Internalization of *Staphylococcus aureus* and *Pseudomonas aeruginosa* bacterial cells by non-phagocytic, epithelial human cells

Received for publication, March 20, 2008
Accepted, Aprilie 5, 2008

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Abstract

The purpose of this work was to investigate the invasion ability and intracellular survival in non-phagocytic, epithelial cells of some S. aureus and P. aeruginosa strains isolated from clinical cases using qualitative (fluorescence staining) and quantitative (gentamycin protection) assays. The qualitative assay of the bacterial adherence to the cellular substrate demonstrated that all tested strains adhered to HeLa cells, S. aureus exhibiting an aggregative or mixed aggregative-localized adherence pattern the adherence rates ranging from 10 to 90%, whereas P. aeruginosa exhibited diffuse, aggregative or mixed adherence patterns with 20 to 100% adherence rates.

The quantitative assay of the adherence and invasion ability showed that both S. aureus and P. aeruginosa strains proved the ability to invade the epithelial, non-phagocytic cells. The examination of fluorescent labeled HeLa cells infected with S. aureus and P. aeruginosa confirmed the results of the quantitative assay of viable, internalized cells. S. aureus interaction with the host cell induced changes in the epithelial cell membrane, exhibiting membrane veils, aspect demonstrating the existence of an endocytic process triggered by the bacterial cell. P. aeruginosa invasion was consistent with active cellular events such as the cytoplasm wrinkling and the induction of thin, lamellar membrane pseudopodes formation.

Our results come into agreement with other literature data, proving the intracellular survival of staphylococci and pseudomonades in mammalian, non-phagocytic cells, breaking the dogma considering these bacteria the prototypes of extracellular infections and demonstrating the necessity of new models for studying the bacterial infection stages, including an intracellular one, in order to highlight new and efficient strategies to prevent of S. aureus and P. aeruginosa persistent infections.

Key words: *Staphylococcus aureus, Pseudomonas aeruginosa, internalization, non-phagocytic cells, fluorescence microscopy*

Introduction

Staphylococcus aureus is a pathogen with a broad host range responsible for humans and animal infections worldwide. The rapid increase of methicillin-resistant strains and the perspective to vancomycin resistance by these bacteria has recently caused considerable alarm within the medical community. Infections associated with this organism are very common and often life threatening; therefore, there is serious potential for *S. aureus* to cause increased morbidity and mortality (2). The mechanism of persistence of staphylococci in its hosts and consecutive repeated occurrences of staphylococcal infections and toxigenic diseases (such as toxic shock syndrome), in spite of induction of apparently significant levels of humoral and mucosal antibodies, remains unexplained until today (3). While there is some evidence that immunosuppression induced by superantigens is partially responsible for both the persistence of infection and reduced antibody levels against some staphylococcal products, not all human and animal cases are attributed to superantigen-producing organisms. One confirmed mechanism employed by some bacteria to escape from humoral immunity is to become internalized in the host cells. For example, organisms such as *Listeria monocytogenes* and *Mycobacterium tuberculosis* are facultative intracellular pathogens that require cell-mediated immunity to be

eliminated most efficiently; the presence of antibodies alone is ineffective during infection by these pathogens. *S. aureus* is generally not considered to be an intracellular pathogen of the magnitude associated with classical facultative intracellular pathogens (i.e., *Listeria*, *Mycobacterium*, *Salmonella*, and *Shigella* spp.). However, it is well established that *S. aureus* can be internalized in epithelial (1) and endothelial cells (8, 16). Little is known about the mechanisms involved in internalization, the potential role of internalization of *S. aureus* by the host cell, or host cell responses.

Although usually considered as an extracellular pathogenic bacteria, ~50% of all isolates of *Pseudomonas aeruginosa* can be measurably internalized into nonphagocytic cells *in vivo* as well as *in vitro* (5), binding preferentially and entering the cells at the edge and basolateral surfaces with mechanical wounding, due to injured and poorly polarized cells (7, 10). The internalization of *P. aeruginosa* has been demonstrated by using the genetically accessible host model of *Dictyostelium discoideum* (14). Since the discovery of type III and IV secretion, progress in analysis of bacteria- host cells interaction has been done, with special consideration targeting macrophages, in the purpose to understand the mechanism of bacterial persistence in pulmonary secretions (6, 15).

The purpose of this work was to investigate the invasion ability and intracellular survival in non-phagocytic, epithelial cells of some *S. aureus* and *P. aeruginosa* strains isolated from clinical cases.

Material and Methods

i) Bacterial strains and culture conditions 16 *Staphylococcus aureus* and 9 *Pseudomonas aeruginosa* strains recently isolated from different clinical specimens (bronchial secretions, blood cultures, wound exudates and nasal swabs) were investigated for their adherence and invasive potential by quantitative and qualitative assays.

ii) Adherence and invasion assay

Bacterial suspensions were prepared from bacterial cultures of 24 hours in nutrient broth, harvested by centrifugation, resuspended in Eagle MEM and used to be inoculated on HEp-2 monolayers of 48 hours with 80% confluence cultivated in 6 - multiwell plates (3 plates/strain). The plates were incubated for 3 h at 37^{o}C in 5% CO_2 atmosphere. Thereafter the wells from one plate were washed 4 times in PBS, Giemsa stained and examined in optic microscopy adapted after Cravioto et al., 1979 (4).

One of the remaining two plates was washed 4 times in PBS and permeabilized with Triton X-100. Serial dilutions were seeded on solid media in order to establish the adhesion and the invasion rates (C.F.U./ml). The number of invasive bacteria was assayed in the third remaining plate. After infection, the cells were incubated with 100 μg/ml gentamycin for 1 h to kill the extracellular bacteria, the supernatant was removed and cells were washed three times with DMEM (without serum) and lysed with 0.1% Triton X-100. Serial dilutions of the lysates were plated onto agar to quantify the intracellular bacteria (12).

iii) Fluorescent actin staining (FAS) Bacterial suspensions in nutrient broth prepared from cultures of 24 hours on agar plates were used for being inoculated into the subconfluent, HEp-2 monolayers of 24 hours cultivated in 6-multiwell plates with coverslips. After 3 hours of incubation at 37^{o}C, the plates were washed 3 times in PBS and the cells were briefly fixed by glutaraldehyde and permeabilized with PBS-Sap-BSA. The coverslips were removed, stained with DAPI or PI (propidium iodide), mounted in glycerol-PBS and examined by incident-light fluorescence using an Olympus Bx40 fluorescence microscope with adequate filters (11).

Results and discussion

The qualitative assay of the bacterial adherence to the cellular substrate demonstrated that all tested strains adhered to HeLa cells, *S. aureus* exhibiting an aggregative or mixed aggregative-localized adherence pattern and adherence rates ranging from 10 to 90%, whereas *P. aeruginosa* a diffuse, aggregative or mixed diffuse-adherence patterns and 20-100% adherence rates.

The quantitative assay of the adherence and invasion ability showed that both *S. aureus* and *P. aeruginosa* strains possess the ability to invade the epithelial, non-phagoytic cells.

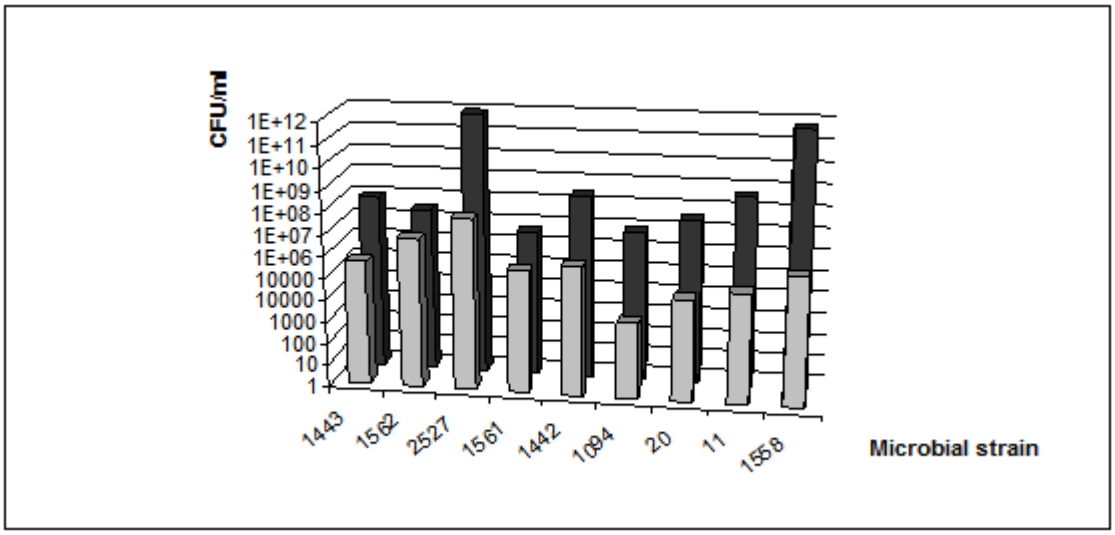

Figure 1. Representation of the adherence (black) and invasion (grey) levels in *P. aeruginosa* strains assessed by the gentamycin protection assay method and expressed in CFU/ml.

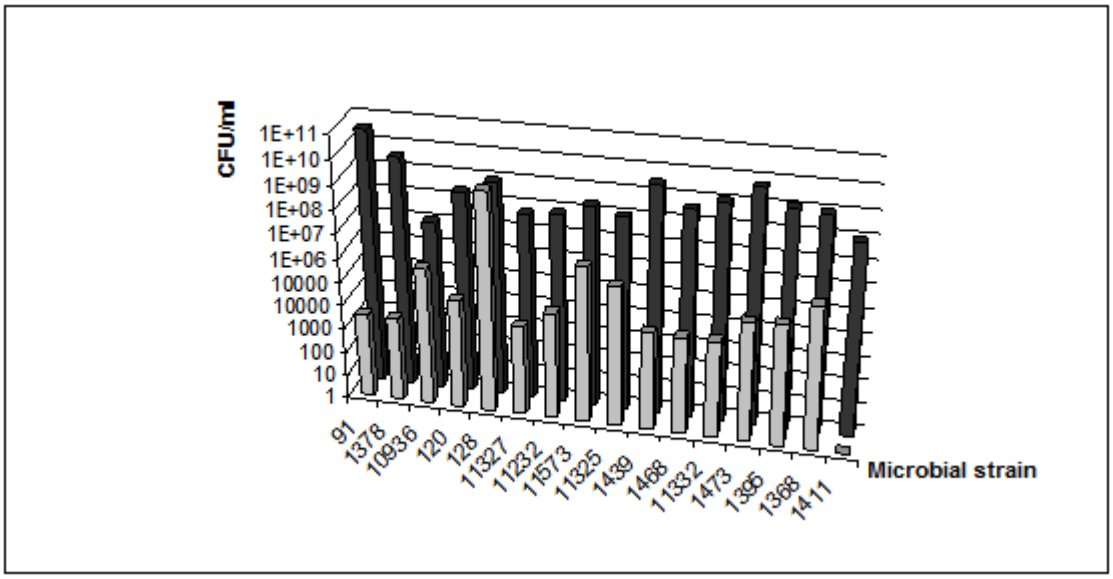

Figure 2. Representation of the adherence (black) and invasion (grey) levels in *S. aureus* strains assessed by the gentamycin protection assay method and expressed in CFU/ml

The examination of fluorescent labeled HeLa cells infected with *S. aureus* and *P. aeruginosa* confirmed the results obtained in the quantitative assay of viable, internalized cells.

Thus, in case of *S. aureus* strains, the fluorescence microscopy aspects showed the presence of single bacterial cells or associated in microcolonies inside the mammalian cells (Fig. 3-5). As the quantitative assay of the invasive ability is based on the counting of viable cells, it is possible that the internalized bacterial cells be metabolically active, playing an important role in the intracellular survival and antibiotic resistance, factors favouring the persistence of staphylococcal infections.

The bacterial cell interaction with the host cell induced changes in the epithelial cell membrane, which exhibited membrane veils, aspect demonstrating an endocytic process triggered by the bacterial cell (Fig. 4.). Previous literature data demonstrated that *agr* regulating factors dependent on bacterial density are implicated in the survival of staphylococci in the intracellular compartment, by modulating the apoptosis of the infected cells, process that could be interpreted as bacterial - host cells communication system (2, 8, 13); it must be noticed that mutant *agr* and *sar* of staphylococcal strains are not able to survive intracellularly and to induce apoptosis in the host cells.

In accordance with this model, in case of the host contaminated with *S. aureus*, the host cells are expressing adhesins for staphylococci. In the formed intracellular endosoms, due to the limited volume, the *agr* octapeptides will rapidly accumulate reaching the threshold density and triggering the exoproteins synthesis by the staphylococcal cells. Some of the released exoproteins (e.g. haemolysins) could destabilize the endosomal

membrane releasing the staphylococcal cells in the cytosol where the octapeptides concentration is rapidly decreasing and the bacterial metabolism is shifting from exoproteins to the synthesis of proteins associated with the bacterial wall.

Once present in cytosol, *S. aureus* can: 1) induce the apoptosis of the host cells resulting the apoptotic bodies that will be ingested by the resident macrophages; the apoptotic bodies protect staphylococci against the macrophage bactericidal systems (Trojan horse); 2) form intracytoplasmatic microcolonies implicated in the genesis of persistent and chronic infections; 3) infect host cell that is submitted to lysis when the octapeptide level in cytoplasm is shifting the metabolic profile to the exotoxin releasing, followed by the contamination of the nearby healthy tissue and dissemination of the infection in the entire body.

Our previous studies (in press) also demonstrated that the tested strains produced pore-forming toxins (i.e. haemolysins, lipases, lecithinases) accounting for their ability to escape from phagosomal or endosomal ingestion and fulfill the infectious cycle inside the infected host.

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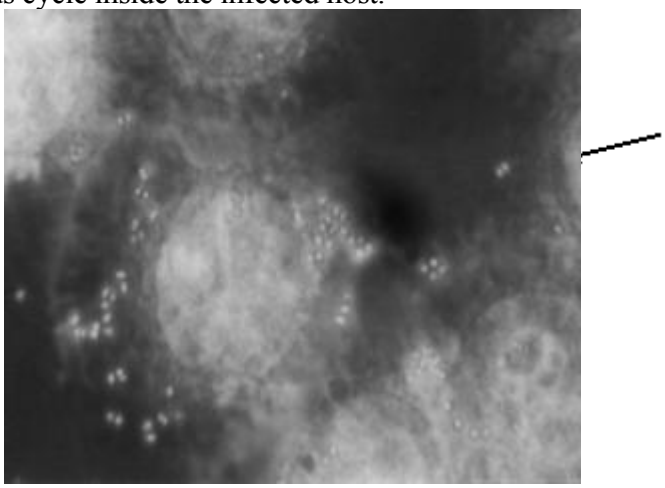

Figure 3. DAPI fluorescence staining showing the internalization of *S. aureus* cells as single cells or as microcolonies in HeLa cells (2500 x)

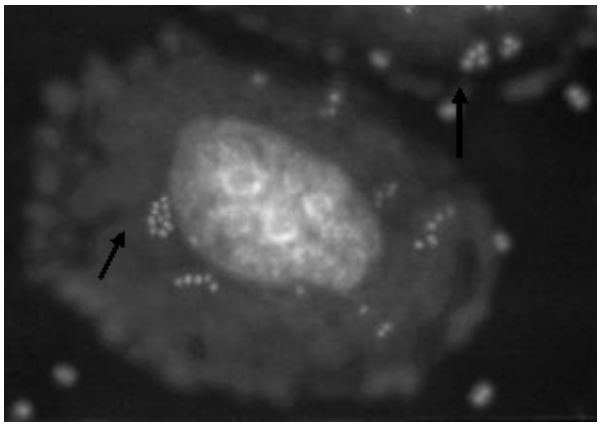

Figure 4. PI fluorescence staining showing the internalization of *S. aureus* cells as single cells or as microcolonies in HeLa cells. The HeLa cells exhibit membrane veils (2500 x)

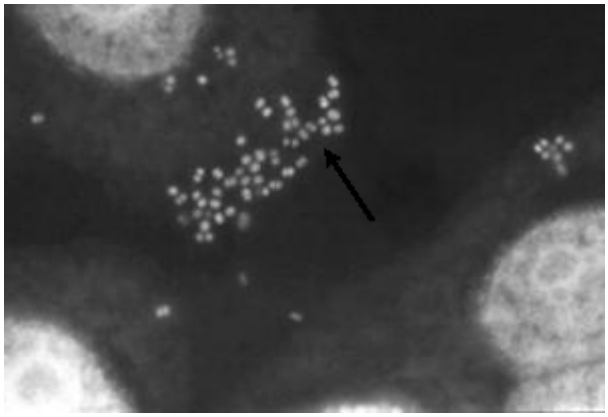

Figure 5. PI fluorescence staining showing internalized clusters of *S. aureus* cells inside HeLa cells (2500 x).

Whereas *P. aeruginosa* has not been traditionally considered as an intracellular pathogen, our fluorescence microscopy studies have revealed that *P. aeruginosa* may be also internalized by endocytosis and capable of intracellular survival. In further characterization of *P. aeruginosa* invasion, it was noticed that bacterial invasion was consistent with the active cellular events, such as the cytoplasm contraction (Fig. 6) and the induction of thin, lamellar membrane pseudopodes formation (Fig. 7). Further studies are necessary to determine the *Pseudomonas* adhesion factor(s), responsible for the signal transduction process initiating pseudopodes formation. In concordance with the quantitative assay of internalized, viable bacterial cells remained after the gentamycin treatment, the fluorescence microscopy also demonstrated the possible active multiplication of bacterial cells inside the host cell (Fig. 8).

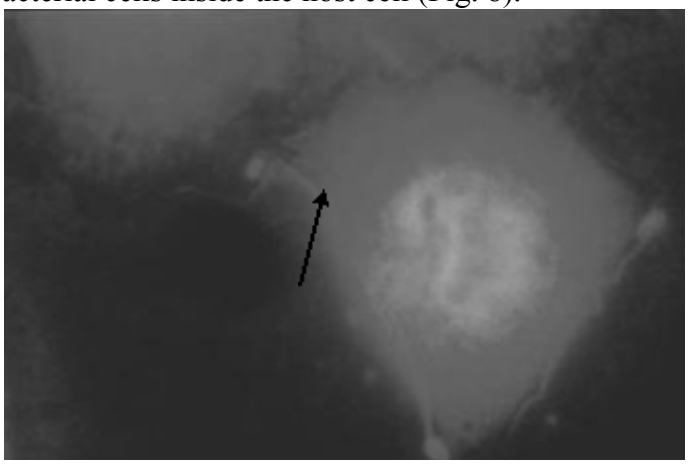

Figure 6. PI fluorescence staining showing initial phase of *P. aeruginosa* adhesion to the HeLa cell membrane, accompanied by the contraction of the epithelial cell cytoplasm (2500 x).

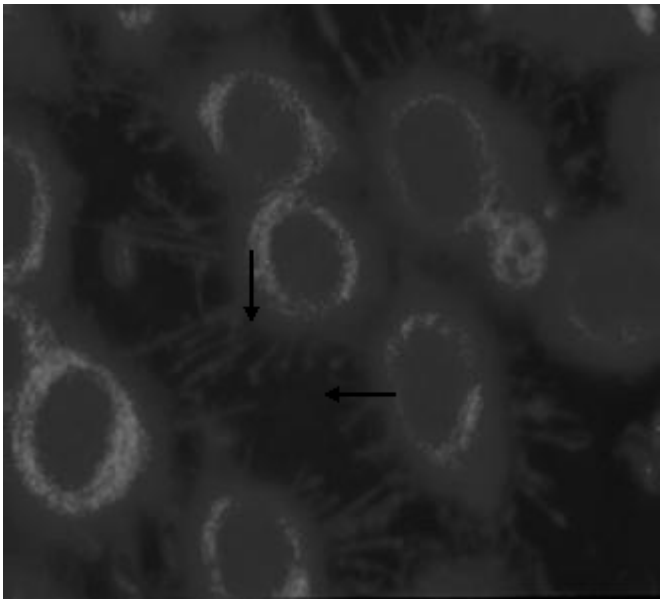

Figure 7. PI fluorescence staining showing *P. aeruginosa* cells adhered to HeLa cells and the induction of thin, lamellar pseudopodes (2500 x).

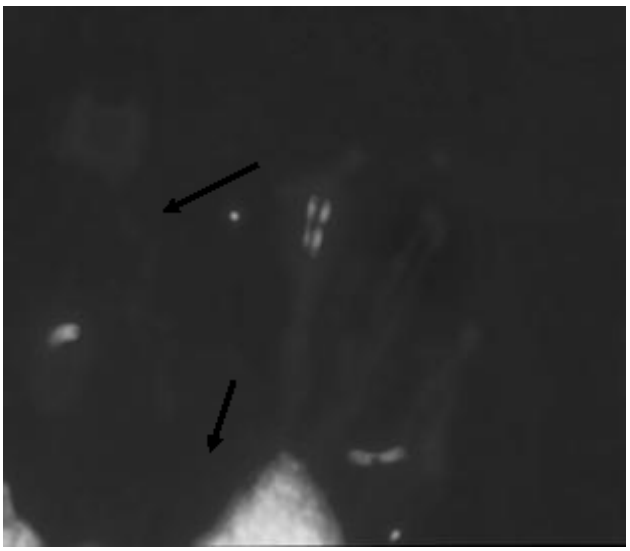

Figure 8. PI fluorescence staining showing internalized cells and the possible active division of the *P. aeruginosa* cells inside HeLa cells (2500 x).

The ability of *S. aureus* and *P. aeruginosa* to survive inside the host cell could explain several aspects of host-pathogen relationships as they pertaining to chronic recurrent diseases and long-term colonization, the internalization process providing probably protection against host defenses and antibiotic treatment. Our results come into agreement with literature data, proving the intracellular survival of staphylococci and pseudomonades in mammalian non-phagocytic cells, and breaking the dogma of considering these bacteria as prototypes for extracellular infections, demonstrating the necessity of accepting new models concerning the stages of bacterial infection, including in this sense an intracellular step of this process, in order to highlight new and efficient strategies to prevent *S. aureus* and *P. aeruginosa* persistent opportunistic infections.

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