Adult cystic fibrosis: association of acute pulmonary exacerbations and increasing severity of lung disease with auxotrophic mutants of *Pseudomonas aeruginosa*

Rowena F H Taylor, Margaret E Hodson, Tyrone L Pitt

Abstract

**Background—** *Pseudomonas aeruginosa* has been located in the endobronchial spaces of patients with cystic fibrosis where nutrients may be limited. In these sites it is thought that adaptation of the pathogen might occur and growth factors, present in relative excess, may thus promote survival of the organism. Auxotrophy of pulmonary isolates of *P aeruginosa* has previously been shown to be a feature of cystic fibrosis and chronic lung sepsis; auxotrophic isolates have additional nutritional requirements to the prototrophic “wild types” of the species. A study was therefore carried out to determine whether the proportion of auxotrophs differs between stable and acutely ill patients, or correlates with the extent of underlying disease.

**Methods—** Sputum samples were cultured for *P aeruginosa* and tested for auxotrophy by spreading serial dilutions of homogenised sputum on to a minimal medium which supports only prototrophs, and a complete medium which supports both nutritional types. The proportion of auxotrophs to prototrophs was determined and growth factors of confirmed auxotrophs were identified.

**Results—** Thirty-two (86%) of 37 adults with cystic fibrosis infected with *P aeruginosa* harboured auxotrophs; methionine dependent mutants were isolated from seven of 16 patients tested (44%). More than 50% of the total number of colonies were auxotrophic in 19 of 26 samples (73%) from patients with acute exacerbations and in only six of 15 samples (40%) from clinically stable patients. In four patients from whom samples in both the acute and stable states were available, the proportion of auxotrophs fell in the sample taken when stable. Auxotrophs predominated in all samples from 11 of those patients with very severe underlying lung disease, in contrast to 13 of 30 samples from patients with less severe disease. There was no association between the percentage of auxotrophs and the presence of other respiratory pathogens.

**Conclusions—** The majority of adults with cystic fibrosis infected with *P aeruginosa* harbour auxotrophs in the sputum. A significant proportion of acutely ill patients and those with severe underlying disease have a preponderance of auxotrophs in the sputum compared with stable patients and those with less severe disease.

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the proportion of auxotrophs differs between stable and acutely ill patients and correlates with the extent of underlying lung disease and the presence of other respiratory pathogens. We have also surveyed the auxotrophic requirements of isolates from single sputum samples and from repeated specimens from the same patient.

Methods

Patients

Forty one samples were studied from 37 patients (19 men) with cystic fibrosis (mean age 28, median 26, range 16–67 years). Samples were taken from 22 randomly selected inpatients with acute infective pulmonary exacerbations, 11 stable outpatients, and four patients who were tested both as acutely ill inpatients and stable outpatients. Eleven patients had very severe lung disease (died or received heart-lung transplants within 12 months), 17 had severe lung disease (forced expiratory volume in one second (FEV,) <40% predicted normal value),10 five had moderate disease (FEV, 40–60%), and four had only mildly impaired lung function (FEV, >60%). Of the 26 inpatient samples the numbers from patients with very severe, severe, moderate and mild disease were eight, 12, two, and four respectively, and of the 15 outpatient samples the numbers were three, seven, four, and one respectively. Twenty six patients were receiving long term nebulised antibiotics (aminoglycosides, colistin, or both), 17 were receiving inhaled steroids, and all inpatients were receiving intravenous antipseudomonal agents (usually a combination of a penicillin derivative with an aminoglycoside) in conjunction with oxygen therapy, bronchodilators, and physiotherapy. The mean duration of intravenous antibiotic therapy before sampling was nine days (range 2–14, median nine days). All patients were taking pancreatic enzyme supplements and eight were diabetic.

Sputum Culture

Manually homogenised sputum was serially diluted (10⁻² to 10⁻⁶) in Ringer’s solution and 100 µl aliquots of each dilution were spread uniformly on to both a minimal salt medium11 which supports only the growth of prototrophs, and King’s “A” agar12 which supports both nutritional types. After aerobic incubation at 37°C for 48 hours the colonies on each plate were counted to determine the total colony count and the proportion of auxotrophs to prototrophs. Isolates which produced the characteristic pigment pyocyanin were accepted to be P aeruginosa and non-pigmented isolates were tested for their ability to oxidise glucose, produce cytochrome oxidase, hydrolyse arginine, and reduce nitrate.13

Sputum samples from 24 patients were taken at the same time as the auxotrophy evaluation and cultured for other respiratory pathogens; inpatient samples from a further five patients were processed within 24 hours.

Specific Growth Factors

Individual colonies were tested for auxotrophy as previously described9 and specific requirements of confirmed auxotrophs were identified by modification14 of Holliday’s experimental method.14

Statistical Methods

Comparison of proportions was made by the x² test with Yates’ correction where appropriate.

Results

Auxotrophic mutants of P aeruginosa were present in the fresh sputum of 35 of 41 (85%) sputum samples from 37 patients with cystic fibrosis. In a comparison of stable with acutely ill patients, the majority (>50%) of Pseudomonas colonies from each sputum sample were auxotrophic in 19 of 26 inpatients with acute pulmonary exacerbations (73%), and in six of 15 stable outpatients (40%) (p < 0.05).

Four patients with cystic fibrosis sampled at random when acutely ill and also when stable harboured a greater proportion of auxotrophs when ill than when stable. The proportion of auxotrophs ranged from 56% to 98% in samples taken during acute exacerbations and from 20% to 52% in samples taken when stable (fig).

Auxotrophs accounted for more than 50% of the total Pseudomonas count in all of the 11 patients with very severe underlying lung dysfunction (including three who were tested when stable) and in only 13 of 30 samples from 26 patients with less impairment of lung function (p < 0.01). Total colony counts of P aeruginosa ranged from 1×10⁷ to >1×10⁹ cfu/ml; there was no relation between the proportion of auxotrophs and the total Pseudomonas colony count or the presence of other sputum pathogens isolated concurrently from 29 of the 37 patients. S aureus was cultured from five patients, Candida albicans >10⁹/ml from 16, P cepacia from three, and Aspergillus fumigatus from three others.

Percentage of auxotrophs in the sputum of four patients with cystic fibrosis sampled when acutely ill and when stable. The time intervals between sampling were 49, 51, 69, and 23 weeks for patients A, B, C, and D respectively. All patients were receiving intravenous and nebulised antibiotics when acutely ill and only nebulised agents when stable.
Table 1  Intraspum variation of growth requirements of
pairs of isolates of P aeruginosa from single sputum
samples of 12 patients with cystic fibrosis

<table>
<thead>
<tr>
<th>Source of sample</th>
<th>Growth requirements*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Same/different</td>
</tr>
<tr>
<td>Inpatient</td>
<td>Different</td>
</tr>
<tr>
<td>Inpatient</td>
<td>Alternative in both</td>
</tr>
<tr>
<td>Inpatient</td>
<td>Same Proline</td>
</tr>
<tr>
<td>Inpatient</td>
<td>Same Multiple</td>
</tr>
<tr>
<td>Inpatient</td>
<td>Same Methionine</td>
</tr>
<tr>
<td>Inpatient</td>
<td>Different 1 alternative, 1 methionine</td>
</tr>
<tr>
<td>Inpatient</td>
<td>Same Methionine</td>
</tr>
<tr>
<td>Inpatient</td>
<td>Different 1 not identified, 1 aspartic acid</td>
</tr>
<tr>
<td>Inpatient</td>
<td>Same Methionine</td>
</tr>
<tr>
<td>Inpatient</td>
<td>Unknown Not identified</td>
</tr>
<tr>
<td>Inpatient</td>
<td>Same Methionine</td>
</tr>
<tr>
<td>Inpatient</td>
<td>Same Methionine</td>
</tr>
</tbody>
</table>

*Growth requirements (after Holiday): alternative—
multiple—require either one or another factor, for example,
serine or methionine; single—require only one factor;
not—require two or more factors; not identified—require absent in factors tested.

Discussion
We have found for the first time that most adults with cystic fibrosis infected with
P aeruginosa harbour auxotrophic mutants in the sputum (86%). Furthermore, a significant
proportion of acutely ill patients and those with very severe underlying lung disease have a preponderance of auxotrophs in the sputum
compared with stable patients and those with less severe disease.

In the light of our findings two further questions arise. Firstly, are auxotrophs of
P aeruginosa induced by a relative excess of certain substrates in the lungs of patients with
cystic fibrosis and non-cystic fibrosis bronchiectasis, or by antibiotic activity in
such patients, or both? Secondly, are the pathogenic effects of auxotrophs greater than
those of prototrophs?

At present the mechanisms underlying the selection of auxotrophic mutants of P aerugi-
osa in cystic fibrosis are poorly understood but the answers to the above questions,
together with the knowledge that auxotrophs are more resistant than prototrophs to
antipseudomonal agents, might explain how
this pathogen persists despite antipseudomonal therapy.

The need for additional growth factors by some members of a bacterial species implies
that a specific biosynthetic defect has developed. It follows that auxotrophs can survive in
vivo only if the end product of the defec-
tive pathway is present in excess within the host environment. Indeed, the relative excess
of such factors may, by a negative feedback
mechanism, inhibit an enzyme within its own
synthetic pathway, and mutants which depend on the substrate in excess may be
selected. It is therefore of interest that in pul-
monary secretions of patients with suppura-
tive lung disease, including bronchiectasis,
there is an excess of glycoprotein and
human DNA.

The growth requirements of isolates from non-cystic fibrosis bronchiectatic patients
have not, as yet, been evaluated. In both non-
cystic fibrosis and cystic fibrosis bronchiecta-
isis, antibiotic treatment may contribute to the
selection of auxotrophic P aeruginosa in a similar
way to the selection of thymidine depend-
ent strains of S aureus in patients with cystic fibrosis after long term treatment with
cotrimoxazole. It has been postulated that
thymidine, a major end product of the folate
pathway, is procured by S aureus from
degraded DNA, thus overriding the trimetho-
prim effect and permitting survival of the
pathogen. In order to delineate the effect of
disease severity on the induction of auxotro-
phy and differentiate this from the effect of
antibiotic exposure, sequential sputum sam-
plestaken before and after antibiotic therapy
need to be tested. Auxotrophy of P aeruginosa
isolates in non-cystic fibrosis bronchiectasis
needs to be evaluated further.

The amino acid composition of sputum from cystic fibrosis and non-cystic fibrosis
patients needs to be evaluated in order to
account for the preponderance of methionine

Table 2  Constancy of auxotrophic factors of P aeruginosa with time in sputum samples
from nine patients with cystic fibrosis

<table>
<thead>
<tr>
<th>Source of paired samples</th>
<th>Sampling interval (weeks)</th>
<th>Growth requirements*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Both outpatient</td>
<td>52</td>
<td>Different 1 methionine, 1 leucine</td>
</tr>
<tr>
<td>Both outpatient</td>
<td>1</td>
<td>Same Methionine</td>
</tr>
<tr>
<td>Both outpatient</td>
<td>37</td>
<td>Same Thiamine</td>
</tr>
<tr>
<td>1 isolate, 1 outpatient</td>
<td>12</td>
<td>Same Alternative</td>
</tr>
<tr>
<td>Both outpatient</td>
<td>35</td>
<td>Same Methionine</td>
</tr>
<tr>
<td>Both isolate</td>
<td>32</td>
<td>Same Methionine</td>
</tr>
<tr>
<td>Both isolate</td>
<td>1</td>
<td>Unknown Not identified</td>
</tr>
<tr>
<td>Both isolate</td>
<td>12</td>
<td>Same Alternative</td>
</tr>
</tbody>
</table>

*For definition of growth requirements see footnote to table 1.
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dependent P. aeruginosa auxotrophs in sputum from patients with cystic fibrosis. It is of interest that serum levels of the key methionine precursor, sulphonamidomethionine, are elevated in cystic fibrosis. Moreover, a report by Tower et al of in vitro growth suppression of Klebsiella pneumoniae by microbial methionine synthetase inhibitors invites similar experimentation with P. aeruginosa. As methionine deficient colonies of P. aeruginosa are now shown to be present in one third of acutely ill cystic fibrosis patients and in nearly half (44%) of all patients with cystic fibrosis, the therapeutic possibility of inhibition of microbial methionine synthesis, acting synergistically with conventional antibiotics, should be explored in patients with this disease.

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