Staphylococcus aureus Isolates Associated with Necrotizing Pneumonia Bind to Basement Membrane Type I and IV Collagens and Laminin

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To investigate how Panton-Valentine leukocidin (PVL)–positive *Staphylococcus aureus* (PPSA) strains associate with specific bronchial lesions during community-acquired necrotizing pneumonia, we examined PPSA strains and PVL-negative *S. aureus* (PNSA) strains for their binding behavior to extracellular matrix (ECM) proteins, primary human airway epithelial cell (HAEC) cultures, and human airway mucosa damaged ex vivo. Compared with PNSA strains, PPSA strains exhibited increased affinity for damaged airway epithelium and especially for exposed basement membrane. PPSA strains, compared with PNSA strains, showed stronger affinity for type I and IV collagens and laminin, a property associated with the presence of the *cna* gene. PPSA and PNSA culture supernatants similarly damaged HAEC layers, whereas recombinant PVL had no effect, suggesting that an *S. aureus* exoprotein other than PVL might contribute to the observed airway epithelial damage. These results suggest that epithelial damage, possibly due to viral infection (which usually precedes necrotizing pneumonia) and/or to a non–PVL *S. aureus* exoproduct action, may permit binding of PPSA to exposed type I and IV collagens and laminin—the PVL cytotoxin being involved later during necrotizing pneumonia.

Staphylococcus aureus possesses a variety of virulence factors that can be divided into 3 functional categories. Factors mediating adherence to tissue and extracellular matrix (ECM) components are grouped together in a single family named "microbial surface components recognizing adhesive matrix molecules" (MSCRAMMs) [1], whose ligands include fibrinogen, fibronectin, collagen, laminin, elastin, and bone sialoprotein. Other factors, such as cytotoxins (hemolysins and leukocidins), promote tissue damage and bacterial spread. Finally, factors such as capsular polysaccharide and superantigenic toxins protect *S. aureus* from the host immune system. The precise roles that most individual staphylococcal factors play in invasive infections are difficult to determine [2].

The synergohymenotropic toxin family is composed of γ-hemolysin, LukE-LukD leukocidin, and Panton-Valentine leukocidin (PVL) [3, 4]. PVL has been epidemiologically correlated to specific human diseases caused by *S. aureus,* such as primary skin infections and severe necrotizing pneumonia [5, 6]. Necrotizing pneumonia was first described in France in 2002 [6], and other cases have since been reported worldwide [7–11]. Contrary to PVL-negative *S. aureus* (PNSA) pneumonia, PVL-positive *S. aureus* (PPSA) pneumonia is preceded by influenza-like symptoms (with or without isolation of influenza virus), hemoptysis, pleural effusion, rapid onset of acute respiratory distress, leukopenia, and high mortality [6]. Moreover, histopathological examination of lung tissue from patients who died of PPSA pneumonia showed specific lesions with

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extensive necrotic ulceration of the tracheal and bronchial mucosae (covered with numerous gram-positive cocci) and massive hemorrhagic necrosis of the interalveolar septa [6].

To investigate why PPSA strains were systematically associated with bronchial lesions in necrotizing pneumonia, we examined PPSA and PNSA isolates for their affinity for ECM proteins, primary human airway epithelial cell (HAEC) cultures, and human airway mucosa damaged ex vivo. We also examined the effects of PPSA and PNSA culture supernatants and recombinant PVL on HAECs, to determine the role of PVL itself. Our results indicate that recombinant PVL alone does not affect the integrity of the airway epithelium, whereas PPSA affinity for type I and IV collagens, laminin, and damaged airway epithelium is greatly enhanced, compared with PNSA affinity.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

S. aureus reference strains ATCC 49775 (LukS-PV LukF-PV$^+$), 8325-4 (FnBPs$^+$), and DU5883 (FnBPs$^-$) were a gift from Tim Foster (Department of Microbiology, Trinity College, Dublin, Ireland) [12]. Centre National de Référence des Staphylocoques (Lyon, France) provided isolates from patients described by Gillet et al. [6]: (1) 16 PPSA strains were from patients with community-acquired necrotizing pneumonia (only 1 strain was methicillin resistant), and (2) 16 PNSA strains were from 9 patients with nonnecrotizing community-acquired pneumonia (2 strains were methicillin resistant) and 7 patients with nonnecrotizing hospital-acquired pneumonia (4 strains were methicillin resistant). The strains were stored at -20°C in trypticase soy broth (TSB; Institut Pasteur) containing 20% (vol/vol) glycerol. Bacteria were cultured in TSB overnight at 37°C, were harvested by centrifugation, and were resuspended in PBS (0.1 mol/L) or RPMI 1640 culture medium (Seromed; Biochrom KG) containing 20 mmol/L HEPES (Sigma). The bacterial suspension was adjusted to a final density of 10^9 cfu/mL.

Preparation of Bacterial Supernatant

The strains were grown in brain-heart medium for 17 h at 37°C with vigorous shaking [4]. After centrifugation, culture supernatants were sterilized by filtration through 0.25-μm filters and were stored at -20°C until use.

Production and Purification of Recombinant LukS-PV and LukF-PV

The pIVEX2.4d vector (Roche Applied Science) was used to produce recombinant His-tagged LukS-PV and LukF-PV proteins (the 2 components of PVL). Primers were designed after identification of suitable hybridization sites in the *lukS-PV* and *lukF-PV* genes. The 5′ primers (rlukS-1, 5′-ACC CTT AAT TAA AGA ATC TAA AGC TGA TAA CAA TAT TGA GAA TAT TG-3′ and rlukF-1, 5′-ACC CTT AAT TAA AGC TCA ACA TAT CAC ACC TGT AAG-3′) were chosen within the coding region of each gene (GenBank accession number X72700), omitting the region predicted to encode the signal peptide, as verified by hydrophobicity analysis, as described by Kyte and Doolitttle [13], and the SignalP V1.1 World Wide Web Prediction Server (http://www.cbs.dtu.dk/services/SignalP/). The 3′ primers (rlukS-2, 5′-ACG CGG ATC CTC AAT TAT GTC CTT TCA CTT TAA TTT CAT GAG- 3′ and rlukF-2, 5′-ACG CGG ATC CTT AGC TCA TAG GAT TTT TTT CCT TAG ATT G-3′) were chosen to encompass the stop codon of each gene. Restriction sites (*Pac*I for rlukS-1 and rlukF-1 and *Bam*H1 for rlukS-2 and rlukF-2) were included in each primer. DNA was extracted from *S. aureus* reference strain ATCC 49775 and used as a template for polymerase chain reaction (PCR) amplification. PCR products and plasmid DNA were prepared by use of the Qiagen plasmid kit (Qiagen). PCR fragments were digested with *Pac*I (Ozyme) and *Bam*H1 (Promega) and ligated (T4 DNA ligase; Promega) with the pIVEX2.4d expression vector, which was digested with the same restriction enzymes. The resulting plasmids were transformed in *Escherichia coli* DH5α (Stratagene) before transformation in *E. coli* BL21star(DE3)pLys (Invitrogen) for gene expression. The integrity of the open-reading frame of each construct was verified by DNA sequencing. The fusion proteins (LukS-PV and LukF-PV) were purified from lysates of transfected *E. coli* cells by affinity chromatography on Ni-NTA columns, in accordance with the supplier's instructions (Qiagen). The purity of the fusion proteins was controlled by 7.5% SDS-PAGE. The leukotoxic activity of recombinant LukS-PV and LukF-PV proteins on human leukocyte preparations was verified by fluorescence-activated cell sorter analysis of cellular ethidium-bromide incorporation, as described elsewhere [14]. Fusion proteins were lyophilized and stored at 4°C before use.

HAEC and Tissue Culture Models

Analysis of the effect of bacterial extracellular products on the integrity of the airway epithelium. The transformed, immortalized human bronchial epithelial cell line 16HBE14o$^-$ [15] was cultured in modified Eagle's medium (MEM; Gibco) supplemented with 10% fetal calf serum (Gibco), L-glutamine (Gibco), and antibiotics (Sigma), at 37°C with 5% CO_2, to obtain highly confluent cell layers. Cells were placed in nonsupplemented MEM before use. Confluent cultures were exposed to *S. aureus* supernatants for 10 min to 4 h and to recombinant LukS-PV and LukF-PV proteins, alone and in combination (final concentration, 200 nmol/L), for 10 min to 24 h. Changes in confluence because of the effect of bacterial supernatants or PVL itself were monitored in comparison with control cell layers exposed to cell culture medium alone. In parallel, the cytotoxic effect of LukS-PV and LukF-PV proteins

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Table 1. Adherence of Panton-Valentine leukocidin (PVL)–positive *Staphylococcus aureus* (PPSA) and PVL-negative *S. aureus* (PNSA) strains to extracellular matrix (ECM) components.

ECM component used for preincubation of wells, concentration	Level of adherence to ECM components,[a] median (IQR)		P^b
	PPSA strains (n = 16)	PNSA strains (n = 16)	
cFN, 5 μg/mL	0.67 (0.48–0.84)	0.28 (0.04–0.69)	.02
cFN, 50 μg/mL	0.81 (0.66–0.95)	0.63 (0.31– 0.85)	NS
pFN, 5 μg/mL	0.76 (0.57–0.88)	0.74 (0.44–0.86)	NS
pFN, 50 μg/mL	0.92 (0.67–1.00)	0.88 (0.64–1.02)	NS
Type I collagen, 5 μg/mL	1.13 (0.95–1.27)	0.01 (0–0.04)	<.001
Type I collagen, 50 μg/mL	0.97 (0.75–1.19)	0.005 (0–0.006)	.001
Type IV collagen, 5 μg/mL	0.31 (0.11–0.45)	0.004 (0–0.03)	.008
Type IV collagen, 50 μg/mL	0.77 (0.49–0.93)	0.007 (0–0.02)	.003
Laminin, 5 μg/mL	0.60 (0.34–0.78)	0.02 (0–0.03)	<.001
Laminin, 50 μg/mL	0.76 (0.47–1.02)	0.02 (0–0.03)	<.001

NOTE. cFN, human cellular fibronectin; IQR, interquartile range; pFN, human plasma fibronectin; NS, not significant.

[a] Values represent absorption at 570 nm.

[b] Kruskal-Wallis test.

both alone and in combination was monitored by use of the lactate dehydrogenase (LDH) activity detection kit (Roche).

Interaction of* S. aureus *with human bronchi damaged ex vivo. Bronchi were collected from patients undergoing surgery for bronchial carcinoma, from areas that were found to be normal by microscopy and were distant from the tumor. Pieces of bronchial tissue were damaged ex vivo, as described elsewhere [16], by use of a metallic probe (diameter, 2 mm) cooled by immersion in liquid nitrogen and applied at a pressure of 33 kPa for 10 s. This treatment damaged a zone of ~15 mm^2 in which only cells of the surface epithelium were damaged and desquamated immediately. Within a few hours, cells edging the wound began to migrate. The tissue was then rinsed with RPMI 1640 culture medium at 37°C with 5% CO_2. Bacterial suspensions were added for 1 h at 37°C, and the tissues were fixed with paraformaldehyde before being embedded in paraffin. Paraffin sections were counterstained with hematoxylin and Gram methods.

Interaction of* S. aureus *with primary HAEC cultures. Fresh human airway tissues collected from nasal polyps were immersed in RPMI 1640 culture medium supplemented with 20 mmol/L HEPES containing antibiotics (200 U/mL penicillin and 200 μg/mL streptomycin; Sigma). The tissues were cut into small explants (width, 2 mm^2) and seeded onto a type I collagen matrix in 24-well plates, in defined culture medium composed of RPMI 1640 supplemented with insulin (1 μg/mL; Sigma), apotransferrin (1 μg/mL; Serva), epidermal growth factor (10 ng/mL; Serva), hydrocortisone (0.5 μg/mL; Sigma), retinoic acid (10 ng/mL; Sigma), penicillin (100 U/mL), and streptomycin (100 μg/mL) and were cultured for 3–4 days at 37°C with 5% CO_2 [17]. On day 3 or 4, antibiotics were removed from primary HAEC cultures by repeated rinsing with RPMI 1640 culture medium. Bacterial suspensions were added to HAEC cultures for 1 h at 37°C. Cultures were then rinsed 3 times with 0.5 mL of PBS (0.1 mol/L; pH 7.2) to remove nonadherent bacteria, fixed with 2.5% glutaraldehyde in 0.1 mol/L PBS for 2 h at 4°C, rinsed 3 times in PBS, postfixed with 2% osmium tetroxide, dehydrated through graded ethanol concentrations, critical point–dried with CO_2, affixed onto stubs, and coated with 15 nm of gold-palladium. Adherence of *S. aureus* to HAECs was observed by use of a Philips XL30 scanning electron microscope operating at 10 kV and was scored by blinded observation of the behavior of the different strains (semiquantified by count of cells) for their affinity for HAECs and the underlying type I collagen matrix. Scanning electron microscopy (SEM)–determined affinity for HAECs and the underlying type I collagen matrix was scored as very strong, strong, medium, and very low. For illustration, the very low score was attributed to strains for which only a few or no bacteria, on different SEM fields, were observed to be bound to HAECs and to the underlying type I collagen matrix on which cells were seeded.

Adherence of* S. aureus *to ECM components. Ninety-six–well microtiter plates were incubated, under constant agitation, overnight at 4°C with either 100 μL of ECM components in PBS (5 and 50 μg/mL) or PBS supplemented with 1% bovine serum albumin (BSA; Sigma) as a control for nonspecific binding. The following ECM components were screened: human cellular fibronectin (cFN) (Sigma), human plasma fibronectin (pFN) (Sigma), type I collagen from rat tail (prepared as described elsewhere [17]), and type IV collagen and laminin from basement membrane of Engelbreth-Holm-Swarm mouse sarcoma (Sigma). The purity and integrity of 15 μL of these ECM components (dilution, 50 μg/mL) were assessed on appropriate

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Figure 1. *Staphylococcus aureus* isolates from necrotizing pneumonia (Panton-Valentine leukocidin [PVL]–positive *S. aureus* [PPSA]) and from nonnecrotizing community-acquired pneumonia (PVL-negative *S. aureus* [PNSA]), examined for their binding capacity (*Y*-axis) to type I collagen from rat tail *(A),* to type IV collagen *(B),* and to laminin, coated on 96-well plates, at concentrations of 5 μg/mL *(white bars)* and 50 μg/mL *(black bars).*

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Table 2. Distribution of microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) genes among 32 Panton-Valentine leukocidin (PVL)–positive *Staphylococcus aureus* (PPSA) and PVL-negative *S. aureus* (PNSA) clinical isolates.

	Source, no. (%)		
Gene	PPSA isolates (n = 16)	PNSA isolates (n = 16)	P^a
cna	14 (87)	4 (25)	<.001
eno	16 (100)	16 (100)	NS

NOTE. NS, not significant.

[a] The χ^2 test was used to simultaneously analyze the distribution of 9 MSCRAMM genes among 32 *S. aureus* isolates from patients with necrotizing pneumonia and from patients with nonnecrotizing community-acquired pneumonia.

SDS gels stained with R250 Coomassie blue (Biorad). After overnight incubation, the wells were washed 3 times for 20 min with 1% PBS-BSA and then with bacterial suspension (100 μL/well; 10^9 cfu/mL in PBS) for 30 min at 37°C under mild agitation. Wells were then washed 3 times with PBS to remove nonadherent bacteria. Bacteria were fixed with 2.5% glutaraldehyde in 0.1 mol/L PBS for 2 h at 4°C, stained with 0.1% crystal violet for 30 min at room temperature, and rinsed in water. The stain was then extracted from bacteria by use of 0.2% Triton X100 (Sigma) for 30 min at room temperature and read by use of a spectrophotometer (MR5000; Dynatec) at 570 nm. A blank value corresponding to BSA-coated wells was automatically subtracted; each assay point was determined in duplicate, and experiments were repeated twice. Values obtained with *S. aureus* strain DU5883 (FnBPs mutant) on 5 μg/mL fibronectin were taken as reference values (bacterial adherence, 0.1).

Detection of Adhesin Genes by Use of PCR

To identify the *eno* (laminin-binding protein) and *cna* (collagen-binding protein) genes, genomic DNA was extracted from staphylococcal cultures [18] and used as an amplification template for multiplex PCR with primers 5′-ACGTGCAGCAGCTGACT-3′ (ENO-1) and 5′-CAACAGCATYCTTCAGTACCTTC-3′ (ENO-2) and 5′-GTCAAGCAGTTATTAACACCAGAC-3′ (CNA-1) and 5′-AATCAGTAATTGCACTTTGTCCACTT-3′ (CNA-2), as described elsewhere [19]. The thermal-cycling conditions consisted of an initial denaturation step (5 min at 94°C) followed by 25 cycles of amplification (denaturation for 1 min at 94°C, annealing for 1 min at 55°C, and extension for 1 min at 72°C). The reaction ended with a 10-min incubation step at 72°C. PCR products were analyzed by electrophoresis through 1% agarose gels (Sigma).

Statistical Analysis

Continuous variables were summarized by use of medians and interquartile ranges, and categorical variables were summarized by use of frequencies and percentages. Nonparametric tests were used to compare distributions. The χ^2 test was used to analyze the presence of adhesin genes and the source of clinical strains, and analysis of variance was used to correlate gene detection and binding capacity to ECM components. Two-tailed $P < .05$ was considered to be significant. Statistical analyses were performed with SPSS software (version 11.5; SPSS).

RESULTS

Adherence of* S. aureus *to ECM components in vitro. ECM-binding capacities, according to the median values for the PPSA and PNSA strains, are presented in table 1. Most of the tested strains showed significant binding capacity to 5 and 50 μg/mL cFN and pFN, compared with the $FnBPs^-$ mutant used as a reference. Adherence to cFN, but not to pFN, differed significantly between PPSA and PNSA strains, specifically on preincubation of wells with cFN at a concentration of 5 μg/mL (table 1). PPSA strains significantly differed from PNSA strains with respect to their type I ($P < .001$ and $P < .01$, for 5 and 50 μg/mL, respectively) and type IV ($P < .01$ and $P < .01$, for 5 and 50 μg/mL, respectively) collagen- and laminin-binding capacities ($P < .01$ and $P < .01$, for 5 and 50 μg/mL, respectively). Fifteen of the 16 PPSA strains bound avidly to type I and IV collagens and laminin ($P < .05$), compared with only 3 of the 16 PNSA strains (figure 1*A*–*C*). The binding capacities of PPSA strains to type IV collagen and laminin were, in general, concentration dependent, although they were already maximal with 5 μg/mL type I collagen. Binding capacities of the strains to type I and IV collagens and laminin were similar and independent of their membership in the PPSA group. After adjusting for PVL, no significant difference was observed between methicillin-sensitive or -resistant isolates and community- or hospital-acquired isolates, in multivariate analysis (P = .4 and P = .9, respectively).

MSCRAMM gene detection by use of PCR. Among *S. aureus* MSCRAMMs, the *eno* and *cna* genes encode for laminin and collagen adhesins. All clinical isolates tested by use of multiplex PCR were found to be positive for the *eno* gene (table 2). By contrast, only 18 of the 36 isolates were found to be positive for the *cna* gene, which was significantly associated with PPSA strains (87% in PPSA vs. 25% in PNSA; $P < .001$). The PPSA strain LY1999053 and the PNSA strains LY1999604, LY19990108, and LY19990176 were found to be negative and positive, respectively, for the *cna* gene and exhibited low and high binding, respectively, to type I and IV collagens. PPSA strains for which the *cna* gene could not be detected did not exhibit a high binding capacity to type I and IV collagens. The presence of the *cna* gene correlated with binding capacity to type I and IV collagens ($P < .001$, for

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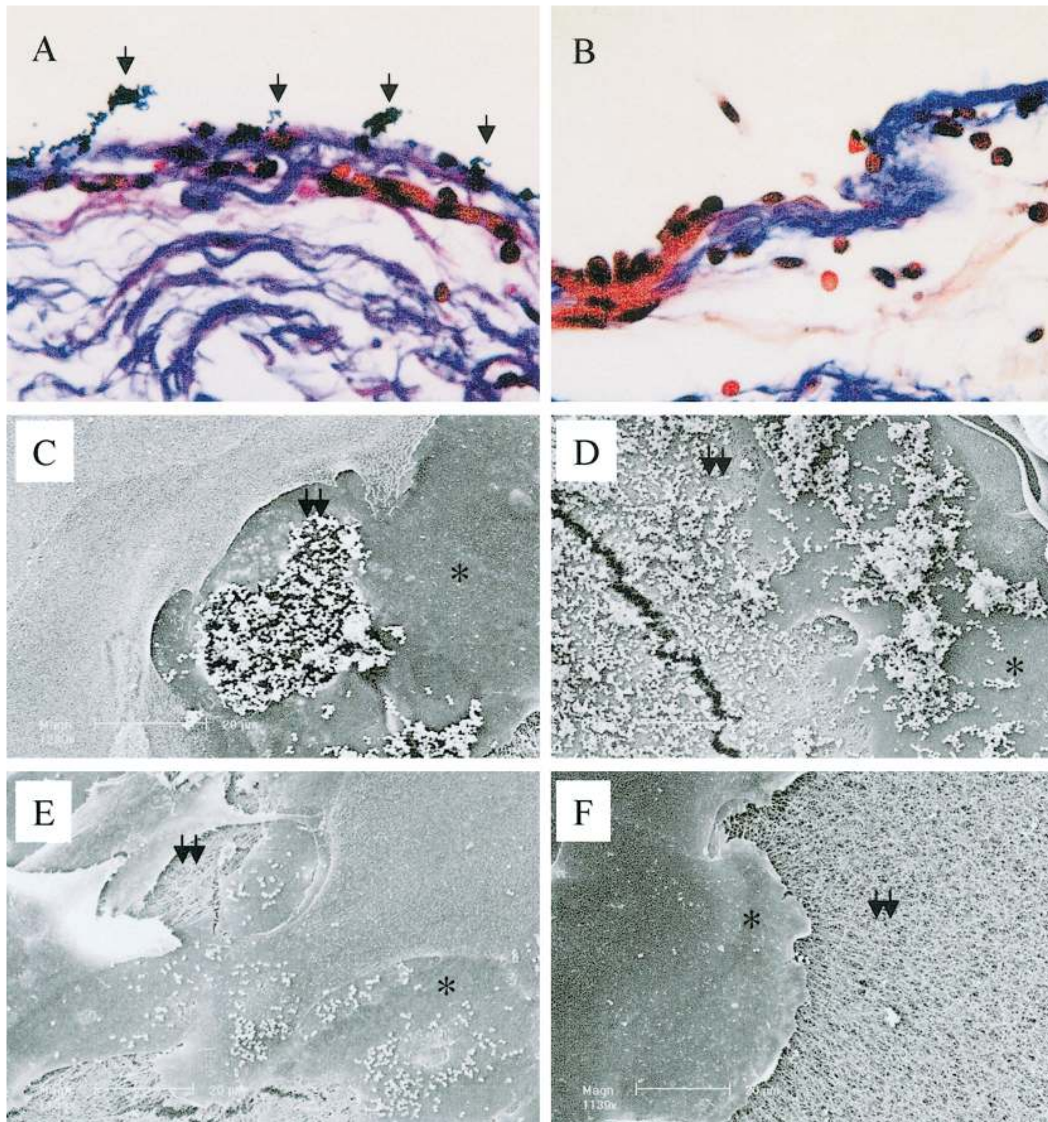

Figure 2. Panton-Valentine leukocidin (PVL)–positive *Staphylococcus aureus* (PPSA) and PVL-negative *S. aureus* (PNSA) strains, tested ex vivo and in vitro for their interaction with damaged human airway tissue (*A* and *B*) and with primary human airway epithelial cell (HAEC) cultures *(C–F).* Human bronchi damaged ex vivo were infected with PPSA strain A980557 *(A)* and PNSA strain LY19990333 *(B),* and cocci *(arrow)* were detected by Gram staining. PPSA strains A980557 *(C)* and LY19990053 *(F)* and PNSA strains LY19990604 *(D)* and LY19990084 *(E)* were further examined in a primary culture model of HAECs, for adherence to both HAECs *(asterisk)* and the underlying type I collagen matrix on which cells were seeded *(double arrow).*

the 2 proteins and 2 concentrations), whereas the presence of other genes, including the *eno* gene, did not correlate with their respective ligand-binding capacities, independent of the PVL analyzed (data not shown).

Interaction of* S. aureus *clinical isolates with human airway tissue and primary HAEC cultures damaged ex vivo and in vitro. One randomly selected strain from each group (PPSA strain A980557 and PNSA strain LY19990333) was first tested for behavior toward human bronchial tissue damaged ex vivo. Abundant gram-positive cocci were detected on damaged bronchial tissue infected with strain A980557 (figure 2*A*), especially on areas of exposed basement membrane, whereas few cocci were seen on damaged bronchial tissue infected with strain LY19990333 (figure 2*B*).

Four PPSA and 5 PNSA strains were then tested for adherence (semiquantified by count of bacteria by microscopy) to HAECs and to the collagen matrix on which the cells were seeded. As summarized in table 3, adherence of PPSA and PNSA strains to HAECs was similar. PPSA strains, compared with PNSA strains, displayed higher affinity for the type I collagen matrix. However, in accordance with ECM-binding experiments performed in 96-well microtiter plates coated with ECM components, PPSA strain A980557 (figure 2*C*) adhered strongly to the type I collagen matrix, whereas strain LY19990053 (figure

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Table 3. Adherence of *Staphylococcus aureus* to primary human airway epithelial cell (HAEC) cultures.

Origin of isolates	Adherence phenotype on HAECs	Affinity for type I collagen matrix
Necrotizing pneumonia isolates (PPSA)		
LY19990025	+	+++
A980557	++	+++
LY19990053	+/−	+/−
LY19991509	++	+++
Nonnecrotizing community-acquired pneumonia isolates (PNSA)		
LY19990604	++	+++
LY19990084	+	+/−
LY19990333	+	+/−
LY19990045	+	+
LY19990096	+/−	+/−

NOTE. Four Panton-Valentine leukocidin (PVL)–positive *Staphylococcus aureus* (PPSA) strains and 5 PVL-negative *S. aureus* (PNSA) strains, selected for their in vitro affinity for extracellular matrix proteins, were further examined for their adherence phenotype, in an HAEC culture model, by semiquantified count of bacteria by microscopy. Scanning electron microscopy determined affinity for HAECs and the underlying type I collagen matrix. +++, very strong affinity; ++, strong affinity; +, medium affinity; +/−, very low affinity.

2*F*) did not. PNSA strain LY19990604 adhered strongly to type I collagen matrix (figure 2*D*), whereas strain LY19990084 (figure 2*E*) adhered weakly.

Effect of PVL on human airway epithelium. Confluent 16HBE140 cell layers exposed to PPSA and PNSA strain supernatants were damaged to equal degrees after 3 h of contact (figure 3*A* and 3*B*, respectively), whereas diluted sterile bacterial growth medium had no effect (figure 3*C*). The presence of PVL in PPSA strain supernatants and its absence in PPSA strain supernatants were verified by Western blot, by use of monoclonal antibodies against LukF-PV (data not shown). Dose-kinetic analysis of PPSA or PNSA strain supernatants was performed and did not identify different possible thresholds of concentrations of bacterial overnight-culture supernatant causing epithelial disruption (data not shown). Cells layers exposed to PPSA or PNSA strain supernatants lost their cell-cell contacts, and the cell layers showed exposed basement membrane areas. Such alterations were not observed after the cells were exposed to recombinant LukF (figure 3*D*) or LukS alone (figure 3*E*) or in combination (figure 3*F*), compared with control cells (figure 3*G*), even after 24 h of exposure, suggesting that PVL itself was not responsible for damaging airway mucosa or exposing basement membrane. Last, we investigated a possible cytotoxic effect of staphylococcal supernatant by measurement of release of LDH by 16HBE140 cells exposed to PPSA and PNSA strain supernatants. No cytotoxic activity was observed when HAECs were exposed to PPSA and PNSA strain supernatants and to recombinant PVL.

DISCUSSION

In the absence of an appropriate animal model of necrotizing pneumonia, we used ex vivo and in vitro models based on HAECs and tissues to investigate how PVL-producing strains associate with the specific bronchial lesions observed during *S. aureus* necrotizing pneumonia. Our results show ex vivo that PPSA strains are strongly adherent to denuded areas of basement membrane in damaged bronchi and that this behavior can be attributed to strong specific binding to type I and IV collagens and laminin. The presence of the *cna* gene was significantly associated with collagen binding and, therefore, with PPSA strains, whereas the presence of the *eno* gene did not correlate with high binding of PPSA strains to laminin. Interestingly, staphylococcal supernatants disrupted the integrity of the airway epithelium, even though recombinant PVL alone did not seem to be directly responsible for this damage. It appears that, compared with PNSA strains, PPSA strains possess additional genetic material, including the *lukS-PV lukF-PV* and *cna* genes, that contributes to high virulence during necrotizing pneumonia; however, PVL itself is not responsible for damaging airway mucosa and exposing basement membrane.

Necrotizing pneumonia is preceded by an influenza-like syndrome [6, 9, 10], possibly due to viral infection associated with desquamation of bronchial ciliated cells [20]. Since *S. aureus* does not adhere to intact airway epithelium but only to damaged or remodeled epithelium [21], viral infection could be a key step in necrotizing pneumonia. We mimicked such bronchial injury by physical damage of human bronchi explants before incubation with *S. aureus* strains [22]. Both PPSA and PNSA strains adhered, but only PPSA strains adhered to histological lesions in a manner similar to that observed on necropsy specimens from patients with necrotizing pneumonia [6], in particular in areas of denuded basement membrane.

To investigate the molecular mechanisms of adherence of PPSA strains to damaged bronchial tissue, we used SEM to study the binding affinity for primary HAEC cultures seeded on type I collagen. Adherence of PPSA and PNSA strains to epithelial cells was not clearly different, but PPSA strains differed from PNSA strains by their strong binding to denuded areas of type I collagen.

Fibronectin-binding proteins, but not collagen-binding proteins, are thought to account for the ability of *S. aureus* to adhere to human airway epithelium [23]. Interestingly, we found that the fibronectin-binding capacities of the 2 groups of strains were similar, suggesting that, in necrotizing pneumonia, this FnB-dependent binding capacity does not account for specific traits of the pathological abnormality. PPSA strains showed a higher affinity for type I and IV collagens and laminin than did PNSA strains. Moreover, the *cna* gene was significantly associated with PPSA strains, and its presence correlated with avid binding to type I and IV collagens. The collagen-binding

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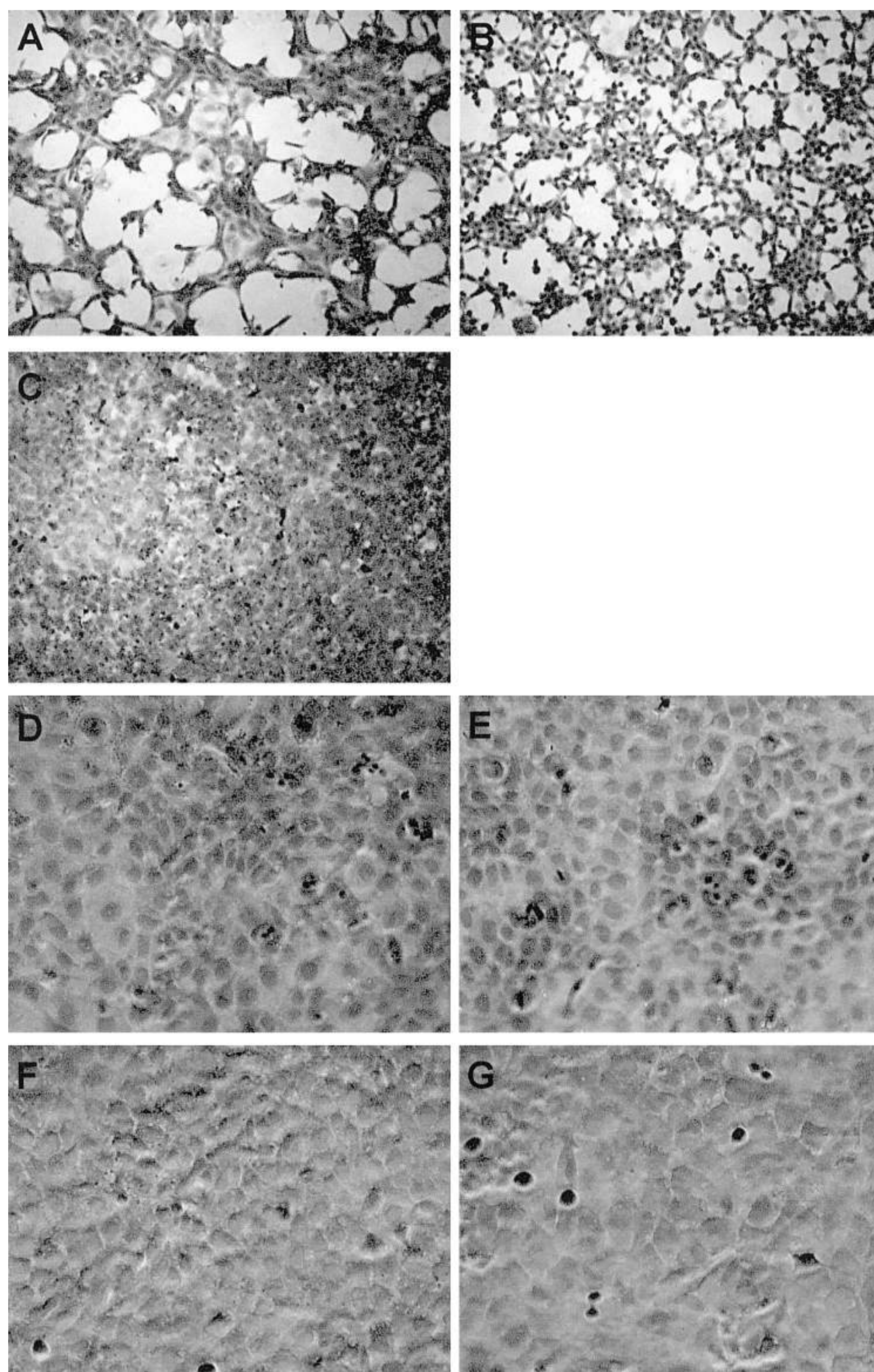

Figure 3. Contribution of extracellular products (including Panton-Valentine leukocidin [PVL]) from strains of both groups (PVL-positive *Staphylococcus aureus* [PPSA] and PVL-negative *S. aureus* [PNSA]) to airway epithelial injury. 16HBE140 confluent cell layers were incubated with PPSA *(A)* and PNSA *(B)* strain supernatants, compared with control cell cultures *(C)* exposed to diluted sterile bacterial growth medium after 3 h, and with LukF *(D)* and LukS alone *(E)* and in combination *(F)*, compared with control cells *(G)* exposed for up to 24 h. Loss of the integrity of the airway epithelium (appearance of holes in the cell layer) (*arrows*) was monitored visually.

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activity of *S. aureus* isolates and the presence of the *cna* gene have been associated with only osteomyelitis and arthritis [19, 24]. In addition to the common *S. aureus* core genome, necrotizing pneumonia–associated *S. aureus* appear to have acquired specific pathogenic and epidemic traits, among which the *lukS-PV lukF-PV* and *cna* genes may play an important role. Other PPSA strains associated with furunculosis exhibited neither such ECM binding nor the *cna* gene (data not shown). Conversely, the specific binding of PPSA strains to laminin did not correlate with the presence of the *eno* gene, although the *eno* gene may be differentially regulated in PPSA strains, compared with PNSA strains. From our results, it appears that the *eno* gene is specifically switched on during necrotizing pneumonia. For other MSCRAMM genes, we observed several discrepancies between ECM-binding behavior and the presence of the corresponding MSCRAMM gene (nondetection of the MSCRAMM gene but ECM-binding ability) (data not shown), suggesting that these genes, including the *fnbp* genes, are sufficiently genetically divergent to not be detected by PCR or that other, not-yet-identified MSCRAMM genes account for these ECM-binding properties.

Finally, we examined the possibility that PVL itself contributes to human airway epithelium damage, with which PPSA strains are clearly associated. Although our recombinant PVL was previously shown to be cytotoxic for human leukocytes [14], no PVL-dependent cytotoxicity or alteration in cell confluency was observed on a human bronchial cell line, independent of the concentration or contact time applied. This is in agreement with our hypothesis that the initial lesions in *S. aureus* necrotizing pneumonia are caused by viral infection [6, 9, 10]. In contrast, crude supernatants from both PPSA and PNSA strains induced alteration of cell-cell contact in confluent layers of our human bronchial cell line, pointing to a role for other, non-PVL exoproteins. For instance, protease and α-toxin, which is cytotoxic for epithelial cells [25, 26] and is produced by most *S. aureus* isolates ([27] and data not shown), could be responsible for the airway epithelial alteration observed. Expression of these proteins, like that of most *S. aureus* exoproteins (including toxins such as PVL and exoenzynes), is controlled by a quorum-sensing system called *agr* (accessory gene regulator), which induces their gene expression at high cell density [28]. In necrotizing pneumonia, the high affinity of PPSA strains for type I and IV collagens and laminin may contribute to adherence of numerous cocci to damaged airway epithelium, thereby triggering *agr* activation through the quorum-sensing mechanism and induction of toxin production. Among these toxins, PVL may play a role in extending tissue damage and altering PMN, both of which constitute a key element of the immune response. Finally, host susceptibility is certainly involved in the outcome of PPSA infection, and polymorphism in Toll-like receptor 2 and MyD88 are putative candidates, since they clearly contribute to host susceptibility to *S. aureus* infection in experimental models [29].

In conclusion, our results strongly suggest that necrotizing pneumonia–associated *S. aureus* strains possess a strong ability for formation of biofilm to ECM components, in particular to type I and IV collagens and laminin, which either previous viral infection or non-PVL exoproducts may expose. These properties may lead, via *agr*-dependent production of PVL, to a dramatically altered immune response resulting in the patient's rapid deterioration and death, as is generally observed for PPSA infection, although not for PNSA infection [6].

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