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An Alternative Role of C1q in Bacterial Infections: Facilitating *Streptococcus pneumoniae* Adherence and Invasion of Host Cells

Vaibhav Agarwal, * Jonas Ahl, † Kristian Riesbeck, † and Anna M. Blom*

*Streptococcus pneumoniae* (pneumococcus) is a major human pathogen, which evolved numerous successful strategies to colonize the host. In this study, we report a novel mechanism of pneumococcal–host interaction, whereby pneumococci use a host complement protein C1q, primarily involved in the host-defense mechanism, for colonization and subsequent dissemination. Using cell-culture infection assays and confocal microscopy, we observed that pneumococcal surface-bound C1q significantly enhanced pneumococcal adherence to and invasion of host epithelial and endothelial cells. Flow cytometry demonstrated a direct, Ab-independent binding of purified C1q to various clinical isolates of pneumococci. This interaction was seemingly capsule serotype independent and mediated by the bacterial surface-exposed proteins, as pretreatment of pneumococci with pronase E but not sodium periodate significantly reduced C1q binding. Moreover, similar binding was observed using C1 complex as the source of C1q. Furthermore, our data show that C1q bound to the pneumococcal surface through the globular heads and with the host cell-surface receptor(s)/glycosaminoglycans via its N-terminal collagen-like stalk, as the presence of C1q N-terminal fragment and low m.w. heparin but not the C-terminal globular heads blocked C1q-mediated pneumococcal adherence to host cells. Taken together, we demonstrate for the first time, to our knowledge, a unique function of complement protein C1q, as a molecular bridge between pneumococci and the host, which promotes bacterial cellular adherence and invasion. Nevertheless, in some conditions, this mechanism could also be beneficial for the host as it may result in uptake and clearance of the bacteria. *The Journal of Immunology*, 2013, 191: 4235–4245.

Although humans have a highly developed defense mechanism, during evolution majority of human bacterial pathogens including the Gram-positive human species *Streptococcus pneumoniae* (the pneumoccus) has developed multiple evasion strategies to counteract the host. Pneumococci colonize asymptomatically the human upper respiratory tract. However, depending upon the host susceptibility, pneumococci can cause mild local infections such as otitis media, sinusitis, as well as severe life-threatening invasive diseases such as lobar pneumoniae, sepsis, and meningitis (1). The burden of pneumococcal infections is highest among the youngest and elderly population and in patients with immunodeficiency.

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Abbreviations used in this article: C4BP, C4b-binding protein; CPS, capsular polysaccharide; CRP, C-reactive protein; FH, factor H; GMFI, geometric mean fluorescence intensity; GVB++, gelatin-veronal buffer; HBEpC, primary human bronchial epithelial cell; NHS, normal human serum; THY, Todd-Hewitt-Yeast extract broth.

Pneumococci have evolved numerous successful strategies such as expression of a variety of virulence factors to colonize its host (2, 3). These virulence factors function either as adhesins that interact directly with the host cell-surface receptors or use host protein(s) as a molecular bridge between bacteria and the host. These host proteins include extracellular matrix and serum proteins such as fibronectin, thrombospondin, vitronectin, plasminogen, factor H (FH), and C4b-binding protein (C4BP) (4–9). Pneumococcal interactions with complement inhibitors FH or C4BP protect bacteria from complement-mediated attacks (6, 10). In addition, association of FH promotes pneumococcal adherence to and invasion of host cells (4, 7).

The human complement system is a major part of the innate immune system and the first line of defense against invading pathogens (11). Complement system consists of ∼35 proteins, and depending on the initiating agent, it can be classified into three different pathways (classical, lectin, and alternative). The alternative pathway is activated by autoactivation of the complement component C3 and its subsequent deposition on the surface of the pathogen, whereas specific carbohydrates and acetylated ligands on the bacterial surface initiate the lectin pathway. Binding of the first component of complement C1 to the Ig-s recognizing invading pathogens or C-reactive protein (CRP) usually triggers the classical pathway. All three pathways lead to the formation of the C3 and C5 convertases with subsequent deposition of opsonin C3b, release of proinflammatory anaphylatoxin C3a and C5a, and finally formation of a lytic membrane attack complex, which disrupts membrane integrity.

The complement component C1 is a multimolecular complex composed of C1q, the recognition molecule, and two Ca2+-dependent dimeric serine proteases, Clr and Cls (tetramer Clr2-Cls2) (12). C1q is a hexamer of three protein subunits—A, B, and C—with molecular masses of 29, 26, and 22 kDa, respectively, and...
consists of an N-terminal collagen-like stalk and a C-terminal globular protein head. C1q, as part of the C1 complex, is central to activation of the classical complement pathway, which is initiated upon recognition of Ag-aggregated Ig (IgM and IgG) Fc domains by the C-terminal globular heads. Moreover, C1q can also recognize CRP, serum amyloid P component protein, and pentraxin (13–15). This initiates sequential activation of C1q-bound serine protease Clr and Cls, which in turn mediates proteolysis and activation of C4 and C2 and to the formation of C3 and C5 convertases.

The direct activation of the classical pathway by pneumococci is not unique, as direct and Ab-independent binding of C1q was demonstrated also for group B streptococci (16), Salmonella minnesota (17), Escherichia coli (18, 19), Klebsiella pneumoniae (20), Legionella pneumophila (21), and Streptococcus pyogenes (22). In this study, we have investigated the Ab-independent binding of C1q to the respiratory tract pathogen S. pneumoniae. We demonstrate for the first time, to our knowledge, a unique function of complement protein C1q as a molecular bridge between pneumococci and the host that promotes pneumococcal cellular adherence and invasion.

Materials and Methods

Cultivation of pneumococci

All pneumococcal strains used in this study, except for NCTC10319 and D39, were clinical isolates collected at the Clinical Microbiology laboratory, Skåne University Hospital (Malmö, Sweden) (Table I). Pneumococci were cultured on blood agar plates at 37°C and 5% CO2, or in Todd-Hewitt-broth (Oxoid) supplemented with 0.5% yeast extract (Todd-Hewitt-Yeast extract broth [THY]) to a density of 5 × 10^8 CFU ml^-1 (OD600 of ~0.5). The pneumococcal D39 strain deficient in capsular polysaccharide (CPS) was kindly provided by Prof. Sven Hammerschmidt (Department of Genetics, University of Greifswald, Greifswald, Germany). All other bacterial species used in this study (Table II) were cultured on chocolate agar plates at 37°C and 5% CO2 atmosphere.

Proteins and Abs

C1q was purified from human plasma as described (23). The stalk and head regions of C1q were prepared by partial proteolytic digestion of intact C1q as described (24, 25). Pepsin (Worthington Biochemicals) was used for preparation of the stalk region and purified collagenase (Clostridium histolyticum, code CLSPA) for preparation of the head region was purchased from the same company. C1- and C1q-depleted serum were purchased from Complement Technology. Pronase E and sodium periodate (NaIO4) were purchased from Sigma-Aldrich, whereas low m.w. heparin was from the National Institute for Biological Standards & Control. Rabbit anti-human C1q and FITC-conjugated C1q Abs from DakoCytomation, whereas polyclonal rabbit Abs against S. pneumoniae were purchased from NordicBioSite.

Normal human serum (NHS) was prepared from freshly drawn blood obtained from six healthy volunteers with informed consent and permission of the ethical board of Lund University. The pooled blood was allowed to clot for 30 min at room temperature and then incubated for 1 h on ice. After two centrifugations, the serum fraction was frozen in aliquots and stored at −80°C. C1q purified from the plasma was added to the depleted serum to compensate for the C1q that bound to the IgG coupled to the protein G column. The concentration of C1q in NHS and IgG-depleted NHS was determined using ELISA.

Flow cytometry analysis of C1q binding to bacteria

Binding of C1q to viable pneumococci or other bacterial species was measured using flow cytometry. Bacteria (1 × 10^8) either cultivated in THY (for pneumococci) or collected directly from culture plates were incubated with purified C1q, at indicated concentrations, in PBS for 1 h at 37°C. For investigating the binding from serum, bacteria were incubated with gelatin-veronal buffer (GVB+) (5 mM veronal buffer [pH 7.3], 140 mM NaCl, 0.1% porcine gelatin, 1 mM MgCl2, and 0.15 mM CaCl2) 1 h at 37°C or depleted incubation, bacteria were washed, and bound C1q was detected with FITC-conjugated rabbit anti-human C1q Abs (DakoCytomation). To investigate the role of pneumococcal surface–exposed proteins or bacterial cell glycoconjugates, pneumococci were treated with Pronase E and NaIO4, respectively, as described previously (9). Briefly, bacteria (1 × 10^7 CFU/ml) were treated with 100 μl 1 mg/ml Pronase E or 5 μl 1 mg/ml NaIO4, the concentration that does not affect bacterial viability, for 15 min at 37°C, followed by washing with PBS and used in the C1q binding assay. Finally, bacteria were fixed using 1% paraformaldehyde (Sigma-Aldrich), and the flow cytometry analysis was performed using CyFlow space (Partec) to detect the binding of C1q. Bacteria were detected using log-forward and log-side scatter dot plot, and a gating region was set to exclude debris and larger aggregates of bacteria. A total of 15,000 bacteria/events were analyzed for fluorescence using log-scale amplifications. The geometric mean fluorescence intensity (GMFI) was used as a measure for binding activity.

Cell lines and culture conditions

Cultivation of host cell lines was performed as described (7). Briefly, human A549 cells (lung alveolar epithelial cells, type II pneumocytes; American...
Type Culture Collection) were cultured in DMEM (PAA Laboratories) supplemented with 10% of heat-inactivated FCS (Invitrogen), 2 mM glutamine (PAA Laboratories), penicillin G (100 units ml\(^{-1}\)) and streptomycin (0.1 mg ml\(^{-1}\); both from Hyclone) at 37°C under a 5% CO\(_2\) atmosphere. Detroit 562 cells, human nasopharyngeal epithelial cells (American Type Culture Collection), were cultivated in RPMI 1640 supplemented with 10% FCS, 2 mM glutamine, and 1 mM sodium pyruvate (PAA Laboratories). NCI-H292, human lung epithelial cells (American Type Culture Collection), were cultivated in RPMI 1640 (HyClone) supplemented with 10% FCS. HUVECs were obtained from Invitrogen and cultivated in M200 media supplemented with low serum growth supplement (Invitrogen). Primary human bronchial epithelial cells (HBBePc) were purchased from PromoCell and cultured according to the instructions of the manufacturer. The cells were used for experiments between passages 1 and 5.

**Pneumococcal host cell adherence and invasion assay**

A549, NCI-H292, or Detroit 562 cells were seeded at a density of 5 × 10\(^4\) cells/well in plain medium either on 24-well tissue culture plates (Nunc) or glass coverslips (diameter 12 mm) when assayed by immunofluorescence and cultivated for 48 h. HBBePc and HUVECs were seeded at a density of 3 × 10\(^4\) cells/well. Confluent monolayers were washed thoroughly and infected for 3 h with pneumococci in 500 µl respective medium, supplemented with 1% FCS for A549, Detroit-562, and NCI-H292 cells only (infection medium) at 37°C using a multiplicity of infection of 25. Bacteria were incubated for 20 min with plasma purified C1q (10 µg) in a total volume of 100 µl infection medium at 37°C prior to infections. The infection assays were carried out in a total volume of 500 µl after adding the bacteria. Postinfection, cells were washed three times with PBS to remove unbound bacteria. The total number of adherent and intracellular recovered bacteria was monitored after detachment and lysis of cells with saponin (1.0% w/v) and plating the bacteria on blood agar. The number of viable intracellular bacteria was quantified by employing the antibiotic protection assay (4). Briefly, epithelial or endothelial cells were incubated with pneumococci (multiplicity of infection of 25) preincubated with or without C1q. After 3 h, the infected cells were washed and further incubated for 1 h with infection medium containing 100 µg ml\(^{-1}\) gentamicin (Sigma-Aldrich) and 100 units ml\(^{-1}\) penicillin G (Sigma-Aldrich) at 37°C and 5% CO\(_2\) to kill extracellular and nonadherent pneumococci. Invasive and viable pneumococci were recovered from the intracellular compartments of the host cells by a saponin-mediated host cell lysis (1.0% w/v), and the total number of invasive pneumococci was monitored after plating sample aliquots on blood agar plates, followed by colony formation and enumeration. In inhibition experiments, infection assays were carried out in the presence of C-terminal blocking sites were blocked with 10% FCS, and before incubating, the cells with pneumococcal Abs (1:100), the infected cell layer was thoroughly washed with PBS. Bound Abs were detected with an Alexa Fluor–labeled goat anti-rabbit Ig conjugate (Invitrogen). The glass coverslips were embedded upside down in mounting media (DakoCytomation), sealed with nail polish, and stored at 4°C. A confocal laser scanning microscope (Zeiss LSM 510 META; Carl Zeiss), and the appropriate software was used for the image acquisition.

**Fluorescence microscopy**

Pneumococci attached to host epithelial or endothelial cells were stained using polyclonal pneumococcal Abs (IgG) (Nordic Biosite) in combination with a secondary goat anti-rabbit IgG coupled with Alexa Fluor 488 (green) or Alexa Fluor 546 (red) (Invitrogen) (26). Postinfection, nonspecific binding sites were blocked with 10% FCS, and before incubating, the cells with pneumococcal Abs were incubated with NHS (40%) for 60 min at 37°C in a total volume of 3 ml containing 5 × 10\(^7\) CFU in NHS w/o C1q, and C1q-depleted serum replenished with 10 µg/ml of purified C1q (NHS w/o C1q + 10 µg/ml C1q) (B) or with NHS and IgG-depleted serum (C) for 60 min at 37°C. The bacteria were then washed and incubated with FITC-conjugated anti-C1q Abs followed by flow cytometry analysis. One representative dataset from three independent experiments is shown in (B), whereas the percentage binding of C1q from three independent experiments is shown in (C). Statistical significance was calculated using Student t test. (D) Dose-dependent binding of plasma-purified C1q to pneumococci as determined by flow cytometry. GMFI is shown as mean of three independent experiments ± SD as a measure of C1q binding. One-way ANOVA test was performed to calculate statistical difference of C1q binding compared with Ab control. (E) Percentage binding of (5 µg/ml) plasma-purified C1q or C1 complex to pneumococci. Statistical significance was calculated using Student t test. ***p < 0.001.
Additionally, binding of C1q to pneumococci was investigated from NHS, C1q-depleted serum, and C1q-depleted serum replenished with 10 μg/ml of purified C1q. Flow cytometry analysis revealed the binding of C1q to pneumococci from NHS, whereas no such deposition was observed from the C1q-depleted serum (Fig. 1B). However, replenishing the depleted serum with plasma purified C1q restored the binding to the level of NHS (Fig. 1B).

Taken together, the data suggest that C1q interacts with pneumococci but do not clarify the nature of this interaction, whether it is direct or mediated via the known ligands of C1q such as IgG. Therefore, the binding of C1q from NHS was compared with the binding from IgG-depleted NHS. Surprisingly, depletion of IgG did not affect significantly the binding of C1q when compared with NHS (100%). The binding from IgG-depleted serum was just moderately reduced to 80.7 ± 13.5%, indicating that the interaction of C1q with pneumococci from NHS does not require bacterial surface-bound IgGs and could therefore be a direct interaction (Fig. 1C). To confirm this, binding of purified C1q to pneumococci was tested at concentrations <80 μg/ml present in serum. Flow cytometric analysis indicated a dose-dependent binding of plasma-purified C1q (Fig. 1D).

Finally, we compared the binding efficiency of plasma-purified C1q with that of C1q as a part of the C1 complex. As observed, compared with the binding of free C1q (100%), the binding of whole C1 complex to pneumococci was only slightly reduced (74.1 ± 17.9%) (Fig. 1E). Taken together, the results suggest that both purified and as part of C1 complex, C1q binds directly and in an Ab-independent manner with pneumococci.

Interference of the pneumococcal capsular polysaccharides with binding of C1q

It has been previously shown that the pneumococcal CPS interferes with pneumococcal interaction with host cells and protein (5, 7, 9, 27). To elucidate whether CPS affects the interaction of C1q with pneumococci, we compared the binding of C1q to S. pneumoniae strain D39 (serotype 2) and its isogenic CPS-deficient strain D39Δcps. Flow cytometric analysis indicated a dose-dependent binding of plasma-purified free C1q to the pneumococcal strains used. However, the results suggested a significantly increased binding of C1q to the capsule knockout strain D39Δcps compared with the wild-type strain D39 (Fig. 2A), suggesting that the CPS masks the bacterial surface-exposed ligand(s) of C1q.

Characterization of C1q ligand on the pneumococcal surface

To characterize the bacterial ligand(s) recognized by C1q, S. pneumoniae were treated with pronase E or sodium periodate followed by plasma-purified C1q, and the binding was investigated by flow cytometry. Oxidation of pneumococcal glycoconjugates by periodate treatment did not affect C1q binding (Fig. 2B, 2D, 2E). In contrast, proteolytic pretreatment of pneumococci with pronase E significantly reduced the binding of C1q (Fig. 2B, 2C, 2E). These findings suggest that the bacterial receptor recognized by C1q is C1q itself, and periodate treatment of pneumococci on C1q binding. To characterize the bacterial receptor recognized by C1q, S. pneumoniae, 1×10^7 CFU, were treated with or without pronase E (1 mg/ml) or sodium periodate (NaIO4) (1 mg/ml) for 15 min at 37°C. The bacteria were washed and the binding of C1q (5 μg/ml) to untreated (none) (B), NaIO4 pretreated (C), and pronase E (D) pretreated was investigated. Representative flow cytometry histograms of binding of C1q are shown. (E) The GMFI values of C1q binding to untreated and pretreated pneumococci are shown. Results are presented as the means ± SD for at least three independent experiments. One-way ANOVA test was performed to calculate statistical difference with in groups for C1q binding. *p < 0.05 compared with in the absence of C1q.
data suggest that the surface-exposed proteins function as the major pneumococcal ligand for C1q.

C1q binding to pneumococci is serotype independent

The CPSs are essential virulence factor of pneumococci, and to date, >91 serologically distinct CPSs have been described (28, 29). However, not all serotypes cause serious infections. Therefore, we wanted to study whether the binding of C1q is dependent on the capsule serotype. Collection of 50 clinical isolates of S. pneumoniae (42 blood isolates and 8 nasopharyngeal isolates; Table I) belonging to 13 different serotypes was analyzed. Flow cytometry analyses demonstrated that although all the clinical isolates bind C1q to variable extents, there is no statistically significant correlation between the degree of C1q binding and particular serotypes (Fig. 3). Taken together, the data suggest that the binding of C1q is a general mechanism that is not influenced by the capsular serotype but that the capsule does restrict the interaction as revealed by the CPS-deficient S. pneumoniae D39Δcps (Fig. 2A).

C1q facilitates pneumococcal adhesion and invasion of host epithelial and endothelial cells

C1q is primarily involved in the initiation of classical complement pathway by recognizing immune complexes. However, lately there have been reports suggesting that the complement proteins have additional functions in conjunction to their role in host defense. For example, upregulation of C1q in the CNS has a novel neuroprotective effect (30). Recently, it has been shown that in addition to other serum factors, C1q is also involved in the entry of Bacillus anthracis spores into epithelial cells (31). Therefore, we asked if C1q could have an additional role in pneumococcal–host interactions.

To elucidate whether pneumococci can exploit the surface-bound C1q as a molecular bridge between host cells and bacteria, infection experiments were performed with nasopharyngeal epithelial cells. Pretreatment of pneumococci (NCTC10319) with plasma-purified

FIGURE 3. C1q binding to various clinical isolates of S. pneumoniae. Binding of C1q (10 μg/ml) to various clinical isolates, blood isolates, and nasopharyngeal isolates (NP) of S. pneumoniae representing different serotypes was analyzed by flow cytometry. Results were expressed as GMFI × percentage of gated bacteria as a measure of C1q binding.

FIGURE 4. Pneumococcal surface-bound C1q mediates adhesion and invasion of human epithelial cells. (A) Adherence of S. pneumoniae strain NCTC10319 was determined after cell culture infection assay on epithelial cells Detroit 562, A549, and NCI H292. The infection assays were conducted with or without preincubation of pneumococci with C1q. Results are shown as the fold increase in the adherence of pneumococci that were pretreated with C1q relative to untreated pneumococci (B). Immunofluorescence microscopy of adherent pneumococci. Scale bars, 10 μm. Invasion and intracellular survival of S. pneumoniae NCTC10319 in host cells Detroit 562 (C), A549 (D), and NCI H292 (E) as determined by the antibiotic protection assay. (F) C1q-mediated adherence of pneumococcal strain NCTC10319 to HBEpC as determined by cell-culture infection assay. Student t test was performed to calculate statistical differences compared with untreated bacteria. (G) Immunofluorescence microscopy of adherent pneumococci to endothelial cells. Scale bars, 10 μm. (H) Invasion and intracellular survival of pneumococci were determined by the antibiotic protection assay. Student t test was performed to determine the statistical difference between the groups. Results present the means ± SD of at least three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001 relative to infections carried out in the absence of C1q.
C1q significantly increased adherence of pneumococci to Detroit 562 (Fig. 4A, 4B). Similarly, bacterial adherence was significantly increased to A549 and NCI H292 lung epithelial cells (Fig. 4A, 4B).

In addition, the internalization of *S. pneumoniae* into epithelial cells was quantified. After the infection, the attached bacteria were killed by antibiotics treatment and intracellular pneumococci were recovered. In accordance to the increased adherence, C1q treatment significantly increased the number of internalized pneumococci (Fig. 4C–E). To further assess the biological significance of C1q-mediated pneumococcal adherence and invasion, primary human airway epithelial cells were infected for 3 h with *S. pneumoniae* pretreated with or without C1q. The presence of C1q significantly increased the bacterial adherence and invasion of HBEpC (Fig. 4F–H). Furthermore, we tested whether a similar mechanism is functional for endothelial cells. Preincubation of bacteria with C1q significantly enhanced both adherence and invasion of HUVECs (Fig. 5A–C). Taken together, our infection experiments showed that C1q acts as a molecular bridge between host cells and pneumococci and hence facilitates adherence and invasion in both epithelial and endothelial cells.

**Intracellular fate of C1q-mediated invasive pneumococci**

To assess the role of C1q in survival or killing of internalized pneumococci postinfection, the intracellular fate of pneumococci were analyzed. The A549 lung epithelial cells were infected with pneumococci pretreated with or without C1q, and postinfection, extracellular and nonadherent pneumococci were killed by antibiotic treatment. After removing the antibiotics, the viability of intracellular pneumococci was investigated for the indicated time points by plating sample aliquots on blood agar plates. At the time point zero, C1q-treatment significantly increased the number of internalized pneumococci compared with untreated bacteria. Further, a significant decrease in the number of viable intracellular bacteria was observed for both C1q pretreated and nonpretreated pneumococci with increasing time postinfection. However, at all time points, there was a tendency for the presence of more intracellular bacteria when these were treated with C1q (Fig. 6). Taken together, the data indicate that the number of recovered and viable pneumococci decreases time-dependently when pneumococci are internalized by host cells independent of the presence of C1q.

**Role of C1q globular heads and collagenous tail in pneumococcal adherence**

To identify the orientation in which the C1q molecule binds pneumococci and thus mediates adherence, cell–culture inhibition experiments were performed using purified C-terminal globular heads or the N-terminal collagen-like stalks of C1q as a specific competitor. The Detroit-562 epithelial cells were preincubated with or without C1q collagen-like stalks or C1q globular heads (both at 2 μg/well) prior to infection with pneumococci that had been incubated in the presence or absence of C1q. As observed, the presence of C1q collagen-like stalks blocked the C1q-mediated increase in pneumococcal adherence (Fig. 7A), whereas the presence of C1q globular heads showed no inhibition. In conclusion, C1q interacts with the pneumococcal surface exposed proteins likely via the C-terminal globular heads, whereas the increase in adherence is mediated by the interaction of C1q N-terminal collagen-like stalk with the host cell-surface receptor(s).

**Influence of heparin on C1q-mediated pneumococcal adherence to host cells**

A previous experiment demonstrates the pivotal role played by the N-terminal collagen-like stalk of C1q in facilitating pneumococcal adherence. Moreover, C1q interacts with heparin via its N-terminal collagen-like stalk (32). Therefore, we investigated the role of small m.w. heparin on C1q-mediated adherence of pneumococci to human lung epithelial cells A549. Competitive inhibition experiments were performed in the presence of soluble heparin (10 μg/ml). Presence of heparin significantly inhibited C1q-mediated pneumococcal adherence to epithelial cells, whereas the basal level of adherence in the absence of C1q was not affected (Fig. 7B).

To demonstrate that the inhibition observed is not a consequence of altered C1q binding to pneumococci in the presence of heparin, the impact of heparin on C1q binding was analyzed by using heparin as a competitor in a binding experiment. Flow cytometry analysis

![FIGURE 5](http://www.jimmunol.org/)

**FIGURE 5.** Pneumococcal surface-bound C1q facilitates adhesion and invasion of human endothelial cells. (A) C1q-mediated adherence of pneumococcal strain NCTC10319 to HUVECs as determined by cell–culture infection assay. One-way ANOVA test was performed to calculate statistical difference compared with untreated bacteria. (B) Immunofluorescence microscopy of adherent pneumococci to endothelial cells. Scale bars, 10 μm. (C) Invasion and intracellular survival of pneumococci were determined by the antibiotic protection assay. Student t test was performed to determine the statistical difference between the groups. Results present the means ± SD of at least three independent experiments. **p < 0.01, ***p < 0.001 relative to infections carried out in the absence of C1q.

![FIGURE 6](http://www.jimmunol.org/)

**FIGURE 6.** Intracellular fate of C1q-coated internalized pneumococci. Intracellular fate of pneumococci as determined by the enumeration of the viable invasive bacteria. Unbound extracellular bacteria were washed away 3 h postinfection, and after killing of extracellular pneumococci by antibiotic (time point 0), the infections were continued for the indicated time points. The results are expressed as CFU recovered per well of 24-well plate (mean ± SD) obtained from two independent experiments performed in duplicates. Statistical significance between the C1q-treated and untreated pneumococci was calculated using two-way ANOVA test, whereas one-way ANOVA test was used to calculate statistical significance within the respective group. *p < 0.05, **p < 0.01, ***p < 0.001.
demonstrated that the acquisition of C1q by pneumococci is not influenced by soluble heparin (Fig. 7C). In conclusion, the presence of heparin did not interfere with pneumococcal acquisition of C1q but blocks the N-terminal collagen-like stalk of C1q, which is involved in C1q-mediated adherence.

**C1 bound to pneumococcal surface is functionally active**

Because our data indicate that C1q interacts via its C-terminal globular heads with the pneumococcal surface-exposed proteins and because activation of the classical complement pathway is initiated upon binding of the globular heads with the Igs, we tested if the C1 complex bound to pneumococci would also be functionally active. Pneumococci were preincubated with purified C1 complex prior to their incubation with purified C4. The functional activity of the bound C1 complex was determined by flow cytometry after incubation of 10 μg/ml plasma-purified C1q with indicated amounts of heparin per reaction. Bacterial-bound C1q was determined by flow cytometry, and results were expressed as GMFI of FITC-labeled bacteria. One-way ANOVA test was used to calculate statistical significance. Pneumococcal surface-bound C1 complex is functionally active. Bacteria were preincubated with or without C1 complex (2 μg/ml) for 20 min at 30°C. After washing, C4 (5 μg/ml) was added and incubated for 20 min at 37°C. Thereafter, deposited C4/C4b was detected using anti-C4c and Alexa 488–conjugated secondary Ab by flow cytometry. As positive control, bacteria were preincubated with 0.25% NHS, whereas bacteria treated with only C4 or C1 complex were used as negative control. (D) A representative flow cytometry histogram from three independent experiments is shown. (E) The GMFI values of C4/C4b deposition on pneumococci are shown. One-way ANOVA test was used to calculate statistical significance. ***p < 0.001.
complex–pretreated pneumococci. This could be attributed to some level of nonspecific binding of the detection Abs. Nevertheless, the C4b deposition signal obtained in the presence of both C1 and C4 was significantly higher than that of C4 or C1 complex alone. In addition, pneumococci pretreated with NHS, corresponding to ∼10-fold less C1 complex concentration, showed an ∼4-fold increase in C4b deposition. However, this enormous increase in C4b deposition could also be due to the naturally occurring anti-pneumococcal Abs as well as complement activation via the lectin pathway. Taken together, our data indicate that the C1 bound to the pneumococcal surface is functionally active and can initiate a basal level of classical complement pathway activation.

C1q binding and its effect on host cell adherence of other pathogenic bacteria

To test whether, besides pneumococci, other pathogenic bacteria also have the ability to directly bind C1q, we investigated the binding of three clinical isolates of Gram-positive (S. pyogenes) and Gram-negative (E. coli) pathogens to plasma-purified C1q by flow cytometry (Table II). In addition, C1q binding was also analyzed for Moraxella catarrhalis RH4 and nontypeable Haemophilus influenzae (NTHi 3655) strains (Table II). Similar to pneumococci, flow cytometry analysis indicated a significant binding of C1q to all the clinical isolates of S. pyogenes and all but one of the E. coli isolates (Fig. 8A). In addition, we demonstrated for the first time, to our knowledge, the direct and Ab-independent binding of C1q to M. catarrhalis RH4 and nontypeable H. influenzae (Fig. 8A).

To determine whether these other bacterial species can also use C1q as a bridging molecule to facilitate adherence to host cells, cell-culture infection assays were carried out using A549 cells. The presence of C1q did not enhance the number of host cell–associated bacteria, as determined by counting the CFU obtained after plating the total number of cell-associated and recovered intracellular bacteria (Fig. 8B). Taken together, the data indicate that even though the direct binding of C1q is a generalized host defense mechanism against the invading pathogens, its ability to function as a bridging molecule that facilitates bacterial host cell colonization is rather specific for pneumococci (Fig. 9).

Discussion

Colonization of the host is a critical step in the pathogenesis of any infectious agent. Therefore, one of the most promising strategies in controlling pathogens is to have a detailed understanding of the molecular mechanism of interaction and identification as well as selective targeting of the essential colonization factor(s) that facilitates colonization and subsequent dissemination. The pneumococcus is a major human pathogen, affecting both adults and children, and has developed multiple strategies such as expression of adhesins or acquisition of various host proteins to facilitate colonization (2–5, 7–9, 33). In this study, we describe a novel mechanism of pneumococcal–host interaction, in which pneumococci use a host protein, C1q, primarily involved in the host-defense mechanism (complement system), for colonization and subsequent internalization of host cells. We demonstrate that C1q interacts directly in an Ab-independent manner with pneumococci and functions as a bridging molecule, facilitating pneumococcal

Table II. Other Gram-positive and Gram-negative bacterial strains used in this study

<table>
<thead>
<tr>
<th>Strains</th>
<th>Reference No</th>
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<tbody>
<tr>
<td>KR714</td>
<td>E. coli</td>
</tr>
<tr>
<td>KR715</td>
<td>E. coli</td>
</tr>
<tr>
<td>KR716</td>
<td>E. coli</td>
</tr>
<tr>
<td>KR717</td>
<td>S. pyogenes</td>
</tr>
<tr>
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<td>S. pyogenes</td>
</tr>
<tr>
<td>KR719</td>
<td>S. pyogenes</td>
</tr>
<tr>
<td>M. c. RH4</td>
<td>M. catarrhalis RH4</td>
</tr>
<tr>
<td>NTHi 3655</td>
<td>Nontypeable H. influenzae</td>
</tr>
</tbody>
</table>

FIGURE 8. Binding of human C1q to other Gram-positive and Gram-negative pathogens and its impact on host cell adherence. (A) Binding of C1q (10 μg/ml) to various clinical isolates of S. pyogenes and E. coli and M. catarrhalis and nontypeable H. influenzae was analyzed by flow cytometry. Results were expressed as GMFI × percentage of gated bacteria as a measure of C1q binding. One-way ANOVA test was used to calculate statistical significance. (B) Adherence of other Gram-positive and Gram-negative pathogens was determined after cell-culture infection assay on A549 lung epithelial cells. The infection assays were conducted with or without preincubation of pathogens with C1q. Results are shown as the fold increase in the adherence of pathogen that were pretreated with C1q relative to untreated bacteria. Statistical significance was calculated using two-way ANOVA test. ***p < 0.001.

FIGURE 9. Schematic model of the C1q-mediated pneumococcal adherence and invasion of host cells. C1q interacts via its C-terminal globular heads with pneumococcal surface-exposed proteins and with host cell-surface receptor(s)/glycosaminoglycans via its N-terminal collagen-like stalk, thereby facilitating pneumococcal adherence and invasion of host cells.
adherence to and invasion of host epithelial and endothelial cells (Fig. 9).

Complement is a crucial part of innate immunity, protecting us from infections and cleaning the body from unwanted debris such as dying cells. C1q plays an important role during the activation of the classical complement pathway by recognizing the Ag–Ab complex via its C-terminal globular heads. In case of pneumococcal infections, the activation of the classical pathway has been suggested to be a major host-defense mechanism (34). Moreover, classical pathway activities have been detected in the lung and the airway epithelium (35, 36). Apart from the naturally occurring Abs, acute-phase proteins such as CRP and serum amyloid P component protein that bind both S. pneumoniae and C1q have been suggested to play a role in complement-mediated immunity against this pathogen (37–39). Additionally, a new mechanism of complement activation has been identified involving C-type lectin receptor SIGN-R1, present on the surface of macrophages and interacting with pneumococcal polysaccharides and C1q (40). Thus, complement plays an important role against pneumococcal infection both at the early stages of colonization as well as in the bacteremic phase of infection. However, pneumococci have evolved numerous strategies for attenuation or escaping such attacks, and these mechanisms are one of the key determinants for their survival within the human host. These include recruitment of complement inhibitors such as C4BP or FH, which inhibit the classical and alternative pathway activation, respectively (6, 7, 41), or the expression of thick capsule, which not only inhibits the deposition of C3b but also complement-mediated opsonophagocytosis (42).

There have been reports describing direct binding of C1q to pathogens, independent of the presence of recognizing Abs or other ligands (16–22). Accordingly, direct and Ab-independent binding of C1q was observed for clinical isolates of E. coli, S. pyogenes, M. catarrhalis, and nontypeable H. influenzae. Therefore, we tested whether a similar Ab-independent C1q binding is also relevant in pneumococci, and if yes, does it have any additional function distinct from its conventional role in host defense. Indeed, we show direct and dose-dependent binding of plasma-purified C1q to pneumococci. In agreement with this, depletion of IgG from NHS did not significantly diminish the interaction of C1q with pneumococci, suggesting that the presence of specific anti-pneumococcal Abs may not be pivotal for the basal level of classical complement pathway initiation but rather to augment the effect. In conjunction, even though C4b deposition on pneumococci in the presence of purified C1 complex and C4 was detected, it was significantly lower compared with NHS alone. Another role of this direct C1q-pneumococcal interaction may be to influence the complement-mediated immune responses at initial stages of infection, perhaps immediately following pulmonary exposure to pneumococci.

The C-terminal globular head region of C1q functions as a pattern-recognition molecule and mediates binding to various molecules exposed on the surface of apoptotic cells such as DNA and calreticulin (43, 44) and thus acts as a bridging molecule between apoptotic cells and APCs such as macrophages and dendritic cells (45–47). Although, a number of C1q ligands have been characterized on apoptotic cells, the list is relatively short in the case of bacterial pathogens. The only reported bacterial ligand of C1q is the OmpK36 porin of K. pneumoniae (20), whereas still uncharacterized outer membrane proteins have been suggested to be the ligands for other bacterial species (16, 17, 19, 21, 22). In accordance, in this study, we showed that C1q interacts via its C-terminal globular heads primarily with the pneumococcal surface-exposed protein(s). To corroborate this, higher C1q binding was observed in a D39Δcps strain deficient in CPS as compared with the wild-type D39 strain, as the deletion of the capsule gene locus might result in higher availability of uncovered bacterial surface proteins, which would otherwise be masked by the CPS. Our data are in agreement with the study of group B streptococci, in which the target is probably not the CPS, but rather protein(s) (48). Indeed, we could not find any significant difference in C1q binding among the tested clinical isolates of pneumococci, representing common serotypes causing infections. This suggests that the binding of C1q to pneumococci is a general phenomenon independent of the capsule serotype; however, one role of the capsule seems to protect the bacteria against direct deposition of C1q on their surface.

Some of the recently published studies have indicated that the complement proteins have functions in addition to their role in host defense. For example, C1q alone promotes ingestion of apoptotic cells by phagocytes, modulates inflammation, and promotes neuronal survival (30, 49). Similarly, complement inhibitor FH bound to the surface of pneumococci plays a dual role in pneumococcal infection. On one hand, it promotes pneumococcal adherence and invasion of mucosal surfaces, while on the other hand, in invasive infection, it improves survival by inhibiting complement activation (4, 7). Consistent with this, we showed that C1q bound to the pneumococcal surface significantly enhanced the adherence and internalization of host epithelial and endothelial cells. A somewhat similar role for C1q has been suggested in the entry of spores of B. anthracis into epithelial cells, where C1q is involved in linking of spores surface protein BclA with host cell receptors via its collagen-like stalk region (31). However, the C1q did not augment the host cell adherence of other Gram-positive and -negative bacteria, as has been the case for pneumococci. Furthermore, our data show that C1q bind to the surface of pneumococci through the globular head region and with the host cell-surface receptor(s) via its N-terminal collagen-like stalk, as the presence of C1q N-terminal fragment and not the C-terminal globular heads blocked C1q-mediated pneumococcal adherence to host cells. Additionally, C1q-mediated adherence of pneumococci to host cells was inhibited by heparin. Taken together, the data suggest that the interaction of C1q-coated pneumococci is mediated by the interaction of the heparin-binding site on the N-terminal collagen-like stalk region with cell-surface receptor(s) such as glycosaminoglycans (32). It is indeed intriguing, whether under in vivo conditions; C1 bound to pneumococcal surface would favor complement activation or C1q-mediated bacterial colonization and internalization. Because complement is an important arm of innate immunity, it is highly likely that the protective role of C1q resulting in complement activation and opsonization of the pathogen would be preferred. However, considering the fact that pneumococci have other mechanisms to circumvent the complement attack, they can simultaneously afford to use C1q as a bridging molecule for colonization and internalization of host cells. Taken together, C1q has a dual role in pneumococcal infections, one as complement activator/opsonin and second as a bridging molecule. In line with this, our data suggest that the C1q-mediated pneumococcal uptake by host cells may also promote the intracellular clearance of bacteria, thus facilitating host protection. Most likely, both outcomes are possible, but under certain conditions, one will prevail. However, to dissect whether and when the direct C1q binding mainly facilitates pneumococcal pathogenesis or promotes host protection, additional in vivo studies are necessary.

In conclusion, C1q interacts directly even in the absence of specific Abs to S. pneumoniae. This binding results from the interaction between the bacterial surface-exposed protein(s) and C-terminal globular head of C1q, as is evident by the fact that C1 bound to pneumococci is functionally active. In contrast, the enhanced bacterial adherence and invasion of host cells is mediated by the N-terminal collagen-like stalk, which interacts with the host
cell-surface receptor(s)/glycosaminoglycans. Our study demonstrates that complement protein C1q, which is primarily involved in the initiation of the classical complement pathway, indeed has an auxiliary role as a molecular bridge and hence mediates pneumococcal adhesion to host cells.

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Disclosures

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References


