Inhaled corticosteroids have a high level of topical anti-inflammatory activity. However, in patients with COPD these drugs have been reported to exert limited effects. A reduction in histone deacetylase (HDAC) activity is suggested to prevent the anti-inflammatory action of corticosteroids. Cigarette smoke is known to reduce HDAC expression. The aim of this study is to compare the outcome of corticosteroid therapy in both smoking and non-smoking COPD patients. Twenty-three smoking patients and 18 ex-smoking patients with COPD were treated with inhaled corticosteroids for a period of 2 months. Blood and induced sputum samples were collected before and after treatment. Values of FEV\(_1\) %-predicted did not change upon the therapy, but there was a trend to improve in the ex-smokers (63.1 ± 64.8%-pred.), compared with a decrease in the smokers (63.3 ± 61.6%-pred.). The levels of the pro-inflammatory cytokine IL-8 increased in the group of smokers from 379 ± 78 to 526 ± 118 ng/ml. Although not significant, a slight decrease from 382 ± 70 to 342 ± 62 ng/ml was observed in the group of ex-smokers. The neutrophil related elastase activity showed similar effects after steroid treatment, it went up from 36.4 ± 12.0 to 113.5 ± 9.7 nmol/l in smokers, and decreased from 346.2 ± 72.1 to 131.1 ± 6.5 nmol/l in ex-smokers with COPD. These results support the evidence that inhaled corticosteroids have no anti-inflammatory effects in COPD patients, but only when these patients are still smoking. Smoking cessation seems the best therapy for COPD patients.

**Key words:** COPD, corticosteroid, ex-smokers, smoking cigarettes, therapy
INTRODUCTION

Corticosteroids are among the most widely used drugs and are effective in many inflammatory and immune diseases. Inflammatory diseases are characterized by the infiltration and activation of many inflammatory and immune cells, which in their turn release multiple inflammatory mediators that interact with and activate structural cells at the site of inflammation. The pattern of inflammation clearly differs between these inflammatory diseases, but all are characterized by increased expression of inflammatory proteins, some of which are common to all inflammatory diseases, whereas others are more specific to a particular disease (1-5). Chronic obstructive pulmonary disease (COPD) and asthma are both associated with airway inflammation (6, 7), but the underlying inflammatory conditions are considered of a different nature (8, 9). In asthma, corticosteroids are most commonly used as a first-line therapy and these drugs are by far the most effective anti-inflammatory therapy. However, in COPD they provide relatively little therapeutic benefit (10), and the treatments with steroids has been shown to have no input on the cellular inflammation or increased protease burden seen in COPD (10, 11). Persistent smoking-induced inflammation is thought to play an important role in the pathogenesis of COPD (12, 13). To reduce the progression of the disease smoking cessation seems the only successful intervention (14). Moreover, patients who quit smoking experience less respiratory symptoms and hyperresponsiveness than those who continue to smoke (15), but there is insufficient evidence that smoking cessation reduces the inflammation. Current COPD therapy is limited to treatment of symptoms, with drugs that have been developed to treat asthma, long-acting bronchodilators and corticosteroids (16-18), with the above described outcome. In vitro studies have shown that cytokine release from alveolar macrophages obtained from smoking COPD patients is markedly resistant to corticosteroid effects compared to cells obtained from normal smokers, and these, in turn, are more resistant than alveolar macrophages from non-smokers (19, 20). Thus, the lack of response may be, in part, explained by an inhibitory effect of cigarette smoking. In asthmatic patients who smoke, more severe and resistant disease occurs in response to corticosteroid therapy (21, 22).

The aim of the present study was to investigate the outcome of a treatment with inhaled corticosteroids for a period of two months in patients with COPD and to compare this outcome with the current smoking status of these patients.

MATERIAL AND METHODS

Patients

Forty-one stable COPD patients (31 males, 10 females) were recruited at the out-patient clinics of the National Institute of Tuberculosis and Lung Diseases, Warsaw, Poland and of the University
Hospital, Antwerp, Belgium. Inclusion criteria for entry were smoker or ex-smoker, not fully reversible airway obstruction with 40%<\text{FEV}_1<80% and chest x-ray compatible with COPD. Patients with any history of atopy, asthma, renal and/or hepatic failure, major cardiac disease, cystic fibrosis as well as patients who suffered an exacerbation of COPD in the three months preceding inclusion or used non-steroid anti-inflammatory medication, theophylline, long-acting beta stimulants, and oxygen therapy were excluded from the study. All patients were fully informed about the purpose of the study and gave written consent before inclusion. The study protocol was approved by the Ethics Committees of both hospitals.

**Study Design**

The study consisted of 2 consecutive periods of 10 wk and involved at least 3 visits. At each scheduled visit induced sputum and blood samples were collected and spirometry was performed. During the first period no medication was allowed, except for short-acting beta-agonists and/or cholinergics (salbutamol or fenoterol and/or ipratropium bromide), which were allowed to use in case of necessity during the whole study period. During the second period, patients were treated with inhaled corticosteroid (fluticasone, 1000 µg in two daily dosages of 500 µg by dry powder inhaler, Glaxo-Smith Kline; or budesonide, 800 µg in two daily dosages of 400 µg by Turbuhaler©, Astra-Zeneca). Patients were withdrawn from the study if they required treatment with either anti-inflammatory agents, oral steroids, theophylline, and/or oxygen, and thus compromised the study protocol.

**Sputum Collection and Processing**

Sputum induction and processing were performed according to the guidelines of the Task Force Induced Sputum of the European Respiratory Society (23, 24). After \text{FEV}_1 and FVC baseline measurements, salbutamol was given by inhalation (200 µg by metered-dose inhaler), and sputum was induced by inhalation of 4.5% sterile hypertonic saline solution. The saline was nebulised with an ultrasonic nebuliser (DeVilbiss, Somerset, PA, USA) and inhaled for up to 20 min during subsequent periods of 5 min. After each period, \text{FEV}_1 was remeasured for safety purposes. After each 5-min inhalation period the subject was asked to rinse the mouth, blow the nose, and cough sputum into a polypropylene container, which was kept on ice. The procedure was terminated after four inhalation periods or after a fall in \text{FEV}_1 >20% from baseline.

The collected sputum was processed as soon as possible, but always within 2 h after induction (24). The expectorate was poured into a polypropylene Petri dish and sputum plugs were selected free of salivary contamination. Weight of the sputum plugs was obtained in a pre-weighed polystyrene tube. Complete homogenization was achieved by incubation with freshly prepared 0.1% dithiothreitol (DTT) in PBS (6.5 mM). The volume of DTT used (in µl) during processing was equal to four times the weight of the selected plugs (in mg). The suspension was aspirated and dispensed several times with a disposable pipette, and mixed for 30 s on a Vortex® mixer before placing on a tube rocker for 15 min at room temperature. The same volume of PBS was added and the suspension was replaced on the tube rocker for another 15 min. To remove mucus and debris, filtration through a 40-µm nylon mesh (Cell Strainer, Becton Dickinson Labware, Franklin Lakes, NJ, USA) was performed. Total cell count was performed manually using a Bürker counting chamber and cell viability was determined by trypan blue exclusion method. Subsequently, the cells were separated from the fluid phase by centrifugation (400 x g, 10 min, room temperature). The supernatant was aspirated and stored at -80°C until assay. The cell pellet was resuspended in PBS supplemented with 0.1% glucose and 0.1% bovine serum albumin at a concentration of 1x10° neutrophils/ml. Four cytospin slides were prepared at 50 x g for 5 min, using aliquots of cell
suspension equivalent to $6 \times 10^4$ cells per slide. The slides were stained according to May-Grünwald Giemsa for differential cell counts. To determine cell differentials, at least 400 cells were counted. The percentage of squamous cells was determined, but not included in the differential cell count. Cytospins containing <80% squamous cells were considered adequate.

**Laboratory Assays**

Free neutrophil elastase activity in serum samples was determined using N-methoxysuccinyl-Ala-Ala-Pro-Val-$p$-nitroanilide (Sigma-Aldrich, St. Louis, MO) as a substrate (25). The stability of this substrate allows the measurement of low levels of elastase by monitoring the change in absorbance ($E_{405}$) after 20 h of incubation at 37°C. Reagent solution was prepared by dissolving 6 mg of substrate in 600 µl 1-methyl-2-pyrrolidinone (Sigma), which was diluted with a buffer solution containing 0.1 M hydroxy-ethylpiperazine ethanesulfonic acid, 0.5 M sodium chloride, and 0.1% Brij-35. Human leukocyte elastase (Sigma) was used as a standard.

Myeloperoxidase (MPO) was assayed spectrophotometrically by incubation with 0.167 mg/ml o-dianisidine dihydrochloride (fast blue B) (Sigma-Aldrich, St. Louis, MO) in the presence of 0.0005% hydrogen peroxide. The change in absorbance at 460 nm was measured. Human MPO (Sigma-Aldrich, St. Louis, MO) was used as a standard. One unit of MPO activity was defined as that degrading one micromole of peroxide per minute at 25°C (26). Albumin was measured spectrophotometrically, monitoring the change in absorbance of bromcresol green as described previously (27). The ratio between albumin in sputum and in serum was calculated ($Q_{\text{albumin}}$). Soluble ICAM-1 and the interleukins IL-8 and IL-10 were assayed using commercially available enzyme-linked immunosorbent assays (ELISA) (Flexia<sup>©</sup>, Biosource Europe, Nivelles, Belgium).

**Statistical Analysis**

All data are presented as mean ±SE. Assuming Gaussian distribution, data were analyzed for differences by Student’s $t$-test for paired samples. Data were compared between baseline and the outcome of the treatment after 10 wk. For non-parametric data (IL-10, neutrophil elastase activity, sputum cells, MPO, sputum production, and $Q_{\text{albumin}}$) the Wilcoxon test was used. At a $P$-value <0.05 differences were considered significant.

**RESULTS**

The COPD patients could be divided in two groups, 23 smokers (17 male, 6 females) and 18 ex-smokers (14 male, 4 female). The mean age of the smokers was 61 ±2 yr and of the ex-smokers 66 ±2 yr ($P=0.09$). Sputum production upon induction was 727 ±159 mg in the group of smokers, which was not significant different from the group of ex-smokers where 885 ±196 mg was obtained. After corticosteroid treatment, the amounts of induced sputum were 940 ±215 mg and 1037 ±228 mg, respectively; the increases being insignificant.

The values of FEV$_1$ %-predicted did not change significantly upon therapy with corticosteroids, but there were two opposite trends, a slight increase in ex-smokers from 63.1 ±3.1 to 64.8 ±2.9%-predicted, and a decrease in smoking COPD patients from 63.3 ±2.3 to 61.6 ±2.9%-predicted (Fig. 1). The level of the pro-inflammatory cytokine interleukin-8 (IL-8) increased in the group of smokers.
from 379 ±78 to 526 ±118 ng/ml (P<0.05), but no change was observed in the group of ex-smokers, from 382 ±70 to 342 ±62 ng/ml (Fig. 2).

The neutrophils-related elastase activity showed similar effects after steroid treatment, it went up from 36.4 ±12.0 to 113.5 ±89.7 nmol/l (P<0.0001) in current smoking COPD-patients, and decreased from 346.2 ±72.1 to 131.1 ±6.5 nmol/l (P<0.05) in ex-smokers with COPD (Fig. 3).

Although the number of cells per gram of induced sputum decreased significantly in the group of smoking patients (from 3.78 ±0.80 x10^6 to 2.19 ±0.39 x10^6; P<0.05) (Fig. 4), no significant change was observed in both the absolute total number and the relative number of sputum neutrophils. In ex-smokers no changes in both cell numbers per gram of induced sputum and neutrophil composition could be observed. The levels of IL-10 were before and after corticosteroid treatment, respectively, 38.0 ±6.1 and 62.0 ±29.0 ng/ml in current smoking COPD-patients compared with 46.7 ±3.5 and 21.0 ±4.1 ng/ml, respectively, in ex-smokers with COPD (P<0.01). Levels of ICAM-1 in plasma and in sputum were similar in all groups and did not change upon steroid therapy. The levels of MPO showed a trend to decrease in both groups of patients, but no
significant change was observed. Also the ratio between albumin in sputum and in serum ($Q_{\text{albumin}}$) showed a non-significant decreasing trend after steroid treatment in both groups, suggesting that steroids may have some small beneficial effect on the leakage of albumin from the blood vessels into the lungs.

**DISCUSSION**

Recently some reports have been published that show the persistence of large airway inflammation in COPD long after smoking cessation (28, 29). These reports support our earlier findings that the levels of inflammatory mediators are not significantly different between current smoking and ex-smoking patients with COPD before the onset of steroid therapy. In general, we were not able to observe any anti-inflammatory effects in COPD-patients, regardless if they were smoking or not (30). That we are not alone with such observations is proven by several other reports that have appeared on the poor outcome of steroid therapy and the lack of response of inflammatory processes to steroids (11, 31, 32). Although the mechanisms for steroid resistance are unclear yet, histone deacetylase (HDAC)
activity is decreased in the lungs and airways of patients with COPD (33). The HDAC activity seems to be inversely related to disease severity. A proposed mechanism of corticosteroid resistance in COPD is the inhibitory effect of cigarette smoke and the subsequent oxidative stress on the function of HDAC, and thus an interference with the anti-inflammatory action of steroids (33).

Normally stimulated alveolar macrophages activate NF-κB and other transcription factors to switch on histone acetyltransferase (HAT), leading to histone deacetylation and subsequently to transcription of genes encoding for inflammatory proteins, such as TNF-α, IL-8, and GM-CSF. In the normal situation steroids can reverse this pathway by binding to glucocorticoid receptors that translocate to the nucleus and bind co-activators to inhibit HAT activity directly and by recruiting HDAC 2 to the activated transcriptional complex, resulting in deacylation of acylated histones and a decrease in inflammatory gene transcription (34). Cigarette smoke contains, among others, aldehydes, as acrolein, and it generates oxidative stress through the formation of peroxynitrite, leading to the impairment of HDAC 2 activity (35-37). This amplifies the inflammatory response to NF-κB activation, but also reduces the anti-inflammatory effect of corticosteroids as HDAC is now unable to reverse histone acylation.

Such a mechanism supports the outcome of our present study in which we made a clear distinction between smoking and ex-smoking COPD-patients. We observed but meager beneficial effects of corticosteroid therapy in the COPD-patients who had stopped smoking. These patients were ex-smokers for more than 2 years, with the exception of 2 patients who quitted smoking only approximately 4 months before inclusion in the study. One of these patients did not have a significant sputum production upon induction and was excluded from further analysis of sputum cells and sputum mediators. The second patient delivered results that were not different from the group of ex-smokers as a whole. In addition, we did not confirm smoking status by laboratory tests and therefore we cannot exclude the possibility that some ex-smokers were still smoking.

Smoking cigarettes not only impairs the effects of corticosteroid therapy, but it also results in an influx of inflammatory cells into the airways and chromatin modifications in the lungs (13, 35). Lack of effect of corticosteroid treatment was also demonstrated on smoke-induced pro-inflammatory mediator release (35). Beside this mechanism, other pathways could play their part in the generation of inflammatory markers, as has recently been reported (38, 39). It was demonstrated that early growth response gene 1 (Egr-1) is significantly up-regulated in the lungs of smokers with COPD (40). Cigarette smoke induces Egr-1 in pulmonary epithelial cells, and via Egr-1-mediated mechanisms, IL-1β and TNF-α are up-regulated (38). Furthermore, cigarette smoke activates human monocytes and macrophages to release IL-8. It also synergizes with IL-1β and TNF-α, and it is this interaction that confers steroid resistance to smoke-induced IL-8 release (39). These alternative pathways for the induction of inflammatory
mediators may explain the increase in inflammation we observed in the smoking patients with COPD. The involvement of the above mechanisms may also play a role in smoking patients with asthma, who give even more insights in the results of our study. Asthmatics who smoke have more severe disease and are also resistant to the anti-inflammatory effects of corticosteroids (21, 22, 41). In smokers without COPD, and those who stopped smoking, lung inflammation is at least partially reversible (42), but it persists in patients with COPD after smoking cessation. The persistence of apoptosis of airway epithelial cells after smoking cessation in COPD may lead to continuation of the inflammation (29). Smoking cessation and reduction of oxidative stress by means of anti-oxidant therapy (30) might be expected to be effective in restoring corticosteroid responsiveness, and finally to the reduction of inflammation in COPD.

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