Coll. Antropol. **28** (2004) 2: 675–680
UDC 616.98:616.24-008.4
Original scientific paper

Adherence Ability of *Pseudomonas aeruginosa* Strains Isolated from Patients with Cystic Fibrosis to Two Different Epithelial Cell Lines

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ABSTRACT

The ability of 59 wild-type strains of Pseudomonas aeruginosa to adhere to the HeLa and Buffalo Green Monkey Kidney (BGMK) cells was investigated. Twenty strains were isolated from sputa of cystic fibrosis patients, while 19 strains were isolated from tracheal aspirates and 20 from bronchial secretions of patients without cystic fibrosis, and they were used as a control group of strains. The statistically significant difference between adherence ability of strains was observed ($p<0.01$). While most of the tracheal and bronchial isolates were hyperadhesive (51–110 bacteria per cell) most of the cystic fibrosis isolates adhered poorly to the HeLa and BGMK cells (1–10 bacteria per cell). The bacterial binding to the cells was blocked when bacteria were incubated at 80°C for 20 min before the adherence assay. These results indicate that alginate is not involved in the adherence of P. aeruginosa to the used epithelial cell lines, and, because of that, mucoid strains isolated from persistently colonized cystic fibrosis patients showed poor adherence ability.

***Key words**: Pseudomonas aeruginosa, adherence, alginate, cystic fibrosis, chronic lung infection*

Introduction

Cystic fibrosis (CF) is the most common lethal genetic disease in humans[1]. Most of the patients with CF are defective in the cystic fibrosis transmembrane con-

Received for publication August 9, 2004

ductance regulator protein, which results in altered secretion in the secretory epithelia[2]. The produced hyperviscous mucus increases the incidence of bacterial lung infections in CF patients. *Staphylococcus aureus* is usually the first pulmonary isolate from these patients and together with *Haemophilus influenzae* predisposes the CF affected lung to colonization with *Pseudomonas aeruginosa*. Although *Burkholderia cepacia* has also been shown to infect the lungs of CF patients with lethal consequences, it has never attained the 80% colonization rate of *P. aeruginosa*[3].

The exact mechanism of *P. aeruginosa* colonization of the lungs of patients with CF is not known[4]. There is evidence that enhanced pseudomonal receptors on the respiratory epithelia and impaired mucociliary clearance may be responsible[2]. During initial colonization, the bacteria are nonmucoid. One of the most striking and clinically important features of infection by *P. aeruginosa* is the tendency of this bacterium to change to a mucoid phenotype, probably initiating the chronic--infection stage of the disease[1]. The mucoid phenotype results from bacterial production of polysaccharide known as alginate and plays an important role in bacterial resistance to the host immune response and antibiotics. Lam et al. first observed this mucoid phenotype in postmortem specimens of infected lung tissue and bronchoscopy material from infected patients[5]. The mucoid material was shown to be an acetylated random copolymer of mannuronic acid and guluronic acid[6], correlated to poor prognosis in CF patients[7]. The bacteria are capable of colonizing the lungs of CF patients for years due to alginate overproduction and biofilm formation[8,9].

The ability of *P. aeruginosa* to adhere to the epithelial cells is considered the initial step in its colonization and subsequent infection. This study was undertaken to examine the adherence ability of *P. aeruginosa* strains isolated from children with CF to two different epithelial cell lines.

Material and Methods

Bacteria and patients: Twenty investigated strains were isolated from sputa of CF patients, while 19 strains were isolated from tracheal aspirates and 20 from bronchial secretions of patients without cystic fibrosis, and they were used as a control group of strains originated from respiratory tract. Bacteria were characterized biochemically as *P. aeruginosa* using the API 20E System (API, Analytab Products, Plainview, NY). All strains were stored in deep-agar tubes at +4 °C (1.5% nutrient agar, Difco Lab., Detroit, MI) and were subcultured by passaging on Tryptic Soy agar (Difco Lab., Detroit, MI) before use. We collected *P. aeruginosa* isolates from seven persistently colonized CF patients being treated at University Medical Center Zagreb from June 2000 to May 2001, aged between eight and 23 years (mean age 13.71 years, four males and three females). The CF patients were chronicaly colonized with *P. aeruginosa*, at least for two years (2–11 years). All strains isolated from CF patients produced alginate and slime positivity was determined for each strain by the tube method as described previously[10]. The isolates from bronchial secretions and tracheal aspirates were isolated in the same period from patients without CF at University Hospital Osijek, which were colonized with *P. aeruginosa* during their hospitalization. The strains were isolated from patients hospitalized at Intensive Care Unit (seven females and 12 males, mean age 50.21 years) and at Pulmonology Division (four females and 16 males, mean age 58.65 years). All patients had symptoms and signs of underlying infection, and cultures were taken during standard diagnostic procedures. Slime production

was detected in seven out of 39 strains by the use of tube method.

Adhesion assay: Buffalo Green Monkey Kidney (BGMK) and HeLa, standard continuous cell lines, were used for the adhesion assay, which has been described previously[11]. These cell lines were used because they proved to be a good experimental model for *in vitro* testing the adherence ability of different wild-type *P. aeruginosa* strains, including the isolates originated from respiratory tract[12]. Briefly, BGMK and HeLa cells were grown as a monolayer in Eagle's minimal essential medium (MEM) supplemented with 10% heat-inactivated fetal calf serum, penicillin and streptomycin in 75 cm^3 cell culture flashes. When confluent, they were incubated with 0.1% trypsin, washed in MEM, and distributed into 16x125 mm tubes containing cover slips size 12x20 mm, in which they were incubated again at 37 °C until about 80% confluent. Monolayers developed on the cover slips were washed three times in phosphate-buffered saline (PBS; pH 7.2) before adherence studies in order to remove the remains of MEM and of antimicrobials, and dipped into tubes containing the investigated strain suspension in PBS adjusted to a concentration of 1.5 x 18^8 bacteria/ml (McFarland Standard, bioMerieux, Lyon, France). After incubation lasting 1h at 37 °C with shaking (60 rpm), the cover slips were removed from tubes and washed thoroughly several times in PBS to eliminate bacteria which did not adhere to the cells. The cells were fixed for 15 min in acetone and then indirect fluorescent antibody test was performed. Fluorescent staining facilitated the counting of bacteria which adhered to the cells, thus avoiding misinterpretation of artefacts produced by disruption of extracellular matrix due to agitation. The adhesion assay for each strain was repeated three times. All samples were identified in code and read by the same person.

For adhesion inhibition bacterial suspensions were incubated at 80 °C for 20 min before the adhesion assay.

Statistical method: The mean adherence to 40 cells for each strain constituted the unit of observation. Statistical testing was done with chi-square analysis with Yeate's correction.

Results

Most strains adhered well to the used epithelial cell lines. There was no significant difference between adherence ability of investigated *P. aeruginosa* strains to the HeLa and to the BGMK cells (Table 1 and 2). The adherence ability of the strains was interpreted as a poor (1–10 bacteria bound per cell), a good (11–50 bacteria per cell), and a very good (more than 50 bacteria per cell).

The statistically significant difference between adherence ability of strains isolated from sputa of patients with cystic fibrosis and strains from tracheal aspirates and bronchial secretions of patients without cystic fibrosis was observed ($p< 0.01$). While most of the tracheal and bronchial isolates were hyperadhesive (51–110 bacteria per cell), most of the mucoid cystic fibrosis isolates adhered poorly to the used epithelial cell lines (1–10 bacteria per cell; Table 1 and 2).

The bacterial binding to the cells was almost completely blocked when hyperadhesive bacterial strains were incubated at 80 °C for 20 min before the adhesion assay (1–5 bacteria bound per 40 cells, compared with 51–110 bacteria per cell for the untreated bacteria, depending on strain).

Discussion

In present study, the statistically significant difference was found between *in vitro* adherence ability of *P. aeruginosa*

strains isolated from respiratory tract of patients with and without cystic fibrosis. *P. aeruginosa* strains isolated from tracheal and bronchial aspirates of patients without cystic fibrosis adhered significantly better to the used epithelial cell lines than mucoid strains isolated from sputa of CF patients. The ability of bacteria to adhere to mucosal surfaces of human body is considered to be a crucial step in the pathogenesis of most infections. Attachment of *P. aeruginosa* to epithelial cells is mediated by surface adhesins. The adherence of *P. aeruginosa* is a much more complex process than previously envisioned[13], consisting of the interactions of multiple adhesins such as fimbriae, exoenzime S, alginate, hemagglutinin and other nonfimbrial adhesins[14–19]. While the flagelar cap protein and nonfimbrial proteinaceous adhesins of *P. aeruginosa* are important for the initial binding of the organism to the respiratory mucus[20,21], type IV fimbriae are the most important *P. aeruginosa* adhesin for binding to host cells. Numerous studies have demonstrated a role for type IV fimbriae in the pathogenesis of *P. aeruginosa* infection, particularly in the CF lung[22,23]. As a further indication of their role in infec-

TABLE 1
ADHERENCE ABILITY OF *P. AERUGINOSA* STRAINS TO THE BGMK CELLS

Specimens*	Adherence ability						Total	
	Poor		Good		Very good			
	N	%	N	%	N	%	N	%
Sputa	15	25.42	4	6.78	1	1.70	20	33.90
Tracheal aspirates	3	5.08	4	6.78	12	20.34	19	32.20
Bronchial aspirates	1	1.70	6	10.17	13	22.03	20	33.90
Total	19	32.20	14	23.73	26	44.07	59	100.00

$\chi^2 = 28.55$, $p<0.01$

Poor adherence ability = 1–10 bacteria/cell; Good adherence ability = 11–50 bacteria/cell; Very good adherence ability = 51–110 bacteria/cell

* Sputa were collected from patients with cystic fibrosis, and tracheal aspirates and bronchial secretions from patients without cystic fibrosis

TABLE 2
ADHERENCE ABILITY OF *P. AERUGINOSA* STRAINS TO THE HELA CELLS

Specimens*	Adherence ability						Total	
	Poor		Good		Very good			
	N	%	N	%	N	%	N	%
Sputa	16	27.12	3	5.08	1	1.70	20	33.90
Tracheal aspirates	4	6.78	4	6.78	11	18.64	19	32.20
Bronchial aspirates	2	3.39	6	10.17	12	20.34	20	33.90
Total	22	37.29	13	22.03	24	40.68	59	100.00

$\chi^2 = 25.63$, $p<0.01$

Poor adherence ability = 1–10 bacteria/cell; Good adherence ability = 11–50 bacteria/cell; Very good adherence ability = 51–110 bacteria/cell

* Sputa were collected from patients with cystic fibrosis, and tracheal aspirates and bronchial secretions from patients without cystic fibrosis

tion, nonfimbriated mutants have been shown to have decreased virulence relative to their parental strains in animal models of pulmonary infections[24,25]. Also, *P. aeruginosa* adherence to epithelial cells was successfully inhibited by treatment with an antibody to a GM1 – the glycosphingolipid receptor for *P. aeruginosa* type IV fimbriae contained within epithelial cell membranes[26]. Thus, type IV fimbriae are considered to be factors significant in the early stages of *P. aeruginosa* chronic and acute infections. That is consistent with the results of present study and our observation that the *P. aeruginosa* strains isolated from tracheal aspirates of patients hospitalized at Intensive Care Unit and the strains isolated from bronchial aspirates of patients without cystic fibrosis hospitalized at Pulmonology Division mostly adhered well to the used cell lines (Table 1 and 2). There was no significant difference between adherences of these strains to the HeLa and to the BGMK cells, and based on that observation it could be concluded that both epithelial cell lines express the glycosphingolipid receptor for *P. aeruginosa*. Since the bacterial adherence to the cell was almost completely blocked when bacteria were incubated at 80 °C before the adhesion assay, it is obvious that alginate is not involved in the adherence of *P. aeruginosa* to HeLa and BGMK cells and that proteinaceous adhesins, such as fimbriae, are important for binding of these bacterial strains to the epithelial cell lines. Similar results were observed in previous study comparing the hemagglutination and adherence ability of *P.* aeruginosa strains isolated from a variety of clinical sites[12].

Also, we noted that the mucoid strains isolated from persistently colonized cystic fibrosis patients showed poor adherence ability to the used epithelial cell lines. It seems that proteinaceous adhesins are important for the initial adherence of *P. aeruginosa*, while alginate and biofilm formation enable the bacteria to establish a chronic pulmonary infection in patients with cystic fibrosis. Recently, Hausssler et al. identified a hyperpiliated, nonmucoid, small-collony variant morphotype of *P. aeruginosa* isolated from respiratory tract of cystic fibrosis patient, which exhibited increased twitching motility and attached strongly to the pneumocytic cell line A 549[27]. The emergence of these highly adherent small-colony variants within the cystic fibrosis lung might play a key role in the pathogenesis of *P. aeruginosa* lung infection, where a biofilm mode of growth and a mucoid morphotype of strains are thought to be responsible for persistent infection.

In summary, the results of present study indicate that alginate is not involved in the initial adherence of *P. aeruginosa* to the used epithelial cell lines, and, because of that, mucoid strains isolated from persistently colonized cystic fibrosis patients showed poor adherence ability.

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SPOSOBNOST ADHERENCIJE NA DVIJE RAZLIČITE EPITELNE STANIČNE KULTURE SOJEVA *PSEUDOMONAS AERUGINOSA* IZOLIRANIH U BOLESNIKA S CISTIČNOM FIBROZOM

S A Ž E T A K

Istražena je sposobnost adherencije 59 kliničkih izolata bakterije *Pseudomonas aeruginosa* na HeLa staničnu kulturu i Buffalo staničnu kulturu bubrega zelenog majmuna (BGMK). Dvadeset sojeva izolirano je iz iskašljaja bolesnika oboljelih od cistične fibroze, dok je 19 sojeva izolirano iz aspirata traheje a 20 iz ispirka bronha bolesnika koji nemaju cističnu fibrozu i upotrijebljeni su kao kontrolna skupina sojeva. Opažena je statistički značajna razlika u sposobnosti adherencije istraživanih sojeva ($p<0.01$). Dok je većina trahealnih i bronhalnih izolata bila hiperadhezivna (51–110 bakterija po stanici), većina sojeva izoliranih iz iskašljaja bolesnika oboljelih od cistične fibroze slabo je adherirala na HeLa i BGMK stanice (1–10 bakterija po stanici). Bakterijsko vezivanje na stanice bilo je zapriječeno kada su bakterije prije testa adherencije izlagane temperaturi od 80 °C kroz 20 minuta. Ovi rezultati pokazuju da aginat nije uključen u adherenciju bakterije *P. aeruginosa* na upotrijebljene stanične kulture, te su stoga mukoidni sojevi izolirani u kronično koloniziranih bolesnika oboljelih od cistične fibroze pokazali slabu sposobnost adherencije.