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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Pulmonary disease in patients with cystic fibrosis is characterised by viscid mucosal secretions which impair natural pulmonary host defence mechanisms and present a mechanical barrier to endogenous and exogenous antibacterial agents. The range of bacterial species which infect patients with cystic fibrosis is curiously restricted and consists primarily of *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Haemophilus influenzae*, and *Pseudomonas cepacia*. These opportunistic pathogens adapt to the abnormal pulmonary environment and the *Pseudomonas* species, in particular, are seldom eradicated despite appropriate therapy. In response to infection, inflammatory changes occur which are themselves implicated in further structural damage. The patient is thereby rendered more susceptible to infection and a cycle of infection and inflammation is established.

*P aeruginosa* has been found in the endobronchial spaces in which Speert *et al* have proposed that nutrients are limited. We speculated that multiple adaptation of *P aeruginosa* might occur in an environment of nutrient imbalance and that growth factors may be present in relative excess within the bronchial mucosa of patients with cystic fibrosis and promote survival of the adaptive organism.

Auxotrophs are bacteria that require growth substances additional to those required by the wild type of the same species (known as prototrophs); in the case of *P aeruginosa*, prototrophs are supported by minimal media alone. We have shown previously that auxotrophy of *P aeruginosa* is a frequent characteristic of sputum isolates from patients with cystic fibrosis and non-cystic fibrosis bronchiectasis, that in cystic fibrosis isolates methionine is the single additional factor most often required, and that, in vitro, auxotrophic isolates are more resistant to antipseudomonal agents than are prototrophs. We have now examined whether...
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 auxotropic 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 (forced expiratory volume in one second (FEV<sub>1</sub>) <40% predicted normal value)<sup>10</sup> five had moderate disease (FEV<sub>1</sub> 40–60%), and four had only mildly impaired lung function (FEV<sub>1</sub> >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. 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<sup>−2</sup> to 10<sup>−6</sup>) in Ringer’s solution and 100 µl aliquots of each dilution were spread uniformly on to both a minimal salt medium<sup>11</sup> which supports only the growth of prototrophs, and King’s “A” agar<sup>2</sup> 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.<sup>13</sup>

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 described<sup>4</sup> and specific requirements of confirmed auxotrophs were identified by modification<sup>8</sup> of Holliday’s experimental method.<sup>14</sup>

Statistical Methods

Comparison of proportions was made by the χ<sup>2</sup> 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 × 10<sup>7</sup> to >1 × 10<sup>9</sup> 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 >10<sup>6</sup>/ml from 16, P cepacia from three, and Aspergillus fumigatus from three others.
<table>
<thead>
<tr>
<th>Source of sample</th>
<th>Growth requirements*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inpatient</td>
<td>Same/different</td>
</tr>
<tr>
<td>Inpatient</td>
<td>Specific</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>Proline</td>
</tr>
<tr>
<td>Inpatient</td>
<td>Multiple</td>
</tr>
<tr>
<td>Outpatient</td>
<td>Methionine</td>
</tr>
<tr>
<td>Inpatient</td>
<td>Methionine</td>
</tr>
<tr>
<td>Inpatient</td>
<td>1 alternative, 1 methionine</td>
</tr>
<tr>
<td>Inpatient</td>
<td>Methionine</td>
</tr>
<tr>
<td>Inpatient</td>
<td>1 not identified, 1 aspartic acid</td>
</tr>
<tr>
<td>Inpatient</td>
<td>Methionine</td>
</tr>
<tr>
<td>Inpatient</td>
<td>Methionine</td>
</tr>
<tr>
<td>Inpatient</td>
<td>Methionine</td>
</tr>
<tr>
<td>Inpatient</td>
<td>Not identified</td>
</tr>
<tr>
<td>Inpatient</td>
<td>Methionine</td>
</tr>
<tr>
<td>Inpatient</td>
<td>Methionine</td>
</tr>
</tbody>
</table>

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

**SPECIFIC GROWTH FACTORS**

**Intrasputum variation**

At least two single colonies of auxotrophs, irrespective of colonial morphology, were selected from the primary culture of 12 patients with cystic fibrosis (nine inpatients) for growth factor identification to assess intrasputum variation. Of 12 auxotrophic pairs tested, eight required the same growth factor; as in our previous study methionine was the most frequently required single amino acid (table 1).

**Variation with time**

The constancy of auxotrophic *P aeruginosa* over time was examined in the sputum of nine patients with cystic fibrosis. Table 2 shows that for seven patients there was no variation in growth factor requirements in the two samples taken one or more weeks apart. However, different auxotrophs were identified in the patient from whom samples were taken over the longest time interval (52 weeks).

**Methionine dependency**

Overall, methionine dependent strains were present in seven of 16 patients with cystic fibrosis (44%) and in four of 12 with acute pulmonary exacerbations (33%).

**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>37</td>
<td>Same Methionine</td>
</tr>
<tr>
<td>Both outpatient</td>
<td>12</td>
<td>Same Methionine</td>
</tr>
<tr>
<td>Both outpatient</td>
<td>12</td>
<td>Same Methionine</td>
</tr>
<tr>
<td>Both outpatient</td>
<td>35</td>
<td>Same Methionine</td>
</tr>
<tr>
<td>Both outpatient</td>
<td>32</td>
<td>Same Methionine</td>
</tr>
<tr>
<td>Both outpatient</td>
<td>1</td>
<td>Unknown Not identified</td>
</tr>
<tr>
<td>Both outpatient</td>
<td>12</td>
<td>Same Alternative</td>
</tr>
</tbody>
</table>

*For definition of growth requirements see footnote to table 1.

**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 aeruginosa* 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 defective 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 pulmonary secretions of patients with suppurrative 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 bronchiectasis, antibiotic treatment may contribute to the selection of auxotrophic *P aeruginosa* in a similar way to the selection of thymidine dependent 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 trimethoprim effect and permitting survival of the pathogen. In order to delineate the effect of disease severity on the induction of auxotrophy and differentiate this from the effect of antibiotic exposure, sequential sputum samples taken 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...
Auxotrophic mutants of Pseudomonas aeruginosa in cystic fibrosis

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