Bacterial cyanogenesis occurs in the cystic fibrosis lung

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ABSTRACT: The cystic fibrosis (CF) lung environment is poorly defined, but data suggest that bacteria may encounter reduced oxygen tensions and possibly an anaerobic environment. *Pseudomonas aeruginosa* produces the potent toxin cyanide under strictly microaerobic conditions. Evidence of bacterial cyanogenesis in the CF lung was investigated in the present study by measuring sputum cyanide concentrations.

Sputum cyanide was measured in seven stable CF patients, as well as before and after intravenous antibiotic therapy during a hospital admission in a further eight patients experiencing acute exacerbations. All patients were chronically infected with *P. aeruginosa*. Comparative sputum data were obtained from nine CF patients with no documented *P. aeruginosa* infection and 10 healthy, nonsmoking normal controls.

High levels of cyanide were detected in all the *P. aeruginosa*-infected stable CF patients (median (range) 0.56 (0.37–2.81) $\mu g \cdot mL^{-1}$), and in seven out of eight acute sputum samples (0.73 (0–1.43) $\mu g \cdot mL^{-1}$). In contrast, cyanide was not detectable in sputum from eight out of nine CF patients without *P. aeruginosa* infection or in any of the normal controls. Intravenous antibiotic treatment significantly reduced sputum cyanide levels (median 0.73 to median 0.0 $\mu g \cdot mL^{-1}$).

The cyanide detected indicates that the cystic fibrosis lung provides a predominantly microaerobic environment for *Pseudomonas aeruginosa*. Cyanide is likely to be a potentially important virulence factor in *Pseudomonas aeruginosa*-infected cystic fibrosis patients.

KEYWORDS: Cyanide, microaerobic, *Pseudomonas aeruginosa*, pulmonary disease

In individuals with cystic fibrosis (CF) and other destructive lung diseases, *Pseudomonas aeruginosa* is an opportunistic bacterium that causes chronic airway sepsis. In CF, it becomes the predominant pathogen by late childhood [1]. This early acquisition of *P. aeruginosa* in relatively normal lungs is unique to CF. Much of the lung damage that occurs in CF is thought to be due to ineffective host factors [2]. Bacterial virulence factors undoubtedly also contribute to lung damage, but they have been poorly characterised *in vivo*. In CF airways, *P. aeruginosa* is thought to grow in anaerobic/reduced oxygen pockets situated within thickened and tenacious mucus plugs that occlude small airways [3, 4].

This environment may be suitable for cyanide production by *P. aeruginosa* [5–7]. However, to date, it is not known whether bacteria are residing in strictly anaerobic or microaerobic pockets within the CF lung. *P. aeruginosa* is one of only a few bacterial species (*P. fluorescens*, *Chromobacterium violaceum* and *Rhizobium leguminosarum*) known to produce cyanide [8]. The *P. aeruginosa* cyanide synthase enzyme is cell associated and requires molecular oxygen as an electron acceptor. Cyanide is only produced over a very narrow spectrum of environmental oxygen tensions (microaerobic) and synthesis is rapidly inactivated under both atmospheric oxygen and strictly anaerobic conditions [8]. Therefore, cyanide levels were measured in sputum samples from CF patients as both an *in vivo* marker of *P. aeruginosa* microaerobic growth and to determine if cyanide production could be a potential virulence mechanism in CF lung disease.

METHODS

The Southern Tasmania Health and Medical Human Research Ethics Committee approved the study and all subjects gave written informed consent before participating. In total, 15 adult CF patients with chronic *P. aeruginosa* lung infection (confirmed by repeated routine microbiological testing) were recruited at the time of a routine clinic appointment for stable patients ($\geqslant$1 month from most recent exacerbation), or when unwell with worsening cough, breathlessness and sputum purulence and admitted to hospital (acute patients). In the acute patients, a repeat sputum sample was obtained $\geqslant$1 week into the intravenous antibiotic course (median (range) 8.5

TABLE 1	Subject demographics			
	CF patients with *P. aeruginosa*		CF patients without *P. aeruginosa*[¶]	Controls
	Acute[#]	**Stable**		
Subjects n	8	7	9	10
Age yrs	23 (19–48)	23 (17–33)	10 (4–17)	43 (26–63)
Sex F/M n	5/3	3/4	3/6	5/5
Sputum cyanide $\mu g \cdot mL^{-1}$	0.73 (0.0–1.43)***	0.56 (0.37–2.81)***	0.0 (0.0–0.49)	0.0 (0.0–0.0)
FEV_1 L	1.55 (0.5–2.84)	1.98 (0.78–4.51)	2.08 (1.06–3.21)	3.57 (2.27–5.12)
FEV_1 % pred	42 (16–67)[+]	52 (26–98)[+]	88 (59–104)[+,§]	106 (99–127)
FVC	2.62 (1.00–4.63)	3.02 (1.39–5.50)	2.54 (1.17–4.24)	4.79 (3.06–6.72)
FVC % pred[+,§]	64 (30–83)	72 (40–103)	95 (73–113)	116 (95–141)

Data are presented as median (range), unless otherwise stated. CF: cystic fibrosis; *P. aeruginosa*: *Pseudomonas aeruginosa*; F: female; M: male; FEV_1: forced expiratory volume in one second; % pred: % predicted; FVC: forced vital capacity. [#]: lung function values achieved within 48 h of admission; [¶]: lung function results are based on seven patients, as two patients aged 4 and 5 yrs could not manage reproducible spirometry; [+]: CF patients *versus* healthy controls, $p<0.01$; [§]: *P. aeruginosa*-infected (stable and acute patients) *versus* CF patients without *P. aeruginosa* infection, $p<0.01$. ***: *P. aeruginosa*-infected (stable and acute patients) *versus* cystic fibrosis (CF) patients without *P. aeruginosa* infection and healthy controls, $p<0.001$.

(7–14) days) at a time-point when symptoms were subjectively improving. In total, 14 patients were receiving long-term anti-*P. aeruginosa* antibiotic treatment regimes by inhalation (data not shown). One patient was not receiving any form of antibiotic therapy directed against *P. aeruginosa* at the time of sampling. Nine patients who had not grown *P. aeruginosa* previously also provided sputum samples. Spontaneously expectorated sputum was collected and processed as described previously [9]. Induced sputum samples were obtained from 10 healthy volunteers using the method of PIN *et al.* [10] and processed in the same way. All CF patients and normal controls were current nonsmokers.

Sputum processing

An aliquot of raw sputum was weighed and an equivalent volume to weight of dithiothreitol (10%) was added. The sample was gently vortexed and placed in a water bath (38°C) for 30 min. At 10-min intervals the sample was removed and gently vortexed again. If the sample appeared particularly tenacious, further mixing was undertaken with gentle pipetting to ensure homogenisation. The sample was diluted a further five times with PBS, ensuring a constant final dilution effect of 10 times, and then centrifuged at $350 \times g$ for 15 min. Following centrifugation, the cell-free supernatant was decanted and stored at -80°C in 1-mL aliquots for further analysis.

Cyanide detection

Cyanide was liberated from the processed sputum supernatants by acid treatment and assayed using a standard method (4500-CN; American Public Health Association, Washington, DC, USA). The lower limit of detection of the method was $0.05 \mu g \cdot L^{-1}$.

Statistical analysis

Differences between groups were assessed using the Kruskal–Wallis test for nonparametric data. The Wilcoxon's rank test was used to compare sputum cyanide levels in samples obtained at the start of an exacerbation and at the end of an intravenous antibiotic treatment course. A two-tailed p-value of $\leqslant 0.05$ was considered to be statistically significant.

RESULTS

Healthy controls were significantly older and had better lung function than the CF patients (table 1). Not surprisingly, CF patients without *P. aeruginosa* infection were younger and had better lung function than those with chronic *P. aeruginosa* infection. Cyanide was present in all of the sputum samples from stable CF patients with *P. aeruginosa* (median (range) 0.56 (0.37–2.81) $\mu g \cdot mL^{-1}$), and in seven out of eight samples from acute patients 0.73 (0.0–1.43) $\mu g \cdot mL^{-1}$. There was no statistical difference between sputum cyanide levels in acute and stable patients ($p=0.9$). Cyanide was not detected in eight out of nine sputum samples from CF patients without *P. aeruginosa* or in

TABLE 2	Demographic details and sputum culture results in stable cystic fibrosis patients without *Pseudomonas aeruginosa* infection		
Subject	Age yrs	Sex	Sputum microbiology
1[#]	3	M	*S. aureus*
2	16	M	MRSA, *S. prolificans*
3	15	M	*S. aureus, A. fumigatus*
4	10	F	*S. aureus, H. influenzae*
5	16	F	*S. aureus*
6	12	M	*S. aureus, A. xylosoxidans, S. prolificans*
7	8	M	*A. fumigatus*
8	4	M	*S. aureus, H. influenzae, A. fumigatus, S. maltophilia*
9	6	F	*S. aureus*

M: male; F: female; *S. aureus*: *Staphylococcus aureus*; MRSA: methicillin-resistant *Stapylococcus aureus*: *S. prolificans*: *Scedosporium prolificans*; *A. fumigatus*: *Aspergillus fumigatus*; *H. influenzae*: *Haemophilus influenzae*; *A. xylosoxidans*: *Alcaligenes xylosoxidans*; *S. maltophilia*: *Stenotrophomonas maltophilia*. [#]: the only patient with detectable sputum cyanide levels.

any of the sputum samples from normal controls (table 1 and fig. 1). The only CF patient without *P. aeruginosa* who had detectable sputum cyanide was <5 yrs of age. This patient had isolated *Staphylococcus aureus* in the only two sputum samples obtained to date (table 2).

Intravenous antibiotic treatment for an acute exacerbation reduced sputum cyanide levels (median (range) 0.73 (0.0–1.43) $\mu g \cdot mL^{-1}$ to 0.0 (0.0–1.00) $\mu g \cdot mL^{-1}$, p=0.05). In four of the seven acute patients with detectable cyanide levels on admission, the levels were reduced to below the detection limit of the assay following intravenous antibiotic treatment.

Interestingly, the highest sputum cyanide concentration was found in a stable patient who was the only individual chronically infected with *P. aeruginosa* and not receiving any form of routine antibiotic therapy directed against the bacterium. There was no relationship between sputum cyanide levels and lung function.

DISCUSSION

This is the first report of cyanide in CF sputum and the present results support previous speculation that the CF lung environment may be suitable for *P. aeruginosa* cyanogenesis. Both clinically stable CF patients and those presenting with acute exacerbations of CF had high levels of cyanide, whereas cyanide was undetectable in sputum samples from normal healthy controls and nearly all sputum samples obtained from CF patients from whom *P. aeruginosa* had not previously been isolated. Following intravenous antibiotic treatment for an acute exacerbation, sputum cyanide levels were significantly reduced in patients infected with *P. aeruginosa*.

Cyanide is a very potent poison that causes cell death through irreversible inhibition of mitochondrial oxidative phosphorylation. Cyanide toxicity is well described following smoke inhalational injuries in humans, but the role of cyanide in human disease in the setting of *P. aeruginosa* infection is poorly characterised. However, cyanide production has been noted in *P. aeruginosa*-infected burn eschars [11–13]. The cellular toxicity of cyanide is greatly influenced by the chemical milieu but, in humans, blood cyanide levels of 0.5–1.0 $\mu g \cdot mL^{-1}$ are associated with cardiac effects, levels of 2.5–3.0 $\mu g \cdot mL^{-1}$ result in reduced consciousness and levels >3.0 $\mu g \cdot mL^{-1}$ cause death [14]. Therefore, the sputum levels detected in the present study are well within the toxic range for cell metabolism and will probably contribute to long-term lung damage in CF.

The capacity of *P. aeruginosa* to produce cyanide has been recognised for some time [12]. Most of the current understanding of the genetics, biochemistry and regulation of cyanide production by *P. aeruginosa* comes from studying its interactions in soil communities, where *P. aeruginosa* uses cyanide to protect its niche and eradicate competing organisms [8]. The current finding of high cyanide levels in CF sputum has several disease implications. First, cyanide may be used by *P. aeruginosa* in the lung in the same way that it is utilised in soil, that is, to protect its niche. This may partly explain why *P. aeruginosa* rapidly becomes the predominant pathogen in CF, apparently displacing other bacterial species. Secondly, cyanide will be directly toxic to airway cells and, finally, cyanide may also contribute to the relative inability of neutrophils to clear *P. aeruginosa* infection in the CF lung [15, 16]. In the
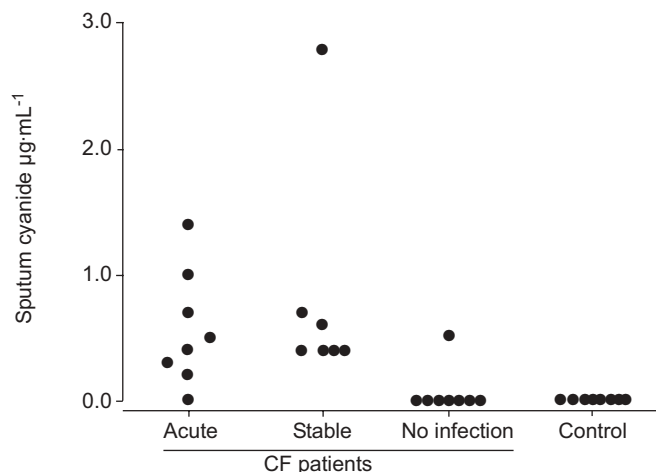

FIGURE 1. Cyanide concentrations in sputum samples from cystic fibrosis (CF) patients with chronic *Pseudomonas aeruginosa* infection experiencing an acute exacerbation (n=8), stable CF patients with chronic *P. aeruginosa* infection (n=7), stable CF patients with no documented *P. aeruginosa* infection (n=9) and healthy controls (n=10). Each point represents one sputum sample.

concentrations reported, cyanide has been shown to inhibit the function of the enzyme myeloperoxidase and prevent production of hypochlorous acid during the oxidative burst [17]. Cyanide may also impair neutrophil migration and cause morphological changes related to damage to the cell cytoskeleton [18]. Interestingly, neutrophils can produce very small amounts of cyanide during chlorination of bacterial cell membranes [19], but this contribution is likely to be negligible in comparison with the high cyanide levels reported herein. Furthermore, the present authors were unable to detect cyanide in eight out of nine sputum samples from CF patients not yet infected by *P. aeruginosa*, although other bacterial pathogens were isolated from the sputum and studies have demonstrated that these subjects will also have a florid airway neutrophilia [20, 21]. Cyanide was detected in sputum from a very young patient who had no isolated *P. aeruginosa* on routine culture. This patient rarely produces sputum and obtaining the sample was extremely fortunate; however, at present there is no explanation for the cyanide detected. There is the potential that the few culture results available from this individual have been false negatives for *P. aeruginosa*, but this has not been confirmed with more invasive techniques. The patient's clinical progress is being closely monitored continuously and, to date, the patient appears well.

There has been recent debate on the nature of the microbial habitat in the CF lung, particularly with respect to whether conditions are aerobic or anaerobic [3, 4]. Understanding lung environmental conditions is clinically important, as the efficacy of several antibiotics is dependent on the chemical milieu, *i.e.* the aminoglycosides function very poorly under anaerobic conditions [22]. The current findings suggest that a substantial proportion of the *P. aeruginosa* population in the CF lung exists in microaerobic (O_2 <5%) rather than aerobic or anaerobic conditions because *P. aeruginosa* can only produce cyanide under strictly microaerobic conditions [8]. Given the oxygen gradient demonstrated in CF mucus, it is also possible that there are

populations of *P. aeruginosa* dwelling within aerobic or anaerobic regions in the CF lung; however, further studies with other metabolic markers are required to confirm this [4]. This habitat diversity may partly explain the refractory nature of infection, as microbial populations in different physiological states may respond quite differently to therapeutic interventions.

In the relatively small number of sputum samples tested, cyanide levels were similar whether patients were stable or acute, but levels in the latter patients were substantially reduced by a course of intravenous antibiotics. A valid criticism of the study is that the small numbers of patients studied reduced the present authors' ability to detect a difference in sputum cyanide between individuals based on clinical status. Ideally, patients should have been followed from the clinically stable situation into an exacerbation and then been reassessed at the end of treatment, as well as 1 month later when they were once again stable. Such a study clearly needs to be undertaken and correlated with changes in bacterial numbers. The confirmation of a relationship between cyanide and clinical status would open up the development of tools, such as exhaled breath condensates to monitor cyanide as a potential biomarker. Despite the small number of subjects studied, it is interesting to note that the highest cyanide level detected occurred in a stable patient who was the only *P. aeruginosa*-infected patient not receiving any form of anti-*P. aeruginosa* therapy. Chronic suppressive inhaled antibiotic treatment strategies directed against *P. aeruginosa* may, therefore, be partially effective at inhibiting cyanide production, while more aggressive treatment in the present study reduced cyanide in the CF lung to undetectable levels in almost half of the cases. Thus, routine intravenous treatment courses may be of particular benefit in treating this disease. Whether the reduction in cyanide levels following in-patient antibiotics was due to alterations in bacterial numbers or to other nonbactericidal effects was not assessed in this study, but it is worth speculating that the excellent outcomes reported in some countries may be due in part to suppression of cyanide production through regular admissions, irrespective of clinical status, for intravenous antibiotic courses [23, 24].

In vitro data suggests that mucoid *P. aeruginosa* isolates produce more cyanide than nonmucoid laboratory strains [5], but such a relationship was not identified in the present study as all patients were colonised with mucoid strains (data not shown). Whether strains that produce more cyanide have a survival advantage in the CF lung environment or whether the alginate layer in mucoid strains simply increases cyanide output by limiting oxygen diffusion are questions that need to be examined further.

In conclusion, the present study demonstrates large amounts of cyanide in cystic fibrosis sputum. Cyanide is likely to be an important virulence factor employed by *Pseudomonas aeruginosa* in cystic fibrosis and almost certainly contributes directly to long-term lung damage and impairment of the local host immune system. Of clinical importance, it has been shown that cyanide production can be reduced to very low levels by aggressive antibiotic therapy. The present findings suggest that a substantial proportion of the bacterial population in the cystic fibrosis lung exists under microaerobic rather than anaerobic conditions. Whether cyanide in either sputum or

exhaled breath condensates may be a reliable marker of bacterial activity that could be used to assess treatment efficacy in cystic fibrosis requires further study.

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