Dynamic interaction between airway epithelial cells and *Staphylococcus aureus*

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Da Silva, Mauricio C. A., Jean-Marie Zahm, Delphine Gras, Odile Bajolet, Michel Abely, Jocelyne Hinnrasky, Magali Milliot, Maria Cristina de Assis, Coralie Hologne, Noël Bonnet, Marc Merten, Maria Cristina Plotkowski, and Edith Puchelle. Dynamic interaction between airway epithelial cells and *Staphylococcus aureus*. Am J Physiol Lung Cell Mol Physiol 287: L543–L551, 2004. First published May 14, 2004; 10.1152/ajplung.00256.2003.—*Staphylococcus aureus* is a major cause of pulmonary infection, particularly in cystic fibrosis (CF) patients. However, few aspects of the interplay between *S. aureus* and host airway epithelial cells have been investigated thus far. We investigated by videomicroscopy the time- and bacterial concentration-dependent (104, 106, and 108 CFU/ml) effect of *S. aureus* on adherence, internalization, and the associated damage of the airway epithelial cells. The balance between the secretion by *S. aureus* of the α-toxin virulence factor and by the airway cells of the antibacterial secretory leukoproteinase inhibitor (SLPI) was also analyzed. After 1 h of interaction, whatever the initial bacterial concentration, a low percentage of *S. aureus* (<8%) adhered to airway cells, and no airway epithelial cell damage was observed. In contrast, after 24 h of incubation, more bacteria adhered to airway epithelial cells, internalized bacteria were observed, and a bacterial concentration-dependent effect on airway cell damage was observed. At 24 h, most airway cells incubated with bacteria at 108 CFU/ml exhibited a necrotic phenotype. The necrosis was preceded by a transient apoptotic process. In parallel, we observed a time- and bacterial concentration-dependent decrease in SLPI and increase in α-toxin expression. These results suggest that airway cells can defend against *S. aureus* in the early stages of infection. However, in later phases, there is a marked imbalance between the bactericidal capacity of host cells and bacterial virulence. These findings reinforce the potential importance of *S. aureus* in the pathogenicity of airway infections, including those observed early in CF patients.

videomicroscopy; secretory leukoproteinase inhibitor; α-toxin; airway cell damage; bacterial adherence and internalization

*STAPHYLOCOCCUS AUREUS* is a major cause of nosocomial pulmonary infection, being associated with primary pneumonia in infants and young children and secondary pneumonia following viral infection. *S. aureus* is also frequently involved in early infectious airway disease in cystic fibrosis (CF) patients (2, 3). However, only a few aspects of the interplay between *S. aureus* and host airway epithelial cells have been investigated thus far.

*S. aureus* has been shown to adhere to (27, 28) and enter into dedifferentiated airway cells in vitro. After an initial lag period, bacteria begin to replicate within the cells, leading to apoptosis of infected cells (19). One of the key virulence factors of *S. aureus*’s implication in host cell apoptosis is α-toxin, a pore-forming protein that was shown to bind to specific as yet unidentified cell surface receptors and to produce small heptameric pores. However, it appears that the mode of cell death critically depends on the concentration of the toxin (8).

*S. aureus* infections have also been shown to activate signaling pathways in airway epithelial cells in vitro, resulting in NF-κB activation and IL-8 expression (31). The ability to induce IL-8 expression is independent of the adherence properties of intact bacteria, indicating that shed and/or secreted bacterial components are sufficient to evoke the airway cell response (13). Although these studies cast light on some aspects of the pathophysiology of *S. aureus*-induced respiratory disease, our understanding is still limited because the interaction between host cells and bacteria has been analyzed only for short periods of time. Moreover, the concentration-dependent effect of *S. aureus* on the integrity of the airway cells, as well as on the balance between host defense molecules and *S. aureus* virulence factors, has not been assessed.

The airway epithelium represents a primary site for the introduction of potentially pathogenic microorganisms into the body. To prevent colonization and infection by inhaled microorganisms, the respiratory epithelium maintains an effective antimicrobial environment by producing constitutively antimicrobial peptides and proteins. Among these antimicrobial agents, secretory leukoproteinase inhibitor (SLPI), which is produced locally in bronchi by serous cells in the submucosal glands, has been demonstrated to cause marked killing of both gram-negative and gram-positive bacteria (14).

Because secretory products from both bacteria and host cells can modulate the behavior of the other, the present study was designed to investigate, using fluorescent probes and videomicroscopic techniques, the dynamic interaction between human airway epithelial cells and *S. aureus* at different concentrations. We analyzed alterations in membrane integrity of *S. aureus*

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and the induction of apoptosis and necrosis in airway epithelial cells over a 24-h incubation period, as well as the number of host cells with adherent or internalized bacteria and the bactericidal activity of surface liquid collected during the host-bacteria interaction. The changes in the release of SLPI antimicrobial protein by airway epithelial cells and the bacterial release of a-toxin were analyzed in parallel.

**MATERIALS AND METHODS**

**Airway epithelial cells culture.** A transformed human tracheal gland cell line, MM-39 (26), was cultured at 37°C under a 5% CO₂ atmosphere on culture plates coated with 2% type I collagen. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) F-12 (Sigma Chemical, St. Louis, MO) supplemented with 1% UltraSer G serum substitute (Biosepa; Villeneuve, La Garenne, France), glucose (10 g/l), sodium pyruvate (0.33 g/l), penicillin (100 IU/ml), streptomycin (100 μg/ml), and amphotericin B (2 μg/ml). Cells were seeded at a density of 10⁵ cells/ml (10⁶ cells/cm²), and experiments were performed 3 days after confluence.

**Bacterial strain.** S. aureus strain 8325-4, a wild-type laboratory strain [fibronectin-binding protein (FnBP) A and FnBP B; ATCC 8325-4], and S. aureus strain 9630 (FNBP), was kindly provided by T. J. Foster (Department of Microbiology, Trinity College, Dublin, Ireland). Bacteria were stored at −20°C in tryptcysoy broth (TSB; bioMérieux, Marcy l’Etoile, France) containing 20% (vol/vol) glycerol. Before each assay, bacteria were cultured in TSF for 16–18 h at 37°C under agitation (120 rpm) to obtain stationary growth-phase cultures and then centrifuged (3,000 rpm) for 15 min at 4°C. Bacterial pellets were washed once with sterile phosphate-buffered saline pH 7.2 (PBS) and resuspended in DMEM/F-12 antibiotic-free culture medium to final concentrations of 10⁷, 10⁶, or 10⁵ colony-forming units (CFU/ml).

**Real-time monitoring of S. aureus-airway epithelial cell interaction.** A fluorescence staining method using the LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes, Eugene, OR) was used to study the interaction between airway epithelial cells and S. aureus. BacLight is composed of two nucleic acid binding stains: SYTO 9 and propidium iodide. SYTO 9 penetrates into bacteria and host cells with both intact or damaged membranes, staining the cells green, whereas propidium iodide only penetrates into cells with damaged membranes, staining the cells red (18). Briefly, after 3 days of cell culture confluence, medium was removed from the culture plates, and cells were washed three times with sterile PBS and incubated with 1 ml of a S. aureus suspension previously treated with SYTO 9 (3 μl) and propidium iodide (3 μl) for 20 min in the dark at 4°C. Infected culture dishes were placed on the stage of an inverted microscope (Axiovert 200M; Zeiss, Le Pecq, France) equipped with an environmental chamber (37°C, 5% CO₂, 100% relative humidity) and with a charge-coupled device video camera (CoolSnap Fx; Roper Scientific, Tucson, AZ). Using Metamorph (Universal Imaging, Downingtown, PA) software, we recorded time-lapse images every 5 min for 1 h. At each time point, a phase-contrast image and two fluorescent images were recorded. At the end of the first hour and 24-h incubation period, fluorescent images were also recorded at different Z-levels (at 1-μm intervals), from the bottom to the top of the cells, to assess the presence of intracellular bacteria. Green fluorescence staining corresponding to SYTO 9 and red fluorescence staining corresponding to propidium iodide were obtained through a 480-nm excitation filter/500-nm emission filter and a 490-nm excitation filter/635-nm emission filter, respectively. After the first 1-h incubation period, the supernatants containing nonattached bacteria were removed and analyzed to determine the percentage of live (green-stained) or dead (red-stained) bacteria, as described below. In parallel, infected cultures were rinsed three times with sterile PBS and treated with 1 ml of fresh antibiotic-free culture medium containing the two BacLight probes. The percentage of airway epithelial cells with attached and/or internalized bacteria after the first 1-h incubation period was determined by fluorescence microscopy. The culture dishes were then kept for a further 23 h in the environmental chamber, and time-lapse images from the infected cultures were recorded every hour, as described above. At the end of the 23-h incubation period, cell supernatants were removed and analyzed to determine the percentage of green and red fluorescent nonattached bacteria. The cell cultures, on the other hand, were rinsed twice with sterile PBS and exposed to 1 ml of fresh culture medium containing the two fluorescent probes, and the percentages of airway epithelial cells with attached and/or internalized bacteria at 24 h were determined. From the fluorescent images recorded, the evolution of the staining was analyzed in both airway epithelial cells and bacteria. Three different videomicroscopic fields were analyzed per well for bacteria at each concentration, and, in each microscopic field, 100–150 cells were numbered. To control cell survival during fluorescence imaging (20a), we performed time-lapse imaging on airway cells without S. aureus.

**Analysis of the SYTO 9/propidium iodide-stained bacteria present in the airway epithelial cell supernatants.** To determine the percentage of nonadherent bacteria exhibiting either damaged or undamaged membranes (red- and green-stained bacteria, respectively) after 1 and 24 h of airway cell-bacteria interaction, we removed cell culture supernatants from the culture dishes and carefully homogenized them. We then deposited three aliquots of 10 μl on glass slides and analyzed them by fluorescence microscopy (AxioPhot, Zeiss) to count the number of red- and green-stained bacteria, which was then expressed as a percentage of the total number of bacteria present in the observation field (100 bacteria/field).

**Bacterial activity of the airway epithelial cells supernatants.** Samples from the cell culture supernatants were collected either from cells that had never been in contact with S. aureus or from airway cells kept in contact with S. aureus (at 10⁸ CFU/ml) for 1 and 24 h, respectively. The supernatants were filtered (0.22-μm filter unit; Millipore, Molsheim, France) and stored at −20°C. The bactericidal activity of cell supernatants was assessed as described elsewhere (35). In brief, suspensions containing 500 CFU of S. aureus in 2 μl of sterile water were mixed with 30 μl of the airway cell supernatants in 96-well microplates, incubated for 2 h at 37°C under low agitation (60 rpm), and seeded on trypticase soy agar (TSA, bioMérieux) plates. As control, S. aureus was incubated with fresh culture medium. After incubation overnight at 37°C, the number of bacteria was counted and expressed as a percentage of bacterial growth inhibition by reference to the control number of bacteria obtained after the incubation of S. aureus with native culture medium.

**Quantification of S. aureus adherence to and internalization by airway epithelial cells.** Adherence to and invasion of cultured cells were assayed by a modification of the gentamicin exclusion method, as described (20). In brief, after 3 days of cell culture confluence, the supernatants from four different culture plates were removed, and the airway cells were washed three times with sterile PBS. The airway cells were then exposed to 1 ml of the S. aureus suspension containing 10⁸ CFU/ml. After 1 h at 37°C under 5% CO₂, the bacterial suspensions were removed from the culture plates, serially diluted in sterile PBS, and plated in TSA to quantify nonadherent bacteria. In parallel, infected cells were rinsed three times with sterile PBS. Cells from two culture plates were then incubated in 1 ml of fresh culture medium containing 200 μg/ml of gentamicin to kill extracellular (EC) adherent bacteria. Cells from the two remaining plates were incubated with 1 ml of gentamicin-free culture medium. After 1 or 24 h at 37°C, cells from cultures exposed to the gentamicin-containing and to the gentamicin-free culture medium were rinsed twice with sterile PBS and lysed with 1 ml of Triton X-100 (vol/vol) 0.05% in PBS. Cell lysates were serially diluted and plated in TSA to quantify intracellular (IC) and total (IC plus EC) bacteria associated with airway epithelial cells, respectively. Finally, the concentration of EC bacteria adhering to airway epithelial cells was calculated by subtracting the concentration
of IC bacteria from the concentration of total bacteria associated with host cells.

**Evaluation of airway epithelial cell death by apoptosis or necrosis.** Two different approaches were used to assess the mechanism of *S. aureus*-induced cell death. We first used two nucleic acid fluorescent stains: YOPRO-1 (Molecular Probes), which selectively passes through the plasma membranes of apoptotic cells and labels them with moderate green fluorescence, and propidium iodide (Molecular Probes), which stains the necrotic cells with red fluorescence. Three days after challenge, airway epithelial cells were incubated with *S. aureus* and the YOPRO-1 (10 μg/ml) and propidium iodide (1 μl/ml) probes. During the 24 h of interaction, fluorescent images were recorded as previously described. From these time-lapse images, we quantified the mean gray level vs. time, corresponding to the fluorescence intensity variations of the YOPRO-1 stain. These variations were expressed as the ratio of the fluorescence intensity at a given time to the initial fluorescence intensity. Each experiment was performed in triplicate.

In the second approach, we looked for the exposure of phosphatidyl serine (PS) residues at the surface of airway cells (22) and the permeability of cell membranes through flow cytometric analysis of cell labeling with biotinylated annexin-V and propidium iodide, respectively, using an annexin V-biotin detection kit (Roche Molecular Biochemicals, Mannheim, Germany). Annexin V-staining precedes the loss of membrane integrity, which occurs during the latest stages of cell death resulting from either apoptotic or necrotic processes. Briefly, cells were incubated with *S. aureus* suspension at 10^6 CFU/ml for 1 h, washed, and reincubated with culture medium. After 4 or 8 h, cell supernatants were transferred to 15-ml conical tubes. Cells were then detached from the microplate wells with a trypsin-EDTA solution. Detached cells were resuspended in the supernatants kept in the conical tubes, treated with culture medium containing 10% fetal calf serum to neutralize trypsin, and centrifuged at 200 g for 10 min. Cell pellets were resuspended in the binding buffer containing 20 μl of annexin V and 1 μg/ml of propidium iodide, according to the manufacturer’s instructions. After incubation for 15 min at room temperature in the dark, cells were centrifuged and incubated with FITC-streptavidin conjugate (Amersham, Arlington Heights, IL) for 20 min at 4°C. Cells were then washed and analyzed with a FACScanCalibur flow cytometer (Becton Dickinson, Mountain View, CA). Assays were repeated twice and in each case at least 10,000 cells were analyzed.

**Analysis by transmission electron microscopy.** For transmission electron microscopy (TEM), at the 1-, 4-, and 24-h time points of the airway cell-*S. aureus* (10^6 CFU/ml) interaction, assay cells were rinsed, fixed for 1 h with 2.5% glutaraldehyde in 0.15 M PBS, and postfixed in 2% OsO₄. After dehydration in ethanol, cells were embedded in Agar 100 resin (Agar Scientific, Stansted, UK), cut as ultrathin sections (Ultracut E; Leica, Rueil Malmaison, France), and observed under a TEM (Hitachi H300; Elexience, Verrières le Buisson, France) at 75 kV.

**Western blot analysis of *S. aureus* α-toxin.** Samples from the cell culture supernatants were collected from airway cells kept in contact with the different concentrations of *S. aureus* for 1 and 24 h, respectively. Supernatants were mixed with Laemmli buffer (Bio-Rad, Hercules, CA) containing 5% 2-mercaptoethanol (vol/vol), boiled for 5 min, separated on a 15% SDS-PAGE gel, and transferred to a polyvinylidene difluoride membrane. The membrane was then blocked with 5% (wt/vol) nonfat dry milk in PBS containing 0.1% Tween 20 (wt/vol) for 2 h at room temperature, before exposure to anti-α-toxin primary antibody overnight at 4°C (1:2,000; Sigma, Saint Quentin Fallavier, France). The blot was then incubated with a horseradish peroxidase-conjugated goat anti-rabbit antibody (1:1,000; Dako, Glostrup, Denmark) for 1 h at room temperature. Signals were detected with an enhanced chemiluminescence (ECL+)-kit (Amersham Pharmacia Biotech, Buckinghamshire, UK). We analyzed Western blot bands after scanning the membranes (Fuji, LAS-1000, Raytest).

**ELISA analysis of airway cell SLPI.** The SLPI concentrations in the culture medium collected at 1 and 24 h of cell interaction with 10^4, 10^6, and 10^8 CFU/ml of *S. aureus* were measured with an ELISA kit according to the manufacturer’s protocol (R&D Systems Europe, Abington, UK).

**Statistical analysis.** Results are expressed as means ± SD of data obtained in at least three independent experiments carried out in triplicate. Statistical significance was set at a *P* value of <0.05, determined by the Mann-Whitney U-test.

**RESULTS**

**Bacterial adherence, internalization, and airway cell damage.** Only a few bacteria were detected near host cell apical membranes at the beginning of the interaction assay, but the number of microorganisms increased progressively throughout the first hour, particularly during the interaction with bacterial suspensions at 10^8 CFU/ml (Fig. 1). With time, a marked shift in the fluorescence of the bacteria was observed, with a color change from green (Fig. 1, A and B) to red (Fig. 1, C–F). Quantitative analysis showed that at the 1-h time point of the incubation period with the different concentrations of bacteria, 44.6 ± 4.8% of the bacteria detected in cell supernatants were labeled by propidium iodide, suggesting an altered permeability of the bacterial membranes by secretory products from the airway epithelial cells. In contrast with the bacterial alterations, airway epithelial cell maintained their green fluorescent staining during the first hour of incubation with the *S. aureus* suspensions, reflecting no change in the integrity of their plasma membrane whatever the bacterial concentration (Fig. 1).

After the first hour of airway cell exposure to *S. aureus* suspensions, cell cultures were rinsed to discard nonadherent bacteria and covered with culture medium containing the two fluorescent probes (see MATERIALS AND METHODS). Bacterial association with host cells was then quantitatively analyzed by fluorescence microscopy. We observed that the percentage of cells exhibiting adherent bacteria varied with the concentration of the bacterial suspensions used to infect the cells and represented 1.0 ± 0.6, 2.7 ± 2.6, and 7.9 ± 5.1% of the total number of airway epithelial cells exposed to *S. aureus* at 10^3, 10^6, and 10^8 CFU/ml, respectively. After the first hour of the assays, no internalized bacteria could be detected. Cell cultures were then kept in the environmental chamber on the inverted microscope, and the behavior of infected airway epithelial cells was followed for an additional 23 h. Typical images obtained throughout this incubation period with 10^8 CFU/ml of *S. aureus* showed that, with time, the fluorescent staining of airway cells shifted from green (Fig. 2A) to red (Fig. 2, B–E). It is noteworthy that green-stained internalized bacteria could be observed in red-stained airway epithelial cells (Fig. 2F). Quantitative analysis showed that after 24 h of incubation with bacterial suspensions at 10^3, 10^6, and 10^8 CFU/ml of *S. aureus* (3.6 ± 1.7 cells/μl), compared with that after 1 h (0.7 ± 0.6 cells/μl), confirmed the extent of damage to the epithelial
cells after 24 h of bacterial contact. Time-lapse imaging of the airway cells in absence of S. aureus showed that the integrity of the airway cells was preserved.

The number of airway epithelial cells with adherent bacteria at 24 h (0, 2.8 ± 2.6, and 27.5 ± 19.8% of total airway cells for cultures incubated with bacteria at 10^4, 10^6, and 10^8 CFU/ml, respectively) was significantly higher (P < 0.05) than at 1 h for the highest bacteria concentration. In addition, after 24 h of interaction with 10^8 CFU/ml of bacteria, 6.5 ± 2.9% of airway epithelial cells had internalized bacteria. No relationship could be established between the presence of cell-associated bacteria (adherent or internalized) and the loss of viability of host cells. After 24 h of interaction between epithelial cell and S. aureus, the percentage of bacteria with an altered membrane in cell culture supernatants (18.1 ± 5.1%) was significantly lower (P < 0.001) than that observed at 1 h (44.6 ± 4.8%).

Bactericidal activity of airway cell supernatants. Cell culture supernatants collected at 1 and 24 h of the cell interaction with S. aureus at 10^8 CFU/ml were filtered to eliminate bacteria and incubated with freshly prepared S. aureus suspensions. The bactericidal activity of airway cell supernatant is shown in Fig. 3. As control we incubated freshly prepared S. aureus with supernatant collected from airway cells that had never been in contact with S. aureus, and under these conditions we observed a 92.9 ± 11.2% inhibition of the bacterial growth. After 1 h of interaction with S. aureus, the bactericidal activity detected in the airway cell supernatant was not significantly different from that of control supernatant. A significant decrease (P < 0.05) in the bacterial growth inhibition was observed for the cell culture supernatant collected after 24 h of interaction.
the interaction assay. These results demonstrate that, early in the interaction process, airway epithelial cells secrete bactericidal molecules that act on *S. aureus* that have not been previously in contact with the airway cells.

*S. aureus* adherence to and internalization by airway epithelial cells. To quantify the number of bacteria associated with airway cell cultures, we used the gentamicin exclusion assay. Due to the low number of cells with adherent bacteria and the absence of cells with internalized bacteria after the interaction with 10^6 or 10^8 CFU/ml of bacteria, the gentamicin exclusion test was applied only to the interaction of airway cells with bacteria at 10^8 CFU/ml. After 1 h, 1.3 ± 0.4 × 10^8 and 2.0 ± 0.2 × 10^5 CFU had adhered to the cells and were present in the intracellular compartment of airway epithelial cells, respectively. In other words, 16.4% of the bacteria that had been added to the airway epithelial cells adhered to the cells, whereas 0.02% had become internalized. After 24 h of the interaction assay, the number of adherent bacteria (1.0 ± 0.8 × 10^8 CFU) was similar to that detected at 1 h; however, in contrast, the number of intracellular bacteria (1.6 ± 0.9 × 10^5 CFU) had decreased significantly (*P* < 0.001).

Evaluation of airway epithelial cell death by apoptosis or necrosis. We used time-lapse videomicroscopy and vital fluorescent probes to test whether airway epithelial cell death could be related to apoptosis or necrosis. Figure 4, A–C, displays fluorescent images of the cell nuclei at 6, 7, and 8 h of interaction with *S. aureus* at 10^5 CFU/ml. At 6 h a small proportion of cells was labeled with the fluorescent green YOPRO-1 (apoptosis) or the fluorescent red propidium iodide (necrosis) stains. It is noteworthy from these images that the cell nuclei were first stained by the YOPRO-1 stain, indicating that the cells entered in an apoptotic process, which was rapidly followed by necrosis, as assessed by the propidium iodide staining of the cell nuclei. Figure 4D displays the relative fluorescence variations vs. time of the YOPRO-1 probe. We observed that the maximal increase of the relative fluorescence was progressively delayed in relation with the decrease in the bacterial concentration: 11, 16, and 20 h for 10^5, 10^6, and 10^8 CFU/ml of *S. aureus*, respectively (Fig. 4D).

In the second method used to assess the mechanism of death of *S. aureus*-infected cells, we looked for PS residues at the surface of airway cells through cell interaction with annexin V. Cells that had lost membrane impermeability, secondary to either late apoptosis or necrosis, were labeled with propidium iodide. Figure 4, E–G, shows the results obtained during a representative FACS analysis. The mean percentage of 8 h-infected cells reactive with annexin V (38.7%) was much higher than the percentage of reactive control noninfected cells (9.7%), indicating the induction of apoptosis by bacterial infection. However, a higher percentage of infected cells was labeled simultaneously by annexin V and propidium iodide (20.2 and 30.5% for cells at 4 and 8 h postinfection, respectively, vs. 2.5% for control cells). These results further suggest that during *S. aureus* infection, cells entered in an apoptotic process that was rapidly followed by necrosis.

Ultrastructural changes of airway epithelial cells. Using TEM, we could see bacteria adhering to the airway epithelial cells after 1 h of interaction with 10^8 CFU/ml of *S. aureus* (Fig. 5A). At 4 h, bacteria were observed in vacuoles within airway epithelial cells, but no obvious alteration in host cell or in bacterial cell ultrastructure was observed (Fig. 5B). At 24 h, dramatic ultrastructural alterations were detected, with all airway epithelial cells exhibiting a necrotic phenotype. A complete disorganization of the epithelial cell layer was observed, and numerous intracellular bacteria were still visible in the necrotic epithelial cells (Fig. 5C).

Quantitation of SLPI in cell culture medium. The SLPI concentration of the medium was measured by ELISA in the airway cell culture medium after 1 and 24 h of interaction with 10^4, 10^6, and 10^8 CFU/ml of *S. aureus*. The results presented in Fig. 6 show that after the first hour of interaction with the different concentrations of *S. aureus*, no significant variation of the SLPI content was observed whatever the bacterial concentration. After 24 h of interaction, a significant concentration-dependent (*P* < 0.05) decrease in SLPI was observed in presence of *S. aureus* compared with the control without bacteria.

α-Toxin expression. To analyze the time- and bacterial concentration-dependent expression of α-toxin during the host cell-*S. aureus* interaction, we performed Western blot analysis of the culture medium collected at 1 and 24 h of interaction with 10^8, 10^6, and 10^4 CFU/ml of *S. aureus*. As shown in Fig. 7, no α-toxin was detected in the control lanes or in the supernatants of cells exposed to 10^8 or 10^6 CFU/ml of *S. aureus* at 1 h. In contrast, an increased amount of the toxin was detected in the supernatants of cells exposed to *S. aureus* at 10^8 CFU/ml. At 24 h, α-toxin was detected in all the supernatants of cells, whatever the bacterial concentration.

DISCUSSION

Over the last decade, important advances in research have been achieved in relation to the cellular and molecular processes involved in pathogen-host cell interplay. Although *S. aureus* is a major human respiratory pathogen, its interaction with airway epithelium has been poorly documented. The present study was therefore carried out to assess aspects of the reciprocal effects resulting from the interaction between *S. aureus* and airway epithelial cells.

The results obtained by time-lapse microscopy clearly showed that the airway cells exhibit efficient self-defense mechanisms against *S. aureus*, which accounted for the permeabilization of plasma membranes in almost 50% of the bacteria detected in cell culture supernatants at 1 h postinfection. This result is in accordance with those obtained in the in vitro bactericidal assay carried out with supernatants recovered from cell cultures previously incubated with *S. aureus*. After freshly prepared bacterial suspensions were exposed to supernatants of cells conditioned by *S. aureus* infection for 1 h, the percentage of bacterial growth inhibition was in excess of 85%. Because a similar percentage of inhibition was obtained with supernatants from control noninfected cells, this anti-*S. aureus* activity is likely to have resulted from a constitutive secretory activity of airway cells rather than from the production of an inducible molecule. Our finding that the supernatants from cells conditioned for 24 h exhibited a lower capacity to inhibit *S. aureus* growth led us to speculate that during infection the self-defense mechanisms of airway cells may have been overwhelmed.

The antimicrobial activity of the airway surface liquid stems from different cationic proteins and peptides including lysozyme, lactoferrin, phospholipase A₂ (PLA₂), β-defensins (5),
and SLPI, a protein constitutively produced by the airway submucosal gland cells (10). Because SLPI has been shown to exhibit a broad-spectrum antibiotic activity that includes anti-

S. aureus activity (14), we wondered whether the microbicidal activity detected in the supernatants of the airway cell cultures could be associated with this protein. To address this point, we compared the amount of SLPI in the supernatants of control noninfected and of airway cells infected with S. aureus at different concentrations for 1 and 24 h. Our results showed that at 24 h, besides exhibiting reduced capability to inhibit S. aureus growth, the supernatants from infected cultures exhibited also significantly reduced concentrations of SLPI compared with the amounts detected in control cultures, which were dependent on the bacterial concentration. When cells were kept in contact with bacteria for 24 h, <20% of bacteria detected in the culture supernatants was stained by propidium iodide. S. aureus in log-phase has been shown to be more susceptible to cationic peptides (11) and PLA₂ (21) than stationary-phase cells, because stationary-phase bacteria typically have a substantially lower transmembrane potential than

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**Fig. 4.** Dynamic imaging and flow cytometric analysis of apoptosis and necrosis of airway epithelial cells over the course of 24 h of interaction with S. aureus. For dynamic imaging, 2 nucleic acid fluorescent stains were used: YOPRO-1, which selectively passes through the plasma membranes of apoptotic cells and labels them with moderate green fluorescence, and PI, which stains the necrotic cells with red fluorescence. We observed that early during the interaction, unstained cells at 7 h of interaction (arrowheads 1 and 2 in A) shifted 1 h later to a green staining characteristic of apoptosis (arrowheads 1 and 2 in B), and 1 h later green staining (arrowhead 3 in A and arrowhead 2 in B) shifted to a red staining, characteristic of necrosis (arrowhead 3 in B and arrowhead 2 in C) or did not change (arrowhead 1 in C). The ratio of the fluorescence intensity at time/initial fluorescence intensity (relative fluorescence) of the apoptotic YOPRO-1 stain is displayed vs. time in D. The maximal value of the relative fluorescence was progressively delayed in relation with the decrease in the bacterial concentration. By flow cytometry, we analyzed the reactivity with annexin V (FL1) and PI (FL3) of control noninfected airway epithelial cells (E) and of cells infected with S. aureus for 4 h (F) and 8 h (G). Annexin V-labeled and PI-unlabeled cells are early apoptotic cells whereas annexin V- and PI-labeled cells are late apoptotic or necrotic cells. The figure is representative of 2 assays with similar results.
logarithmic-phase cells (21), as well as a different membrane phospholipid composition (9) and a thicker peptidoglycan layer (17). Structural and physiological changes of staphylococci during the transition from a growing to a nongrowing state may explain the low percentage of propidium iodide-stained bacteria detected in the supernatant of cell cultures at the 24-h time point in the interaction assay, because \textit{S. aureus} growth is likely to have reached the stationary phase in the latter stages of the assay. In addition, the dramatic decrease in antimicrobial proteins such as SLPI secreted by the airway epithelial cells after 24 h of interaction with \textit{S. aureus} may also have accounted for the decrease in the bacterial killing.

\textit{S. aureus} is generally not considered to be a significant intracellular pathogen. However, there is growing evidence that it can enter into nonprofessional phagocytic cells such as fibroblasts (34), osteoblasts (16), and endothelial (6, 30, 33) and epithelial cells (4, 7, 15, 23). It has also been reported that bacteria can persist intracellularly (29) and induce apoptosis in infected cells as early as 4 h after internalization (25). In a study carried out by Kahl et al. (19), respiratory cells infected by the \textit{S. aureus} strain RN6390 exhibited features of apoptosis at 24 h, but not at 4 h after bacterial internalization. The induction of apoptosis was clearly associated with replication of intracellular bacteria, because Cowan I, an \textit{S. aureus} laboratory strain that was internalized but failed to replicate, was unable to induce apoptosis (19). In our study, although most of the cells died after 24 h of interaction with bacteria at high concentration, no relationship could be established between the presence of adherent and/or internalized 8325-4 \textit{S. aureus} strain and airway epithelial cell death. We observed that airway cell membranes became permeable to propidium iodide, a characteristic of necrotic rather than apoptotic cells. The differences between our results and those obtained by Kahl et al. (19) are likely to have resulted from the differences in our experimental conditions. In our study, all the assays were carried out in the presence of extracellular bacteria, whereas Kahl et al. (19) treated infected cells with antibiotics to kill extracellular bacteria. Moreover, the epithelial cell type used in the assays (tracheal vs. alveolar cells), the \textit{S. aureus} strain, and the bacterial capacity to replicate inside the host cells likely represent important factors determining the induction of cell death by apoptosis or necrosis. In our assays, cells were kept continuously exposed to \textit{S. aureus} in suspension in the culture medium. Under these conditions, extracellular bacteria are likely to have replicated and released virulence factors into the culture medium. We speculate that bacterial exoproducts might...
have accounted for the death of airway epithelial cells, either by enzymatic activity or by direct toxic effects on host cells. 

α-Toxin is a pore-forming protein released by most S. aureus strains. Its main activity is to cleave phospholipids of the host cell membranes, and its toxic effects are thought to be primarily the result of its rapid action, which destroys the membrane more quickly than it is replaced by the cell’s biosynthetic pathways, resulting in cell lysis and necrosis. Because in our study a dramatic alteration of airway cell permeability was detected at 24 h postinfection in both real-time monitoring of bacteria-host cell interaction and TEM assays, we assessed α-toxin in cell culture supernatants by Western blot assays. High amounts of the toxin were detected in the supernatants of cells exposed to S. aureus at 10^8 CFU/ml. At 24 h, α-toxin was detected in all the supernatants of cells, whatever the bacterial concentration. Although it would be tempting to attribute the killing of airway cells to the marked expression of α-toxin in cell culture supernatants, further assays will need to be carried out using S. aureus clinical strains. 

Besides its capability to induce necrosis, α-toxin has been reported to generate, at subcytotoxic concentrations, small, monovalent cation-selective pores leading to cell activation and death through the activation of the intrinsic apoptotic pathway (1, 12). We looked for features of apoptosis in cells exposed to S. aureus for periods shorter than 24 h. Excitingly, by two different approaches, we showed that at 8 h postinfection a high percentage of cells exhibited characteristics of apoptotic cells and that during S. aureus infection, cells entered in an apoptotic process that was rapidly followed by necrosis. These data are in agreement with a recent study showing that in the induction of cell death by S. aureus α-toxin, necrosis predominates despite the apoptotic activation of caspase (1). 

A potential pathway involved in α-toxin-mediated cell death could be related to an increase of intracellular nitric oxide (NO) induced by the toxin (24). The endogenous mediator NO has been shown to block apoptosis and to switch death to necrosis. During airway epithelial cell-S. aureus interaction in our experiments, this latter mechanism could have been involved in the onset of cell necrosis, which rapidly followed the first steps of the apoptotic cascade (32).

In late phases of the real-time interaction assays, high concentrations of nonadherent bacteria were observed in cell culture supernatants. Given that airway epithelial cell cultures were rinsed to eliminate nonadherent microorganisms after the initial 1-h exposure of airway epithelial cells to S. aureus suspensions, free bacteria are likely to have originated both from the replication of adherent extracellular staphylococci and from bacteria escaping from damaged host cells. Despite the presence of free bacteria in cell supernatants, no difference was detected in the CFU counts of adherent staphylococci at 1 and 24 h. This may result from the saturation of cell membrane receptors for bacterial adhesins or from the reduced adhesive-ness of S. aureus in the stationary-growth phase. The FnBPs, which are major S. aureus adhesins, have been shown to take place during the first hour of bacterial growth and then decrease dramatically between 2 and 4 h (27). In our study, the gentamicin exclusion assay highlighted a significant decrease in the number of intracellular bacteria at 24 h vs. that at 1 h. Although this could suggest that host cells have killed intracellular bacteria, the accuracy of the gentamicin assay is known to depend on the selective permeability of the host cell membranes. Given that an increasing number of airway cells exhibited damaged membranes during the 24 h of the interaction assay, the lower counts of intracellular staphylococci detected at 24 h are likely to be the result of antibiotic entry into damaged airway cells leading to the killing of bacteria. 

Together, these results suggest that the innate capacity of airway epithelial cells to defend themselves against bacteria such as S. aureus may be overcome after exposure to bacteria. The signaling events that control the host response to S. aureus clinical strains from non-CF and CF origin will represent an important challenge in the understanding of the S. aureus-induced cytotoxicity and may provide a rationale for future cytoprotective therapeutic interventions. 

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