

The Inhibitory Effect of C-Reactive Protein on Bacterial Phosphorylcholine Platelet-Activating Factor Receptor–Mediated Adherence Is Blocked by Surfactant

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Numerous major bacterial pathogens in the human respiratory tract, including *Streptococcus pneumoniae* and *Haemophilus influenzae*, express cell-surface phosphorylcholine (ChoP), a ligand for the receptor for platelet-activating factor (rPAF). ChoP is also bound by C-reactive protein (CRP), which, in the presence of complement, may be bactericidal. This study found that CRP can block the attachment of bacteria expressing cell-surface ChoP to host cells. Concentrations of CRP equivalent to those on the mucosal surface of the human airway blocked most adherence of both *S. pneumoniae* and *H. influenzae* to human pharyngeal epithelial cells. ChoP-mediated adherence was also reduced in the presence of an rPAF antagonist. The antiadhesive effects of the rPAF antagonist and CRP were not additive, suggesting that CRP activity is specific to the area of adherence mediated by the receptor. The binding of CRP to ChoP and the effect of CRP on adherence were inhibited by human surfactant (primarily ChoP). The antiadhesive effect of CRP may be diminished in the terminal airway, where surfactant is abundant.

Bacterial infection of the lower respiratory tract is commonly caused by organisms that asymptotically colonize the upper respiratory tract. Here, we examine the hypothesis that surfactant, a host molecule synthesized by type II lung epithelial cells lining the alveolar spaces, may contribute to the increased susceptibility of the normally sterile lung to infection by colonizing bacteria derived from the oro- and nasopharynx [1]. Surfactant lowers the surface tension at the air-fluid interface in the lung and prevents alveolar collapse during expiration. Surfactant is composed of ~80% phospholipids, 10% neutral lipids, and 10% associated proteins [2]. The major lipid component of lung surfactant is dipalmitoyl phosphatidylcholine (DPPC), which has a polar head group consisting of phosphorylcholine (ChoP). The collectin glycoproteins associated with surfactant (e.g., surfactant proteins A and D) enhance the antimicrobial activity of alveolar macrophages [3–6]. In contrast, the phospholipid component of surfactant also has both immune-enhancing and inhibitory effects [7]

Bacterial infection of the lower respiratory tract is caused by a diverse group of gram-positive and gram-negative species and

Mollicutes. Within each group, the most common etiologic agents of pneumonia, including *Streptococcus pneumoniae* (the pneumococcus), *Haemophilus influenzae*, and *Mycoplasma* species, respectively, may express ChoP on their cell surfaces [8, 9]. ChoP, otherwise an unusual structural feature of prokaryotes, is now known to be a cell-surface component of a number of species that reside primarily in the mammalian airway [10–13]. For both *S. pneumoniae* and *H. influenzae*, the expression of ChoP allows for bacterial interaction with the receptor for platelet-activating factor (rPAF), whose natural ligand also contains ChoP [14–16]. This molecular mimicry of PAF increases adherence and invasion of host epithelial cells and thus has been proposed as a factor in promoting colonization.

An additional consideration in the expression of ChoP is that, for many of the species with this cell-surface characteristic, it undergoes phase variation. In *H. influenzae*, the on-off display of ChoP attached to hexose residues on the lipopolysaccharide is controlled by a translational switch generated by slip-stranded mispairing of multiple tandem repeats of 5'-CAAT-3' within the *licA* gene encoding a putative choline kinase [17]. When resident in the human respiratory tract, most *H. influenzae* have an in-frame number of 5'-CAAT-3' repeats and, therefore, are expected to express ChoP in this host environment. Further evidence that the expression of ChoP contributes to colonization comes from analysis of phase variation in *S. pneumoniae*. In most pneumococci, variation between transparent and opaque colony phenotypes may be observed [18]. One of the major differences distinguishing these forms is the higher amount of ChoP linked to the cell-associated teichoic acid or C-polysaccharide in transparent pneumococci [19]. Analysis of paired colonizing and invasive isolates from the same person

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showed that the transparent variant predominates during human carriage [20].

Although observations on phase variants support a role for ChoP in bacterial survival on the mucosal surface of the airway, the ability to down-regulate this structure suggests there are conditions under which its expression is disadvantageous to the organism. Opaque pneumococcal variants expressing less ChoP, for example, are selected for infection beyond the respiratory tract in animal models and in natural human disease [20, 21]. One explanation for the requirement for these pathogens to decrease expression of cell-surface ChoP is that there is a host protein that specifically targets this structure and appears to be a component of innate host defense [22]. C-reactive protein (CRP), so named for its ability to bind to C-polysaccharide, is found in serum, where its expression in humans may increase up to 1000-fold in response to tissue injury and inflammation [23, 24]. This acute-phase reactant has been implicated in protection against invasive pneumococcal infection on the basis of the diminished susceptibility of mice carrying the transgene for human CRP (hCRP) [25]. The protective effect of CRP is thought to be mediated by its ability to act as an opsonin and, when bound, to activate complement by the classical pathway through interaction with C1q [26–28]. The direct antimicrobial effect of CRP is demonstrated by its bactericidal activity in the presence of complement against *H. influenzae* expressing ChoP [17].

We recently reported that, in addition to synthesis by hepatocytes to generate serum CRP, this protein is also expressed on the epithelial surface of the human respiratory tract [29]. Furthermore, CRP is present in airway-surface fluid in concentrations that, although lower than those normally found in serum, may allow it to contribute to innate immunity at this mucosal site, where ChoP-expressing bacteria reside [30]. An additional factor in the antimicrobial activity of CRP in the lower respiratory tract is that, during inflammatory states, when there is increased capillary alveolar permeability, plasma proteins, including CRP, leak into the alveolar spaces [31, 32]. CRP binds to DPPC by its specific interaction with its ChoP head group and has a detrimental effect on the surface activity of surfactant.

Materials and Methods

Bacterial strains and media. *S. pneumoniae* strains used in this study have been described elsewhere [21]. They included opaque and transparent phase variants of P303 (a type 6A clinical isolate), D39 (a type 2 clinical isolate), and R6 (an unencapsulated mutant of D39). *S. pneumoniae* was grown in semisynthetic casein plus yeast medium (pH 6.8) or on tryptic soy agar onto which 5000 U of catalase (Worthington Biochemical) was applied [34]. The *H. influenzae* strains used also have been described elsewhere [35] and included H233 (a nontypeable clinical isolate) and defined mutants of Rd. H233 was separated into populations that were predominantly expressing or not expressing ChoP by using colony immunoblotting with TEPC-15, a monoclonal antibody (MAb) to ChoP (Sigma-Aldrich Chemical). Strain Rd was modified by in-

sertion of a kanamycin resistance cassette in the *licD* gene or in-frame deletion of the tandem 5'-CAAT-3' repeats in *licA*, resulting in constitutive nonexpression (Rd/ChoP-off) and expression (Rd/ChoP-on) of ChoP, respectively, as described elsewhere [36]. *H. influenzae* was grown in brain-heart infusion medium (Difco) with or without 1% agar supplemented with 2 $\mu\text{g}/\text{mL}$ NAD (Sigma-Aldrich) and 2% Fildes enrichment (Becton Dickinson).

Cell culture. Detroit 562 cells (ATTC CCL-138), a human pharyngeal carcinoma cell line, and A549 cells (ATTC CCL-185), a human lung carcinoma cell line, were grown in MEM with L-glutamine (Gibco BRL) supplemented with sodium pyruvate (1 mM) and 10% fetal bovine serum (FBS; HyClone, VWR Scientific) along with penicillin (10 $\mu\text{g}/\text{mL}$) and streptomycin (10 $\mu\text{g}/\text{mL}$) to confluence and then harvested by using trypsin (final concentration, 0.25%) and EDTA (final concentration, 0.02%) (both from Gibco BRL). FBS lacked detectable CRP in Western blots by use of a polyclonal antiserum to hCRP (Sigma-Aldrich) that cross-reacts with CRP in adult bovine serum. Cells were frozen in FBS with dimethylsulfoxide (final concentration, 10%), placed overnight at -70°C in a 1°C freezer container, and stored in liquid nitrogen.

Adherence assays. Confluent monolayers of Detroit 562 (D562) pharyngeal epithelial cells or A549 cells in 6-well plates were washed 3 times with PBS and maintained in MEM without antibiotics until ready to infect. Bacteria were grown in liquid culture to the midlogarithmic phase (absorbance at 620 nm, 0.4) and added to each well at a density of 10^6 cfu/well unless otherwise specified. Where indicated, *S. pneumoniae* or *H. influenzae* were pretreated for 60 min at 4°C in Hanks' buffer (Gibco BRL) to provide a source of calcium with or without 2–4 $\mu\text{g}/\text{mL}$ purified serum hCRP (Sigma-Aldrich) prior to inoculation of the washed monolayers. The bacteria were applied to the monolayers by centrifugation (1300 g for 5 min) and then incubated at 37°C for 60 min to allow for adherence.

To determine the percentage of bacteria from the inoculum that were adherent, the monolayer was washed 5 times with PBS, and the cells and adherent bacteria were lifted off by treatment with 500 $\mu\text{L}/\text{well}$ 0.25% trypsin–1 mM EDTA, vortexed, and maintained at 4°C . Serial dilutions of the inoculum or adherent bacteria were quantified by plating on selective media in duplicate. Percentage of adherence was calculated as the portion of the inoculum that was adherent to the target cells. Results showing $>100\%$ adherence were due to bacterial replication while in tissue culture. We expressed results as the mean \pm SD in ≥ 3 separate wells per condition in representative experiments. Data were analyzed for significance by Student's *t* test. Where indicated, the rPAF binding antagonist (PAF-Ra; [1]-hexadecyl-2-acetyl-sn-glycerol-3-phospho-[N,N,N-trimethyl]-hexanolamine; Calbiochem) was used to treat confluent D562 cells at the concentration specified for 30 min at 37°C before and during the course of the adherence assay.

Human surfactant was purified from therapeutic pulmonary lavage specimens obtained from patients with alveolar proteinosis, a condition characterized by overproduction of surfactant, as described elsewhere [7, 37]. The purified surfactant contained a 1:1 ratio of protein to phospholipid (A. Fisher, personal communication). After lyophilization, the surfactant was reconstituted in Hanks' buffer to a final concentration of 1 mg/mL, on the basis of its protein content, as measured by the Micro BCA assay (Pierce Chemical), and stored at -20°C . Surfactant or soluble ChoP (03078; Sigma), at a final

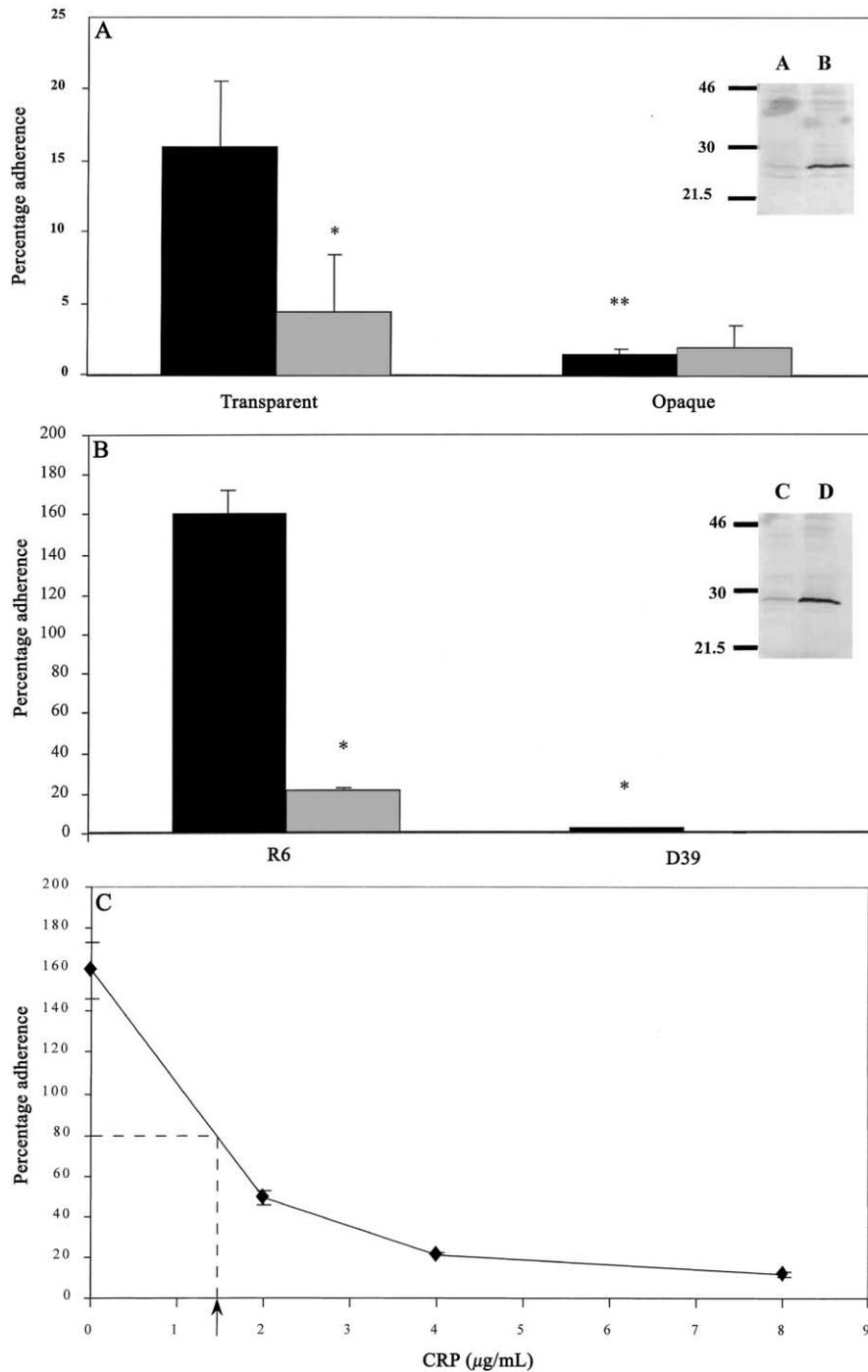


Figure 1. Adherence of *Streptococcus pneumoniae* to Detroit 562 pharyngeal cells. Bacteria were pretreated with (gray bars) or without (black bars) hCRP. Adherence is expressed as a percentage of inoculum binding to epithelial cells over the course of 60 min. Data are means of ≥ 3 determinations in duplicate (\pm SD) in a representative experiment. *A*, Adherence of P303, a type 6A clinical isolate divided into its opaque and transparent phenotypic variants. * $P < .01$ and ** $P < .0005$, vs. transparent variant without C-reactive protein (CRP). *B*, Adherence of D39, a type 2 clinical isolate, and R6, an unencapsulated mutant of D39. * $P < .0001$, vs. R6 without CRP. Inserts compare hCRP binding to pneumococcal cells that were then lysed and analyzed by Western immunoblotting with a monoclonal antibody to hCRP to detect bound protein. P303 opaque variant (insert *A*), P303 transparent variant (insert *B*), D39 (insert *C*), and R6 (insert *D*). The nos. in the inserts are kilodaltons. *C*, Dose-response assay shows effect of purified human CRP (hCRP) on adherence of R6. Arrow, concentration of hCRP calculated to reduce adherence by 50%.

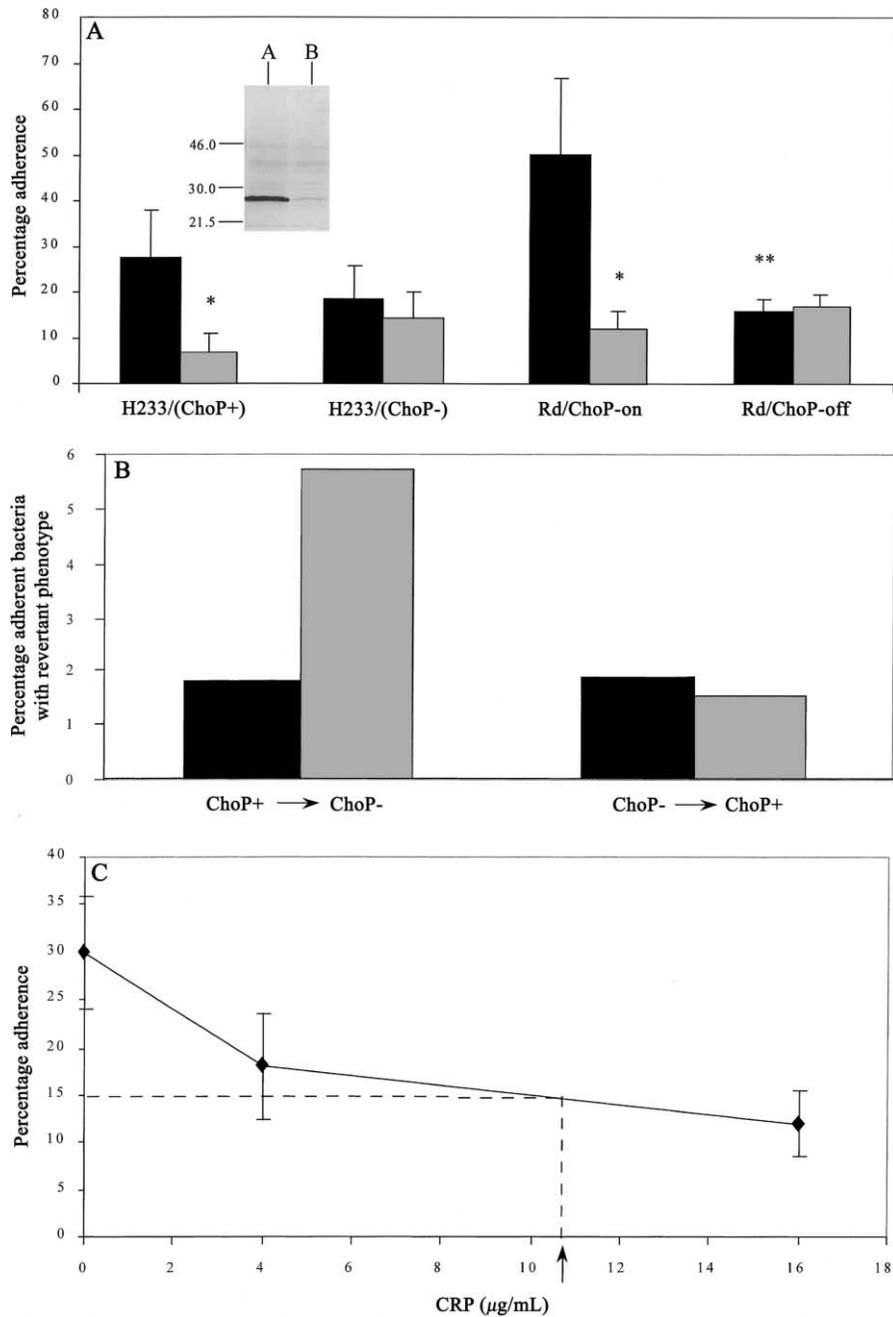


Figure 2. Adherence of *Haemophilus influenzae* to Detroit 562 pharyngeal cells. Adherence is expressed as a percentage of the inoculum binding to epithelial cells after 60 min. Data are means of ≥ 3 determinations in duplicate (\pm SD) in a representative experiment. *A*, Adherence of H233, a nontypeable clinical isolate divided into its phosphorylcholine (ChoP) expressing (+) and nonexpressing (-) phase variants and constitutive mutants Rd/ChoP-on and Rd/ChoP-off. Bacteria were pretreated with (gray bars) or without (black bars) 4 μ g/mL purified serum human C-reactive protein (hCRP). Insert compares hCRP binding in H233 phase variants with (insert *A*) or without (insert *B*) ChoP that were then lysed and analyzed by Western immunoblotting with a monoclonal antibody to hCRP to detect bound protein. The nos. in the inserts are kilodaltons. * $P < .01$, vs. same bacteria without CRP; ** $P < .005$, vs. Rd/ChoP-on without CRP. *B*, Selection for revertant phenotype among adherent H233 after inoculation with populations that were predominantly ChoP+ or ChoP- phase variants. *C*, Dose-response assay shows effect of hCRP on adherence of Rd/ChoP-on. Arrow, concentration of hCRP calculated to reduce adherence by 50%.

concentration of 0.3 mg/mL, was used to pretreat hCRP for 30 min at 4°C before it was added to adherence experiments as described above. Controls showed no detrimental effect of treatment with trypsin-EDTA, hCRP, soluble ChoP, PAF-Ra, or surfactant on bacterial clumping or viability.

Western blot analysis. Comparison of the binding of hCRP to equal numbers of whole *S. pneumoniae* or *H. influenzae* by Western blot analysis has been described elsewhere [17, 28]. To assess the ability of surfactant to interfere with the binding of CRP to bacteria, hCRP (300 ng/mL) was treated with or without 0.3 mg/mL human surfactant in Hanks' buffer for 30 min at 4°C before incubation by rotation with 10^7 cfu of bacteria for 60 min at 4°C. In control experiments, an equivalent amount of soluble ChoP was substituted for surfactant. The CRP bound to bacteria was then removed by centrifugation at 10,000 *g* for 5 min, and the supernatants were analyzed by Western blot analysis for the presence of unbound CRP.

To determine the presence of rPAF, D562 cells were harvested by using 500 μ L of trypsin-EDTA, as described above, vortexed, and maintained at 4°C. Samples for Western blot analysis were treated at 100°C for 5 min in gel loading buffer (10% glycerol, 2% SDS, 50 mM Tris-Cl [pH 6.8], 100 mM β -mercaptoethanol, and bromophenol blue). Proteins were separated by PAGE in 12.5% polyacrylamide gels, transferred to Immobilon-P membranes (Millipore), immunoblotted with an MAb to human rPAF (a murine IgG2a; Alexis Biochemicals) at a final concentration of 1.7 μ g/mL of MAb to human CRP (CRP-8, a murine IgG1; Sigma-Aldrich) at a dilution of 1:10,000 and detected with alkaline phosphatase conjugated to anti-mouse IgG (Sigma-Aldrich) at a dilution of 1:10,000. Colorimetric detection was performed with NitroBT and 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt (Fisher Scientific).

Immunocytochemistry. D562 cells were seeded onto sterile coverslips and allowed to adhere overnight at 37°C. The adherent cells then were washed with PBS and fixed by using 4% paraformaldehyde for 10 min at 25°C. After being washed twice with PBS, the cells were permeabilized with methanol for 2 min at 25°C and treated with 1% bovine serum albumin (BSA) for 10 min at 25°C. An MAb to human rPAF (Alexis Biochemicals) or an IgG2a isotype control (HASP-11; Statens Seruminstitut) was diluted in 1% BSA to a final concentration of 1.25 μ g/well. A control coverslip incubated with only the secondary antibody was also examined to exclude the possibility of nonspecific binding. After incubation overnight at 37°C in a humidity chamber, the slides were washed 10 times with PBS, and anti-mouse IgG fluorescein isothiocyanate (FITC) conjugate (Sigma-Aldrich) diluted 1:100 in 1% BSA was added for 30 min at 37°C. The coverslips were washed 10 times in PBS, mounted with Vectashield (Vector Laboratories), and stored in the dark until they were ready to be examined under UV fluorescence.

Immunohistochemistry. For immunohistochemistry, 7- μ m-thick slices of human nasopharyngeal tissue removed with nasal polyps were deparaffinized through a series of xylene and graded ethanol baths and permeabilized by using 0.5% Triton X-100 in PBS, followed by a rinse with PBS. The sections then were treated twice for 3 min with sodium borohydride (1 mg/mL) in PBS to prevent autofluorescence and reduce background binding, followed by a rinse in PBS for 5 min. The sections then were blocked with goat serum (Sigma-Aldrich), in PBS for 20 min at 25°C and rinsed in PBS. MAb to human rPAF or the IgG2a isotype control was

diluted in goat serum to a final concentration of 2.5 μ g/mL, applied to each slide, covered with paraffin, and incubated overnight in a humidity chamber at 4°C. A control incubated with only the secondary antibody was also examined to rule out nonspecific binding. The secondary antibody, anti-mouse IgG FITC conjugate (Sigma-Aldrich), was diluted 1:100 in goat serum and allowed to incubate with the tissue section at 25°C for 60 min in a humidity chamber. The sections then were washed twice with PBS and counterstained with methyl green (Vector Laboratories) for 5 min at 25°C, rinsed, and mounted with Vectashield. The slides were stored in the dark until they were ready to be examined under UV fluorescence.

Results

CRP blocks adherence. The effect of CRP on adherence to D562 cells was examined by use of *S. pneumoniae* and *H. influenzae*. The concentrations of hCRP used in these assays corresponded to amounts previously shown to be present in human airway surface fluid (0.6–10.5 μ g/mL) [29]. The addition of purified serum hCRP (2 μ g/mL) resulted in a 73% decrease in the number of pneumococci of a type 6A clinical isolate adhering to D562 cells (figure 1A). The antiadhesive effect of CRP, however, was specific to the transparent variant of this isolate and correlated with the increased binding of hCRP to the transparent compared with the opaque form of the same strain (figure 1A, insert). Because the ability of pneumococci to bind to CRP depends on amounts of the variably expressed capsular polysaccharide, subsequent studies were done with R6, an unencapsulated mutant of strain D39 (figure 1B, insert) [28]. For R6, the presence of hCRP (4 μ g/mL) resulted in an 87% decrease in adherence (figure 1B). A dose-response assay revealed that 1–2 μ g/mL hCRP blocked 50% adherence of R6 (figure 1C). Experiments were done with an MOI of 1 bacterial/host cell, to model nasopharyngeal carriage in which $<10^6$ cfu/mL of nasal airway-surface fluid is measured [17, 18]. A similar effect of CRP was observed with an MOI of 10 bacteria/host cell (data not shown).

Similar results were observed with a clinical nontypeable *H. influenzae* isolate (H233), where hCRP (4 μ g/mL) blocked 75% of adherence to D562 cells (figure 2A). For H233, the effect of CRP was specific to the ChoP-expressing phase variant and correlated with the ability of hCRP to bind to whole bacteria (figure 2A, insert). After inoculation of the cell culture with a predominately ChoP-expressing population, the addition of hCRP led to an increased selection for ChoP-nonexpressing revertants among the adherent bacteria (figure 2B). The selection for revertants among adherent bacteria was not observed when a ChoP-nonexpressing population was used in the inoculum. These results provide further evidence that the antiadhesive effects of CRP are specific to ChoP-expressing organisms. To avoid working with a variable population of bacteria, subsequent studies were done with constitutive ChoP-on and ChoP-off mutants constructed in strain Rd for which hCRP (4 μ g/mL) resulted in a 76% reduction in adherence, a result that

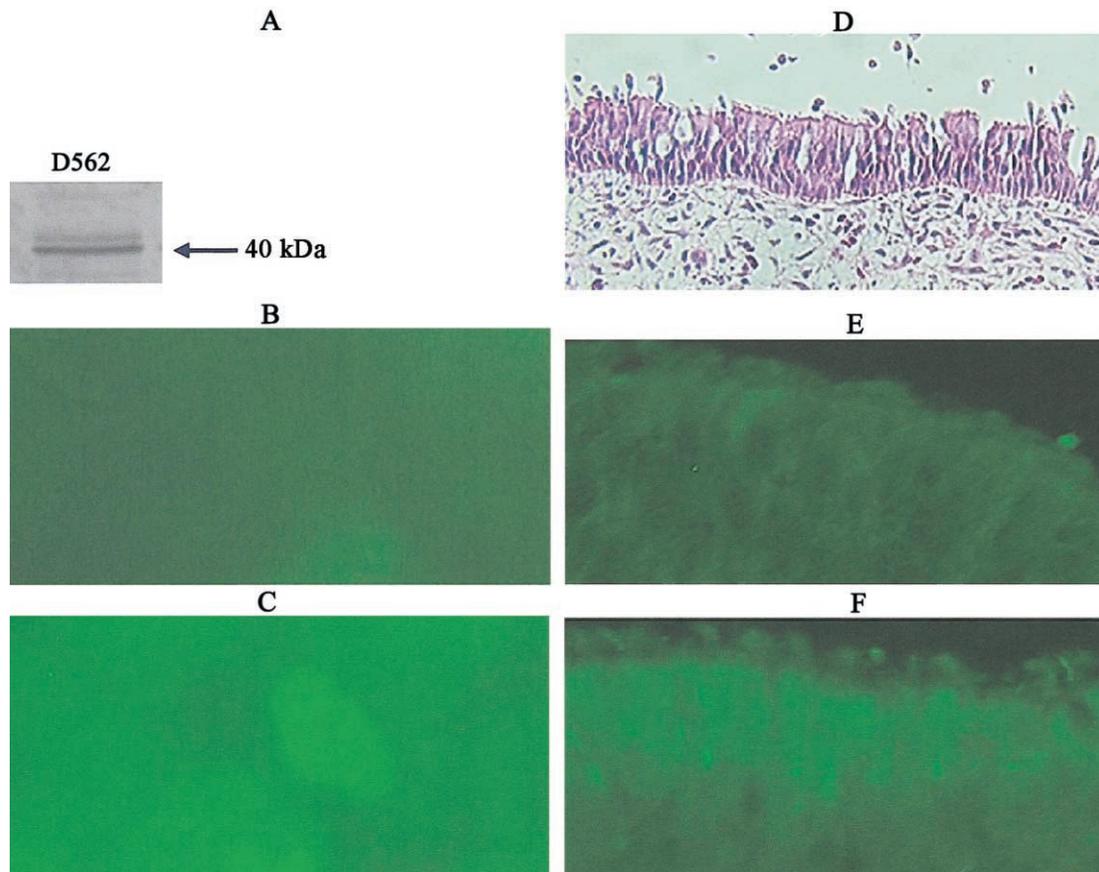


Figure 3. Expression of receptor for platelet-activating factor (rPAF). *A*, Western blot analysis of Detroit 562 (D562) pharyngeal cell homogenates with a monoclonal antibody (MAb) against human rPAF shows an immunoreactive band of the predicted size for rPAF. *B* and *C*, Immunocytochemistry of D562 pharyngeal cells with an isotype control MAb and with an MAb against human PAF, respectively. Original magnification, $\times 1000$. *D*, Hematoxylin-eosin staining of section from excised human nasal polyp shows normal ciliated columnar architecture of the epithelial surface. *E* and *F*, Immunohistochemistry of human nasal polyp tissue with an isotype control MAb and with an MAb to human rPAF. *D–F*, Original magnification, $\times 400$.

was specific for the mutant expressing ChoP (figure 2*A*). In a separate dose-response experiment, 50% inhibition of adherence of Rd/ChoP-on was observed with an hCRP concentration of 10–12 $\mu\text{g}/\text{mL}$, and a significant effect on adherence was seen at a concentration as low as 4 $\mu\text{g}/\text{mL}$ (figure 2*C*).

rPAF is expressed on human nasopharyngeal epithelium. Since rPAF has been shown to interact with ChoP on both *S. pneumoniae* and *H. influenzae*, we sought to determine whether the epithelial cells derived from the upper respiratory tract used in this study and the tissues lining the airway that would be exposed to these organisms *in vivo* express this receptor. D562 cells were tested in immunocytochemistry experiments by using an MAb to rPAF (figure 3*C*) or isotype control MAb (figure 3*B*) to demonstrate the presence of this receptor. This result was confirmed in Western blots of D562 cell lysates by using the rPAF MAb to demonstrate a 40-kDa immunoreactive band of the predicted size for denatured rPAF (figure 3*A*). The analysis of these immortalized cells in culture was then extended

by immunohistochemistry showing expression of the rPAF on the epithelial surface of tissue sections of human nasal polyps by comparing the binding of the rPAF MAb (figure 3*F*) and isotype control MAb (figure 3*E*).

CRP interferes with binding to rPAF. We next sought to determine whether the rPAF plays a role in the observed anti-adhesive properties of CRP. These experiments used PAF-Ra, an analogue of PAF, in adherence assays. The relative effect of hCRP and the PAF-Ra on adherence was examined for both *S. pneumoniae* R6 and *H. influenzae* Rd/ChoP-on strains. Treatment of the D562 cells with PAF-Ra (1 μM) caused a decrease in adherence of R6 from $92\% \pm 5.1\%$ to $56.5\% \pm 26.4\%$, suggesting that at least some of the detectable adherence in these assays was due to the interaction of rPAF and bacterial ChoP (figure 4*A*).

In the presence of PAF-Ra (1 μM), there was no significant additional antiadhesive effect of hCRP (4 $\mu\text{g}/\text{mL}$) on R6 ($42.4\% \pm 6.9\%$ adherence). The lack of an additive effect of CRP

and PAF-Ra suggested that both antiadhesive molecules act through a common rather than separate pathway, a result consistent with the hypothesis that CRP blocks rPAF-mediated pneumococcal adherence. Similar results were seen with *H. influenzae* where there was a dose-related effect of PAF-Ra with a 63.6% and 91.2% decrease in adherence in the presence of the antagonist at concentrations of 1 and 10 μM , respectively. This dose-related effect of the PAF-Ra was specific to the ChoP-expressing mutant, indicating that, like the pneumococcus, at least a portion of the adherence of *H. influenzae* to these cells is mediated by the binding of ChoP to rPAF. The inclusion of hCRP (4 $\mu\text{g}/\text{mL}$) provided no significant additional antiadhesive effect in the presence of 1 or 10 μM of PAF-Ra (46.7% \pm 4.2% adherence for hCRP without PAF-Ra, compared with 40.0% \pm

6.0% adherence for hCRP with 1 μM PAF-Ra; figure 4B). The lack of an additive antiadhesive effect of CRP and PAF-Ra suggested that CRP acts by interfering with the binding of ChoP on *H. influenzae* to rPAF on epithelial cells.

Human surfactant blocks the antiadhesive effect of CRP. The effect of surfactant on the activity of CRP was tested by using purified human surfactant. To demonstrate that surfactant could compete with ChoP-expressing bacteria for CRP binding, CRP was pretreated with human surfactant (final concentration, 0.3 mg/mL) prior to incubation with ChoP-expressing bacteria. The effect of surfactant on binding of CRP to bacterial ChoP was determined by Western blotting with a MAb to hCRP (CRP-8; figure 5). Both *S. pneumoniae* R6 and *H. influenzae* Rd/ChoP-on were able to absorb and deplete the hCRP from supernatants

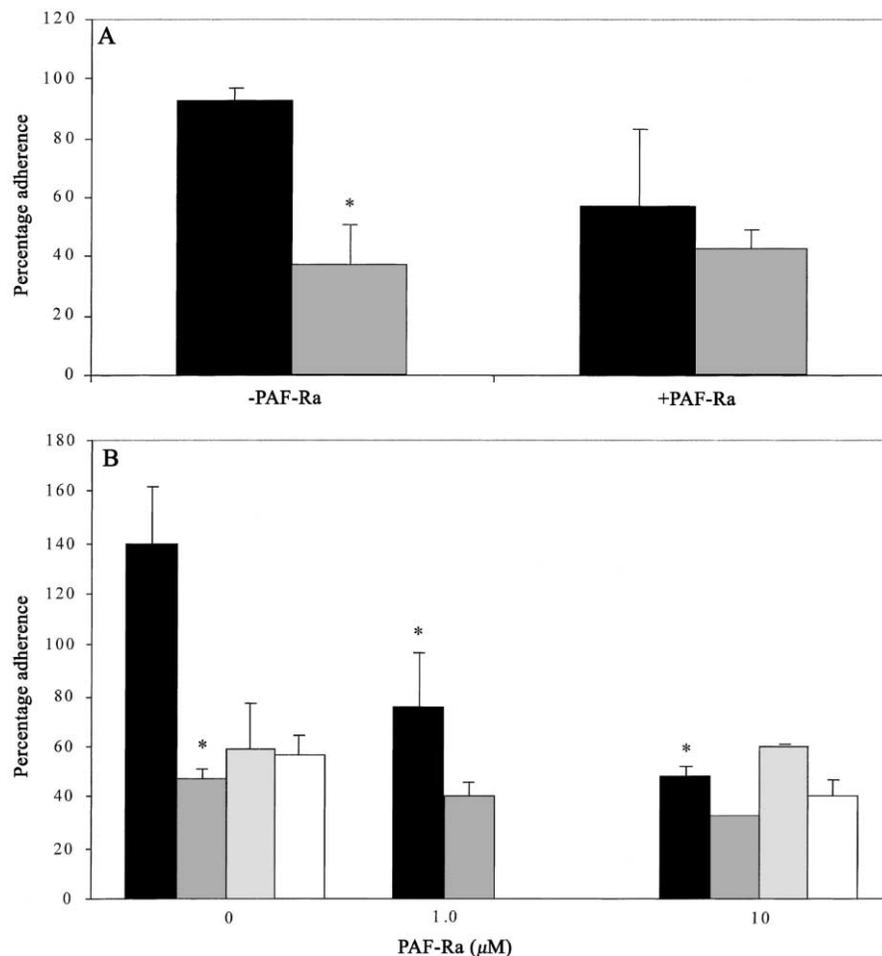


Figure 4. Effect of a platelet-activating factor receptor antagonist (PAF-Ra) on bacterial adherence. Adherence is expressed as a percentage of the inoculum binding to epithelial cells after 60 min. Data are means of ≥ 3 determinations in duplicate (\pm SD) in a representative experiment. *A*, Adherence of *Streptococcus pneumoniae* R6 pretreated (dark-gray bars) or not pretreated (black bars) with C-reactive protein (CRP; 4 $\mu\text{g}/\text{mL}$) to Detroit 562 pharyngeal cells in the presence (+) or absence (-) of 1 μM PAF-Ra. * $P < .01$, vs. same condition without CRP. *B*, Adherence of *Haemophilus influenzae* Rd/ChoP-on (black and dark-gray bars) and Rd/ChoP-off (light-gray and white bars) pretreated (dark-gray and white bars) or not pretreated (black and light-gray bars) with CRP (4 $\mu\text{g}/\text{mL}$) to Detroit 562 pharyngeal cells in the concentration of PAF-Ra indicated. * $P < .025$, vs. same condition without CRP or PAF-Ra.

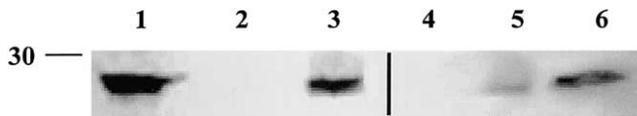


Figure 5. Effect of human surfactant on binding of C-reactive protein (CRP) to bacteria. Western blot analysis with a monoclonal antibody to human CRP shows the ability of bacteria to bind and deplete CRP (300 ng/mL) from supernatants. *Lane 1*, Purified CRP (6 ng); *lane 2*, *Streptococcus pneumoniae* R6 incubated with CRP without human surfactant; *lane 3*, R6 incubated with CRP with human surfactant (0.3 mg/mL); *lane 4*, *Haemophilus influenzae* H233 expressing phosphocholine (ChoP) incubated with CRP without human surfactant; *lane 5*, H233 expressing ChoP incubated with CRP with human surfactant (0.3 mg/mL); and *lane 6*, H233 expressing ChoP incubated with CRP in the presence of soluble ChoP (0.3 mg/mL).

in these assays. Preincubation of hCRP with surfactant partially or completely blocked CRP binding to the bacteria. An equivalent amount of soluble ChoP (based on a 1:1 ratio of surfactant protein to phospholipid) was used as a positive control and had a similar effect to surfactant. This suggests that the ChoP component of surfactant is sufficient to account for the ability of surfactant to bind to CRP and inhibit its interaction with bacteria.

Finally, we examined the surfactant's effect on the antiadhesive properties of CRP in adherence assays with D562 cells. Human surfactant (0.3 mg/mL) was used to pretreat hCRP (4 μ g/mL) before incubation with bacteria. This concentration of surfactant completely inhibited the antiadhesive effect of CRP when examined with *S. pneumoniae* R6 (43.3% \pm 2.3% adherence with CRP alone vs. 182.0 \pm 44.8% mean adherence with surfactant-pretreated CRP; figure 6A). A similar inhibitory effect of surfactant was observed with *H. influenzae* Rd/ChoP-on (12.1% \pm 3.6% adherence with CRP alone vs. 30.6% \pm 17.3% adherence with surfactant-pretreated CRP; figure 6B). Pretreatment of hCRP with the equivalent concentration of soluble ChoP (final concentration, 0.3 mg/mL) also reduced its inhibitory effect on adherence (data not shown). Because surfactant is found in the lower, but not the upper, airway, these studies were repeated by examining adherence to A549 cells, a cell line with some characteristics of type II alveolar pneumocytes previously shown to express rPAF-dependent pneumococcal binding [14]. For adherence of R6 to A549 cells, surfactant completely blocked the antiadhesive effect of CRP (5.3% \pm 4.3% adherence with CRP alone vs. 76.3% \pm 57.7% mean adherence with surfactant-pretreated CRP; figure 6C). Similar results were obtained when Rd/ChoP-on adherence to A549 cells was tested (47.2% \pm 9.6% adherence for CRP alone vs. 78.4% \pm 14.8% mean adherence for surfactant-pretreated CRP; figure 6D). Together, results with D562 and A549 cells with both *S. pneumoniae* and *H. influenzae* confirmed that the antiadhesive effect of CRP is diminished in the presence of human surfactant.

Discussion

There is increasing evidence that, among the varied functions attributed to CRP, an antimicrobial activity augments host defense by specifically targeting organisms expressing cell-surface ChoP [17, 25]. Innate defense may be particularly important against this group of organisms, because many are encapsulated and able to rapidly overwhelm other aspects of humoral immunity before the development of type-specific protective antibody directed against capsular polysaccharide.

The proposed mechanisms for CRP-mediated protection have emphasized the ability of bound CRP to interact with complement and to enhance opsonization and/or bactericid-

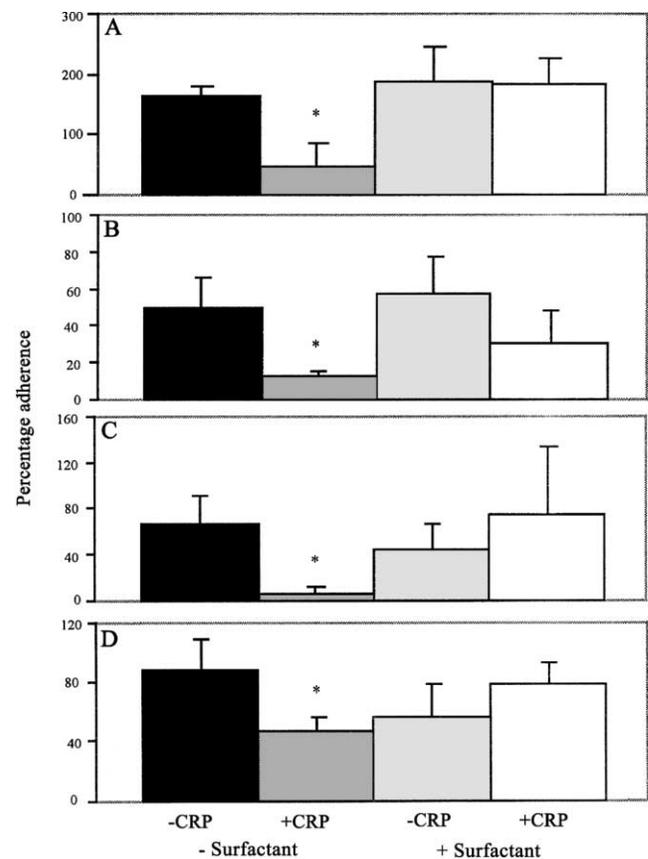


Figure 6. Effect of human surfactant on bacterial adherence. Bacteria were pretreated with (+; dark-gray and white bars) or without (-; black and light-gray bars) 4 μ g/mL purified human C-reactive protein (CRP) in the presence (light-gray and white bars) or absence (black and dark-gray bars) of purified human surfactant (0.3 mg/mL). Adherence is expressed as a percentage of the inoculum binding to epithelial cells after 60 min. Values are means of ≥ 3 determinations in duplicate (\pm SD) in a representative experiment. *A*, *Streptococcus pneumoniae* R6 adherence to Detroit 562 pharyngeal cells. *B*, *Haemophilus influenzae* Rd/ChoP-on adherence to Detroit 562 pharyngeal cells. *C*, *S. pneumoniae* R6 adherence to A549 type II pneumocytes. *D*, *H. influenzae* Rd/ChoP-on adherence to A549 type II pneumocytes. * $P \leq .05$, vs. CRP treated with surfactant, except for panel *B*, where $P = .07$.

al activity [17, 26, 27, 38]. In this study, we demonstrated that an additional function not previously described for CRP is its ability to inhibit the attachment of bacteria to host cells. This complement-independent activity of CRP may be especially relevant on the mucosal surface, where attachment to epithelial cells is a critical step in bacterial colonization and there are lesser amounts of complement components in the absence of serum extravasation that would occur during inflammation or tissue damage [30]. The antiadhesive effect of CRP shown utilized amounts of CRP previously documented to be present on the airway surface, where there is local production by the respiratory epithelium and alveolar macrophages [29, 39]. Furthermore, the effect of CRP on attachment was seen only for bacteria capable of binding to this host protein. This suggests that CRP functions by inhibiting the ability of bacteria to interact with host tissues rather than by an indirect effect, such as the modification of the host cell.

It is now clear that a number of species found predominately in association with the respiratory tract use a common pathway for adherence or invasion that involves bacterial ChoP binding to rPAF [14, 15]. The antiadhesive effect of CRP, therefore, acts through its specific targeting of this receptor ligand interaction. This study examined bacterial attachment to the respiratory epithelium for the 2 most common pathogens of the human respiratory tract, *S. pneumoniae* and *H. influenzae*, both of which express cell-surface ChoP [8, 10–12].

The role of CRP in diminishing adherence was demonstrated for the transparent but not the opaque form of the pneumococcus. More transparent pneumococci adhere to epithelial cells than do opaque pneumococci [40], because they have more of the teichoic acid–ChoP ligand, which allows for increased adherence to the rPAF, and diminished amounts of capsular polysaccharide, a material that interferes with efficient attachment [21, 41]. Although variable amounts of bacterial ChoP also affect CRP binding, levels of expression of the capsular polysaccharide is the predominant factor in allowing CRP binding to pneumococci [28]. Our findings are consistent with the possibility of a CRP effect in the airway, since the transparent phenotype that efficiently binds this protein predominates on the mucosal surface *in vivo* [18, 20].

Pneumococcal adherence to epithelial cells appears to be multifactorial. Zhang et al. [42] reported that choline-binding protein A (CbpA) binding to the polymeric immunoglobulin receptor was the major determinant of pneumococcal strain R6 adherence and invasion of D562 cells. In contrast, our adherence assay results show that most but not all attachment of strain R6 is inhibited by CRP, indicating that the binding of teichoic acid–ChoP to rPAF is responsible for at least a substantial proportion of pneumococcal adherence. An alternative explanation is that CRP also interferes with binding of the CbpA adhesin, which requires anchoring to the pneumococcal cell surface by noncovalent interaction with ChoP [43]. The possibility that CRP also inhibits CbpA-mediated adherence,

however, would not account for the decrease in attachment to D562 cells seen with the PAF-Ra in the absence of CRP.

For *H. influenzae*, a role for the LPS–ChoP interaction with rPAF leading to a low-frequency internalization into primary bronchial epithelial cells has been described elsewhere [15]. Our current study also demonstrates that the interaction of LPS–ChoP and rPAF contributes to the ability of *H. influenzae* to attach to host epithelial cells. *H. influenzae* Rd, expressing LPS–ChoP, was more efficient than the controls lacking this feature at adhering to D562 pharyngeal cells. Moreover, an rPAF antagonist caused a dose-related decrease in adherence, an effect that required the presence of bacterial ChoP. The concentration of PAF-Ra required to observe a decrease in adherence was significantly greater than that previously reported to be sufficient to inhibit invasion [15]. The addition of PAF-Ra was also sufficient to eliminate most, but not all, attachment to these cells. This is most likely because of the presence of other *H. influenzae* adhesins, although strain Rd, used in this study, does not express most of the known adhesins described for this species, including high-molecular-weight proteins (HMW1 and HMW2), Hia, Hap, and fimbriae [44].

Our findings are consistent with earlier reports showing that bacterial ChoP allows for binding to rPAF [14, 15]. CRP appears to inhibit only that portion of attachment mediated by this interaction. rPAF appears to be widely distributed in the human lung and other tissues [45, 46]. The availability of this receptor has been noted on A549 cells derived from type II pneumocytes lining the alveolar spaces and used in this study [47].

The environmental niche for both *S. pneumoniae* and *H. influenzae* is the mucosal surface of the upper respiratory tract. In this regard, we demonstrate that D562 cells derived from the epithelial surface of the pharynx, as well as the epithelial surface of the human nasopharynx, *in situ* express this receptor. Since it is predominantly the phenotypic variants expressing cell-surface ChoP that colonize the human upper airway, where CRP is also present, the additional observation that rPAF is expressed on the mucosal surface at this site suggests that the antiadhesive effects of CRP may be a factor in limiting colonization [17, 20, 29].

If CRP is important in maintaining the balance between the host and microbe in the upper airway, the situation in the lower airway may be different. Surfactant, found in airway-surface fluid in alveolae but not in the oro- and nasopharynx, contains abundant quantities of ChoP and readily binds to CRP [30, 33]. Although the precise concentration of surfactant in the alveolar spaces has not been determined, on the basis of amounts in the whole rat lung and its volume of distribution, it is estimated that the concentration of the phospholipid component of surfactant is 5 mg/mL, a level far in excess of that shown in this study to block the antiadhesive activity of CRP (A. Fisher, personal communication). In this respect, we show that surfactant effectively blocked the antiadhesive activity of CRP for both *S. pneumoniae* and *H. influenzae*. This CRP-inhibiting effect of surfactant was

initially shown by using D562 cells and then demonstrated for epithelial cells derived from the alveolae (A549 cells). These results suggest that surfactant may potentiate bacterial attachment in the lower airway. We propose that surfactant may not always have a protective function, as reported elsewhere for several respiratory pathogens [3–6], but may in some situations inactivate the innate defense involving CRP and allow ChoP-expressing bacteria to adhere to and gain access to the lower airway. This effect of surfactant could, at least in part, provide an explanation for the prominence of organisms expressing cell-surface ChoP among the etiologic agents of bacterial pneumonia.

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