Bronchial Hyperresponsiveness in Chronic Obstructive Pulmonary Disease

Functional and inflammatory characteristics

Effects of treatment with inhaled fluticasone propionate

Bronchiale hyperreactiviteit bij patiënten met een chronisch obstructieve longaandoening (COPD)

Functionele en inflammatoire kenmerken

Effecten van behandeling met fluticasone propionaat per inhalatie

Bronchial Hyperresponsiveness in Chronic Obstructive Pulmonary Disease

Functional and inflammatory characteristics

Effects of treatment with inhaled fluticasone propionate

Bronchiale hyperreactiviteit bij patiënten met een chronisch obstructieve longaandoening (COPD)

Functionele en inflammatoire kenmerken

Effecten van behandeling met fluticasone propionaat per inhalatie

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam
op gezag van de Rector Magnificus
Prof.dr.ir. J.H.van Bemmel
en volgens besluit van het College voor Promoties

De openbare verdediging zal plaatsvinden op
woensdag 16 mei 2001 om 15.45 uur

door

Gerrit Teunis Verhoeven
geboren te Rijswijk (NBr)

PROMOTIECOMMISSIE

Promotoren: Prof.dr. H.C. Hoogsteden
Prof.dr. J.M. Bogaard

Overige leden: Prof.dr. H.M. Jansen
Prof.dr. J.C. de Jongste
Prof.dr. Th.H. van der Kwast

Dit proefschrift is tot stand gekomen binnen de afdelingen Longziekten en Intensive Care, Immunologie, Farmacologie, Biochemie en Biostatistiek van de Erasmus Universiteit, Rotterdam.

Het onderzoek werd mede mogelijk gemaakt door financiële bijdragen van het Nederlands Astma Fonds en GlaxoWellcomeKline B.V., Nederland. Het drukken van dit proefschrift werd bekostigd door GlaxoWellcomeKline B.V., Nederland.

Het proefschrift werd gedrukt door Ridderprint B.V. te Ridderkerk.

Aan Dicky,
Jan-Willem,
Anne-Martine

CONTENTS

Chapters

Chapter 1

Introduction and aim of this thesis

In recent years the disease entity of COPD has become accepted widely. One of the major steps forward has been the publication by the American and the European Societies of their statements on the diagnosis and management of COPD in 1995 (1,2). This was accompanied or followed by national standards (*eg,* British Thoracic Society) and recommendations or guidelines for specific groups of health-care workers, for instance the family doctors 'NHG-standaard COPD: behandeling' (3). Although differences exist with regard to the management of patients with COPD, the definitions of COPD have become quite uniform. Initially, COPD was considered as composed of two diseases, which often occurred concomitantly: chronic bronchitis and emphysema. Chronic bronchitis is defined as "the presence of chronic or recurrent increases in bronchial secretions sufficient to cause expectoration. The secretions are present on most days for a minimum of 3 months a year, for at least two successive years and cannot be attributed to other pulmonary or cardiac causes". Emphysema is defined not by clinical characteristics, but anatomically by "a permanent, destructive enlargement of airspaces distal to the terminal bronchioles, without obvious fibrosis". Nowadays, a more comprehensive definition has gained popularity: COPD is defined as "a disorder characterized by reduced expiratory flow and slow forced emptying of the lungs, which does not change markedly in time". This definition also reminds of the classical work of Fletcher and Peto, who found that FEV_1 gradually falls over lifetime and clinically significant airflow obstruction occurs in a small group of susceptible smokers (4,5). In daily practice, for establishing the diagnosis of COPD, the physician combines the results from the patient's history, physical examination, chest X-ray and lung function data. If necessary, additional investigations, such as allergy tests and high resolution computed tomography (HRCT) scan are completed. Still, it may be difficult in some cases to differentiate COPD from asthma. This is particularly true for older patients with more or less stable expiratory flow limitation and significant increases of their FEV_1 after inhalation of a beta-adrenergic or an anticholinergic drug. Terms like "chronic asthma", "chronic asthmatic bronchitis" and "asthma with persistent airflow limitation" have been applied for categorizing these patients. A popular way of clarifying the interface of asthma and chronic bronchitis/emphysema is a picture with three overlapping circles. Nowadays, there is a tendency to depict the relation of asthma and COPD as a flat ellipse with on the one side COPD and on the other asthma. On the one side there is "classical COPD" interpreted as a disease with airflow limitation that is (in a stable state) impossible to be influenced by any kind of intervention, and on the other side, asthma is depicted as a disorder with (fully) reversible airways obstruction.

In this introduction three items are discussed which are important for the intervention study that we will describe in this thesis: COPD and bronchial hyperresponsiveness, inflammation in COPD, and glucocorticoid therapy in COPD.

COPD and bronchial hyperresponsiveness

Excessive airways narrowing by allergens, pharmacological agents and physical stimuli is most extensively investigated in asthma. Those patients who have experienced this phenomenon tend to avoid these stimuli, of which cigarette smoke is the most notorious. Not only subjects with asthma but also COPD patients, even those who have been categorized as emphysematous, have this health problem.
Provocative airway narrowing by means of inhalation of a variety of substances has been applied extensively, both for purposes of research and patient care. In 1921, Alexander and Paddock noted that subcutaneous injections of pilocarpine produced asthmatic attacks in patients with asthma (6). In 1946, Curry presented a study in which the effects of histamine on vital capacity were recorded after nebulization (7). In nine patients with 'bronchitis, emphysema and asthma' bronchoconstriction was noticed, while there was no effect on vital capacity in normal subjects and in patients with a history of allergy. Responses to inhaled pharmacological stimuli like histamine and methacholine appeared to be highly reproducible (8).
Later on, the FEV_1 was accepted as the standard outcome measure. PC_{20} is the provocative concentration that causes a 20% fall of the FEV_1, and a PC_{20} below or at a concentration of 8 mg/ml histamine or methacholine in a two-minute provocation test is considered abnormal: bronchial hyperresponsiveness (BHR) (9, 10). The establishment of a low PC_{20} is considered to be a confirmation of the diagnosis of asthma. From epidemiologic studies it appears that the positive and negative predictive values of the PC_{20} in the absence of respiratory symptoms are low (11, 12). A PC_{20} below 2 mg/ml is a strong predictor for the diagnosis of asthma, but was also present in "chronic bronchitis" and "respiratory symptoms"(11).
The highest chance of finding BHR was in patients with upper or lower respiratory tract symptoms (12-14). BHR was found in approximately 20% of apparently normal (non-asthmatic) children (15).
Orie and co-workers proposed in 1961 that BHR is a genetically determined risk factor for the development of COPD (16). This 'Dutch hypothesis' has been a subject of debate for many years (17).
There is some evidence that BHR is related to smoking (18-20). However, this relationship is complex (20-22).
With regard to COPD it was estimated in one study that approximately half of the subjects with COPD in a general population have BHR (23). In the Lung Health Study, BHR was noted in 85.1% of the women and 58.9% of the men with mild to moderate airflow limitation and cigarette smoking (21). Clinical studies suggest that BHR in COPD differs from BHR in asthma (24-26). In COPD patients with hyperresponsiveness for pharmacological agents (e.g. histamine), bronchoconstriction

could usually not be provocated by physiologic stimuli (*eg*, cold air), whereas in asthma both types of stimuli cause bronchoconstriction (25). Increased diurnal variation of peak expiratory flow is related to BHR in patients with asthma, but this relation is less clear in patients with chronic bronchitis and airflow obstruction (27). Contrary to patients with asthma, smokers with mild airflow limitation are significantly less responsive to methacholine than to equimolar doses of histamine (28). Usually the degree of BHR, with respect to both PC_{20} and maximal bronchoconstriction, is less in chronic bronchitis as compared to asthma (28,29).
In COPD, contrary to mild asthma, there is a relationship between baseline FEV_1 and the level of PC_{20} (13,23,24,26,30-33). In accordance with this, the change of PC_{20} in smokers over a period of four years was related to the change of FEV_1 (32). The presence of obstructed airways leading to a low FEV_1 makes a provocative fall in FEV_1 more likely to occur (34). This provides a mathematical problem. This has not yet been investigated in detail.
The explanation for the differences between asthma and COPD with regard to BHR might be found in the different pathologic changes in the airways and in the lung parenchyma of asthma and COPD patients (35). This subject will be discussed in more detail in the next paragraph. The main and most clear functional difference is based on the destruction of lung parenchyma in COPD, leading to emphysema and loss of lung elasticity (36). Decrease of parenchymal elastic recoil pressure is one of the mechanisms leading to enhanced bronchoconstriction (table 1) (37,38). In one study, in patients with α -1-antitrypsine deficiency, it was demonstrated that elastic recoil indeed influenced maximal airways narrowing (39).
Otherwise, it is possible that the presence of BHR in COPD is a reflection of a different inflammatory infiltrate. In asthma, BHR has been associated with eosinophil cell infiltrates of bronchial walls (41, 42). Patients with COPD and BHR could have eosinophilic airways inflammation and therefore constitute "hidden asthmatics". Studies concerning the inflammatory characteristics of BHR in patients with COPD will be discussed in the next paragraph.
In a general population, smoking in combination with respiratory symptoms constitutes a worse prognosis (43,44). Several epidemiologic studies have shown that patients with COPD and BHR (particularly those who continue smoking) are prone to an even more accelerated decline of their FEV_1 as compared to those without (45-51). This could indicate a more severe or a different inflammatory infiltrate and/or higher levels of markers of inflammation.

The presence of BHR in patients with COPD predicts a worse prognosis, especially if these patients continue smoking. Cessation of smoking is important as an attempt to improve the prognosis in COPD, but also for reducing symptoms and reducing the other risks of health associated with smoking. However, whether BHR in COPD can be influenced by either smoking cessation or therapy, and thereby improve the prognosis, is uncertain. In parallel with the findings in asthma it is tempting to speculate that anti-inflammatory therapy can modulate BHR and subsequently improve life expectancy of patients with COPD. The need for intervention studies

addressing this idea was proposed for instance by O'Connor et al. (48). Data from earlier intervention studies concerning BHR in COPD will be discussed later on.

Table 1.
Mechanisms that are potentially involved in bronchial hyperresponsiveness (adapted from 38 and 40):

A: amplified stimulation by bronchoconstrictor stimuli, "pre-synaptic mechanisms", leading to a leftward shift of the dose-response curve:

1. defects of epithelium: malfunction, epithelial shedding, open tight junctions
2. inflammation: increased number of cells, increased cell cytokine/mediator release
3. neural control: cholinergic activity, excitatory nonadrenergic noncholinergic
4. reduced breakdown or removal of stimuli

B: amplified bronchoconstrictor response, "post-synaptic mechanisms", leading to an increased plateau value of the dose-response curve:

1. increased smooth muscle contractility and/or hyperplasia/ hypertrophy
2. swelling of the submucosal and/or adventitial layer
3. increased collagen in the subepithelial reticular layer
4. reduced mucosal folding
5. intraluminal exudates and secretion
6. decreased parenchymal elastic recoil pressure (reduced alveolar attachments)
7. uncoupling of forces of interdependence between parenchyma and airway wall

Inflammation in COPD

Initially it was believed that obstuction of the small airways by mucus caused a mechanical damage to the alveoli resulting in enlarged airspaces (52). Mucus hypersecretion is related to both an increased number of glands in the bronchial wall and in the epithelial layer, and to an increased secretory activity. Additionally, the composition of mucus is abnormal (53). Mechanisms involved in mucus hypersecretion are autonomic nervous system imbalance, local inflammation and exogenous stimuli from cigarette smoke.
Cigarette smoking is by far the most important factor in the pathogenesis of COPD (4,5,54,55). Smoking induces many changes of inflammatory cell number and function (56-62). Smokers with airflow obstruction have higher numbers of cells in

their BAL fluid as compared to smokers without airflow obstruction (63). Interestingly, there are several studies, which were unable to demonstrate significant differences with regard to inflammation between patients with COPD who continue smoking and patients with COPD who stopped smoking (64-66).

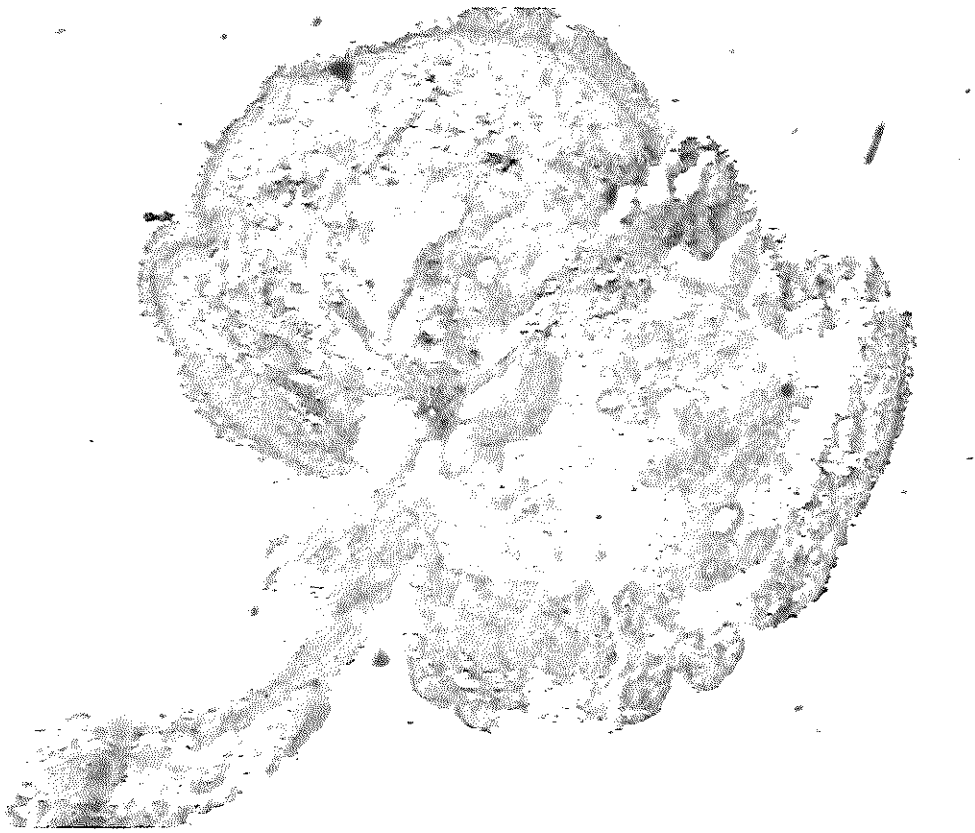


Figure 1. Biopsy from one of the patients with COPD and BHR described in this thesis. Epithelium is intact at the top of the specimen. In the submucosal layer, at the right, mucous secreting glands are visible. Preparation of the biopsy specimen is described in Chapter 4. Picture taken by Joost Hegmans.

The oldest observations on pathologic changes in chronic bronchitis showed hypertrophy of **submucosal glands** and an increased number of **Goblet cells** (68-73). The volume of expectorated sputum correlates with the proportion of mucous glands (74). The number of serous acini of the submucosal glands is reduced, unlike in to asthma (68). Increased numbers of monocytes were found in the mucosa of airways with a diameter greater than 2 millimeter and in surrounding glands and gland ducts of bronchi larger than 4 millimeter, indicating markedly inflamed

airways (75). Neutrophil and mast cell proteases could be involved in the pathogenesis of mucous secretion (76).
In young smokers there is an increased number of intraluminal **macrophages** in the respiratory bronchioles (77). In BAL fluid the number of inflammatory cells is raised five- to tenfold, especially alveolar macrophages and neutrophilic granulocytes (78-80). Functional characteristics of cells from smokers are different. Macrophages from smoking COPD patients released higher levels of elastase (81,82) or had higher elastinolytic activity (83).

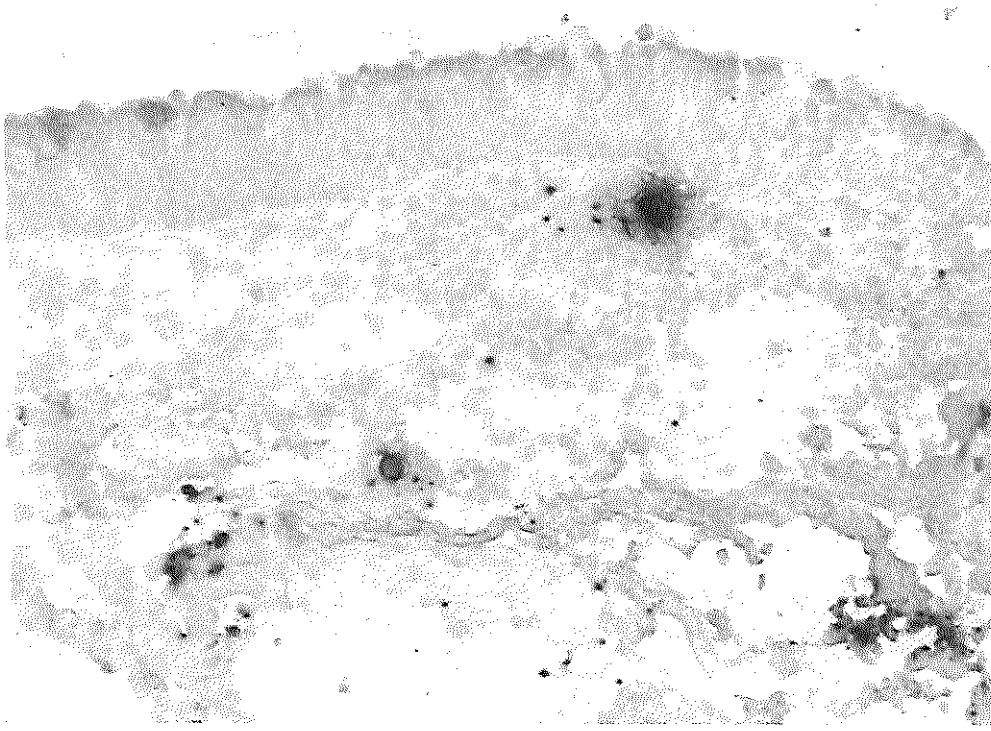


Figure 2. The positively staining cells (in red) in the submucosal layer express BMK-13 (eosinophils). Picture taken by Joost Hegmans.

A large number of **neutrophils** was found in the lungs of patients with COPD who smoke (84), especially during COPD exacerbation (85). Neutrophils in BAL fluid are found predominantly in the first lavage aliquot, suggesting their presence in airways rather than in the alveolar compartment (78,79). Also in induced sputum the percentage of neutrophils and the levels of their activation markers are increased (86). Even in patients with COPD who have never smoked the percentage of neutrophils in BAL was increased (87). In the lamina propria increased numbers of

neutrophils, monocytes/macrophages as well as plasma cells and sporadically eosinophils were found (68,71). In biopsies taken from lobar bronchi significant increases were reported in the numbers of macrophages, total leukocytes (CD45+), T lymphocytes (CD3+) and activated T-cells (CD25+ and VLA-1+) (88). In another biopsy study there was an increased expression of E-selectin on vessels and of ICAM-1 on basal epithelial cells (89). Levels of circulating ICAM-1 and E-selectin were also increased in serum and BAL fluid of patients with COPD (90). These adhesion molecules are involved in neutrophil recruitment. The expression of several adhesion molecules appears to be influenced rather by (heavy) smoking than by the disease process of COPD (91).
The function of circulating neutrophils is different in patients with COPD as compared to normals: for instance, the number of formyl-peptide receptors expressed on their surface is significantly increased (92).

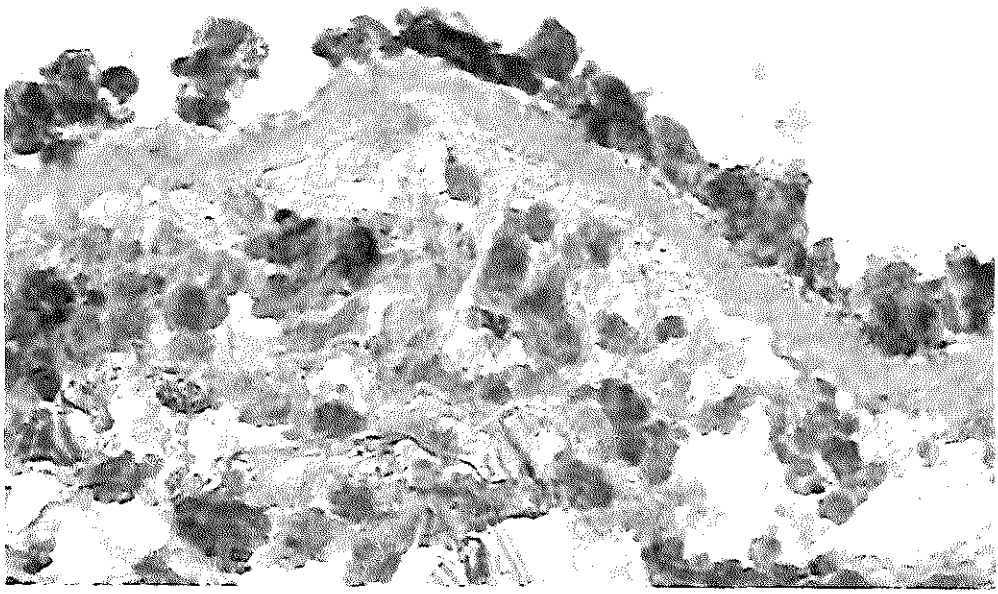


Figure 3. Above the thickened basement membrane the epithelium has almost completely disappeared, due to epithelial shedding and/or mechanical damage. Picture taken by Joost Hegmans.

The central airways of smokers with chronic bronchitis have a higher number of **lymphocytes** (93,94). In the submucosa, greater than normal lymphocyte numbers were counted (95). Increased numbers of B lymphocytes were present in the adventitia of smokers, with a positive correlation with the number of smoked cigarettes (96). T lymphocytes are present in both the epithelial and the subepithelial layer, and the predominant type is the CD8+ (cytotoxic/suppressor) lymphocyte (97). CD8+ T-cells are also more abundant in the lung parenchyma and in pulmonary arteries (98). In BAL fluid the CD4/CD8 ratio was lower in smokers with or without chronic bronchitis, compared with healthy individuals (58). The number of CD8+ T-cells is negatively correlated with FEV_1, and positively correlated with mucus-secreting cells (97-99). Furthermore, the number of T lymphocytes has a negative correlation with the degree of alveolar destruction (100). Circulating CD4+ T-lymphocytes in patients with COPD produce predominantly TNFα and less Interleukin-4, pointing to a T_H1-like immune response (101).

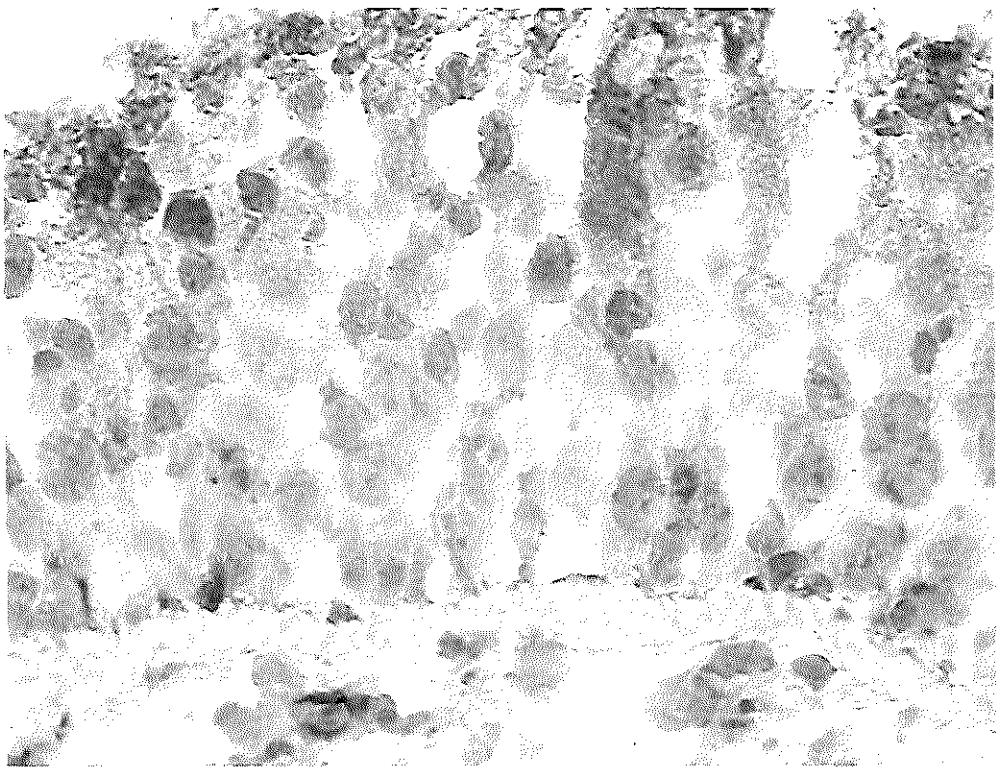


Figure 4. A detailed view showing metaplasia and dysplasia of the epithelial layer in a bronchial biopsy of a patient with COPD and BHR. Staining is positive (red) for CD8+ T-lymphocytes. Picture taken by Joost Hegmans.

Eosinophilic granulocytes are present in slightly increased numbers, and do not seem to degranulate (80,102). Their numbers increase markedly during an exacerbation (103,104). The presence of eosinophilia seems to be an early sign of larger declines of lung function as compared to those chronic bronchitis patients without eosinophilia (55).

Several studies indicate that **mast cells** are involved in COPD as their number is increased (95,105-107). Perhaps the higher proportion of mast cells in airway epithelium is related to smoking (108).

An increased total amount of airway **smooth muscle** was noted in small bronchi and bronchioli of COPD patients (109,110). In the small airways (< 3 mm diameter), there may be, next to smooth muscle hypertrophy, mural edema, peribronchiolar fibrosis, peribronchiolar inflammation and loss of alveolar attachments (77,110-115). These peripheral airways are the major site of airflow resistance in patients with obstructive airways disease (116,117). The airflow limitation in small airways seems to be caused to a larger extent by airway pathology than by decreased elastic recoil or a check-valve in the small airways (118-122). Surprisingly, there is at least one study showing a relation between the severity of airway inflammation in biopsies from large bronchi and the severity of airflow limitation (123).
It should be noted that airflow is also limited by intraluminal material such as mucus, inflammatory cells and shedded epithelial cells.

The inflammatory infiltrate in the bronchioli and in the alveoli consists mainly of pigmented macrophages, neutrophils, and CD8+ T-lymphocytes (77,110,111,115, 124). These inflammatory changes lead to expiratory airflow limitation and disturbance of the protease-antiprotease balance. Next to a possible direct cytotoxic activity of T cells, secretory products from inflammatory cells (*eg*, neutrophil elastase) and an excessive oxidant burden, all of which contribute to the protease-antiprotease imbalance with subsequent destruction of alveolar walls (125-127).

The **immunopathology of BHR** in COPD has not been studied as extensively as in asthma. Several studies in asthma have shown a correlation between severity of BHR and inflammatory changes in the bronchial walls (128,129). In asthma the most prominent inflammatory cell types are eosinophils, lymphocytes and mast cells (41,42,68,95,128). Especially eosinophilic inflammation seems to be involved in the pathogenesis of BHR in asthma (130,131). In patients with chronic bronchitis, the level of airways hyperresponsiveness was not correlated with blood or sputum eosinophilia (131). In one study of patients with COPD undergoing lung surgery, pre-operative PC_{20} was correlated with the severity of inflammation in bronchioli and not with changes in airways containing cartilage (75). In the same study, PC_{20} was correlated with cigarette consumption (pack years).

In COPD, the most prominent cell types are monocytes/macrophages, neutrophils and CD8+ lymphocytes. If BHR in COPD, as in asthma, is related to inflammation, than BHR can be associated with different inflammatory processes. It is also possible that we will find yet a different inflammatory profile in well-defined patients with COPD and BHR.
Another potential causative mechanism of BHR is epithelial shedding. In asthma the surface epithelial layer of the bronchi is fragile and parts have been lost (133). The extent of such loss shows negative correlation with BHR (134-136). Loss of epithelial cells occurs also in patients with COPD (72,95). One study describes the mucosal permeability of aerosol radioactive particles in smokers and in nonsmokers (137). Mucosal permeability was increased in smokers, but there was no relation to BHR. Taylor et al. investigated airway responsiveness in another way. They tested bronchial smooth muscle strips from surgical specimens of patients with COPD and a wide range of PC_{20} values, and were unable to find correlations with several contractile or relaxation mechanisms (138). Their conclusion was that *in vivo* airway responsiveness was not solely due to changes of *in vitro* smooth muscle function.

In summary, the pathologic and immunologic changes of COPD are an increase of mucus secreting cells, loss of alveolar walls, and the presence of increased numbers of neutrophils, monocytes/macrophages and CD8+ lymphocytes, with altered cell properties (139). The pathologic basis of airflow limitation consists of reduced airway lumen due to inflammation of small airway walls, increased collapsibility of small airways due to loss of alveolar attachments, and the presence of intraluminal secretions (140). The presence of BHR in COPD may be due to an increased severity of inflammation, to a more asthma-like inflammatory profile or to defects of the epithelial layer.

Glucocorticoid therapy in COPD

There is a large discrepancy between daily practice and guidelines with regard to glucocorticoid therapy in COPD. In most guidelines the institution of glucocorticoid therapy has been discouraged. In those guidelines in which inhaled glucocorticoids are not rejected, the importance of a thorough evaluation of a trial with oral or inhaled glucocorticoids is emphasized.
The percentage of patients with COPD enrolled in bronchodilator studies being treated with inhaled glucocorticoids has grown from 13.2% in 1987 to 41.4% in 1995 (141). In an American study the percentages of patients with unstable and stable COPD admitted to hospital who had a current prescription of inhaled glucocorticoids were 48 and 26, respectively (142). In the Netherlands, the number of COPD patients receiving inhaled glucocorticoid therapy is approximately 70%. Apparently clinicians are inclined to a "benefit of the doubt" strategy when being confronted with patients who experience therapy-resistant symptoms and limitations. Certainly, the successes of inhaled glucocorticoids in asthma, together

with negligible side effects, may contribute to this way of prescribing. It is also possible that "asthmatic features" are more frequent in patients with COPD than generally assumed. It is somewhat surprising that patients with COPD adhere to therapy that provides little or no subjective improvement in the short term. This is especially true if one takes into account that the compliance to inhaled therapy is low.

Perhaps the earliest study on glucocorticoid effects in COPD was published in 1978 by Shim et al (143). They found that oral prednisone caused improvement of FEV_1 in 7 out of 24 patients with chronic bronchitis. Remarkably, these seven patients showed eosinophils in their sputum. The same author reported in a later study that effects of oral steroids could not be reproduced by inhaled beclomethasone (144). Mendella et al reported that 8 out of 46 patients with COPD responded to a short course of prednisolone (145). The response to prednisolone was present in those patients who also responded significantly to a bronchodilator, suggesting that these patients had asthmatic features (145). In one other study, eight of 29 patients with COPD had an improved FEV_1 and FVC after oral steroid treatment (146). The amount of steroid responsive patients with COPD was, however, inversely related to FEV_1 suggesting that patient selection markedly influenced the results (147). Oral prednisolone did not improve exercise performance of patients with COPD (148). One study showed an increase of the protease inhibitor α-1-antichymotrypsin in sputum after oral steroid treatment (149).
The earliest studies on the effect of glucocorticoids on FEV_1 decline showed that prednisolone at doses above 7.5 mg/day could slow down the progression of the disease (150,151). A meta-analysis showed that a minority of patients with stable COPD benefit slightly from oral corticosteroid treatment (152). Three separate studies have shown that a beneficial effect of an oral glucocorticoid might occur in a subset of patients with COPD and with features of asthma, particularly larger numbers of eosinophils and higher levels of eosinophil-derived cationic protein in BAL fluid or the presence of sputum eosinophilia (153-155).
There are several short-term studies with inhaled glucocorticoids, looking at different parameters of treatment effect. These are summarized in tables 2 and 3. Overall, these results give the impression that inhaled glucocorticoids have at least some effect, due to their ability to modulate the inflammatory process in COPD.

Recently, the results of four large multicenter trials have been reported. The aim of these studies was to examine whether inhaled glucocorticoids could reduce, in patients with COPD, the FEV_1 decline in the long term and thereby improve prognosis. The EUROSCOP trial showed that the use of inhaled budesonide in patients with mild COPD gave a small improvement of the FEV_1 in the first three months, but the long-term decline was not influenced (171). The Copenhagen study was unable to show clinical benefit from inhaled budesonide treatment in patients with mild COPD (172). Results from the ISOLDE study indicate that patients with COPD and moderate or severe airflow obstruction benefit from high-dose inhaled

fluticasone, mainly because of a decreased frequency of exacerbations (173). Also, quality of life scores showed a significant beneficial effect of inhaled fluticasone in these patients with COPD (173).

Table 2:
Summary of placebo-controlled trials with inhaled glucocorticoids in COPD.

Study/first author (reference number) [number of patients]*	*Parameter*	*Beneficial effect*	*No effect*
Engel (156) [8 vs 10]	Symptom scores Ventilatory capacity PC_{20} histamine		✔ ✔ ✔
Weir (157) [34 vs 35]	FEV_1, FVC, PEF	✔	
Auffarth (158) [12 vs 12]	Dyspnoea Other symptom scores Spirometry, PC_{20}	✔	✔
Thompson (159) [20 vs 10]	FVC, FEF_{25-75} FEV_1 BAL cells (bronchial sample) Lactoferrin, lysozyme	✔ ✔ ✔	 ✔
Llewellyn-Jones (160) [8 vs 8]	Sputum: chemotactic activity Sputum: elastase inhibitory activity Superoxide generation	✔ ✔	 ✔
Renkema (161) [21 vs 18]	Drop outs Symptoms FEV_1 decline	✔ ✔	 ✔
Bourbeau (162) [39 vs 40]	FEV_1, PEF, exercise capacity, dyspnoea, Quality of Life, respiratory symptoms		✔ ✔ ✔
Confalonieri (163) [17 vs 17]	Sputum cells Spirometry	✔	 ✔
Van Grunsven (164) [144 vs 88]	FEV_1 decline Exacerbation rate	✔	 ✔

* number of actively treated patients versus placebo

If patients with COPD were included in the ISOLDE study and their inhaled glucocorticoids were discontinued, 38% experienced an exacerbation, whereas in those without previous inhaled glucocorticoid therapy, there was an exacerbation rate of 6% (174). The Lung Health Study on inhaled triamcinolone in patients with mild-to-moderate COPD showed no effect on FEV_1 decline and on quality of life scores (175). Beneficial effects of triamcinolone inhalation were improvement of airway reactivity, less dyspnea and a reduction of unscheduled medical respiratory care.

Table 3:
Summary of crossover and other trials with inhaled glucocorticoids in COPD

Study/first author (reference number) [number of patients]	*Parameter*	*Beneficial effect*	*No effect*
Watson (165) [14, crossover]	FEV_1, VC, PC_{20}		✔
Weiner (166) [30, crossover]	FEV_1	✔ (25%)	✔ (75%)
Culpitt (167) [13, crossover]	Sputum cells Sputum proteases		✔ ✔
Llewellyn-Jones (168) [8, *ex vivo* tests]	Neutrophil functions Superoxide production	✔	 ✔
Keatings (169) [13]	Lung function Symptom scores Cells and cell activation markers		✔ ✔ ✔
Balbi (170) [8]	IL-8, MPO, cells in BAL Symptoms, bronchitis index	✔ ✔	

One of the conclusions that can be drawn from the results of these four studies is that, overall, patients with COPD do not seem to benefit from inhaled glucocorticoid therapy, but there is a strong indication that patients with a worse FEV_1 experience a beneficial effect. Remarkably, the findings of the inhaled glucocorticoid studies are similar to the older oral glucocorticoid trials. In these trials there was also a higher chance of a beneficial effect in those patients with a worse lung function (147). The multicentre trial of Paggiaro et al demonstrated significant beneficial effects of inhaled fluticasone with regard to a reduction of

moderate or severe exacerbations, with regard to several functional parameters as well as cough and sputum volume (176).

Analysis of these studies leads to the conclusion that there are at least some patients with COPD for whom inhaled glucocorticoid therapy is useful. It is not yet possible to specify with certainty which subgroups of COPD patients should be prescribed inhaled glucocorticoids. It may be that those patients with "asthmatic characteristics" and those with a low FEV_1 benefit from inhaled glucocorticoid therapy.

Aim of this thesis

We intended to study in detail a well-defined group of patients with COPD, those with bronchial hyperresponsiveness. They attracted our attention for two reasons. First was the fact that these patients with COPD had an even more accelerated decline of their FEV_1, and that might offer the opportunity to detect an effect of an anti-inflammatory therapy on lung function parameters. Those patients with COPD who had a steep decline of their FEV_1 showed indeed an improvement of lung function after inhaled glucocorticoid therapy (177). From a Dutch multicenter trial it appeared that one of the parameters predicting a beneficial effect of an inhaled glucocorticoid was bronchial hyperresponsiveness: the lower the PC_{20}, the greater the chance of improvement (178,179). The second reason for studying BHR in COPD is that this phenomenon has not been studied in detail before. Whereas BHR in asthma improves after glucocorticoid therapy (180, 181), most studies in COPD indicate that PC_{20} was not influenced by anti-inflammatory therapy (156,158,165). One of our approaches involves taking into account not only the PC_{20}, as well as also other parameters from a dose-response curve (182), which could well be be influenced by anti-inflammatory therapy. Furthermore, we wanted to investigate in detail the inflammatory processes in this particular group of patients, before and after anti-inflammatory therapy, in order to gain insight in the pathogenetic mechanisms underlying this disease. Therefore, we examined cell numbers in bronchial biopsies, in bronchoalveolar lavage fluid and in blood, and also determined concentrations of mediators of inflammation, and the capability of cells to produce reactive oxygen species.

References

1. Standards for the diagnosis and care of patients with chronic obstructive pulmonary disease (ATS Statement). Am J Respir Crit Care Med 1995; 152: S77-S120.
2. Siafakas NM, Vermeire P, Pride NB, et al., on behalf of the Task Force. Optimal assessment and management of chronic obstructive pulmonary disease (COPD). Eur Respir J 1995; 8: 1398-1420.
3. Geijer RMM, van Schayck CP, van Weel C, et al. NHG-standaard COPD: behandeling. Huisarts Wet 1997; 40: 430-442.

4. Fletcher C, Peto R, Tinker C, Speizer FE. The natural history of chronic bronchitis and emphysema. Oxford University Press, Oxford,1976.
5. Fletcher C, Peto R. The natural history of chronic airflow obstruction. Br Med J 1977; 1: 1645-1648.
6. Alexander HL, Paddock R. Bronchial asthma: response to pilocarpine and epinephrine. Archiv Intern Med 1921; 27: 184.
7. Curry JJ. The action of histamine on the respiratory tract in normal and asthmatic subjects. J Clin Invest 1946; 25: 785-791.
8. Juniper EF, Frith PA, Dunnett C, et al. Reproducibility and comparison of responses to inhaled histamine and methacholine. Thorax 1978; 33: 705-710.
9. Cropp GJA, Berstein IL, Boushey HA, et al. Guidelines for bronchial inhalation challenges with pharmacologic and antigenic agents. ATS News, Spring 1980, 11-19.
10. Sterk PJ, Fabbri LM, Quanjer PhH, Cockroft DW, O'Byrne PM, Anderson SD, Juniper EF, Malo JL. Airway responsiveness. Standardized challenge testing with pharmacological, physical and sensitizing stimuli in adults. Eur Respir J 1993; 6(suppl 16):53-83.
11. Enarson DA, Vedal S, Schulzer M, et al. Asthma, asthmatic symptoms, chronic bronchitis and the degree of bronchial hyperresponsiveness in epidemiological surveys. Am Rev Respir Dis 1987; 136: 613-617.
12. Cockcroft DW, Berscheid BA, Murdock KY. Unimodal distribution of bronchial responsiveness to inhaled histamine in a random human population. Chest 1983; 83: 751-754.
13. Cockcroft DW, Killian DN, Mellon JJA, et al. Bronchial reactivity to inhaled histamine: a method and clinical survey. Clin Allergy 1977; 7: 235-243.
14. Rijcken B, Schouten JP, Weiss ST, et al. The relationship of nonspecific bronchial responsiveness to respiratory symptoms in a random population sample. Am Rev Respir Dis 1987; 136: 62-68.
15. Peat JK, Salome CM, Xuan W. On adjusting measurements of airway responsiveness for lung size and airway caliber. Am J Respir Crit Care Med 1996; 154: 870-875.
16. Orie NGM, Sluiter HJ, de Vries K, et al. The host factor in bronchitis. In: Orie NGM, Sluiter HJ, eds. Bronchitis: an international symposium. Assen, Netherlands: Royal van Gorcum, 1961.
17. Vestbo J, Prescott E. Update on the "Dutch hypothesis" for chronic respiratory disease. Thorax 1998; 53 Suppl 2: S15-19.
18. Gerrard JW, Cockroft DW, Mink JT, et al. Increased nonspecific bronchial reactivity in cigarette smokers with normal lung function. Am Rev Respir Dis 1980; 122: 577-581.
19. Oosterhoff Y, de Jong JW, Jansen MAM, et al. Airway Responsiveness to adenosine 5'-monophosphate in chronic obstructive pulmonary disease is determined by smoking. Am Rev Respir Dis 1993; 147: 553-558.
20. Rijcken B, Schouten JP, Mensinga TT, et al. Factors associated with bronchial responsiveness to histamine in a population sample of adults. Am Rev Respir Dis 1993; 147: 1447-1453.
21. Tashkin DP, Altose MD, Bleecker ER, et al. The Lung Health Study: airway responsiveness to inhaled methacholine in smokers with mild to moderate airflow limitation. Am Rev Respir Dis 1992; 145: 301-310.
22. Finkelstein R, Ma HD, Ghezzo H, et al. Morphometry of small airways in smokers and its relation to emphysema type and hyperresponsiveness. Am J Respir Crit Care Med 1995; 152: 267-276.
23. Yan K, Salome CM, Woolcock AJ. Prevalence and nature of bronchial hyperresponsiveness in subjects with chronic obstructive pulmonary disease. Am Rev Respir Dis 1985; 132: 25-29.
24. Ramsdale EH, Morris MM, Roberts RS, et al. Bronchial responsiveness to methacholine in chronic bronchitis: relationship to airflow obstruction and cold air responsiveness. Thorax 1984; 39: 912-918.
25. Ramsdale EH, Roberts RS, Morris MM, et al. Differences in responsiveness to hyperventilation and methacholine in asthma and chronic bronchitis. Thorax 1985; 40: 422-426.
26. Verma VK, Cockcroft DW, Dosman JA. Airway responsiveness to inhaled histamine in chronic obstructive airways disease. Chronic bronchitis vs emphysema. Chest 1988; 94: 457-461.
27. Ramsdale EH, Morris MM, Hargreave FE. Interpretation of the variability of peak flow rates in chronic bronchitis. Thorax 1986; 41: 771-776.

28. DuToit JI, Woolcock AJ, Salome CM, et al. Characteristics of bronchial hyperresponsiveness in smokers with chronic air-flow limitation. Am Rev Respir Dis 1986; 134: 498-501.
29. Ramsdell JW, Nachtwey FJ, Moser KM. Bronchial hyperreactivity in chronic obstructive bronchitis. Am Rev Respir Dis 1982; 126: 829-832.
30. DeVries K, Booij-Noord H, Goei JT, et al. Hyperreactivity of the bronchial tree to drugs chemical and physical agents. In *Bronchitis II*, ed. Orie NGM, Suiter NGM, 1964: 167-180. Assen : Royal Van Gorcum.
31. Mullen JBM, Wiggs BR, Wrigbt JL, et al. Nonspecific airway reactivity in cigarette smokers. Relation to airway pathology and baseline lung function. Am Rev Respir Dis 1986; 133: 120-125.
32. Lim TK, Taylor RG, Watson A, et al. Changes in bronchial responsiveness to inhaled histamine over four years in middle aged male smokers and ex-smokers. Thorax 1988; 43: 599-604.
33. Kanner RE, Connett JE, Altose MD, et al. Gender difference in airway hyperresponsiveness in smokers with mild COPD. The Lung Health Study. Am J Respir Crit Care Med 1994; 150: 956-961.
34. Rijcken B, Schouten JP, Weiss ST, et al. The relationship between airway responsiveness to histamine and pulmonary function level in a random population sample. Am Rev Respir Dis 1988; 137: 826-832.
35. Kerrebijn KF, ed. Similarities and discrepancies between asthma and chronic obstructive pulmonary disease. Am Rev Respir Dis 1991; 1149-1196 (part 1) and 1419-1472 (part 2).
36. Bogaard JM, Overbeek SE, Verbraak AFM, et al. Pressure-volume analysis of the lung with an exponentional and linear-exponentional model in asthma and COPD. The Dutch CNSLD study group. Eur Respir J 1995; 8: 1525-1531.
37. Moreno RH, Hogg JC, Paré PD. Mechanics of airway narrowing. Am Rev Respir Dis 1986; 133: 1171-1180.
38. Sterk PJ, Bel EH. Bronchial hyperresponsiveness: the need for a distinction between hypersensitivity and excessive airway narrowing. Eur Respir J 1989; 2: 267-274.
39. Cheung D, Schot R, Zwinderman, et al. Relationship between loss in parenchymal elastic recoil pressure and maximal airway narrowing in subjects with α_1-antitrypsine deficiency. Am J Respir Crit Care Med 1996; 155:135-140.
40. Chueng D. The determinants of excessive airway narrowing. Thesis, 1996, Leiden. Pasmans Offsetdrukkerij b.v., 's Gravenhage.
41. Bradley BL, Azzawi M, Jacobson M, et al. Eosinophils, T-lymphocytes, mast cells, neutrophils, and macrophages in bronchial biopsy specimens from atopic subjects without asthma and from atopic subjects without asthma and normal control subjects and relationship to bronchial hyperresponsiveness. J Allergy Clin Immunol 1991; 88: 661-674.
42. Chetta A, Foresi A, Del Donno M, et al. Bronchial responsiveness to distilled water and methacholine and its relationship to inflammation and remodeling of the airways in asthma. Am J Respir Crit Care Med 1996; 153: 910-917.
43. Burrows B, Bloom JW, Traver GA, et al. The course and prognosis of different forms of chronic airways obstruction in a sample from the general population. N Engl J Med 1987; 317: 1309-1314.
44. Camilli AE, Burrows B, Knudson RJ, et al. Longitudinal changes in forced expiratory volume in one second in adults. Effects of smoking and smoking cessation. Am Rev Respir Dis 1987; 135: 794-799.
45. Taylor RG, Joyce H, Gross E, et al. Bronchial reactivity to inhaled histamine and annual rate of decline in FEV1 in male smokers and ex-smokers. Thorax 1985; 40: 9-16.
46. Postma DS, de Vries K, Koëter GH, Sluiter HJ. Independent influence of reversibility of air-flow obstruction and nonspecific hyperreactivity on the long-term course of lung function in chronic air-flow obstruction. Am Rev Respir Dis 1986; 134: 276-280.
47. Parker DR, O'Connor GT, Sparrow D, et al. The relationship of nonspecific airway responsiveness and atopy to the rate of decline of lung function. The Normative aging study. Am Rev Respir Dis 1990; 141: 589-594.

48. O'Connor GT, Sparrow D, Weiss ST. A prospective longitudinal study of methacholine airway responsiveness as a predictor of pulmonary-function decline: the Normative aging study. Am J Respir Crit Care Med 1995; 152: 87-92.
49. Rijcken B, Schouten JP, Xu X, et al. Airway hyperresponsiveness to histamine associated with accelerated decline in FEV_1. Am J Respir Crit Care Med 1995; 151: 1377-1382.
50. Villar MTA, Dow L, Coggon D, et al. The influence of increased bronchial responsiveness, atopy, and serum IgE on decline in FEV_1. A longitudinal study in the elderly. Am J Respir Crit Care Med 1995; 151: 656-662.
51. Hospers J. Histamine airway hyper-responsiveness and mortality from chronic obstructive pulmonary disease. Lancet 2000; 356: 1313-1317.
52. Coope R. Diseases of the chest. Chapters 15 and 16. Livingstone Ltd, Edinburgh, 1948.
53. Reid LM. The pathology of obstructive and inflammatory airway diseases. Eur J Respir Dis 1986; 69 (suppl 147): 26-37.
54. Krzyzanowski M, Jedrychowski W, Wysocki M. Factors associated with the change in ventilatory function and the development of chronic obstructive pulmonary disease in a 13-year follow-up of the Cracow Study. Am Rev Respir Dis 1986; 134: 1011-1019.
55. Lebowitz MD, Postma DS, Burrows B. Adverse effects of eosinophilia and smoking on the natural history of newly diagnosed chronic bronchitis. Chest 1995; 108: 55-61.
56. Holt PG. Immune and inflammatory function in cigarette smokers [review]. Thorax 1987; 42: 241-249.
57. Burke WMJ, Roberts CM, Bryant DH, et al. Smoking-induced changes in epithelial lining fluid volume, cell density and protein. Eur Respir J 1992; 5: 780-784.
58. Costabel U, Maier K, Tescler H, Wang YM. Local immune components in chronic obstructive pulmonary disease. Respiration 1992; 59 (suppl 1): 17-19.
59. Hasday JD, McCrea KA, Meltzer SS, Bleeker ER. Dysregulation of airway cytokine expression in chronic obstructive pulmonary disease and asthma. Am J Respir Crit Care Med 1994; 150: S54-S58.
60. Nakamura Y, Romberger DJ, Tate L, et al. Cigarette smoke inhibits lung fibroblast proliferation and chemotaxis. Am J Respir Crit Care Med 1995; 151: 1497-1503.
61. Schaberg T, Klein U, Rau M, et al. Subpopulations of alveolar macrophages in smokers and nonsmokers : relation to the expression of CD11/CD18 molecules and superoxide anion production. Am J Respir Crit Care Med 1995; 151: 1551-1558.
62. Pesci A, Balbi B, Majori M, et al. Inflammatory cells and mediators in bronchial lavage of patients with chronic obstructive pulmonary disease. Eur Respir J 1998; 12: 380-386.
63. Garcia-Garcia JM, Hernandez JR, Martinez-Muniz MA, et al. Airways reactivity, atopy and bronchoalveolar lavage in male smokers with airflow obstruction. Respiration 1996; 63: 199-204.
64. Hobson JE, Wright JL, Wiggs BR, Hogg JC. Comparison of the cell content of lung lavage fluid with the presence of emphysema and peripheral airways inflammation in resected lungs. Respiration 1986; 50: 1-8.
65. Wright JL, Hobson J, Wiggs BR, Hogg JC. Comparison of inflammatory cells in bronchoalveolar fluid with those in the lumen and tissue peripheral airways and alveolar airspace. Lung 1988; 166: 75-83.
66. Turato G, DiStefano A, Maestrelli P, et al. Effect of smoking cessation on airway inflammation in chronic bronchitis. Am J Respir Crit Care Med 1995; 152: 1262-1267.
67. Reid L. Pathology of chronic bronchitis. Lancet 1954; i: 275-279.
68. Glynn AA, Michaels L. Bronchial biopsy in chronic bronchitis and asthma. Thorax 1960; 15: 142-153.
69. Reid L. Measurement of the bronchial mucous gland layer: a diagnostic yardstick in chronic bronchitis. Thorax 1960; 15: 132-141.
70. Carlile A, Edwards C. Structural variation in the named bronchi of the left lung. A morphometric study. Br J Dis Chest 1983; 77: 344-348.
71. Tos M, Moller K. Goblet-cell density in human bronchus in chronic bronchitis. Arch Otolaryngol 1983; 109: 673-676.

72. Spurzem JR, Thompson AB, Daughton DM, et al. Chronic inflammation is associated with an increased proportion of Goblet cells recovered by bronchial lavage. Chest 1991; 100: 389-393.
73. Trevisani L, Sartori S, Bovolenta MR, et al. Structural characterization of the bronchial epithelium of subjects with chronic bronchitis and in asymptomatic smokers. Respiration 1992; 59: 136-144.
74. Jamal K, Cooney TP, Fleetham JA, Thurlbeck WM. Chronic bronchitis. Correlation of morphologic findings in sputum production and flow rates. Am Rev Respir Dis 1984; 129: 719-722.
75. Mullen JBM, Wright JL, Wiggs BR, et al. Reassessment of inflammation of airways in chronic bronchitis. Br Med J 1986; 1: 587-591.
76. Nadel JA. Role of enzymes from inflammatory cells on airway submucosal gland secretion. Respiration 1991; 58(supplement): 3-5.
77. Niewoehner DE, Kleinerman J, Rice DB. Pathologic changes in the peripheral airways of young cigarette smokers. N Engl J Med 1974; 291: 755-758.
78. Martin TR, Raghu G, Maunder RJ, Springmeyer SC. The effects of chronic bronchitis and chronic air-flow obstruction on lung cell populations recovered by bronchoalveolar lavage. Am Rev Respir Dis 1985; 132: 254-260.
79. Thompson AB, Daughton D, Robbins RA, et al. Intraluminal airway inflammation in chronic bronchitis. Characterization and correlation with clinical parameters. Am Rev Respir Dis 1989; 140: 1527-1537.
80. Lacoste J-Y, Bousquet J, Chanez P, et al. Eosinophilic and neutrophilic inflammation in asthma, chronic bronchitis, and chronic obstructive pulmonary disease. J Allergy Clin Immunol 1993; 92: 537-548.
81. Laviolette M, Chang J, Newcombe DS. Human alveolar macrophages: a lesion in arachidonic acid metabolism in cigarette smokers. Am Rev Respir Dis 1981; 124: 397-401.
82. Mcleod R, Mack DG, Mcleod EG, et al. Alveolar macrophage function and inflammatory stimuli in smokers with and without obstructive lung disease. Am Rev Respir Dis 1985; 131: 377-384.
83. Muley T, Wiebel M, Schulz V, Ebert W. Elastinolytic activity of alveolar macrophages in smoking-associated pulmonary emphysema. Clin Investig 1994; 72: 269-276.
84. Hunninghake GW, Crystal RD. Cigarette smoking and lung destruction: accumulation of neutrophils in the lungs of cigarette smokers. Am Rev Respir Dis 1983; 128: 833-838.
85. Selby C, Drost E, Lannan S, et al. Neutrophil retention in the lungs of patients with chronic obstructive pulmonary disease. Am Rev Respir Dis 1991; 143: 1359-1363.
86. Keatings VM, Barnes PJ. Granulocyte activation markers in induced sputum: comparison between chronic obstructive pulmonary disease, asthma, and normal subjects. Am J Respir Crit Care Med 1997; 155: 449-453.
87. Lusuardi M, Capelli A, Cerutti CG, et al. Airways inflammation in subjects with chronic bronchitis who have never smoked. Thorax 1994; 49: 1211-1216.
88. Saetta M, DiStefano A, Maestrelli P, et al. Activated T-lymphocytes and macrophages in bronchial mucosa of subjects with chronic bronchitis. Am Rev Respir Dis 1993; 147: 301-306.
89. Di Stefano A, Maestrelli P, Roggeri A, et al. Upregulation of adhesion molecules in the bronchial mucosa of subjects with chronic obstructive bronchitis. Am J Respir Crit Care Med 1994; 149: 803-810.
90. Riise GC, Larsson S, Löfdahl C-G, Andersson BA. Circulating cell adhesion molecules in the bronchial lavage and serum in COPD patients with chronic bronchitis. Eur Respir J 1994; 7: 1673-1677.
91. Gonzalez S, Hards J, Van Eeden S, Hogg JC. The expression of adhesion molecules in cigarette smoke-induced airways obstruction. Eur Respir J 1996; 9: 1995-2001.
92. Stockley RA, Grant RA, Llewellyn-Jones CG, et al. Neutrophil formyl-peptide receptors. Relationship to peptide-induced responses and emphysema. Am J Respir Crit Care Med 1994; 149: 464-468.
93. Fournier M, Lebargy F, Le Roy Ladurie F, et al. Intraepithelial T-lymphocyte subsets in the airways of normal subjects and of patients with chronic bronchitis. Am Rev Respir Dis 1989; 140: 737-742.

94. Di Stefano A, Turato G, Maestrelli P, et al. Airflow limitation in chronic bronchitis is associated with T-lymphocyte and macrophage infiltration of the bronchial mucosa. Am J Respir Crit Care Med 1996; 153: 629-632.
95. Ollerenshaw SL, Woolcock AJ. Characteristics of the inflammation in biopsies from large airways of subjects with asthma and subjects with chronic airflow limitation. Am Rev Respir Dis 1992; 145: 922-927.
96. Bosken CH, Hards J, Gatter K, Hogg JC. Characterization of the inflammatory reaction in the peripheral airways of cigarette smokers using immunocytochemistry. Am Rev Respir Dis 1992; 145: 911-917.
97. O'Shaughnessy TC, Ansari TW, Barnes NC, Jeffery PK. Inflammation in bronchial biopsies of subjects with chronic bronchitis: inverse relationship of CD8+ T lymphocytes with FEV1. Am J Respir Crit Care Med 1997; 155: 852-857.
98. Saetta M, Baraldo S, Corbino L, et al. CD8+ve cells in the lungs of smokers with chronic obstructive pulmonary disease. Am J Respir Crit Care Med 1999; 160: 711-717.
99. Saetta M, Turato G, Facchini FM, et al. Inflammatory cells in the bronchial glands of smokers with chronic bronchitis. Am J Respir Crit Care Med 1997; 156: 1633-1639.
100. Finkelstein R, Fraser RS, Ghezzo H, Cosio MG. Alveolar inflammation and its relation to emphysema in smokers. Am J Respir Crit Care Med 1995; 152: 1666-1672.
101. Majori M, Corradi M, Caminati A, et al. Predominant TH1 cytokine pattern in peripheral blood from subjects with chronic obstructive pulmonary disease. J Allergy Clin Immunol 1999; 103: 458-462.
102. Balzano G, Stefanelli F, Iorio C, et al. Eosinophilic inflammation in stable chronic obstructive pulmonary disease. Am J Respir Crit Care Med 1999; 160: 1486-1492.
103. Fabbri L, Beghe B, Caramori G, et al. Similarities and discrepancies between exacerbations of asthma and chronic obstructive pulmonary disease. Thorax 1998; 53: 803-808.
104. Saetta M, Di Stefano A, Maestrelli P, et al. Airway eosinophilia in chronic bronchitis during exacerbations. Am J Respir Crit Care Med 1994; 150: 1646-1652.
105. Overveld FJ van, Houben LAMJ, Terpstra GK, Kreukniet J. Patients with chronic bronchitis differ in their mast cell subtypes as compared with normal subjects. Agents and Actions 1989; 26: 60-62.
106. Pesci A, Rossi GA, Bertorelli G, et al. Mast cells in the airway lumen and bronchial mucosa of patients with chronic bronchitis. Am J Respir Crit Care Med 1994; 149: 1311-1316.
107. Grashoff WFH, Sont JK, Sterk PJ, et al. Chronic Obstructive Pulmonary Disease. Role of bronchiolar mast cells and macrophages. Am J Pathol 1997; 151: 1785-1790.
108. Lamb D, Lumsden A. Intra-epithelial mast cells in human airway epithelium: evidence for smoking-induced changes in their frequency. Thorax 1982; 37: 334-342.
109. Kuwano K, Bosken CH, Pare PD, et al. Small airways dimensions in asthma and in chronic obstructive pulmonary disease. Am Rev Respir Dis 1993; 148: 1220-1225.
110. Saetta M, Di Stefano A, Turato G, et al. CD8+ T-lymphocytes in peripheral airways of smokers with chronic obstructive pulmonary disease. Am J Respir Crit Care Med 1998; 157: 822-826.
111. Cosio MG, Hale KA, Niewoehner DE. Morphologic and morphometric effects of prolonged cigarette smoking on the small airways. Am Rev Respir Dis 1980; 122: 265-271.
112. Mitchell RS, Stanford RE, Johnson JM, et al. The morphologic features of the bronchi, bronchioles and alveoli in chronic airway obstruction: a clinicopathologic study. Am Rev Respir Dis 1976; 114: 137-145.
113. Hale KA, Ewing SL, Gosnell BA, et al. Lung disease in long-term cigarette smokers with and without chronic air-flow obstruction. Am Rev Respir Dis 1984; 130: 716-721.
114. Saetta M, Ghezzo H, Kim WD, et al. Loss of alveolar attachments in smokers: a morphometric correlate of lung function impairment. Am Rev Respir Dis 1985; 132: 894-900.
115. Wright JL, Hobson JE, Wiggs B, Paré JC. Airway inflammation and peribronchiolar attachments in the lungs of nonsmokers, current and ex-smokers. Lung 1988; 166: 277-286.
116. Gelb AF, Gobel PH, Fairshter R, Zamel N. Predominant site of airway resistance in chronic obstructive pulmonary disease. Chest 1981; 79: 273-276.

117. Woestijne KP van de. Are the small airways really quiet? Eur J Respir Dis 1982; 63/suppl.121: 19-25.
118. Hogg JC, Macklem PT, Thurlbeck WM. Site and nature of airway obstruction in chronic obstructive lung disease. New Eng J Med 1968; 278:1355-1360.
119. Leaver DG, Tattersfield AE, Pride NB. Contributions of loss of lung recoil and of enhanced airways collapsibility to the airflow obstruction of chronic bronchitis and emphysema. J Clin Invest 1973; 52: 2117-2128.
120. Gelb AF, Schein M, Kuei J, et al. Limited contribution of emphysema in advanced chronic obstructive pulmonary disease. Am Rev Respir Dis 1993; 147: 1157-1161.
121. Hogg JC, Wright JL, Wiggs BR, et al. Lung structure and function in cigarette smokers. Thorax 1994; 49: 473-478.
122. Tiddens HAWM, Bogaard JM, de Jongste JC, et al. Physiological and morphological determinants of maximal expiratory flow in chronic obstructive lung disease. Eur Respir J 1996; 9: 1785-1794.
123. Di Stefano A, Capelli A, Lusuardi M, et al. Severity of airflow limitation is associated with severity of airway inflammation in smokers. Am J Respir Crit Care Med 1998; 158: 1277-1285.
124. Lams BEA, Sousa AR, Rees PJ, Lee TH. Immunopathology of the small-airway submucosa in smokers with and without chronic obstructive pulmonary disease. Am J Respir Crit Care Med 1998; 158: 1518-1523.
125. Gadek JE, Fells GA, Crystal RG. Cigarette smoking induces functional antiprotease deficiency in the lower respiratory tract of humans. Science 1979; 206: 1315-1316.
126. Cantin A, Crystal RG. Oxidants, antioxidants and the pathogenesis of emphysema. Eur J Respir Dis 1985; 139(suppl): 7-17.
127. Repine JE, Bast A, Lankhorst I, and the oxidative stress study group. State of the art: oxidative stress in chronic obstructive pulmonary disease. Am J Respir Crit Care Med 1997; 156: 341-357.
128. Reed CE. Basic mechanisms of asthma. Role of inflammation. Chest 1988; 94: 175-177.
129. Ackerman V, Marini M, Viottori E, et al. Detection of cytokines and their cell sources in bronchial biopsy specimens from asthmatic patients. Relationship to atopic status, symptoms, and level of airway hyperresponsiveness. Chest 1994; 105: 687-696.
130. Bentley AM, Menz G, Storz Chr, et al. Identification of T-lymphocytes, macrophages, and activated eosinophils in the bronchial mucosa in intrinsic asthma. Relationship to symptoms and bronchial responsiveness. Am Rev Respir Dis 1992; 146: 500-506.
131. Möller GM, Overbeek SE, Van Helden-Meeuwsen CG, et al. Eosinophils in the bronchial mucosa in relation to methacholine dose-response curves in atopic asthma. J Appl Physiol 1999; 86: 1352-1356.
132. Bahous J, Cartier A, Ouimet G, et al. Nonallergic bronchial hyperexcitability in chronic bronchitis. Am Rev Respir Dis 1984; 129: 216-220.
133. Laitinen LA, Heino M, Laitinen A, et al. Damage of the airway epithelium and bronchial reactivity in patients with asthma. Am Rev Respir Dis 1985; 131: 599-606.
134. Laitinen LA, Jeffery PK, Wardlaw A, et al. Bronchial biopsies in asthma: an ultrastructural quantification study and correlation with hyperreactivity. Am Rev Respir Dis 1989; 140: 1745-1753.
135. Naylor B. The shedding of the mucosa of the bronchial tree in asthma. Thorax 1962; 17: 69-72.
136. Beasly R, Roche W, Roberts JA, et al. Cellular events in the bronchi in mild asthma and after bronchial provocation. Am Rev Respir Dis 1989; 139: 806-817.
137. Kennedy SM, Elwood RK, Wiggs BJR, et al. Increased airway mucosal permeability of smokers. Relationship to airway reactivity. Am Rev Respir Dis 1984; 129: 143-148.
138. Taylor SM, Paré PD, Armour CL, et al. Airway reactivity in chronic obstructive pulmonary disease. Failure of in vivo methacholine responsiveness to correlate with cholinergic, adrenergic, or nonadrenergic responses in vitro. Am Rev Respir Dis 1985; 132: 30-35.
139. Jeffery PK. Comparison of the structural and inflammatory features of COPD and asthma. Gilles F. Filley Lecture. Chest 2000; 117(Suppl 1): 251S-260S.
140. Saetta M, Finkelstein R, Cosio MG. Morphological and cellular basis for airflow limitation in smokers [review]. Eur Respir J 1994; 7: 1505-1515.

141. Van Andel AE, Reisner C, Menjoge SS, Witek TJ. Analysis of inhaled corticosteroid and oral theophylline use among patients with stable COPD from 1987 to 1995. Chest 1999; 115: 703-707.
142. Jackevicius C, Joyce DP, Kesten S, Chapman KR. Prehospitalization inhaled corticosteroid use in patients with COPD or asthma. Chest 1997; 111: 296-302.
143. Shim C, Stover DE, Williams MH. Response to corticosteroids in chronic bronchitis.J Allergy Clin Immunol 1978; 62: 363-367.
144. Shim CS, Williams MH. Aerosol beclomethasone in patients with steroid-responsive chronic obstructive pulmonary disease. Am J Med 1985; 78: 655-658.
145. Mendella LA, Manfreda J, Warren CPW, Anthonissen NR. Steroid response in stable chronic obstructive pulmonary disease. Annals of Internal Medicine 1982; 96: 17-21.
146. Blair GP, Light RW. Treatment of chronic obstructive pulmonary disease with corticosteroids. Comparison of daily vs alternate-day therapy. Chest 1984; 86: 524-528.
147. Eliasson O, Hoffman J, Trueb D, et al. Corticosteroids in COPD. A clinical trial and reassessment of the literature. Chest 1986; 89: 484-490.
148. Strain DS, Kinasewitz GT, Franco DP, George RB. Effect of steroid therapy on exercise performance in patients with irreversible chronic obstructive pulmonary disease. Chest 1985; 88: 718-721.
149. Wiggins J, Elliott JA, Stevenson RD, Stockley RA. Effect of corticosteroids on sputum sol-phase protease inhibitors in chronic obstructive pulmonary disease. Thorax 1982; 37: 652-656.
150. Postma DS, Steenhuis EJ, van der Weele LTh, Sluiter HJ. Severe chronic airflow obstruction: can corticosteroids slow down progression? Eur J Respir Dis 1985; 67: 56-64.
151. Postma DS, Peters I, Steenhuis EJ, Sluiter HJ. Moderately severe chronic airflow obstruction. Can corticosteroids slow down obstruction? Eur Respir J 1988; 1: 22-26.
152. Callahan CM, Dittus RS, Katz BP. Oral corticosteroid therapy for patients with stable chronic obstructive pulmonary disease. A meta-analysis. Ann of Int Med 1991; 114: 216-223.
153. Chanez P, Vignola AM, O'Shaugnessy, et al. Corticosteroid reversibility in COPD is related to features of asthma. Am J Respir Crit Care Med 1997; 155: 1529-1534.
154. Pizzichini E, Pizzichini MMM, Gibson P, et al. Sputum eosinophilia predicts benefit from prednisone in smokers with chronic obstructive bronchitis. Am J Respir Crit Care Med 1998; 158: 1511-1517.
155. Fujimoto K, Kubo K, Yamamoto H, et al. Eosinophilic inflammation in the airway is related to glucocorticoid reversibility in patients with pulmonary emphysema. Chest 1999; 115: 697-702.
156. Engel T, Heinig JH, Madson, et al. A trial of inhaled budesonide on airway responsiveness in smokers with chronic bronchitis. Eur Respir J 1989; 2: 935-939.
157. Weir DC, Gove RI, Robertson AS, Sherwood Burge P. Corticosteroid trials in non-asthmatic chronic airflow obstruction: a comparison of oral prednisolone and inhaled beclomethasone dipropionate. Thorax 1990; 45: 112-117.
158. Auffarth B, Postma DS, de Monchy JGR, et al. Effects of inhaled budesonide on spirometric values, reversibility, airway responsiveness, and cough threshold in smokers with chronic obstructive lung disease. Thorax 1991; 46: 372-377.
159. Thompson AB, Mueller MB, Heires AJ, et al. Aerosolized beclomethasone in chronic bronchitis. Improved pulmonary function and diminished airway inflammation. Am Rev Respir Dis 1992; 146: 389-395.
160. Llewellyn-Jones CG, Harris TAJ, Stockley RA. Effect of Fluticasone propionate on sputum of patients with chronic bronchitis and emphysema. Am J Respir Crit Care Med 1996; 153: 616-621.
161. Renkema TE, Schouten JP, Koëter GH, Postma DS. Effects of long-term treatment with corticosteroids in COPD. Chest 1996; 109: 1156-1162.
162. Bourbeau J, Rouleau MY, Boucher S. Randomised controlled trial of inhaled corticosteroids in patients with chronic obstructive pulmonary disease. Thorax 1998; 53: 477-482.
163. Confalonieri M, Mainardi E, Della Porta R, et al. Inhaled corticosteroids reduce neutrophilic bronchial inflammation in patients with chronic obstructive pulmonary disease. Thorax 1998; 53: 583-585.

164. Van Grunsven PM, Van Schayck CP, Derenne JP, et al. Long terms effects of inhaled corticosteroids in chronic obstructive pulmonary disease: a meta-analysis. Thorax 1999; 54: 7-14.
165. Watson A, Lim TK, Joyce H, Pride NB. Failure of inhaled corticosteroids to modify bronchoconstrictor or bronchodilator responsiveness in middle-aged smokers with mild airflow obstruction. Chest 1992; 101: 350-355.
166. Weiner P, Weiner M, Azgad Y, Zamir D. Inhaled budesonide therapy for patients with stable COPD. Chest 1995; 108: 1568-1571.
167. Culpitt SV, Maziak W, Loukidis S, et al. Effect of high dose inhaled steroid on cells, cytokines and proteases in induced sputum in chronic obstructive pulmonary disease. Am J Respir Crit Care Med 1999; 160: 1635-1639.
168. Llewellyn-Jones CG, Hill SL, Stockley RA. Effect of fluticasone propionate on neutrophil chemotaxis, superoxide generation, and extracellular proteolytic activity in vitro. Thorax 1994 ; 49 : 207-212.
169. Keatings VM, Jatakanon A, Worsdell YM, Barnes PJ. Effects of inhaled and oral glucocorticoids on inflammatory indices in asthma and COPD. Am J Respir Crit Care Med 1997; 155: 542-548.
170. Balbi B, Majori M, Bertacco S, et al. Inhaled corticosteroids in stable COPD patients. Do they have effects on cells and molecular mediators of airway inflammation? Chest 2000; 117: 1633-1637.
171. Pauwels RA, Löfdahl C-G, Laitinen LA, et al., on behalf of the European Respiratory Society Study on Chronic Obstructive Pulmonary Disease. Long-term treatment with inhaled budesonide in persons with mild chronic obstructive pumonary disease who continue smoking. N Engl J Med 1999; 340: 1948-1953.
172. Vestbo J, Sorensen T, Lange P, et al. Long-term effect of inhaled budesonide in mild and morate chronic obstructive pulmonary disease: a randomised controlled trial. Lancet 1999; 353: 1819-1823.
173. Burge PS, Calverley PMA, Jones PW, et al., on behalve of the ISOLDE study investigators. Randomised, double blind, placebo controlled study of fluticasone propionate in patients with moderate and severe chronic obstructive pulmonary disease: the ISOLDE trial. BMJ 2000; 320: 1297-1303.
174. Jarad NA, Wedzicha JA, Burge PS, Calverley PM. An observational study of inhaled corticosteroid withdrawal in stable chronic obstructive pulmonary disease. ISOLDE Study Group. Respir Med 1999; 93: 161-166.
175. The Lung Health Study Research Group. Effect of inhaled triamcinolone on the decline in pulmonary function in chronic obstructive pulmonary disease. N Engl J Med 2000; 343: 1902-1909.
176. Paggiaro PL, Dahle R, Bakran I, et al., on behalf of the International COPD Study Group. Multicenter randomised placebo-controlled trial of inhaled fluticasone propionate in patients with chronic obstructive pulmonary disease. The Lancet 1998; 351: 773-780. [Erratum, Lancet1998; 351: 1968.]
177. Dompeling E, Van Schayck CP, Van Grunsven PM, et al. Slowing the deterioration of asthma and chronic obstructive pulmonary disease observed during bronchodilator therapy by adding inhaled corticosteroids. A 4-year prospective study. Annals of Internal Medicine 1993; 118: 770-778.
178. Kerstjens HAM, Brand PLP, Hughes MD, et al. A comparison of bronchodilator therapy with or without inhaled corticosteroid therapy for obstructive airways disease. N Engl J Med 1992; 327: 1413-1419.
179. Kerstjens HA, Overbeek SE, Schouten JP, et al. Airway hyperresponsiveness, bronchodilator response, allergy and smoking predict improvement in FEV_1 during long-term inhaled corticosteroid treatment. Dutch CNSLD Study Group. Eur Respir J 1993; 6: 868-876.
180. Bel EH, Timmers MC, Hermans J, et al. The long-term effects of Nedocromil sodium and Beclomethasone dipropionate on bronchial responsiveness to methacholine in non-atopic asthmatic subjects. Am Rev Respir Dis 1990; 141: 21-28.
181. Overbeek SE, Rijnbeek, Vons C, et al. Effects of fluticasone propionate on methacholine dose-response curves in non-smoking atopic asthmatics. Eur Respir J 1996; 9: 2256-2262.

182. Sterk PJ, Bel EH. The shape of the dose-response curve to inhaled bronchoconstrictor agents in asthma and in chronic obstructive pulmonary disease. [Review] Am Rev Respir Dis 1991; 143: 1433-1437.

Chapter 2

Influence of lung parenchymal destruction on the different indexes of the methacholine dose-response curve in COPD patients

G.T. Verhoeven, A.F.M. Verbraak, S. Boere-van der Straat, H.C. Hoogsteden, J.M. Bogaard
Department of Pulmonary and Intensive Care Medicine, University Hospital Dijkzigt, EMCR, Rotterdam, The Netherlands

(Published in: Chest 2000; 117: 984-990)

Abstract

Study objectives: The interpretation of nonspecific bronchial provocation dose-response curves in COPD is still a matter of debate. Bronchial hyperresponsiveness (BHR) in COPD could be influenced by the destruction of the parenchyma and the augmented mechanical behavior of the lung. Therefore, we studied the interrelationships between indexes of BHR, on the one hand, and markers of lung parenchymal destruction, on the other.
Patients and methods: COPD patients were selected by clinical symptoms, evidence of chronic, nonreversible airways obstruction and BHR, which was defined as a provocative dose of a substance (histamine) causing a 20% fall in FEV_1 (PC_{20}) of ≤ 8 mg/ml. BHR was subsequently studied by methacholine dose-response curves to which a sigmoid model was fitted for the estimation of plateau values and reactivity. Model fits of quasi-static lung pressure-volume (PV) curves yielded static compliance (Cstat), the exponential factor KE and elastic recoil at 90% of total lung capacity (P90TLC). Carbon monoxide (CO) transfer was measured with the standard single-breath method.
Results: Twenty-four patients were included in the study, and reliable PV data could be obtained from 19. The following mean values (±SD) were taken: FEV_1, 65 ±12% of predicted; reversibility, 5.6 ±3.1% of predicted; the PC_{20} for methacholine, 4.3 ±5.2 mg/mL; reactivity, 11.0 ±5.6% FEV_1/doubling dose; plateau, 48.8 ±17.4% FEV_1; transfer factor, 76.7 ±17.9% of predicted; transfer coefficient for carbon monoxide (KCO), 85.9 ±22.6% of predicted; Cstat, 4.28 ±2.8 L/kPa; shape factor (KE) 1.9 ±1.5 kPa^{-1}; and P90TLC, 1.1 ±0.8 kPa. We confirmed earlier reported relationships between Cstat, on the one hand, and KE ($p<0.0001$), P90TLC ($p=0.0012$), and KCO percent predicted ($p = 0.006$), on the other hand. The indexes of the methacholine provocation test were not related to any parameter of lung elasticity and CO transfer.
Conclusion: BHR in COPD patients who smoke most probably is determined by airways pathology rather than by the augmented mechanical behavior caused by lung parenchymal destruction.

Introduction

Bronchial hyperresponsiveness (BHR) is present in patients with asthma and COPD (1). Approximately half of the subjects with COPD in a general population have BHR (2). In the Lung Health Study, BHR was noted in 85.1% of the women and 58.9% of the men with mild-to-moderate airflow limitation (3). The estimation of BHR is important for the diagnosis of asthma and for determining asthma severity, whereas the meaning of BHR for the clinical management of COPD is still unclear (4). COPD patients with BHR appear to be prone to a more rapid decline of their FEV_1 (5).

Clinical studies suggest that BHR in patients with COPD differs from BHR in patients with asthma (6-9). For example, in patients with COPD, BHR for physiologic stimuli (*eg,* cold air) usually is not found in the presence of BHR for pharmacologic agents (*eg,* histamine). In patients with asthma, both types of stimuli cause bronchoconstriction (6,7). The explanation for these differences might be found in the different pathologic changes in the airways and in the lung parenchyma of asthma and COPD patients (1). The main and clearest difference between asthma and COPD is destruction of the lung parenchyma in COPD, leading to emphysema and loss of lung elasticity. In patients with COPD, compared to those with asthma, there is a relationship between baseline FEV_1 and the level of BHR (8,9). The FEV_1 is, however, not a good predictor of the amount of parenchymal destruction and loss of elastic recoil (10-13). The most reliable test for lung elasticity is the direct estimation of quasi-static esophageal pressure-volume (PV) curves (10,11,14). The destruction of parenchymal tissue also is shown by impairment of carbon monoxide (CO) transfer (15-18). We performed these lung function tests in COPD patients who smoked, who fulfilled the established clinical and functional criteria for COPD, and who also had a provocative concentration of a substance (histamine) causing a 20% fall in FEV_1 (PC_{20}) of $\leq$ 8 mg/ml. In subsequent methacholine dose-response curves, not only PC_{20} (sensitivity) but also maximal bronchoconstriction (plateau) and the slope of the curve (reactivity) were estimated, because these factors should yield additional information on the causative mechanisms of BHR (19).

The aim of our study was to investigate the influence of the impairment of lung parenchymal structure on BHR by the estimation of the interrelationships between indexes related to lung parenchymal destruction (lung elasticity and CO transfer) and indexes from methacholine log-dose response curves.

Materials and methods

Patients

COPD patients were recruited according to generally accepted clinical and functional criteria (20). The inclusion criteria were the following: chronic productive cough; age between 40 and 70 years; current smokers; negative skin tests for standard inhalation allergens; FEV_1 or FEV_1/inspiratory vital capacity

(IVC) ratio ≤ 70% of the predicted normal value; reversibility of FEV_1 of <10% predicted after 750 μg terbutaline administered by metered-dose inhalation; and nonspecific BHR, defined by a PC_{20} for histamine of ≤ 8 mg/ml. Exclusion criteria were the following: a history of asthma; complaints of wheezing; radiographic signs of bullous emphysema; recent respiratory tract infection; and recent or concurrent usage of anti-inflammatory drugs. Eligible patients refrained from oral anti-inflammatory medication at least 3 months and from inhaled glucocorticoids at least 6 weeks before the start of the study.
The study was approved by the Medical Ethics Committee of the University Hospital Dijkzigt, and written informed consent was obtained from all participants.

Lung function tests
Functional tests included the following: spirometry (total lung capacity [TLC], functional residual capacity [FRC], RV, IVC, FEV_1); reversibility test (FEV_1 percent predicted); single-breath CO transfer (transfer factor of the lung for CO [T_{LCO}] and transfer coefficient for CO [KCO]; and PV curves. Spirometry was measured with the multiple-breath, closed Helium wash-in method, using a water-sealed spirometer (model D35R; Lode; Groningen, The Netherlands) and with the patient in sitting position. The lung volumes were corrected to body temperature and ambient pressure, saturated with water vapor. All reference values were derived from the standards of the European Community for Steel and Coal (21,22).

Bronchial provocation tests
Histamine and methacholine provocation tests were performed according to the 2-min tidal breathing method that was first described by Cockcroft et al (23,24). The PC_{20} for histamine was used as an inclusion criterion. Because of lesser side effects, methacholine was used for obtaining as complete as possible dose-response curves (25). After inhalation of an isotonic saline solution, doubling concentrations of histamine-sulphate or acetyl-β-methylcholine-bromide were administered, starting with doses of 0.03 mg/mL. Methacholine was prepared by our hospital pharmacy department. Solutions were stored at 4°C and were used at room temperature. Aerosols were generated by a nebulizer (model 646; DeVilbiss Co; Somerset, PA) (measured output 0.13 mL/min) and were inhaled by tidal breathing over a 2-min period at 5-min intervals. The response to methacholine or histamine was measured as the change in FEV_1 expressed as a percentage of the initial value. The histamine provocation test was interrupted if a 20% fall in FEV_1 occurred before or at a dose of 8 mg/mL. The methacholine tests were continued up to a concentration of 256 mg/mL but were interrupted if the FEV_1 fell by > 60% or if unpleasant side effects occurred.
From the methacholine provocation tests, the log2 concentration and the measured percentage of changes in FEV_1 were imported to a computer program that fitted a sigmoid function (cumulative Gaussian distribution) to the data (26). Reactivity (the slope of the curve), and plateau values (maximal bronchoconstriction) were taken

from this model fit, whereas the PC_{20} was calculated from the measured data by linear interpolation of adjacent data points.

Pressure-volume curves

The lung elasticity measurements were performed immediately after the reversibility test. This sequence was chosen for reasons of standardization and in order to minimize the potential disturbing effect of airway closure on lung elasticity. Quasi-static deflation exercises were performed according to the method that has been described before (27). In short, the transpulmonary pressure was measured via a transducer (model P45; Validyne Engineering Corp; Northridge, CA) coupled to a balloon that was positioned in the lower third of the esophagus. The simultaneous recording of volume changes was obtained during a slow expiration (*ie*, expiration not exceeding 0.3 L/s). Selected curves were smoothed by drawing a line by hand through katacrotic points of the cardiac pulsations on the curve. Volume data were obtained at equal transpulmonary pressure intervals, yielding an average 10 to 30 data points up to the TLC level. A linear-exponential (LE) and an exponential model were fitted to the measured data. The LE model gave the most accurate fit to experimental curves and was, therefore, used for the estimation of static lung compliance (Cstat) and volume- dependent recoil pressures (27,28). For this fit, the curve was considered to be composed of a linear part, from the first data points on starting at the FRC level, and an exponential part, starting at the higher volume level. Cstat was obtained from the linear part if four or more data points contributed to that part. In a minority of cases, we calculated Cstat by hand as the slope between FRC and FRC + 0.5 L. The elastic recoil pressure at 90% of TLC (P90TLC) also was derived from the LE model fit. This pressure index was considered to be the elastic recoil index with the lowest variation coefficient (29). Additionally, we prefer to use the P90TLC values above the recoil pressure at TLC because P90TLC is less dependent on inspiratory muscle force. The shape factor KE was determined from the following generally used exponential equation:

$$V = Vmax \{1 - \exp [KE (P - P0)]\}$$

where Vmax is the asymptotic value (in liters) and P0 is the intercept with the P axis at V = 0 kPa.

KE can be considered as an elasticity index, independent of lung size (28). For the fit with the exponential model, we used the same (measured) input data as for the LE model fit. KE was considered as an additional elasticity index.

Statistics

Linear regression analysis between variables, pairwise multivariate correlation, and statistical significance were calculated with the use of a package of statistical software (Statistical Graphics Corp; Rockville, MD). Test results were considered statistically significant at $p < 0.05$.

Results

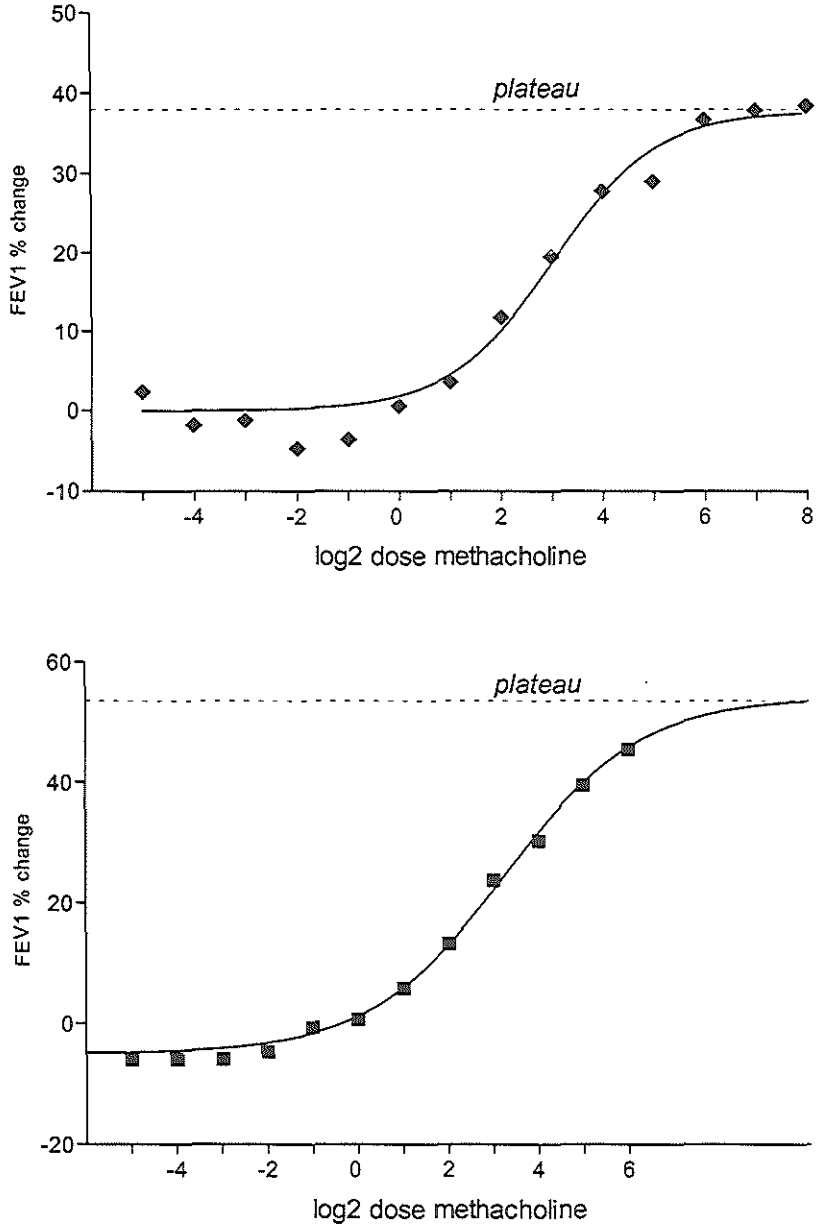
Baseline characteristics

Twenty-four patients were included in the study. From 19 patients, we obtained reliable PV curves; the remaining patients did not tolerate the esophagus balloon long enough or showed effects of swallowing that hampered an accurate interpretation of the data. The mean age of the patients was 56 years (Table 1). The mean FEV_1 was 65% of predicted. One patient had an FEV_1 > 70% of predicted, but was included because his FEV_1/IVC ratio was 0.51. The mean reversibility of FEV_1 after terbutaline inhalation was 5.6%. Four patients showed no reversibility at all.

Table 1.
Smoking habits, FEV_1, reversibility, BHR, CO transfer and lung elasticity data.

	n	Mean ±SD	median	Range
Age, yr	24	55.5 ±8.5	54	42 - 69
Actual smoking, cigarettes/d	24	15.6 ±6.8	13	6 - 30
Pack-years	24	23 ±10.5	23	5 - 50
FEV_1, % predicted	24	64.5 ±11.9	65	34 - 93
Reversibility, % predicted	24	6.8 ±4.0	7.3	0 - 13.8
PC_{20}				
Histamine	24	1.66 ±2.00	0.87	0.11 - 8
Methacholine	24	4.27 ±5.2	1.46	0.4 - 17.4
log2 PC_{20} methacholine	24	1.07 ±1.74	0.53	-1.3 - 4.1
Reactivity, %FEV_1/doubling dose	24	11.0 ±5.6	8.98	3.9 - 26.8
Plateau, %FEV_1	24	48.8 ±17.4	48.3	20.8 - 95.7
Cstat, L/kPa	19	4.6 ±2.8	4.1	1.1 - 10.5
KE, kPa^{-1}	19	2.5 ±1.5	2.2	0.7 - 6.3
P90TLC, kPa	19	1.1 ±0.8	0.8	0.4 - 2.7
T_{LCO}, % predicted	24	76.7 ±17.9	75	34 - 106
KCO, % predicted	24	85.9 ±22.6	86	43 - 139

Figure 1. Two examples of sigmoid fitting of the methacholine provocation tests.



Top: the measured data (diamonds) are almost equal to the fitted plateau.
Bottom: the measured data (squares) show that the plateau was not reached during the test.

The patients had moderate or severe BHR (Table 1). The mean PC_{20} for methacholine was higher than that for histamine (4.3 vs 1.7 mg/mL, respectively). This difference was also reported in an earlier study of smokers with mild chronic airflow limitation (30). After correction for the difference in molecular weight (1 mg of the bromide compound is equivalent to 0.82 mg of the chloride compound), the corrected mean bromide value of the PC_{20} became 3.5 mg/mL.

The mean plateau value was 48.8 % of the FEV_1. In Figure 1, we present a curve in which the fitted plateau is almost equal to the measured data (Fig.1, *top*) and a curve in which the plateau value is derived from extrapolation (Fig.1, *bottom*). If the experimental plateau estimate was defined by the mean value of the last two provocative concentrations with a variation of <5%, we observed that the fitted plateau was almost equal to the experimental plateau estimate in 13 of the 24 dose-response curves.

Cstat ranged from 1.06 to 10.52 L/kPa, which indicates a range from moderately low to clearly increased if a normal range of 1.5 to 2.5 L/kPa is taken into account (21). Mean Cstat was 4.6 L/kPa (Table 1).

T_{LCO} was between 34% of predicted to 106% of predicted and K_{CO} ranged from low (43% of predicted) to higher than normal (139% of predicted).

Table 2. Correlations of the indexes of the PV curves, CO transfer, and smoking by pairwise multivariate analysis.

Variable 1	Variable 2	Number of patients	*R* Value	p Value
Cstat	KE	19	0.821	<0.0001
Cstat	P90TLC	19	-0.687	0.0012
T_{LCO}, % predicted	Cstat	19	-0.290	0.23
T_{LCO}, % predicted	KE	19	-0.254	0.29
T_{LCO}, % predicted	P90TLC	19	0.123	0.62
KCO, % predicted	Cstat	19	-0.604	0.006
KCO, % predicted	KE	19	-0.414	0.077
KCO, % predicted	P90TLC	19	0.438	0.061
KE	Act smoking*	19	0.534	0.019

*Act smoking = actual smoking (cigarettes/day).

Correlation studies

Pressure-volume curves

Statistically significant correlations existed among all the parameters of the PV curve (Table 2). The strongest correlation was between Cstat and KE ($R = 0.81$; $p < 0.0001$).

The KCO percent predicted correlated strongly with Cstat ($R = -0.60$; $p = 0.006$; Table 2) but not with KE and P90TLC. The T_{LCO} percent predicted showed no significant correlation with Cstat, KE or P90TLC.

Bronchial hyperresponsiveness

The indexes of BHR (PC_{20}, reactivity, and plateau value) were tested for correlation with the indexes of the PV curve (Cstat, KE, P90TLC), CO transfer (T_{LCO} and KCO), and FEV_1. In Table 3, we present the correlations between Cstat and the BHR indexes. No significance was found and no significance was found for the additional elasticity indexes and diffusion parameters.

Table 3. Correlations of the indexes of BHR with quasi-static compliance and FEV_1 by pairwise multivariate analysis.

Variable 1	Variable 2	No. of patients	*R* Value	p Value
PC_{20} histamine	Cstat	19	0.282	0.24
PC_{20} methacholine	Cstat	19	0.061	0.82
Reactivity	Cstat	19	0.291	0.24
Plateau	Cstat	19	0.321	0.19
PC_{20} histamine	FEV_1, % predicted	24	0.483	0.01
PC_{20} methacholine	FEV_1, % predicted	24	0.470	0.02
Reactivity	FEV_1, % predicted	24	-0.522	0.0075
Plateau	FEV_1, % predicted	24	-0.194	0.35

There was a significant correlation between the FEV_1 percent predicted, on the one hand, and $log2PC_{20}$ for histamine and $log2PC_{20}$ for methacholine, on the other hand ($R = 0.44$; $p = 0.024$; and $R = 0.46$, $p = 0.023$, respectively; Table 3). There was also a significant (negative) correlation between the FEV_1 percent predicted and reactivity ($R = -0.52$; $p = 0.008$; Table 3). The correlation between the FEV_1 percent predicted and plateau value was not significant (Table 3).

Because smoking is the most important risk factor for emphysema, we looked at paired correlations among smoking data, CO transfer and indexes of the PV curve. There was a significant correlation only between KE and the actual number of cigarettes smoked per day ($R = 0.53$; $p = 0.019$; Table 2).

Discussion

The aim of our investigations was to study the interrelationships among indexes describing BHR, lung elasticity, and CO transfer in patients with COPD. For the estimation of BHR and the degree of impairment of lung mechanics, detailed information was obtained by fitting models of methacholine dose-response curves and quasi-static PV curves.

Several mechanisms have been proposed for explaining enhanced bronchoconstriction as a reaction to inhaled stimuli (1,19,31). Detailed analysis of methacholine log-dose response curves is supposed to offer additional information on the causative mechanisms of BHR (19). PC_{20} and reactivity are considered to be determined by prejunctional mechanisms, and the plateau value is more dependent on postjunctional mechanisms (19). In patients with COPD, both prejunctional (*ie*, epithelial damage, neural control, and inflammation) and postjunctional mechanisms (*ie*, loss of lung elasticity, swelling of airway wall, and intraluminal secretions) can be responsible for the occurrence of BHR. Because lung elasticity in stable patients with asthma is not appreciably disturbed, this would be an attractive explanation for the occurrence of BHR in COPD and to relate it to the loss of elastic recoil. Theoretically, a decrease in lung elasticity can facilitate an amplified bronchomuscular response (31).

PV-curves and lung parenchyma impairment

First, we have studied the functional indexes of lung parenchymal destruction. Some degree of emphysema, which is present in patients with mild COPD, already influences the PV relationships (10,11,14). A PV curve can be obtained with relatively simple techniques but has the disadvantage of being an invasive test. The reproducibility of estimates, especially of KE, was reported to be good, at least for healthy adults (29,32,33). KE was found to be a good indicator for the presence of mild emphysema (10,15,32). We found that Cstat and P90TLC from the LE model fit and KE from the exponential model fit correlated well with each other, indicating that these indexes were linked to elastic properties of the lung (Table 2).

Elasticity and CO diffusion

An additional index of lung parenchymal destruction is CO diffusion. Berend et al were the first to report a correlation between CO transfer and severity of emphysema (16). Others have confirmed the relationship between emphysema and KCO (15,17,18). We found also a significant correlation of Cstat with KCO (Table 2). KCO can be considered as an index, related to structural aspects of lung parenchyma, whereas T_{LCO} is a measure of overall gas transport.

Lung elasticity and smoking

In this study, we also tested the indexes of the impairment of lung parenchymal structure for correlation with cigarette smoke exposure, pack-years and actual smoking. There was a significant correlation of KE only with actual smoking (Table 2). There are few data about the correlation of smoking with parenchyma impairment. In one study, there was no detectable effect of smoking on lung recoil in healthy men (34). Other investigators have reported a quantitative relationship between the total exposure to cigarette smoke and both alveolar and airway pathologic features in a necropsy study (35). So, although the assumption is plausible that there is a relationship between cigarette smoke exposure and loss of elastic recoil, it is not yet clear how this influences the derivatives of the PV curve. We have assumed that differences in vulnerability of the lung parenchyma to cigarette smoke influence the measured loss of elastic recoil more than the amount of cigarette smoke exposure.

Impairment of lung parenchymal structure and bronchial hyperresponsiveness

In patients with α_1-antitrypsine deficiency, Cheung et al found a relationship between the loss of elastic recoil and maximal airway narrowing (plateau) (36). It should be noted that their patient group was selective; five of eight patients had an FEV_1 >80% of predicted, patients were clinically stable, and patients were ex-smokers or nonsmokers. These patients seemingly had only parenchymal disease. The effect of the involvement of airways disease was shown in a study by Eidelman et al (13). They described different patterns of mechanical abnormalities between smoking and nonsmoking patients with α_1-antitrypsine deficiency. In COPD patients, especially in those who smoke, it is likely that there are both parenchymal and airway changes. In our study and in the study by Koyama et al (37), no significant correlations were found among indexes of the PV curve, on the one hand, and BHR, on the other hand. This means either that PV curves do not represent elastic recoil changes or that BHR is also influenced by airway pathology. There are several arguments that support the last mechanism. First, as discussed above, indexes of PV curves have been found to correlate with pathologic assessment of lung parenchyma. Second, the significant correlations among the different indexes of the PV curve, and between elasticity and KCO, indicate that our results are a good reflection of the loss of elastic recoil of the lung. Third, there were significant relationships between the determinant of airways obstruction (FEV_1) and PC_{20} (Table 3).

Relationship of FEV_1 and the indices of BHR

In the present study, not only were the PC_{20} for histamine and the PC_{20} for methacholine correlated with the FEV_1 percent predicted, but also with reactivity. The first correlation was reported elsewhere (2,8,9) and was found also by Cheung et al (36) and Koyama et al (37). This indicates that the definition of PC_{20} as a 20% fall of the starting FEV_1 makes the outcome highly dependent on measurements of FEV_1 in patients with a low FEV_1. Our finding that reactivity (the slope of the

dose-response curve) is steeper at lower FEV_1 percent predicted, indicates that reactivity also was hampered by the way in which the response is expressed. This appeared to be distinct for the plateau value, which was not correlated with the starting FEV_1 (Table 3). The clinical significance of the level of a plateau value is that it is a measure of the maximal acute bronchoconstriction that can be provoked in an individual. The application of the plateau value in combination with the PC_{20} for methacholine has been suggested for the distinction between asthma and COPD (1). While BHR can be found both in patients with asthma and patients with COPD when considering PC_{20}, the plateau value is usually not reached in patients with moderately severe or severe asthma. In our study of patients with COPD who have moderately severe BHR, the plateau was reached in the majority of the patients, but not in all. It appears, therefore, that the estimation of the plateau does not always provide a clear distinction between asthma and COPD.

BHR and airway pathology in COPD

Because none of the indexes of BHR is related to any of the functional data of lung elasticity or CO transfer in COPD patients who smoke, airway pathology determines the response to methacholine at least to such an extent that it overrules a possible correlation with parenchymal destruction. The nature and extent of airways disease seem to be more important for the occurrence of BHR in patients with COPD than does parenchymal pathology. Taylor et al have compared PC_{20} for methacholine *in vivo* with the function of bronchial smooth muscle strips from surgical specimens (38). No correlation was found, which led to their conclusion that smooth muscle pathophysiologic changes were not responsible for BHR in COPD. One other study provided evidence that BHR in patients with emphysema is related to differences among types of emphysema and to the cell infiltrate in the airway walls (39).

In conclusion, we found no relationship between the impairment of lung parenchymal structure, either from PV curves or CO diffusion, and indexes of BHR. Nonspecific BHR in COPD patients who smoke is determined by small airway pathology to such an extent that it overrules a possible correlation with parenchymal impairment. The combination of our findings with those from clinicopathologic studies suggest that the plateau value (maximal airway constriction) is a better indicator of small airways pathologic changes than are PC_{20} and reactivity.

Acknowledgments:

The authors wish to thank J.P.Dekker-Oosterom, K. van der Velden-van Etten and L.Verdooren for their technical assistance. This study was financially supported by the Netherlands Asthma Foundation and Glaxo-Wellcome, The Netherlands.

References

1. Kerrebijn KF, ed.. Similarities and discrepancies between asthma and chronic obstructive pulmonary disease: a symposium; october 19-21, 1989, Lunteren, The Netherlands. Am Rev Respir Dis 1991; 143: 1419-1472
2. Yan K, Salome CM, Woolcock AJ. Prevalence and nature of bronchial hyperresponsiveness in subjects with chronic obstructive pulmonary disease. Am Rev Respir Dis 1985; 132: 25-29.
3. Tashkin DP, MD Altose, ER Bleeker, et al. The Lung Health Study: airway responsiveness to inhaled methacholine in smokers with mild to moderate airflow limitation. Am Rev Respir Dis 1992; 145: 301-310.
4. Siafakas NM, Vermeire P, Pride NB, et al. Optimal assessment and management of chronic obstructive pulmonary disease (COPD); The European Respiratory Society Task Force. Eur Respir J 1995; 8: 1398-1420.
5. Postma DS, De Vries K, Koëter GH, et al. Independent influence of reversibility of airflow obstruction and non-specific hyperreactivity on the long-term course of lung function in chronic airflow obstruction. Am Rev Respir Dis 1986; 134: 176-180.
6. O'Byrne PM, Ramsdale EH, Hargreave FE. Isocapnic hyperventilation for measuring airway hyperresponsiveness in asthma and in chronic obstructive pulmonary disease. Am Rev Respir Dis 1991; 143: 1444-1445.
7. Ramsdale EH, Morris MM, Roberts RS, et al. Differences in responsiveness to hyperventilation and methacholine in asthma and chronic bronchitis. Thorax 1985; 40: 422-426.
8. Ramsdale EH, Morris MM, Roberts RS, et al. Bronchial responsiveness to methacholine in chronic bronchitis : relationship to airflow obstruction and cold air responsiveness. Thorax 1984; 39: 912-918.
9. Verma VK, Cockcroft DW, Dosman JA. Airway responsiveness to inhaled histamine in chronic obstructive airways disease: chronic bronchitis vs emphysema. Chest 1988; 94: 457-461.
10. Paré PD, Brooks LA, Bates J, et al. Exponential analysis of the lung pressure-volume curve as a predictor of pulmonary emphysema. Am Rev Respir Dis 1982; 126: 54-61.
11. Petty TL, Silvers GW, Stanford RE. Mild emphysema is associated with reduced elastic recoil and increased lung size but not with air-flow limitation. Am Rev Respir Dis 1987; 136: 867-871.
12. Osborne S, Hogg JC, Wright JL, et al. Exponentional analysis of the pressure-volume curve: correlation with mean linear intercept and emphysema in human lungs. Am Rev Respir Dis 1988; 137: 1083-1088.
13. Eidelman DH, Ghezzo H, Kim WD, et al. Pressure-volume curves in smokers. Comparison with alpha-1-antitrypsine deficiency. Am Rev Respir Dis 1989; 139: 1452-1458.
14. Silvers GW, Petty TL, Stanford RE. Elastic recoil changes in early emphysema. Thorax 1980; 35: 490-495.
15. Gugger M, Gould G, Sudlow MF, et al. Extent of pulmonary emphysema in man and its relation to the loss of elastic recoil. Clin Sci (Colch) 1991; 80: 353-358.
16. Berend N, Woolcock AJ, Marlin GE. Correlation between the function and structure of the lung in smokers. Am Rev Respir Dis 1979; 119: 695-705.
17. Eda S, Kubo K, Fujimoto K, et al. The relations between expiratory chest CT using helical CT and pulmonary function tests in emphysema. Am J Respir Crit Care Med 1997; 155: 1290-1294.
18. Morrison NJ, Abboud RT, Ramadan F, et al. Comparison of single breath carbon monoxide diffusing capacity and pressure-volume curves in detecting emphysema. Am Rev Respir Dis 1989; 139: 1179-1187.
19. Sterk PJ, Bel EH. Bronchial hyperresponsiveness : the need for a distinction between hypersensitivity and excessive airway narrowing. Eur Respir J 1989; 2: 267-274.
20. American Thoracic Society. Standards for the diagnosis and care of patients with chronic obstructive pulmonary disease. Am J Respir Crit Care Med 1995; 152(suppl): S77-S120.
21. Quanjer PhH, Tammeling GJ, Cotes JE, et al. Lung volumes and forced ventilatory flows. Eur Respir J 1993; 6(suppl 16): 5-40.

22. Cotes JE, Chinn DJ, Quanjer PhH, et al. Standardization of the measurement of transfer factor (diffusing capacity): report of working party standardization of lung function tests, European Community for Coal and Steel; official statement of the European Respiratory Society. Eur Respir J 1993; 6(suppl 16): 41-52.
23. Cockcroft DW, Killian DN, Mellon JJA, et al. Bronchial reactivity to inhaled histamine: a method and clinical survey. Clin Allergy 1977; 7: 235-243.
24. Sterk PJ, Fabbri LM, Quanjer PhH, et al. Airway responsiveness: standardized challenge testing with pharmacological, physical and sensitizing stimuli in adults. Eur Respir J 1993; 6(suppl 16): 53-83.
25. Juniper EF, Frith PA, Dunnett C, et al. Reproducibility and comparison of responses to inhaled histamine and methacholine. Thorax 1978; 33: 705-710.
26. Aerts JGJV, Bogaard JM, Overbeek SE, et al. Extrapolation of methacholine log-dose response curves with a cumulative gaussian distribution function. Eur Resp J 1994; 7: 895-900.
27. Bogaard JM, Overbeek SE, Verbraak AFM, et al. Pressure-volume analysis of the lung with an exponentional and linear-exponentional model in asthma and COPD. Eur Respir J 1995; 8: 1525-1531.
28. Gibson GJ, Pride NB, Davis J, et al. Exponential description of the static pressure-volume curve of normal and diseased lungs. Am Rev Respir Dis 1979; 120: 799-811.
29. Yernault J-C, Noseda A, Van Muylem A, et al. Variability in lung elasticity measurements in normal humans. Am Rev Respir Dis 1983; 128: 816-819.
30. DuToit JI, Woolcock AJ, Salome CM, et al. Characteristics of bronchial hyperresponsiveness in smokers with chronic air-flow limitation. Am Rev Respir Dis 1986; 134: 498-501.
31. Moreno RH, Hogg JC, Paré PD. Mechanics of airway narrowing. Am Rev Respir Dis 1986; 133: 1171-1180.
32. Greaves IA, Colebatch HJ. Elastic behavior and structure of normal and emphysematous lungs post mortem. Am Rev Respir Dis 1980; 121: 127-136.
33. McCuaig KE, Vessal S, Coppin K, et al. Variability in measurements of pressure-volume curves in normal subjects. Am Rev Respir Dis 1985; 131: 656-658.
34. Yernault JC, Baran D, Englert M. Effect of growth and aging on the static mechanical lung properties. Bull Eur Physiopathol Respir 1977; 13: 777-788.
35. Mitchell RS, Stanford RE, Johnson JM, et al. The morphologic features of the bronchi, bronchioles and alveoli in chronic airway obstruction: a clinicopathologic study. Am Rev Respir Dis 1976; 114: 137-145.
36. Cheung D, Schot R, Zwinderman, et al. Relationship between loss in parenchymal elastic recoil pressure and maximal airway narrowing in subjects with α_1-antitrypsine deficiency. Am J Respir Crit Care Med 1996; 155: 135-140.
37. Koyama H, Nishimura K, Ikeda A, et al. Influence of baseline airway calibre and pulmonary emphysema on bronchial responsiveness in patients with chronic obstructive pulmonary disease. Respir Med 1996; 90: 323-328.
38. Taylor SM, Paré PD, Armour CL, et al. Airway reactivity in chronic obstructive pulmonary disease. Failure of in vivo methacholine responsiveness to correlate with cholinergic, adrenergic, or nonadrenergic responses in vitro. Am Rev Respir Dis 1985; 132: 30-35.
39. Finkelstein R, Ma HD, Ghezzo H, et al. Morphometry of small airways in smokers and its relation to emphysema type and hyperresponsiveness. Am J Respir Crit Care Med 1995; 152: 267-276.

Chapter 3

'Normalized' PC_{20} values are unrelated to baseline FEV_1 but correlate with eosinophilic airways inflammation in patients with Chronic Obstructive Pulmonary Disease

Joachim G.J.V. Aerts, Gert T. Verhoeven, Joost P.J.J. Hegmans, Jan-Bas Prins, Henk C. Hoogsteden, Jan M. Bogaard
Department of Pulmonary and Intensive Care Medicine, Erasmus University Medical Center Rotterdam, The Netherlands.

(Submitted for publication)

Abstract

Study objectives: The interpretation of bronchial provocation tests in COPD is hampered by the correlation of $log2PC_{20}$ with the baseline FEV_1 (FEV_1pre): a lower FEV_1 makes the patient more prone to an abnormal test result. To avoid the influence of FEV_1pre on $log2PC_{20}$ we expressed the response by an alternative expression ($log2nPC_{20}$) based on FEV_1 response as percent predicted and compared the relationships between $log2PC_{20}$ and $log2nPC_{20}$ with eosinophilic airways inflammation.
Patients and methods: 21 patients with COPD were included. Mean (±SD) FEV_1, 65 ± 11% of predicted, mean $log2PC_{20}$ for methacholine equalled 1.1 ±1.7. $Log2PC_{20}$ was calculated as log dose of the percentage change of the FEV_1pre (FEV_1pre – FEV_1post / FEV_1pre), and $log2nPC_{20}$ as the log dose of the quotient of the absolute decrease in FEV_1 and the FEV_1pred (FEV_1pre – FEV_1post/ FEV_1pred). Bronchial biopsy specimens were stained with monoclonal antibodies for major basic protein (MBP), and immunostained areas were measured with an image analysis system. Correlation studies were performed with linear regression analysis.
Results: $Log2PC_{20}$ was positively correlated to FEV_1pre ($p = .04$), whereas $log2nPC_{20}$ did not correlate. $Log2nPC_{20}$ was correlated to MBP count ($p = .004$), while $log2PC_{20}$ failed to reach significance.
Conclusions: $Log2nPC_{20}$ was not correlated to baseline FEV_1 but related to eosinophilic airways inflammation both in contrast to $log2PC_{20}$. $Log2nPC_{20}$ may, therefore, be a more adequate indicator of inflammation in patients with COPD.

Introduction

In patients with asthma, bronchial hyperresponsiveness is related to eosinophilic airways inflammation. Bronchial hyperresponsiveness (BHR) is usually defined as a 20% decrease in FEV_1 from the baseline value ($log2PC_{20}$) at a provocative concentration lower than 8 mg/mL histamine or methacholine (1). In asthma, corticosteroids are known to have a beneficial effect on eosinophilic airways inflammation and consequently on bronchial hyperresponsiveness (2,3). It is therefore generally accepted as standard therapy in these patients (2,3). The use of corticosteroids, either inhaled or oral, in patients with stable COPD is controversial. It is suggested that those patients with COPD who have eosinophilic airways inflammation would benefit from corticosteroid therapy (4-7). In others, the use of corticosteroids may be detrimental (8). Consequently, it is of great value to properly establish the level of eosinophilic airways inflammation in patients with COPD. Until now only measures like bronchoscopy and induced sputum could be used to determine the level of eosinophilic airways inflammation.
In patients with COPD, similar to patients with asthma, bronchial provocation tests are performed to diagnose BHR. In patients with COPD, a positive correlation between the baseline FEV_1 and PC_{20} has been described (9-12). This indicates that at a lower FEV_1, due to the mathematical expression of $log2PC_{20}$, patients are prone to be indicated as hyperresponsive, while for the purpose of a clinical estimate of eosinophilic airways inflammation, the significance of the $log2PC_{20}$ is unknown.
We tested whether a deduced variable from the bronchial provocation test was independent of FEV_1 but related to airways inflammation. $Log2PC_{20}$ was estimated in patients with COPD and related to baseline FEV_1 values, and eosinophilic airways inflammation. Eosinophilic airways inflammation was determined as the number of eosinophils in the lamina propria present in bronchial biopsy specimens from these patients.
We also calculated an alternative expression of $log2PC_{20}$: the provocative concentration at which a 20% decrease in FEV_1 predicted values was obtained. This '$log2nPC_{20}$' was also tested for correlation with the baseline FEV_1 and the number of eosinophils in the airway wall.

Patients and Methods

Patients
Patients with COPD were included according to the following criteria: chronic productive cough, age between 40 and 70 yrs, current smokers, negative skin tests for inhalation allergens, FEV_1 < 70% of predicted normal value (pred) or FEV_1/VC <0.70, reversibility of FEV_1 <10% pred after 750 µg terbutaline inhalation and BHR defined as PC_{20} for histamine < 8 mg/mL (Table 1). Exclusion criteria were a history of asthma, complaints of wheezing, recent respiratory tract infection and recent or concurrent usage of anti-inflammatory drugs. Oral anti-inflammatory

medication was discontinued for at least 3 months and inhaled glucocorticoids at least 6 weeks before the start of the study.
The study was approved by the Medical Ethics Committee of the Erasmus University Medical Center Rotterdam, and written informed consent was obtained from all participants.

Methacholine provocation tests
Methacholine provocation tests were performed according to the 2-min tidal breathing method at 5-min intervals (1,13). A nebulizer (model 646; DeVilbiss Co; Somerset PA, USA) was used to generate aerosols at a measured output of 0.13 mL/min. Methacholine provocation tests were continued up to a concentration of 128 mg/mL or when the FEV_1 fell by more than 60% of the baseline value, or when a plateau was reached (defined as a no further decline in FEV_1 (± 5%) at two consecutive concentrations of methacholine).

Processing of methacholine provocation tests
$Log2PC_{20}$ was calculated by linear intrapolation of the log2 dose of methacholine before and after a 20% decrease in the baseline value of the FEV_1 (FEV_1pre).
In formula:

$$\log2PC_{20} = \log2 \frac{FEV_1pre - FEV_1post}{FEV_1pre}$$

$Log2nPC_{20}$ was calculated by linear extrapolation of the log2 dose of methacholine before and after 20% decrease in predicted FEV_1 values.

In formula:

$$\log2nPC_{20} = \log2 \frac{FEV_1pre - FEV_1post}{FEV_1pred}$$

Predicted FEV_1 values were obtained from published data (European Community for Coal and Steel [14]). When a plateau was reached at FEV_1 values lower than 20% of predicted values patients were excluded. When bronchial provocation tests were interrupted either because of a decline in FEV_1 of more than 60% of the baseline value, or when the maximal provocation dose was reached, the $log2nPC_{20}$ was extrapolated from a cumulative Gaussian distribution curve fitting to the measured data, which we have described earlier (15).

Bronchoscopy
All patients received 0.5 mg atropine intramuscularly. Pulse rate and oxygen saturation was monitored by pulse-oxymetry. Lidocaine spray was used to anaesthetize the pharynx. Oxybuprocaine solution (5mg/mL) was nebulized to anaesthesize vocal cords, trachea and bronchial tree (maximum 20mL). Bronchoscopy was performed

with an Olympus BF 1T10. At least 6 biopsies were taken from the bronchi of the right and the left upper and lower lobes using a fenestrated forceps (FB-18C or FB-20C). All according to published guidelines (16).

Estimation of eosinophilic airways inflammation

Bronchial biopsies were snap-frozen in Tissue-Tek II OCT embedding medium (Miles, Naperville, Illinois, USA) in liquid nitrogen within 10 minutes after collection, and stored at -80°C. Once all biopsy specimens were collected, 6 μm serial tissue sections were cut on a cryostat (model HM-560, Microm, Heidelberg, Germany). At least two sections from one biopsy specimen, 120 μm apart, were placed on a poly-L-lysine-coated microscopic slide (Sigma Diagnostics; St.Louis, MO, USA). Next, sections were air dried for 30 min and stored at -80°C until use. Immunostaining was carried out with a monoclonal antibody directed against Major Basic Protein (MBP) (BMK13 from Dako, Glostrup, Denmark). Binding of the antibodies was detected by the immuno-alkaline phosphatase anti-alkaline phosphatase (APAAP) method (Dako).

Immunostained sections were analyzed with an image analysis system by an investigator unaware of the other subject information (JPJJH). The image analysis hardware consisted of a 3CCD (charge-coupled device) color video camera (DXC-950; Sony, Tokyo, Japan) mounted on a microscope (DM RBE, Leica, Rijswijk, The Netherlands) and connected to a Matrox Meteor frame grabber in a Pentium 200 MHz personal computer. Illumination was provided by a halogen light-source, connected to a stabilized adjustable power supply (12V/100W). Images were taken at 10x10 magnification.

The image analysis software consisted of the QWin Standard software (Leica) running under Microsoft Windows environment. Images were analyzed by interactively setting the reticular basement membrane. The program was set to analyze 100 μm deep into the lamina propria. The outer perimeter of the intact epithelium was interactively set. Detection of the color of interest was done by setting thresholds for hue, saturation and intensity. During the image analysis procedure, hue, saturation and intensity were kept constant during the analysis of the entire study population. This approach allows for a densitometric analysis. The immunostained areas (mm^3) were divided by the field measurement (mm^3).

Statistics

Results are expressed as means ±standard deviations, unless otherwise mentioned. Linear regression analysis between variables and statistical significance were calculated with the use of a package of statistical software (Statistical Graphics Corp; Rockville, MD). Test results were considered statistically significant at $p < 0.05$.

Results

Twenty-one patients were included in the study. From all participants lung function tests and bronchial biopsy specimens were obtained. They had moderate-to-severe COPD and moderate or severe bronchial hyperresponsiveness (Table 1). The mean age of the patients was 54 ± 8 years (17 male, 4 female). The mean FEV_1 equalled 2.07 ±0.47 L. MBP count equalled 0.86 ±1.16 (mm^3/mm^3).

Table 1. Patient characteristics.

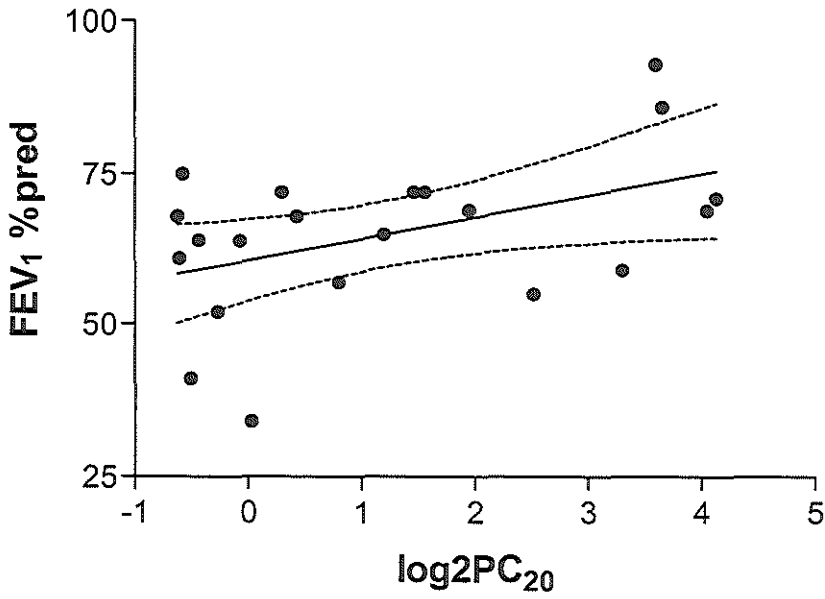
	N	mean ±SD	median	Range
Age, yrs	21	56.3 ±8.9	60	42 – 69
Actual smoking, cigarettes/day	21	15.4 ±7.4	13	6 – 30
Pack-years	21	25.3 ±11.2	21	5 – 50
FEV_1, % predicted	21	62.5 ±12.9	65	34 – 93
Reversibility, % predicted	21	5.3 ±3.1	5	0 - 9.0
PC_{20}, mg/mL				
For histamine	21	1.7 ±2.1	0.87	0.11 – 8
For methacholine	21	4.6 ±5.5	1.72	0.6 - 17.4
Eosinophils, MBP count, mm^3/mm^3	18	0.86 ±1.16	0.39	0.03 – 4.46

In three patients no $log2nPC_{20}$ could be calculated because in these patients a plateau of the measured decline in FEV_1 was reached below the level necessary to obtain a 20% decrease in predicted FEV_1. These patients were excluded from further analysis.

Mean (±SD) $log2PC_{20}$ for methacholine was 1.1 ±1.7. A positive correlation between $log2PC_{20}$ and FEV_1 starting value was obtained (Figure 1, $R = 0.53$, $p = 0.04$). There was no significant correlation between $log2PC_{20}$ and MBP count ($p > .2$).

$Log2nPC_{20}$ for methacholine equalled 3.9 ±1.9. No correlation between $log2nPC_{20}$ and FEV_1 was obtained ($p > .1$). A statistically significant negative correlation was found between MBP count and $log2nPC_{20}$ (Figure 2, $R = -0.58$, $p = 0.04$).

Figure 1. Correlation of $log2PC_{20}$ and FEV_1 in percentage of predicted normal values (FEV_1pred) in 21 patients with COPD; $R = 0.53$, $p = 0.04$.

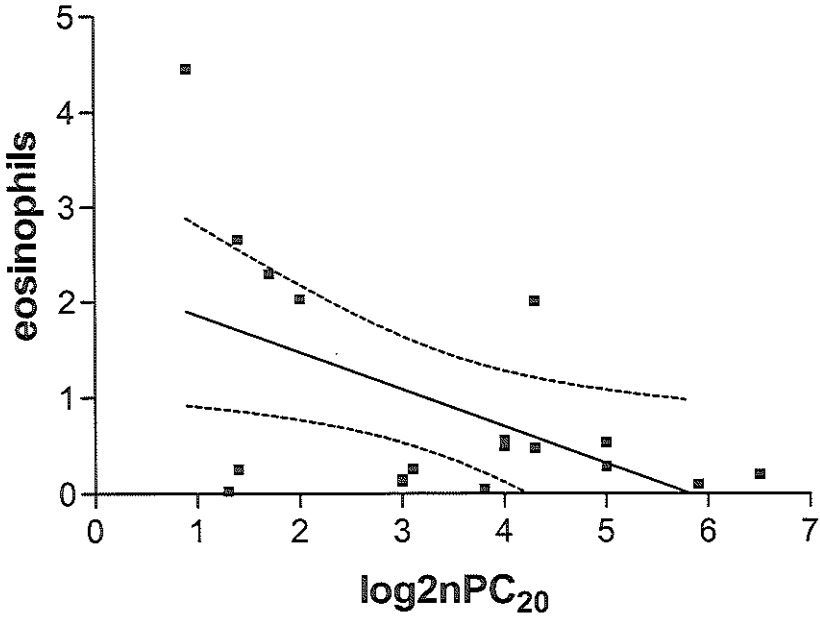


Discussion

This study confirms the earlier described relationship between baseline FEV_1 and the provocative concentration at which a 20% decrease of the baseline FEV_1 is reached ($log2PC_{20}$), in patients with COPD (2-5). An alternative expression of bronchial hyperresponsiveness, the concentration of a provocative agent at which a 20% decrease in predicted FEV_1 was reached ($log2nPC_{20}$), was found to be irrespective of the baseline FEV_1. This log2nPC20 was found to be correlated with eosinophilic airways inflammation whereas $log2PC_{20}$ was not.
Three patients were excluded from further analysis as no $log2nPC_{20}$ could be measured. A plateau of the decline in FEV_1 during the test was reached at a level below a 20% decrease in predicted values. In all three patients low levels of eosinophilic airways inflammation were found which is another indication for the relationship between eosinophilic airways inflammation and $log2nPC_{20}$.
Low $(log2)PC_{20}$ values were found in more than half of the subjects with chronic airflow limitation, who were current smokers (6,9,16). Due to the known positive correlation between FEV_1 and $log2PC_{20}$, the level of $log2PC_{20}$ could be influenced to a greater extent by the baseline FEV_1, than by the contractility of smooth muscles in the walls of the small airways. Small absolute changes become large percentage changes in subjects with a low baseline FEV_1. This problem is comparable to the

difficulties in the interpretation of the measurements of reversibility (17). The relation between FEV_1 and $log2PC_{20}$, and the lack of studies regarding this subject are the reasons why the clinical meaning of a low $log2PC_{20}$ in patients with COPD is still unclear (18).

Figure 2. Correlation of $log2nPC_{20}$ and the number of eosinophils in bronchial biopsies in 18 patients with COPD; $R = -0.58$, $p = 0.04$.



Bronchial hyperresponsiveness, established with pulmonary function testing with a provocative agent ($log2PC_{20}$), is one of the keystones for the diagnosis of asthma. In patients with asthma, BHR is related to eosinophilic airways inflammation. The standard estimate of BHR, $log2PC_{20}$, was related to the number of eosinophils in the airway wall (19,20), although there also have been published negative results (21).
As far as we know, no study has been published concerning the relationship between eosinophilic airway inflammation and $log2PC_{20}$ in patients with COPD. Several recent studies in patients with COPD show a relationship between eosinophilic airways inflammation and beneficial effects of glucocorticosteroids (4-7).
In these studies the level of eosinophilic airways inflammation was determined by using induced sputum. Induced sputum is now regarded as a minimal-invasive technique for the estimation of the number of eosinophils involved in the

inflammatory process in the lungs. This technique has some major drawbacks (22). It has an invasive character and is not suitable in every patient. In patients with BHR, the inhalation of hypertonic saline may lead to uncontrolled and dangerous levels of bronchoconstriction. Next to the difficulties with obtaining a proper specimen, the laboratory work on determining eosinophil counts is complex (22).
Bronchial provocation testing, using either methacholine or histamine, is a uniformly used technique for establishing BHR. If we could obtain from this test information regarding the level of eosinophilic airways inflammation, it may be possible to identify those patients with stable COPD who benefit from long-term glucocorticosteroid therapy and, even so important, to preclude glucocorticosteroid therapy in others.
In this study we have normalized $log2PC_{20}$ measurements by taking into account predicted FEV_1 values. This $log2nPC_{20}$ was irrespective of baseline FEV_1 and was related to eosinophilic airways inflammation as analyzed in bronchial biopsy specimens, both in contrast to $log2PC_{20}$. Additional studies are needed for establishing whether COPD patients with low $log2nPC_{20}$ levels are more prone to benefit from long-term glucocorticosteroid therapy.

Acknowledgements

This study was financially supported by Glaxo-Wellcome, the Netherlands (FLIL44/FMS40060) and the Netherlands Asthma Foundation.

References

1. Sterk PJ, Fabbri LM, Quanjer PhH, et al. Airway responsiveness. Standardized challenge testing with pharmacological, physical and sensitizing stimuli in adults. Eur Respir J 1993; 6(suppl 16): 53-83.
2. Boulet L-P, Turcotte H, Laviolette M, et al. Airway hyperresponsiveness, inflamation, and subepithelial collagen deposition in recently diagnosed versus long-standing asthma. Am J Respir Crit Care Med 2000; 162:1308-1313.
3. Giannini DA, Di Franco A, Cianchetti, et al. Analysis of induced sputum before and after withdrawal of treatment with inhaled corticosteroids in asthmatic patients. Clin Exp Allergy 2000; 30:1777-1784.
4. Chanez P, Vignola AM, O'Shaugnessy T, et al. Corticosteroid reversibility in COPD is related to features of asthma. Am J Respir Crit Care Med 1997; 155:1529-34.
5. Pizzichini E, Pizzichini MM, Gibson P, et al. Sputum eosinophilia predicts benefit from prednisone in smokers with chronic obstructive bronchitis. Am J Respir Crit Care Med 1998; 158:1511-17.
6. Fujimoto K, Kubo K, Yamamoto H, et al. Eosinophilic inflammation in the airway is related to glucocorticoid reversibility in patients with pulmonary emphysema. Chest 1999; 115:697-702
7. Brightling C, Monteiro W, Ward R, et al. Sputum eosinophilia and short-term response to prednisolone in chronic obstructive pulmonary disease: a randomised controlled trial. Lancet 2000; 356:1480-1485.
8. Barnes PJ. Inhaled corticosteroids are not beneficial in chronic obstructive pulmonary disease. Am J Respir Crit Care Med 2000; 161:342-343.
9. Yan K, Salome CM, Woolcock AJ. Prevalence and nature of bronchial hyperresponsiveness in subjects with chronic obstructive pulmonary disease. Am Rev Respir Dis 1985; 32:25-29

10. Ramsdale EH, Morris MM, Roberts RS, et al. Bronchial responsiveness to methacholine in chronic bronchitis: relationship to airflow obstruction and cold air responsiveness. Thorax 1984; 39:912-18.
11. Verma VK, Cockcroft DW, Dosman JA. Airway responsiveness to inhaled histamine in chronic obstructive airways disease. Chronic bronchitis vs. emphysema. Chest 1988; 94:457-61.
12. Verhoeven GT, Verbraak AFM, Boere-van der Straat S, et al. Influence of lung parenchymal destruction on the different indices of the methacholine dose-response curves in COPD patients. Chest 2000; 117:984-90.
13. Cockcroft DW, Killian DN, Mellon JJA, et al. Bronchial reactivity to inhaled histamine: a method and clinical survey. Clin Allergy 1977; 7:235-43.
14. Quanjer PhH, Tammeling GJ, Cotes JE, et al. Lung volumes and forced ventilatory flows. Eur Respir J 1993; 6(suppl 16): 5-40.
15. Aerts JGJV, Bogaard JM, Overbeek SE, et al. Extrapolation of methacholine log-dose response curves with a Cumulative Gaussian Distribution function. Eur Respir J 1994; 7:895-900.
16. Tashkin DP, Altose MD, Bleeker ER, et al. The Lung Health Study: airway responsiveness to inhaled methacholine in smokers with mild to moderate airflow limitation. Am Rev Respir Dis 1992; 145:301-10.
17. Weir DC, Burge S. Measure of reversibility in response to bronchodilators in chronic airflow obstruction: relation to airway calibre. Thorax 1991; 46:43-45.
18. Siafakas NM. ERS-Consensus statement: Optimal assessment and management of chronic obstructive pulmonary disease (COPD). Eur Respir J 1995; 8:1398-1420.
19. Bradley BL, Azzawi M, Jacobson M, et al. Eosinophils, T-lymphocytes, mast cells, neutrophils and macrophages in bronchial biopsy specimens from atopic subjects without asthma and normal control subjects and relationship to bronchial hyperresponsiveness. J Allergy Clin Immunol 1991; 88:661-74.
20. Chetta A, Foresi A, Del Donno M, et al. Basement thickness is related to epithelial cellular infiltrate, bronchial responsiveness and airway patency parameters in asthma. Am J Respir Crit Care Med 1995; 151:A132
21. Möller GM, Overbeek SE, van Helden-Meeuwsen CG, et al. Eosinophils in the bronchial mucosa in relation to methacholine dose-response curves in atopic asthma. J Appl Physiol 1999; 86:1352-6.
22. Rytila PH, Lindqvist AE, Laitinen LA. Safety of sputum induction in chronic obstructive pulmonary disease. Eur Respir J 2000; 15:1116-1119.

Chapter 4

Effects of Fluticasone propionate in COPD patients with bronchial hyperresponsiveness

Gert T.Verhoeven[1], Joost P.J.J. Hegmans[1], Paul G.H. Mulder[2], Jan M. Bogaard[1], Henk C. Hoogsteden[1], and Jan-Bas Prins[1]
[1]Department of Pulmonary and Intensive Care Medicine, and [2]Department of Biostatistics, Erasmus Medical Center Rotterdam, The Netherlands.

(Submitted for publication)

Abstract

Background - Treatment of chronic obstructive pulmonary disease (COPD) with inhaled glucocorticoids does not appear to be as effective as similar treatment of asthma. It appears that only certain subgroups of patients with COPD benefit from steroid treatment. Therefore, we examined whether inhaled fluticasone propionate (FP) had an effect on lung function and on indices of inflammation in a subgroup of COPD patients, with bronchial hyperresponsiveness (BHR) in particular.
Methods - We included 23 patients with COPD based on the following criteria, chronic productive cough, forced expiratory volume in one second (FEV_1) ≤ 70% of the predicted normal value (%pred), reversibility of FEV_1 < 10% predicted after inhalation of 750 mcg terbutaline, negative serology (Phadiatop test) and negative skin prick test for standard inhaled allergens. Patients had to be persistent current smokers between 40 and 70 years of age. Nonspecific BHR was defined by a PC_{20} for histamine of ≤ 8 mg/ml. Patients received either 2 x 500 mcg FP or placebo for 6 months. Expiratory volumes were measured at monthly visits, BHR was determined at the start, after 3 and after 6 months, bronchial biopsies were taken at the start and after 6 months. Biopsies from healthy smokers served as controls.
Results - Unlike in asthma, indexes of BHR were not significantly influenced by FP treatment. There was a steep decline of the FEV_1 in the placebo group, while the FEV_1 in the FP treated patients remained stable. FEV_1/FVC, MEF50 and MEF25 were significantly increased in the FP treated patients compared to the control group, while FVC and PEF were not affected. Biopsy specimens were analyzed for the presence of $CD3^+$, $CD4^+$, $CD8^+$, MBP^+, $CD15^+$, $CD68^+$, $CD1a^+$ and $tryptase^+$ cells. After FP treatment, the numbers of $CD8^+$, MBP^+ and $CD68^+$ cells were significantly reduced in the lamina propria. In the epithelium, the number of $tryptase^+$ cells was significantly reduced.
Conclusion – In patients with COPD and BHR, FP compared to placebo, has a positive effect on indexes of lung function while bronchial inflammation as analyzed in bronchial biopsy specimens is reduced.

Introduction

Chronic obstructive pulmonary disease (COPD) is a disorder characterized by reduced maximum expiratory flow and slow forced emptying of the lungs. About 15% of the smoking population develops COPD. The airways in COPD are markedly inflamed. However, the predominant types of inflammatory cells and the main anatomical site of the lesions appear to differ from those in asthma (1-3).
Inhaled glucocorticoids (GCs) are the most prominent medication used in the treatment of asthma and COPD. However, while treatment with GCs is the potent therapy available for patients with asthma, the beneficial effects of steroid treatment in COPD are subject of debate. The improvements of lung function parameters, which are characteristic in case of steroid treatment in asthma, have not been unequivocally found in COPD (4-7). It has been postulated that if steroids are important in the treatment of COPD, they act via down- regulation of cytokine and adhesion molecule expression, with a consequent reduction in cell migration and activation (8). To date, the number of studies reported dealing with the effect of steroid treatment on inflammation in COPD is limited.
Approximately 25% of patients with stable COPD could, however, benefit from continuous steroid treatment (9). Earlier, it was suggested that inhaled glucocorticoids were more likely to have beneficial effects in patients with COPD when asthmatic features were present (10). Most recently, a beneficial effect of prednisolone was shown in patients with COPD who had increased numbers of sputum eosinophils (11). In a large multicenter trial with patients with respiratory symptoms, the effect of an inhaled steroid on the course of FEV_1 was found to be most prominent in patients with more severe bronchial hyperresponsiveness (BHR) (12). BHR is usually regarded as a distinguishing characteristic of asthma, but is surprisingly often found in patients with COPD and in smokers (13,14). The features of BHR, however, differ between asthma and COPD. In COPD, the presence of BHR is associated with more severe disease, leading to an even more accelerated decline of lung function (FEV_1) resulting in reduced life expectancy (15-18). It could be an indicator of a more serious and/or a different inflammatory process in the airways of these patients with COPD (19-21).
Therefore, we speculated that patients with COPD and BHR would benefit from inhaled steroid treatment. We analyzed indexes of lung function and markers of inflammation in the larger airways before and after treatment in this group of patients. Indexes of lung function were determined at the start and at four weeks intervals up to 6 months into the study during which the patients received either FP, twice daily 500 mcg, or placebo. Bronchial biopsies were obtained at the start and 6 months later at the end of the study. Immunohistochemical analysis of biopsy specimens was performed to identify and quantify markers of inflammation.

Participants, materials and methods

Patients

Patients with COPD (n = 23) were selected according to generally accepted criteria (22). In short, these criteria were chronic productive cough, $FEV_1 \leq 70\%$ of the predicted normal value (%pred), reversibility of FEV_1 of < 10 % predicted after 750 µg terbutaline administered by metered-dose inhalation, negative serology (Phadiatop test) and negative skin prick tests for standard inhaled allergens. Also, patients with an FEV_1/inspiratory vital capacity (IVC) ratio ≤ 0.70 were included, provided their TLC was greater than the predicted value plus 1.64 SD. Reference values were obtained from EGKS standards (23). Participants had to be current and persistent smokers between 40 and 70 years of age. Nonspecific BHR was defined by a PC_{20} for histamine ≤ 8 mg/mL. FEV_1 had to be > 1.2 L to ensure complete safety in view of the bronchial provocation tests. Patients' characteristics are listed in table 1.

Subjects were excluded if they had a history of asthma characterized by attacks of dyspnoea, chest tightness or wheezing; respiratory tract infection in the four weeks preceding the first visit or were suffering from serious or unstable concomitant disease.

Eligible patients using anti-inflammatory therapy including NSAIDs were asked to refrain from oral prescriptions for at least three months and from inhaled glucocorticoids, sodium cromoglycate or nedocromil sodium for at least six weeks prior to the start of the study. Also, longacting β2-agonists, xanthine derivatives and anti-histamine drugs had to be stopped at least six weeks before the start of the study. If after discontinuation of drug therapy deterioration of symptoms or lung function occurred, patients were excluded from the study.

Asymptomatic smokers with a normal maximal expiratory flow-volume (MEFV) curve, PC_{20} for histamine > 8 mg/mL and absence of allergy (negative Phadiatop test) were selected and served as control group (n = 6; Table 1).

After an overnight fast, blood was drawn for measurement of circulating cortisol levels early in the morning prior to lung function tests. The concentration of cortisol was determined using kits supplied by Diagnostics Products Corporation (Los Angeles, California, USA) at the Department of Internal Medicine of our University Hospital (24).

The hospital's Medical Ethics Committee approved the study. Written informed consent was obtained from all participants.

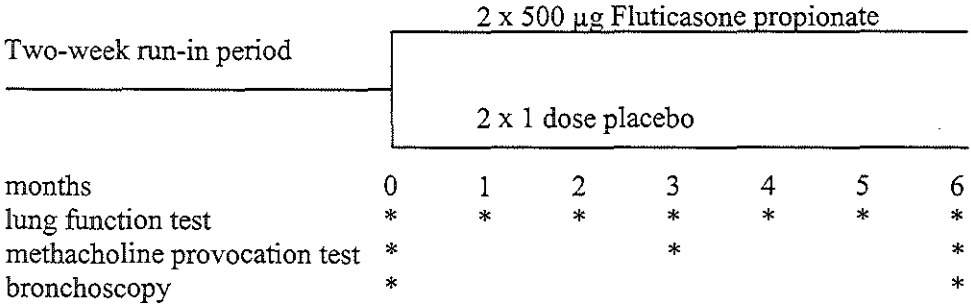
Study design

We performed a double blind, placebo-controlled study with a treatment period of six months. The design is schematically presented in figure 1.

During the two-weeks' run-in period, subjects underwent lung function tests. Eligible patients were randomly allocated to either the treatment group (n = 10), which received twice-daily 500 µg FP via a diskhaler, or the placebo group (n = 13), which received their prescription via a similar device. Salbutamol via diskhaler

was given as rescue medication. Inhalation of ipratropium bromide was allowed at constant dosages. At the start of the treatment period and after 6 months, bronchial biopsies were collected. Every four weeks, participants visited our outpatient clinic for a follow-up, to have their used and unused medication of the preceding period taken in, and to have their MEFV curve determined, before and after inhalation of 750 µg terbutaline. BHR was tested with methacholine at the start, three months into and at the end of the treatment period.

Figure 1. Study design



Lung function tests

At baseline, spirometry and the closed circuit Helium wash-in method were used to obtain spirometric volumes and lung capacities (total lung capacity [TLC], functional residual capacity [FVC], IVC, FEV_1). At the start and at each fourth week visit, MEFV curves were recorded with a heated pneumotachometer (Jaeger, Wurzburg, Germany). From the MEFV curve, the forced vital capacity (FVC), the peak expiratory flow (PEF), the maximal expiratory flow at 50% of FVC (MEF50), and the maximal expiratory flow at 25% of FVC (MEF25), were derived. All MEFV curves were recorded at the same time of the day. Patients were instructed not to take any bronchodilators at least 8 hours prior to lung function tests.

Histamine and methacholine provocation tests were performed according to the 2-min tidal breathing method. Histamine was used to determine PC_{20} (inclusion criterion), while methacholine was used to establish a full dose-response curve. Solutions of histamine-sulphate and acetyl-ß-methylcholinebromide were prepared at the hospital's Pharmacy Department. Solutions were stored at 4°C and were used at room temperature. Aerosols were generated by a nebulizer (model 646; DeVilbiss Co; Somerset, PA, USA) (measured output 0.13 mL/ min) and were inhaled by tidal breathing over a 2-min period at 5-min intervals. After inhalation of an isotonic saline solution, doubling concentrations (0.03 – 256 mg/ml) of histamine or methacholine were administered. The response to methacholine or

histamine was measured as the change in FEV_1 expressed as a percentage of the initial value. FEV_1 was derived from the pneumotachograph readings. PC_{20} was calculated from these data. Histamine provocation tests were interrupted if a 20% fall of FEV_1 was achieved at a histamine concentration ≤ 8 mg/mL. Methacholine tests were interrupted if the FEV_1 fell by more than 60%, or if unpleasant side effects occurred. After the test, the patient inhaled a bronchodilator to restore the FEV_1 to the initial value.
From the methacholine provocation tests, the log2 concentration and the measured percentage changes in FEV_1 were imported to a computer program that fitted a sigmoid function (cumulative Gaussian distribution) to the data (25). Reactivity (the slope of the curve), plateau values (maximal bronchoconstriction), and EC_{50} (the concentration of the stimulus causing 50% of maximal bronchoconstriction) were taken from this model fit.

Bronchoscopy
The procedure was carried out essentially as described in the ATS guidelines (26). At the start of the procedure, the patient received 0.5 mg atropine intramuscularly. A pulse-oxymeter was applied to a fingertip of the patient to monitor pulse rate and oxygen saturation. Mouth and pharynx were anaesthetized with a lidocaine spray. Next the vocal cords, trachea and bronchial tree were anaesthetized with aerosolized oxybuprocaine-solution (5 mg/mL) up to a maximum of 20 mL. Bronchoscopy was performed with the Olympus BF 1T10. At least 6 biopsies were taken from the subcarinae of both the right and the left upper and lower lobes with a fenestrated forceps (FB-18C or FB-20C). Sedative drugs and/or oxygen were administered whenever thought necessary by the physician or requested by the patient.

Processing of the bronchial biopsies
Bronchial biopsies were snap-frozen in Tissue-Tek II OCT embedding medium (Miles, Naperville, Illinois, USA) in liquid nitrogen within 10 min after collection, and stored at -80°C. Once all biopsy specimens were collected, 6 µm serial tissue sections were cut on a HM-560 cryostat (Microm, Heidelberg, Germany). At least two sections 120 µm apart from one biopsy specimen were placed on a poly-L-lysine-coated microscopic slide (Sigma Diagnostics, St.Louis, MO, USA). Next, sections were air dried for 30 min and stored at -80°C until use. Immunostaining was carried out with: αCD3 (UCHT1), αCD4 (MT310), αCD8 (DK25), and αCD25 (ACT-1) (Becton Dickinson, San Jose, CA, USA); αCD1a (NA1/34), αCD15 (C3D-1), αCD68 (EMB11), and αMBP (BMK13) (Dako, Glostrup, Denmark); α-tryptase (Chemicon Brunschwig Chemie, Temecula, CA, USA); αEG2 (Pharmacia).
Binding of the antibodies was detected by the immuno-alkaline phosphatase anti-alkaline phosphatase (APAAP) method. Sections were fixed in acetone for 10 min at RT, rinsed in phosphate buffered saline (PBS) pH 7.2 and placed in a semi-automatic stainer (Shandon, Pittsburgh, PA, USA). Slides were sequentially incubated with bovine serum albumin (BSA) 2% in PBS for 10 min, incubated with

10% normal rabbit serum in PBS (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands) for 10 min. Next, they were incubated with the appropriate mAbs for 60 min at RT. Subsequently, sections were rinsed twice in PBS for 5 min and incubated with a rabbit anti-mouse (RαM) (1:50) immunoglobulin serum for 30 min. Next, slides were rinsed twice in PBS, incubated with APAAP (1:50) (Dako, Glostrup, Denmark) for 30 min at 20°C, rinsed in PBS and Tris buffer (pH 8.0), and incubated for 30 min with New Fuchsin substrate (Chroma, Stuttgart, Germany) as chromogen. Sections were counterstained with Mayers hematoxylin.

Immunostained sections were analyzed with an image analysis system. The image analysis hardware consisted of a 3CCD (charge-coupled device) color video camera (model DXC-950; Sony, Tokyo, Japan) mounted on a microscope (model DM RBE; Leica, Rijswijk, The Netherlands) and connected to a Matrox Meteor frame grabber in a Pentium 200 MHz personal computer. Illumination was provided by a halogen light-source, connected to a stabilized adjustable power supply (12V/100W). Since the emission of light-sources and the noise and sensitivity of CCD cameras are known to vary with temperature, all hardware was allowed a 1 hour warming-up period to minimize unwanted thermal effects. Images were taken at 10x10 magnification.

The image analysis software consisted of the QWin Standard software (Leica) running under Microsoft Windows environment. Images were analyzed by interactively setting the reticular basement membrane. The program was set to analyze 100 μm deep into the lamina propria. The outer perimeter of the intact epithelium was interactively set. Detection of the color of interest was done by setting thresholds for hue, saturation and intensity. During the image analysis procedure, hue, saturation and intensity were set for each immunohistochemical staining and kept constant during the analysis of the entire study population for that particular staining. This approach allows for a densitometric analysis. However, this makes a comparison between marker molecules impossible.

The immunostained areas were divided by the field measurement, which consisted of the total area, either of the epithelium or the lamina propria. If the total area studied was less than 100.000 μm^2 for a given subject, this subject was excluded from further analysis of that particular inflammatory marker.

Statistics

Results are expressed as mean ± SD, unless otherwise mentioned. Differences between groups were analyzed with the unpaired Student's *t* test or the Mann-Whitney U-test where appropriate. Paired data were tested for significance with the paired Student's *t* test or, after ln-transformation, the Wilcoxon matched pairs test where appropriate. A rmANOVA was performed for the dependent variables FEV_1 (as percent predicted), FVC, FEV_1/FVC, PEF, MEF50 and MEF25, after ln-transformation. From each of these variables, one baseline measurement at the start of the intervention and 6 repeated measurements during treatment were available. In the rmANOVA model, the independent variables are time (a within-subject

categorical factor with 6 levels), treatment (a between-subjects factor with 2 levels), and the baseline measurement of the outcome variable considered as a between-subjects continuous and constant variable. The within-subject residual covariance structure is assumed to be of the type "compound symmetry". Interest is in the effect of treatment and its interaction with time. The question of interest is whether there is a difference between the two treatment groups and whether this difference changes over time. Significance is accepted if $p < 0.05$.

Results

Patients

Participants of the study had a mild to moderate degree of COPD according to ERS criteria and moderately to severe BHR. At baseline, characteristics were not significantly different between the FP and placebo group. Asymptomatic smokers and patients with COPD could be discriminated based on a significantly different FEV_1 and FEV_1/ FVC (Table 1).

Compliance with study medication was high. Percentages of returned used blisters were not different between the groups, 92.5 in the FP group and 90.7 in the placebo group.

During the treatment period, no subjects dropped out of the study. Periodically, several participants noticed worsening of disease-related symptoms, like coughing and production of sputum. In all cases, these symptoms could be resolved with rescue medication while not violating the inclusion criteria.

Adverse events were reported 25 times in the FP treated group and 28 times in the placebo group ($p > 0.05$). Adverse events related to airways' disease and/or study medication were reported more often by patients receiving placebo as compared to FP treated patients (18 vs 7, $p = 0.02$). These adverse events included candidiasis of mouth (in the FP treated group), stomatitis, throat pain, irritation of pharynx, upper respiratory tract infection, acute bronchitis without decrease in FEV_1, and mild increase of airways' obstruction.

The use of rescue medication was not different between the two groups of patients ($p > 0.05$). The mean numbers of used salbutamol dosages were 32.5 ±27 and 40.4 ±42.7 per month in the FP group and in the placebo group, respectively. The mean numbers of used ipratropium bromide dosages were 2.2 ±4.7 and 9.6 ±3.0 per month in the FP group and in the placebo group, respectively.

Levels of circulating cortisol were monitored. At the start of the study, mean levels were 360.9 ±112 and 335 ±87 nMol/L in the FP and the placebo group, respectively. After 6 months, mean levels were 414.6 ±156 and 430 ±104 nMol/L. Levels were not significantly different between groups at a given time-point, nor were they significantly different between time-points.

Lung function

During the treatment period of six months, the mean PC_{20} for methacholine had increased in the FP treated patients relative to the placebo group (Fig. 2). This

increase was, however, not statistically significant. No significant changes of reactivity, maximal bronchial constriction (plateau) and EC_{50} were detected either (Fig. 3).

Table 1. Subject characteristics (mean ±SD)

	Asymptomatic smokers (n = 6)	*Fluticasone propionate* (n = 10)	*Placebo* (n = 13)
Male/ female ratio	3/3	8/2	11/2
Age (yrs)	44 ±9	54 ±8	56 ±8
Actual smoking	21 ±10.0	15 ±8.2	16 ±6.7
Pack years	23 ±12.4	25.2 ±12.3	26 ±9.7
Serum IgE (U/L)	N.D.	72 ±64	81 ±127
Blood eosinophils ($x10^3$/L)	N.D.	163 ±98	195 ±91
FEV_1 (L)	2.78 ±0.48*	2.09 ±0.45	2.02 ±0.48
FEV_1 (%pred)	93 ±11¶	66 ±13	61 ±12
Reversibility (%pred)	4.0 ±3.0	5.3 ±3.7	5.6 ±2.7
FEV_1/ FVC	0.79 ±0.03	0.56 ±0.12	0.56 ±0.10
PC_{20} for histamine (mg/ mL)	> 8	2.6 ±2.4	0.8 ±0.6
PC_{20} methacholine (mg/ mL)	N.D.	6.3 ±5.9	2.6 ±4.3

* p = 0.02 and ¶ p = 0.01 (asymptomatic smokers vs COPD patients), N.D. = not done.

Changes of mean FEV_1 after bronchodilation are shown (Fig. 4A). A significant decline of the FEV_1 was detected in the placebo group while the FEV_1 in the FP treated group remained almost unchanged. The mean decline of the FEV_1 in the placebo group compared to the FP treated patients was 6.92 %pred before bronchodilation and 7.97 %pred after bronchodilation. The course of the pre- and that of the post-bronchodilator FEV_1 were significantly different between the FP treated patients and the placebo group (pre-bronchodilator, p = 0.015; post-bronchodilator, p = 0.0002 (rmANOVA); Fig. 4B). The starting values were taken as co-variants for the rmANOVA tests. The FEV_1/ FVC ratio (pre-bronchodilator) showed a similar pattern of change, mean difference was 0.065, p = 0.024 (rmANOVA). There were significant changes of MEF50 from the second month onwards [0.29 ±0.07 L (mean ±SD)], and of MEF25 from the third month onwards [0.32 ±0.09 L (mean ±SD)] (*t*-test for equality of mean changes) (Fig. 5). Both FVC and PEF were not affected and remained unchanged in both groups.

Figure 2. Course of the mean $log2PC_{20}$ for methacholine during the treatment period of 6 months with either Fluticasone propionate (closed circles) or placebo (open circles). $P > 0.05$ (rmANOVA).

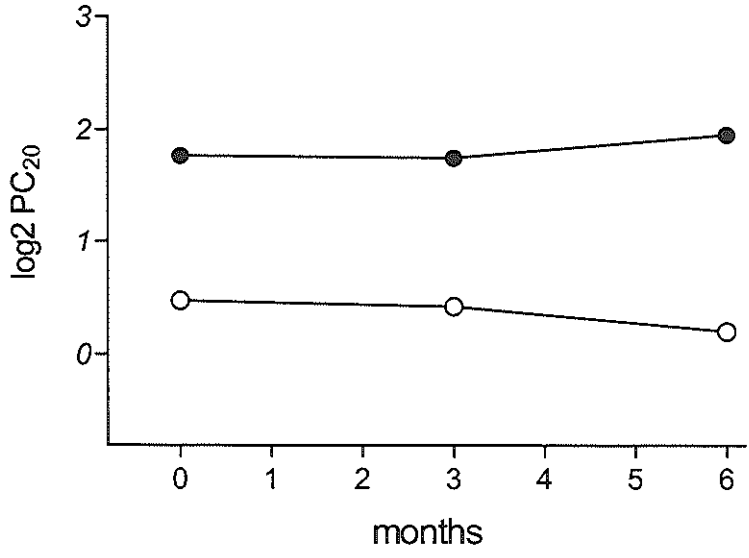
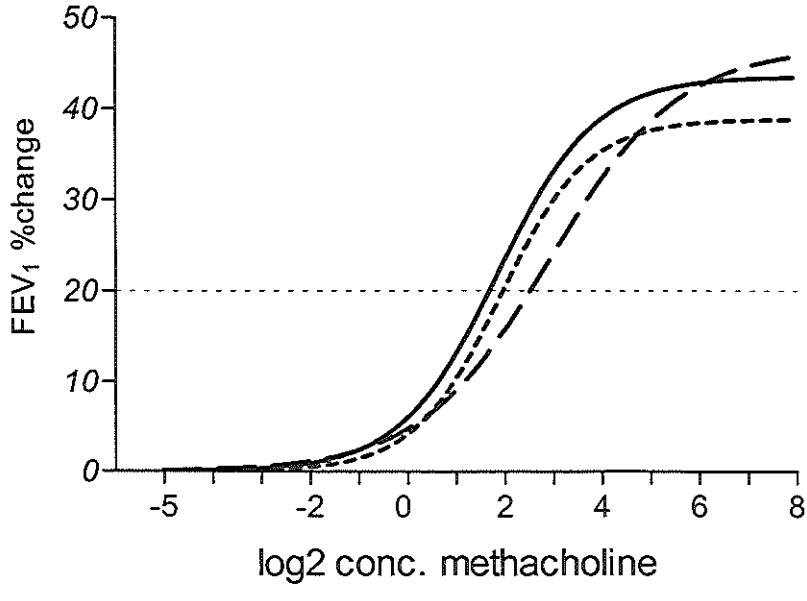


Figure 3. Fitted methacholine dose-response curves, mean of the Fluticasone propionate treated patients, at the start of the treatment (------), 3 months into the treatment (———), 6 months into the treatment (end of study, – – – –).



Bronchial inflammation

Bronchial biopsy specimens were analysed for the presence of inflammatory cells characterized by cell surface molecules and/or cytoplasmic proteins.

First, COPD patients were compared with 'healthy' smoking controls. Inflammation was analyzed separately in the bronchial epithelium and 100 μm deep into the lamina propria. None of the marker molecules were significantly increased or decreased in the patients with COPD as compared to 'healthy smoking' control subjects. There was, however, less CD3 detectable in the lamina propria in COPD patients compared with the control group (Table 2).

Next, the effects of six months' treatment with FP or placebo were analyzed (Table 3). CD8 was significantly decreased in the lamina propria in both placebo ($p = 0.006$) and FP treated ($p = 0.028$) patients. In contrast, MBP ($p = 0.015$) and CD68 ($p = 0.021$) were significantly decreased in the FP treated patients only. In the epithelial layer, levels of tryptase were significantly decreased after FP treatment ($p = 0.027$). Other markers of inflammation were not or only marginally affected by the treatment regimen.

Discussion

We treated mild-to-moderate COPD patients who were hyperresponsive and who continued smoking, with FP (500 μg twice daily) for 6 months in a double-blind, placebo-controlled study. Analysis of the methacholine dose-response curves showed that not only PC_{20}, but all other indexes of BHR did not change as a result of FP treatment. Earlier we have shown that an equal dose of FP in patients with asthma with a similar degree of BHR, resulted in a rightward shift of the methacholine dose-response curve (27). Studies concerning patients with COPD in which PC_{20} was determined showed that inhaled steroid treatment had no effect on BHR (28-30). To our knowledge, we show for the first time that also additional indexes of BHR (reactivity, plateau, and EC_{50}) were not affected by steroid treatment.

There was, however, a significant reduction of the decline in both pre- and post-bronchodilator FEV_1 (%pred) (Fig. 4). Our results are supported by a recent study (31). Here, the investigators reported that FP treatment (500 μg twice daily for 6 months) of COPD patients resulted in improvement of FEV_1 and FVC. The FP treated patients had less frequent, but also less severe exacerbations. Clinical symptoms improved significantly, while sputum production decreased. Markers of inflammation were not analysed in that study. Three years treatment with inhaled budesonide in subjects with mild COPD who continued smoking (EUROSCOP) showed an improvement of FEV_1 of 17 mL per year, as compared with a decline of 81 mL per year in the placebo group during the first six months of the study (5).

Figure 4. Change of FEV_1 (**A**) and the course of FEV_1 (**B**) [post-bronchodilation], expressed as percentages of predicted normal values (% pred), during the treatment period of 6 months with either Fluticasone propionate (closed squares) or placebo (open squares).

Figure 4A. *mean difference: 7.97% pred, p = 0.0002 (rmANOVA)*

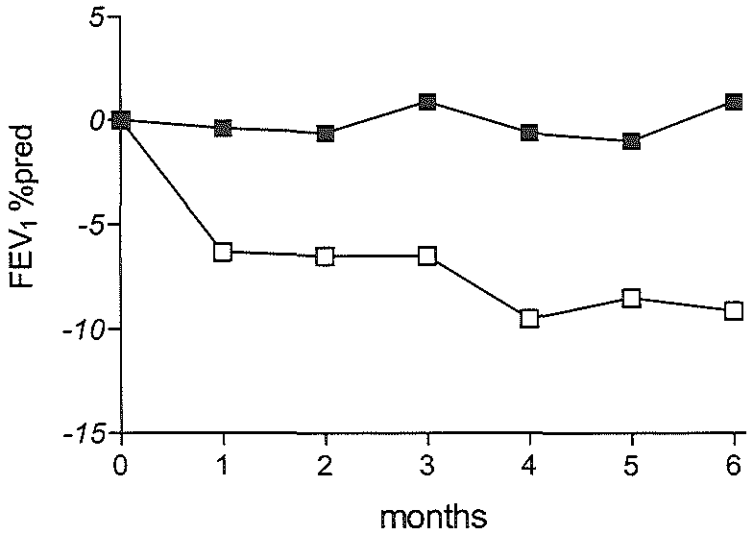


Figure 4B

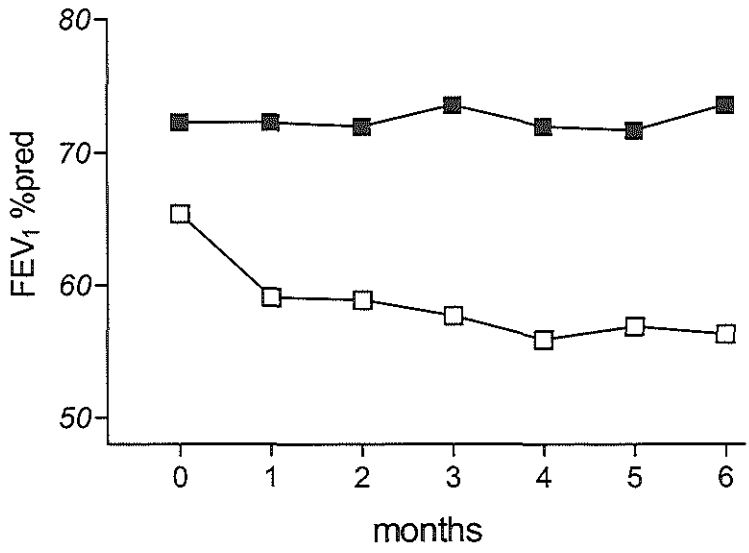


Figure 5. Course of MEF50 (**A**) and MEF25 (**B**), expressed as percentages of predicted normal values (% pred) during the treatment period of 6 months with either Fluticasone propionate (closed triangles) or placebo (open triangles).

Figure 5A. *p = 0.0006 (rmANOVA)*

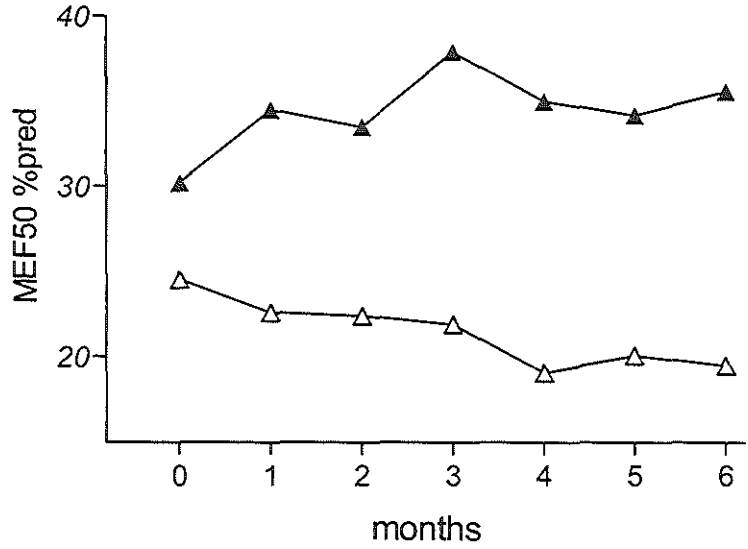


Figure 5B. *p = 0.0016 (rmANOVA)*

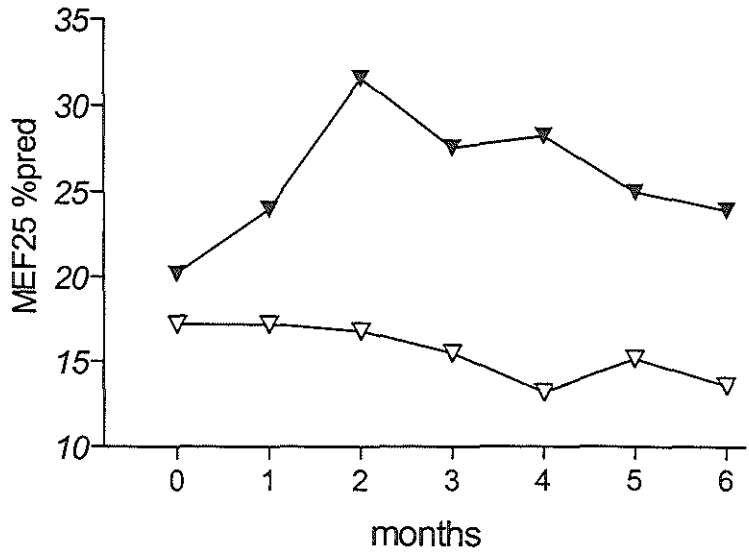


Table 2. Baseline scores of inflammatory markers in epithelium and lamina propria of biopsies taken from the central airways of smoking patients with COPD and 'healthy', smoking control subjects.

Data are expressed as median (range) ratios of immunostained areas and field measurement, which consisted of the total area, either of the epithelium or the lamina propria. Statistical analysis by Mann-Whitney U test after log transformation.

	EPITHELIUM		LAMINA PROPIA	
Marker	Patients with COPD	'healthy', smoking control subjects	Patients with COPD	'healthy', smoking control subjects
CD3	0.72 (0.10-4.52)	0.63 (0.24-0.93)	0.36 (0.04-5.71)	0.74 (0.61-1.19)
CD4	1.07 (0.14-4.0)	0.94 (0.40-2.0)	0.74 (0.07-9.0)	0.64 (0.27-1.63)
CD8	0.08 (0.03-0.60)	0.05 (0.02-0.09)	0.04 (0.03-0.42)	0.04 (0.01-0.13)
CD25	0.22 (0.03-3.28)	0.10 (0.03-0.22)	0.15 (0.03-2.39)	0.12 (0.04-0.26)
MBP	0.12 (0.03-5.27)	0.03 (0.01-0.67)	0.38 (0.00-6.45)	0.25 (0.01-5.13)
EG2	0.12 (0.01-1.79)	0.12 (0.01-0.55)	0.25 (0.03-0.68)	0.46 (0.14-8.01)
CD15	3.43 (0.08-17.4)	3.39 (0.68-8.96)	0.62 (0.14-4.73)	0.81 (0.01-1.40)
CD68	1.53 (0.24-11.4)	1.09 (0.46-1.98)	2.49 (0.44-20.1)	2.95 (1.13-4.74)
CD1a	0.12 (0.04-0.45)	0.08 (0.03-0.18)	0.14 (0.04-0.35)	0.07 (0.02-0.12)
Tryptase	0.14 (0.03-0.63)	0.10 (0.05-0.29)	1.30 (0.12-4.03)	1.23 (0.20-2.49)

Table 3. Comparison of baseline and post-intervention scores of inflammatory markers in epithelium (**A**) and lamina propria (**B**) of biopsies taken from the central airways of patients with COPD, treated with either Fluticasone propionate or placebo. Data are expressed as median ratios (range) of immunostained areas and field measurement, which consisted of the total area, either of the epithelium or the lamina propria.
Statistical analysis by paired Wilcoxon test after log transformation.

Table 3A.

	EPITHELIUM			
	Fluticasone propionate		Placebo	
Marker	Baseline	post-intervention	Baseline	post-intervention
CD3	0.57 (0.10-4.52)	0.28 (0.10-1.54)	0.79 (0.35-2.23)	0.45 (0.147-3.25)
CD4	1.03 (0.14-4.0)	0.70 (0.40-2.03)	1.11 (0.19-1.79)	1.19 (0.70-2.17)
CD8	0.09 (0.05-0.60)	0.09 (0.01-0.12)	0.07 (0.03-0.26)	0.07 (0.026-0.23)
CD25	0.16 (0.07-3.28)	0.20 (0.03-0.31)	0.27 (0.03-1.28)	0.15 (0.004-1.79)
MBP	0.06 (0.01-1.22)	0.03 (0.01-0.25)	0.20 (0.03-5.27)	0.15 (0.001-1.05)
EG2	0.16 (0.01-0.45)	0.06 (0.02-0.31)	0.08 (0.03-1.79)	0.07 (0.008-0.47)
CD15	3.31 (1.52-17.4)	2.12 (0.08-8.68)	4.32 (0.15-14.6)	1.64 (0.529-27.7)
CD68	1.42 (0.24-11.4)	0.81 (0.23-2.64)	1.64 (0.39-9.86)	1.19 (0.368-5.12)
CD1a	0.14 (0.04-0.45)	0.13 (0.05-0.29)	0.10 (0.05-0.30)	0.10 (0.013-0.19)
Tryptase	0.10 (0.03-0.63)	0.02 (0.00-0.18) ¶	0.18 (0.03-0.61)	0.097 (0.023-0.59)

¶ Reduction of tryptase$^+$ cells compared to baseline in FP treated group ($p<0.03$)

Table 3B.

	LAMINA PROPIA			
	Fluticasone propionate		Placebo	
Marker	baseline	post-intervention	baseline	post-intervention
CD3	0.23 (0.04-5.71)	0.20 (0.03-1.58)	0.50 (0.20-1.40)	0.31 (0.02-1.97)
CD4	0.68 (0.07-5.61)	0.54 (0.18-1.02)	0.80 (0.14-9.0)	0.58 (0.47-1.48)
CD8	0.06 (0.03-0.42)	0.03 (0.01-0.07) *	0.04 (0.03-0.16)	0.02 (0.01-0.10) †
CD25	0.17 (0.05-2.39)	0.15 (0.02-0.51)	0.13 (0.03-0.47)	0.06 (0.00-0.46)
MBP	0.29 (0.05-2.30)	0.08 (0.01-0.64) ‡	0.46 (0.03-4.46)	1.87 (0.00-6.45)
EG2	0.22 (0.13-0.68)	0.28 (0.01-1.01)	0.28 (0.03-2.5)	0.72 (0.01-4.69)
CD15	0.40 (0.19-4.73)	0.90 (0.19-3.73)	0.68 (0.14-3.95)	0.73 (0.15-3.98)
CD68	1.77 (0.44-20.1)	0.98 (0.26-2.77) §	2.67 (0.52-4.35)	1.76 (0.58-5.64)
CD1a	0.16 (0.04-0.35)	0.14 (0.04-0.59)	0.12 (0.05-0.31)	0.11 (0.02-0.21)
Tryptase	0.67 (0.19-3.26)	0.44 (0.02-0.74)	1.95 (0.12-4.03)	

* reduction of $CD8^+$ cells compared to baseline in FP treated group ($p<0.03$)
† Reduction of $CD8^+$ cells compared to baseline in placebo group ($p<0.01$)
‡ Reduction of MBP^+ cells compared to baseline in FP treated group ($p<0.02$)
§ Reduction of $CD68^+$ cells compared to baseline in FP treated group ($p<0.03$)

A similar treatment period with FP (ISOLDE) resulted in a significant reduction in the fall of FEV_1 (32). The rate of decline in FEV_1 in the group receiving placebo was markedly higher than that of the steroid treated group over the initial 6 months of treatment comparable to our findings. In contrast, the Copenhagen City Lung Study could not demonstrate an effect of long-term treatment with inhaled steroids on decline in lung function in patients with mild to moderate irreversible airflow limitation (6). In a recent study, patients with COPD were treated with triamcinolone for three years (7). A subgroup of these patients were hyperreactive. Among this subgroup the rate of decline in lung function did not slow down when evaluated over the entire three years' period. Over the initial six months of the trial, however, a beneficial treatment effect of triamcinolone, as compared to placebo, on the rate of decline of the FEV_1 can be deduced. This is in line with our results showing abolishment of FEV_1 decline in the FP treated group while the placebo group had an extrapolated decline of 120 ml per year. During the first 2 months of treatment, the decline was most marked and leveled off thereafter. The initial rapid decline among the patients receiving placebo may have resulted from the withdrawal of steroids. Steroids were withdrawn at least 6 weeks before the actual treatment period, which should have been ample time to at least overcome such a steroid weaning effect. A more likely explanation for our findings may be found in that BHR itself makes the patients subject to a decline in lung function in the absence of steroids. The beneficial effect of steroid treatment in this group of COPD patients leads to relieving patients from this decline and keeping FEV_1 constant.

It is unlikely that the treatment period of six months was not long enough to achieve a detectable response. Based on results of other studies including EUROSCOP, the most marked effects of inhaled steroid treatment should have been achieved during this time period (5,6,31,33). Therefore, it is unlikely that an extended FP treatment would have resulted in an increase of the FEV_1 in the steroid treated patients. On the other hand, a meta-analysis of three studies published between 1983 and 1996 showed a significant beneficial effect of inhaled corticosteroids compared with placebo on the course of the pre-bronchodilator FEV_1 during two years of treatment (34).

From our analysis of the derivatives of the MEFV curves, we propose that the anatomical location of the anti-inflammatory effect of the inhaled steroid is in the smaller airways. Significant changes were observed in MEF50 and MEF25. In contrast to others, we found FVC and PEF not to be affected (31). This discrepancy can be attributed to differences in patient selection criteria. Although MEF50 and MEF25 are less accurate parameters of expiratory flow, the significance and consistency of this finding indicate that the effect of FP on lung function is most probably caused by an effect on the small airways rather than by an effect on the larger airways. Bronchial biopsy specimens were taken from the central airways.

Eosinophils are important in the inflammatory process of asthma and correlate with BHR (35). We found eosinophils to be also present in the inflammatory infiltrate in COPD comparable with that in asymptomatic smokers. It is likely that smoking and other mechanisms that recruit neutrophils into the airways in COPD cause a degree of eosinophil influx (36). Recent findings show that eosinophilic airway inflammation was common among patients who had stable moderate and severe COPD (11). In our study, no significant differences in changes of cellular indices between treatment groups were detected. However, significant changes were detected within groups. Treatment with FP resulted in a significant decrease of mast cells in the epithelium and of $CD8^+$ lymphocytes, eosinophils (MBP^+) and macrophages ($CD68^+$) in the lamina propria. The significant treatment effect on $CD68^+$ cells may be explained by a coincidental regression to the mean. In the placebo treated group, an increase in eosinophils was detected, which coincided with a decrease of $CD8^+$ cells. The persistent inflammation is likely to cause the accelerated decline of the FEV_1 in patients with COPD.

Previously, we have shown that the number of dendritic cells was elevated in the asthmatic airways compared to the airways of healthy control subjects (37). In the airway mucosa of COPD patients as well as in that of 'healthy' smoking controls, $CD1a^+$ dendritic cells were hardly detectable. Consequently, a reduction of the number of DCs in airway biopsy specimens from COPD patients as a result of FP treatment could not be demonstrated. Our findings are supportive of a limited contribution of DCs to the pathology of COPD.

After a two weeks course of oral prednisolone, no changes of inflammatory indexes as measured in sputum samples of COPD patients were detected (8). Our study illustrates that FP treatment for 6 months leads to a reduction of cellular infiltrates in the bronchial mucosa. This discrepancy may be explained by the fact that sputum cell counts have been found to be related to bronchial wash and bronchoalveolar lavage fluid, but to a lesser extent to the counts in bronchial biopsies (38). Others have reported, that sputum is derived from a different compartment altogether (40). We were not able to detect a treatment effect on eosinophil counts in the epithelium. In contrast, we observed a significant reduction of eosinophils in the sub-epithelium. These results are also reflected by the absence of a treatment effect on eosinophil numbers in bronchial wash and bronchoalveolar lavage fluid (data not shown). This may be explained by the fact that eosinophilic inflammation seemed to be much more pronounced in the sub-epithelium than in the epithelium of the larger airways in our population of patients with COPD and BHR.

Overall this study shows that in COPD patients with BHR, FP treatment has a positive effect on indexes of airflow limitation. In contrast, indexes of hyperresponsiveness were not affected, while inflammation was only marginally reduced. Our results suggest that COPD patients with BHR benefit from inhaled steroid treatment.

Acknowledgement

The authors wish to thank GlaxoWellcome (FLIL44/FMS40060), Zeist, The Netherlands for providing study medication and financial support.

References

1. O'Shaughnessy TC, Ansari TW, Barnes NC, *et al.* Inflammation in bronchial biopsies of subjects with chronic bronchitis: inverse relationship of CD8+ T lymphocytes with FEV_1. Am J Respir Crit Care Med 1997; 155: 852-857.
2. Saetta M. Airway inflammation in chronic obstructive pulmonary disease. Am J Respir Crit Care Med 1999; 160: S17-S20.
3. Jeffery PK. Comparison of the structural and inflammatory features of COPD and asthma. Giles F. Filley Lecture. Chest 2000; 117: 251S-260S.
4. Postma DS, Peters I, Steenhuis EJ, *et al.* Moderately severe chronic airflow obstruction. Can corticosteroids slow down obstruction? Eur Resp J 1988; 1: 22-26.
5. Pauwels RA, Lofdahl CG, Laitinen LA, *et al.* Long-term treatment with inhaled budesonide in persons with mild chronic obstructive pulmonary disease who continue smoking. European Respiratory Society Study on Chronic Obstructive Pulmonary Disease. N Engl J Med 1999; 340:1948-1953.
6. Vestbo J, Sorensen T, Lange P, *et al.* Long-term effect of inhaled budesonide in mild and moderate chronic obstructive pulmonary disease: a randomised controlled trial. Lancet 1999; 353: 1819-1823.
7. The Lung Health Study Research Group. Effect of inhaled Triamcinolone on the decline in pulmonary function in chronic obstructive pulmonary disease. N Engl J Med 2000; 343: 1902-1999.
8. Keatings VM, Jatakanon A, Worsdell YM, *et al.* Effects of inhaled and oral glucocorticoids on inflammatory indices in asthma and COPD. Am J Respir Crit Care Med 1997; 155: 542-548.
9. Weiner P, Weiner M, Rabner M, *et al.* The response to inhaled and oral steroids in patients with stable chronic obstructive pulmonary disease. J Intern Med 1999; 245: 83-89.
10. Chanez P, Vignola AM, O'Shaugnessy T, *et al.* Corticosteroid reversibility in COPD is related to features of asthma. Am J Respir Crit Care Med 1997; 155: 1529-1534.
11. Brightling CE, Monteiro W, Ward R, *et al.* Sputum eosinophilia and short-term response to prednisolone in chronic obstructive pulmonary disease: a randomised controlled trial. Lancet 2000; 356: 1480-1485.
12. Kerstjens HA, Overbeek SE, Schouten JP, *et al.* Airways hyperresponsiveness, bronchodilator response, allergy and smoking predict improvement in FEV_1 during long-term inhaled corticosteroid treatment. Dutch CNSLD Study Group. Eur Resp J 1993; 6: 868-876.
13. Woolcock AJ, Anderson SD, Peat JK, *et al.* Characteristics of bronchial hyperresponsiveness in chronic obstructive pulmonary disease and in asthma. Am Rev Respir Dis 1991; 143: 1438-1443.
14. Tashkin DP, Altose MD, Bleecker ER, *et al.* The Lung Health Study: airway responsiveness to inhaled methacholine in smokers with mild to moderate airflow limitation. The Lung Health Study Research Group. Am Rev Respir Dis 1992; 145: 301-310.
15. Taylor RG, Joyce H, Gross E, *et al.* Bronchial reactivity to inhaled histamine and annual rate of decline in FEV_1 in male smokers and ex-smokers. Thorax 1985; 40: 9-16.
16. Rijcken B, Schouten JP, Xu X, *et al.* Airway hyperresponsiveness to histamine associated with accelerated decline in FEV_1. Am J Respir Crit Care Med 1995; 151: 1377-1382.

17. Kerstjens HA, Brand PL and Postma DS Risk factors for accelerated decline among patients with chronic obstructive pulmonary disease. Am J Respir Crit Care Med 1996; 154: S266-S272.
18. Tashkin DP, Altose MD, Connett JE, *et al.* Methacholine reactivity predicts changes in lung function over time in smokers with early chronic obstructive pulmonary disease. The Lung Health Study Research Group. Am J Respir Crit Care Med 1996; 153: 1802-1811.
19. Finkelstein R, Ma HD, Ghezzo H, *et al.* Morphometry of small airways in smokers and its relationship to emphysema type and hyperresponsiveness. Am J Respir Crit Care Med 1995; 152: 267-276.
20. Nagai A, Thurlbeck WM and Konno K Responsiveness and variability of airflow obstruction in chronic obstructive pulmonary disease. Clinicopathologic correlative studies. Am J Respir Crit Care Med 1995; 151: 635-639.
21. Riess A, Wiggs B, Verburgt L, *et al.* Morphologic determinants of airway responsiveness in chronic smokers. Am J Respir Crit Care Med 1996; 154: 1444-1449.
22. Fabbri L, Caramori G, Beghe B, *et al.* Chronic obstructive pulmonary disease international guidelines. Curr Opin Pulm Med 1998; 4: 76-84.
23. European Respiratory Society. Standardized lung function testing. Official statement of the European Respiratory Society. Eur Resp J 1993; Suppl. 16: 1-100.
24. Lamberts SW, Koper JW, Biemond P, *et al.* Cortisol receptor resistance: the variability of its clinical presentation and response to treatment. J Clin Endocrin Metab 1992; 74: 313-321.
25. Aerts JG, Bogaard JM, Overbeek SE, *et al.* Extrapolation of methacholine log-dose response curves with a Cumulative Gaussian Distribution function. Eur Resp J 1994; 7: 895-900.
26. Pesci A, Balbi B, Majori M, *et al.* Inflammatory cells and mediators in bronchial lavage of patients with chronic obstructive pulmonary disease. Eur Resp J 1998; 12: 380-386.
27. Overbeek SE, Rijnbeek PR, Vons C, *et al.* Effects of fluticasone propionate on methacholine dose-response curves in nonsmoking atopic asthmatics. Eur Respir J 1996; 9: 2256-2262.
28. Engel T, Heinig JH, Madsen O, *et al.* A trial of inhaled budesonide on airway responsiveness in smokers with chronic bronchitis. Eur Respir J 1989; 2: 935-939.
29. Auffarth B, Postma DS, de Monchy JG, *et al.* Effects of inhaled budesonide on spirometric values, reversibility, airway responsiveness, and cough threshold in smokers with chronic obstructive lung disease. Thorax 1991; 46: 372-7.
30. Watson A, Lim TK, Joyce H, *et al.* Failure of inhaled corticosteroids to modify bronchoconstrictor or bronchodilator responsiveness in middle-aged smokers with mild airflow obstruction. Chest 1992; 101: 350-355.
31. Paggiaro PL, Dahle R, Bakran I, *et al.* Multicentre randomised placebo-controlled trial of inhaled fluticasone propionate in patients with chronic obstructive pulmonary disease. International COPD Study Group. Lancet 1998; 351: 773-780.
32. Burge PS, Calverley PM, Jones PW, *et al.* Randomised, double blind, placebo controlled study of fluticasone propionate in patients with moderate to severe chronic obstructive pulmonary disease: the ISOLDE trial. BMJ 2000; 320: 1297-1303.
33. Niewoehner DE, Erbland ML, Deupree RH, *et al.* Effect of systemic glucocorticoids on exacerbations of chronic obstructive pulmonary disease. Department of Veterans Affairs Cooperative Study Group. N Engl J Med 1999; 340: 1941-1947.
34. van Grunsven PM, van Schayck CP, Derenne JP, *et al.* Long term effects of inhaled corticosteroids in chronic obstructive pulmonary disease: a meta-analysis. Thorax 1999; 54: 7-14.
35. Möller GM, Overbeek SE, van Helden-Meeuwsen CG, *et al.* Eosinophils in the bronchial mucosa in relation to methacholine dose- response curves in atopic asthma. J Appl Physiol 1999; 86: 1352-1356.
36. Matsumoto K, Aizawa H, Inoue H, *et al.* Eosinophilic airway inflammation induced by repeated exposure to cigarette smoke. Eur Respir J 1998; 12: 387-394.
37. Möller GM, Overbeek SE, Van Helden-Meeuwsen CG, *et al.* Increased numbers of dendritic cells in the bronchial mucosa of atopic asthmatic patients: downregulation by inhaled corticosteroids. Clin Exp Allergy 1996; 26: 517-524.

38. Grootendorst DC, Sont JK, Willems LNA, *et al.* Comparison of inflammatory cell counts in asthma: Induced sputum vs bronchoalveolar lavage and bronchial biopsies. Clin Exp Allergy 1997; 27: 769-779.
39. Rutgers SR, Timens W, Kaufmann HF, *et al.* Comparison of induced sputum with bronchial wash, bronchoalveolar lavage and bronchial biopsies in COPD. Eur Respir J 2000; 15: 109-115.

Chapter 5

Effects of fluticasone propionate inhalation on levels of arachidonic acid metabolites in patients with chronic obstructive pulmonary disease.

Gert T.Verhoeven[1], Ingrid M. Garrelds[2], Henk C. Hoogsteden[1], Freek J. Zijlstra[2].
[1]Department of Pulmonary and Intensive Care Medicine, [2]Department of Pharmacology, University Hospital Dijkzigt and Erasmus University, EMCR, Rotterdam, The Netherlands.

(Published in: Mediators of Inflammation 2001; 10: 21-26)

Abstract

Background: In smoking COPD patients the bronchoalveolar lavage (BAL) fluid contains high numbers of inflammatory cells. These cells might produce Arachidonic Acid (AA) metabolites, which contribute to inflammation and an increased bronchomotor tone.
Aim of the study: investigate levels of AA metabolites in BAL fluid, before and after inhaled glucocorticoid therapy: fluticasone propionate (FP) 1 mg per day, or placebo.
Methods: double-blind placebo controlled trial lasting six months. COPD patients were selected by clinical criteria and the presence of bronchial hyperresponsiveness (BHR). Lung function was recorded and in BAL fluid we counted cell numbers and measured LTB_4, $LTC_4/D_4/E_4$, PGE_2, $6kPGF_1\alpha$, $PGF_2\alpha$ and TxB_2. A control group consisted of asymptomatic smokers (n=6).
Results: paired data were obtained from 9 FP treated and 11 placebo patients. BAL cells were almost exclusively alveolar macrophages. In patients and controls both cellularity and levels of AA metabolites were equal. Cell numbers did not change after treatment. Statistically significant decreases after FP therapy were noticed for PGE_2 (30%), $6kPGF_1\alpha$ (41%) and $PGF_2\alpha$ (54%).
Conclusions: in COPD, the capability of inflammatory cells to produce certain AA metabolites was decreased after inhaled FP treatment. This result is discussed in its relation to clinical effects, the influence of smoking, and the results of an earlier, similar study in asthma patients.

Introduction

Leukotrienes (LTs) and prostaglandins (PGs) are mediators of inflammation that have been investigated almost exclusively in asthma, but not in COPD. These metabolites of arachidonic acid (AA) are products of several inflammatory cells and part of the mechanisms leading to bronchoconstriction and increased bronchomotor tone. In chronic obstructive pulmonary disease (COPD) airway narrowing is caused by several mechanisms, including mechanical factors (loss of elasticity of the lung parenchyma), increased thickness of the walls of the conducting airways, increased bronchomotor tone and intraluminal secretions (1,2). Increased numbers of alveolar macrophages, neutrophilic granulocytes and CD8 positive cells constitute the main burden of the cellular infiltrate in the airway walls and alveoli. Their secretory products cause multiple effects, for instance cell influx and cell activation, but also an increase of the contractile status of the smooth muscles in the small bronchi. Among these cell products, AA metabolites are known for their potent bronchoconstrictor activity.
Therefore, we hypothesized that AA metabolites could also be detected in COPD and anti-inflammatory treatment could influence their levels.
Samples of BAL supernatant were collected at the start and after six months of a double-blind trial comparing the effect of fluticasone propionate (FP) with placebo, in smoking COPD patients. We decided to study a subgroup of COPD patients, particularly those with bronchial hyperresponsiveness (BHR), because these patients are subject to an even more accelerated decline of their FEV_1 (3,4). This could indicate a severe inflammatory infiltrate, increased levels of AA metabolites and potentially an effect of an inhaled glucocorticoid. Smokers without symptoms and with normal lung function served as controls.

Material and methods

Patients
COPD patients were selected according to generally accepted clinical and functional criteria (5). Inclusion criteria were: age between 40 and 70 years, current smoker, FEV_1/iVC ratio $\leq$70% of predicted normal values (pred), reversibility of FEV_1 <10%pred after 750 µg terbutaline, non-specific BHR - defined by a PC_{20} histamine $\leq$8 mg/ml - and negative skin tests for standard inhaled allergens. Reference values were obtained from ECCS standards (6). The main exclusion criteria were a history of asthma and recent respiratory tract infection. Any anti-inflammatory therapy, including corticosteroids, non-steroidal anti-inflammatory drugs and theophyllines, was discontinued. Potential candidates for this study were informed about the negative effects of smoking, and we offered them the opportunity to participate in a smoking-cessation program. The Hospital's Medical Ethics Committee approved the study. Written informed consent was obtained from all participants.

Design of the intervention study
We performed a double blind, placebo-controlled study that lasted for six months. Patients were randomly assigned to either twice daily 500 µg FP or placebo, which were delivered via a similar diskhaler. The start of treatment was immediately after the first bronchoscopy. Bronchoscopy was repeated at the end of the study. Follow-up visits were at intervals of four weeks.

Bronchoscopy
The procedure was carried out according to international guidelines (7). For local anaesthesia we used lidocaine spray and aerosolised oxybuprocaine. The bronchoscope (Olympus BF 1T10) was advanced into the lateral segment of the middle lobe, in wedge position. Lavage was performed successively with 1 x 40 ml and 4 x 50 ml sterile phosphate-buffered saline (PBS) solution (Organon Teknika, Boxtel, The Netherlands) at body temperature (37°Celsius) and pH 7.4. Aliquots were aspirated in two siliconized specimen traps, one for the first 'bronchoalveolar fraction' after 40 ml PBS and the second 'alveolar fraction' for the recovery of the 4 x 50 ml PBS. The lavage fluid was transported to the laboratory on ice and processed immediately. The recovery of the first, bronchoalveolar fraction appeared to be low and insufficient for performing the desired experiments.

Processing of BAL fluid
After measurement of volume, the BAL fluid was filtered through sterile nylon gauzes and centrifuged at 400xg at 4°Celsius for 5 minutes. The cell pellet was washed in PBS supplemented with 0.5% bovine serum albumin. The total cell number in the BAL cell suspension was counted in a Coulter Counter Model ZM (Coulter Electronics, Hialeah, FL, USA) and viability was assessed by cellular exclusion of trypan blue. With FACScan flow-cytometry (Becton Dickinson, San Jose, CA, USA) we analysed $CD3^+$ T-cells. Immunocytochemistry of cytospins was carried out with the APAAP (alkaline phosphatase anti-alkaline phosphatase) method and the monoclonal antibodies CD68 (alveolar macrophages), CD15 (VIM-D5, neutrophils), BMK-13 (eosinophils). Two independent observers counted at least 300 cells in each cytospin.

Determination of concentrations of AA metabolites
In an earlier publication this has been described in more detail (8). In short: immediately after the BAL procedure, 20 ml of supernatant was processed on C18 SepPak cartridges (Millipore, Bedford, USA), 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 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 PGE_2 and $PGF_2\alpha$ were determined with commercially available [^{3}H] kits (Amersham, UK) and $6kPGF_1\alpha$ with a [^{125}I] RIA kit (Du Pont de Nemours, Dreieich, Germany), according to the manufacturer's instructions. $LTC_4/D_4/E_4$ was measured at

room temperature in a microtitre enzyme immunoassay according to 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%.

Statistics

Data are expressed as mean ±SD or as median (range), depending on distribution of data. Differences between patient groups were tested with either the (unpaired) Student *t*-test or the Mann-Whitney test, depending upon distribution of data. Pre- and post-intervention data were tested for significant difference with the paired *t*-test or the Wilcoxon signed rank test. Statistical significance was considered at $p < 0.05$.

Results

From the initial 23 participants in the intervention study, paired results were obtained from 9 FP patients and from 12 patients with placebo. The reason for missing paired data was insufficient recovery of either BAL fluid or BAL cells. Characteristics of patients (n=21) and controls are listed in table 1. Baseline characteristics of the FP and the placebo group did not differ significantly. The COPD patients had on average moderately severe obstructive airways disease and severe BHR. FEV_1%predicted and PC_{20} were within the range of normal values in the group of asymptomatic smokers, due to selection.

Table 1. Patient characteristics (mean ±SD).

	Fluticasone Propionate	Placebo	Asymptomatic smokers
N	9	12	6
Age, years	54 ±8	56 ±8	44 ±9
Cigarettes/day	15 ±8	16 ±7	21 ±10
Pack years	25 ±12	26 ±10	23 ±12
FEV_1 %predicted	66 ±13	61 ±12	93 ±11
FEV_1 reversibility, %	5.3 ±3.7	5.6 ±2.7	4.0 ±3.0
PC_{20} histamine, mg/ml	2.6 ±2.4	0.8 ±0.6	>8

Compliance with study medication was high: the percentage of returned used blisters (which contained FP or placebo) was 92.5 in the FP and 92.7 in the placebo group.
In the placebo group, mean FEV_1 declined from 61.4 %pred at the start of the trial to

52.0 %pred after 6 months. On the other hand, in the FP treated patients, lung function remained unchanged; mean FEV_1: 66.4 and 68.1 %pred, respectively. This treatment effect was statistically significant ($p<.05$, rmANOVA test), and considered as a beneficial effect of FP treatment. Parameters of BHR were not influenced by FP treatment.

Immunocytology

BAL fluid recovery was higher in controls as compared to the COPD patients (table 2).

Mean total cell number of the BAL at the start of the trial was 49 x 10^6 for FP treated patients and 44 x 10^6 in the placebo group (table 2, no statistical significant difference). Small changes were noticed in the BAL cell numbers at the end of the trial, which were not significant (table 2). The percentages of fluid recovery, cell viability and cells types were not statistically different between both patient groups at baseline, and did not change significantly after 6 months of intervention (table 2). The low number of eosinophils indicates that COPD was stable in nature (2).

Table 2. Characteristics of bronchoalveolar lavage (mean ±SD).

	Fluticasone propionate		Placebo		'Healthy' smokers
	Start	6 months	Start	6 months	
Fluid recovery, %	55 ±14	57 ±14	55 ±15	50 ±19	69 ±2.2
Cell number, x10^6	49 ±24	47 ±31	44 ±27	40 ±23	53 ±27
Cell viability, %	62 ±12	66 ±12	70 ±13	67 ±12	66 ±14
$CD68^+$ macrophages, %	93 ±8	94 ±4	94 ±4	91 ±7	93 ±7
$CD15^+$ granulocytes, %	2.9 ±2.5	2.8 ±2.7	2.8 ±2.7	3.6 ±6.4	1.7 ±1.6
$BMK13^+$ eosinophils, %	0.6 ±0.4	0.5 ±0.4	0.5 ±0.4	1.0 ±1.3	0.9 ±0.1
$CD3^+$ T-cells, %	1.3 ±1.9	0.7 ±0.6	3.8 ±4.8	5.9 ±5.2	1.8 ±1.9

Arachidonic Acid metabolites

Table 3 shows the levels of protein, albumin and AA metabolites at the start and after six months of intervention, which consisted of either FP inhalation or placebo. The results of the initial measurements in the FP treated patients and the placebo group are comparatively equal ($p>.05$, Mann-Whitney test). There are also no significant differences when comparing the COPD patients with the controls.

Statistical significant differences (comparing post- and pre-intervention results) were found in the FP treated patients with regard to PGE_2, $6kPGF_1\alpha$ and $PGF_2\alpha$ ($p<.05$, Wilcoxon signed rank test). After correction for protein levels, the results from the statistical tests were similar and also significant for the same three mediators (data not shown). The change of levels of $6kPGF_1\alpha$ and $PGF_2\alpha$ differed significantly from that in the placebo group [treatment effect] ($p<0.05$, Wilcoxon signed rank test).

Discussion

In a subgroup of COPD patients, particularly those with bronchial hyperresponsiveness, we were able to measure levels of cyclooxygenase and 5-lipoxygenase products in BAL fluid. We observed significant decreases of three out of six mediators after inhaled fluticasone propionate therapy, in a placebo controlled trial that lasted for six months (table 3).

Arachidonic acid metabolites have gained attention in asthma, in which they play an important role as mediators of inflammation with bronchoconstriction as the most important clinical effect. In a previous study in our institution concerning asthma patients we have used the same techniques for collecting BAL fluid and measuring levels of AA metabolites (9). These patients had a similar degree of BHR (as expressed by PC_{20}) compared to the COPD patients in the present study. However, these asthma patients had a history consistent with asthma, they had allergic reactions for common inhaled allergens and were non-smokers. $LTC_4/D_4/E_4$ levels were comparable, whereas levels of TxB_2, PGE_2 and $6kPGF_1\alpha$ were, respectively, approximately 10-, 5- and 15-fold higher in COPD as compared to asthma. The number of cells in the BAL fluid was, however, about five times higher in COPD as compared to asthma. Taking this in account, it appears that cell secretory activity with regard to $LTC_4/D_4/E_4$ is higher in asthma. This is feasible because asthma is an allergic inflammatory disease, whereas in COPD there is a nonspecific inflammatory infiltrate. The production of $LTC_4/D_4/E_4$ is almost exclusively attributed to mast cells, which are numerous in the airway walls of asthma patients, whereas they are hardly detectable in COPD. On the other hand, inflammatory cells in COPD seem to produce higher amounts of TxB_2 and $6kPGF_1\alpha$. The most obvious explanation seems to be that this is induced by cigarette smoking. In one other study we have shown that levels of TxB_2 and $PGF_2\alpha$ in BAL fluid were significantly elevated in smokers as compared to non-smokers, and this correlated with packyears (8). Smoking also increased the pentagastrin-stimulated gastric luminal release of TxB_2 and $PGF_2\alpha$ (10).

In the present study, we have compared levels of these mediators in smoking COPD patients with the levels in a control group, which consisted of asymptomatic smokers with normal lung function and negative allergy tests. There were no significant differences. These results indicate that smoking (and not some mechanism specific for COPD) could be the single or most important stimulus for an inflammatory infiltrate with high levels of certain cyclooxygenase products (11).

Table 3. Concentrations of protein (μg/ml), albumin (μg/ml) and AA metabolites (pg/ml), before and after intervention, and in the control group (asymptomatic smokers). Data are expressed as median (range). * data incomplete.

	Fluticasone Propionate				Placebo				Asymptomatic smokers	
	n	Start	Post-intervention	p	n	Start	Post-intervention	p	n	
Protein	8	118.1 (13.1-201.9)	111.3 (18.0-205.4)	.6	10	105.4 (33.0-213.7)	84.1 31.4-122.8)	.3	6	*
Albumin	9	23.9 (14.6-55.2)	20.6 (14.7-74.1)	.3	12	32.6 (20.8-47.0)	24.6 (14.7-48.1)	.1	6	16.4 (10.1-27.6)
LTB_4	9	131.2 (54.8-1167)	110.6 (11.4-920.5)	.3	12	282.5 (41.0-1108)	212.7 (1.7-773.9)	.3	6	244.1 (199.1-326.3)
$LTC_4/D_4/E_4$	9	8.05 (4.68-68.3)	5.46 (4.11-11.0)	.055	12	9.31 (4.3-27.7)	9.32 (0.01-31.8)	.1	6	8.26 (6.49-9.0)
PGE_2	9	43.0 (35.0-141.0)	30.0 (12.0-61.0)	***.01***	12	62.0 (15.0-166.0)	48.5 (3.0-75.0)	.3	6	37.0 (32.0-60.0)
$6kPGF_1\alpha$	9	69.0 (44.0-184)	41.0 (33.0-65.0)	***.008***	12	56.5 (41.0-78.0)	54.5 (27.0-81.0)	.7	6	49.0 (40.0-67.0)
$PGF_2\alpha$	9	98.0 (47.0-193.0)	45.0 (27.0-135.0)	***.028***	11	100.0 (36.0-228.0)	107.0 (0.0-347.0)	.6	6	37.0 (28.0-172.0)
TxB_2	9	136.0 (73.0-1269)	141.0 (69.0-350.0)	.1	12	201.5 (54.0-421)	182.5 (0.0-369.0)	.3	6	170.0 (96.0-318.0)

Fluticasone propionate (FP) had no significant effect on BAL cellularity, and also not on protein and albumin levels (table 2). Apparently, the influx and the survival of cells were not affected by this potent topical glucocorticoid. FP also did not influence the levels of the leukotrienes. However, cell secretory activity, particularly the production of PGE_2, $6kPGF_1\alpha$ and $PGF_2\alpha$, was inhibited as their levels were decreased after 6 months exposure to FP (table 3).
In the above-mentioned previous study of asthma patients the same dose of FP was used (9). From the AA metabolites in asthma, only PGD_2 decreased significantly. Levels of $PGF_2\alpha$ were not measured in that study. One other study in asthma patients also showed lower levels of PGD_2 in BAL fluid after inhaled glucocorticoid therapy (12). It was concluded that, in asthma, FP downregulated the capability of mast cells, and perhaps also of alveolar macrophages, to produce PGD_2. From our present study in COPD patients we conclude that FP causes downregulation of the capability of alveolar macrophages to produce certain prostaglandins.
In the present study, the statistically significant clinical effect of FP treatment in COPD patients with BHR was not a reduction of any parameter related to BHR, but the course of the FEV_1: it decreased in the placebo group, whereas it remained stable in the FP treated patients. So, in the placebo group the ongoing inflammation made airways obstruction worse. Anti-inflammatory therapy with FP obliterated FEV_1 decline, and one of the mechanisms underlying this effect could be the reduction of levels of AA metabolites, particularly $PGF_2\alpha$. $PGF_2\alpha$ has strong bronchoconstrictor activity through a direct effect on airway receptors and indirectly through cholinergic-mediated bronchoconstriction (13). Reduction of its level could diminish bronchomoter tone. We also observed significant decreases of the levels of $6kPGF_1\alpha$ and PGE_2. $6kPGF_1\alpha$ is derived from PGI_2, which is, like PGE_2, a mediator with, amongst others, a bronchodilatory effect. Apparently, the activity of these PGs in COPD is less as compared to the activity of $PGF_2\alpha$. In murine lower airways $PGF_2\alpha$ was the most potent bronchial muscle constrictor (14). Alternatively, other inflammatory mechanisms could be more important for explaining the clinical effect of FP, which we observed in this particular group of COPD patients.
In conclusion, the ongoing inflammation with, amongst others, high concentrations of $PGF_2\alpha$ made airways obstruction worse, whereas inhibition of $PGF_2\alpha$ production by FP seems to be one of the mechanisms leading to preservation of lung function.

Acknowledgements

This study was financially supported by the Netherlands Asthma Foundation and by GlaxoWellcome, the Netherlands (FLIL44/FMS40060). The authors wish to thank Dr. M. Galli for critically reviewing the manuscript.

References

1. Saetta M. Airway inflammation in chronic obstructive pulmonary disease. Am J Respir Crit Care Med 1999; 160: S17-S20.
2. Jeffery PK. Comparison of the structural and inflammatory features of COPD and asthma. Gilles F. Filley Lecture. Chest 2000; 117(Suppl 1): 251S-260S.
3. Rijcken B, Schouten JP, Xu X, Rosner B, Weiss ST. Airway hyperresponsiveness to histamine associated with accelerated decline in FEV1. Am J Respir Crit Care Med 1995; 151:1377-1382.
4. Tashkin DP, Altose MD, Connett JE, Kanner RE, Lee WW, Wise RA. Methacholine reactivity predicts changes in lung function over time in smokers with early chronic obstructive pulmonary disease. The Lung Health Study Group. Am J Respir Crit Care Med 1996; 153: 1802-1811.
5. ATS Statement. Standards for the diagnosis and care of patients with chronic obstructive pulmonary disease. Am J Respir Crit Care Med 1995; 152: S77-S120.
6. Quanjer PhH, Tammeling GJ, Cotes JE, Pedersen OF, Peslin R, Yernault JC. Lung volumes and forced ventilatory flows. Eur Respir J 1993; 6(suppl 16): 5-40.
7. Summary and recommendations of a workshop on the investigative use of fiberoptic bronchoscopy and bronchoalveolar lavage in asthmatics. Am Rev Respir Dis 1985; 132: 180-182.
8. Zijlstra FJ, Vincent JE, Mol WM, Hoogsteden HC, Van Hal PT, Jongejan RC. Eicosanoid levels in bronchoalveolar lavage fluid of young female smokers and non-smokers. Eur J Clin Invest 1992; 22: 301-306.
9. Overbeek SE, Bogaard JM, Garrelds IM, Zijlstra FJ, Mulder PGH, Hoogsteden HC. Effects of fluticasone propionate on arachidonic acid metabolites in BAL-fluid and metacholine dose-response curves in nonsmoking atopic asthmatics. Mediators of Inflammation 1996; 5: 224-229.
10. Fedi P, Calabro A, Orsini B, Milani S, Zacchi P, Salvadori G, et al. Cigarette smoking increases gastric luminal prostaglandin F_2 alpha and thromboxane B_2 in healthy smokers. Digestion 1990; 46: 27-34.
11. Lee LY, Hong JL. Involvement of prostanoids in cigarette smoking-induced pathophysiological effects in the lung. Prostaglandins Leukot Essent Fatty Acids 1999; 61: 145-155.
12. Oosterhoff Y, Overbeek SE, Douma R, Noordhoek JA, Postma DS, Hoogsteden HC, et al. Lower leukotriene C4 levels in bronchoalveolar lavage fluid after 2.5 years inhaled corticosteroid therapy. Mediators of Inflammation 1995; 4: 426-430.
13. Beasly R, Varly J, Robinson C, Holgate ST. Cholinergic-mediated bronchoconstriction induced by prostaglandin D_2, its initial metabolite $9_\alpha,11_\beta$-$PGF_2\alpha$ and $PGF_2\alpha$ in asthma. Am Rev Respir Dis 1987; 136: 1140-1144.
14. Richter M, Sirois P. Effects of eicosanoids, neuromediators and bioactive peptides on murine airways. Eur J Pharmacol 2000 ; 389: 225-234.

Chapter 6

Effect of an inhaled glucocorticoid on reactive oxygen species production by bronchoalveolar lavage cells from smoking COPD patients.

Gert T. Verhoeven[1,2], Annemarie J.M. Wijkhuijs[2], Herbert Hooijkaas[2], Henk C. Hoogsteden[1], Wim Sluiter[3].
[1] Department of Pulmonary and Intensive Care Medicine, [2]Department of Immunology and [3]Department of Biochemistry, Erasmus University Medical Center Rotterdam, The Netherlands.

(Published in: Mediators of Inflammation 2000; 9: 109-113)

Abstract

Oxidative stress in the lung is important in the pathogenesis of COPD. Published data indicate that glucocorticoids inhibit blood cells in their capability of producing reactive oxygen species (ROS). We investigated the effect of Fluticasone propionate (FP) on the ROS production capability of pulmonary cells.
Bronchoalveolar lavage (BAL) was performed in smoking COPD patients, before and after a six month, placebo-controlled treatment with FP. BAL cells were stimulated with phorbol myristrate acetate (PMA) alone, and together with superoxide dismutase (SOD). From kinetic plots of ferricytochrome-c conversion we calculated the maximal rate of superoxide production (V_{max}). Also, we examined BAL cell subsets and performed correlation analyses on ROS production and relevant clinical determinants.
Paired results were obtained from 6 FP and 9 placebo treated patients. No significant change of V_{max} was found in both patients groups. Also BAL cellularity was unchanged. Correlation analyses showed a significant (inverse) association of V_{max} with the number of cigarettes smoked per day.
We concluded that a potent inhaled glucocorticoid had no effect on the ROS production capability of BAL cells from smoking COPD patients. Apparently, heavy smoking impaired the ability of alveolar macrophages to produce ROS, which was not further decreased by FP.

Introduction

The airways and alveoli of patients with chronic obstructive airways disease (COPD) are invaded with numerous inflammatory cells, which have the ability of producing reactive oxygen species (ROS) (1). It has been established that an increased oxidative stress is one of the major pathogenic mechanisms (2,3). Oxidative stress may occur either by an increased exposure to ROS or by a decreased capability of the involved tissue to neutralise the ever-emerging production of ROS. Both mechanisms seem to be involved in the development of COPD. The smoking of cigarettes is clearly related to COPD. Cigarette smoke contains several molecules, which are ROS by themselves, and it contains molecules and particulates, which generate chemical reactions and inflammation, with subsequent ROS production (4).
A major goal in the treatment of COPD is the reduction of the number and/or the activity of inflammatory cells. In asthma patients, glucocorticoids have been applied successfully with this intention. In the present study, we have investigated if a potent inhaled glucocorticoid (Fluticasone propionate, FP) could reduce the ROS production in COPD patients. We have selected COPD patients with bronchial hyperresponsiveness (BHR) because these patients have an exaggerated decline of their lung function. We hypothesized that inflammation and ROS production in this subgroup of COPD patients were severe, and could be influenced by an inhaled glucocorticoid. Inflammatory cells were collected by means of bronchoalveolar lavage (BAL), before and after treatment with inhaled FP or placebo. Besides cell counts, the ROS production capability was determined *in vitro*. Furthermore, we analyzed the association of ROS production capability with smoking behaviour, airways obstruction and BHR.

Material and methods

Patients
COPD patients were selected according to the clinical and functional criteria, which are generally accepted (5). Inclusion criteria were: age between 40 and 70 years, current smoker, FEV_1/iVC ratio 70% of predicted normal values (pred), reversibility of FEV_1 <10%pred after 750 µg terbutaline, non-specific BHR - defined by a PC_{20} histamine 8 mg/ml - and negative skin tests for standard inhaled allergens. Reference values were obtained from ECCS standards (6). The main exclusion criteria were a history of asthma and recent respiratory tract infection. Any anti-inflammatory drugs, including steroids and NSAIDs, and theophyllines were discontinued. Potential candidates for this study were informed about the negative effects of smoking, and we offered them the opportunity to participate in a smoking-cessation program. The Hospital's Medical Ethics Committee approved the study. Written informed consent was obtained from all participants.

Design of the intervention study
We performed a double blind, placebo-controlled study that lasted for six months. Patients were randomly assigned to either twice-daily 500 μg FP or placebo, which were delivered via a similar diskhaler. The start of treatment was immediately after the first bronchoscopy. Bronchoscopy was repeated at the end of the study. At intervals of four weeks, the study participants had a check-up.

Bronchoscopy
The procedure was carried out according to international guidelines (7). For local anaesthesia we used lidocaine spray and aerosolised oxybuprocaine. The bronchoscope (Olympus BF 1T10) was advanced into the lateral segment of the middle lobe, in wedge position. Lavage was performed successively with 1 x 40 ml and 4 x 50 ml sterile phosphate-buffered saline (PBS) solution (Organon Teknika, Boxtel, The Netherlands) at body temperature (37°Celsius) and pH 7.4. Aliquots were aspirated in two siliconized specimen traps, one for the first 'bronchoalveolar fraction' after 40 ml PBS and the second 'alveolar fraction' for the recovery of the 4 x 50 ml PBS. The lavage fluid was transported to the laboratory on ice and processed immediately. The recovery of the first, bronchoalveolar fraction appeared to be low and insufficient for performing the desired experiments.

Processing of BAL fluid
After measurement of volume, the BAL fluid was filtered through sterile nylon gauzes and centrifuged at 400xg at 4°Celsius for 5 minutes. The cell pellet was washed in PBS supplemented with 0.5% bovine serum albumin. The total cell number in the BAL cell suspension was counted in a Coulter Counter Model ZM (Coulter Electronics, Hialeah, FL, USA) and viability was assessed by cellular exclusion of trypan blue. With FACScan flow-cytometry (Becton Dickinson, San Jose, CA, USA) we analyzed $CD3^+$ T-cells. Immunocytochemistry of cytospins was carried out with the APAAP (alkaline phosphatase anti-alkaline phosphatase) method and the monoclonal antibodies CD68 (alveolar macrophages), CD15 (VIM-D5, neutrophils), BMK-13 (eosinophils). Two independent observers counted at least 300 cells in each cytospin.

Superoxide production assay
ROS production of BAL cells was determined by the kinetic microplate assay of superoxide dismutase (SOD)-inhibitable ferricytochrome-c (0.16 mM horse heart cytochrome-c, Sigma) reduction in a 96-well microplate (Falcon 3072, Becton Bickinson, Lincoln Park, NJ). The cells, at a density of $1x10^5$/well, were stimulated, in the absence and the presence of SOD, with 8 nM and 80 nM phorbol 12-myristate 13-acetate (PMA) at 37 °C. At 18 seconds intervals the change in absorbance at 550 nm (1 nm bandwith) was read during 20 minutes in a Thermomax microplate reader (Molecular Devices, Sopachem, Lunteren, The Netherlands), using 540 nm (10 nm bandwith) as a reference wavelength. All measurements were performed in duplo. Using the software programme SOFTmax PRO the maximum

velocity (V_{max}) of ferricytochrome-c reduction was determined by calculating the first derivative of the absorbance time course in each well, and next expressed as nmol of superoxide/min/10^6 cells, using the difference in V_{max} in the presence and absence of SOD, the reduced minus oxidized cytochrome-c extinction coefficient of 21.1 $mM^{-1}cm^{-1}$, and the empirically determined light path length. Since we found the V_{max} upon stimulation with 80 nM PMA did not increase further as compared to 8 nM PMA, apparently the cells were already maximally stimulated at the lowest PMA concentration. Therefore, we present these data as the mean and do not refer to the PMA concentration.

Statistical analysis

Pre- and post-intervention data were tested for significant difference with the paired t-test. Differences between patient groups were tested with either the (unpaired) Student t-test or the Mann-Whitney test, depending upon distribution of data. Correlation studies were performed with the Spearman rank test. Statistical significance was considered at $p < 0.05$.

Results

From the initial 23 participants in the intervention study, paired results were obtained from 6 FP patients and from 9 patients with placebo. The reasons for missing paired data were insufficient recovery of either BAL fluid or BAL cells. The patient characteristics (n=15), listed in table 1, show that on average these patients had moderately severe obstructive airways disease and severe BHR. Compliance with study medication was high: the percentage of returned used blisters was 92.5 in the FP and 92.7 in the placebo group.

Table 1. Patient characteristics (mean ±SD).

	Fluticasone propionate	Placebo
N	6	9
Age	56 ±7	57 ±9
Cigarettes/day	17 ±8	15 ±5
Packyears	25 ±12	27 ±10
FEV_1 %predicted	72 ±12	68 ±13
FEV_1 reversibility	4.6 ±2.4	5.3 ±2.4
PC_{20} for histamine	3.3 ±3.2	0.8 ±0.6

Differences of baseline characteristics between the FP and the placebo group were not statistically significant. In the placebo group, mean FEV_1 declined from 68.5 %pred at the start of the trial to 52.0 %pred after 6 months. However, lung function remained unchanged in the FP group (mean FEV_1: 72.4 and 69.1 %pred, respectively). This treatment effect was statistically significant, and considered as a beneficial effect of FP treatment.

Immunocytology

Mean total cell number of the BAL at the start of the trial was 53.0 x 10^6 for FP treated patients and 45.3 x 10^6 in the placebo group (table 2). Small changes were noticed in the BAL cell numbers at the end of the trial, which were not significant (table 2). The percentages of fluid recovery, cell viability and cells types were not statistically different between both patient groups, before intervention, and did not change significantly after 6 months of intervention (table 2). The low number of eosinophils indicates that COPD was stable in nature.

Table 2. Characteristics of bronchoalveolar lavage (mean ±SD).

	Fluticasone propionate		Pacebo	
	Start	6 months	Start	6 months
Fluid recovery, %	60 ±13	62 ±11	54 ±16	51 ±19
Cell number, x10^6	53 ±13.5	59 ±31	45.3 ±22.7	36.4 ±18
Cell viability, %	62 ±12	66 ±12	70 ±13	67 ±12
$CD68^+$ macrophages, %	95 ±3	95 ±4	95 ±4	91 ±5
$CD15^+$ granulocytes, %	2.4 ±2.8	1.9 ±1.2	3.1 ±2.8	4.2 ±7.5
$BMK13^+$ eosinophils, %	0.4 ±0.3	0.8 ±0.5	0.4 ±0.3	0.8 ±1.0
$CD3^+$ T-cells, %	2.0 ±2.9	0.7 ±0.9	3.0 ±2.9	3.0 ±6.5

ROS production capability

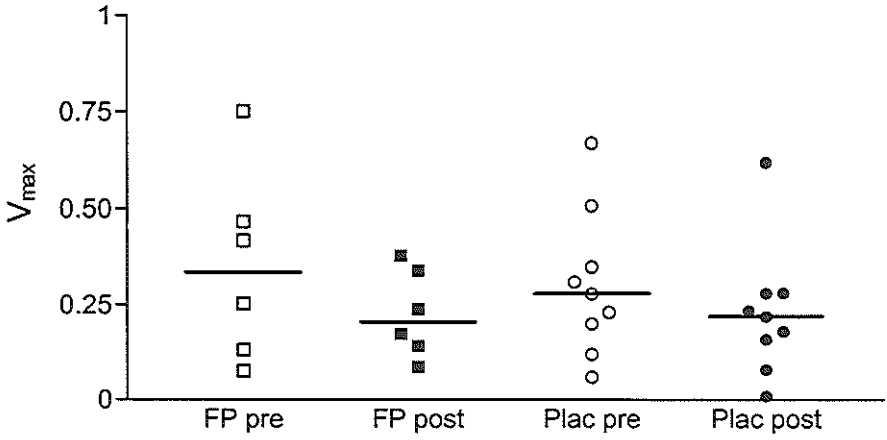
Figure 1 shows V_{max} of the patients from the FP and the placebo group, before and after intervention. V_{max} ranged from 0.076 to 0.753 (mean 0.35) nmol superoxide/min/10^6 cells in the FP treated patients, and from 0.06 to 0.67 (mean 0.303) in the placebo group. Statistical analysis showed that there were no significant changes after FP or placebo.

Association of V_{max} with airways obstruction, BHR and smoking status

There were no significant correlations between pre-treatment V_{max} on the one hand, and FEV_1 and PC_{20} on the other. However, the actual number of cigarettes smoked

per day correlated significantly with pre-treatment V_{max} (figure 2, R = -0.67, p = 0.007, Spearman rank test). There was no significant correlation with packyears of smoking.

Figure 1. Results of the superoxide production assay.



V_{max}: maximum velocity of reactive oxygen species production, nmol/min/10^6 cells;
FP: Fluticasone propionate; Plac: placebo; pre: pre-intervention; post: post-intervention.

Discussion

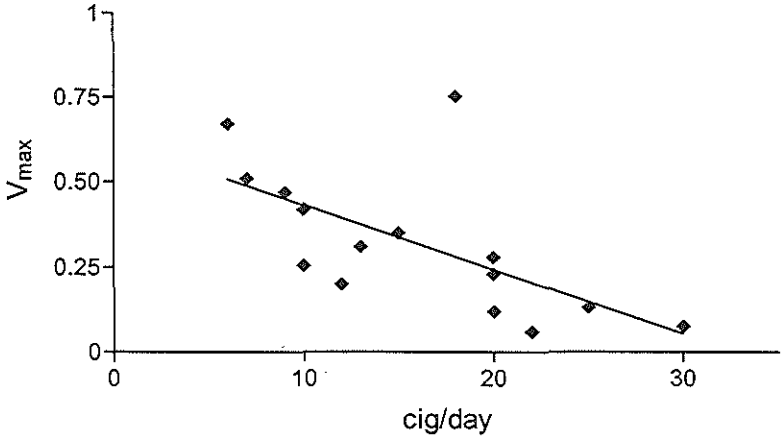
In this double blind, placebo controlled trial, we observed no significant effect of a potent inhaled glucocorticoid on the ROS production capability (V_{max}) of BAL cells derived from a subgroup of COPD patients, namely those with bronchial hyperresponsiveness. At first glance, this result appears to be related to the absence of a decrease of the number of inflammatory cells in the BAL. Our correlation studies indicated that actual smoking status is an important confounding factor. With increasing number of cigarettes smoked per day, V_{max} decreases.

As there has not been published a similar study in COPD patients before, we compared our results with studies concerning the effects of glucocorticoids on ROS production capability of blood derived cells, and with studies concerning the effect of smoking.

In one study, the superoxide production by PMA-stimulated blood granulocytes, taken from 18 miscellaneous patients on oral glucocorticoid treatment, was decreased as compared to healthy controls (8). Superoxide production by blood granulocytes of patients with emphysema was decreased after *in vivo* prednisolone

treatment (9). However, dexamethasone, both *in vitro* and *in vivo*, did not alter the spontaneous superoxide release of blood granulocytes from healthy volunteers (10).

Figure 2. Correlation of reactive oxygen species production and smoking.
$R = -0.67$, $p < 0.01$ (Spearman rank test).



V_{max}: maximum velocity of reactive oxygen species production, nmol/min/10^6 cells
Cig/day: number of cigarettes smoked per day.

The reported studies concerning the effect of smoking on ROS production capability are not consistent. Alveolar macrophages (AM) from healthy smokers produced more superoxide than AM from nonsmoking control subjects (11 - 13). Exposure to tobacco smoke *in vitro* increased the oxidative metabolism of AM (14), while it reduced superoxide generation by blood granulocytes (15). Hoidal et al. used almost the same lavage method as in our study, and compared young asymptomatic smokers with nonsmokers (11). They reported that BAL cells from smokers, stimulated with PMA, produced on average 1.93 nmol ROS/min/10^6 cells, while BAL cells from nonsmokers produced 0.945 nmol ROS/min/10^6 cells. We found an average (pre-intervention) ROS production of 0.322 nmol/min/10^6 cells (n = 15). The low ROS production capability in our study could either be due to patient selection (COPD), the presence of BHR, different BAL cell counts or to smoking. ROS production by BAL cells from smokers without COPD was not different from smokers with COPD (16). So, the diagnosis of COPD seems to be unimportant. Furthermore, since the level of PC_{20} was not significantly correlated with V_{max}, and the cell profile in BAL resembles the reported findings in 'average' COPD patients and smokers, the first three options are not likely. However, our

correlation analyses indicate that cigarette smoking influences ROS production capability. A possible explanation could be that viable macrophages loose their ability to release superoxide after repeated stimulation (17). This was attributed to ligand-induced desensitisation of specific receptors. This phenomenon might also have occurred in our heavy smoking COPD patients.
In conclusion, the BAL cells from smoking COPD patients with BHR, consisting mainly of alveolar macrophages, have a low ROS production capability. This was not further decreased by *in vivo* exposure to a potent inhaled glucocorticoid. The low ROS production capability might be one of the causative mechanisms of the increased susceptibility of respiratory tract infections and the increased frequency of bacterial colonisation of the airways of COPD patients.

Acknowledgements

The authors would like to thank dr. R.J. van Klaveren for critically reading the manuscript, and GlaxoWellcome, the Netherlands (FLIL44/FMS40060), for their financial support.

References

1. Jeffery PK. Comparative morphology of the airways in asthma and chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 1994; 150: S6-S13.
2. Cantin AM, Crystal RG. Oxidants, antioxidants and the pathogenesis of emphysema. *Eur J Respir Dis* 1985; 66: 7-17.
3. Repine JE, Bast A, Lankhorst I, and the oxidative stress study group. State of the art: oxidative stress in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 1997; 156: 341-357.
4. Pryor WA, Stone K. Oxidants in cigarette smoke: radicals, hydrogen peroxides, peroxynitrate, and peroxynitrite. *Ann N Y Acad Sci* 1993; 686: 12-28.
5. ATS Statement. Standards for the diagnosis and care of patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 1995; 152: S77-S120.
6. Quanjer PH. Lung volumes and forced expiratory flows. Report Working Party Standardization of lung function tests, European Community for Steel and Coal. *Eur Respir J* 1993; 6, Suppl. 16: 5-40.
7. Workshop summary and guidelines: investigative use of bronchoscopy, lavage and bronchial biopsies in asthma and other airways diseases. *Am Rev Respir Dis* 1991; 135: 250-263.
8. Fukushima K, Ando M, Ito K, *et al.* Stimulus- and cumulative dose-dependent inhibition of O2-production by polymorphonuclear leukocytes of patients receiving corticosteroids. *J Clin Lab Immunol* 1990; 33: 117-123.
9. Renkema TE, Postma DS, Noordhoek JA, *et al.* Influence of in vivo prednisolone on increased in vitro O2- generation by neutrophils in emphysema. *Eur Respir J* 1993; 6: 90-95.
10. Lomas DA, Ip M, Chamba A, Stockley RA. The effect of in vitro and in vivo dexamethasone on human neutrophil function. *Agents Actions* 1991; 33: 279-285.
11. Hoidal JR, Fox RB, LeMarbe PA, *et al.* Altered Oxidative metabolic response in vitro of alveolar macrophages from asymptomatic cigarette smokers. *Am Rev Respir Dis* 1981; 123: 85-89.
12. Nakashima H, Ando M, Sugimoto M, *et al.* Receptor-mediated O_2^- release by alveolar macrophages and peripheral blood monocytes from smokers and nonsmokers. *Am Rev Respir Dis* 1987; 136: 310-315.

13. Schaberg T, Haller H, Rau M, *et al.* Superoxide anion release induced by platelet-activating factor is increased in human alveolar macrophages from smokers. *Eur Respir J* 1992; 5: 387-393.
14. Drath DB, Karnovsky ML, Huber GL. The effects of experimental exposure to tobacco smoke on the oxidative metabolism of alveolar macrophages. *Journal of the Reticuloendothelial Society* 1970; 25: 597-604.
15. McLeod RG, Mack DG, McLeod EG, *et al.* Alveolar macrophage function and inflammatory stimuli in smokers with and without obstructive lung disease. *Am Rev Respir Dis* 1985; 131: 377-384.
16. Tsuchiya M, Thompson DF, Suzuki YJ, *et al.* Superoxide formed from cigarette smoke impairs polymorphonuclear leukocyte active oxygen generation activity. *Arch Biochem Biophys* 1992; 299: 30-37.
17. Berton G, Gordon S. Desensitization of macrophages to stimuli which induce secretion of superoxide anion. Down-regulation of receptors for phorbol myristate acetate. *Eur J Immunol* 1983; 13: 620-627.

Chapter 7

Glucocorticoids hamper the *ex vivo* maturation of lung dendritic cells from their low autofluorescent precursors in the human bronchoalveolar lavage: decreases in allostimulatory capacity and expression of CD80 and CD86

G. T. Verhoeven[1,2], *J. M. W. van Haarst*[1], *H. J. de Wit*[1], *P. J. Simons*[1], *H. C. Hoogsteden*[2], *H. A. Drexhage*[1].
Departments of [1]Immunology and [2]Pulmonary and Intensive Care Medicine, University Hospital Dijkzigt, EMCR, Rotterdam, the Netherlands.

(Published in: Clin Exp Immunol 2000; 122: 232-240)

Abstract

Dendritic cells (DCs) were prepared from human bronchoalveolar lavage (BAL) cells. We previously reported that in particular the CD1a fraction of the low autofluorescent (LAF) cells contains the precursors for DCs: after overnight culture, 40% of the LAF cells change into functionally and phenotypically prototypic dendritic/veiled cells. There are, as yet, no data on the modulatory effects of glucocorticoids (GC) on the maturation and function of such DCs isolated from the human lung.
Functional tests (allogeneic mixed lymphocyte reaction: allo-MLR) were therefore performed with $CD1a^+$ LAF cells at different stimulator-to-T-cell ratios and after preincubation with different dexamethasone (DEX) concentrations. DEX caused suppression of the T-cell stimulatory capacity of $CD1a^+$ LAF cells, which was dose-dependent, and more evident at the higher stimulator-to-T-cell ratios.
Here, we also show that CD80 and CD86 are normally expressed at low levels on $CD1a^+$ LAF cell-derived DCs as compared to other DC populations. This low-level expression of co-stimulatory molecules is discussed here in relation to the previously reported low-level expression of CD80 (and CD86) on lung DCs in experimental animals. This appears to play a role in a predominant Th2 cell stimulating potential of DC from the lung environment. DEX exposure of $CD1a^+$ LAF cells prevented the upregulation of even this low-level expression of CD80 and CD86.
The veiled/dendritic morphology and the expression of other relevant cell surface markers and adhesion molecules were not affected by DEX exposure.
It is concluded that DEX hampers the maturation of $CD1a^+$ LAF cells into active lung DCs.

Introduction

Dendritic cells (DCs) are professional antigen-presenting cells (APC) and, as such, the most potent accessory cells for the stimulation of T cells (1). This function makes the pulmonary DC a target cell for studying the initiation and regulation of the immune processes underlying various lung diseases (2,3). In humans and in experimental animals, pulmonary DCs form a contiguous network within - and directly below - the airway epithelium and throughout the alveolar interstitium. Pulmonary DCs situated just underneath the bronchial epithelium are often seen in small clusters with T-cells (4,5). In allergic asthma, increased numbers of $CD1a^+$ DCs have been observed at this location (6,7).

Glucocorticoid (GC) therapy, considered to be the most effective treatment in asthma (8), reduces the number of DCs in the bronchial mucosa (6,7,9). Studies in rats have suggested that GCs cause this reduction in number by inhibiting the influx of DCs into the airway wall (10). Moreover, GCs induce apoptosis of DCs in rat tracheal mucosa (11). Studies using human monocytes, monocyte-derived DCs or human epidermal Langerhans cells, indicate that GCs can directly affect the maturation of these cells, and can downregulate their accessory function (12-17). There are, to our knowledge, no data available on the direct effects of a GC on the *ex vivo* function and marker expression of DCs isolated from the human lung.

Recently, we described (18) the isolation, via FACS-sorting, of low autofluorescent (LAF) cells from human bronchoalveolar lavage (BAL). These LAF cells matured, after being cultured overnight, into veiled/dendritic cells with the morphology and the immunophenotype of immature DCs. The cells showed a strong potency to stimulate naive T-cells, and both contained and released biologically active IL-1 and IL-6. We also described the marked differences between the $CD1a^+$ and $CD1a^-$ subsets of LAF cells (19). The $CD1a^+$ subset exhibited a higher accessory capability than the $CD1a^-$ subset. $CD1a^+$ LAF cells were very poor producers of IL-1, IL-6 and TNF-α, whereas $CD1a^-$ LAF cells were potent producers of these cytokines. $CD1a^+$ LAF cells were – after the overnight maturation period – both positive for and producers of S100; $CD1a^-$ LAF cells were negative in this respect. We therefore concluded that the $CD1a^+$ LAF cells could be regarded as examples of typical DCs from the lung environment, because they can rapidly assume all the characteristics of Langerhans cell-like immature DCs (19).

The above-described method of obtaining typical DCs from human BAL enabled us to study, *ex vivo*, the function and phenotype of human pulmonary DCs, with or without preincubation with GCs.

We report an investigation on the effect of various concentrations of GCs on the allogeneic T-cell stimulatory capacity of both the LAF cells and the $CD1a^+$ LAF cells isolated from the human BAL. We also studied cell morphology and the expression of cell surface markers, adhesion molecules and co-stimulatory molecules in the various DC populations, both before and after incubation with GCs. Markers and molecules were chosen that are known to be relevant for T cell stimulation (e.g. MHC-class-II, CD80, CD86, CD11, CD54, etc.) and can be affected by GCs (12-17).

Materials and methods

Bronchoalveolar lavage

After we obtained informed consent, BAL was performed on individuals during anesthesia for routine elective surgery. Subjects with a history of pulmonary or serious systemic disease were excluded. BAL was carried out with a flexible bronchoscope placed in the right middle lobe with the tip in wedge position. Four aliquots of 50ml isotonic saline were, subsequently, instilled and aspirated with gentle suction. BAL fluid was collected in siliconized bottles. The selected subjects were predominantly active smokers (n = 24, smoking 17 ±7 (mean ±SD) cigarettes per day), because the yield of cells in smoking individuals is much larger and more workable than in nonsmoking individuals. We also studied the BAL cells of a few subjects who had never smoked (n = 3). The mean age of the lavaged subjects was 36 years (range 18-53 years). The procedure was approved by the Medical Ethics Committee of the Erasmus University and University Hospital Dijkzigt.

Isolation and purification of DC

BAL cells were kept at 4°C, washed twice in phosphate buffered saline (PBS) containing 0.5% bovine serum albumin and 0.45% glucose and, subsequently, filtered through a 55μm and a 30μm gauze. BAL cells were sorted on a FACS-Vantage (Becton-Dickinson, Erembodegem, Belgium) with a 488-nm laser. Sort windows were generated on autofluorescence (FL1, 530 nm) to create a population of cells with low autofluorescence (LAF) in order to exclude alveolar macrophages (AM), on forward scatter (FSC) to exclude small cells (lymphocytes) and debris and on sideward scatter (SSC) to exclude cells with a high SSC (granulocytes and AMs). As we described previously, this procedure yields a population of LAF cells, comprised of DCs and their precursors, with small contaminations of AMs (± 10%) and lymphocytes (± 5%) (18).

A further purification was performed in experiments by sequential labeling of LAF cells with OKT6 (CD1a) conjugated with phycoerythrin and an additional sort window on fluorescence channel 2 (FL2, 585 nm). Flow cytometry results were reported in an earlier publication (19). This method yields $CD1a^+$ DCs with a contamination of AMs of ≤ 2%.

Blood monocytes were purified according to techniques described in detail elsewhere (20). In short, monocytes were isolated from heparin blood or buffy coats by subsequent Ficoll-Paque (1.079 g/ml; Pharmacia, Uppsala, Sweden) and Percoll (1.063 g/ml; Pharmacia) density gradient separation.

Glucocorticoid exposure

After isolation, the various BAL DC populations and the blood monocytes were incubated overnight under non-adherent conditions in RPMI containing 10% FCS (16 hours, 37°C, 5% CO_2, polypropylene tubes, Falcon, Lincoln Park, NJ, USA) with or without various concentrations of dexamethasone (DEX) (Sigma, St. Louis, MO, USA). To prevent contamination of the MLR with DEX, cells were washed four times

after this incubation period. Between each wash, the cells were incubated at 37°C for 30 min.
In selected experiments, beclomethasone dipropionate (BDP) or fluticasone propionate (FP) replaced DEX. These GCs are widely used as inhalation therapy for asthma patients.

Monoclonal antibodies
The following monoclonal antibodies (MoAb) were used: OKIa (HLA-DR) and OKM1 (CD11b) (Ortho Diagnostics Systems, Beerse, Belgium); LFA-1/2 (CD11a) and LFA-1/1 (CD18) (CLB, Amsterdam, the Netherlands); LeuM5 (CD11b) (Becton & Dickinson, San Jose, CA, USA); My4 (CD14) and 4B4 (CD29) (Coulter Immunology, Hialeah, FL, USA); HP2/1 (CD49d) (Immunotech, Marseille, France); BBA3 (anti-ICAM-1, CD54) (British Biotechnology, Oxon, UK); OKT6 (CD1a) (American Type Culture Collection, Rockville, MD, USA); B7-24 (anti-B7-1, CD80) and B70/B7-2 (CD86) (Pharmingen GmbH, Hamburg, Germany). L25 is a MoAb directed against B cells and DC (21,22) and was a generous gift from Dr T. Takami (Gifu, Japan); RFD1 recognizes a class II-associated epitope present on activated B cells, DC and alveolar macrophages (23) and was kindly provided by Dr L.W. Poulter (London, UK). HB15a (CD83) was provided by T. F. Tedder (Boston, MA, USA). TS2/9 (anti-LFA3, CD58) was a gift from T. Schumacher (Amsterdam, the Netherlands).

Immunocytology
Cytocentrifuge preparations were prepared from the FACS-sorted LAF and $CD1a^+$ cell fractions, immediately after sorting, as well as after overnight culturing in RPMI/FCS with or without 10^{-6}M DEX. The cytocentrifuge preparations were fixed in acetone (Merck, Darmstadt, Germany) for 10 min, and then incubated with normal rabbit serum 10% (Dako, Gostrup, Denmark) in PBS for 10 min. Next, the slides were incubated for 1 h with the appropriate MoAb. Subsequently, the slides were either incubated with rabbit-anti-mouse antiserum conjugated with horseradish peroxidase (RαMHRP; Dako) for diaminobenzidine (Sigma Chemicals, Axel, the Netherlands) staining, or with alkaline phosphatase anti-alkaline phosphatase for Fast Blue BB Base (Sigma) staining. Most slides were stained for acid phosphatase and selected slides were stained for non-specific esterase. Counterstaining was performed with hematoxylin.

Allogeneic T-cell stimulation
Responder T-cells were isolated from buffy coats of healthy blood donors. Buffy coats were diluted 1 : 1 with PBS, and mononuclear cells were obtained by Ficoll-Paque (1.079 g/ml; Pharmacia) density gradient separation (15 min, 1000 *g*). Monocytes and lymphocytes were separated by Percoll (1.063 g/ml; Pharmacia) density gradient centrifugation. B-cells and residual monocytes were removed from the lymphocytes by adherence to a nylon wool column (Polyscience, Warrington, PA, USA) (1 h, 37°C, 5% CO_2). The purified T-cell populations were stored in liquid nitrogen until use.

For the allogeneic mixed lymphocyte reaction (MLR) (18, 19), variable numbers of stimulator cells (e.g. 950, 1900, 3750, and 7500 $CD1a^+$ LAF cells) were cultured with 150 000 allogeneic T-cells (giving a stimulator-to-T-cell ratio of ranging from 1 : 160 to 1 : 20). Cultures were always performed in triplicate in a flat bottom 96-well plate (Falcon) with a total volume of 200 µl per well. The culture medium consisted of RPMI 1640 (Gibco, Breda, the Netherlands) to which 10% human A^+ serum, penicillin and streptomycin were added. Before the MLR, the stimulator cell populations had been irradiated with 2000 Rad. After 5 days, 0.5 µCi ^{3}H-thymidine (20 µl from a stock of 250 mC/ml ^{3}H-thymidine, Amersham, Buckinghamshire, UK) was added. The cells were harvested 16 h later. Scintillation was counted with an LKB 1205 Betaplate liquid scintillation counter (Wallac, Turku, Finland). ^{3}H-thymidine incorporation in T cells alone, as well as in the various stimulator cell fractions without T cells, was always less than 500 c.p.m.

Statistical Analysis

The Wilcoxon matched pairs test was used for the statistical analysis of data which were obtained in the DEX dilution series. In order to compare the results of other data sets, either Student's-*t*-test or the Mann-Whitney test were applied, when appropriate. *P*-values < 0.05 were considered significant.

Results

Allogeneic T cell stimulation by LAF cells and $CD1a^+$ pulmonary DC

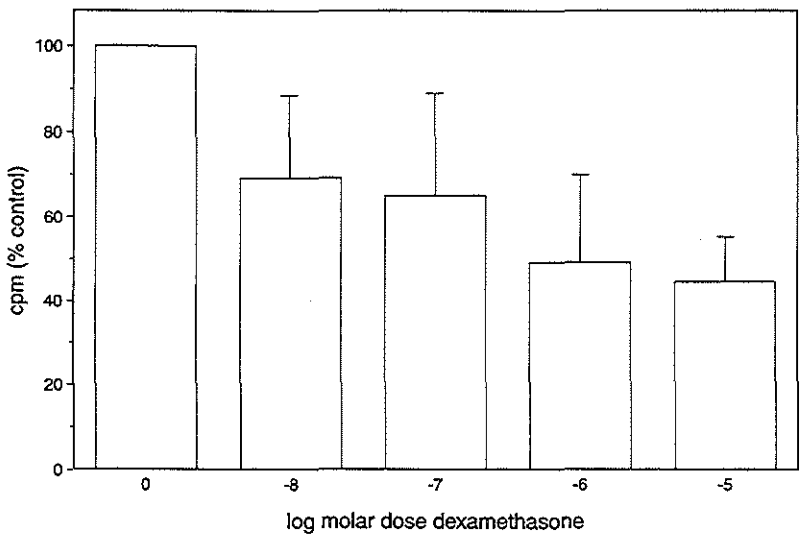
With regard to the yields of the various LAF cell populations in smokers: our starting population of BAL cells, after the first washing procedures, ranged from 4.0-7.5 x 10^6 cells ($n = 24$). The yield of LAF cells after FACS sorting was 1.8% (SD 0.8, range 0.9-3.5) of the initial population, and that of high autofluorescent (HAF) cells, i.e. alveolar macrophages, 13.4% (SD 5.3, range 6.1-23.7). The yield of $CD1a^+$ LAF cells was 0.47% (SD 0.31, range 0.1-1.0) of the total BAL cells. That of $CD1a^-$ LAF cells was 1.33% (SD 0.74, range 0.5-2.7). Because the yield of the $CD1a^-$ LAF cells was so low and involved such a cumbersome methodology, we developed a strategy in which experiments were first carried out on unseparated LAF cells before verifying interesting data on purified $CD1a^+$ LAF cells.

With regard to nonsmoking subjects: yields of BAL and LAF cells were found to be no more than one tenth of those from smoking subjects. Therefore, only a few experiments were carried out in this group (see below).

In the first series of experiments, the optimal *in vitro* dosage of DEX for influencing BAL DCs was investigated. For this purpose, unseparated LAF cells were exposed to graded concentrations of DEX for 16 h (overnight). The cells were then washed thoroughly and used as stimulator cells in the MLR. The recovery of the cells after overnight DEX exposure was established via simple cell counting; the viability of LAF cells after overnight exposure to DEX was tested via trypan blue exclusion. DEX neither affected the cell survival (the recovery with or without DEX was identical: 40-60%), nor affected the viability of the cells (80-90%). Figure 1 gives the MLR data.

10^{-10}M DEX had no significant effect on LAF cell-induced T-cell proliferation and T-cell stimulations were found to be 40-80 x 1000 c.p.m. in our assay system.
10^{-8}M DEX significantly suppressed the LAF cell-induced T-cell response to 69.2 ±19.2% (mean ±SD) of the value found in the absence of DEX. Suppression to 65.0 ±23.9% was reached with 10^{-7} M, suppression to 49.1 ±21.0% with 10^{-6} M and suppression to 44.5 ±10.5% with 10^{-5} M DEX (n = 5-7, $p < 0.05$ for all concentrations tested, Wilcoxon matched pairs test).

Figure 1. Effect of increasing concentrations of dexamethasone (DEX) preincubation (16 h) on accessory function of low-autofluorescent (LAF) cells.
Data represent mean ±SD of 5-7 experiments of ^{3}H-thymidine incorporation expressed as percentage of ^{3}H-thymidine incorporation of the situation without DEX-exposure.



We checked to see if a DEX contamination, after the washing procedure, had influenced these MLR results by directly influencing the responder T-cell population, thus accounting for the suppression. For this purpose, LAF cells were exposed to 10^{-6} M ^{3}H-labelled DEX overnight. ^{3}H-DEX concentrations in the supernatant and in the cell pellet, after the above-described washing procedure, was as low as 10^{-13} M. At concentrations lower than 10^{-10} M, we found that DEX did not affect PHA-driven T-cell stimulation. Hence, contaminating quantities of residual DEX, directly influencing T-cells, could not have accounted for the described suppression of the LAF cell driven MLRs. On the basis of the above-described data using LAF cells, we decided to use 10^{-10} through 10^{-6} M DEX in further experiments with CD1a$^+$ and CD1a$^-$ populations, purified from the LAF cell fraction.

Figure 2. Effects of increasing concentrations of dexamethasone (DEX) on the allogeneic T cell stimulatory capacity of CD1a$^+$ and CD1a$^-$ LAF cells derived from human BAL, and blood monocytes.
Results are expressed as counts per minute (c.p.m.) of ^{3}H-thymidine incorporation in the mixed lymphocyte reaction (MLR) of 5–15 interindividual experiments (mean ±SD). A few data are from single, duplicate experiments (in this case, SD is not given). Intraindividual variation was negligible. Open bars express DEX-free experiments, the wide-striped bars 10^{-10} M DEX, the narrow-striped bars 10^{-8} M and the closed bars 10^{-6} M. Asterisks indicate significance of group results ($p < 0.05$, Mann-Whitney or Student's *t*-test).

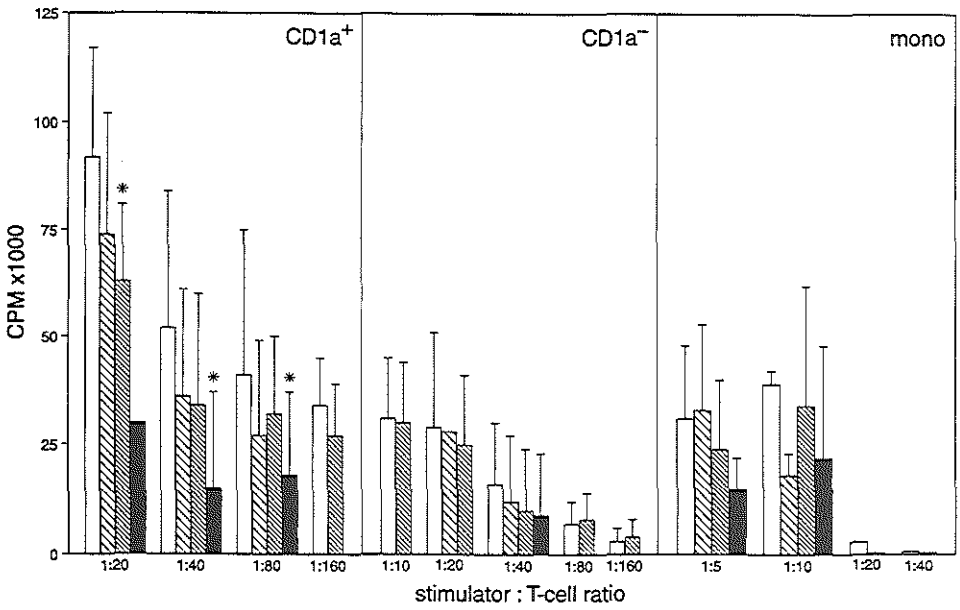


Figure 2 shows the results of the MLRs with blood monocytes and CD1a$^+$ and CD1a$^-$ LAF cells as stimulators at various DC-to-T-cell ratios, as well as with graded concentrations of DEX. In the ratios of 1 : 40 and 1 : 80, 15 individual BAL derived CD1a$^+$ and CD1a$^-$ cells could be used. For the other ratios, fewer BAL isolated cells were available (Fig. 2). For all stimulator-to-T-cell ratios, the CD1a$^+$ cells were the most potent T-cell stimulators. Statistically significant differences between the (non-GC exposed) CD1a$^+$ DCs and CD1a$^-$ LAF cells were found for the stimulator-to-T-cell ratios of 1 : 20 through 1 : 60 ($p < 0.05$, Mann-Whitney test).
DEX exposure had no effect on the recovery and the survival of the cells, although it appeared to have a dose-dependent suppressive effect on CD1a$^+$ LAF cells in the MLR when used at various stimulator-to-T-cell ratios. The suppressive effect of DEX on CD1a$^+$ DCs was statistically significant for all concentrations used. When compared to cultures without DEX, $p = 0.044$ for 10^{-10} M; $p = 0.004$ for 10^{-8} M; $p = 0.031$ for 10^{-6}

M, Wilcoxon test). Interestingly, DEX exposure had no significant suppressive effect on the intrinsically low stimulatory capacity of the $CD1a^-$ cells. The DEX exposure effect on blood monocytes with regard to T-cell stimulatory capacity was limited and not significant. In ratios of 1 : 10 and 1 : 5 at a concentration of 10^{-6} M DEX: p = 0.0625 (n = 6, Wilcoxon test).

As smoking has a negative effect on the allogeneic T-cell stimulatory capacity of DCs (5,18), we compared the suppressive effect of DEX in smokers and in nonsmokers. We found that DEX induced comparable effects on LAF cells isolated from the BAL of both smokers and nonsmokers. Preincubation overnight with 10^{-6} M DEX suppressed the proliferative T-cell response to 52.6% in nonsmokers (n = 3) and to 46.5% in smokers (n = 4). We therefore did not extend these experiments to include $CD1a^+$ fractionated cells, because, among other reasons, it is difficult to obtain sufficient LAF cells from the BAL of nonsmoking subjects.

GCs other than DEX, such as beclomethasone dipropionate (BDP) and fluticasone propionate (FP), also suppressed LAF cell-induced T-cell proliferation in the MLR. In a limited series of six experiments, mean (±SD) c.p.m. x1000 was 90.4 (±11.0) without GC, 71.7 (±3.2) with DEX, 66.7 (±4.5) with BDP and 37.1 (±16.2) with FP. In these experiments, we used a LAF-to-T-cell ratio of 1 : 20 and the concentration of the GCs was 10^{-6} M. This shows that steroids other than DEX also reduce the accessory capability of LAF cells, albeit at other potencies. We did not extend these studies on the suppressive effects of steroids other than DEX to include $CD1a^+$ fractionated cells.

Immunocytology of unseperated LAF cells

It was clear that the overnight DEX-exposure affected the T cell stimulatory capacity of the unseparated LAF cells and the $CD1a^+$ LAF cells. We, therefore, embarked on a study of the effects of DEX exposure on the expression of relevant molecules on the pulmonary APCs. Again, we started by studying the effects of DEX on the unseparated LAF cells (Table 1 and Fig. 3). First of all, we confirmed our earlier observations by demonstrating that 40% of the LAF cells develop a typical veiled morphology after an overnight maturation in the absence of DEX (Table 1).

The DC-like cells are negative for acid phosphatase, and have many typical DC markers when studied in immunocytochemistry: the MHC class II molecules are expressed by the vast majority of the LAF cells, while the MHC class II related DQ molecule L25 is expressed by ± 30% of the cells (Table 1). CD1a is expressed by ± 15%, and CD83 by ± 7% of the LAF cells (Table 1). The monocytic marker CD14 is expressed by ± 50% of the cells, but there was a large variation between experiments (SD, Table 1). In FACS analysis the intensity of CD14 expression decreased after overnight maturation of freshly isolated LAF cells (without veils) to DC-like cells (see representative example of three experiments, Fig. 3).

Noteworthy is the low level of CD80 expression by freshly isolated LAF cells and LAF cell derived veiled/dendritic cells: values of 2-5% to ± 12% were found in immunocytochemistry, respectively (Table 1). Again, there was a large variability between experiments. In immunocytochemistry, the CD80 expression was mainly intracellular, thus it is not surprising that in FACS analysis an even smaller percentage

(range 0 – 6 %) of the LAF cell derived veiled/dendritic cells expressed CD80 on their cellmembrane (see Fig. 3). CD86 expression was tested in one experiment. The expression was low in both freshly isolated LAF cells and their maturation products (± 20% of the cells) both in immunocytochemistry and FACS analysis (Table 1, Fig. 3).

Table 1. Immunocytochemistry of the LAF cells and the subpopulation of $CD1a^+$ LAF cells after overnight exposure to 10^{-6} M dexamethasone (DEX).
Control exposure was overnight exposure of the cells to the vehiculum only (culture fluid without DEX). For the LAF cells, $n = 6$; for the $CD1a^+$ LAF cells, $n = 4$. Means ±SD are given.

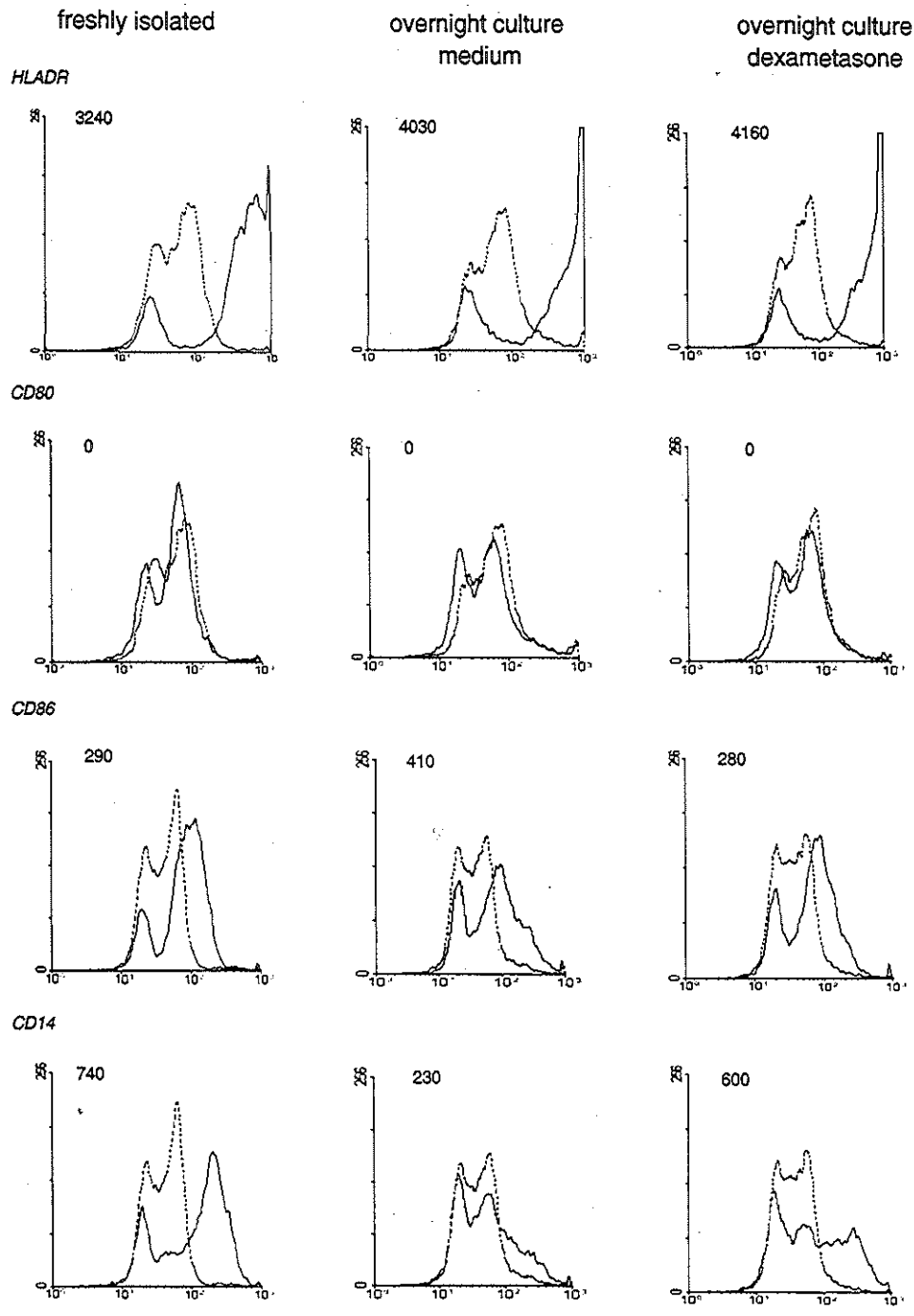
		Overnight culture without dexamethasone		Overnight culture with dexamethasone	
	Freshly isolated LAF cells	LAF cell derived DC	$CD1a^+$ LAF cell Derived DC	LAF cell derived DC	$CD1a^+$ LAF cell derived DC
DC morphology	11.0 ±3.0	39.0 ±16.8	-	36.5 ±15.6	-
HLA-DR	86.0 ±11.0	93.0 ±3.7	-	95.0 ±2.6	-
RFD1	-	29.3 ±16.1	-	25.3 ±16.7	-
L25	-	72.5 ±15.2	-	74.3 ±12.2	-
CD1a	20.0 ±14.0	14.8 ±11.7	-	14.3 ±10.8	-
CD83	4.0 ±1.0	7.0 ±0.8	-	5.0 ±2.5	-
CD14	63.0 ±14.0	52.5 ±30.3	-	52.0 ±26.2	-
CD80	2.0 -5.0	12.4 ±7.4	14.0 ±3.7	4.2 ±3.9*	11.3 ±3.4*
CD86	(17)	(23)	29.0 ±2.4	(15)	20.0 ±3.0*
CD11a	-	17.8 ±11.7	-	16.6 ±13.2	-
CD11b	45.0 ±17.0	39.3 ±15.2	31.0 ±11.3	16.2 ±10.7	34.6 ±9.0
CD11c	-	57.4 ±24.5	-	56.2 ±22.4	-
CD18	-	81.4 ±18.3	-	89.4 ±7.6	-
CD54	60.0 ±31.0	62.9 ±19.1	-	64.3 ±15.7	-
CD29	-	84.2 ±14.8	-	92.4 ±4.1	-
CD49d	-	15.3 ±4.1	-	13.7 ±5.6	-

* $p < 0.05$ versus control, Wilcoxon matched pairs test. –, not tested.

We also studied the marker expression after overnight maturation of LAF cells in the presence of DEX. This exposure affected neither the veiled morphology nor the expression of HLA-DR. The latter was observed both in terms of number of positive cells (Table 1), and intensity of marker expression (Fig. 3). Such exposure did also not affect the expression of the MHC class II-related molecules RFD1 and L25, and the expression of the typical DC markers CD1a and CD83.
In immunohistochemistry, the number of CD14 positive cells was not affected by DEX exposure (Table 1). In FACS analysis, the level of CD14 expression remained somewhat higher (for a representative example of three experiments, see Fig. 3).
Despite the low CD80 expression, DEX prevented the upregulation during the overnight maturation (Table 1, immunocytochemistry: 12.4% versus 4.2% positive cells, $n = 6, p < 0.05$; Wilcoxon test). In the one experiment in which CD86 expression was tested on unseperated LAF cells, DEX exposure also prevented the upregulation of this costimulatory molecule both in terms of number of positive cells (Table 1) and intensity of expression (Fig. 3).
Although CD11b expression on LAF cell-derived DCs was reduced after DEX exposure from 39.3 to 16.2% (mean, Table 1), it was not statistically significant due to large interindividual differences in expression. The expression of the other adhesion/costimulatory molecules CD11a, CD11c, CD18, CD29, CD49d, CD54 and CD58 was not affected by overnight DEX exposure, either in numerical terms (Table 1), or in terms of expression levels (data not shown).

CD80 and CD86 expression of $CD1a^+$ LAF cells
Because of the effects of DEX exposure on the costimulatory molecules CD80 and CD86 on LAF cells, similar studies were performed on the $CD1a^+$ and $CD1a^-$ LAF cell populations. The CD80 and CD86 expression was studied by FACS analysis, after overnight culture with or without DEX (10^{-6} M). We also studied the CD11b expression of these cells. Table 1 gives the data, while Fig. 4 gives a representative example of a FACS intensity staining used in such an experiment. It also appeared that the $CD1a^+$ LAF cells had a limited expression of CD80 and CD86, but slightly higher in comparison to the $CD1a^-$ cells, *ie,* CD80: 14 ±4% versus 5 ±3%; CD86: 29 ±2% versus 13 ±5%, both $p < 0.05$, $n = 4$). Exposure of $CD1a^+$ LAF cells to DEX gave a significant, but very small, decrease of CD80 expression (Table 1). CD86 expression was clearly diminished (Table 1). Overnight incubation with DEX had no effect on CD11b expression in $CD1a^+$ LAF cells (Table 1).

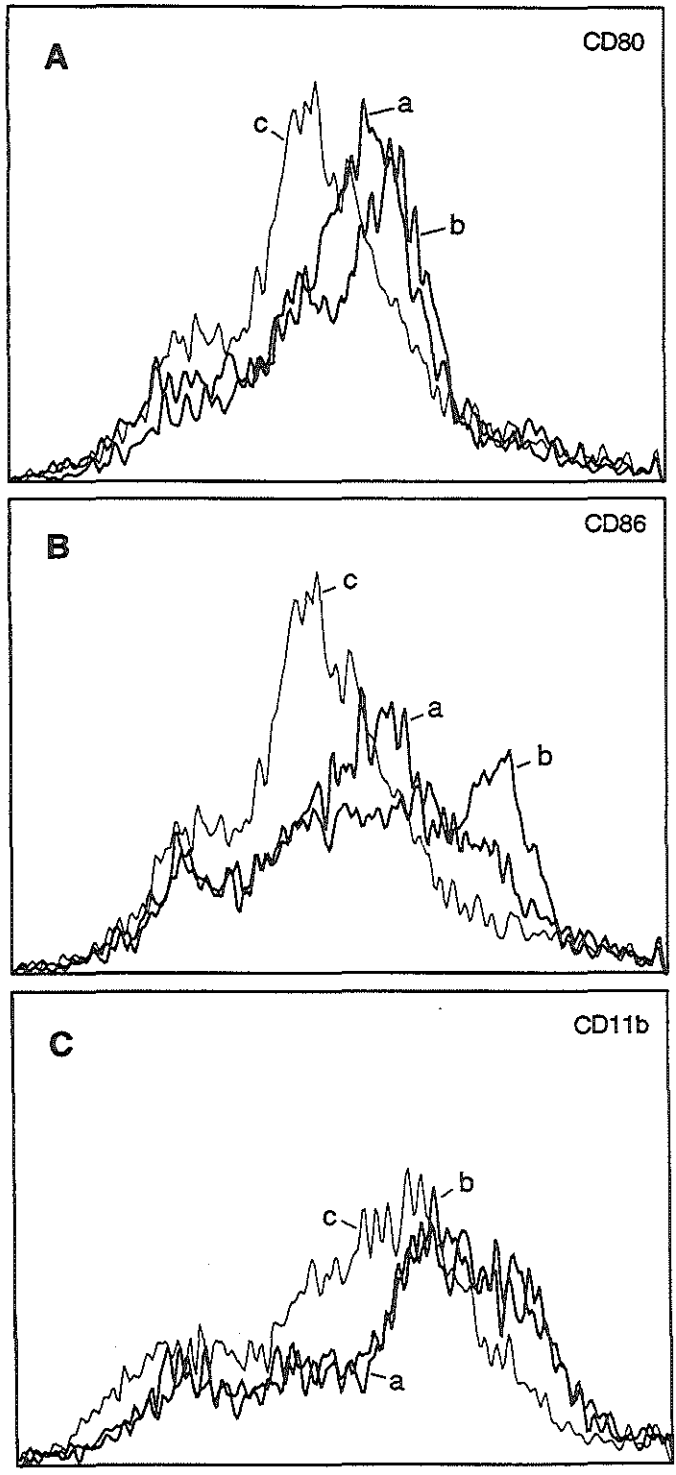
Figure 3. (next page) Flow cytometry results of a representative experiment (from three experiments) showing the effect of overnight maturation and the modulatory effect of dexamethasone (DEX) during this overnight process on the cell surface expression of HLA-DR, CD80, CD86 and CD14 on LAF cell derived dendritic cells.
Differences in mean fluorescence intensities (MFI) between staining with the specific antibody (solid line) and the IgG control (dotted line) are given in the upper left part of each graphs. Note that with the IgG control, there is a relatively high background of immunofluorescence, *ie,* autofluorescence.


freshly isolated
overnight culture medium
overnight culture dexametasone
HLADR
3240
4030
4160
CD80
0
0
0
CD86
290
410
280
CD14
740
230
600

Discussion

Costimulation via CD80 and CD86 is essential for full T-cell activation. The costimulatory molecules CD80 and CD86 expressed in antigen presenting cells (APC) interact for such activation with CD28 and CTLA-4 on T cells, thereby delivering essential second signals. CD80 and CD86 expression is also essential for lung and other DC populations: there are various reports showing that antiCD80 and/or antiCD86 MoAbs interfere with T cell stimulation by such DC populations (24-26).
DCs isolated from the respiratory tract of rats express relatively low levels of the costimulatory molecule CD80, which could indicate extreme immaturity (27). Our data on human BAL derived DCs agree with these animal data, and show that only a small fraction of human BAL-derived $CD1a^+$ LAF cells express CD80, i.e. 14%. CD86 was expressed in a larger number of the $CD1a^+$ LAF cells, though in no more than ± 30% of the cells. It is our experience and that of others (28), that 'immature' human monocyte-derived DCs (e.g. those generated by a 7-day culture of monocytes in GM-CSF/IL-4) express CD80 in ± 25% and CD86 in ± 50% of the cells. Mature DC populations (e.g. those from the spleen of mice, or after propagation of immature monocyte-derived DC in LPS and TNF-α containing culture fluids) express higher and equal levels of CD80 and CD86, i.e. on 50-60% of the cells (29).
CD80 and CD86 expression in APC have, in some models, different roles in influencing the commitment of the stimulated T cells to a Th1 or Th2 pathway of development. For example, according to Kuckroo *et al.* (30) and Freeman *et al.* (31), CD86 more effectively directs the differentiation of T-cells towards a Th2-like phenotype, whereas CD80 favours a Th1 development. For this reason, the relatively low CD80 expression of pulmonary DCs in rats, compared with the CD86 expression, could indicate that DCs at this location are in Th2 default; indeed, such pulmonary DCs have been proven to stimulate primarily Th2 pathways (27). Upon maturation of such rat lung DCs with GM-CSF, the cells first increase in CD80 expression up to the level of CD86 expression and then increase in IL-12 production capacity. At the same time, they loose their Th2 default (27). Since our data on human $CD1a^+$ BAL-DC are analogous to the rat pulmonary DC with regard to the relatively low CD80 and CD86 expression, it is tempting to speculate that the T-cells generated by such $CD1a^+$ BAL-DC are skewed towards a Th2 phenotype, but this needs further investigation.

Figure 4 (next page). Flow cytometry results of a representative experiment (from four experiments) showing the modulatory effect of dexamethasone (DEX) on the cell surface expression of CD80 (top, a), CD86 (middle, b) and CD11b (bottom, c) in $CD1a^+$ BAL derived dendritic cells, after 16 h culture. The expression of CD86, and to a lesser extend CD80, was decreased. Curve a: with addition of 10^{-6} M DEX, curve b: without DEX, and curve c: IgG-control (note with this control the relatively high background immunofluorescence, i.e. autofluorescence).


A
CD80
c
a
b
B
CD86
c
a
b
C
CD11b
b
c
a

Interestingly, the other cell population of the BAL, i.e. the alveolar macrophages, has also been described as being defective in its expression of costimulatory cell surface molecules (32). This might indicate that the BAL environment is exceptional in that it prevents full expression of costimulating molecules on lung APC. It is of interest to note that our $CD1a^+$ DC population isolated from the BAL was contaminated with less than 2% alveolar macrophages.

This report, moreover, shows that $CD1a^+$ DCs, *ex vivo*, generated from LAF precursors in the presence of GCs had a reduced capability to stimulate allogeneic T-cells. This suppression was dose-dependent and occurred at various stimulator-to-T cell ratios (Fig. 2). The exposure to GCs also prevented the, already minor, upregulation of the costimulatory molecules CD80 and CD86 during the overnight culture of freshly isolated LAF cells to $CD1a^+$ LAF cells. Neither the level of CD14 and MHC-class II expression, nor the dendritic morphology was affected significantly by GC. Collectively, these data could indicate that GCs force LAF cells to retain precursor characteristics during overnight culture.

There are no earlier reports available on the *ex vivo* effect of GC exposure on the allostimulatory function of human BAL-derived DCs. In a rat model, *in vivo* DEX treatment caused a 50% reduction of the immunostimulatory capacity of pulmonary DC (33). In mice, the immunostimulatory properties of splenic DCs were reduced by DEX exposure *in vitro* (34). The mouse DCs also showed that GCs caused a selective downregulation of the expression of CD80 and CD86.

With regard to human DC populations, GCs were reported to suppress the accessory function of epidermal Langerhans cells *ex vivo* (12). Using human monocyte-derived DCs, van den Heuvel *et al.* (16) and Piemonti *et al.* (17) reported that GCs affected the differentiation and maturation of DCs, resulting in APC populations less able to stimulate T cells. Piemonti *et al.* (17) also reported that a DC-specific marker (CD1a) and various costimulatory markers (CD40, CD86) were downregulated, while macrophage markers were increased (CD14, CD16). Van den Heuvel *et al.* (16) could not confirm these latter effects. With regard to GC effects on previously differentiated immature human monocyte-derived DCs, exposure to GCs inhibited the production of IL-1, IL-6, TNF-α and IL-12p70 when the cells were additionally stimulated with LPS or other stimuli (14,15). One report (15) indicates that such GC exposure had no effect on DC marker expression and T cell stimulatory capacity of the immature DCs. Other reports, however, found downregulating effects on the costimulatory molecules CD40, CD80 (17) and CD86 (14) on the DCs, and on the T cell stimulatory capacity of the cells (17). Collectively, these data indicate that GCs prevent the further maturation and function of DCs.

Our data on DEX exposure on the relatively immature lung $CD1a^+$ DC are by and large in agreement with the literature and show a hampering of the maturation of BAL-derived LAF cells to DCs due to GC exposure. The overnight DEX exposure had, in our hands, no effect on the survival and viability of the $CD1a^+$ LAF cells, the MHC-class II expression or the expression of adhesion molecules such as CD11, CD18 and CD54. Other reports indicate that GCs induce apoptosis of pulmonary DC *in situ* (11), as well as in their precursors, the monocytes, *in vitro* (16). It should be noted that our

results do not completely rule out an effect of DEX on apoptosis of $CD1a^+$ lung DCs in our system (more appropriate apoptosis assays other than those involving simple survival and viability need to be performed for this). It seems, nevertheless, less likely that survival problems of the DC would be solely responsible for the reductions in T-cell stimulatory capacity found here.

Acknowledgements

This study was supported by a grant of the Netherlands Asthma Foundation (grant number 94.20). We thank coworkers in the Department of Anesthesiology for their assistance during the bronchoalveolar lavages, Mascha Verheggen for her support on the ^{3}H-DEX estimations and Tar van Os for the preparation of the figures. The secretarial assistance of Geertje de Korte is greatly acknowledged.

References

1. Van Voorhis WC, Valinsky J, Hoffman E, Luban J, Hair LS, Steinman RM. Relative efficacy of human monocytes and dendritic cells as accessory cells for T cell replication. J Exp Med 1983; 158: 174-191.
2. McWilliam AS, Stumbles PA, Holt PG. Dendritic cells in the lung. In: Lotze MT, Thomson AW, Eds. Dendritic cells, biology and clinical applications. San Diego, Academic Press, 1998; 123-140.
3. Nicod LP. Role of antigen presenting cells in lung immunity. Eur Respir J 1996; 6: 142-150.
4. Holt PG, Schon-Hegard MA, Philips MJ, McMenamin PG. Ia positive dendritic cells form a tightly meshed network within the human airway epithelium. Clin Exp Allergy 1989; 19: 597-601.
5. Van Haarst JMW, de Wit HJ, Drexhage HA, Hoogsteden HC. Distribution and immunophenotype of dendritic cells and mononuclear phagocytes in the human lung. Am J Respir Cell Mol Biol 1994; 10: 487-492.
6. Burke C, Power CK, Norris A, Condez A, Schmekel B, Poulter LW. Lung function and immunopathological changes after inhaled corticosteroid therapy in asthma. Eur Respir J 1992; 5: 73-79.
7. Möller GM, Overbeek SE, van Helden-Meeuwsen CG, van Haarst JMW, Prens EP, Postma DS, Hoogsteden HC. Increased numbers of dendritic cells in the bronchial mucosa of atopic asthmatic patients: downregulation by inhaled glucocorticoids. Clin Exp Allergy 1996; 26: 224-229.
8. Barnes PJ, Pedersen S. Efficacy and safety of inhaled corticosteroids in asthma. Am Rev Respir Dis 1993; 148: S1-26.
9. Djukanovic R, Wilson JW, Britten KM, Walls AF, Roche WR, Howarth PH, Holgate ST. Effect of inhaled corticosteroid on airway inflammation and symptoms in asthma. Am Rev Respir Dis 1992; 145: 669-674.
10. Holt PG, Haining S, Nelson DJ, Sedgwick JD. Origin and steady-state turnover of class II MHC-bearing dendritic cells in the epithelium of the conducting airways. J Immunol 1994; 153: 256-261.
11. Brokaw JJ, White GW, Baluk P, Anderson GP, Umemoto EY, McDonald DM. Glucocorticoid-induced apoptosis of dendritic cells in the rat tracheal mucosa. Am J Respir Cell Mol Biol 1998; 19: 598-605.
12. Rhodes J, Ivanyi J, Cozens P. Antigen presentation by human monocytes: effects of modifying major histocompatibality complex class II antigen expression and interleukin 1 production by using recombinant interferons and corticosteroids. Eur J Immunol 1986; 16: 370-375.
13. Braathen LR, Hirschberg H. The effect of short-term corticosteroid incubation on the alloactivating and antigen-presenting capacity of human epidermal Langerhans cells. Br J Dermatol 1984; 11: 295-302.

14. Kitajima T, Ariizumi K, Bergstresser PR, Takashima A. A novel mechanism of glucocorticoid-induced immune suppression: The inhibition of T cell-mediated terminal maturation of a murine dendritic cell line. J Clin Invest 1996; 98: 142-147.
15. Vieira PL, Kalinska P, Wierenga EA, Kapsenberg ML, De Jong EC. Glucocorticoids inhibit bioactive IL-12p70 production by in vitro-generated human dendritic cells without affecting their T cell stimulatory potential. J Immunol 1998; 161: 5245-5251.
16. Van den Heuvel MM, Van Beek NMA, Broug-Holub E, Postmus PE, Hoefsmit ECM, Beelen RHJ, Kraal G. Glucocorticoids modulate the development of dendritic cells from blood precursors. Clin Exp Immunol 1999; 115: 577-583.
17. Piemonti L, Monti P, Allavena P, Sironi M, Soldini L, Eugenio Leone B, Socci C, Di Carlo V. Glucocorticoids affect human dendritic cell differentiation and maturation. J Immunol 1999; 162: 6473-6481.
18. Van Haarst JMW, Hoogsteden HC, de Wit HJ, Verhoeven GT, Havenith CEG, Drexhage HA. Dendritic cells and their precursors isolated from the human bronchoalveolar lavage, immunocytologic and functional properties. Am Respir Cell Mol Biol 1994; 11: 344-350.
19. Van Haarst JMW, Verhoeven GT, de Wit HJ, Hoogsteden HC, Debets R, Drexhage HA. $CD1a^+$ and $CD1a^-$ accessory cells from human bronchoalveolar lavage differ in allostimulatory potential and cytokine production. Am J Resp Cell Mol Biol 1996; 15:752-759.
20. Mooij P, Simons PJ, de Haan- Meulman M, de Wit HJ, Drexhage HA. The effect of thyroid hormones and other iodinated compounds on the transition of monocytes into veiled/dendritic cells. A. Role of GM-CSF, TNFα and IL-6. J Endocrinol 1994; 140: 503-512.
21. Ishii Y, Takami T, Kokai Y, Yuasa H, Fujimoto J, Takei T. A novel human B cell antigen shared with lymphoid dendritc cells: characterization by a monoclonal antibody. Clin Exp Immunol 1985; 61: 624-632.
22. Kabel PJ, de Haan M, vd Gaag RD, Drexhage HA. Intrathyroidal dendritic cells. J Clin Endocrin Metab 1987; 66: 199-207.
23. Poulter LW, Campbell DA, Munro C, Janossy G. Discrimination of human macrophages and dendritic cells by means of monoclonal antibodies. Scan J Immunol 1986; 24: 351.
24. Masten BJ, Yates JL, Polard Koga AM, Lipscomb MF. Characterization of accessory molecules in murine lung dendritic cell function: roles for CD80, CD86, CD54, and CD40L. Am J Respir Cell Mol Biol 1997; 16: 335-342.
25. Inaba K, Witmer-Pack M, Inaba M, Hatchcock KS, Sakuta H, Azuma M, Yagita H, Okumura K, Linsley PS, Ikehara S, Muramatsu S, Hodes RJ, Steinman RM. The tissue distribution of the B7-2 costimulator in mice: abundant expression on dendritic cells in situ and during maturation in vitro. J Expir Med 1994; 180: 1849-1860.
26. Rattis F-M, Peguet-Navarro J, Staquet J, Dezutter-Dambuyant C, Courtellemont P, Redziniak G, Schmitt D. Expression and function of B7-1 (CD80) and B7-2 (CD86) on human epidermal Langerhans cells. Eur J Immunol 1996; 26: 449-453.
27. Stumbles PA, Thomas JA, Pimm CL, Lee PT, Venaille TJ, Proksch S, Holt PG. Resting respiratory tract dendritic cells preferentially stimulate T helper cel type 2 (Th2) responses and require obligatory cytokine signals for induction of Th1 immunity. J Exp Med 1998; 188: 2019-2031.
28. Caux C, Lebeque S, Liu YJ, Banchereau J. Developmental pathways of human myeloid dendritic cells. 1999. In: Lotze MT, Thomson AW, Eds. Dendritic cells: biology and clinical applications. San Diego, Academic Press, 1998; 63-92.
29. Delemarre FG, Simons PJ, de Heer HJ, Drexhage HA. Signs of immaturity of splenic dendritic cells from the autoimmune prone bio breeding rat: consequences for the *in vitro* expansion of regulator and effector T cells. J Immunol 1999; 162: 1795-1801.
30. Kuchroo VK, Das MP, Brown JA, Ranger AM, Zamvil SS, Sobel RA, Weiner HL, Nabavi N, Glimcher LH. B7-1 and B7-2 costimulatory molecules activate differently the Th1/Th2 developmental pathways: application to autoimmune disease therapy. Cell 1995; 80: 707-718.
31. Freeman GJ, Boussiotis VA, Anumanthan A, Bernstein GM, Ke XY, Rennert PD, Gray GS, Gribben JG, Nadler LM. B7-1 and B7-2 do not deliver identical costimulatory signals, since B7-2 but not B7-1 preferentially costimulates the initial production of IL-4. Immunity 1995; 2: 523-532.

32. Chelen CJ, Fang Y, Freeman GJ, Secrist H, Marshall JD, Hwang PT, Frankel LR, DeKruyff RH, Umetsu DT. Human alveolar macrophages present antigen ineffectively due to defective expression of B7 costimulatory cell surface molecules. J Clin Invest 1995; 95: 1415-1421.
33. Lim TK, Chen GH, McDonald RA, Toews GB. Granulocyte-macrophage colony-stimulating factor overrides the immunosuppressive function of corticosteroids on rat pulmonary dendritic cells. Stem Cells 1996; 14: 292-299.
34. Moser M, de Smedt Th, Sornasse Th, Tielemans F, Chentoufi AA, Muraille E, van Mechelen M, Urbain J, Leo O. Glucocorticoids down-regulate dendritic cell function *in vitro* and *in vivo*. Eur J Immunol 1995; 25: 2818-2824.

Chapter 8

General discussion

The results of recent large clinical studies (1-4) give argument to those who believe that inhaled glucocorticoid therapy offers no beneficial effect to patients with stable chronic obstructive pulmonary disease (5). Yet, inhaled glucocorticoid therapy in COPD is not and will not be abandoned. There are three conditions which make this therapy worth considering in patients with COPD.
The first is the inconclusive definition of COPD, which makes a clear distinction from asthma difficult. Terms like 'asthmatic bronchitis' have been used to fill this gap between asthma and COPD. If records of patients with chronic respiratory symptoms are presented to experienced clinicians, their choices of asthma or COPD diagnoses are not always identical. The differentiation between asthma and COPD can be facilitated by additional investigations, such as high-resolution CT-scan and bronchial provocation testing, or by trials with oral or inhaled glucocorticoids. All these tests have their pros and cons.
Secondly, the primary outcome parameter in most studies was FEV_1, more particularly FEV_1 decline. This was thought to be the most important objective, because FEV_1 decline determines the prognosis of these patients. Nowadays, we are inclined to also taking into account other parameters, like quality of life, the frequency of exacerbations, and exercise tolerance. There are studies showing beneficial effects of inhaled glucocorticoids on one or more of these parameters (3,6). Future intervention studies should take into account these outcomes as well.
The third condition is based on the understanding that COPD is a heterogeneous disease. Both reversible and irreversible changes contribute to the pathophysiology of COPD. Reversible changes are airway smooth muscle contraction, mucus hypersecretion and inflammation. Irreversible changes are remodelling of small airways, loss of alveolar attachments and proteolytic destruction of the elastic-collagen framework. There are subgroups of COPD with asthmatic features, such as some degree of bronchodilator responsiveness or an increased eosinophilic airways inflammation. Whether the latter findings are caused by a subclinical degree of exacerbation is unclear. Eosinophilic granulocytes do not seem to contribute substantially to the inflammatory process in stable COPD, but are present in large numbers during COPD exacerbation (7-9). These observations have led to the idea that there may be subgroups of COPD patients for whom long-term inhaled glucocorticoid therapy is beneficial (10-12).

The Copenhagen City Lung Study (1) included mild, asymptomatic patients with COPD (mean FEV_1 2.36 L or 86% predicted; only 39% of the participants had an FEV_1 of <80% predicted). Inhaled budesonide at a moderately high dose had no effect on FEV_1 decline, on respiratory symptoms and on the number of exacerbations.

The EUROSCOP study (2) compared the effects of a moderate dose of inhaled budesonide and placebo on lung function in 912 patients with mild COPD (mean FEV_1 of 77% predicted). A significant treatment effect on FEV_1 decline was noticed in the first three months.
The ISOLDE study (3) was conducted in 990 patients with moderate-to-severe COPD (mean FEV_1 of 50% predicted). A high dose of fluticasone (2 x 500 μg) was compared with placebo. Fluticasone treated subjects showed significantly increased FEV_1 during the entire three year treatment period, improved morning peak expiratory flow rate, less patients drop out of the study due to respiratory causes, reduced number of exacerbations, and diminished decline in health status. Some of the differences between fluticasone and placebo increased over time, in favor of fluticasone. The effect of the inhaled glucocorticoid was greater in those patients who had deteriorated faster.
The Lung Health Study Research Group investigated whether inhaled triamcinolone could slow down the decline of lung function in patients with mild-to-moderate COPD (4). Approximately 90 percent of the 1116 participants were current smokers. There appears to be no effect on FEV_1 decline and also none on quality of life scores. In the first six months of therapy, there was a small increase of the FEV_1, comparable to the course of the FEV_1 in the EUROSCOP study. Beneficial effects of the inhaled glucocorticoid were fewer respiratory symptoms and fewer unscheduled consultations to physicians for respiratory symptoms. Airway reactivity to methacholine improved in the triamcinolone treated patients, but this could be explained by the presence of patients with a diagnosis of asthma, who constituted almost 10 percent of the participants. In this study, the results of the bone density measurements showed a significant adverse effect of triamcinolone, as compared to placebo, on bone mineral density after three years of therapy.
The different findings in those four studies are not necessarily contradictory; the results provide varying insights. First, a lower dose of anti-inflammatory therapy seems to evoke no or lesser effects. Second, a beneficial effect of inhaled glucocorticoid therapy seems to occur most likely in patients with more severe COPD. Remarkably, the findings of recent inhaled glucocorticoid studies are similar to the older oral glucocorticoid trials, because these also showed a better effect in those patients with COPD who had a worse lung function (13). Thes findings underscore the importance of further research, leading to definitions of subgroups of COPD patients in whom inhaled glucocorticoid therapy is likely to be beneficial.

Design of our trial
When designing a study on inhaled glucocorticoids in COPD, we chose a subgroup of patients with bronchial hyperresponsiveness (BHR). Epidemiological studies showed a steeper decline of the FEV_1 in patients with COPD and BHR, suggesting a more severe inflammation (14). A large multicenter study on patients with respiratory symptoms and BHR showed a beneficial effect of an inhaled glucocorticoid in patients with the worst degree of BHR [expressed by PC_{20}] (15).

We decided to investigate these patients in detail in order to characterize them as much as possible and to detect subtle changes after inhaled glucocorticoid therapy or placebo. A treatment period of six months was chosen as a compromise between a minimal length for obtaining a steady state effect and a maximal treatment period for having patients motivated and for the least drop outs due to exacerbations or intercurrent diseases.

Patient recruitment

Potential candidates were recruited from the University Hospital Dijkzigt, the Sint Franciscus Ziekenhuis, Rotterdam, the Holy Ziekenhuis, Schiedam, the Zuiderziekenhuis, Rotterdam, and the Schieland Ziekenhuis, Schiedam. Approximately 200 patients were considered to fulfill the inclusion criteria of the study and received an invitation for participating in our trial. Eventually, there were 23 patients who completed the trial, having visited our hospital 15 times in the 6 months study period. There was no patient dropping out of the study after he or she was found to be eligible. The reasons for not participating in the study were (in order of frequency): concomitant disease or medication (especially NSAIDs and anticoagulant medication for heart disease), lack of time, severity of the study and exclusion because of lung function results. There was only one patient in whom BHR was no longer present at the first visit, after having had a low PC_{20} several years before. In two patients, meeting the clinical criteria for COPD, PC_{20} for histamine was above 8 mg/ml.

Initial characterization of trial participants

We have started with lung function testing in order to characterize the participants of the trial in terms of pathophysiology. The patients had on average moderately severe obstruction (mean FEV_1 65% predicted) according to ERS criteria (16). The degree of BHR was moderate or severe: mean PC_{20} for methacholine: 3.5 mg/ml. One of our major interests was the interrelationship of the mechanical behavior of the lungs and the different indexes of the methacholine log dose-response curve. Estimates of the mechanical behavior of the lungs and of the integrity of the lung parenchyma were obtained from pressure-volume curves with the aid of an esophageal balloon, and the single-breath carbon monoxide (CO) transfer. In accordance with earlier observations, correlations were significant between indexes of the pressure-volume curve and CO diffusion. Bronchial provocation tests were performed as complete as possible in order to obtain a reliable plateau value. A model fit (17) was applied to it for the estimate of the slope of the curve (reactivity) and maximal airways constriction (plateau). PC_{20} was calculated from the interpolation of two adjacent points in the log dose-response curve. None of the indexes of the bronchial provocation test was correlated with any parameter of lung parenchymal destruction. This means that in patients with COPD who continue smoking, there is no straightforward influence of loss of elasticity on contractile mechanisms. There is one study showing a correlation between lung parenchyma destruction and maximal airways constriction in patients with α-1-antitrypsine

deficiency who did not smoke (18). The participants in our study were moderate or heavy smokers. So, it appears that smoking and the following small-airways inflammation were confounders with respect to the degree of BHR. In other words, inflammation of small airways seems to be the most important mechanism contributing to the degree of BHR in COPD.

We found a significant correlation of both PC_{20} and reactivity (the slope of the curve) with the starting FEV_1. The correlation of the starting FEV_1 and PC_{20} is well known and predictable from a mathematical point of view. First, the definition of PC_{20} (as a percentage and not an absolute change of FEV_1) makes that small changes in narrow airways give a greater percent change as compared to similar changes in larger airways. Secondly, resistance to airflow depends on airway lumen and according to the law of Poisseuille the (laminar) resistance is inversely proportional to the radius with power four. From this, large increases of resistance result from small decreases of the diameter of the small airways. So, PC_{20} is not only a determinant of airways pathology but is also influenced by physical mechanisms, depending on severity of airway obstruction.
There are several methods of expressing the effect of a bronchodilatory drug on airflow, particularly the FEV_1, by taking into account the starting FEV_1. We have, in a similar way, made calculations with the bronchoconstrictory responses provoked by methacholine. We found that the correlation of PC_{20} and the starting FEV_1 is lost if the change of FEV_1 is expressed as percentage of the predicted value: "normalized PC_{20}". When correlating PC_{20} with eosinophilic airways infiltration, as determined by the number of eosinophils in bronchial biopsies, there was no significance. However, there was a significant (negative) correlation between eosinophil numbers and normalized PC_{20}. This implies two things: first, the normalized PC_{20} is a better indicator of eosinophilic airways inflammation, and secondly, for predicting a beneficial response of (inhaled) glucocorticoid therapy the normalized PC_{20} appears to be a better tool than the standard PC_{20}. The last statement is based on the observation that a beneficial effect of inhaled glucocorticoids in COPD can be expected with increasing numbers of eosinophils in sputum (10).

We have also tested correlations of other cell types with PC_{20}, the slope and the plateau of the methacholine dose-response curve. There were no correlations, suggesting that it is not the number of individual inflammatory cells but perhaps cell activity or airway remodeling, such as swelling of the small-airways walls, that contributes to BHR in COPD.

Effects of inhaled fluticasone propionate
In accordance with earlier studies, there was no significant effect of an inhaled glucocorticoid on PC_{20} (19-21). There was also no significant effect on the slope of the curve, the plateau and EC_{50} (= dose of methacholine causing 50% of the maximal effect), which has not been published before. The rightward shift of the

methacholine dose-response curve and the decrease of the maximal degree of bronchoconstriction (plateau), which occur in patients with asthma after inhaled glucocorticoid therapy, were not observed in patients with COPD (fig. 3, chapter 4). This, again, indicates that BHR in COPD and in asthma are different phenomena.
In the placebo treated subjects lung function declined fast, which is in accordance with the observations from several epidemiological studies (*eg.* ref. 14). The withdrawal of glucocorticoids could also have contributed to this exaggerated decline. Similarly, investigators of the ISOLDE trial noticed during the run-in period a significant number of exacerbations after discontinuation of inhaled corticosteroids (22). The FEV_1 of the patients with FP therapy remained almost unchanged, and this difference between FP and placebo was highly significant. When comparing the different maximal expiratory flow rates, the impression arises that the treatment effect originates from an effect on the small airways. Unfortunately, we were unable to collect small airways for immunopathological investigations. A surrogate measurement of the events in the small airways could be bronchoalveolar lavage (BAL). We performed BAL at the start and after six months of the intervention period, and harvested two fractions: the first as a bronchial fraction and the second as a bronchoalveolar fraction. We measured cell numbers and cell activation markers in both samples and were unable to find significant changes after placebo or FP therapy. So, these results provided us with no clues for the effect of fluticasone on lung function.
Also, we measured cell numbers and cell activation markers in blood. There were no significant changes attributable to either glucocorticoid therapy or glucocorticoid withholding.
However, there were significant changes of cell numbers in biopsy specimens taken from the larger airways. The most obvious observation was the increase of the number of eosinophils in the patients with placebo and the decrease of eosinophils in the patients with FP therapy, suggesting a state of exacerbation in the first and a glucocorticoid effect in the last ones. Decreased influx of cells and accelerated apoptosis of eosinophils in tissue are well known effects of glucocorticoid therapy in subjects with asthma and this also seems to happen in our patients with COPD.
We were unable to detect an effect on symptoms, probably because we used a questionnaire (adapted from the questionaire of the Medical Research Council), that turned out to be inappropriate for this study. The use of rescue medication was not appreciably different between the two groups of patients. Disease related adverse events were significantly more often reported by placebo patients as compared to FP treated patients. These adverse events included candidiasis of mouth, stomatitis, irritation of pharynx, upper respiratory tract infection, acute bronchitis, and (mild) increase of airways obstruction.

Mediators of inflammation
We measured arachidonic acid metabolites in bronchoalveolar fluid, before and after inhaled fluticasone therapy or placebo. We observed significant decreases of the concentrations of PGE_2, $6kPGF_1\alpha$ and $PGF_2\alpha$ after fluticasone therapy. $PGF_2\alpha$

has strong bronchoconstrictor activity and reduction of its concentration could be one of the mechanisms by which FEV_1 decline was abolished during FP therapy. On the other hand, $6kPGF_1\alpha$ and PGE_2 are known in asthma for causing bronchodilation and edema. Reduction of edema and decrease of swelling of the walls of small airways could have contributed to the observed effect on FEV_1. There is little information about the relative effects of these mediators on the bronchomotor tone, and none about their contribution to airway small muscle tone in COPD. The concentrations of the mediators in our COPD patients were compared with those of a control group consisting of smokers without symptoms and normal lung function, and also with the results of an earlier study of our department using the same methodology in asthma patients and in asymptomatic smokers. There were clear differences with asthma, in which concentrations of 5-lipoxygenase products (predominantly leukotrienes) were higher, while in COPD and in smokers the concentrations of cyclooxygenase products (predominantly prostaglandins) were increased. From this we concluded that the function of inflammatory cells in COPD patients with BHR differs in a qualitative manner from asthma and resembles the responses induced by smoking.

Oxygen radicals

In an attempt to quantify the capability of inflammatory cells to produce reactive oxygen species (ROS), bronchoalveolar cells were separated in order to perform kinetic assays. These cell fractions consisted almost exclusively of alveolar macrophages. Cells from the bronchoalveolar lavage were tested for stimulated ROS production, before and after the 6-months intervention period with fluticasone or placebo. We found neither effect of fluticasone therapy nor placebo on ROS production capability. Other studies were also unable to demonstrate a significant effect of an inhaled glucocorticoid on ROS production by sputum derived cells (23,24). In our study the concentrations of ROS were low as compared to previous studies in which macrophages were tested. The correlations of ROS production and patient characteristics were therefore calculated. Actual smoking (the number of cigarettes being smoked per day) was negatively correlated with ROS production, indicating that heavy smoking had either stimulated intracellular counter effects against excessive ROS production, or had exhausted macrophages after repetitive stimulation. While the general feeling tends towards increased capability of ROS production as an effect of smoking, the last thought seems to be the most applicable. The finding of a decreased ROS production capability in heavy smokers could be an attractive explanation for the vulnerability to bacterial infections or bacterial colonization. The oxidative processes in the walls of the small airways and in the alveolar walls could however be different (higher?) from what we observed in our study.

Role of dendritic cells

In asthma, dendritic [or: antigen-presenting] cells are important for the initiation and regulation of the characteristic inflammatory response. Dendritic cells are

present in the bronchial wall of patients with asthma in significant numbers. After glucocorticoid therapy their number decreases, possible due to a decreased influx. In bronchial biopsies derived from COPD patients, dendritic cells were hardly detectable, whereas phagocytic cells were present in large numbers. It appears,

Table 1. Overview of the investigated items in the patients with COPD and BHR, and the effects of treatment with inhaled fluticasone propionate. No negative effects occurred.

		Beneficial effect	No effect
Blood	Cells		✔
	Cells activation markers		✔
	Cortisol		✔
Lung function	PC_{20}		✔
	Reactivity		✔
	Plateau		✔
	EC_{50}		✔
	FEV_1	✔	
	FEV_1/FVC	✔	
	MEF50	✔	
	MEF25	✔	
	FVC		✔
	PEF		✔
Broncho-Alveolar Lavage	Cellularity		✔
	Dendritic cells		✔
	Reactive oxygen species		✔
	LTB_4		✔
	$LTC_4/D_4/E_4$		✔
	TxB_2		✔
	PGE_2	✔	
	$6kPGF_1\alpha$	✔	
	$PGF_2\alpha$	✔	
Immunostain-positive cells in bronchial biopsies	CD3		✔
	CD4		✔
	CD15		✔
	CD1a		✔
	CD8	✔	
	MBP	✔	
	CD68	✔	
	tryptase	✔	

therefore, that the inhaled toxic substance, namely cigarette smoke, is handled in a different way in COPD, as compared to asthma, in which the causative inhaled particles are allergens. Next to particle size, intrinsic factors seem to play an important role. In asthma it is the inherited tendency to react with an allergic response to allergens and in COPD it is the susceptibility to cigarette smoke and/or similar particles that causes the disease. Asthma could be a disorder resulting from an abnormal dendritic cell function, while COPD is a macrophage or neutrophil driven disease. One of the most intriguing problems to be solved is whether there are intrinsic defects in dendritic cells or macrophages or their environment, which causes either one disease.

Effect of glucocorticoids on dendritic cell function
The collection, selection and sorting of dendritic cells from bronchoalveolar lavage cells enabled us to perform *ex vivo* functional studies of dendritic cells. We have started with investigating cells derived from smoking subjects without lung disease. Typical dendritic cells, which were sorted with the aid of cell fluorescence characteristics and relevant cell surface markers, showed the highest potency for activating T-lymphocytes. After incubation with dexamethasone this function was decreased in a dose-dependent way, and this decrease was associated with a reduction of the expression of cell surface markers involved in co-stimulation. These *ex vivo* studies were performed prior to our *in vivo* studies with inhaled glucocorticoids in both asthma and COPD patients. The results from the patients with COPD and BHR indicate that dendritic cells derived from the bronchoalveolar lavage fluid are not influenced by inhaled glucocorticoid therapy.

Conclusion

Taking the above-mentioned results into account, our study of bronchial hyperresponsiveness in patients with COPD has given some intriguing new findings. BHR in COPD differs from BHR in asthma in several respects. Pathophysiological mechanisms, apart from FEV_1, are not responsible for the degree of BHR. There is an abundant inflammatory response, which is reflected, for instance, by a high number of cells in BAL fluid. The inflammatory characteristics of COPD and BHR show greater resemblances to "healthy smokers" than to asthma. Heavy smoking influenced several functions of the inflammatory cells from the patients with COPD. The finding of high levels of cyclooxygenase products is of interest because the release of eicosanoids from various inflammatory cells is involved in the observed BHR induced by repeated exposure of airways to cigarette smoke. This deserves further attention. Next to the beneficial effects of inhaled fluticasone, which we observed, prostaglandin antagonists could be important for the long-term management of COPD. The lung function of placebo treated patients deteriorated while FEV_1 remained stable in the patients with fluticasone therapy. This was accompanied by changes in cell numbers in bronchial biopsies, of which the effect on eosinophils was most significant. BHR appears to be a characteristic of

COPD, which predicts a beneficial response to long-term inhaled glucocorticoid therapy. Future intervention studies in COPD should incorporate the measurement of BHR.

References

1. Vestbo J, Sorensen T, Lange P, et al. Long-term effect of inhaled budesonide in mild and morate chronic obstructive pulmonary disease: a randomised controlled trial. Lancet 1999; 353: 1819-1823.
2. Pauwels RA, Löfdahl C-G, Laitinen LA, et al., on behalf of the European Respiratory Society Study on Chronic Obstructive Pulmonary Disease. Long-term treatment with inhaled budesonide in persons with mild chronic obstructive pumonary disease who continue smoking. N Engl J Med 1999; 340: 1948-1953.
3. Burge PS, Calverley PMA, Jones PW, et al., on behalve of the ISOLDE study investigators. Randomised, double blind, placebo controlled study of fluticasone propionate in patients with moderate and severe chronic obstructive pulmonary disease: the ISOLDE trial. BMJ 2000; 320: 1297-13.
4. The Lung Health Study Research Group. Effect of inhaled triamcinolone on the decline in pulmonary function in chronic obstructive pulmonary disease. N Engl J Med 2000; 343: 1902-1909.
5. Barnes PJ. Inhaled corticosteroids are not beneficial in chronic obstructive pulmonary disease. Am J Respir Crit Care Med 2000; 161: 342-344.
6. Paggiaro PL, Dahle R, Bakran I, et al., on behalf of the International COPD Study Group. Multicenter randomised placebo-controlled trial of inhaled fluticasone propionate in patients with chronic obstructive pulmonary disease. The Lancet 1998; 351: 773-780.
7. Balzano G, Stefanelli F, Iorio C, et al. Eosinophilic inflammation in stable chronic obstructive pulmonary disease. Am J Respir Crit Care Med 1999; 160: 1486-1492.
8. Saetta M, Di Stefano A, Maestrelli P, et al. Airway eosinophilia in chronic bronchitis during exacerbations. Am J Respir Crit Care Med 1994; 150: 1646-1652.
9. Fabbri L, Beghe B, Caramori G, et al. Similarities and discrepancies between exacerbations of asthma and chronic obstructive pulmonary disease. Thorax 1998; 53: 803-808.
10. Chanez P, Vignola AM, O'Shaugnessy, et al. Corticosteroid reversibility in COPD is related to features of asthma. Am J Respir Crit Care Med 1997; 155: 1529-1534.
11. Pizzichini E, Pizzichini MMM, Gibson P, et al. Sputum eosinophilia predicts benefit from prednisone in smokers with chronic obstructive bronchitis. Am J Respir Crit Care Med 1998; 158: 1511-1517.
12. Eliasson O, Hoffman J, Trueb D, et al. Corticosteroids in COPD. A clinical trial and reassessment of the literature. Chest 1986; 89: 484-490.
13. Fujimoto K, Kubo K, Yamamoto H, et al. Eosinophilic inflammation in the airway is related to glucocorticoid reversibility in patients with pulmonary emphysema. Chest 1999; 115: 697-702.
14. Rijcken B, Schouten JP, Xu X, et al. Airway hyperresponsiveness to histamine associated with accelerated decline in FEV_1. Am J Respir Crit Care Med 1995; 151: 1377-1382.
15. Kerstjens HA, Overbeek SE, Schouten JP, et al. Airway hyperresponsiveness, bronchodilator response, allergy and smoking predict improvement in FEV_1 during long-term inhaled corticosteroid treatment. Dutch CNSLD Study Group. Eur Respir J 1993; 6: 868-876.
16. Siafakas NM, Vermeire P, Pride NB, et al., on behalf of the Task Force. Optimal assessment and management of chronic obstructive pulmonary disease (COPD). Eur Respir J 1995; 8: 1398-1420.
17. Aerts JGJV, Bogaard JM, Overbeek SE, Verbraak AFM, Thio P. Extrapolation of methacholine log-dose response curves with a cumulative gaussian distribution function. Eur Resp J 1994; 7: 895-900.

18. Cheung D, Schot R, Zwinderman, Zagers H, Dijkman JH, Sterk PJ. Relationship between loss in parenchymal elastic recoil pressure and maximal airway narrowing in subjects with α_1-antitrypsine deficiency. Am J Respir Crit Care Med 1996; 155:135-140.
19. Engel T, Heinig JH, Madson, et al. A trial of inhaled budesonide on airway responsiveness in smokers with chronic bronchitis. Eur Respir J 1989; 2: 935-939.
20. Auffarth B, Postma DS, de Monchy JGR, et al. Effects of inhaled budesonide on spirometric values, reversibility, airway responsiveness, and cough threshold in smokers with chronic obstructive lung disease. Thorax 1991; 46: 372-377.
21. Watson A, Lim TK, Joyce H, Pride NB. Failure of inhaled corticosteroids to modify bronchoconstrictor or bronchodilator responsiveness in middle-aged smokers with mild airflow obstruction. Chest 1992; 101: 350-355.
22. Jarad NA, Wedzicha JA, Burge PS, Calverley PM. An observational study of inhaled corticosteroid withdrawal in stable chronic obstructive pulmonary disease. ISOLDE Study Group. Respir Med 1999; 93: 161-166.
23. Llewellyn-Jones CG, Harris TAJ, Stockley RA. Effect of Fluticasone propionate on sputum of patients with chronic bronchitis and emphysema. Am J Respir Crit Care Med 1996; 153: 616-621.
24. Llewellyn-Jones CG, Hill SL, Stockley RA. Effect of fluticasone propionate on neutrophil chemotaxis, superoxide generation, and extracellular proteolytic activity in vitro. Thorax 1994 ; 49 : 207-212.

Chapter 9

Summary

This thesis describes a detailed investigation of a subgroup of smoking COPD patients, particularly those with bronchial hyperresponsiveness (BHR). We speculated that inhaled fluticasone propionate could have beneficial effects in these patients, who suffer from an exaggerated FEV_1 decline and a worse prognosis.

In **chapter 1** the current knowledge about BHR in patients with COPD is summarized in the first section. Clinical studies indicate that BHR in patients with COPD differs in several ways from the BHR observed in patients with asthma. Next, inflammation in COPD is discussed, especially pathologic changes and the contribution of various cell types. In COPD the most prominent cells in the peripheral airways are monocytes/macrophages, neutrophils and CD8+ lymphocytes. The inflammatory infiltrate in patients with COPD and BHR has gained almost no attention. In the last section of chapter 1, the results of previous studies with glucocorticoid therapy in patients with stable COPD are summarized. One of the conclusions is that glucocorticoids appear to have at least some beneficial effects in certain patients with COPD. At this moment, the patients benefitting from longterm glucocorticoid therapy are those with 'asthmatic features', those with severe airways obstruction and those with rapidly declining lung function (FEV_1).

In **chapter 2** the results of the functional studies of patients with COPD and BHR are presented. Our main interest was the interrelationship of the mechanical behavior of the lungs with the different indexes of the methacholine log dose-response curve. In accordance with earlier observations, correlations were significant between indexes of the pressure-volume curve and CO diffusion, be it exclusively with the CO diffusion after correction for alveolar volume, which is a better estimate for the condition of the lung parenchyma than CO transfer as such. A model fit was applied to the results of the methacholine dose-response curves for the estimate of the slope of the curve, the plateau value, and EC_{50}. None of the indexes of the bronchial provocation test correlated with any parameter of lung parenchymal destruction. This means that, in patients with COPD who smoke, there is no straightforward influence of loss of elasticity on contractile mechanisms. It appears that inflammation of small airways is the most important mechanism contributing to the presence and the degree of BHR in patients with COPD.

In **chapter 3** we performed calculations on the expression of the most often used index of BHR, PC_{20}. We confirmed earlier observations of a correlation between PC_{20} and the baseline FEV_1. This correlation is predictable from a mathematical point of view. By expressing the FEV_1 response as percent predicted, we defined

the log2nPC$_{20}$. The log2nPC$_{20}$ showed no correlation with the baseline FEV$_1$. Next, we compared the levels of log2PC$_{20}$ and log2nPC$_{20}$ with eosinophilic airways inflammation, as determined by the number of eosinophils in bronchial biopsies. There was a significant (negative) correlation only between eosinophil numbers and log2nPC$_{20}$. The log2nPC$_{20}$ appeared to be a good indicator of eosinophilic airways inflammation. Several studies have shown that (inhaled) glucocorticoid therapy in patients with COPD is more likely to be beneficial when increased numbers of eosinophils are present in induced sputum. The calculation of log2nPC$_{20}$ could replace the procedure of sputum induction.

In **chapter 4** the effects of treatment with fluticasone propionate (FP) inhalation on lung function and inflammation are shown. At the start of the intervention, the inflammatory profile in bronchial biopsies from the patients with COPD and BHR was compared to a control group, which consisted of smokers without symptoms and with normal lung function. There were no significant differences, which indicates that the inflammatory profile of BHR in COPD is similar to smoking-induced changes. We observed that dendritic cells were hardly detectable in the bronchial biopsy specimens, contrary to our previous findings in patients with asthma. Dendritic cells do not seem to be involved in the inflammatory process in COPD.
In accord with earlier studies, there was no significant effect of the intervention on PC$_{20}$. There was also no significant effect on the other derivatives of the methacholine dose-response curve (Fig. 3, Chapter 4: the slope of the curve, the plateau value and the EC$_{50}$).
In the placebo treated patients, lung function declined fast, while FEV$_1$ of the FP treated patients remained almost unchanged. This difference was statistically and clinically significant. Comparison of the different maximal expiratory airflows suggests that the treatment effect originates from an effect in the small airways. This was accompanied by changes in cell numbers in bronchial biopsies, of which the effect on eosinophils was most significant: their number increased in placebo treated patients and decreased in FP treated subjects.
We conclude that inhaled fluticasone causes beneficial effects in patients with COPD and BHR during a six-month treatment period.

In **chapter 5**, results are described of the measurements of arachidonic acid metabolites in bronchoalveolar fluid, before and after inhaled FP therapy and placebo, in the same group of patients with COPD and BHR. We observed significant decreases of the concentrations of PGE$_2$, 6kPGF$_1\alpha$ and PGF$_2\alpha$ after FP therapy. PGF$_2\alpha$ has strong bronchoconstrictor activity and reduction of its concentration could be one of the mechanisms by which FEV$_1$ decline was abolished during FP therapy (chapter 3). We compared the levels of the mediators in our patients with COPD with the results of earlier studies in our institution using the same methodology in patients with asthma and in asymptomatic smokers with normal lung function. There were clear differences between COPD and asthma.

In asthma, concentrations of 5-lipoxygenase products were higher, while in COPD and in 'healthy' smokers the levels of cyclooxygenase products were increased. The function of inflammatory cells in COPD patients with BHR appears to differ from asthma in a qualitative manner, and resembles the changes induced by smoking.

In **chapter 6** we reported the results of an attempt to quantify the process of reactive oxygen species (ROS) production in COPD. Oxidative stress is one of the important pathogenic mechanisms in COPD. Bronchoalveolar lavage cells, mainly consisting of alveolar macrophages, were tested for PMA-stimulated ROS production, before and after the trial with FP and placebo. We found neither FP therapy nor placebo had any effect on ROS production. In our patients the concentrations of ROS were low compared to previous studies. Actual smoking (the number of cigarettes smoked per day) was negatively correlated with ROS production, indicating that heavy smoking had either stimulated intracellular counter effects against excessive ROS production, or had exhausted macrophages after repetitive stimulation. The finding of a decreased ROS production capability in heavy smokers could be an explanation for the vulnerability to bacterial infections or bacterial colonization. The oxidative processes in the walls of the small airways and in the alveolar walls could nontheless be different from what we observed in our assay.

The **last chapter** (7) describes functional studies of *ex vivo* dendritic cells, derived by means of bronchoalveolar lavage from subjects who smoke and have no lung disease. Typical dendritic cells, which were sorted with the aid of cell fluorescence characteristics and typical cell surface markers, showed the highest potency for activating T lymphocytes. After incubation with dexamethasone this function was decreased in a dose-dependent way, and this decrease was associated with a reduction of the expression of cell surface markers involved in co-stimulation. These *ex vivo* studies were performed prior to our studies with inhaled glucocorticoids in patients with asthma and in patients with COPD. The results from the patients with COPD and BHR indicate that the appearance and the function of dendritic cells derived from bronchoalveolar lavage are not influenced by inhaled glucocorticoid therapy which lasted for six months.

In short, we have investigated in detail a subgroup of smoking COPD patients, particularly those with bronchial hyperresponsiveness, because we speculated that therapy with inhaled fluticasone propionate could have beneficial effects. We observed that the inflammatory infiltrate in bronchial biopsies and the cellularity of BAL had characteristics comparable to smoking-induced changes rather than those observed in asthma. BHR was not influenced by mechanical factors related to reduced elasticity of the lung parenchyma. None of the indexes of BHR were affected by fluticasone therapy. The lung function of placebo treated patients deteriorated while FEV_1 remained stable in the patients with fluticasone therapy.

This was accompanied by changes in cell numbers in bronchial biopsies, of which the effect on eosinophils was most significant. We were unable to find significant effects of fluticasone on the *ex vivo* reactive oxygen species production of BAL cells. The concentrations of cyclooxygenase products involved in bronchial narrowing were significantly reduced.

These results indicate that a subgroup of patients with COPD, particularly those with bronchial hyperresponsiveness, benefit from therapy with inhaled fluticasone. Our results are a strong argument for testing bronchial responsiveness in COPD patients, if one is in doubt of prescribing inhaled glucocorticoids. However, this policy should be confirmed by a placebo-controlled trial in which bronchial responsiveness, among other characteristics, is estimated at the start of the study.

Samenvatting

In dit proefschrift wordt een onderzoek beschreven bij patienten met COPD (Chronic Obstructive Pulmonary Disease; chronisch obstructieve longaandoening). Onder COPD worden die long- aandoeningen verstaan met een algehele vernauwing van de luchtwegen, die in de loop van de tijd geleidelijkaan verslechtert en niet duidelijk verbetert na gebruik van medicijnen die de luchtwegen verwijden. Algemeen bekend zijn de ziektebeelden 'chronische bronchitis' en 'emphyseem'/'uitgerekte longen', die vaak samen voorkomen en tegenwoordig COPD genoemd worden. Bij patienten met COPD, vooral als zij blijven roken, verslechtert de longfunctie sneller dan bij de leeftijd past. Als patienten met COPD ook nog een bronchiale hyperreactiviteit hebben, gaat hun longfunctie nog sneller achteruit, wat betekent dat zij meer klachten hebben en eerder zullen overlijden. Bronchiale hyperreactiviteit is een overmatige prikkelbaarheid van de luchtwegen. Dit kan door de patienten ervaren worden, bijvoorbeeld een scherpe lucht die hoesten of kortademigheid geeft. Het kan ook in het longfunctie-laboratorium gemeten worden, door de reactie op de uitademing te bepalen na het inademen van histamine of methacholine. Bronchiale hyperreactiviteit wordt bij vrijwel alle patienten met astma gevonden en bij ongeveer de helft van alle patienten met COPD. Het hier beschreven onderzoek richt zich op de kenmerken van bronchiale hyperreactiviteit bij patienten met COPD, en op het effect van een zes maanden durende, ontstekingsremmende behandeling, namelijk met een glucocorticoid per inhalatie: fluticasone propionaat.

In **hoofdstuk 1** wordt de huidige kennis omtrent bronchiale hyperreactiviteit (BHR) bij COPD samengevat. Er bestaan verschillen tussen de BHR die bij COPD wordt gevonden en de BHR bij astma. Vervolgens wordt het ontstekingsproces in de longen van patiënten met COPD besproken. Er zijn duidelijke verschillen tussen het ontstekingsproces bij COPD en bij astma. Over het ontstekingsproces bij patiënten met COPD en BHR is nauwelijks iets bekend. Tenslotte worden in het eerste hoofdstuk de effecten beschreven van (inhalatie) glucocorticoiden bij patiënten met COPD. Deze effecten zijn beduidend minder uitgesproken dan bij astma. Echter bij bepaalde patiënten met COPD worden wel gunstige effecten waargenomen. Dit zijn COPD patiënten met astmatische kenmerken, met een ernstige luchtweg vernauwing en met een snel verslechterende longfunctie.

In **hoofdstuk 2** wordt beschreven wat de relatie is tussen enerzijds BHR en anderzijds afwijkingen van de longfunctie die specifiek zijn voor patienten met COPD. De meest opvallende daarvan is het verlies van elasticiteit van het longweefsel. Wij vonden geen relatie tussen de mate van BHR en de ernst van het verlies van long elasticiteit. Dit betekent dat BHR bij patienten met COPD veroorzaakt wordt door een overmatige contractiliteit van de gladde spieren in de kleine luchtwegen, onafhankelijk van de elasticiteit van het longweefsel.

In **hoofdstuk 3** hebben wij onderzocht hoe de relatie is tussen enerzijds BHR en anderzijds de ontsteking in de luchtwegen van patiënten met COPD. In tegenstelling tot bij astma vonden wij geen verband tussen de mate van BHR (de PC_{20}) en ontsteking, die uitgedrukt werd in het aantal eosinofiele granulocyten in biopten uit de grote luchtwegen. Conform eerdere bevindingen was er een correlatie tussen de PC_{20} en de longfunctie (FEV_1 bij aanvang van de hyperreactiviteitstest). Daarom hebben wij een alternatieve manier van uitdrukken van de PC_{20} gedefinieerd: de $log2nPC_{20}$. Deze waarde was onafhankelijk van de aanvangs-FEV_1, en wel gerelateerd aan het aantal eosinofiele granulocyten in de wand van de luchtwegen. De $log2nPC_{20}$ kan dus als een indicator voor de eosinofiele luchtweg-ontsteking worden gebruikt.

Hoofdstuk 4 handelt over de effecten van fluticasone propionaat, vergeleken met placebo behandeling, op ontsteking en longfunctie bij COPD patiënten met BHR. Eerst werden de uitgangswaarden vergeleken met een controle groep van rokers zonder luchtweg-klachten en met een normale longfunctie. Het ontstekingsinfiltraat in de grote luchtwegen bij de patiënten met COPD was gelijk aan dat in de controle groep, dus vooral door het roken bepaald. Fluticasone had geen enkel significant effect op de BHR. De FEV_1 van de placebo groep daalde sterk, terwijl de FEV_1 bij de patiënten die fluticasone inhaleerden vrijwel onveranderd bleef. De sterke daling van de FEV_1 werd dus door de actieve behandeling gestopt. Bestudering van de overige longfunctie parameters geeft aanwijzingen dat er een effect op de kleine luchtwegen is. Er waren kleine, significante veranderingen in het ontstekingsinfiltraat, waarvan het meest opvallend was de stijging van het aantal eosinofiele granulocyten in de biopten van de grote luchtwegen in de placebo groep, terwijl dit aantal daalde in de met fluticasone behandelde patiënten. Fluticasone heeft dus een beschermend effect op de longfunctie op basis van een afname van het aantal ontstekingscellen in de wand van de luchtwegen.

Hoofdstuk 5 beschrijft de metingen van ontstekingsmediatoren, afkomstig van ontstekingscellen. Bij astma spelen deze stoffen een belangrijke rol bij het ontstekingsproces, en veroorzaken o.a. vernauwing van de luchtwegen. Bij COPD is nog nauwelijks onderzoek gedaan naar het vóórkomen van ontstekingsmediatoren. Wij hebben deze stoffen gemeten in de bronchoalveolaire lavage vloeistof van patiënten met COPD en BHR, vóór en na behandeling gedurende zes maanden met fluticasone of placebo. De concentraties van drie van de gemeten stoffen, namelijk de prostaglandines E_2, $F_1\alpha$ en $F_2\alpha$ namen af na behandeling met fluticasone. Van deze is prostaglandine $F_2\alpha$ een sterke bronchoconstrictor, en de daling in concentratie kan dus één van de verklaringen zijn voor de effecten, die wij zagen op de FEV_1 (zie hoofdstuk 4). Bij de patiënten met COPD waren de concentraties van de mediatoren vergelijkbaar met die gevonden bij rokers zonder luchtweg-klachten en een normale longfunctie. Bij patiënten met COPD en BHR zijn de concentraties van de prostaglandines verhoogd, terwijl bij astma vooral de leucotrienes verhoogd aanwezig zijn.

In **hoofdstuk 6** worden de resultaten besproken van de metingen van de productie door ontstekingscellen van zuurstofradicalen. Effecten van zuurstofradicalen spelen een belangrijke rol bij het ontstaan van COPD. Wij hebben cellen uit de bronchoalveolaire lavage vloeistof (voornamelijk alveolaire macrophagen) gestimuleerd tot het produceren van zuurstofradicalen, vóór en na een zes maanden durende behandeling met fluticasone of placebo. Het bleek dat de aldus verkregen cellen betrekkelijk geringe hoeveelheden zuurstofradicalen produceerden, en dat dit niet veranderde na fluticasone of placebo. Onderzoek naar correlaties tussen zuurstofradicaalproductie en klinische kenmerken van de patiënten met COPD toonde dat (veel) roken een negatieve invloed had op het vermogen tot productie van zuurstofradicalen. Het is mogelijk dat dit een verklaring is voor het feit dat COPD patiënten een verhoogde vatbaarheid hebben voor bacteriele infecties en voor bacteriële kolonisatie van de luchtwegen. Een open staande vraag blijft natuurlijk wat er gebeurt in de wanden van de luchtwegen en de alveoli van patiënten met COPD.

Het **zevende en laatste hoofstuk** beschrijft het onderzoek naar de functies van dendritische (of: antigeen-presenterende) cellen bij gezonde proefpersonen, voornamelijk rokers. Dit onderzoek ging vooraf aan onderzoeken bij patiënten met COPD en BHR, en bij patiënten met astma. Dendritische cellen werden verkregen uit de bronchoalveolaire lavage vloeistof door het sorteren op specifieke cel eigenschappen. De typische (CD1a positieve) dendritische cellen waren de sterkste stimulatoren voor de deling van T lymfocyten. Wanneer dexamethason aan het cel-medium werd toegevoegd, vonden wij een remming van dit stimulerende effect, wat afhankelijk was van de dosering van dexamethason.
Dezelfde onderzoeken werden gedaan bij de eerder beschreven patiënten met COPD en BHR, vóór en na behandeling met fluticasone of placebo per inhalatie. Hier werden geen significante effecten gevonden van fluticasone en placebo.

Conclusie

In dit proefschrift worden de resultaten beschreven van het onderzoek van een subpopulatie van patiënten met COPD, namelijk die met bronchiale hyperreactiviteit (BHR). Wij veronderstelden dat deze patiënten een ernstige mate van ontsteking van de luchtwegen hebben waarvoor onderhoudsbehandeling met een inhalatie glucocorticoid (fluticasone propionaat) significant gunstige effecten heeft.
Er werd een ontstekingspatroon gevonden wat niet significant verschilde van rokers zonder luchtweg-klachten en met een normale longfunctie, terwijl er wel verschillen waren met eerder gepubliceerde gegevens over ontsteking bij patiënten met astma.
Behandeling met fluticasone gaf geen verandering van één van de parameters van bronchiale hyperreactiviteit. Volgens verwachting was er een sterke daling van de longfunctie van de patiënten die placebo kregen. De FEV_1 van de patiënten met fluticasone bleef echter vrijwel onveranderd. In de biopten van de beide groepen patiënten werden na behandeling significante veranderingen gezien ten gunste van

fluticasone. Ook werden er significant effecten van fluticasone gevonden op bepaalde ontstekingsmediatoren.
Deze resutaten tonen aan dat er een gunstig effect te verwachten is van onderhoudsbehandeling met fluticasone propionaat bij COPD patiënten met bronchiale hyperreactiviteit. Onze resultaten zijn een argument voor het testen van bronchiale reactiviteit wanneer men twijfelt over het voorschrijven van een inhalatie glucocorticoid bij patiënten met COPD. Deze aanpak verdient bevestiging in interventie-onderzoeken bij patiënten met COPD, waarbij de bronchiale reactiviteit als een van de uitkomstparameters wordt genomen, of minimaal bij aanvang wordt gemeten.

Dankwoord

Bij de totstandkoming van een proefschrift zijn velen direct of indirect betrokken. Al diegenen wil ik van harte bedanken voor hun bijdrage.
Ik begin met degenen die als vrijwilligers aan de diverse onderzoeken hebben deel genomen. Zonder hen was dit niet mogelijk geweest. Vooral de COPD patiënten, die 15 keer en soms vaker het ziekenhuis bezochten, verdienen veel dank. Zij ondergingen twee maal een uitvoerige bronchoscopie, vier maal een inhalatie-provocatie test, enzovoorts. Er waren er die bij herhaling "de moord stikten" maar er waren ook deelnemers die het jammer vonden dat het onderzoek afgelopen was. Ook de andere deelnemers die longfunctie-onderzoeken en/of bronchoscopiën ondergingen wil ik van harte bedanken.

Professor Hoogsteden, beste Henk, vanaf het prille begin in 1992 tot nu zijn er grote veranderingen geweest die van invloed waren op de totstandkoming van dit proefschrift. Ik heb je leren kennen als iemand die de puntjes op de i's zet. Ik wil je bedanken voor de mogelijkheid om dit onderzoek te verrichten.
Professor Bogaard, beste Jan, ik heb veel van je geleerd en wil je bedanken voor de hartelijke betrokkenheid, belangstelling en steun, vooral in de slotfase.
Professor Hilvering wil ik bedanken voor de steun bij de aanvang van de onderzoeken beschreven in dit proefschrift. Ik heb dan wel niet "het voorrecht om in Groningen te hebben gestudeerd" gehad, maar wel het voorrecht om door U te zijn opgeleid tot longarts. Daarbij denk ik ook met dankbaarheid terug aan de helaas te jong overleden Siang Kho, van wie ik vele technische vaardigheden leerde.
Het onderzoek werd aangevangen in het kader van een "research medewerker" project, gesubsidueerd door het Nederlands Astma Fonds. Van GlaxoWellcome, Nederland, werd aanvullende steun verkregen zodat het onderzoek ook daadwerkelijk uitgevoerd kon worden. Beiden wil ik voor hun steun bedanken, in het bijzonder Katja van Geffen en Peter Jagt.
Vervolgens wil ik de dames van de longfunctie-afdeling bedanken voor het vele werk wat zij gedaan hebben. En zo ook de verpleegkundigen, die bij de bronchoscopiën hebben geassisteerd.
De mede-auteurs ben ik dank verschuldigd voor hun bijdrage, die ik in volgorde van de hoofdstukken in dit proefschrift nader wil noemen: Ton Verbraak voor de computer-ondersteuning en de gezellige praatjes, Sandra Boere voor de logistieke ondersteuning en het vertalen van Hongaarse brieven, Joachim Aerts voor zijn enthousiaste meedenken, Joost Hegmans voor het excellente snijden, kleuren en digitale tellen van de biopten, Jan-Bas Prins voor de discussies over de interpretatie van de bevindingen rond de biopten, Paul Mulder voor zijn pogingen om mij iets bij te brengen over statistiek, Ingrid Garrelds voor het meten van de arachidonzuur metabolieten, Freek Zijlstra voor zijn kritische en deskundige blik, Annemarie Wijkhuijs voor het geduldige tellen van de vele cytospins en de gesprekken op de fiets, Herbert Hooijkaas voor zijn onverstoorbare steun, Wim Sluiter voor zijn scherp denkvermogen en gevoel voor humor, Jan-Maarten van Haarst voor het lab

werk wat je gedaan hebt en mij hebt geleerd, Harm de Wit voor het vele sorteerwerk wat jij als enige zo goed in de vingers hebt, Peter Simons voor zijn kritische opmerkingen en, last but not least, Hemmo Drexhage, van wie ik veel geleerd heb en die in moeilijke tijden een grote steun was.
Martha Galli wil ik hartelijk bedanken voor de correcties van de Engelse taal en grammatika.
Voor hun hulp bij het recruteren van patiënten wil ik bedanken de collegae Yoeke Tan, Jaap Bouvy, Jaap de Goeij, Mick Heijsteeg en Rob Slingerland.
Omdat het promotie onderzoek naast de klinische taken en onderwijs verricht moest worden, bedank ik de collegae stafleden van het Dijkzigt ziekenhuis, in het bijzonder Alice Kars, en de assistenten, in het bijzonder Hans Grotjohan, voor hun bereidheid om werk van mij uit handen te nemen. Evenzo wil ik mijn huidige collega's van de maatschap Inwendige Geneeskunde (internisten en arts-assistenten) en collega Jan Brouwers bedanken voor hun coöperatieve opstelling tijdens de slotfase. De poli-assistentes Marijke en Wilma zijn een belangrijke steun geweest voor het goed laten verlopen van dat deel van mijn werk wat anders een chaos zou zijn geworden.
Ik ben mijn ouders dank verschuldigd voor het scheppen van de voorwaarden voor datgene wat ik in mijn leven heb kunnen doen. Tenslotte, last but not least, Dicky, Jan-Willem en Anne-Martine, jullie hebben het nodige te lijden gehad onder mijn werkzaamheden en ik beloof beterschap. Het is prettig om op zo'n gezin terug te kunnen vallen en het leert je dat er naast het werk nog zoveel andere dingen in het leven zijn die o zo belangrijk zijn.

Curriculum vitae

Gerrit Teunis Verhoeven werd geboren op 12 januari 1956 te Rijswijk (NBr). Middelbare school: Atheneum-B, Gorinchem. Studie geneeskunde: Rijksuniversiteit Utrecht, van 1974 tot 1981. Wetenschappelijke stages: o.a. Meting van Closing Volume bij CARA (supervisor: dr. J. Kreukniet). Gedurende enkele jaren arts-assistent in ziekenhuizen in Oosterhout (NBr) en Breda. Opleiding Interne Geneeskunde: Diaconessenhuis Refaja, Dordrecht, opleider: C. Verdoorn†, juli 1986 tot juli 1988. Opleiding tot longarts in het Academisch Ziekenhuis-Rotterdam, Dijkzigt, opleider: prof.dr.C. Hilvering, januari 1989 tot januari 1992.
Van januari 1992 tot mei 1994 longarts-onderzoeker in het kader van het project "Research-medewerker" van het Nederlands Astma Fonds, AZR Dijkzigt, aanvang promotie-onderzoek. Van 1 mei 1994 tot 1 mei 2000 staflid afdeling Longziekten, AZR Dijkzigt. Aanvullende opleidingen: oa. medische lasertoepassing en klinische besliskunde. Sinds 1 mei 2000 longarts in Medisch Centrum Rijnmond-Zuid, locatie Clara.