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PSEUDOMONAS AERUGINOSA ADHESION TO NORMAL AND INJURED RESPIRATORY MUCOSA

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Human nasal polyps in outgrowth culture were used to study the adhesion of Pseudomonas aeruginosa *to respiratory cells. By transmission electron microscopy, bacteria associated with ciliated cells were identified trapped at the extremities of cilia, usually as aggregates of several bacterial cells. They were never seen at the interciliary spaces or attached along cilia. Bacteria were also seen to adhere avidly to migrating cells of the periphery of the outgrowth culture. Using a model of repair of wounded respiratory epithelial cells in culture, we observed that the adhesion of* P. aeruginosa *to migrating cells of the edges of the repairing wounds was significantly higher than the adhesion to non-migrating cells and that adherent bacteria were surrounded by a fibronectin-containing fibrillar material. The secretion of extracellular matrix components is involved in the process of epithelium repair following injury. To investigate the molecular basis of* P. aeruginosa *adhesion to migrating cells, bacteria were treated with a fibronectin solution before their incubation with the respiratory cells.* P. aeruginosa *treatment by fibronectin significantly increased their adhesion to migrating cells. Accordingly, we hypothesize that during cell migration, fibronectin secreted by epithelial cells may favour* P. aeruginosa *adhesion by establishing a bridge between the bacteria and the epithelial cell receptors. Such a mechanism may represent a critical step for* P. aeruginosa *infection of healing injured epithelium.*

Key words: *Pseudomonas aeruginosa* – bacterial adhesion – wound repair – respiratory mucosa – fibronectin – cell migration

PSEUDOMONAS AERUGINOSA ADHESION TO NORMAL AND INJURED ANIMAL RESPIRATORY MUCOSA

Current evidences suggest that a crucial step in the development of infectious diseases is the molecular interaction between bacterial adhesive structures and receptors from the glycocalyx of mammalian cells. This specific adhesion to host tissues protects bacteria from being eliminated by natural host defense mechanisms (Sharon, 1987).

Pseudomonas aeruginosa, a virtually nonvirulent microorganism in immunocompetent hosts, is a major pathogen in patients with chronic respiratory diseases such as chronic bronchitis, bronchiectasis and more typically, cystic fibrosis. Over 90% of death in these patients results from respiratory insufficiency associated with overwhelming pulmonary infections by *P. aeruginosa.*

Different experimental models have been used in studies aiming to determine the mechanisms allowing *P. aeruginosa* to adhere to the respiratory mucosa, but the results obtained in many of them have been contradictory. Studies carried out on animal models, in which functionally active ciliated cells were exposed to nonmucoid *P. aeruginosa* suspensions, have shown that bacteria do not adhere to respiratory epithelium, even after long incubation periods, unless it has been first injured someway (Baker & Marcus, 1982; Ramphal & Pyle, 1983; Marcus et al., 1989; Zoutman et al., 1991). In contrast, *P. aeruginosa* have been shown to adhere to cilia of human cells obtained by tracheal brushing (Nierdman et al., 1983; Franklin et al., 1987; Doig et al., 1988). This discrepancy may be attributed to differences between animal and human respiratory cells in their susceptibility to the bacterial adhesins. Alternatively, it may result from the fact that epithelial cells in suspension exspose receptors for bacterial adhesion different from those exposed *in vivo.* Recent data published

by Baltimore et al. (1989), on the immunohistopathological localization of *P. aeruginosa* in airways from patients with cystic fibrosis, futher support the concept that these microorganisms do not adhere to healthy respiratory mucosa. In their study, *P. aeruginosa* interaction with intact epithelium was never noted, neither was bacteria entanglement within cilia. Bacteria were always seen to remain intraluminaly, sequestred within a surrounding exsudate. In contrast, everywhere there were erosion of the epithelium, *P. aeruginosa* were found attached to the denuded membranes. In a prior *in vitro* study, we could also observed the affinity of *P. aeruginosa* for basement membranes (Plotkowski et al., 1989). Respiratory secretions from patients suffering from chronic pulmonary diseases may present a high proteolytic activity, mainly due to the presence of free leukocyte elastase, released by neutrophils during phagocytosis (Stockley & Burnett, 1979). To investigate the effect of proteolytic enzymes on bacterial adhesion to respiratory mucosa, the frog palate mucosa was exposed to human leukocyte elastase and, later, to *P. aeruginosa* suspensions. Respiratory mucosa responded to leukocyte elastase with a marked hypersecretion of mucus and exfoliation of the ciliated epithelium. *P. aeruginosa* were found to adhere to mucus granules, to exfoliated cells and to the exposed extracellular membrane, but not to intact ciliated cells (Plotkowski et al., 1989). *P. aeruginosa* have also been shown to bind to a matrix obtained from the tumor of EHS (Plotkowski et al., 1990), known to contain the main components of basic membranes, that is, laminin, type IV collagen and proteoglycans (Orkin et al., 1977), as well as to type I collagen, a major component of submucosal underlying connective tissues (Plotkowski et al., 1991c). Accordingly, it is conceivable that, in chronic respiratory diseases, *P. aeruginosa* may bind to specific receptors exposed following tissue injury, such as extracellular matrix components, and that this affinity may account for the opportunistic character of the bacteria. However, maybe injured epithelium exhibits specific receptors for *P. aeruginosa* adhesins different from the extracellular matrix components.

Epithelial regeneration is a basic response to injury, fundamental for the maintenance of epithelial barrier function. Regeneration of hamster tracheal epithelium following mechanical injury has been shown to begin by spreading and migration of viable cells neighbouring the wounds. In small lesions, cell migration was enough to cover the denuded lesion in such a way that by in a few days following injury the wounded sites were covered by a layer of undifferentiated cells, which later differentiated in ciliated and secretory cells, rendering the regenerating epithelium indistinguishable from the normal one (McDowell et al., 1979).

Cell surface glycoconjugates are shown to be altered during cellular differentiation (Chailley et al., 1982; Mann et al., 1987). Therefore, it may hypothesized that cells migrating and differentiating to repair injured tissues may present receptors for microbial adhesins which are not present in stationnary differentiated cells.

The differentiation of respiratory epithelium in primary cell culture has been shown to recapitulate the epithelial repair following injury (McDowell et al., 1987). Chevillard et al. (1991) have recently developed a human nasal polyp primary culture allowing the study of ciliogenesis and *in vitro* functional activity of respiratory cilia during their maturation. This model provides an exciting approach for the study of *P. aeruginosa* adhesion to human respiratory cells during the process of growth and differentiation.

PSEUDOMONAS AERUGINOSA ADHESION TO HUMAN EPITHELIAL RESPIRATORY CELLS IN PRIMARY CULTURE

In the culture model developed by Chevillard et al. (1991) human nasal polyp explants are cultured on a type I collagen matrix, in a serum free defined culture medium. Under these conditions, epithelial cells from the explants proliferate and migrate, giving origin to an outgrowth are around the explants. In this outgrowth area both ciliated and non ciliated cells, at different steps of differentiation, are found. Moreover, cells of the periphery of the outgrowth are migrating cells, as revealed by the presence of lamelipodia extensions toward the collagen matrix and unfolded apical cell membranes. To investigate whether the presence of receptors for bacteria may vary during cell differentiation, we exposed respiratory cells in culture to suspensions of a nonmucoid *P. aeruginosa* strain. Bacterial adhesion was investigated by scanning and transmission electron microscopy (Plotkowski et al., 1991c).

Fig. 1: scanning electron micrographs of respiratory cell cultures exposed to *Pseudomonas aeruginosa* suspensions showing: A – large aggregate of bacteria associated with the extremities of cilia (arrow); B – bacteria adherent to a migrating cell of the periphery of the outgrowth and to the collagen matrix (CM). Arrows point to the leading edge of the migrating cell. Bars = 3 µm.

We could never observe the presence of *P. aeruginosa* adherent along the ciliary membranes or at the interciliary spaces. Bacteria associated with ciliated respiratory cells were mainly present as aggregates of several bacterial cells, trapped at the extremities of cilia (Fig. 1A), while microorganisms associated with non ciliated cells were non aggregated. By transmission electron microscopy, aggregated bacteria were seen to be surrounded by a matrix-like material, which appeared to establish the interaction of the aggregates with the tips of cilia. When *P. aeruginosa* adhesion to the respiratory cells was quantitated, by using an image analyzer connected to the scanning electron microscope, we observed that it was the migrating cells of the periphery of the outgrowth, rather than the differentiating non ciliated cells or differentiated ciliated cells, which appeared as a pole of attraction for *P. aeruginosa* adhesion (Figs 1B, 2).

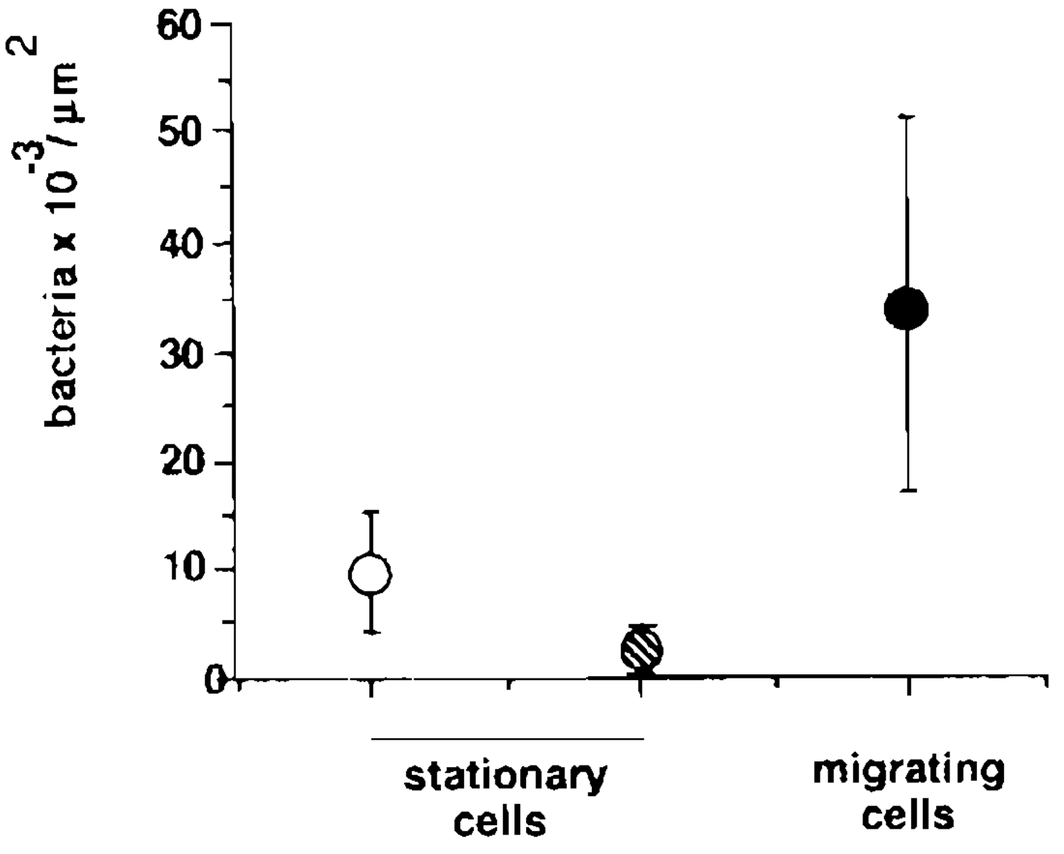

Fig. 2: mean adhesion of *Pseudomonas aeruginosa* to stationary cells (○, ciliated; ◍, non ciliated) and to migrating cells (●). Data are mean ± standard deviation of adherent bacteria in at least 150 different scanning electron microscopic fields.

Cell surface carbohydrates from migrating cells have been shown to differ from sugars found on stationary cells (Gipson et al., 1983; Zieske et al., 1986; Ball et al., 1989). So, we hypothesized that during the regeneration of injured respiratory tissues, epithelial cells migrating to cover the wounds could express receptors for which *P. aeruginosa* exhibit a higher affinity. To ascertain the veracity of this hypothesis, we worked on the *in vitro* model of repair of wounded respiratory epithelium developed by Zahm et al. (1991).

PSEUDOMONAS AERUGINOSA ADHESION TO RESPIRATORY CELLS MIGRATING TO REPAIR EPITHELIAL WOUNDS

In the *in vitro* model used, cell culture was performed as described by Chevillard et al. (1991) and wounds were made by detaching about 50 cells from the collagen matrix on

which they have grown, by repeated stroking the cells with a glass capilary guided by a micromanipulator. The wounded were incubated at 37 °C on the heat stage of a phase-contrast inverted microscope and the repairing process was analyzed by time-lapse videomicroscopy. After about 2 hr of the cell culture wounding, when epithelial cells at the edges of the wounded surfaces were seen to migrate, the defined culture medium was removed and replaced by a suspension of a non mucoid *P. aeruginosa* strain. The cultures were then kept at 37 °C on the heat stage of the inverted microscope and video recorded for 30 min. Thereafter, cultures were fixed and bacterial adhesion to the respiratory cells was quantitated by scanning electron microscopy (Plotkowski et al., 1991b). Almost immediately following the *P. aeruginosa* suspension addition to the wound-repairing cultures, many bacteria were seen to adhere to the collagen matrix on which cells had been cultured. The remaining microorganisms were seen to be propulsed by the ciliary activity. Later, as described previously (Plotkowski et al., 1991c), bacterial aggregates were seen to appear in the culture medium and to be trapped at the tips of cilia, which continued to beat with trapped aggregates. The adhesion to the migrating cells, both from the margins of the outgrowth and from the borders of the wounds, began a little later than the adhesion to the collagen matrix and the aggregation of the bacterial cells, and continued throughout the incubation period. Excitingly, since the beginning of the incubation of *P. aeruginosa* with the injured epithelial cell cultures, a remarkable proportion of non-aggregated bacterial cells was seen to concentrate upon the repairing area, as if they had been attracted by some chemotactic factor. Occacionally, some foci of concentrated bacteria were seen at the periphery of the cell outgrowth. On late phases of the incubation period (30 min), when most bacterial cells had either adhered to the collagen matrix or to migrating cells, or had been aggregated and trapped by cilia, countless non-aggregated bacteria were still seen to remain in suspension upon the repairing area of the wound. Quantitative studies showed that *P. aeruginosa* adhesion to migrating cells of the borders of the repairing wounds was significantly higher than the adhesion to non-migrating ciliated or non-ciliated cells and was not different from the adhesion to the marginal cells of the outgrowth culture (Table).

TABLE

Pseudomonas aeruginosa adhesion to human epithelial respiratory cells in primary culture[a]

Bacteria x 10^{-3} per μm^2			
Migrating cells		Nonmigrating cells	
Outgrowth margins	Wound edges	Ciliated cells	Non ciliated cells
22.8 ± 9.3	28.3 ± 10.9	5.0 ± 2.3	0.8 ± 0.9
26.2 ± 9.3	20.5 ± 9.3	0.8 ± 0.9	0.8 ± 1.0
31.0 ± 13.8	45.8 ± 25.4	4.2 ± 1.3	0.6 ± 0.3
54.8 ± 21.4	80.1 ± 25.0	6.7 ± 1.5	1.4 ± 0.6
93.0 ± 16.0	92.0 ± 24.1	5.5 ± 1.4	1.0 ± 0.4
78.1 ± 17.0	84.0 ± 14.0	4.3 ± 3.0	0.8 ± 0.7

a: data are mean ± standard deviation of bacteria present in at least 20 SEM field, for each assay.

MOLECULAR BASIS OF *PSEUDOMONAS AERUGINOSA* ADHESION TO MIGRATING EPITHELIAL RESPIRATORY CELLS

Cell migration is a very complex process requiring cell adhesion to extracellular matrix and controlled detachment as the cell move. Fibronectin (FN) is a multifunctional glycoprotein present in an insoluble form at cell surface and on extracellular matrix and in a soluble form in plasma and other body fluids (Ruoslahti et al., 1981). It binds to a number of macromolecules, such as those forming the supportive framework of interstitial spaces and basement membranes, and molecules present on both eukaryotic and procaryotic cell surfaces. Because of its adhesive properties, FN plays a major role in securing epithelial cells to their underlying substrate (Yamada, 1989). Both the number and the distribution of cell receptors for FN are modulated as cells move (Akiyama et al., 1989; Yamada, 1989). Moreover, migrating cells from the edges of epithelial wounds rapidly synthetize FN and deposit it at the cell-basement membrane interface, providing a matrix over which cells can easily move (Grinnell et al., 1981; Suda et al., 1981; Clark et al., 1983).

Fibronectin has been found, in a soluble form, in cultures of human epithelial respiratory cells (Shoji et al., 1989) and Sutter et al. (1988) have shown human bronchial epithelium to be intensely labeled by an anti-FN antibody, revealing the presence of polimerized insoluble FN at the apical surface of bronchial cells.

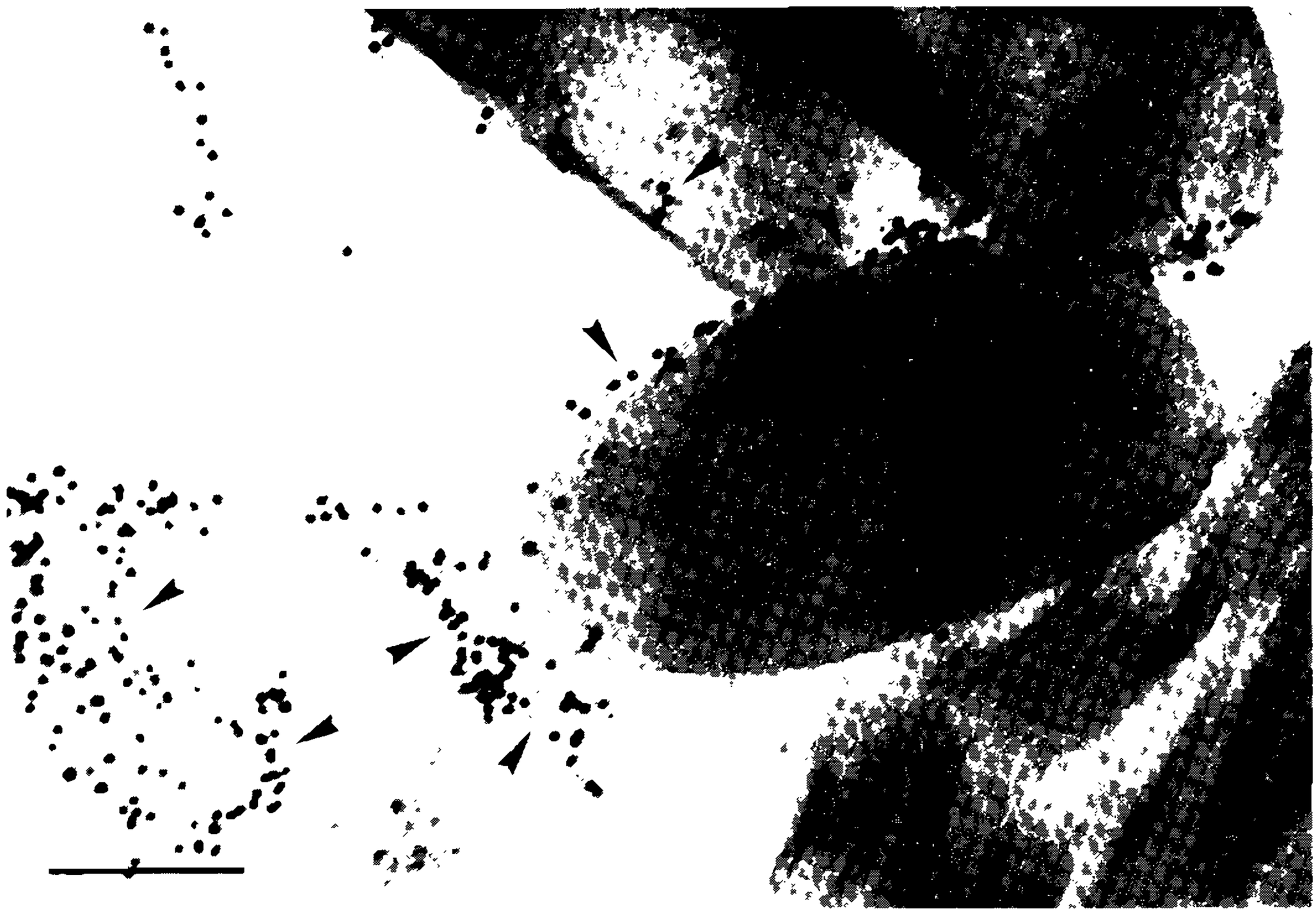

Fig. 3: transmission electron micrograph of a thick section of aggregated *Pseudomonas aeruginosa* adherent to an epithelial respiratory cell showing the labeling of the surrounding matrix (arrowheads) by the anti-FN antibody and the protein A-colloidal gold complex. Bar = 0.25 µm.

The presence of insoluble polimerized fibronectin at the apical surface of human buccal cells has been shown to correlate inversely with *P. aeruginosa* adhesion (Woods et al., 1981; Woods, 1987). As the expression of FN on epithelial cells is known to be modulated during cell migration, we hypothesized that the preferential adhesion of *P. aeruginosa* to migrating cells could result from a decrease in the concentration of insoluble fibronectin at the apical surfaces of moving respiratory cells. To ascertain the veracity of this assumption we carried out a double labeling study in which epithelial respiratory cells in culture were exposed to *P. aeruginosa* suspensions and later to an anti-fibronectin-fluorescein complex and to an anti-*P. aeruginosa*-Texas Red complex. The observation of the specimens in an epifluorescent microscope showed no difference in the content of insoluble fibronectin at the apical surface of migrating and stationary respiratory cells, as both were indistinguishably poorly labeled by the anti fibronectin antibody. Accordingly, *P. aeruginosa* adhesion to migrating cells could not be correlated with a low content of insoluble fibronectin at the surface of migrating epithelial respiratory cells. However, in contrast with the poor labeling of epithelial cells with the anti-FN antibody, a fibrillar material, intensely fluorescent was seen unevenly distributed over the cell culture. Excitingly, in our double labeling assay, we noticed that this FN-containing fibrilar material systematicaly surrounded aggregated bacteria associated with respiratory cells. The presence of FN in the matrix surrounding aggregated *P. aeruginosa* adherent to respiratory cells was also observed by transmission electron microscopy, by treating the specimens with an anti-FN antibody and a protein A-colloidal gold complex (Fig. 3). This finding led us to hypothesize that FN released by respiratory cells could interact with bacterial receptors and mediate bacterial adhesion by establishing a bridge between bacteria and cell surface receptors. To ascertain the veracity of this hypothesis we treated *P. aeruginosa* with FN solutions and the adhesion of FN-treated bacteria to migrating cells was compared with the adhesion of control bacteria by scanning electron microscopy (Plotkowski et al., 1991a). The adhesion of FN-treated *P. aeruginosa* to migrating respiratory cells was significantly higher than the adhesion of control microorganisms, as determined in two different assays (Fig. 5).

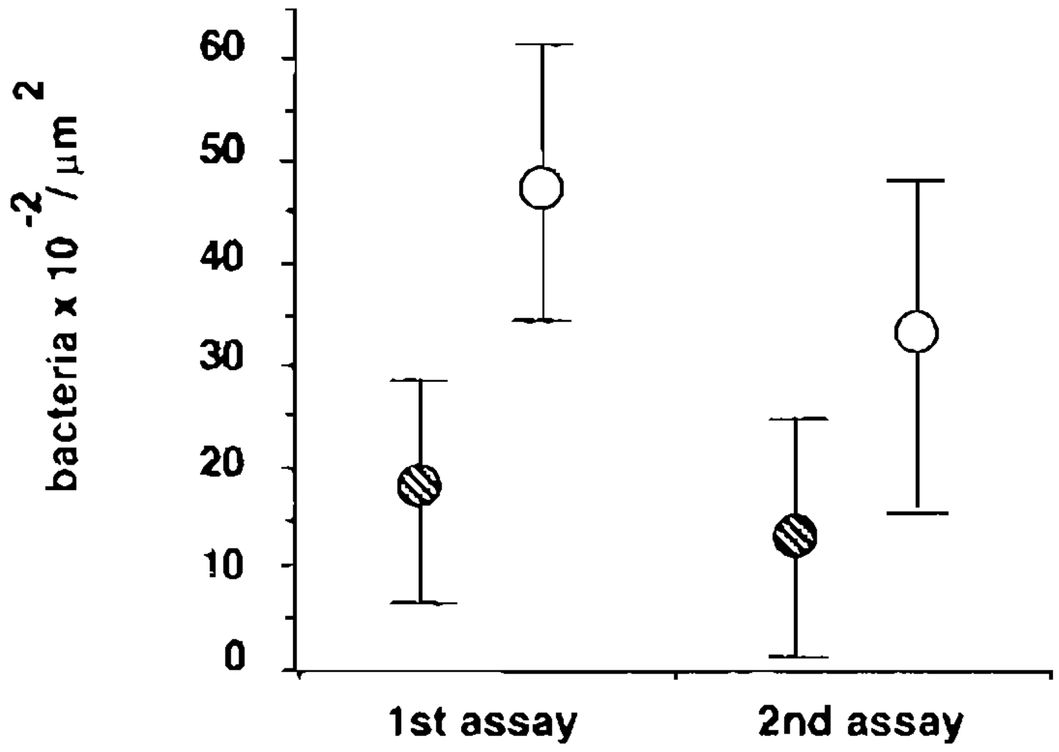

Fig. 4: mean adhesion of *Pseudomonas aeruginosa* treated with FN at 500 µg/ml (○) and of control untreated bacteria (◍) to migrating epithelial respiratory cells, as determined in two different assays. Data are mean ± standard deviation of adherent bacteria in at least 20 different scanning electron microscopic fields; $p < 0.05$.

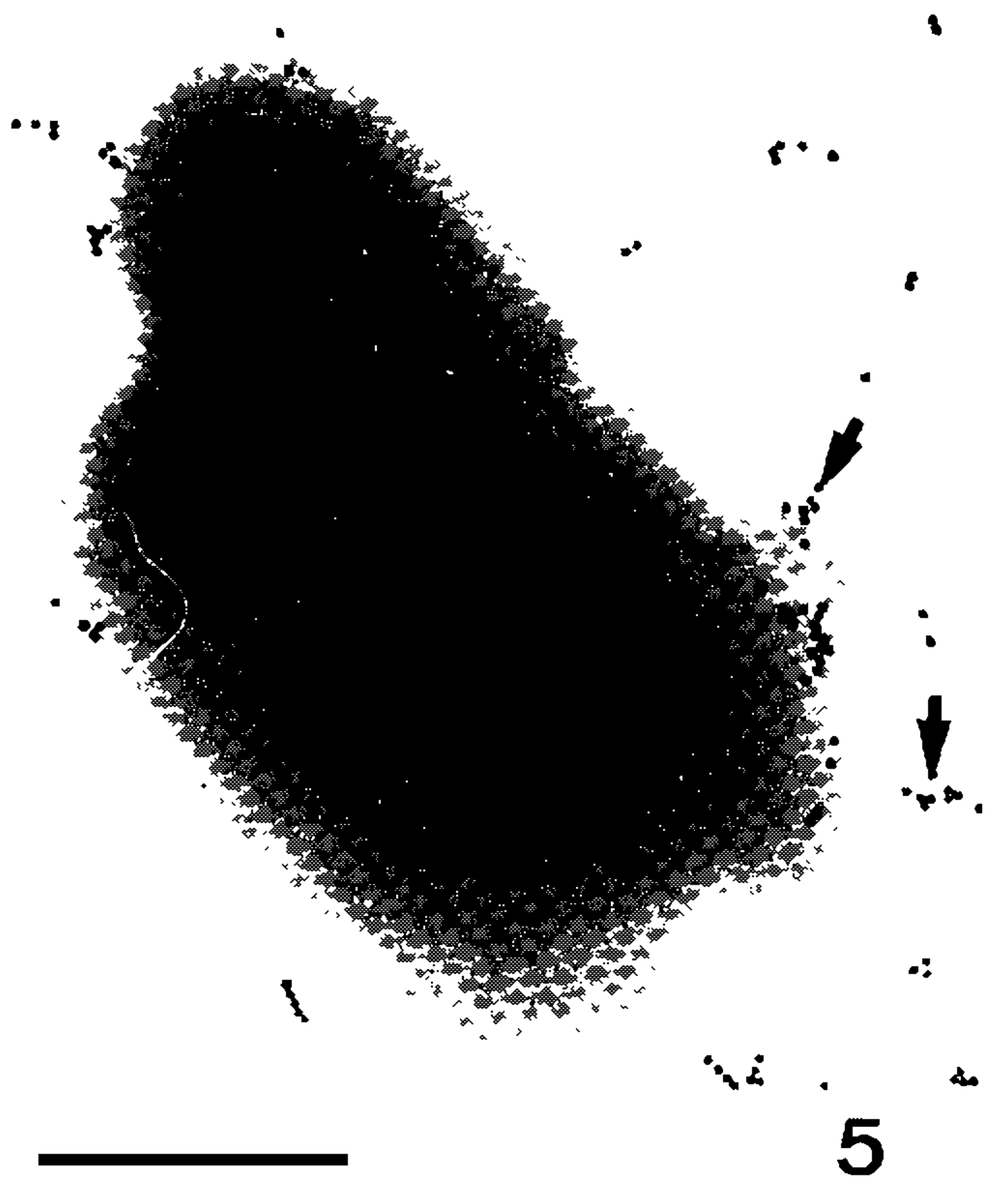

Fig. 5: transmission electron micrograph of *Pseudomonas aeruginosa* deposited on Parlodion-coated grids and labeled with the cellular FN-colloidal gold complex. Bar = 0.5 µm.

Several different microorganisms present specific ligands for FN which facilitate their attachment to both mammalian cells and to FN-coated bioplymers (Kuusela, 1978; Switalski et al., 1982; Thomas et al., 1985; Calderone & Scheld, 1987; Hermann et al., 1988). In contrast, polymerized FN was reported to serve as a barrier that blocks the adhesion of *P. aeruginosa*. Accordingly, to support our *P. aeruginosa*-soluble FN reactivity hypothesis, two different explanations may be proposed. The first one depends on studies from Ffrench-Constant et al. (1989) in which the FN secreted by cell at the base of epithelial wounds were shown to present an embryonic pattern. The authors suggested that, during wound healing, an alternative splicing of FN mRNA may occur as a mechanism to generate forms of FN that may be functionally more appropriate for cell migration and proliferation associated with tissue repair. As embryonic FN presents domains that are not present in the normal adult tissue and plasma FN (Matsuura & Hakomori, 1985), it is conceivable that these two FN forms may react differently with microbial ligands.

The second explanation to support our hypothesis is based on the dramatic physical conformation change that soluble FN suffers as it polymerises and on the fact that these changes may uncover and/or block potential receptor sites for pathogens (Ruoslahti et al., 1981). Our results showing the increase of the adhesion to migrating cells following *P. aeruginosa* treatment by soluble FN suggest that bacteria-FN interaction occured someway. However, *P. aeruginosa* affinity for plasmatic FN should be low, since high protein concentrations were required.

PSEUDOMONAS AERUGINOSA INTERACTION WITH SOLUBLE AND INSOLUBLE POLIMERIZED FIBRONECTIN

To investigate whether polimerization of soluble FN may interfere with its reactivity with the bacterial cells, we looked for the *P. aeruginosa* interaction with both polimerized insoluble and soluble FN.

P. aeruginosa affinity for insoluble FN was investigated by incubating 99m Tc labeled bacteria with coverslips coated with cellular FN at 100 µg/ml or with BSA at 10 mg/ml (control) and by calculating the percentage of radioactivity associated with each one, following rinsing to eliminate non adherent bacteria. To evaluate *P. aeruginosa* affinity for soluble FN we adopted two different approaches. In the first one, 99m Tc labeled bacteria were treated for 30 min. with cellular FN at 400 µg/ml or with PBS, before their exposure to coverslips coated with type I collagen matrix, a substrate for which FN presents a high affinity. In the second approach, we analysed the

labeling of *P. aeruginosa* cells by cellular FN absorbed to colloidal gold granules, by transmission electron microscopy.

Bacterial affinity for coverslips coated with insoluble cellular FN (31.6 ± 1.6%) was not different from their affinity for BSA-coated coverslips (35.2 ± 2.6%). We confirmed, therefore, that *P. aeruginosa* do not interact specifically with insoluble FN. In contrast, the adhesion of *P. aeruginosa* treated with soluble FN to the collagen matrix was higher than the adhesion of control PBS-treated bacteria (19.4 ± 7.2% and 14.4 ± 4.4%, respectively; $p < 0.05$). By transmission electron microscopy we confirmed the reactivity of *P. aeruginosa* with cellular soluble FN, as bacterial cells were seen to be labeled by gold granules (Fig. 5).

During the study of the kinetics of the interaction of *P. aeruginosa* with the wounded respiratory cell cultures, we observed bacteria to concentrate themselves upon the repairing area, as if they had been attracted by some chemoctactic factor. Following epithelial wound, different secreted products can induce the migration of cells, which participate in the tissue repair process, to the site of injury. For instance, FN secreted by bronchial epithelial cells has been reported to attract fibroblasts (Shoji et al., 1989), whereas FN fragments induce both monocyte chemotaxis and chemokinesis (Norris et al., 1982). Bacterial locomotion in response to host attractive substances is supposed to play an important role in the colonization by bacteria of certain sites of predilection (Nelson et al., 1990). To our knowledge, the chemoctactic activity of FN towards microorganisms has never been described. Nevertheless, we wonder whether soluble FN, or any other product secreted during the repairing process, would not have favoured *P. aeruginosa* adhesion to migrating cells by attracting them to the sites of injury.

CONCLUSION

The interaction of *P. aeruginosa* with epithelial respiratory cells seems to be a very complex phenomenon. Based in our results, we speculate that the release of cellular FN and changes in cell surface glycoconjugates related with cell migration to repair epithelial injury may favour *P. aeruginosa* adhesion and, therefore, be parttly responsible for the persistence of infections in patients with chronic lung diseases.

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