Comparison of human bronchiolar smooth muscle responsiveness in vitro with histological signs of inflammation

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ABSTRACT A study was carried out to test the hypothesis that chronic inflammation is associated with increased sensitivity or contractility of human airway smooth muscle. Bronchiolar strips from 30 patients, 12 of whom had chronic bronchitis, were examined in the organ bath for their response to histamine, methacholine, and leukotriene (LT) C₄. The same airways were also studied histologically and small airway disease was quantified by subjective grading of the degree of inflammatory cell infiltration, smooth muscle hypertrophy, fibrosis, and goblet cell hyperplasia. The degree of small airway disease varied widely among patients both with and without chronic bronchitis. Multiple regression analysis failed to show increased sensitivity ($-\log EC_{50}$) to histamine, methacholine, or LTC₄ in relation to small airway disease. In contrast, the only significant correlations found were between a decreased $-\log EC_{50}$ to histamine and methacholine and an increased small airway disease score. Contractile responses (T_{max}) to histamine and methacholine in peripheral airways tended to be higher in patients with chronic bronchitis than in those without. T_{max} was not related to small airway disease scores. These results suggest that chronic airway inflammation does not cause in vitro hyperresponsiveness of human small airway smooth muscle.

Introduction

Clinical observations have led to the view that airway inflammation may be an important modulator of non-specific bronchial responsiveness in patients with asthma or chronic bronchitis and even in normal individuals.¹⁻⁵ Moreover, we recently found increased histamine responsiveness in isolated peripheral airways from patients with chronic bronchitis and airflow obstruction.⁶ The mechanisms by which inflammation could enhance bronchial responsiveness are not clear. Few studies have examined the responsiveness of isolated human airways in relation to the histological appearance of the bronchial tissue, and these studies have shown no relation between the degree of airway disease and function.^{7,8}

In the present study we measured the functional responses to pharmacological stimulation of isolated

peripheral airways from patients with and without chronic bronchitis or airflow obstruction (or both) and quantified various histological aspects of small airway disease in the same preparations by means of a subjective grading method. Functional data were related to histological findings to determine whether inflamed peripheral airways are more responsive in vitro than non-inflamed airways.

Methods

PATIENTS

Thirty patients who underwent lobectomy or pneumonectomy—29 for bronchial carcinoma and one for carcinoid tumour—were included in the study. All patients had lung function tests before surgery, including inspiratory vital capacity (VC) and forced expiratory volume in one second (FEV₁) before and after 10 mg nebulised isoprenaline. Chronic bronchitis was diagnosed according to the Medical Research Council criteria of cough and phlegm production for more than two years and more than three months in a year.⁹ Subjects were classified

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as "normal" when they reported no respiratory symptoms, apart from recent symptoms caused by their tumour, and had normal lung function with an FEV₁/VC of at least 70%. In 11 patients bronchial responsiveness was measured in vivo before surgery. Nebulised histamine was administered via a Wiesbadener Doppel Inhalator in doubling concentrations, and the provocative concentration that caused a 20% fall in FEV₁ from baseline (PC₂₀ FEV₁) was calculated.¹⁰ The study protocol was approved by the ethical committee of the University Hospital.

IN VITRO STUDIES

Immediately after surgical resection a macroscopically normal piece of lung tissue was collected in ice cold Krebs-Henseleit buffer (composition in mmol/l: NaCl 118, KCl 4, 7, CaCl₂ 2, 5, MgSO₄ 1, 2, KH₂PO₄ 1, 2, NaHCO₃ 25, glucose 5, 55), aerated with 5% carbon dioxide and 95% oxygen, and rapidly transported to the laboratory. Bronchiolar strips (10th-13th generation) were dissected, stored overnight, and studied isometrically, with Grass FTO3D transducers, in a 10 ml organ bath containing aerated Krebs-Henseleit buffer at 37°C, as described previously.¹¹ A baseline tension of 500 mg was applied. Histamine hydrochloride, methacholine bromide (Janssen pharmaceuticals, Belgium), and leukotriene (LT) C₄ (a gift from Dr J Rokach, Merck Frosst, Canada) were used as contracting agents, and three cumulative concentration-response curves were made in succession on a single bronchiolar strip from each patient. The sequence of the agonists was determined by a random digits table. From each concentration-response curve the EC₅₀ value (the molar agonist concentration that produced 50% of the maximal effect on a given strip) was derived as a measure of smooth muscle sensitivity. We have previously shown that for EC₅₀ measurements this procedure does not lead to interactions between these three agonists.¹² The maximal force (T_{max}, contractility) developed by a strip was calculated only for the first concentration-response curve on each strip because interactions occur with T_{max} when agonists are applied successively.¹²

HISTOLOGY

After dissection of the bronchiolar strips a proximal segment was removed, fixated in formalin, and processed for histological examination. We used a simplified version of Cosio's subjective grading method,¹³ adapted for bronchiolar strips, to assess the severity of small airway disease. The items scored were (1) inflammatory cell infiltration, (2) smooth muscle hypertrophy, (3) goblet cell hyperplasia, and (4) fibrosis. Each item was assigned a score (0-3) by

comparison with pictorial reference standards, 0 representing the normal picture and 1-3 reflecting increasing degrees of abnormality (fig 1). The total small airway disease score was the sum of scores for the four items, so 12 was the maximum attainable score. The validity of the scoring system was confirmed in a preliminary study (the mean (SEM) kappa coefficients for within and between observer agreement were 0.77 (0.07) and 0.66 (0.12)).

STATISTICAL ANALYSIS

Data for patients with and without chronic bronchitis were compared by means of Student's *t* test (two tailed). For each agonist the EC₅₀ and T_{max} values of 30 strips from 30 patients were related to the total small airway disease score by linear regression analysis. The relation between EC₅₀ and T_{max} values and the four separate small airway disease items was examined by multiple regression analysis. The significance of the regression was determined by analysis of variance and the F test ($\alpha = 0.05$).

Results

Of the 30 patients, 12 had chronic bronchitis as defined, and 18 reported no cough or phlegm. None of the patients had a history of asthma. The clinical findings are summarised in table 1. Eight of the 18 subjects without symptoms of chronic bronchitis had airflow obstruction (FEV₁/VC < 70%). Although this subgroup had a higher mean age and may have had airway obstruction or compression by tumour, we analysed the results for this subgroup separately.

Current cigarette smoking was reported by eight of the 12 patients with symptoms of chronic bronchitis and by five of those without symptoms. Three subjects without chronic bronchitis smoked a pipe or cigars. Only one subject had never smoked; all others had ceased smoking at least two years previously (table 1).

The response to histamine in vivo was measured in eight patients without and three with chronic bronchitis. The PC₂₀FEV₁ was greater than 64 mg/ml in all patients except one of the three with chronic bronchitis (32 mg/ml) (table 1). A wide range of small airway disease was found in patients both with and without chronic bronchitis. Severe disease was slightly more common in chronic bronchitis, but group mean small airway disease scores did not differ significantly (table 2). Current cigarette smoking was not associated with the small airway disease score within groups. The scoring system did not take account of the composition of the inflammatory cells in the airway wall, but most were neutrophilic granulocytes and mononuclear cells (fig 1); eosinophils were rare.

The results of the in vitro experiments are sum-

(a)

(b)

(c)

Fig 1 Photomicrographs showing various degrees of inflammatory cell infiltration in bronchiolar strips. a, b, and c are increasingly abnormal pictures of inflammatory cell accumulation corresponding to cellular infiltration scores 1, 2 and 3 respectively. (May-Grünwald-Giesma.)

Table 1 Characteristics of the patients (mean (SEM) values)

	No chronic bronchitis, no airflow obstruction (n = 10)	No chronic bronchitis, airflow obstruction (n = 8)	Chronic bronchitis, airflow obstruction (n = 12)
Age (years)	49 (5)	65 (3)	55 (5)
Inspiratory vital capacity (VC) (% predicted)	105 (4)	108 (7)	97 (5)
FEV ₁ as % of VC	77 (2)	59 (3)	55 (4)
% increase in baseline FEV ₁ after isoprenaline	1 (1)	4 (1)	10 (3)
Smoking habit (n)			
Current smoker	4	4	8
Ex-smoker (> 2 years)	5	4	4
Never smoked	1	0	0
Response to inhaled histamine (PC ₂₀ FEV ₁ , mg/ml)	> 64 (n = 6)	> 64 (n = 2)	> 64 (n = 2) 32 (n = 1)

Table 2 Histological findings (mean (SEM) values)

	Small airways disease scores*		
	No chronic bronchitis, no airflow obstruction (n = 10)	No chronic bronchitis, airflow obstruction (n = 8)	Chronic bronchitis, airflow obstruction (n = 12)
Small airway disease feature			
Cellular infiltration	0.9 (0.4)	0.5 (0.3)	0.7 (0.2)
Muscle hypertrophy	0.9 (0.3)	0.4 (0.2)	0.7 (0.2)
Fibrosis	1.2 (0.2)	1.9 (0.2)	1.8 (0.3)
Goblet cell hyperplasia	0.7 (0.3)	1.0 (0.3)	1.3 (0.3)
Total small airways disease score	3.7 (0.9)	3.8 (0.5)	4.5 (0.7)

*No significant differences existed between the groups.

marised in table 3. The $-\log EC_{50}$ values of methacholine, histamine, and LTC₄ were similar in patients with and without chronic bronchitis or airflow obstruction. As a consequence of the randomisation procedure, histamine was applied as the first drug to 15 of the 30 airway preparations, and methacholine and LTC₄ to nine and six strips respectively. Because of the relatively large variation of T_{max} within subjects,¹² no conclusions about possible differences in T_{max} between groups of patients can be drawn from the small numbers of observations. There was, however, a tendency for histamine and methacholine T_{max} to be higher in airways from patients with chronic bronchitis and airflow obstruction (table 3). Because no differences were found in small airway disease scores or EC_{50} values between groups of patients, the relation between small airway disease and EC_{50} was analysed on the total population. The methacholine $-\log EC_{50}$ showed a weak but significant negative correlation with total small airway disease scores ($p < 0.05$)—that is, severely diseased airways tended to be less sensitive to methacholine. No relation between histamine or LTC₄ EC_{50} and total small airway disease scores was found (fig 2). Multiple regression analysis showed that inflammatory cell infiltration was the only single variable that correlated negatively with both methacholine $-\log EC_{50}$

($p < 0.05$) and histamine $-\log EC_{50}$ ($p < 0.05$) (fig 3). T_{max} values for histamine, methacholine, and LTC₄ were not related to the severity of small airway disease.

Discussion

Our results indicate that small airway disease and, in particular, inflammatory cell infiltration of the airway wall in patients with and without chronic bronchitis is not associated with increased sensitivity or contractility of small airway smooth muscle to histamine, methacholine, and LTC₄ in vitro. Infiltration of inflammatory cells was negatively correlated with histamine and methacholine sensitivity, which was the opposite of what we expected. In accordance with our earlier findings,⁶ histamine T_{max} tended to be higher in airways from patients with chronic bronchitis and airflow obstruction than in airways from normal subjects. In this study mean T_{max} for methacholine was also higher in the patients with chronic bronchitis. Statistical analysis was not carried out because of the small numbers of observations and the large intrinsic variation in T_{max} .¹¹ There was no correlation between T_{max} and small airway disease scores. Airway inflammation scores were determined before tissue was stored and possibly the storage procedure and

Table 3 Results of *in vitro* concentration-response studies

		No chronic bronchitis, no airflow obstruction (n = 10)	No chronic bronchitis, airflow obstruction (n = 8)	Chronic bronchitis, airflow obstruction (n = 12)
Histamine	-log EC ₅₀ *	5.77 (0.09)	5.96 (0.07)	5.79 (0.11)
	T _{max} †	51 (8) [4]	73 (21) [5]	113 (21) [6]
Methacholine	-log EC ₅₀ *	6.22 (0.13)	6.22 (0.13)	6.04 (0.10)
	T _{max} †	89 (13) [4]	78, 91 [2]	176 (68) [3]
LTC ₄	-log EC ₅₀ *	8.24 (0.08)	8.00 (0.06)	7.89 (0.08)
	T _{max} †	53, 79 [2]	18 [1]	40 (12) [3]

*Mean (SEM) of 30 measurements.

†Expressed in mg isometric force per mg dry tissue weight. The number of observations is indicated in square brackets; only the first concentration-response curve for each strip is included (see under "Methods"). Mean (SEM) values are given for three observations or more; otherwise individual values are given.

Fig 2 Relation between $-\log EC_{50}$ values for histamine, methacholine, and LTC₄ and total small airways disease scores for 30 bronchiolar strips from 30 patients with and without chronic bronchitis. Fig 3 Relation between $-\log EC_{50}$ values for histamine, methacholine, and LTC₄ and inflammatory cell infiltration scores for 30 bronchiolar strips from 30 patients with and without chronic bronchitis.

further processing of the bronchiolar strips altered smooth muscle function, conceivably by removing humoral factors or inflammatory cells. We did not repeat the histological examination after the challenge experiments, but preliminary investigation did not show any deleterious effects of storage or organ bath studies on the histological appearance of the airways. Moreover, we and others have shown that prolonged storage in cold buffer does not affect *in vitro* human airway smooth muscle responsiveness to histamine or methacholine.^{11 14} It therefore seems unlikely—though the possibility cannot be excluded—that we have missed an effect of inflammation on smooth muscle function as a result of factors relating to tissue processing.

In the dog, ozone induced acute airway inflammation is associated with airway hyperresponsiveness,^{4 15} and this has been linked to granulocyte infiltration in the airway wall. In the guinea pig, ozone and cigarette smoke also produce acute airway inflammation and hyperresponsiveness,^{16 17} though in this model hyperresponsiveness preceded cellular infiltration and the granulocytes persisted longer than the increased responsiveness. Possibly in chronic inflammation airway hyperresponsiveness does not persist if inflammatory cells continue to be present after the acute phase, and this would explain the contradictory results in human and animal airways regarding inflammation and responsiveness.

Our findings are in agreement with the clinical observation that patients with severe chronic non-asthmatic airway inflammation, such as occurs in cystic fibrosis or bronchiectasis, usually have only mild bronchial hyperresponsiveness or none at all, despite large numbers of leucocytes in their airways. Our results confirm and extend the findings of Armour *et al*, who did not find an association between inflammation and *in vitro* responsiveness of human airways to histamine and carbachol in a smaller group of patients.^{7 8}

In the present study and in other reports on *in vivo* and *in vitro* responsiveness of human airways there has been only a weak correlation or none between the amount of airway smooth muscle and airway responsiveness.^{3 7 18 19} This suggests that not only the amount but also the intrinsic properties of airway smooth muscle may determine airway responsiveness. In addition, the arrangement of smooth muscle, rather than its amount, is likely to be responsible for a large part of the high intrinsic variability of $T_{\max}$ in spirally cut strips. Mullen *et al* found that in non-asthmatic smokers inflammation of membranous bronchioles correlated with *in vivo* airway responsiveness.³ These authors also found a relation between bronchiolar inflammation and *in vivo* airway calibre, but the effects of these factors on airway

responsiveness seemed to be independent.³ Our present findings do not support a direct effect of inflammation on bronchiolar smooth muscle function. It remains possible, however, that peripheral airway inflammation modulates airway responsiveness *in vivo* by other mechanisms—for example, by altering autonomic regulation processes. Furthermore, our results do not exclude an effect of inflammation on smooth muscle function in central airways. Such an effect seems unlikely, however, as in the study by Mullen *et al* no relationship was found between inflammatory changes in central airways and *in vivo* bronchial responsiveness.³ In the tissues we studied we found no small airway disease scores above eight. Although our results do not point in this direction, we cannot exclude the possibility that smooth muscle sensitivity or contractility would have been abnormal in bronchioles with a higher score. This seems very unlikely, however, because severe small airway disease is not a characteristic of subjects with a greatly increased *in vivo* airway responsiveness.³

We, like others,²⁰ found small airway disease to a variable degree in lungs from both patients with and patients without chronic bronchitis and airflow obstruction, with a large overlap. This indicates that histological signs of small airways disease are not specific for chronic bronchitis, and may occur in otherwise healthy, non-smoking symptomless subjects with normal lung function. This, together with the finding that small airway inflammation had no relation to *in vitro* small airway smooth muscle function, suggests that airway hyperresponsiveness in chronic bronchitis is not due to a direct effect of chronic inflammation on airway smooth muscle function.

Our present findings do not exclude the possibility that the inflammation may potentiate the mechanisms that underly airway hyperresponsiveness in asthma. We have recently reported increased contractility in isolated airways showing eosinophilic infiltration of the bronchial mucosa and smooth muscle hypertrophy from an asthmatic subject.²¹ Airway hyperresponsiveness in asthma and chronic bronchitis has different characteristics, however, and may have a different pathogenesis.²²

More studies are needed to establish to what extent airway inflammation or alternative mechanisms, such as mucosal oedema, changes in central airways, potentiation of local or central nervous reflexes, or other abnormalities in neural control, contribute to the pathogenesis of airway hyperresponsiveness in chronic bronchitis and asthma.

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