

Effects of cysteinyl leukotrienes and leukotriene receptor antagonists on markers of inflammation

Anthony P. Sampson, PhD,^a Emilio Pizzichini, MD, PhD,^b and Hans Bisgaard, MD^c

Southampton, UK, Santa Catarina, Brazil, and Copenhagen, Denmark

The understanding that asthma pathophysiology includes an inflammatory component has spurred the more aggressive use of anti-inflammatory therapies and created a need for effective tools to measure inflammation. Biomarkers of airway inflammation proposed are obtained by methods that are direct but highly invasive (bronchial biopsy, bronchoalveolar lavage), moderately direct, and less invasive (indirect sputum, exhaled air, breath condensate) or indirect and least invasive (blood, urine). Several studies described in this review have implicated the cysteinyl leukotrienes (CysLTs) as inflammatory mediators in a wide range of diseases, implying that their biological activities reach far beyond acute bronchoconstriction, the activity traditionally ascribed to them. The validity of examining sputum for "biomarkers" has improved the understanding of asthma pathophysiology, optimization of asthma treatment and management, and investigation of the relation between CysLTs and airway inflammation in asthma. Nitric oxide is also a surrogate marker of asthma and reflects airway inflammation. The anti-inflammatory effects of the leukotriene receptor antagonists and the markers of their activity continue to grow. (*J Allergy Clin Immunol* 2003;111:S49-61.)

Key words: Airway inflammation, cysteinyl leukotrienes, leukotriene receptor antagonists, biomarkers of inflammation, nitric oxide

For a long time, measures of pulmonary function were the mainstay of asthma diagnosis and treatment, reflecting the traditional view of asthma as a disease of airway smooth muscle. Such objective measures of asthma control show relatively poor relationships to subjective assessments. Thus, outcome measures such as the use of β_2 -agonists, symptom scores, and global evaluations are now more commonly included in current clinical trials than in the past. Furthermore, the understanding that asthma pathophysiology includes an inflammatory component has spurred the more aggressive use of anti-inflammatory therapies.

Therefore, it seems logical that indexes of airway inflammation should be included as an outcome to docu-

Abbreviations used

BAL: Bronchoalveolar lavage
CysLTs: Cysteinyl leukotrienes
ECP: Eosinophil cationic protein
5-LO: 5-Lipoxygenase
FeNO: Nitric oxide in exhaled air
FVC: Forced vital capacity
ICS: Inhaled corticosteroids
iNOS: Inducible nitric oxide synthase
LTRAs: Leukotriene receptor antagonists
NO: Nitric oxide
NOS: Nitric oxide synthase
NSAIDs: Nonsteroidal anti-inflammatory drugs
PAF: Platelet aggregating factor
PEF: Peak expiratory flow

ment the presence, type, and severity of airway inflammation to monitor the airway inflammatory processes and to investigate the cause of exacerbations and the effects of therapy. The need for effective tools to measure inflammation was initially accomplished with fiberoptic bronchoscopy to obtain tissue from the proximal bronchi or to perform a bronchoalveolar lavage (BAL) and obtain cells residing in the airway lumen and molecular mediators.¹ More recently, research has emphasized less invasive techniques for examining markers of airway inflammation such as induced sputum² and exhaled air.³

The biomarkers of airway inflammation that have been studied are both direct and indirect measures and require varied levels of invasiveness: The most direct but highly invasive are endobronchial biopsies and BAL.¹ The most indirect and least invasive come from urine and blood. In between are induced sputum,⁴ exhaled air,³ and breath condensate.

Cysteinyl leukotrienes (CysLTs) have traditionally been viewed as potent bronchoconstrictors implicated in acute airway narrowing after exposure to allergic and other stimuli. This perspective has been validated by numerous clinical trials of oral leukotriene receptor antagonists (LTRAs).⁵ The CysLTs also have chronic roles in other aspects of airway inflammation as well, particularly in recruiting inflammatory leukocytes and in vascular responses. In addition, because it has been recognized that chronic airway remodeling plays a role in long-term asthma outcomes, measures of remodeling are needed that can be obtained in clinical settings as well as in research.

From ^athe Respiratory Cell and Molecular Biology Divisions at Southampton General Hospital, Southampton, UK; ^bthe Department of Respiriology, University of Santa Catarina, Santa Carina, Brazil; and ^cthe Department of Pediatrics, Copenhagen University Hospital, Copenhagen, Denmark.

Reprint requests: Anthony P. Sampson, PhD, Dermatopharmacology Unit, Mail Point 825, Level F, South Block, Southampton General Hospital, Southampton SO16 6YD, UK.

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TABLE I. Diseases in which CysLTs have been implicated by detection of enhanced urinary LTE₄ levels

Acute myocardial infarction
Acute severe asthma
Adult respiratory distress syndrome
Anaphylaxis
Atopic dermatitis
Chronic liver disease in neonates
Crohn's disease
Cystic fibrosis
Dublin-Johnson syndrome
Hepatorenal syndrome
Kawasaki vasculitis
Kwashiorkor
Mevalonate kinase deficiency
Mountain sickness
Nocturnal asthma
NSAID-intolerant asthma
Psoriasis
Rheumatoid arthritis
Surgical trauma (CABG)
Systemic lupus erythematosus
Unstable angina
Upper respiratory tract infection in infants

Adapted from Westcott et al. 1998.

This review will focus first on evidence that CysLTs are implicated in the chronic airway inflammation in asthma and that treatment with oral LTRAs has anti-inflammatory effects. This will include evidence of the validity of using sputum to better understand asthma pathophysiology and to optimize asthma treatment and management. Finally, it will examine the role of nitric oxide (NO) as a surrogate marker of asthma, how it reflects airway inflammation, and the benefits of treatment with anti-inflammatory drugs, including LTRAs.

BLOOD AND URINE

The advantages of relatively noninvasive measurements of blood or urine include the ability to make measurements repeatedly in individual patients. Their principal drawback is that most of the inflammatory cells and important molecules never emerge from the inflamed airways into the systemic circulation in a way that provides any meaningful indication of the inflammatory processes occurring in the lung. The precise tissue and cellular origins of downstream markers can only be surmised, and their dilution, metabolism, and intermixing within large volumes of blood or urine sometimes creates insurmountable problems of assay sensitivity and reproducibility.

CYSTEINYL LEUKOTRIENES IN INFLAMMATION

Urinary LTE₄ nevertheless has been used extensively as a marker of whole-body CysLT production.⁶ Several studies have implicated CysLTs as inflammatory mediators in a wide range of inflammatory conditions including respiratory diseases other than asthma, skin diseases,

immune disorders, and cardiovascular diseases (Table I). Some of these studies were completed in the 1980s with relatively basic methodologies. The wide spectrum of diseases in which CysLTs are implicated does suggest that their biological activities reach far beyond acute bronchoconstriction.

Nevertheless, numerous bronchoprovocation studies in susceptible subjects have shown significant acute increases in urinary LTE₄ after challenges including allergen, exercise/cold air, nonsteroidal anti-inflammatory drugs (NSAIDs), SO₂, and platelet aggregating factor (PAF).⁶ Compounds that induce comparable bronchoconstriction but do not cause leukocyte activation, such as histamine and methacholine, do not lead to increases in urinary LTE₄. Increases in urinary LTE₄ during the early response to inhaled allergen did not correlate with the concomitant fall in pulmonary function except when bronchial reactivity also was factored into the equation.⁶ Treatment with β_2 -agonists, or with anti-inflammatory drugs including oral or inhaled corticosteroids (ICS), sodium cromoglycate (cromolyn), or LTRAs themselves, does not reduce urinary LTE₄ levels. Circumstantial evidence suggests that urinary LTE₄ does not reflect airway inflammation (Table II).

Classically, the late response to inhaled allergen is regarded as a simple model of chronic inflammation. Clinical trials with LTRAs clearly showed significant inhibition of at least 50% of the area under the curve of the late response, but elevated CysLT levels have been difficult to detect during this phase. In one study, modest elevations in urinary LTE₄ levels were in the late response associated with increases in urinary metabolites of histamine and prostaglandin (PG)D₂, but not eosinophil protein X (EPX).⁷ In clinical asthma, a reversible and significant rise in urinary LTE₄ was observed in adults with asthma who had acute severe exacerbations.⁸ Increased urinary LTE₄ has been more difficult to demonstrate in mild-to-moderate persistent asthma, with only one study showing a difference compared with normal subjects.⁹ Urinary LTE₄ is slightly elevated in nocturnal asthmatic patients compared with patients without nocturnal exacerbations and with normal subjects, and the nocturnal rise in LTE₄ correlated weakly with the morning dip in peak expiratory flow (PEF).¹⁰ The European Network for Understanding Mechanisms of Severe Asthma (ENFUMOSA) study reported particularly high LTE₄ levels associated with high levels of the eosinophil activation marker EPX in the urine of severe uncontrolled asthmatic subjects compared with stable patients. Asthmatic patients intolerant of NSAIDs consistently demonstrated elevated baseline levels of urinary LTE₄ compared with other asthmatic or normal subjects, with further increases after NSAID exposure.¹¹ Overall, urinary LTE₄ shows broad associations with the severity of persistent asthma but usually no simple correlations with objective measures of pulmonary function, bronchial responsiveness, or anti-inflammatory therapy; thus, its value as a biomarker of inflammation is limited.

Measurements of CysLTs in blood are fraught with technical difficulty: LTC₄ and LTD₄ are undetectable, levels of LTE₄ are theoretically very low, and extensive purification of samples is required.⁶ Increases in blood LTE₄ have been described in acute severe asthma in children, but large differences between the results from different laboratories cast doubt on the validity of this approach.

ANTI-INFLAMMATORY EFFECTS OF LTRAs

Objective measures of pulmonary function and subjective measures including symptom scores, medication use, and quality of life have shown significant therapeutic benefits of oral LTRA therapy in asthma.⁵ Much less information is available on the impact of LTRAs on airway inflammation. The most promising studies suggesting anti-inflammatory effects of LTRAs involved leukocyte counts in sputum, BAL fluid, bronchial biopsies, and NO in exhaled air.

With respect to blood, the strongest data come from eosinophil counts in the blood of asthmatic patients in clinical trials of oral LTRAs, mostly during a period of 8 to 12 weeks. In contrast to improvements in pulmonary function, which occurred within hours, blood eosinophil counts fell linearly over a period of 8 to 12 weeks of LTRA therapy in adults and children.^{12,13}

A meta-analysis of 7 clinical trials with montelukast in adults and children with asthma demonstrated, however, that reductions in blood eosinophils averaged only 19% from baseline (Fig 1) compared with analogous reductions in biopsy specimens, BAL fluid, and sputum of about 40% to 75%. The relative lack of sensitivity of blood eosinophils to LTRA therapy makes it unlikely that this relatively noninvasive approach will be useful in monitoring the anti-inflammatory efficacy of LTRAs in asthma management.

A humanized monoclonal anti-IL-5 antibody depleted eosinophils in the blood of asthmatic patients without affecting their responses to inhaled allergen challenge or nonspecific bronchial responsiveness, further degrading blood eosinophil counts as markers of inflammatory processes within the bronchial mucosa.¹⁴

NOVEL BLOOD-DERIVED BIOMARKERS OF LTRA ACTIVITY

Other potential biomarkers of inflammation accessible in blood might include autacoid mediators and their metabolites (histamine, PGD₂, tryptase, eosinophil cationic protein [ECP], major basic protein), plasma cytokine concentrations, expression of cytokines, adhesion molecules, and other surface markers (including CysLT₁ receptors) in blood leukocytes ex vivo and ex vivo leukocyte chemotaxis or apoptosis. To date, few studies of these markers have been reported in clinical asthma, although LTRAs modify at least some of these processes in vitro and in animal models.¹⁵ Allergen-stimulated synthesis of T_H2 cytokines (interleukin [IL]-4, IL-5, and granulocyte macrophage-colony stimulating fac-

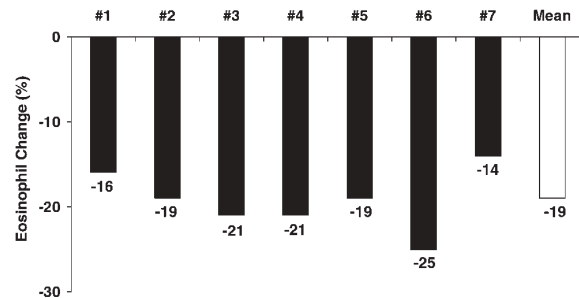


FIG 1. Percent change in blood eosinophil counts after treatment of asthmatic adults and children with oral montelukast. Studies included are (1) Altman et al. *J Allergy Clin Immunol* 1998;102:50-6; (2) Noonan et al. *Eur Respir J* 1998;11:1232-9; (3) Malmstrom et al. *Ann Intern Med* 1999;130:487-95; (4) Reiss et al. *Arch Intern Med* 1998;158:1213-20; (5) Leff et al. *N Engl J Med* 1998;339:147-52; (6) Pizzichini et al. *Eur Respir J* 1999;14:12-8; (7) Knorr et al. *JAMA* 1998;279:1181-6.

TABLE II. Responses of urinary LTE₄ levels to exogenous stimuli and drug therapies

Increased by	Decreased by	Unaffected by
Allergen	Leukotriene synthesis inhibitors	Methacholine
Exercise/cold air	Fish oil	Histamine
NSAIDs	CSF	LTRA
GM-CSF	Vitamin E	Adenosine
PAF	Glutathione S-transferase deficiency	β ₂ -agonists
SO ₂		Corticosteroids
Smoking		Cromones

tor [GM-CSF]) by blood mononuclear cells ex vivo has been shown to be reduced in asthmatic patients treated with pranlukast.¹⁶ Such studies provide valuable new insights into the biological activities of CysLTs, but the complexity of the required experimental approaches makes them unlikely candidates as clinical biomarkers in the foreseeable future. Crucially, the plethora of inflammatory processes in the asthmatic airway means that no single biomarker is likely to provide a clinically useful tool for monitoring the overall level of airway inflammation for diagnosis or in response to treatment. Instead, novel biomarkers, both immunologic and genetic, may help to delineate subgroups of asthmatic subjects in which specific inflammatory anomalies can be targeted with specific anti-inflammatory therapies.

GENETIC MARKERS OF LTRA RESPONSIVENESS

The relative efficacy of LTRA therapy in different patients is likely to depend, at least in part, on the capacity of their airway tissues to synthesize CysLTs, although other factors, including airway sensitivity to these mediators, are also likely to be important. Variant forms of a number of the genes that encode leukotriene pathway proteins have been described, of which a promoter poly-

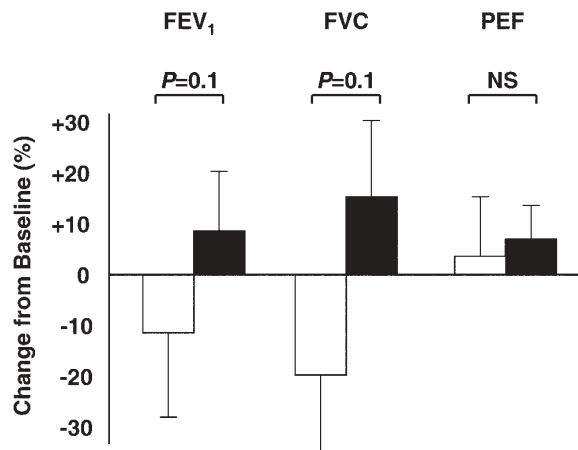


FIG 2. LTC₄ synthase genotype and pulmonary function response to zafirlukast in patients with severe asthma. Pulmonary function responses to 2 weeks of treatment with oral zafirlukast (20 mg twice daily for 2 weeks) in 23 persons with chronic severe asthma, stratified by their wild-type (AA) (white bars, n = 10) or mutant (AC/CC) (black bars, n = 13) genotype for the LTC₄ synthase A-444C promoter polymorphism. From Sampson et al. *Thorax* 2000;55(Suppl 2):S28-31.

morphism in the LTC₄ synthase gene is the most common.¹⁷ This consists of a substitution of cytosine for adenosine at a position 444 bases upstream of the transcription initiation site (A₄₄₄C) at a site that may affect the probability that the enzyme is expressed in inflammatory cells. The prevalence of the variant genotypes with 1 or 2 of the mutant C₄₄₄ alleles is approximately 40% in the normal population; one study found a higher prevalence (75%) in Polish patients with NSAID-intolerant asthma,¹⁷ whereas other researchers found no difference in US and Japanese populations.¹⁸

Irrespective of prevalence, however, the variant LTC₄ synthase C₄₄₄ allele may cause a propensity to overproduce CysLTs in the subgroup of individuals expressing it. In normal human eosinophils in vitro, LTC₄ synthesis after ionophore stimulation is 3-fold higher in cells from subjects with 1- or 2-variant LTC₄ synthase alleles compared with those with 2 wild-type alleles.¹⁸ Furthermore, in 317 mild-to-moderate asthmatic patients, basal pulmonary function of FEV₁ was significantly lower in patients with the variant LTC₄ synthase genotypes (92.7% of predicted) than in patients with the wild-type genotype (FEV₁ 97.5% predicted, $P = .004$), and methacholine bronchial responsiveness was higher in the mutant group.¹⁹ Thus, the LTC₄ synthase polymorphism may determine much of the impairment of baseline pulmonary function and bronchial hyperreactivity in patients with mild-to-moderate asthma.

In an open study in 23 persons with chronic severe asthma, their pulmonary function responses to 2 weeks of treatment with oral zafirlukast (20 mg bid) were compared with prestudy baseline values. In 13 patients with variant LTC₄ synthase genotypes, the postzafirlukast improvements in FEV₁ (+9%) and forced vital capacity (FVC) (+15%) tended to be greater than in the 10

patients with wild-type genotypes (falls of -12% and -18%, respectively, $P = .1$),²⁰ suggesting that the LTC₄ synthase genotypes can predict the responses of pulmonary function to LTRA therapy (Fig 2). This outcome has been confirmed by a larger study investigating responses to 4 weeks of treatment with oral pranlukast in 45 patients with mild-to-moderate asthma.²¹ Mean FEV₁ improved by only 2.8% in wild-type patients, but those with the variant LTC₄ synthase genotypes showed an improvement of 12.4% ($P = .05$), with half of the patients increasing their FEV₁ by at least 10% ($P = .03$). Univariate analysis showed that pranlukast responders and nonresponders were not predictable on the basis of clinical demographics (age, sex, serum immunoglobulin E [IgE], atopic status), although patients with poorer pulmonary function tended to respond better to pranlukast and to salbutamol than those with better pulmonary function. Intriguingly, pranlukast response was not predictable on the basis of blood eosinophil counts or urinary LTE₄ levels, strongly supporting the evidence already described that such inflammatory biomarkers would be of little value in monitoring LTRA efficacy. Most importantly, the variant LTC₄ synthase genotype was a significant predictor of pranlukast response with a multivariate analysis, indicating that patients with 1 or 2 mutant LTC₄ synthase alleles were 8-fold more likely than the wild-type genotype to show a significant (> 10%) improvement in FEV₁.²¹

SPUTUM AND BRONCHOALVEOLAR LAVAGE

Origin of sputum

Sputum is defined as expectorated lower respiratory secretions.²² Results of studies using fractional analysis of sequential samples of induced sputum, bronchial wash lavage, BAL, or bronchial segmental lavage performed at different depths within the airways suggest that induced sputum samples inflammatory cells and soluble markers present in the airway lumen of the proximal airways, although prolonged induction can also sample the more distal parts.²³ In contrast, BAL samples more peripheral airways and the alveolar compartment.¹ Therefore, differences between induced sputum and BAL are expected. Induced sputum is richer in neutrophils, whereas BAL is richer in macrophages and lymphocytes. Another important difference is the lower and variable concentration of soluble markers in the supernatant of BAL fluid. The concentration of various markers is severalfold higher in sputum than in BAL fluid. These differences may indicate a complementary role for these methods in assessing airway inflammation.²⁴

One of the most important advances for studying airway inflammation was the introduction in 1992 of sputum induction to obtain airway secretions in asthma directly by the inhalation of an aerosol of hypertonic saline.²⁵ This noninvasive method has a number of advantages over invasive methods: It is safe, and the methods of inducing and processing sputum have pro-

vided a valid and repeatable method of assessing airway inflammation.²⁶ In addition, sputum induction can be applied randomly in severe asthma and repeatedly in the same subject. It is also suitable for use in large-scale clinical studies. Furthermore, indexes of inflammation have been shown to discriminate different conditions and respond to intervention. Therefore, it is not surprising that the examination of induced sputum has now become the most clinically applicable method for assessing airway inflammation directly.

Biomarkers in induced sputum

Induced sputum can be used to examine cells in cytopins or flow cytometry and several fluid-phase markers. Immunocytochemistry or special stains (trypan-blue, hemosiderin staining, oil-red-O) can further stain the cells on cytopins. The fluid-phase measurements enable the study of adhesion molecules, cytokines and tachykinins, microvascular leakage markers (fibrinogen, α_2 -macroglobulin, and albumin levels), mucus secretion (mucin-like glycoprotein), and several other mediators of inflammation and airway smooth muscle constriction such as eosinophilic and neutrophilic proteins and leukotrienes.²⁷ Cell counts have been used chiefly to study the effects of drugs. As with any new method, emphasis initially has been placed on evaluating the procedures and measurements. The relevance and accuracy of measurements of some soluble phase markers (particularly cytokines) have not yet been fully appraised and understood, however.²⁸

Three models, using induced sputum markers, have been applied in clinical research to study the anti-inflammatory effects of drugs on airway inflammation. These models are (1) uncontrolled asthma, (2) allergen inhalation, and (3) treatment reduction (Fig 3).²⁹ Because the investigation of the anti-inflammatory effects of a drug requires the presence of inflammation in the airways, the signal most frequently used in any of these models is sputum eosinophilia because it is discriminative and responsive to interventions. Although not exclusively characteristic of asthma, sputum eosinophils distinguish asthma from other conditions and discriminate different levels of asthma severity (Fig 4). Sputum eosinophils are also more accurate than sputum ECP, blood eosinophils, and serum ECP.³⁰ They are a marker that easily can be studied longitudinally.³¹

Uncontrolled asthma

Subjects with uncontrolled asthma have active inflammation (sputum eosinophilia), and the reversal of this inflammation by an anti-inflammatory drug can be studied. Up to 40% of the symptomatic, uncontrolled asthmatic subjects may not have an increase in sputum eosinophils, however.³² Recent data suggest that glucocorticoid treatment helps those patients with eosinophilic bronchitis but not those without sputum eosinophilia in asthma,³³ chronic cough,^{34,35} or chronic obstructive pulmonary disease.³⁶ In subjects with severe exacerbations of asthma,³¹ treatment with prednisone reversed the spu-

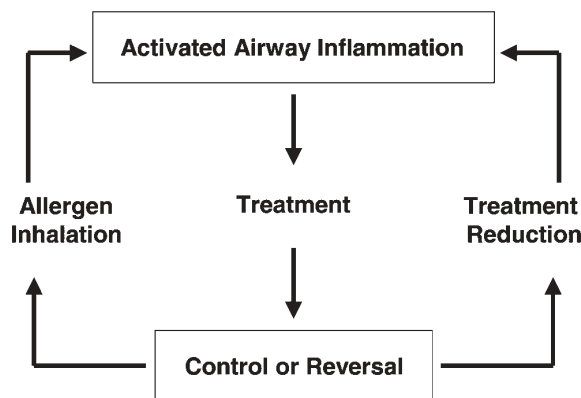


FIG 3. Diagram showing 3 models to investigate the anti-inflammatory effects of intervention using examination of induced sputum. Activated airway inflammation can be presented spontaneously (uncontrolled asthma) or induced by an allergen challenge or by a controlled reduction of therapy.

tum eosinophilia within 1 week. The kinetics of different biomarkers' responses are unique, however.

The uncontrolled asthma model has also been used to investigate the anti-inflammatory effects of salmeterol or montelukast. For example, patients with a mild exacerbation of their asthma characterized by sputum eosinophilia were randomly assigned to a 3-week treatment with either salmeterol (100 μ g/d), beclomethasone (1000 μ g/d), or placebo.³⁷ When compared with placebo, salmeterol improved symptoms and airway function but had no effect on the eosinophilia, whereas beclomethasone improved both clinical outcomes and suppressed sputum eosinophilia.

Allergen inhalation

Allergen inhalation by sensitized atopic asthmatic subjects results in an increase in airway inflammation, bronchoconstriction, and subsequent airway hyperresponsiveness. This model is used to investigate the protective effect of different drugs on allergen-induced late response and airway inflammation. For example, this model was able to show that pretreatment with a single dose of formoterol³⁸ and salmeterol³⁹ abolished the late asthmatic response and heightened methacholine airway response at 24 hours but had no effect on the allergen-induced eosinophilic bronchitis. In contrast, pretreatment with a single dose of beclomethasone (500 μ g) partly inhibited the late asthmatic response but did not inhibit the early asthmatic response or the allergen-induced eosinophilic bronchitis at 24 hours. The latter was inhibited, however, by inhaled budesonide (400 μ g/d) by Turbuhaler given for 1 week before the allergen inhalation.⁴⁰

Steroid reduction

This model can be used to identify overtreatment with inhaled steroids, minimum requirements of steroid treatment, or the steroid-sparing effects of different drugs.⁴¹ Patients requiring glucocorticoids to control their asthma can be followed with the addition of the test drug during

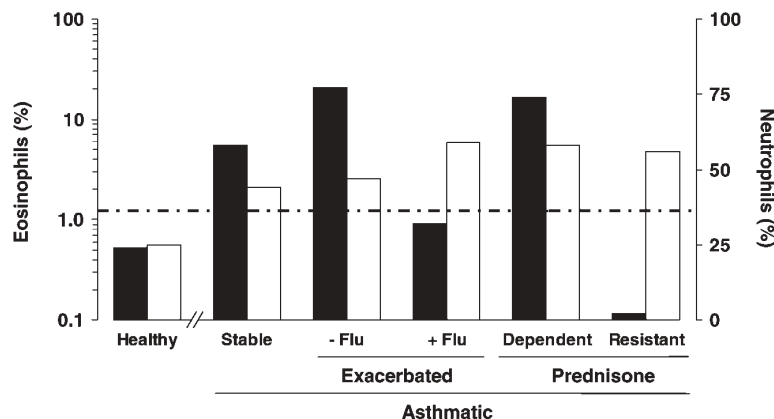


FIG 4. Sputum cell counts for eosinophils (black bars) and neutrophils (white bars) in healthy subjects and in subjects with asthma of varying severities. Results are the mean values from the results of several studies. Dashed line represents the upper limit (+90% CI) of the mean for eosinophils from healthy subjects, 1.1%. Higher proportions of eosinophils and neutrophils were measured in asthmatic patients compared with healthy subjects.

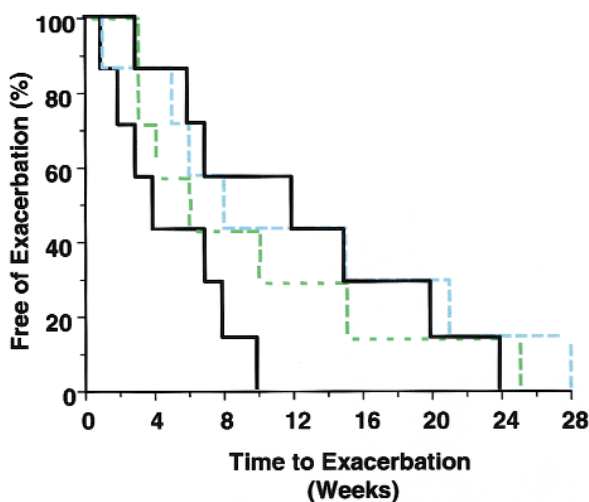


FIG 5. Time course of effects of prednisone reduction on markers of airway functional inflammation. Blue indicates symptoms; gray, FEV₁; green, blood eosinophils; black, sputum eosinophils. Adapted from data in Pizzichini et al. Eur Respir J 1999;13:15-21.

steroid reduction. In one double-blind, 2-phase, crossover, placebo-controlled study, McIvor et al⁴² applied a stepwise steroid-reduction to allow inflammation to increase gradually and compared the effects of added salmeterol with placebo.⁴² They assessed the steroid-sparing and masking effects of salmeterol (50 µg bid) versus matching placebo in 13 asthmatic persons who were initially well controlled with a high-dose inhaled steroid regimen (1500 µg beclomethasone daily). The results showed that corticosteroid was reduced further during salmeterol treatment than with placebo. The regular use of salmeterol, however, masked the deterioration of airway inflammation as detected by increasing sputum eosinophilia and delayed the awareness of an exacerbation judged by usual clinical and pulmonary function criteria.

This model also allowed for the investigation of the kinetics of changes in sputum and clinical parameters. In a group of patients with prednisone-dependent asthma, their glucocorticosteroids were reduced to produce a mild exacerbation of their asthma.⁴³ Clinical parameters deteriorated much later than the increased eosinophilia in the sputum (Fig 5). In contrast, additional treatment with prednisone resulted in the improvement of clinical outcomes before complete resolution of airway inflammation. Using this model in patients with less severe asthma, sputum eosinophilia has been shown to predict the loss of asthma control or asthma exacerbations.⁴⁴

CYSTEINYL LEUKOTRIENES AND AIRWAY INFLAMMATION

The CysLTs are not only one of the most important mediators causing exercise-induced bronchoconstriction, but they are, to a large degree, responsible for bronchoconstriction that occurs during the early and late responses after allergen inhalation. In addition, they are involved in allergen-induced airway inflammation.

The study of the relation between CysLTs and airway inflammation in asthma has been performed chiefly by using allergen inhalation models, inhalation of the mediators or, indirectly, by blocking the effect of the mediator using receptor antagonists and leukotriene synthesis inhibitors. These studies used bronchoscopy before and after intervention. The results have shown that inhalation of LTE₄ increased the number of eosinophils and neutrophils in the lamina propria of airway mucosa⁴⁵ and that treatment with pranlukast decreased the number of bronchial inflammatory cells in asthma.⁴⁶ With the use of segmental bronchial lavage, the effects of allergen on leukotriene generation and inflammation were examined within the airways of allergic asthmatic patients: The effects of the 5-lipoxygenase (5-LO) inhibitor zileuton were evaluated.⁴⁷ After a segmental allergen chal-

lenge, those subjects who generated an increase in CysLTs had more eosinophils and inflammatory cytokines (IL-5, IL-6, and tumor necrosis factor- α) in their BAL fluid and responded to treatment with a significant decrease in sputum eosinophils and the cytokines. Antileukotriene treatment may be more effective in these asthmatic subjects.

The role of CysLTs in asthma was recently reported in a study that measured CysLTs in induced sputum from asthmatic and control subjects.² The concentration of CysLTs was significantly higher in asthmatic subjects, with more persistent asthma and in those seen within 48 hours of an exacerbation. Allergen challenge significantly increased the concentration of CysLTs and correlated with an increased sputum eosinophilia 24 hours after challenge, suggesting that allergen-induced airway inflammation could, in part, be caused by the release of CysLTs.⁴⁸

More recently, inhaled LTE₄ but not LTD₄ was shown to increase sputum eosinophilia significantly at 7 and 24 hours and sputum basophils only at 7 hours.⁴⁹ The accumulation of sputum eosinophils caused by the LTE₄ inhalation was relatively small, however, when compared with the effect of allergen inhalation. Furthermore, inhalation of LTE₄ did not result in the development of late responses or methacholine airway hyperresponsiveness, suggesting that although CysLTs are important in allergen-induced bronchoconstriction and accumulation of airway eosinophils, they are not the only proinflammatory mediators involved in the late response.

A study using induced sputum in 12 patients with mild asthma⁵⁰ found that 3 doses of montelukast (10 mg administered 36 hours and 12 hours before and 12 hours after allergen) significantly inhibited the early and late asthmatic responses compared with placebo; they did not, however, decrease allergen-induced sputum eosinophilia nor the concentrations of sputum ECP. Inadequate dosing or additional mechanisms leading to eosinophilia after allergen challenge may explain these results.

A randomized, double-blind, placebo-controlled, parallel-group study examined the effects of 4 weeks of LTRA treatment on sputum cell counts in 40 adults with mildly uncontrolled asthma and sputum eosinophilia.⁵¹ Montelukast significantly reduced the mean sputum eosinophil count from 7.5% to 3.9%. This was paralleled by an improvement in asthma symptoms, β -agonist use, and morning PEF. In contrast, placebo treatment was associated with an increase in sputum eosinophils from 14.5% to 17.9%. These results suggested an anti-inflammatory effect of montelukast.

Finally, the anti-inflammatory effect of pranlukast, another LTRA, was examined in 32 subjects with mild-to-moderate asthma during a 4-week trial.⁵² Pranlukast decreased symptoms and sputum eosinophils. Although the results of these and other studies have shown a benefit of LTRAs on airway inflammation, additional studies are required to compare the anti-inflammatory effects of LTRAs with that of ICS.

Nitric oxide

Nitric oxide is a freely diffusible gas that functions as a mediator. It was first seen as a signaling molecule in the cardiovascular system but has since been shown to perform critical functions in the central and peripheral nervous system and in inflammatory cells. NO is derived from L-arginine by the enzyme NO synthase (NOS), of which at least 3 isoforms exist.⁵³ The isoforms NOS1 and NOS3 are dependent on intracellular Ca²⁺ concentration, whereas the third isoform, inducible NOS (iNOS or NOS2), is independent of Ca²⁺ concentration and is induced by inflammatory cytokines.⁵⁴ All 3 isoforms are expressed in the normal airway epithelium, whereas airway epithelial cells from asthmatic subjects have shown increased expression of iNOS probably induced by proinflammatory cytokines.^{55,56} Very high expression of mRNA for iNOS has been found in lower airways⁵⁷ and airway epithelial cells from asthmatic patients.⁵⁵ Thus, it seems likely that the respiratory epithelium is an important source of exhaled NO. Furthermore, it seems to originate primarily in the conducting airways, and airway concentrations peak before carbon dioxide has reached its maximum level.⁵⁸ Furthermore, iNOS may also be expressed in other cells of the airway, such as macrophages, eosinophils, and other inflammatory cells.⁵⁹ The exact functional role of NO in asthma is uncertain, but its presence has been linked to airway hyperresponsiveness in animal models of allergic asthma.⁶⁰

Nitric oxide itself diffuses through cell membranes and targets guanylate cyclase to upregulate intracellular cyclic guanine monophosphate (cGMP), which mediates specific effects in the cell. NO in exhaled and inhaled air is considered to be of little physiologic significance, since it rapidly binds to hemoglobin. In high local concentrations, NO may cause bronchodilation, affect ciliary activity, and provide antimicrobial effects.⁶¹

NO is used therapeutically for its vasodilator properties. High concentrations (parts per million) have been used successfully in pulmonary hypertension. The local release of NO from drugs such as nitroglyceride and sildenafil produces their therapeutic actions. Airway obstruction in asthma may be associated with endogenous NO deficiency, but inhaled NO did not improve pulmonary function, nor did attempts to improve its endogenous production (by administering analogues of the NO synthase substrate L-arginine) improve airway hyperresponsiveness to histamine, although the dose-response curve was slightly reduced in patients with asthma.⁶² Treatment with an NO synthase inhibitor reduced the NO in exhaled air (FeNO), but pulmonary function did not change.⁶³ The FeNO from the nose are orders of magnitude higher than FeNO from lower airways.⁶⁴ It is unknown if this peculiar difference is a secondary phenomenon or of physiologic importance.

Studies using polymorphic markers within the NOS1 gene have established a genetic association between NOS1 and the diagnosis of asthma.⁶⁵ The size of an aspartate amino transferase (AAT)_n repeat polymorphism in intron 20 of NOS1 was significantly associated with FeNO in patients with mild asthma.⁶⁶

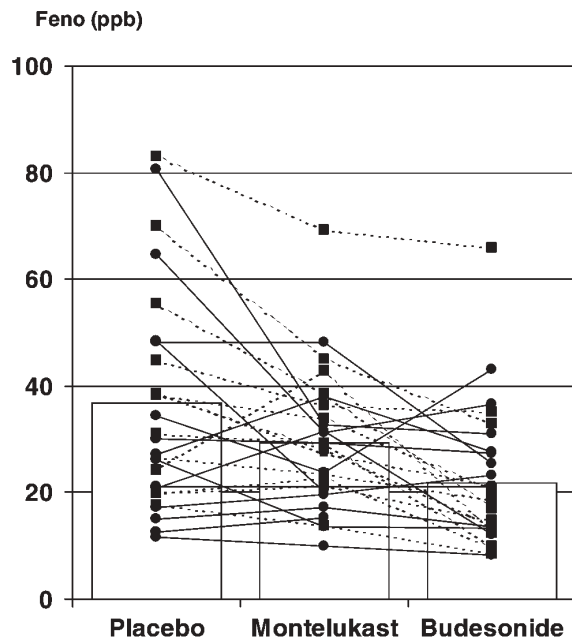


FIG 6. NO in exhaled air (Feno, in parts per billion) was significantly reduced after treatment with montelukast and budesonide as compared with placebo. Such treatment effects were independent of concurrent treatment with inhaled steroid (circles, solid lines) or no concurrent treatment with inhaled steroid (squares, dashed lines). From Bisgaard H, et al. *Am J Respir Crit Care Med* 1999;160:1227-31.

Relation of NO to other inflammatory biomarkers

Only recently has the measurement of FeNO become standardized through the access of dedicated instrumentation and the development of guidelines from the American Thoracic Society (ATS) and the European Respiratory Society (ERS).⁶⁷ On-line measurements at fixed flow rates are now increasingly recognized as the method of choice, which can be performed easily in children from 6 to 8 years of age, and adaptations for use of on-line measurements during tidal breathing in young children are appearing.⁶⁸

As a surrogate marker for asthma, FeNO has attracted considerable interest. It is measured in healthy subjects in parts per billion, whereas asthmatic subjects have several-fold increased FeNO, although with some overlap.⁶⁹⁻⁷¹ The importance of atopy is disputed with some reports finding higher FeNO in atopic than in nonatopic asthmatic persons^{72,73}; other reports show no difference between nonatopic asthmatic subjects and healthy control subjects.⁷³ Still others find no difference in FeNO between atopic and nonatopic subjects without asthma.⁷⁴ During the late but not the early phase of an allergic asthmatic response, FeNO increases.⁷⁵ It correlates with symptoms,⁷⁶ airway responsiveness,^{77,78} blood eosinophils,⁷⁹ sputum eosinophils,^{77,80} BAL eosinophils,⁸¹ and eosinophils in airway biopsies.⁸² FeNO increases during exacerbations⁸³ and during natural pollen exposure.⁸⁴ It may become a convenient method for detecting and monitoring asthma.

Correlations against established standard measurements of asthma may be a misleading approach to document a new measure of asthma disease activity. Correlations address how well two outcomes measure the same thing. No definitive measure for asthma exists, however, and all known outcomes are only surrogate markers reflecting different aspects of the disease. Even pulmonary function only reflects a certain static and mechanical aspect of airway patency and not the underlying inflammation. Pulmonary function shows poor correlation with asthma symptoms,⁸⁵ but it can still be trusted to measure that particular aspect of asthma. It is therefore difficult to prove the excellence of any new asthma outcome measure. Even though FeNO generally correlates with the above measures of symptoms and inflammatory cells, airway patency, hyperresponsiveness, and inflammation, the correlations may seem poor at times.⁸⁶ The gold standard for documenting the meaningfulness of FeNO is yet to be established.

To detect loss of control of asthma therapy in asthmatic subjects, FeNO appears to be more sensitive than standard pulmonary function testing and as sensitive as sputum eosinophil levels and bronchial hyperresponsiveness.⁸⁷ Exhaled NO was elevated in adolescents with normal pulmonary function who were in apparent clinical remission,⁸⁸ but FeNO was more sensitive than pulmonary function at detecting disease activity during pollen exposure.⁸⁴ It even seemed to be able to separate mildly asthmatic young preschool children from healthy control children.⁶⁸ In Pacific Islanders with a high risk for development of asthma, asymptomatic subjects sensitized to home dust mite have higher FeNO, and this correlated with the severity of their sensitivity.⁸⁹ Formaldehyde exposure, which is a suspected risk factor for asthma, correlated with FeNO in a group of healthy asymptomatic children.⁹⁰

EFFECTS OF LEUKOTRIENE RECEPTOR ANTAGONISTS

Leukotriene receptor antagonists have been found to reduce FeNO in asthmatic children^{91,92} (Fig 6) and adults⁹³⁻⁹⁵: FeNO fell by 15% within 2 days and eventually by 20% in 14 days.⁹¹ A 30% reduction was seen after 4 weeks of treatment in an open trial.⁹² The LTRAs controlled the usual increase in FeNO subsequent to steroid reduction,⁹⁶ and the effect on FeNO was additive to the effect of ICS.⁶⁸ The mechanism of this complementary anti-inflammatory effect of LTRAs would appear to act through the blocking of leukotrienes unchecked by steroids in the asthmatic airway inflammatory process,^{97,98} probably by blocking some of the inflammatory effects of leukotrienes. Reducing the number of airway tissue eosinophils is suspected to be one important mechanism of the anti-inflammatory effect of LTRAs. The CysLTs are specific chemoattractants to eosinophils,^{45,99} and LTRAs consistently have reduced eosinophils in blood, sputum, and airway biopsies.¹⁰⁰ Another mechanism by which CysLTs may increase FeNO is through increased microvascular blood flow¹⁰¹ and venular permeability.¹⁰²

This allows the escape of macromolecules into the tissue, providing the source for plasma protein-derived inflammation mediators, including the kinins and the complement and clotting systems fueling the inflammatory process. In one report, inhaled LTE_4 did not increase FeNO, although sputum eosinophils were increased.¹⁰³ An unusually high flow rate, however, was used in that study and likely hampered the sensitivity of the FeNO measurements. Nevertheless, the mechanism by which CysLTs increase FeNO is unknown.

CONCLUSIONS

Biomarkers of airway inflammation are obtained by methods that have varying levels of invasiveness as well as of effectiveness. Urinary LTE_4 shows broad associations with the severity of persistent asthma but usually no simple correlations with objective measures of asthma; its value as a biomarker of inflammation is limited. Blood CysLT levels are insensitive to anti-inflammatory therapy and are difficult to measure. Blood eosinophils measured over a period of 8 to 12 weeks decreased with LTRA therapy, but they are not a sensitive marker compared with more invasive measures. A variant gene for LTC_4 synthase assessed by blood genotyping may be a predictor of good response to LTRA therapy. Induced sputum is a simple, valid, reliable, and noninvasive way of examining airway inflammation, and it has been used increasingly in clinical trials of asthma. Characterization of the inflammatory effects of CysLTs and the anti-inflammatory effects of LTRAs in asthmatic airways using induced sputum may improve the understanding of these agents' role in asthma management. Finally, FeNO is a sensitive surrogate marker of concurrent allergy and airway inflammation and is therefore useful for diagnosing and monitoring subclinical asthma. Increased FeNO in asymptomatic subjects also may be an early marker of emerging asthma.

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QUESTIONS AND DISCUSSION

Redwan Moqbel: Tony, are the relatively small reductions in blood eosinophils in patients treated with LTRAs due to effects in the bone marrow or on vascular adherence in the periphery?

Anthony Sampson: Eosinophil counts in the blood reflect the net balance of their release from the bone marrow and their egress into inflamed tissues. It seems unlikely that LTRAs would enhance the adherence and migration of eosinophils into the lung, and only a small proportion of the total body population of eosinophils is within the peripheral tissues. The ability of LTRAs to reduce blood eosinophil counts is probably dependent on effects on the proliferation and/or release from the bone marrow. These processes probably have very little direct relationship to what is happening to eosinophils within the airways.

Marc Peters-Golden: Transfection experiments by Tim Bigby's group suggest that the C₄₄₄ polymorphism in LTC₄ synthase actually downregulates transcription. How is this reconciled with the clinical data?

Anthony Sampson: The effect of this polymorphism (and others) in transfection or reporter gene experiments depends on the cell system investigated. There are conflicting data in monocytic cell lines, HeLa cells, and basophilic KU812F cells. In particular, in these experiments, it is difficult to replicate the cytokine microenvironment that induces expression of the 5-LO/LTC₄ synthase pathway in maturing leukocytes in vivo. However, in blood eosinophils from aspirin-induced asthmatic persons, levels of LTC₄ synthase mRNA are elevated in patients with mutant genotypes compared to wild-type, suggesting a transcriptional effect is unmasked in vivo.¹

Marc Peters-Golden: Does body weight contribute to determining the response to LTRAs? Do leptin levels matter? My reason for bringing this up is that we found an association between leukotriene synthesis and leptin levels.² In leptin-deficient mice, lung cells underproduce leukotrienes. If leptin is added to the cells, there is increased leukotriene production. That would suggest that obese individuals would have higher leptin levels compared with malnourished individuals who would have low leptin levels. Therefore, it is possible that obesity and leptin might have an effect on the leukotriene pathway.

Anthony Sampson: Data indicating a link between leukotriene synthesis and leptin levels are fascinating, but I don't know of a study relating body weight to LTRA response.

Stephen Peters: Can the LTC₄ synthase genotyping help to resolve the confounding data that urinary LTE₄ did not relate to pranlukast response in the Asano study?

Anthony Sampson: It would certainly be interesting to reassess published work on the variability in leukotriene production and in clinical responses to LTRA in the light of the LTC₄ synthase genotyping. However, in the Asano study, neither clinical response to pranlukast nor LTC₄ synthase genotype related in any way to urinary LTE₄ levels, suggesting that urinary LTE₄ analysis is insufficiently sensitive to pick up the genetic influence on CysLT levels within the lung. In addition, we found no relationship between LTC₄ synthase genotype and urinary LTE₄ in normal subjects, although in these same subjects, ex vivo LTC₄ production by stimulated blood eosinophils did show a difference.³ Perhaps BAL fluid or sputum CysLT levels may show a relationship where urinary LTE₄ does not.

Mark Liu: How prevalent is the mutant LTC₄ synthase genotype in aspirin-sensitive asthmatic persons?

Anthony Sampson: The original paper by the Krakow group⁴ showed a higher prevalence of the mutant LTC₄ synthase genotype in aspirin-intolerant asthmatic persons from a Polish population, but Drazen's data in US white and Japanese populations⁵ indicated no difference compared with aspirin-tolerant asthmatic persons or normal persons. The polymorphism may be related to asthma severity not aspirin sensitivity, so the Sanak data may have been confounded by a higher proportion of severe patients in the aspirin-intolerant group. My view is that although the prevalence may not be different in asthmatic populations compared with normal populations, it does not predispose people to have asthma but rather it can predispose asthmatic persons to a form of the disease in which CysLTs play a disproportionate role. These asthmatic persons are probably the best responders to LTRAs.

Hans Bisgaard: Were the siblings with no genetic association between baseline FEV₁ and LTC₄ synthase genotype younger than the siblings who showed the relationship?

Anthony Sampson: The strong association was seen in a cohort (n > 300) of the eldest asthmatic sibling in each family but disappeared in the cohort of younger asthmatic siblings. However, the age range was 5 to 21 years, so in some families, the older sibling was younger (and had had asthma for a shorter time) than the younger siblings in other families. Because of the wide range of ages in the cohorts as a whole, it is unlikely that the effect is directly related to age or to duration of asthma. It seems to be a genuine birth order effect, but the mechanism is entirely unclear.

Stephen Peters: Hans, if you follow patients longitudinally from the very high NO quartile, do they have a difference in exacerbation rate than the ones of the lower quartile?

Hans Bisgaard: We don't have these data. However, there is one study ongoing in Stockholm where about 1200 children, 9 to 10 years of age, were screened. In these children, a subgroup of patients had no symptoms, no suspicion of airway disease, but very high levels of NO. After a prospective follow up, it was shown that these children were hyperreactive compared with the others. These children are being followed longitudinally, and your question will eventually be answered.

Qutayba Hamid: When you measure NO, can you tell from which compartment of the lung the NO comes?

Hans Bisgaard: There have been some studies correlating NO and CO₂, and results from these measurements suggest that you measure NO from small airways.

Jonathan Edelman: Are you satisfied with the definition that NO production is a marker of inflammation as opposed to a marker of asthma, but not necessarily related to inflammation?

Hans Bisgaard: I think it is a very sensitive marker of asthma control, which I believe is related to inflammation, but whether it is inflammation or not, I do not know. It appears to reflect clinically defined asthma control. NO correlates with eosinophils, exacerbations, lung function, and symptoms. By relating to all of these indi-

cators of asthma control, NO is very useful, but I do not know if it relates specifically to inflammation.

Mark Liu: Are there 2 components to exhaled NO, as suggested by Permutt and Silkoff⁶? One component is related to airway wall concentration of NO, and the other is related to the surface area over which NO actually contributed to the exhaled gas. There is a steroid-responsive component to the exhaled NO concentration, but the diffusing capacity (D_{NO}), which relates to the surface area over which NO is produced during exhalation, is not steroid sensitive. If the inflammatory component is eliminated, there seems to be a relation between reactivity and D_{NO}. Therefore, NO may be a good and a bad player. There is evidence that high NO may be protective of airway tone, and this extended area over which NO is produced in the lower airway may actually be protective. In people who are more symptomatic, or who have more severe disease, this protective function is lost and the D_{NO} becomes lower, leading to clinical disease.

Hans Bisgaard: If NO has any endogenous effect, it has to be through very high local concentrations, not the <1 part per million concentration found in exhaled gas. This latter concentration has no effect when you inhale it. However, NO certainly could have a local effect due to higher local concentration in the tissue (from release of NO from possible stored depots, or activation of NO synthase), but this is purely speculative. The airways NO concentration is not a bronchodilating concentration.

Marc Peters-Golden: Emilio, your data looked primarily at eosinophil numbers and suggests that only measuring numbers seem to provide useful, predictive information. Therefore, is the number of eosinophils as good, better, or worse than measuring secretory products of eosinophils?

Emilio Pizzichini: We do not yet have a specific marker for eosinophil activation. We have compared sputum eosinophils (%) and sputum and serum ECP as a marker of eosinophil activation. We have found that in relation to sputum eosinophils, sputum or serum ECP did not provide additional information.⁷ We also found high concentrations of ECP in the sputum of patients with COPD⁸ and in patients with noneosinophilic asthma.⁹ The source for ECP in these situations could be neutrophils. It has been suggested that neutrophils can produce or store ECP. Asthmatic patients in comparison with healthy non-smoking subjects have increased proportions of neutrophils. When there is an infectious exacerbation, for example, the number of total cell counts per gram of sputum is markedly increased, the majority of these being neutrophils. It seems reasonable to assume that the ECP from neutrophils might be the source of part of the increased levels of ECP. In conclusion, we still need a good and specific marker of eosinophil activation, since ECP, major basic protein, and eosinophil-derived neurotoxin seem not to be very helpful. A recent report using immunocytochemistry to stain cells obtained from BAL and sputum has shown that eosinophil peroxidase (EPO) is a specific marker for eosinophils. Similarly, the study showed that human neutrophils lipocalin (HNL) is unique for neutrophils.¹⁰

Redwan Moqbel: Perhaps more important markers are the combination of eosinophil peroxidase interacting with the superoxide and the NO, forming a tyrosine complex derivative. It actually appears to be a sensitive marker and it can be measured in sputum.

Hans Bisgaard: In pediatric patients, in whom obtaining sputum is not possible, do you have any data or any suggestions as to what we should do or any of the markers that would be useful?

Emilio Pizzichini: No. We can only induce sputum on children older than 6 to 7 years of age. In this age group, the induction is successful and safe, and different types of inflammatory response occur.¹¹ In younger children and infants with a diagnosis of asthma, there is a recent report using BAL to evaluate the airway inflammation. The results of this study showed that eosinophilic and neutrophilic inflammation are present and that they are associated with different asthma phenotypes.¹² Because of the invasiveness of this method, it cannot be largely used. Perhaps other noninvasive methods such as exhaled NO, although unspecific, might be useful in these children.

Stephen Peters: In the asthmatic population that we studied in the Asthma Clinical Research Network, it was hard to find asthmatic persons with more than 2% to 3% eosinophils in their sputum. That was a very tough marker to use because of the low numbers in many asthmatic persons. Any comment?

Emilio Pizzichini: The presence of eosinophilic bronchitis (sputum eosinophils >3%) depends on the population studied. In steroid naive asthmatic persons with mild asthma, about 60% of them presented with eosinophilic bronchitis.¹³ In asthmatic persons with an exacerbation without a cold (no evidence of infection and clear, not green or yellow, sputum), most of them have sputum eosinophilia.¹⁴ In random exacerbations or uncontrolled asthma, again, 40% are noneosinophilic.¹⁵ In stable asthmatic persons controlled with steroids, the prevalence of sputum eosinophilia is variable.

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