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INDUCED SPUTUM FOR ASSESSMENT OF AIRWAY INFLAMMATION IN PATIENTS WITH COPD, ASTHMA AND ASTHMA-LIKE SYMPTOMS

by

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Academic dissertation

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To my family

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, referred to in the text by means of the Roman numerals. The original communications are reproduced by permission of the copyright holders. Some unpublished data are also included in the thesis.

I Rytilä PH, Lindqvist AE, Laitinen LA. Safety of sputum induction in chronic obstructive pulmonary disease of various degrees of severity. Eur Resp J 2000; 15: 1116-1119.

II Peleman RA, Rytilä PH, Kips JC, Joos GF, Pauwels RA. Cellular characteristics of induced sputum in COPD. Eur Resp J 1999; 13: 839-843.

III Rytilä P, Metso T, Heikkinen K, Saarelainen P, Helenius IJ, Haahtela T. Airway inflammation in patients with symptoms suggesting asthma but with normal lung function. Eur Res J 2000; 16: 824-830.

IV Rytilä P, Pelkonen AS, Metso T, Nikander K, Haahtela T, Turpeinen M. Inflammatory cells in induced sputum in children with newly detected asthma and the effect of anti-inflammatory treatment. Submitted.

V Rytilä P, Metso T, Petäys T, Sohlman A, Työlahti H, Kohonen-Jalonen P, Kiviniemi P, Haahtela T. Eosinophilic airway inflammation as an underlying mechanism of undiagnosed prolonged cough in primary health care patients. Resp Med 2002; 96: 52-58.

ABBREVIATIONS

APAAP	Alkaline phosphatase anti-alkaline phosphatase
ATS	American Thoracic Society
AUC	Area under curve
BAL	Bronchoalveolar lavage
BDP	Beclomethasone dipropionate
COPD	Chronic obstructive pulmonary disease
CTAB	Cetyl-N,N,N-trimethylammonium bromide
DLCO/VA	Diffusion capacity for carbon monoxide per litre/lung (alveolar) volume
DTE	Dithioethreitol
DTT	Dithiothreitol
ECP	Eosinophilic cationic protein
EPO	Eosinophil peroxidase
EPX	Eosinophil protein X
ERS	European Respiratory Society
FEV_1	Forced expiratory volume in one second
FVC	Forced vital capacity
HNL	Human neutrophilic lipokalin
MBP	Major basic protein
MDI	Metered dose inhaler
MGG	May-Grünwald-Giemsa
MPO	Myeloperoxidase
NO	Nitric oxide
NOS	Nitric oxide synthase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
$PD_{15}FEV_1$	Dose of histamine provoking a 15% fall in FEV_1
$PD_{20}PEF$	Dose of histamine provoking a 20% fall in PEF
PEF	Peak expiratory flow
PIF_{THB}	Peak inspiratory flow though Turbuhaler®
ROC	Receiver operator characteristics
SEM	Standard error of mean
TBS	Tris-buffered saline
VC	Vital capacity

ABSTRACT

Sputum induction by inhalation of hypertonic saline using an ultrasonic nebulizer is a non-invasive procedure allowing assessment of airway inflammation. In the studies described in this thesis, we used a method of sputum induction in patients with chronic obstructive pulmonary disease (COPD), asthma, respiratory symptoms suggestive of asthma, prolonged cough, and healthy persons. Subjects were recruited from the outpatient clinics of two University Hospitals and from primary care. Both adults and children took part. Sputum cells associated with inflammation on cytospins or smears and markers of eosinophil and neutrophil activation were studied at baseline and after anti-inflammatory treatment.

Sputum induction was found to be a safe and reproducible method allowing study of airway inflammation even when disease was severe. Since inhalation of hypertonic saline can result in bronchoconstriction, there should be pre-treatment with a bronchodilator, and lung function should be monitored during sputum induction.

Marked sputum neutrophilia was noted in patients with COPD. However, there was a subgroup of COPD patients with eosinophilic airway inflammation. Immunostaining revealed very few lymphocytes.

A patient group with symptoms suggestive of asthma and signs of airway eosinophilia but no significant airflow limitation was characterised. The degree of eosinophilic airway inflammation was less pronounced than in asthma. The patients responded to treatment with inhaled steroids. There is no agreed definition or diagnostic criteria for this condition. Its occurrence and the effects of various kinds of treatments need systematic studies.

The method of sputum induction studied can be safely and successfully used in assessing airway inflammation in children with newly detected asthma. Six months of treatment with inhaled budesonide improved lung function, controlled asthma well, and decreased numbers of sputum eosinophils. Regular treatment with low doses of inhaled steroid for 18 months resulted in further clinical improvement but the results were not significantly different from those obtained using treatment during exacerbations only.

Eosinophilic airway inflammation is fairly commonly associated with prolonged cough in primary care, even in patients not suffering from asthma or COPD, or in whom no other cause of cough is known to be present. Induced sputum samples obtained in health centres could be studied in a central laboratory. Detection of eosinophilic airway inflammation could aid decisions regarding treatment.

INTRODUCTION

A recent advance to extend our understanding of asthma, COPD and other airway diseases has been introduction of a direct, relatively non-invasive, method of induction of sputum production involving subjects inhaling hypertonic saline (Pin et al. 1992a). The cellular and non-cellular compositions of sputum depend on many biological processes within the respiratory system. Determination of sputum composition is a valuable tool in studying such processes (Gibson et al. 1989a). Since the late 1980s sputum induction has become one of the most widely used methods for the study of airway inflammation in disease. A PubMed search of abstracts containing the key words asthma or COPD and induced sputum resulted in identification of more than 400 articles published during the last 10 years. They include several editorials and reviews. An international group of researchers has formed a task force, supported by the European Respiratory Society (Djukanovic 2000), to evaluate the merits of techniques of sputum induction and processing of samples and suggest ways of improving them.

The importance of measuring airway inflammation in chronic respiratory conditions such as asthma, COPD and prolonged cough has recently been emphasised (Gibson 2000). The Finnish National Asthma Programme in 1994 and the COPD Programme in 1998 stressed the importance of early diagnosis and treatment of these diseases (Haahtela and Laitinen 1996, Laitinen and Koskela 1999). Asthma often begins in childhood and may become persistent by adulthood. It is important to evaluate airway inflammation in children to determine what role it may play in the genesis of asthma. A simple method allowing characterisation of airway inflammation is therefore likely to be valuable.

Several research groups have used sputum induction in characterising patients with chronic cough (Gibson et al. 1989b, Brightling et al. 1999a, Fujimura et al. 2000). Eosinophilic airway inflammation characteristic of asthma is common in such patients. However, little is known about the occurrence of this condition, its natural history, or the effects of various anti-inflammatory treatments.

The studies described in this thesis were designed to allow investigation of clinical applications of sputum induction method in various patient populations. We wanted to assess how safe, successful and reproducible the method might be, and to compare inflammatory cells and mediators in sputum from patients with COPD, asthma and chronic respiratory symptoms suggesting asthma. We also investigated use of the method in children with asthma and analysed the effects of various anti-inflammatory treatments on markers in sputum. One aim was to relate inflammatory changes to such clinical characteristic of patients as their symptoms and airway function.

REVIEW OF THE LITERATURE

DEFINITIONS

Chronic obstructive pulmonary disease (COPD)

COPD is a major cause of morbidity and mortality. The main factor associated with it is cigarette smoking. Clinically, COPD is defined as a disorder characterised by the existence of airflow obstruction associated with chronic bronchitis or emphysema. The airflow obstruction is generally progressive and irreversible but may be accompanied by airway hyperresponsiveness, and may be partly reversible (ATS 1987a and 1995). Three disorders have, historically, been included in COPD: emphysema, peripheral airways disease and chronic bronchitis. Any patient can have from one to three of these conditions. Emphysema is defined anatomically as destructive enlargement of the airspace distal to the terminal bronchiole, primarily the alveolar area, without obvious fibrosis (Snider et al. 1985). Various morphological abnormalities have been identified in peripheral airways disease. They include inflammation of the terminal and respiratory bronchioles, fibrosis of airway walls with consequent narrowing, and goblet cell metaplasia of the bronchial epithelium (ATS 1995). Chronic bronchitis has been defined clinically as the condition of subjects with cough and excessive mucus secretion occurring on most days for at least three months of the year for at least two successive years (ATS 1987a).

Chronic bronchitis can occur with or without airway obstruction. Approximately 50% of heavy smokers develop mucous hypersecretion. A minority, perhaps only 10 to 15%, develops chronic airflow obstruction (US Surgeon General 1984). Bronchoscopic studies of subjects with COPD have shown increased total numbers of cells and neutrophils in bronchoalveolar lavage (BAL) fluid (Martin et al. 1985, Thompson et al. 1989). In studies of bronchial biopsy samples, subjects with chronic bronchitis were found to have increased numbers of macrophages and activated T lymphocytes (Saetta et al. 1993). It has recently been have suggested that eosinophilic airway inflammation contributes to airflow obstruction in some patients with COPD, and that the short-term effects of oral prednisolone are a result of modification of this feature of the inflammatory response (Chanez et al. 1997, Pizzichini et al. 1998a, Brightling et al. 2000a).

Asthma

Asthma is a major chronic airway disease, the prevalence of which is increasing in Finland and throughout the world (ISAAC 1998, Haahtela et al. 1990, Pallasaho et al. 2000, Kilpeläinen 2001). Asthma affects people of all ages and places considerable burdens on health-care budgets (Weiss et al. 1992, Haahtela et al. 2001). Asthma is currently defined as a chronic inflammatory airway disease in which many cells, in particular mast cells, eosinophils, and T lymphocytes, play roles. In susceptible individuals, the inflammation causes recurrent episodes of wheezing, breathlessness, chest tightness and cough. The symptoms are usually associated with variable airflow limitation that is at least partly reversible, spontaneously or with treatment. Chronic airway inflammation is associated

with increases in airway responsiveness to various stimuli (NHLBI/WHO 1995). Airway inflammation in asthma is characterised by vascular leakage, mucus hypersecretion, epithelial shedding, and extensive airway narrowing. Apart from cells conventionally associated with inflammation, structural tissue cells, such as epithelial cells, fibroblasts and smooth muscle cells, play significant roles in relation to airway inflammation, through release of a variety of mediators (Chung and Barnes 1999). Chronic airway inflammation stimulates mechanisms of airway healing and repair that can lead to progressive, potentially irreversible, tissue destruction and airway remodelling (Vignola et al. 2000). Some patients with asthma and COPD share pathological features (Barnes 2000). Some investigators believe it is impossible to regard the two conditions as wholly separate (Sluiter et al. 1991) (Fig. 1).

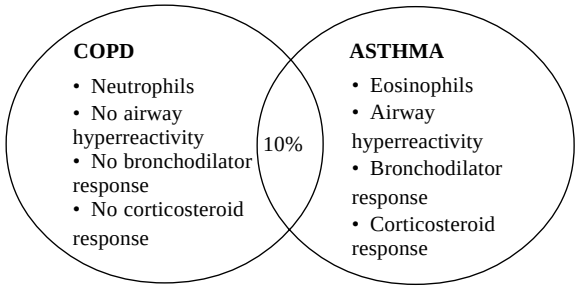


Fig. 1
Overlap between COPD and asthma (Barnes 2000).

Asthma-like symptoms, chronic cough with eosinophilic bronchitis

Diagnosis of asthma is based on establishing variable airway obstruction. Improvement of airway narrowing, as indicated by an increase of more than 12 to 15% in forced expiratory volume in one second (FEV_1) or in peak expiratory flow values (PEF), spontaneously or after using a bronchodilator, indicates asthma (NHLBI/WHO 1995). Reversibility in relation to bronchodilators and variability of lung volumes or PEF values exhibit, however, continuous distributions (Higgins et al. 1989), and the cut-off limit of 15% is fairly arbitrary. Within the Finnish National Asthma Programme it has been estimated that roughly 10% of the population, apart from asthma patients proper, occasionally exhibit symptoms suggestive of mild asthma even though results of lung-function tests remain normal or close to normal (Haahtela and Laitinen 1996). The symptoms in such subjects include cough, chest tightness with wheezing, shortness of breath, sputum production, and wheezing or cough during exercise. Chronic cough is a problem commonly encountered in primary health-care. It can indicate or precede asthma. In practice, many patients exhibit symptoms that give rise to suspicion of asthma but results of lung-function tests do not vary significantly from normal. In such patients no specific diagnosis is usually reached.

Eosinophilic bronchial inflammation is found in patients with newly detected or mild intermittent asthma (Laitinen et al. 1993, Vignola et al. 1998a) but also in some patients with chronic cough and normal lung function (Gibson et al. 1989b and 1995, Brightling

et al. 1999a, Carney et al. 1997, Brightling and Pavord 2000). The term eosinophilic bronchitis has also been used in relation to subjects with asthma-like symptoms related to work but no asthma proper, and with sputum eosinophils (Lemière et al. 1997). Other researchers have used the term eosinophilic tracheobronchitis (Fujimura et al. 2000). Patients with chronic cough responsive to corticosteroids have been found to resemble patients with asthma in relation to gene expression of some cytokines found in cells in BAL fluid (Gibson et al. 1998a), and in relation to various inflammatory mediator concentrations (Brightling et al. 2000b).

ASSESSMENT OF AIRWAY INFLAMMATION

Airway inflammation is considered to be a major cause of exacerbations in asthma and COPD and of permanent structural alterations of the airways in these conditions (Vignola et al. 2000). Consensus guidelines state that treatment of asthma should be directed primarily towards minimisation of inflammation (NHLBI 1997). However, it has not so far been possible in clinical practice to measure airway inflammation directly. The presence and nature of airway inflammation have been deduced from indirect measurements, e.g. of lung function, and from symptoms, results of physical examination and the effects of treatment. The association between degree of lung-function abnormality and underlying inflammation is not straightforward (Crimi et al. 1998). Correspondingly, in patients with COPD there is a need to discover markers that would allow prediction of long-term response to treatment with inhaled corticosteroids (Pauwels et al. 1999, Brightling et al. 2000a). Patients with chronic cough cannot be diagnosed as suffering from eosinophilic bronchitis unless airway inflammation has been studied (Carney et al. 1997).

Asthma often begins in childhood. It may become persistent by adulthood. There is an increasing tendency to treat it preventively with inhaled corticosteroids, in both children and adults (NHLBI 1997). The rationale is that the inflammatory process is similar in children and adults. However, airway inflammation in childhood asthma has not been so well characterised as in adults.

Direct measurement of airway inflammation

Bronchial biopsies and bronchoalveolar lavage (BAL)

Use of rigid or fibre-optic bronchoscopy to obtain bronchial biopsy samples or perform BAL, segmental allergen challenge, and bronchial brushing has contributed significantly to current knowledge relating to asthma and COPD (Jarjour et al. 1998). It has been shown to be feasible to measure airway inflammation via bronchial biopsy samples and BAL fluid. Results of measurements have been shown to be responsive to change (Laitinen et al. 1992, Djukanovic et al. 1992a). However, it is difficult to quantify changes observed in relation to bronchial biopsy samples, and reproducibility has been reported to be poor (Ward et al. 1995, Richmond et al. 1996). The invasiveness of the procedure, associated costs, and restriction of its use to subjects with mild or moderate and stable disease make bronchoscopy unsuitable for monitoring airway inflammation in clinical practice (Djukanovic et al. 1991, Workshop Summary and Guidelines 1991, Jarjour et al. 1998) (Table 1).

Table 1. Advantages and disadvantages of bronchial biopsy, BAL fluid examination, induced sputum examination and exhaled NO determination (modified from Workshop Summary and Guidelines 1991, Jarjour et al. 1998, Djukanovic 2000, Jatakanon et al. 1999a)

	Advantages	Disadvantages

Bronchoscopy	• Allows biopsy and BAL: samples can be obtained from mucosal tissue and of cells and mediators from airway lumen
	• Provides information on structural changes (relating to epithelium, basement membrane, lamina propria)
	• Can be followed by immunohistochemistry, *in situ* hybridisation, electron microscopy
	• Can be followed by segmental allergen challenge
	• Allows the use of bronchial wash (cells for *in vitro* study)
		• Requires trained personnel and expensive equipment
		• Invasive
		• Cannot be undertaken repeatedly or in severe disease
		• BAL fluid
		  • relates to only one segment of the distal lung
		  • there is mixing of contents of alveolar and bronchial compartments
		  • is primarily saline
		  • can be contaminated with blood
		• Biopsy samples
		  • can only be obtained from the larger airways
		  • cell count reproducibility is low

Induced sputum	• Relatively non-invasive • Allows samples to be obtained from several proximal airways • Can be undertaken repeatedly • Safe even in severe disease • No expensive equipment required • Allows study of large patient populations	• Risk of bronchoconstriction • Success rate around 80% • Processing methods fairly laborious • Results not available immediately
Exhaled NO determination	• Non-invasive • Use of rapid detectors allows results to be obtained immediately • Can be done repeatedly • Safe even in severe disease • Quick • Allows study of large patient populations	• Equipment needed is expensive • Determination is flow-dependent • Contamination with nasal material is possible • Only one mediator is detected • Method is very sensitive to steroid administration

Exhaled air

Exhaled air contains many substances. In the past few years there has been intense research into the role played by nitric oxide (NO) in the physiology and pathology of airway diseases and its clinical significance in respiratory diseases (Kharitonov and Barnes 2000). The NO molecules originate from L-arginine and are converted to L-citrullin by the NO-syn-

thase enzyme (NOS). One of the isoforms of this enzyme, inducible NOS, is induced by cytokines. Levels of NO, which can be determined using chemiluminescence analysers, are high in air exhaled by asthmatic patients (Alving et al. 1993, Kharitonov et al. 1994) but decrease after steroid treatment (Kharitonov et al. 1996, Berlyne et al. 2000). Preliminary reports relating to measurements of other inflammatory mediators and markers in exhaled breath condensate has recently been published (Montuschi et al. 1999).

Sputum

History

Sputum is defined as expectorated lower respiratory secretions (Hargreave et al. 1997, Dorland's Illustrated Medical Dictionary 1988). Hippocrates considered sputum to be one of the four essential humours of the body. Sputum contains bronchial secretions from the respiratory epithelium and submucosal gland cells. Ciliary beating and the cough mechanism primarily control clearance of these secretions. Sputum also contains inflammatory cells that have migrated from the blood through the vascular endothelium and across respiratory tissue into the bronchial lumen (Hansel and Walker 1992).

Over 100 years ago, Gollasch found eosinophilic leukocytes in sputum from asthmatic patients (Gollasch 1889, Ellis 1908). Sputum from asthmatic patients occasionally also contains clusters formed by eosinophils, so-called Charcot-Leyden crystals (Dor et al. 1984). It has been known for a long time that bronchial epithelium is damaged in asthma. Curschmann found clusters of shedded epithelial cells in acute asthma in 1885 (Curschmann 1885). He also noted the presence of corkscrew-shaped twists of condensed mucus (Curschmann's spirals). The clusters of epithelial cells were later called Creola's bodies (Naylor 1962). From the 1950s to 1980s, many investigators analysed sputum microscopically and measured fluid-phase components of sputum in attempts to diagnose asthma and chronic bronchitis, and assess disease severity (Brown 1958, Chodosh et al. 1962, Chodosh 1970, Turnbull et al. 1977, O´Connell et al. 1978, Viera and Prolla 1979, Dor et al. 1984). However, the methods used were considered difficult and unreliable.

In the 1950s, sputum testing was developed in connection with diagnosis of lung cancer and tuberculosis. Since not all patients could provide sputum samples spontaneously, some were asked to inhale hypertonic saline, to allow demonstration of malignant cells (Bickermann et al. 1958) or tuberculosis bacteria in sputum specimens. In relation to asthma, international interest in sputum testing arose after it was observed that epithelial damage and inflammatory changes are present even in early-stage asthma (Laitinen et al. 1985). In 1992, Pin et al. induced sputum production with the help of hypertonic saline to allow determination of eosinophilic inflammation in asthmatic patients.

Cleland (1964) identified dithiothreitol (DTT) and its isomer, dithioethreitol (DTE), as agents with low redox potentials, which could reduce and split mucoprotein disulphide bonds. Shah and Dye (1965) showed treatment with DTT and DTE allowed dispersion of sputum before processing of smears and cultures for mycobacteria. The agents were not used to process sputum for cell examination until 1978 (Wooton and Dulfano 1978). From 1989, Gibson et al. demonstrated that cell counts using smears of sputum selected from saliva were reproducible and allowed demonstration of asthmatic inflammation. However, it is difficult to identify different types of cell in smears without using special stains (Pin et al. 1992a). In subsequent developments, DTT or DTE have been used to

disperse mucus, cytospins have been made from cell suspensions, and fluid phases have been collected for measurement of molecular components (Hansel et al. 1991, Virchow et al. 1992, Fahy et al. 1993a, Popov et al. 1994).

Induced sputum

Sputum production is induced to allow collection of adequate samples of secretions from the lower airways for assessment of airway inflammation in patients unable to produce sputum spontaneously. It has been shown that inhalation of isotonic or hypertonic solutions via an ultrasonic nebulizer induce production of small amounts of secretions from the airways. These secretions can be expectorated and analysed. The mechanisms underlying the process are largely unknown. It is believed that increased osmolarity of airway lining fluid increases vascular permeability in the bronchial mucosa and production of mucus by submucosal glands. Hypertonic saline has been shown to increase bronchovascular permeability in rat trachea (Umeno et al. 1990) but there is no information about how the mucous-secreting cells in the airways are stimulated. It has been shown that clearance of secretions from the airways in human beings is increased after administration of a hypertonic saline aerosol (Pavia et al. 1978). Our clinical experience is that saline inhalation induces coughing, which helps clear the pre-existing sputum from airways. Details of the induced sputum method are discussed below.

Indirect measurement of airway inflammation

Clinical features

Direct indices of airway inflammation can correlate with clinical parameters such as symptoms, degree and variability of airflow limitation, and airway responsiveness (Virchow et al. 1992, Pizzichini et al. 1996a, Gibson et al. 1992, Pin et al. 1993, Keatings et al. 1997a). However, correlation is not universal and changes in these parameters may not be similar or simultaneous (Pizzichini et al. 1997a). Correlations between clinical parameters can vary considerably (Kendrick et al. 1993) or be conflicting (Siersted et al. 1996). It is difficult to judge the nature or degree of airway inflammation from clinical parameters (Prameswaran et al. 2000a).

Peripheral blood

Peripheral blood eosinophilia is often found in asthma. Correlations have been observed between it and severity of symptoms, degree of airflow limitation and airway responsiveness to methacholine or histamine (Bousquet et al. 1990, Ulrik 1995). More recently, it has been suggested that the state of activation of blood eosinophils, as measured by levels of eosinophil activation markers, e.g. eosinophilic cationic protein (ECP), might be a useful indirect marker of airway inflammation (Griffin et al. 1991, Juntunen-Backman et al. 1993). However, results of analyses relating to sputum may reflect inflammatory processes more accurately than results of blood and serum measurements (Pizzichini et al. 1996b, Metso et al. 1996, Sorva et al. 1997, Piacentini et al. 1999).

INDUCED SPUTUM

Sputum induction

Induction of sputum production involves the subject inhaling saline solution. The subject is instructed to expectorate the small amount of secretions produced in the airways into a sterile plastic container. Various issues that need to be considered in relation to this procedure are listed in Table 2. Although no standard method for the induction of sputum has been agreed, consensus regarding induction of sputum production in asthma has recently been proposed (Kips et al. 1998). A future European Respiratory Society Task Force document will review the matter (Djukanovic 2000). One reason why standardisation of procedure is desirable is that inhalation of hypertonic saline can cause asthmatics to suffer airway constriction (Smith and Anderson 1989). The mechanism of the effect is unknown but it may involve activation of airway mast cells (Gravelyn et al. 1988) or sensory nerve endings (Makker and Holgate 1993). Safety procedures include measurement of lung function before and during sputum induction, and pre-treatment with a short-acting β_2-agonist.

Table 2. Issues relating to sputum induction (based on unpublished data of the European Respiratory Society Task Force on Induced Sputum)

Facilities, equipment and personnel	• Quiet room with water supply and ventilation • Ultrasonic nebulizer, spirometer or peak-expiratory-flow (PEF) meter, safety equipment, • Sterile saline solutions, sputum cup, freezer in which to store the sample • Experienced nurse, medical supervision
Pre-treatment with bronchodilators	• For safety reasons pre-treat with 200 to 400 μmg of inhaled salbutamol
Pulmonary function monitoring	• For safety reasons monitor FEV_1 or PEF • No agreed protocol: monitor before and after induction of sputum production, at least, and always if adverse effects appear
Nebulizer output	• Ultrasonic nebulizer preferred, several models exist • Output varies between 0.21 and 4.6 ml/min
Concentration of saline solution	• Different concentrations have been used (0.9, 3, 4, 5%) • Increasing concentrations or constant concentration
Duration of inhalation	• Should be standardised and reported • Duration of between 10 and 30 minutes have been reported

Pre-treatment with bronchodilators

Although some investigators have not pre-treated patients, because they wished to study the functional response of airways to the bronchoconstricting stimulus (Iredale et al. 1994, Bacchi et al. 1997, Gibson et al. 1998b), most have given short-acting β_2-agonists beforehand, to prevent excessive bronchoconstriction (Pin et al. 1992a, Jatakanon et al. 1998a, Wong and Fahy 1997). Severe asthma exacerbation, leading to death in an asthmatic subject undergoing a distilled-water challenge, has been reported (Saetta et al. 1995). Safety reasons apart, excessive bronchoconstriction after a few minutes of inhalation of hypertonic solution can necessitate interruption of induction and collection of an insufficient amount of sputum.

In the original studies of sputum induction, few side effects were reported, and no falls in FEV_1 (Pin et al. 1992a, Fahy et al. 1993a). The safety of sputum induction has been assessed in patients with asthma in a number of studies (Pizzichini et al. 1997a, Wong and Fahy 1997, de la Fuente et al. 1998, Grootendorst et al. 1999, Hunter at al. 1999, ten Brinke et al. 2001) but information on patients with COPD is scarce (Maestrelli et al. 1996, Keatings et al. 1996, Bhowmilk et al. 1998). Pre-treatment with inhaled salbutamol has not always prevented bronchocontriction in asthma (de la Fuente et al. 1998). It has been shown that pre-treatment with a β_2-agonist did not alter the cellular constituents of induced sputum (Popov et al. 1995, Cianchetti et al. 1999).

Pulmonary function monitoring during induction

No standard approach to pulmonary-function monitoring during sputum induction has been suggested but many authors measure pulmonary function every five to 10 minutes, and every time symptoms occur (Iredale et al. 1994, Bacchi et al. 1997, Pin et al. 1992a, Jatakanon et al. 1998b, Maestrelli et al. 1994). Various methods have been used: most investigators have used spirometers, some PEF meters (Wong and Fahy 1997). It has been suggested that lung function should be measured within the first minute of sputum induction, to identify subjects who may be unusually sensitive to hypertonic saline (Pizzichini et al. 1997a).

Concentration of saline solution and nebulizer output

The concentration of saline used for sputum induction has ranged from normal to 5% (Iredale et al 1994, Wong and Fahy 1997, Bacchi et al. 1997). Some investigators change concentration during the procedure, starting with 3%, increasing to 4% and 5% (Pin et al. 1992a, Pizzichini et al. 1996a). It has been suggested that sputum production should not be induced in asthmatic patients with an FEV_1 following bronchodilator administration of less than 1 l or 60% of the predicted value (Wong and Fahy 1997, Kips et al. 1998). An aerosol of normal saline followed by one of hypertonic saline has, however, been safely used in patients with severe exacerbations of asthma (Pizzichini et al. 1997a). Saline concentration and nebulizer output would be expected to influence the safety, tolerability and success rate of the procedure, and the cellular and biochemical characteristics of the sputum induced. Hypertonic saline seems to be more effective than normal saline in relation to induction of sputum production (Popov et al. 1995). No differences have been found

between cell compositions of sputum induced by use of isotonic saline, hypertonic saline and different concentrations of saline (Popov et al. 1995, Bacchi et al. 1997). In one study, jet nebulizers were compared with ultrasonic nebulizers. The success rate was highest with the latter (Popov et al. 1995). Success with ultrasonic nebulizers with relatively low outputs has been found to be similar to those with high-output nebulizers (Hunter et al. 1999).

Duration of inhalation

It has been found in at least two studies that the cellular and biochemical constituents of induced sputum change during induction (Holtz et al. 1998a, Gershman et al. 1999a). Neutrophils and eosinophils are prominent in samples collected early during induction. Lymphocytes and macrophages are more prominent in samples collected later. Mucin concentrations are higher in samples collected early than in those collected late, and surfactant concentrations are higher in samples collected later than in earlier samples (Holtz et al. 1998a, Gershman et al. 1999). The results of these studies suggest that different compartments of the respiratory tract are sampled at different times during sputum induction. Central airways are sampled early, peripheral airways and alveoli later (Moodley et al. 2000).

Protocols for sputum induction differ in relation to schedules of sputum collection. Subjects may be asked to stop inhalation at regular intervals to produce sputum, or to stop only when an urge to cough is felt. Methods of sputum expectoration also differ. In some protocols subjects are required to spit saliva into one container before coughing sputum into another (Gershman et al. 1996). Some authors have suggested that the first sputum sample should be discarded and that only subsequent samples obtained after saline inhalation should be collected and analysed (Jatakanon et al. 1998a).

Sequential sputum induction

In several studies sputum production has been induced sequentially, after intervals of only a few hours (Pin et al. 1992b, Nordenhäll et al. 2000). However, it has been suggested that frequent repetition of sputum induction can result in late airway inflammation and affect differential cell counts in the later inductions (Pavord 1998, Richter et al. 1999). Some authors have reported that induction of sputum eight to 24 hours after a first induction results in more neutrophils being found in the second sputum sample (Kips et al. 1995, Nightingale et al. 1998, Holtz et al. 1998b). No effects on eosinophils or other cells associated with inflammation were seen. It has been shown that methacholine challenge does not alter sputum eosinophil counts but may increase sputum neutrophil percentages (Spavanello et al. 1999, Gershman and Fahy 1999).

Sputum processing

Methods of sputum processing for cell counting and fluid-phase measurements

Two methods have mainly been used for processing induced sputum (Fig. 2). The first in-

volves selecting all of the more dense portions (plugs) from expectorated samples (Pin et al. 1992a, Pizzichini et al. 1996a), the second processing the entire expectorate, consisting of sputum plus saliva (Fahy et al. 1993a). Recent modifications to this method include separate collection of saliva and sputum (Gershman et al. 1996, Keatings et al. 1996, Louis et al. 1997). Data on whether differential cell counts differ between the two methods are conflicting. In one study a higher percentage of eosinophils in the sputum processed using the selection method than in sputum processed using the entire expectorate was reported (Spanevello et al. 1998). However, in other studies this was not found (Peleman et al. 1995, Gershman et al. 1996, Pizzichini et al. 1996b). Both methods have shown to yield reproducible cell counts and results relating to certain fluid phase markers (in´t Veen et al. 1996, Pizzichini et al. 1996a).

Total and differential cell counts

Total numbers of cells have been counted manually, in a haemocytometer, and cell viability has been determined using the trypan-blue exclusion method by most investigators (Fahy et al. 1993a, Pizzichini et al. 1996a). Automated methods for total cell counting and differential cell counting have been investigated and found to be unreliable (Hansel et al. 1991, Popov et al. 1994). Differential cell counting involves counting a minimum of 400 non-squamous cells on May-Grünwald-Giemsa (MGG)-stained slides. Results are reported as proportions of eosinophils, neutrophils, macrophages, lymphocytes and bronchial epithelial cells. Metachromatic staining, in which both basophils and mast cells are identified, has been performed using the toluidine blue staining method (Fahy et al. 1993a, Pizzichini et al. 1996a, Popov et al. 1996a, Spanevello et al. 1998). Immunocytochemistry can be used in relation to sputum cells (Girbis-Gabardo et al. 1994), and can discriminate between basophils and mast cells (Gavreau et al. 2000).

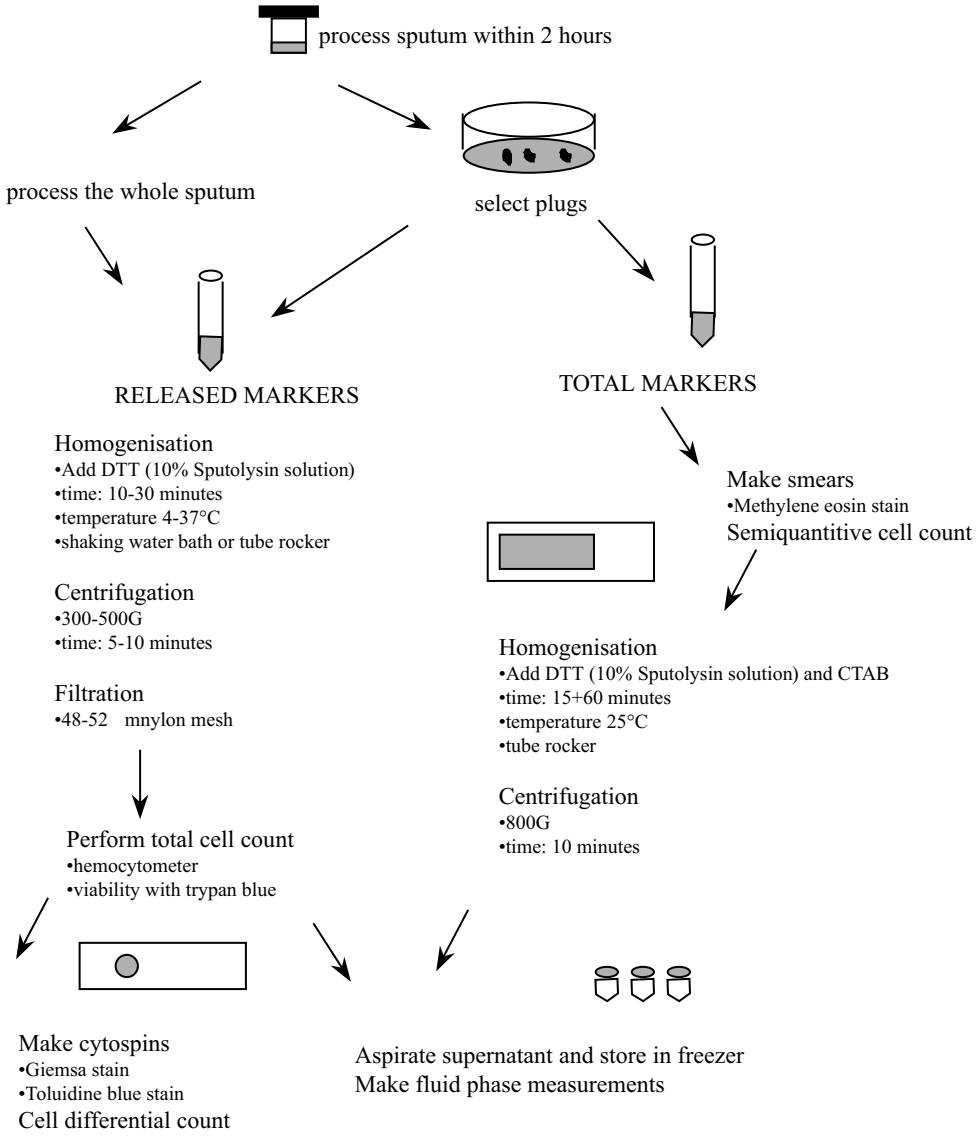
Sputum cell counts have been shown to be valid, reliable and responsive to change (Pin et al 1992a, Popov et al. 1994, Claman et al. 1994, Pizzichini et al. 1996b, in´t Veen et al. 1996, Spanevello et al. 1997, Ward et al. 1998). Recently, normal values for a large population of healthy controls have been obtained (Belda et al. 2000, Spavenello et al. 2000). In some studies in situ hybridisation has been undertaken in relation to cells in induced sputum samples (Olivenstein at al. 1999, Profita et al. 2000). Polymerase chain reaction (PCR)-technique has also been employed (Gelder et al. 1995, Fireman et al. 1999a). Flow cytometry has been used to study lymphocyte subsets in sputum (Kidney et al. 1996, Louis et al. 1997).

Fluid-phase measurements

Numerous inflammatory mediators have been measured in the fluid phase of sputum. They include cytokines, chemokines, granulocyte proteins, markers of vascular leakage, eicosanoids and proteases (Fahy et al. 1993b, Tomaki et al. 1995, Keatings et al. 1996, Vignola et al. 1998b and 1999, Louis et al. 1997, Pavord et al. 1999, Tanaka et al. 2000, Purokivi et al. 2000). Many of the assay methods used to measure these soluble mediators have been developed for serum or culture fluid. The reproducibilities, precisions and validities of many of these measurements in sputum have not been investigated (Kelly et al. 2000, Stockley and Bayley 2000).

In many studies the roles played by eosinophils and neutrophils in asthma and COPD

Fig. 2.
Sputum processing



References: Pin et al. 1992a, Fahy et al. 1993, Pizzichini et al. 1996, in´t Veen et al. 1996, Keatings et al. 1996, Louis et al. 1997, Metso et al. 2001.

have been studied. After activation, eosinophils can release granule-derived proteins, the most cytotoxic of which are eosinophilic cationic protein (ECP) and major basic protein (MBP). Other markers of eosinophil activation include eosinophil protein X (EPX) and eosinophil peroxidase (EPO) (Venge et al. 1999). Myeloperoxidase (MPO) is a protein released from primary (azurophil) granules of neutrophils, and can serve as a marker of neutrophil activation. Human neutrophil lipokalin (HNL) is another protein that has recently been isolated and purified from secondary neutrophil granules (Xu et al. 1994). Its function remains unknown but it may prove a better and more specific marker of neutrophil activation than MPO.

Simplification of sputum-processing

Sputum processing to allow preparation of cytospin slides and differential cell counting is laborious, and can only be undertaken in larger laboratories. A prerequisite for reliable preparation of cytospin slides and measurement of inflammatory markers is immediate processing of sputum (Grebski et al. 1998, Louis et al. 1999). We therefore modified a previously validated sputum-processing method (Pizzichini et al. 1996a) to improve its clinical applicability (Metso et al. 2001) (Fig. 2). Sputum induction and collection of sputum plugs is the same for both methods. Sputum plugs are first treated with DTT then with the cationic detergent CTAB (cetyl-N,N,N-trimethylammonium bromide). DTT treatment is necessary because liquefaction of sputum plugs prior to CTAB treatment is wanted. Detergent is added to lyse cells and liberate any biochemical markers engulfed by cells. We chose CTAB as detergent because sticky proteins like ECP can be efficiently recovered from cell pellets by a one-hour CTAB treatment, as shown by Carlson et al. (1994).

Our method allows sputum specimens for measurement of biochemical markers to be frozen and stored after collection. Specimens can therefore be obtained in one place (e.g. a health-care centre), and processed and analysed elsewhere (e.g. in a central laboratory). This method for sputum processing gives results similar to those obtained using the reference method (Metso et al. 2001). By measuring one eosinophil activation marker, ECP, and one neutrophil marker, MPO, using commercially available kits, eosinophilic and neutrophilic airway inflammation can be studied with reasonable accuracy. Incubation of reference protein with DTT and CTAB showed no negative effects on marker assays. Recovery of four markers (eosinophil markers ECP and EPO, neutrophil markers MPO and HNL) ranged from 96.6% to 102.1%. The advantage of our method (which measures total markers) over the reference method (which measures released markers) is its relative simplicity. Even a small laboratory which does not have cytocentrifuge can use it. Numbers of cells associated with inflammation can be studied semiquantitatively in smears prepared using eosin/methylene-blue staining prior to freezing (Hansel 1949).

Spontaneous versus induced sputum

Some asthmatics, particularly those suffering acute exacerbations or experiencing severe symptoms, and COPD patients can produce sputum spontaneously. It has been shown that spontaneous sputum samples contain percentages of cells and mediators associated with inflammation similar to those found in induced sputum samples (Pizzichini et al. 1996c, Bhowmik et al. 1998). However, the viabilities of cells in spontaneous sputum samples is considerably lower than that of cells in induced sputum samples (Pizzichini et

al. 1996, Bhowmik et al. 1998), perhaps because mucus secretion stays longer in the airways in the first case. Distinction between different types of cells associated with inflammation is therefore more difficult.

Comparison between direct measurements of airway inflammation

There have been few direct comparisons in relation to asthma and COPD of results obtained following bronchoscopy and through study of induced sputum samples (Fahy et al. 1995a, Maestrelli et al. 1995, Grootendorst et al. 1997, Keatings et al. 1997a, Pizzichini et al. 1998b, Rutgers et al. 2000). The results of these studies allow the conclusion that there are marked differences between the two methods, i.e. they complement each other as research tools (Djukanovic 2000) (Table 1). Differential cell counts obtained using BAL fluid and induced sputum samples differ even when the volume of physiological saline is limited to achieve so-called bronchial wash (Fahy et al. 1995a, Keatings et al. 1997). However, in asthma, eosinophil counts correlate with each other (Grootendorst et al. 1997). Higher concentrations of several mediators have been found in sputum samples than in BAL fluid (Fahy et al.1995a) but they correlate better than differential cell counts (Rutgers et al. 2000).

In several studies in asthmatics good correlations have been found between numbers of sputum eosinophils and levels of exhaled NO both in adults and children (Jatakanon et al. 1998b, Mattes et al. 1999). Correlation was poorer in steroid-treated asthmatics (Piacentini et al. 1999, Berlyne at al. 2000). Poor correlation between numbers of eosinophils in bronchial biopsy samples and levels of exhaled NO has been reported (Lim et al. 2000).

Clinical applications of assessment of induced sputum

Induced sputum cell counts can accurately discriminate between eosinophilic and non-eosinophilic airway inflammation, and help guide therapy (Jayaram et al. 2000). Studies on large numbers of subjects can be conducted using induced-sputum methods (Louis et al. 2000a), something that is clearly not easy when bronchoscopy is used. By studying subjects with varying degrees of disease severity, insights into determinants of disease persistence and severity can be gained.

It has been argued that sputum eosinophils are more responsive to intervention than blood measurements but not as hypersensitive as exhaled NO levels (Jatakanon et al. 1999a). Induced sputum samples have been used to study anti-inflammatory effects of oral and inhaled corticosteroids in adult asthma (Claman et al. 1994, Fahy and Boushey 1998, Lim et al. 1999, Jatakanon et al. 1999a, Gershman et al. 2000, Metso et al. 2000). They have also been used in studies of other anti-inflammatory drugs, such as leukotriene antagonists (Pizzichini et al. 1999a), theophylline (Louis et al. 2000b) and novel agents such as anti-IgE antibodies (Fahy et al. 1997). They have been employed in clinical trials of various combinations of treatment (Kips et al. 2000). Reports of studies of effects of treatment in COPD patients have been published (Keatings et al. 1997b, Pizzichini et al. 1998a, Brightling et al. 2000a).

Induced sputum in children

Induced sputum has been used to study asthma in children since the technique was first described, in 1992 (Pin et al. 1992b). The method has particular advantages in the study

of asthma in childhood because it is relatively non-invasive. In some studies (Gibson et al. 1998b, Oh et al. 1999) hypertonic saline challenge has been used to assess airway responsiveness and to induce sputum production. In other studies, pre-treatment with bronchodilators has been employed, and sputum induction has been found safe.

Most studies in which sputum production has been induced in children have been cross-sectional, with the asthma in a stable state or the patients suffering acute exacerbations (Twaddel et al. 1996, Cai et al. 1998, Gibson et al. 1999, Grootendorst et al. 1999, Piacentini et al. 1999, Wilson et al. 2000, Norzila et al. 2000). In children with stable asthma, numbers of eosinophils and mast cells were found to be higher than normal. During acute exacerbations, numbers of neutrophils, mast cells and eosinophils were higher than normal (Twaddel et al. 1996). No data relating to airway inflammation shortly after diagnosis of childhood asthma or from controlled trials of the effects of various anti-inflammatory treatments are available. Children with cystic fibrosis have been found to have intense sputum neutrophilia (DeBoeck et al. 2000).

Induced sputum in other diseases

Processes like gastroesophageal reflux and left ventricular heart failure lead to accumulations of lipids or haemosiderin in sputum macrophages. It has recently been suggested that detection of these markers in sputum macrophages could indicate the existence of one or other of these diseases (Parameswaran et al. 2000b, Leigh et al. 1999). In interstitial lung diseases, such as sarcoidosis and idiopathic pulmonary fibrosis, lymphocytes obtained from BAL fluid and induced sputum samples have been studied by means of flow-cytometry. Results were similar with the two methods of obtaining the lymphocytes (Fireman et al. 1999b, D´Ippolito et al. 1999). Induced sputum has been also been used to assess for example exposure to hazardous dust, and pulmonary involvement in Crohn´s disease (Fireman et al. 1999a and 2000).

AIMS OF THE STUDY

1. To assess the safety, success rate and reproducibility of sputum induction in patients with COPD of varying severities, and to characterise airway inflammation in them.

2. To test the hypothesis that eosinophilic airway inflammation is common in patients with prolonged respiratory symptoms suggestive of asthma, irrespective of lung function, and to investigate the effects of anti-inflammatory treatment with inhaled corticosteroid in such patients.

3. To assess airway inflammation in children of five to 10 years of age shortly after diagnosis of asthma, and to investigate the effects of treatment with regular or periodic inhaled corticosteroid.

4. To test a simplified method of analysis of induced sputum in primary health-care and to assess airway inflammation in patients with prolonged cough.

SUBJECTS AND METHODS

STUDY POPULATIONS AND DESIGNS

Three hundred and eighty-one subjects participated in the studies. All of the patients and controls were Caucasian. Local Ethics Committees had approved the studies. All subjects gave consent to participation in the studies. In study IV, informed consent was given by signatures of parents and children over seven years of age. The main characteristics of the study populations are shown in Table 3.

Table 3. Diagnoses, clinical data and baseline lung function in patients and control subjects.

Studies and Diagnoses	Sex (f/m)	Age (years)	Atopic*	Smokers*	FEV_1 (% of predicted)†
Study I					
COPD (n = 28)	9/19	60 (51 - 68)	1 (4%)	28 (100%)	53 (28 - 60)
Study II					
COPD (n = 21)	2/19	61 (45 - 76)	0 (0%)	21(100%)	54 (23 - 80)
Healthy controls (n = 16)	2/14	30 (22 - 53)	0 (0%)	0 (0%)	nd
Study III					
Respiratory symptoms (n = 36)	33/3	39 (19 - 60)	19 (53%)	8 (22%)	95 (74 - 119)
Asthma (n = 25)	16/9	38 (15 - 75)	14 (56%)	9 (36%)	79 (57 - 105)
Healthy controls (n = 43)	27/16	36 (23 - 54)	10 (23%)	4 (9%)	98 (77 - 212)
Study IV					
Asthma (n = 60)	25/35	8 (5 - 10)	37 (62%)	0 (0%)	91 (69 - 116)
Healthy controls (n = 17)	10/7	9 (5 - 13)	7 (41%)	0 (0%)	95 (79 - 118)
Study V					
Prolonged cough (n = 82)	25/57	46 (16 - 85)	12 (15%)	23 (28%)	82 (41 - 111)
Healthy controls (n = 53)	43/10	42 (25 - 61)	9 (17%)	3 (6%)	nd

Data are expressed as means and ranges, and numbers and percentages*, COPD = chronic obstructive pulmonary disease, f = female, m = male, nd = not done

†Reference values from Viljanen (Viljanen 1982) studies I, III and V, European Community for Coal and Steel (Quanjer et al. 1993) study II, Polgar and Prodmadhat (Polgar and Prodmadhat 1971) study V.

Study I

The study population consisted of 28 patients, clinically diagnosed as suffering from symptomatic COPD (ATS 1995), with an average smoking history of 52 pack-years (range 20 to 103 pack-years) (Table 3). Two (7%) were former smokers, all of the others current smokers. For inclusion in the study patients had to meet the following criteria: 1) FEV_1 before bronchodilator administration of less than 70% of the predicted value (Viljanen 1982), 2) less than 10% reversibility from FEV_1 before bronchodilator administration in response to a short-acting β_2-agonist (200 μg of Ventoline®, 0.1 mg, GlaxoSmithKline, UK). Patients who were being treated for disease exacerbation or who had needed antibiotic treatment for a respiratory-tract infection during the six

weeks preceding the study were excluded. Four patients (14%) used inhaled steroids (beclomethasone or budesonide, mean dose 900 μg/day, range 800 to 1200 μg/day) and two (7%) took theophylline orally. None were on regular oral steroid treatment. Five (18%) had been given a short-acting β_2-agonist and three (11%) an anticholinergic as rescue medication. None of the subjects used long-acting bronchodilators.

Two weeks before sputum induction, spirometry was undertaken and reversibility of FEV_1 assessed. Patients measured PEF every morning and evening for one to two weeks before sputum induction. They also recorded daily symptoms (cough, sputum production, shortness of breath) on a scale ranging from 0 to 4. Patients were allowed to continue their usual medication for COPD except that short-acting bronchodilators were not allowed during the four hours before induction.

Study II

Twenty-one patients with COPD took part in this prospective study. The FEV_1 of each before bronchodilator administration had to be less than 80% of the predicted value (Quanjer et al. 1993) with less than 10% reversibility from FEV_1 before bronchodilator administration in response to a short-acting β_2-agonist (400 μg of salbutamol, Ventoline, 0.1 mg). All of the patients were former smokers, who had stopped smoking at least one year before entry into the study. They had smoked for a mean of 37.0 pack-years (range 20 to 60 pack-years). All except one of the patients were on inhaled steroid treatment (beclomethasone or budesonide, < 1000 μg/day). The 16 healthy controls were selected from hospital employees, had never smoked, and had normal pulmonary function. None of the subjects had had a respiratory-tract infection within the four weeks preceding the study.

Subjects visited the laboratory three times. During the first visit, subject characteristics were documented and lung function (spirometry and diffusion capacity) measurements were made. Subsequently, subjects underwent sputum induction, at the same time of the day, on 2 days, 10 days apart. Results of spirometry were recorded before and after the induction.

Study III

The study population consisted of 36 consecutive patients with respiratory symptoms suggestive of asthma referred to the Outpatient Clinic of the Department of Allergy, Skin and Allergy Hospital, Helsinki University Central Hospital during the period October 1996 to March 1997. Twenty-five patients who had been diagnosed as asthmatic during the same period and 43 healthy individuals were recruited into control groups.

For inclusion in the study, patients with respiratory symptoms suggestive of asthma had to be symptomatic at the time they were studied. We included only patients who had reported at least two of six respiratory symptoms (cough, chest tightness with wheezing, shortness of breath, sputum production, wheezing or cough on exercise, disturbed sleep) for more than two months but not for more than one year. Each of the six symptoms was graded on a scale ranging from 0 (asymptomatic) to 9 (most severe discomfort). Patients who had been treated with anti-inflammatory asthma medication (corticosteroids, disodium cromoglycate, nedocromil sodium or theophylline) or who had used H_2-blockers were excluded. Patients had to have normal chest and sinus X-rays, and normal serum C-reactive protein values. The healthy subjects exhibited no respiratory symptoms and had no history of chronic pulmonary disease. Patients or healthy subjects who had had a clin-

ically diagnosed respiratory infection during the preceding eight weeks were excluded. None of the smokers included had a history of chronic bronchitis (ATS 1995).

Patients were considered asthmatic in this study if they exhibited, during resting flow-volume spirometry, an increase of 12% or more in FEV_1 15 minutes after inhalation of 200 μg of salbutamol (Buventol Easyhaler® 100 μg/dose, Orion Pharma, Espoo, Finland), or if PEF varied by more than 12% between morning and evening on at least three days during a two-week follow-up period. They also had to exhibit increased bronchial responsiveness to inhaled histamine (Sovijärvi et al. 1993). Patients who did not display significant airflow variability and were not hyperresponsive were diagnosed as exhibiting respiratory symptoms. All of the healthy subjects had normal lung function.

Subjects visited the laboratory three times. During the first visit, subject characteristics were documented and lung function measured. Patients measured PEF every morning and evening for one to two weeks. One week after the first visit, bronchial responsiveness to inhaled histamine was measured. A week later sputum was induced.

Follow-up

To study the effects of treatment with inhaled steroid and the course of respiratory symptoms, the 36 patients without variable airway obstruction were allotted at random to inhale, on a single-blind basis, beclomethasone dipropionate (BDP), 400 μg b.i.d., from a multidose powder inhaler (Beclomet Easyhaler® 200 μg/dose, Orion Pharma, Espoo, Finland), or placebo (lactose) for the first three months. Randomisation was performed using a computerised list based on a block size of 10. Compliance was checked by collecting the dry powder inhalers after the three-month treatment period and checking whether they had been used adequately. After the first three-month period patients were allowed to take symptomatic medication if needed (inhaled salbutamol, Buventol Easyhaler® 100 μg/dose). Symptoms, lung function, and blood and sputum samples were studied at three months and after one year. Patients with asthma and normal subjects were not followed-up.

Study IV

The study population consisted of 60 children (five to 10 years of age) with newly diagnosed asthma, and 17 healthy control subjects (Table 3). The asthmatic children were randomly selected from 180 subjects participating in a study of early pharmacological treatment of childhood asthma (Turpeinen 2000). All of the asthmatic children had to have a history of asthma symptoms, defined as cough, wheeze or decreased tolerance to exercise, during the preceding month at least. They had to meet at least one of the following inclusion criteria during the preceding month:

1) Abnormal diurnal variation in PEF (≥ 20%). Assessment of variation was based on records in written diaries kept by the patient or the patient's parents. Variation in PEF was calculated by the investigator as 100 x (PEF evening - PEF morning)/ average PEF . 2) Bronchial reversibility of ≥ 15% as measured by PEF or FEV_1 in response to 0.50 mg of inhaled terbutaline (Bricanyl Turbuhaler® 0.25 mg/dose, AstraZeneca, London UK, Sweden). 3) A fall of ≥ 15% in FEV_1 during an exercise test conducted in accordance with methods used at the clinic. Patients were excluded if they exhibited asthmatic symptoms only during the pollen season, if there was a history of passive smoking at home, or if hairy pets had been kept at home during the previous months. They were also excluded

if they had previously required maintenance treatment for asthma, had ever been treated with inhaled corticosteroids for more than 60 days, or had had moderate to severe atopic dermatitis (extent more than 15%). Before the study, patients were not allowed to have been given an inhaled, nasal or oral corticosteroids, inhaled disodium cromoglycate or inhaled nedocromil during the preceding two months, or a long-acting β_2-agonist during the preceding month. The healthy control subjects exhibited no respiratory symptoms and had normal lung function. None of the children had had symptoms of a respiratory tract infection within the four weeks preceding the study.

Cross-sectional study (baseline)

Patients and healthy controls were evaluated in relation to various baseline parameters during a four-week run-in period. During this time all of the children paid three visits to the outpatient unit. During the first visits, a physical examination was performed, and the children and parents were interviewed. Flow-volume spirometry and skin-prick tests were performed. The children were instructed about use of an electronic home spirometer. Lung function and symptom scores were monitored twice daily at home throughout the study. During the second visit, a histamine challenge test was performed. During the third visit, at least one week after the second visit, flow-volume spirometry was undertaken and sputum was induced, blood samples were taken, and data for baseline measurements were downloaded from the home spirometer.

Longitudinal study (treatment)

After the baseline evaluation, children with asthma were randomised to three treatment groups. In the double-blind study, children received 1) budesonide 400 µg b.i.d. for one month (Pulmicort Turbuhaler® 400 µg/dose, AstraZeneca), 200 µg b.i.d. for five months (Pulmicort Turbuhaler® 200 µg/dose), followed by 100 µg b.i.d. for 12 months (Pulmicort Turbuhaler® 100 µg/dose) (n=19) or 2) the same treatment as the first group for the first six months, thereafter placebo for 12 months (n=20). A third group of patients received in an open study disodium cromoglycate 10 mg t.i.d. for 18 months (pressurised metered dose inhaler with a valved spacer device, Lomudal® with Fisonair®, Fisons, UK) (n=21). A patient in any treatment group who experienced an asthma exacerbation was treated with budesonide, 400 µg b.i.d (Pulmicort Turbuhaler® 400 µg/dose), for two weeks, as a replacement for their normal treatment. Exacerbation of asthma was suspected if asthma symptoms were not controlled by up to six inhalations of terbutaline (Bricanyl Turbuhaler®, 0.25 mg/dose, AstraZeneca) over 24 hours. In such cases, the patient was examined at the clinic, where lung function measurements were performed. A decision about whether a patient needed treatment for an exacerbation was made by the investigator, who was blind in relation to budesonide treatment but not to cromoglycate treatment. Compliance with dosing regimes was checked by recording peak inspiratory flow by means of a Turbuhaler (PIF_{TBH}) with a pneumotachograph, and by weighing returned Lomudal® canisters during each visit to the clinic. During the study period of 18 months, compliance with treatment instructions gradually declined in each group, from 90% to 55% (Turpeinen 2000).

Study V

Eighty-two consecutive patients not known to suffer from asthma or any other chronic respiratory disease were enrolled into the study in six health-care centres in the Helsinki area of Finland, between November 1997 and February 1998. Each centre serves a population of about 30 000. One physician from each centre participated into the study and was specially trained in the Skin and Allergy Hospital, Helsinki University Central Hospital. Patients complaining of coughing daily for more than one month were eligible for enrolment into the study. Patients had to be symptomatic at the time they were studied and were carefully questioned about respiratory symptoms other than cough. Patients being treated with anti-inflammatory asthma medication (corticosteroids, disodium cromoglycate, nedocromil sodium, theophylline, leukotriene antagonists) or who were using angiotensin-converting-enzyme inhibitors were excluded. Patients who had suffered from a respiratory infection during the preceding six weeks or who had symptoms of rhinitis or gastro-oesophageal reflux were also excluded. Patients had to have normal chest and sinus X-rays, and normal serum C-reactive protein values. Fifty-three healthy control subjects with no history of asthma or other respiratory symptoms were recruited from the staff of the Skin and Allergy Hospital, Helsinki University Central Hospital.

Physicians were asked to complete a questionnaire regarding the symptoms and medical history of each patient. Participants were subjected to careful physical examination. PEF values were recorded for all patients and control subjects (highest value from three successful attempts in each case). If the physician suspected asthma on the basis of the results of these examinations, variability of PEF values measured mornings and evenings over a two-week period was recorded. When feasible, spirometry was performed. Sputum was induced within a week of the consultation.

METHODS

Lung function measurements

Spirometry

Various spirometers were used (Studies I and IV Spirotrac III®, Vitalograph, Ltd., Buckingham, UK; study II Expirograph®, Godart, Bilthoven, Netherlands; studies III and V Medikro 905®, Medikro Oy, Kuopio, Finland). At least three forced expiratory volume curves were obtained that were technically correct according to ATS acceptability criteria (less than 5% variability, ATS 1987b) or ERS acceptability criteria (less than 4% variability, Quanjer et al. 1993). Reference values were those of Viljanen (1982) in studies I, III and V, European (Quanjer et al. 1993) in study II, and those of Polgal and Promadhat (1971) in study IV. Reversibility of FEV_1 as determined by means of spirometry was measured in studies I, III and V 15 minutes after inhalation of 200 μg of salbutamol (Ventoline® MDI with Volumatic or Buventol Easyhaler®).

Diffusing capacity

Single breath carbon monoxide diffusing capacity (DLCO/VA) was measured (Masterscreen PFT®, Erich Jaeger BmbH, Hoechberg, Germany) as described (ATS 1987c) in study II.

Histamine challenge

Airway responsiveness to histamine, expressed as the histamine dose (mg) resulting in a 15% decrease in FEV_1 (PD_{15} FEV_1) or a 20% decrease in PEF (PD_{20}PEF), was determined using an automated, inhalation-synchronised, dosimetric jet nebulizer (Spira Electro 2®, Respiratory Care Centre, Hämeenlinna, Finland) as previously described (Sovijärvi et al. 1993, Pelkonen et al. 1997). The maximum dose inhaled histamine was 1.6 mg. PD_{15} FEV_1 >1.6 mg of histamine is considered normal for adults and PD_{20} PEF >1.6 mg of histamine for children.

Peak expiratory flow (PEF)

PEF values were measured at home every morning and evening by means of a Mini Wright peak-flow meter (Clement Clarke Int., London, UK) over a two-week follow-up period (studies I, III to V). Three measurements were made and the highest value was recorded. Variation in PEF was calculated by the investigator using the formula 100 x (PEF evening - PEF morning) / average PEF evening and morning.

Home recordings

In study IV, lung function was recorded at home using a Vitalograph Data Storage Spirometer (Vitalograph) specially designed for long-term recording and storage of lung-function parameters, as previously described (Pelkonen et al. 1997 and 2000). The device consists of a pneumotachograph with a built-in electronic diary. Lung function parameters, including FVC (forced vital capacity), FEV_1, PEF and PIF_{TBH} were monitored twice daily. Measurement was repeated up to five times, until the two best sets of values met ATS (1995) reproducibility criteria. Children were asked to record symptom scores twice daily (scales 0 to 10) and use of rescue medication (Bricanyl® Turbuhaler 0.25 mg/dose). The median PIF_{TBH} during the last run-in week had to be at least 40 l/min as measured and recorded using a Vitalograph Data Storage Spirometer and judged by the investigator.

Skin prick tests

Skin prick tests were made using 10 common inhalant allergens (Soluprick SQ, 10 HEP, ALK, Denmark), and positive (histamine dihydrochloride, 10 mg/ml) and negative (solvent) control solutions. The allergens used were birch, timothy, meadow fescue, and mugwort pollen; horse, cat, dog and cow danders; the mite *Dermatophagoides pteronyssimus*; and spores of the mould *Cladosporium herbarum*. A subject was classified as at-

opic if any allergen caused a weal 3 mm or more in diameter and the control solutions produced the expected results (Dreborg 1989).

Sputum induction

Various ultrasonic nebulizers and different concentrations of saline were used for sputum induction (Table 4). Before induction, subjects were asked to blow their noses and rinse their mouths. Pre-treatment with a bronchodilator was used in four of the five studies (Table 4). Lung function was monitored by means of FEV_1 or PEF before and after induction in all studies except study I.

In study I, 10 minutes after bronchodilator pre-treatment, spirometry was undertaken. If FEV_1 after bronchodilator administration was less than 1 l, normal (0.9%) saline was used, and FEV_1 was checked every three minutes. If FEV_1 after bronchodilator administration was more than 1 l, induction was started with 3% hypertonic saline, and FEV_1 was measured every seven minutes. Inhalation was discontinued if lung function fell by more than 20% or if troublesome symptoms occurred.

Patients were urged to interrupt inhalation repeatedly to cough sputum into a sterile plastic cup. To minimise salivary contamination, saliva was collected in a separate cup in study IV (Gershman et al. 1996). Inhalation times in studies III to V were 15 to 20 minutes. In studies I and II they varied from two to 21 minutes.

Sputum processing

Various sputum-processing methods were used (Table 5). The methods were modified from those described by Pin et al. (1992a) and Fahy et al. (1993a) and validated by Pizzichini et al. (1996a) and in´t Veen et al. (1996).

Studies I and III

The method of sputum examination described by Pizzichini et al. (1996) was used. Briefly, all sputum macroscopically free of salivary contamination was selected and treated with dithiothreitol (Sputolysin®, Boehringer-Calbiochem Corp., LaJolla, Ca, US, diluted 10-fold in distilled water). The mixture was incubated for 15 minutes on a roller mixer at room temperature. An equal volume of phosphate-buffered saline (PBS) was added and incubation was continued for a further five minutes. The mixture was filtered using a 52-µm mesh nylon filter (Nybolt PA-53/35, Seidengaze, Germany). Sputum cells were separated by centrifugation at 800 g for 10 minutes and sputum supernatant was collected for determinations of fluid-phase markers. Cells obtained after centrifugation were resuspended in 1 ml of PBS and total cell numbers were determined using a haemocytometer. Viability was measured using the trypan-blue (Sigma Chemical, St.Louis, MO, US) exclusion test. The cell suspension was cytocentrifuged (Cytospin 3®, Shandon, Astmoor, UK) on to Vectabond-treated (Vectabond® Reagent, Vector Laboratories Inc., CA, US) microscope slides at 300 rpm for five minutes. Slides were air-dried for at least 30 minutes. One slide was used for differential staining by means of the May-Grünwald-Giemsa (MGG) method. Two slides were fixed using Carnoy's fixative and stained using toluidine blue (Fluka AG, Buchs, Switzerland) for detection of metachromatic cells (basophils, mast cells). At least 400 nonsquamous cells were counted on MGG-stained slides, 1500 on toluidine blue-stained slides, using a standard light microscope (Axioskop 20, Carl Zeiss, Heidenheim, Germany).

Table 4. Sputum induction methods, safety and success of induction in different studies.

Nebulizer type	Diagnosis (number of subjects)	Output mean (ml/min)	Particle size mean (µm)	Pre-treatment	Saline conc (%)	Induction time mean (min)	Monitored lung function	Change in lung function mean, range (%)	Success rate (%)
Study I									
Ultra-neb 2000® (DeVilbiss Health Care Inc., Somerset, PA, US)	COPD (28)	2.5	4.5	200 µg salbutamol	0.9,3,4,5	8	FEV_1	-8.5 (-23-11)	96
Study II									
Ultra-neb 2000®	COPD (21) Healthy controls (16)	2.5	4.5	400 µg salbutamol	3,4,5	10	FEV_1	7.1 (-24-24)	100 100
Study III									
Spira Ultra®	Respiratory symptoms (36)	0.6	2	none	5	15	PEF	-4.9 (-17-2.2)	94
(Respiratory Care Centre,	Asthma (25)							-5.1 (-12-2.8)	84
Hämeenlinna, Finland)	Healthy controls (43)							-1.0 (-8-12)	86
Study IV									
Spira Ultra®	Asthma (60)	0.6	2	0.5 mg terbutaline	5	15	PEF	3.8 (-22-38)	77
	Healthy controls (17)							nd	100
Study V									
Omron U1®	Prolonged cough (82)	0.25	7	200 µg salbutamol	3	15	PEF	-1.1 (-13-32)	90
(Omron, Tokyo, Japan)	Healthy controls (53)							-3.0 (-17-19)	92

nd=not determined

Table 5. Sputum processing

Study I	Study II	Study III	Study IV	Study V
Plugs selected	Plugs selected	Plugs selected	Whole sample	Plugs selected, smears made frozen and thawed
↓	↓	↓	↓	↓
Four times volume of 10% DTT	1 ml 10% DTT	Four times volume of 10% DTT	Four times volume of 10% DTT	Four times volume of 10% DTT
↓	↓	↓	↓	↓
15 minutes of incubation	Five minutes of incubation vortex	15 minutes of incubation	15 minutes of incubation	15 minutes of incubation
↓	↓	↓	↓	↓
Four times volume of PBS	Aspiration through 21G needle vortex	Four times volume of PBS	Four times volume of PBS	Five times volume of CTAB
↓	↓	↓	↓	↓
Five minutes of incubation		Five minutes of incubation	Five minutes of incubation	One hour of incubation
↓	↓	↓	↓	↓
Filtration through 52 µm gauze	Filtration through 52 µm gauze	Filtration through 52 µm gauze	Filtration through 52 µm gauze	
↓	↓	↓	↓	↓
Centrifugation 800 g, 10 minutes	Centrifugation 450 g, 10 minutes	Centrifugation 800 g, 10 minutes	Centrifugation 800 g, 10 minutes	Centrifugation 800 g, 10 minutes
↓	↓	↓	↓	↓
Aspiration of supernatant	Aspiration of supernatant	Aspiration of supernatant	Aspiration of supernatant	Aspiration of supernatant
↓	↓	↓	↓	
Resuspension in PBS	Resuspension in PBS	Resuspension in PBS	Resuspension in PBS	
↓	↓	↓	↓	
Preparation of cytospins 300 rpm, five minutes	Preparation of cytospins 450 rpm, six minutes	Preparation of cytospins 300 rpm, five minutes	Preparation of cytospins 300 rpm, five minutes	

DTT = dithiotreitol (Sputolysin®, CalbioChem, La Jolla, CA, US), CTAB = cetyl-N,N,N-trimethylammonium bromide

Study II

Sputum was analysed as described by Pin et al. (1992a), with slight modifications. Sputum plugs (total weights 70 to 200 mg) were transferred into an Eppendorf tube and 1 ml of a 10% solution of the mucolytic agent dithiothreitol (Sputolysin®) was added. The sample was rapidly mixed for two minutes, aspirated and expelled through a 21 G needle five times, and mixed for a further 30 seconds. The cell suspension was filtered through a 52-µm gauze and its weight was recorded. The sample was spun for 10 minutes at 450 g at 25°C. The cell pellet was resuspended in 0.01 PBS, pH 7.2, containing 2% of bovine serum albumin (Behringwerke AG, Marburg, Germany) and the cells were counted by means of a haemocytometer. Viability was determined by means of trypan blue exclusion.

Cytocentrifuge preparations were made by adding 75-µl portions of a cell suspension (0.75×10^6cells/ml) to Shandon II cytocentrifuge cups and centrifuging for six minutes at 450 rpm. Two slides were stained with Wright-Giemsa stain (BDH, Poole, UK) and examined by light microscopy to allow differential counting. Slides were coded and counted blind by two investigators. Four hundred nucleated cells were counted. To identify metachromatic cells two slides were stained with toluidine blue and 600 nucleated cells

per slide were counted. The remaining slides were kept at -80°C for further immunocytochemical staining.

Immunocytochemical analysis

Cell surface markers were demonstrated by immunocytochemical staining with the monoclonal mouse antibodies CD3 (all T cells), CD4 (helper T cells), CD8 (suppresser T cells), CD 20 (all B cells), CD25 (activated T cells), CD 45 (all leukocytes), CD 68 (macrophages), and Ber-Mac3 (activated macrophages) (all from Dako, Dakopatts, Copenhagen, Denmark) using the alkaline phosphatase anti-alkaline phosphatase (APAAP) method in accordance with the suppliers' instructions (Cordell et al. 1984). Briefly, slides bearing frozen cells were thawed and fixed with 4% paraformaldehyde, pH 7.2, for five minutes at room temperature. After fixation, preparations were rinsed with 0.05 M TRIS-buffered saline, pH 7.35, (TBS) and incubated with primary antibody diluted in TBS (1:100 for all antibodies expect 1:50 for CD20) in a moist chamber at room temperature for 30 minutes. Secondary rabbit anti-mouse antibody and mouse APAAP complex (both from Dako) diluted 1/25 in TBS were applied to slides for 30 minutes in each case. Additional incubations of secondary antibody and APAAP-complex were then undertaken for 10 minutes. Washing between steps was done with TBS. Endogenous alkaline phosphatase activity was blocked with levamisole and the signal was developed with Fast Red Violet substrate for 20 minutes, giving an intense red coloration. The substrate was prepared from 0.2 M TRIS/HCL-buffer, pH 8.5, 25 ml; distilled water 15 ml; levamisole 15 mg; naphthol AS-BI phosphate 5 mg; dimethylformamide 0.5 ml, and Fast Red Violet 40 mg (all from Sigma Chemical, St. Louis, MO, US). Mayer's haematoxylin (Sigma Chemical) was used for counterstaining, and the slides were mounted with a water-based mounting medium (Glycergel, Dako). Four hundred nucleated cells were counted using bright-field microscopy. Positive cells were expressed as percentages of intact, round, nucleated cells, ignoring squamous epithelial cells. Mouse IgG1 (Dako) was used as a negative control in all of the experiments. Cytocentrifuge preparations made from peripheral blood leukocytes separated using a Ficoll-Paque (Pharmacia, New Jersey, US) gradient were used as positive controls. For anti-CD25 staining positive controls, leukocytes were stimulated with phytohaemagglutinin in culture for 48 hours.

Study IV

We used a sputum processing method modified from the one described by Fahy and colleagues (Fahy et al. 1993) and validated by in´t Veen et al. (1996). Briefly, an entire sputum sample was weighted and treated with an equal volume of dithiothreitol (Sputolysin 10%, Calbiochem Corp.) and PBS. The samples were gently mixed and incubated in a shaker at room temperature. The resulting suspension was centrifuged at 800 g for 10 minutes, and the supernatant was aspirated and stored in Eppendorf tubes at -20^{0}C for later assay. The cell pellet was resuspended, and viabilities and absolute numbers of cells per milligram of sputum processed were calculated by means of trypan-blue exclusion, using a haemocytometer. Coded cytospins were prepared and stained using MGG stain and toluidine blue, to obtain differential cell counts. Four hundred nonsquamous cells were counted on the MGG-stained slides, 1500 cells on toluidine-blue-stained slides. A sputum sample was considered adequate if it had less than 80% of squamous epithelial cell contamination from saliva (in´t Veen et al. 1996).

Study V

Sputum handling in primary health care

Sputum samples were transferred to Petri dishes and examined against a dark surface. The more viscous parts were collected, using forceps, and mixed. Some of each sample was used to make two smears, which were air-dried. The rest was transferred into a pre-weighed tube and frozen at –20 °C.

Sputum analysis in the laboratory

Frozen sputum samples and air-dried slides were sent to the Skin and Allergy Hospital, where further analyses were undertaken. Samples were processed using the method previously described and validated (Metso et al. 2001). Briefly, each sample was thawed and treated with a DTT (diluted 10-fold with distilled water) and a detergent (0.5% cetyl-N,N,N-trimethylammonium bromide, 0.4% human serum albumin, 100 mM PBS, pH 7.2). After incubation for one hour cells were lysed, and markers inside and outside the cells released and solubilized. Sputum supernatant was separated from cell debris by centrifugation and frozen at -20^{0}C for later assay.

Air-dried slides were stained using eosin and methylene blue. Cell proportions were assessed semi-quantitatively, on a scale from 0 to 4, modified from previously described scales (O´Connell et al. 1978). Eosinophils were graded as 0 = none or occasional, 1 = scanty, 2 = moderate, 3 = numerous, or 4 = predominant. The approximate values of the grades were 0 = eosinophils fewer than 1% of all non-squamous cells, 1 = eosinophils one to 5% of all non-squamous cells, 2 = eosinophils five to 10% of all non-squamous cells, 3 = eosinophils 10 to 50% of all non-squamous cells, and 4 = eosinophils more than 50% of all non-squamous cells. A sample was considered adequate and originating from the lower airways if it contained macrophages and fewer than 50% of squamous epithelial cells.

Fluid-phase measurements

Concentrations (μg/l) in thawed serum and sputum supernatants of two markers of eosinophil activation, eosinophil cationic protein (ECP) and eosinophil peroxidase (EPO), and of two markers of neutrophil activation, myeloperoxidase (MPO) and human neutrophil lipocalin (HNL), were measured. ECP and MPO concentrations were determined using commercially available immunoassay kits (Pharmacia). EPO and HNL concentrations were determined using prototype kits (Pharmacia CAP System FEIA, Pharmacia) as previously described (Helenius et al. 1998). All analyses were blind in relation to clinical characteristics of subjects.

Statistical analyses

Data are expressed as means plus or minus the standard error of the mean (SEM), medians or ranges. In the case of bronchial responsiveness following histamine challenge, geometric means were calculated. Significances of differences between several groups were assessed using Kruskal-Wallis one-way analysis by ranks. Significances of differences between two groups were assessed using the Mann-Whitney U-test or the χ^2-test, as ap-

propriate. Wilcoxon´s test relating to paired data was used in determining significances of differences for follow-up data relating to a given patient. Significances of correlations were assessed using Spearman´s test. Two-tailed p values below 0.05 were considered to indicate significance.

The reference (normal) range for sputum eosinophils in study III was calculated as three standard deviations of mean values for the healthy control group. The control group was used to establish upper limits of normal ranges (90^{th} percentiles) for sputum ECP, EPO, MPO and HNL values in study V and for numbers of eosinophils in blood and sputum in study IV.

In study IV, sensitivities and specificities of results of determinations of numbers of eosinophils in blood and sputum in relation to diagnoses of asthma were assessed, using cut-off values relating to the healthy control group. However, since the cut-off values were selected arbitrarily (Pizzichini et al. 1997b), the reliability of determinations of numbers of blood and sputum eosinophils was assessed by preparing a receiver-operating-characteristic (ROC) curve for each test (Hanley and McNeil 1982). Areas under curves (AUCs) were compared using the method of Hanley and McNeil (1983). AUCs closest to 1.0 indicate greatest reliability of diagnosis.

The reproducibility of sputum cell counts was examined by means of repeated-measures analysis of variance with calculation of an intraclass correlation coefficient (the ratio of the variance of cell counts between subjects to the total variance in cell counts, including observer and error variance) (Kramer and Feinstein 1981). Values above 0.75 indicate high reliability. Intrapatient variability at various time points was also assessed, using a coefficient of reproducibility representing limits within which 95% of the differences are expected to be found (Bland and Altman 1986).

RESULTS

CLINICAL CHARACTERISTICS OF SUBJECTS

Baseline clinical characteristics of subjects are shown in Table 3. Most patients with COPD were male. There were more women than men in the other groups of adults. COPD patients were older than other adult subjects ($p < 0.0001$). Subjects were regarded as atopic if at least one result of skin-prick testing was positive (see Methods). Numbers and percentages of atopic subjects are shown in Table 3.

Reversibility of lung-function measurements and airway responsiveness

Results of lung-function measurements at baseline from all studies are shown in Table 3. Results of reversibility testing, PEF follow-up and histamine challenge in studies I, III and IV are shown in Table 6.

In study II, mean diffusion capacity was 62 ± 3.4% of that predicted. In study V in primary care, 13 of 82 patients (16%) were diagnosed as suffering from asthma on the basis that PEF values varied by more than 20% between morning and evening on three days during the two-week follow-up period, or that reversibility of FEV_1 exceeded 15%.

In the asthmatic children in study IV, PD_{20}PEF correlated with baseline FEV_1 ($r = 0.43$, $p = 0.001$), morning PEF (% of predicted) ($r = 0.39$, $p = 0.01$), and evening PEF (% of predicted) ($r = 0.40$, $p = 0.01$). Atopic asthmatic children were more responsive to histamine than non-atopic asthmatic children (PD_{20}PEF 0.84 ± 0.10 versus 1.21 ± 0.10 mg, $p = 0.009$). These two subgroups of asthmatics did not differ from each other in respect of other lung function measurements.

Table 6. Bronchodilator responses, PEF-variability and airway responsiveness at baseline in studies I, III and IV

Studies and Diagnoses	Reversibility of FEV_1 (%)	Variability of PEF (%)	Airway responsiveness mg*
Study I			
COPD (n = 28)	2.5 (-7.4 - 9.9)	8.9 (1.9 - 39)	nd
Study III			
Respiratory symptoms (n = 36)	2.5 (0 - 6.0)	5.2 (0 - 11)	> 1.6
Asthma (n = 25)	11.2 (11 - 60)	21.1 (11 - 60)	0.40 (0.02 - 1.25)
Healthy controls (n = 43)	nd	nd	> 1.6
Study IV			
Asthma (n = 60)	nd	10.4 (3.1 - 25)	0.92 (0.02 - 1.6)
Healthy controls (n = 17)	nd	0.3 (0 - 1)	1.50 (0.41 - 1.6)

Data expressed as means and ranges, nd = not determined

* In study III PD_{15} is the dose of histamine provoking a 15% fall in FEV_1. In study IV PD_{20}, is the dose of histamine provoking a 20% fall in PEF (Sovijärvi et al. 1993)

Symptoms

Different questionnaires and scales relating to symptoms were used in each study. Results are therefore not comparable between studies. In study III, patients with asthma had higher total symptom scores ($p = 0.005$) than patients with respiratory symptoms suggestive of asthma. They also had higher individual scores relating to wheezing ($p < 0.0001$), shortness of breath ($p = 0.006$), and exercise symptoms ($p = 0.0003$). Duration of symptoms was similar in both groups (mean nine months).

In study IV, at baseline 23 asthmatic children (38%) complained only of cough. Thirty-seven (62%) had experienced both coughing and wheezing. Children who wheezed were significantly more hyperresponsive to histamine than children who did not (PD_{20}PEF of 1.23 ± 0.10 versus 0.84 ± 0.01 mg, $p = 0.009$). Children who wheezed also had more sputum eosinophils than those with just cough (7.8 ± 2.0 versus 1.2 ± 0.5 %, $p = 0.02$). The mean duration of symptoms for all asthmatic children was 10 months (range one to 72 months). The three treatment groups in study IV did not differ from each other at baseline.

In study V, the mean duration of cough in primary health care patients was 11 months (range one to 96 months). It was 15 months for asthmatics, and 10 months for other patients. Forty patients (49%) reported marked sputum production, 38 (46%) wheezing or cough on exercise, 30 (37%) disturbed sleep, 28 (34%) shortness of breath, and 23 (28%) chest tightness with wheezing. Asthmatics more often experienced sputum production ($p = 0.03$, χ^2-test), and chest tightness with wheezing ($p = 0.04$) than patients with prolonged cough without asthma.

Measurements relating to blood and serum

In study III, asthma patients had significantly higher blood eosinophil counts and higher concentrations of serum EPO than patients with respiratory symptoms ($p = 0.002$, $p = 0.008$), and healthy individuals ($p < 0.0001$ in both cases) (Table 7). Patients with respiratory symptoms also had higher blood eosinophil counts and higher concentrations of serum EPO than healthy individuals ($p = 0.01$, $p = 0.02$, respectively). Atopic asthmatics had higher blood eosinophil counts than non-atopic asthmatics (0.51 versus 0.27 x 10^9/l, $p = 0.04$). In study V, serum ECP was highest (mean 12.0 mmg/l, range 4-26 mmg/l) in patients with chronic cough who had been diagnosed as suffering from asthma.

Table 7. Measurements relating to blood and serum.

Studies and Diagnoses	Blood eosinophil counts (x 10^9/l)	Serum ECP (µg/l)	Serum EPO (µg/l)	Serum MPO (µg/l)	Serum HNL (µg/l)
Study III					
Respiratory symptoms (n = 36)	0.17 (0 - 0.61)	11.8 (3.5 - 39)	13.8 (1.3 - 50)	254 (167 - 555)	112 (44 - 293)
Asthma (n = 25)	0.41 (0.66 - 1.0)	15.2 (3.3 - 50)	30.0 (2.3 - 111)	251 (155 - 535)	104 (54 - 171)
Healthy controls (n = 43)	0.11 (0.02 - 0.27)	8.1 (1.0 - 18)	10.6 (1.2 - 81)	200 (145 - 283)	94.5 (56 - 139)
p-value	*< 0.0001*	*= 0.003*	*< 0.0001*	*ns*	*ns*
Study IV		nd	nd	nd	nd
Asthma (n = 60)	0.51 (0.02 - 3.54)				
Healthy controls (n = 17)	0.17 (0.03 - 0.36)				
p-value	*=0.0028*				
Study V					
Prolonged cough (n = 82)	nd	10.5 (2-71)	nd	nd	nd
Healthy controls (n = 53)		6.7 (2-17)			
p-value		*= 0.01*			

Data are expressed as means and ranges. P-values relate to between-group comparisons in each study (Kruskall-Wallis), ns = not significant, nd = not determined

INDUCED SPUTUM

Safety of sputum induction

Sputum induction procedure was well tolerated in all studies. One child in study IV reported nausea but otherwise there were no notable side effects. The mean changes in lung function, and ranges, are shown in Table 4.

COPD

Studies I and II related to COPD patients. In both studies, a fairly high-output nebulizer, the DeVilbiss Ultra-neb®, was used. In study II, spirometry was performed before and after induction in 16 of 21 patients. After pre-treatment and induction, there was an increase in FEV_1 (p = 0.007) (Table 4). Study I was specifically designed to study the safety of sputum induction. The mean change during induction from FEV_1 before bronchodilator treatment was -162 ml (-541 to 205 ml), i.e. -8.5% (-23 to 11%) (p = 0.001). The mean change during induction from FEV_1 after bronchodilator treatment was -202 ml (-625 to 95 ml), i.e. -10.7% (-25 to -5%) (p < 0.0001). In three subjects (11%) the fall in FEV_1 from baseline before bronchodilator treatment was more than 20%. In a further 10 (36%) it was between 10 and 20%. All patients in whom the fall in FEV_1 was 20% or more were followed-up carefully and treated with salbutamol by inhalation. A statistically significant inverse relationship existed between reversibility in FEV_1 and fall in FEV_1 during induction (r = -0.4, p = 0.03). Otherwise, no correlation was found between levels of inflammatory markers in sputum, baseline clinical characteristics and changes in FEV_1 during induction. No significant differences were found in relation to any parameter be-

tween female and male subjects, or between patients on inhaled steroids and steroid-naive patients. Three female patients (11%) had FEV_1 values after bronchodilator treatment of less than 1 l (mean 0.87 l, range 0.81 to 0.94 l, on average 36% of predicted values, range 34 to 38%), and sputum was induced in these patients using only 0.9% saline. Mean changes in FEV_1 in these patients were -60 ml (-118 to 53 ml), i.e. -7.2% (-15 to 7%) from baseline before bronchodilator treatment and -120 ml (-188 to 17 ml), i.e. -13.7% (-20 to 2%) from baseline after bronchodilator treatment.

Asthma

Studies III to V involved patients with asthma (Table 4). Two types of low-output ultrasonic nebulizers were used, the Omron U1® and Spira Ultra®. In study III, there was no pre-treatment. Asthma patients exhibited small but statistically significant ($p = 0.007$) falls in PEF values during induction. In studies IV and V, pre-treatment was undertaken. No significant fall in PEF values was noted. In study IV, there were weak correlations between percentage change in PEF during sputum induction and mean PEF (% of predicted) as recorded by patients at home ($r = -0.32$, $p = 0.03$) and variability between morning and evening PEF values ($r = 0.35$, $p = 0.01$).

Respiratory symptoms suggestive of asthma, chronic cough, and healthy controls

Study III involved patients with respiratory symptoms suggestive of asthma. The low-output nebulizer Spira Ultra® was used, with no pre-treatment. Like the patients with asthma proper, patients with respiratory symptoms suggestive of asthma exhibited a small but statistically significant fall in PEF during induction ($p = 0.005$). In study V, patients with chronic cough exhibited no fall in PEF values (Table 4). Healthy controls were included in all but study II. All three types of ultrasonic nebulizer were used. No significant fall in lung function was observed (Table 4).

Success of sputum induction

In studies I, II, III and IV, a sputum sample was considered adequate if squamous epithelial cell contamination was less than 80% on MGG-stained cytospin slides (in‘t Veen et al. 1996). In study V smears were examined. A sample was considered adequate and originating from the lower airways if it contained macrophages and fewer than 50% of squamous epithelial cells. Overall, sputum induction at baseline was successful in 82% of cases. Success rates in individual studies and for different patient groups at baseline are shown in Table 4. In study III, the success rate in patients with respiratory symptoms after three months of treatment was 87%. After one year it was 81%. In study IV, the success rate in relation to five-year old children was 67%. It was 75% for six-year old children, 86% for children from seven to 10 years of age, and 100% for children over 10 years of age. After six months of treatment it was 68%, after 18 months 60%. There was no significant correlation between age and extent of squamous epithelial cell contamination, and there were no significant differences between boys and girls. Success rates did not differ significantly between different types of nebulizer.

Sputum total and differential cell counts at baseline

Various methods of sputum induction and processing of sputum were used, making comparisons between studies difficult. In study II, patients with COPD had higher total cell counts (mean 9.5 10^3/mg, range 1.4 to 84 10^3/mg) than healthy controls ($p < 0.002$), and higher percentages of neutrophils (mean 81.3, range 19 to 24) ($p < 0.001$), and lower percentages of macrophages (mean 17.8, range 6.5 to 65) ($p < 0.001$). The median percentage of eosinophils in COPD patients was 0.25 (range 0 to 33).

Sputum was induced using different types of nebulizer in studies I and III. The methods of sputum processing were, however, similar. Total and differential cell counts in studies I and III are shown in Table 8 and Figure 3. Patients with respiratory symptoms exhibited highest total cell counts ($p = 0.02$ in relation to comparison with asthma patients, $p = 0.0009$ in relation to comparison with COPD patients, and $p = 0.0008$ in relation to comparison with healthy controls). Eosinophil counts were highest in asthma patients ($p = 0.01$ in relation to comparison with respiratory-symptom patients, $p < 0.0001$ in relation to comparison with healthy controls). COPD patients did not differ significantly from asthmatics in relation to sputum eosinophil counts but sputum eosinophil counts were higher than in patients with respiratory symptoms ($p = 0.04$) and healthy controls ($p < 0.0001$). Patients with respiratory symptoms also had higher sputum eosinophil counts than healthy controls ($p < 0.0001$). Sputum neutrophil counts were highest in COPD patients ($p = 0.03$ in relation to comparison with asthma patients, $p = 0.004$ in relation to comparison with respiratory-symptom patients, and $p < 0.0001$ in relation to comparison with healthy controls). Sputum macrophage counts were highest in healthy controls (Table 8). Metachromatic cell counts were highest in asthma ($p = 0.04$ in relation to comparison with respiratory symptoms, $p = 0.01$ in relation to comparison with healthy controls). However, COPD patients also exhibited higher metachromatic cell counts than patients with respiratory symptoms ($p = 0.04$) and healthy controls ($p = 0.0007$). Differential cell counts showed that COPD subjects in study I had higher sputum eosinophil counts ($p = 0.01$) and macrophage counts ($p = 0.0002$) and lower neutrophil counts ($p = 0.01$) than COPD patients in study II.

Total and differential cell counts in sputum at baseline in study IV are shown in Table 8 and Figure 4. Asthmatic children had higher sputum eosinophil counts and sputum metachromatic cell counts than healthy children. Atopic asthmatics had higher sputum eosinophil counts ($p < 0.0001$) than non-atopic asthmatics. No differences were seen between atopic and non-atopic healthy children, or between the three kinds of treatment at baseline. Children who wheezed had significantly higher sputum eosinophil counts than those with just cough (mean 2.8%, range 0 to 39%, versus mean 0.4%, range 0 to 5.6%, $p = 0.02$). Cell viability, as assessed by means of trypan-blue exclusion, always exceeded 70%.

In study V, a semiquantitative scale relating to sputum eosinophil numbers was used (Table 9). The score relating to sputum eosinophils was higher in patients with chronic cough than in healthy subjects ($p = 0.001$). No healthy subject had a sputum-eosinophil score greater than 1 (5% eosinophils). A sample was defined as positive for eosinophils if the score relating to eosinophils was 2, 3 or 4. Using this criterion, 14 patients with prolonged cough (19%) had eosinophil-positive sputum samples. Five of these 14 patients (36%) were diagnosed as having asthma, while nine of the 14 patients (64%) were not.

Table 8. Sputum measurements in the four adult study groups (studies I and III) and in children (study IV).

	ADULTS					CHILDREN		
	COPD (n=27)	Asthma (n=21)	Respiratory symptoms (n=34)	Healthy persons (n=37)	p-value*	Asthma (n=46)	Healthy persons (n=17)	p-value†
Total cells, (10^6)	0.28 (0.04 - 12)	0.40 (0.01 - 3.6)	0.74 (0.1 - 27)	0.29 (0.04 - 4.0)	<0.001	2.18 (0.10 - 40)	1.80 (0.06 - 15)	ns
Eosinophils (%)	1.7 (0 - 11)	2.8 (0 - 90)	0.4 (0 - 21)	0 (0 - 0.9)	<0.0001	1.1 (0 - 39)	0 (0 - 0.4)	0.0001
Neutrophils (%)	60 (38 - 89)	26 (0 - 82)	35 (0 - 90)	28 (1.5 - 86)	0.0005	19 (0 - 87)	26 (12 - 88)	ns
Lymphocytes (%)	0 (0 - 4)	0.3 (0 - 1.9)	0 (0 - 2.9)	0 (0 - 3.6)	ns	0.3 (0 - 2.9)	0 (0 - 1.3)	ns
Macrophages (%)	36 (9.3 - 62)	32 (7 - 99)	55 (10 - 99)	65 (13 - 98)	0.003	74 (11 - 98)	72.3 (12 - 86)	ns
Epithelial cells (%)	0 (0 - 16)	0 (0 - 20)	0 (0 - 10)	0.3 (0 - 47)	0.006	0 (0 - 4)	0.3 (0 - 3.3)	ns
Metacromatic cells (%)	0 (0 - 1)	0 (0 - 4.2)	0 (0 - 0.13)	0 (0 - 0.07)	0.005	0 (0 - 0.6)	0 (0 - 0)	0.04
Squamous cells (%)	5.5 (0 - 35)	4.6 (0 - 70)	9.4 (0 - 70)	6.3 (0 - 67)	ns	22 (0 - 75)	40 (1 - 76)	ns
ECP (µg/l)	382 (36 - 4460)	476 (23 - 5647)	155 (11 - 6419)	73.4 (18 - 1530)	<0.0001	-	-	-
EPO (µg/l)	-	30 (3.2 - 2927)	15 (2.5 - 1004)	4.5 (2.5 - 401)	<0.0001	-	-	-
MPO (µg/l)	9652 (531 - 45540)	72 (72 - 2340)	72 (72 - 1359)	72 (72 - 103)	<0.0001	-	-	-
HNL (mg/l)	-	6.6 (1.2 - 23)	6.4 (0.8 - 76)	4.0 (0.8 - 21)	ns	-	-	-

Data are expressed as medians and range , *p-value between all adult groups, Kruskal-Wallis test, †p-value between the two children groups, Mann-Whitney U- test,
-=not determined, ns=not significant

Figure 3. Sputum eosinophils in the four adult patient groups (studies I and III). Horizontal lines represent mean values ($p < 0.0001$).

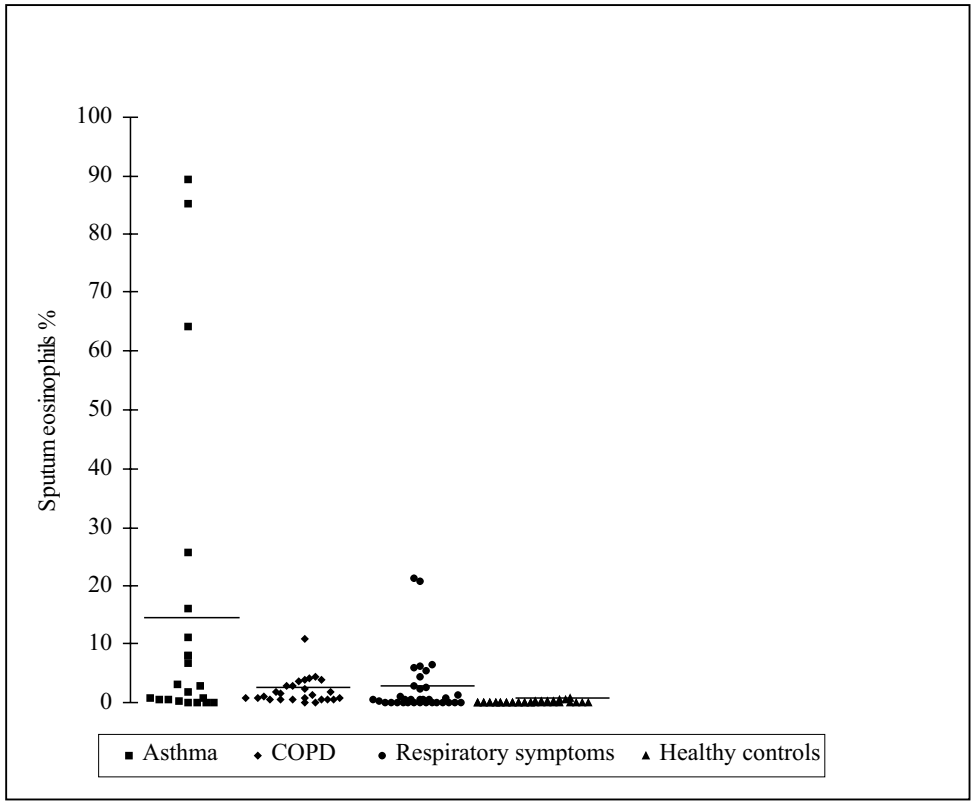


Figure 4. Sputum eosinophils in children (study IV). Horizontal lines represent mean values ($p = 0.0001$).

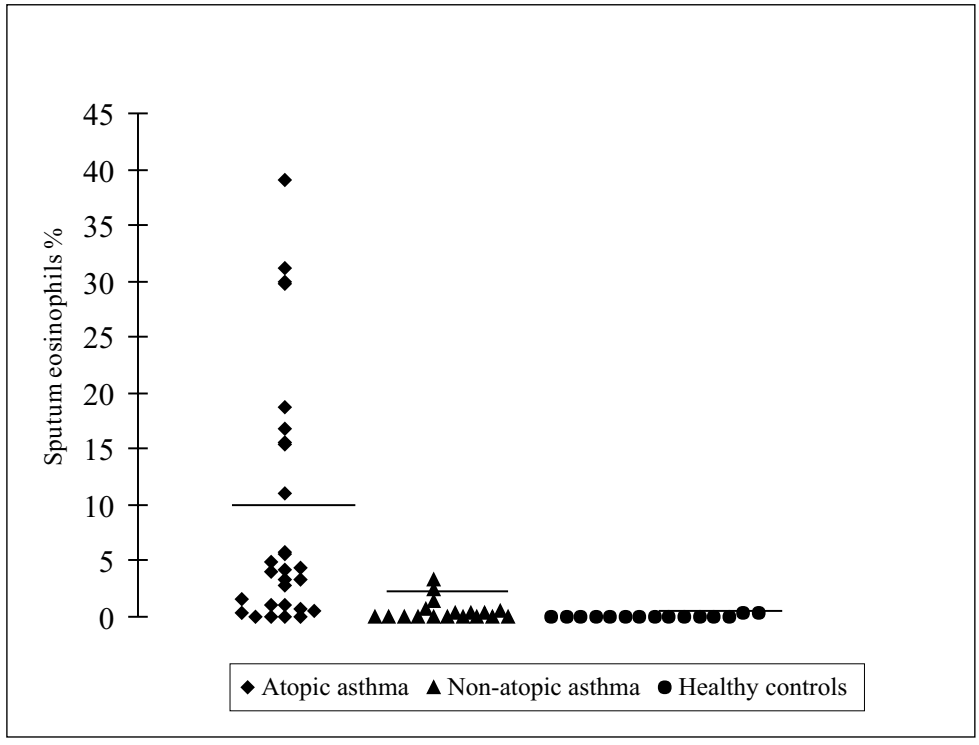


Table 9. Measurements relating to induced sputum in study V

		Prolonged cough (n = 82)	Healthy persons (n = 53)
Sputum			
	Weight, mg	290 (10 - 2980)	110 (3 - 913)
	Eosinophils *		
	0 - 1	59 (81%)	49 (100%)
	2	7 (10%)	0 (0%)
	3	4 (5%)	0 (0%)
	4	3 (4%)	0 (0%)
	ECP (µg/L) †	1015 (44 - 65062)	498 (68 - 2706)
	EPO (µg/L) ‡	297 (55 - 33408)	63 (25 - 6830)
	MPO (µg/L)	93 (80 - 34300)	138 (80 - 1524)
	HNL (mg/L)	7.3 (0.5 - 98)	5.4 (1.1 - 19)

Data are expressed as medians and ranges or numbers and percentages (in the case of eosinophils).
Sputum samples were obtained from 73 patients with chronic cough and 49 healthy subjects.
* Semi-quantitatively, on a scale from 0 to 4, $p = 0.001$ (χ^2-test)
† $p = 0.02$ (Mann-Whitney U-test), ‡ $p = 0.005$ (Mann-Whitney U-test)

Reproducibility of sputum cell counts

Reproducibility of sputum cell counts was examined in studies II and III. In patients with COPD, reproducibility was high for the main types of cells, neutrophils and macrophages, both on a within investigator basis (PR) ($r = 0.99$ for neutrophils, $r = 0.99$ for macrophages) and between investigators (PR and JK) ($r = 0.95$ for neutrophils, $r = 0.77$ for macrophages). Reproducibility was lower for other cell types, both on a within investigator basis ($r = 0.33$ for lymphocytes, $r = 0.30$ for eosinophils) and between investigators ($r = 0.16$ for lymphocytes, $r = 0.16$ for eosinophils). To allow assessment of within-subject variability, differential cell counts in two sputum samples, induced 10 days apart, were compared. Reproducibility was high for major cell types ($r = 0.84$ for neutrophils, $r = 0.67$ for macrophages, $r = 0.90$ for eosinophils), but lower for lymphocytes ($r = 0.30$) and total cell count ($r = 0.13$). In patients with asthma (study III, data not shown in manuscript) within-investigator reproducibility (PR) ($r = 0.93$ for neutrophils, $r = 0.92$ for macrophages, $r = 0.89$ for lymphocytes, $r = 0.98$ for eosinophils, $r = 0.94$ for bronchial epithelial cells, $r = 0.85$ for squamous cells) and between-investigator reproducibility (PR and TM) ($r = 0.86$ for neutrophils, $r = 0.44$ for macrophages, $r = 0.34$ for lymphocytes, $r = 0.81$ for eosinophils, $r = 0.9469$ for bronchial epithelial cells, $r = 0.98$ for squamous cells) were assessed.

Immunocytochemical analysis

In an attempt to characterise further the inflammatory nature of COPD, good quality cytospin preparations from both COPD patients (n = 8) and healthy controls (n = 8) were studied by means of immunocytochemistry. Very few CD3+, CD4+, and CD8+ and no CD20+ or CD25+ cells were found (Table 10). The percentage of CD45+ cells was significantly higher in COPD patients (Table 10) than in healthy subjects. In both groups, most macrophages (stained for CD68) were activated, as indicated by positive staining with Ber-Mac3.

Table 10. Cell-surface markers in induced sputum in study II.

Parameter	COPD patients	Healthy subjects
CD3 (%)	1.8 ± 0.7	2.1 ± 0.5
CD4 (%)	0.1 ± 0.1	2.1 ± 0.2
CD8 (%)	0.3 ± 0.1	0.4 ± 0.2
CD45 (%)*	80.5 ± 5.5	52.6 ± 6.1
CD68(%)*	17.9 ± 4.8	71.2 ± 4.5
Ber-Mac3 (%)	10.1 ± 2.2	47.4 ± 7.4
Ber-Mac3/CD68	56	66

Values are expressed as means ± SEMs, n = 8 for both groups, calculated as percentages of non-squamous cells. Percentages of activated macrophages are expressed in relation to total numbers of macrophages. * $p < 0.05$ Mann-Whitney U-test

Sputum fluid-phase measurements

Results of fluid-phase measurements in studies I and III are shown in Table 8. ECP values were highest in asthma patients ($p = 0.002$ in comparison with healthy controls). COPD patients also had high ECP values ($p < 0.0001$ in comparison with healthy controls). Both asthmatics and patients with respiratory symptoms had higher sputum EPO levels than healthy controls ($p < 0.0001$) but they did not differ significantly from each other. MPO values were highest in COPD patients ($p < 0.0001$ in comparison with all other groups).

In study V, sputum EPO and ECP levels were higher in patients with prolonged cough than in healthy subjects (Table 9). Values for the sputum neutrophil markers MPO and HNL were not significantly different between patients and controls. There were no significant differences in levels of inflammatory markers between smokers and non-smokers or atopic and non-atopic subjects in any group. If smokers were excluded from the analysis differences were essentially unaffected.

Normal values for inflammatory markers: sensitivity and specificity

In study III, the normal upper limit (mean + 3 SD) for percentage of sputum eosinophils (0.7) was calculated on the basis of values in sputum from healthy subjects. Fourteen asthma patients (67%) who produced samples for sputum analysis exhibited high percentages of sputum eosinophils, as opposed to 13 patients with respiratory symptoms (38%) and one healthy subject (3%). Receiver operating characteristic curves for percentages of eosinophils, and ECP and EPO levels in sputum and blood were examined. Areas under curves relating to each test revealed that percentage of sputum eosinophils (AUC 0.88) was the most sensitive and specific marker in the case of asthmatics. AUCs for other markers were Ex-EPO 0.87, B-eos 0.84, S-EPO 0.83, S-ECP 0.76, and Ex-ECP 0.75. In patients with respiratory symptoms the best marker was sputum EPO level (AUC 0.80). AUCs for other markers were Ex-eos 0.67, S-EPO 0.67, B-eos 0.66, ex-ECP 0.61, and S-ECP 0.58. None of the differences were statistically significant, however.

In study IV, the upper reference limit in healthy subjects for blood eosinophils was 0.35×10^9/l, for the percentage of eosinophils in sputum 0.31. On the basis of these values the sensitivity of determination of blood eosinophils for diagnosis of asthma was 42% and the specificity was 93%. The corresponding figures relating to determination of sputum eosinophils were 70% and 88% (Table 11). ROC curves relating to blood and sputum eosinophil determinations were examined. AUCs relating to sputum eosinophil determination (0.77) and blood eosinophil determinations (0.76) were similar.

In the healthy controls in study V, the upper reference limit for ECP in serum was 16.5 μg/l. For ECP in sputum it was 1987 μg/l, for EPO in sputum 3899 μg/l, for MPO in sputum 1012 μg/L, and for HNL in sputum 14286 μg/L. Patients with respiratory symptoms more often had high serum ECP levels, high numbers of sputum eosinophils, and high sputum ECP, MPO and HNL levels.

Table 11. Numbers and percentages of asthmatic children in study IV at baseline with results of blood and sputum eosinophil determinations above reference values (results of both determinations were available for 43 patients).

		Result of blood eosinophil determination	
		Positive	Negative
Sputum eosinophils	Positive	13 (30%)	16 (37%)
	Negative	3 (7%)	11 (26%)

Correlation between results

Correlation between clinical data and inflammatory markers

In study II, in patients with COPD, percentages of sputum neutrophils correlated inversely with FEV_1 ($r = -0.48$, $p < 0.05$). No correlation of this kind was found in COPD patients in study I. In study III, there was a correlation between symptom scores relating to wheezing and numbers of blood eosinophils ($r = 0.50$, $p = 0.002$) and sputum eosinophils ($r = 0.54$, $p < 0.0001$). In smokers in study V, numbers of pack-years correlated weakly with serum ECP levels ($r = 0.2$, $p = 0.05$). In study IV, numbers of blood eosinophils ($r = -0.41$, $p = 0.002$) and numbers of sputum eosinophils ($r = -0.53$, $p = 0.0002$) correlated with airway responsiveness. The correlation between PEF (mean of morning and evening values at baseline, % of predicted) and numbers of blood eosinophils was on the margin of significance ($r = -0.29$, $p = 0.05$). When only data relating to atopic asthmatics were analysed the correlation was significant ($r = -0.44$, $p = 0.02$). Numbers of blood eosinophils correlated with mean symptom score ($r = 0.28$, $p = 0.04$).

Correlation between results relating to inflammatory markers

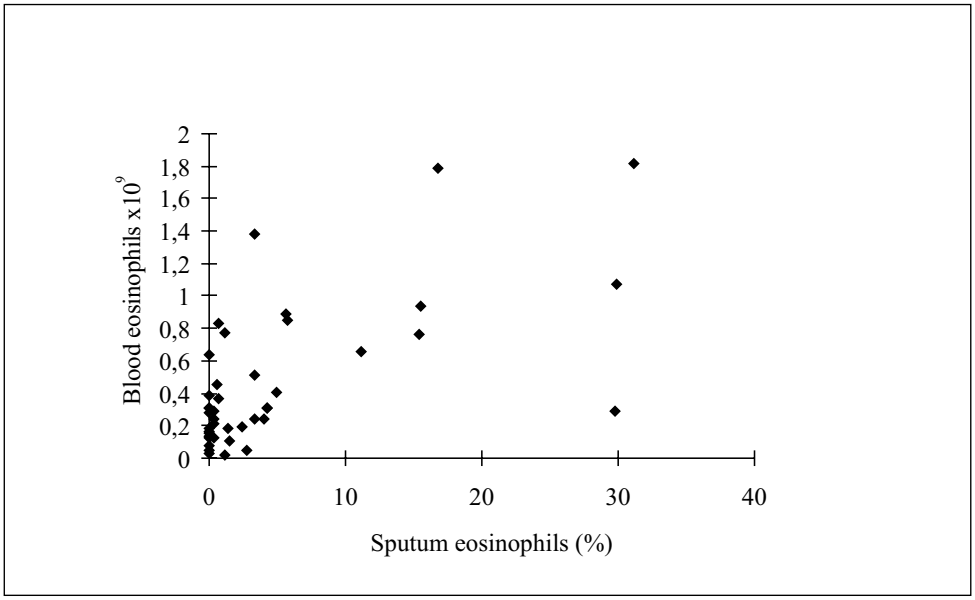
Correlations between results relating to inflammatory markers in studies I and III are shown in Table 12. In study IV, there was a significant correlation between numbers of blood eosinophils and sputum eosinophil percentages (Fig. 5) in relation to atopic asthmatics ($r = 0.65$, $p = 0.0003$) but not in relation to non-atopic asthmatics ($r = 0.22$, $p = 0.39$). Numbers of sputum eosinophils correlated with numbers of sputum metachromatic cells ($r = 0.55$, $p < 0.0001$), and numbers of sputum lymphocytes ($r = 0.37$, $p = 0.01$). In study V, semiquantitative scores relating to sputum eosinophils on smears correlated with sputum ECP levels ($r = 0.33$, $p = 0.008$) and sputum EPO levels ($r = 0.39$, $p = 0.02$). Serum ECP levels correlated with sputum ECP levels ($r = 0.33$, $p = 0.0004$).

Table 12. Correlations between different inflammatory markers in studies I and III.

	S-ECP	S-EPO	Ex-eos	Ex-ECP	Ex-EPO	Ex-neutro	Ex-MPO	Ex-HNL
B-eos	C: - A: r=0.45, p=0.03 R: ns H: r=0.40, p=0.01 ***ALL:*** ***r=0.49, p<0.0001***	C: - A: r=0.84, p<0.001 R: r=0.50, p=0.005 H: r=0.64, p<0.0001 ***ALL:*** ***r=0.74, p<0.0001***	C: - A: r=0.52, p=0.02 R: r=0.56, p=0.0008 H:ns ***ALL:*** ***r=0.59, p<0.0001***	ns	ns	ns	ns	ns
S-ECP		A: r=0.63, p=0.002 R: r=0.61, p=0.004 H: r=0.69, p<0.0001 ***ALL:*** ***r=0.89, p<0.0001***	C: - A: ns R: ns H: ns ***ALL:*** ***r=0.30, p=0.007***	C: -	ns	ns	ns	ns ns
S-EPO			A: r=0.53, p=0.02 R: ns H: ns ***ALL:*** ***r=0.45, p<0.0001***	C: -	ns A: ns R: ns H: ns ***ALL:*** ***r=0.35, p=0.001***	C: -	ns	ns ns
Ex-eos				C:ns A: r=0.87, p<0.0001 R: ns H: ns ***ALL:*** ***r=0.43, p<0.0001***	C:ns A: r=0.57, p=0.01 R: ns H: ns ***ALL:*** ***r=0.50, p<0.0001***	ns	ns	ns
Ex-ECP					C: - A: r=0.63, p=0.002 R: r=0.70, p<0.0001 H: ns ***ALL:*** ***r=0.63, p<0.0001***	C: r=0.43, p=0.03 A: ns R: r=0.51, p=0.002 H: r=0.53, p=0.001 ***ALL:*** ***r=0.48, p<0.0001***	C: r=0.82, p<0.0001 A: ns R: r=0.42, p=0.02 H: ns ***ALL:*** ***r=0.61, p<0.0001***	C: - A: ns R: r=0.84, p<0.0001 H: r=0.53, p=0.001 ***ALL:*** ***r=0.61, p<0.0001***
Ex-EPO							ns A: ns R: ns H: ns ***ALL:*** ***r=0.528, p=0.0003***	C: - C: - A: ns R: ns H: ns ***ALL:*** ***r=0.28, p=0.01***
Ex-neutro							C: r=0.43, p=0.03 R: ns H: ns ***ALL:*** ***r=0.47, p<0.0001***	C: - A: ns A: ns R: r=0.62, p=0.0006 H: r=0.60, p=0.0002 ***ALL:*** ***r=0.54, p<0.0001***
Ex-MPO								C: - A: ns R: ns H: ns ***ALL:*** ***r=0.29, p=0.05***

C = COPD, A = asthma, R = patients with respiratory symptoms, H = healthy controls, r = Spearman rank correlation, - = not determined, ns= not significant

Fig. 5. Correlation between numbers of blood eosinophils and numbers of sputum eosinophils in asthmatic children at baseline (study IV), $r = 0.56$, $p < 0.0001$.



TREATMENT EFFECT

Study III : Follow-up in patients with respiratory symptoms

Of the 36 patients with respiratory symptoms, 31 (86%) were re-examined at three months and one year. Four of the five patients unavailable for re-examination had received placebo.

At three months, cough and total symptom scores, and numbers of blood eosinophils in the BDP-treated group had decreased significantly from baseline (Fig. 6). In the placebo-treated group only total symptom score had decreased significantly. Cough score decreased to a significantly greater extent in the BDP-treated group than in the placebo-treated group ($p < 0.05$).

After one year, 17 of 31 patients with respiratory symptoms (55%) continued to exhibit symptoms but have normal lung function. Four patients (13%), two in the BDP group and two in the placebo group, had developed asthma. Another 10 (32%), six in the BDP and four in the placebo group, were free from symptoms, i.e. they did not meet the criteria relating to symptoms. In the BDP-treated group total symptom score had decreased from 9.9 to 5.7, in the placebo group from 11.8 to 7.0. Two of the patients (both in the placebo group) who had developed asthma had received treatment with inhaled steroid after the first three-month period and were omitted from the one-year analysis. At one year, the mean percentage of eosinophils in sputum had decreased in the BDP-treated group from 3.0 at baseline to 1.3, in the placebo group from 2.3 at baseline to 0.3 (Fig. 6). Of the 29 patients that were included in one-year analysis, 11 of those 28 with adequate sputum sample (39%) had increased sputum eosinophils at the start of the study. At

Fig 6.

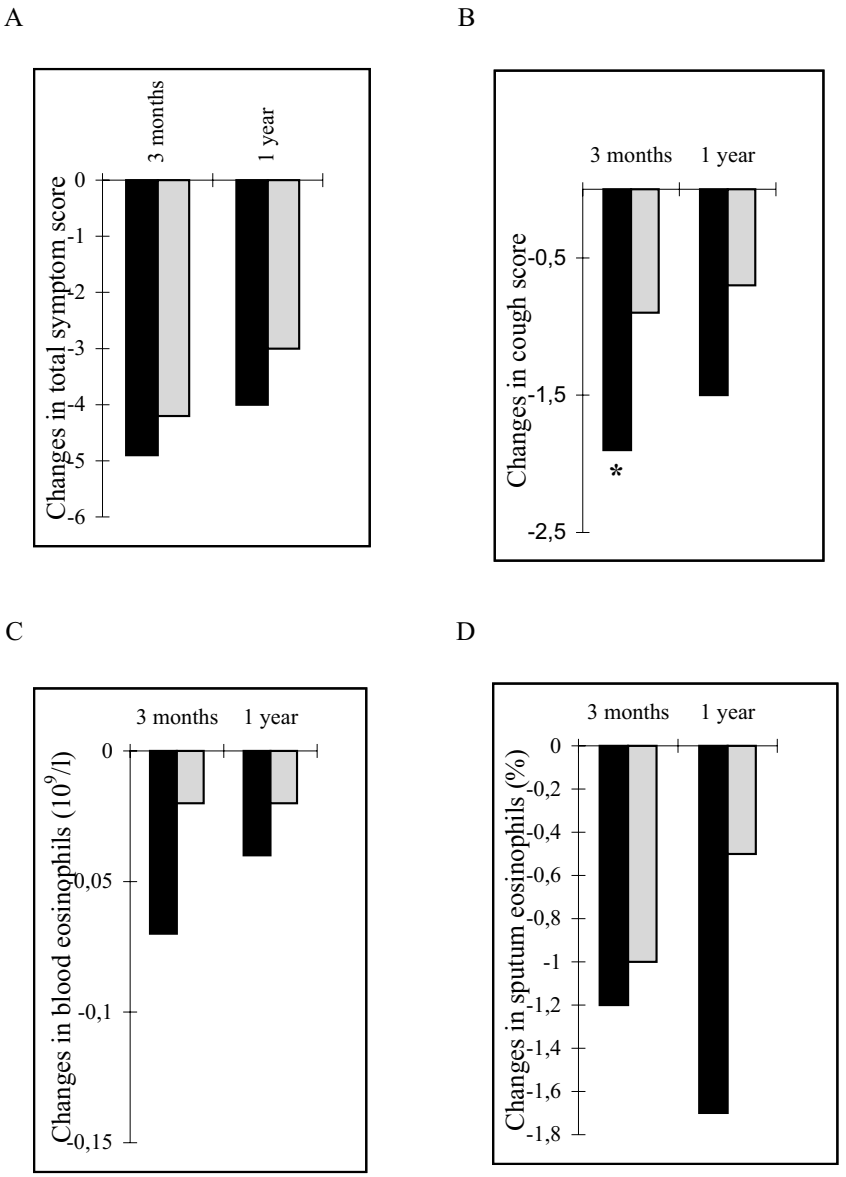


Fig. 6. Changes from baseline in total symptom score (A), in cough score (B), in numbers of blood eosinophils (C), and in percentages of sputum eosinophils (D) after three months (left panel) and one year (right panel) in patients with respiratory symptoms (Study III). Each bar represents the mean change from baseline. Solid bars indicate BDP treatment, shaded bars indicate placebo treatment.

*$p < 0.05$ in relation to differences between treatment groups

one year, in 50% of them eosinophils persisted. Of the 17 patients (61%) that had no sputum eosinophils at the first examination, 33% showed sputum eosinophils at one year.

Study IV

Six months of treatment

After six months of treatment with budesonide, children had significantly fewer exacerbations than those treated with cromoglycate ($p < 0.01$, Table 13). FEV_1 had improved in both groups but only in the budesonide group was the predicted change from baseline in terms of percentage of predicted FEV_1 (with adjustment for child's height) significant. Symptoms ($p < 0.001$) and use of rescue medication ($p < 0.01$) decreased significantly from baseline in the budesonide-treated groups (Table 13).

At six months, adequate sputum samples were obtained from 41/60 children (68%). Representative samples at both baseline and six months were obtained from 34 children (23 in the two budesonide groups, 11 in the cromoglycate group). Numbers of sputum eosinophils decreased significantly in the budesonide groups ($p = 0.003$), but not in the cromoglycate group (Table 13). Numbers of blood eosinophils and of sputum cells other than eosinophils did not change significantly in any group. The three children who had had more than 10% of eosinophils in sputum at baseline in the cromoglycate group experienced three, four and four exacerbations over the six-month period, and therefore received treatment with budesonide by inhalation for about two months. Of the five patients with high percentages (> 10) of eosinophils in sputum in the budesonide groups, one experienced one exacerbation during the months of treatment, the others none.

Eighteen months of treatment

After 18 months of treatment, the number of exacerbations was highest in the cromoglycate group (Table 13), significantly different from the number in the group that had received regular treatment with budesonide at low doses ($p < 0.05$). No subject in the latter group had to withdraw from the study because of hospitalisation as a result of asthma. Two children in the placebo group, in which budesonide was used only during exacerbations, were hospitalised between six and 18 months of treatment, and three children in the cromoglycate group.

FEV_1 had improved in all groups but only in the group that had received regular treatment with budesonide was the change from baseline in terms of percentage of predicted FEV_1 significant ($p = 0.02$, Table 13). Symptoms and use of rescue medication had decreased in all groups in comparison with baseline but not in comparison with findings at six months. Hyperresponsiveness to histamine did not decrease in any group in comparison with baseline.

At 18 months, adequate sputum samples were obtained from 36/60 children (60%). Representative samples at both baseline and 18 months were obtained from 32 children (10 in the group treated regularly with budesonide, 12 in the group that received budesonide during exacerbations, and 10 in the cromoglycate group). Numbers of blood eosinophils and differential counts of sputum cells were not significantly different from those at baseline or six months (Table 13).

Table 13. Study IV: Effects of treatment

	Budesonide (n = 19)	Budesonide-placebo (n = 20)	Cromoglycate (n = 21)
FVC, l			
Baseline	1.90 (1.03 - 2.96)	1.79 (1.18 - 2.40)	1.99 (1.30 - 3.11)
Change after six months	0.16 (-0.02 - 0.40)*	0.11 (-0.22 - 0.30)†	0.12 (-0.21 - 0.44)†
Change after 18 months	0.37 (0.15-0.58)*	0.34 (0.01-0.71)*	0.36 (0.09 - 0.71)*
FVC, % of predicted			
Baseline	99.2 (88 -119)	101.2 (81 - 123)	101.7 (87 - 127)
Change after six months	0.86 (-23 - 16)	1.50 (-21 - 17)	-0.31 (-15 - 14)
Change after 18 months	1.20 (-12 - 14)	0.79 (-15 - 16)	-1.12 (-13 - 8.8)
FEV_1, l			
Baseline	1.60 (0.97 - 2.35)	1.49 (1.027 - 2.00)	1.69 (1.02 - 2.30)
Change after six months	0.15 (-0.70 - 0.50)†	0.09 (-0.70 - 0.40)‡	0.05 (-0.91 - 0.30)†
Change after 18 months	0.33 (-0.53 - 0.80)†	0.29 (0.01 - 0.50)*	0.26 (-0.80 - 0.60)†
FEV_1, % of predicted			
Baseline	88.6 (71 - 102)	92.8 (75 - 111)	91.9 (74 - 113)
Change after six months	5.14 (-18 - 28)‡	2.2 (-31 - 18)	-0.38 (-11 - 12)
Change after 18 months	5.08 (-8.4 - 22)‡	-0.17 (-13 - 14)	-0.57 (-8.2 - 6.3)
PEF, l/min			
Baseline	177 (123 - 231)	174 (98 - 230)	204 (114 - 280)
Change after six months	14.2 (-17 - 62)‡	-3.6 (-89 - 59)	-3.4 (-76 - 57)
Change after 18 months	32.4 (2.4 - 55)†	28.3 (-40 - 95)‡	24.3 (-20 - 106)‡
Symptoms (average score/day)			
Baseline	4.2 (0.5 - 12)	4.4 (0.5 - 10)	4.4 (0.9 - 12)
Change after six months	-2.2 (-6.4 - 2.7)†	-2.1 (-9.3 - 3.6)‡	-1.7 (-8.0 - 5.6)
Change after 18 months	-2.8 (-11.9 - 1.8)†	-1.5 (-8.4 - 4.0)‡	-1.8 (-9.4 - 2.9)‡
Rescue medication (puffs/day)			
Baseline	1.3 (0 - 4.9)	1.3 (0 - 3.1)	1.7 (0 - 6.3)
Change after six months	-0.5 (-3.6 - 2.0)	-0.8 (-3.1 - 0.7)†	-0.5 (-4.4 - 3.7)
Change after 18 months	-0.9 (-4.4 - 2.0)‡	-0.5 (-2.0 - 1.4)‡	-0.7 (-2.7 - 1.6)‡
Blood eosinophils, 10^9			
Baseline	0.43 (0.13 - 1.38)	0.71 (0.02 - 3.54)	0.39 (0.03 - 1.79)
Change after six months	0.02 (-0.40 - 0.55)	-0.31 (-1.94 - 0.40)	-0.07 (-0.80 - 0.43)
Change after 18 months	-0.002 (-0.73 - 0.69)	-0.19 (-2.30 - 0.22)	-0.06 (-0.69 - 0.83)
Sputum eosinophils, %			
Baseline	5.9 (0 - 39)	6.9 (0 - 31)	4.5 (0 - 30)
Change after six months	-2.0 (-14.7 - 0.9)‡	-5.9 (-29.9 - 0.3)†	-3.6 (-22.0 - 2.2)
Change after 18 months	-2.9 (-15.5 - 3.9)	-3.8 (-29.9 - 3.5)	-3.4 (-21.0 - 2.0)
Number of exacerbations			
After six months**	0.5 (0 - 2)	0.7 (0 - 2)	1.4 (0 - 4)
After 18 months	1.6 (0 - 4)	2.8 (0 - 10)	3.1 (0 - 7)

Data are expressed as mean and range. *p<0.001, †p<0.01, ‡p<0.05, differences Vs baseline (Wilcoxon´s signed rank test).**p<0.05 between treatment groups (Kruscall-Wallis test).

DISCUSSION

STUDY POPULATIONS AND METHODS

Clinical characteristics of subjects

We used internationally accepted criteria to select COPD and asthma patients (ATS 1995, NHBI 1997, Warner et al. 1998). These criteria are based on findings relating to symptoms and results of lung-function measurements. In the case of patients with chronic respiratory symptoms suggestive of asthma, the criteria for inclusion were symptoms that made the clinician suspect asthma even though lung function did not meet the criteria allowing a diagnosis of asthma. It could be argued that the patients classed as suffering from respiratory symptoms were in fact suffering from mild asthma. Diagnosis of mild asthma can be difficult, since results of measurements of lung function can remain normal for long periods, or may be conflicting (Siersted et al. 1996). To exclude asthmatics from the group of patients classed as suffering from respiratory symptoms, low PEF and FEV_1 variability criteria (12%) and absence of increased bronchial responsiveness were used. In patients diagnosed as suffering from respiratory symptoms, FEV_1 increased on average by only 2.5% 15 minutes after inhaling salbutamol in the bronchodilatation test. Mean variability between morning and evening was 5.2% during PEF monitoring, and no increased responsiveness to inhaled histamine was detected.

In study V, on the basis of results of clinical examinations and lung-function tests, 13 of 82 patients with persistent cough (16%) were diagnosed for the first time as suffering from asthma. However, since no PEF follow-up or spirometry was undertaken in 15 subjects, the possibility cannot be excluded that some of these subjects would have met the criteria for asthma. The incidence of asthma may therefore have been slightly underestimated. Taking into account the prevalence of asthma in the study population that underwent lung-function testing, two or three subjects with asthma might have been missed. Lung function was however tested in all patients with eosinophilic airway inflammation. Since the study was carried out in patients attending for primary care, airway responsiveness could not be measured, nor could gastroscopy with 24-hour pH measurement be undertaken (McGarvey et al. 1999).

All of the COPD patients were previous or current smokers. Mean pack-years were 52 and 37 in studies I and II, respectively. Most COPD patients were male. Smoking has been found to be commoner in men than in women in both Belgium and Finland. COPD patients were also significantly older than the patients in the other groups. In study II, the healthy controls were matched for sex but not for age. We found no correlation between age and results of any measurements made. In the other studies, age- and sex-matching of patients and controls were better. In study IV, the control children were slightly older than the asthmatic children. This is one reason why the success rate in relation to induction of sputum production was better in the healthy controls than in the asthmatic children.

No differences were found between smokers and non-smokers in any other disease groups or in the healthy controls. Numbers of pack-years in relation to these subjects were significantly lower than numbers for the COPD patients, and none of the smokers in studies III and V were diagnosed (on the basis of results relating to lung function and duration of symptoms) as suffering from chronic bronchitis or COPD (ATS 1995).

Sputum induction

Safety

One of the aims of the studies reported here was to assess the safety of sputum induction, especially in patients with COPD. In study I, the mean fall in FEV_1 during induction from baseline before bronchodilator administration was 8.5%, that from baseline after bronchodilator administration 10.7%. In study II, there were no falls in lung function during sputum induction. In both studies all patients tolerated the procedure for induction of sputum production well. No major side effects were reported but airway obstruction occurred as a result of the induction procedure. Similar high-output nebulizers and similar concentrations of hypertonic saline were used in the two studies. In study II, subjects were pre-treated with 400 μg of inhaled salbutamol. In study I, 200 μg of inhaled salbutamol were used. Baseline lung function in the COPD patients was similar in both studies. However, in study II all patients except one were treated with inhaled steroids. Only four of the patients (14%) in study I were treated in this way. All patients in the second study were former smokers. Only two patients in study I had stopped smoking. These differences could partly explain the different responses in the two studies. It is not clear whether extent of bronchial obstruction depends on the amount of expectorate, the degree of hypertonicity of the inhaled saline, the total amount inhaled or the rate of delivery, or if bronchconstriction could be prevented by pre-treatment with higher doses of inhaled salbutamol (Popov et al. 1995, Bacchi et al. 1997, Pavord 1998).

Pre-treatment with a bronchodilator did not prevent declines in FEV_1, especially in COPD patients with little or no FEV_1 reversibility. Falls in FEV_1 during sputum induction correlated inversely with degree of reversibility in FEV_1. This indicates that COPD patients with appreciable FEV_1 reversibility may gain most from bronchodilator treatment to prevent bronchoconstriction. Airway hyperreactivity and reversibility of airway obstruction are thought to be characteristic of asthma but can also be present in COPD. Increased airway responsiveness in smokers is a risk factor for accelerated decline in FEV_1 with time, and hence for development of COPD (Ricjken et al. 1995). Disease severity as assessed by symptom scores or results of lung-function measurements was not associated with severity of bronchoconstriction. No correlations were found between degrees of neutrophilic or eosinophilic airway inflammation and falls in FEV_1 during induction.

Our results relating to safety of sputum induction in COPD patients are similar to results reported by others. Keatings et al. (1996) found a small but statistically significant fall in FEV_1 during induction in 17 COPD patients. Maestrelli et al. (1996) studied 33 patients with at least 10 pack-years of smoking history, 14 of whom exhibited airway obstruction. Falls in FEV_1 of more than 20% were detected in only two subjects following inhalation of hypertonic saline. In a study by Bhowmik et al. (2000) in 27 COPD patients a mean fall of 11.7% in FEV_1 was found during induction. Only one patient experienced a fall in FEV_1 of more than 20%.

In study III, sputum production was induced without pre-treatment in patients with asthma or chronic respiratory symptoms suggestive of asthma. In these patients there were also small falls in lung function during sputum induction. The greatest fall in PEF was 17%. De la Fuente et al. (1998) found sputum induction to be safe even in patients with uncontrolled asthma, with falls in FEV_1 that never exceeded 20%. However, the procedure had to be stopped in 23% of cases because of the patients reporting side effects. Changes in FEV_1 were significantly more marked in patients with severe asthma than in those less affected but there was no association between extent of control of the disease

and poor tolerance of the procedure. In a study by Wong et al. (1997) 78 asthmatics with baseline FEV_1 after bronchodilator administration of more than 60% of predicted FEV_1 were studied. Fourteen per cent exhibited bronchocontriction of more than 20%, another 8% falls of 10 to 20%. The greatest fall in FEV_1 in their study was 69%. Changes in FEV_1 during sputum induction correlated significantly with baseline lung function and percentages of eosinophils in induced sputum. In a study by Hunter et al. (1999), a low-output ultrasonic nebulizer was used by 79 patients. A fall of 20% was seen in only three subjects. In a later study by ten Brinke et al. (2001) it was shown that additional use of short-acting β_2-agonist as rescue medication during the days preceding sputum induction increase the risk for bronchoconstriction.

In all of these previous studies on the safety of sputum induction in adult asthmatics pre-treatment with a bronchodilator was used. We did not treat before induction because we were studying patients with mild or moderate asthma (FEV_1 more than 57% in all patients, mean 79%). The fact that significant falls in lung function occurred during induction of sputum even in mild disease highlights the importance of pre-treatment and monitoring of lung function during induction.

Sputum induction was found to be safe when asthmatic children were pre-treated with a β_2-agonist. This had previously been demonstrated in adolescents with severe stable asthma (Grootendors et al. 1999), even in acute childhood asthma (Gibson et al. 1999, Cai et al. 1998). Like Grootendors et al., we found a weak correlation between baseline PEF values and PEF change during sputum induction. Sputum induction has been shown to be safe in children with cystic fibrosis (De Boeck et al. 2000). When inducing sputum in children, those concerned should have experience of evaluation of lung function, and of identification and management of adverse effects.

Success of sputum induction

The overall baseline success rate of 82% is similar to rates reported by others (Pizzichini et al. 1996a, int´Veen et al. 1996, Belda et al. 2000, Spavenello et al. 2000). It was lower after anti-inflammatory treatment. In primary care, the rate of success in obtaining adequate sputum samples was high (91%).

Sputum production has previously been induced mainly in children of six or more years of age. We included 15 five-year-old children. Ten of them (67%) produced adequate sputum samples. Success rate improved with age. It was 77% for the entire group at baseline, 68% after six months of treatment and 60% after 18 months of treatment. These rates are slightly better than rates reported by others in children on asthma medication (Wilson et al. 2000).

Sputum processing

Various sputum-processing methods were used in the studies described in this thesis. The main difference related to selection of sputum masses from saliva as opposed to use of entire expectorates. Selection of sputum results in squamous cell contamination being less than that found when whole expectorate is used. In consequence, cell counting is easier and quicker. Slides prepared from selected masses of sputum are, for example, better suited to immunocytochemical analysis than slides prepared from entire expectorate.

Concentrations of substances in the fluid phase are unaffected by the presence of saliva, and accurate correction for dilution is possible. A disadvantage is that selection takes time. Use of entire expectorate is quicker. We used selection in every study except study IV. The latter was a longitudinal study to assess the effects of various anti-inflammatory treatment strategies in children with asthma over 18-month periods. We felt it would be difficult to select sputum after effective treatment over such a lengthy period. Contamination of sputum with squamous epithelial cells was relatively high. The reproducibility of sputum cell counts was high in all patient groups using the selection method.

Recently, reference values for sputum differential cell counts in sputum selected from saliva have been published (Belda et al. 2000, Spavenello et al. 2000). Induced sputum samples from more than 200 control subjects in the studies mentioned were rich in macrophages and neutrophils, poor in eosinophils, lymphocytes, metachromatic cells and bronchial epithelial cells. Percentages of various types of cells were similar to those found in our control groups.

INFLAMMATORY MARKERS

COPD

Differential cell counts relating to COPD patients revealed very high numbers of neutrophils and high concentrations of MPO compared to those relating to other patient groups and healthy controls. Our findings are in line with previous findings of high percentages of intraluminal neutrophils (Thompson et al. 1989). It has been shown in studies conducted using BAL that this neutrophilia increases significantly with severity of airway obstruction (Martin et al. 1985, Linden at al. 1993). This observation is in agreement with our finding of an inverse correlation between percentages of neutrophils in induced sputum and findings relating to FEV_1 (study II). Similar results have been published by others (Keatings et al. 1996, Balzano et al. 1999). However, findings in the other COPD study reported here (study I) were different. It has also been shown that increased levels of neutrophils in sputum correlate with rapid declines in lung function over 15-year follow-up periods (Stânescu et al. 1996). Neutrophils probably pass into the airway lumen because chemotactic factors are released by other airway cells, such as macrophages, T lymphocytes and epithelial cells (Richman-Eisenstat et al. 1993, Keatings et al. 1996). It has been shown that there is increased expression of adhesion molecules, such as E-selectin on vessels and ICAM-1 on basal epithelial cells, in the bronchial mucosa of patients with chronic bronchitis with airway obstruction. This suggests such molecules are involved in origination of the disease (DiStefaro et al. 1994).

When cytocentrifuge preparations were stained with a macrophage marker (CD68), the percentage of positive cells was lower in COPD patients (17.9 ± 4.8) than in healthy controls (71.2 ± 4.5). In both groups most macrophages were activated, as evidenced by the high percentages of macrophages expressing the antigen M130, as recognised by the antibody Ber-Mac 3 (Law et al. 1993)

Numbers of eosinophils in sputum were high in stable COPD patients. We and others have previously published similar results (Balzano et al. 1999, Pizzichini et al. 1998a,

Brightling et al. 2000a, Metso et al. 2001). Lacoste et al. (1993) have demonstrated higher numbers of eosinophils in blood, BAL fluid and bronchial biopsy specimens from COPD patients than in normal subjects. However, the eosinophils were not degranulated as they are in asthma. The mechanisms by which eosinophils pass into airways in asthma and COPD may differ, since IL-5 expression has been found only in asthma patients (Saetta et al. 1996).

The role played by eosinophils and value of anti-inflammatory medication in COPD remain unclear, despite large multicentre trials relating to use of inhaled steroids (Pauwels et al. 1999, Burge et al. 2000). Results of some studies suggest that COPD patients with airway eosinophilia may respond better to corticosteroid treatment than COPD patients without airway eosinophilia (Chanez et al. 1997, Pizzichini et al. 1998a, Brightling et al. 2000a). In another study on inhaled and oral glucocorticoids in COPD no clinical effects were seen, and none of the levels of inflammatory markers that were measured altered (Keating et al. 1997c). Some of the stable chronic bronchitis patients evaluated in their study also exhibited sputum eosinophilia. Eosinophils accounted in some cases for as many as 10% of all inflammatory cells present. In a later study by the same group (Culpitt et al. 1999), no changes in clinical parameters, numbers of sputum cells, and levels of IL-8, proteases or antiproteases were found after one month of steroid treatment at high dose by inhalation. All of the 13 subjects exhibited low numbers of sputum eosinophils.

Saetta et al. (1994) have demonstrated marked airway eosinophilia during exacerbations of chronic bronchitis. Patients with frequent exacerbations have higher baseline IL-6 and IL-8 cytokine levels than other patients (Saetta et al. 1996). Determination of cytokine levels may allow prediction of frequency of future exacerbations (Bhowmik et al. 2000).The effect of glucocorticoids has been shown to be better in relation to treatment of exacerbations of COPD (Niewoehner et al. 1999). Further studies are needed to establish if there are sub-populations of COPD patients in whom eosinophils play a role in origination of the disease, and in whom exacerbations might be worse than in other patients. The role of airway eosinophilia in disease progression is also unclear (Lebowitz et al. 1995).

The low numbers of lymphocytes on Wright-Giemsa-stained slides is reflected in the results of immunocytochemical analyses: only 2.1% and 1.8% of CD3-positive lymphocytes were seen in healthy controls and COPD patients, respectively. These low percentages of lymphocytes make it difficult to draw conclusions about the role played by lymphocytes in origination of the disease. In addition, very few CD4- and CD8- and no CD20- or CD25-positive cells were detected. It has been shown that dithiothreitol does not reduce the reactivity of target molecules for immunocytochemistry after 10 minutes of treatment (Girbis-Gabardo et al. 1994) but treatment for more than 20 minutes reduces numbers of EG-2 positive cells (Popov et al. 1994). Since we exposed the cells to dithiothreitol for no more than five minutes, the low numbers of positively staining cells are not likely to have been a result of mucolytic treatment. Flow-cytometric analysis of sputum lymphocytes is probably the best approach in relation to induced sputum.

Asthma

High numbers of eosinophils and markers of eosinophil activation (EPO and ECP) were found in blood and sputum from patients with asthma, both adult and children. All of the asthma patients we studied had been newly diagnosed as suffering from the disease, which had therefore not been previously treated with anti-inflammatory medication. We found good correlation between numbers of blood and sputum eosinophils in both adults and children with asthma. Peripheral blood eosinophilia discriminated at baseline between patients with asthma and healthy controls almost as well as discovery of high numbers of sputum eosinophils. Results of previous studies in adult asthmatics suggest that the presence of high numbers of eosinophils in induced sputum samples discriminates patients with asthma from control subjects better than results of determination of peripheral blood eosinophils (Pizzichini et al. 1997b). We found determination of numbers of eosinophils in sputum to be better than determination of numbers of eosinophils in blood for detecting asthma in children but the difference is not statistically significant. At baseline, 30% of asthmatic children exhibited normal numbers of eosinophils in blood but had unusually high numbers of eosinophils in sputum. Only 5% of asthmatic children exhibited normal numbers of eosinophils in sputum and high levels of eosinophils in blood.

Atopic patients with asthma had significantly higher numbers of eosinophils in blood than non-atopic asthmatics. Numbers of eosinophils in sputum did not differ significantly between adult asthmatics with and without atopy. However, atopic children with asthma had significantly larger numbers of eosinophils in sputum than non-atopic children with asthma. It has been shown in adults that atopic subjects without asthma and patients with seasonal allergic rhinitis can exhibit airway eosinophilia even without natural exposure to the allergens concerned (Djukanovic et al. 1992b, Foresi et al. 1997, Vignola and Chanez 2001). Avoidance of allergens by children with asthma can significantly reduce the eosinophilic phase of airway inflammation, and bronchial hyperresponsiveness (Piancentini et al. 1996). The atopic children in our study IV were not exposed to any animals or tobacco smoke at home. Only three (8%) were allergic to house-dust mites. Sixteen (43%) suffered from allergic rhinitis. However, it has been shown that children can be exposed to significant amounts of cat and dog allergens in the environment (Lönnqvist et al. 1999, Partti-Pellinen et al. 2000). Most baseline blood and sputum samples were collected outside the pollen season, but 12 samples (15%) were collected during summer months. The latter were found not to differ significantly from samples collected during winter. We found no significant differences between atopic and non-atopic healthy controls.

The role played by neutrophils in the progress of asthmatic inflammation is controversial. In some studies it has been reported that neutrophils are important in chronic severe asthma in adults (Jatakanon et al. 1999c) and children (Marquet et al. 1999). It has even been suggested that there are at least two phenotypes of severe asthma, in which eosinophils are either present or absent (Wenzel et al. 1999). Children with acute asthma exhibit intense airway inflammation, in which neutrophils, eosinophils and mast cells are involved (Twaddel et al. 1996, Gibson et al. 1999). In the studies reported here, which relate to mild or moderate asthma, numbers of sputum neutrophils were not higher than normal. We found no correlation between numbers of sputum neutrophils and severity of disease. In a study by Turner et al. (1995) asthma patients who had suffered clinical exacerbation exhibited no greater than 4% airway eosinophilia but signs of neutrophilic inflammation were evident. Similar results were found by Fahy et al. (1995b). Sputum HNL and MPO values were not higher than normal in asthmatic patients.

Numbers of metachromatic cells in sputum were higher than normal in patients with

asthma and in patients with COPD, and correlated with numbers of eosinophils. Tryptase is a serine endoprotease selectively released from mast cells. It has been shown that asthmatics with increased levels of tryptase in sputum had higher sputum eosinophil counts than normal (Bettiol et al. 1999).

Eosinophilic bronchial inflammation without asthma or COPD

We have suggested use of the term ”asthma-like inflammation” to describe the condition suffered by patients with chronic respiratory symptoms suggestive of asthma, but normal lung function, and signs of eosinophilic bronchial inflammation. The term would cover the same kind of characteristics of inflammation (e.g. presence of eosinophils) as those found in asthma but with a lesser degree of eosinophilic inflammation than in patients diagnosed as suffering from asthma.

Gibson et al. (1989b and 1995) described a condition in which chronic cough was associated with eosinophilic bronchitis. The condition presents as chronic productive cough without no other asthma symptoms and normal spirometric findings. Airway responsiveness to methacholine was normal but sputum eosinophil counts were abnormally high. They could be restored to normal by treatment with corticosteroid. The study population was chosen on the basis of percentage of eosinophils in sputum (minimum 10). The condition was fairly rare. Of 180 new referrals to the clinic concerned only seven met the criteria established. The main difference between our patients and patients with eosinophilic bronchitis would seem to be the existence of other symptoms suggestive of asthma. Patients with eosinophilic bronchitis produce sputum but experience no wheezing, dyspnoea, or chest tightness. Cough was the main symptom in our patients but all also exhibited some other respiratory symptom. The differences may seem minor but serve to emphasise the need for thorough clinical characterisation of patients in descriptive studies.

Recently, it has been suggested that the limit for diagnosis of eosinophilic bronchitis should be 3% of eosinophils in sputum (Carney et al. 1997, Brightling et al. 1999a). Only seven of 34 patients with respiratory symptoms suggestive of asthma in the study reported here met this criterion indicating marked eosinophilia. In our experience percentages of eosinophils in the sputum of healthy, truly asymptomatic individuals are very low (mean 0.07). Thirty-eight per cent of patients with respiratory symptoms and 67% of patients with asthma in the study reported here exhibited eosinophilia, when this was defined as a percentage (0.7) of eosinophils in sputum more than three standard deviations above the normal mean. Recently published reference values for percentages of eosinophils in sputum have been around 1% (Belda et al. 2000, Spanevello et al 2000).

In a recent study, the prevalence of eosinophilic bronchitis in 91 patients with isolated chronic cough was 12% (Brightling et al. 1999a). Brightling et al. modified a previously validated protocol for the study of chronic cough (Irwin et al. 1990). In study V, eosinophils were found in sputum in 19% of patients with chronic cough in primary care. Concentrations of the markers of eosinophil activation, ECP and EPO, were more often abnormally high in serum and sputum in patients with chronic cough than in healthy individuals. Thirty-six per cent of those who had eosinophils in sputum were diagnosed as suffering from asthma, 64% were not.

Several groups have studied patients with chronic respiratory symptoms (mainly cough) and airway eosinophilia. A Japanese group has used the term atopic cough and eosinophilic tracheobronchitis to describe the conditions suffered by patients with corti-

costeroid-responsive non-productive cough, eosinophilia in induced sputum and bronchial biopsy samples, normal spirometric values, normal airway responsiveness and increased cough response to inhaled capsaicin (Fujimura et al. 2000). The term eosinophilic bronchitis has also been used in relation to subjects with asthma-like symptoms related to work and eosinophils in sputum but no asthma proper (Lemière et al. 1997). Higher than normal numbers of mast cells and eosinophils have been found in BAL fluid from patients with chronic non-productive cough caused by gastro-oesophageal reflux (McGarvey et al. 1999). A Swedish group has used the term ”sensory hyperreactivity” in relation to patients with cough, asthma-like symptoms and a positive response to the capsaicin provocation test (Millqvist et al. 1998). The subjects had normal lung function. The nature of any airway inflammation present in such patients has yet to be studied.

All of our asthma patients exhibited increased bronchial responsiveness to inhaled histamine except for those with respiratory symptoms suggestive of asthma. This finding is in accordance with the results of inflammatory cells in sputum and suggests that relatively severe inflammatory processes in asthmatic subjects could have led to airway restructuring (remodelling) and increased bronchial responsiveness that were mostly lacking in the patients with just asthma-like inflammation. In ”classic” asthma, two factors may affect functional outcome: a Th-2-mediated inflammation involving mast cells and eosinophils, and airway-wall restructuring involving changes in airway epithelial, vascular, fibroblast, neural and smooth-muscle cell phenotypes. It has been shown that eosinophilic bronchitis is associated with airway inflammation and increased release of vasoactive and bronchoconstricting mediators (Brightling et al. 2000b). Levels of exhaled NO have been shown to be higher than normal in eosinophilic bronchitis (Berlyne et al. 2000). Gibson et al. (1998a) have shown similar extents of BAL eosinophilia and cytokine gene expression in patients with asthma and eosinophilic bronchitis. Niimi et al. (1998) showed that numbers of eosinophils in bronchial biopsy samples from patients with increased bronchial responsiveness were the same in both so-called ”cough variant asthma” and in traditional asthma. It has been suggested that a possible reason for the difference in airway responsiveness in patients with eosinophilic bronchitis and asthma is that the epithelium remains intact in eosinophilic bronchitis (Brightling et al. 2000b). We found no differences in numbers of epithelial cells in induced sputum samples in patients with asthma and respiratory symptoms suggestive of asthma. Further studies of bronchial biopsy material are required.

Airway eosinophilia can occur in otherwise healthy individuals during and after viral infection (Trigg et al. 1996). We tried to exclude the effects of viral infection by including only patients with no signs of respiratory infection during the preceding eight weeks. Numbers of neutrophils, and levels of the markers of neutrophil activation HNL and MPO, were no higher than normal in our patients with respiratory symptoms suggestive of asthma. It has been shown that chronic dry cough can exist with higher than normal numbers of neutrophils and levels of cytokines associated with neutrophil chemotaxis (Jatakanon et al. 1999b).

Correlations between different inflammatory markers

In adults, numbers of blood eosinophils correlated best with serum EPO levels, in all groups studied (no data available for COPD patients). Numbers of blood eosinophils correlated less well with serum ECP levels and numbers of eosinophils in sputum but the correlations were statistically significant. In children with asthma, we found good corre-

lation between numbers of eosinophils in blood and sputum. Numbers of eosinophils in sputum correlated significantly with sputum EPO and ECP levels. When patients were divided into groups according to diagnosis, a correlation was found only in relation to asthmatics. Numbers of neutrophils in sputum correlated with sputum MPO and HNL levels. They correlated with ECP levels better than numbers of eosinophils in sputum overall, in all groups of patients except asthmatics. Levels of sputum ECP correlated strongly with sputum MPO and HNL levels. These correlations suggests that factors which prime neutrophils may also affect eosinophils. Venge et al. (1997) have reported uptake of ECP by neutrophils. This could partly explain the correlation observed, since it has been suggested that ECP occupies the same locations as MPO in secondary granules of neutrophils (Sur et al. 1998).

Levels of sputum EPO and HNL have been measured in only a few previous studies. Keatings et al. (1997b) found values similar to ours in asthma and COPD patients. A slightly different sputum-handling method was used. It has been suggested that ECP and EPO are released by different mechanisms, and that EPO might be the more sensitive indicator of allergic inflammation (Karawajczyk et al. 1995, Metso et al. 2002).

The value of measuring inflammatory markers is questionable, given that counting inflammatory cells yields similar results. Sputum processing to obtain cytospin slides and differential cell counting are, however, laborious, and suitable only for research purposes. Counting from smears gives only semiquantitative results. We have therefore been trying to develop sputum processing and devise a simple method of measuring ECP and MPO levels that could be applied in primary health-care settings (Metso et al. 2001).

EFFECTS OF TREATMENT

In the patients with respiratory symptoms suggestive of asthma, three months of treatment with inhaled BDP suppressed symptoms and resulted in declines in numbers of eosinophils to greater extents than treatment with placebo. The difference between results of treatment was significant in relation to the most important symptom, cough. In asthma, eosinophilic inflammation has been found to be reversed or suppressed contemporaneously with improvement in lung function through treatment with inhaled steroid (Djukanovic et al. 1992a, Jatakanon et al. 1999a). In eosinophilic bronchitis, treatment with inhaled steroid has been shown to suppress symptoms and decrease numbers of eosinophils in sputum (Gibson et al. 1995, Brightling et al. 2000c). However, the studies in eosinophililic bronchitis were not placebo controlled.

The 60 asthmatic children who participated in study IV had been randomly selected from 180 subjects participating in a study of early pharmacological intervention in childhood asthma (Turpeinen 2000). In the latter study, treatment with budesonide resulted in 50% fewer exacerbations than treatment with disodium cromoglycate treatment over an 18-month period. In our study, lung function, symptoms, use of rescue medication and numbers of eosinophils in sputum did not alter statistically significantly in the cromoglycate group during the first six months. In some patients in the cromoglycate group there were marked decreases in numbers of eosinophils in sputum. However, there had been supportive treatment with inhaled budesonide by inhalation during exacerbations. Eighteen of 21 children (86%) who started with cromoglycate needed at least one two-week course of budesonide during the 18 months of the study.

Six months of treatment with inhaled budesonide decreased numbers of eosinophils

in sputum with contemporaneous improvement clinical status but no significant changes in numbers of eosinophils in blood. This finding suggests that determinations of numbers of eosinophils in sputum may be a better means of following-up results of treatment than determinations of numbers of eosinophils in blood.

After six months of treatment, children who had received budesonide were divided into two groups of equal sizes. In one group the children continued with a low dose of budesonide regularly, in the other the children received placebo and inhaled budesonide only during exacerbations. No significant differences were seen between the two groups at 18 months. This suggests that after clinical improvement has been seen during regular treatment with inhaled steroid, periodic treatment might be sufficient to keep mild childhood asthma under control. However, all these patients should controlled regularly by their physician.

CLINICAL IMPLICATIONS

There is general consensus as regards how moderate and severe asthma should be treated, less agreement concerning treatment of mild asthma. Commencement of anti-inflammatory therapy is not usually recommended until the patient has tried the effect of a short-acting β_2-agonist several times per week (NHLBI 1995). This situation has arisen largely because not enough is known about the natural history of astma. It is also not clear whether patients with just respiratory symptoms suggestive of asthma will develop asthma proper. In the study reported here, four patients (13%) developed asthma during a year of follow-up. The first three months of treatment received by these subjects differed. In a Finnish study of children from seven to 12 years of age who had symptoms suggestive of asthma but normal lung function, 33% developed clinical asthma during a two-year follow-up period (Remes at al. 1998). Early detection of eosinophilic inflammation would improve results of treatment with anti-inflammatory medication. This could affect disease progress and the risk of developing chronic asthma. In general practice, bronchial inflammation that may underlay symptoms is usually not characterised. Clinical judgement is based on indirect information concerning bronchial status. In consequence, the condition from which symptomatic patients with no apparent lung-function abnormality are suffering is not diagnosed appropriately and courses of antibiotics, expectorants, antitussives, antihistamines and β_2-agonists, which will essentially have no effect on processes involving eosinophils, are prescribed.

The natural course of eosinophilic airway inflammation is not yet known. Many patients may never develop asthma but follow-up studies are needed (Brightling et al. 1999b). Three months of inhaled BDP in patients with respiratory symptoms did not prevent the development of asthma in all patients in our study. On the other hand, eosinophilic inflammation can heal spontaneously, especially if it has been caused by exposure to an allergen, which ceases. Effective treatment of these patients may require prescription of a course of inhaled steroids for two to three months. COPD patients in whose eosinophils are found experience relatively rapid declines in lung function (Lebowitz et al. 1995) and can benefit from anti-inflammatory therapy (Pizzichini et al. 1998a, Brightling et al. 2000a).

Inflammatory changes such as infiltration of inflammatory cells into the mucosa, and thickening of the basement membrane are visible in mucosal biopsy specimens taken during endoscopic examination of the lungs in early stages of asthma, before dysfunction

has become permanent (Laitinen et al. 1993). Mucosal inflammation obviously exists well before development of pulmonary dysfunction. The interval between mucosal inflammation and initial symptoms and development of dysfunction is unknown. There are indications that symptoms of mucosal inflammation need to exist for at least a year before increased bronchial susceptibility to constriction can be demonstrated by means of challenge testing (Sovijärvi et al. 1993). We assessed asthmatics in whom duration of symptoms had been fairly short (less than a year). It has been found in our clinic that asthma can be diagnosed somewhat late in both adults and children (Haahtela et al. 1999). Even in the early stages of asthma in the studies reported here, there was marked eosinophilic airway inflammation, which demonstrates the importance of early intervention in asthma (Haahtela et al. 1991 and 1994).

We found no correlations between duration or severity of symptoms and levels of inflammatory markers in sputum. The only symptom that correlated with sputum eosinophilia was wheezing, seen in both asthmatics and patients with respiratory symptoms suggestive of asthma. Children with just recurrent cough are at lesser risk of developing asthma than children with both cough and wheeze (Wright et al. 1996). In the study reported here recurrent cough without wheeze was present in 38% of asthmatic children. Although there were no significant differences in atopic status or baseline lung function between the children who had both cough and wheezing and children who had just cough, children in the latter group were less responsive to histamine and exhibited less eosinophilic airway inflammation. However, children with just cough met the reversibility criterion for asthma, and did not respond differently to the anti-inflammatory asthma medication used in this study from children with wheezing.

Determination of changes in numbers of eosinophils in sputum has been shown to be a potentially useful means of predicting loss of control of asthma, as reflected by loss of airway function (Jatakanon et al. 2000). However, some exacerbations appear to occur via an eosinophil-independent mechanism (in´t Veen et al. 1999). Assessment of the nature of an exacerbation could improve decisions regarding treatment.

A wide variety of relationships between clinical and functional parameters and results of measurements on induced sputum samples from patients with asthma has been described (Rosi and Scano 1999). The relationship between severity of asthma as defined by symptoms and results of lung-function tests and numbers of eosinophils in sputum is at best weak (Iredale et al. 1994, Ronchi et al. 1997). Symptoms, results of lung-function tests and bronchial hyperresponsiveness cannot predict determinations relating to cells in sputum or biochemical parameters. This demonstrates the specific role that sputum analysis can play in the assessment and monitoring of asthma.

FUTURE DIRECTIONS

Attempts have been made to devise a simplified method of sputum induction for use in epidemiological studies (Gibson et al. 1998c). The procedure needs to be simplified further if it is to be applicable in clinical practice (Metso et al. 2001). It remains to be seen whether sputum analysis could be used daily at general practitioner level. Results of sputum analysis can lead to better characterisation of asthma and COPD phenotypes, and of mechanisms underlying chronic cough. They can be used to assess or predict responses to treatments. Future studies will show whether asthma management with the aim of normalising numbers of eosinophils in sputum results in better outcomes than standard ap-

proaches, in which the aim of treatment is suppression of symptoms. (Sont et al. 1999). Analysis of induced sputum samples can help with choice of additional therapy for asthma patients with persistent symptoms who are taking inhaled steroids (Pizzichini et al. 1999b). Sputum induction could be used in relation to other airway diseases, such as tuberculosis, other infections and lung cancer, to evaluate inflammatory mechanisms and altered gene expression, for example (Fleming et al. 1999). Induced sputum samples can also be used to obtain airway cells for *in vitro* culture, allowing study of their functional and phenotypic characteristics (Hamzaoui et al. 2000).

SUMMARY

The aim in study I was to assess safety of sputum induction in patients with COPD of different severities. The subjects were 28 smokers with a mean baseline FEV_1 of 1.8 L (53% of the predicted) and mean reversibility of 2.5%. After pre-medication with 200 µg of salbutamol, sputum was induced using increasing concentrations of saline in an ultrasonic nebulizer. The procedure was well tolerated. No patient reported a major side effect. However, the mean change during induction from FEV_1 before bronchodilator administration was -8.5% and from the FEV_1 after bronchodilator administration -10.7%. Three subjects (11%) exhibited falls of more than 20% from baseline FEV_1. A further 10 (36%) experienced falls of 10 to 20%. Patients with the greatest reversibility of airway obstruction benefited most from bronchodilator pre-treatment because a statistically significant inverse relationship between reversibility in FEV_1 and fall in FEV_1 during induction was found.

In study II, sputum was induced in 21 COPD patients with a mean FEV_1 of 1.6 L (54% of the predicted) and 16 healthy controls. Success rates, safety of the method, reproducibility of cell counts and differences in cell counts were investigated in both groups. All subjects produced adequate sputum samples. Induction did not alter spirometric values. Marked sputum neutrophilia was noted in patients with COPD. Both within-investigator and between-investigator cell-count reproducibility were high for major cell types. In patients with COPD, an inverse correlation was noted between percentages of neutrophils and FEV_1 values. Immunostaining revealed very few lymphocytes.

In study III, we tested the hypothesis that eosinophilic airway inflammation is present in many patients who present with respiratory symptoms suggestive of asthma but normal lung function. Thirty-six consecutive patients of this kind were studied. Twenty-five asthmatics and 43 healthy volunteers served as controls. Signs of eosinophilic inflammation were studied in blood and induced sputum samples. Patients with respiratory symptoms were treated on a single-blind basis with inhaled beclomethasone dipropionate (BDP) (800 µg daily) or with placebo for three months. They were re-examined at three months and one year. Patients with respiratory symptoms had higher numbers of blood and sputum eosinophils than healthy individuals but degrees of eosinophilic inflammation were less than in asthmatics. Three months of treatment with BDP significantly reduced total symptom scores, cough scores, and numbers of eosinophils in blood. For cough, the improvement was significantly greater than that achieved with placebo. Of 31 patients followed-up for one year, 17 (55%) still exhibited symptoms but had normal lung function. Four patients (13%) had developed asthma. Ten (32%) had become symptom-free.

Study IV took the form of a double-blind randomised study in 60 children, five to 10 years of age, with newly diagnosed asthma Thirty-nine children received budesonide (800 µg/day) for one month, 400 µg/day for five months, and budesonide (n = 19) (200 µg/day) or placebo (n = 20) for 12 months. Patients in a third group (n = 21) were treated with disodium cromoglycate (30 mg/day) for 18 months. Asthma exacerbations in all groups were treated with budesonide (800 µg/day) for two weeks. At baseline, a control group of 17 healthy children was studied. Diaries were used to record respiratory symptoms and lung function throughout the study. Airway inflammation was assessed by examination of induced sputum samples. At baseline, the asthmatic children had more eosinophils in blood and sputum than the controls, and more metachromatic cells in sputum.

Eosinophilic inflammation was most pronounced in children with atopic asthma. After six months of treatment, the children who had been treated with budesonide had had significantly fewer exacerbations than those treated with cromoglycate. Treatment with budesonide significantly improved lung function, decreased symptoms, use of rescue medication and numbers of eosinophils in sputum. Regular treatment with inhaled budesonide for 18 months resulted in further improvements in lung function and symptoms but the changes were not significantly different from those in the other two groups.

In study V, using simplified methods of sputum induction and processing of sputum samples, we determined whether eosinophilic airway inflammation was associated with prolonged cough. Eighty-two patients who had had cough for more than one month were enrolled into the study, from six primary-health-care centres. Patients with known pulmonary disease, including asthma and COPD, whose cough was known to have another cause, or who had recently suffered a respiratory infection were excluded. Fifty-three healthy individuals served as controls. Sputum production was induced by inhalation of 3% saline. Inflammatory cells in smears were studied semi-quantitatively. Concentrations of eosinophil cationic protein (ECP), eosinophil peroxidase (EPO), myeloperoxidase (MPO) and human neutrophilic lipocalin (HNL) were determined. Sputum induction proved safe and adequate samples were obtained from 91%. Sputum eosinophilia (eosinophils accounting for more than 5% of all cells in smears) was present in 14 patients with prolonged cough (19%) but in no healthy individual. Five of the 14 individuals (36%) who exhibited sputum eosinophilia appeared to have asthma, while nine of the 14 (64%) did not. Concentrations of ECP and EPO were higher in patients with prolonged cough than in healthy individuals.

CONCLUSIONS

- Sputum induction by inhalation of hypertonic saline can result in bronchial obstruction. Our results highlight the importance of pre-treatment with bronchodilators and monitoring of lung function during sputum induction. If appropriate methods are used sputum induction is a safe and reproducible method allowing to study airway inflammation in various respiratory diseases.

- Marked sputum neurophilia is present in patiens with COPD. However, there is a subgroup of COPD patiens with eosinophilic airway inflammation.

- Patients exist who have symptoms suggestive of asthma and signs of eosinophilic bronchial inflammation but no significant airflow limitation. Such patients respond to treatment with inhaled steroid. The condition from which they suffer has no agreed definition or diagnostic criteria. Its occurrence and effects of treatments on it have not been systematically studied.

- Sputum induction can be safely and successfully used in studying airway inflammation in children with newly diagnosed asthma. Evidence of eosinophilic inflammation can be seen in induced sputum samples, especially in atopic asthmatics. Six months of treatment with inhaled budesonide controlled asthma well, decreased numbers of eosinophils in sputum and improved lung function. Regular treatment with low doses of inhaled steroid for 18 months resulted in further clinical improvement but results were equally good with periodic treatment during exacerbations only. The results may indicate a new strategy for treatment of newly detected mild childhood asthma, in which regular therapy with inhaled steroids may, if effective, be followed by treatment at intervals.

- Eosinophilic airway inflammation is a fairly common cause of chronic cough in primary care, even in patients not suffering from asthma or COPD, or in whom no other cause of cough is known to exist. Induced sputum samples obtained in health centres can be studied in central laboratories. Detection of eosinophilic airway inflammation could aid decisions regarding treatment.

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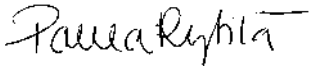

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