**Bordetella pertussis** Binds the Human Complement Regulator C4BP: Role of Filamentous Hemagglutinin

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C4BP (C4b-binding protein) is a high-molecular-weight plasma protein that inhibits the classical pathway of complement activation. Recent experiments have demonstrated that C4BP binds to many strains of the gram-positive bacterium *Streptococcus pyogenes*, a major respiratory tract pathogen. Binding to *S. pyogenes* was shown to be due to members of the M protein family, a group of surface proteins important for virulence. Here we report that human C4BP also binds to all clinical isolates of the gram-negative bacterium *Bordetella pertussis*, the etiologic agent of whooping cough. In addition, binding of C4BP was demonstrated for other *Bordetella* species that can cause disease in humans. Characterization of different *B. pertussis* mutants showed that the binding of C4BP is strongly dependent on the expression of the cell surface protein filamentous hemagglutinin, a well-known virulence factor. Inhibition experiments suggested that *B. pertussis* and *S. pyogenes* bind to the same region in C4BP. The finding that *B. pertussis* and *S. pyogenes* both have the ability to bind human C4BP suggests that these two unrelated respiratory tract pathogens may use a common mechanism during the establishment of an infection.

The complement system is an important part of the human defense against pathogenic microorganisms. Activation of this system by a pathogen may result in opsonization for phagocytosis, release of anaphylatoxins, and formation of a membrane attack complex (12). Excess activation is inhibited by various regulatory proteins, fluid phase and cell bound, which protect the host from complement attack. Many of these regulators are members of a family of proteins, the RCA (regulators of complement activation) proteins, which regulate the C3 convertases and interact with fragments of C3 and/or C4. The RCA proteins include the plasma proteins C4BP (C4b-binding protein) and factor H and the membrane proteins CR1, CR2, CD46 (MCP), and CD55 (DAF) (22).

Several pathogenic microorganisms are known to bind to host cells via RCA proteins or to bind RCA proteins present in plasma. For example, Epstein-Barr virus binds to CR2, measles virus binds to CD46, many echoviruses bind to CD55, and human immunodeficiency virus type 1 binds factor H (2, 6, 15, 41). Among bacteria, strains of the gram-positive bacterium *Streptococcus pyogenes* (group A streptococcus), a major respiratory tract pathogen, have been reported to use CD46 as a cellular receptor and to bind factor H or C4BP (21, 32, 41). The binding of C4BP to CD46 (MCP), and CD55 (DAF) (22).

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Our studies focus on interactions between bacteria and the plasma protein C4BP, a ~570-kDa protein with a spider-like structure, that is composed of seven α-chains and one β-chain (5, 8). The main biological function of C4BP is believed to be its ability to inhibit the C3 convertase (C4b2a) of the classical pathway. C4BP causes dissociation of this convertase and also acts as a cofactor in the degradation of C4b by the plasma protease factor I. In addition, C4BP may also have some inhibitory effect on the C3 convertase of the alternative pathway (39).

In *S. pyogenes*, the binding of C4BP was shown to be due to members of the M protein family, a family of surface proteins important for virulence (41). When bound to the bacterial surface, C4BP retained its inhibitory activity, suggesting that the bacteria may use this ligand for protection against complement attack. Interestingly, the binding of C4BP takes place in a hypervariable N-terminal region of the bacterial surface proteins, a region that is believed to be a major target for the immune system (24).

Here we demonstrate that the gram-negative bacterium *Bordetella pertussis*, the etiologic agent of whooping cough, also binds C4BP. Thus, the unrelated respiratory tract pathogens *B. pertussis* and *S. pyogenes* both have the ability to bind C4BP. The binding of C4BP to *B. pertussis* was found to be strongly dependent on the expression of the surface protein filamentous hemagglutinin (FHA), a major virulence factor of this bacterium.

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions.** Clinical isolates of *B. pertussis* were obtained from the Clinical Microbiology Laboratory at Lund University Hospital. All other *B. pertussis* strains used in this study are listed below (see Table 1). Strains of other *Bordetella* species were obtained from the collections at the National Institute of Health and the Environment, Bilthoven, The Netherlands, and from the Culture Collection, Department of Clinical Bacteriology, University of Göteborg, Göteborg, Sweden. Strains of *B. pertussis* were grown at 37°C for 2 days on Bordet-Gengou plates supplemented with 10 g of peptone per liter and 10 ml of glycerol per liter, and strains of *Bordetella parapertussis* and *Bordetella bronchiseptica* were grown on the same type of plates for 1 day. Strains of *Bordetella holmesii*, *Bordetella hinzii*, and *Bordetella avium* were cultured on horse blood plates for 1 to 2 days. Fifteen isolates of *S. pyogenes*, from cases of human septicemia, and 10 isolates of *Haemophilus influenzae*, from throat cultures, were obtained from the Clinical Microbiology Laboratory at Lund University Hospital. The *S. pyogenes* strains were cultured overnight at 37°C in Todd-Hewitt broth (Difco, Detroit, Mich.), and the *H. influenzae* strains were grown on chocolate agar plates. The *Escherichia coli* col strain LE392, used as a negative control in binding studies, was grown overnight at 37°C in LB medium. Liquid cultures of *B. pertussis* were cultivated in modified Stainer-Scholte medium (19).

**Proteins.** Purified preparations of human C4BP and C4(H2O) were the kind gift of Bjørn Dahlbäck, Department of Clinical Chemistry, Lund University, Malmö General Hospital, Malmö, Sweden. C4BP (free of protein S) was pre-

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pared from plasma as described previously (7, 20), and C4(H2O) was prepared from C4 (4), which was repeatedly frozen and thawed to hydrolyze the internal thiolester. Unlike C4b, the C4(H2O) molecule still contains the C4a part of the molecule. However, C4(H2O) is functionally equivalent to the unstable C4b in its interactions with C4BP and was used instead of this molecule (42). Two preparations of purified FHA (220 kDa) were used; one of these was obtained from List Laboratories (Campbell, Calif.), and the second was kindly provided by Hans Hallander, Swedish Institute for Infectious Disease Control, Stockholm. Streptococcal protein Arp4, a member of the M protein family, was purified as described previously (11, 24). Bovine serum albumin was from Sigma (St. Louis, Mo.). Proteins were radiolabeled with carrier-free125I (Amersham International, Little Chalfont, United Kingdom) by the chloramine-T method (16).

Binding assays. Bacteria were cultivated as described above and washed in PBSAT (0.12 M NaCl, 0.03 M phosphate, 0.02% NaN3, 0.05% Tween 20 [pH 7.2]), and the bacterial concentration was adjusted to 1010 organisms/ml. Different bacterial concentrations of Bordetella strains were achieved by dilution in a suspension of the non-C4BP-binding E. coli strain LE392. Duplicate samples of bacteria (200 μl) were mixed with 25 μl of 125I-C4BP (~2 ng, 12,000 cpm) and incubated at room temperature for 90 min. After the addition of PBSAT (2 ml), the samples were centrifuged at 3,100 × g for 20 min, allowing efficient sedimentation of the bacteria, and the supernatants were discarded. Radioactivity in the pellets was determined, and binding was expressed as the percentage of radioactivity added that remained in the pellet. The average of the duplicate samples was calculated, and nonspecific binding (~4%), recorded with strain LE392, was subtracted.

To analyze the binding of C4BP to B. pertussis as a function of time, duplicate samples of 100 μl of bacterial suspension (1010 bacteria) were mixed with 25 μl of 125I-C4BP (~2 ng, 12,000 cpm) and the mixture was incubated for different time periods. After the addition of PBSAT (1.5 ml), the samples were centrifuged and binding was determined as described above. Reversibility of the binding was analyzed by adding 30 μg of unlabeled C4BP to samples incubated for 100 min.

Inhibition experiments. To analyze whether the binding of C4BP to B. pertussis can be inhibited by known C4BP-binding proteins, samples of C4(H2O) and streptococcal protein Arp4 were mixed with radiolabeled C4BP (25 μl, ~2 ng, 12,000 cpm) and incubated for 30 min, and the mixture was then used for binding assays with B. pertussis strain BP338 as described above. Bovine serum albumin and unlabeled C4BP were used as negative and positive controls, respectively. All inhibitors were added in equimolar amounts (10.6 pmol), and duplicate samples were analyzed in each experiment.

RESULTS

C4BP binds to B. pertussis. The binding of C4BP to clinical isolates of B. pertussis was compared to the binding of C4BP to two other respiratory tract pathogens, S. pyogenes and H. influenzae (Fig. 1A). In agreement with previously published data, strains of H. influenzae did not bind C4BP, whereas most strains of S. pyogenes bound the ligand (41). All strains of B. pertussis bound C4BP, but the fraction bound was smaller than that of those S. pyogenes isolates that bound C4BP. In addition to the 18 B. pertussis isolates for which results are shown in Fig. 1A, another 28 clinical isolates were analyzed and were all found to bind C4BP, indicating that all strains of B. pertussis bind the ligand. These binding assays were performed with bacteria harvested from Bordet-Gengou plates, but bacteria cultured in the synthetic medium of Stainer-Scholte (19) also bound C4BP, excluding that the binding of the ligand was mediated by some factor present in the plates.

When the binding of radiolabeled C4BP to wild-type B. pertussis was analyzed as a function of time, maximum binding was reached after about 60 min (Fig. 1B). To analyze whether B. pertussis binds native C4BP, an excess of unlabeled C4BP was added to samples before incubation with bacteria, which strongly reduced the binding. However, the lack of complete inhibition suggests that a minor fraction of the binding observed with radiolabeled C4BP does not represent binding of native C4BP. The addition of unlabeled C4BP after 100 min of incubation resulted in dissociation of about 10% of the bound material, indicating that the binding is largely irreversible. This finding might be explained by the spider-like structure of the C4BP molecule, which has seven α-chains that could provide several binding sites and cause binding of very high avidity to the bacterial surface.

Binding assays with other species of the genus Bordetella showed that B. parapertussis, B. holmesii, and B. bronchiseptica, all of which have been isolated from humans (33), also bind human C4BP (Fig. 2). The highest degree of binding was observed with strains of B. parapertussis. Isolates of the bird pathogen B. avium and the closely related B. hinzii were unable to bind human C4BP.

The binding of C4BP to B. pertussis is controlled by the bvg locus: role of FHA. In an attempt to identify the C4BP-binding component(s) of B. pertussis, strains carrying mutations in genes for known virulence factors were tested for binding ability. Representative experiments are shown in Fig. 3, and binding data for all mutants tested are summarized in Table 1.

The expression of most virulence factors of B. pertussis is regulated by a single genetic locus, bvg (3). A mutant affected in this locus completely lacked ability to bind C4BP (Fig. 3A). Mutants affected in different bvg-controlled genes were therefore analyzed. Binding was normal in mutants lacking pertussis toxin, adenylate cyclase toxin, pertactin, fimbriae, and tracheal colonization factor but was strongly reduced in strains lacking FHA.
It was of particular interest to analyze the C4BP-binding ability of B. pertussis mutants affected in the bvg-regulated brk locus, which contributes to complement resistance in B. pertussis (9). Since brk mutants have been described to be more sensitive to classical pathway complement-dependent killing than wild-type B. pertussis, it seemed possible that the brk locus was responsible for the residual binding observed with fha mutants. However, mutants affected in any of the two linked brk genes, brkA and brkB, bound C4BP as well as the wild type did, indicating that the brk locus is not involved in the binding of C4BP to B. pertussis (Fig. 3B).

The binding assays described above suggested that C4BP binds to FHA on the surface of B. pertussis. However, it was not possible to demonstrate binding of C4BP to purified FHA by use of Western blot analysis or assays in which either ligand was immobilized in microtiter wells.

Inhibition experiments with other C4BP-binding ligands. The spider-like C4BP molecule was originally discovered due to its ability to bind C4b and to inhibit the classical pathway C3 convertase (14, 38). C4BP is composed of seven α-chains and one β-chain, and the binding site for the C4b molecule has been mapped to the most amino-terminal short consensus repeat of the eight short consensus repeats of the C4BP α-chain (1, 18). C4BP also binds many proteins in the streptococcal M protein family, including the well-characterized protein Arp4 (24, 41). Recent evidence indicates that the streptococcal proteins and C4b bind to the same or overlapping site(s) in the C4BP α-chain (1). To analyze whether B. pertussis binds to the same region of C4BP as C4b and streptococcal proteins do, inhibition experiments were performed (Fig. 4). In agreement with the data reported above (Fig. 1B), the binding of radiolabeled C4BP to B. pertussis could be strongly, but not completely, inhibited by unlabeled C4BP. The inhibition could not be increased by the addition of fivefold-larger amounts of unlabeled C4BP (data not shown). Interestingly, both C4(H2O) (functionally equivalent to C4b) and streptococcal protein Arp4 inhibited the binding of C4BP to B. pertussis, suggesting that C4b, Arp4, and B. pertussis bind in the same region of the C4BP α-chain.

Since the data reported above indicate that two surface structures of B. pertussis, one of which is FHA, contributes to
the binding of C4BP, it was of interest to test whether the residual C4BP binding of fha mutants (Fig. 3) could be inhibited by C4(H2O) or Arp4. This residual binding was indeed inhibited by C4(H2O) and Arp4, indicating that the second, and unknown, C4BP-binding surface structure of B. pertussis interacts specifically with C4BP (data not shown). This finding also implies that the second C4BP-binding surface component binds to the same region in the C4BP α-chain as the other ligands.

**DISCUSSION**

In this report, we have described a novel interaction between pathogenic bacteria and the human complement system, the ability of B. pertussis to bind the complement regulator C4BP. Previous studies have shown that C4BP binds to most strains of S. pyogenes (24, 41). Except for being important respiratory tract pathogens, B. pertussis and S. pyogenes have little in common. Since B. pertussis is a gram-negative bacterium and S. pyogenes is a gram positive one, the cell walls of these bacteria are very different. Yet both of these pathogens have the ability to bind human C4BP.

It could be argued that respiratory tract pathogens like B. pertussis and S. pyogenes would normally not come into contact with serum proteins or complement regulators like C4BP. However, several studies have indicated that complement proteins and regulators are indeed present in the human respiratory tract (17, 34), suggesting that pathogens like B. pertussis and S. pyogenes would encounter such molecules in the course of the infectious process.

The C4BP-binding ligands of S. pyogenes are members of the M protein family, a group of extensively studied surface proteins implicated in virulence (26). Similarly, the binding of C4BP to B. pertussis depends on the expression of a virulence factor, in this case, the surface protein FHA, a high-molecular-weight molecule that is structurally unrelated to the streptococcal M proteins (28). Although it has not yet been possible to demonstrate binding between purified FHA and C4BP, our data on the binding ability of different B. pertussis mutants are most easily explained by a direct binding of C4BP to FHA, when FHA is present on the bacterial cell surface. It should be noted that the FHA-negative mutants used in our binding assays with bacteria lacked expression of the entire FhaB gene.
product, which is synthesized as a 367-kDa precursor molecule. However, for binding assays with purified FHA, it was only possible to use the mature 220-kDa protein, which corresponds to the N-terminal two-thirds of FhaB (30). It therefore seems possible that the binding of C4BP to B. pertussis is due to the C-terminal third of FhaB.

Comparison of different Bordetella species (Fig. 2) supports the conclusion that C4BP binds to FHA, since FHA-related molecules have been described for B. pertussis, B. parapertussis, and B. bronchiseptica (28), all of which bind C4BP. In contrast, B. avium, which causes disease in birds and expresses a hemagglutinin unrelated to FHA, did not bind C4BP. Concerning the other two Bordetella species studied here, B. holmesii and B. hinzii, it is not known whether they express FHA-related molecules.

Since C4BP inhibits complement activation, in particular, activation via the classical pathway, the ability of B. pertussis and S. pyogenes to bind C4BP suggests that these pathogens use C4BP for the prevention of complement attack. Indeed, C4BP bound to the surface of S. pyogenes retains its complement inhibitory activity (41). The result of such inhibition close to S. pyogenes should be reduced formation of C4b and C3b and reduced opsonization. In B. pertussis, binding of C4BP would be expected to contribute to virulence mainly through reduced formation of the membrane attack complex, to which this gram-negative bacterium is sensitive, unlike the gram-positive bacterium S. pyogenes (25). However, attempts to demonstrate that C4BP on the surface of B. pertussis retains its inhibitory function have to date yielded inconclusive results, possibly because B. pertussis binds fewer C4BP molecules per cell than S. pyogenes.

Apart from protecting B. pertussis against complement attack, bacterium-bound C4BP could contribute to virulence by other mechanisms. For example, the spider-like C4BP molecule could promote adhesion by binding simultaneously to a bacterium and a human cell (41). C4BP might also modify coagulation near the bacterial cell, due to its ability to bind the plasma component protein S, which has antiocoagulant properties (8). It also seems possible that the binding of C4BP to B. pertussis reflects a potential ability of this bacterium to bind other members of the RCA protein family; e.g., B. pertussis not only binds C4BP but also uses CD46 as a cellular receptor, as reported for S. pyogenes (32).

The C4BP molecule, which owes its name to its ability to bind C4b, has now been described to bind to the surface of two important human pathogens, S. pyogenes and B. pertussis. The binding site for C4b in C4BP has been mapped to the N-terminal part of the a-chain, and recent studies indicate that surface proteins of S. pyogenes also bind to this region (1, 18). Moreover, the inhibition experiments reported here indicate that B. pertussis binds to the same region of C4BP as C4b and streptococcal proteins do (Fig. 4). These results suggest that the different ligands that bind to the a-chain might have sequence similarities. However, streptococcal proteins bind to C4BP via an N-terminal region that is highly variable, excluding any sequence similarity with the other ligands (24). With regard to FHA, computer-assisted analysis did not demonstrate any obvious sequence similarities between this molecule and C4b. The structural basis for the ability of these different ligands to bind to C4BP therefore remains to be determined. In this context, it should be noted that overlapping binding sites in C4BP, for C4b and a bacterial ligand, would not exclude that bacterium-bound C4BP retains its ability to bind C4b, since some of the a-chains in the spider-like C4BP molecule might bind to the bacterial surface, while other a-chains bind C4b.

In summary, we have shown that all clinical isolates of B. pertussis bind the human complement inhibitor C4BP. This ability is controlled by the regulatory locus bvg and is strongly dependent on the expression of FHA, a major virulence factor and a component of novel acellular vaccines against B. pertussis (35).

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