Characterization of *Bartonella bacilliformis* Flagella and Effect of Antiflagellin Antibodies on Invasion of Human Erythrocytes

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Bartonella bacilliformis is the etiologic agent of Oroya fever in humans. Flagellum-mediated motility has been postulated as a major virulence factor for invasion of host cells. To address this hypothesis, we purified and characterized flagella from strain KC584 and then assessed their role in human erythrocyte association and invasion. Electron microscopy of the flagellar preparation showed a high concentration of filaments with a mean wavelength of 800 nm. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis, immunoblot analysis, and KBr density gradient centrifugation indicated that the flagellar filament is composed of a polypeptide of 42 kDa. The flagellin is partially (ca. 50%) resistant to treatment with trypsin. The first 17 amino acid residues of the N terminus of the mature flagellin protein are GAAILTNDNAMDALQDL and show approximately 46% sequence identity to the residues of the N termini of two *Caulobacter crescentus* flagellin proteins. A monospecific polyclonal antibody to the flagellin protein was generated, and its specificity was verified by both immunoblot and immunogold analyses. Human erythrocyte invasion assays performed with bartonellae exposed to the antiflagellin antiserum showed a significant decrease in bacterial association with and invasion of human erythrocytes in comparison with that in bartonellae exposed to preimmune rabbit serum or phosphate-buffered saline (PBS) controls. These results suggest that flagella are an important component in the invasiveness of *B. bacilliformis*.

Bartonella bacilliformis is an intracellular bacterial parasite of humans. The agent is transmitted by the bite of nocturnal sand flies of the *Phlebotomus* genus and is endemic to the Andes regions of Peru, Ecuador, and Columbia (15, 23). Thus far, only humans have been identified as reservoirs for the bacterium (8). The disease caused by this organism is known as Carrión's disease and manifests itself in two distinct phases. In the primary (hematic) phase of the disease, nearly all of the circulating erythrocytes are invaded, culminating in a severe hemolytic anemia which reduces the density of erythrocytes by nearly 80% (8). In the preantibiotic era, this phase of the disease, known as Oroya fever, was fatal in approximately 40% of cases (15). In the secondary (tissue) phase of the disease, organisms which have invaded capillary endothelial cells induce cellular proliferation (7), resulting in hemangioma-like nodules, termed verruga peruana, on the extremities and face. This phase of the disease is rarely fatal and presents within 1 to 2 months of the primary infection (7, 23). Recovery from bartonellosis is complete, but persistent carrier states sometimes occur (6). *B. bacilliformis* is placed in the *Rickettsiales* family on the basis of its 16S rRNA sequence homology (4, 5), cell size, and route of transmission (4). However, within this family, *B. bacilliformis* is uniquely motile, possessing 1 to 10 polar flagella 3 to 10 μ m in length (15).

The mechanism by which *B. bacilliformis* gains entry into erythrocytes has not been studied extensively. Previous studies showed that there is a bacterial energy requirement for host cell invasion to occur, indicating that the

organism plays an active role in the process (26). It has also been shown that treatment of host cells with cytochalasin D partly reduces the efficiency of invasion of human dermal fibroblasts, laryngeal epithelium, and umbilical vein endothelium, suggesting that the host cell may play a microfilament-dependent role in the uptake of bartonellae (12). *B. bacilliformis* also produces an extracellular proteinaceous factor that deforms the erythrocyte membrane, causing deep invaginations (17). This deformation factor may be associated with the ability of the bacterium to invade erythrocytes. However, even with deformation of the erythrocyte membrane, bartonellae cannot invade unless they are motile (17). It is likely that flagellum-based motility and the deformation factor act in concert to facilitate entry into erythrocytes.

To gain a better understanding of the factors necessary for host cell invasion, we isolated and characterized the flagellin of *B. bacilliformis* and determined that antiflagellin antibodies significantly decrease the ability of the organism to associate with and gain entry into human erythrocytes.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *B. bacilliformis* KC583 and KC584 were obtained from the American Type Culture Collection, Rockville, Md. Bacteria were routinely grown on heart infusion agar (Difco Laboratories, Detroit, Mich.) supplemented with 5% defibrinated sheep erythrocytes and 5% sheep serum (Colorado Serum Co., Denver) at 30°C in a water-saturated atmosphere. Growth was usually observed by 3 days, and cultures were used at 5 days postinoculation for experiments.

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Purification of flagella. The contents of approximately 50 petri plates of *B. bacilliformis* KC584 were harvested into 25 ml of phosphate-buffered saline (PBS) (pH 7.4), placed in a 50-ml centrifuge tube, and vortexed vigorously for 2 min. This preparation was centrifuged at $6,000 \times g$ for 25 min at 4°C to remove cellular debris. The supernatant was then centrifuged at $100,000 \times g$ in an SW28 rotor (Beckman Instruments, Fullerton, Calif.) for 3 h at 4°C . The resulting pellet was resuspended in 0.5 ml of distilled water. This crude flagellar preparation (CFP) was observed by transmission electron microscopy and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (16). For further purification, the CFP was fractionated with KBr density gradients (0.5 g of KBr per ml of H_2O) as described by Kalmokoff et al. (14). Approximately 125 μg of protein from the CFP was layered onto a KBr gradient and centrifuged for 24 h at $260,000 \times g$ in a Beckman SW60 rotor at 4°C . Two visible bands were subsequently removed from the gradient by aspiration and analyzed by transmission electron microscopy and SDS-PAGE with silver staining.

Trypsin treatment. The trypsin sensitivity of the flagellin was determined by incubating 10 μl (approximately 30 μg of total protein) of intact *B. bacilliformis* KC584 cells suspended in Hanks buffered saline solution (HBSS) with high-pressure liquid chromatography-purified trypsin (Sigma Chemical Co., St. Louis, Mo.) at a final concentration of 2 mg/ml for 2 h at 30°C . Molecular mass standards (0.5 μg of total protein) were similarly digested with trypsin as a positive control, and bartonellae incubated in HBSS without trypsin served as a negative control. The reaction was stopped by adding 20 μl of Laemmli sample buffer (16), and the entire reaction mixture was run in SDS-PAGE and stained with Coomassie brilliant blue. SDS-polyacrylamide gels of the trypsin digest were also analyzed by use of immunoblots developed with rabbit anti-flagellin antiserum to confirm the identity of the trypsin-resistant 42-kDa protein band on SDS-polyacrylamide gels. Densitometry scans of Coomassie brilliant blue-stained gels were performed with an E-C 910 densitometer (E-C Apparatus Corp., St. Petersburg, Fla.) interfaced with a Perkin-Elmer LCI-100 laboratory computing integrator (Perkin-Elmer, Norwalk, Conn.).

SDS-PAGE and immunoblotting. SDS-PAGE was carried out by the method of Laemmli (16) with 10 or 12.5% (wt/vol) polyacrylamide gels. To visualize protein bands, the gels were stained with silver (27) or Coomassie brilliant blue (22). Immunoblotting was carried out essentially as described by Towbin et al. (25). Unfixed SDS-polyacrylamide gels were blotted to nitrocellulose (0.45- μm -pore size; Schleicher & Schuell, Inc., Keene, N.H.) in 20 mM sodium phosphate buffer (pH 8.0). The blots were electrophoresed overnight at a constant voltage of 15 V with constant cooling. The nitrocellulose was incubated for 1 h at 22°C in blocking solution (PBS [pH 7.4], 0.3% [vol/vol] Tween 20, 2% [wt/vol] nonfat milk) and then with rabbit anti-flagellin or rabbit anti-*B. bacilliformis* antiserum at a 1:2,000 dilution in PBS (pH 7.4)–0.3% Tween 20 for 2 h. The blots were subsequently washed five times in PBS for 10 min and then incubated for 1 h with a 1:2,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Sigma) in PBS. The blots were washed five times in PBS for 10 min and then developed in a solution containing 50 ml of PBS, 10 ml of 4-chloro-1-naphthol in methanol (3 mg/ml), and 75 μl of 30% H_2O_2 . Development was stopped by rinsing

FIG. 1. Comparison of the CFP of *B. bacilliformis* with bacterial cell lysates on Coomassie brilliant blue-stained SDS-polyacrylamide gels (10% acrylamide). Lanes: 1, molecular mass standards in kilodaltons; 2, *B. bacilliformis* KC583 whole-cell lysate; 3, *B. bacilliformis* KC584 whole-cell lysate; 4, CFP isolated from strain KC584. The arrowhead indicates the position of the 42-kDa band identified as flagellin.

the blots thoroughly in distilled water. The blots were stored in the dark to prevent fading of the bands.

N-terminal sequencing. A preparative SDS-polyacrylamide gel was run with a Tricine running buffer system (0.1 M Tris base, 0.1 M Tricine, 0.1% SDS). The gel was blotted to an Immobilon polyvinylidene difluoride membrane (Millipore Corp., Bedford, Mass.) as described above for the immunoblot procedure. The membrane was then fixed for 30 min in a fixer containing 25% isopropanol and 7% acetic acid, stained for 30 min in this fixer containing 0.25% Coomassie brilliant blue, and destained in this fixer until the bands were visible. The membrane was subsequently rinsed several times in distilled water, and the 42-kDa flagellin band was removed and sequenced on a model 473A automated protein sequencer (Applied Biosystems, Foster City, Calif.).

FIG. 2. Immunoblot analysis of a *B. bacilliformis* cell lysate and the CFP with anti-*B. bacilliformis* antiserum. (A) Coomassie brilliant blue-stained SDS-polyacrylamide gel (12.5% acrylamide). (B) Corresponding immunoblot. Lanes: 1, molecular mass standards in kilodaltons; 2, *B. bacilliformis* KC584 whole-cell lysate; 3, CFP. The arrows indicate the 42-kDa flagellin band.

FIG. 3. (A) Coomassie brilliant blue-stained SDS-polyacrylamide gel (12.5% acrylamide) containing trypsin-treated flagella. Lanes: 1, molecular mass standards in kilodaltons (0.5 μ g); 2, the same molecular mass standards digested for 2 h at 30°C with trypsin at 2 mg/ml; 3, whole-cell lysate of intact KC584 cells incubated as in lane 4 but without trypsin; 4, trypsin digest (2 h at 30°C with 2 mg of trypsin per ml) of intact KC584 cells showing the residual flagellin band. (B) Corresponding immunoblot developed with rabbit anti-flagellin antiserum. The arrows indicate the 42-kDa flagellin band.

Transmission electron microscopy. Samples (10 μ l) were placed on Formvar-coated grids and allowed to incubate for 15 min at 22°C. The grids were then rinsed twice for 2 min each time with distilled water and negatively stained with 0.33% (wt/vol) aqueous uranyl acetate for 15 s. The grids were then air dried and observed at 75 kV with a model 7100 transmission electron microscope (Hitachi, Mountain View, Calif.).

Antibody production. To prepare monospecific polyclonal antiserum to the flagellin, the 42-kDa flagellar filament protein was isolated from the CFP by preparative SDS-PAGE. The CFP (100 μ g) was run on preparative gels and stained with Coomassie brilliant blue, and the 42-kDa flagellin protein band was removed with a razor blade, minced, and emulsified with 0.5 ml of PBS–0.5 ml of Freund's incomplete adjuvant (Sigma). The sample was subcutaneously injected into an adult male New Zealand White rabbit at three depots above the shoulder. After 3 weeks, the rabbit was given a booster of an equal quantity of the flagellin sample in a similar manner. Antiserum was collected 2 and 3 weeks following the booster and stored in 0.5-ml aliquots at –20°C until used.

To prepare anti-*B. bacilliformis* antiserum, bacteria were suspended in sterile PBS containing formalin (0.1% [vol/vol]) to a final density of approximately 10^9 cells per ml and incubated for 4 h at 22°C. Bacterial death was ascertained by plate counts. An adult male New Zealand White rabbit was injected intravenously with approximately 3×10^8 cells of the formalin-killed bacteria. Six injections were administered at 48-h intervals. Antiserum was collected 2 weeks following the sixth injection.

Immunogold assays. Immunogold assays were performed by a modification of the procedure of Timmerman et al. (24). In brief, approximately 3 mg (wet weight) of *B. bacilliformis* was resuspended in 0.5 ml of heart infusion broth containing

FIG. 4. KBr density gradient fractionation of the CFP. (A) Silver-stained SDS-polyacrylamide gel (12.5% acrylamide) containing fractions from the KBr gradient. Lanes: 1, molecular mass standards in kilodaltons; 2, CFP; 3, band 1 (low-density band) from the KBr gradient; 4, band 2 (high-density band) from the KBr gradient. The arrow indicates the 42-kDa flagellin band. (B) Transmission electron micrograph of KBr band 2 showing a high density of flagella.

0.5 ml of 0.5% glutaraldehyde–2% paraformaldehyde in PBS (pH 7.4) and allowed to incubate for 1 h at 22°C. The cells were pelleted at $1,500 \times g$ in a microcentrifuge for 10 min, decanted, resuspended in 0.5 ml of 50 mM glycine in PBS, and incubated for 1 h. The cells were pelleted, resuspended in 0.45 ml of PBG (0.5% bovine serum albumin [BSA]–1% gelatin in PBS), and incubated for 1 h. The cells were pelleted, resuspended in 0.45 ml of PBG with 20 μ l of rabbit anti-flagellin serum, and incubated for 1 h. The cells were rinsed four times in 0.5 ml of PBG for 10 min each time and resuspended in 0.45 ml of protein A-gold buffer (0.15 M NaCl [pH 7.0], 0.5% BSA, 0.5% Tween 20). To this mixture, 20 μ l of protein A-gold (Sigma) was added and incubated for 1 h. The cells were washed four times for 10 min each time in 0.5 ml of PBG and then four times for 10 min each time in distilled water. Finally, the bacteria were negatively stained and observed by transmission electron microscopy.

Invasion assays. One petri plate of *B. bacilliformis* was overlaid with 5 ml of liquid bartonella growth medium (3) for 5 min at 22°C. A 1-ml aliquot of the growth medium was gently removed, and the bacteria were enumerated on a Petroff-Hausser cell counter. Approximately 10^5 bacteria were placed into microcentrifuge tubes, adjusted to a final volume of 0.1 ml, and treated with 0.1 ml of rabbit anti-flagellin serum, PBS (pH 7.4), or preimmune rabbit serum. After 1 h, human erythrocytes (American Red Cross) which had been washed twice in PBS-GI (PBS supplemented with 10 mM glucose and 5 mM inosine) were added to the tubes to yield a bacterium/erythrocyte multiplicity-of-infection ratio of 500:1. The tubes were then centrifuged at $1,500 \times g$ for 5

FIG. 4—Continued.

min to increase the association of the bacteria with the erythrocytes and incubated for 10 h at 30°C to allow for invasion.

For enumeration of bacteria associated with erythrocytes via Giemsa staining, 0.1 ml of each invasion reaction mixture was centrifuged at $1,500 \times g$ on a 70% Percoll gradient as described by Benson et al. (3) to separate free bacteria from

erythrocytes after the 10-h incubation. Gradients were made by adding 0.3 ml of 0.51 M NaCl to 0.7 ml of Percoll (Sigma) and centrifuging the mixtures at $10,000 \times g$ for 10 min in a microcentrifuge. The gradients were briefly stored at 4°C until needed. The erythrocyte band was removed from the gradients with a syringe, and the residual Percoll was removed from the erythrocytes by three brief washes in

FIG. 5. Immunoblot analysis showing the specificity of the anti-flagellin antiserum for a *B. bacilliformis* cell lysate and the CFP. (A) Coomassie brilliant blue-stained SDS-polyacrylamide gel (12.5% acrylamide). (B) Corresponding immunoblot developed with the anti-flagellin antiserum. Lanes: 1, molecular mass standards in kilodaltons; 2, CFP; 3, *B. bacilliformis* KC584 whole-cell lysate. The arrows indicate the 42-kDa flagellin band.

PBS-GI, with centrifugations between the washes. Erythrocyte-associated bacteria were observed and counted by light microscopy following Giemsa staining.

Enumeration of intracellular bacteria was achieved by a modification of an invasion assay described by Jones et al. (13). In brief, 10-h invasion reaction mixtures (multiplicity of infection ratio, 5:1) were pelleted at $1,500 \times g$, resuspended in PBS-GI containing gentamicin sulfate (250 $\mu\text{g/ml}$ [Sigma]), and incubated for 3 h at 30°C. This antibiotic kills only extracellular bacteria, since it cannot enter erythrocytes. The cells were then pelleted at $1,500 \times g$ in a microcentrifuge and briefly washed three times in PBS-GI, with centrifugations between the washes. The pelleted cells were resuspended in 0.2 ml of sterile PBS, twofold serially diluted, lysed by adding 3 volumes of sterile distilled water, and plated. Colonies representing intracellular (invasive) bacteria were observed after 8 days of incubation.

Statistical analysis. Numerical values are expressed as means $\pm$ standard errors of the means. Statistical significance of the data was ascertained by use of Student's *t* test. A *P* value of <0.05 was considered significant.

RESULTS

Isolation and identification of the flagellin protein. When the CFP of *B. bacilliformis* was visualized on Coomassie brilliant blue-stained SDS-polyacrylamide gels two prominent bands of 33 and 42 kDa plus minor bands of 34, 45, and 65 kDa were fractionated from the total cell lysate (Fig. 1). Electron microscopic examination of the CFP revealed that it contained a high density of flagellar filaments with a mean wavelength of 800 nm, suggesting that one of the major protein bands was the flagellar filament protein.

Since flagella are known to be highly antigenic polypeptides, immunoblot analyses with rabbit anti-*B. bacilliformis* antiserum were performed to identify highly reactive proteins in the CFP. Figure 2B shows the proteins recognized by the anti-*B. bacilliformis* antiserum in both the bartonella whole-cell lysate (lane 2) and the CFP (lane 3). Along with a

variety of proteins, the 42-kDa protein was strongly recognized in both the *B. bacilliformis* whole-cell lysate and the CFP.

Invasion of erythrocytes by *B. bacilliformis* was previously shown to be resistant to trypsin treatment (17). Since motility appears to play a central role in invasion, we hypothesized that the trypsin resistance of the flagellin protein might account for this observation. Indeed, digestion of intact bartonellae with trypsin showed that a 42-kDa protein of the bacterium was partially resistant (ca. 50%, as determined by densitometry) to degradation by trypsin, whereas the majority of the proteins in the gel were digested (Fig. 3A). Immunoblot analysis of the trypsin digest with rabbit anti-flagellin antiserum subsequently confirmed the identity of the 42-kDa protein band as flagellin (Fig. 3B).

With these data, we tentatively identified the 42-kDa protein band in the CFP as the flagellin protein. To confirm this identification, additional purification steps were taken. Further purification of flagella was achieved by fractionation of the CFP by KBr buoyant density gradient centrifugation. KBr density gradient centrifugation of the CFP yielded two distinct bands of low and high density, designated bands 1 and 2, respectively. The presence of the 42-kDa protein on silver-stained SDS-polyacrylamide gels correlated with the appearance of flagella in transmission electron micrographs. Only gradient band 2 contained the 42-kDa protein, as seen on silver-stained SDS-polyacrylamide gels (Fig. 4A, lane 4), and flagella, when observed by transmission electron microscopy (Fig. 4B). Gradient band 1 did not show a 42-kDa protein in SDS-PAGE (Fig. 4A, lane 3) and contained no flagella, as determined by transmission electron microscopy (data not shown).

Immunoblot analysis. The specificity of the anti-flagellin polyclonal antiserum generated against the 42-kDa protein band was initially assessed by an immunoblot analysis. The 42-kDa protein was specifically recognized by the anti-flagellin antiserum in both the CFP and the whole-cell lysate of *B. bacilliformis* KC584 (Fig. 5). This serum also specifically recognized a 42-kDa protein in strain KC583 of *B. bacilliformis* but did not cross-react with *Escherichia coli* or *Salmonella typhimurium* flagellin or other cellular proteins in immunoblot analyses (data not shown).

Immunogold analysis. Immunogold analysis was used to verify that the 42-kDa protein used to generate the anti-flagellin antiserum was indeed the flagellar filament protein as well as to demonstrate the specificity of the anti-flagellin polyclonal antiserum. This analysis showed that the antiserum recognized the flagella of *B. bacilliformis* and did so with a high specificity. Figure 6A shows that the flagella are strongly recognized by the anti-flagellin polyclonal antiserum, and Fig. 6B demonstrates its specificity for this structure, as indicated by the absence of gold particles on the surface of the bacterial cell. This analysis, together with the immunoblot analysis (Fig. 5), showed that the 42-kDa protein used to generate the antiserum was indeed the flagellin protein of *B. bacilliformis* KC584 and that the anti-flagellin polyclonal antiserum was monospecific.

N-terminal sequence of the flagellin. The N-terminal sequence of the 42-kDa flagellar filament protein was determined in triplicate and found to be GAAILTNDNAM DALQDL. This sequence showed 52.9 and 41.2% sequence identity to the N termini of the 25-kDa flagellin (18) and 28.5-kDa flagellin (9) from *Caulobacter crescentus*, respectively, when analyzed by PC Gene 8.0 release 24 (Intelligence, Mountain View, Calif.). Figure 7 shows the alignment

of the first 17 amino acids from the *B. bacilliformis* flagellin and the two flagellins from *C. crescentus*.

Invasiveness of *B. bacilliformis* in the presence of anti-flagellin antibodies. To assess whether anti-flagellin antibodies could inhibit the invasiveness of *B. bacilliformis*, invasion assays were performed in the presence or absence of anti-flagellin antiserum. Invasiveness was scored by two methods.

The first protocol was to observe invaded human erythrocytes by Giemsa staining and enumerate the invaded erythrocytes. Since it was impossible to determine whether the *B. bacilliformis* cells were intra- or extracellular by this assay, infected erythrocytes were referred to as having intimately associated bacteria. Two separate invasion assays were run with anti-flagellin antiserum, preimmune rabbit serum, and a PBS control. Two sets of 1,000 erythrocytes were scored for associated bacteria after Giemsa staining. The staining revealed considerable agglutination of bacteria in the anti-flagellin antiserum-treated invasion assays that was absent in PBS and preimmune rabbit serum control assays. The anti-flagellin antiserum significantly reduced the number of erythrocytes with associated bacteria by approximately 52% ($P < 0.003$) and 41% ($P < 0.005$) in comparison with PBS and preimmune rabbit serum control assays, respectively (Fig. 8A). Differences between the PBS and preimmune serum control assays were not statistically significant (Fig. 8A).

The second method used to enumerate invaded human erythrocytes was done with the antibiotic gentamicin. In this assay, a gentamicin solution was added after the 10-h invasion incubation to kill any extracellular bacteria. The reductions in bacterial invasion conferred by the anti-flagellin antiserum were approximately 99.8 and 99.7% in comparison with PBS and preimmune rabbit serum control assays, respectively (Fig. 8B). Both of these reductions were highly significant ($P < 0.0001$ and $P < 0.001$, respectively). Preimmune rabbit serum could also significantly reduce invasion, by approximately 31.5% relative to that in PBS controls ($P < 0.03$) (Fig. 8B).

DISCUSSION

B. bacilliformis, a member of the *Rickettsiales* family, is an intracellular parasite of human erythrocytes which causes one of the most severe hemolytic anemias known (8). Although the threat of *B. bacilliformis* as a serious health problem has been reduced with the introduction of antibiotics and vector control within regions of endemicity, documented epidemics have occurred as recently as 1987, with serious consequences (10). In addition to the ultimate goal of preventing bartonellosis, studies of this organism will provide insight into the biology of intracellular parasites, more specifically hemotrophic organisms.

This study provides the first molecular characterization of flagella from *B. bacilliformis* and the potential role that they play in the invasion process. We have identified a 42-kDa protein in *B. bacilliformis* KC584 as the flagellin protein through the use of fractionation, transmission electron microscopy, and a monospecific antiserum generated against the protein. The anti-flagellin antiserum cross-reacts with a 42-kDa protein from *B. bacilliformis* KC583 but shows no cross-reactivity with either *E. coli* or *S. typhimurium* proteins in immunoblot analyses. Immunogold assays showed that the anti-flagellin antiserum is specific for the flagella of *B. bacilliformis* and no other cell surface structures. Although

the evidence presented here shows that the flagella of *B. bacilliformis* KC584 are composed of a single 42-kDa flagellin subunit, our results do not preclude the possibility that a second flagellin protein of 42 kDa and with a conserved N terminus also exists.

A number of previous studies on *B. bacilliformis* have implicated the importance of motility, i.e., functional flagella, in invasiveness. Benson et al. (3) observed that *B. bacilliformis* was able to deform the erythrocyte membrane in an irreversible fashion and attributed this ability to the motility of the bacterium. They postulated that the motile bacteria could act in a manner similar to that of a drill and, in conjunction with other factors, permanently alter the erythrocyte membrane. They also found that nonmotile mutants (or old cultures with reduced motility) bound poorly, if at all, to erythrocytes. It was hypothesized either that the loss of motility reduced the number of interactions between the bacterium and host cells and abrogated the "drill" effect of the bacterium or that the nonmotile *B. bacilliformis* cells lacked a surface receptor necessary for attachment (3).

A more recent study by Mernaugh and Ihler (17) on the invasive attributes of *B. bacilliformis* identified a proteinaceous factor released by the bacterium during growth in vitro and with the ability to deform the erythrocyte membrane independently of the pathogen. This factor, termed the deformation factor, irreversibly causes deformations in the erythrocyte membrane much like those originally attributed to flagella (3). While motility is apparently not responsible for the invaginations and trenches on the erythrocyte surface (17), it is still essential for invasion by *B. bacilliformis* to occur (17). Therefore, the deformation factor and motility probably act in concert to promote membrane deformation and entry, respectively, into erythrocytes. Mernaugh and Ihler (17) also found that the invasion process was insensitive to trypsin at a concentration of 1 mg/ml. The partial resistance (approximately 50%) of flagellin to trypsin at twice that concentration in this report (Fig. 3) is in keeping with their observations.

Hill et al. (12) found that invasion by *B. bacilliformis* of human dermal fibroblasts, laryngeal epithelial cells, and umbilical vein endothelial cells was diminished by approximately 50% when the bacteria were treated with *B. bacilliformis* whole-cell antiserum. This reduction in invasion corresponds very well with the 41 to 99.8% reduction that we report here with anti-flagellin antiserum (Fig. 8). Given this similarity, it is possible that the reduction in invasion seen by Hill et al. (12) was at least in part due to the presence of anti-flagellin antibodies in their antiserum. Cytochalasin D can also reduce invasion by *B. bacilliformis* of endothelial, epithelial, and fibroblast cells, indicating microfilament-dependent activity on the part of the host cell (12). However, prevention of invasion in the presence of cytochalasin D is not complete, suggesting that *B. bacilliformis* plays an active role in the invasion process. Since erythrocytes are nonendocytic, entry would necessarily be pathogen dependent and passive with respect to the host cell. In any case, it seems that invasion by *B. bacilliformis* of host cells is a complex interaction that relies upon several factors, including the recently characterized deformation factor, host cell microfilaments, and functional bacterial flagella.

In addition to supplying the mechanical force for invasion, the flagellin of *B. bacilliformis* has been speculated to be involved in attachment to erythrocytes. Walker and Winkler (26) noted that a significant number of bacteria were attached to erythrocytes via fiber-like projections from one pole of the

FIG. 6. Transmission electron micrographs of the immunogold assays. (A) KBr-purified flagella from *B. bacilliformis* KC584 labelled with the antFLAGELLIN antiserum and protein A-gold. (B) Immunogold analysis of an intact *B. bacilliformis* KC584 cell showing the specificity of the antFLAGELLIN antiserum for flagella on the bacterial cell surface.

organism when observed under electron microscopy. This observation is of obvious interest, since *B. bacilliformis* has multiple polar flagella. The attachment of bacteria to host cells via flagella is known to occur in *Vibrio cholerae*, for which attachment to host cells is apparently dependent on the presence of flagella rather than their functional state (20). Our data do not preclude this possible role for flagella in *B. bacilliformis*. The reduction in invasiveness that we report here in the presence of antFLAGELLIN antiserum (Fig. 8) could be due to a masking of attachment sites on the flagella in addition to a reduction in motility by agglutination of the bacteria.

It is clear that invasion (Fig. 8B) is inhibited to a much greater extent than erythrocyte association (Fig. 8A) when bartonellae are treated with antFLAGELLIN antiserum. Assuming that flagella are involved in host cell attachment, these observations suggest that antibody blocking of flagellar receptor sites is only partial or, alternatively, that other adhesins are involved. Our data also suggest that preimmune rabbit serum reduces the invasiveness of *B. bacilliformis* by approximately 31.5% in comparison with PBS controls (Fig. 8B). While this is a significant reduction in invasiveness ($P < 0.03$), it is obvious that antFLAGELLIN antiserum is significantly more effective at reducing invasiveness and results in an

FIG. 6—Continued.

BBFLAG	G	A	-	-	A	I	L	T	N	D	N	A	M	D	A	L	Q	D	L
FLA 1	M	A	S	N	S	I	N	T	N	A	G	A	M	I	A	L	Q	-	-
FLA 2	-	A	L	-	S	V	N	T	N	Q	P	A	L	I	A	L	Q	N	L

FIG. 7. Comparison of the N-terminal amino acid sequences of *B. bacilliformis* KC584 flagellin and two *C. crescentus* flagellins. The first 17 amino acid residues from the *B. bacilliformis* sequence (BBFLAG) are compared with the first 17 amino acids of published sequences for the 25-kDa flagellin (FLA 1) (18) and the 28.5-kDa flagellin (FLA 2) (9) from *C. crescentus*.

approximately 68.3% greater reduction in CFU than preimmune serum, relative to PBS controls (Fig. 8B).

The reduction in erythrocyte association and invasion obtained by treatment of *B. bacilliformis* with anti-flagellin antiserum is not surprising. In studies with *Proteus mirabilis*, investigators found that mutants which lack flagella could not invade host cells (1). Even *P. mirabilis* mutants deficient in swarming ability and with only partial motility had significantly reduced invasiveness (1). Flagellin-specific antibodies are produced in *P. mirabilis*-infected mice and can prevent the spread of the bacterium as well as decrease the ability of the bacterium to initiate infection (2, 11). Flagellin preparations used as vaccines also confer protection against a lethal challenge with *P. mirabilis* in mice (2). Inhibition of

FIG. 8. Histograms quantifying invasion of human erythrocytes by *B. bacilliformis* KC584 pretreated with flagellar immune serum (FIS), preimmune rabbit serum (PIS), or PBS (pH 7.4). (A) Giemsa stain assay results. Values represent the means $\pm$ standard errors of the means for two separate invasion assays in which two sets of 10^3 erythrocytes (RBC's) per treatment were evaluated for associated bacteria. (B) Gentamicin assay results. Values represent the mean CFU $\pm$ standard error of the mean for three separate invasion assays, calculated from serial dilution plates inoculated with 30 μ l of the invasion reaction mixture.

motility by monoclonal antibodies against *Pseudomonas aeruginosa* flagellin can prevent invasion of host cells in vitro (19) as well as prevent mortality from lethal challenges with the organism in experimental mouse infections (21). These data and the results from this report indicate that the flagellin from *B. bacilliformis* might eventually serve as a vaccine candidate.

Since we generated a monospecific antiserum to flagella, we were able to inhibit motility to determine its effect upon *B. bacilliformis* association with and invasion of human erythrocytes. Whether the antibodies exert their effect by reducing the motility of the organism via agglutination, by

masking an attachment site on the flagella, or both is currently under investigation.

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