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Increased Virulence of a Fibronectin-Binding Protein Mutant of *Staphylococcus aureus* in a Rat Model of Pneumonia

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Fibronectin-binding proteins mediate *Staphylococcus aureus* internalization into nonphagocytic cells in vitro. We have investigated whether fibronectin-binding proteins are virulence factors in the pathogenesis of pneumonia by using *S. aureus* strain 8325-4 and isogenic mutants in which fibronectin-binding proteins were either deleted (DU5883) or overexpressed [DU5883(pFnBPA4)]. We first demonstrated that fibronectin-binding proteins mediate *S. aureus* internalization into alveolar epithelial cells in vitro and that *S. aureus* internalization into alveolar epithelial cells requires actin rearrangement and protein kinase activity. Second, we established a rat model of *S. aureus*-induced pneumonia and measured lung injury and bacterial survival at 24 and 96 h postinoculation. *S. aureus* growth and the extent of lung injury were both increased in rats inoculated with the deletion mutant (DU5883) in comparison with rats inoculated with the wild-type (8325-4) and the fibronectin-binding protein–overexpressing strain DU5883(pFnBPA4) at 24 h postinfection. Morphological evaluation of infected lungs at the light and electron microscopic levels demonstrated that *S. aureus* was present within neutrophils from both 8325-4- and DU5883-inoculated lungs. Our data suggest that fibronectin-binding protein-mediated internalization into alveolar epithelial cells is not a virulence mechanism in a rat model of pneumonia. Instead, our data suggest that fibronectin-binding proteins decrease the virulence of *S. aureus* in pneumonia.

In a recent survey, *Staphylococcus aureus* was found to be the most common cause of lower respiratory tract infections in Europe, the United States, Canada, Latin America, and the Western Pacific region (9). In view of the fact that *S. aureus* infections are increasingly difficult to treat because of the high percentage of antibiotic-resistant strains (34), a better understanding of the molecular basis of *S. aureus* virulence in pneumonia may help in the design of new therapeutic strategies.

*S. aureus* has long been regarded as an extracellular pathogen because it is rarely observed inside cells in vivo and because it secretes a range of toxins that are cytolytic to many host cell types (14, 29). However, recent in vitro studies demonstrate that *S. aureus* is internalized and survives inside nonphagocytic cells (1, 2, 12, 18, 19, 21). Fibronectin-binding proteins present on the surface of *S. aureus* (16, 19, 26) mediate internalization into nonphagocytic cells. *S. aureus* fibronectin-binding proteins bind β1-integrins on the surface of the host cells by means of a fibronectin bridge (16).

Survival of internalized *S. aureus* within nonphagocytic cells may be an additional virulence mechanism in *S. aureus* infections (20). Internalized *S. aureus* may be able to evade or delay elimination by the host’s immune system and avoid extracellular antibodies (20). If internalization contributes to *S. aureus* persistence in vivo, then drugs which interfere with fibronectin binding to host cell integrins may have a role to play in treatment of *S. aureus* infections (6).

Alveolar epithelial type I cells are large squamous cells that cover over 95% of the lungs’ surface area; the remaining 5% is covered by alveolar epithelial type II cells. Both alveolar epithelial type I and II cells have a number of potential fibronectin-binding receptors on their cell surfaces (7, 28, 32). The overall objective of our study was to investigate whether fibronectin-binding protein–mediated internalization into alveolar epithelial cells is a virulence mechanism in *S. aureus*-induced pneumonia.

MATERIALS AND METHODS

All reagents and materials were supplied by Sigma, Dorset, United Kingdom, unless stated otherwise.

**Bacteria.** The *S. aureus* strains used in this study are derivatives of the wild-type strain 8325-4. Strain DU5883 is an isogenic mutant of strain 8325-4 disrupted in the *fnbA* (*fnbA::Tc*) and *fnbB* (*fnbB::Em*) genes (17). DU5883(pFnBPA4) is the deletion mutant with *fnbA* expressed on a high copy plasmid (17). All strains were grown overnight in Todd Hewitt broth (B. D. Biosciences, Oxord, United Kingdom); DU5883(pFnBPA4) was selected with 10 μg of chloramphenicol per ml. The identity of each *S. aureus* strain was regularly checked by using antibiotic disks (B. D. Biosciences). Overnight cultures were washed twice with endotoxin-free phosphate-buffered saline (PBS) before resuspension in PBS for all experiments.

**Alveolar epithelial cell line.** Simian virus 40 (SV40)-transformed strain AT2 neonatal alveolar epithelial cells were used for the in vitro internalization assays (4). SV40-AT2 cells retain the sodium transport properties of alveolar type II cells and express RT1α (rat alveolar epithelial type I cell protein; molecular mass, approximately 40 kDa) (25, 31). SV40-AT2 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Labtech International, East Sussex, United Kingdom), penicillin (100 U/ml), and streptomycin sulfate (100 μg/ml) (Invitrogen, Paisley, United Kingdom). SV40-AT2 cells were maintained at 37°C in a 5% CO₂ humidified incubator (31). SV40-AT2 cells were prepared for internalization assays by seeding semiconfluent 75-cm² flasks onto six-well plates (Fred Baker Scientific, Cheshire, United Kingdom) and incubating overnight in DMEM plus 10% FCS.

Control SV40-AT2 cells were checked during the experimental period for mycoplasmas by immunofluorescence staining with Hoechst 33258 DNA-binding
dye (only the SV40-AT2 nuclei stained with the DNA-bonding dye in mycoplasma-negative cells).  

Internalization assay. Confluent SV40-AT2 cells were incubated for 1 h in serum-free DMEM and then washed twice in PBS with calcium and magnesium. DMEM (1 ml) containing 10⁶ CFU of S. aureus per ml was added to each well that contained SV40-AT2 cells (approximately 1.5 × 10⁶ SV40-AT2 cells per well). S. aureus was cocultured with SV40-AT2 cells for 2 h to 6 h. At the end of the coculture period, SV40-AT2 cells were washed twice with PBS and then incubated for 1 h in 100 µl of 2% (wt/vol) glutaraldehyde buffer (3%) containing sodium cacodylate (0.1 M). Lung blocks were then dehydrated in a graded series of alcohol and embedded in polyethylene capsules in fresh Araldite epoxy resin. Lung tissue  

Experimental design. Lung tissue was obtained from rats inoculated with 8325-4 (n = 3), DU5883 (n = 3), or PBS (vehicle) (n = 3) at 24 h postinfection for analysis at the light microscopic level. For morphological analysis of lung tissue at 24 h postinfection (24). Frozen lung sections (3 μm) were placed on glass microscope slides, air dried, and stained with Giemsa (for light microscopy) or for electron microscopic studies. Sections were viewed with a Hitachi H-500 electron microscope.  

Collection of lung tissue, blood, and BAL fluid samples. At the end of the experimental period, rats were anaesthetized with pentobarbital (45 mg/kg intraperitoneally) containing heparin (500 U/kg intraperitoneally) (22-24). Blood was collected from the ascending aorta. The trachea was cannulated and the lungs were lavaged two times with sterile PBS. The lungs were then removed. BAL fluid was centrifuged at 900 × g for 5 min (23, 24). BAL fluid leukocytes were quantified by hemacytometric counting. Differential cell counts on BAL fluid cells were performed on cytocentrifuged preparations fixed with methanol and stained with Diff-Quick. A total of 300 cells were counted per cytospin.  

Protein assay. BAL fluid protein concentration was determined by using the Bio-Rad assay (Bio-Rad Laboratories, Hertfordshire, United Kingdom) with bovine serum albumin as a standard (Pierce Warriner, Chester, United Kingdom) (24). Data are expressed as the total amount of protein recovered in BAL fluid (i.e., milligrams of protein per milliliter multiplied by the total volume of BAL fluid) and then expressed as a percentage of the control value.  

Lung homogenates. Lungs were weighed and homogenized in Tris-HCl (2.42 mM)-buffered saline (TBS), pH 8.2, containing NaCl (154 mM) and proteasome inhibitor cocktail (Roche Diagnostics). Lungs were homogenized by using a blender (Ultra Turrax Y25; IKA-Werke) on ice at approximately 4,000 rpm twice for 5 s each. Lung homogenates were serially diluted and plated out on blood agar plates to determine the CFU.  

Electron microscopic analysis. Lungs were fixed with paraformaldehyde (4% wt/vol in PBS) to determine the extent of alveolar epithelial necrosis in S. aureus pneumonia at 24 h postinfection (24). Frozen lung sections (3 μm) were incubated with monoclonal antibodies (MAB) against alveolar epithelial type I cells (anti-RTI40 MAB) (10) and two different MAB against alveolar epithelial type II cells (anti-MMC4 antigen and anti-RTI160) (11), followed by isotype-specific secondary antibodies. Fluorescein isothiocyanate (FITC) conjugated to anti-immunoglobulin G1 (IgG1) was used to detect the RTI40 protein (Lorre Laboratories, Reading, United Kingdom) (3), Rhodamine-conjugated to anti-IgG2 (Lorre Laboratories) was used to detect the MMC4 protein (3), and Alexa Fluor 350 conjugated to anti-IgG3 was used to detect the anti-RTI160 antigen (Molecular Probes, Lieder, Netherlands). The nucleus was stained with the DNA-binding dye Hoechst 33258. Immunofluorescence-stained sections were observed by epifluorescence illumination (Axiovert S100; Zeiss). Images were captured with a Cool Snap camera, and the images were processed by using Improvision OpenLab software (version 2.2).  

Statistical analysis. All data are expressed as means ± standard error of the mean. Data from the in vitro internalization assays were transformed to reflect the total number of S. aureus recovered per well or transformed to fractions of control values. Multiple sample means were compared by using one-way analysis of variance with the Student-Newman-Keuls posttest. Multiple sample means from data generated from the pneumonia models (CFU, leukocyte numbers, and BAL protein and RTI40 concentrations) were compared by using Kruskal-Wallis analysis of variance (nonparametric analysis of variance), with Dunn’s multiple comparison posttest. P < 0.05 was considered significant. Tests were performed by using GraphPad InStat v3.00 for Windows 95 (www.graphpad.com).
The ratio of S. aureus inoculated with 8325-4. Data from one experiment are performed twice with similar results. Data from one experiment are shown. **, P < 0.01 versus control; ***, P < 0.001 versus control.

FIG. 1. Effect of fibronectin-binding proteins on S. aureus internalization into alveolar epithelial cells. SV40-AT2 cells were cocultured with either 8325-4, DU5883, or DU5883(pFnBPA4) for 6 h. Extracellular S. aureus cells were killed with gentamicin. The experiment was performed three times; data from one representative experiment are shown. **, P < 0.01 versus control; ***, P < 0.001 versus control.

Morphological changes to the lung in 8325-4-infected rats. Distal airway instillation of 8325-4 into rat lungs induced pneumonia in the left lung in the majority of experiments. At autopsy, the involved lobe turned from red at 24 h postinfection to grey at 96 h postinfection. Light microscopic analysis of lung tissue at 24 h postinfection demonstrated that leukocytes had filled the distal air spaces (Fig. 3C).

The morphological integrity of alveolar epithelial type I and II cells in 8325-4-infected lungs was determined by immunofluorescence analysis with the aid of cell-selective antibodies (3, 10, 11). Type I cells were visualized with the aid of an anti-RTI40 MAb (green staining) (Fig. 3 and 4). In control lungs, the anti-RTI40 MAb bound to the apical surface of alveolar epithelial type I cells (Fig. 3 and 4). In 8325-4-infected lungs, the pattern and extent of anti-RTI40 binding were similar to those in control lungs in most regions of the lung (Fig. 3 and 4).

Alveolar epithelial type II cells were identified by using two different MAbs. The anti-RTI70 MAb recognizes the apical surface of type II cells (blue apical staining) (Fig. 4) (11), while the MMC4 MAb recognizes the apical surface of alveolar epithelial type II and Clara cells (red staining) (Fig. 4) (3). The pattern and extent of both anti-type II MAb binding in 8325-4-infected lungs 24 h postinfection were comparable to that in control sections (Fig. 4). Occasionally, MMC4- and RTI70- positive cells (type II cells) were rounded-up and appeared to be in the process of shedding from their basement membranes in 8325-4-infected lungs at 24 h postinfection (data not shown).

FIG. 2. Effects of cytochalasin D and genistein on S. aureus internalization into alveolar epithelial cells. SV40-AT2 cells were cocultured with either 8325-4, DU5883, or DU5883(pFnBPA4) for 6 h. Extracellular S. aureus cells were killed with gentamicin. The experiment was performed three times; data from one representative experiment are shown. **, P < 0.01 versus control; ***, P < 0.001 versus control.

Effect of fibronectin-binding proteins on recovery of S. aureus in lung tissue and BAL fluid. The number of CFU recovered in lung tissue 24 h postinfection decreased by over 97% in rats inoculated with either 8325-4 or DU5883(pFnBPA4) in comparison to the number of CFU instilled (Fig. 5). In contrast, the number of CFU recovered in lung tissue from DU5883-infected rats was increased by 4.6-fold relative to the instillate at 24 h postinfection (Fig. 5). Moreover, the number of CFU recovered in DU5883-infected lungs at 24 h was increased by over 190-fold relative to the number in 8325-4-infected rats (P < 0.05) and by 1,700-fold (P < 0.01) in comparison to the number of CFU recovered from DU5883(pFnBPA4)-infected lungs (Fig. 5). By 96 h postinfection, the number of CFU recovered from DU5883-infected lungs was approaching the number of CFU obtained from both 8325-4- and DU5883(pFnBPA4)-infected lungs (Fig. 5).

In contrast to the lung data, the number of CFU recovered in BAL fluid at 24 h was not significantly different between the three S. aureus strains (Fig. 5). No CFU were recovered in BAL fluid at 96 h postinfection in any of the S. aureus-infected lungs.

Effect of fibronectin-binding proteins on leukocyte recruitment to the lungs in pneumonia. The total number of leukocytes recovered in BAL fluid from 8325-4-infected rats was elevated 57-fold above control values at 24 h (Fig. 6A). At 24 h postinfection, most of the leukocytes in BAL fluid from 8325-4-infected rats were neutrophils (90%) (Fig. 6B). However, by 96 h postinfection the percentage of neutrophils had decreased, while that of both monocytes/macrophages and lymphocytes had increased (Fig. 6B).

The total number of leukocytes recovered in BAL fluid from DU5883- and DU5883(pFnBPA4)-infected rats was significantly elevated over control values, by 37-fold and 33-fold, respectively (Fig. 6A). However, there were no significant differences in the total number of leukocytes in BAL fluid between the different strains at either 24 or 96 h postinfection. Nor was the percentage of neutrophils, macrophages/monocytes, and lymphocytes recovered in BAL fluid significantly different between S. aureus strains at either 24 or 96 h postinfection (data not shown).
The amount of protein recovered in BAL fluid from 8325-4-infected rats was elevated by 6.8-fold above control values at 24 h postinfection but by only 2.7-fold at 96 h postinfection (Fig. 7A). The amount of protein recovered in BAL fluid from DU5883-infected rats was significantly elevated over values from both control and DU5883(pFnBPA4)-inoculated rats at 24 h postinfection (Fig. 7A). However, by 96 h postinfection, the amount of protein recovered in BAL fluid was not significantly different between the three different strains (Fig. 7A).

To determine the extent of alveolar epithelial type I cell necrosis in *S. aureus* pneumonia, the amount of RTI40 was also measured in BAL fluid. The amount of RTI40 recovered in BAL fluid from DU5883-infected rats was significantly elevated over both control and DU5883(pFnBPA4)-infected rats at 24 h postinfection (Fig. 7B). There were no differences in the amount of RTI40 recovered in BAL fluid from rats inoculated with *S. aureus* at 96 h postinfection (data not shown).

**Cellular location of *S. aureus* in 8325-4- and DU5883-infected lungs.** Gram stains of both BAL fluid cells (cytospins) and infected lung tissue (paraffin sections) demonstrated that *S. aureus* was predominantly associated with neutrophils from infected rats (data not shown). In addition, the percentage of neutrophils containing one or more Gram stain particles was not different between 8325-4- and DU5883-infected lungs (10.3 ± 1.45 versus 11.8 ± 6.08, respectively). Macrophages occasionally contained a few gram-positive particles in their cytoplasm. Gram-positive particles were very occasionally as-

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**FIG. 3.** Immunofluorescence visualization of leukocyte influx and alveolar epithelium in *S. aureus* 8325-4-inoculated lungs at 24 h postinfection. Alveolar epithelial type I cells were stained with the anti-RTI40 MAb (green), while alveolar epithelial type II cells were stained with the MMC4 MAb (red). Nuclei were stained with the Hoechst DNA-binding dye. (A) Phase-contrast image of control lung. (B) Corresponding immunofluorescence image. (C) Phase-contrast image of 8325-4-inoculated lung. (D) Corresponding immunofluorescence image. Many of the air spaces (stars) are filled with leukocytes (Hoechst 33258-only-positive cells) in 8325-4-inoculated lungs in comparison with control lungs. Original magnification, ×100.
associated with the alveolar wall in both 8325-4- and DU5883-infected lungs (data not shown). However, it was not possible to resolve the location of the gram-positive particles by light microscopy.

Electron microscopic analysis confirmed that neutrophils from both 8325-4- and DU5883-infected lungs contained *S. aureus* (Fig. 8A and B). Electron microscopic analysis of lung tissue failed to demonstrate any *S. aureus* internalized with alveolar epithelial cells in 8325-4-infected rats. However, *S. aureus* was observed in one necrotic alveolar epithelial type II cell from DU5883-infected rats (Fig. 8C and D).

**DISCUSSION**

We have demonstrated that *S. aureus* is internalized into rat alveolar epithelial cells in vitro and that internalization is mediated by fibronectin-binding proteins located on the surface of the bacteria. *S. aureus* internalization into alveolar epithelial cells also requires an active cytoskeleton, since internalization was inhibited by cytochalasin D, which disrupts actin structures. Furthermore, our data suggest that *S. aureus* internalization is a regulated process, since the inhibition of tyrosine kinases by genistein prevented uptake. Similar results have been reported previously for *S. aureus* internalization into other nonprofessional phagocytic cells, including mammary gland epithelial cells, endothelial cells, and osteoblasts (1, 12, 15, 18, 24, 26).

To determine whether the expression of fibronectin-binding proteins contributes to virulence in vivo, we first established a rat model of *S. aureus*-induced pneumonia (strain 8325-4). Instillation of *S. aureus* (10⁶ CFU) into the distal airways of rat lungs induced an acute inflammatory reaction that was resolving by 96 h postinfection. The total number of leukocytes and the percentage of neutrophils in BAL fluid were both elevated at 24 h postinfection but had returned to near control values at 96 h postinfection. Similarly, the amount of protein recovered...
The amount of RTI40 in BAL either the parental 8325-4 or the complemented mutant strain. In comparison with values obtained from rats inoculated with increased at 24 h postinfection in DU5883-infected rats in fl

in BAL fluid, which is a measure of serum flux into the air spaces, was elevated at 24 h but returned to near control levels at 96 h postinfection. However, despite the elevated number of leukocytes and protein in BAL fluid, our data did not reveal major necrosis of alveolar epithelial type I cells in 8325-4-induced pneumonia.

To determine whether fibronectin-binding proteins contribute to S. aureus virulence in pneumonia, we compared the survival of three S. aureus strains. These three strains were the wild-type 8325-4, a mutant (DU5883) in which both the fnbPA and fnBPB genes were deleted, and the same mutant containing the gene for FnBPA on a high-copy-number plasmid [DU5883(pFnBPA4)] (17). The extent of acute lung injury, as measured by the total amount of protein and RTI40 recovered in BAL fluid and BAL fluid (horizontal bars).

Many studies demonstrate that highly encapsulated forms of bacterial pathogens, including S. aureus, are more virulent than the weakly encapsulated forms (5, 35, 36). This difference in virulence is thought to be due to the fact that encapsulated forms are more resistant to internalization by both professional (e.g., neutrophils) and nonprofessional (e.g., epithelial cells) phagocytes in vivo (5, 35, 36). We have not determined why strain DU5883 survives better in lung tissue than 8325-4 and DU5883(pFnBPA4). Our data demonstrate that the number of neutrophils recovered in BAL fluid at 24 h postinfection is not significantly different among the three strains. Moreover, neutrophil recruitment to lung tissue, as measured by myeloperoxidase activity, was not significantly different between 8325-4- and DU5883-infected lungs (unpublished observations).

Soluble fibronectin, which is present in BAL fluid, facilitates neutrophil phagocytosis and killing of S. aureus in vitro assays (13, 31, 41). The fibronectin-binding protein-deficient S. aureus strain may be less efficiently cleared by neutrophils in comparison with fibronectin-binding protein-expressing strains because it does not bind fibronectin. Alternatively, fibronectin bound to fibronectin-binding protein-expressing strains of S. aureus may enhance alveolar macrophage activation in comparison with the knockout mutant (39). We are currently investigating these two possibilities.

The exact role of fibronectin-binding proteins in S. aureus virulence is unclear. On one hand, all of the clinical strains of S. aureus tested so far contain one or both fibronectin-binding protein genes (27). No S. aureus strains have been identified which do not contain either of the fibronectin-binding protein genes (27). On the other hand, data from experimental animal studies demonstrate that the expression of fibronectin-binding proteins is not important for S. aureus virulence and may in fact decrease the extent of virulence (8, 15; this study). Interestingly, adhesion of clinical strains of S. aureus to fibronectin-coated slides is variable and does not correlate with the number of fibronectin-binding protein genes (27). Some methicillin-resistant S. aureus strains are also characterized by their low adherence to fibronectin-coated slides.
Low adherence of these methicillin-resistant *S. aureus* strains is associated with the expression of a cell surface protein called Pls (plasmin sensitive) (33). It is possible that the antivirulence effects of fibronectin-binding proteins may be masked or modified during infection by the expression of antiadhesion proteins such as Pls.

Integrin antagonists have been proposed as possible new therapeutic agents to aid in the elimination of gram-positive organisms by preventing fibronectin-binding protein-mediated internalization into host cells (6). Our study suggests that such drugs are unlikely to be useful in the treatment of *S. aureus*-induced pneumonia. While we were able to detect *S. aureus* within neutrophils at 24 h postinfection in rats inoculated with strain 8325-4, we were not able to detect any *S. aureus* internalized into alveolar epithelial cells. Our data suggest that *S. aureus* internalization into alveolar epithelial cells by a fibronectin-binding protein-mediated mechanism is unlikely to be a virulence mechanism in our rat model of pneumonia.

In summary, despite the fact that fibronectin-binding proteins are widely expressed by *S. aureus*, our data suggest that they reduce *S. aureus* virulence in a rat model of pneumonia. Our data raise the possibility that anti-fibronectin-binding proteins, such as Pls, may contribute to virulence by preventing *S. aureus* internalization by both professional and nonprofessional phagocytes.

**FIG. 6.** Number and profile of leukocytes recovered in BAL fluid from *S. aureus*-inoculated lungs at 24 and 96 h postinfection. (A) Total number of leukocytes recovered in BAL fluid from 8325-4-, DU5883-, and DU5883(pFnBPA4)-inoculated lungs. (B) Percentage of neutrophils (black bars), macrophages and monocytes (diagonally striped bars), and lymphocytes (horizontally striped bars) in BAL fluid from control and 8325-4-inoculated lungs. *P* < 0.05 versus control; **P** < 0.01 versus control; ***P** < 0.001 versus control.

**FIG. 7.** Amount of protein and RTI40 recovered in BAL fluid at 24 and 96 h postinfection. (A) The amount of protein recovered in BAL fluid from 8325-4-, DU5883-, and DU5883(pFnBPA4)-inoculated rats at 24 and 96 h postinfection. (B) The amount of RTI40 recovered in BAL fluid at 24 h postinfection. *P* < 0.05 versus control; **P** < 0.01 versus control; ***P** < 0.001 versus control; #*P* < 0.05 versus DU5883(pFnBPA4); ##*P* < 0.01 versus DU5883(pFnBPA4).
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