: Pauwels et al (Pauwels, 1985) showed a clear inhibitory effect of a single intravenous dose of aminophylline and enprofylline, but not placebo, on the LAR, but not the EAR in a group of 5 asthmatics. Fabbris' group (Mapp, 1987) evaluated the effect of theophylline, verapamil, chromoglycate and beclomethasone on toluene diisocyanate EAR & LAR:

theophylline, like the corticosteroids had little effect of the EAR but significantly reduced the LAR (the other 2 drugs had little or no effect). Ward has shown that pre-treatment with oral theophylline reduces the magnitude of the LAR significantly and to a greater degree than the EAR (which did not change significantly) (Ward 1993) and Crescioli has shown that 7 days pre-treatment with theophylline has a greater effect on the LAR than the EAR (Crescioli 1991). Other workers have, however, demonstrated an effect during the EAR but not the LAR. (Cockroft, 1989).

1.11 IN VITRO EVIDENCE

One source of information on the potential effect of theophylline on the inflammatory response in asthma comes from experiments which have examined the effect of pre-incubation with theophylline upon the activity of immunocompetent cells. Another important source of information, albeit indirect, is the demonstration of the effects of an elevation of intracellular cyclic AMP on inflammatory cells, since theophylline has been shown to have such an effect at therapeutic concentration in polymorphonuclear leukocytes (Neilson, 1988) and in purified eosinophils (Kita, 1991). The mechanism of action of theophylline is, however, complex and multiple (Howell, 1990), and is therefore not necessarily reflected by the second type of experiment.

1.12 EOSINOPHILS

Using preparations of eosinophils taken from inflamed guinea pig peritoneum and from patients with peripheral blood eosinophilia who were receiving no medication, Yukawa has shown that theophylline reduces superoxide generation at supra therapeutic concentration. There was no effect at a concentration of 100 μ M which is close to the upper end of the therapeutic range. At 10 μ M there was an enhancement of eosinophil

superoxide production. The eosinophil preparations were specified as greater than 80% pure (Yukawa, 1989). Using >85% pure preparations of eosinophils from the peripheral blood of normal volunteers, Kita has stimulated cells with sepharose beads coated with immunoglobulins IgG or secretory IgA and measured the subsequent release of eosinophil derived neurotoxin (EDN). Addition of theophylline led to a concentration-dependent inhibition of stimulated EDN release with a 50% inhibition at 16 $\mu\text{g/ml}$ for IgG stimulation. Addition of cholera toxin to eosinophils causes a progressive rise in the intra-cellular cAMP concentration which is accompanied by a reduction in degranulation (Kita, 1991) suggesting that phosphodiesterase inhibition, which also leads to an increase in cAMP concentration within cells, might the potential to suppress eosinophil function.

1.13 NEUTROPHILS

Neilson has examined the effects of theophylline on un-separated peripheral blood polymorphonuclear leukocytes. The vast majority of these cells are neutrophils. The cellular respiratory burst, which generates oxygen radicals, was quantified by chemiluminescence. Theophylline, at concentrations above 1.8 $\mu\text{g/ml}$, reduced the magnitude of the respiratory burst after stimulation with either FMLP, a peptide receptor agonist, or a calcium ionophore. 40% inhibition was achieved at therapeutic concentrations (Neilson, 1986). The same group has also shown in separate work that therapeutic concentration of theophylline reduce leukotriene production by peripheral blood polymorphonuclear leukocytes from normals. At 9 $\mu\text{g/ml}$ theophylline reduced leukotriene B4 production by 50% and at 18 $\mu\text{g/ml}$, a concentration that is achievable in clinical practice, there was 90% reduction of leukotriene B4 production (Neilson, 1988). Radermaker assessed neutrophil chemotaxis by counting the number of purified peripheral blood neutrophils that crossed a millipore filter per unit time. Chemotaxis was reduced after incubation with theophylline, with a maximal effect of 70%

inhibition at very low theophylline concentration of only 0.2 µg/ml (Radremaker, 1992).

Neutrophils are recruited to the lung after local allergen challenge in asthma and they are present in increased numbers in the airway wall in people who have died after a short-lived, severe final asthma attack (Sur, 1993) but the role of these cells in asthma is unknown. The neutrophil is, however, by far the most abundant of the granulocytes in the peripheral blood and therefore one of the most accessible cells for the purpose of research. The fact that theophylline inhibits neutrophil activity in vitro is of interest in that it shows that theophylline has the potential to affect inflammatory cells and is more than a pure bronchodilator. Furthermore the processes that are inhibited in the neutrophil, such as leukotriene and oxygen radical generation, are common to the neutrophil and the eosinophil and it is surmised that theophylline may have similar inhibitory effects upon eosinophil function.

1.14 MAST CELLS.

Theophylline reduces in-vitro histamine release from rat peritoneal mast cells (i.e. connective tissue type mast cells similar to those found in the lungs) after stimulation by antigen or by anti IgE receptor antibodies (Pearce, 1982) and it reduces histamine release from human basophils and lung fragments (Louis, 1990). An increase in intracellular cAMP after treatment of mast cells with forskolin causes a marked reduction of PGD₂, LTB₄ and LTC₄ release after antigen stimulation and the addition of a phosphodiesterase inhibitor abolishes their release, lessens histamine release and impairs ionophore induced liberation of platelet activating factor and free arachidonic acid. These effects are seen after a cAMP elevation of less than 20% (Undem, 1990).

1.15 MONOCYTES AND MACROPHAGES

Peripheral blood mononuclear cells and bronchoalveolar lavage alveolar macrophages from normal subjects secrete less superoxide anion after exposure to low therapeutic concentrations of theophylline (5 µg/ml) in vitro (Calhoun, 1991). In vitro theophylline exposure leads to a blunting of the proliferation response to con A in normal adult monocytes and an increase in the concentration of cAMP within the cells. Incubation with theophylline leads to a significant inhibition of leukotriene B₄ production by peripheral blood monocytes. There is 48% reduction of LTB₄ release after calcium ionophore stimulation when cells have been incubated with theophylline at a concentration of 18 µg/ml and a 38% reduction at 1.8 µg/ml. Pre-incubation with theophylline also causes an increase in intracellular cAMP at concentrations above 1.8 µg/ml. There is an increase in the production of prostaglandin E₂, an eicosanoid which may have beneficial actions in asthma, at 18 µg/ml (Juergens, 1993). Other work has shown that increasing the concentration of cAMP causes a reduction in mouse macrophage IL1 production and a decrease in bone marrow macrophage proliferation and DNA synthesis (Brandwein, 1986; Vairo, 1990), which suggests that phosphodiesterase inhibition may have similar effects.

1.16 LYMPHOCYTES

If peripheral blood lymphocytes are incubated with theophylline they begin to suppress autologous cell responses (Zocchi, 1985) and this phenomenon can be shown to be due to the presence of a subgroup of T cells which is sensitive to in vitro stimulation by theophylline (Shore, 1978). This property was once used to define the T-suppressor sub-population of lymphocytes in the laboratory. Incubation with theophylline reduces lymphocyte expression of the IL2 receptor, which is involved in T cell activation (Rao,

1991) and manoeuvres that increase intracellular cAMP concentration inhibit the proliferation of T lymphocytes in vitro (Foresi, 1990).

1.17 NATURAL KILLER CELLS

Theophylline modulates the activity of natural killer cells. These cells are not currently thought to be involved in the inflammatory response in asthma and the inhibitory action of theophylline is of interest in so far as it illustrates a potential for inhibition of other cell types. Yokoyama and colleagues have shown that incubation with aminophylline leads to a dose dependent reduction in natural killer activity which is apparent at concentrations above 1.8 µg/ml (Yokoyama, 1990).

1.18 IN VIVO AND EX VIVO EVIDENCE

1.19 EOSINOPHILS

Inhaled allergen challenge in sensitised guinea pigs is followed by a period of airway eosinophilia which persists for at least 1 week. Theophylline has been shown to have an inhibitory effect on such an eosinophilic response. Sanjar treated ovalbumin sensitised guinea pigs with a continuous infusion of aminophylline for 6 days prior to challenge. The bronchoalveolar lavage eosinophilia after ovalbumin challenge was reduced significantly by 49% and the effect was similar in magnitude to the reduction by 57% after pre-treatment with dexamethasone (Sanjar, 1990). Tarayre has similarly shown a reduction in airway eosinophilia after allergen challenge in sensitised guinea pigs pre-treated with aminophylline (Tarayre, 1991). Yamada administered oral theophylline 1 hour before inhalation challenge in ovalbumin sensitised guinea pigs. There was a 66% reduction in lavage eosinophilia in theophylline treated guinea pigs,

although the difference did not achieve statistical significance (Yamada, 1992). Other work has shown theophylline and isbufylline to inhibit eosinophil recruitment into the airways after challenge with a range of stimuli (Llupia, 1991). In man, six months of treatment with theophylline in asthma has been shown to lead to a significant reduction in the concentration of eosinophil cationic protein in peripheral blood, suggesting a decrease in eosinophil activity and degranulation (Venge, 1992). Since eosinophils degranulate in tissue this finding suggests a decrease in the activity and/or number of eosinophils within the airway.

1.20 NEUTROPHILS.

In vivo theophylline has been shown to increase polymorphonuclear leukocyte intracellular cAMP concentration in normal subjects (Neilson, 1986) and to reduce the chemotaxis of neutrophils ex vivo obtained from asthmatic children treated for ten days with theophylline (Condino-Neto, 1991). Neilson has examined the effect of either seven days dosing with oral theophylline (mean theophylline concentration 9.4 μ g/ml) or 40 minutes intravenous infusion (mean concentration 7.3 μ g/ml) on the respiratory burst in peripheral blood polymorphonuclear leukocytes ex vivo. Five minutes after phlebotomy, when the blood had been diluted 100 fold to reduce the extra cellular theophylline concentration to a negligible level, there was enhancement of isoprenaline induced inhibition of the respiratory burst which had disappeared after 90 minutes, suggesting an effect due to residual intracellular theophylline (Neilson, 1986; Neilson, 1988).

1.21 MAST CELLS.

There is a reduction in histamine release when subjects with allergic rhinitis are challenged with antigen after a one week course of theophylline (Naclerio, 1985). There is also a reduction in the early response to allergen in the skin during treatment with theophylline but no change in the skin's response to injected histamine, indicating that theophylline has an effect on endogenous histamine release (Gronneberg, 1985).

1.22 MONOCYTES AND MACROPHAGES.

Alveolar macrophages have been shown to generate less superoxide *ex vivo* after *in vivo* theophylline treatment (Calhoun, 1991). Oral theophylline also reduces alveolar macrophage intracellular killing, bactericidal activity and hydrogen peroxide release and the degree of impairment correlates with bronchoalveolar lavage theophylline concentration (O'Neill, 1986). In pre-term human neonates the same effect is seen after *in vivo* aminophylline therapy (Bessler, 1988) and in asthmatic children theophylline treatment has also been shown to inhibit mononuclear cell chemotaxis *ex vivo* (Condino-Neto, 1991).

1.23 LYMPHOCYTES.

Fink has shown that when compared with asthmatics on other treatments, asthmatics on long term theophylline have more peripheral blood T suppresser cells, defined as cells that are able to suppress plaque formation in lymphocyte culture by the method of Shore (Shore, 1978). When lymphocytes from asthmatic subjects or from normals were injected into the skin of rats there was a graft-versus-host reaction in 90% of cases. When lymphocytes from subjects receiving theophylline were injected there was

a graft-versus-host reaction in only 10% of cases. Furthermore, in the group of normals whose cells did provoke a response incubation of lymphocytes with theophylline in vitro prior to injection into the rats abolished the graft versus host reaction in two thirds of cases (Fink, 1987). In other work, asthmatics have been shown to have abnormal T suppresser function: ConA is a substance that enhances T cell suppression of co-cultured lymphocytes from normal volunteers. This response to conA is impaired in asthmatics and oral theophylline partially restores the response at serum levels above 5 µg/ml (Ilfield, 1985). Two studies in asthmatic children have reported a decrease in the number of peripheral blood T lymphocytes which express suppresser activity and one of these found a correlation between T suppresser numbers and the severity of asthma. In both studies theophylline treatment led to a restoration of normal T suppresser numbers (Shohat, 1983; Lahat, 1985). Barnes' group has recently shown that withdrawal of chronic theophylline treatment in asthma is followed by clinical deterioration and an increase in the numbers of circulating activated CD4+ and CD8+ T- lymphocytes (Kidney, 1994a).

The immunomodulatory action of theophylline has been used successfully in the treatment of renal transplant rejection. When aminophylline was added to conventional prophylactic drug therapy after renal transplant there was a decrease in the dose of steroid required to control rejection episodes. There was also a fall in the CD4/CD8 T cell ratio (Guillou, 1984). Of particular importance was the finding that theophylline had an immuno suppressive action which may be separate from and additional to that of gluco-corticosteroids. In 4 patients with rejection episodes that were not responding to steroids the addition of aminophylline arrested the rejection and increased T suppresser cell numbers (Shohat, 1983). Theophylline also increases T suppresser cell function during renal transplant rejection episodes, increasing the degree of inhibition of the GVH response (Shapira, 1983). Rats, after experimental heart transplant, similarly exhibit increased graft survival with theophylline treatment (Rugarli, 1983).

1.24 THEOPHYLLINE AND AIRWAY INFLAMMATION

The extensive body of evidence that theophylline inhibits a range of different leukocytes both in vitro and in vivo and the attenuation of the LAR that follows pre-treatment with theophylline suggest that theophylline may have an effect on cellular infiltration in the airway wall in asthma. Withdrawal of chronic oral theophylline treatment from patients with asthma has recently been shown to be accompanied by clinical deterioration and an increase in the number of lymphocytes seen in bronchial biopsies (Kidney, 1994b) but as yet there are no data on the effect of addition of theophylline treatment on inflammatory changes within the lung in man.

CHAPTER 2: METHODS

2.1 HYPOTHESIS

Previous work has demonstrated that low serum theophylline concentrations reduce the magnitude of the late asthmatic response after allergen inhalation and it has been suggested that this effect may be mediated by an anti-inflammatory action. The present study has investigated the hypothesis that low dose oral theophylline has anti-inflammatory effects during the LAR. The inflammatory infiltrate in asthma consists largely of T lymphocytes and eosinophils. The initial hypothesis that the study was designed to address was that theophylline affects the number of T-lymphocytes or eosinophils within the sub-epithelial region airway 24 hour after allergen challenge when there has been a LAR. The hypothesis was tested by measurement of the CD3 positive T lymphocyte count and count of eosinophils identified on H+E sections in sub-epithelial bronchial tissue. Changes in these values after treatment were compared between the active theophylline and the placebo groups in a parallel group study.

The effect of theophylline on a number of other variables was also assessed during the study. These included the number of activated eosinophils within the airway wall, the number of mast cells, the number of lymphocyte subsets and activated subsets in tissue, bronchoalveolar lavage and peripheral blood and the number of inflammatory cells and concentration of mediators in bronchoalveolar lavage fluid. All comparisons except for those involving the treatment effect of theophylline on T-lymphocyte counts and total eosinophil counts in bronchial tissues are regarded as exploratory.

2.2 SUBJECTS

Subjects were non smoking mild atopic asthmatics aged between 18 and 45 years with an FEV₁ greater than 80% of the predicted value. They were all allergic to house dust

mite antigen, Der P1, with a positive weal reaction of at least 3mm to skin prick testing. They all exhibited both an early asthmatic response and late asthmatic response after inhalation of Der P1 antigen represented by a fall in FEV₁ of at least 15% and 20% respectively. All subjects were taking only inhaled salbutamol as required for the treatment of asthma before the study.

The diagnosis of atopic asthma was based on the presence of :

- i. a diagnosis of asthma by medical practitioner at least 1 year prior to the study,
- ii. a history of intermittent breathlessness, chest tightness and/or wheezing,
- iii. a history of relief of the above symptoms after inhaled beta agonist treatment.
- iv. a positive skin weal response to intra-dermal Der P1 allergen

Whilst it is recognised that *atopic non-asthmatic* individuals may develop bronchoconstriction after allergen challenge (Miadonna, 1990), the presence of a classical history of asthma, including a clear history of a clinical response to inhaled bronchodilators and a dual response to inhaled allergen were regarded as sufficient criteria for entry into the study: it was not felt that additional tests such as histamine challenge were necessary.

2.3 INCLUSION CRITERIA

1. Clinical history consistent with asthma, including a history of clear symptomatic benefit from inhaled bronchodilators,
2. FEV₁ at least 80% of predicted value,
3. Age between 18 and 55 years,
4. Able to give informed consent.

2.4 EXCLUSION CRITERIA

1. history of hypersensitivity to methyl xanthines, salbutamol, atropine, alfentanil, midazolam lignocaine or corticosteroids.
2. history of severe renal, hepatic or cardiac disease, convulsions or of dyspepsia which has required medical treatment.
3. currently taking any medication other than inhaled salbutamol, oral contraceptive preparations or paracetamol.
4. an exacerbation of asthma lasting more than 12 hours in the past six weeks.
5. any steroid medication in the last 6 weeks.
6. is pregnant, lactating or likely to become pregnant during the study.
7. is a smoker or has smoked a cigarette in the last 12 months.
8. is likely to travel outside Europe during the treatment period

2.5 SCREENING

Volunteers attended the department at 8:30 am having taken no caffeine containing foods or beverages for at least 12 hours and no inhaled medication for at least 4 hours.

Subjects were interviewed to establish that their symptoms were compatible with asthma. The study procedures were explained in detail to the subjects, who were also required to read printed information describing the study. A consent form was then signed.

A Wright Spirometer was calibrated with a 1 litre calibration syringe. The same spirometer was used for all measurements throughout the study and it was always accurate within 2% during calibration. The subject performed 3 forced expiratory manoeuvres at 1 minute intervals. The subject's height was measured with a standard

clinical height bar and the predicted value for the forced expiratory volume in the first second (FEV_1) was calculated according to Cotes (Cotes, 1979). Each subject performed three forced expiratory manoeuvres using the calibrated spirometer. The mean of three values was taken as the study base-line. Subjects were allowed to enter the study if all three readings were at least 80% of their predicted value.

2.6 SKIN TESTING

Skin tests were performed for allergy to house dust mite. A single drop of commercial "Soluprick" house dust mite allergen extract was applied along the extensor surface of a forearm and the skin was breached gently with a fine needle. The excess solution was removed and the size of the palpable weal response was measured after 10 minutes. Two measurements were made, one across the widest part of the weal and one along a line perpendicular to the first. If the mean of the two measurements was above 3mm the subject was considered to be allergic to house dust mite.

2.7 CONTROL INHALATION CHALLENGE.

The FEV_1 was recorded three times to ensure that bronchoconstriction had not occurred as a result of the skin testing. 2ml of ALK-DIL, the vehicle for inhaled house dust mite antigen, was measured into a Wright Nebuliser which had been calibrated to deliver 0.26 mg of nebulised solution over 2 minutes when driven by air at a flow rate of 7 litres per minute. For each subject the same nebuliser and cylinder head were used before and after treatment. The subject was instructed to take normal breaths from the mask of the nebuliser through an open mouth for a timed period of 2 minutes.

Three FEV₁ recordings were made after 5 minutes and the mean value recorded. This was repeated at 10 and 15 minutes. In order to continue in the study the mean FEV₁ on all three occasions was required to be within 10% of the baseline.

2.8 ALLERGEN INHALATION CHALLENGE

2ml of a solution of Der P1 antigen in ALK-DIL at a concentration of 800 Sq units per ml (see *materials*) was measured into a clean nebuliser bottle. The subject was seated in an enclosed glass fronted cubicle with an air extraction system and nebulised solution was administered as above for exactly 2 minutes. FEV₁ was measured as the mean of 3 values 5, 10 and 15 minutes after antigen administration.

If there was not a 15% fall in FEV₁ and at the end of the 15 minute period the FEV₁ was within 5% of baseline, the subject received nebulised house dust mite antigen which was 5 times more concentrated than the previous solution. If, after 15 minutes, the FEV₁ was not within 5% of the baseline spirometry was repeated at 5 minute intervals until the value had returned to within 5% before proceeding. This process was repeated until the subject had demonstrated an early asthmatic reaction or had inhaled house dust mite antigen at a concentration of 100,000 SQ units per ml. If no early response occurred after administration of this solution the subject was excluded from the study.

Once a 15% fall in FEV₁ had occurred spirometry was repeated 20 minutes after antigen administration and then at 10 minute intervals up to 1 hour after challenge. Spirometry was then performed every 30 minutes until the FEV₁ fell below the baseline value by 20%. If there was no secondary fall within 8 hours of antigen challenge the subject was excluded from further study. If a 20% fall occurred within 8

hours of challenge a late asthmatic reaction was said to have occurred. The subject then received 2.5 mg of salbutamol via a nebuliser driven by air at 6 litres per minute.

A peak flow meter (MINI-WRIGHT) and diary card were issued and the subject was instructed in their use. The diary card included records of thrice daily peak flow readings (on waking, early evening, on retiring), as the best of three measurements on each occasion, number of doses of rescue medication used, and a simple asthma symptom score for each day and night. The subject was given a portable nebuliser and a supply of salbutamol nebuliser solution to take away.

2.9 NUMBER OF SUBJECTS AND RANDOMISATION

At this stage the subject was allocated a subject number. These were consecutive numbers, starting at "1". Each number had previously been designated as part of either the "ACTIVE" or "PLACEBO" group. 23 subjects entered and 19 subjects completed the study. Some data were not available for all subjects for technical reasons (equipment failure or inadequate samples) and the number of subjects therefore varies for different measurements. The actual 'n' value for each parameter is given in the relevant tables.

2.10 PRE-TREATMENT ALLERGEN CHALLENGE

After a run in period of at least 3 weeks the subjects came back to the department in the morning having taken no caffeine for 12 hours and no bronchodilators for 4 hours. Three forced expiratory manoeuvres were performed using the Wright Vitalograph, as

above, at 1 minute intervals. In order to continue in the study all FEV₁ readings were required to be greater than 80% of the predicted value. The diary card was checked and compliance with instructions assessed. The subject was asked an open question: "*Have you recently had any symptoms of any kind other than those of asthma?*" If any symptoms were reported the subject was asked about the duration and severity and the latter was recorded as "mild", "moderate" or "severe".

The subject then received an inhalation challenge with ALK-DIL solution by the method outlined above. The mean of three FEV₁ readings was recorded at 5, 10 and 15 minutes. In order to continue in the study the FEV₁ had to remain within 5% of the baseline. The lowest FEV₁ recorded after this control challenge was taken as the baseline for the rest of the day. Nebulised house dust mite antigen was then administered for exactly 2 minutes, at the concentration which had produced a satisfactory late response during the initial visit. Spirometry was performed at 5 minute intervals for 20 minutes, then at 10 minute intervals during the first hour and subsequently at 30 minute intervals for a further 5 hours.

Salbutamol, 2.5 mg via a nebuliser, was administered after 12 hours or earlier if the investigator considered it necessary.

Samples of peripheral blood were obtained by venesection with the subject seated from a vein in the antecubital fossa. A 18fg Butterfly cannula was inserted into the vein. The first 5ml of blood were discarded and two samples for lymphocyte analysis were taken into containers prepared with EDTA. Blood samples were taken before and after treatment with theophylline or placebo;

- i) at baseline, immediately before allergen challenge,
- ii) 15 minutes after allergen challenge, during the EAR,

- iii) during the LAR, 6.5 hours after allergen challenge.
- iv) 24 hours after allergen challenge.

The subject remained in the hospital overnight in a special research department supervised by a nurse trained in intensive care. Emergency intubation equipment was available and there was a cardiac arrest team on site. An investigator was available by aircall during the night.

The following morning the subject underwent bronchoscopy. Inhaled salbutamol was allowed until 6 hours before the bronchoscopy and no food or drink was allowed within 4 hours of the procedure. Caffeine containing foods and beverages were avoided for 12 hours before allergen challenge and 36 hours before bronchoscopy.

2.11 BRONCHOSCOPY

Spirometry was performed as above. In order to continue in the study the subject was required to have an $FEV_1 > 80\%$ of predicted *or of the baseline during the screening visit*.

The subjects received 2.5 mg of salbutamol via a nebuliser driven by air at a flow rate of 6 litres per minute 15 minutes before bronchoscopy. Venous access was established with a Venflon intravenous cannula, and the subject was connected to an ECG monitor and a finger pulse oximeter. Throughout the procedure the subject received supplemental oxygen via nasal prongs at a flow rate of at least 4 litres per minute. Full resuscitation equipment was available in the bronchoscopy room and was checked before every procedure.

4% lignocaine spray was applied to the posterior part of the oropharynx and to the nasopharynx. The subject received 600µg of atropine and either 500-1000µg of alfentanil or 5-10mg of midazolam intravenously. Alfentanil was used unless the subject had a history of unpleasant reactions after opiates.

The same bronchoscope was used for every procedure (see appendix). The bronchoscope was inserted into a nostril and passed through the nasopharynx. The true vocal cords were visualised and 4 ml of 4% lignocaine was applied to the cords. The bronchoscope was then advanced into the trachea and 2ml of 2% lignocaine was applied to each of the main carina, the opening of the right middle lobe segmental bronchus and the lower end of the left main bronchus.

Biopsies were taken with Olympus 15C alligator biopsy forceps which were never used more than 5 times to ensure sharpness. 4 biopsies were taken from the sub-carinas separating the sub-segmental bronchi of the left lower lobe. The bronchoscope was then withdrawn into the left main bronchus and 5ml of saline followed by 10 ml of air were injected through the biopsy channel to remove any blood. The saline was aspirated through the suction channel and air was then aspirated for 15 seconds to clear the suction channel prior to bronchial lavage. This procedure was repeated until clear fluid was aspirated, with no macroscopically visible blood staining.

The bronchoscope was then passed into the right middle lobe and wedged in an airway in the medial segment. If it was possible to advance the bronchoscope to the next division it was passed into the most anterior of the subdivisions. Bronchoalveolar lavage was performed by injecting three aliquots of 60ml of sterile normal saline which had been kept at 37 degrees centigrade in a water bath. As much fluid as possible was aspirated after each injection, using intermittent low pressure suction to avoid trauma and airway collapse. After the third aliquot had been aspirated the bronchoscope tip

was withdrawn into the right main bronchus. If there was any visible local bronchoconstriction 2.5mg of salbutamol, as nebuliser solution, dissolved in 10ml of saline was injected through the bronchoscope into the right middle lobe. The bronchoscope was then removed.

Supplemental oxygen and pulse oximetry were continued for at least 15 minutes and subjects were supervised for at least 3 hours after the bronchoscopy. If there was any evidence of bronchospasm nebulised salbutamol was administered as required.

2.12 TREATMENT

The following day the subject commenced treatment with either Uniphyllin continus tablets, 200mg 12 hourly, or identical placebo tablets: neither the subject nor the investigator knew which treatment had been allocated. Randomisation had taken place before the study and the medications sealed into containers, numbered for each individual subject.

2.13 ASSESSMENT OF EFFICACY

In order to assess the clinical efficacy of the maintenance of a serum theophylline concentration of less than 10µg/ml subjects were asked to complete a daily diary card. Peak expiratory flow measurements were recorded using a Mini-Wright peak flow meter on waking, in the early evening and before retiring to bed; the best of three values was recorded on each occasion. The subjects were also asked to record the number of puffs of salbutamol rescue medication that they used each day and night and to assess the severity of asthma on a simple scoring system during the day and the

night. The subjects also underwent spirometry, having taken no rescue medication for at least 4 hours, before and after 6 weeks of treatment with theophylline or placebo.

2.14 ASSESSMENT OF ADVERSE EFFECTS.

Subjects were asked an open question ("have you recently had any symptoms of any kind other than those of asthma") during the baseline visit, after 1 week of treatment, after 2 weeks of treatment and at the end of the treatment period. (At the end of the treatment period they were also asked specifically whether they had experienced **nausea, vomiting, tremor, insomnia or rash**). Any positive responses were followed by an enquiry as to whether the symptom was **mild, moderate or severe**, and the approximate duration of the symptom was recorded. In the interim, subjects were instructed to contact the investigator via an emergency air-call should they develop any problems.

2.15 DOSAGE TITRATION

After 5-10 days the subject attended the department. The subject was asked whether he or she had experienced any symptoms other than those of asthma and, if so, the severity was recorded as mild, moderate or severe. A finger prick capillary blood sample was obtained and tested for theophylline by the Acculevel test. The investigators were not present during the test and did not see the result. Instead, an unblinded member of staff passed the result on to an investigator if the subject was on active treatment. If the subject was on placebo treatment she selected a bogus value from a list of results which had been prepared before the study, and gave it to the investigator. A computer programme, PREDICTOR (Napp Laboratories, Cambridge), was used to calculate the dose of theophylline required to achieve a mean serum

theophylline concentration of 5 µg/ml. If that dose was less than or equal to 200mg 12 hourly the subject continued with that dose. Otherwise the dose was adjusted. The subject was issued with the relevant combination of 200mg and 300mg tablets and written dosage instructions. Unused medication was collected and the tablets were counted. After 5-10 days the same procedure was repeated and a further supply of drugs was issued. Unused medication was collected and counted.

2.16 POST-TREATMENT ALLERGEN CHALLENGE

After a period of at least 6 weeks the subject returned to the department having taken no caffeine for 12 hours or inhalers for 4 hours. He or she was reminded to continue to take the medication that morning and evening.

Spirometry was recorded and the subject continued in the study only if the FEV₁ was greater than 80% of the predicted value. Antigen challenge, blood testing and spirometry were performed in exactly the same way as they had been during visit 2. At the appropriate time in the evening the subject was reminded to take the theophylline/placebo tablets. If a salbutamol nebuliser had been given earlier than 12 hours after challenge during visit 2 a nebuliser was given at the same time post challenge on visit 5, even if the subject was clinically well.

The procedures on the following day were exactly as for visit 2, except that the subject was allowed to take the trial medication with 30 ml of water at the usual time and that blood was taken for theophylline level at the time of bronchoscopy and 4 hours after the last dose.

2.17 SERUM THEOPHYLLINE CONCENTRATION MEASUREMENTS

At the end of the study a sample of blood was taken by venipuncture at the time of bronchoscopy and again 4 hours after the last dose of trial medication. The serum was separated by centrifugation at 3000 rpm for 5 minutes, stored at minus 20 degrees centigrade for up to 6 months and then analysed by a EMIT assay by Napp Laboratories, Cambridge.

2.18 BIOPSIES

Biopsies were always taken before bronchoalveolar lavage was performed. Four biopsies were taken from left lower lobe basal segmental sub-carinae with alligator type forceps (Olympus FB-15C): sampling continued until it appeared that 4 adequate biopsies had been obtained. The tissue was removed immediately from the forceps by gentle agitation in phosphate buffered saline cooled on ice. Occasionally a needle was used to carefully dislodge tissue from the jaws. The forceps were rinsed in sterile normal saline before reintroduction to the airway.

A vessel containing liquid isopentane was lowered into liquid nitrogen and cooled until most of the isopentane became solid. The vessel was removed from the nitrogen and the isopentane allowed to partially melt. A drop of Optimum Cutting Temperature (OCT) medium was placed on a cork disc and immersed in cold isopentane until frozen. Another drop of OCT medium was added to the first and a single biopsy was carefully removed from the saline on a spatula and placed in the liquid OCT. The disc, OCT and biopsy were plunged into the isopentane and held there for 15 seconds. They were then wrapped in aluminium foil and placed in an airtight container surrounded by liquid nitrogen.

Biopsies were transferred to a freezer and stored at minus 80° centigrade. They were transferred in liquid nitrogen to a cryostat at minus 24° centigrade and 6µm sections were cut and discarded until tissue was visible in the cut edge of the block. A 6µm section was then taken and placed on a glass microscope slide. It was immersed for 2 minutes in a filtered Harris Haematoxylin stain, washed for 30 seconds in running tap water, immersed for 30 seconds in Eosin stain and washed in tap water for 5 seconds. 1 drop of 50% glycerine and 50% phosphate buffered saline solution was placed on the section and a glass cover slip was applied. The section was then examined by light microscopy and the quality of the tissue section was assessed. If no basement membrane was present, 5 further sections, each 6µm thick were discarded and the process was repeated until an adequate section with basement membrane was obtained. Consecutive sections, 6µm thick, were then cut. Each section was allowed to rest on the cryostat knife for 10 seconds to prevent curling. It was then picked up onto a glass microscope slide. The commercially available slides are coated with teflon material with three wells etched to expose clear glass. A tissue section was placed in the centre of each well. Every fifteenth section was placed alone on a glass slide and stained with Haematoxylin and eosin (H+E), as above, and it was examined immediately. When the area of the tissue sections became so small that there was not a zone 60µm wide below the basement membrane the biopsy was discarded. The H+E stained sections were labelled and retained. The unstained sections were placed in slide racks and covered loosely with aluminium foil. They were allowed to dry overnight before fixation for exactly 2 minutes in a mixture of 50% acetone and 50% methanol at room temperature. After a further 1 hour air drying, the slides were wrapped in aluminium foil and stored at minus 80° centigrade for up to 16 weeks.

2.19 CONTROL TISSUE

Fresh inflamed human tonsils were collected from children undergoing routine tonsillectomy in King's College Hospital. Tissue was suspended immediately at the time of operation in phosphate buffered saline cooled on ice and subsequent processing was as for bronchial biopsy tissue.

2.20 STAINING

Aqueous H+E stained sections were examined and those with a 60µm band of intact tissue beneath the basement membrane were noted. Unstained, fixed sections that had been taken from within 30µm of acceptable H+E sections were selected randomly. Sections were examined by phase contrast microscopy to ensure that the tissue had not folded upon itself. For each subject specimens taken before and after the treatment period were included in the same staining "run".

2.21 HAEMATOXYLIN AND EOSIN - PERMANENT STAINING

1. Slides were removed from the freezer 1 hour before staining. They were immersed in the following solutions:
2. Freshly filtered Harris Haematoxylin for 60 seconds
3. Running water for 60 seconds
4. Acid alcohol for 10 seconds
5. Running water for 60 seconds
6. Eosin for 45 seconds
7. Running water for 10 seconds
8. Ethyl Alcohol, 70%, for 5 seconds

9. Ethyl Alcohol, 90%, for 5 seconds
10. Ethyl Alcohol, 100%, for 5 seconds
11. Ethyl Alcohol, 100%, in a separate container, for 5 seconds
12. CNP-30 clearing agent for 5 seconds
13. CNP-30 clearing agent, in a separate container, for 5 seconds
14. CNP-30 clearing agent, in a separate container, until mounting
15. The sections were set in DPX polymer mountant which was miscible with the clearing agent and a cover slip was applied.
16. Slides were stored at 4 degrees celcius.

2.22 IMMUNO-HISTOCHEMISTRY

Immuno-histochemistry is a technique which allows visualisation of the distribution of molecules within a two dimensional section of tissue. A monoclonal antibody which is specific for a particular marker, for example a cell surface receptor, is first added to a very thin tissue section. Various methods have been devised to translate the localisation of such antibody to a visible image which can be examined by light microscopy. In the present study the APAAP technique is used. Normal human serum is added to block non-specific tissue protein binding. The tissue is incubated with mouse monoclonal IgG immunoglobulin which recognises one of a variety of cell markers. The next step involves the addition of a polyclonal rabbit antibody directed against mouse IgG. A number of rabbit anti mouse (RAM) antibodies can bind to one mouse antibody because their polyclonal nature allows binding to a number of different molecular sites. A mouse IgG antibody bound to alkaline phosphatase, known as alkaline phosphatase anti alkaline phosphatase complex (APAAP), is added next. The RAM which was bound to marker antibody can also bind the APAAP, forming a cross link between tissue marker and APAAP and because the RAM is polyclonal more than one APAAP antibody can bind to each anti-marker antibody. This is known as

amplification. The chromagen, fast blue salt, is converted to a visible blue compound by alkaline phosphatase enzyme, creating an image of tissue marker distribution. Levamisole is added to the stain to neutralise any native alkaline phosphatase which would otherwise produce false positive staining.

2.23 IMMUNO-STAINING BY APAAP TECHNIQUE

The slides were removed from the freezer 1 hour before immuno-histochemical staining and allowed to warm to room temperature. Slides were labelled and placed in a damp-chamber in which tap water was allowed to evaporate in order maintain a humid atmosphere and prevent evaporation of the solutions on the slides and subsequent concentration of the reagents.

Tris buffered saline was prepared as described in the "materials" section at the end of this thesis and the pH adjusted to 7.6 by the addition of 1 Molar sodium hydroxide solution or hydrochloric acid. Commercial normal human serum (NHS), which had been aliquoted and stored at minus 20° centigrade, was taken from the freezer and allowed to thaw. Mouse monoclonal antibodies to cell surface markers were diluted in a mixture of 90% tris-buffered saline at pH 7.6 and 10% normal human serum: the dilution factors had previously been determined for each antibody by titration of staining quality in sections of inflamed human tonsil and human bronchial wall. Rabbit anti mouse (RAM) antibodies and alkaline phosphatase anti-alkaline phosphatase antibodies (APAAP) were diluted according to the manufacturers instructions in a mixture of 80% tris-buffered saline at pH 7.6 and 20 % normal human serum immediately prior to use.

50µl of the appropriate mouse monoclonal antibody solution was added to the well containing each biopsy section. Care was taken to ensure that the tissue sections were

completely covered with solution and that the solution was contained entirely by the wells and was not likely to run off the sections. One tonsil section was stained with each monoclonal antibody, as a positive control for the run. One tonsil section was also incubated with normal human serum/tris buffered saline solution rather than a monoclonal antibody solution to act as a negative control. Bronchial tissue sections from the same patients were also included as a negative control, treated with normal human serum/Tris buffered saline solution.

After 40 minutes the slides were tipped to 45° to remove most of the antibody solution and washed for 2 minutes in agitated tris-buffered saline at pH 7.6. Slides were removed from the wash solution three at a time and tapped to remove excess solution from the wells. Excess liquid was wiped from the teflon surface of the slides and the slides returned to the humidity chamber. 50µl of rabbit anti-mouse solution was added to each section. Care was taken to ensure that none of the sections were allowed to dry between the washing stage and the addition of the antibody solution.

After a further 30 minutes the slides were tipped to remove excess antibody and washed for 2 minutes in tris buffered saline at pH 7.6. They were returned to the humidity chamber and 50 µl of a solution of "alkaline phosphatase anti-alkaline phosphatase" monoclonal antibody/enzyme complex in 80% tris buffered saline and 20% normal human serum was added to each section, as above.

The fast-blue stain was prepared during the final incubation. 10mg of fast blue B salt was added to 10 ml of tris buffered saline with its pH adjusted to 8.2: 2mg of naphthol AsMx phosphate were dissolved in 200µl of dimethylformamide and the resulting solution was added to the tris buffered saline: 10µl of levamisole was added to the mixture immediately before tissue staining and the solutions were mixed by inversion.

The slides were washed for 3 minutes in tris buffered saline, pH 7.6 and returned to the humidity chamber. The fast blue stain was poured onto the sections through a filter paper. After 15 minutes the slides were washed in running tap water for 1 minute and immersed in fast neutral red stain for 3 minutes. They were washed in tap water for 1 minute and mounted in Aquamount medium under a glass cover slip.

Negative control tonsil sections were examined by light microscopy to ensure that no background staining had occurred. Positive control tonsil sections were examined to assess the staining pattern and clarity for each monoclonal primary antibody. Anti-CD25 stained bronchial biopsy tissue was also used as a negative control since only a small number of cells were positive and background staining would be easily detected.

The slides were stored at 4° centigrade and cell counts were made within 2 weeks.

2.24 CELL COUNTING

All of the counts of each marker were performed by the author using exactly the same microscope, lenses and graticules in the manner described below.

2.25 BLINDING

A coded plain opaque paper label was applied to all slides in such a way that the identification marks were completely covered and that the origin of the tissue could not be determined without removing the label. This was an additional precaution, since at the time of counting the investigators did not know which subjects had received active medication. At the end of each day's counting the labels were removed and the individual cell counts were only then attributed to the relevant subjects. The

investigators remained ignorant of which subjects had received active theophylline treatment until all of the cell counts were completed.

2.26 SELECTION OF AREA FOR TO BE COUNTED.

Lymphocytes , eosinophils and the total cell number were all counted in a band 60µm deep below the basement membrane. Initial examination of the stained tissue sections showed that the majority of these cells were close to the basement membrane. The 60µm depth was selected because in many areas the lamina propria had split along a line that was parallel to the basement membrane. When this occurred there was evidence of local oedema, with a fine reticular appearance, suggesting that the tissue might be particularly inflamed in these areas and therefore that to exclude these areas from the cell counts would risk the introduction of artefact. Virtually all of the lamina propria was intact to 60µm in this study and for every selected tissue section all such lamina propria was counted in order to avoid selection bias.

Areas of tissue were rejected :

- i) if there was not a full 60µm depth of intact lamina propria below the basement membrane, either because of damage to the tissue or because smooth muscle or gland tissue was close to the basement membrane.
- ii) if there were appearances on the corresponding H+E slide to suggest that cells had been crushed by the biopsy forceps (i.e. the presence of large numbers of crowded elongated nuclei with little cytoplasm visible).
- iii) if there was a prominent band of fibroblast like cells below the basement membrane to a depth of more than 30µm. If there was a band of these cells less than

30 μ m wide counts were performed in a band extending 60 μ m from these cells. Infiltrating cells did not penetrate the bundles of these fibroblasts or myofibroblasts and therefore to include them in the counting area would lead to artificially low counts in some cases.

Mast cells, interleukin 4 (IL4) and IL5 (IL5) positive cells were counted in the whole tissue section, since these cells did not appear to be concentrated under the basement membrane, but seemed to be distributed evenly throughout the sections. Additionally, mast cells were counted in a 60 μ m sub-epithelial band.

Cells were counted at 480 times magnification. When counting below the basement membrane a square eyepiece graticule, measuring 60 μ m X 60 μ m at 480 times magnification, was used in conjunction with a focusing eye-piece. The section was moved so that adjacent squares were counted along the basement membrane: when "turning a corner" care was taken not to miss cells or count them twice. When counting the entire section a large graticule that measured 200 μ m square was used. The tissue was moved so that adjacent squares were counted one by one, in strips 200 μ m wide.

A detailed sketch was made of each section at 120 times magnification, indicating the stretches of basement membrane that had been counted. After the cell counts were read the section was transferred to an image analysis system (Improvision UK). A monochrome video camera fed an image of the section to a digitalisation board. The resulting picture was displayed on the monitor of a Macintosh computer running appropriate software (Image 1.38). Grey-scale contrast enhancement was used to improve the image quality. The exact length of the basement membrane over the lamina propria where the cells had been counted was measured by moving the computer mouse to trace the image of the basement membrane. The result was given in arbitrary pixel units and was converted to millimetres by calibration with an object

micrometer. The cell count was then expressed per millimetre of basement membrane. When cells had been counted in the entire section a similar technique was used to trace around the outline of the tissue. The image analysis system then calculated the area of tissue and the cell count was expressed as cells per mm².

Extracellular EG2 staining was quantified by point counting. A graticule that measured 200µm square at 480X magnification was used. The graticule had 100 crosses within the outer square arranged regularly in 10 rows of 10. The crosses were fine and the position of the centre of each cross was very precise. The section was viewed through the graticule and the number of crosses that were over tissue was noted. The number of crosses lying over extracellular and the number over intracellular positive staining was recorded (see below). The section was moved on and the next square counted. The process was then repeated in a band 100µm wide below the basement membrane.

2.27 CRITERIA FOR POSITIVE STAINING

Specific criteria were used throughout the study to identify cells that should be counted as "positive" for each marker. Criteria were based upon knowledge of the morphology of the relevant cells but were to a certain extent arbitrary. The same criteria were strictly applied throughout the study.

Lymphocytes

The same criteria were used to select positive cells for all of the lymphocyte surface markers stained with the APAAP technique (CD3, CD4, CD8 and CD25). A cell was counted as positive if it gave the appearance of a single nucleus (stained red by the counterstain) surrounded by a thin blue line which covered at least 50% of the cell circumference.

Mast Cells

A cell was counted as positive only if there was an area of blue (positive) staining which surrounded a nucleus and which was clearly demarcated from the surrounding tissue. The shape of the cell was not considered as mast cells may be round or elongated. If there was an area of blue staining in apposition to, but not surrounding a nucleus, it was considered to be a mast cell only if there was a definite appearance of granularity with a clearly demarcated outer border.

Eosinophils

Cells were counted as EG2 positive eosinophils if they were dark blue and were clearly demarcated from any diffuse, extracellular staining in the surrounding tissue and a nucleus could be seen in the cell. Cells which looked like lymphocytes with a thin line of blue stain surrounding a round nucleus were occasionally seen. These were not counted as EG2+ cells.

Point counting for EG2 staining.

The colour of the tissue under each cross on the point counting graticule was assessed as either blue (positive) or pink (counterstain). The crosses were made up of very thin lines and the "point" to be counted was very small and clear. The colour of the underlying tissue was not difficult to assess. Positive points were considered to be over an eosinophil if the criteria for EG2+ cells above were satisfied, otherwise a blue point was counted as extracellular EG2+. If the cross fell on a cell that looked like a lymphocyte, with a thin blue line around a round nucleus, it was counted as extracellular EG2+ only if the cross fell exactly over the blue line.

Interleukin 4

Cells were regarded as positive if there was dark blue staining in a clearly demarcated area around a nucleus.

Interleukin 5

Interleukin 5 staining was not as well defined as that for interleukin 4. Positive cells had a pale blue appearance which surrounded a nucleus.

Eosinophil counts on H+E stained material

A cell was counted as an eosinophil if it had a eosinophilic cytoplasm, which stood out from the surrounding tissue and cells, and an irregular nucleus. Cells with a round nucleus were also included if they appeared to be no larger than other eosinophils *and* the cytoplasm appeared granular. Elongated eosinophilic cells lying in bundles immediately below and with their long axis parallel to the basement membrane were not counted as eosinophils.

Total Cell Count on H+E stained material

All nuclei stained by Harris Haematoxylin were counted, regardless of shape.

2.28 PERIPHERAL BLOOD CELL SAMPLES

One sample was analysed by a Coulter counter in the routine hospital laboratory for total lymphocyte count at each time point. The second sample was processed for FACS scan analysis in order to determine lymphocyte subset concentrations.

2.29 FACS SCAN ANALYSIS

The FACS scanner is an automated analyser which feeds a stream of separated discrete cells through a beam of light from a laser, in this case an argon based laser. The degree of scatter that is produced in two perpendicular directions, forward scatter within a few degrees of the axis of flow and side scatter at 90 to the axis, by each

individual cell is detected and the results for the whole population are displayed as a scatter-graph, with forward and side scatter forming the x and y axes. The forward scattering of laser light is a function of the size of an individual cell and the side scatter is a function of granularity. Each cell type in peripheral blood exhibits a characteristic combination of size and granularity and therefore occupies a particular area on the scatter-graph. The operator is able to select a particular group of cells on the basis of their position on the graph by drawing a "gate" around them, using computer software. There is a reproducible pattern of cell types on the scatter-graph when peripheral blood is analysed and the lymphocytes cloud can be identified and selected (Figure 21).

The cells are labelled with two different monoclonal antibodies, each directed against a different cell surface marker and each conjugated to a fluorescent molecule which emits light at a specific wavelength when excited by laser light. The fluorescent molecules used in these experiments are PHYCOERYTHRIN (PE) and FLUORESCINE ISOTHIOCYANATE (FITC). The intensity emission at the wavelengths specific for PE and for FITC is recorded for every individual cell. When the cells have been gated for their scatter characteristics the intensity of light emitted at the two relevant wavelengths by all of the cells within the gate can be calculated and displayed as a second scatter-graph, this time with intensity of emission at the PE emission wavelength on the x axis and the intensity at the FITC specific wavelength on the y axis (Figure 22).

There is a degree of fluorescence on all cells because of auto-fluorescence and background, non-specific antibody staining. There are therefore four groups of cells on the second scatter-graph: cells staining with both antibodies emitting high intensity light at both wavelengths, cells staining with neither antibody but emitting a low, background, intensity light at both wavelengths and two groups of cells which stain with only one of the antibodies and which emit at high intensity at one wavelength at low at the other. In order to set an objective cut-off between "low" and "high" intensity

negative control antibodies, directed against plant derived antigen, is used to stain the first sample from each blood-test. These antibodies are of the same immunoglobulin subclass as the test antibodies and are conjugated with PE and FITC in the same way. The degree of staining is therefore identical to the non-specific staining for all of the subsequent samples i.e. the staining accounted for by auto-fluorescence and by all binding to cells except that between the variable region of the monoclonal antibody and its specific epitope. A gate is set so that any staining at higher intensity is regarded as positive, or in other words, when a monoclonal antibody against a specific marker is added, any cell staining with a greater number of antibodies than were attached to non specific binding sites on similar cells during negative control staining is regarded as positive. Once the gates are set the FACS analyser calculates the number cells staining with both antibodies, with one antibody and with no antibody.

There is inevitably a degree of cross talk between the fluorescence transducers so that cells stained with only PE will produce a small signal in the FITC channel and *vice versa*. In order to account for this phenomenon cells similar to those used in the study are labelled with single PE or FITC conjugated and put through the machine. The sensitivity of the transducers is altered until there is no positive signal for the absent marker. These compensation settings are then stored on computer disk and used during all subsequent analyses. All samples were processed within 3 hours of venesection. 100µl of whole blood (anticoagulated with EDTA) was incubated with 10 µl of each of two specific monoclonal antibodies at room temperature for 15 minutes.

The following combinations of markers were employed;

CD3/CD4; CD3/CD8; CD3/ICAM; CD4/VLA; CD8/VLA; CD4/HLA DR;
CD8/HLA DR; CD4/CD25; CD8/CD25.

3ml of FACS-LYSE solution (Beckton-Dickinson, USA) was then added. After 10 minutes the solution was centrifuged at a force of 300G for 5 minutes. The resulting cell pellet was suspended in a mixture of bovine serum albumin and 1% sodium azide. The solution was re-centrifuged and suspended in bovine serum albumin/ sodium azide solution and finally centrifuged and fixed by re-suspension in 1% para-formaldehyde.

FACS analysis was performed using a Beckton-Dickinson FACS sorter with CONSORT 3.0 software. 10,000 gated events were analysed for each marker. FACS scan results, which are given as percentages were multiplied by the circulating lymphocyte count as appropriate to obtain the absolute circulating cell count for all surface markers.

2.30 BRONCHOALVEOLAR LAVAGE CELL SAMPLES: LIGHT MICROSCOPY CELL COUNTS.

Bronchoalveolar lavage fluid was aspirated into a siliconised container cooled on ice and processed immediately. The volume of return was measured and the lavage was then poured through a 100µm nylon mesh in order to remove mucus. The filtrate was gently agitated and a 3ml sample removed for cell counts. 100µl of the fluid was deposited in the well of a haemocytometer and total cell concentration determined at 100X magnification by light microscopy. The number of viable cells was assessed by trypan blue exclusion. 150µl of BAL fluid was deposited in a Shandon cytopsin, after agitation of the sample, with an appropriate disposable filter paper well and a glass microscope slide which had previously been coated in 10% poly-L-lysine (PLL) (one drop of PLL on slide; a second slide placed on top and moved from side to side until entire surface covered with liquid; slides separated and air dried for 30 minutes). The sample was centrifuged for 15 minutes at 800 revolutions per minute in the standard Shandon cytopsin centrifuge and the resulting preparation was dried in room air for 30

minutes. The slide was stained by immersion for 5 seconds in each of the three solutions that make up the commercially available Diff-Quick kit. Individual cell counts were calculated by multiplying the proportional cell count on the cytopsin slide by the total cell count from the haemocytometer.

The remaining fluid was centrifuged immediately after filtration at a force of 300G for 10 minutes and the supernatant was decanted, separated into 2ml aliquots and immediately snap frozen by plunging into liquid nitrogen. The cell pellet was suspended in bovine serum albumin solution containing 1% sodium azide and 100µl aliquots were separated and incubated with 10µl of each of two conjugated monoclonal antibodies (one to PE and one to FITC) for 15 minutes at room temperature. The solution was diluted in 3ml of FACS-LYSE fluid and left for 10 minutes. The fluid was then centrifuged at 150G for 5 minutes and the cell pellet suspended in a solution of bovine serum albumin containing 1% sodium azide. The centrifugation/re-suspension was repeated and the cells were then centrifuged and suspended in 1% paraformaldehyde. FACS scan analysis was then performed as described above.

2.31 BAL MEDIATOR CONCENTRATION

The aliquots of BAL supernatant obtained by centrifugation of filtered fresh raw BAL were stored for up to 10 weeks at minus 80 degrees centigrade. Analysis for histamine, tryptase, PGD₂ and ECP were performed by radio-immuno-assay (RIA) using commercially available kits.

Histamine assay

Because histamine is such a small molecule, the radio-immuno-assay that was used determined the concentration of acylated histamine. Acylating agent supplied as part of the RIA kit was added to BAL fluid and incubated for 30 minutes at room temperature. 50µl of acylated sample was added to a special anti-histamine coated tube, followed by 500µl of ¹²⁵I labelled acylated histamine. An identical procedure was followed using standard histamine solutions. Uncoated tubes were also filled with 500 µl of iodinated tracer. The tubes were incubated at 4 degrees centigrade for 18 hours and the coated tubes were then emptied by pipette aspiration. The total counts per minute (cpm) was determined for the uncoated, fluid filled tubes and bound cpm determined for the coated, emptied tubes by a gamma-counter. Bound cpm were expressed as a fraction of total cpm and a standard curve constructed from the values for the standard histamine solutions. The histamine concentrations for the samples were then derived by interpolation of the standard curve.

Prostaglandin D2 assay

100µl of BAL fluid was mixed with 100µl of tracer solution (³H labelled PGD₂), 100 µl of anti-PGD₂ antibody solution and 100µl of specific buffer supplied as part of the commercial kit in polypropylene tubes. Other tubes were filled with only 100µl tracer and made up to the same volume as the sample tubes by addition of extra buffer. The tubes were incubated for 18 hours at 4 degrees centigrade. 500 µl of charcoal suspension was then added to each tube in an ice water bath. After 10 minutes the tubes were centrifuged at 2000g for 10 minutes and the supernatant decanted into scintillation vials. 10ml of scintillation fluid was added to each tube and radioactivity measured for 4 minutes in a β-counter. An identical procedure was followed using standard concentrations of PGD₂ and a standard curve was constructed, using β-counts expressed as a fraction of the total count read from tubes containing only tracer and buffer. PGD₂ sample concentrations were derived by interpolation.

ECP assay

50 µl of sample or standard ECP solution was mixed with 50µl of ¹²⁵I labelled ECP and 50µl of anti ECP antibody. The tubes were incubated for 3 hours at room temperature. 2ml of decanting solution, containing sepharose beads coated with anti IgG was added and incubation continued for a further 30 minutes. The tubes were centrifuged for 10 minutes at 15000g and the supernatant decanted by inversion. The tubes were left upside down for 30 seconds and radioactivity counted by gamma counter for 1 minute. ECP concentrations were derived by the standard curve method.

Tryptase assay.

100µl of standard tryptase solution or 50µl of BAL and 50µl of sample diluent were added to tubes coated with anti tryptase antibody. 50 µl of radiolabelled tryptase was also added and the tubes were incubated for 18 hours at room temperature. The tubes were emptied, washed three times with saline and finally emptied completely by pipette aspiration. Uncoated tubes filled only with tracer were also processed. Radioactivity was measured and results calculated from a standard curve, as above.

2.32 STATISTICAL ANALYSES

A probability level of 95% ($p=0.05$) was assumed as the threshold for statistical significance for all tests.

Changes in cell counts of CD3+ T lymphocytes on immunostained sections and eosinophil counts on H+E stained sections of bronchial tissue were regarded as primary outcome measures which tested the hypothesis that theophylline has an anti-inflammatory effect during the late asthmatic response. Both variables were analysed and used to test the same hypothesis. The acceptable p value for statistical significance was corrected the method of Bonferroni for non dependant variables. The acceptable probability threshold for statistical significance, 95%, was maintained by adopting a

value for p lower than 0.05 to allow for the increased risk of type I error resulting from multiple comparisons. The corrected p value was derived from the formula: $p_c = 1 - (1 - p')^{1/N}$ where p_c is the corrected value and p' the initial value, in this case 0.05. This gives a corrected p value for the threshold of statistical significance of $p_c = 0.025$. All other comparisons were regarded as exploratory, or hypothesis generating and Bonferroni corrections were not applied.

2.33 CELLULAR DATA

Biopsy cell counts and bronchoalveolar cell counts: for each individual subject the change in cell count during the study was expressed as a percentage of the baseline count. This was felt to be more meaningful comparison than comparison of absolute cell counts because there was a wide variation in baseline counts. The changes within the theophylline group were compared to the changes within the placebo group non-parametrically using the Mann Whitney U test.

Bronchoalveolar T cell subset and activated T cell subset counts were compared as percentages to assess the proportion of cells that were activated. This was intended to detect a change in the proportion of cells that were activated from a change in the absolute number resulting from a change in the overall number of lymphocytes i.e. not necessarily an effect on activation *per se*.

Peripheral blood cell counts were compared at individual time points in relation to allergen challenge before and after treatment (for example the CD3 cell count during the EAR before treatment was compared with the CD3 count during the EAR after treatment for both the theophylline and the placebo group). T cell activation marker expression was analysed as both absolute cell count per millilitre and as percentage of subset cells which co-express activation the marker. The treatment effects, as a percentage of pre-treatment values, were compared between groups, as above, using the Mann Whitney U test.

Bronchoalveolar lavage mediator concentrations were expressed as concentration in the returned fluid. No attempt was made to correct for airway lining fluid volume or dilution during dwell time. For each individual subject the change in concentration during the study was expressed as a percentage of the respective baseline concentration. The changes within the theophylline group were compared to the changes within the placebo group non-parametrically using the Mann Whitney U test.

Associations between bronchial biopsy cell counts, extracellular tissue EG2 staining, bronchoalveolar lavage cell counts and bronchoalveolar lavage mediator concentrations were examined by correlation, using Spearman's rank test and calculating statistical significance with reference to a standard table. Bonferroni type correction was not applied, and all analyses of correlation were regarded as exploratory.

2.34 CLINICAL DATA

Spirometry was performed at the same time of day before any challenge procedures before and after the treatment period. Changes during treatment were expressed as percentage of the baseline value for each subject and the resulting values for the theophylline and placebo groups were compared by student's t test.

Diary card entries were analysed during two periods; during run-in, for 14 days before the first visit of the study proper (i.e. not the screening visit) and at the end of the treatment period, for 14 days prior to the final visit. Mean morning and evening PEFRs for each subject were calculated for each period and comparison was made by paired t-test. Diurnal PEFr variability was calculated as the mean of

$$\frac{\text{morning PEFR} - \text{previous evening PEFR}}{\text{previous evening PEFR}}$$

for each day in the assessment period and compared similarly.

Day-time and night-time symptom scores for the two 14 day periods were compared in each treatment group by Chi square test. Expected values for the severe symptom category frequencies were too low to allow meaningful analysis (Clark, 1992). Symptoms were therefore grouped into two categories, "absent" and "present". Rescue beta agonist inhaler use during the two periods was compared non parametrically by Wilcoxon rank sign test within treatment groups.

The number of subjects studied was too small to allow meaningful statistical analysis of side effect data. The frequency of possible adverse treatment effects are therefore presented descriptively.

2.35 PROSPECTIVE POWER CALCULATIONS

Power calculations were based upon the assumption of normally distributed data. The actual power will be different. Power calculations were based upon the probability of detecting differences in biopsy counts during treatment for eosinophils and T-lymphocytes. It was not possible to perform power calculations for the treatment effect (i.e. difference between the theophylline and placebo groups) because the variance of biopsy counts over time in asthma is not available.

Mean values and standard errors of the means for biopsy eosinophil counts and CD43+ cell counts (mainly T lymphocytes) were derived from a study of patients who had died from asthma (Azzawi, 1992). The study was chosen as the basis for power

calculation because i) the severity of disease might be closer to the state after allergen challenge than that seen in mild stable asthma; ii) the techniques used in biopsy processing were similar to those used in the current study and iii) results were given in table form as mean and SEM. Standard deviation (SD) was calculated by multiplying the standard error of the mean (SEM) by the square root of the number of subjects. The mean (SD) eosinophil count per mm of basement membrane was 62 (62) and the lymphocyte count was 73 (50). It should be noted that this data cannot be normally distributed, since the magnitude of the SD would imply that a significant proportion of patients have negative cell counts. This power calculation is therefore only regarded as a rough guide.

Power calculation was performed by using the formula (Chatfield, 1983):

$$n > \frac{2 \cdot \sigma^2 (z_{\alpha} + z_{\beta})^2}{\delta^2}$$

where data are assumed to be normal and:

n = minimum number of subjects required per group to satisfy the power requirement

σ = predicted standard deviation of the data

δ = expected treatment difference

z_{α} = two-tailed z value derived from standard table for α

z_{β} = one tailed z value for β

α = p value accepted as significant (=0.05)

β = (1- power) and power describes the chance of detecting a true difference,

i.e. of rejecting the null hypothesis when it is false(Chatfield, 1983).

In other words, if there is 80% chance of detecting a true difference, $\beta=0.2$

For eosinophils:

Given a standard deviation which is 100% of the mean (from Azzawi, 1992) and a significance level set at $p=0.05$ and assigning a required power of 80% to detect an arbitrary difference of 50% of baseline the calculated value for the size of each group is at least 84 subjects for a two tailed test.

For lymphocytes:

Given an estimated standard deviation which is 68% of the mean and a significance level set at $p=0.05$ and assigning a required power of 80% to detect a difference of 50% of baseline the calculated minimum value for $n = 40$ for a two tailed test.

In order to design a study with reasonable power to detect a positive result, given the above assumptions, the number of subjects would need to be at least 80 for a parallel group design. Recruitment is a major limitation in this type of clinical study, particularly when an invasive procedure is involved, and it would be considered impossible to complete a study of 80 subjects in a reasonable time. It was therefore decided to accept a smaller number. As many subjects as possible were recruited but the acceptable sample size was chosen as two groups of 10, based upon the expected rate of recruitment. This meant that the power of the study was likely to be low; in other words, if a true difference occurred the study might not detect it, and conversely, if the study did not demonstrate a statistically significant difference, it would not mean that there was likely to be no difference. However, the power calculations were based upon standard deviations of tissue cell counts, and not the standard deviations of the change in cell counts with time which is an important distinction as the latter value will be used in analyses this study: the statistical analyses will compare the effect of

two treatments over a period of time. The relevant data was not available before the study. It was therefore not possible to estimate the *true* power of the study and there were therefore no firm grounds to abandon the project on the basis of low power. The confidence for positive results is not affected by the power of the study.

CHAPTER 3: RESULTS

3.1 BIOPSY COUNTS

Primary parameters

There was a difference between the effect of theophylline and that of placebo in terms of the number of eosinophils on H+E sections in sub epithelial tissue in favour of reduction after theophylline (Table 7 and figure 1 ($p=0.02$)). When Bonferroni correction is applied this difference is statistically significant ($p<0.025$). Whilst initially higher, the median pre-treatment eosinophil count was similar statistically in the theophylline and placebo groups. There was no significant difference between groups in terms of CD3 positive T lymphocytes either before treatment or as a result of treatment (Table 3 and figure 2).

Secondary parameters

Comparisons were made between groups for baseline values for all parameters using Mann Whitney U test. The theophylline and placebo group were not statistically different for any parameter before treatment. There was a treatment effect in terms of a reduction in the number of EG2 positive activated cells after theophylline (Table 6; $p=0.04$) but this effect was not a test of the primary hypothesis. These findings are regarded as a basis for the generation of a hypothesis for a future investigation. After theophylline treatment the number of EG2+ cells correlated with the amount of extracellular EG2 staining within the lamina propria ($r_s=0.82$; $p<0.05$) and there was a positive correlation between the change in the number of EG2 positive cells in the sub-epithelial zone, expressed as a percentage of baseline, and the change in the extent of extra cellular EG2 staining as a percentage of baseline in the sub-epithelial zone during theophylline treatment ($r_s=0.73$; $p<0.05$). There was, however, no significant treatment effect after theophylline (compared with placebo) in terms of extracellular EG2 staining.

There were no statistically significant changes in the number of cells staining positively for tryptase. There were no statistically significant changes in the number of cells staining for IL4 or IL5. If positive control material becomes available for these antibodies the cell counts will be repeated on freshly stained tissue. Results for all biopsy parameters are included in tables 3-7.

3.2 PERIPHERAL BLOOD

Peripheral blood T cell subset analyses are listed in Tables 8-11. The total lymphocyte concentration was elevated during the LAR in both groups before and after treatment and theophylline had no effect either on lymphocyte count at any time point or on the pattern of changes after challenge. After theophylline treatment there was an increase in the percentage of CD4 cells which also expressed the activation marker HLA-DR before allergen challenge and during the EAR and LAR ($p < 0.05$) (Figures 25 and 26). These differences were not corrected for multiple variable comparison, but instead were regarded as exploratory rather than hypothesis-testing.

3.3 BRONCHOALVEOLAR LAVAGE CELL COUNTS

The absolute lymphocyte count in BAL fluid, expressed as cells per volume of fluid returned, was reduced after theophylline and the inter-group comparison showed a significant treatment effect (Table 14; $p < 0.05$). T cell subset analysis (Table 12) showed a reduction after theophylline in the absolute number of CD3+ and CD8+ cells with a significant treatment effect ($p < 0.05$). Activation marker analysis showed reduction in the absolute concentration of CD3+, ICAM+ cells and CD4+, VLA+ cells and a statistically significant inter-group treatment effect for both cells ($p < 0.05$).

. There were no statistically significant changes in sub-sets activation expressed as percentages. These differences were not corrected for multiple variable comparison, but instead were regarded as exploratory rather than hypothesis-testing.

3.4 BRONCHOALVEOLAR LAVAGE MEDIATOR CONCENTRATIONS

There were no statistically significant differences in BAL PGD₂, ECP, histamine or tryptase concentrations resulting from treatment with theophylline (Table 13).

3.5 CLINICAL DATA

Demographic details of subjects are shown in table 1. FEV₁ was recorded in the morning before allergen challenge both before and after the 6 week treatment period. There was a small increase in the theophylline group; the mean (SEM) value was 3.34 (0.20) litres before treatment and 3.47 (0.24) litres after treatment. In the placebo group there was a fall in FEV₁ during treatment, from 3.67 (0.29) litres to 3.46 (0.31) litres. The difference in treatment effect between the two groups was statistically significant ($p < 0.05$). There were no statistically significant differences between groups in terms of peak expiratory flow rate (PEFR) or PEFR diurnal variability.

3.6 AIRWAY RESPONSE AFTER ALLERGEN - EAR AND LAR

Figs 4 and 5 show the EAR and LAR before and after treatment with theophylline and placebo respectively. There was no statistically significant treatment effect when the groups were compared. Figure 3 shows the median size of the effect of treatment on the magnitude of the EAR and LAR. The treatment effect was not distributed

normally and non parametric 95% confidence intervals are therefore shown. These confidence intervals emphasise the low power of this study to detect a treatment effect for theophylline compared with placebo. Figures 4 and 5 show mean FEV1 after allergen challenge against time.

3.7 SYMPTOM SCORES

Symptoms were regarded either as absent or present for the purpose of statistical analysis, since the expected frequencies of some of the scores was too low to allow Chi square analysis of the "raw" data. Two-by-two Chi square analysis revealed a statistically significant improvement in both the theophylline and placebo group ($p < 0.05$). One subject in the placebo group and none in the theophylline group suffered an acute exacerbation of asthma during the treatment period.

3.8 ADVERSE EFFECTS

Table 2 shows the profile of symptoms not due to asthma in both treatment groups.

The following symptoms are not thought to have been caused by theophylline:

- i. one subject developed a rash during hot weather which resolved whilst theophylline treatment continued; he reported that he often developed a rash in hot weather.
- ii. one subject experienced epigastric pain, on and off for a few days, in the middle of the treatment period. He had experienced similar pains before and on this occasion the pain resolved despite continuing theophylline treatment.
- iii. one subject in the placebo group developed persistent nausea immediately after the first dose of study medication which persisted for over 2 weeks.

No subjects reported vomiting, anorexia, tremor, diarrhoea or palpitations.

	Theophylline	Placebo	p value
Number	12	10	-
Age (years)	26.3 (20-43)	27.8 (22-36)	0.66
Height (cm)	173 (163-192)	169 (155-185)	0.38
Weight (kg)	75.8 (54-105)	67.7 (55-80)	0.19
Duration of symptoms of asthma (years)	13.6 (2-40)	20.0 (6-30)	0.17
FEV1 (litres)	3.63 (2.38-4.84)	3.33 (2.68-4.88)	0.42
FEV1 as percentage of predicted value.	93.8 (81.4-114.2)	97.4 (81.2-115.6)	0.49
Male / Female	7 / 5	2 / 8	*

* number too small for Chi square analysis.

Table 1: Demographic characteristics of subjects entering the study

Treatment	Timing in relation to treatment.	Symptom	Duration (weeks)	Severity, as assessed by subject.
<i>Theophylline</i>	<i>Before treatment</i>	<i>Nausea abdominal pain</i>	<i>?</i>	<i>Moderate</i>
Theophylline	During first week.	Nausea	< 1	Moderate
Theophylline	During first week	Nausea	5/7	Moderate
<i>Theophylline</i>	<i>Latter part</i>	<i>Pain in one leg</i>	<i>3</i>	<i>Moderate</i>
<i>Theophylline</i>	<i>Middle</i>	<i>Rash</i>	<i>2</i>	<i>Severe</i>
Theophylline*	First week	Insomnia	?	Mild
Theophylline*	Second week	Nausea	?	Mild
Theophylline*	Latter part	Irritable	4	Moderate
<i>Placebo</i> §	<i>During first week</i>	<i>Nausea</i>	<i>1</i>	<i>Mild</i>
<i>Placebo</i> §	<i>During second week</i>	<i>Nausea</i>	<i>1</i>	<i>Mild</i>

Theophylline	Fifth week	Epigastric pain	<1	Severe
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Table 2:

Symptoms other than those of asthma and rhinitis during treatment; the upper table refers to the responses to open questioning at the beginning of treatment, after 1 and 2 weeks and at the end of treatment. The lower table refers to responses to a symptom seeking question i.e. "did you experience epigastric pain, nausea, insomnia or tremor during treatment?"

Italics indicate symptoms thought unlikely to be related to treatment (see text).

* identifies symptoms occurring in one particular subject, as does §.

Subject	Group	CD3		CD4		CD8	
		Before	After	Before	After	Before	After
12	T	72.8	51.1	11.6	18.2	18.1	5.6
13	T	60.0	38.0	33.8	9.0	23.9	15.8
15	T	82.5	75.3	53.3	36.3	9.9	22.9
18	T	103.0	73.0	102.7	24.7	46.7	46.7
20	T	59.2	19.4	38.0	19.5	8.0	16.3
22	T	25.9	34.9	9.9	10.9	5.6	25.5
23	T	32.8	54.9	14.9	60.0	8.6	25.5
n		7	7	7	7	7	7
Median (95% CI)		60.0 (32.8-87.9)	51.1 (28.7-73.0)	33.7 (11.6-70.4)	19.5 (10.9-42.3)	9.9 (7.1-32.4)	22.9 (10.9-36.1)

9	P	73.3	28.2	17.0	12.7	6.7	5.5
11	P	54.0	66.0	80.1	69.7	49.5	61.2
16	P	77.8	149.3	40.9	29.7	9.2	32.4
17	P	73.0	134.7	69.4	111.0	12.4	11.1
19	P	50.0	22.2	50.5	8.8	28.7	12.3
n		5	5	5	5	5	5
Median (95% CI)		73.0 (50-77.8)	66 (22.0-149)	50.5 (17.0-80.1)	29.7 (9.0-111)	12.4 (6.7-49.5)	12.3 (5.5-61.2)

Table 3:

Individual and median biopsy cell counts with 95% confidence intervals.

All counts as cells/mm BM to a depth of 60µm.

Group T= theophylline; group P = placebo.

No significant differences.

Subject	Group	CD25		IL4		IL5	
		Before	After	Before	After	Before	After
12	T	2.9	0.8	174.0	0.0	6.6	0.0
13	T	8.2	0	6.7	5.3	2.5	2.6
15	T	1.8	0.8	17.3	0.0	0.0	0.0
18	T	7.0	2.6	19.8	1.7	0.0	0.0
20	T	3.5	0.0	17.2	12.0	2.0	4.8
22	T	2.4	0.0	33.4	6.9	1.9	9.8
23	T	0.3	3.7	2.1	8.7	0.0	0.0
n		7	7	7	7	7	7
Median (95% CI)		2.9 (1.4-7.0)	0.8 (0-2.6)	17.3 (6.7-97.0)	5.3 (0.0-9.5)	1.9 (0.0-4.3)	0.0 (0.0-6.2)

9	P	0.0	0.7	25.0	3.3	0.0	0.0
11	P	1.5	1.7	5.1	23.7	2.2	6.0
16	P	2.2	2.4	25.3	3.4	5.9	1.6
17	P	0.0	1.9	30.4	3.2	4.5	4.4
19	P	1.2	0.0	0.9	2.0	5.9	1.6
n		5	5	5	5	5	5
Median (95% CI)		1.2 (0-2.2)	1.7 (0-2.4)	25.0 (5.1-30.4)	3.3 (3.2-23.7)	4.5 (0.0-5.9)	1.6 (0.0-6.0)

Table 4:

Individual and median biopsy cell counts with 95% confidence intervals.

CD25 counts as cells/mm BM to a depth of 60µm; IL4 and IL5 counts as cells/mm² in entire section.

No significant differences

Subject	Group	Tryptase (A)		Tryptase (B)	
		Before	After	Before	After
12	T	10.9	2.8	17.7	7.5
13	T	4.6	0.9	59.5	4.3
15	T	3.5	4.6	17.4	15.1
18	T	1.7	6.8	5.6	26.2
20	T	1.9	0.4	14.6	7.9
22	T	9.4	1.8	1.8	27.0
23	T	7.4	0.0	9.0	1.6
n		7	7	7	7
Median (95% CI)		4.6 (1.9-9.6)	1.8 (0.4-4.8)	14.6 (5.4-38.5)	7.9 (4.2-26.2)

9	P	0.9	2.1	4.7	11.8
11	P	7.2	13.8	19.7	37.1
16	P	9.5	3.4	54.1	42.4
17	P	6.9	9.5	38.7	20.5
19	P	1.8	1.8	6.3	9.7
n		5	5	5	5
Median (95% CI)		6.9 (0.9-9.5)	3.4 (1.8-13.8)	19.7 (4.7-54.0)	20.5 (9.7-42.4)

Table 5:

Tryptase (A) refers to tryptase positive cells/mm BM to a depth of 60 μ m.

Tryptase (B) refers to tryptase positive cells/mm² of tissue in the entire section.

No significant differences.

Subject	Group	EG2+cells		Free EG2 (A)	
		Before	After	Before	After
6	T	8.6	4.9	1	4
10	T	4.6	3.3	7	5
12	T	18.1	6.7	47	29
13	T	5.9	2.6	14	2
15	T	8.5	2.1	19	1
18	T	5	0	45	0
20	T	5.8	0.5	11	1
22	T	4.8	1.2	2	2
23	T	5.9	0.8	6	3
n		9	9	9	9
Median (95% CI)		5.9 (5.0-11.9)	2.1 (0.7-4.4)	10.7 (4.0-30.5)	2.0 (0.8-15.3)

8	P	2.4	1.8	*	*
9	P	12.6	8.4	22	4
11	P	6.3	9.8	10	20
16	P	5.5	5.4	0	4
17	P	9.5	2.3	16	2
19	P	4.8	2.3	5	4
n		6	6	6	6
Median (95% CI)		5.9* (3.6-11.1)	3.9 (2.0-9.1)	10.0 (0.0-22.0)	4.0 (2.2-19.9)

Table 6:

EG2+ cell count as cells/mm BM to a depth of 60 μ m. Free EG2 refers to the percentage of the tissue area staining for extracellular EG2 in a 100 μ m band below the epithelial basement membrane.

*p<0.05 for comparison of treatment effect between groups (Mann Whitney U)

Subject	Group	Eosinophils		All cells	
		Before	After	Before	After
6	T	25.2	27.8	506	911
10	T	16.7	8.9	556	507
12	T	23.6	4.7	407	617
13	T	62.1	7.6	706	493
15	T	93.9	9.7	703	247
18	T	6.3	2.8	717	333
20	T	8.9	3.9	692	425
22	T	1.5	8.7	476	406
23	T	13.6	5.8	627	600
n		9	9	9	9
Median (95% CI)		16.7 (7.6-55.3)	7.6 (4.3-16.8)	627 (491-705)	459 (333-702)

8	P	6.0	16.5	567	221
9	P	4.3	6.1	200	413
11	P	12.4	16.1	894	692
16	P	12.5	7.2	433	557
17	P	9.2	11.4	861	423
19	P	5.1	7.4	437	422
n		6	6	6	6
Median (95% CI)		7.6** (4.7-12.5)	9.4 (6.6-16.3)	502 (316-878)	422 (317-625)

Table 7:Haematoxyllin and Eosin section cell counts as cells/mm BM to a depth of 60 μ m

** p<0.025 for intergroup treatment effect

Marker	Theophylline n=10		Placebo n=6	
	Week 0	Week 6	Week 0	Week 6
CD3+	69.8 (66.4-72.3)	69.4 (64.9-74.1)	72.4 (68.7-77.2)	71.1 (67.5-78.1)
CD3+CD4+	62.7 (57.3-67.7)	63.4 (56.7-71.6)	58.8 (47.2-68.6)	60.1 (49.3-68.8)
CD3+CD8+	32.4 (27.0-38.4)	36.2 (26.8-39.3)	38.7 (30.0-48.2)	37.5 (30.3-48.9)
CD3+ ICAM+	12.4 (5.6-19.0)	13.3 (7.8-19.9)	16.1 (9.5-22.7)	17.7 (9.7-26.2)
CD4+VLA+	1.3 (0.7-2.0)	1.4 (0.7-2.3)	1.2 (0.3-2.4)	0.8 (0.4-1.7)
CD8+VLA+	2.2 (0.8-4.2)	1.4 (0.8-1.9)	1.0 (0.5-1.6)	1.0 (0.2-1.1)
CD4+HLADR+	5.6 (4.3-6.8)	6.9 ** (5.1-8.2)	7.6 (5.1-9.9)	8.1 (5.4-9.5)
CD8+HLADR+	14.2 (8.4-20.4)	12.6 (9.0-21.9)	20.8 (11.9-31.2)	21.4 (10.5-33.3)
CD4+CD25+	24.8 (22.0-28.0)	28.5 (23.6-31.6)	30.3 (22.7-40.1)	27.6 (24.1-44.4)
CD8+CD25+	5.8 (2.9-12.1)	5.3 (3.1-7.6)	5.8 (2.0-8.3)	7.6 (3.2-22.3)

Table 8: FACS analysis of blood lymphocytes prior to allergen challenge before and after theophylline and placebo treatments. Results are median values (95% non parametric confidence intervals). CD3+ cells as % of all lymphocytes. Other cell counts are given as a percentage of the total number of lymphocytes staining with the first of the two markers.

e.g. CD3+CD4+ = Percentage of CD3+ cells which are also CD4+.

**= $p < 0.05$ for comparison for intergroup treatment effect;

Marker	Theophylline n=10		Placebo n=6	
	Week 0	Week 6	Week 0	Week 6
CD3+	69.1 (67.9-73.0)	69.6 (65.5-71.6)	71.1 (69.1-75.5)	72.4 (68.0-75.6)
CD3+CD4+	62.4 (58.6-67.6)	62.8 (59.5-71.1)	60.2 (46.9-67)	58.2 (48.1-68.9)
CD3+CD8+	33.5 (27.8-37.2)	33.4 (27.4-37.0)	38.1 (30.3-48.3)	39.3 (30.1-48.7)
CD3+ ICAM+	12.6 (5.5-16.2)	11.7 (6.7-18.4)	14.1 (8.5-24.8)	22.8 (10.7-25.3)
CD4+VLA+	1.7 (1.0-2.3)	1.4 (0.6-2.2)	0.8 (0.6-1.8)	0.7 (0.3-1.5)
CD8+VLA+	1.4 (0.8-2.7)	1.3 (0.8-2.0)	0.9 (0.6-1.2)	0.9 (0.4-1.0)
CD4+HLADR+	6.0 (4.5-6.7)	6.7 ** (5.5-8.4)	8.2 (6.2-9.8)	7.2 (5.8-9.6)
CD8+HLADR+	10.7 (7.3-20.4)	13.5 (8.7-20.5)	21.0 (11.7-32.9)	21.6 (13.8-36.5)
CD4+CD25+	26.6 (22.3-29.7)	28.0 (24.3-33.6)	29.5 (21.2-38.5)	27.9 (22.5-42.6)
CD8+CD25+	4.7 (2.9-10.6)	4.7 (3.1-8.6)	7.7 (1.1-10.0)	8.0 (2.9-22.5)

Table 9: Results of FACS scan of peripheral blood lymphocytes during EAR.
For explanation see table 9.

**= p<0.05 for intergroup treatment effect;

Marker	Theophylline n=10		Placebo n=6	
	Week 0	Week 6	Week 0	Week 6
CD3+	68.7 (64.9-73.8)	70.5 (65.0-74.8)	76.6 (69.1-78.0)	72.4 (69.1-77.7)
CD3+CD4+	63.2 (58.2-68.5)	63.6 (59.7-70.3)	61.0 (50.1-70.5)	70.0 (56.7-71.4)
CD3+CD8+	33.4 (26.3-37.0)	32.9 (27.6-36.7)	37.2 (28.5-46.5)	37.4 (27.9-47.5)
CD3+ ICAM+	10.7 (5.9-16.3)	10.0 (5.4-16.5)	13.7 (4.6-21.7)	17.7 (9.8-26.3)
CD4+VLA+	1.4 (0.8-2.3)	1.2 (0.8-2.4)	0.7 (0.4-1.9)	1.1 (0.7-2.2)
CD8+VLA+	1.3 (0.9-2.5)	1.7 (1.1-2.2)	1.4 (0.6-1.6)	1.3 (0.2-2.2)
CD4+HLADR+	6.1 (4.4-6.9)	6.5 ** (5.0-8.4)	7.2 (5.6-8.3)	6.2 (5.4-7.4)
CD8+HLADR+	12.1 (8.0-22.4)	11.6 (7.7-19.8)	17.7 (11.5-26.3)	17.3 (10.9-35.6)
CD4+CD25+	26.6 (21.6-33.5)	28.7 (24.7-33.6)	30.8 (22.7-39.7)	26.2 (21.9-45.3)
CD8+CD25+	5.3 (3.0-15.0)	4.2 (2.8-8.7)	8.8 (2.4-11.7)	5.8 (3.2-22.4)

Table 10: Results of FACS scan of peripheral blood lymphocytes during LAR.

For explanation see table 9.

** p<0.05 for comparison with pre-treatment value and for intergroup treatment effect;

Marker	Theophylline n=10		Placebo n=6	
	Week 0	Week 6	Week 0	Week 6
CD3+	70.5 (65.0-74.8)	66.2 (60.2-70.8)	72.4 (69.1-77.7)	66.0 (57.7-71.4)
CD3+CD4+	63.6 (59.7-70.3)	62.4 (55.9-70.3)	61.0 (50.7-71.4)	51.5 (39.9-63.6)
CD3+CD8+	32.9 (27.6-36.7)	34.7 (28.6-38.3)	37.4 (27.9-47.5)	45.8 (35.0-55.9)
CD3+ ICAM+	10.0 (5.4-16.5)	8.6 (6.1-19.8)	17.7 (9.8-26.3)	19.0 (17.4-25.5)
CD4+VLA+	1.2 (0.8-2.4)	1.2 (0.4-3.6)	1.1 (0.7-2.2)	1.0 (0.4-1.8)
CD8+VLA+	1.7 (1.1-2.2)	1.3 (0.7-2.0)	1.3 (0.2-2.2)	0.4 (0.3-0.8)
CD4+HLADR+	6.5 (5.0-8.4)	7.0 (5.3-7.7)	6.2 (5.4-7.4)	7.5 (5.5-10.3)
CD8+HLADR+	12.0 (7.7-19.8)	12.2 (8.6-22.9)	17.3 (10.9-35.6)	18.2 (13.2-37.4)
CD4+CD25+	28.7 (24.7-33.6)	29.4 (24.5-32.2)	26.2 (21.9-45.3)	26.8 (21.4-42.1)
CD8+CD25+	4.2 (2.8-8.7)	3.6 (2.8-7.6)	5.8 (3.2-22.4)	5.6 (2.2-24.8)

Table 11: Results of FACS scan of peripheral blood lymphocytes 24 hours after allergen challenge. For explanation see table 9.

Marker	Before	After
<i>THEOPHYLLINE</i> n=10		
CD3	0.28 (0.14-0.42) *	0.11 (0.07-0.26)
CD3, CD4	0.16 (0.07-0.25)	0.07 (0.04-0.14)
CD3, CD8	0.13 (0.09-0.17) *	0.08 (0.05-0.13)
CD3, ICAM	0.06 (0.02-0.11) *	0.01 (0.005-0.07)
CD4, VLA	0.01 (0.005-0.06) *	0.006 (0.001-0.027)
CD8, VLA	0.05 (0.01-0.09)	0.024 (0.014-0.061)
CD4, HLA DR	0.07 (0.02-0.11)	0.024 (0.014-0.049)
CD8, HLA DR	0.03 (0.02-0.06)	0.023 (0.017-0.047)
CD4, CD25	0.02 (0.01-0.03)	0.009 (0.004-0.017)
CD8, CD24	0.001 (0.0005-0.004)	0.002 (0.0004-0.035)
<i>PLACEBO</i> n=6		
CD3	0.20 (0.08-0.34)	0.20 (0.11-0.56)
CD3, CD4	0.11 (0.05-0.18)	0.10 (0.07-0.34)
CD3, CD8	0.107 (0.03-0.18)	0.13 (0.06-0.27)
CD3, ICAM	0.05 (0.01-0.17)	0.04 (0.02-0.80)
CD4, VLA	0.05 (0.01-0.17)	0.02 (0.005-0.06)
CD8, VLA	0.03 (0.003-0.07)	0.06 (0.02-0.15)
CD4, HLA DR	0.05 (0.01-0.60)	0.04 (0.21-0.09)
CD8, HLA DR	0.05 (0.01-0.11)	0.04 (0.02-0.07)
CD4, CD25	0.01 (0.005-0.02)	0.01 (0.006-0.07)
CD8, CD24	0.0003 (0.0002-0.003)	0.0007 (0.0001-0.0015)

Table 12: Bronchoalveolar lavage FAC scan analysis results. Median absolute lymphocyte counts (95% confidence intervals) in returned BAL fluid. ($\times 10^5$ cells/ml).

* $p < 0.05$ for inter-group comparison of treatment effect (Mann Whitney).

	Theophylline n=10		Placebo n=7	
	Before	After	Before	After
Histamine	4.6 (2.2-11.4)	3.3 (1.6-19.5)	1.3 (0.6-5.0)	2.9 (1.0-4.0)
Tryptase	2.7 (1.5-6.3)	2.1 (1.0-7.8)	1.9 (0.5-2.5)	2.0 (0.8-5.3)
PGD2	11.0 (7.2-40.5)	11.8 (4.0-29.0)	7.6 (3.0-23.0)	12.0 (3.0-40.0)
ECP	17.0 (10.1-38.5)	12.2 (5.9-39.2)	16.0 (6.6-55.0)	20.0 (10.5-101.7)

Table 13: Median concentration of mediators (95% confidence interval) in BAL expressed as pg/ml.

Theophylline (n=10)**Placebo (n=7)**

Cell	Before	After	Before	After
Leukocyte	1.6 (1.2-2.0)	1.5 (1.2-2.1)	1.4 (1.0-1.9)	1.8 (1.2-4.7)
Macrophage	1.3 (0.8-1.5)	1.2 (0.8-1.4)	0.9 (0.6-1.1)	1.3 (0.9-2.2)
Lymphocyte	0.3 (0.2-0.4)	0.2 (0.2-0.3)	0.2 (0.1-0.4)	0.3 (0.2-0.7)
Neutrophil	0.05 (0.02-0.09)	0.02 (0.01-0.42)	0.03 (0.01-0.19)	0.06 (0.01-0.34)
Eosinophil	0.09 (0.03-0.07)	0.06 (0.01-0.36)	0.19 (0.04-0.43)	0.17 (0.03-1.490)
Epithelial Cell	0.1 (0.0-0.4)	0.1 (0.0-0.3)	0.05 (0.02-0.09)	0.09 (0.03-0.20)

Table 14: BAL counts (10^5 cells/ml) as median (95% CI);

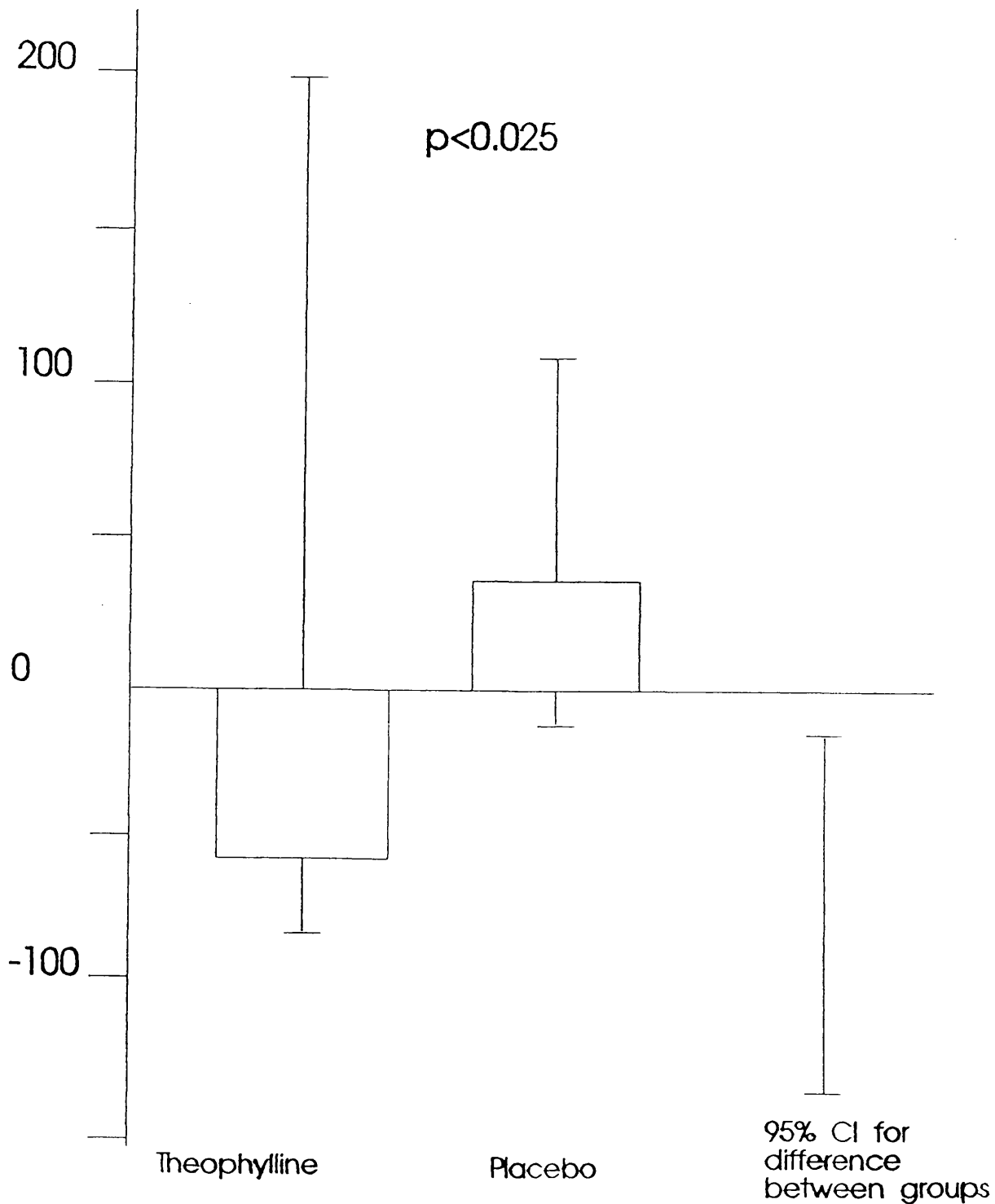


Figure 1: Median (non parametric 95% CI) change in the subepithelial eosinophil count during six weeks treatment with theophylline or placebo expressed as % of the initial value. The error bar on the right of the graph is the non parametric 95% CI for the difference between the groups.

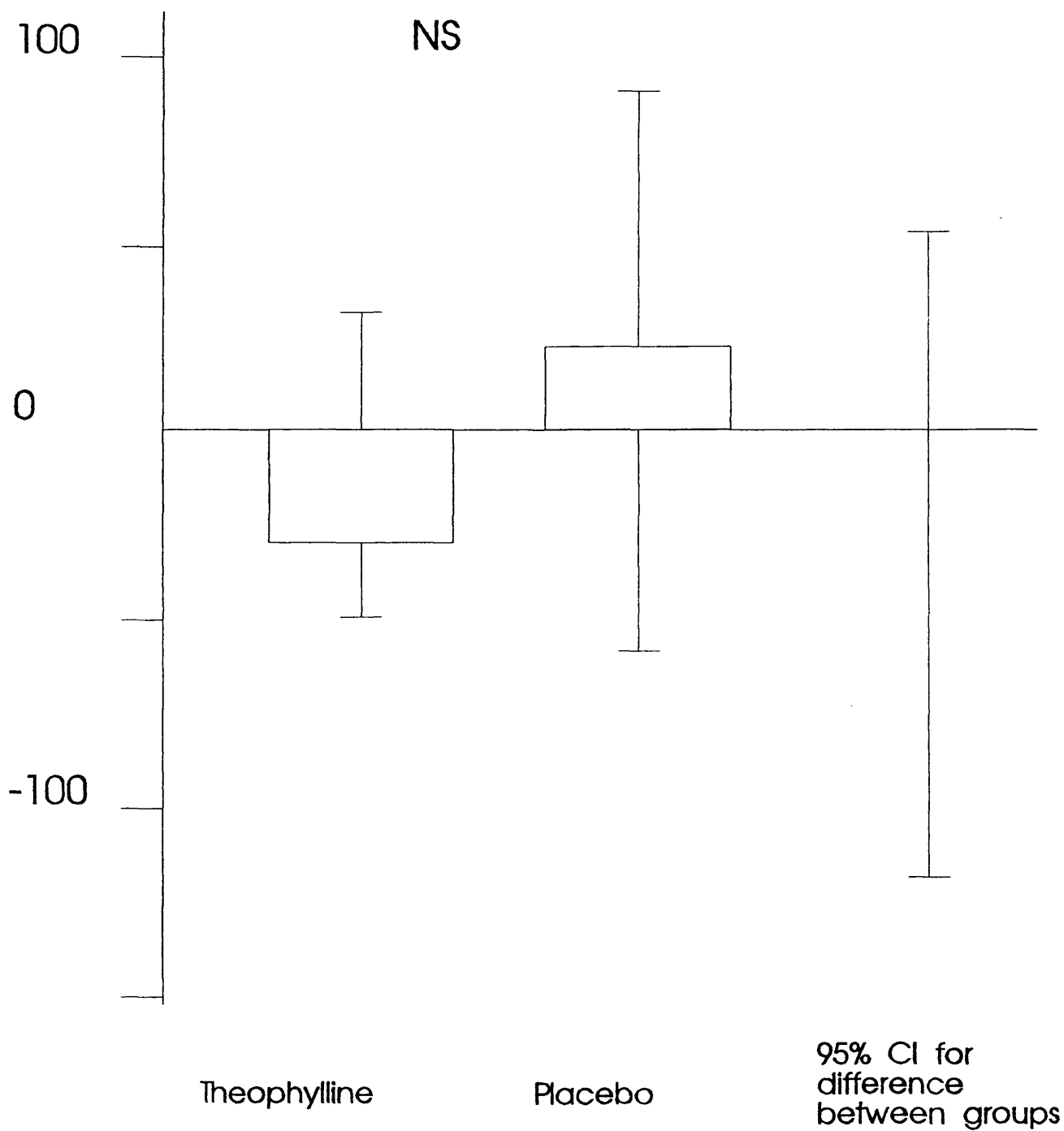


Figure 2: Median (non parametric 95% CI) change in the subepithelial CD3+ T-lymphocyte biopsy count during six weeks treatment with theophylline or placebo expressed as % of the initial value. The error bar on the right of the graph is the non parametric 95% CI for the difference between the groups.

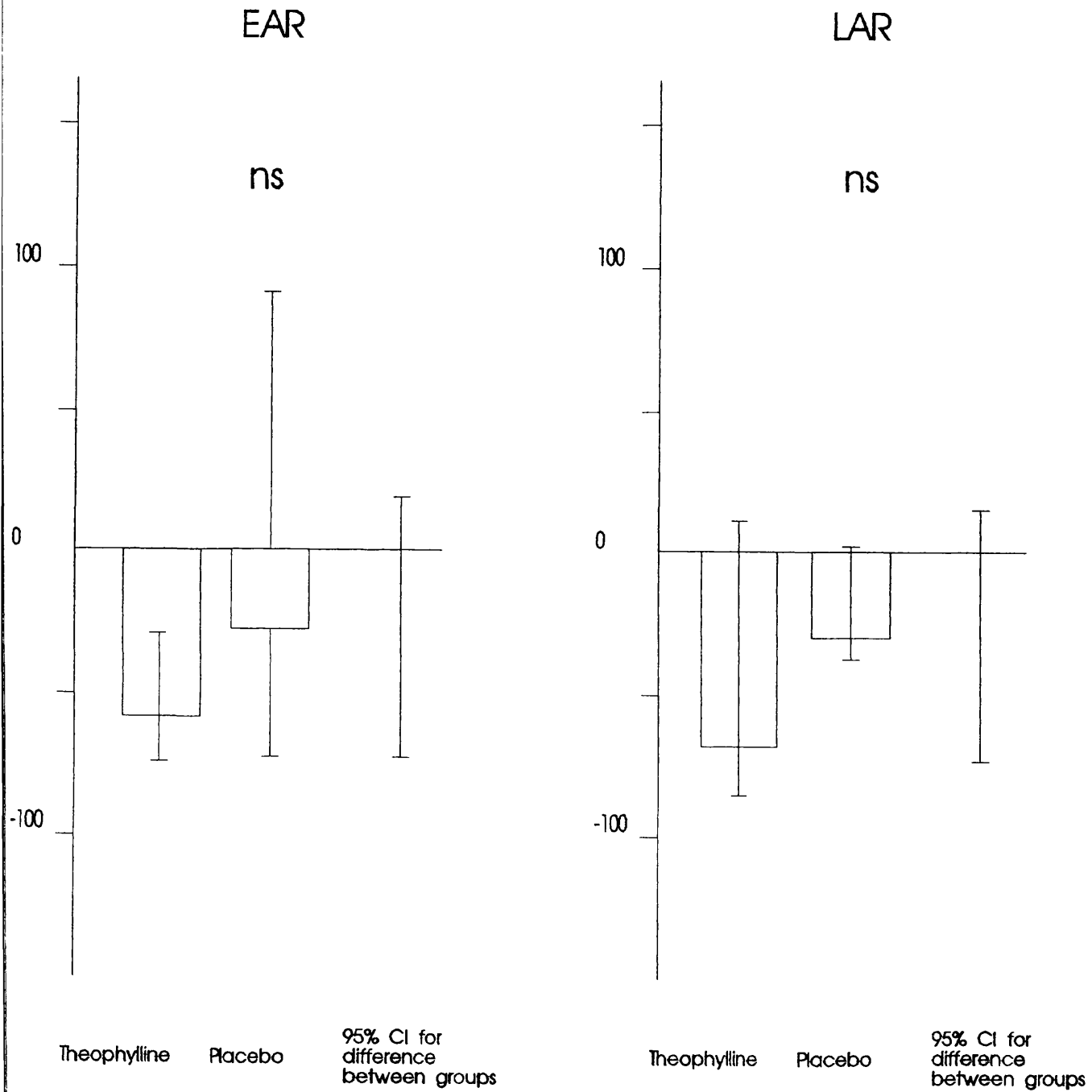


Figure 3: Median (non parametric 95% CI) change in the EAR (defined as fall in FEV1 at 30 minutes) and LAR (at 6 hours) during six weeks treatment with theophylline or placebo expressed as % of the initial value. The error bar on the right of each graph is the non parametric 95% CI for the difference between the groups.

Figure 4: EAR and LAR before and after six weeks treatment with theophylline.

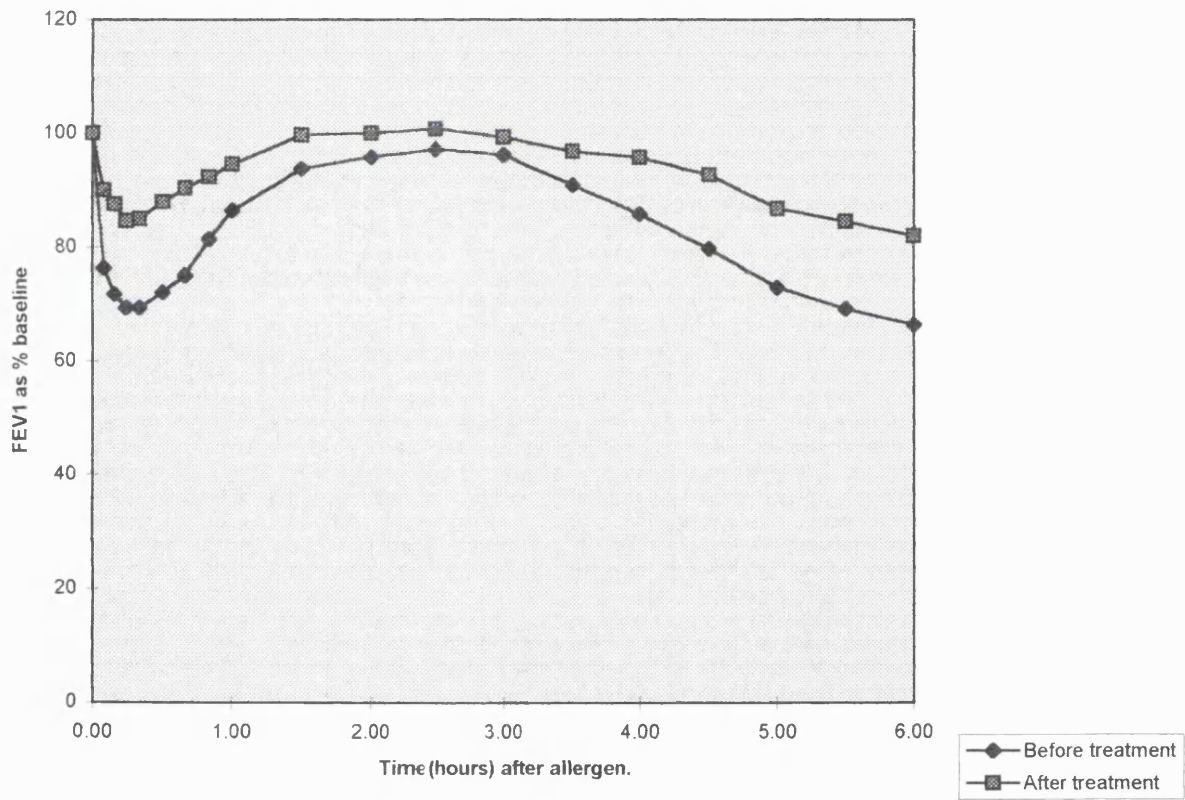
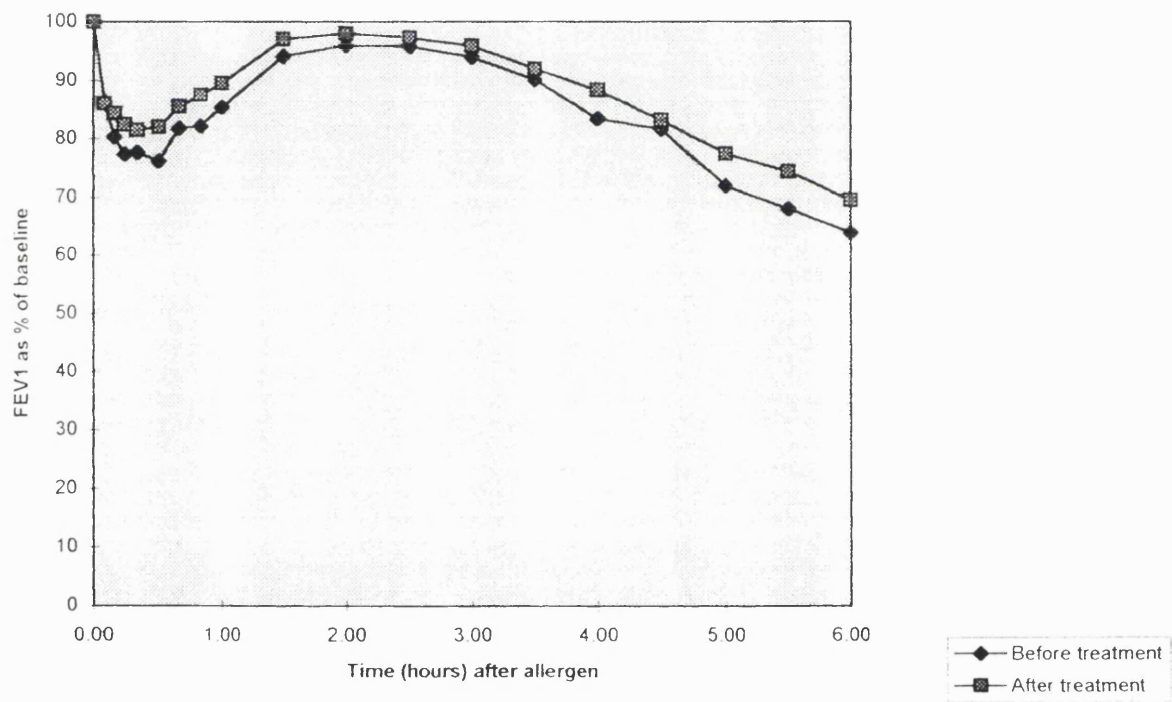


Figure 5: EAR and LAR before and after six weeks treatment with placebo.



3.9 RETROSPECTIVE ASSESSMENT OF POWER

This study has used relatively small sample numbers. No data were available before the study to assess variance in cell counts with time; a power calculation was performed based on the variance of cell counts within a group of asthmatics at one time point.

It is therefore not possible to use the outcome of this power calculation to make any assessment of the value of the negative findings in this study. Confidence intervals can, however, be used to assess the strength of negative results and this method is useful with non parametric data. Examination of confidence intervals for the percentage change in cell counts over six weeks in the theophylline group shows that the results of this study are consistent with large changes which could be clinically significant. 95% confidence intervals for the change in counts as percent of baseline were: CD3 -114% to +45%; CD4 -73% to +157%; CD8 -35% to +244%; CD25 -100% to +575%; tryptase -90% to 118%. In other words, quite large changes as a result of theophylline treatment could have occurred and the negative findings of this study do not imply absence of effect.

Another way of interpreting the strength of negative findings is to perform a retrospective power calculation based upon the observed variance in the placebo group. Assuming normally distributed data, analysis of the percentage-of-baseline change over six weeks in the placebo group reveals a standard deviation of 74% for CD3+ T-lymphocytes and 71% for eosin positive eosinophils. Power calculation based upon a standard deviation of 70% of the baseline mean, which approximates to the value for both cell types, reveals that for a power of 90% to detect a change of 50% from baseline 40 subjects would be needed in each group. To be 90% certain of detecting a 100% change from baseline would require 10 subjects in each group.

This means that although the study did not show any treatment effect upon T-lymphocytes, it cannot be claimed with confidence that no such effect occurred during the study.

Examination of the data from the placebo group shows that the *mean* value for FEV1 as a percentage of baseline at 30 minutes and 6 hours after allergen challenge did not change over the course of the study but there was considerable individual variation. The size of the fall in FEV1 was calculated at 30 minutes and 6 hours and expressed as a percentage of the baseline value. The change over six weeks was calculated and expressed as a percentage of the fall in FEV1 during the first challenge. These data were assumed to be normal for the purpose of this calculation, and standard deviation was calculated. The value of the standard deviation was 100% of the initial fall in FEV1 during EAR and 170% during LAR. Power calculation based upon the above values shows that in order to be 90% certain of detecting a 50% change during LAR the groups would need to include at least 244 subjects. To be 90% certain of detecting a 100% change in LAR would require groups of 60. Again, the study has a less than 90% chance of detecting important changes in LAR.

For bronchoalveolar lavage measurements, retrospective power calculations were made for ECP concentration and eosinophil counts. The standard deviation of the changes with time was 144% of the baseline value for ECP and 30% for eosinophil count. Power calculation showed that two groups of 174 subjects would be needed to be 90% certain of detecting a 50% change in ECP concentration. Only 8 subjects per group would be required to be 90% certain of detecting a 50% change in eosinophil count. It is therefore possible to be reasonably certain that there was not a 50% fall in eosinophil count in BAL after theophylline.

All of the above calculations assume that the data was normal, which was not the case. The results of these power calculations therefore serve only as a rough guide.

CHAPTER 4: LIMITATIONS OF METHODS

4.1 BIOPSY CELLS COUNTS: PRECISION

Examination of the biopsy cell counts in the placebo group in this study reveal that there is a large variance with time.

The high variability of cell counts limits the power of the study; this has two disadvantages, firstly the chance of detecting a true treatment effect is low and the resources of the study may be wasted and secondly, if no effect is shown it cannot be claimed with any reasonable confidence that no effect has occurred.

Imprecision is regarded as deviation of a measured value from the true value in the absence of any systematic error, i.e. without a bias in any direction. In order to assess the degree of precision of cell counts, three different biopsy sections stained for CD3 were each counted five times by the author. This exercise demonstrated a high level of intra-observer repeatability with a coefficient of variance of less than 2% in every case.

In order to assess the imprecision related to variability between sections from the same set of biopsies 5 sections separated by at least 18 μ m were stained for CD3 counted from each of three different subjects before treatment. The mean counts (SD) per mm basement membrane were 75 (26), 42 (23) and 46 (19). In other words, the SD was 26%, 55% and 42% of baseline cell count. It is therefore likely that variability of

cell counts between sections from the same biopsy leads to imprecision and contributes significantly to the variation seen over a period of time. This could be improved by counting a greater number of sections for each subject. Assuming normally distributed data,

$$z = \frac{SD}{\sqrt{n}}$$

where SD=standard deviation of repeat measurements and n = number of

measurements.

It can be stated there is 95% certainty that the true mean lies within 2z of the mean of the measurements.

This means that, if the SD is 55% of baseline and a precision of 10% is required it is necessary to count 120 biopsy sections for each subject and for precision of 20% (i.e. the true value is within 20% of the measured value) it would be necessary to count 30 sections.

It is, however, likely that the actual population SD is not as high as estimated in this study, since only a small number of measurements were made. Azzawi has presented similar data on the variance of cell counts within a biopsy and between biopsies from the same subject. The standard deviation was 32% between biopsies for CD45+ cells (leukocytes) and 44% for CD4+ cells in asthmatics.

The implications are that it would be advisable in future to:

1. include a pilot study to assess the variance between sections in one subject at a given time and the variance over time;
2. attempt to count a large number of sections for each cell;

3. include sufficient resources to allow a large number of sections to be counted,

4.2 BRONCHOALVEOLAR LAVAGE

There is a similar variation over time in BAL absolute cell counts and mediator concentrations. The standard deviation of the change over time was as high as 208% for ECP and 440% for PGD2 concentrations and 102% for total leukocytes and 213% for BAL eosinophil count. These differences may have been due to different distribution of lavage fluid on different occasions. There was a degree of visible bronchospasm during BAL in most subjects. This might lead to random variation in the number of small airways lavaged and in the number of airways from which fluid could be aspirated, since bronchoconstriction would increase the chance of airway closure under negative pressure. After allergen challenge the subjects are more likely to cough, and this could affect the distribution of lavage fluid. Any change in the proportion of lavage return fluid from distal versus proximal airways may affect cell and mediator concentrations in two ways: first, cell populations may differ down the bronchial tree and second there is a shift in the ratio between epithelial surface area and lumen volume down the bronchial tree.

An increase in the number of cells per millilitre of lavage fluid has been demonstrated after local allergen challenge (Beasley, 1989) and after allergen inhalation by BAL with small aliquots of 20 ml (De Monchy, 1985) but other studies using larger volumes have failed to show any statistically significant change in lavage cellularity after inhaled allergen challenge. Smith performed BAL with 5 consecutive 60ml aliquots 12 hours after allergen challenge and was unable to demonstrate any statistically significant change despite a 213% increase in eosinophil count during the LAR (Smith, 1992). In

future studies it may therefore be advisable to use small aliquots, particularly after allergen challenge.

A number of different methods have been proposed to correct for the dilution of mediators that inevitably occurs during lavage and to calculate the actual mediator concentration within the lung. These methods have attempted to derive a value for mediator concentration in the epithelial lining fluid (ELF) of the airway. The BAL concentration of urea has been used as a marker for dilution to calculate the ELF volume by assuming that the urea concentration in ELF is equal to that in plasma, through equilibration, and that the final concentration of urea in the returned lavage is simply a function of dilution of the initial volume of ELF in the large volume of instilled lavagate (Rennard, 1971). However, urea is able to diffuse into bronchial lavage fluid with relative ease: in foetal lamb lung urea in the blood equilibrates with airway fluid within 30 minutes (Normand, 1971). When saline is instilled into the lung the urea concentration in the lumen of the airway becomes much lower than that in the tissue and the blood. This creates a concentration gradient down which urea is free to diffuse. Urea may also enter the lumen as a solute in exuded plasma. Animal work suggests that urea flux may be rapid: in rat lung preparations the half time for passage from lung to blood was only 4 minutes (Effros, 1990). In man, too, it has been shown that the concentration of urea in lung lavage fluid rises with dwell time (Marcy, 1987), which makes it difficult to quantify the lung fluid volume. The situation is further complicated by the finding that during lavage there is a net movement of water from the tissue into the lumen by mechanisms that remain unknown (Baughman, 1983; Kelly, 1988). Other endogenous and exogenous markers such as albumin, methylene blue and inulin (Jones, 1990; Von Wichert, 1993) have been used to estimate the degree of dilution of mediators in the lavage fluid and some workers have employed novel and ingenious techniques, including microlavage of a very peripheral wedge of lung (Baldwin, 1991) and reintroduction and re-aspiration of lavage fluid (Peterson, 1991). There is, however, no gold standard by which to assess the accuracy of the

concentration values that are Oderived by such techniques, and no consensus as to which, if any, should be used.

4.3 ALLERGEN INHALATION CHALLENGE

There appears to be considerable variation over time in the response to allergen in late responders. Whilst the mean values for the size of the EAR and LAR were similar in the placebo group after six weeks, the standard deviation of the difference over time was 100% of baseline for EAR and 170% for LAR. If this represents the true standard deviation of the overall population, it will be difficult to demonstrate a treatment effect as such in a parallel group study without including a large number of subjects. If these figures were used in a power calculation for a future parallel group study and if it were predicted that there would be a reduction of the LAR by 50% the minimum number of subjects per group for a power of 90% would be 242! Whilst there was no treatment effect for theophylline when the two groups were compared, the 95 % confidence interval for the difference between the groups included a 100% reduction in the size of the LAR or a 100% increase in the size of the LAR as a result of treatment with theophylline.

CHAPTER 5: DISCUSSION OF RESULTS

5.1 LABORATORY DATA

This study tested the hypothesis that theophylline reduces inflammation in asthmatic airways after allergen challenge. Lymphocytes and eosinophils are thought to be key cells in the pathogenesis of asthma: together they form a large part of the bronchial inflammatory infiltrate. The effect of theophylline on the concentration of these cells in tissue was therefore studied. A placebo group was included and the effect of six weeks pre-treatment with placebo was compared with that of theophylline. Statistical analysis compared the change T-lymphocyte counts between the two groups. A confidence level of 95% was considered appropriate for the test of the hypothesis. In other words, the hypothesis would be considered to be true only if it could be stated with 95% certainty. In order to achieve this level of confidence a p value was chosen as the threshold for statistical significance which accounted for the fact that more than one comparison was used to test the hypothesis.

There was a significant treatment effect in terms of reduction in the number of eosinophils counted in biopsies of the airway wall 24 hours after allergen inhalation when the effect after 6 weeks in the theophylline group was compared with the placebo group ($p < 0.025$). The eosinophil is an important effector of the inflammatory component of asthma which releases a number of harmful products into the surrounding tissue. Eosinophil granules contain cytotoxic basic proteins such as major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil derived neurotoxin (EDN) (Weller, 1991) and other toxic molecules such as eosinophil peroxidase (Jong, 1980). MBP is cytotoxic at very low concentrations and has been shown to damage human bronchial epithelium ex vivo (Frigas, 1981) and the toxicity of ECP has also been demonstrated in guinea pig tracheal tissue (Djukanovic, 1990). Both proteins

have activity as ribonucleases (Gleich, 1986). Eosinophils also produce and release toxic free oxygen radicals (Kanofsky, 1988) and generate products of lipid metabolism including platelet activating factor and sulphidopeptide leukotrienes (Henderson, 1991). The eosinophil, then, has a potential role in a number of the phenomena that constitute asthma. Platelet activating factor and sulphidopeptide leukotrienes exert a potent effect on the airways, causing immediate bronchoconstriction and persistent bronchial hyper-responsiveness (Stimler, 1983; Smith, 1988; Arm, 1990). Platelet activating factor and sulphidopeptide leukotrienes induce vascular permeability and oedema experimentally (Henderson, 1991). BAL fluid in stable asthma (Van Der Graff, 1991) and in asthma following allergen challenge (Fick, 1987) contains a greater than normal serum protein concentration suggesting enhanced vascular permeability: PAF and sulphidopeptide leukotrienes are therefore likely to contribute to vascular permeability and mucosal oedema in asthma. In vitro work has also shown that eosinophils release a substance or substances which stimulate fibroblast proliferation: they may therefore play a part in the genesis of sub-epithelial fibrosis in chronic asthma. The processes involved in recruiting cells and maintaining inflammation after allergen may be the same as those which are active in chronic asthma. If so, the effect of a drug after allergen challenge will be similar to its effect in chronic asthma. Reduction in the density of eosinophils in the bronchial wall after six weeks pre-treatment with low dose oral theophylline may represent an important beneficial effect.

There was no apparent change in the number of T-lymphocytes. Given the small sample size, it is not possible to state with any degree of confidence that there was no effect: the confidence intervals were wide and it is not possible to exclude a substantial decrease or increase in T lymphocyte count after theophylline treatment.

A number of other parameters were evaluated: the findings were not regarded as hypothesis testing. They were not part of the initial hypothesis which gave rise to the study. Changes that were shown could be regarded as generating new hypotheses.

There was a significant reduction in the number of activated eosinophils in the bronchial wall. Of interest is the fact that there was a positive correlation between the number of EG2+ eosinophils and the amount of extracellular EG2 staining after theophylline treatment and between the change in the number of EG2+ cells and the change in the extent of extracellular EG2 staining, expressed as percentages of their respective baselines. This implies that the fall in the number of EG2 positive cells during theophylline treatment is not a result of increased eosinophil degranulation and subsequent exhaustion of intracellular EG2 or of cytolytic degranulation. This also implies that the reduction in tissue eosinophilia that has been demonstrated was not caused by accelerated degranulation.

In peripheral blood there was a significant treatment effect of six weeks pre-treatment with theophylline in terms of an increase in the proportion of circulating CD4+ cell that were CD4+,HLA-DR+ activated T helper cells at baseline and during EAR and LAR but not 24 hours after allergen challenge. The CD4+,HLADR+ cell population did not appear to change after allergen challenge and it may be the case that the apparent lack of effect at 24 hours is due to type II error. The suggested increase in circulating activated T lymphocytes is difficult to interpret. The circulating lymphocyte pool is only a small proportion of the total body lymphocyte population and lymphocytes are involved in a complicated re-circulation. A possible explanation is that theophylline affects the traffic of lymphocytes from blood to tissue, thereby increasing the number of cells remaining in the circulation. This is consistent with the finding by Kidney and colleagues that withdrawal of theophylline is followed by clinical deterioration and an increase in the numbers of circulating activated CD4+ and CD8+ T- lymphocytes

(Kidney, 1994a) and an increase in the number of lymphocytes seen in bronchial biopsies (Kidney, 1994b).

In keeping with these findings, in the current study in BAL fluid there was a significant reduction of the number of lymphocytes per unit volume recovered and a reduction in the number of CD3+, CD3+,CD8+, CD3+, ICAM+ and CD3+,VLA+ cells. There was, however, no change in the proportion of cells expressing activation markers and it is possible that the latter changes are simply the result of an overall reduction in lymphocyte count rather than a differential effect on cell activation.

It is of note that while there was a reduction in biopsy eosinophilia there was no reduction in the BAL eosinophil count or ECP concentration. The power of this study to detect a difference in ECP was low and it cannot be stated that the ECP concentration did not change. Similarly, the fact that the BAL eosinophil count was not shown to be affected cannot be interpreted as showing absence of treatment effect, although as discussed above, it is reasonably certain that if there is a reduction in BAL eosinophilia after theophylline it is by less than 50% of baseline.

A number of studies have examined the effect of theophylline upon the physiological response to allergen inhalation in asthma. When a susceptible asthmatic inhales an antigen to which he or she is sensitive, there is a fall in lung function within a few minutes called the "early asthmatic response" (EAR). The EAR is thought to be due in the main to the release of pre formed mediators, such as histamine, from mast cells. This recovers but may be followed 4-6 hours later by a delayed fall in lung function, termed the "late asthmatic reaction" (LAR). The LAR occurs in association with an increase in the number of inflammatory cells within the airway and it is thought of as a useful model of airway inflammation in asthma. Steroids, which have proven anti-inflammatory effects in terms of the magnitude of the cellular infiltrate in asthma abolish the late response although they have little effect on the EAR when given as a

single dose. The LAR has been used to measure the putative "anti-inflammatory" effects of a wide variety of potential anti-asthmatic agents. Several studies of the effect of theophylline on the LAR have been reported: Pauwels et al (Pauwels, 1985) showed a clear inhibitory effect of a single intravenous dose of aminophylline and enprofylline, but not placebo, on the LAR, but not the EAR in a group of 5 asthmatics. Fabbris group (Mapp, 1987) evaluated the effect of theophylline, verapamil, chromoglycate and beclomethasone on toluene diisocyanate EAR & LAR: theophylline, like the corticosteroids had little effect of the EAR but significantly reduced the LAR (the other 2 drugs had little or no effect). Ward has shown that pre-treatment with oral theophylline reduces the magnitude of the LAR significantly and to a greater degree than the EAR (which did not change significantly) (Ward 1993) and Crescioli has shown that 7 days pre-treatment with theophylline has a greater effect on the LAR than the EAR (Crescioli 1991). Other workers have, however, demonstrated an effect during the EAR but not the LAR. (Cockroft, 1989) and it has been suggested that the differential attenuation of the EAR and LAR by theophylline may be the result of an anti-inflammatory action. The current study was designed to test the hypothesis that theophylline reduces the bronchial inflammatory infiltrate after allergen challenge in late responders and not the hypothesis that theophylline attenuates the magnitude of the LAR. In this study no effect on the size of the LAR was shown. The power of the study to detect such an effect was, however, low because of the number of subjects included. No conclusion can be made regarding the effect of theophylline on the LAR on the basis of this study.

5.2 CLINICAL DATA

We have examined the clinical outcome of treatment with theophylline at serum concentrations below 10 µg/ml in young mild atopic asthmatics. The symptom scores showed a significant improvement in the proportion of symptom free days in both the

placebo and the treatment groups, which is likely to be a period effect related to a change in diary card reporting. The FEV1 prior to antigen challenge increased slightly in the theophylline group but it fell in the placebo group. A significant treatment effect was demonstrated ($p < 0.05$). It is possible that the initial allergen challenge led to a persistent decline in lung function and that theophylline has a protective effect. Alternatively, the deterioration in the placebo group may have been a chance occurrence. Overall the present study has shown a small increase in resting lung function but no effect on diary card symptom scores. The subjects in the study had very mild disease and it is unlikely that a beneficial effect would become apparent in such a group since the baseline frequency of symptoms was low. The present study was not intended as a clinical trial of low dose theophylline: the study was designed to investigate the effects of theophylline on the inflammatory response after allergen challenge. Further work, in the form of a prospective trial with a larger number of subjects, is necessary to elucidate the clinical efficacy of low dose oral theophylline.

The dose of theophylline used in this study was well tolerated and the incidence of persistent side-effects was low. Only one subject (8%) experienced an attributable symptom, irritability, after the second week of treatment. Two subjects (17%) reported nausea and one (8%) reported nausea and insomnia during the first two weeks of theophylline treatment: all of these symptoms resolved completely without any alteration of dose and none of the subjects opted to discontinue medication. Transient side effects that occur at the beginning of theophylline treatment have been reported elsewhere (Jacobs, 1976). Many of the adverse actions of theophylline appear to be mediated by adenosine receptor antagonism and there is in vitro evidence for rapid up-regulation of adenosine receptors during methyl xanthine exposure (Szot, 1987). Conversely, the bronchodilator effect of theophylline is unlikely to be mediated by the adenosine antagonism since enprofylline, a methyl xanthine without any effect at the adenosine receptor at clinical doses has similar bronchodilator action. Enprofylline also has a similar effect during the LAR as theophylline (Pauwels, 1985), suggesting

that the putative anti inflammatory effects of theophylline are not due to actions at the adenosine receptor. A possible explanation for the transient nature of the side effects of low dose theophylline is that adenosine receptor up-regulation abolishes the adverse effects without affecting the therapeutic action in the lung. It is of note that one subject in the placebo group experienced constant nausea during treatment and one subject in the theophylline group complained of nausea during the run-in, drug free, phase. The side effect profile of low dose theophylline compares very favourably with reports of the effects of higher doses. In a group of 47 patients receiving long term theophylline 30% experienced side effects and 19% reported vomiting. 78% of patients with a serum concentration above 25 $\mu\text{g/ml}$ were affected but no side effects occurred where the concentration was less than 15 $\mu\text{g/ml}$ (Jacobs, 1976). In conclusion, there was not an important excess of adverse effects in the theophylline group. Symptoms attributable to treatment were transient and virtually completely confined to the first two weeks of the study and none required discontinuation of the medication.

The dose of theophylline used throughout the study was 200mg twelve hourly, (as Uniphyllin Continus tablets), for all subjects. The intention was to alter the dose after the first week to achieve a mean steady state theophylline concentration of 5 $\mu\text{g/ml}$ and the initial dose chosen was the minimum possible dose of Uniphyllin Continus tablets. All subjects had an adequate serum concentration after 1 week on that dose and none required an increase: a decrease of dose was not practical since Uniphyllin Continus tablets cannot be accurately divided. The serum theophylline concentrations attained at the end of the study period were surprisingly similar in all of the subjects, with a mean of 6.2 $\mu\text{g/ml}$ and a range of 4-8.3 $\mu\text{g/ml}$. The non-parametric 95% confidence intervals for the population median theophylline concentration attained when otherwise healthy asthmatics take Uniphyllin Continus tablets, 200mg twice daily, was 5.35 - 6.9 $\mu\text{g/ml}$. The need for dose titration and serum monitoring of theophylline concentration derives both from the current practice of attempting to maintain a theophylline concentration

above 10µg/ml and the concern that the concentration might drift inadvertently beyond the upper permissible level of 20µg/ml: in one series 26% of patients with toxic theophylline concentrations had no risk factors for metabolic impairment and had taken the correct prescribed dosages (Sessler, 1990). The demonstration of a beneficial action of theophylline at low serum concentration would allow the use of a fixed low dose with little risk of toxicity in selected asthmatics, since the maintenance of a lower serum concentration would reduce the risk of accidental toxicity. The subjects in the present study were all young and healthy, apart from asthma and none were taking medications known to interfere with theophylline metabolism. Caution should be exercised in the interpretation of the above findings when considering the treatment of patients with conditions which might impair theophylline metabolism, such as heart failure and liver disease (Sullivan, 1994).

The present study demonstrates that in asthmatics with no concurrent disease or medication a simple fixed dose regimen of twice daily slow release theophylline achieves serum concentrations which are without lasting side effects and which may have a beneficial effect on inflammatory cell recruitment.

5.3 FUTURE STUDIES SUGGESTED BY THIS WORK

Allergen challenge is regarded as a model of the inflammation seen in chronic asthma. The finding of reduced tissue eosinophilia as a result of theophylline treatment in this situation, however, does not necessarily apply to chronic disease. It will be necessary to repeat the study, without allergen challenge, in subjects with different degrees of severity of asthma: two questions need to be answered. First, does theophylline have an anti-inflammatory effect in mild disease and, second, is there an effect in patients with severe chronic asthma. These studies would require adequate sample sizes based on power calculations.

An alternative approach to the above questions would be to examine the prophylactic effect of low dose theophylline in a group of adult patients with asthma. If theophylline has an anti-inflammatory effect which is of clinical benefit it might be expected to prevent exacerbations and/or attenuate the progressive impairment in lung function that can be seen in asthma. This type of study would require large sample sizes and would probably have to be of multi-center design.

There is also a need for research into the inherent variance with time of a number of different parameters in asthma; this information is not currently available. It would be helpful to perform a study of variance in cell counts in biopsies and BAL over time with a larger sample of subjects.

Similarly, there is no reliable data on the variance of cell counts between biopsy sections or the distribution (i.e. are cell counts from different sections from the same patient normally distributed?). This information would allow investigators to select a number of sections for each marker in a rational way and to allocate adequate time and resources for cell counting in advance. Again, larger numbers of sections would need to be assessed in order to arrive at a sample variance which is similar to that of the overall population (i.e. all asthmatics or all normals). This study is currently being undertaken by the author at King's College Hospital.

5.4 CONCLUSION

Six weeks pre-treatment with oral theophylline, a drug which has been used as a bronchodilator for many years, has an additional effect of reducing airway eosinophilia after allergen challenge. This effect is achieved at a dose which appears to be associated with few side effects. This may translate into a beneficial effect in the

treatment of chronic asthma: further work is required to investigate this. Other immunomodulatory effects are suggested by this study. There was an increase in circulating HLA-DR positive - CD4 positive lymphocytes (activated T helper cells) and a decrease in the concentration of lymphocytes and activated lymphocytes in bronchoalveolar lavage. This may reflect an effect upon lymphocyte traffic from blood to lung. The findings relating to lymphocytes were regarded as exploratory: further work is necessary to confirm these findings.

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