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Sputum and ciliary inhibition in asthma

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ABSTRACT Twenty-eight sputum samples collected from 20 patients with chronic bronchial asthma of atopic and intrinsic clinical types were incubated with human bronchial explants to study their influence on ciliary motility. Of these, 19 (68%) of the sputa exerted a ciliary inhibitory effect of varying degree in a two-hour period. Analysis of the data indicates that (1) the ciliary inhibitory effect was invariably present when patients produced a distinctive slurry sputum; (2) this occurred more frequently during clinical exacerbations; (3) the induced ciliary inhibition was reversible on removal of the sputum; (4) the intensity of the ciliary inhibitory effect decreased with clinical improvement of the patient; (5) the inhibitory effect was unrelated to the medications used; (6) it was equally common in the atopic and the intrinsic types of asthmatic patients; (7) the effect was not pH dependent or related to the degree of eosinophilia. The ciliary inhibitory factor in sputum was identified as having a molecular weight of 6000–8000. It may play a part in the pathogenesis of asthma and recognition of sputum containing it carries implications for treatment.

Recent studies suggest that certain human fluids may modulate ciliary beating frequency in tissues from lower animal species and mammals. A ciliary dyskinesia factor has been reported in the serum of patients with cystic fibrosis,[1] various respiratory and autoimmune diseases,[2] and asthma.[3] This factor was demonstrated by biological assays in oyster gills[4 5] and rabbit trachea.[2 6–8] Wilson and Fudenberg found specific biochemical differences between the ciliary dyskinesia factor in serum from patients with cystic fibrosis and that from asthmatic patients.[3] Others, however, have noted that human sera from both normal people and patients with cystic fibrosis could induce cytolysis of the mucosal surface in rabbits.[6 8] These observations suggested that species incompatibility may be responsible for tissue damage, which then leads to ciliary dysfunction. Recently, Gleich and his colleagues, pursuing long-term studies with a major basic protein isolated from eosinophils of asthmatic patients, found it capable of inducing ciliostasis, cytolysis, and exudation in man and pig.[9 10] This major basic protein is present in large quantities in asthmatic patients.

Studies in our laboratory have shown that sputum from some asthmatic patients can produce ciliary inhibitory effects when incubated with strips of frog palate mucosa.[11 12] This effect was not accompanied by anatomical disruption of the mucosa and was reversible. The present study was undertaken to determine whether sputum from patients with asthma would have the same ciliostatic effect on explants of human bronchial mucosa.

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Methods

THE SUBSTRATUM

After informed consent had been given, biopsy specimens of bronchial mucosa were obtained in the course of routine diagnostic fibreoptic bronchoscopies of patients with various respiratory diseases. The biopsy site was an area of mucosa that appeared normal by visual examination. Previous experience with this technique assured satisfactory results and no significant differences in ciliary activity from various bronchial areas.[13] Immediately on removal the explant, which was to be used as substratum for bioassay, was placed in tubes containing culture medium 199 (Gibco, NY) and the mixture was incubated at 37°C for half an hour to an hour before the test.

THE SPUTUM

The sputum to be tested came from 20 patients suffering from chronic bronchial asthma.[14] Clinically, the group included 10 patients with "atopic" asthma

Table 1 *Clinical and physiological data on the 20 asthmatic patients studied*

Sputum donor*	*Clinical type†*	*Steroid treatment*	*Clinically worse*					*Clinically better*				
			FEV_1‡	*Sputum cytology***				*FEV_1‡*	*Sputum cytology***			
				E	*P*	*H*	*BEp*		*E*	*P*	*H*	*BEp*
1	I	+	15/29	65	10	17	8	40/55	10	27	35	28
2	I	+	25/36	82	6	5	7	43/69	38	22	7	33
3	A	+	66/80	64	21	7	8	63/80	15	56	14	15
4	A	+	45/55	61	19	10	10	48/55	22	58	15	5
5	A	+	35/39	48	36	12	4	50/62	3	52	21	24
6	I	+	31/34	55	26	9	10	31/40	37	20	18	25
7	I	+	31/35	72	8	14	6	31/39	33	12	35	20
8	I	+	43/43	70	15	7	8	46/52	30	14	15	41
9	A	+	30/36	67	20	7	6	39/51	10	40	28	22
10	I	+	59/66	45	28	19	8	—	17	21	15	47
11	I	+	18/24	59	19	14	8	32/37	11	31	22	36
12	A	+	10/22	53	28	12	7	40/53	—	—	—	—
13	I	+	25/36	54	21	15	10	50/62	25	46	11	18
14	I	−	39/45	69	13	8	10	45/52	11	25	46	18
15	A	−	40/42	45	30	9	16	60/68	56	10	25	9
16	A	+	42/54	47	14	26	13	61/64	45	16	33	6
17	A	+	13/19	62	20	9	9	19/27	9	78	5	8
18	A	+	38/38	73	9	7	11	57/73	25	48	9	18
19	A	+	38/38	53	10	26	11	46/54	8	34	37	21
20	I	+	45/48	81	10	3	6	68/80	57	10	25	8

*Nos 1–8 studied during both "worse" and "better" periods; 9–14 during "worse" periods only; and 15–20 during "better" periods only (see text).
†A — atopic; I — intrinsic.
‡Percentage of predicted values before/after bronchodilator aerosol.
**E — eosinophil; P — polymorphonuclear leucocyte; H — histiocyte; BEp — bronchial epithelial cells (figures are percentages of total white blood cell count).

and 10 with the "intrinsic" variety (table 1). In addition to having the expected symptoms and signs they all had airflow obstruction, which at some time had been partially reversible by administration of bronchodilators or steroids.

Since the degree of clinical exacerbation at a given moment is difficult to express for an individual asthmatic patient, we decided to classify the patients into two broad categories, "worse" and "better." This indicated for each individual the extent of his relative clinical severity at the time of the collection of the sample[15] (table 1). An attempt was made to collect and test the sputum of these patients at both the "worse" and the "better" clinical stages. Some of the collected sputa, however, could not be tested for the presence of ciliary inhibitory effect either because bronchoscopic explants were not available on the same day or because the pH of the sputum was unsuitable ($\leq 6{\cdot}5$ or $\geq 9{\cdot}6$).

The sputum was collected in the early morning at room temperature. Gram stain and Papanicolaou smears were prepared from each sample to ascertain the degree of purulence and site of origin in the lower respiratory tract (table 1). The samples were centrifuged at 30000 *g* in an IEC B-20A refrigerated centrifuge for 45 minutes; this yields a viscous pellet and a "sol" layer. The gel pellet was discarded and the sol used to study the effects on the ciliary activity of the mucosa. The pH of the sputum sol was measured with a Beckman model SS-2 pH meter at 24°C. All samples with a pH less than 6·5 were excluded from analysis in view of the findings of Holma *et al* that in bovine trachea ciliary inhibition develops at pH $\leq 6{\cdot}5$ or $\geq 9{\cdot}6$.[16]

CILIARY BIOASSAY

The bronchial explant was placed in a flat-bottomed, welled slide of Fisher-Littman type containing Medium 199, and kept at 37°C by air curtain incubator. Ciliary beating frequency was measured with a photoelectric system developed in this laboratory.[13] The actual field scanned by the photomultiplier is about 30 μm at ×200 magnification. The number of deflections per second are recorded as ciliary beating frequency.[13] Under these conditions our control ciliary beating frequency values in the explant are $12{\cdot}7 \pm 1{\cdot}3$ in Medium 199 and $13{\cdot}8 \pm 1{\cdot}3$ in buffered saline with pH 7·2. Over a two-hour period these values remain within 10% of initial ciliary beating frequency values.

After we had obtained normal control ciliary beating frequency values (10–16 beats a second),[17] Medium 199 was carefully aspirated from the well with filter paper and replaced with sputum sol, care being taken not to disturb the underlying tissue. Thus ciliary beating frequency could be measured repeatedly in the same area of the explant. The presence of a ciliary inhibitory effect was accepted only if the ciliary beating frequency was decreased by 25% or more from the control value within two

Table 2 *Consistency of ciliary inhibitory effect of sputum in different explants*

Source of sputum	*Source of explant*	*Ciliary inhibition (%)*
JO	VO	100
	VT	100
GJ	JN	100
	CD	100
JT	PV	36
	MC	34
ON	ZR	55
	NB	56
VO	MR	31
	CA	48
FO	MG	100
	KO	100
	BW	100
VT	VV	100
	CJ	100
FJ	HJ	30
	HT	45

hours of incubation with the sputum sol.

To further minimise experimental artefacts additional precautions were taken. (1) Each ciliary beating frequency value was the average of 10 consecutive readings of acceptable quality. (2) To test the reversibility of the ciliary inhibitory effect in each case, where a significant decrease of ciliary beating frequency took place the sputum sol was replaced with Medium 199 until control values were restored. (3) Eight sputum sols with a demonstrated ciliary inhibitory effect were again tested on different explants, obtained from new donors, on different days. The presence of a ciliary inhibitory effect was repeatedly confirmed, with only minor differences in degree of inhibition (table 2). (4) Five explants were exposed to sputum samples with and without the ciliary inhibitory factor. The test showed that each explant was capable of yielding the correct positive or negative response to each sputum sol (table 3).

Table 3 *Specificity of explant response to sputum ciliary inhibitory effect*

Source of explant	*Source of sputum*	*Ciliary inhibition (%)*
MC	JO	34
	SL	7
	HW	13
MR	KA	28
	VO2	29
	BP	8
SA	KT	100
	BJ	44
	ON	100
EJ	KO	100
	KF	26
VV	RR	0
	WA	53
	VO1	100
	VO2	31

Table 4 *Dose-response relationship of ciliary inhibitory effect (CIE)*

CIE containing fraction concentration (%)	*Degree of inhibition (%)*	*Time of appearance (min)*
100	100	20
50	100	60
25	30	60
16	15	60

*Refers to material obtained after Sephadex G-50 fractionation.

Preliminary experiments were carried out to characterise the nature of the material responsible for the ciliary inhibitory effect. Sputum sols were subjected to gel chromatography fractionation on Sephadex G-200 and G-50. The fractionation was done on several batches of sputum in which the ciliary inhibitory effect was known to be present or absent. Fractions were pooled into six groups. Each group's pool was concentrated and assayed for the ciliary inhibitory effect by the method described above for single sol speciments. In addition, the fraction containing ciliary inhibitory effect was diluted with isotonic sodium chloride to various concentrations and a dose-response effect was elicited (table 4).

Bronchial explants were prepared for scanning electron microscopy within two hours after a ciliary inhibitory effect had been demonstrated. This was to determine whether the observed effect resulted from histological damage to the mucosa with secondary entrapment of the cilia.

Results

Twenty-eight sputum samples from the 20 patients were tested for effects on ciliary activity. On gross examination the tested sputa appeared to be of two kinds, one being of the well-known mucoid type and the other appearing as a more liquid material somewhat resembling the secretions of allergic rhinitis. This slurry type of sputum was seen more frequently during the periods of clinical exacerbation of asthma.[12 15] Of the 28 sputum samples, 14 were of the slurry type and 14 of the mucoid variety. No purulent samples were produced. During the "worse" clinical phase, 10 patients produced slurry expectorates and four produced mucoid sputa, whereas during the "better" clinical period there were 10 mucoid and four slurry samples (table 5). Nineteen of the 28 sputum sols (68%) prepared from the collected samples inhibited ciliary activity. Examination of the data showed no correlation between ciliary inhibitory effect and the clinical type of asthma (intrinsic or atopic).

All the "slurry" sputum samples induced a ciliary

Table 5 *Ciliary inhibitory effect (CIE), clinical state, and sputum type*

	Total number of samples (number with CIE)	
	"Worse"	*"Better"*
Slurry	10 (10)	4 (4)
Mucoid	4 (1)	10 (4)

inhibitory effect, whereas this effect was observed in only five of the 14 mucoid sputum samples. The difference is statistically significant by the Fisher probability test ($p < 0.003$). Although the ciliary inhibitory effect was seen more frequently during the "worse" clinical periods, the difference was not statistically significant ($p < 0.16$) (table 5). The relation between clinical activity of disease and the presence of a ciliary inhibitory effect in the sputum was examined in seven patients whose clinical condition changed from "worse" to "better". In all cases the ciliary inhibitory effect disappeared or diminished with clinical improvement (table 6). The presence of the inhibitory effect was not related to the type or amount of medication (including steroids).

As the onset of an asthmatic exacerbation in untreated patients generally coincides with an increase of eosinophils, we sought a correlation between sputum eosinophilia and percentage of ciliary inhibition. This could not be shown statistically ($r = 0.2$).

The pH of the sputum samples ranged from 6·5 to 9·0, most values being in the range 6·8–8·0. From 6·5 to 9·0 no direct correlation between pH and degree of ciliary inhibition was found ($r = 0.15$) (fig 1).

In every case ciliary inhibition was reversed after substitution of Medium 199 for the sputum sol. Ultrastructural studies using scanning and transmission electron microscopy failed to show signs of cell damage on the bronchial mucosa.

Fractionation of sputum sols containing the ciliary inhibitory factor on Sephadex G-200 and G-50 indicated that the inhibitory effect was found predominantly in fractions with a molecular weight of 6000–9000 (fig 2).

Table 6 *Sputum ciliary inhibitory effect and clinical changes**

Patient	*Diagnosis*	*Ciliary inhibition (%)*	
		"Worse"	*"Better"*
AA	Intrinsic asthma	49	17
ON	Intrinsic asthma	100	42
PM	Intrinsic asthma	80	5
FJ	Intrinsic asthma	100	37
EI	Atopic asthma	54	30
GJ	Atopic asthma	100	27
NJ	Atopic asthma	64	33

*"Worse" and "better" indicate relative degree of clinical severity of asthma for each patient.

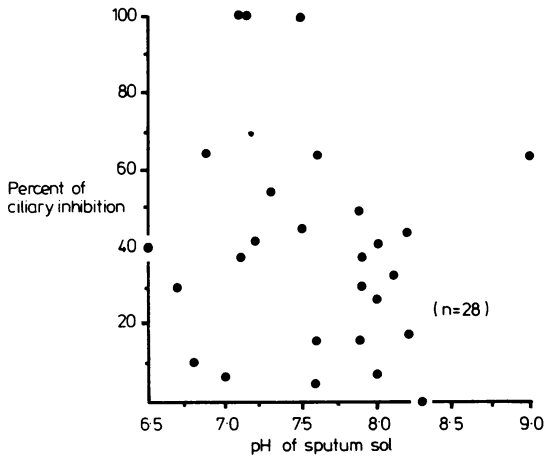

Fig 1 *pH and degree of ciliary inhibitory effect of 28 sputum samples from asthmatic patients.*

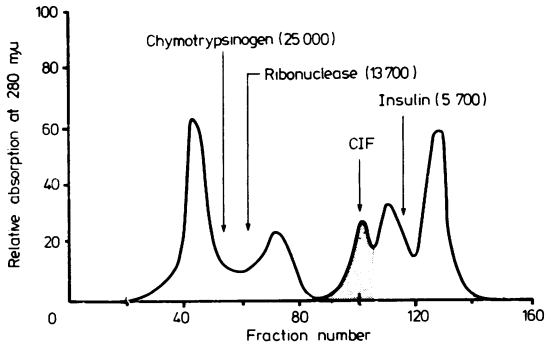

Fig 2 *Chromatogram of sputum sol containing ciliary inhibitory factor (CIF) (Sephadex G-50 in phosphate buffered saline, pH = 7·2; 5×90 cm column).*

Discussion

The results of this study suggest that the ciliary inhibitory effect results from a specific interaction between a substance sometimes present in the sputum of some asthmatic patients and the cilia of the human bronchial mucosa. This confirms the results of earlier studies by our group[11 12] in which frog palates were used as substrate. The ciliary impairment does not result from lack of nutrients in the medium. This ciliary inhibitory effect appears to be a functional phenomenon not secondary to structural damage to the cilia or mucosal cells and it is

reversible. The functional nature of the inhibitory effect was also expressed in clinical terms, by the decrease or total disappearance of the ciliary inhibition after the amelioration of symptoms seen in some patients. These characteristics are different from the ciliotoxic effects reported to be produced by serum from patients with cystic fibrosis in lower species of animals.[1-8] The differences may be real or be related to the method of testing.[6 8]

Differences in reported results may be related to whether serum or respiratory secretions are studied. In our study, where sputum and blood samples were obtained from our asthmatic patients at the same time inhibition of ciliary activity was detected only in the sputum.

Frigas and colleagues, in comprehensive work dealing with the major basic protein of the eosinophil, have reported the presence of a protein with a molecular weight of about 9000 in the blood and sputum of asthmatic patients capable of inducing mucosal desquamation and cytolysis as well as ciliary inhibition in bronchial explants of man and guinea-pig.[9 10] They have also reported that this major basic protein is present in much higher quantities in the sputum of asthmatic patients than in the sputum from patients with other respiratory disease.[9] In our studies gel fractionation of sputum containing the ciliary inhibitory factor on Sephadex G-200 and G-50 points to a protein of similar molecular weight. But radioimmunoassays of our sputum fractions containing ciliary inhibitory factor performed in Dr Frigas's laboratory at the Mayo Clinic were negative for major basic protein and bioassays of their material containing major basic protein performed in our laboratory failed to inhibit human cilia (personal communications). This leaves open the question of whether major basic protein and the ciliary inhibitory factor we describe are identical. Possibly large-molecular-weight proteins carrying the inhibitory factor break down into smaller complexes and these may then appear in more than one analytical fraction. This might also be the case with the ciliary dyskinesia factor that Wilson and Fudenberg found in sera of asthmatic patients.[3] This was identified as a very-high-molecular-weight protein, which was different from the ciliary dyskinesia factor that they found in serum of patients with cystic fibrosis. The mechanism of action of the ciliary inhibitory effect remains uncertain. In our material the effect appeared to be purely functional, affecting only ciliary dynamics, whereas the major basic protein of Gleich and his colleagues seems to induce ciliary inhibition in the context of a wider effect on the mucosa characterised by desquamation and cytolysis.[10]

Holma *et al*[16] found that at pH $\leq 6{\cdot}5$ or $\geq 9{\cdot}6$ there is ciliary inhibition. Careful examination of the relation between pH and ciliary activity, however, in all our samples with a pH in the range 6·5–9·0 indicated no significant correlation. This conclusion is supported by a recent study in our laboratory showing that human cilia develop the highest ciliary beating frequency in the pH range 6·5–8·0.[18]

The results of this study and our previous observations[11 12] suggest that the ciliary inhibitory effect in the sputum of asthmatic patients occurs more frequently during acute or subacute clinical exacerbations than during stable periods, and that the sputum produced during exacerbations (slurry sputum) frequently differs in appearance from that produced during clinical remissions (mucoid sputum). We followed for two years the ciliary inhibitory activity in the sputum of an asthmatic patient on continuous steroid treatment and observed such "waxing" and "waning" of the inhibitory activity with clinical and spirometric variations. Interestingly, sputum from this patient induced complete inhibition of ciliary activity whereas his serum, tested simultaneously, failed to affect ciliary beating on a control assay.

Previous investigators have noted the association between slurry sputum and asthma, and labelled the production of such sputum bronchorrhoea.[19 20] Some years ago we became interested in this sputum and reported that when placed in contact with ciliated mucosa (from the frog) it was transported at a very slow rate or not at all.[15] Thus recognition of this type of sputum has clinical significance since it always indicates, in asthmatic patients, the presence of the ciliary inhibitory factor and its potential consequences.

A decrease in mucociliary transport in asthmatic patients has been reported by several investigators.[21 22] We do not know whether this results from the special rheological characteristics of the mucus in asthmatic patients (very low viscosity and elastic modulus). the currently described ciliary inhibitory effect, or both. Clearly, the rheological characteristics, though they greatly influence the rate of transport of sputum by ciliated mucosa,[23] could not explain the ciliary inhibitory effect since the sol fraction of sputum was used and the viscoelastic properties of these sols is uniformly low.

All these considerations led us to form the hypothesis that in asthmatic patients a functional alteration takes place, characterised by a change in the physicochemical character of the mucus secreted that initiates or contributes to a decrease in clearance of the mucus. Initially, this mucus has very low viscoelasticity and it contains a ciliary inhibiting factor. Such mucus may interact in some as yet unknown fashion with other factors responsible for the exudation and bronchoconstriction characteris-

tic of the asthmatic exacerbation. Clinically, the "benign" gross appearance of this watery sputum may be deceptive since persistent impairment of mucociliary clearance can easily lead to stagnation of the secretion, converting the slurry mucus to the hard intra-airway plugs characteristic of advanced stages of clinical asthma. Elucidation of the exact nature of the ciliary inhibitory factor found in the secretions of asthmatic patients would form an important link in understanding of the pathogenesis of the asthmatic process. Hitherto most of the emphasis has been directed towards explaining the mechanisms leading to bronchoconstriction and bronchial congestion or oedema. It is now apparent that the production of a specific secretion into the bronchial lumen could carry important pathogenetic implications.

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