

Deleterious effects of purulent sputum sol on human ciliary function in vitro: at least two factors identified

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ABSTRACT Patients with chronic bronchial sepsis have impaired mucociliary clearance. A study was carried out on the effect of sputum sol (obtained by rapid centrifugation of purulent sputum) from 20 patients with chronic bronchial sepsis on the beating of human nasal cilia in vitro by a photometric technique. Thirteen sols caused significant ($p < 0.001$) ciliary slowing. Two patterns of slowing were observed: firstly, a gradual onset associated with epithelial disruption (inhibited by α_1 antiprotease) and, secondly, an immediate onset associated with ciliary dyskinesia and ciliostasis (inhibited by chloroform extraction). The ciliary slowing activity of sputum sols was associated with the isolation of *Pseudomonas aeruginosa* ($p < 0.01$). It is concluded that purulent sputum contains at least two factors that impair ciliary beating—one a serine protease, which is probably a product released by the host's phagocytic defences, and the other, which is chloroform extractable and probably a bacterial product.

Mucociliary clearance is an important defence mechanism that protects the human respiratory tract against inhaled particles, including bacteria. This is shown by the development of recurrent or chronic chest infections and sinusitis in patients with primary ciliary dyskinesia.¹ Primary ciliary dyskinesia is rare (one in 15–30 000 births), but chronic bronchial sepsis of other aetiologies is not. Such patients also have evidence of impaired mucociliary clearance: clearance of radioactive particles is delayed in patients with bronchiectasis² and cystic fibrosis,³ the movement of Teflon discs observed bronchoscopically in the trachea of patients with cystic fibrosis is slow,⁴ and, in the upper respiratory tract, nasal mucociliary clearance is delayed in patients with mucopurulent sinusitis, especially if the patient also has bronchiectasis.⁵

There are probably several reasons for delayed clearance in the presence of purulent secretions. We examined the effect of the sol phase of purulent sputum, obtained from patients with chronic bronchial sepsis, on human cilia beating in vitro.

Patients and methods

PATIENTS AND SPUTUM SOLS

Twenty patients with daily purulent sputum production were studied. Seventeen had bronchiectasis, two had cystic fibrosis, and one produced purulent sputum daily but did not have bronchiectasis (bronchogram was normal). Bronchiectasis was diagnosed by history, examination, and chest radiograph (with additional bronchographic information in 11 patients). Cystic fibrosis was diagnosed by history, examination, chest radiograph, and sweat sodium excretion greater than 70 mmol/l. Sputum production was collected over four hours from each patient in a sterile container. Each sputum sample was examined by standard and selective bacteriological culture techniques.⁶ The sputum samples were then centrifuged at 50 000 g for 90 minutes at 4°C. The watery sol phase was removed by pipette and retained and the gel phase discarded. Before the effect of these sputum sols on ciliary beating was assayed, each sample was assayed for elastolytic activity. To standardise the ciliary function assay the pH of each sputum sol was tested using a pH meter (pH range of sputum sols before adjustment 6.9–9.2) and adjusted to 7.4 by addition of molar sodium hydroxide or molar hydrochloric acid. This adjustment required addition of no more than 5% of the original volume of the sputum

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sols, so there was little dilution of the sputum sols before ciliary function assay.

ASSAY OF ELASTOLYTIC ACTIVITY

We modified a fluorescein-elastin technique,⁷ using microtitre plates. A total volume of 200 μ l diluted standard (porcine pancreatic elastase (PPE) Sigma Type IV E0258) or sputum in 0.2M Tris hydrochloric acid buffer pH 8.8 was added to each well which contained 1.6 mg of washed and dried fluorescein-elastin (Elastin Products Company, United States). Each plate was covered and then incubated at 37°C for 16 hours. The reaction was then stopped by addition of 50 μ l of 50% acetic acid to each well. The fluorescence of an aliquot of the supernatant was measured (excitation wavelength 468 nm and emission wavelength 512 nm). The lower limit of detection with this method was equivalent to 40 μ g/l PPE. The coefficient of variation of this assay was 6% within plate and 26% between plates. The assay was standardised by measuring elastolytic activity at pH 8.8, although we recognise that the measurement may not reflect elastolytic activity *in vivo*.

ASSAY OF THE EFFECT OF SPUTUM SOLS ON CILIARY FUNCTION

Human nasal cilia were obtained from normal volunteers, without local anaesthesia, by brushing the lateral aspect of the inferior turbinate of the nostril with a cytology brush and dispersing the strips of ciliated epithelium that were obtained by agitation in phosphate buffered saline (PBS). The suspension of ciliated epithelium was then divided into two and both centrifuged at 100 g for 10 minutes at 4°C. The PBS was then aspirated and discarded and one pellet of ciliated epithelium resuspended in 500 μ l sputum sol and the other pellet in 500 μ l PBS to act as control. Each suspension was placed in a sealed microscope coverslip slide preparation at 37°C for measurement of ciliary beat frequency (CBF) by a photometric technique,⁸ using an automated ciliary beat frequency processor unit.⁹ Each preparation was allowed to equilibrate to 37°C over 10 minutes. During this period usually 10, but at least six, strips of ciliated epithelium were identified and the slide marked so that serial readings of CBF could be taken from the same strips throughout the experiment. Single or small groups of ciliated cells were ignored because readings from such areas were inconsistent. Ten CBF readings were then taken (after the 10 minute equilibration phase) and were designated as being taken at time 0. Subsequent readings were taken hourly for four hours. At each time point readings were taken using all the marked strips and the mean of these 10 readings calculated. When all cilia on a marked strip stopped beating the CBF was recorded as zero. When

some cilia on a strip were still beating, however, the CBF of the moving cilia was measured. During CBF recordings a note was made of any ciliary dyskinesia (disturbance of the normal ordered pattern of ciliary beating), ciliostasis, or epithelial disruption (break up of an epithelial strip into smaller groups of cells) present on the slide.

In the first part of the study a sputum sol from each of the 20 patients was assayed in this way and the results analysed for any association of ciliary slowing with elastolytic activity of the sols or with the bacteria isolated from the sputum. Ciliary inhibition was expressed as the percentage reduction of the mean CBF at each time point in sol compared with the mean CBF at the same time point in PBS.

INHIBITION/EXTRACTION TESTS ON SPUTUM SOLS BEFORE ASSAY OF CILIARY FUNCTION

Initial results suggested that sol elastolytic activity was not the only cause of the ciliary slowing activity. We therefore studied the sputum sol from five patients (cases 1, 5, 6, 8, and 10) in greater detail.

These sputum sols were retested in the CBF assay after the addition of sufficient α_1 antiprotease (Sigma) to inhibit all the sol elastolytic activity (confirmed subsequently using the fluorescein-elastin technique). In these experiments CBF was measured in parallel on three microscope slide-coverslip preparations. In the first experiment, cilia were suspended in 600 μ l PBS; in the second, cilia were suspended in 500 μ l sputum sol with 100 μ l PBS, in which the appropriate amount of α_1 antiprotease had been dissolved; and in the third, cilia were suspended in 500 μ l sputum sol with 100 μ l PBS. In these experiments the cilia were suspended in the sputum sols immediately after mixing the sols with PBS or PBS and α_1 antiprotease. The cilia were then allowed to equilibrate to 37°C over 10 minutes before the first CBF readings were taken, which were designated as being taken at time 0.

Sols from the same five sputum samples were retested in the CBF assay after treatment with chloroform. Firstly, 500 μ l sputum sol was agitated vigorously with 1 ml chloroform for 30 minutes at room temperature. The mixture was then allowed to separate and the bulk of the chloroform removed by pipette and retained and the residual chloroform removed by exposure to an air stream for 15 minutes. The extracted sputum sol was then assayed for ciliary slowing activity. The material extracted by chloroform was also tested in the CBF assay after evaporating the chloroform using an air stream and resuspending the material in 500 μ l PBS.

Results

Table 1 shows the mean CBF at each assay time point

Table 1 Human nasal ciliary beat frequency (CBF) in sputum sols from patients with chronic bronchial sepsis compared with control readings in phosphate buffered saline

Test (T) or Control (C)		Mean CBF (Hz) at each time point (hours) in assay					% reduction in CBF by sol after		Pattern of ciliary slowing*	Elastolytic activity of sol (mg/l)	Bacteria isolated from sputum
		0	1	2	3	4	0 h	4 h			
1†	T	3.1	0	0	0	0	79	100	a	0	<i>P aeruginosa</i>
	C	14.5	14.6	13.3	13.3	12.6					
2†	T	7.6	6.6	6.6	0	0	33	100	a	2.9	<i>P aeruginosa</i>
	C	11.4	11.0	11.2	11.0	10.8					<i>H influenzae</i>
3†	T	7.5	6.4	4.2	0	0	35	100	a	4.0	<i>P aeruginosa</i>
	C	11.6	11.9	11.4	12.4	12.4					
4†	T	9.2	7.8	7.8	6.2	6.2	24	52	a	6.7	<i>P aeruginosa</i>
	C	12.2	12.8	13.0	12.8	12.9					
5†	T	6.7	6.9	5.7	3.4	4.1	46	69	a	1.7	<i>P aeruginosa</i>
	C	12.5	12.8	12.5	13.4	13.2					
6†	T	14.2	10.2	7.8	2.1	0	0	100	b	2.4	<i>P aeruginosa</i>
	C	13.6	12.4	12.0	12.8	12.4					<i>H influenzae</i>
7†	T	12.5	12.1	8.9	7.6	4.1	2	69	b	0.6	<i>H influenzae</i>
	C	12.8	13.5	13.6	13.4	13.6					
8†	T	13.0	10.1	10.6	7.8	4.2	7	63	b	0.8	<i>P aeruginosa</i>
	C	14.1	14.2	13.0	12.5	11.4					<i>H influenzae</i>
9†	T	12.0	12.2	10.3	9.5	6.0	0	51	b	1.6	<i>H influenzae</i>
	C	11.5	12.2	12.1	12.0	12.2					<i>S aureus</i>
10†	T	15.2	9.7	9.9	7.7	6.8	0	45	b	1.2	<i>P aeruginosa</i>
	C	14.0	13.0	12.4	12.6	12.3					<i>H influenzae</i>
11†	T	12.2	13.7	12.0	9.6	8.3	3	40	b	6.0	<i>S aureus</i>
	C	12.6	13.8	13.6	14.0	13.9					<i>H influenzae</i>
12†	T	12.0	12.7	11.9	11.3	10.8	2	15	b	2.8	<i>P aeruginosa</i>
	C	12.2	13.0	13.1	12.9	12.7					<i>H influenzae</i>
13†	T	12.3	11.9	11.2	11.4	11.0	2	12	b	0.04	<i>P aeruginosa</i>
	C	12.5	12.5	13.1	12.6	12.5					<i>H influenzae</i>
14	T	12.4	13.8	13.5	13.0	13.5	4	2		1.5	<i>S aureus</i>
	C	12.9	13.6	13.9	13.7	13.8					<i>H influenzae</i>
15	T	12.1	12.6	10.9	10.8	11.5	0	4		4.6	<i>P aeruginosa</i>
	C	11.7	12.2	12.0	12.1	12.0					<i>H influenzae</i>
16	T	13.5	12.6	12.6	12.2	12.0	0	3		0.1	<i>H influenzae</i>
	C	13.0	12.7	12.3	12.3	12.4					
17	T	12.5	13.0	13.3	13.0	13.2	2	0		10.0	<i>H influenzae</i>
	C	13.5	12.8	13.3	13.1	13.1					
18	T	14.2	13.8	13.6	13.5	13.9	0	0		1.1	<i>H influenzae</i>
	C	13.8	13.5	13.6	13.0	13.4					
19	T	12.0	13.3	13.6	13.3	13.4	4	0		0.9	Nil
	C	12.5	13.4	13.5	13.2	13.2					
20	T	11.9	12.5	12.5	12.4	12.6	0	0		3.2	<i>H influenzae</i>
	C	11.9	12.4	12.2	12.2	12.3					<i>S aureus</i>

*Pattern of ciliary slowing refers to figure 1(a) and (b).

†Sputum sol caused significant ciliary slowing ($p < 0.001$, unpaired Student's t test).

for all sputum sols with control readings in PBS. Also shown is the percentage reduction in CBF caused by each sputum sol at time 0 and after four hours, calculated as:

$$\frac{\text{Mean CBF in PBS} - \text{mean CBF in sputum sol}}{\text{Mean CBF in PBS}} \times 100$$

Elastolytic activity of each sputum sol and the bacteria isolated from each sputum sample are included in table 1. The two patterns of ciliary slowing referred to in table 1 are described below.

CBF in PBS did not decrease significantly over four hours in 16 of 20 experiments. In the other four experiments there was a small but significant decrease in CBF over four hours in PBS ($p < 0.05$, unpaired Student's t test).

In seven of 20 sputum sols CBF was not significantly slower than CBF in control PBS, the pattern of ciliary beating was normal, and there was no epithelial disruption. In the remaining 13 sputum sols, however, there was significant ciliary slowing ($p < 0.001$, unpaired Student's t test). Two patterns of ciliary slowing were observed. The first was characterised by a significantly slower CBF in sputum sol than in PBS at time 0 and four hours ($p < 0.001$, unpaired Student's t test). This immediate slowing of CBF by sputum sol was associated with ciliary dyskinesia and areas of static cilia without epithelial disruption. Five sputum sols caused this pattern of slowing, which is represented in figure 1(a). This shows the results using sputum sol from case 5 and is referred to as pattern a in the tables. The second pat-

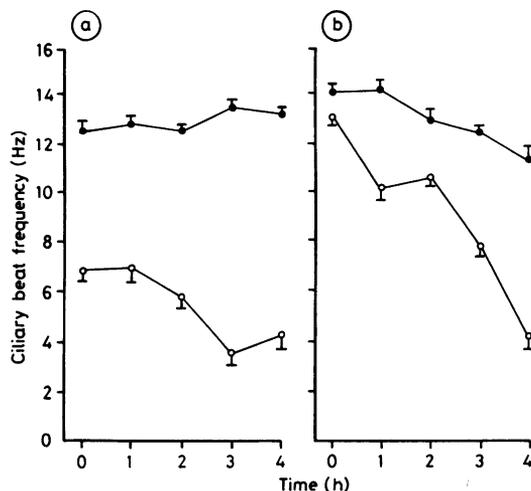


Fig 1 Human nasal ciliary beat frequency in sputum sols from (a) case 5 (chloroform extractable) and (b) case 8 (α_1 antiprotease inhibitable) compared with control readings in phosphate buffered saline. ●—● = Ciliary beat frequency in phosphate buffered saline (control); ○—○ = ciliary beat frequency in sputum sol. Bars represent standard errors.

tern of slowing was characterised by a significantly slower CBF in sputum sol than in PBS at four hours ($p < 0.001$, unpaired Student's *t* test) but no significant difference between CBF in sputum sol and PBS at time 0. This gradually progressive slowing of CBF by sputum sol was associated with epithelial disruption. Eight sputum sols caused this pattern of slowing, which is represented in figure 1(b). This shows the results using sputum sol from case 8 and is referred to as pattern b in the tables. Table 2 shows the changes in ciliary inhibitory activity of five sputum sols treated by α_1 antiprotease and chloroform extraction. Pattern a slowing was inhibited by chloroform extraction but unaffected by α_1 antiprotease. The material extracted by chloroform reproduced the immediate ciliary slowing effect. Pattern b ciliary

slowing was inhibited by α_1 antiprotease but unaffected by chloroform extraction.

Sputum sol elastolytic activity (mg/l) did not correlate with ciliary slowing activity expressed as the percentage reduction of CBF after four hours. This was true for the whole group ($r = -0.118$, linear regression analysis) and also when sputum sols that caused immediate slowing (pattern a) were excluded ($r = -0.184$, linear regression analysis). The latter analysis was performed because the immediate slowing is not a serine protease effect (not α_1 antiprotease inhibitable) and would therefore be unlikely to correlate with elastolytic activity. Of particular note was the lack of ciliary slowing activity or epithelial disruption caused by sputum sols from cases 14, 15, 17, and 20 in which there was considerable elastolytic activity (1.5, 4.6, 10.0, and 3.2 mg/l, respectively).

Pseudomonas aeruginosa was isolated from 10 of 13 sputum samples whose sols had ciliary slowing activity but from only one of six sputum samples whose sols had no such activity. This association of *P. aeruginosa* with ciliary slowing activity was significant ($\chi^2 = 7.2$ $p < 0.01$).

Discussion

The impairment of mucociliary clearance in patients with chronic bronchial sepsis but without primary ciliary dyskinesia may be due to alteration of the properties of mucus or alteration in ciliary function, or both. It has been reported that mucus with low viscosity and high elasticity is ideal for ciliary transport¹⁰ but that purulent sputum becomes more viscous and has increased crosslinking by hydrogen bonding within the gel.¹¹ Loss of ciliated epithelium may occur due to virus infection¹² or at sites of purulent secretions.¹³ Impaired ciliary beating occurs at sites of purulent secretions in the respiratory tract¹⁴ and may also contribute to the secondary impairment of mucociliary clearance seen in the presence of purulent infection.

Two groups of workers have shown that purulent

Table 2 Effect of α_1 antiprotease and chloroform extraction on ciliary slowing activity of sputum sols

Case No from whom sol taken	% Reduction CBF by sol after adding 100 μ l PBS to 500 μ l sol at		Pattern of ciliary slowing*	% Reduction CBF by sol after treatment with α_1 antiprotease at		% Reduction CBF by sol after chloroform extraction at		% Reduction in CBF by chloroform extracted material at	
	0h	4h		0h	4h	0h	4h	0h	4h
1	81	95	a	76	100	3	0	58	89
5	43	74	a	50	68	2	5	22	57
6	0	100	b	0	2	0	100		
8	5	56	b	0	0	0	45		
10	0	41	b	0	4	0	36		

CBF = Ciliary beat frequency; PBS = phosphate buffered saline.

*Pattern of ciliary slowing refers to figure 1(a) and (b).

sputum slows ciliary beating. Sputum sols obtained by ultracentrifugation of purulent sputum from patients with bronchiectasis have been reported to slow human nasal ciliary beating in vitro.¹⁵ The pattern of ciliary slowing reported was progressive over six hours from a normal initial CBF and the effect was inhibited by α_1 antiprotease, suggesting that a serine protease is one factor in purulent sputum that slows cilia. Mucociliary wave frequency on the frog palate was slowed after application of purulent sputum from patients with chronic bronchitis and cystic fibrosis.¹⁶ This property of purulent sputum may contribute to the impairment of mucociliary clearance seen in patients with chronic bronchial sepsis. It is still unclear exactly what factor(s) is responsible for this ciliary slowing activity in purulent sputum, although a serine protease such as neutrophil elastase or cathepsin G is probably one of the factors.¹⁵ Leucocyte enzymes are known to affect rabbit ciliated epithelium,¹⁷ but the effect is predominantly disruptive.

Products from the bacteria *P aeruginosa* and *Haemophilus influenzae* isolated from patients with chronic bronchial sepsis have been shown to slow human nasal cilia in vitro.¹⁸ Other bacterial products that have been shown to slow cilia in vitro include a heat stable, trypsin resistant factor released by *H influenzae*¹⁹ and chloroform extractable factors released by *P aeruginosa*, which are likely to be phenazine pigments and possibly haemolysin.²⁰ *P aeruginosa* pigment production has been reported to correlate with human nasal ciliary slowing activity in vitro²¹ and the purified *P aeruginosa* pigments pyocyanin and 1-hydroxyphenazine have been shown to slow human nasal cilia in vitro.²¹ These bacterial products are therefore candidates for at least part of the ciliary slowing activity of purulent sputum.

Our results confirm that purulent sputum slows human ciliary beating in vitro. Two patterns of ciliary slowing were observed. The chloroform extractable factor(s) that cause immediate ciliary slowing (pattern a) has not been described previously. The pattern of effect on ciliary beating is similar to that reported for bacterial products of organisms from patients with chronic bronchial sepsis.¹⁸ In particular, crude supernatants from overnight culture of *P aeruginosa* caused a pattern of ciliary slowing that closely matched that caused by sputum sols (pattern a), although filtering the supernatants (0.2 μ m Gelman) altered the pattern of slowing so that some recovery of ciliary beating occurred after four hours.¹⁸ The chloroform extractability of the factor(s) that caused pattern a slowing would be compatible with the reported properties of the *P aeruginosa* cilioinhibitory phenazine pigments, pyocyanin and 1-hydroxyphenazine.^{20 21} The isolation of *P aeruginosa* from all five sputum samples that caused immediate ciliary slowing would also be

compatible with the effect being due to *P aeruginosa* pigments. Isolation of *P aeruginosa* from six sputum samples that did not cause immediate ciliary slowing does not exclude these phenazine pigments from having an important ciliary slowing role, as different isolates of *P aeruginosa* obtained from patients with chronic bronchial sepsis have a range of pigment producing activity.²¹ Strains that produce larger amounts of pigment may therefore be those responsible for pattern a ciliary slowing. The factor(s) that cause progressive ciliary slowing with epithelial disruption (pattern b) is likely to be a serine protease, as it is inhibited by α_1 antiprotease (a serine protease inhibitor). This agrees with other findings,¹⁵ although epithelial disruption was not mentioned in this study. The enzyme(s) responsible for this effect are most likely to have been released by phagocytes at the site of infection—neutrophil elastase or cathepsin G or both are strong candidates, both being serine proteases. Neutrophil elastase activity has been shown to be present in purulent sputum.²²

Poor correlation between a sputum sol's elastolytic activity and its CBF slowing potency is evidence against elastase being the only factor(s) that cause pattern b ciliary slowing. We draw this conclusion with the slight reservation that our method involves recording CBF from moving cilia when they are on the same epithelial strip as static cilia, which may on occasions lead to underestimation of ciliary slowing activity and therefore contribute to a lack of correlation between ciliary slowing and elastolytic activity. Nevertheless, this would hardly explain the complete lack of ciliary slowing activity in sputum sols from cases 14, 15, 17, and 20, which all contained considerable elastolytic activity (1.5, 4.6, 10.0, and 3.2 mg/l, respectively). We do not believe that measurement of elastolytic activity at pH 8.8 compared with measurement of ciliary slowing at pH 7.4 affects this conclusion, as we have been able to measure elastolytic activity in 17 sputum sols at pH 7.4. The correlation between elastolytic activity and CBF slowing potency was still poor (linear regression analysis, $r = 0.19$).

The strong association of *P aeruginosa* with sputum sol ciliary slowing activity is probably due to a combination of reasons. Firstly, *P aeruginosa* may be directly responsible for pattern A ciliary slowing by the release of cilio-inhibitory phenazine pigments.^{20 21} Secondly, the organism may also contribute indirectly to pattern b ciliary slowing by the release of potent chemotactic factors,²³ which resulted in increased neutrophil traffic to the bronchial tree and consequent release of neutrophil proteases. Thirdly, the patients from whom sputum sols cause ciliary slowing may have more advanced disease and be more likely to be colonised by *P aeruginosa*.

The detection of these cilio-inhibitory activities in purulent sputum lends further support to the 'vicious circle' hypothesis of the pathogenesis of chronic bronchial sepsis.²⁴ This holds that an initial damaging insult to the respiratory tract allows microbial colonisation to occur, after which the clearance mechanisms are further damaged by microbial release of cilio-inhibitory factors¹⁸ and by products of the host inflammatory response against such colonising microbes. The strong association shown here of *P. aeruginosa* with such ciliary slowing properties of purulent sputum underlines the importance of reducing the colonising microbial load in patients with chronic bronchial sepsis to minimise progressive impairment of mucociliary clearance, which may predispose to chronicity and increasing severity of infection.

In conclusion, purulent sputum in chronic bronchial sepsis contains at least two factors that slow human nasal ciliary beating in vitro. One is a serine protease activity and may be released by neutrophil host defences and the other is chloroform extractable and may be a bacterial product.

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