Adherence of *Pseudomonas aeruginosa* to cystic fibrosis buccal epithelial cells

To the Editor:

The cystic fibrosis (CF) respiratory tract is inhabited by complex polymicrobial communities [1–4]. The sinonasal site has been proposed as a gateway and reservoir for subsequent pulmonary infection with the major CF pathogens like *Pseudomonas aeruginosa* [5, 6]. To colonise and infect the lungs, the bacteria must first colonise the oropharynx and then, on occasion, pass this barrier. Here, we report on the adherence of *P. aeruginosa* to oropharyngeal epithelial cells, namely buccal epithelial cells that were collected from CF patients during the initial and chronic phase of *P. aeruginosa* lung colonisation, and from *P. aeruginosa*-negative patients. Based on an assay with radioactively labelled bacteria published in the late 1970s [7], we established a low-cost, nonradioactive assay of *P. aeruginosa* adherence to buccal epithelial cells that may be useful for patient monitoring or as a biomarker for clinical studies.

The study was approved by the Ethics Committee of Hannover Medical School (number 4656).

Reliable data with low random and systematic errors were obtained after the size of the inoculum of the *P. aeruginosa* test strain, its culture and the spectrophotometric adjustment of bacterial cell number had been standardised as follows. Buccal epithelial cells were collected by thorough rubbing with a sterile cotton swab (diameter 12 mm, length 150 mm). Cells were transferred into PBS, precipitated by centrifugation at 200g for 10 min and resuspended in 1 mL PBS. Aliquots of 3×10^4 cells in 3 mL PBS were mixed with either 3×10^6 *P. aeruginosa* bacteria grown to late logarithmic phase in 3 mL PBS or vehicle alone (negative control for the endogenous bacterial flora attached to the buccal cells). The suspensions were incubated at 37°C for 2 h in a shaking waterbath with 600 rpm. The numbers of bound and free bacteria were determined by filtration of 2 mL of the suspension through either filters of pore size 5 µm (epithelium-bound bacteria) or filters of pore size 0.05 µm (bound and free bacteria), and subsequent counting of colony-forming units from serial dilutions on agar plates. Assays were performed in triplicate and tests with cells from a healthy donor were repeated once for each strain on another day. As a positive control of maximal bacterial binding, assays were performed with buccal epithelial cells that had been pre-incubated at 37°C for 10 min with 25 µg·mL⁻¹ trypsin in PBS. After addition of 250 µg·mL⁻¹ trypsin inhibitor, the trypsinised cells were mixed with bacteria.

Three healthy subjects provided buccal epithelial cells to test the binding of four *P. aeruginosa* reference strains (PAO1, and IATs serotypes 4, 6 and 9) and 10 isolates from CF airways. On average, two or three bacteria bound to one buccal cell. The adherence never exceeded 10 bacteria per cell, indicating that *P. aeruginosa* is a poor binder to intact buccal epithelial cells. The median (range) was 2.3 (0.1–5.0) bacteria per buccal cell from donor A, 2.2 (0.3–9.7) from donor B and 2.7 (0.2–4.3) from donor C. Bacterial adherence to buccal epithelial cells was not significantly different between donors or between *P. aeruginosa* strains. Nevertheless, a trend of differential bacterial binding was noted. The IATs serotype 6 and 9 strains were the strongest binders, and three CF isolates were the weakest binders, with less than one bacterium per epithelial cell.

Cell damage enhanced bacterial adherence. Treatment of buccal epithelial cells with trypsin increased the adherence to 50–200 bacteria per cell, indicating that almost all bacteria had attached to the trypsinised cells. Similarly, when healthy subjects experienced an upper airway infection on the day of investigation, their buccal epithelial cells became more susceptible to adherence by *P. aeruginosa* (table 1).

After the binding assay had been standardised, buccal epithelial cells retrieved from 36 individuals with CF and 24 healthy controls were compared in their binding to the *P. aeruginosa* CF isolate TBCF121838 [9]

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***Pseudomonas aeruginosa* cystic fibrosis buccal adherence assay** <http://ow.ly/lPtB306VKIQ>

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TABLE 1 Adherence of *Pseudomonas aeruginosa* TBCF121838 to cystic fibrosis (CF) buccal epithelial cells

Subjects	Bound TBCF121838 bacteria per buccal epithelial cell			
	0–10	10–20	20–40	>40
CF patients				
<i>P. aeruginosa</i> negative	10	2		
<24 months <i>P. aeruginosa</i> positive	7	2		
>36 months <i>P. aeruginosa</i> positive	1 [#] +1 [†]	2	5	2
Acute upper airway infection				4
Non-CF controls				
Healthy	19			
Acute upper airway infection			2	3
Trypsinised buccal cells				6

Data are presented as numbers of subjects. [#]: single patient of the cohort who chronically inhaled tobramycin; no *P. aeruginosa* had been detected in patient's sputum at the day of investigation. [†]: single patient in this group classified as having a CF transmembrane conductance regulator-related disorder [8].

(table 1). *P. aeruginosa* adhered to buccal cells from most cystic fibrosis donors as poorly as to cells from healthy non-CF controls if the cells had been collected from *P. aeruginosa*-negative patients or during the first 2 years of *P. aeruginosa* CF lung colonisation. However, in seven of these 21 CF donors, bacterial adherence exceeded the upper range of controls so that overall the binding of TBCF121838 to buccal cells was significantly higher in the groups “*P. aeruginosa* negative” and “<24 months *P. aeruginosa* positive” than in the control group ($p=0.004$, Fisher's exact test). Bacterial adherence to buccal cells was consistently increased during the phase of chronic airway infection with *P. aeruginosa* ($p=0.0001$ in comparison to controls, Fisher's exact test) (table 1). Bacterial attachment could be as high as that seen during an acute upper airway infection (40–60 bacteria per buccal cell), suggesting that the oropharyngeal epithelium is substantially damaged during the chronic *P. aeruginosa* infection in CF. Acute or chronic inflammatory processes cause secondary proteolytic damage of the epithelium and thus unmask epitopes for bacterial attachment [10].

We have evidence that despite long-lasting colonisation with *P. aeruginosa*, the oropharyngeal barrier can be restored. *P. aeruginosa* has meanwhile been eradicated from four of the 12 chronically colonised CF patients listed in table 1 by repetitive courses of 2-week intravenous antipseudomonal chemotherapy and chronic administration of aerosolised tobramycin [11]. When, 6 months after the last detection of *P. aeruginosa* in the patients' respiratory secretions, the buccal adherence assays were repeated, the binding of TBCF121838 had returned to the normal non-CF range, suggesting that the damaged oropharyngeal barrier can be repaired as is the case in non-CF subjects after an acute upper airway infection.

The noninvasive adherence assay may be a worthwhile adjunct to the existing portfolio of laboratory procedures. The assay is admittedly low throughput, and demands a skilful and dedicated operator who meticulously follows the protocol, but it is low cost and does not require any sophisticated equipment. With the exception of handling the biohazardous *P. aeruginosa*, which is probably more problematic on the ward than in the laboratory, the procedure is harmless. In contrast to the forerunner protocol that utilised radioactive metabolic labelling [7], no hazardous chemicals or procedures are employed. We implemented the sampling of buccal cells into the routine visit to the doctor's office and particularly the younger CF patients enjoyed the procedure.

The surveillance of the integrity of the oropharyngeal barrier is the primary application of the assay. Acute or chronic infections damage the CF epithelium, which becomes evident by increased bacterial binding in the buccal assay. This damage of the epithelial barrier seems to be reversible even after years of chronic *P. aeruginosa* infection if the bacteria have been eradicated. According to these preliminary data, the buccal adherence assay could represent an informative biomarker of the *Pseudomonas* colonisation status of a CF subject. However, prior to the introduction of the assay into the clinic, more longitudinal data from a larger cohort need to be collected in order to evaluate whether the assay can predict the colonisation status, particularly the successful eradication of *P. aeruginosa* from CF airways. If the assay is validated, numerous further applications to patient care or clinical research can be envisaged. Patients at risk for lower airways colonisation can be identified and the impact of therapeutic measures on the oropharyngeal barrier may be followed on a case-to-case basis or in the context of clinical trials. Considering its broad applicability, the acceptance of the assay by the community would be appreciated.

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