INHALED CORTICOSTEROIDS IN ASTHMA

Effects on inflammation and lung function

INHALATIECORTICOSTEROIDEN BIJ ASTMA

Effecten op ontsteking en longfunctie

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The everlasting universe of things
Flows through the mind, and rolls its rapid waves,
...where from secret springs
The source of human thought its tribute brings

Shelley, *Mont Blanc.*

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Chapter 1

General Introduction

Asthma

1.1 Clinical aspects

Many clinicians are frequently confronted with an adolescent who comes to the first aid department in the middle of the night, complaining of breathlessness and chest tightness. While he was in a smoky environment he became wheezy and felt out of breath. After taking some bronchodilator puffs his complaints did not improve but got even worse. Others are more familiar with the picture of the infant, out of breath sitting on the bench during gymnastics whereas other kids are busy doing their exercises. All clinicians will immediately recognize the clinical symptoms of an asthma patient. But what exactly is going on within the airways?

Asthma is one of the most common disorders, affecting approximately 10% of the population in the Western countries. Asthma, coming from the Greek word ασθμα, meaning panting, was used to describe several disorders characterized by breathlessness or pain in the chest. Sir John Floyer wrote in his "treatise of the asthma" in 1698[59]: "I have assigned the immediate cause of asthma to the straitness, compression, or constriction of the bronchi". Laennec in the eighteenth century attributed asthma to a spasm of the smooth muscle fibers of the bronchi. In spite of the fact that our knowledge of the disease has increased since then and asthma is now considered as a chronic inflammatory disease, we still do not know the fundamental cause of asthma and all the factors that induce airway inflammation.

Airway inflammation in asthma is characterized by redness and swelling of the mucosa[14]. These classical signs of inflammation are easily visible at bronchoscopic examination. Bronchial biopsies not only show activated mast cells, eosinophils and lymphocytes[49], but also epithelial shedding and fragility. Structural changes include hypertrophy and hyperplasia of airway smooth muscle, and thickening of the basement mem-brane[82] due to the deposition of collagen in the lamina reticularisb (figure 1).

Acute exacerbations of asthma are associated with increased eosinophilia in peripheral blood and sputum. What we do know is that there is accumulating evidence that the prevalence[58], severity[9, 158] and mortality of asthma are rising[102].

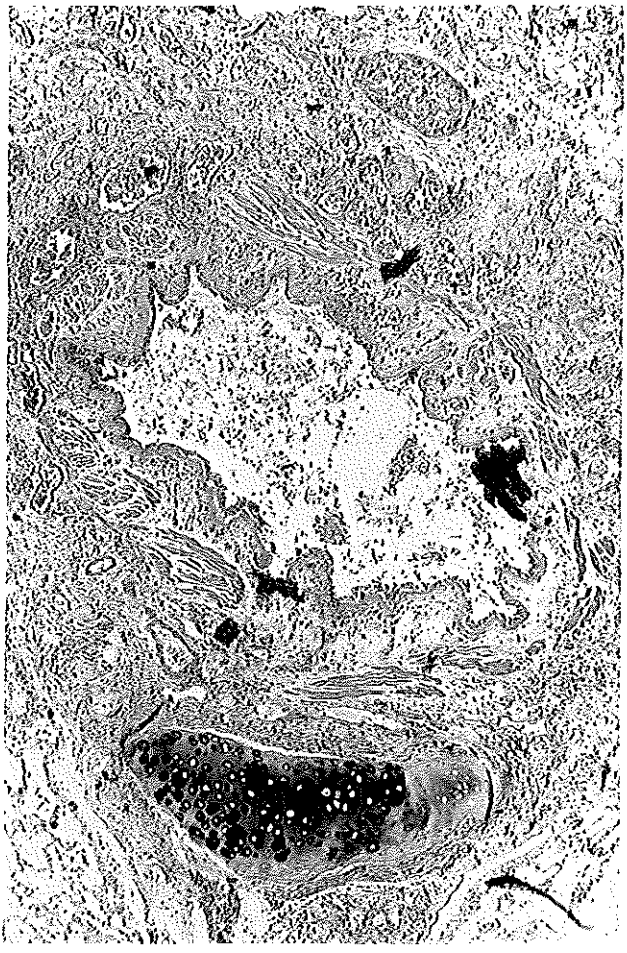

Figure 1. Obduction specimen of an asthmatic patient (magnification 25x). Mucus plugging of the lumen is present. Hypertrophy of smooth muscle, vasodilatation and mucosal inflammation can be observed around the obstructed airway. At the bottom of the figure cartilage can be seen.

Clinically, asthma is characterized by intermittent episodes of reversible airway obstruction that are separated by periods of stability, which may vary in length from hours to weeks[89]. In attack symptoms of obstruction such as cough and wheezing are evident. The most common problem encountered in paediatric practice is exercise-induced asthma, whereas in adults, exercise represents just one of the provoking stimuli. A significant number of asthmatic patients has a poor perception of the disease[151]. Signs and symptoms often do not correlate with the intensity of an attack[39, 95]. Asthma can be differentiated in an atopic ("extrinsic") and non-atopic

("intrinsic") form. The extrinsic form is encountered more frequently in childhood and adolescence. Atopy is the inherited predisposition[133] to become sensitized to specific airborne allergens[41] such as house dust mite, pollen or animal danders. Binding of allergen to specific IgE-molecules present on mast cells and eosinophils induces secretion of mediators[98, 175], causing bronchoconstriction, vasodilation and secretion of mucus, resulting in clinical symptoms. Quite characteristically, almost immediately after exposure to certain allergens, such as pollen or cats, patients experience a period of chest tightness, which becomes maximal 10-20 min after allergen inhalation and resolves spontaneously in one to two hours, and is often associated with symptoms of rhinitis and itchy eyes. This response (early asthmatic response = EAR) is often followed by a second phase of airflow limitation, which becomes maximal at 6 to 12 hours (late asthmatic response = LAR). This LAR is mainly due to airway inflammation[2]. Some subjects experience both an EAR and LAR, others may only experience an isolated single response[118].

Subjects with atopic astma usually have a positive family history, high IgE and positive skin tests for allergens such as house dust mite, pollen or animal danders. Blood eosinophilia is often but not necessarily present, being a prominent feature in both intrinsic and extrinsic asthma[52]. On the basis of the relation between airway hyperreactivity and serum IgE levels, it has been suggested that all patients with asthma have an atopic component[33, 157]. Other estimates however, indicate that as many as one third of patients with asthma are not atopic[121].

The intrinsic, non-atopic, form of asthma is found in adolescence, in subjects with a negative family history, and is often associated with eosinophilia and nasal polyps. The etiology of non-atopic asthma is more difficult to demonstrate[81, 124]. Viral infections[108] have been proposed as a pathogenic mechanism. Although the precise mecha-nisms that cause atopic or intrinsic asthma are still not fully understood[98], both types of asthma have in common an eosinophil-dominated inflammatory reaction of the bronchial tissue[98] with T lymphocyte activation[21].

It is highly unusual for a patient who has been diagnosed as suffering from asthma not to demonstrate bronchial hyperresponsiveness (BHR) at least at some time during the course of the disease. BHR is a characteristic feature of asthma and refers to the increased sensitivity of the airways to a wide variety of physical stimuli[77] such as exercise[10, 85], fog, or isocapnic hyperventilation of cold dry air[125]. These stimuli are believed to act through release of bronchoconstrictor mediators from cells within the airways. In addition, BHR can also be provoked by a number of pharmacological agonists such as histamine, adenosine[182], methacholine[86], acetylcholine[129], serotonin, leukotrienes[3] and the prostaglandins PGD_2[73] and $PGF_{2\alpha}$[166].

Several studies have shown that BHR is not a fixed phenomenon, but may be induced or enhanced by certain triggers such as viral infections of the upper airways[34, 35, 47], exposure to allergens[31, 42], air pollutants (tobacco smoke included)[70] or during occupational exposure to sensitizing agents[114]. Clinically, BHR can become manifest as an episode of airway obstruction, rather frequently coinciding with unproductive coughing. BHR is related to the severity of the disease as indicated by respiratory symptoms[30], the level of inflammation in the airway wall[40], and the need for therapy as indicated by the minimum drug requirements to keep symptoms well controlled[75]. The precise link between symptoms, BHR and airway inflammation is, however, still uncertain[13]. BHR also appears to be of prognostic significance in prospective clinical studies both in children[65] and in adults[153].

From the end of the 19th century untill the early 1980s asthma was considered as a disease of paroxysmal dyspnoea with narrowing of the airways by inappropriate constriction of airway smooth muscle[163]. With the introduction of the fibre-optic bronchoscopy, it became possible to perform bronchoalveolar lavage (BAL) and to take bronchial biopsies safely in individuals with asthma[183]. These studies have

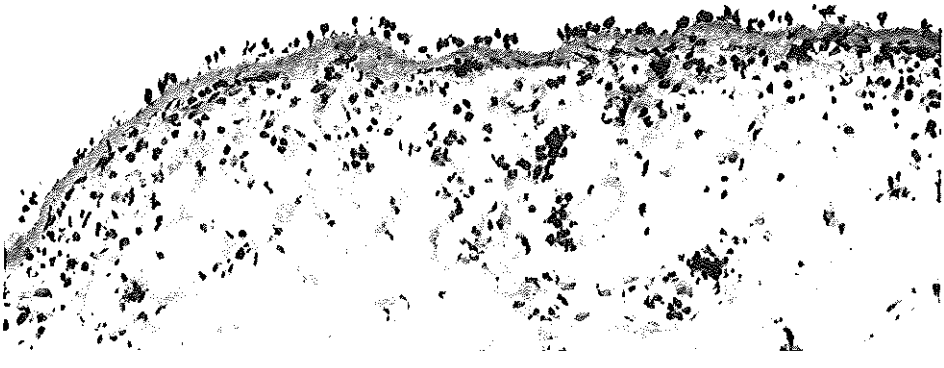

Figure 2. Bronchial biopsy of an asthmatic patient (magnification 63x). There is severe shedding of the epithelium. Parts of the basement membrane are not covered by epithelium. (Biopsy S.E. Overbeek, staining G.M. Möller)

considerably enlarged our knowledge about the disease[104, 105, 111] and taught us, somewhat unexpectedly, that not only in patients with very severe asthma[17, 104] but also in patients with all grades of asthma[62, 82] inflammatory changes can be found in the airways[40, 79, 140]. Biopsies of bronchial tissue from patients with asthma have demonstrated that infiltration by inflammatory cells, particularly eosinophils, mast cells and lymphocytes, and epithelial shedding (figure 2) are prominent features[49, 82]. BAL has also revealed an increased proportion of inflammatory cells, particularly eosinophils[26, 32]. Based upon the above insight in the pathophysiology of asthma, it has become acceptable to define asthma as a disease that is characterized by a history of episodic wheezing, by physiologic evidence of reversible airflow obstruction, either spontaneously or following bronchodilator therapy, and by pathologic evidence of inflammatory changes in the bronchial mucosa[123].
Despite the quite clear clinical picture of asthma, we still do not know exactly what is going on in the airways during an asthmatic attack. What is, or are, the most important cell(s) participating in the inflammatory response, which mediators are produced first and do they stimulate the production of others in a later phase?

1.2 BRONCHOALVEOLAR LAVAGE AND BRONCHIAL BIOPSY

Invasive bronchoscopy with BAL and biopsy is of little clinical value in asthma, but it can be used as a tool for clinical studies. Since we know that asthma is not only characterized by reversible airway obstruction, due to contraction of airway smooth muscle, but also by inflammatory changes in the bronchial mucosa, the interest in investigative bronchoscopy has increased. Combined with bronchial biopsy, the study of BAL material enabled us to better characterize the pathology in asthma and understand its pathogenesis. The application of the modern techniques of cell and molecular biology further enlarged our knowledge. In this chapter the technique of BAL and biopsy, that we used for our studies, will be outlined together with the problems we and other investigators had to cope with.
Several investigators have performed BAL studies in asthma to obtain the fluid, lining the airways and alveoli to study inflammatory cells and mediators[69, 94, 119]. Because it is likely that both airway and alveolar material are present in the recovered lavage samples, with alveolar material predominating in the most commonly used techniques, a number of modified techniques have been proposed to enrich lavage samples for airway contents[53, 142]. Using fractionated lavage, Van Vyve et al.[170] demonstrated that the cell content of bronchial and more distal segments of the lung were not

comparable, indicating that studies should not give pooled data in asthmatics. Because the small airways may play an important role in asthma, BAL fluid samples of the "alveolar" fraction may give data that are just as significant as those obtained from "bronchial" samples.

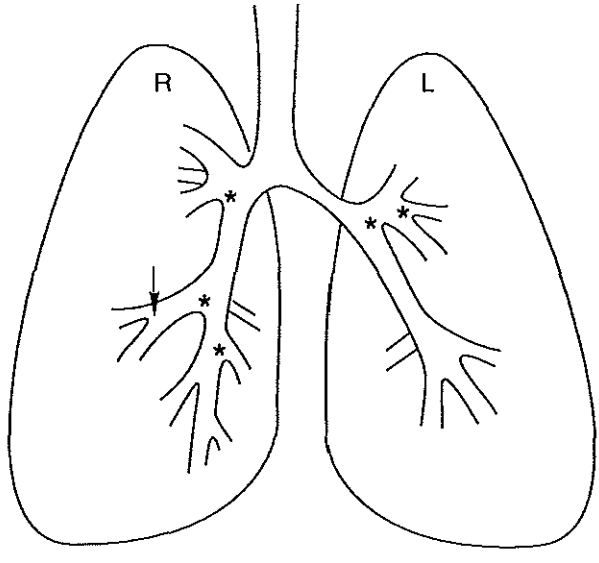

Figure 3: Using the fibre-optic bronchoscope, mucosal biopsies were taken from the carinae of the segmental and subsegmental divisions of the main bronchi (*). The lavage site is indicated with an arrow (↓)

Although there is not one universally accepted standard technique for BAL in asthmatics, many guidelines are currently available[23, 55, 183]. In general, the procedure is as follows: a full explanation of the procedure and possible complications is given to the patient. Premedication, consisting of atropine 0.5 mg intramuscularly and bronchodilator medication per nebulizer, is then administered. The nose, throat and vocal cords are then anaesthesized with topical anaesthetic. The patient is then attached to a pulse oxymeter to monitor oxygen saturation during the procedure. Oxygen is supplied when oxygen saturation is below 90%. Firstly, the bronchoscope is placed in wedge position in one of the segments of the right middle lobe and BAL is performed. In the literature the quantity of fluid used in lavages varies according to the needs of the protocol. Lavages have been performed with as little as 5 ml of fluid, and with as much as 400 ml[141, 183]. We usually have used 30-40 ml for the bronchial fraction and 4 x 50 ml for the alveolar portion. Lavage fluid should be aspirated with gentle suction. Secondly, biopsies from segmental and subsegmental divisions of the main bronchi can be taken, three to six in total (figure 3).

Immediately after the procedure extra bronchodilator can be supplied, when indicated. In over 125 bronchoscopies with lavage and biopsy in our hospital in asthmatic patients, only one patient needed to be hospitalized for one day because of an acute asthmatic attack. Only minor complications were seen in some other patients (minor bleeding, some wheezing, transient hypoxaemia). A few patients suffered from fever and transient pulmonary infiltrates, sometimes associated with chest pain, within hours after the procedure. These complications are identical to the potential hazards of BAL reported in the literature[23, 143]. Nevertheless, when performed by skilled investigators and even when combined with biopsies, BAL can be safely performed for research purposes in asthma[62, 93].

It is a major problem to retrieve mucosal biopsies of good quality. This has also been experienced by other investigators[82, 173]. We had to cope with the size of the bronchoscope in relation to the size of the biopsy forceps. When performing a BAL well, the scope needs to be placed in wedge position. The smaller the diameter of the scope, the better the procedure can be performed. This limits, however, the size of the biopsy forceps, thereby directly influencing the size of the biopsy specimen.

The biopsy forceps need to be large enough to retrieve biopsy samples of sufficient size and quality. However, it should not artifactually damage tissue samples. We experienced these problems in earlier studies when our biopsy samples were very small indeed, sometimes even too small to allow investigation, or were partially damaged by the biopsy forceps. But, with increasing experience, these problems were resolved.

In asthmatics, biopsy taking is difficult for two reasons. Firstly the patient can become very bronchospastic during the procedure despite adequate broncho-dilator therapy, therefore one has to work very fast. Secondly the bronchial mucosa in asthmatics can be very weak and bleeds easily because of its fragility[82]. When biopsy is preceded by BAL, mucosal weakness makes it especially difficult to retreave a good biopsy sample.

BAL studies have shown that many different inflammatory cells and mediators are involved in asthma[17]. BAL findings have been related to various clinical features of asthma, such as symptoms[175], spirometric measurements[94] as well as to the degree of airway responsiveness to histamine or methacholine[37, 93, 175]. Cells and mediators present in BAL fluid only represent an indirect estimation of the bronchial inflammation. It is evident that no single inflammatory cell type is responsible for the complex pathophysiology of asthma, although certain particular cells are predominant in asthmatic inflammation. The most characteristic features of these cells will be outlined in chapter 1.3.

1.3 PATHOPHYSIOLOGY

As mentioned earlier, it is gradually appreciated that asthma is a chronic inflammatory disease of the airways, with, in atopic subjects at least, IgE-mediated mechanisms producing intermittent exacerbations of this chronic inflammatory process. Despite the quite clear clinical features of asthma we still, however, do not know the exact relationship between the symptoms and the chronic inflammation that is so characteristically present in all grades of asthma. In the first part of this chapter the present knowledge of the possible events and sequence of events taking place in asthma will be outlined; in the second part the most striking features of the different cells, involved in these events, will be discussed. Most, if not all, of these cells are known to be producers of different inflammatory mediators such as leukotrienes or cytokines. We decided to look into the role of arachidonic acid metabolites in particular. Therefore, in the third part the metabolism and properties of the various arachidonic acid metabolites will be outlined.

Postulated mechanisms

The initial event in allergic asthma is the presentation of antigen to T lymphocytes by antigen presenting cells, dendritic cells (Dc) in particular (see figure 4). After having recognized the antigen as "foreign" material, T cells become activated by the Dc. Subsequently, not only the T lymphocytes, but also the Dc start the production of a wide variety of mediators. T lymphocytes will secrete a mixture of lymphokines characteristic for either the Th1 or the Th2 CD4+ T-lymphocyte phenotype. Th1 cells mainly secrete IL-2 and interferon γ, whereas Th2 cells mainly produce IL-4, IL-5, IL-6, IL-10 and IL-13. IL-3 and GM-CSF are produced by both cell types. The mechanisms which determine the expression of Th1 or Th2 phenotypes are not completely understood, but we do know that products of the Th1 cells (through interferon-γ) have the capacity to inhibit growth of the Th2 cells[144], and vice versa (through IL-10) [56, 57]. Th1 cells are thought to elicit delayed-type hypersensitivity; Th2 cells are supposed to stimulate allergic responses.

The cytokines produced by the Th2 subpopulation modulate the function of several other cell types. Physical coupling of the T and B cell is required[64] to bring about an IL-4 induced B cell isotype switch leading to the production of IgE. IL-13 has been shown to affect B cell isotype switching via an IL-4 independent mechanism[185]. Cross-linking of IgE on mast cells and/or basophils leads to the release of inflammatory mediators and cytokines. IL-5 has been shown to promote the recruitment and activation of eosinophils[98, 110].IL-3 and GM-CSF are important in the activation of

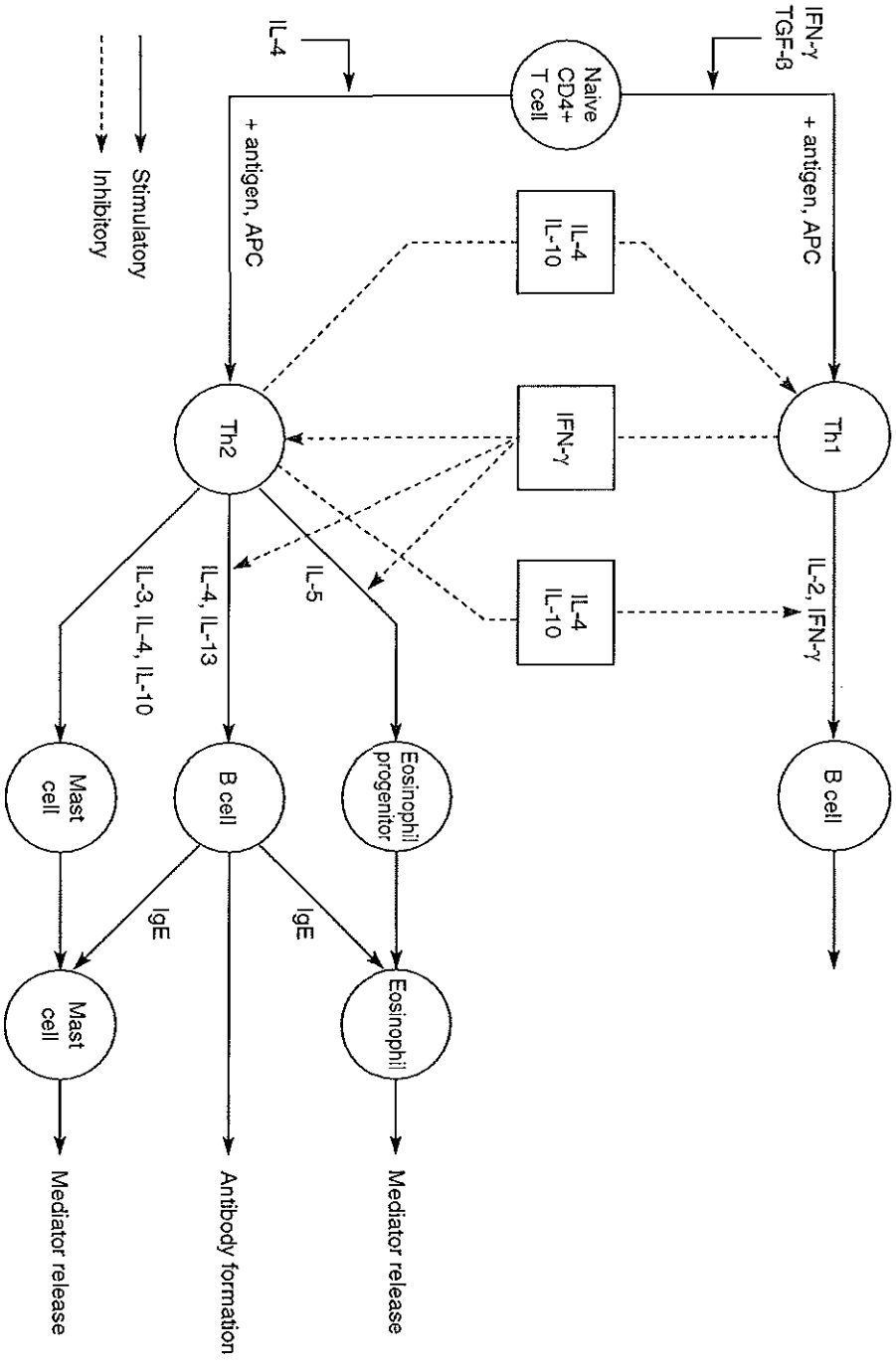

Figure 4: Regulatory interactions between CD4 T cell subsets

eosinophils[150, 165]. The entire sequence of events will eventually lead to the accumulation and local activation in particular of eosinophils, mast cells and T lymphocytes in the mucosa. There is circumstantial evidence that it is the secretory products of eosinophils which are responsible for the ultimate mucosal damage (figure 5) found in asthmatics[26]. Nowadays, it is believed that the EAR is largely due to the IgE-dependent release of mast cell spasmogens such as histamine, prostaglandin (PG) D_2 and its active metabolites (9α,11ß - PGF_2)[16], and the leukotrienes (LT) C_4, D_4 and E_4[176] (see later in this chapter). The T cells are considered to orchestrate the inflammation seen during LAR and to encourage the recruitment of eosinophils through the production of IL5.

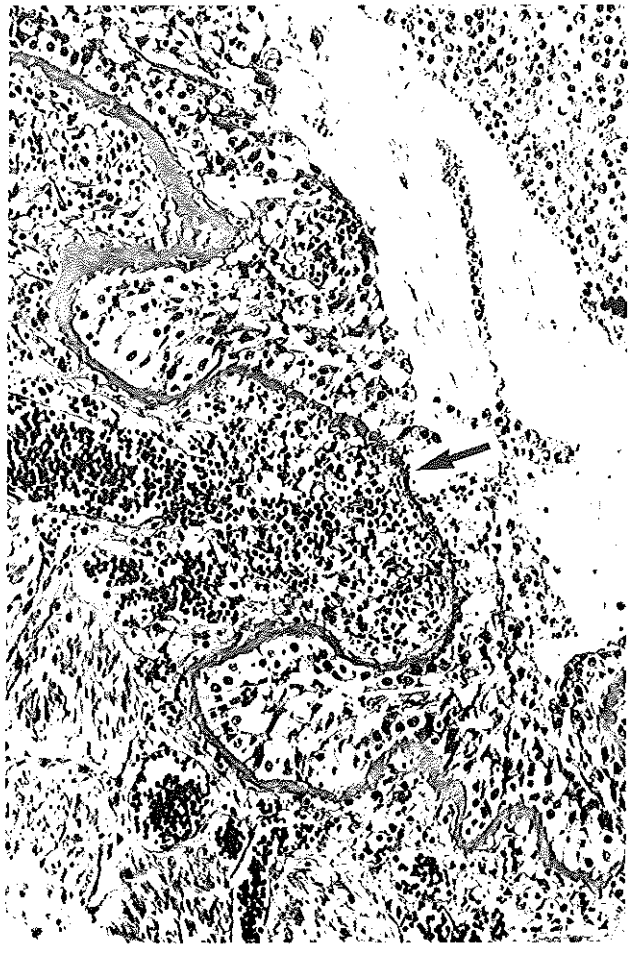

Figure 5. An example of thickened basement membrane of the bronchial mucosa in an asthmatic patient (arrow).

Cells important in asthma

Eosinophils

Elevated numbers of eosinophils in BAL-fluid and in bronchial tissue are a constant feature in asthma[12, 26, 175] although the increase in numbers seems of less importance than their activity[4, 50]. Activated eosinophils play a crucial role in the astmatic airway, already being significantly elevated at 3 h following allergen inhalation[1]. In activated state they can interact with T cells[117].

Both in atopic and non-atopic asthma, most studies have shown an increase of eosinophil numbers in BAL-fluid[26] and biopsies[103]. The correlation between BAL-fluid eosinophilia and BHR is controversial: several studies reported a significant correlation between BAL-fluid eosinophilia and BHR[90, 175], whereas others could not confirm these findings[4]. Also in bronchial biopsies of both atopic and non-atopic asthmatics eosinophilic inflammation is present[21, 26]. Again the correlation between the number of eosinophils and BHR is controversial[50, 126].

Eosinophils, at the site of inflammation, secrete major basis protein (MBP), eosiniphilic cationic protein (ECP), eosinophil peroxidase (EPO) and eosinophil derived neurotoxin (EDN) which induce desquamation of the respiratory epithelium (figure 6).

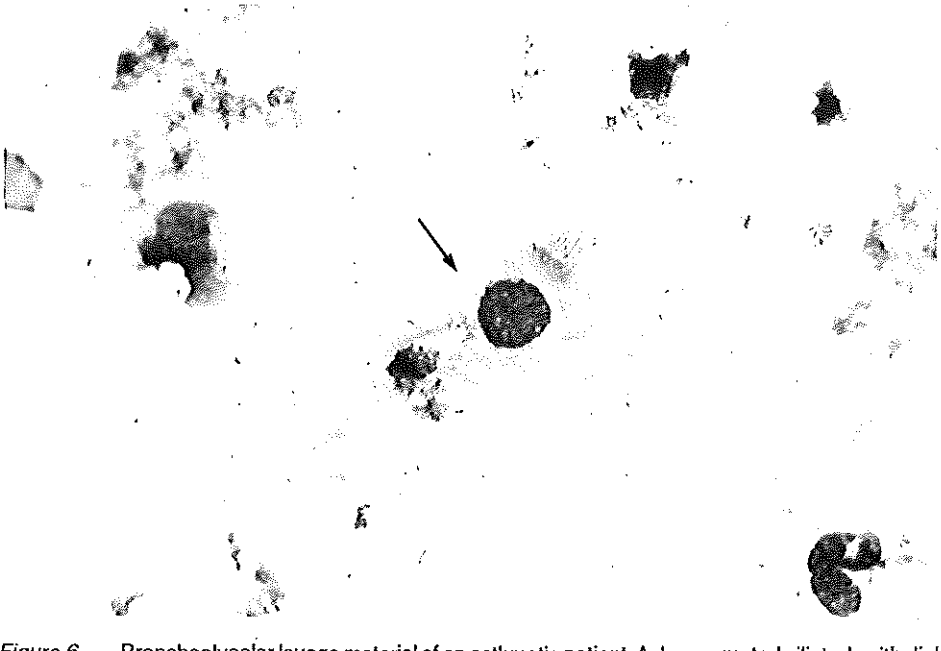

Figure 6. Bronchoalveolar lavage material of an asthmatic patient. A desquamated ciliated epithelial cell can be seen (arrow).

They may also release a range of newly formed mediators, such as LTC_4, a potent bronchoconstrictor, PAF, prostaglandins[159, 174] and many cytokines as will be discussed later in this chapter.

Mast Cells

Mast cells have been found in slightly increased numbers in BAL of both atopic and non-atopic asthmatics[132, 175, 178]. Spontaneous release of mediators from mast cells such as granule-associated histamine and tryptase is increased in asthmatics[32, 175], suggesting that these mast cells are in an activated state[139]. Most of the mast cells in human airways are of the tryptase-containing subtype[156].

Mast cells are an important source of membrane-derived mediators: small amounts of arachidonic acid metabolites such as LTC_4, PGD_2 and TxB_2 as well as PAF are released by human mast cells. Various cytokines such as IL-3, IL-4, IL-5, IL-6, GM-CSF and TNF-α[27, 28] are also generated whereas, as mentioned above, histamine and tryptase are secreted from the mast cell granules.

The demonstration that mast cells may serve as a source of IL-4, IL-5, in addition to GM-CSF and TNF-α, suggests that they may play an important part in initiating and maintaning the inflammatory response in asthma[50]. The importance of both IL-4 and IL-5 in the pathogenesis of asthma is well established[36]. IL-4 stimulates the differentiation of Tlymphocytes into the Th2-phenotype[8, 184] and is responsible for B cell isotype switching to IgE production[135]. IL-5 has been shown to be a potent and specific growth factor for eosinophils[88] also prolonging their survival[128].

Lymphocytes

Lymphocytes are the only cells which, through the CD3/antigen receptor complex, directly recognize and respond to processed antigens, and are now suggested to play a major role in asthma. The initial event is thought to be the presentation of antigen by dendritic cells to naïve CD4+ T cells. After activation by antigen, CD4+ T lymphocytes have the capacity to elaborate a wide variety of protein mediators called cytokines. According to the pattern of cytokines they secrete, CD4+ cells can be subdivided into two major phenotypes[122], namely Th1 and Th2. Also a third phenotype, Th0, secreting a mixture of Th1- and Th2-derived lymphokines is supposed to exist[137]. Th1 cells predominantly secrete IL-2 and interferon-γ, whereas Th2 lymphocytes are the producers of IL-4, IL-5, IL-6, IL-10 and IL-13[8, 45]. Both Th1 and Th2 cells produce IL-3 and GM-CSF[8]. IL-4 and IL-13 promote IgE production by B cells[66] whereas interferon (IFN)-γ is a strong inhibitor of this process[144]. By prolonging its survival

and stimulating its activity, IL-5 is a potent regulator of various eosinophil activities[68, 99]. Asthma is generally considered to be a Th2 cell mediated disorder[145]. In BAL fluid from asthmatic patients IL-4 and IL-5 were increased in comparison to IL-2 and IFN-γ, this profile being compatible with synthesis of the Th2 subset[147, 148].

Antigen presenting cells, dendritic cells in particular

Antigen must be presented by antigen presenting cells, Dc in particular, to T cells before it can be recognized. Dc are found in all the tissues and organs of the body, the lung included. Immature Dc differentiate in the lung under the influence of locally produced GM-CSF into a more mature type. Möller et al[120] showed increased numbers of Dc in the bronchial submucosa of atopic asthmatics when compared to normals, that decreased after inhaled glucocorticoid therapy. Increased numbers of Dc were also found in the nasal mucosa of patients with isolated grass-pollen allergy [60, 61]. Certain chemokines may contribute to the recruitment of Dc to the lung such as monocyte-chemotactic protein (MCP)-3, macrophage inflammatory protein (MIP)-1α and RANTES[161]. TNF-α may be a general stimulus for Dc migration[113]. In sensitized asthmatics, Dc may play a role in the maintenance of the mucosal inflammation by ensuring local activation of specific memory T cells in response to allergen. Further studies are needed, however, to elucidate the exact role of Dc in asthma.

Macrophages

Macrophages are the predominant cells in the airways accounting for 80% to 90% of the airway cells in lavage fluid. Macrophages are in activated state thought to contribute to airway inflammation in asthma because of their capacity to secrete preformed inflammatory mediators, to produce reactive oxygen products such as superoxide, and to secrete PAF and arachidonic acid metabolites[13, 69]. Following allergen challenge, macrophages become activated through low-affinity IgE-receptors[13, 69]. Cytokines secreted by macrophages, such as IL-1, IL-6, IL-8 and TNF-α, are potential contributors to inflammation of the late asthmatic response[24].

Basophils

Basophils are suggested to play an important role in the LAR, based on their release of lipid mediators such as LTC_4 (and its metabolites LTD_4 and LTE_4) and PAF[7]. Unlike the mast cell, the basophil is unable to make LTB_4 or PGD_2[177]. Although tryptase is present, it does not exceed 1% of the level seen in mast-cell granules[38]. The precise role of the basophil in asthma needs to be further elucidated.

Neutrophils

The role of neutrophils in asthma is still poorly understood, as neutrophil infiltration is not a prominent feature. Increased numbers have inconsistently been found in asthmatics[116, 119]. In normal subjects, however, neutrophils can also be found in the BAL-fluid[134] and in the bronchial mucosa.

Arachidonic acid metabolism

Arachidonic acid, released from membrane bound phospholipids during cell activation, may be transformed into biologically active lipids, the so called eicosanoids, via two major pathways. Prostaglandins and thromboxane (Tx) are generated through the cyclooxygenase pathway, whereas leukotrienes are formed via the lipoxygenase pathway (see figure 7).

By catalyzation and subsequent reduction PGH_2 is formed, which is then transformed to PGI_2, PGE_2, TxA_2, PGD_2, and $PGF_{2\alpha}$[107]. PGI_2 and TxA_2 are unstable mediators and rapidly metabolize into 6-keto-$PGF_{1\alpha}$ and TxB_2 respectively[101]. Almost all cells are able to generate cyclooxygenase products, although the specific product varies from cell to cell. Human mast cells preferentially generate PGD_2[154], macrophages and airway epithelial cells generate $PGF_{2\alpha}$, PGE_2 and Tx[80], whereas vascular endothelial cells produce PGI_2[152]. Cyclooxygenase has two isoforms: cyclooxygenase-1 is involved in physiological actions of prostaglandins in the stomach and kidney, whereas cyclooxygenase-2 is associated with inflammatory processes[180].

TxA_2, PGD_2, and $PGF_{2\alpha}$ are potent bronchoconstrictors[18, 43, 146]. Increased concentrations of PGD_2 and TxA_2 were found in BAL of atopic asthmatics after allergen provocation[109, 179]. PGD_2 levels in BAL are also increased in patients with nocturnal asthma[127]. In addition, both PGD_2 and TxA_2 have been shown to induce airway hyperreactivity in asthmatics[18, 63]. PGE_2 may relax or contract airway smooth muscle, depending on which receptor subtype is involved[44]. PGE_2, PGI_2 (prostacyclin) and $PGF_{2\alpha}$ are potent inducers of cough, possibly through stimulation of irritant receptors and C-fibers[46]. Although usually acting as bronchodilator, PGI_2 may have bronchoconstrictor properties in mild asthmatics[74].

Free arachidonic acid may be metabolized via the 5-lipoxygenase pathway to leukotrienes and 5-hydroxy-eicosa-tetranoic acid (HETE), to 15-HETE via the 15-lipoxygenase pathway, or to 12-HETE via the 12-lipoxygenase pathway (see figure 7) with 5-, 12- or 15-hydroxyperoxy eicosatetraenoic acids (HPETE's) as instable intermediates. The unstable LTA_4 is further converted into LTC_4, LTD_4 and LTE_4. LTC_4, LTD_4 and LTE_4 are formed by eosinophils, basophils and mast cells.

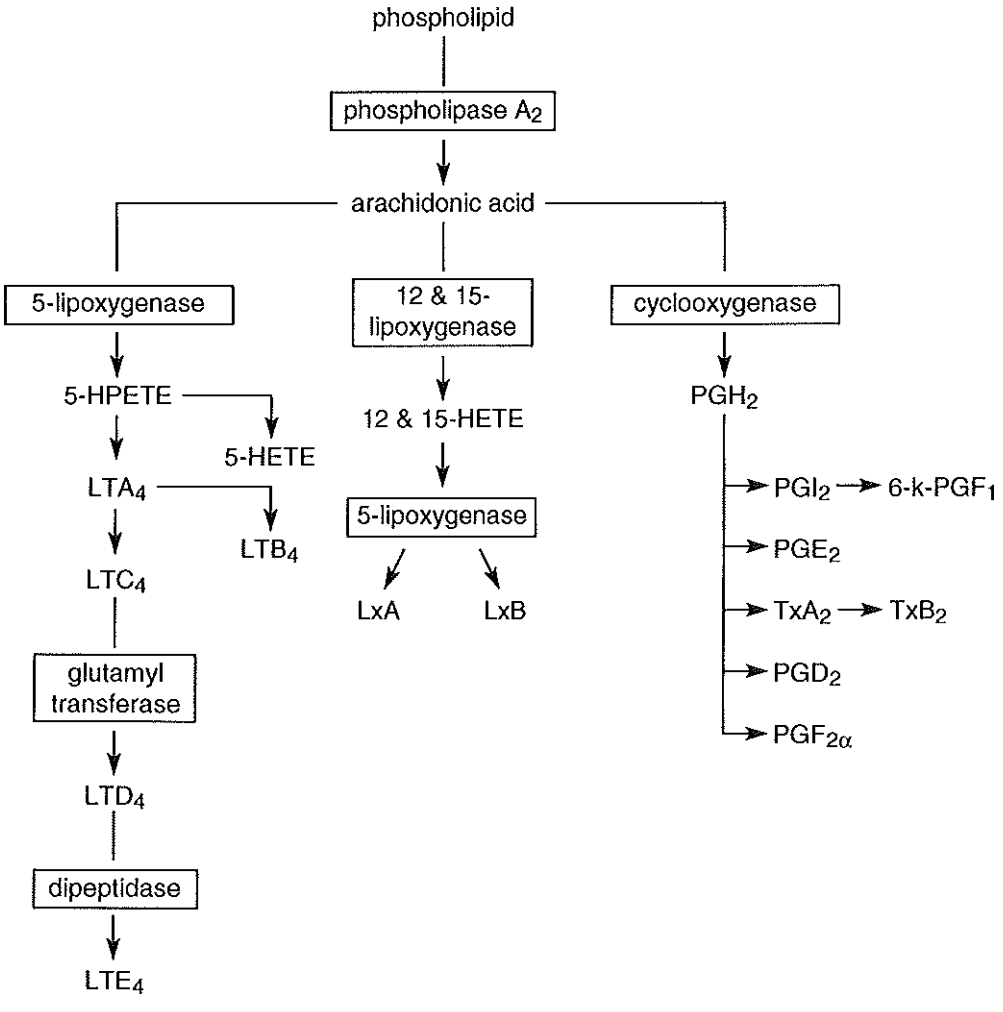

Figure 7: Metabolism of arachidonic acid

They induce bronchoconstriction, reduce sputum clearance, and increase vascular permeability[48]. LTB_4 and LTE_4 are potent chemoattractants for inflammatory cells[106, 115]. LTB_4 is produced by neutrophils, airway epithelial cells and macrophages. Enhanced urinary LTE_4 can be recovered from the urine after allergen challenge[130, 165]. HETE's are only produced in small amounts. Their role in asthma is unclear[80].

1.4 LUNG FUNCTION MEASUREMENTS

Several lung function measurements have been used for our studies. A short description of each test will be given in this chapter, preceded by some general information.
Eight hours before each lung function test all pulmonary medication was discontinued. Reference values are those of Quanjer et al[136]. Patients refrained from drinking tea or coffee and from smoking before measurements.
Methacholine, and not histamine, was chosen as bronchoconstrictor stimulus for the dose-response curves, because it produces less systemic side effects when given in high doses[162].

Spirometry
The forced expiratory volume in one second (FEV_1) was measured using water-sealed spirometers (Lode, D53R, the Netherlands) until at least 3 reproducible (less than 5% difference) recordings were obtained; the highest value was then used for analyses. Residual volumes (RV) and total lung capacity (TLC) were measured with the closed circuit helium dilution method[136] and the expiratory flow at 50% of the actual forced vital capacity ($MEF_{50\%}$) was derived from a maximal expiratory flow volume (MEFV) curve, using a pneumotachometer (Jaeger, Germany).

Bronchodilator response
FEV_1 was measured before and 20 minutes after 4 separate inhalations of 250 µg of terbutaline sulphate from a metered dose inhaler, administered through a 750 ml spacer device (Nebuhaler, Astra Pharmaceuticals, Rijswijk, the Netherlands). Reversibility was expressed as % predicted normal[29].
Maximal reversibility was tested by administering cumulative doses of terbutaline starting with one single puff of 250 µg while measuring FEV_1 and forced vital capacity (FVC) after 15 minutes. Measurements were stopped after a maximum of 4 puffs at a time, were given, or, at any time when the difference between two consecutive measurements was less than 5%.

Provocation tests
Histamine provocation tests were performed using a 2 minute tidal breathing method [42] using a nose clip. For analysis purposes, patients already responding with more than 20% to saline or to the lowest concentration of histamine (0.03 mg/ml) were assigned a PC_{20} value of 0.015, being half the lowest concentration applied[29].

Methacholine was administered according to a standardized tidal breathing method [76]. Dose-response curves were obtained after inhalation of doubling concentrations of acetyl-ß-methylcholine-bromide (0.03-256 mg/ml^{-1}) in normal saline. Usually acetyl-ß-methylcholine-chloride is used as provocative agent. Although there is a difference between the molecular weights of the bromide (1 mole = 240 grams) and the chloride (1 mole = 196 grams) compound, we made no correction for this difference in our studies. If however comparison of absolute values with chloride data has to be made, this difference must be taken into account. Solutions of methacholine were stored at 4°C and administered at room temperature. The aerosols were generated by a De Vilbiss 646 nebulizer (output 0.13 ml/min) and inhaled by tidal breathing for 2 min. The response to methacholine was measured as change in FEV_1 expressed as percentage of initial value and related to $\log_2$ dose. A test was interrupted if the FEV_1 fell by more than 60%, or if unpleasant side-effects or dyspnea compelled the patient to stop.
For the interpretation of the entire dose-response curve a recently developed and validated sigmoid function (Cumulative Gaussian Distribution=CGD function) was fitted to the data[5]. This CGD function is described in Chapter 2.1. Although the sensitivity ($\log_2 PC_{20}$) was obtained by linear interpolation of two successive $\log_2$ concentration values, the reactivity was defined as the slope at the 50% response point of the CGD function [%/doubling dose (= dd)], the effective concentration at that point (EC_{50}) and the plateau value were obtained as additional fit parameters.

The model fit was used for two reasons: firstly because it enabled us to minimize random fluctuations due to *e.g.* varying patient cooperation; secondly it facilitated extrapolation of the whole curve where it was impossible to obtain direct estimates of plateau values. Sometimes this situation occurred when we were, due to feelings of discomfort or severe dyspnea of the patient, forced to interrupt the measurements.

1.5 MECHANISMS OF ACTION OF CORTICOSTEROIDS

Inhaled corticosteroids (ICS) rapidly enter airway cells where they bind to cytosolic receptors in the cytoplasm. The so formed glucocorticoid-receptor complexes then move quickly into the nucleus and bind to the glucocorticoid-responsive elements of certain genes, thereby regulating gene transcription in a positive or negative manner. In addition to their interactions with DNA within the nucleus, ICS can also interact with certain proteins, such as the transcription factor activator protein (AP)-1, either in the nucleus or the cytoplasm. A third supposed working mechanism of ICS is their influence on the stability of mRNA, that codes for certain cytokines.

ICS are known to control the rate of synthesis and also the release of a number of regulatory proteins, by interacting with the genes that regulate their production. One of these proteins is lipocortin-1 [78, 131]. Corticosteroids may enhance the production of lipocortin-1, which appears to inhibit the activity and synthesis of phospholipase A_2 (PLA_2). Inhibition of PLA_2 leads to a reduced production of arachidonic acid (AA) and mediators. Platelet activating factor (PAF) production is also reduced[138]. Recently, it has been shown that ICS also selectively inhibit the expression of cyclooxygenase-2 [100, 181].
ICS may have direct inhibitory effects on many of the cells involved in the inflammatory process in asthma, including macrophages, T lymphocytes, eosinophils, and airway epithelial cells[155]. ICS inhibit the production of a number of pro-inflammatory mediators such as histamine from basophils, and cytokines such as interleukin (IL)-1, IL-2, IL-4, IL-6, IL-8, TFN-α, and IFN-γ. Prednisolone decreases the number of cells expressing IL-4 and IL-5 mRNA in asthma[149].
ICS decrease the number and activation of mast cells, macrophages, T lymphocytes, and eosinophils in bronchial biopsies[103] and BAL-fluid of asthmatic patients[51]. A recent study supported the view that beneficial effects of corticosteroids in asthma may result from the reduction in the number of inflammatory cells infiltrating the bronchial mucosa with inhibition of cytokine gene expression[22]. Furthermore, ICS reverse the shedding of epithelial cells in bronchial biopsies from patients with asthma[83, 103]. In addition to their suppressive effects on inflammatory cells, ICS may also inhibit plasma exudation[25] and mucus secretion in asthma[160].

Given either orally or by inhalation, CS are able to reduce bronchial hyperreactivity (BHR) both in children[92] and in adults[96] with asthma. In usual doses, however, ICS have an effect on BHR that is not seen with doses of prednisone that can be used

safely in the long term[84]. ICS have been shown to be effective in decreasing BHR not only after short-term treatment[96] but also after long-term treatment[71, 87] in atopic[96] as well as in non-atopic asthmatic patients[19]. Both in children[54] and in adults[71, 91], ICS have been shown to be more effective in decreasing BHR than bronchodilator treatment alone. Frequently, maximum reduction in BHR may only be achieved after months of inhaled therapy. In spite of this, the normal range is not usually reached in both adults[87, 91] and children[54, 92, 172].
Early treatment with ICS results in long-lasting control of mild asthma[72]. Maintenance therapy with ICS can usually be given at a reduced dose[72] to maintain control. Cessation of treatment often results in deterioration. Data suggest that, depending upon the duration of the treatment period, this occurs within weeks[67, 169] or months[87, 172]. Delaying the start of ICS therapy after diagnosis may reduce the overall improvement in lung function both in adults and children[6, 72].
ICS not only reduce BHR, but also limit the maximal airway narrowing response[20]. Although, as mentioned above, the effects of ICS on BHR may take several months to reach a plateau, the improvement in symptoms and on FEV_1 is more rapid[91, 169]. ICS have shown to be effective in mild but also in severe disease[71, 167]. High doses[11] of ICS are now widely used to control more severe asthma. This treatment markedly reduces the need for oral glucocorticoid therapy and improves the control of more severe and unstable forms of the disease[15, 167].

1.6. AIMS OF THE STUDIES

The concomitant use of bronchial lavage and biopsy techniques enabled us to investigate some of the interrelationships between cellular and biochemical events both on the airway surface and in bronchial tissue. Combining these investigational methods with lung function measurements made it possible to examine physiological, cytological and histological manifestations of inflammation in asthma at the same time. The effects of anti-inflammatory therapy on all these manifestations were studied with a new topical ICS, fluticasone propionate (FP).
The effect of late introduction of anti-inflammatory therapy with beclomethasone dipropionate (BDP) was also investigated. Some of these studies, all conducted in adult asthmatics, are presented in this thesis.

In **Chapter 2.1** a new mathematical model, describing the shape of the methacholine dose-response (MDR) curves in normal individuals as well as in asthmatic patients,

is studied. Usually only attention is paid to the provocative concentration, producing a fall of 20% in FEV_1 (PC_{20}), and not to the overall shape of the curve. This curve also presents a slope in the mid-part and a maximal response plateau. The presence and level of a maximal response plateau provides relevant information on the potential severity of airways obstruction. The model facilitated extrapolation of the whole curve where it was impossible to obtain direct estimates of plateau values. It was used for the studies described in chapter 2.2, 3.1 and 4.1.

In **Chapter 2.2** the relation between airway inflammation in the bronchial mucosa and indices of the MDR curves in atopic asthmatics is investigated. The study was focussed on the role of the total number of eosinophils and the number of activated eosinophils.

In **Chapter 3.1** we investigated the effects of FP on MDR curves in nonsmoking atopic asthmatics. The findings are compared with results in the literature.

In **Chapter 3.2** the results are shown of delayed introduction of ICS. The study was designed to investigate whether a 2.5 years delay of ICS administration led to smaller improvements in FEV_1 and airways hyperresponsiveness compared to immediate prescription in patients with mild to moderately severe obstructive airways disease.

In **Chapter 4.1** we tested whether short-term therapy (12 weeks) with FP not only affected AA metabolites but also influenced the position and shape of the MDR curves in nonsmoking atopic asthmatics. We also investigated the relation between the various parameters studied.

In **Chapter 4.2** the influence of long-term therapy (2.5 years) with beclomethasone dipropionate (BDP) on AA metabolites is studied in patients with mild to moderately severe obstructive airways disease.

In **Chapter 4.3** the cellular effects of 2.5 years of treatment with BDP and smoking on eicosanoid and lipocortin-1 levels in BAL fluid of atopic asthmatics are investigated.

In the **General Discussion** the results of the previous chapters are discussed with regard to the data presented in the literature.

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Chapter 2

Dose response-curve

2.1 Extrapolation of methacholine dose-response curves with a Cumulative Gaussian Distribution function
Adapted from: Eur Respir J 1994;7:895-900

2.2 Eosinophils in the bronchial mucosa in relation to indices from methacholine dose response (MDR) curves in atopic asthmatics
Submitted for publication

Chapter 2.1

Extrapolation of methacholine dose-response curves with a Cumulative Gaussian Distribution function.*

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INTRODUCTION

Bronchial hyperresponsiveness (BHR) is a characteristic feature of asthma. Non-specific bronchoconstrictor stimuli such as histamine or methacholine are widely used to demonstrate this BHR and to generate dose-response curves in asthmatics. In practice, the provocative concentration at a fall of 20% of the response variable (PC_{20}), has been used to diagnose BHR with the FEV_1 as response variable because of its high reproducibility and repeatability[1]. Less attention has been paid to the overall shape of the dose response curve and the plateau value. Usually these curves are sigmoid in shape, with a distinct threshold, linear slope in the midpart and a maximum response[2]. The PC_{20} is called the sensitivity, whereas the slope in the midpart is defined as reactivity. Initially it was felt that slope measurements could provide relevant information[3] but as yet the clinical relevance of the reactivity is unclear. Asthmatics show an increased reactivity[2, 4, 5] when compared with normals. The plateau value of the curve reflects maximal airway narrowing. Asthmatics not only show a leftward shift of the dose-response curve, but also higher or even unmeasureable plateau values when compared to normals[2].

When interpreting data of the entire dose-response curve, one may need the use of a model fit to the experimental data for the following reasons: firstly because it enables smoothing of the curve, thereby minimizing fluctuations due to varying patient co-operation or other causes; secondly, because it facilitates extrapolation of the whole curve where it is impossible to obtain direct estimates of plateau values, for example when severe dyspnea of the patient or feelings of general discomfort forces one occasionally to interrupt the patient measurements. It has been shown that direct estimates of the plateau values are possible in the majority of normal subjects and mild asthmatics, but not in moderate or severe cases of asthma[6-8], where reaching of a plateau can be associated with a large drop in FEV_1. Extrapolation may therefore be necessary.

The aim of our investigation was to test a new mathematical model, describing the shape of methacholine dose-response curves in normal individuals, as well as in asthmatic patients. In addition we tested the accuracy of extrapolated plateau values compared to directly measured plateau values.

MATERIAL AND METHODS

Subjects

Three normal volunteers (2 males and 1 female; mean age 41 years, range 40-42 years), and seven patients with mild to moderate asthma (2 males and 5 females; mean age 40 years, range 28-52 years) volunteered to participate in the study (table 1).

Subject No.	Sex	Age yrs	Height cm	Weight kg	FEV_1 L	FEV_1 % pred	VC L	VC % pred	PC_{20} $mg.ml^{-1}$
1	M	40	178	65	4.33	108	6.10	120	*
2	M	42	175	78	4.06	106	5.76	119	*
3	F	40	158	53	3.10	117	3.92	125	*
4	F	31	165	60	2.71	86	3.44	94	5.0
5	F	45	167	58	2,42	84	3.29	99	2.8
6	M	52	170	82	2.08	63	3.58	88	0.7
7	F	26	165	57	3.50	107	4.27	114	9.9
8	M	51	168	75	3.34	103	4.59	115	7.5
9	F	46	164	63	2.60	94	3.38	106	7.7
10	F	28	160	55	2.74	90	3.49	100	7.1

Table 1. Anthropometric data and FEV_1, VC and PC_{20} values of the three normal volunteers and seven patients with bronchial asthma. Pred = predicted.
Number 1-3 were normal volunteers. Subjects number 4-10 had mild to moderate asthma. FEV_1: forced expiratory volume in one second; VC: vital capacity; PC_{20}: provocative concentration producing a 20% fall in FEV_1.

Mean FEV_1 in the normal subjects was 110% of reference (range 106-117%), and in the asthmatics 86% of reference (range 63-107%). Reference values were according to Quanjer et al.[9].

Asthma was diagnosed as a positive history of episodic dyspnea and wheezing, and mildly to moderately increased airway responsiveness. As determined from a standardized method (see below) the geometric mean PC_{20} (methacholine) was 5.6 $mg.ml^{-1}$; range 0.7-9.9 $mg.ml^{-1}$. In the normal subjects, no PC_{20} could be detected. In all subjects, a plateau in the methacholine dose-response curve could be obtained experimentally.

The study was approved by the Ethics Committee of the Dijkzigt hospital and informed consent was given by the volunteers.

Inhalation test

Methacholine was administered according to a standardized tidal breathing method[10]. Dose-response curves were obtained after inhalation of doubling concentrations of acetyl-ß-methylcholine-bromide (0.04 -314 $mg.ml^{-1}$) in normal saline.

The aerosols were generated by a De Vilbiss 646 nebulizer (output 0.13 $ml.min^{-1}$) and inhaled by tidal breathing for 2 min.

The response to methacholine was measured as change in FEV_1 expressed as percentage of the initial value and related to log_2 dose. A test was interrupted if the FEV_1 fell by more than 60%, or if unpleasant side-effects or dyspnea compelled the patient to stop. In all cases, a plateau was reached according to the criterion that the last three response values showed a variation coefficient of less than 5% of the mean value.

Mathematical model

Before a mathematical model can be applied, it must fulfil certain criteria. Firstly, the model should describe the entire sigmoid-shaped curve. Secondly, the plateau value and reactivity should be obtainable from the fitted model parameters.

The Cumulative Gaussian Distribution (CGD) function.

A recently developed and validated sigmoid CGD function was fitted to the data[4]. The CGD model is explained in figure 1.

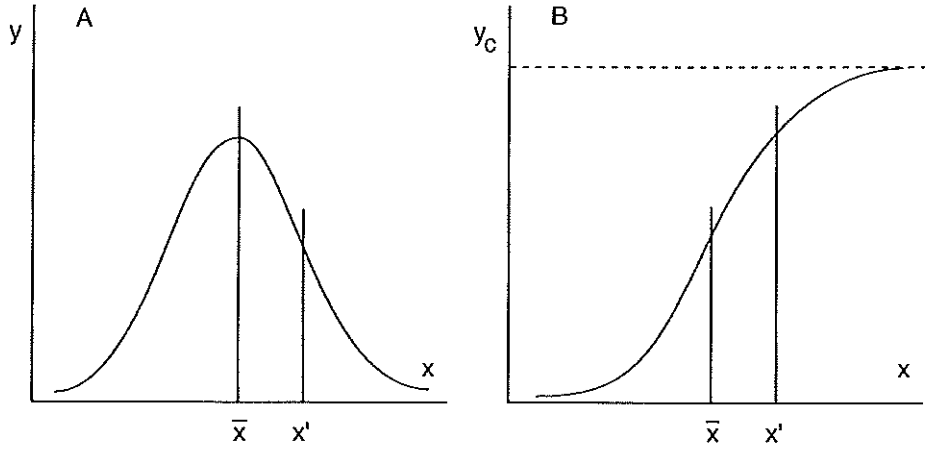

Figure 1. Example of Gaussian distribution (A) and the sigmoid shaped cumulative distribution function (B). At the mean of the distribution ($\bar{x}$) the slope in B gives the reactivity. The ordinate value at x' in B is equal to the area under the curve up to x' in A. For further explanation see text.

A continuous Gaussian distribution (figure 1A) is defined by three parameters, a mean value $\bar{x}$, a standard deviation and a normalizing factor equal to the area under the curve. In figure 1B the CGD curve is denoted, being equal to the cumulative area under the curve as function of the abscissa. The CGD curve was chosen because it reflects the pattern of the dose-response curves quantitatively, $\bar{x}$ denoting the response and y_c the plateau.

CGD fit procedure

Because the equation describing the CGD function is nonlinear, we used a nonlinear regression technique, as first described by Marquard[11]. Data from the methacholine dose-response curve ($\log_2$ concentration and ΔFEV_1 (%)) were imported off-line. The $\log_2 PC_{20}$ was determined by linear interpolation of adjacent data points. The fit procedure yielded estimates for plateau and reactivity, defined as the percentage change from baseline FEV_1 per doubling dose (dd) at the steepest point of the CGD curve. The goodness of fit was expressed by the coefficient of determination R^2.

Truncation of the curve

The reliability of the extrapolation of the dose-response curves in order to estimate a plateau value was also studied. Therefore, up to four last data points were left out. The fitted plateau estimates were compared with the measured values, being the average response of three data points, with a variation coefficient <5% of the mean value, at the highest methacholine concentrations[12].

Results

Examples of the dose-response curves of the asthma patients and normal volunteers are shown in figure 2A and B, together with the CGD fit. The numerical data concerning the fits are presented in table 2. By one-way analysis of variance, a significant difference was found between normal subjects and asthmatic patients in both the plateau value ($p=0.005$) and the reactivity estimates ($p=0.01$).

Truncation of the curves, by omitting the last four data points, enabled a plateau extrapolation for all subjects with the CGD model. Figure 3 shows the extrapolated plateaus *versus* the experimentally determined plateaus.

The regression line [ΔFEV_1 (%, extrapolated)] = -0.06 + 0.99 x [ΔFEV_1 (%, measured)] was nearly identical with the identity line, the linear correlation coefficient ($R=0.93$) indicating a highly significant relationship ($p<0.001$). The mean deviation of the fitted plateau in percentage of the measured plateau was -2.6% (sd 18.2%).

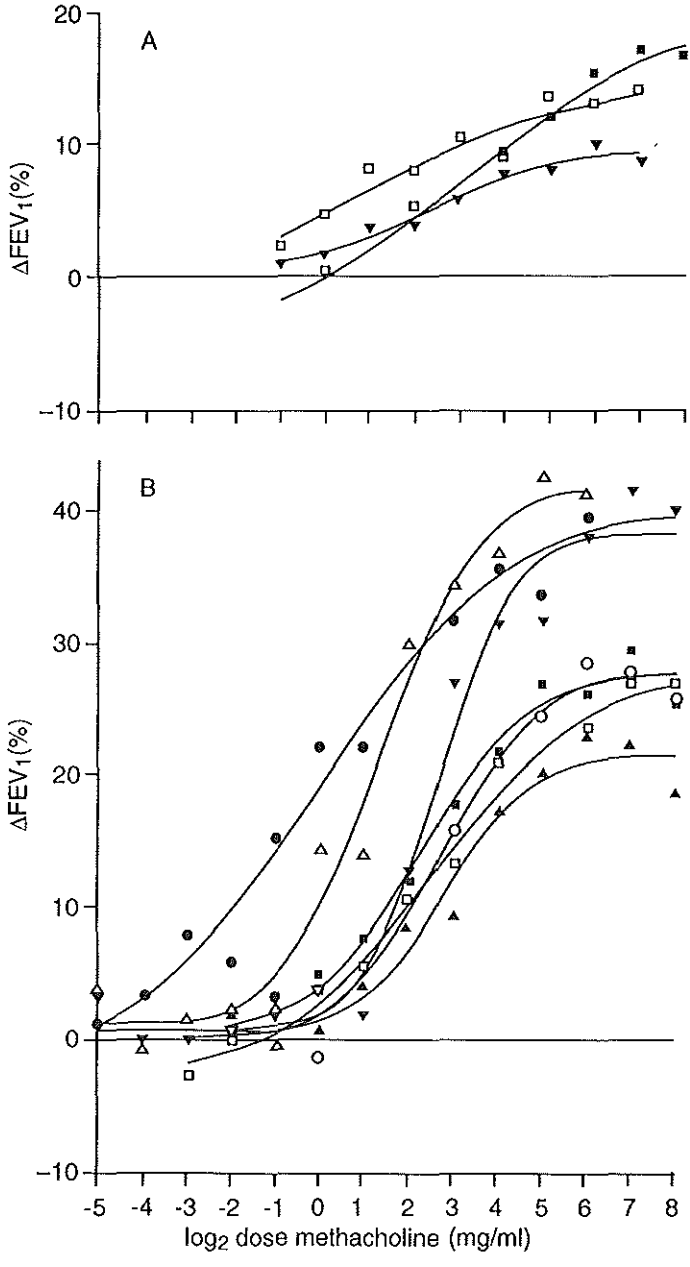

Figure 2. A. Experimental data and Cumulative Gaussian Distribution (CGD) model fits for the normal volunteers: No. 1 (▼); No. 2 (□); No. 3 (■).
B. Experimental data and CGD model fits for the asthmatic patients No. 4 (▼); No. 5 (△); No. 6 (●); No. 7 (▲); No. 8 (○); No. 9 (□); No. 10 (■). FEV_1 forced expiratory volume in one second.

Table 2. Measured plateau values and last provocative dose. Plateau and reactivity were estimated by the CGD fit using all available data. CGD: Cumulative Gaussian Distribution. Relative deviation of fitted versus measured plateau value is also presented.

			CGD model		
Subject No.	Plateau % measured	Last dose $Mg.ml^{-1}$	Plateau % fit	Relative deviation	Reactivity % FEV_1/dd
1	8.5	157	9.0	0.06	1.6
2	13.1	157	13.9	0.06	1.7
3	15.8	314	18.8	0.19	2.7
4	39.9	314	39.2	-0.02	10.2
5	40.3	78	41.9	0.04	9.1
6	39.9	314	40.3	0.01	4.8
7	21.1	314	21.7	0.03	5.2
8	26.8	314	27.7	0.03	6.2
9	26.0	314	27.8	0.07	4.3
10	27.0	314	27.7	0.03	5.4
mean				0.05	
SD				(0.06)	

Discussion

Methacholine dose-response curves were obtained in 3 normal volunteers and 7 patients with mild to moderate asthma in a standard manner, in order to evaluate the fit ability of a new model, the Cumulative Gaussian Distribution (CGD) function. In all curves, an experimental plateau could be reached.

The highly significant correlation between experimental and fitted response data, as derived from the R^2 values, showed a good fit ability of the CGD function in our group of volunteers. The CGD function showed a slight overestimation of the measured plateaus if all data points were used.

As far as the truncation of the curves was concerned: even when data from the last four provocation doses were left out, a reasonable accurate plateau estimate proved to be possible (figure 3).

The reactivity can be derived from the CGD function halfway plateau value, *i.e.* in the steepest part of the curve. Chung at al[13] defined reactivity as the slope in the steepest part of the curve, using the sGaw instead of FEV_1 as response parameter.

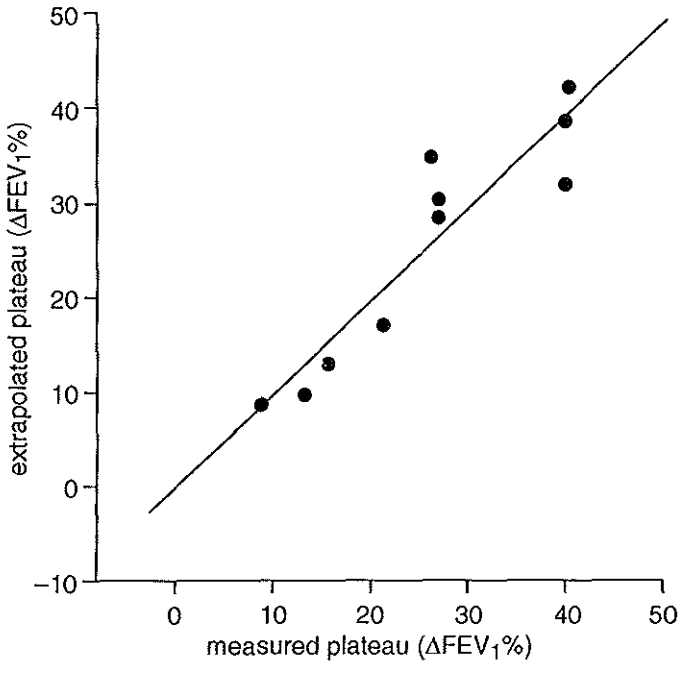

Figure 3. Relationship between the CGD extrapolations of the plateaus in case of omitting data from the last four provocative doses. FEV_1: forced expiratory volume in one second.

Some studies determined the slope by linear regression analysis of the data from the threshold (PC_{20}) upward and considered this slope as reactivity[14, 15]. This last procedure becomes inaccurate when the dose-response curve is extended, such that a curve-linear part is reached at higher doses. We think, therefore, that the slope should be preferably defined as model parameter, as we did in our experiments. This would enable comparison between different studies. Otherwise, the steepest part of an experimental curve should be used as definition.

We found the mean reactivity of the normal volunteers to be significantly lower than in the asthmatics, which was also found in a previous investigation[15]. The experimentally determined plateaus in our study were significantly lower in the normal subjects, as was also shown by others[5]. The CGD model proved to be significantly more accurate in the extrapolation of truncated curves[14] when compared with generally applied hyperbolic model as described among others by Sterk et al[12].

We conclude that the CGD function is promising in describing the entire methacholine dose-response curve in normal volunteers as well as in patients with mild to moderate asthma.

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Chapter 2.2

Eosinophils in the bronchial mucosa in relation to indices from the methacholine dose-response (MDR) curve in atopic asthmatics

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INTRODUCTION

Atopic asthma is characterized by local infiltration of several activated inflammatory cells in the bronchial mucosa, even in mild asthmatics[1]. Eosinophils in particular, have been associated with epithelial shedding[2, 3] through their release of different basic proteins with cytotoxic properties such as major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil derived neurotoxin (EDN) and eosinophil peroxidase (EPO). The number of eosinophils in the bronchial mucosa and in bronchoalveolar lavage (BAL) fluid has been correlated with the severity of asthma and the mechanisms which underlie airway hyperresponsiveness[4-7], one of the most prominent functional characteristics of asthma[8].

Airway hyperresponsiveness is defined as a decreased threshold of airway narrowing in response to a variety of non-specific stimuli[9]. At present airway responsiveness is usually measured in dose-response curves as the provocative concentration of histamine or methacholine causing a fall of 20% in the forced expiratory volume in one second (FEV_1) and is called the sensitivity (log_2PC_{20}). Previous studies[10, 11] have focussed on the importance of characterising the entire histamine or methacholine dose-response (MDR) curve not only by sensitivity, but also by reactivity, defined as the slope at 50% of the curve (% FEV_1/doubling dose) and the maximal airway narrowing response value (plateau; % FEV_1). It was found that dose-response curves from asthmatics show a shift to the left, have a steeper slope and higher maximal response value as compared to normals[12].

To interpret the entire dose-response curve well, a model fit to the data is often necessary. Reaching an experimental plateau may be associated with a large drop in FEV_1, causing feelings of severe dyspnea and making the measurement very uncomfortable to the patient. To avoid actual measured plateau values, extrapolation of the data may therefore be necessary. Moreover, the model gives a smoothing of the individual data fluctuations, due to varying patient co-operation or other causes. In our investigation indices were obtained as parameters of a Cumulative Gaussian Distribution (CGD) function fitted to the dose-response curves[13].

Airway inflammation produces hyperemia in the lamina propria[14]. The process of vasodilation and plasma leakage may increase airway wall thickness, which may directly contribute to airway narrowing[14, 15]. An indicator for the size of the airway lumen is the specific (volumic) airway conductance (s*G*aw)[16], which can be derived from bodyplethysmography.

In this study we tested the hypothesis that in mild to moderate asthma indices from the entire MDR curves and baseline sGaw are related to the presence and activation of eosinophils in the lamina propria.

PATIENTS AND METHODS

Patients

Twenty non-smoking atopic asthmatics (15 M; median age 26 years (21-56)), who were on bronchodilators only, entered the study. Median FEV_1 was 2.98L (range 1.94-5.36L); in % predicted: median 87 (range 47-108).

Bronchial biopsies

Bronchial biopsies were obtained from segmental divisions of the main bronchi by fiber-optic bronchoscopy. Cryostat sections (6 μm) were stained with two monoclonal antibodies using the alkaline-phosphatase anti-alkaline phosphatase (APAAP) technique: EG1, recognizing both resting and activated eosinophils and EG2, recognizing the cleaved epitope of eosinophil cationic protein of activated eosinophils (Pharmacia, Uppsala, Sweden). Cell counts were expressed as the median number of positive cells (ranges) per unit length (1mm) of basement membrane (BM).

Methacholine dose-response curves

Methacholine challenge test was performed using the standardized 2 min tidal breathing technique[17]. Dose-response (FEV_1%) curves were obtained after inhalation of doubling concentrations of acetyl-ß-methylcholine-bromide (0.03-256 mg/ml) in normal saline. Dose was expressed as $\log_2$ concentration.

Curve fitting

Although sensitivity was obtained by linear interpolation of adjacent data points of the dose-response curve according to international standards[17], other indices of the sigmoid curve were obtained by fitting with a cumulative Gaussian Distribution (CGD) function[13]. This fit yielded the reactivity as the slope at 50% of the curve (% FEV_1/ doubling dose) and the plateau value. If the last three provocative concentration (PC) values showed a variation coefficient of less than 5% of the mean value, this mean value was considered as experimental plateau estimate; otherwise the model fit was taken from which the extrapolated plateau value was obtained.

Specific airway conductance

Specific airway conductance (s*G*aw) was measured in a volume-constant body-plethysmograph (Jaeger bodytest Würzburg, Germany). The value was derived from the ratio between the inverse of the effective Resistance (Reff) and the intrathoracic gas volume during the measurement. Bodyplethysmography was assessed with a humidified and thermostatted (37°C) rebreathing bag during normal breathing whereas panting frequency during the volume measurement was about 0.5 - 1 Hz.

Data analysis

Correlation coefficients between cellular and pulmonary function indices were obtained by Spearman's Rank method; $p<0.05$ was considered significant.

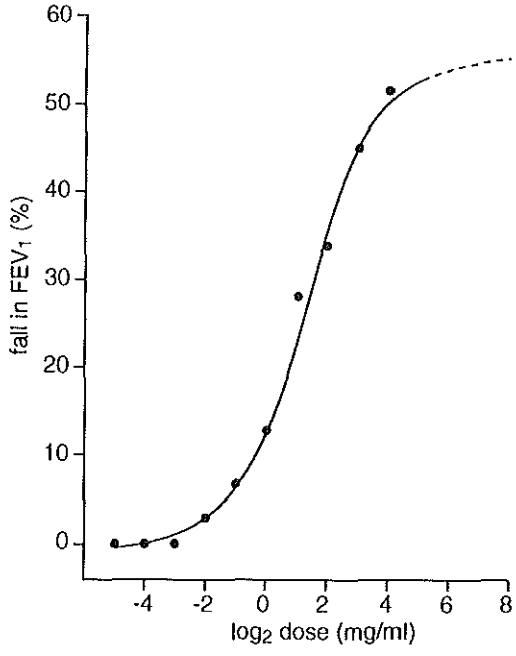

Figure 1. Example of curve fit with the Cumulative Gaussian Distribution (CGD) function. Plateau value = 56%, reactivity = 11.3% FEV_1/doubling dose. The plateau was obtained by extrapolation

RESULTS

Curve fitting and sGaw

In 12 patients the plateau value had to be obtained with the model fit because experimental determination was not sufficiently accurate. An example of a fit in which extrapolation was necessary is given in figure 1.

Median values and range of reactivity and plateau values were 9.06 (3.31-16.7) % FEV_1 / doubling dose and 52.95 (23.8-80.5) % FEV_1 respectively. Median and range of log_2PC_{20} and sGaw were 0.08 (-5.18-7.98) mg/ml and 0.63 (0.25-228) $s^{-1}kPa^{-1}$ respectively.

Correlation between cellular indices, hyperresponsiveness and sGaw

The median values and range for $EG1^+$ (total) eosinophils and $EG2^+$ (activated) eosinophils were 11.6 (1.6-68)/mm BM, and 3.5 (0-29.3)/mm BM respectively, as indicated in Table 1, where the relationship between the lung function variables and activated eosinophils ($EG2^+$) is also shown. There was no significant correlation between the total number of eosinophils ($EG1^+$) and log_2PC_{20}, nor with reactivity or plateau value. The number of activated eosinophils ($EG2^+$) was significantly related

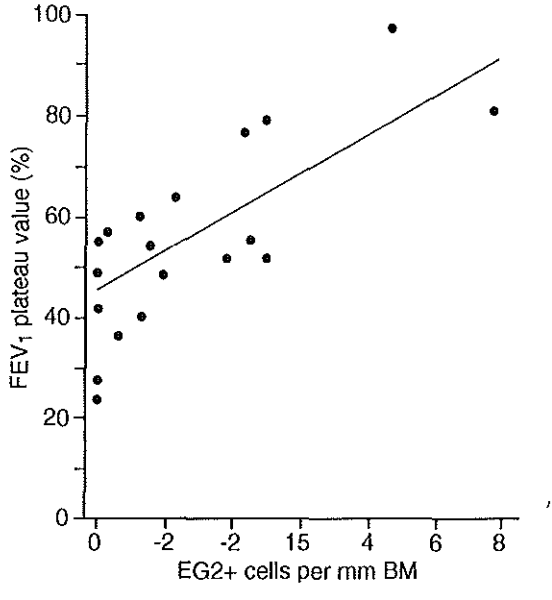

Figure 2. Plateau values in relation to the number of activated ($EG2^+$) eosinophils per mm basement membrane (BM), ($r=0.62$, $p<0.05$).

Table 1. Spearman Rank correlation coefficients between firstly, the total number of eosinophils ($EG1^+$) and sensitivity (log_2PC_{20}), reactivity (%/dd), plateau value and specific airway conductance (s*G*aw) and secondly, the number of activated eosinophils ($EG2^+$) in relation to the same parameters.

	Median (ranges)	$log_2 PC_{20}$	reactivity	plateau	s*G*aw
$EG1^+$ (n=20)	11.6 (1.6 - 68)	- 0.10	0.28	0.37	-0.40
$EG2^+$ (n=20)	3.5 (0 - 29.3)	- 0.32	0.12	0.62*	-0.52*

(* $p < 0.05$)

to the plateau value (Table 1; $r = 0.62$, $p<0.05$) (figure 2). The s*G*aw was negatively correlated to the number of activated eosinophils (Table 1; $r = -0.52$, $p<0.05$) but not to the total eosinophil number.

DISCUSSION

In the present study we have demonstrated a significant positive correlation between the number of activated eosinophils in the lamina propria, an important cellular marker in bronchial mucosal inflammation, and the plateau value. Furthermore, the s*G*aw was significantly negatively correlated with the number of activated eosinophils. We have shown in an earlier investigation[13] that our CGD model was superior in fitting ability when compared with other methods, using only a part of the data points. The CGD model not only enabled smoothing out of random fluctuations in the data points, it also enabled us to obtain reliable curve indices (reactivity and plateau value) where no experimental plateau values could otherwise be reached. In 8 of the MDR curves, in this study, a plateau could be accurately estimated by averaging the last three PC values. In the other cases the value had to be extrapolated with the CGD model fit.

Interestingly, only the number of activated and not the total number of eosinophils was significantly correlated with the plateau value. In previous studies with bronchial biopsies from asthmatic patients a possible relationship between eosinophils and

the degree of airway hyperresponsiveness (PC_{20} methacholine) has been investigated [18,21]. A negative correlation was found between the number of activated eosinophils in the lamina propria and sensitivity[18]. Djukanovic et al.[21], however, were unable to demonstrate a correlation between the number of activated eosinophils and PC_{20} methacholine, and neither were we. A recent study showed a significant negative correlation between the total number of intraepithelial eosinophils and PC_{20} methacholine values[19]. These findings were in line with another study[20] which showed that the total number of inflammatory cells and mast cells were significantly negatively correlated with PC_{20} methacholine. Apparently, the number of eosinophils in the lamina propria, either total or activated, does not reflect all the inflammatory events in the airway wall.

The maximal response plateau has been considered as determined by "postjunctional" mechanisms such as smooth muscle contractility, viscous and elastic loads on airway smooth muscle shortening, swelling of the airway wall and intraluminal exudate[10]. We used s*G*aw, derived from Reff and intrathoracic gas volume, as being representative for the size of the airway lumen. No unique index for resistance exists in cases of unequal ventilation which may be present in this patient catagory. Mean resistance within the breathing cycle is then best represented by Reff[22]. Moreover, s*G*aw is less dependent upon long volume than other resistance indices. The significant relation we found between s*G*aw and the number of activated eosinophils, indicating that a decrease in specific conductance coincides with an increase in the number of activated eosinophils, can also possibly be explained by swelling of the airway wall and the presence of intraluminal exudate.

Our findings therefore support the hypothesis that activated eosinophils and/or their mediators cause bronchoconstriction, enhance airway inflammation and epithelial damage, and possibly cause an increase in airway hyperresponsiveness.

In conclusion, the plateau value and also baseline s*G*aw are significantly related to the number of activated eosinophils in the bronchial mucosa. These results suggest a direct relationship between bronchial mucosal inflammation, characterized by eosinophil activity, and decrease in airway lumen. Our results stress the importance of modelling the entire MDR curve when characterizing airway hyperresponsiveness in patient follow-up.

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Chapter 3

Effects of steroids on lung function

3.1 Effects of fluticasone propionate on methacholine dose-response curves in nonsmoking atopic asthmatics
Eur Respir J 1996;9:2256-2262

3.2 Is delayed introduction of inhaled corticosteroids harmful in patients with obstructive airways disease?
Chest 1996;110:35-41

Chapter 3.1

Effects of fluticasone propionate on methacholine dose-response curves in nonsmoking atopic asthmatics

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Eur Respir J 1996;9:2256-2262

ABSTRACT

Methacholine is frequently used to determine bronchial hyperresponsiveness (BHR) and to generate dose-response curves. These curves are characterized by a threshold (provocative concentration of methacholine producing a 20% fall in forced expiratory volume in one second (PC_{20} = sensitivity), slope (reactivity) and maximal response (plateau). We investigated the efficacy of 12 weeks of treatment with 1,000 µg fluticasone propionate in a double-blind, placebo-controlled study in 33 atopic asthmatics.
The outcome measures used were the influence on BHR and the different indices of the methacholine dose-response (MDR) curve. After 2 weeks run-in, baseline lung function data were obtained and a MDR curve was measured with doubling concentrations of the methacholine from 0.03 to 256 mg/ml. MDR curves were repeated after 6 and 12 weeks. A recently developed, sigmoid Cumulative Gaussian Distribution function was fitted to the data. Although sensitivity was obtained by linear interpolation of two successive $\log_2$ concentrations, reactivity, plateau and the effective concentration at 50% of the plateau value (EC_{50}) were obtained as best fit parameters.

In the fluticasone group, significant changes occurred after 6 weeks with respect to means of PC_{20} (an increase of 3.4 doubling doses), plateau value fall in forced expiratory volume in one second (FEV_1) (from 58% at randomization to 41% at 6 weeks) and baseline FEV_1 (from 3.46 to 3.75L) in contrast to the placebo group. Stabilization occurred after 12 weeks. Changes for reactivity were less marked, whereas changes in $\log_2 EC_{50}$ were not significantly different between the groups.
We conclude that fluticasone is very effective in decreasing the maximal airway narrowing response and in increasing PC_{20}. However, it is likely, that part of this increase is related to the decrease of the plateau of maximal response.

INTRODUCTION

Bronchial hyperreactivity (BHR) is a hallmark of asthma. Nonspecific broncho-constrictor stimuli, such as histamine or methacholine, are widely-used to demonstrate this BHR and to generate dose-response curves in asthmatics. These curves are sigmoid in shape, with a distinct threshold, linear slope in the midpart, and maximum response[1]. The provocative concentration producing a fall of 20% in the FEV_1 (PC_{20}) is called the sensitivity, which is lowered in asthmatics and is associated with a leftward

shift of the dose-response curve[2]. The slope in the mid-part is defined as reactivity. Initially it was felt that slope measurements could provide relevant information[3] but as yet the clinical relevance of the reactivity is unclear. Asthmatics show an increased reactivity[1, 4, 5] as compared with normals. Also, a significant correlation has been found between reactivity and $\log_2 PC_{20}$ in these patients[4, 6]. Plateau values reflect maximal airway narrowing. Asthmatics not only show a leftward shift of the dose-response curve but also higher or even unmeasurable plateau levels as compared to normals[1]. Moreno et al[7] postulated that any augmentation of airway narrowing stimuli ("prejunctional" mechanisms) can result in a leftward shift of the curve, while any increase in response of the effector organ ("postjunctional" mechanisms)[5, 7] theoretically results in an increase in the maximal plateau level.

In practice, PC_{20} has been used to diagnose BHR and less attention has been paid to the overall shape of the dose-response curve and the plateau value. Previous investigations[1, 4, 5] have focused on the importance of measuring parameters of the entire dose-response curve, *i.e.* the sensitivity, reactivity and plateau value.

Recognition and distinction of these components of hyperresponsiveness may have implications for the diagnosis and therapy of asthma. Although PC_{20} is generally used as an index for the shift of the curve along the concentration axis, the fit of the sigmoid function also enables calculation of the effective concentration at half of the plateau response (EC_{50}). This index is commonly used in pharmacology[8], and is less dependent on the absolute value of the plateau response.

Anti-inflammatory therapy with inhaled corticosteroids (ICS) both shifts the dose-response curve to the right, *i.e.* decreases sensitivity[9] and reduces the maximum response[10], presumably by preventing the fixed element of airway obstruction caused by inflammation.

A new topically active glucocorticoid, fluticasone propionate (FP), has been shown to be more clinically effective than other ICS and to have fewer systemic side-effects in equi-effective doses at the upper end of the dose range[11, 12]. The aim of the present study was to investigate the efficacy of FP, 1,000 μg/day, on BHR and its *in vivo* influence on the different characteristics of the methacholine dose-response curve in atopic asthmatics.

METHODS

Subjects

Thirty-three nonsmoking atopic asthmatics (23 male, 10 female; median age 26 yr; range 18 to 56 yr) were selected if they met the following criteria during the run-in

period: PC_{20} histamine ≤ 8 mg/ml and ≥ 9% reversibility in forced expiratory volume in one second (FEV_1), relative to baseline, following inhalation of 1000 µg terbutaline. Atopy was defined by at least one positive skin-prick test to a panel of 16 common aero-allergens in the presence of positive and negative controls.
In the month preceding the run-in period, patients were allowed to take only inhaled short-acting $beta_2$-agonists, on an as needed basis. All other medication was stopped. Patients with a history suggesting respiratory infection or exacerbation of asthma in the month prior to the study were excluded.
All subjects gave informed written consent to the study, which was approved by the local ethics committee.

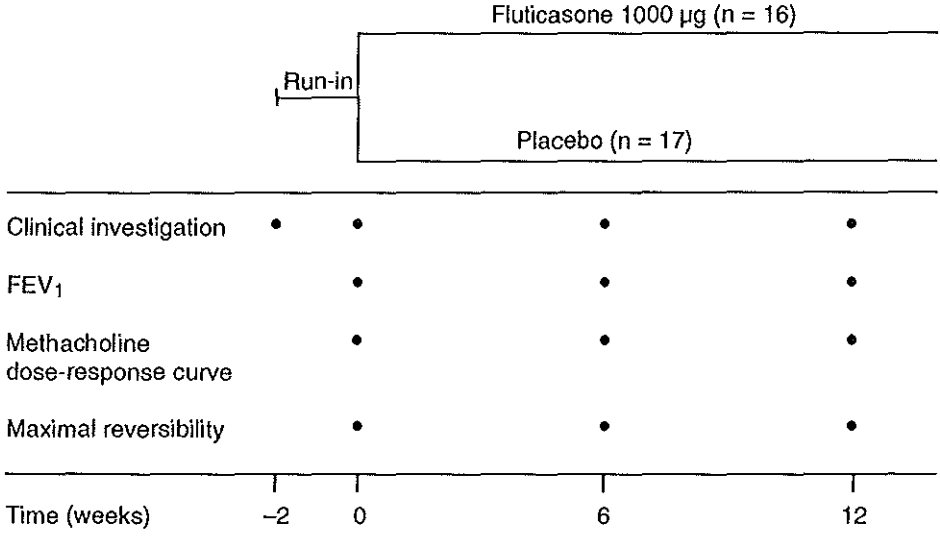

Figure 1. Design of the study.

Study design
The study was of a randomized, double-blind, placebo-controlled design, which is schematically presented in figure 1.
After a run-in period of two weeks there was a twelve week treatment period, and then a follow-up period of two weeks. During the two week run-in period patients discontinued use of their usual inhaled bronchodilator which was replaced with salbutamol 400 µg taken as a dry powder via the Diskhaler p.r.n. up to a maximum of 8 doses daily. Baseline data were obtained on two visits with an interval of two weeks. Each baseline visit consisted of two morning or afternoon sessions. At one of these

sessions a flow volume curve was constructed and bronchodilator response was measured; at the other session a provocation test was carried out. In addition, a standardized history regarding respiratory symptoms was obtained in addition to flow volume curve, bronchodilator response, and provocation test (see below). At the second baseline visit, also intradermal skin testing was performed.
During the run-in period, patients noted the severity of their asthma symptoms on a daily record card. Symptoms related to interference with daily activities were rated as follows: 0 = none; 1 = wheezing or shortness of breath during one episode; 2 = wheezing or shortness of breath during two or more episodes; 3 = wheezing or shortness of breath most part of the day, not interfering with normal daily activities; 4 = wheezing or shortness of breath for most of the day, normal activities difficult; 5 = unable to carry out normal activities because of shortness of breath. Symptoms during the night related to sleep disturbances were rated as follows: 0 = none; 1 = cough / wheeze / breathlessness causing once or early wakening; 2 = woken two or three times by symptoms; 3 = awake most of the night because of cough / wheeze / breathlessness; 4 = awake all night because of asthma symptoms. Patients also recorded their coughing, sputum production and use of the study medication and salbutamol inhaler, each day and night. Additionally, they noted an overall 24-hours symptom score concerning dyspnea.
Following the run-in period, the patients were randomized to treatment with either inhaled FP 500 µg or placebo, both given twice daily as dry powder via the Diskhaler. Patients continued salbutamol 400 µg p.r.n., but could take up to 8 doses as needed for symptomatic relief.
After 6 weeks and 12 weeks of treatment patients attended the clinic on 2 separate days (within 3 successive days): on one occasion maximal reversibility was tested, while on the other a methacholine dose-response curve (see below) was performed. Two weeks after the end of the actual treatment period a follow-up visit was scheduled.

Lung function testing
Where possible all measurements were made at the same time of day at each visit, and patients were asked not to use their bronchodilator or the study medication for eight hours before attending the clinic.

Inclusion measurements. FEV_1 was derived from a maximal expiratory flow volume curve, using a pneumotachometer (Jaeger, Würzburg, Germany). Reversibility was tested 20 minutes after 4 separate inhalations of 250 µg of terbutaline sulphate from a metered dose inhaler, administered through a 750 ml spacer device.

A histamine provocation test was performed using a 2 min tidal breathing method[13] using a nose clip.

Study measurements. Maximal reversibility was tested by administering cumulative doses of terbutaline starting with one single puff of 250 µg while measuring FEV_1 and FVC after 15 min. Measurements were stopped after a maximum of 4 puffs at a time were given or at any time when the difference between two consecutive measurements was less than 5%.

Methacholine was administered according to a standardized tidal breathing method[2]. Dose-response curves were obtained after inhalation of doubling concentrations of acetyl-ß-methylcholine-bromide (0.03 -256 mg ml-in normal saline). 1 mg ml^{-1} is equivalent to 0.82 mg ml^{-1} of methylcholine-chloride solution. Therefore a fixed conversion constant of 0.29 should be subtracted from the log_2 dose values for comparison with methylcholine-chloride data. Methacholine and not histamine was chosen as bronchoconstrictor stimulus because it produces less systemic side effects when given in high doses[14]. Solutions of methacholine were stored at 4°C and administered at room temperature. The aerosols were generated by a De Vilbiss 646 nebulizer (output 0.13 ml min^{-1}) and inhaled by tidal breathing for 2 min. The response to methacholine was measured as change in FEV_1 expressed as percentage of initial value and related to log_2 dose. A test was interrupted if the FEV_1 fell by more than 60%, or if unpleasant side-effects or dyspnea compelled the patient to stop.

A recently developed and validated sigmoid Cumulative Gaussian Distribution (CGD) function was fitted to the data[4]. Although the sensitivity (log_2PC_{20}) was obtained by linear interpolation of two successive log_2 concentration values[13], the plateau value and the reactivity (defined as the slope at the 50% point of the CGD function) and the effective concentration at this point (EC_{50}) were obtained as best fit parameters. Hence, reactivity denotes the percentage change from baseline FEV_1 per doubling dose (dd) at the steepest point of the CGD function. Details of the fit procedure and validation of the CGD fit are according to Aerts et al[4].

Statistical analysis

The paired t-test was used to analyse changes in FEV_1, reversibility and indices of bronchial hyperresponsiveness with respect to baseline. The unpaired t-test was used for comparisons between groups. The patient recorded diary data were averaged over all days; the Mann-Whitney test was used for comparison between the groups. Group means and standard error of the mean (± SEM) at the various time points were calculated.

Table 1. Baseline characteristics of the study patients

Patient characteristics	Fluticasone		Placebo	
n	16		17	
withdrawals	1		1	
sex M/F	12/4		11/6	
age, yr, range	18-51		18-56	
age, yr	28	(11)	35	(14)
FEV_1 (% pred)	84	(15)	86	(18)
reversibility*	16	(8)	18	(14)
$PC_{20}H^{\dagger}$ mg/ml	0.7	(0.6)	0.9	(0.9)
$\log_2 PC_{20}H^{\dagger}$ mg/ml	-1.1	(1.6)	-1.0	(1.7)
$\log_2 EC_{50}M^{+}$ mg/ml	1.3	(1.4)	0.0	(2.0)
plateau value#	59	(20)	51	(13)
reactivity (%/dd)	12	(5)	9	(4)

All values are expressed as mean ± standard deviation (between brackets).
*reversibility: change in FEV_1 expressed as % baseline, after 1,000 µg terbutaline.
†H = histamine; +M = methacholine. # plateau value expressed as % fall in FEV_1

RESULTS

Sixteen patients were randomized into the FP group and 17 patients into the placebo group. Baseline values were comparable in both groups on entry to the study; only a larger mean $\log_2 EC_{50}M$ was found in the FP group ($p = 0.05$). Thirty-one of the 33 subjects completed the study (table 1).

One patient receiving placebo and one receiving FP were withdrawn after experiencing a pulmonary exacerbation. Data of these 2 patients have not been included in the analysis. In one patient only a reliable PC_{20} methacholine ($PC_{20}M$) could be obtained during all methacholine dose-response measurements; no reliable reactivity or plateau values were measured. All parameters ($PC_{20}M$, reactivity, EC_{50} and plateau value) were obtained in 90 out of 93 curves. Although in all cases only the fitted plateau value was used for the analysis (as mentioned above), in 61 out of 91 measurements also an experimental plateau could be obtained. In the remaining curves the flattening enabled us to estimate the plateau value with reasonable accuracy.

Mean values for reactivity (%/dd) and plateau (% fall in FEV1) before, during and after treatment are shown in figure 2. Examples of dose-response curves to methacholine in the FP and placebogroup are shown before and after 12 weeks of treatment in figure 3.

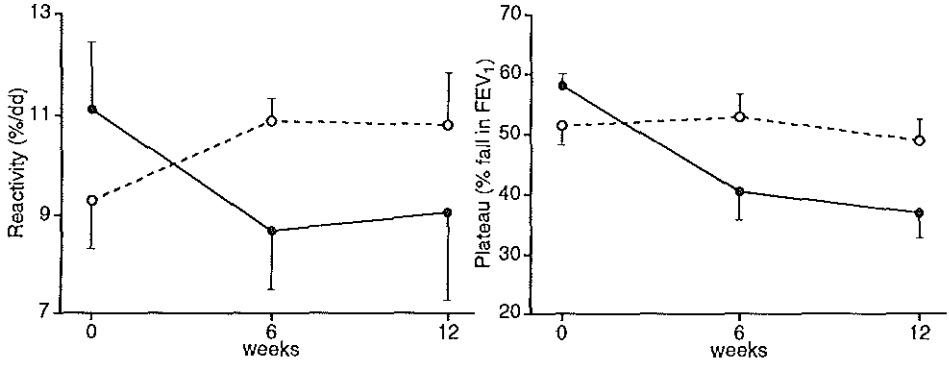

Figure 2. Mean values (± SEM) of reactivity (percent change per doubling dose, %/dd) and plateau value (% fall in FEV_1 from baseline) before, during and after treatment with placebo (O) and fluticasone (●).

During placebo treatment mean $log_2PC_{20}M$ hardly changed from 0.04 (SEM 0.78) at baseline to 0.34 (SEM 0.69) after 6 weeks and to 0.26 (SEM 0.46) after 12 weeks. During treatment with FP, however, mean $log_2PC_{20}M$ increased from 0.31 (SEM 0.59) to 3.73 (SEM 0.69) after 6 wks and to 3.77 (SEM 0.72) after 12 wks. After 6 weeks the mean difference in change from baseline between the FP and the placebo group was 3.12 (SEM = 0.76) ($p<0.0005$ and 95% CI:1.57 to 4.67). After 12 weeks this was 3.25 (SEM = 0.75) ($p<0.0005$ and 95% CI:1.71 to 4.78).

During placebo treatment the mean plateau value hardly changed as can be seen in figure 2. During treatment with FP, however, mean plateau value decreased from 58.1% (SEM 5.24) to 40.6% (SEM 5.06) after 6 weeks and to 36.5% (SEM 4.05) after 12 weeks. After 6 weeks the mean difference in change from baseline between the FP and the placebo group was -19.3 (SEM 4.68) ($p<0.0005$ and 95% CI:-28.9 to -9.72). After 12 weeks this difference was -18.8 (SEM = 5.67) ($p = 0.003$ and 95% CI:-30.4 to -7.15).

In neither of the treatment groups did mean reactivity change substantially (figure 2). However, there were significant differences when the results of the treatment groups were compared. After 6 weeks the mean difference in change from baseline between the FP and the placebo group was -4.05 (SEM = 1.60) ($p = 0.017$ and 95% CI:-7.32

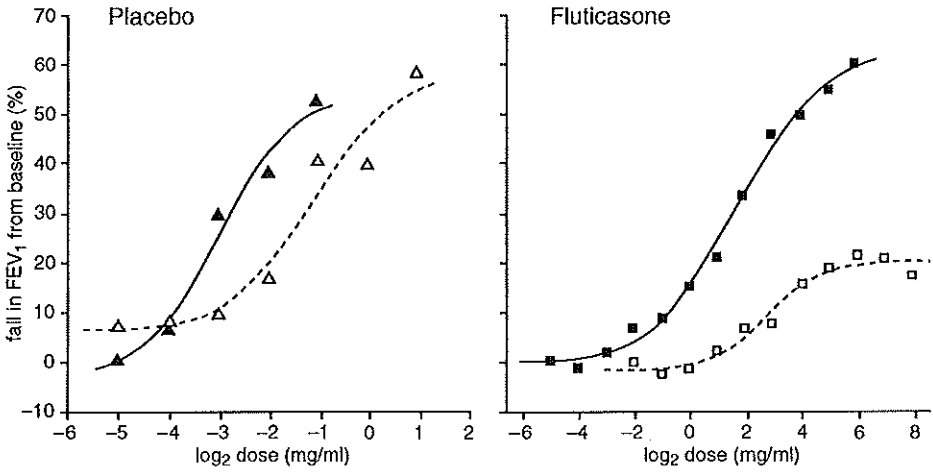

Figure 3. Examples of dose-response curves to methacholine before (open symbols) and after 12 weeks of treatment (closed symbols) with placebo and fluticasone

to -0.78). After 12 weeks this was -3.69 (SEM = 1.87) (p = 0.058 and 95% CI:-7.52 to 0.13).

Log_2EC_{50} changes with respect to baseline were not significantly different between groups. The mean values were 0.0 (SEM 0.5), 0.8 (SEM 0.5), 0.2 (SEM 0.4) mg/ml for the placebo group and 1.3 (SEM 0.4), 2.9 (SEM 0.4), 2.4 (SEM 0.3) for the FP group, at baseline, 6 and 12 weeks, respectively. No significant correlation was found between log_2EC_{50} and plateau estimates.

Baseline values for FEV_1 as absolute values hardly changed during placebo treatment. FEV_1 decreased from 3.27 (SEM 0.24) at baseline to 3.02 (SEM 0.23) after 6 weeks and to 3.07 (SEM 0.24) after 12 weeks. In the FP group FEV_1 increased significantly from 3.46 (SEM 0.25) at baseline to 3.75 (SEM 0.24) after 6 weeks and to 3.73 (SEM 0.22) after 12 weeks. After 6 weeks the mean difference in change from baseline between the FP and the placebo group was 0.54 (SEM 0.17) (p = 0.003 and 95% CI: 0.19 to 0.88) after 6 weeks and 0.47 (SEM 0.16) (p = 0.007 and 95% CI: 0.14 to 0.81) after 12 weeks.

Because of the skewed distribution of the data, maximal reversibility was analyzed after log transformation. After 6 weeks, maximum reversibility in the FP group was 68% lower in relative comparison to the placebo group (95% CI:37 to 84%; p = 0.002). After 12 weeks this was 54% (95% CI:11 to 76%; p = 0.022).

FP, compared with placebo, significantly improved individual nighttime symptom score (sleeping disturbances) at endpoint (p = 0.01), whereas individual daytime symptom score (interfering with normal daily activities) almost reached significance (p = 0.06).

The overall 24-hour symptom score also significantly improved in the FP treated group compared with placebo ($p = 0.01$).

DISCUSSION

The aim of the present study was to investigate the efficacy of a new inhaled corticosteroid, fluticasone propionate (FP), on BHR and its *in vivo* influence on the different characteristics of the methacholine dose-response curve in atopic asthmatics. The results of the study show that FP is very effective in decreasing the maximal degree of airway narrowing after 6 weeks of treatment, and that this effect is sustained after 12 weeks. An improvement in FEV_1 was accompanied by an increase in PC_{20} methacholine and a decrease in plateau value in the FP group but not in the placebo group. Although log_2EC_{50} increased more in the FP group than in the placebo group the difference between these changes was not significant. Reactivity improved only slightly in the first 6 weeks of treatment with FP.

For the interpretation of the entire dose-response curve a recently developed and validated sigmoid function (Cumulative Gaussian Distribution = CGD function) was fitted to the data[4]. Although the sensitivity (log_2PC_{20}) was obtained by linear interpolation of two successive log_2 concentration values, the reactivity defined as the slope at the 50% point of the CGD function (%/dd), EC_{50} and the plateau value were obtained as additional fit parameters. The model fit was used for two reasons: firstly, because it enabled smoothing of the curve, thereby minimizing fluctuations due to varying patient co-operation or other causes; and secondly, because it facilitated extrapolation of the whole curve, where it was impossible to obtain direct estimates of plateau values, because of, for example, severe dyspnea of the patient or feelings of general discomfort, which occasionally forced an interruption of the measurements[4]. In our present investigation, however, prolonged administration of methacholine was possible beyond the fitted plateau value in 67% of the cases, which further contributed to the reliability of reactivity and plateau estimates.

In contrast to other studies[9, 15-17] the improvement in PC_{20} methacholine in our study is high (approximately 3.5 doubling doses after 6 and 12 weeks). This may be due to differences between the patients studied (atopic asthmatics[15] *versus* non-atopic asthmatics[9]) or to a difference in study medication prescribed[9, 15-17], or dosage used [9, 15-17]. Also, the difference in provocative concentration used[18] makes comparison difficult, although we do know that in asthma PC_{20} is similar for histamine and methacholine[18, 19].

The greater increase in PC_{20} methacholine found in the present study may be due to the relatively high dose and/or high efficacy of FP prescribed. The normal daily dose of inhaled steroids in mild-to-moderate asthma varies somewhere between 800-1,200 µg. One recent investigation demonstrated that 200 µg FP was as effective as 400 µg beclomethasone dipropionate with respect to peak expiratory flow (PEF), symptom scores, percentage of symptom-free days and nights, use of rescue $ß_2$-agonist medication and clinical lung function[20]. This was also shown by others[11, 21]. Taking these results into account, the daily dose of 1,000 µg FP used in the present study may be considered to be relatively high; this could, in part, explain the large improvement in PC_{20} methacholine. True comparisons with the above-mentioned studies remain difficult, however, because none of them investigated the differences in effect on PC_{20} between FP and the other inhaled steroids.

Another reason for careful interpretation of the study results is the interaction between changes in plateau estimation (a postjunctional index[5]) and PC_{20} (sensitivity, presumed to be a prejunctional index). EC_{50}, as a pharmacologically well-based index for the horizontal shift of sigmoid curves, independent of the plateau value, was found to change similarly in both groups. This means that part of the PC_{20} changes may be due to changes in plateau values, which in turn makes comparisons between different studies difficult.

It is possible that, since PC_{20} methacholine was not measured sooner than after 6 weeks of treatment, the effect measured may have occurred earlier than this time. After 12 weeks of treatment, no further improvement was found. This is in keeping with the findings of others[16, 22]. Kerstjens, et al[22] demonstrated in patients with chronic obstructive lung disease (asthma and COPD) that the largest improvement in PC_{20} histamine occurred after 6 months of treatment. Continuation of the therapy in their study resulted in only a slight but not significant further increase. Haahtela et al[16] also showed, in patients with newly detected asthma, that the marked decrease in bronchial responsiveness was already apparent after 6 weeks of treatment; although this decrease continued over the two years of the study, the trend over time was not significant.

FP also influenced the plateau value of the methacholine dose-response curve, whereas no change was observed during placebo treatment. FP induced a significant decrease in percentage fall FEV_1 after 6 weeks. A further, although not significant, decrease was found after 12 wks. This finding may be of clinical importance. A maximal response that is increased to a severe or even unmeasurable degree of airway narrowing is potentially dangerous. The major reason why asthmatics get into trouble is not primarily the increased sensitivity of their airways to bronchoconstrictor stimuli,

but the excessive degree of airway narrowing[23, 24]. Successful treatment of their asthma, therefore, should be directed towards preventing or at least diminishing this excessive response. Like other steroids, FP may result in diminished airway wall thickness by its effect on inflammatory mediators, decreasing mucosal swelling or plasma exudation[5]. Since we have shown FP to be very effective in diminishing the plateau value, it may, in our opinion, be considered a useful addition to the already existing arsenal of ICS.

Reactivity has been defined in some studies as the slope of the line fitted to the data upward from the threshold (PC_{20})[6, 25, 26]. In our opinion such an approach becomes inaccurate because of the curvilinear shape reached at higher doses. Therefore reactivity is better defined as the steepest slope of the dose-response curve (50% point of the CGD function), thus enabling comparison of reactivity estimates between different investigations. In the present study, FP also influenced reactivity. Although small changes were found after placebo as well as after FP treatment, only the change caused by FP from baseline to 6 weeks reached significance in comparison with placebo. After 6 weeks, a stabilization of reactivity seemed to occur. The change in reactivity during the first 6 weeks of FP treatment may be because, in the presence of an increasing stimulus, airway obstruction develops less progressively after treatment with FP than without treatment. Although reactivity is certainly coupled to pharmacodynamic properties, its interpretation is as yet unclear[6, 26, 27]. Further investigations will be needed in this respect to fully understand the clinical implications of the present finding.

Like other ICS[16, 22], treatment with FP increased FEV_1 and decreased maximal reversibility, as can be expected when baseline values increase.

Improvements in asthma symptom scores were not as consistent as the changes in lung function, with only the night-time and overall 24 h scores being significant. The present results confirmed those found by Ayres[12]. An explanation may be that some symptom questions are more sensitive measures for the disease than others. The finding that lung function parameters had already significantly improved after 6 weeks of treatment with FP, whereas symptom scores only showed significant changes after 12 weeks of treatment, was interesting. This discrepancy may be explained by the fact that in a short-term study lung function changes precede symptom improvements.

In conclusion, fluticasone propionate proved to be very effective in decreasing the maximal response of airway narrowing in atopic asthmatic patients. The changes in provocative concentration of methacholine causing a 20% fall in forced expiratory volume in one second could suggest that an effect is exerted on sensitivity.

However, the lack of significance in changes in the effective concentration at half of the plateau response makes this doubtful. Further studies are, therefore, warranted on this aspect.

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Chapter 3.2

Is delayed introduction of inhaled corticosteroids harmful in patients with obstructive airways disease (asthma and COPD)?

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Chest 1996; 110:35-41

ABSTRACT

Background: The institution of inhaled corticosteroids is generally advocated for effective treatment of patients with asthma. It is yet unknown what is the best time to start inhaled corticosteroids and especially whether delayed introduction is harmful.
Phase 1: In a previous study in patients with asthma and COPD we found that 2.5 years of treatment with a ß2-agonist plus inhaled corticosteroid (BA+CS) was more effective in improving the forced expiratory volume in one second (FEV_1) and the provocative concentration of histamine causing a 20% reduction in FEV_1 (PC_{20}) than treatment with a ß2-agonist plus anticholinergic (BA+AC) or placebo (BA+PL).
Phase 2: We extended this study with 6 months to investigate whether delayed introduction of inhaled CS (800 µg beclomethasone dipropionate) in the groups previously not treated with inhaled CS (BA ± AC) could also improve FEV_1 and PC_{20} to the same degree. A distinction was made between patients with predominantly asthma (high baseline reversibility, $\Delta FEV_1 \geq 9\%$ of predicted), and predominantly COPD (low baseline reversibility, $\Delta FEV_1 < 9\%$ of predicted).
Results: Improvement of FEV_1 %predicted by inhaled CS was comparable both in the asthmatics between phase 1 (13.8 %predicted) and phase 2 (8.5 %predicted, p=0.13) as well as in the patients with COPD (2.5 and 1.5 %predicted, respectively). PC_{20}, however, increased significantly more in the asthmatics in phase 1 (1.77 doubling concentration (DC)) than in phase 2 (0.79 DC, p= 0.03). Improvement of PC_{20} in the COPD patients was not significantly higher in phase 1 (0.74 DC) than in phase 2 (0.00 DC, p=0.19).
Conclusions: Our study indicates that although delayed introduction of inhaled CS in asthmatics leads to similar improvements in FEV_1, improvements in PC_{20} are significantly smaller. These findings in patients with longer-existing asthma concur with the findings of Haahtela et al. in newly detected asthma. We suggest that institution of inhaled corticosteroids should not be postponed in asthmatics with documented airways obstruction and reversibility.

INTRODUCTION

Inhaled corticosteroids (ICS) have been demonstrated to be effective in improving symptoms, airways hyperresponsiveness, and airways obstruction in patients with asthma[1-9]. It is, however, unclear when ICS should be started in early or mild disease.

Current guidelines advocate the timing of institution to be dependent upon the amount of bronchodilators daily used[10-12]. This may vary considerably between patients, since there are marked differences in individual perception of breathlessness[13-15], probably leading to differences in the amount of bronchodilators used. Moreover, this recommendation is not based on studies comparing the effects of delayed versus early institution. It is conceivable that delayed institution could lead to the irreversible lung function loss that some patients with asthma demonstrate[16]. This question is especially important given the suggestion that continuous use of bronchodilators without ICS leads to accelerated decline in lung function[17].
The value of ICS in patients with COPD is currently unclear. Short-term studies have not shown a beneficial effect[18-20]. A few long-term reports suggest that ICS slow down the progressive deterioration of lung function in at least some patients with COPD[7, 9, 21-23]. Here again, the effect of delayed institution of ICS is uncertain.
The present study was designed to investigate whether a 2.5 years delay of ICS administration leads to smaller improvements in FEV_1 and airways hyperresponsiveness in patients with mild to moderately severe obstructive airways disease (asthma and COPD).

PATIENTS AND METHODS

The current investigation is an extension of a 2.5 years multicentre study[7]. Patients aged 18-60 years with mild to moderately severe obstructive airways disease (asthma and COPD) were selected from six university outpatient clinics if they met the following two criteria: 1) a concentration of histamine causing a 20% decrease in FEV_1 (PC_{20}) of 8 mg/ml or less[24, 25]. 2) Baseline FEV_1 more than 1.2 liters and 1.64-4.5 residual standard deviations (RSD) below the predicted value, or the FEV_1/inspiratory vital capacity (IVC) ratio more than 1.64 RSD below the predicted value, provided that total lung capacity (TLC) was higher than 1.64 RSD below the predicted level[7]. Patients with conditions or medication likely to interfere with the purpose of the study were excluded. Further details of the study methods have been described elsewhere[24].

Study design

The study consisted of two parts as depicted in figure 1 and 2.
Phase 1: Two hundred seventy four patients were randomly allocated to one out of three double-blind treatment regimens: all patients received from identical metered dose inhalers an inhaled β_2-agonist (terbutaline 250 µg 2 puffs q.i.d.) combined with

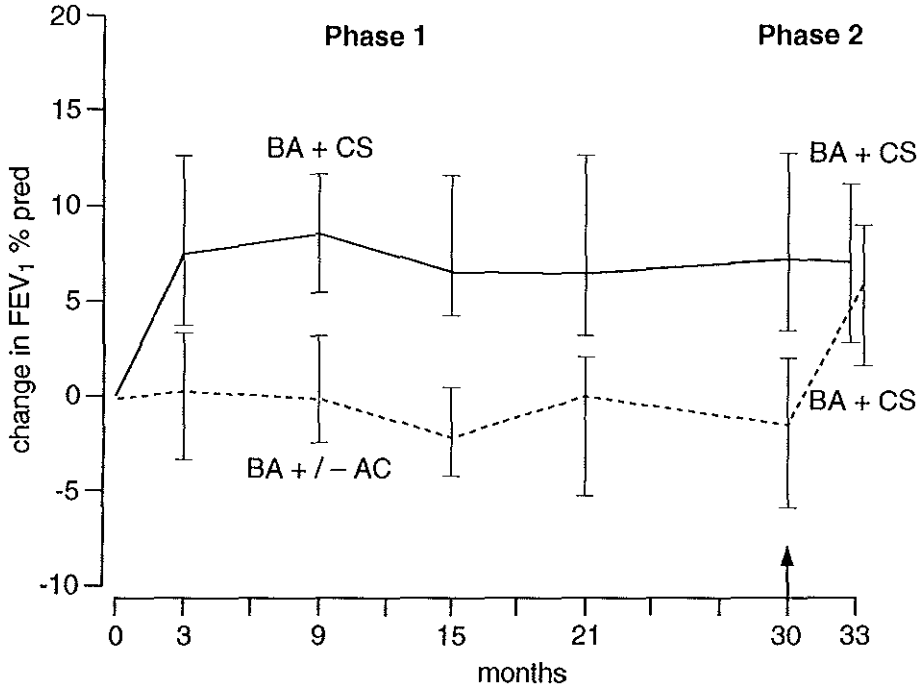

Figure 1. Change in FEV_1(%pred) for all patients followed up for at least 33 months. Treatment was changed at 30 months (solid arrow). In the present study, only changes in phase 1 (0-3 months) and phase 2 (30-33 months) are compared. Medians and 95% confidence intervals are presented.

either an inhaled corticosteroid [beclomethasone 100 μg 2 puffs q.i.d. (BA+CS)], or an inhaled anticholinergic [ipratropium bromide 20 μg 2 puffs q.i.d. (BA+AC)], or an inhaled placebo 2 puffs q.i.d. (BA+PL). Additional bronchodilator medication was supplied as salbutamol dry powder inhalations (400 μg) on demand. No other concomitant pulmonary medication was allowed, except during exacerbations, when a 12-day course of oral prednisolone was prescribed. Follow-up visits were scheduled every 3 months during 2.5 years, at which the level of airways obstruction and, at alternate visits, PC_{20} were measured.

Phase 2: All patients of group BA+AC and BA+PL who had completed phase 1 and were willing to participate in phase 2, were switched after 2.5 years to the same treatment the BA+CS group had been receiving from the start of phase 1: beclomethasone (100 μg 2 puffs, q.i.d.) combined with terbutaline (250 μg 2 puffs q.i.d.). The BA+CS group continued their medication. The reallocation of medication was performed in a double-blind fashion, *i.e.* the prior code was not broken for patients or

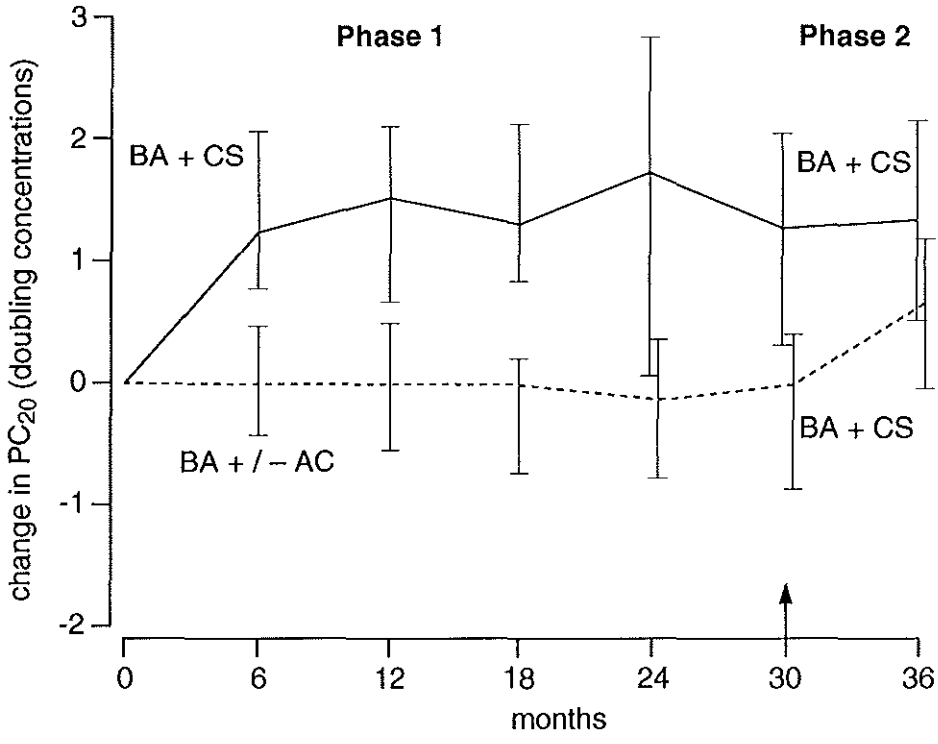

Figure 2. Change in log_2PC_{20} for all patients followed for at least 36 months. Treatment was changed at 30 months (solid arrow). In the present study, only changes in phase 1 (0-6 months) and phase 2 (30-36 months) are compared. Medians and 95% confidence intervals are presented.

their physicians. By design, however, it was clear to all patients that from the start of phase 2 they were all treated with inhaled corticosteroids. Informed consent was obtained from all patients. The level of airways obstruction was measured at baseline and 3 months after the start of phase 2, whereas PC_{20} was measured at baseline and after 6 months.

Lung function

FEV_1 and PC_{20} were only measured during clinically stable periods, and not within 4 weeks after the end of a prednisolone course. Eight hours before these tests all pulmonary medication was discontinued. FEV_1 was measured using water-sealed spirometers until at least 3 reproducible (less than 5% difference) recordings were obtained; the highest value was then used for analyses. Reference values are those of the European Community for Coal and Steel[25]. For bronchodilator response testing,

FEV_1 was measured before and 20 minutes after 4 separate inhalations of 250 µg of terbutaline sulphate from a metered dose inhaler, administered through a 750 ml spacer device (Nebuhaler, Astra Pharmaceuticals, Rijswijk, The Netherlands). Reversibility was expressed as % predicted normal[26]. Residual volumes (RV) and TLC were measured with the closed circuit helium dilution method[25] and the expiratory flow at 50% of the actual forced vital capacity ($MEF_{50\%}$) was derived from a maximal expiratory flow volume (MEFV) curve, using a pneumotachometer.' Histamine provocation tests were performed using a 2 minute tidal breathing method[27]. For analysis purposes, patients already responding to saline or to the lowest concentration of histamine (0.03 mg/ml) were assigned a PC_{20} value of 0.015, being half the lowest concentration applied[24]. Patients refrained from drinking tea or coffee and from smoking between measurements.

Symptom scores

After instruction at the outpatient clinic, symptom scores were noted daily at home for 14 consecutive days before every visit to the clinic on a 4-point scale (0 = no symptoms, 3 = severe symptoms) for wheeze, dyspnea, cough, and phlegm, separately. Symptom scores over 14 days were averaged for each of the symptoms and then added to obtain a mean symptom score (up to a maximum of 12).

Classification of patients

A distinction was made between those patients with high reversibility ($\Delta FEV_1 \geq 9\%$ of predicted) considered as having predominantly asthma, and those with low reversibility ($\Delta FEV_1 < 9\%$ of predicted) considered as having predominantly COPD. Patients were categorized as atopic on the basis of skin prick testing[7].

Statistical analysis

Data are presented as medians [plus 95% confidence interval (CI) of the median] unless stated otherwise. All calculations with PC_{20} were performed using the base-2 logarithm, one unit difference reflecting 1 doubling dose. Because no significant differences were found between the BA+AC and BA+PL groups with regard to FEV_1 and PC_{20} during phase 1 both at baseline and in response to their respective treatments[7], the data of these groups were subsequently pooled for analysis during phase 2. Reversibility was measured both in phase 1 and 2 at baseline and used for patient classification. Improvement with therapy was assessed as change from baseline of phase 1 in the group receiving CS from the start of the study and in the group receiving CS only from the start of phase 2 as change from baseline of phase 2.

Mann-Whitney U tests were employed and p-values < 0.05 were considered significant.

RESULTS

Of the 274 patients randomised in phase 1, 101 patients were withdrawn before the end of the study. The withdrawal rate was significantly larger in the BA+PL and BA+AC groups (44 and 45 patients respectively) than in the original BA+CS group (12; $p < 0.0001$). Seventy percent of this withdrawal was related to an increase in pulmonary symptoms[7]. Of the remaining 94 patients not treated with ICS in phase 1 (47 in both the BA+AC and BA+PL group) 76 agreed to continue in phase 2. Baseline characteristics of all patients at the start of phase 1 (table 1) were comparable among the original BA+CS, BA+AC and BA+PL groups[7]. There were no significant differences in baseline characteristics between the group originally treated with BA + CS and the group treated with BA+ CS only in phase 2. When the baseline characteristics of the patients treated with BA + CS in phase 2 only were compared between the start of phase 1 and phase 2, the MEF_{50} had significantly deteriorated ($p=0.02$), but not FEV_1 and PC_{20}.

Table 2 shows the characteristics of the patients subdivided in asthma and COPD according to reversibility. In phase 1, 49 patients in the BA+CS group had high reversibility (ΔFEV_1 to terbutaline $\geq$ 9% of predicted, considered to have predominantly asthma) and 42 had low reversibility (ΔFEV_1 < 9% of predicted, considered to have predominantly COPD). In phase 2, 53 patients had high reversibility and 23 low reversibility. There were no significant differences between the baseline characteristics of the asthmatic groups at the start of phase 1 and 2, or between the COPD groups. There was a median rise in FEV_1 of 8.6% predicted (95% CI 4.9-12.6) in the group treated with BA+CS at the start of phase 1 (0-3 months), compared with 4.5% predicted (1.8-10.0) in the group receiving corticosteroids only from the start of phase 2 (30-33 months). This difference was not significant ($p = 0.24$). Changes in FEV_1 % predicted for all patients who reached phase 2 are presented in figure 1.

Figure 3 shows the changes in FEV_1 % predicted in the first 3 months of phase 1 and phase 2 for patients with high and low reversibility separately. In the patients with reveribility treated with BA+CS from the start of phase 1, the median rise in FEV_1 was 13.8% predicted (7.7 to 18.7) compared with 8.5% predicted (3.3 to 15.9) in those who received CS only from the start of phase 2 ($p = 0.13$). In the patients with low reversibility treated with BA+CS from the start of phase 1, there was a median

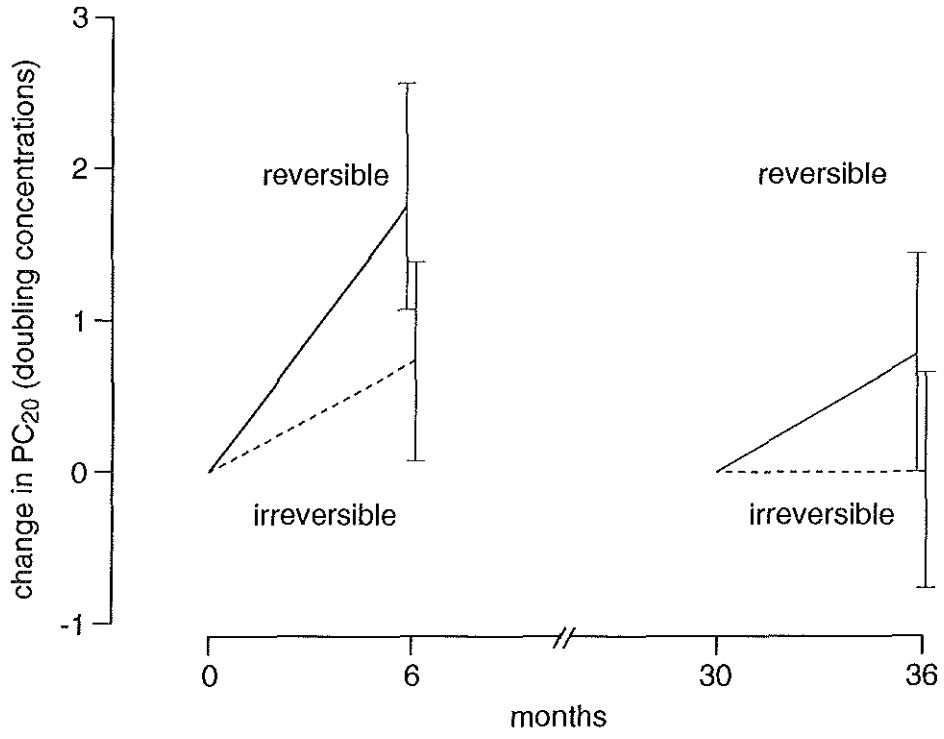

Figure 4. Change in PC_{20} histamine with ICS in the first six months of phase 1 and phase 2 for patients with high and low reversibility separately. Solid lines are for patients with a reversibility of ≥ 9%predicted (asthmatics) and dotted lines for patients with a reversibility < 9%predicted (COPD).

rise in FEV_1 of 2.5% predicted (-1.2 to 9.0), compared with 1.5 % predicted (-2.3 to 4.8) in the group that received CS only from the start of phase 2 (p=0.50).

There was a median rise of 1.30 doubling concentrations (DC) (0.91-1.81) with BA+CS in phase 1 (0-6 months) compared with 0.52 (0-0.94) DC in phase 2 (30-36 months). This difference was significant (p=0.04). In figure 4 the changes in PC_{20}, expressed as DC for all patients reaching phase 2 are depicted.

In figure 4, the changes in PC_{20} during the first 6 months of treatment in phase 1 and phase 2 are presented for the asthmatic and COPD patients separately. There was a median rise of 1.77 DC (1.07 to 2.56) in the asthmatics treated with BA+CS from phase 1, as compared with 0.79 DC (0.00 to 1.44) in those who received steroids only in phase 2. This difference was significant (p = 0.03). In the COPD patients treated with BA+CS in phase 1 there was a median rise of 0.74 DC (0.08 to 1.39) compared with 0.00 DC (-0.77 to 0.65) in phase 2 (p=0.19).

Table 1. Baseline characteristics (means ± SD) at the start of phase 1 and phase 2

	start phase 1		start phase 2
	BA + CS* (n=91)	BA ± AC (n=183)	BA + CS** (n=76)
age (yr)	40.2 (12.3)	39.2 (12.1)	42.1 (12.2)
Sex (m/f)	59/32	117/66	55/21
FEV_1 (%pred)	64.6 (15.4)	63.3 (15.3)	61.7 (15.8)
FEV_1/VC (%)	56.5 (11.5)	54.7 (10.8)	53.0 (11.6)
ΔFEV_1 (%pred)	11.3 (8.6)	12.3 (9.0)	13.6 (9.9)
$MEF_{50\%}$ (%pred)	35.3 (14.8)	33.4 (14.7)	9.8 (13.4)
RV/TLC (%pred)	118.3 (24.7)	118.9 (24.4)	117.5 (24.5)
log_2PC_{20} (mg/ml)	-1.62 (2.23)	-2.11 (2.32)	-1.81 (2.66)
- geometric mean PC_{20} (mg/ml)	0.33	0.23	0.29
atopic (%)	56.0	55.7	56.6
current smoker (%)	36.3	35.5	34.2

BA+CS: $ß_2$-agonist + inhaled corticosteroid; * treated with BA+CS at the start of phase 1 (0-3 months); ** treated with BA+CS only from the start of phase 2 (30-33 months); BA±AC: $ß_2$-agonist + either anticholinergic or placebo; ΔFEV1 (% pred): bronchodilator response to $ß_2$-agonist (terbutaline 1000 µg); $MEF_{50\%}$: maximal forced expiratory flow after 50% of the forced vital capacity; RV/TLC: ratio of residual volume and total lung capacity; log_2PC_{20}: base 2 logarithm of provocative concentration of histamine producing a fall of 20% in FEV_1

Table 2 Baseline characteristics (means ± SD) of the groups receiving ICS at the start of phase 1 and 2, with a subdivision in patients with high reversibility (≥ 9% pred) and low reversibility (< 9% pred)

	start phase 1		start phase 2	
	high n=49	low n=42	high n=53	low n=23
age (yr)	36.8 (11.9)	44.2 (11.7)	37.7 (12.6)	43.9 (10.6)
sex (m/f)	26/23	33/9	34/19	21/2
FEV_1 (%pred)	64.6 (14.1)	64.5 (17.0)	61.2 (15.6)	70.8 (14.6)
ΔFEV_1/VC (%)	55.4 (8.4)	57.9 (14.2)	54.0 (10.7)	56.7 (10.7)
ΔFEV_1 (%pred)	17.5 (6.5)	4.0 (3.5)	16.6 (5.8)	4.4 (3.6)

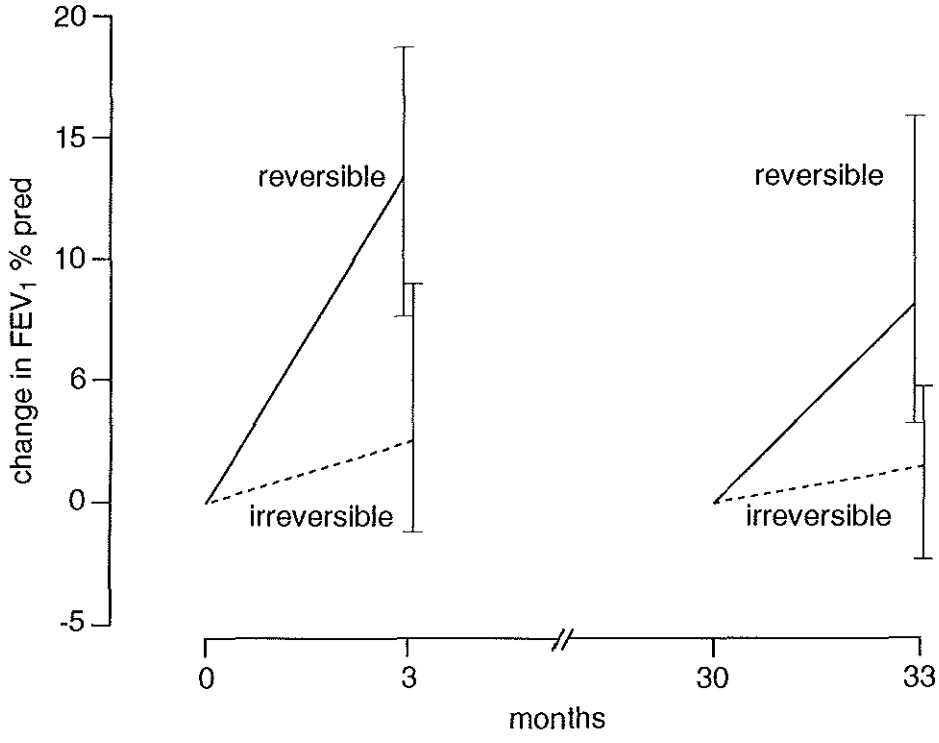

Figure 3. Change in FEV_1 (% pred) with ICS in the first 3 months of phase 1 and phase 2 for patients with high and low reversibility separately. Solid lines are for patients with a reversibility of ≥ 9% predicted (asthmatics) and dotted lines for patients with a reversibility < 9% predicted (COPD).

Although COPD patients, as shown in table 2, had a significantly higher mean symptom score at baseline than asthmatic patients in phase 1 and in phase 2, there was no significant difference in change in symptom scores between phase 1 and phase 2 in the two patient groups.

DISCUSSION

This study suggests that a delay in the introduction of ICS in patients with obstructive airways disease and marked reversibility may blunt their benificial effect. When ICS were instituted after 2.5 years of therapy with inhaled bronchodilators only, FEV_1 in asthmatics as well as in COPD patients improved to a similar degree as in the group that received this treatment 2.5 years earlier. Improvement of airways hyper-res-

ponsiveness, however, was smaller after delayed institution, the difference being significant only in the group with high reversibility.

The difference we found in improvement in airways hyperresponsiveness between the asthmatic patients in whom ICS were administered at the start of phase 1 and those who had delayed institution might have been due to selection bias. Because the withdrawal rate in phase 1 in the groups treated with bronchodilators alone had been as high as 49%, we thought it very likely that the patients entering into phase 2 had a "survivor effect". We did not, however, find such an effect when comparing the baseline characteristics at the start of phase 1 and 2, made on the basis of those factors found to be of importance in predicting response to ICS[7,8]. Additionally, when comparing these baseline characteristics for asthmatics and COPD patients separately, no significant differences were found.

One explanation for the smaller improvement in PC_{20} in phase 2 may be ongoing airway wall inflammation in patients not having received ICS during the first 2.5 years. Airway inflammation is assumed to be an important determinant in the pathophysiology of asthma[28] and perhaps also in COPD[29]. Corticosteroids have potent anti-inflammatory properties[30], such as inhibition of the release of mediators from macrophages and eosinophils and the influx of inflammatory cells into the lungs[31]. Several studies have shown beneficial effects of ICS on airways responsiveness and ventilatory function[1,2,4-9,32,33]. After delayed introduction of CS therapy, smaller increases in hyperresponsiveness can thus be expected especially in asthmatics, in whom airway inflammation is the major feature.

Another explanation for our finding could be that bronchodilators, in the absence of corticosteroids, have deleterious effects[34,35]. They relax smooth muscle, but do not have anti-inflammatory properties. It has been suggested that they promote an increase in exposure to allergens, cigarette smoke or other irritants[36,37]. Furthermore, hyperresponsiveness may deteriorate slightly and transiently during treatment with ß-agonists only[1,2,38]. The explanation for this phenomenon is still controversial[23,34,35].

In our study, PC_{20} at baseline in phase 1 for the BA ± AC groups and for phase 2 measured 2.5 years later, was not significantly different from PC_{20} in the group initially treated with ICS. Furthermore, there was no significant difference in PC_{20} when the asthmatics and COPD patients were considered separately. Thus, our finding does not confirm a deleterious effect of treatment with bronchodilators only. Nevertheless, PC_{20} is an important parameter of disease in asthma, having a close relation with the degree of inflammation. ICS administration improves PC_{20}, whereas discontinuation leads to worsening of PC_{20}[39,40].

Our findings in a group of patients with longer existing asthma are similar to those found in a recent study by Haahtela et al[40] in a group of newly detected asthmatics. This may suggest that the effects of late introduction of ICS are not dependent on the stage of the disease. In current guidelines for the management of asthma, prescription of ICS therapy depends on the number of puffs of β_2-agonists and on symptoms. This symptom indication could conceivably lead to a later introduction of ICS therapy in certain patients. Because in our study the significant change in PC_{20} in the asthmatics was not accompanied by a change in symptom score, our findings and those of Haahtela et al[40] would question the wisdom of this aspect of the guidelines. They also suggest that lung function parameters and symptom score do provide different information about disease activity[41].
The fact that ICS showed a more pronounced effect on FEV_1 and PC_{20} - with earlier as well as with delayed institution - in asthmatics than in patients with COPD is compatible with numerous studies in the literature showing ICS to be effective in asthma, whereas their value in COPD is still unclear[1-9, 18-23]. The four-item symptom score with sputum and cough as two separate items probably favoured higher reported average symptom scores in COPD. This was true for phase 1 and phase 2. The difference in change in neither of the patient groups was, however, significant between phase 1 and 2.

In conclusion, we have shown in both asthma and COPD that ICS administered in the later stages of the disease may improve FEV_1 to the same extent as when prescribed early. However, our results suggest that delayed institution of ICS therapy in patients with airways obstruction and high reversibility leads to a smaller improvement in airways hyperresponsiveness than earlier introduction. Further prospective studies with longer follow-up are needed to show whether the damage caused by delayed institution of inhaled corticosteroids is permanent or can still be reversed by longer periods of treatment with ICS than the 6 months, duration of this study.

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Chapter 4

Effects of steroids on arachidonic metabolites

4.1 Effects of fluticasone propionate on arachidonic acid metabolites in BAL-fluid and methacholine dose-response curves in nonsmoking atopic asthmatics
Mediators of inflammation 1996;5:224-229

4.2 Lower leukotriene C4 levels in bronchoalveolar lavage fluid of asthmatic subjects after 2.5 years inhaled corticosteroid therapy
Mediators of inflammation 1995;4:426-430

4.3 Eicosanoids and lipocortin-1 in BAL-fluid in asthma: effects of smoking and inhaled glucocorticoids
J Appl Physiol 1996;81(2):548-555

Chapter 4.1

Effects of fluticasone propionate on arachidonic acid metabolites in BAL-fluid and methacholine dose-response curves in nonsmoking atopic asthmatics

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ABSTRACT

Hyperresponsiveness of the airways to nonspecific stimuli is a characteristic feature of asthma. Airway responsiveness is usually characterized in terms of the position and shape of the dose-response curve to methacholine (MDR). In this study we have investigated the influence of fluticasone propionate (FP), a topically active glucocorticoid, on arachidonic acid (AA) metabolites in bronchoalveolar lavage (BAL) fluid (i.e. TxB_2, PGE_2, PGD_2, $6kPGF_1\alpha$ and LTC_4) on the one hand and MDR curves on the other hand. The effect of FP was studied in a randomized, double-blind, placebo-controlled design in 33 stable non-smoking asthmatics; 16 patients received FP (500 µg b.i.d.) whereas 17 patients were treated with placebo. We found that the forced expiratory volume in one second (FEV_1 % predicted) increased, the log_2PC_{20} methacholine increased and the plateau value (% fall in FEV_1) decreased after a 12 week treatment period.

No changes in AA-metabolites could be determined after treatment except for PGD_2 which decreased nearly significantly ($p= 0.058$) within the FP treated group, whereas the change of PGD_2 differed significantly ($p=0.05$) in the FP treated group from placebo. The levels of the other AA-metabolites (i.e. TxB_2, PGE_2, $6kPGF_1\alpha$ and LTC_4) remained unchanged after treatment and were not significantly different from the placebo group. Our results support the hypothesis that although FP strongly influences the position, the shape and also the maximum response plateau of the MDR curve, this effect is not mainly achieved by influence on the level of AA-metabolites. Other pro-inflammatory factors may be of more importance for the shape of the MDR curve. It is suggested that these pro-inflammatory factors are downregulated by FP.

INTRODUCTION

Bronchial hyperresponsiveness (BHR), a prominent feature of asthma, can be demonstrated by generating dose-response curves through inhalation of histamine or methacholine. Usually these curves are sigmoid in shape, with a distinct threshold, linear slope in the midpart and a maximum response[1]. The provocative concentration producing a fall of 20% in the FEV_1 (PC_{20}) is called the sensitivity, whereas the slope in the midpart is defined as reactivity. The plateau value of the curve reflects maximal airway narrowing.

Asthmatics not only show a leftward shift of the dose-response curve, but also higher or even unmeasureable plateau values as compared with normal subjects[1]. Activation

of inflammatory cells and release of mediators, such as arachidonic acid (AA) metabolites in bronchoalveolar lavage (BAL)-fluid, may be present in asthma and may influence the shape of the methacholine dose-response (= MDR) curve by enhancing BHR[2-5].
Although it is known that anti-inflammatory therapy with inhaled corticosteroids (ICS) shifts the dose-response curve to the right[6] and reduces the maximum response[7], data about the influence of inflammatory mediators such as AA metabolites are scarce[4]. One may expect that inhibition of their release by ICS may lead to a change in the shape of the dose-response curve.
Therefore, in asthmatics, we tested the hypothesis that fluticasone propionate (FP), a new topically active ICS, downregulates AA metabolites in BAL-fluid on the one hand, and influences the different characteristics of the MDR curves on the other hand. We also investigated the relation between the various parameters studied.

METHODS

Subjects

Thirty-three nonsmoking atopic asthmatics (23 male; median age 26 yr; range 18 to 56 yr) fulfilled the following criteria: PC_{20} histamine $\leq$ 8 mg/ml and $\geq$ 9% reversibility in forced expiratory volume in one second (FEV_1), relative to baseline, following inhalation of 1000 µg terbutaline sulphate. Atopy was defined by at least one positive skin-prick test to a panel of 16 common aero-allergens in the presence of a positive histamine and negative control.
In the month preceding the run-in period, patients were only allowed to take inhaled short-acting $beta_2$-agonists, on an as needed basis. All other medication was stopped. Patients with a history suggesting respiratory infection or exacerbation of asthma in the month prior to the study were excluded. All subjects gave informed written consent to the study, which was approved by the local ethics committee.

Study design

The study was of a randomized, double-blind, placebo-controlled design. After a run-in period of 2 weeks there was a twelve week treatment period. During the 2o week run-in period patients discontinued use of their usual inhaled bronchodilator which was replaced with salbutamol 400 µg as dry powder via the Diskhaler 4 times daily. Up to 4 additional doses were allowed as needed. Baseline data were obtained on 2 visits with an interval of 2 weeks. At each baseline visit that consisted of two morning

or afternoon sessions, a flow volume curve was constructed, bronchodilator response was measured and a provocation test was carried out. At the second baseline visit, intradermal skin testing was performed. When patients fulfilled the mentioned criteria, bronchoalveolar lavage (BAL) was performed 1 week after the last baseline visit. Following the BAL, the patients were randomized to treatment with either inhaled 500 μg FP or placebo, both given twice daily as dry powder via the Diskhaler. Patients continued salbutamol 400 μg 4 times a day, but could take up to four additional doses as needed for symptomatic relief.
After 6 and 12 weeks of treatment patients attended the clinic on which occasion a MDR curve (see below) was performed. After 13 weeks, one week after the last dose-response curve was obtained, the BAL procedure was repeated.

LUNG FUNCTION TESTING

Inclusion measurements
FEV_1 was derived from a maximal expiratory flow volume curve, using a pneumotachometer (Jaeger, Würzburg, Germany). Reversibility was tested 20 min after four separate inhalations of 250 μg of terbutaline sulphate.
A histamine provocation test was performed with a 2 min tidal breathing method[8] using a nose clip. Reference values of lung function measurements are according to Quanjer et al[9].

Study measurements
Methacholine was administered according to a standardized tidal breathing method[10]. Dose-response curves were obtained after inhalation of doubling concentrations of acetyl-ß-methylcholine-bromide (0.03-256 mg ml^{-1}) in normal saline. Methacholine, and not histamine, was chosen as bronchoconstrictor stimulus during the study, because it produces less systemic side effects when given in high doses[11]. Solutions of methacholine were stored at 4°C and administered at room temperature. The aerosols were generated by a De Vilbiss 646 nebulizer (output 0.13 ml min^{-1}) and inhaled by tidal breathing for 2 min. The response to methacholine was measured as change in FEV_1 expressed as percentage of initial value and related to log_2 dose. A test was interrupted if the FEV_1 fell by more than 60%, or if unpleasant side effects or dyspnea compelled the patient to stop.
A recently developed and validated sigmoid Cumulative Gaussian Distribution (CGD) function was fitted to the data[12]. Although the sensitivity (log_2PC_{20}) was obtained by

linear interpolation of two successive $\log_2$ concentration values[8], the plateau value and the reactivity (defined as slope in the 50% point of the CGD function) were obtained as best fit parameters. Hence, reactivity denotes the percentual change from baseline FEV_1 per doubling dose (%/dd) in the steepest point of the CGD function. Details of the fit procedure and validation of the CGD fit are according to Aerts et al[12].

Bronchoalveolar lavage

Fiber-optic bronchoscopy was performed according to guidelines of the American Thoracic Society[13]. After premedication with inhaled terbutaline and atropine i.m., the bronchoscope (Olympus B1 IT 10, Tokyo, Japan) was introduced into the lateral segment of the right middle lobe under local anaesthesia and placed in wedge position. BAL was performed with four 50-ml aliquots of sterile phosphate buffered saline (PBS) warmed to 37°C. The fluid was then immediately aspirated by gently suctioning with - 40 cm H_2O into a siliconized specimen trap placed on melting ice and transported to the laboratory for processing and analysis.

The BAL-fluid was centrifuged at 400 x g for 5 minutes at 4°C. The supernatants were decanted and stored. The cell pellets were then washed in PBS supplemented with 0.5% heat-inactivated bovine serum albumin (BSA). For total leukocyte numbers in BAL-fluid, cell suspensions were counted in a Coulter Counter and viability was assessed by cellular exclusion of trypan blue. Cytospin preparations were stained with May-Grünwald-Giemsa stain, and the differential counts were performed by counting at least 500 cells.

Determination of AA metabolites

Immediately after the BAL procedure, 20 ml of supernatant was processed on C18 SepPak cartridges (Millipore, Bedford, USA) as described previously[14], eluated with 2.5 ml methanol and stored at -80°C until analysis. Samples of 200 µl BAL eluted fluid were pipetted into polypropylene tubes and dried with a Savant sample concentrator. After dissolving in 300 µl assay buffer, levels of thromboxane B_2 (TxB_2) were determined by means of a [^{3}H] RIA with antisera from Advanced Magnetics Inc. (Cambridge, Mass.) and [^{3}H] labelled compounds from Amersham International (Buckinghamshire, UK). Levels of prostaglandin PGD_2 and PGE_2 were determined with commercially available [^{3}H] kits (Amersham, UK) and 6k$PGF_1\alpha$ with a [^{125}I] RIA kit (Du Pont de Nemours, Dreieich, Germany), according to the manufacturer's instructions. Leukotriene $C_4/D_4/E_4$ (LTC_4) was measured at room temperature in a microtitre enzyme immunoassay according to protocol (Biotrak, Amersham, UK).

Statistical analysis

The paired t-test was used to analyze within-treatment changes in FEV_1 and bronchial hyperresponsiveness. The unpaired t-test was used for comparisons between groups. In case of non-normally distributed variables non-parametric tests were used (paired and unpaired Wilcoxon test). A p-value smaller than 0.05 was supposed to indicate statistical significance. Spearman-Rank correlations (r_s) were used for testing intercorrelation between outcome variables within the groups. Values of $|r_s| > 0.6$ were considered relevant only if they reached a significance level ($p < 0.01$). Group means and standard error of the mean (± SEM) at the various time points were calculated.

RESULTS

Sixteen patients (12 men) were randomized into the FP group and 17 patients (11 men) into the placebo group. Baseline values such as FEV_1, reversibility and PC_{20}histamine were not significantly different between the groups on entry to the study (table 1).

Table 1. Baseline characteristics of the study patients

Characteristics	Fluticasone	Placebo
n	16	17
sex M/F	12/4	11/6
age, yr	28.4 (10.8)	34.5 (13.5)
FEV_1 (% predicted)	84.2 (15.4)	86.0 (17.6)
reversibility*	15.5 (7.8)	18.1 (14.3)
$PC_{20}H$† mg/ml	0.71 (0.59)	0.88 (0.87)
$log_2PC_{20}H$†	-1.12 (1.62)	-0.96 (1.70)
plateau value**	58.8 (19.8)	50.8 (12.7)
reactivity (%/doubling dose)	11.6 (5.3)	9.4 (3.7)
Total IgE (IU/ml)	311 (288)	299 (289)

All values are expressed as mean ± standard deviation (between brackets).
* reversibility: change in FEV_1 expressed as % baseline, after 1000 µg terbutaline sulphate.
†$PC_{20}H$ = PC_{20} histamine; $log_2PC_{20}H$ = log_2PC_{20} histamine.
**plateau value expressed as % fall in FEV_1.
No statistical significant differences were present between the treatment groups.

Table 2. MDR-curve indices of the study patients

	Fluticasone	
lung function parameters	before treatment	after 12 weeks
FEV_1 (% predicted)	82.9 (4.1)	91.0 (3.8)*,**
$log_2PC_{20}M^+$	0.18 (0.56)	3.77 (0.72)*,**
reactivity (%/doubling dose)	11.6 (1.3)	9.0 (1.8)
plateau (% fall in FEV_1)	58.8 (4.9)	36.5 (4.1)*,**
	Placebo	
lung function parameters	before treatment	after 12 weeks
FEV_1 (% predicted)	82.9 (4.2)	80.1 (5.1)
$log_2PC_{20}M^+$	-.05 (0.74)	0.26 (0.46)
reactivity (%/doubling dose)	9.4 (0.9)	10.8 (1.0)
plateau (% fall in FEV_1)	50.8 (3.2)	48.8 (3.6)

All values are expressed as mean ± S.E.M. (between brackets)
+ $log_2PC_{20}M$= log_2PC_{20} methacholine
No statistical significant differences were present between the indices before treatment. Significance of changes during treatment are indicated (* $p<0.05$). Also significant differences in changes between the treatment groups are indicated (** $p<0.01$).

Thirty-one of the 33 subjects completed the study. One patient receiving placebo and one receiving FP were withdrawn after experiencing a pulmonary exacerbation. Data of these two patients have not been included in the analysis.
Mean values for FEV_1 (as % predicted), sensitivity (log_2PC_{20}methacholine), reactivity (%/dd) and plateau (% fall in FEV_1) before and after treatment are shown in table 2. No statistical significant differences were present between the indices before treatment. In the FP group significant changes occurred after 12 weeks with respect to means of PC_{20} (an increase of 3.6 doubling doses), plateau value (from 58.8% at randomisation to 36.5% fall in FEV_1) and baseline FEV_1 (from 82.9% to 91.0%) in contrast to the placebo group. Changes for reactivity were less marked.
Mean values for AA metabolites and total cell recovery before and after treatment are presented in table 3. Values were comparable in both treatment groups on entry to the study.

Table 3. AA metabolites and cell levels in BAL fluid

	Fluticasone	
AA metabolites	before treatment	after 12 weeks
TxB_2 pg/ml	34.1 (4.2)	36.1 (4.2)
PGE_2 pg/ml	11.3 (0.6)	10.2 (0.8)
PGD_2 pg/ml	25.8 (10.9)	7.3 (1.8)
$6kPGF_1\alpha$ pg/ml	4.0 (0.4)	3.2 (0.2)
LTC_4 pg/ml	12.1 (3.8)	6.7 (2.0)
total cell recovery x 10^6	10.8 (0.08)	15.2 (0.11)
	Placebo	
AA metabolites	before treatment	after 12 weeks
TxB_2 pg/ml	32.2 (4.8)	29.2 (3.8)
PGE_2 pg/ml	11.3 (0.6)	11.3 (0.9)
PGD_2 pg/ml	15.4 (4.0)	17.9 (4.5)
$6kPGF_1\alpha$ pg/ml	4.2 (0.4)	3.3 (0.5)
LTC_4 pg/ml	11.6 (4.1)	12.1 (3.0)
total cell recovery x 10^6	12.4 (0.09)	11.4 (0.06)

All values are expressed as mean ± S.E.M. (between brackets).
AA= Arachidonic acid; TXB_2= Thromboxane B_2; PGE_2= Prostaglandin E_2; PGD_2= Prostaglandin D_2; $6kPGF_1\alpha$= 6 keto-Prostaglandin $F_1\alpha$; LTC_4= Leukotriene C_4. The decrease in PGD_2 within the fluticasone group was nearly significant (p=0.058). With respect to the difference in changes between both groups only the change in PGD_2 differed significantly (p=0.05) between the fluticasone and the placebo group.

Significant results were found only for PGD_2: it decreased nearly significantly in the FP group from 25.8 (SEM ± 10.9) to 7.3 (SEM ± 1.8)(p=0.059), whereas its change in the FP group differed significantly from that in the placebo group (p=0.05) after 12 weeks of treatment.

We also determined if there were correlations between changes in either treatment group and investigated whether these correlations were different between the treatment groups. In neither of the treatment groups, however, relevant and significant correlations were found between the parameters investigated.

DISCUSSION

We showed that after 12 weeks of treatment fluticasone propionate (FP) significantly decreased both sensitivity to methacholine and the maximal airway narrowing response, whereas it substantially decreased PGD_2 levels in BAL-fluid. The change in PGD_2 level after treatment with FP was significantly larger than the change in the placebo group. We were unable, however, to demonstrate a correlation between these changes in sensitivity and plateau level with the change in PGD_2 or one of the other arachidonic acid (AA) metabolites.

To date several studies have described the relation between airway inflammation, the subsequent release of AA metabolites and bronchial responsiveness. It has to be kept in mind, however, that the bronchial responsiveness in those studies has never been measured by the entire methacholine dose response (MDR) curve. Bronchial responsiveness as determined by the MDR curve is defined as the sensitivity of the airways to a wide variety of nonsensitizing bronchoconstricting stimuli. It has been demonstrated that the curves from asthmatics could be differentiated from those of normals by their position, slope and maximal response[1]. MDR curves in asthma have a steeper slope and a higher maximal response at high doses of methacholine as compared to normals[1]. A leftward shift of the curve can be regarded as being the result of any augmentation of airway narrowing stimuli (i.e. prejunctional mechanisms) such as activation of inflammatory cells and release of mediators such as AA metabolites[15-17]. An upward movement of the plateau is the result of an increase in the response of the effector organ (i.e. postjunctional mechanisms) such as smooth muscle contraction and swelling of the airway wall.

To our knowledge this is the first study that investigated the possible correlation of sensitivity, reactivity and plateau value of the MDR curve on the one hand and AA metabolites in the BAL-fluid on the other hand. However, as mentioned above, we could not demonstrate such a correlation, nor could we find a significant correlation between the FP-induced decrease in BHR and the levels of AA metabolites in BAL-fluid. This suggests that in addition to AA metabolites also other factors, such as epithelial damage, numbers of eosinophils and neutrophils in BAL-fluid, platelet activating factor, histamine and major basic protein, are important as was shown by some authors[18-22]. Our findings are in contrast with those of Oosterhoff, et al. who have demonstrated that the levels of PGD_2 in BAL-fluid are inversely correlated with the PC_{20} histamine. A possible explanation for this observed discrepancy could be the difference in treatment period; they found a correlation between AA metabolite levels and PC_{20} histamine after long-term treatment (2.5 years) with ICS.

Prostaglandins D_2 and $F_2\alpha$, thromboxane B_2, and leukotrienes B_4, C_4 and D_4 are potent pro-inflammatory mediators with a wide variety of biologic activities, including smooth muscle contraction, mucus hypersecretion and leukocyte activation[23-25]. In a previous study, we demonstrated that the subclinical inflammation in smokers was associated with higher levels of $PGF_2\alpha$ and TxB_2 in BAL-fluid as compared to non-smokers[14]. It is conceivable that the increased amounts of $PGF_2\alpha$ and TxB_2 are due to activation of alveolar macrophages in the airways of smokers. $PGF_2\alpha$ and TxB_2 induce airway secretion, constrict isolated human airways and increase the sensitivity to contractile stimuli[26]. In a study of Wardlaw et al. levels of leukotrienes (LTC_4 and LTB_4) were higher in BAL-fluid of symptomatic asthmatics with bronchial hyper-responsiveness as compared to asymptomatics[27]. Although their methodology to measure AA metabolites differed from ours, which makes comparison of the results rather difficult, the levels of LTC_4 in our study appeared to be equal to those of the asymptomatics in their study. The fact that these levels were already low at the start of the study may explane why we were unable to demonstrate an effect of treatment.

Others did allergen challenges and measured BAL-fluid and urinary levels of LTE_4, the end product of enzymatically converted LTC_4 and LTD_4. It was found in asthmatics that the basal levels of leukotrienes were not elevated, but increased *in vivo* after allergen challenge[28-31]. Christie et al. showed that children with atopic asthma who were resident at high altitude, exhibit a fall in FEV_1 and an increase in airway responsiveness to histamine upon visiting regions at sea level. This was associated with a threefold increase in urinary LTE_4 excretion[32]. Thus it seems that in asymptomatic asthma patients or asthmatics with minor symptoms, the levels of leukotrienes in BAL fluid are not increased. Upon stimulation (tobacco smoke, allergen challenge or visit to sea level), however, leukotriene levels rapidly increase resulting in bronchial hyperresponsiveness.

In support of this hypothesis, Bel et al. demonstrated that inhaled LTD_4 not only caused a higher maximal response plateau than methacholine, but also increased the maximal response to methacholine for at least 3 days. These findings could be prevented by the administration of inhaled steroids[33].

Tamaoki et al.[34] found that prednisone reduced the synthesis of eicosanoids by stimulated macrophage-rich BAL-fluid cells *in vitro*. However, Dworski et al. showed that oral prednison reduced symptoms but had no significant effect on BAL-fluid eicosanoid levels *in vivo*[35]. In line with this study, we also failed to find a reduction in AA metabolites in BAL-fluid. Only PGD_2 appeared to be nearly significantly lower ($p= 0.058$) in FP treated subjects, whereas the change in PGD_2 before and after 12

weeks of treatment with FP was significantly larger (p= 0.05). It has been demonstrated in canine airway smooth muscle that PGD_2 prejunctionally augments the parasympathetic contractile response[34]. Stimulation of asthmatics with PGD_2 significantly increased the reactivity to histamine and methacholine[2]. It was suggested that enhanced cholinergic tone underlied these findings. Beasley et al. demonstrated that PGD_2 and its metabolite 9α,11ß-PGF_2 caused a marked increase of methacholine induced bronchoconstriction that could only partially be prevented by an anticholinergic[23]. In addition, several studies showed that allergen challenge resulted in a marked increase in prostaglandin levels in BAL-fluid[36, 37]. This suggests that PGD_2 may augment the histamine or methacholine induced hyperresponsiveness. The effects of prostaglandins on reactivity or plateau value is unknown, because none of the above mentioned authors investigated the entire MDR curve and a possible relation of prostaglandins with reactivity or plateau value.
In our study PGD_2 levels in BAL-fluid of asthmatics substantially decreased after treatment with FP. Since PGD_2 is a product derived from mast cells and to a lesser extent from alveolar macrophages[38, 39], it may be concluded from our results that ICS, particularly, downregulate mast cells to produce PGD_2 although the influence on alveolar macrophages is not excluded.

In conclusion, in our study we demonstrated that BHR as determined by the MDR curve is downregulated by FP. Although FP strongly influences the position, the shape and also the maximum response plateau of the MDR curve, it did not influence levels of AA metabolites in BAL-fluid except for PGD_2. Our results indicate that the effect of FP on BHR is not achieved mainly by its influence on the level of the AA metabolites. It is suggested that other pro-inflammatory factors are of more importance for the shape of the MDR curve.

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Chapter 4.2

Lower leukotriene C_4 levels in bronchoalveolar lavage fluid of asthmatic subjects after 2.5 years inhaled corticosteroid therapy

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ABSTRACT

Longterm treatment with inhaled corticosteroids has been shown to result in improvement of symptoms and lung function in subjects with asthma. Arachidonic acid (AA) metabolites are thought to play a role in the pathophysiology of asthma. It was assessed whether differences were found in bronchoalveolar lavage (BAL) AA metabolite levels between subjects with asthma who were double-blinded treated during 2.5 years with inhaled bronchodilators alone or in combination with inhaled corticosteroids.

Prostaglandin (PG)D_2, $PGF_2\alpha$, 6keto-$PGF_1\alpha$, thromboxane B_2, leukotriene (LT)C_4 and LTB_4 levels and cell numbers were assessed in BAL fluid from 22 nonsmoking asthmatic subjects. They were participating in a randomized, double-blind multicentre drug trial during 2.5 years. Results of the group treated with inhaled corticosteroids (CS+: beclomethasone 200 µg four times daily) were compared with the other group (CS–) which was treated with either ipratropium bromide (40 µg four times daily) or placebo.

BAL LTC_4 levels of asthmatic subjects were significantly lower after 2.5 years inhaled corticosteroid therapy [CS+ : 9 (1-17) pg/ml versus CS– :16 (6-53) pg/ml; p= 0.01]. The same trend was observed for the PGD_2 levels.

The results suggest that inhaled corticosteroids may exert their beneficial effect on lung function via a mechanism in which inhibition of LTC_4 synthesis in the airways is involved.

INTRODUCTION

Recent long-term studies on the prognosis and morbidity of obstructive airways disease have shown that addition of inhaled corticosteroids (ICS) to maintenance treatment with a β_2-agonist in hyperresponsive patients with obstructive airways disease leads to a significant reduction of respiratory symptoms, exacerbation rates, airways obstruction and airway hyperresponsiveness[1,2]. These findings are thought to be a consequence of suppression of inflammatory processes in the airways.

No single cell type or mediator in the inflammatory processes in the airways is responsible for the clinical events in asthma. Nevertheless, there is now substantial evidence that arachidonic acid (AA) metabolites play an important role in the pathophysiology of the disease. They are potent airway constrictors, increase mucus secretion, play a role in chemotaxis and may enhance airway responsiveness, one

of the characteristics of the disease[3]. A role for AA metabolites in the modulation of severity of asthma is suggested by the presence of higher levels in BAL fluid as compared to those in healthy subjects, increasing even more after allergen provocation[4, 5].

One potential mode of action of ICS involves the modulation of AA metabolite levels in the airways[6]. They may decrease AA metabolite synthesis by their inhibitory effect on phospholipase A_2 or by inhibition of synthesis of cytokines that stimulate AA metabolite release. In order to assess the role of AA metabolites in the effects of long-term treatment of asthmatic subjects with corticosteroids, AA metabolite levels in bronchoalveolar lavage BAL-fluid were assessed in a subgroup of non-smoking asthmatic patients who participated in a randomized, double-blind multicentre drug trial during 2.5 years.

MATERIALS AND METHODS

Patient selection

Before entering the multicentre drug intervention trial, patients were selected based on: *1)* Baseline FEV_1 larger than 1.2 litres (ranging between 4.5 and 1.6 residual standard deviations below the predicted value), or an FEV_1/inspiratory vital capacity (IVC) ratio lower than 1.64 RSD below the predicted value provided that total lung capacity was higher than 1.64 RSD below the predicted level[1]; *2)* Airway hyperresponsiveness to histamine, defined as the provocative concentration of histamine that caused 20% decrease in FEV_1 (PC_{20}) $\leq$ 8 mg/ml; *3)* Exclusion of pregnant women, patients with a history of occupational asthma or other serious diseases, patients who used oral corticosteroids, ß-blockers, nitrates, or anti-coagulants, and patients who continuously used antibiotics.

At enrolment, the subjects were assigned to one of three double-blind regimens using identical metered dose inhalers: all patients received an inhaled $ß_2$-agonist (terbutaline 500 µg) combined with either an inhaled corticosteroid (beclomethasone dipropionate 200 µg), an inhaled anticholinergic (ipratropium bromide 40 µg) or an inhaled placebo. All medication was taken four times daily.

Study design

AA metabolites and cell numbers were analysed in BAL-fluid obtained from outpatients from two university pulmonary departments (Groningen and Rotterdam), 2.5 years after participation in the above-indicated randomized, double-blind multicentre drug

intervention trial[1]. At the time of selection of patients for this study and bronchoscopy, the patients were still receiving their trial medication. Therefore, the investigators were blind to the treatment that had been received in the preceding 30 months. Analysis was performed only in BAL-fluid obtained from 22 nonsmoking patients with asthma, diagnosed by criteria from a standardized history on respiratory symptoms[1] and with a reversibility of airways obstruction > 9% of FEV_1 % predicted at entry.
In accordance with findings in all participants of the trial[1], no significant differences in FEV_1 values, reversibility of airway obstruction and airway responsiveness to histamine obtained at the last visit preceeding the bronchoscopy procedure were found between asthmatic patients assigned to the placebo and anticholinergic groups. Therefore, differences in BAL parameters in this study were investigated between the groups without (*i.e.* placebo + anticholinergic therapy) and with inhaled corticosteroid therapy.
Correlations between BAL data and lung function parameters obtained 1 week preceding the bronchoscopy procedure were investigated. The study protocol was approved by the medical ethics committees of the participating hospitals. All patients gave written informed consent.

Pulmonary function and inhalation provocation tests
FEV_1 was performed on water-sealed spirometers according to standardized guidelines[7], before and 20 min after four single inhalations of 250 μg of terbutaline administered through a 750-ml spacer device (Nebuhaler). Histamine provocation tests were performed using a 2 min tidal breathing method, as described previously[1]. Measurements were made only during clinical stable periods, and not within four weeks after the termination of a course of prednisolone. All pulmonary medications were discontinued eight hours before each test.

Bronchoalveolar lavage
Fiber-optic bronchoscopy (Olympus B1 IT10, Tokyo, Japan) was undertaken according to guidelines of the American Thoracic Society[8]. Premedication consisted of intramuscular injection of 0.5 mg atropine and inhalation of 500 μg terbutaline 30 minutes before the procedure. Lidocaine 4% was administered into the upper airways and bronchial tree. Bronchoalveolar lavage was performed with 1x30 ml (pool 1) and 4x50 ml (pool 2) sterile phosphate-buffered saline (PBS) at 37°C with the bronchoscope wedged in the lateral segment of the right middle lobe. After recovery by gentle

suction (-40 cm H_2O), the BAL-fluid was collected in a siliconized specimen trap placed on melting ice.
Immediately after collection of the BAL-fluid the laboratory procedures were carried out. The BAL-fluid was centrifuged at 400 x g at 4°C for 5 minutes. BAL supernatant was separated from the cell pellet. The cell pellets were washed in PBS supplemented with 0.5% heat-inactivated bovine serum albumin (BSA). Total leukocyte numbers in BAL cell suspensions were counted in a Coulter Counter and viability was assessed by cellular exclusion of trypan blue. Cell differentials were done on May-Grünwald-Giemsa stained cytocentrifuge preparations. At least 500 cells were counted.

AA metabolites determination
Immediately after the BAL procedure, 20 ml of BAL supernatant from pool 2 was processed on C18 SepPak cartridges (Millipore, Bedford, USA) as described previously[9], eluated with 2.5 ml methanol and stored at -80°C until analysis. Samples of 200 µl BAL eluted fluid were pipetted into polypropylene tubes and dried with a Savant sample concentrator. After dissolving in 300 µl assay buffer, levels of thromboxane B_2 (TxB_2), and leukotriene B_4 (LTB_4) were determined by means of a [^{3}H] RIA with antisera from Advanced Magnetics Inc. (Cambridge, Mass.) and [^{3}H] labelled compounds from Amersham International (Buckinghamshire, UK). Levels of prostaglandins (PG) D_2 and $PGF_2\alpha$ were determined with commercially available [^{3}H] kits (Amersham, UK) and 6k$PGF_1\alpha$ with a [^{125}I] RIA kit (Du Pont de Nemours, Dreieich, Germany), according to the manufacturer's instructions. Leukotriene $C_4/D_4/E_4$ (LTC_4) was measured at room temperature in a microtitre enzyme immunoassay according to the manufacturer's protocol (Biotrak, Amersham, UK). The cross reactivity of the LTC_4 antibody with LTD_4 was 100% and with LTE_4, 30%. Cross reactivities for the other assays to related compounds were negligible or less than 2% at B/Bo 50%.

Data analysis
Values are presented as medians with ranges. Spirometry data were analysed with the Student's t-test. Bronchoalveolar lavage data were not normally distributed and therefore analysed with the Mann-Whitney U-test. Correlations were made using Spearman's rank correlation tests. All analyses were performed with the SPSS/PC+ V 4.01 software package (SPSS Inc., Chicago, IL). Values of $p < 0.05$ were considered statistically significant.

RESULTS

Subjects

Patient characteristics and lung function parameters at entry in the study in the groups with (CS+, n=9) and without (CS–, n=13) corticosteroid treatment are listed in table 1.

Table I. Patient characteristics and lung function parameters at entry in the study, according to treatment group

Characteristic	CS–	CS+	p
Number	13	9	
Gender, M/F	11/2	5/4	n.s.
Age, years	36 (23-55)	43 (31-60)	n.s.
Diagnosis, asthma/asthmatic bronchitis	10/3	6/3	n.s.
Blood eosinophils (x 10^6/l)	200 (79-631)	251 (18-501)	n.s.
Atopy*	13+	7+/2–	n.s.
Total IgE (IU/ml)	120 (18-1000)	110 (4-1000)	n.s.
FEV_1 % pred.	62 (38-90)	60 (46-84)	n.s.
FEV_1 /VC	55 (38-68)	55 (43-65)	n.s.
Reversibility (FEV_1 % pred.)	16.6 (9-31.2)	16.4 (9-27.3)	n.s.
PC_{20} histamine (mg/ml)	0.14 (0.01-0.79)	0.16 (0.03-3.2)	n.s.

Medians with range.
CS– and CS+ = asthma groups treated without and with inhaled corticosteroids, respectively. *Atopy as determined by positive results of intracutaneous tests against house dust mite or two other tested common aeroallergens.

Comparing group data at entry in the trial retrospectively, no significant differences in patient characteristics and lung function parameters were found between both groups. In contrast, after 2.5 years of double blind, randomized treatment, a significant improvement in FEV_1, reduction in reversibility of airways obstruction and reduction in airway responsiveness to PC_{20} histamine were found in the CS+ as compared to the CS– group (table 2).

BAL cell numbers and levels of AA metabolites

The percentage recovery of BAL-fluid was significantly higher in the CS+ than in the CS– group (p= 0.01). There were no significant differences in median total or differential cell numbers/ ml BAL-fluid between both groups (table 3).

Table 2. Lung function parameters at the last visit just before bronchoscopy, according to treatment group

Parameter	CS⁻	CS⁺	p
Blood eosinophils (x 10^6/l)	152 (59-616)	130 (22-309)	n.s.
FEV_1 % pred.	56 (31-87)	88 (56-99)	0.002
FEV_1 /VC	51.6 (28.9-68.4)	60.6 (53.6-75.4)	0.01
Reversibility (FEV_1 % pred.)	19.0 (–0.6-36.9)	8.5 (–3.5-19.9)	0.01
PC_{20} histamine (mg/ml)	0.06 (0.02-0.87)	1.46 (0.1-14.4)	0.001

Medians with range. CS⁻ and CS⁺ = asthma groups treated without and with inhaled corticosteroids, respectively.

Table 3. Effect of inhaled corticosteroids on BAL-fluid volume and cell numbers (pool 2)

Parameter	CS–	CS+	p
Recovery %	34 (10-68)	63 (33-72)	0.01
Total leukocytes x 10^3/ml	85 (14-212)	123 (26-431)	n.s.
Alveolar macrophages	79 (9-180)	99 (21-349)	n.s.
Lymphocytes	3 (0-15)	7 (0-78)	n.s.
Neutrophils	1 (0-19)	2 (0-7)	n.s.
Eosinophils	0 (0-9)	0 (0-4)	n.s.

Medians with range. CS– and CS+ = asthma groups treated without and with inhaled corticosteroids, respectively.

The median LTC_4 level in the CS+ group was significantly lower than the level in the CS– group (p= 0.01), while the median PGD_2 level showed the same trend (table 4). The levels of the other investigated AA metabolites were not significantly different between the CS+ and CS– groups.

Table 4. BAL AA metabolite levels (pg/ml)

Metabolite	CS–	CS+	p
PGD_2	77 (15-200)	28 (17-138)	0.12
$PGF_2\alpha$	19 (5-25)	15 (5-36)	n.s.
6-k$PGF_1\alpha$	16 (8-30)	13 (7-24)	n.s.
TxB_2	71 (1-141)	42 (3-149)	n.s.
LTC_4	16 (6-53)	9 (1-17)	0.01
LTB_4	75 (15-138)	96 (23-279)	n.s.

Medians with range. CS– and CS+ = asthma groups treated without and with inhaled corticosteroids, respectively.

Correlation with lung function

LTC_4 levels correlated significantly with FEV_1 % pred. (rho= -0.46, p= 0.03)(*figure 1*). PGD_2 levels correlated significantly with FEV_1 % pred. (rho= -0.62, p=0.002), as did PC_{20} histamine (rho= -0.50, p= 0.02) and the reversibility of airways obstruction (rho= -0.52, p= 0.01).

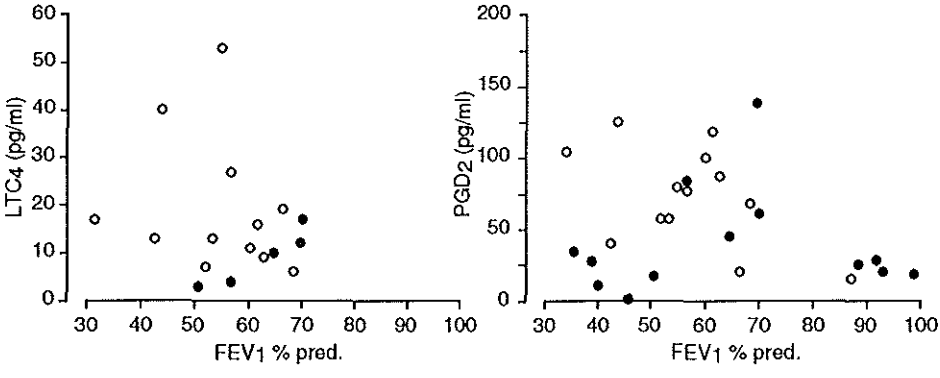

Figure 1. Relation of BAL LTC_4 and PGD_2 levels on FEV_1 % predicted in asthmatic subjects treated with inhaled corticosteroids (●) on asthmatic subjects treated with inhaled β_2-agonists alone (O). For details, see text.

DISCUSSION

This study started from the hypothesis that suppression of inflammatory processes in the airways underlies the improvement in lung function observed after longterm treatment with ICS. AA metabolite levels are considered as biochemical markers of ongoing chronic airway inflammation in the airways of asthmatic subjects[3-5]. Therefore, we investigated whether differences in BAL AA metabolite levels could be detected between subjects treated with $ß_2$-agonists and ICS during 2.5 years and those treated with inhaled $ß_2$-agonists alone. Results from this study show that the BAL LTC_4 levels are significantly lower in asthmatic patients treated with ICS. The same trend is observed for PGD_2 levels. The median BAL AA metabolite values of the corticosteroid treated group were within the same level as those from a control group of eight non-smoking, non-atopic healthy subjects which were concurrently analysed during the same procedure[10].

This is the first study in which AA metabolite levels were measured after long-term treatment with ICS. In contrast to our results, short-term treatment with prednisolone 60 mg/ day has been reported to have no significant effect on BAL-fluid AA metabolite levels in asthmatic subjects, although the *in vitro* synthesis of AA metabolites by BAL cells was decreased[11]. A lower production of LTC_4 by BAL cells was also found in asthmatic subjects who had been treated for more than 2 years with 5-15 mg prednisone[12], which is in line with our results. A role for cysteinyl leukotrienes in the pathophysiology of asthma is suggested by findings that leukotrienes induce airway obstruction, increase airway hyperresponsivenes and increase mucous secretion[3]. After oral treatment with a leukotriene D_4 receptor antagonist a significant reduction in asthma symptoms and improvement of lung function has been reported in asthmatic subjects[13]. In the light of current knowledge, several mechanisms can be postulated to explain the reduced LTC_4 levels in the CS-treated group. First, corticosteroids exert a decreased cellular AA metabolite synthesis by inhibiting phospholipase-A_2 activation through the generation of lipocortin[14]. Results from this study would suggest that cells that predominantly release LTC_4 (eosinophils, alveolar macrophages, mast cells) are more sensitive to the corticosteroid treatment. Secondly, corticosteroids may selectively inhibit the transcription of cytokines from airway cells[6, 15, 16] that may regulate LTC_4 release. IL-3, IL5 and GM-CSF have been shown to prime human basophils, eosinophils and neutrophils for augmented release of LTC_4 after stimulation by a second agonist[17-19].

Results of this study suggest an effect of long-term corticosteroid treatment on BAL PGD_2 levels as well. PGD_2 is a potent airway constrictor and is implicated in the

increase in airway responsiveness[3]. PGD_2 is synthesized by a variety of airway cells. It has been observed that the *in vitro* PGD_2 synthesis by human lung mast cells was not affected by glucocorticoids[20]. The PGD_2 synthesis by human alveolar macrophages upon stimulation by calcium ionophore A23187 was, however, significantly inhibited by methylprednisolone[21]. If *in vitro* results can be extrapolated to the *in vivo* situation, the results favour a role for PGD_2 release by alveolar macrophages in the chronic inflammatory process in asthma.
We did not find a difference in total or differential cell numbers between the groups. Previous findings in studies on the effect of short-term corticosteroid treatment (6 weeks to 4 months) on BAL cell numbers are not consistent, although in the majority a reduction of BAL eosinophil numbers was found[15, 16, 22, 23]. It cannot be excluded that we did not find differences as a consequence of group sizes that were too small. Inflammatory processes in the airways are, however, probably better reflected by cell activation than increased cell numbers in the BAL-fluid, as has been found in this study.

In conclusion, this study shows that BAL LTC_4 levels of asthmatic subjects were significantly lower after 2.5 years inhaled corticosteroid therapy. The results suggest that corticosteroids may exert their beneficial effect on lung function via a mechanism in which inhibition of LTC_4 synthesis in the airways is involved.

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Chapter 4.3

Eicosanoids and lipocortin-1 in BAL-fluid in asthma: LDD effects of smoking and inhaled glucocorticoids

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ABSTRACT

Both smoking and asthma are associated with inflammatory changes in the lung, which may be suppressed with the help of exogenous anti-inflammatory drugs or by the endogenous defence system. Lipocortin-1 (Lc-1; annexin-1) is an anti-inflammatory protein present in respiratory tract secretions. We report an inverse correlation between extracellular Lc-1 concentration and the bronchoconstrictor prostaglandin D_2 (PGD_2) (r_s=-0.597, n=15, p <0.05) in bronchoalveolar lavage fluid (BALF) from allergic asthmatics, together with positive correlations between extracellular Lc-1/ml BALF and the prostacyclin (PGI_2) metabolite, 6-keto-$PGF_{1\alpha}$ (r_s=0.480, n = 15, p <0.05) and between Lc-1/ml BALF and concentration of histamine causing a 20% decrease in forced expired volume in 1 s (PC_{20}) (r_s=0.720, n=15, p<0.01) in these subjects. We found no significant difference between the Lc-1 concentration in BALF from non-smoking asthmatic patients who were receiving inhaled glucocorticoid therapy (2x100 mg beclomethasone q.i.d. for 2.5 y; median 186 ng Lc-1/mg albumin, n = 6) and those who were not (median 126 ng Lc-1/mg albumin, n = 12), perhaps because inhaled drugs deposit predominantly in central airways, which are poorly represented in bronchoalveolar lavage. Both asthmatic and healthy volunteers who smoked had higher levels of Lc-1 in their BALF than their non-smoking counterparts (e.g. asthmatic smokers, median 317 ng Lc-1/mg albumin, n=10; asthmatic nonsmokers, median 162 ng Lc-1/mg albumin, n=18; p <0.05), perhaps because smokers' lungs contain more alveolar macrophages, cells that release Lc-1. We observed a positive correlation between BALF Lc-1 and bronchoalveolar lavage cell number (r_s=0.821, n=16, p<0.001). Increased extracellular Lc-1 may be part of a protective response of the lung to inflammatory insult. Regulation of prostanoid levels might be one mechanism by which Lc-1 suppresses inflammation.

INTRODUCTION

Inflammation is a key feature of many diseases of the respiratory tract and may be induced by exogenous factors such as inhaled allergens or tobacco smoke. Asthma is one example of an inflammatory disease, as evidenced by changes in respiratory tract secretions such as elevated levels of inflammatory mediators[5], and by increased numbers and altered profiles of inflammatory cells in bronchoalveolar lavage fluid (BALF) and bronchial biopsies[16, 22]. Inflammation in the submucosal layers and airway microvasculature is also likely to contribute to the pathology of asthma, but these regions of the respiratory tract are difficult to investigate in vivo in human subjects.

Inhaled glucocorticoids are effective in reducing clinical symptoms and improving lung function in asthmatic subjects[e.g.14]. These effects probably result from their ability to subdue inflammation and prevent the development of irreversible changes such as fibrotic lesions. Therefore, they are now considered an essential component of treatment in all but the mildest asthma. The most important mechanism of action of glucocorticoids is thought to be the modulation of transcription[4] of target genes for both pro-and anti-inflammatory proteins. Thus glucocorticoids can downregulate transcription of the genes for proinflammatory cytokines such as interleukin-1ß whilst upregulating those for anti-inflammatory proteins such as lipocortin-1 (Lc-1). The relative contributions of these actions to the overall anti-inflammatory effect of glucocorticoids are incompletely understood but are likely to depend on a number of factors, including disease state, and to vary between organs. The anti-inflammatory protein Lc-[11, 18, 20, 21] has been demonstrated to be glucocorticoid inducible[19, 33] and to mediate, at least in part, the anti-inflammatory effects of glucocorticoids in several models of inflammation[11, 20].

The anti-inflammatory actions of Lc-1 are thought to be initiated after its secretion and binding to specific cell-surface binding sites[12]. The actions of Lc-1, when given systemically in vivo include inhibition of neutrophil influx[20] and, when applied to an isolated lung preparation, reduction of eicosanoid synthesis[8], including some mediators that may play a role in the pathogenesis of asthma[5]. Lc-1 is present in abundance in the lung. It can be detected in human BALF[3, 28], indicating that it is released onto the epithelial lung surface. Previous studies have shown that treatment with oral glucocorticoids can increase Lc-1 in BALF[3, 28] and in bronchoalveolar lavage (BAL) cells[10], but to date there have been no published studies on the effects of inhaled glucocorticoids on lung Lc-1, despite the importance of this form of therapy. Interestingly, however, a nonspecific inflammatory stimulus, lipopolysaccharide, has been associated with increased Lc-1 levels in the lung of glucocorticoid-depleted (adrenalectomized) animals[29].

The aim of the present study was to examine the relationships between a number of pro- and anti-inflammatory eicosanoids and anti-inflammatory Lc-1 in lung lavage fluid from asthmatic patients and to determine the effect of inhaled glucocorticoids and tobacco smoking on these relationships; in addition, we have examined the effect of smoking on extracellular Lc-1 in lung lavage from subjects with healthy lungs.

The investigation was undertaken as an extension of an earlier published multicenter study[6, 15] to examine the therapeutic effectiveness of a $ß_2$-agonist with an inhaled glucocorticoid compared with a $ß_2$-agonist alone or with the addition of a muscarinic

antagonist in allergic asthma. At the end of the treatment period, some individuals from that study underwent BAL; this material has been used in the present study. Part of the present study has been published in abstract form[26, 32].

MATERIALS AND METHODS

Study A: Patients with allergic asthma

Patient characteristics

Twenty-eight allergic asthmatic patients (8 women and 20 men; median age 41, range 21-62 yr), both smokers (1 woman and 9 men; median age 42, range 21-62 yr) and nonsmokers (11 men and 7 women; median age 40, range 25-61 yr), were randomly sampled in two participating centers, Groningen and Rotterdam, of the Dutch CNSLD study group[6, 15]. Patients who had stopped smoking at least 6 months before the start of the study were included in the nonsmokers group. The diagnosis of asthma was based upon a history of attacks of breathlessness and wheezing without chronic (i.e. for >3 months/year) cough or sputum production, according to the criteria of the American Thoracic Society[31]. Allergy was defined as a positive skin prick test to house-dust mite or to at least 2 out of 12 other common aeroallergens (mean wheal size ≥70% of the histamine wheal size[6]). At entry to the study, all patients showed airway hyperresponsiveness, defined as a 20% decrease in forced expired volume in 1 s (FEV_1) caused by inhalation of a histamine concentration (PC_{20}) of ≥8 mg/ml[6]; reversibility at entry was ≥10% of the baseline value for each individual.

Subjects who fulfilled the entry criteria were randomly assigned to the following treatment groups. Patients were treated double blind with the inhaled β_2-agonist terbutaline (2 puffs of 250 mg q.i.d.) plus either the inhaled glucocorticoid beclomethasone dipropionate (2 puffs of 100 mg q.i.d.; n=12, 6 smokers), the anticholinergic bronchodilator ipratropium bromide (2 puffs of 20 mg q.i.d.; n=8, 2 smokers) or placebo q.i.d. (n=8, 2 smokers). At the end of the 2.5-yr treament period, no significant differences with regard to FEV_1, PC_{20}, or any of the biochemical parameters studied were found among the groups using either the β_2-agonist plus anticholinergic bronchodilator or the β_2-agonist plus placebo. Therefore, these data were eventually pooled for analysis as one single group (n=16, 4 smokers). Patient characteristics at the time of bronchoscopy are shown in Table 1. The study protocol was approved by the Medical Ethics Committee of all participating centers, and all patients gave written informed consent. Further details of the study methods have been described previously[6].

Table 1. Patient characteristics.

Patient number	Sex	Age	Smoking status	Inhaled glucocorticoids	After 2.5 year	
					FEV_1 (% predicted)	PC_{20} (mg/ml)
1	F	44	-	-	45.4	0.04
2	M	45-	-		63.2	0.05
3	M	25	-	-	59.5	0.58
4	M	26	-	-	59.0	0.02
5	F	37	-	-	47.2	0.06
6	M	57	-	-	68.0	0.04
7	M	31	-	-	71.9	0.02
8	M	32	-	-	67.5	0.13
9	M	30	-	-	96.4	0.42
10	M	38	-	-	44.6	0.02
11	M	42	-	-	65.9	0.87
12	M	50	-	-	54.0	0.18
13	F	32	-	+	53.4	0.10
14	M	61	-	+	91.7	5.38
15	M	37	-	+	60.3	1.46
16	F	45	-	+	86.9	0.45
17	F	6	-	+	93.2	0.96
18	F	45	-	+	70.4	0.21
19	M	21	+	-	72.1	0.96
20	M	36	+	-	65.2	0.70
21	M	58	+	-	55.6	2.00
22	M	48	+	-	n.d.	n.d.
23	M	54	+	+	61.4	2.55
24	M	40	+	+	77.4	0.96
25	M	29	+	+	64.4	1.16
26	F	62	+	+	56.1	0.05
27	M	43	+	+	82.1	0.40
28	M	31	+	+	92.4	1.79

M: male; F: female; n.d.: not determined

Bronchoalveolar lavage

At the end of the 2.5-year study, BAL was performed 2-7 days after the last follow-up visit at which FEV_1 and PC_{20} were determined. Thus, by the time of the bronchoscopy, ex-smokers had refrained from smoking for at least 3 years. The BALs were all carried out between August and December in both centers, before the code was broken, and were performed 30 min after premedication with atropine (0.5 mg i.m.) and 2 puffs of 250 mg terbutaline. Local anesthesia was achieved by using a lidocaine (2%, w/v) spray. The bronchoscope was placed in the wedge position in the right middle lobe. Four aliquots of 50 ml sterile and prewarmed (37°C) phosphate- buffered saline were instilled and aspirated immediately into a siliconized specimen trap placed on iced water. Immediately after collection, BALF was strained through a sterile gauze to trap large mucus particles, after which the cells were separated from the fluid by centrifugation at 4°C and 400g. Differential cell counts of the BAL cells were done after May-Grünwald Giemsa staining. Supernatants were stored at -70°C or lower until biochemical analysis.

Quantitation of eicosanoids

Prostaglandin (PG) D_2, PGF_{2a}, 6-keto-PGF_{1a} (a metabolite of prostacyclin, PGI_2), thromboxane (Tx) B_2 (a metabolite of TxA_2), and leukotriene (LT) B_4 were assayed in BALF by using radioimmunoassays as described previously[18]. $LTC_4/D_4/E_4$ was measured using a previously described microtiter enzyme immunoassay[18].

Study B: Subjects with healthy lungs

Subject characteristics

Twelve healthy women (5 smokers, median age 37, range 31-44 yr; 7 nonsmokers, median age 31, range 24-45 yr), who denied symptoms of pulmonary diseases were hospitalized for laparoscopy for reanastomosis of the fallopian tubes. They did not use any steroidal or nonsteroidal anti-inflammatory drugs. BAL was performed during general anesthesia for laparoscopy. The bronchoscope was introduced via the endotracheal tube and placed in the wedge position in the right middle lobe. From this point, BAL was performed as described for the asthma patients. All women gave informed consent; the protocol was approved by the local Medical Ethics Committee.

Both studies

Protein analyses

Lc-1 levels were assayed using a previously described enzyme-linked immunosorbent assay (ELISA) for human Lc-1[23]. The lower limit of sensitivity of the assay is 1 ng/ml and the coefficient of variation between triplicate samples on the same plate is < 5% (between plates this rises to 10%); a standard curve and quality controls were included on every plate. Specificity for Lc-1 is conferred on the assay by the use of a monoclonal antibody which has no cross-reactivity with other human annexins (JL Browning, personal communication). Albumin was determined by rocket immuno-electrophoresis using specific polyclonal antisera[25] and total protein by the Lowry method[17]. All analyses were performed blind to the clinical and treatment status of the subject.

Statistical analysis.

For data from asthmatic subjects, an analysis of variance (ANOVA) was used where multiple conditions were compared. Variables for which significant differences were found or significant trends observed were also analysed by simple comparisons between groups using unpaired Student's t-tests. Data from healthy subjects were analysed by unpaired Student's t-test only. Relationships between parameters were examined using the Spearman rank correlation coefficient (r_s). Statistical significance was taken as $p < 0.05$. Differences which were statistically significant with unpaired t-tests were also significant at the $p < 0.05$ level in a Mann-Whitney U test.

RESULTS

Study A: Asthmatic patients

Standard comparators

More lavage fluid was recovered from the lungs of glucocorticoid-treated non-smokers than from those not inhaling glucocorticoids (Table 2). There were no other significant differences in fluid recovery between groups.

More cells were present in the BALF from smokers than non-smokers and a higher proportion of the cells were alveolar macrophages (AM) in smokers (Table 2). No significant differences were observed in either BAL cell number or cell profile between the groups treated with glucocorticoids and those receiving other treatments (Table 2).

Table 2. Standard comparators for BALF from patients with allergic asthma.

Smoking status Steroid use	Nonsmoker None	Nonsmoker Inhaled	Smoker None	Smoker Inhaled
n	12	6	4	6
Fluid, ml	73 (30-135)	123 (65-144)*	85 (60-160)	98 (60-127)
BAL cells, x 10^6	3.5 (0.4-10.0)	11.0 (0.9-31.0)	21.1 (2.4-30.7)*†	27.4 (4.0-41.8)†
AM, %	90 (62-98)	82 (70-100)	95 (94-97)†	97 (90-98)†
Total protein, µg/ml	130 (25-180)	91 (53-155)	223 (53-810)	76 (48-163)‡
Albumin, µg/ml	28 (12-32)	28 (15-42)	44 (22-65)	22 (14-39)‡
Album./total prot., µg/µg	0.20 (0.11-0.50)	0.28 (0.24-0.53)	0.23 (0.08-0.41)	0.24 (0.20-0.44)

Values are medians with limits of range in parentheses; n, no. of subjects. BALF, bronchoalveolar lavage fluid; BAL, bronchoalveolar lavage; AM, alveolar macrophages. *Significantly greater than untreated nonsmokers, P <0.05 (Student's t-test). †Significantly greater than pooled data from nonsmokers, P <0.05 (analysis of variance). ‡Significantly less than untreated smokers, P <0.05 (Student's t-test).

Table 3. Lipocortin-1 and eicosanoid concentrations in BALF from patients with allergic asthma.

Smoking status Steroid use	Nonsmoker None	Nonsmoker Inhaled	Smoker None	Smoker Inhaled
n	12	6	4	6
LC-1, ng/ml	3 (1-16)	5 (2-9)	13 (4-33)*‡	7 (3-26)‡
PGD_2, pg/ml	77 (20-200)	35 (17-138)	48 (27-54)	31 (17-172)
$PGF_{2\alpha}$, pg/ml	19 (11-25)	14 (7-36)	21 (8-53)	14 (10-29)
TxB_2, pg/ml	76 (1-141)	41 (3-194)	10 (1-112)	38 (7-67)
LTC_4, pg/ml	16 (6-53)†	7 (1-12)	11 (3-12)	13 (5-16)
LTB_4, pg/ml	75 (21-138)	32 (23-279)	48 (10-171)	97 (23-171)
6-keto-$PGF_{1\alpha}$, pg/ml	16 (10-28)	12 (7-21)	31 (15-66)*‡	28 (10-62)†‡

Values are shown as median with limits of range in parentheses; n, no. of subjects. LC-1, lipocortin-1; PGD_2, prostaglandin D_2; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$. TxB_2, thromboxane B_2; LTC_4, leukotriene C_4; LTB_4, leukotriene B_4; 6-keto-$PGF_{1\alpha}$; 6-ketoprostaglandin $F_{1\alpha}$. *Significantly greater than untreated nonsmokers, P <0.05 (Student's t-test). †Significantly greater than treated nonsmokers, P <0.05 (Student's t-test). ‡Significantly greater than nonsmokers when all data are pooled, P <0.05 (analysis of variance).

Concentrations of total protein and albumin were lower in BALF collected from glucocorticoid-treated asthmatic subjects than from those given no glucocorticoids, significantly so in smokers (Table 2). There were no differences in albumin-to-total protein ratios among any of the groups.

Eicosanoids

There was considerable variation between subjects in the concentrations of the eicosanoids measured (Table 3). Smokers had significantly more of the PGI_2 metabolite, 6-keto-PGF_{1a} in their BALF than nonsmokers (Table 3). In addition, glucocorticoid-treated nonsmokers had lower levels of LTC_4 in their BALF than those not receiving glucocorticoid (Table 3). There were no other significant differences in eicosanoids between smoking or treatment groups.

Lipocortin-1

In asthmatic subjects who did not use inhaled glucocorticoids, extracellular Lc-1 was significantly greater in BALF from smokers than from nonsmokers whether the data were expressed per unit of fluid recovered (Table 3) or as a ratio to albumin (Figure 1a) or to total protein (data not shown). When the data were expressed per million

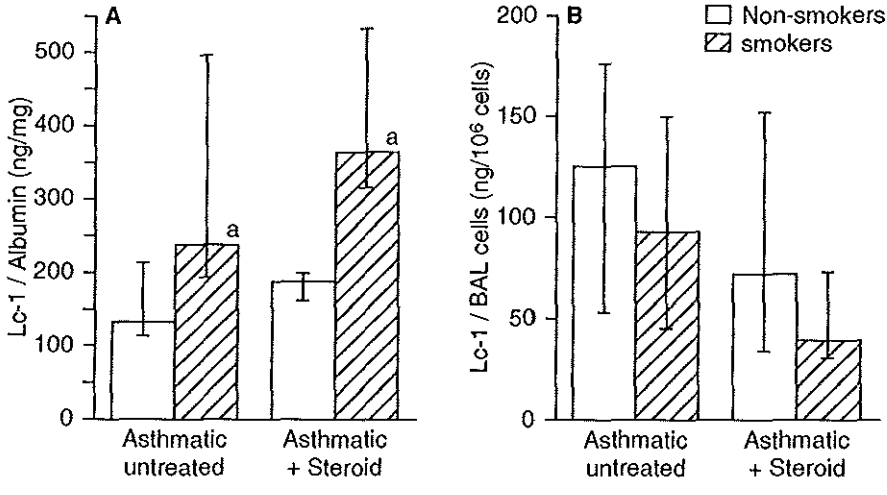

Figure 1. Comparison of extracellular lipocortin-1 (LC-1) in asthmatic smokers and nonsmokers. Values are medians with interquartile ranges indicated by error bars. When LC-1 was expressed per mg of albumin (A), bronchoalveolar lavage fluid of smokers contained significantly more LC-1 per mg of albumin than did that of nonsmokers. When LC-1 was standardized to broncheoalveolar lavage cell number (B), there was no significant difference between asthmatic smokers and nonsmokers. There was no difference in LC-1 between asthmatic patients who had been treated with inhaled glucocorticoids and those who had not. [a]Significantly greater than nonsmokers, $P < 0.05$ (Student's t-test).

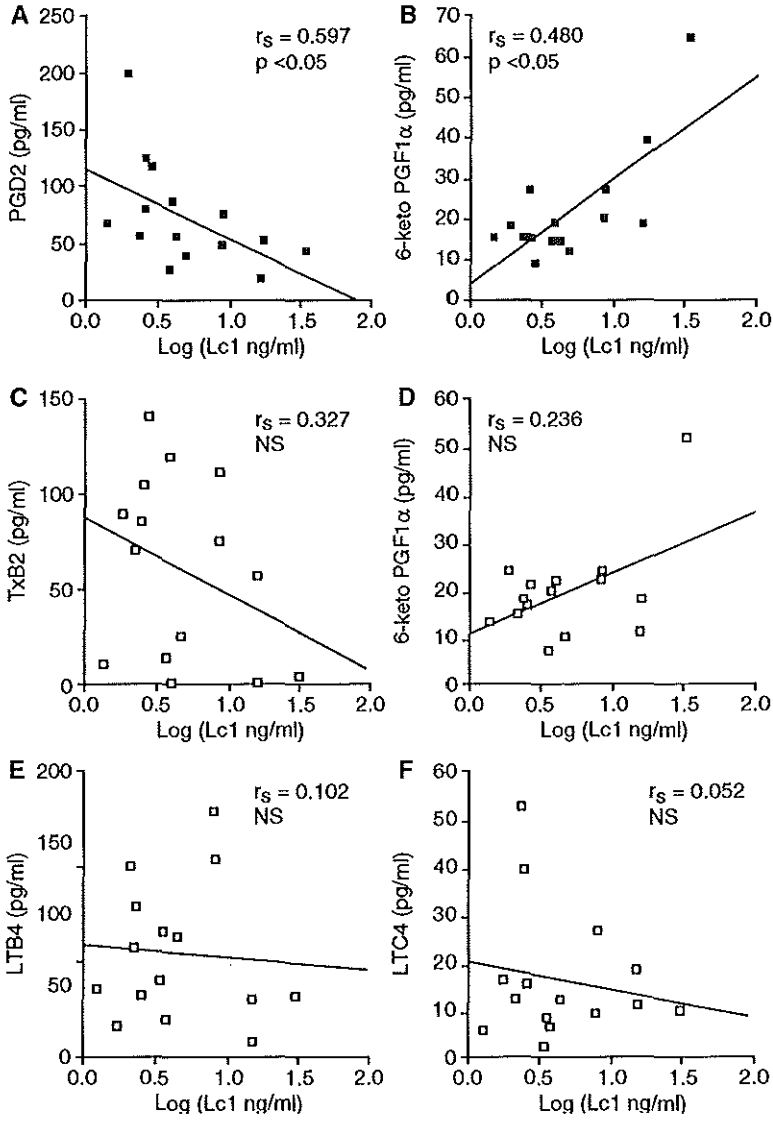

Figure 2. Relationship between extracellular LC-1 and eiosanoids in bronchoalveolar lavage fluid from asthmatic patients not treated with inhaled glucocorticoids. LC-1 concentrations are shown as logarithms for ease of illustration. Data from both smokers and nonsmokers are included (n = 15), A and B: statistically significant correlations. C-F: correlations that are not significantly different at 5% level. PGD_2, prostaglandin D_2; 6-keto-$PGF_{1\alpha}$, 6-ketoprostaglandin $F_{1\alpha}$; TxB_2, thromboxane B_2; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; LTB_4, leukotriene B_4; LTC_4, leukotriene C_4, r_s, Spearman rank correlation coefficient; NS, not significant.

BAL cells, there was no significant difference between smokers and non-smokers (Figure 1b). In contrast to smoking, glucocorticoid treatment had no significant effect on extracellular Lc-1 (Table 3, Figure 1a and 1b).

When the data from all asthmatic subjects were pooled, there was a significant correlation between BAL cell number and both total Lc-1 (n=27, r_s=0.825, p<0.001) and 6-keto-PGF_{1a} (n=26, r_s=0.565, p<0.01). Not suprisingly, therefore, Lc-1 and 6-keto-PGF_{1a} were also positively correlated (n=26; r_s=0.727, p<0.01).

In the absence of glucocorticoid treatment, there were positive correlations between Lc-1 concentration and total protein (n=15, r_s=0.574, p<0.05) and albumin concentrations (n=15, r_s=0.942, p<0.001), fluid recovery (n=15, r_s = 0.796, p<0.01), and BAL cell number (n=16, r_s=0.821, p<0.001). In the subjects not treated with glucocorticoids, Lc-1 (however expressed) was significantly inversely correlated to the PGD_2 concentration (e.g. Lc-1/ml vs PGD_2/ml, n=15, r_s=-0.597, p<0.05; Lc-1/albumin vs PGD_2/ml, r_s=-0.783, p<0.01; Figure 2a). In contrast, there was a positive correlation between Lc-1 concentration and the PGI_2 metabolite, 6-keto-PGF_{1a} (r_s=0.480; Figure 2b). There were no significant correlations between Lc-1 and any other eicosanoid in the BALF from asthmatics not inhaling glucocorticoids (Figures 2c-e). Interestingly, in these subjects, positive correlations were found between FEV_1 and Lc-1 expressed as a ratio to either albumin (n=15, r_s= 0.471, p<0.05) or total protein (n=15,r_s = 0.586, p< 0.05). PC_{20} was also significantly correlated to the ratio

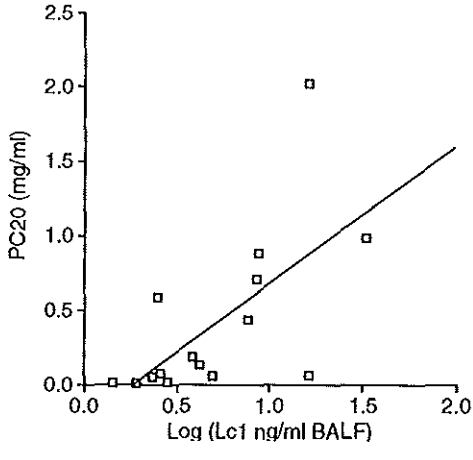

Figure 3. Relationship between extracellular LC-1 in bronchoalveolar lavage fluid (BALF) and concentration of histamine causing a 20% decrease in forced expired volume in 1s (PC_{20}) of asthmatic patients not treated with inhaled glucocorticoids. PC_{20} values were determined at end of 2.5 year study period, 2-7 days before collection of bronchoalveolar lavage. LC-1 data are shown as logarithms for ease of illustration. Data from both smokers and nonsmokers are included (n = 15).

of Lc-1 to albumin (n = 15, r_s = 0.645, p <0.01) and Lc-1 concentration (n = 15, r_s = 0.720, p<0.01; Figure 3).
In patients receiving inhaled glucocorticoid treatment, none of these relationships was significant, except that between Lc-1 and cell number (n=11, r_s=0.838, p<0.01).

Study B: Subjects with healthy lungs

Standard Comparators
Although there were no significant differences in fluid recovery between smokers and nonsmokers with healthy lungs, significantly more BAL cells were recovered from smokers than from nonsmokers (Table 4). Irrespective of smoking status, >90% of the cells were AM.
Neither total protein nor albumin concentrations, nor albumin-to-total protein ratios differed significantly between smokers and non-smokers with healthy lungs (Table 4).

Table 4. BALF data from subjects with healthy lungs

Parameter	Nonsmoker	Smoker
n	7	5
Fluid recovery, ml	125 (110-160)	130 (90-150)
BAL cells, x 10^6	6.5 (4.7-16.8)	46.6 (30.0-71.0)*
Total protein, µg/ml	115 (95-148)	130 (78-212)
Albumin, µg/ml	34.5 (20.7-70.1)	43.3 (20.9-47.5)
Albumin/total protein, µg/µg	0.31 (0.18-0.55)	0.37 (0.16-0.56)
LC-1, ng/ml	7 (5-10)	16 (8-21)*
LC-1/albumin, ng/mg	232 (74-444)	383 (354-451)*
LC-1/BAL cells, ng/10^6 cells	133 (58-173)	39 (30-68)†

Values are medians with limits of range in parentheses; n, no. of subjects. *Significantly greater than nonsmokers, P <0.05 (Student's t-test). †Significantly less than non smokers, P <0.05 (Student's t-test).

Lipocortin-1
There was more extracellular Lc-1 in BALF from healthy smokers than from nonsmokers whether the data were expressed per milliliter or as a ratio to albumin (Table 4) or to total protein (data not shown). In contrast, there was significantly less extracellular Lc-1 per million BAL cells in the BALF from healthy smokers than from nonsmokers (Table 4).

There was a strong positive correlation between total extracellular Lc-1 recovery and BAL cell number (n=12, r_s=0.9091, p<0.001), but no significant relationship with any other parameter.

DISCUSSION

In these two parallel studies, we have demonstrated that, both in subjects without lung disease and in patients with allergic asthma, tobacco smokers have more extracellular Lc-1 in their air spaces than do nonsmokers, perhaps because in the respiratory tract of both healthy and asthmatic individuals there is a positive relationship between extracellular Lc-1 in BALF and BAL cell number. These observations suggest that BAL cells are the principle source of the Lc-1 found in human respiratory tract secretions, even though other lung cell types contain and may secrete this protein[2, 13]. In addition, we observed that, in the lung lavage of asthmatic subjects who were not treated with inhaled glucocorticoids, there was an inverse correlation between extracellular Lc-1 and the bronchoconstrictor PGD_2 and also a positive correlation between Lc-1 and both FEV_1 and PC_{20}, suggesting that there may be a link between these variables.

Lc-1 has been shown to have anti-inflammatory effects in a variety of in vivo and in vitro models, including the isolated perfused lung[8]. It has been suggested that one of the anti-inflammatory mechanisms of Lc-1 may be to reduce the activity of phospholipase A_2, either directly or indirectly[8, 9] and hence the synthesis of inflammatory mediators derived from arachidonic acid. The inverse correlation between Lc-1 and the eicosanoid PGD_2 observed here would support this hypothesis, but the observations that Lc-1 and 6-keto-PGF_{1a} were positively correlated and that none of the other four eicosanoids measured in this study was related to Lc-1 might suggest a rather more selective mechanism of action for the protein. PGD_2 may contribute to the early bronchoconstriction reaction following allergen challenge in allergic asthma[5] and thus could be a determinant of bronchial hyperresponsiveness. This would be supported by the finding that the higher the PGD_2, the more responsive the airways to histamine as measured by the PC_{20} [34]. Thus this study suggests that, in allergic asthmatic subjects given no exogenous glucocorticoids, Lc-1 might contribute to protection of the airways from histamine-induced bronchoconstriction; one possible mechanism for this could be via PGD_2 regulation. Because both Lc-1 and 6-keto-PGF_{1a} concentrations were positively related to BAL cell number, it is possible that they are derived from a common cellular source; this could account for

their apparent correlation. However, there may be a functional link between these parameters; for example, an Lc-1 mediated reduction in PGD_2 synthesis may result in an increased availability of substrate (i.e. PGG_2, PGH_2) for the synthesis of other prostanoids, including PGI_2 and its metabolite 6-keto-PGF_{1a}. If all the additional substrate were converted to PGI_2, one might expect an inverse correlation between 6-keto-PGF_{1a} and PGD_2. However, this was not the case (r_s = -0.071, n = 15). Nor was there a statistically significant inverse relationship between PGD_2 and any of the other eicosanoids quantified in this study (data not shown). It therefore seems likely that the additional substrate generated by the reduction in PGD_2 synthesis can undergo conversion to several prostanoids, including PGI_2, but is not exclusively converted to any single eicosanoid measured in this study. With the exception of PGI_2, which has been reported to prevent PGD_2-induced bronchoconstriction[5], only broncho-constrictors were quantified in this study. However, the excess substrate may be converted to PGE_2 (a bronchodilator not assessed in this study[5]) which would be an additional source of benefit in asthmatic subjects.

Interestingly, there were no significant correlations among Lc-1, any prostanoid and lung function parameters in glucocorticoid-treated subjects, supporting the hypothesis that inhaled glucocorticoids suppress respiratory tract inflammation by multiple mechanisms. The data presented in this paper suggest that one relevant mechanism may be the suppression of LTC_4 release (Table 3). A future longitudinal study in which BAL is performed in the same subject both before and after treatment with inhaled glucocorticoid should enable us to address this problem more fully.

Lc-1 is made both by secretory cells in the lung[2, 13] and by AM[1, 10]. Thus the increase in extracellular Lc-1 in smokers' BALF could result from an increased production of respiratory tract secretions, the raised macrophage population, activation of Lc-1-producing cells, or a combination of these possibilities. However, in both studies, the ratio of Lc-1 to protein was higher in smokers than nonsmokers, significantly so in the subjects with healthy lungs, suggesting that the increased extracellular Lc-1 was not due simply to a nonspecific increase in protein secretion. Furthermore, the strong positive correlation between Lc-1 and BAL cell number in both study groups would suggest the macrophage as a major source of Lc-1 in the respiratory tract secretions, supporting the hypothesis of Ambrose at all[1] to this effect.

The regulation of Lc-1 secretion into the epithelial lining fluid is not fully understood. Oral glucocorticoids have been shown to increase both extracellular Lc-1 in BALF[3, 28] and intracellular levels in BAL cells[10]. In addition, two studies in glucocorticoid-depleted (adrenalectomized) rats have shown that Lc-1 in peritoneal leukocytes and lung homogenates can be increased by nonspecific stimuli, namely paraffin oil[19] and

lipopolysaccharide[29]; a component of tobacco smoke or a mediator activated by it may also non-specifically induce an increase in Lc-1 production and/or secretion. The promoter sequence of the gene for Lc-1 has regions that are similar to those found in some acute-phase proteins[7], so multiple factors could contribute to the control of Lc-1 expression, either directly or indirectly. Alternatively, the high extracellular Lc-1 level observed in smokers' BALF in the present study could result from raised circulating cortisol in these subjects. However, because a previous study showed no difference in circulating cortisol among current, ex-, and nonsmokers[30], this seems unlikely.

In the present investigation, extracellular Lc-1 did not differ between BALF from asthmatic patients who had been taking inhaled glucocorticoids and those who had not, even though both total protein and albumin were reduced in the former, significantly so in smokers. Thus, while inhaled glucocorticoids appear to reduce the levels of other proteins, Lc-1 levels were maintained but, in contrast to previous studies using oral glucocorticoids[3, 10, 28], were not elevated above control values. This confirms a previous preliminary observation made by Smith et al[24]. The doses administered by inhalation may be insufficient to stimulate Lc-1 synthesis or secretion, or the glucocorticoid may be deposited and act in the upper and central airways which are poorly represented by conventional BAL[27]. It is unlikely that Lc-1 synthesis and/or release is entirely glucocorticoid independent in patients with allergic asthma because De Caterina et al[10] observed an increase in cellular Lc-1 in BAL cells from asthmatic subjects after treatment with oral glucocorticoids.

In summary, we have observed an increase in extracellular Lc-1 in asthmatic smokers that we have demonstrated is also found in the healthy lung; we hypothesize this to be part of a self-protecting mechanism to limit respiratory tract inflammation. We have confirmed that, in allergic asthmatic patients, inhaled glucocorticoids down-regulate total protein and albumin but do not alter Lc-1 in BALF. Finally, we present preliminary evidence suggesting that Lc-1 levels may be related to improved FEV_1 and PC_{20} values in allergic asthma, perhaps via regulation of specific eicosanoids, including PGD_2. The effect of inhaled glucocorticoid on Lc-1, and the role of Lc-1 and PGD_2 in the etiology of allergic asthma, may be worthy of a more detailed investigation using subjects as their own controls in a prospective double-blind longitudinal study.

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Chapter 5

General discussion

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Although the clinical picture of asthma is quite evident, we do not know what exactly is going on in the airways of patients, nor what is or are the most important cell(s) participating in the inflammatory response or the sequence of cellular events taking place. The clinician, however, is interested in knowing whether a change in pulmonary symptoms and function on the one hand, is preceded or followed by a change in inflammatory parameters such as numbers of cells or mediators on the other hand, since this knowledge may help him to determine the best therapy. These questions helped define the objectives of the studies in this thesis, which are as follows:

1. to investigate the accuracy and the usefulness of a new mathematical model, describing the entire methacholine dose-response (MDR) curve.
2. to study lung function parameters and cellular parameters of inflammation on the airway surface and in bronchial tissue in asthma concurrently.
3. to examine the effects of inhaled corticosteroids (ICS) on these parameters.

Usefulness of MDR curves

Although usually only attention is paid to the provocative concentration, producing a fall of 20% in FEV_1 (PC_{20}, sensitivity), and not to the overall shape of the MDR curve, also presenting a slope in the mid-part (reactivity) and a maximal response (plateau), the presence and level of a maximal response plateau provide relevant information on the potential severity of airways obstruction[35, 39]. A maximal response that is induces a severe or even unmeasurable degree of airway narrowing is potentially dangerous. The major reason that asthmatic patients get into difficulties is not their increased sensitivity *per sé*, but their excessive degree of airway narrowing[5, 28].

We showed that the new mathematical model, the Cumulative Gaussian Distribution (CGD) function, facilitated extrapolation of the whole curve where no experimental plateau values could otherwise be reached with sufficient accuracy [1, 29]. In all curves of normal volunteers and asthmatic patients an experimental plateau was reached, but even when data from an appreciable number of last provocation data were left out, a reasonably accurate plateau estimate proved to be possible. Our model demonstrated its usefulness in fitting a curve to the observed data points and is preferably used in the analysis of curves of those asthmatics with severe disease,

where reaching an experimental plateau can be associated with a large drop in FEV_1, causing feelings of severe dyspnea and making the measurement very uncomfortable to the patient. It also proved to be superior in fitting ability when compared with a generally applied hyperbolic model[34]. Furthermore, the direct plateau and reactivity estimation give an advantage over models which yield indirect indices related to the sigmoid character of the curve[39].

Although PC_{20} is generally used as an index for the shift of the curve along the concentration axis, the fit of the sigmoid function also enabled us to calculate the EC_{50} as response variable as well, being the concentration at half of the plateau response. This variable is commonly used in pharmacology[26] and is independent of the absolute value of the plateau response. We showed that ICS increased PC_{20} and plateau values significantly, whereas it hardly affected reactivity or EC_{50}. No correlation was found between EC_{50} and plateau estimates, as expected. These findings indicate that part of the PC_{20} changes may be attributed to changes in plateau values. PC_{20} only indicates the provocative concentration causing a fall of 20% in FEV_1, does not provide any further information about the amount of inflammatory changes present in the airways. The plateau value represents the level of *maximal* bronchoconstriction, depending on thickening of the airway wall and mucosal swelling, and can thus be used for comparison between individuals. Being independent of PC_{20} or plateau values, EC_{50} is probably the best determinant of the position of the MDR curve. The diagnostic value of this index, however, still needs to be investigated.

Indices of MDR curves in relation to mucosal inflammation

As mentioned previously, asthmatic patients not only show a leftward shift of the dose-response curve, but also a higher or even unmeasurable plateau value when compared to normals[4]. The most important mechanism leading to an increased plateau level is probably the thickening of the airway wall, especially of the tissues inside the ring of smooth muscle and consisting of the epithelium, sub-epithelium, the mucosa and the submucosa[19]. Asthmatic patients have thickened airway walls[19] due to cellular infiltration, collagen deposition beneath the baseline membrane[9], vascular congestion[36] and tissue edema. Eosinophils, in particular, are known to release different basic proteins with cytotoxic properties at the site of inflammation, leading to epithelial shedding[24, 25]. They may also release a range of newly formed mediators, such as LTC_4, with strong broncho-constrictor properties. Although the total number of eosinophils in the bronchial mucosa has been shown to relate to PC_{20} [6], the relation with reactivity or plateau values has, to our knowledge, never been investigated.

We found that only the number of activated EG2+ eosinophils in the lamina propia and not the total number of eosinophils correlated significantly with the plateau value. This suggests that activated eosinophils and/or their products are at least involved or even responsible for the events in the airway wall, leading to excessive airway narrowing. The fact that we also found a significant correlation between the number of activated EG2+ eosinophils and sGaw, representing airway luminal changes[16], can possibly be explained by swelling of the airway wall and the presence of intraluminal exudate.

We could not demonstrate a relationship between either the total number of eosinophils or the number of activated eosinophils and sensitivity = PC_{20} methacholine. These findings are in contrast to those of other investigators, who demonstrated that the total number of eosinophils in BAL-fluid[9, 37] or bronchial mucosa[6] correlated well with PC_{20} values. Some investigators found a negative relation between the number of activated eosinophils in the lamina propria and PC_{20} [7], whereas others did not [11]. We have no explanation for these inconsistent findings in the literature. Apparently, the number of eosinophils, either total or activated, does not reflect all inflammatory events in the airway wall.

Our findings stress the importance of modelling the entire MDR curve, paying not only attention to sensitivity, but to reactivity and plateau value as well, when follow-up measurements of BHR in research settings are made.

Effects of steroids on lung function

In chapter 3.1 the results of a study related to the effects of fluticasone propionate (FP) on MDR curves in nonsmoking atopic asthmatics are presented. The findings, compared with results in the literature[23], show that FP is very effective in decreasing the maximal airway narrowing response and increasing the PC_{20}, whereas it hardly affected reactivity. The improvements found may be partly explained by the efficacy of FP[27] or by the relatively high daily dose (1000 µg) of FP used in our study. Differences in patient selection (atopic[23] versus non-atopic[3]) or in study medication, may also have influenced the results. Although current international guidelines for the treatment of asthma advocate institution of ICS for effective treatment[8, 18], it is as yet unknown at which time ICS treatment should be initiated. To the clinician it is very important to know the answer to this question.

We therefore investigated whether a 2.5 years delay of ICS administration led to smaller improvements in FEV_1 and airways hyperresponsiveness compared to immediate prescription in patients with mild to moderately severe obstructive airways disease. A distinction was made between patients with high reversibility (ΔFEV_1 to

terbutaline ≥ 9% of predicted), considered to have predominantly asthma, and low reversibility (ΔFEV_1 <9% of predicted), considered to have predominantly COPD. The data in chapter 3.2 indicate that, although delayed introduction of ICS in asthmatics leads to similar improvements in FEV_1, improvements in PC_{20} are significantly smaller. In the past years numerous studies have demonstrated the effectiveness of ICS treatment in asthma in all stages of the disease[13, 20, 21]. Only a few investigators, have addressed the issue of delayed institution. Haahtela et al.[14] demonstrated in a group of newly detected asthmatics that delayed institution of ICS treatment resulted in consistently poorer responses with respect to peakflow values and asthma symptoms when compared to immediate institution. Similar results were seen in children[2]. Taking into account that our study concerned patients with asthma existing for a longer period of time, the results suggest that delayed institution of ICS may blunt their benificial effects and that these effects are not dependent upon the stage of the disease. In our opinion it may therefore be preferable to start ICS treatment in asthma as soon as possible in the course of the disease, although further studies are needed with longer follow-up to investigate whether the damage caused by delayed institution is definite, or can still be reversed by longer periods of treatment.
In contrast to their value in patients with asthma, the role of ICS in the routine treatment of COPD is less clear. Short-term studies have not shown any beneficial effect[38], whereas at present several multicentre-studies are being performed to evaluate the effect of long-term ICS therapy in patients with COPD[32, 40]. The deleterious effect on PC_{20} we found in the patients with asthma after delayed introduction of ICS, was not seen in the COPD patients, possibly because inflammation in COPD is not as predominant or is of a different type as in asthma[10].

AA metabolites in asthma

As mentioned earlier, it is suggested that the maximal level of airway narrowing may result from inflammatory changes in the airway wall, leading to mucosal edema and vascular congestion. Arachidonic acid metabolites, such as prostaglandins (PG) and leukotrienes (LT), are produced by various cell types although the specific product varies from cell to cell. As has been shown by BAL studies, many cells are present in the airways of asthmatic patients in an activated state[11]. Mast cells mainly produce PGD_2 [33], a potent bronchoconstrictor, whereas PGE_2, $PGF_2\alpha$ and Tx are preferentially generated by macrophages and airway epithelial cells[17]. All mediators produced by these cells have pro-inflammatory actions including smooth muscle contraction, mucus hypersecretion and leukocyte activation[12, 22]. They may, therefore, cause a thickening of the airway wall resulting in a change in the plateau level. ICS are supposed to

induce the formation of the messenger protein, lipocortin. This protein belongs to a family of peptides. One of its hypothesized functions concerns the inhibition of phospholipase A_2 (PLA_2) inhibiting proteins[31]. Since PLA_2 liberates arachidonic acid from the phospholipid stores, inhibition of PLA_2 indirectly leads to reduced production of prostaglandins and leukotrienes. Hence, inhibition of PLA_2 by ICS would be expected to inhibit all cellular metabolism of arachidonic acid.
Our results show, however, that FP only nearly significantly decreased the levels of PGD_2, without affecting the level of the other AA metabolites[15]. Our findings indicate that, although FP strongly influences the position, the shape and also the maximum response plateau of the MDR curve, this effect is not achieved mainly by its influence on the level of AA metabolites. It is suggested that other factors such as epithelial damage, platelet activating factor or histamine may be of more importance for the shape of the MDR curve, and that these factors are downregulated by FP.
The findings in this study are in contrast with those of Oosterhoff et al.[30], described in detail in chapter 4.2. They demonstrated that after 2.5 years treatment with beclomethasone dipropionate (BDP), the levels of PGD_2 in BAL-fluid were inversely correlated with the PC_{20} histamine. However, comparison between these two, and also other studies in the literature, is difficult because of differences in populations studied (symptomatic versus asymptomatic patients), in treatment periods (weeks versus years), and in medication used (oral prednisone versus ICS). The main difference between the study of Oosterhoff et al. and ours seems to be the treatment period, being 2.5 years in the former and only 12 weeks in the latter.

Concluding remarks

The studies described in this thesis show that

1. In research settings the new mathematical model, describing and facilitating extrapolation of the entire MDR curve, is very useful in the analysis of BHR. The additional value in clinical settings still needs to be established.
2. PC_{20} and the plateau level are probably partly linked to each other, the plateau level being the primary determinant of airway resistance and representing the level of *maximal* bronchoconstriction. EC_{50}, being independent of both PC_{20} and plateau values, is probably the best determinant of the position of the MDR curve.
3. Eosinophils and/or their products are in someway involved in the inflammatory events in the airway wall, leading to excessive airway narrowing.
4. It may be preferable to start ICS treatment in asthma at the onset of the disease. We showed that FP is very effective in decreasing BHR.
5. ICS decrease only PGD_2 and LTC_4 levels in the asthmatic airway.

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SUMMARY

The main goal of this thesis was to investigate the effects of inhaled corticosteroids (ICS) on inflammatory markers in asthma. The combination of bronchoalveolar lavage (BAL) and biopsy with challenge tests using bronchoconstricting agents enabled us to improve our knowledge about cytological, histological and physiological events in asthma. A possible relationship between these different manifestations was also investigated. We also examined the modulating influence of anti-inflammatory therapy with inhaled corticosteroids on these events.

In **chapter 1** asthma is defined as a common condition characterized by recurrent episodes of dyspnea, wheezing and cough. It can be differentiated in an atopic ("extrinsic") and non-atopic ("intrinsic") form. Subjects with atopic asthma usually have a positive family history, high IgE and positive skin tests for allergens. The intrinsic form is mainly seen in adolescence, in patients with a negative family history, and is often associated with nasal polyps and eosinophilia. In both forms of asthma an eosinophil-dominated inflammatory reaction of the bronchial tissue and T-lymphocyte activation is present.

Since it became possible to perform BAL and to take bronchial biopsies safely in subjects with asthma, it became clear that in the airway of patients with all grades of asthma inflammatory changes could be found. Asthma is now considered as a chronic inflammatory disease of the airway with variable airway obstruction and bronchial hyperresponsiveness, *i.e.* the increased sensitivity of the airways to a wide range of non-specific stimuli including exercise, tobacco smoke, fog and cold air. Bronchial hyperresponsiveness is related to the severity of the disease, the amount of inflammation in the airway wall and the need for therapy. The technique of BAL is outlined, and the problems we and other investigators had to cope with are discussed.

Despite initial concerns regarding risks of bronchospasm, laryngospasm or hypoxaemia, the technique of BAL and bronchial biopsy is now widely used in asthma research. The present knowledge of the possible events taking place in asthma is described, whereas the role of eosinophils, mast cells, lymphocytes, dendritic cells and macrophages are outlined. Finally, the metabolism and properties of arachidonic acid metabolites is described. The lung function measurements used in our studies, such as spirometry, histamine and methacholine provocation tests, and reversibility tests are briefly mentioned. The major mechanisms of action of inhaled glucocorticoids with respect to their inhibition of cellular activities of many cells, including eosinophils, mast cells, and macrophages are described, and that they inhibit the production of a

number of pro-inflammatory mediators such as histamine and various cytokines. The effectiveness in various clinical studies (in children and adults, long-term versus short-term treatment) is discussed. Finally the aims of the studies in this thesis are described.

In **chapter 2.1** a new mathematical model is presented, describing the shape of the methacholine dose-response (MDR) curves in normal individuals as well as in asthmatic patients. Often only the provocative concentration, producing a fall of 20% in FEV_1 (PC_{20}) is measured whereas the overall shape of the curve, also presenting a slope in the mid-part and a maximal response plateau, is ignored. Knowledge about the presence and level of a maximal response plateau may, however, provide extra information about the severity of airway obstruction and may therefore have therapeutical implications. The model facilitated extrapolation of the whole curve where it was impossible to obtain direct estimates of plateau values, and proved to be a good substitution for other models applied up until now.

In **chapter 2.2** the relation between airway inflammation in the bronchial mucosa and indices of the MDR curves in atopic asthmatics is investigated. A positive relationship was found between the number of activated eosinophils in the lamina propria and the plateau value. The number of activated eosinophils was also negatively correlated with the specific airway conductance (sGaw), representing airway luminal changes. These findings suggest that activated eosinophils and/or their products are at least involved in events in the airway wall, leading to excessive airway narrowing.

In **chapter 3.1** the effects of fluticasone propionate (FP) on MDR curves in non-smoking atopic asthmatics are studied. The findings, compared with results in the literature, show that FP is very effective in decreasing the maximal airway narrowing response and increasing PC_{20}. Since changes in EC_{50} (the provocative concentration at 50% of the plateau value) were not significantly different between the FP treated and the placebogroup, it is most likely that part of the increase in PC_{20} is related to the decrease of the plateau.

In **chapter 3.2** the results are shown of delayed introduction of ICS. The study was designed to investigate whether a 2.5 years delay of ICS administration led to smaller improvements in FEV_1 and airway hyperresponsiveness compared to immediate prescription in patients with mild to moderately severe obstructive airways disease. The data indicate that although delayed introduction of ICS in asthmatics leads to similar improvements in FEV_1, improvements in PC_{20} are significantly smaller. Several factors are hypothesized to be responsible for our findings such as ongoing airway wall inflammation, which is a major determinant in asthma.

In **chapter 4.1** we tested whether short term therapy (12 weeks) with FP not only affected arachidonic acid (AA) metabolites, but also influenced the position and shape of the MDR curves in nonsmoking atopic asthmatics. We also investigated the relationship between the various parameters studied. Although the findings indicate that FP strongly influences the position, the shape and also the maximum response plateau of the MDR curve, this effect is apparently not mainly achieved by its influence on the level of AA metabolites. We suggest that other pro-inflammatory factors are more important in affecting the shape of the MDR curve. It is suggested that these pro-inflammatory factors are downregulated by FP.

In **chapter 4.2** the influence of long term therapy (2.5 years) with beclomethasone dipropionate (BDP) on AA metabolites is studied in patients with asthma. The results suggest that ICS may exert their beneficial effect on lung function, at least partially, through inhibition of LTC_4 synthesis.

In **chapter 4.3** the cellular effects of 2.5 years of treatment with BDP and smoking on eicosanoid and lipocortin-1 (Lc-1) levels in BAL-fluid of atopic asthmatics are investigated. In contrast to smoking, which coincided with elevated Lc-1 levels, BDP had no significant effect on Lc-1 levels. Furthermore, in the non-steroid treated group, a negative correlation was found between Lc-1 levels and PGD_2, but a positive relation between Lc-1 levels and PC_{20}. In the BDP group no such correlations were seen. Of all the AA metabolites measured, we only found lower LTC_4 levels in the BDP group. This could be explained by the fact that ICS selectively suppressed the release of LTC_4. Furthermore, BDP treatment was associated with lower total protein and albumen levels without, as mentioned above, affecting Lc-1 levels. It is, therefore, suggested that ICS actively maintain Lc-1 levels, but suppress the expression of other proteins in BAL.

SAMENVATTING

Het onderzoek, beschreven in dit proefschrift, had als doel om de effecten van inhalatie-corticosteroïden (ICS) na te gaan op ontstekingsparameters bij astma. De combinatie van bronchoalveolaire lavage (BAL) en het nemen van biopten, en het verrichten van provocatietesten door middel van luchtwegvernauwende stoffen, maakte het mogelijk de onderlinge samenhang van de cytologische, histologische en fysiologische veranderingen bij astma te onderzoeken. We onderzochten eveneens of ontstekings-remmende therapie met ICS deze gebeurtenissen kon beïnvloeden.

In **hoofdstuk 1** wordt astma gedefinieerd als een vaak voorkomende ziekte, die wordt gekenmerkt door terugkomende episoden van kortademigheid, piepen en hoesten. Astma kan worden onderscheiden in allergisch ("extrinsiek") en niet-allergisch ("intrinsiek"). Personen met allergisch astma hebben gewoonlijk een positieve familie-anamnese, een hoog IgE en positieve huidtesten voor allergenen. De niet-allergische vorm komt vooral voor bij adolescenten, met negatieve familie-anamnese, en gaat vaak gepaard met neuspoliepen en eosinofilie. Bij beide vormen overheerst een eosinofiele ontstekingsreactie van het bronchiale weefsel en is er sprake van activatie van T lymfocyten.

Sinds het mogelijk bleek bij astmapatiënten veilig een BAL te verrichten en slijmvliesbiopten te nemen, werd duidelijk dat er bij astma, in welke graad dan ook, ontstekingsverschijnselen in de luchtwegen kunnen worden gevonden. Astma wordt nu als een chronisch ontstekingsproces van de luchtwegen beschouwd met een variabele luchtwegobstructie en luchtweghyperreactiviteit. Bronchiale hyperreactiviteit is de toegenomen prikkelbaarheid van de luchtwegen voor vele niet-specifieke prikkels zoals inspanning, rook, mist of koude lucht. Bronchiale hyperreactiviteit hangt samen met de ernst van de ziekte, de mate van ontsteking in de luchtwegwand en de therapiebehoefte.

De techniek van BAL met bronchusbiopten is algemeen geaccepteerd in de astmaresearch, ondanks de aanvankelijke bezorgdheid voor het risico op bronchospasme, laryngospasme of hypoxaemie. De techniek wordt ervan besproken, evenals de problemen waar wij en andere onderzoekers mee te kampen hadden betreffende de grootte en kwaliteit van de biopten. De huidige kennis over de mogelijke gebeurtenissen, zoals deze bij astma plaatsvinden, wordt vervolgens uiteengezet, terwijl de rol van eosinofielen, mestcellen, lymfocyten, dendritische cellen en macrofagen wordt

beschreven. Hierna worden het metabolisme en de eigenschappen van arachidonzuur (AA) metabolieten uiteengezet. In het kort worden de voor onze studies gebruikte longfunctie-onderzoeken genoemd, zoals de spirometrie, de histamine- en methacholine provocatie-testen en het meten van de reversibiliteit. De voornaamste werkingsmechanismen van ICS worden beschreven, zoals de remming van cellulaire activiteiten van verschillende cellen, eosinofielen, mestcellen en macrofagen inbegrepen. Ook komt de remming van de productie van een aantal pro-inflammatoire mediatoren zoals histamine en diverse cytokinen ter sprake. De effectiviteit van ICS bij diverse klinische studies zowel bij kinderen als volwassenen, gedurende kortere of langere tijd, wordt opgesomd. Tot slot worden de doelstellingen van de studies in dit proefschrift beschreven.

In **hoofdstuk 2.1** wordt een nieuw wiskundig model getoond, dat zowel bij normale proefpersonen als bij astmatici de vorm van de methacholine dosis-respons (MDR) curve beschrijft. Vaak wordt alleen de provocerende concentratie die een daling van 20% veroorzaakt in de geforceerde een-seconde waarde (PC_{20}) gemeten, en wordt niet naar de gehele curve gekeken, die ook nog een helling in het middengedeelte en een maximaal responsplateau beslaat. Kennis over de aanwezigheid en hoogte van een maximaal responsplateau kan echter aanvullende informatie geven over de ernst van de luchtwegobstructie en kan gevolgen hebben voor de therapie. Het model bood de mogelijkheid van extrapolatie wanneer we geen direct gemeten plateauwaarden konden verkrijgen, en bewees een goede vervanger van tot dusver gebruikte modellen te zijn.

In **hoofdstuk 2.2** wordt bij allergische astmapatiënten de relatie beschreven tussen ontsteking in de bronchiale mucosa en parameters van de MDR curve. Er werd een positieve relatie gevonden tussen het aantal geactiveerde eosinofielen in de lamina propria en de plateauwaarde. Bovendien bleek er een negatieve correlatie te bestaan tussen het aantal geactiveerde eosinofielen en de specifieke luchtwegconductantie (sGaw), een maat voor de verandering in luchtweglumen. Deze bevindingen suggereren dat geactiveerde eosinofielen en/of hun producten betrokken zijn bij gebeurtenissen in de luchtwegwand die leiden tot buitensporige luchtwegvernauwing.

In **hoofdstuk 3.1** worden de effecten van fluticasone-propionaat (FP) op MDR curven van niet-rokende allergische astmapatiënten nagegaan. Er wordt aangetoond dat FP, vergeleken met gegevens uit de literatuur, zeer effectief het maximale responsplateau verlaagt en de PC_{20} doet toenemen. Aangezien de veranderingen in EC_{50} (provocerende concentratie op 50% van de plateauwaarde) niet significant verschillen tussen de met FP en placebo behandelde groep, is het waarschijnlijk dat een

deel van de toename in PC_{20} gerelateerd is aan een afname van de plateauwaarde. In hoofdstuk 3.2 worden de resultaten getoond van vertraagde introductie van ICS. De studie beoogde te onderzoeken of uitstel van 2.5 jaar in de behandeling met ICS van patiënten met matig tot ernstig obstructief longlijden zou leiden tot geringere toename in FEV_1 en hyperreactiviteit dan wanneer meteen met de behandeling gestart werd. Er wordt aangetoond dat uitstel bij astmatici leidt tot minder verbetering in PC_{20}, terwijl de verbetering in FEV_1 identiek is. De hypothese is dat verschillende factoren hiervoor verantwoordelijk kunnen zijn, waaronder de bij astma zo bepalende chronische luchtwegontsteking.

In **hoofdstuk 4.1** wordt getest of kortdurende therapie (12 weken) met FP niet alleen AA metabolieten-spiegels verlaagt, maar ook de positie en de vorm van de MDR curve bij niet-rokende astmapatiënten beinvloedt. Tevens wordt de relatie tussen de diverse parameters onderzocht. Hoewel de resultaten erop duiden dat FP sterk de positie, de vorm en het maximale responsplateau beinvloedt, wordt dit effect blijkbaar niet hoofdzakelijk bereikt via de invloed op AA metabolieten. Wij veronderstellen dat andere pro-inflammatoire factoren een grotere invloed hebben op de vorm van de MDR curve.

In **hoofdstuk 4.2** wordt de invloed van langdurige therapie (2.5 jaar) met beclomethasone dipropionaat (BDP) bestudeerd op AA metabolieten bij patënten met astma. De resultaten veronderstellen dat ICS hun gunstige werking op de longfunctie, in ieder geval ten dele, uitoefenen door belemmering van de LTC_4 synthese.

In **hoofdstuk 4.3** worden de cellulaire effecten van 2.5 jaar therapie met BDP en roken onderzocht op AA metabolieten en lipocortine-1 (Lc-1) spiegels in lavagevloeistof van allergische astmapatiënten. Hoewel wij vonden dat roken gepaard ging met verhoogde lipocortinespiegels, bleek BDP geen significante invloed te hebben op het Lc-1 gehalte. Bovendien werd er in de niet met BDP-behandelde groep patiënten een negatieve correlatie gevonden tussen Lc-1 spiegels en PGD_2, maar een positieve relatie tussen Lc-1 en PC_{20}. In de met BDP-behandelde groep werden deze correlaties daarentegen niet gezien. Omdat wij alleen in de BDP groep verlaagde LTC_4-spiegels vonden, zou de verklaring hiervoor kunnen zijn dat CS selectief het vrijmaken van LTC_4 verhinderen. BDP-behandeling ging bovendien gepaard met lagere spiegels van totaal eiwit en albumen, zonder van invloed te zijn op de Lc-1 spiegels. De veronderstelling is dat ICS de Lc-1 spiegels actief hoog houden, maar de expressie van andere eiwitten in BAL onderdrukken.

Abbreviations

AA	arachidonic acid
AC	anticholinergic agent
AM	alveolar macrophage
ANOVA	analysis of variance
APAAP	alkaline phosphatase anti-alkaline phosphatase
BHR	bronchial hyperresponsiveness
BA	β_2-agonist
BAL(F)	bronchoalveolar lavage (fluid)
BDP	beclomethasone dipropionate
BM	basement membrane
BSA	bovine serum albumin
CD	cluster of differentation
CGD	Cumulative Gaussian Distribution
CI	confidence interval
CNSLD	chronic non-specific lung disease
COPD	chronic obstructive pulmonary disease
CS	corticosteroids
dc	doubling concentration
Dc	dendritic cells
dd	doubling dose
EAR	early asthmatic response
EC_{50}	provocative concentration at 50% of the plateau response
ECP	eosinophilic cationic protein
EDN	eosinophil derived neurotoxin
e.g.	exempli gratia = for example
ELISA	enzyme linked immuno assay
EPO	eosinophil peroxidase
f	female
FEV_1	forced expiratory volume in one second
FP	fluticasone propionate
FVC	forced vital capacity
GM-CSF	granulocyte macrophage-colony stimulating factor
H	histamine
HETE	5-hydroxy-eicosa-tetranoic acid
ICS	inhaled corticosteroids

i.e.	id est
IFN	interferon
Ig	immunoglobulin
IL	interleukin
i.m.	intramuscularly
IVC	inspiratory vital capacity
LAR	late asthmatic response
Lc-1	lipocortin-1
LPS	lipopolysaccharide
LT	leukotriene
log	logarithm
m	male
M	methacholine
MBP	major basic protein
MCP	monocyte-chemotactic protein
MDR	methacholine dose-response
MEFV-curve	maximal expiratory flow volume-curve
$MEF_{50\%}$	expiratory flow at 50% of the actual forced vital capacity
MIP	macrophage inflammatory protein
mRNA	messenger RNA
n	number
n.d.	not determined
n.s.	non significant
P	plateau
PAF	platelet activating factor
PBS	phosphate-buffered saline
PC_{20}	provocative concentration (of bronchoconstricting agent) causing a 20% fall in FEV_1 = sensitivity
PD_{20}	provocative dose (of bronchoconstricting agent) causing a 20% fall in FEV_1
PEF(R)	peak expiratory flow (rate)
PG	prostaglandin
PL	placebo
PL-A	phospholipase-A
pred	predicted
q.i.d.	quater in die= four times daily
R	reactivity

r	linear correlation coefficient
R^2	coefficient of determination
RANTES	regulated upon activation, normal T expressed, and presumably secreted
reactivity	% change from baseline FEV_1 per doubling dose at the 50% point of the CGD function
Reff	effective resistance
rho = r_s	Spearman's non-parametric correlation coefficient
RIA	radio immuno assay
RNA	ribonucleic acid
RSD	residual standard deviations
RV	residual volume
S	sensitivity
SD	standard deviation
SEM	standard error of the mean
sGaw	specific airway conductance
SGO	stimuleringsprogramma gezondheidsonderzoek (health research promotion program)
Th	T helper lymphocyte
TLC	total lung capacity
TNF	tumor necrosis factor
Tx	thromboxane
VC	vital capacity
vs	versus
w/v	weight/volume

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Curriculum vitae

Shelley Eldred Overbeek werd op 16 juli 1950 geboren te Enschede. Het diploma Gymnasium ß behaalde zij in 1970 aan het Praedinius Gymnasium te Groningen. In dat jaar begon zij de studie geneeskunde aan de Vrije Universiteit te Amsterdam. In 1975 slaagde zij voor het doctoraal examen en in 1977 voor het arts-examen. Van december 1977 tot april 1980 was zij werkzaam als arts-assistente Interne Geneeskunde in de Maria Stichting te Haarlem. Vanuit dit ziekenhuis deed zij haar eerste onderzoekservaring op onder leiding van Professor Ph.H. Quanjer in het laboratorium fysiologie van de Universiteit te Leiden. Van april 1980 tot april 1983 werd de opleiding tot longarts voltooid op de afdeling longziekten van het Academisch Ziekenhuis Rotterdam-Dijkzigt onder supervisie van Professor C. Hilvering. Sedertdien is zij werkzaam als staflid op deze afdeling. Haar belangstelling voor klinisch onderzoek kwam vooral tot uiting in het landelijke SGO-CARA onderzoek, waarbij zij namens Rotterdam Dijkzigt de volwassen patiënten recruteerde en jarenlang vervolgde. Tijdens dit multi-centrum onderzoek werd de basis gelegd voor een aantal van de artikelen uit dit proefschrift.

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