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PavA of *Streptococcus pneumoniae* Modulates Adherence, Invasion, and Meningeal Inflammation

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Pneumococcal adherence and virulence factor A (PavA) is displayed to the cell outer surface of *Streptococcus pneumoniae* and mediates pneumococcal binding to immobilized fibronectin. PavA, which lacks a typical gram-positive signal sequence and cell surface anchorage motif, is essential for pneumococcal virulence in a mouse infection model of septicemia. In this report the impact of PavA on pneumococcal adhesion to and invasion of eukaryotic cells and on experimental pneumococcal meningitis was investigated. In the experimental mouse meningitis model, the virulence of the *pavA* knockout mutant of *S. pneumoniae* D39, which did not show alterations of subcellular structures as indicated by electron microscopic studies, was strongly decreased. Pneumococcal strains deficient in PavA showed substantially reduced adherence to and internalization of epithelial cell lines A549 and HEp-2. Similar results were obtained with human brain-derived microvascular endothelial cells and human umbilical vein-derived endothelial cells. Attachment and internalization of pneumococci were not significantly affected by preincubation or cocultivations of pneumococci with anti-PavA antisera. Pneumococcal adherence was also not significantly affected by the addition of PavA protein. Complementation of the *pavA* knockout strain with exogenously added PavA polypeptide did not restore adherence of the mutant. These data suggest that PavA affects pneumococcal colonization by modulating expression or function of important virulence determinants of *S. pneumoniae*.

Streptococcus pneumoniae is a natural resident of the upper and lower respiratory tracts of humans (2). Pneumococci are the most frequent causative agent of community-acquired pneumonia and a leading cause of otitis media in children, bacteremia, and bacterial meningitis (11). Pneumococci bind to and invade cells of the epithelium and endothelium. From the bloodstream, pneumococci can penetrate the vascular cell layer of the blood-brain and blood-cerebrospinal fluid barriers, enter the cerebrospinal fluid, and produce meningitis by subarachnoid bacterial growth (34, 40, 54). Pneumococcal adherence involves the recognition of host cell receptor glycoconjugates (16), but except for SpsA (also referred to as CbpA and PspC), the bacterial adhesins have not been identified so far. The choline-binding protein SpsA mediates pneumococcal adherence to and invasion of mucosal epithelial cells by a human-specific interaction with the polymeric immunoglobulin receptor (pIgR) (21, 27, 59). PspC and the PspC-like Hic protein have been shown to bind the complement factor H (18, 32). Binding of proteins of the extracellular matrix and serum has been shown to contribute to pneumococcal pathogenesis. The PspA protein binds lactoferrin and inhibits deposition of C3b onto cells, thereby inhibiting complement activation (26, 53). The α-enolase of *S. pneumoniae* has been shown to recruit plasmin(ogen) to the bacterial cell surface, which provides proteolytic activity to the cell surface and enhances the virulence potential (4, 5).

Pneumococci also bind to the immobilized form of fibronectin (55). The PavA protein, which shows 69.6% and 79.1% identities to the fibronectin-binding proteins FBP54 of *Streptococcus pyogenes* and FbpA of *Streptococcus gordonii*, respectively, was identified as a pneumococcal adhesin for fibronectin. However, isogenic *pavA* mutants were not devoid of fibronectin binding and retained approximately 50% of wild-type binding activity to fibronectin (30). This suggests that PavA is not the sole fibronectin-binding protein expressed by *S. pneumoniae*. Despite the lack of a signal sequence required for protein export via the general secretory pathway and the lack of a typical LPXTG anchorage motif (22), PavA was localized to the outer cell surface of *Streptococcus pneumoniae* (30). Other proteins of streptococci that also lack these motifs and are nevertheless associated with the outer surface include, e.g., FBP54 (14), streptococcal surface dehydrogenase (43), surface enolase of *S. pyogenes* (44), and the pneumococcal α-enolase (4). These proteins, therefore, constitute a novel class of surface proteins of gram-positive bacteria (12).

In addition to its function as a fibronectin-binding protein, PavA was also identified as a virulence factor and therefore designated pneumococcal adhesion and virulence factor (30). Although the expression of major virulence determinants such as the polysaccharide capsule, pneumolysin, PsaA, and PspA,

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as well as other phenotypic properties, was not affected in *pavA* mutants, these mutants were massively attenuated in the mouse sepsis model (30). PavA was also independently identified as a virulence determinant in pneumococcal infection by signature mutagenesis experiments (35). PavA-deficient strains were attenuated in pneumonia and sepsis models of infection (30, 35). These results suggested that PavA is involved in pneumococcal pathogenesis. In this study, we have elucidated the impact of PavA on adherence and invasion and in a mouse model of bacterial meningitis. Intracranial infection of mice with *pavA* mutants resulted in failure of physical impairment of mice and clearance of bacteria from the central nervous system, indicating the crucial impact of PavA also for survival of pneumococci in the physiologically immunocompromised central nervous system. We therefore addressed the question of whether the attenuation of *pavA* mutants is due to an impairment of adherence. Cell culture infection experiments indicated that adherence of isogenic mutants in which the *pavA* gene was inactivated was strongly reduced.

MATERIALS AND METHODS

Bacterial strains and isogenic mutants. *Streptococcus pneumoniae* was cultured in Todd-Hewitt broth (Oxoid, Basingstoke, England) supplemented with 0.5% yeast extract (THY) to mid-log phase or grown on blood agar. *S. pneumoniae* D39 (Cps^+ type 2), R800 (Cps^-, derived from R36A), NCTC 10319 (Cps^+ type 35A), and corresponding isogenic *pavA* mutants, designated UB1341 for the D39Δ*pavA* mutant and UB1339 for the R800Δ*pavA* mutant, were used in this study (30). The *pavA* mutant of type 35A was generated by transformation of the parental strain with plasmid pMSH14 and designated SPMU65. The pMSH14 plasmid was constructed by ligation of the ApaI-linearized and blunt-ended *pavA* gene, which was formerly cloned in pQE30 (30), with a blunt-ended spectinomycin gene cassette. To construct a complemented *pavA* knockout strain, SPMU65*pavA*$^+$, the entire *pavA* gene (30) cloned in pJDC9 (pJDC9::*pavA*$^+$) was transformed into SPMU65. A wild-type serotype 35A strain transformed with pJDC9::*pavA*$^+$ was used as a control strain. In order to avoid pneumolysin-induced damaging of the cells in extended infections, pneumolysin-negative PavA-expressing strains as well as pneumolysin-negative, PavA-negative strain type 35A were generated by transformation with pJDC9::*ply* (kindly provided by G. Zysk, Düsseldorf, Germany). Functional inactivity was confirmed by the hemolysis assay as described previously (3).

Mouse meningitis model and histopathology. Female C57BL/6 mice (weight, 26 to 37 g; age, 5 to 7 months) were obtained from the Central Animal Care Facility of the Georg-August-University, Göttingen. In order to evaluate the impact of the *pavA* deficiency on mortality in pneumococcal meningitis, mice were infected by injection of 10 μl of 0.9% NaCl containing 10^6 CFU of *S. pneumoniae* D39 or the *pavA* knockout mutant UB1341 of D39 in the right frontal lobe ($n = 9$ for each group). The effect of PavA on physical impairment and meningeal inflammation was evaluated by injection of the lower dose of 10^4 CFU of *S. pneumoniae* D39 or the isogenic *pavA* knockout mutant UB1341 of D39 into the right frontal lobe ($n = 6$ for each group). Physical impairment due to meningitis was assessed by repeated tightrope tests before and 12, 24, and 36 h after infection as described previously (24). At 36 h after infection animals were killed, brains were removed and cortical hemispheres were fixed in 4% paraformaldehyde. Serial dilutions of blood, cerebellum, and spleen homogenates were plated on blood agar plates. Hematoxylin-eosin staining of paraformaldehyde-fixed coronal brain sections was performed to evaluate meningeal inflammation and encephalitic involvement. The degree of meningeal inflammation was estimated by the number of granulocytes in one high-power field (40× objective), with the following scale: no granulocytes, 0; <10 granulocytes, 1; 10 to 50 granulocytes, 2; >50 granulocytes, 3. The following areas were scored: frontal interhemispheric region, hippocampal fissure (both sides), three superficial meningeal regions over the convexities, and the third ventricle. The scores of the individual regions were added (24). The experiments were approved by the Animal Care Committee of the Georg-August-University Göttingen and by the District Government of Braunschweig, Lower Saxony, Germany.

Lysine-acetate-based formaldehyde/glutaraldehyde ruthenium red-osmium fixation procedure for transmission electron microscopy. Wild-type and mutant strains of *S. pneumoniae* were first fixed with 2% formaldehyde and 2.5% glutaraldehyde in cacodylate buffer containing 0.075% ruthenium red and 0.075 M lysine-acetate for 20 min on ice. After washing with cacodylate buffer containing 0.075% ruthenium red, samples were fixed a second time with 2% formaldehyde and 2.5% glutaraldehyde in cacodylate buffer with 0.075% ruthenium red for 3 h, washed again, and further fixed with 1% osmium in ruthenium red-containing cacodylate buffer for 1 h at room temperature. Subsequently, samples were washed several times with ruthenium red-cacodylate buffer and dehydrated with a graded series of acetone solutions. Samples were infiltrated with the acrylic resin LRWhite by applying 1 part 100% ethanol and 1 part LRWhite for 2 h on ice and then 1 part ethanol and 2 parts LRWhite overnight on ice. Pure resin was added and left for 8 h on ice, changed, and left overnight. Finally, samples were placed in gelatin capsules and filled with pure LRWhite resin at room temperature. LRWhite resin was polymerized for 48 h at 60°C. Ultrathin sections were cut and counterstained for 5 min with 4% aqueous uranyl acetate, and air-dried samples were examined in a Zeiss EM 910 transmission electron microscope at an acceleration voltage of 80 kV.

Cell lines and cell culture. Pneumococcal attachment to and internalization in epithelial and endothelial cells, respectively, was examined by using the following cell lines. Human lung alveolar carcinoma epithelial cell line A549 (ATCC CCL-185), a HEp-2 larynx carcinoma cell line (ATCC CCL-23), Calu-3 cells (human lung epithelium; ATCC HTB-55), and Madin-Darby canine kidney epithelial cells, stably transfected with cDNA coding for the human pIgR (MDCK-hpIgR) (52), were cultured as described previously (21, 38). Human brain-derived microvascular endothelial cells (HBMEC) (49) were cultured in RPMI 1640-based medium supplemented with 10% fetal calf serum, 10% Nu-Serum IV (Becton Dickinson), 1% nonessential amino acids, 1% minimal essential medium vitamins (Gibco), 1 mM sodium pyruvate, 2 mM glutamine, penicillin (100 U/ml), and streptomycin (0.1 mg/ml). Human umbilical vein-derived endothelial cells (HUVEC) (Clonetics) were cultured in EBM-2 medium (Clonetics) supplemented with EGM-2 supplement kit (Clonetics). All cells were cultured at 37°C in 5% CO_2. Equivalent amounts of the cells (with or without serum starvation) were analyzed by immunoblotting with antifibronectin antibodies (DakoCytomation, Denmark), or cell surface-bound fibronectin was analyzed by enzyme-linked immunosorbent assay. HUVEC produced fibronectin, and low levels of fibronectin production were indicated for A549. HEp-2 cells and HBMEC showed no detectable levels of fibronectin (50) (data not shown).

Adherence and internalization assays. Pneumococcal adherence and invasion studies were performed in 24-well tissue culture plates (Greiner, Germany) as described previously (21). Briefly, 48 h prior to infection cells were seeded on glass coverslips (diameter, 12 mm) at a density of 5×10^4 cells per well. Confluent cell layers with approximately 2×10^5 cells were washed, prior to infection, with Dulbecco's modified Eagle's medium containing HEPES (PAA Laboratories, Coelbe, Germany) plus 1% fetal calf serum and then inoculated in a standardized assay with 10^7 pneumococci previously grown in THY medium to an optical density at 600 nm of 0.3 to 0.4. After 4 h of incubation at 37°C in 5% CO_2, unbound bacteria were removed from the cells by rinsing several times with phosphate-buffered saline (PBS), fixed, and stained for immunofluorescence microscopy. The number of viable intracellular pneumococci was quantified by antibiotic protection assay. Confluent monolayers were infected as described above. The number of CFU inoculated per well was determined by serial platings on blood agar plates. After 4 h of infection the cells were washed several times with PBS to remove unbound bacteria, and 1 ml of DMEM with HEPES containing 100 μg gentamicin and 100 units penicillin G was added per well. The plates were incubated for 1 h at 37°C to kill extracellular bacteria. The intracellular pneumococci were recovered after washing by a saponin-mediated lysis (1% wt/vol) of the cells and plated on blood agar plates. The number of surviving bacteria per well was determined. In parallel assays, the adherent and invasive bacteria were plated on blood agar plates. The number of invasive bacteria was subtracted from the total number of CFU, resulting in the number of adherent bacteria. In blocking experiments, adherence and invasion were performed in the presence of various amounts of recombinant PavA and C-terminally truncated 42-kDa PavA (1 μg, 10 μg, and 20 μg), or bacteria were preincubated with a 1:5 dilution (approximately 0.43 mg) of polyclonal anti-PavA IgG antibodies prior to infection (30). Infections were also carried out in the presence of increasing or decreasing concentrations of antibodies, with identical results.

Fluorescence microscopy. To visualize the number of adherent and invasive pneumococci by double immunofluorescence, cells and bacteria were fixed on glass coverslips with 1% paraformaldehyde. Extracellular bacteria attaching to cells were incubated for 45 min with rabbit antipneumococcal antiserum, followed by 30 min of incubation with an Alexa Fluor 488-labeled goat anti-rabbit Ig (MoBiTec). After permeabilization of the cells with 0.1% Triton X-100 for 5 min, intracellular and extracellular pneumococci were incubated with antipneumococcal antibody, followed by staining with Alexa Fluor 568-labeled goat anti-

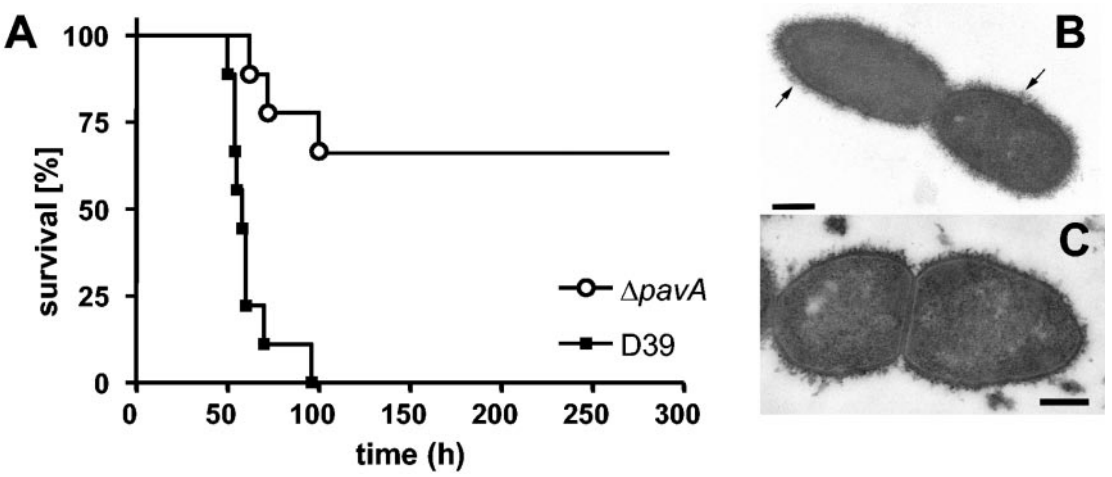

FIG. 1. Kaplan-Meier survival analysis of mice (A) infected with 10^6 CFU *S. pneumoniae* D39 or the *pavA* knockout mutant UB1341 (log rank test, $P = 0.008$). Transmission electron photographs of *S. pneumoniae* D39 (B) and isogenic *pavA* mutant UB1341 (C). Ultrathin sections show that the cell morphology and the expression of the capsule (arrows) of the attenuated mutant are comparable to those of the wild-type strain D39. Bars represent 0.25 μm.

rabbit Ig (MoBiTec). Each experiment was repeated at least three times, and results were expressed as mean ± standard deviation (SD).

Recombinant PavA proteins and anti-PavA antisera. Expression of recombinant PavA proteins was conducted as described previously (30). Purification of His_6-tagged PavA proteins was performed by affinity chromatography on Ni-nitrilotriacetic acid resins according to the protocol of the manufacturer (Qiagen). Deletion of a C-terminal part of PavA resulted in PavA 42-kDa polypeptide (PavA42). In *S. pneumoniae*, C-terminal truncation of PavA protein is sufficient to attenuate virulence in the mouse sepsis model (30). Polyclonal anti-PavA42 antiserum against the 42-kDa His-tagged PavA was raised in rabbits by routine immunogenic procedures (Eurogentec). Purification of anti-PavA antiserum and anti-PavA42 antiserum was performed by preabsorption against *Escherichia coli* lysate and by protein A-Sepharose 4B affinity chromatography (Amersham Pharmacia Biotech). Concentrations of the antibodies were 1.2 mg/ml, 2.3 mg/ml, and 2.14 mg/ml for preimmune IgG, anti-PavA IgG, and anti-PavA42 IgG, respectively. Dose-dependent binding of anti-PavA and anti-PavA42 antisera, respectively, to the surface of live pneumococci expressing PavA was indicated by flow cytometry (data not shown). Briefly, pneumococcal cells incubated with antibodies (approximately 0.4 mg, 0.2 mg, 0.1 mg, and 0.05 mg) for 30 min at 37°C were washed several times with PBS, followed by incubation with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit Ig (Dianova). The cells were washed and suspended in PBS for analysis using a FACSCalibur (Becton Dickinson). Flow cytometry revealed that the amounts of antisera used were sufficient for blocking experiments.

Binding of pneumococci to immobilized fibronectin. A 2.0-μg amount of fibronectin (ICN) per well was applied to a 96-well microtiter plate (polystyrene surface) at 4°C overnight. The surfaces of the wells were subsequently blocked with 1% bovine serum albumin for at least 3 h at room temperature. Labeling of the bacteria with FITC was performed as described previously (5). Extensively washed FITC-labeled pneumococci (2.5×10^7) were added to the wells and incubated for 1 h at 37°C for binding. In blocking experiments pneumococci were pretreated for 20 min at 37°C with anti-PavA antibodies. Protein A-purified antibodies were used in different amounts (approximately 0.2 mg, 0.1 mg, and 0.05 mg), and pneumococci were washed prior to incubation with fibronectin to remove unbound antibodies. Fluorescence was measured at 485 nm/538 nm (excitation/emission) using a Fluoroskan Ascent (ThermoLabsystems).

Statistical analysis. Data were expressed as mean ± SD, and differences in adherence and invasion were analyzed by the two-tailed unpaired Student *t* test. Nonparametric data from the tightrope score and meningeal inflammation score were compared by the two-tailed Mann-Whitney U test. A *P* value of <0.05 was considered statistically significant.

RESULTS

Impact of PavA on pneumococcal meningitis in a mouse meningitis model. In mice infected with the *pavA* knockout mutant UB1341, mortality was substantially lower than that in control animals infected with wild-type *S. pneumoniae* D39. A fatal outcome was observed only in three (33%) of nine mice infected with the *pavA*-deficient strain, whereas nine mice (100%) died within 4 days after infection with D39 (Fisher's exact test, $P = 0.009$; log rank test, $P = 0.008$) (Fig. 1A). Surviving mice fully recovered from infection and showed no impairment of physical activity. The impact of PavA on physical activity and meningeal inflammation was assessed by injection of mice with doses of 10^4 CFU *S. pneumoniae*. Mice infected with the isogenic *pavA* mutant UB1341 showed less impairment of physical activity in comparison to mice infected with wild-type D39 ($P = 0.04$); the median (25th/75th quartiles) scores in the tightrope test 36 h after infection were 2 (1/3.5) in mice infected with the PavA-deficient mutant versus 12 (2.5/20) in control animals infected with the wild-type D39 strain. With the lower inoculum of 10^4 CFU, the *pavA* mutant UB1341 did not grow in mice after intracerebral injection; all mice infected with UB1341 had no detectable bacterial titers in cerebellum, spleen, and blood at 36 h after infection (detection limit, 2 log CFU), whereas bacterial titers in mice infected with the wild-type D39 rose up to 6.5 ± 0.6 log CFU in cerebellum, 6.4 ± 1 log CFU in spleen, and 6.5 ± 0.6 log CFU in blood (mean ± SD) ($P < 0.001$). Similarly, the meningeal inflammation score was higher in mice infected with wild-type D39 than in animals infected with the *pavA*-deficient strain ($P = 0.007$); the median (25th/75th quartile) scores were 11 (5.5/12) versus 1.5 (1/2.5).

Transmission electron microscopic comparison of wild-type and *pavA* mutant *S. pneumoniae*. The cell morphology and subcellular structures were visualized by transmission electron microscopy. Isogenic *pavA* mutants of encapsulated *S. pneumoniae* D39 showed no alterations in cell morphology (Fig. 1B and C). Moreover, photographs of ultrathin sections revealed that capsule expression of *pavA* mutants of D39 serotype 2 (UB1341) and pneumococcal serotype 35A was not affected, as depicted for UB1341 in Fig. 1.

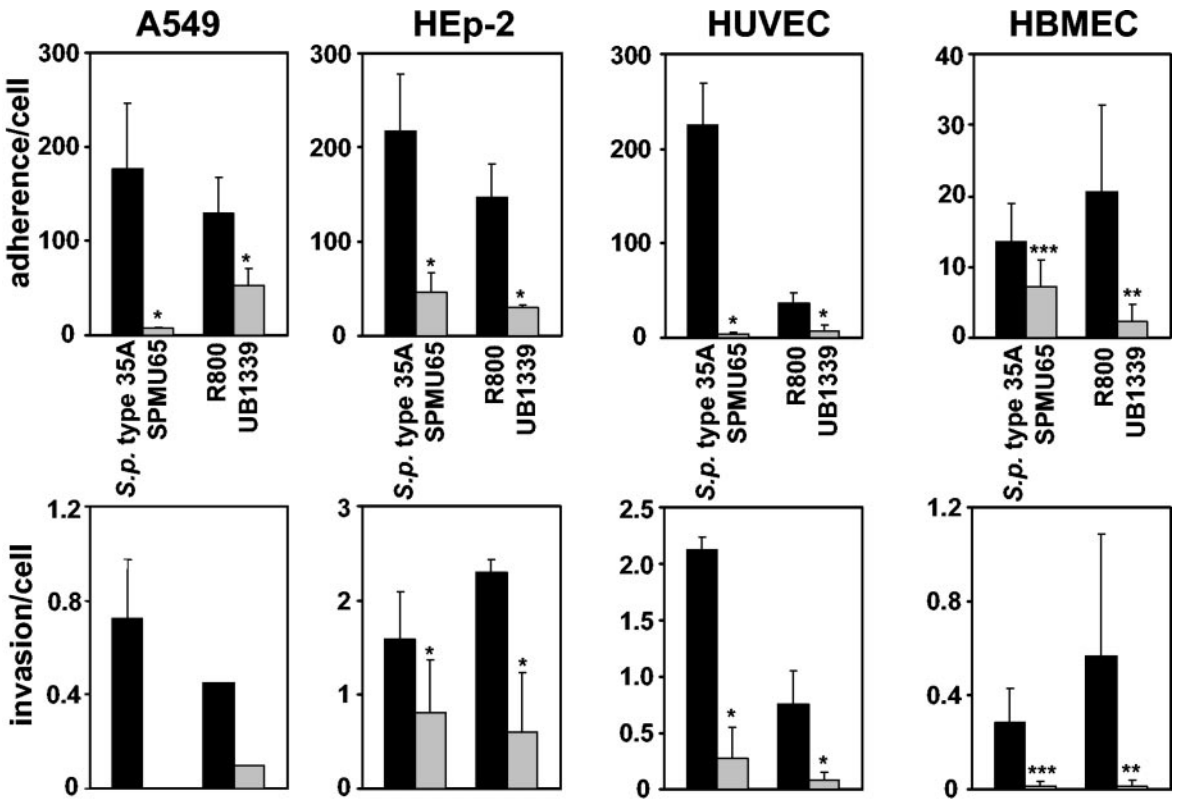

FIG. 2. Infection of human epithelial and endothelial cells depends on PavA expression. *S. pneumoniae* type 35AΔ*ply* (NCTC 10319) and R800 or their isogenic *pavA* mutants SPMU65Δ*ply* and UB1339 were examined for adherence and invasion. Pneumococcal infection experiments with epithelial cell lines A549 and HEp-2 and endothelial cells HBMEC and HUVEC were conducted for 4 h at 37°C in 5% CO_2. Wild-type pneumococci (black bars) and PavA-negative mutants (gray bars) were made devoid of pneumolysin in order to avoid destruction of the monolayer in the in vitro infection system. Scoring the number of adherent and invasive bacteria indicated that adhesion and invasion are substantially reduced for *S. pneumoniae* devoid of PavA. Results are presented as the means ± standard deviations for at least three independent experiments. *, $P < 0.02$ (A549 and HEp-2) or $P < 0.003$ (HUVEC); **, $P < 0.03$; ***, $P < 0.085$ (all with respect to the wild type).

Effect of PavA on adhesion of pneumococci to epithelial and endothelial cells. In order to elucidate whether PavA affects virulence through the role of PavA during the initial infectious processes, *pavA* knockout pneumococci were assayed for their ability to adhere to and invade eukaryotic cells derived from different host tissues. Adhesion and invasion of the *pavA*-deficient encapsulated type 35A (SPMU65) and unencapsulated R800 (UB1339) were scored for the epithelial lung alveolar cell line A549 and larynx carcinoma epithelial cell line HEp-2. To avoid pneumolysin-induced disruption of epithelial and endothelial monolayers (28, 29, 33, 60), for long-term experiments wild-type and *pavA* mutant strains that were devoid of pneumolysin were generated. Inactivation of the gene encoding pneumolysin protects the cells against cytolytic and cytotoxic effects of pneumolysin when infections are carried out for an extended period (up to 4 h). The adherence and invasion of parental strains ($PavA^+$) and corresponding pneumolysin-negative but PavA-positive mutants were not affected as indicated by immunofluorescence (data not shown). In contrast, inactivation of the gene encoding PavA resulted in a substantial reduction of adherence and reduced the number of internalized pneumococci compared to the adherence and invasion of the corresponding parental strains (Fig. 2 and 3). Cell culture infection experiments were also conducted with endothelial cell lines such as HUVEC and HBMEC, which represent a model for the transition of the blood-brain barrier (49). In accordance with the results obtained for the epithelial cells, *pavA* knockout pneumococci were also reduced in their ability to attach to endothelial cells. Similarly, the number of internalized *pavA* knockout bacteria was reduced (Fig. 2 and 3). To confirm that changes in adherence to host cells were due to the mutation in *pavA*, SPMU65 was complemented with plasmid DNA carrying the entire *pavA* gene. Reintroduction of the *pavA* gene in the mutant SPMU65 restored adhesion levels (Table 1). To confirm the effects of PavA on adherence and invasion, *S. pneumoniae* type 35 and its isogenic *pavA* mutant SPMU65 were selected for quantitative assessment of internalization by using a standard antibiotic protection assay. The intracellular survival of the bacteria was determined. Comparison of the percentages of internalized SPMU65 and wild-type bacteria revealed that the numbers of internalized SPMU65 in A549 cells, HEp-2 cells, HBMEC, and HUVEC were reduced to 3.4%, 43.3%, 4.79%, and 55.9%, respectively, of the numbers of the *S. pneumoniae* type 35A strain (Fig. 4). These data are in accordance with the reduced levels of internalization of SPMU65 determined by double immunofluorescence (Fig. 2). The results revealed significantly decreased invasion of the *pavA* mutant. However, the degree of reduction (calculated as

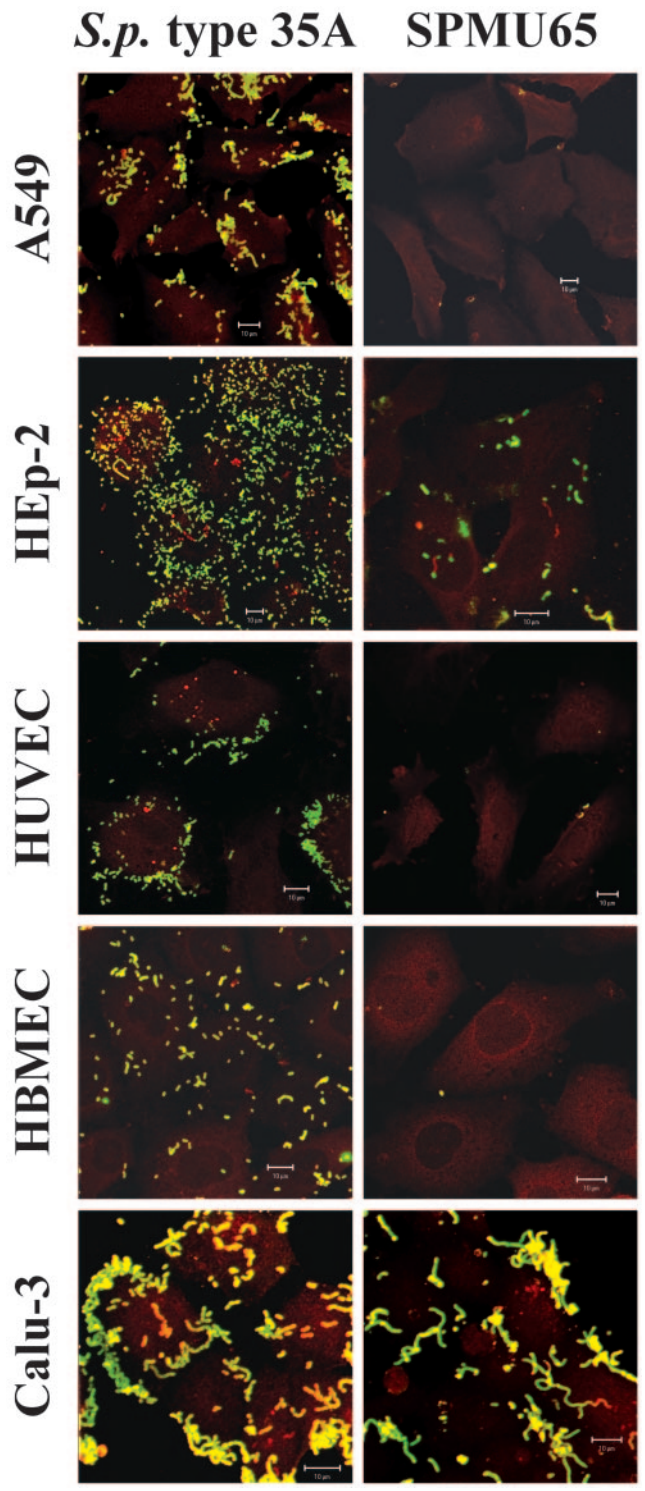

FIG. 3. Immunofluorescence microscopy of adherent (green/yellow) and invasive (red) pneumococci. After infection of the eukaryotic cells with *S. pneumoniae*, here representatively shown for serotype 35AΔ*ply* (NCTC 10319) and SPMU65Δ*ply*, the extracellular bacteria were stained with a polyclonal antibody against pneumococci followed by an Alexa Fluor 488-labeled antibody (green). Intra- and extracellular bacteria were stained red after permeabilization of the cells by incubation with antipneumococcal antibody followed by staining with Alexa Fluor 568-labeled antibody. Adherence (yellow [green plus red]) and invasion (red) of epithelial and endothelial cells by *pavA* mutants were substantially reduced, expect for the pIgR-expressing Calu-3 lung epithelial cells.

TABLE 1. Adhesion levels of *S. pneumoniae* wild-type serotype 35A, isogenic *pavA* mutant SPMU65, control mutant *S. pneumoniae* serotype 35A pJDC9::*pavA*$^+$, and complemented mutant SPMU65 pJDC9::*pavA*$^+$

Strain	No. of bacteria (10^5) attached to HEp-2 cells ± SD (n = 3)[a]
Type 35A wild type	140 ± 31
Type 35A pJDC9::*pavA*$^+$	118 ± 40
SPMU65	42 ± 11
SPMU65 pJDC9::*pavA*$^+$	108 ± 44

[a] The number of adherent bacteria was determined by immunofluorescence microscopy.

a percentage) was equivalent to the decreased adherence of the mutant. The adherence of SPMU65 (A549, 3.8%; HEp-2, 21.4%; HBMEC, 53.4%; HUVEC, 1.5%) was equally reduced or, with the exception of HBMEC, was even more influenced than uptake. In conclusion, these results suggested a pivotal role of PavA in pneumococcal adherence but probably not invasion, which is cell type independent and therefore represents a non-tissue-specific mechanism.

Influence of PavA protein and anti-PavA antisera on pneumococcal adherence and invasion. To determine whether PavA protein directly influences the infection by its function as adhesin, the effects of purified PavA and anti-PavA antisera on pneumococcal adherence were examined. First, PavA antibodies, generated against full-length PavA, and anti-PavA42, generated against C-terminally truncated PavA protein with a molecular mass of 42 kDa and negative for fibronectin binding, were assessed for their ability to block binding of pneumococci to immobilized fibronectin. *S. pneumoniae* type 35A was pretreated with different amounts of protein A-purified preimmune serum, anti-PavA antiserum, and anti-PavA42 antiserum. Binding of pneumococci to immobilized fibronectin was inhibited in a dose-dependent manner by PavA antibodies. In contrast, preimmune serum and anti-PavA42 did not significantly influence binding (Fig. 5). Human epithelial or endo-

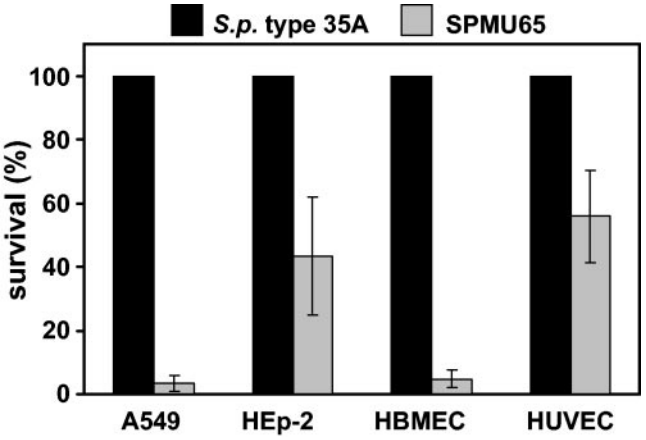

FIG. 4. Intracellular survival of *S. pneumoniae* in eukaryotic cells. Invasion und survival were studied by antibiotic protection assay, and the number of intracellular surviving bacteria of the *pavA* mutant (grey bars) was expressed as percent survival (percent recovered CFU per well). Invasion of the wild type (black bars) was defined as 100%. Error bars indicate standard deviations.

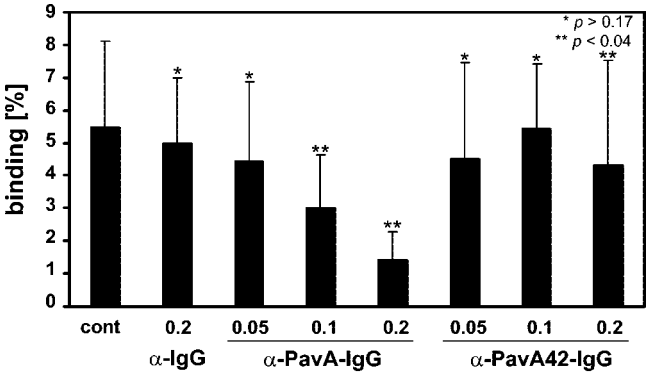

FIG. 5. Inhibition of *S. pneumoniae* binding to immobilized fibronectin by anti-PavA antiserum. FITC-labeled *S. pneumoniae* type 35A was added to wells coated with 2.0 μg fibronectin, and fluorescence was measured after 1 h at 485 nm/538 nm (100%). Specific binding was measured after removal of unbound bacteria. The numbers of adherent bacteria were calculated and are means ± SDs of triplicates from three independent experiments. To demonstrate the effect of anti-PavA antisera, *S. pneumoniae* was preincubated with 0.05 mg, 0.1 mg, or 0.2 mg of protein A-purified anti-PavA or anti-PavA42, washed, and added to the wells. Treatment of *S. pneumoniae* with preimmune serum (α-IgG) and anti-PavA42 showed no effect on binding of *S. pneumoniae* to fibronectin. In contrast, anti-PavA significantly inhibited adherence of pneumococci to fibronectin. *, $P > 0.17$; **, $P < 0.04$ (with respect to the control [cont]).

thelial cells were infected with *S. pneumoniae* which had been pretreated with anti-PavA or anti-PavA42 in order to assess the ability of these antibodies to block the interaction of PavA with its putative host receptor. PavA localization on the cell surface of pneumococci was previously demonstrated by immunogold labeling of bound anti-PavA IgG (30). Preincubation of pneumococci with anti-PavA antisera did not substantially reduce pneumococcal attachment and invasion in infection assays with HEp-2 cells (adherence, $P > 0.13$; invasion, $P > 0.25$), HBMEC (adherence, $P > 0.19$; invasion, $P > 0.25$), and HUVEC (adherence, $P > 0.47$, invasion, $P > 0.35$), as shown in Fig. 6. These data provide evidence that the effect of PavA on adherence in our experiments is independent of direct binding to fibronectin. Binding of pneumococci to immobilized fibronectin was inhibited by purified full-length recombinant PavA (0.25 μg) but not PavA42 (30). Cell culture infection experiments were also performed in the presence of PavA protein. Different amounts of recombinant PavA protein did not inhibit in a dose-dependent manner adherence to HEp-2 cells ($P > 0.16$) and HBMEC ($P > 0.24$), which were used as representative cell lines in the competition assays. Latex beads coated with full-length PavA protein also did not bind to the cell lines (data not shown). The role of PavA was also assessed in infection assays with *pavA* knockout strains complemented with purified PavA protein. In cocultivation infection assays with increasing amounts of PavA, adherence and invasion of the *pavA* mutant were not restored by exogenously added PavA (data not shown). Incubation with up to 20 μg PavA protein did not result in a significant increase of adherence of PavA-deficient pneumococci to HEp-2 ($P > 0.18$) and HBMEC ($P > 0.13$). These results suggest that PavA is not directly involved in the host-pathogen interaction by mediating pneumococcal adhesion to a specific receptor but may modulate expression or function of other adherence and virulence determinants of this pathogen.

Role of PavA in pneumococcal adherence to pIgR-expressing cells. Pneumococci have developed different strategies for adhesion and invasion. Recently, the interaction of the choline-binding protein SpsA (also referred to as CbpA) and the ectodomains of pIgR has been shown to promote attachment to and invasion in mucosal epithelial cells (21, 59). To investigate whether *pavA* inactivation affected *spsA* and *pspA* transcription, Northern blot analysis was performed with PCR-derived digoxigenin-labeled DNA probes of *spsA*, *pspA*, and the enolase gene. Analysis of gene transcription and protein production, conducted by immunoblotting with anti-SpsA, antienolase, and anti-PspA antisera, revealed similar levels of gene expression and protein production in wild-type pneumococci and the isogenic *pavA* mutant (data not shown). Flow cytometry revealed surface localization of SpsA by binding of FITC-labeled secretory IgA, which specifically interacts with SpsA (data not shown) (25). In order to demonstrate that PavA does not affect the biological function of SpsA as a pneumococcal adhesin, adherence and invasion of wild-type serotype 35A and its *pavA* mutant were examined for the pIgR-expressing Calu-3 lung epithelial cells and for MDCK-hpIgR. The results of the in vitro infection experiments demonstrated that adherence to and invasion of hpIgR-expressing epithelial cells were not affected for pneumococci deficient in PavA (Fig. 7). Since this invasion mechanism strongly depends on the expression of the bacterial adhesin SpsA (CbpA) (21, 59), we conclude that the expression of choline-binding proteins in pneumococci, including *pavA* knockout strains, is not modulated by PavA.

DISCUSSION

Pneumococcal adherence to eukaryotic cells of the upper and lower respiratory tracts is a prerequisite for colonization and disease in the host. A number of studies have clearly indicated that pneumococci are able to target and invade nasopharyngeal cells, bronchial epithelial cells, type II pneumocytes (A549), and human endothelial cells (1, 17, 23, 51, 59). These pathogen-host interactions are mediated by the binding of pneumococcal surface-exposed adhesins to specific cellular receptor molecules. Although it is well established that in the initial phase of colonization glycoconjugate-containing receptors contribute to pneumococcal adherence, the major surface adhesins of *S. pneumoniae* are largely unknown. Exceptions are represented by the choline-binding protein SpsA/CbpA and phosphorylcholine. SpsA mediates uptake of pneumococci by interacting with the polymeric immunoglobulin receptor of mucosal epithelial cells (21, 59). Phosphorylcholine, a unique component of the pneumococcal cell wall, promotes attachment to the platelet-activating factor receptor of endothelial cells, which is associated with invasion (16).

S. pneumoniae expresses further adhesins which interact with proteins of the extracellular matrix and serum of the human host. Binding of pneumococci to factor H, lactoferrin, plasmin(ogen), and immobilized fibronectin via specific bacterial adhesins has been described (4, 18, 20, 26, 32, 55). PavA was identified as the first pneumococcal adhesin for fibronectin; mutants, however, retained 50% of fibronectin-binding

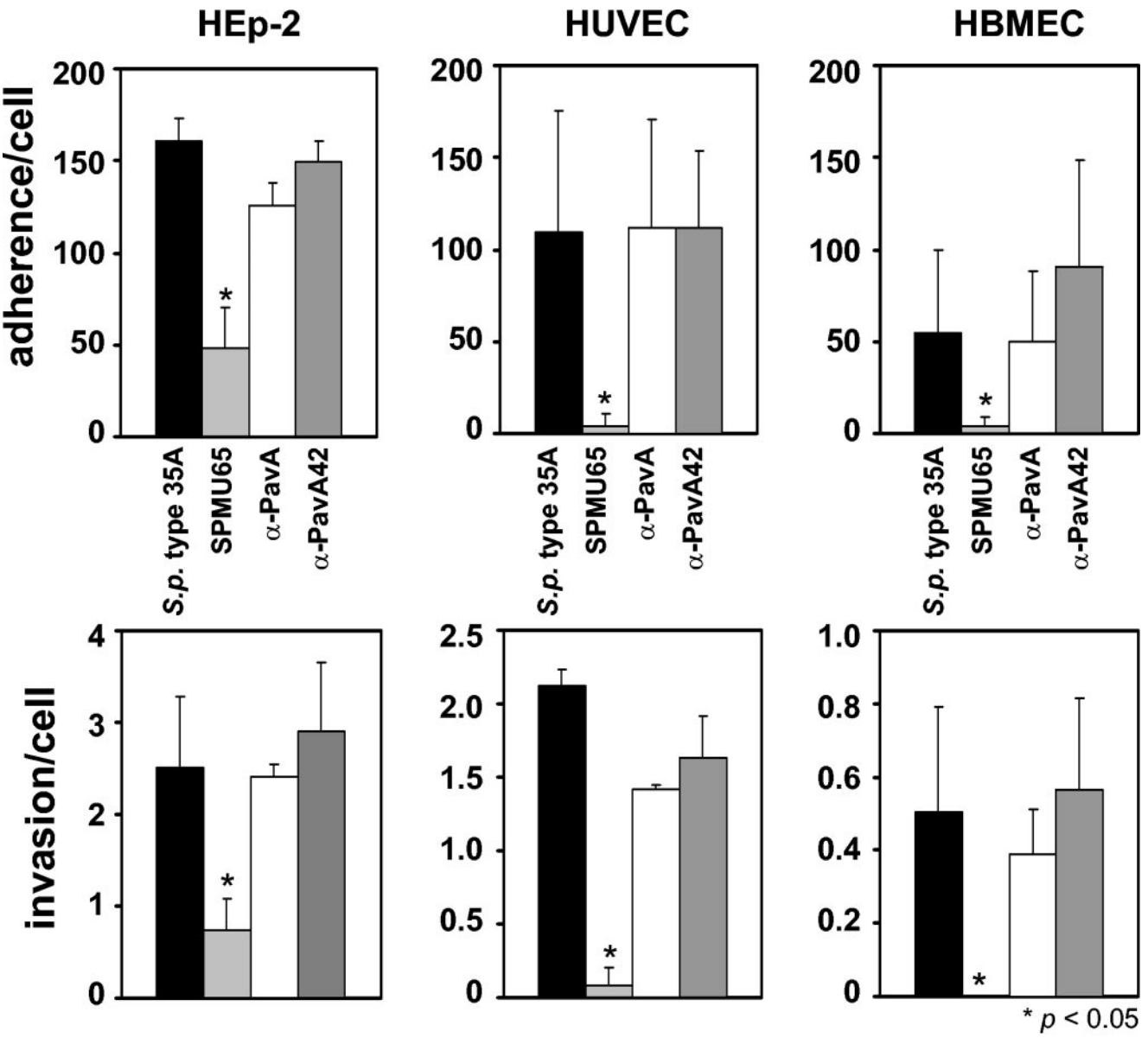

FIG. 6. Effects of anti-PavA antisera on pneumococcal adherence and invasion. For blocking experiments with anti-PavA generated against full-length PavA protein or with anti-PavA42 antiserum which was generated against the C-terminally truncated PavA of 42 kDa, bacteria were treated with a 1:5 (approximately 0.43 mg) dilution of IgG antibodies before infection. The results of the antibody inhibition experiments did not reveal substantial differences in pneumcoccal adherence to and invasion of HEp-2 cells, HUVEC, or HBMEC, respectively (adherence, $P > 0.13$; invasion, $P > 0.25$). Results are presented as the means ± standard deviations for at least three independent experiments. *, $P < 0.05$.

activity (30), suggesting the presence of another fibronectin-binding molecule(s) on *S. pneumoniae*. Fibronectin mediates attachment of bacteria to host cell surfaces by binding to numerous integrins, with the $\alpha_5\beta_1$ integrin as the classical receptor (46, 48, 56, 58). Moreover, the pathogen-fibronectin interaction is associated with uptake of pathogens, as shown extensively for *Streptococcus pyogenes* (31, 38, 39, 41, 42, 50). The pneumococcal PavA protein is highly homologous to the *Streptococcus gordonii* FbpA and the group A streptococcal protein FBP54, both of which bind to fibronectin (13, 14). FbpA of *S. gordonii* modulates gene expression of CshA, another fibronectin-binding protein of S. *gordonii*, thereby affecting the attachment of the bacteria to fibronectin (13, 37). Adherence of group A streptococci to buccal epithelial cells but not to HEp-2 cells was inhibited by FBP54, indicating cell-specific mediation of adhesion via FBP54 (15). In this study we have investigated the effect of PavA on adherence and invasion by using tissue-specific host cells involved in the pathogenesis of pneumococcal disease. Pneumococcal mutants deficient in PavA showed a substantial decrease in adherence and invasion irrespective of whether human epithelial or endothelial cells were infected. In order to elucidate whether PavA is directly involved in binding of bacteria to host cells, blocking experiments were conducted with anti-PavA antibod-

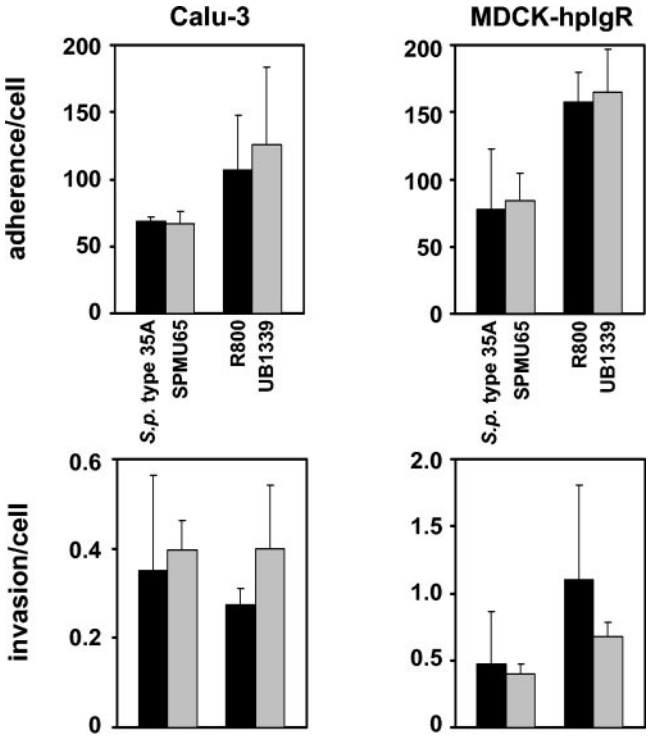

FIG. 7. Adherence and invasion of *S. pneumoniae* deficient in PavA to pIgR-expressing epithelial cells. Cell lines expressing pIgR have been shown to promote adherence and invasion through an interaction of the surface-displayed choline-binding protein CbpA/SpsA and the extracellular domains of pIgR, known as secretory component (21, 59). In contrast to the results of reduced adherence to A549 cells, HEp-2 cells, HUVEC, and HBMEC (Fig. 2 and 3), CbpA/SpsA-pIgR mediated adherence and invasion of pneumococcal *pavA* mutants are not affected as shown for the hpIgR-expressing Calu-3 cells and MDCK-hpIgR cells ($P > 0.25$).

ies and recombinant PavA protein. The antibodies were used to detect cell surface-associated PavA of pneumococci of different serotypes and should, therefore, conceal functional domains of PavA during pneumococcal adherence to cellular receptors (30). The results revealed that anti-PavA antibodies, which are able to detect PavA on the bacterial cell surface, did not affect attachment of pneumococci to both epithelial and endothelial cells. Two lines of evidence suggest that the role of PavA in mediating adhesion and invasion is not directly through interactions with fibronectin. First, except for HUVEC and to a lesser extent A549, the selected cell lines do not produce fibronectin (50) (data not shown), and yet deletion of *pavA* had an inhibitory effect on pneumococcal adherence to A549 cells that was similar to that, e.g., on adherence to HEp-2 cells. Second, anti-PavA antibodies were effective in blocking binding of pneumococci to fibronectin, as shown in Fig. 5, but were ineffective in blocking binding of pneumococci to eukaryotic cell lines.

PavA is a surface-associated protein, although it lacks typical sequence motifs for secretion and anchoring (30). If PavA were directly involved in the interaction with eukaryotic host cells, binding to target molecules on the host cell should have been inhibitable by specific antibodies. This effect was recently shown for anti-SpsA antibodies, which blocked SpsA-mediated adhesion of pneumococci to pIgR-expressing epithelial cells (21). PavA protein, when used in tissue culture coinfections, also did not significantly decrease adherence of pneumococci to host cells. These results are consistent with the lack of binding of PavA-coated latex beads to epithelial and endothelial cells (data not shown). Furthermore, adherence of *pavA* knockout pneumococci to host cells was not increased by cocultivation with PavA protein. These studies suggest that PavA neither has a direct role as an adhesin nor functions as a bridging molecule connecting a surface-exposed adhesin of the pathogen with the cellular receptor. In contrast, the attachment and uptake via the pIgR mediated by SpsA were not affected by disruption of the *pavA* gene, suggesting that choline-binding proteins are expressed. Northern blot analysis indicated that transcription of genes encoding choline-binding proteins was not modulated by PavA.

The data from the cell culture infections were similar to effects observed for pneumococcal mutants with deficiencies in peptide permeases. Mutations in genes encoding protein-dependent peptide permeases resulted in decreased pneumococcal binding to type II pneumocytes and endothelial cells (17). Moreover, mutations in *adc* and *psaA*, representing putative ABC metal permeases of *S. pneumoniae*, resulted in attenuated virulence of the pathogen (8, 19, 36). PsaA was first described as an adhesin because the adherence of a *psaA* mutant to A549 cells was significantly decreased (8). The *pavA* mutant, which was not affected in growth rate and expression of other defined virulence factors such as pneumolysin, was substantially less virulent than the wild type in a systemic mouse infection model (30). The effect of PavA on pneumococcal pathogenesis was independently identified in signature-tagged mutagenesis experiments using a pneumonia model of infection (35). In transmission electron microscopic studies, ultrathin section photographs further suggested that differences in adherence and virulence of isogenic *pavA* mutants are not attributable to visible changes of their cell morphology and/or subcellular structures. The strong attenuation in virulence in the murine sepsis model seems to be even higher than the additive attenuation of virulence caused by a mutation of the gene encoding pneumolysin along with a mutation in a gene encoding a further virulence determinant such as neuraminidase, hyaluronidase, PspA, CbpA, or autolysin (LytA) (9). Pneumolysin belongs to the family of thiol-activated cytolysins and has direct cytotoxic effects on endothelial and epithelial cells (28, 29, 45, 47, 60). Deficiency of pneumolysin has a significant impact on pneumococcal virulence in mice (6, 7, 10, 57). In experimental meningitis, mice infected with an *S. pneumoniae* pneumolysin-deficient strain survived longer than control animals, whereas the survival time was unchanged for animals infected with neuraminidase- or hyaluronidase-deficient strains of *S. pneumoniae*. Interestingly, cerebellum and spleen bacterial titers and meningeal inflammation remained uninfluenced by the lack of any of the virulence factors (57). In this study, however, at an inoculum of 10^4 CFU the *pavA* knockout mutant of *S. pneumoniae* D39 was unable to produce fatal infection after intracerebral injection, as indicated by a lack of bacterial growth and meningeal inflammation after 36 h. Bacterial spread and multiplication in cerebellum, blood, and spleen were detected only for the parental strain. Furthermore, mortality was substantially reduced in mice infected with

a large inoculum of 10^6 CFU of the *pavA*-deficient strain. This is the first investigation observing such a strong attenuation of bacterial virulence in the physiologically immunocompromised compartment of the central nervous system caused by the absence of a single pneumococcal virulence factor.

In conclusion, PavA has a substantial impact on pneumococcal pathogenesis, probably without being directly involved in host cell interactions and inflammatory responses. Most likely, PavA acts directly as a fibronectin adhesin and modulates important, yet-unknown virulence determinants of *S. pneumoniae* which are associated with adherence and survival in vivo. If this modulation occurs through transcriptional regulatory events, then it may be possible to detect changes in gene expression in *pavA*-deficient mutants through DNA microarray analyses. It is also possible that PavA is involved posttranscriptionally in regulating functional levels or subcellular localization of pneumococcal virulence factors, in which case proteomic analyses of subcellular fractions may provide further insights as to how PavA contributes to pneumococcal pathogenesis.

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