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Borrelia burgdorferi Binding of Host Complement Regulator Factor H Is Not Required for Efficient Mammalian Infection▽

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The causative agent of Lyme disease, *Borrelia burgdorferi*, is naturally resistant to its host's alternative pathway of complement-mediated killing. Several different borrelial outer surface proteins have been identified as being able to bind host factor H, a regulator of the alternative pathway, leading to a hypothesis that such binding is important for borrelial resistance to complement. To test this hypothesis, the development of *B. burgdorferi* infection was compared between factor H-deficient and wild-type mice. Factor B- and C3-deficient mice were also studied to determine the relative roles of the alternative and classical/lectin pathways in *B. burgdorferi* survival during mammalian infection. While it was predicted that *B. burgdorferi* should be impaired in its ability to infect factor H-deficient animals, quantitative analyses of bacterial loads indicated that those mice were infected at levels similar to those of wild-type and factor B- and C3-deficient mice. Ticks fed on infected factor H-deficient or wild-type mice all acquired similar numbers of bacteria. Indirect immunofluorescence analysis of *B. burgdorferi* acquired by feeding ticks from the blood of infected mice indicated that none of the bacteria had detectable levels of factor H on their outer surfaces, even though such bacteria express high levels of surface proteins capable of binding factor H. These findings demonstrate that the acquisition of host factor H is not essential for mammalian infection by *B. burgdorferi* and indicate that additional mechanisms are employed by the Lyme disease spirochete to evade complement-mediated killing.

Borrelia burgdorferi is maintained in nature through an infectious cycle between a variety of vertebrate reservoir hosts and infected *Ixodes* sp. ticks. *B. burgdorferi* readily disseminates throughout the vertebrate body from the tick bite site to cause persistent systemic infection. The infectious dose of *B. burgdorferi* is very low, with as few as 20 spirochetes being capable of causing infection and disease in immunocompetent animals (12). The ability of such low numbers of spirochetes to establish infection reflects an innate ability of these bacteria to efficiently overcome vertebrate immune systems.

The complement system is a critical component of vertebrate immune systems that functions to eliminate invading microorganisms. The alternative pathway of complement is activated independent of antibodies and serves as an early defense mechanism before the production of antibodies by the humoral immune response. The activation of the complement system triggers a proteolytic cascade that leads to C3 being cleaved and the deposition of C3b on surfaces of invading microorganisms, resulting in both opsonization and the formation of membrane attack complexes (MAC). The alternative pathway requires a serine protease, factor B, to be cleaved to an active form (Bb) upon binding C3b to form the C3/C5 convertase and ultimately the MAC. Humans and other vertebrates protect their own cells from damage by the complement system by covering their surfaces with complement regulatory proteins that inactivate C3b. Factor H is the major host serum protein involved in the negative regulation of the alternative pathway. Unfortunately for humans, many microbial pathogens have evolved mechanisms to exploit the host's regulatory proteins in order to evade complement-mediated killing, including the binding of factor H (47).

Virulent *B. burgdorferi* strains are highly resistant to complement-mediated killing by the alternative pathway, as evidenced by the low number of spirochetes required for mammalian infection and the abilities of many cultured Lyme disease spirochetes to withstand incubation in >50% normal serum (18, 19, 39, 43, 46, 64). Cultured *B. burgdorferi* is capable of binding host factor H to its surface, which has been proposed to be the mechanism by which it evades the killing effects of complement. Five borrelial proteins that have abilities to interact with factor H, which are collectively termed CRASPs (complement regulator-acquiring surface proteins), have been characterized to date. CRASP-1 is encoded on lp54 by *cspA* and is distinct from the other CRASPs (41). *B. burgdorferi* bacteria deficient in CRASP-1 were found to be sensitive to killing by serum in culture (21). High levels of CRASP-1 are

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expressed by essentially every bacterium during transmission from tick to mammal as well as from mammal to tick (84), although production is repressed within the first month of mammalian infection (54). CRASP-2, encoded on lp28-3 by *cspZ*, can bind factor H (34, 44, 45), and preliminary studies indicate that it is also expressed during mammal-to-tick transmission (T. Bykowski and B. Stevenson, unpublished results). CRASP-3, -4, and -5 are members of the Erp protein family and are encoded on the spirochete's resident cp32 prophages (4, 5, 36, 44, 77). Erp proteins and CRASP-3, -4, and -5 are also expressed during both transmission stages and are produced throughout chronic mammalian infection (58–61). Despite this wealth of information, the functionality of these proteins during mammalian and tick infections has not previously been examined.

To directly test the importance of factor H binding in mammalian infection, we assessed the ability of *B. burgdorferi* to infect and persist within factor H-deficient ($Cfh^{-/-}$) mice. The relative abilities of Lyme disease spirochetes to transmit from $Cfh^{-/-}$ and wild-type mice to feeding ticks were also assessed. The role of the alternative pathway in controlling *B. burgdorferi* infection was further explored through infection studies of both factor B-deficient ($Bf^{-/-}$) and C3-deficient ($C3^{-/-}$) mice. The binding of factor H by bacteria during various stages of infecting mice and ticks was examined using immunofluorescence analysis (IFA).

MATERIALS AND METHODS

Bacteria and growth conditions. Preliminary mouse infection studies used *B. burgdorferi* strain N40, a clonal, infectious, and wild-type isolate (13). All subsequent mouse and tick infection studies utilized *B. burgdorferi* strain B31-MI-16, an infectious clone of the sequenced, nonclonal strain B31-MI (24, 31, 60). Clone B31-MI-16 contains all of the plasmids carried by its parent culture (60). For all purposes, bacteria were grown in Barbour-Stoenner-Kelly II (BSK-II) (9) at 34°C to mid-exponential phase (approximately 10^7 bacteria/ml).

Mouse strains. $Cfh^{-/-}$ mice (factor H deficient, lack regulation of alternative pathway) (66) and $Bf^{-/-}$ mice (factor B deficient, no alternative pathway activity) (53) have been backcrossed onto a C57BL/6 genetic background for 10 generations. The genotypes of these mice were initially confirmed by M. Botto, who developed the $Cfh^{-/-}$ strain, and then reconfirmed by M. E. Woodman following completion of these studies. Both the $Cfh^{-/-}$ and $Bf^{-/-}$ strains have been used extensively by others for a variety of infection and other studies (2, 23, 32, 56, 63, 66, 67, 82, 90, 91). The Botto laboratory provided these mouse strains to the Stevenson and Wooten laboratories, where the experiments described herein were performed. C57BL/6 mice deficient in C3 ($C3^{-/-}$) (no complement activity) were obtained from Jackson Laboratory (Bar Harbor, ME) (86). Wild-type C57BL/6 mice were obtained from Harlan Sprague Dawley (Indianapolis, IN).

Detection of C3 in mouse sera. Serum samples were collected from uninfected wild-type, $Cfh^{-/-}$, $Bf^{-/-}$, and $C3^{-/-}$ mice and examined by immunoblotting for the presence of C3. A 2-μl aliquot of each serum sample was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, electrotransferred to a nitrocellulose membrane, and blocked with 5% nonfat dry milk in Tris-buffered saline–Tween 20 (20 mM Tris [pH 7.5], 150 mM NaCl, 0.05% [vol/vol] Tween 20) overnight at 4°C. C3 was detected by incubation with goat anti-human C3 polyclonal antiserum (Quidel, San Diego, CA), diluted 1:2,000 in Tris-buffered saline–Tween 20, for 1 h at room temperature. The membrane was then washed and incubated with bovine anti-goat horseradish peroxidase conjugate (Santa Cruz Biotechnology, Santa Cruz, CA), and bound antibodies were visualized by enhanced chemiluminescence (Pierce, Rockford, IL).

Mouse infection studies. The doses and route of injection used in these studies result in a progression of infection comparable to that following infection by natural tick bite (28, 74). Needle injection of cultured bacteria was utilized to ensure reproducible infection by known quantities of *B. burgdorferi*, which is impossible to achieve by using a tick bite route of infection.

In a preliminary experiment, 16 each of wild-type and $Cfh^{-/-}$ 4- to 6-week-old female mice were injected intradermally with 2×10^3 *B. burgdorferi* strain N40 bacteria. Groups of 8 $Cfh^{-/-}$ mice were killed after 2 or 4 weeks of infection, as were also 10 wild-type mice after 2 weeks and 6 wild-type mice after 4 weeks of infection. Tibiotarsal joints, ear pinnae, and hearts were removed immediately, and total DNA was extracted as previously described (22).

Based upon the results of the above-described pilot study, a more comprehensive study was undertaken, using a well-characterized derivative of *B. burgdorferi* strain B31. Groups of female wild-type, $Cfh^{-/-}$, $Bf^{-/-}$, and $C3^{-/-}$ mice, 4 to 6 weeks old, were infected by subcutaneous injection with 5×10^4 *B. burgdorferi* B31-MI-16 bacteria. Cohorts of eight mice of each strain were killed at 1, 2, or 4 weeks postinoculation, numbers of mice that have been reported to consistently provide statistically significant values for animal infection studies (22, 49, 70, 88). Ear pinnae, tibiotarsal joints, and hearts were immediately harvested, placed in liquid nitrogen, and stored at −80°C.

All experimental protocols used in the handling and infections of mice were approved by the Institutional Animal Care and Use Committees and the Institutional Biosafety Committees of the University of Kentucky and the University of Toledo Health Sciences Campus. Prior to their use in these studies, mice were tested by institutional veterinarians and determined to be free of pathogenic bacteria, viruses, nematodes, and other infectious agents.

Quantification of *B. burgdorferi* during mammalian infection. Total DNA was extracted from individual tissues using Puregene genomic DNA purification kits (Gentra Systems, Inc., Minneapolis, MN). After purification, the DNA content of each sample was determined by measuring the absorbance at 260 nm and a working concentration of 50 ng/μl water was created for each.

Quantitative PCR was performed using a LightCycler thermal cycler (Roche Applied Science, Indianapolis, IN), as described previously (48, 62). The *B. burgdorferi recA* gene and the mouse *nidogen* gene were targeted for amplification to determine the number of spirochetes in each tissue sample (62). Briefly, each amplification was performed with 25 ng of sample DNA in a 10-μl final volume containing Platinum *Taq* polymerase (Invitrogen, Carlsbad, CA) (final dilution of 1:10 into enzyme diluent; Idaho Technology, Salt Lake City, UT), 0.8 mM deoxynucleoside triphosphates (Idaho Technology), 1× PCR buffer (final concentration, 3 mM $MgCl_2$) (Idaho Technology), 0.5 μM of each oligonucleotide primer, and 1× SYBR green (Molecular Probes, Eugene, OR) (final dilution of 1:10,000 in Tris-EDTA buffer [10 mM Tris-HCl containing 1 mM EDTA-Na_2, pH 8.0]; Promega, Madison, WI). A negative control that lacked template was included with each assay to ensure that reagents were not contaminated with extraneous DNA. Dilutions of B31-MI-16 and uninfected wild-type mouse DNA were also included to generate standard curves to quantify the amount of each respective DNA in the samples. All DNA samples were analyzed in triplicate. Melting curves were analyzed for each reaction to ensure specificity of the PCR product. The amplification program for *B. burgdorferi recA* consisted of an initial denaturation step at 95°C for 2 min, followed by 45 amplification cycles comprised of 95°C for 1 s, 60°C for 4 s, 72°C for 10 s, and 82°C for 3 s. The amplification program for mouse *nidogen* consisted of an initial denaturation step at 95°C for 2 min, followed by 40 amplification cycles comprised of 95°C for 1 s, 60°C for 4 s, 72°C for 10 s, and 84°C for 3 s (48, 62). The oligonucleotide primers used to detect *B. burgdorferi recA* were nTM17.F (5′-GTG GAT CTA TTG TAT TAG ATG AGG CTC TCG-3′) and nTM17.R (5′-GCC AAA GTT CTG CAA CAT TAA CAC CTA AAG-3′). The oligonucleotide primers used to detect mouse *nidogen* were nido.F (5′-CCA GCC ACA GAA TAC CAT CC-3′) and nido.R (5′-GGA CAT ACT CTG CTG CCA TC-3′) (62, 89). Data were reported as numbers of spirochete genomes per 1,000 mouse cell genomes.

Measurement of antibody concentrations during mammalian infection. Serum was prepared from each *B. burgdorferi*-infected mouse at the indicated times postinfection. *B. burgdorferi*-specific immunoglobulin (Ig) was detected in each serum sample by enzyme-linked immunosorbent assay as previously described (48). Briefly, microtiter plates were coated with 5 μg/ml sonicated *B. burgdorferi* B31-MI-16 or goat antibody to mouse IgG, IgM, and IgA (Southern Biotechnology Associates, Birmingham, AL). Serum dilutions were added to the microtiter plates for 90 min at 37°C and then washed to remove unbound material. Bound Ig was detected using horseradish peroxidase-conjugated antibodies to murine IgG or IgM (Southern Biotechnology). Quantification of Ig was determined by comparing standard curves for purified IgG and IgM (Southern Biotechnology) (48).

Tick rearing and infection. *Ixodes scapularis* egg masses were obtained from the Department of Entomology at Oklahoma State University (Stillwater, OK) and held at room temperature in a chamber at 95% relative humidity until hatching to larvae. Larval acquisition of *B. burgdorferi* from infected mice was examined by feeding approximately 200 naïve larvae each on wild-type and $Cfh^{-/-}$ mice that were previously infected with *B. burgdorferi* (see above). Larval ticks were allowed to feed to repletion and naturally detach from the mice. Some ticks were dissected immediately after detachment, while the remaining ticks

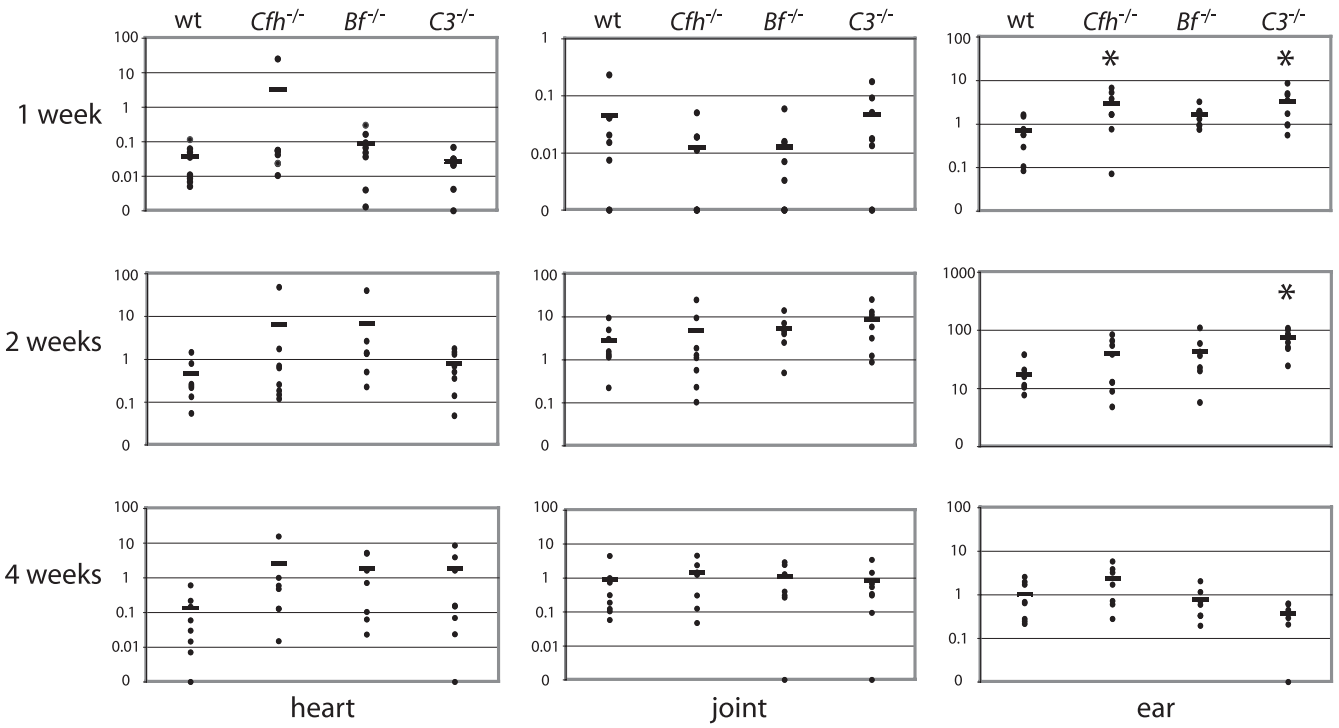

FIG. 1. *B. burgdorferi* numbers in heart, joint, and ear tissues of wild-type (wt), $Cfh^{-/-}$, $Bf^{-/-}$, and $C3^{-/-}$ mice following 1, 2, or 4 weeks of infection, as determined by quantitative PCR. *y* axis values indicate numbers of *B. burgdorferi* bacteria per 1,000 mouse cells. Each dot represents an individual mouse, and the bars indicate the averages. Asterisks indicate tissues of complement-deficient mice in which spirochete numbers were significantly different from those of wild-type mice ($P < 0.05$).

were placed in a humidified chamber to molt into nymphal ticks. Studies of *B. burgdorferi* transmission from infected ticks to mice were then performed by allowing 20 infected nymphs per mouse to feed on naïve wild-type and $Cfh^{-/-}$ mice. Engorging nymphal ticks were forcibly removed with fine forceps after feeding on the mice for 72 h (60, 84).

Analysis of factor H binding in ticks and tick bite sites. After drop-off or forcible removal, larval and nymphal tick midguts were immediately dissected. Often during forcible removal of a feeding tick, a piece of skin remained attached to the hypostome. Those bite-site biopsy samples were carefully dissected away from the tick and also examined. At least six ticks of each life stage were examined. At least two distinct mice were used for tick feeding/transmission of each tick life stage. Multiple tick bite sites from four different wild-type C57BL/6 and $Cfh^{-/-}$ mice were examined. All tick midguts and skin samples were dissected into 10 μl phosphate-buffered saline (PBS) on glass slides and allowed to air dry overnight. Control slides with cultured *B. burgdorferi* were prepared by growing the bacteria to mid-exponential phase and then incubated with either 40% or 100% nonimmune wild-type mouse or human serum for 30 min at 34°C or examined without incubation in any additional serum. Ten-microliter aliquots of such cultured bacteria were spread on glass slides and allowed to air dry overnight.

Slides were then fixed and permeabilized in acetone for 15 min and allowed to air dry. Slides were blocked overnight in PBS containing 0.2% bovine serum albumin (BSA) at 4°C. Slides were then incubated in goat anti-human factor H polyclonal antiserum (Quidel) diluted 1:1,000 in PBS-0.2% BSA for 1 h at room temperature. Slides were washed in PBS-0.2% BSA and incubated for 1 h at room temperature with rabbit polyclonal antiserum raised against *B. burgdorferi* total membrane proteins, diluted 1:40,000 in PBS-0.2% BSA (60). Slides were washed and incubated simultaneously with 1:1,000 dilutions of Alexa Fluor 488-labeled donkey anti-goat IgG and Alexa Fluor 594-labeled donkey anti-rabbit IgG (Molecular Probes) for 45 min at room temperature. Slides were then washed, dried, and mounted in ProLong antifade mounting medium (Molecular Probes) (60, 84). Slides were analyzed at ×400 magnification using a BX51 epifluorescence microscope (Olympus, Melville, NY) and a Retiga 200R Fast 1394 imaging system (QImaging, Burnaby, BC, Canada). To quantify the number of spirochetes acquired by ticks from infected wild-type and $Cfh^{-/-}$ mice, labeled bacteria were counted in 25 random fields per slide, with a minimum of three slides being analyzed per mouse strain (60, 84).

Statistical analysis. All analyses were performed by K. Tucker, a professional statistician (79, 87). An analysis of variance was performed in SAS version 9.1 (SAS Institute, Cary, NC), using the GLM procedure to compare mean spirochete and antibody levels across strains of mice. Spirochete and antibody levels were analyzed separately at each time point, and spirochete values were normalized using a $\log_{10}$ transformation. For those models with significant *P* values ($P < 0.05$), Tukey's test was performed to determine which strains were different from one another.

RESULTS

Quantification of *B. burgdorferi* infection of mouse tissues. To determine the contribution of factor H binding on the ability of *B. burgdorferi* to survive in the mammalian host, spirochete numbers were compared following infection of wild-type and homozygous factor H-deficient ($Cfh^{-/-}$) mice. Although $Cfh^{-/-}$ mice are largely unable to control the activation of C3 (66), they continually produce additional C3 and so contain reduced, but detectable, levels of plasma C3 (66; data not shown).

In a pilot study, 16 each of $Cfh^{-/-}$ and wild-type C57BL/6 mice were infected with *B. burgdorferi* strain N40. Half of the factor H-deficient mice were killed after 2 weeks of infection and the remaining half after 4 weeks of infection. Ten of the wild-type mice were killed after 2 weeks and the remaining six after 4 weeks. Quantitative PCR analyses of the animals' tibiotarsal joints indicated that *B. burgdorferi* successfully infected the factor H-deficient mice, with tissue burdens that were

equal to or greater than those found for wild-type animals (data not shown).

Following that unanticipated result, a more detailed study was undertaken to better characterize *B. burgdorferi* infection of *Cfh*$^{-/-}$ mice. This study utilized the well-characterized *B. burgdorferi* strain B31-MI, a strain for which all of the surface proteins capable of interacting with host factor H have been biochemically and temporally characterized (4, 21, 29, 33, 34, 37, 40–42, 44, 60, 71, 77, 84; Bykowski and Stevenson, unpublished). To further examine the role of the complement system in controlling *B. burgdorferi* infection, mice deficient in either factor B (*Bf*$^{-/-}$) or C3 (*C3*$^{-/-}$) were also examined. Cohorts of eight animals of each strain were examined after 1, 2, or 4 weeks of infection.

At 1 week postinfection, all animals from all tested strains were infected, but the *B. burgdorferi* levels were relatively low in all tissues (Fig. 1). At that time point, only minimal levels of spirochete-specific IgM were detectable (Fig. 2). No significant differences in bacterial loads were found in heart and joint tissues of *Cfh*$^{-/-}$ mice compared to those in wild-type animals, although higher numbers of bacteria were detected within ear tissues ($P < 0.05$). Likewise, *C3*$^{-/-}$ mice also possessed higher *B. burgdorferi* numbers in ear tissues than did wild-type mice ($P < 0.05$), but bacterial levels were similar in both heart and joint tissues of wild-type and *C3*$^{-/-}$ mice. *Bf*$^{-/-}$ mice did not demonstrate statistically significant differences in the bacterial loads of any tested tissue compared to wild-type mice.

By 2 weeks postinfection, the spirochete numbers had increased in all tissues of all mouse strains (Fig. 1) and substantial antibody levels were detected (Fig. 2). Although not statistically significant, the *Cfh*$^{-/-}$ mice trended toward higher levels of *B. burgdorferi*, with 6.55, 4.94, and 39.2 spirochetes, than did wild-type mice, with 0.45, 2.88, and 17.6 spirochetes per 1,000 murine cells in the heart, joint, and ear tissues, respectively. One *Cfh*$^{-/-}$ mouse had exceptionally high bacterial numbers in the heart, which caused the average to increase dramatically; exempting this sample from calculations reduced the average to 0.54 spirochetes per 1,000 murine heart cells. The tissues from *Bf*$^{-/-}$ mice also possessed higher numbers of *B. burgdorferi* bacteria than did those from wild-type mice, although none were significantly different. As in the *Cfh*$^{-/-}$ studies, one *Bf*$^{-/-}$ heart tissue sample contained extraordinarily high numbers of spirochetes. Notably, the joints and ears of the exceptional *Cfh*$^{-/-}$ and *Bf*$^{-/-}$ mice contained spirochete levels similar to those of all other mice of that strain, suggesting that these individual mice were not simply injected with higher doses than the other mice. The *C3*$^{-/-}$ mice had 0.79, 8.75, and 73.7 spirochetes per 1,000 murine cells in the heart, joint, and ear tissues, respectively, but only the numbers in ear tissues were significantly different from those in wild-type mice ($P < 0.05$).

Between 2 and 4 weeks postinfection, the spirochete numbers were shown to decrease in all tissues (Fig. 1) and robust antibody responses were evident (Fig. 2). With the production of antibodies, the classical pathway could play a role in controlling the spirochetes. However, no significant differences were seen in numbers of spirochetes in any tissues of wild-type or complement-deficient mice.

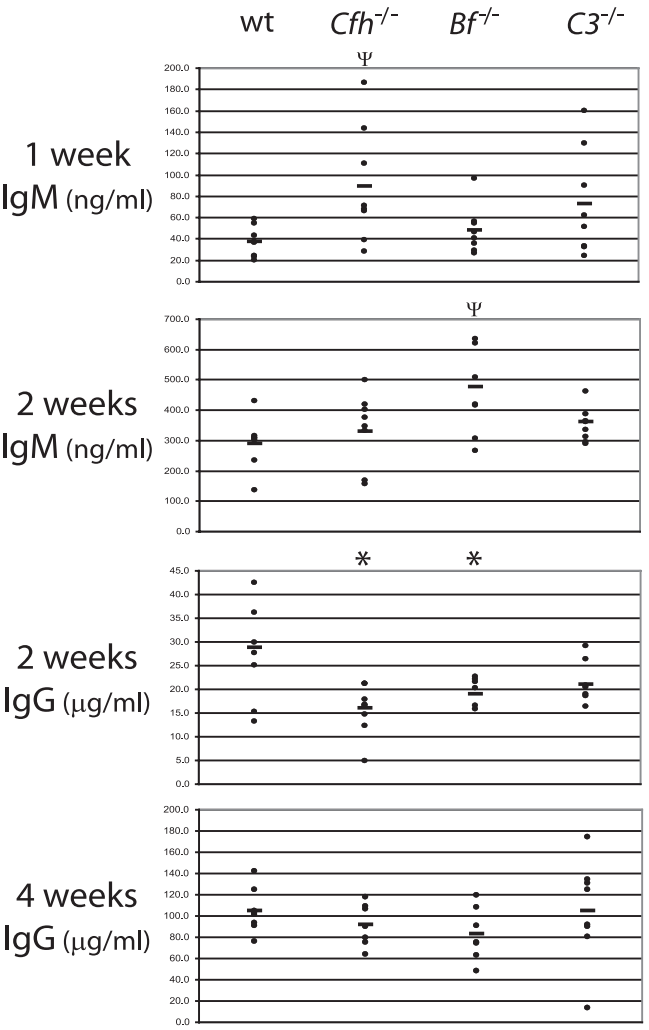

FIG. 2. *B. burgdorferi*-specific antibody levels in wild-type (wt), *Cfh*$^{-/-}$, *Bf*$^{-/-}$, and *C3*$^{-/-}$ mice following 1, 2, or 4 weeks of infection, as determined by enzyme-linked immunosorbent assay. Each dot represents an individual mouse, and the bars indicate the averages. Asterisks indicate tissues of complement-deficient mice in which antibody levels were significantly different from those of wild-type mice ($P = 0.002$). Ψ indicates tissues of complement-deficient mice in which antibody levels were marginally different from those of wild-type mice ($P = 0.053$ or $P = 0.052$).

***B. burgdorferi*-specific antibody responses in mice.** To address any potential influence that the classical pathway of complement activation might have on the observed spirochete levels, the *B. burgdorferi*-specific antibody levels were determined in the infected wild-type, *Cfh*$^{-/-}$, *Bf*$^{-/-}$, and *C3*$^{-/-}$ mice after 1, 2, or 4 weeks of infection. At 1 week postinfection, the levels of *B. burgdorferi*-specific IgM were measured. *Cfh*$^{-/-}$ mice had serum IgM levels that were marginally higher than those of wild-type mice ($P = 0.053$) (Fig. 2). No significant amounts of *B. burgdorferi*-specific IgG were detected at this time in any mouse strain (data not shown). By 2 weeks postinfection, substantial increases were detected in both IgM and IgG levels for all mouse strains. Marginally significant differences in IgM levels ($P = 0.052$) were detected between *Bf*$^{-/-}$ and wild-type mice. *Cfh*$^{-/-}$ and *Bf*$^{-/-}$ mice had significantly lower IgG levels

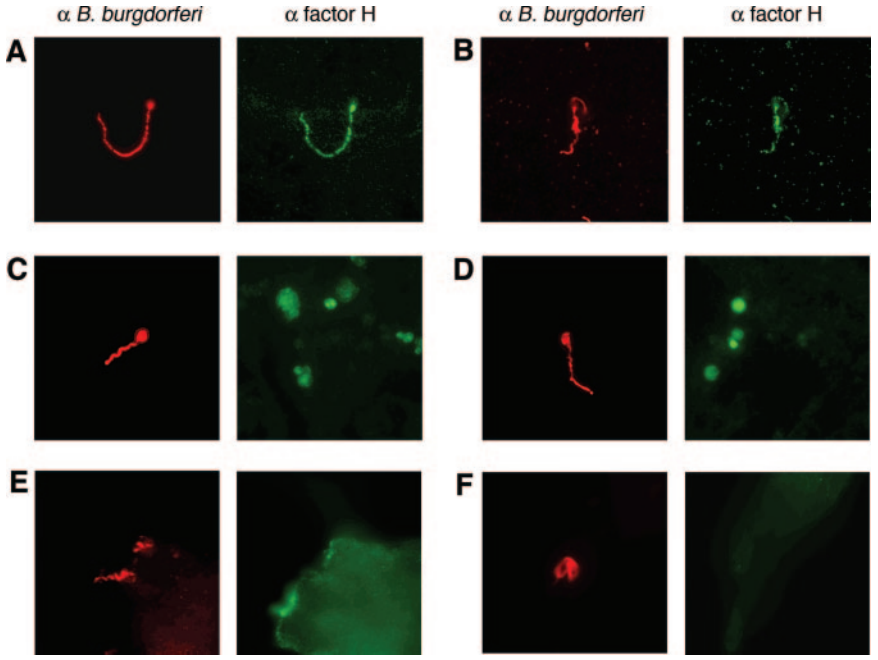

FIG. 3. Representative IFA images of *B. burgdorferi* interactions with factor H in culture and during tick and mammal infections. At least six ticks of each life stage were examined, with an average of 20 bacteria detected in each dissected tick. For each tick life stage, at least two distinct mice were used for tick feeding/transmission. Tick bite sites from at least four different wild-type and $Cfh^{-/-}$ mice were examined. Goat anti-human factor H polyclonal antiserum (α factor H) labeled those spirochetes with bound factor H, while rabbit polyclonal antiserum raised against *B. burgdorferi* total membrane proteins (α *B. burgdorferi*) labeled all spirochetes in the same field. (A and B) Cultured *B. burgdorferi* incubated in 100% mouse serum (A) or in BSK-II culture medium (which contains 6% rabbit serum as an essential ingredient) (B). (C and D) *B. burgdorferi* in midguts of naïve larval *I. scapularis* ticks immediately following completion of feeding on infected mice. Autofluorescent components of the digesting blood meal are visible in the anti-factor H (green light) channel and were also apparent in midguts of larvae fed on factor H-deficient mice (data not shown). (E and F) Skin samples at tick bite sites from wild-type (E) and factor H-deficient (F) mice.

than wild-type mice ($P = 0.002$), but no significant differences were detected in $C3^{-/-}$ mice. By 4 weeks postinfection, no differences in *B. burgdorferi*-specific IgG levels were detected between any mouse strains. Thus, the only distinguishable effect of the complement deficiencies was a slight delay in switching from IgM to IgG, much as was previously observed (30, 81). Importantly, there was no relationship between high antibody levels and low *B. burgdorferi* numbers, or vice versa, in any individual mouse. Uninfected control samples from all mouse strains possessed similar, low levels of IgM (~15.0 ng/ml) that recognized *B. burgdorferi*, most likely indicative of natural antibody (14), but there were no detectable levels of *B. burgdorferi*-specific IgG.

Examination of interactions between *B. burgdorferi* and host factor H in ticks and skin bite wounds. As described above, *B. burgdorferi* is capable of binding factor H from serum through a number of spirochete-encoded proteins. However, all previous analyses of factor H binding were performed in vitro, using either cultured bacteria or recombinant proteins and purified factor H or serum. To determine whether *B. burgdorferi* actually binds factor H during the natural infectious cycle, we examined individual bacteria by IFA during different stages of mammal-to-tick transmission. Previous studies have shown that *B. burgdorferi* CRASPs are able to bind murine factor H (3, 77; J. Hellwage, unpublished results), and consistent with those in vitro studies, IFA of *B. burgdorferi* incubated in either 40% (data not shown) or 100% (Fig. 3A) fresh, normal mouse serum indicated factor H binding. *B. burgdorferi* incubated in human serum also bound factor H, with IFA intensities similar to those for mouse factor H (data not shown). Factor H binding was also detected, albeit to a weaker extent, when we examined *B. burgdorferi* cultured in BSK-II (contains 6% rabbit serum but no additional source), indicating that factor H bound to bacteria can be identified by IFA even at low serum concentrations (Fig. 3B).

During mammalian infection, *B. burgdorferi* bacteria are widely dispersed at low concentrations throughout their host, which prevents the direct assessment of factor H acquisition within the mammalian host (69). However, when larval ticks acquire *B. burgdorferi* from an infected vertebrate host, large numbers of bacteria become concentrated in the tick midgut. Moreover, essentially 100% of the bacteria express high levels of CRASP-1 and Erp proteins (60, 61, 84). Feeding tick larvae, full of fresh blood and newly acquired *B. burgdorferi*, were dissected and examined by IFA for the presence of factor H on the bacteria. Surprisingly, none of the bacteria acquired by tick larvae during natural feeding exhibited factor H binding on their surfaces (Fig. 3C and D and data not shown). *B. burgdorferi* bacteria within midguts of larval ticks that fed on wild-type mice were as devoid of IFA signals as bacteria in ticks fed on $Cfh^{-/-}$ mice (data not shown). Furthermore, the numbers of *B. burgdorferi* bacteria detected in larval ticks that fed on infected wild-type and $Cfh^{-/-}$ mice were similar, indicating that factor H is not critical during mammalian infection and

during acquisition of borreliae from the host and that the bacteria can evade complement-mediated killing by other means.

In the next stage of the *B. burgdorferi* life cycle, infected nymphal ticks transmit bacteria to a new host during their blood meal. Spirochetes within feeding nymph midguts express low levels of all CRASPs on their surfaces (60, 84). Consistent with these data, factor H could not be detected on any *B. burgdorferi* bacteria in the midguts of the feeding, blood-filled nymphs (data not shown).

During transmission from feeding nymphs to the skin of the host, *B. burgdorferi* bacteria dramatically increase the expression of surface proteins capable of binding factor H, such that essentially 100% of transmitted bacteria produce CRASP-1 and Erp proteins (35, 60, 84). However, none of the *B. burgdorferi* bacteria detected in mouse skin during transmission appeared to bind factor H above the background staining in the skin (Fig. 3E). An unavoidable caveat to this final analysis is that mouse tissues are covered in factor H, and bacteria are too infrequently found in the skin to allow analysis separate from the host tissue. Thus, it is possible that newly transmitted *B. burgdorferi* bacteria might bind host factor H to some extent. *B. burgdorferi* bacteria were also detected in the skin of factor H-deficient mice following feeding by infected ticks, demonstrating that the entire *B. burgdorferi* life cycle, from murine infection to acquisition by larval ticks to transmission of tick nymphs back into mice, can occur in the absence of host factor H (Fig. 3F). As would be expected, the *B. burgdorferi* bacteria within the skin of factor H-deficient mice did not yield anti-factor H IFA signals.

DISCUSSION

As are many other pathogenic organisms, virulent *B. burgdorferi* strains are generally resistant to their host's alternative pathway of complement. The spirochete can express several surface proteins capable of binding factor H, which has been hypothesized to afford the bacteria with protection against host complement (4, 5, 21, 34–36, 41, 44, 45, 47, 55, 58–61, 77, 84). We therefore anticipated that *B. burgdorferi* would be impaired in its ability to infect and/or persist within animals deficient in factor H. To the contrary, $Cfh^{-/-}$ mice were infected at levels similar to those of wild-type animals. Infection levels were also similar to those of mice lacking factor B or C3, which cannot utilize the alternative pathway or any pathway of complement activation, respectively. For most mouse tissues and durations of infection, spirochete loads in any complement-deficient animal did not significantly differ from those observed in wild-type mice. After 1 and 2 weeks of infection, elevated numbers of spirochetes were detected in ear tissues of $C3^{-/-}$ mice, but not $Bf^{-/-}$ mice, suggesting the involvement of a complement activation pathway other than the alternative pathway during early stages of mammalian infection. For comparison, a study with complement-deficient mice demonstrated that innate immunity to *Streptococcus pneumoniae* infection is mediated primarily by the classical complement pathway (23), whereas the alternative pathway is essential for murine resistance to *Pseudomonas aeruginosa* (63) and both are important for resistance to *Streptococcus pyogenes* (90). *B. burgdorferi* loads in $Cfh^{-/-}$ mice did not differ significantly from those in wild-type mice, with the exception of those in ear tissues after 1 week of infection, indicating that factor H is not essential for protecting *B. burgdorferi* from clearance by mammalian hosts. One possible explanation for these observations is that the function of factor H is redundant to the functions of other host proteins capable of regulating the alternative pathway. *B. burgdorferi* may produce additional substances that afford it with protection against complement, such as a putative slime layer (43) and an apparent CD59-like protein that obstructs MAC formation (65). Several pathogens are known to bind other host components or produce their own, novel substances to aid in the protection against complement (10, 11, 15, 47, 51, 57, 68). A large number of studies have clearly indicated that *B. burgdorferi* expresses a repertoire of proteins during mammalian infection different from the one it expresses during laboratory cultivation (6, 20, 27, 50, 75, 78), so it is highly probable that other, as-yet-unidentified bacterial factors protect the spirochete from complement in vivo. Supporting those alternative hypotheses are the Lyme disease borreliae that are virulent for humans and other mammals yet lack the ability to bind factor H (5, 45, 55, 85).

Several studies have demonstrated that the relative infectivity of Lyme disease spirochetes for humans and other animals does not always correlate with their ability to withstand the direct bactericidal effects of normal serum in vitro (5, 18, 19, 43, 46, 83). This suggests that serum ex vivo does not necessarily replicate conditions experienced by *B. burgdorferi* during actual infection. For example, murine serum is known to contain inhibitors of both the alternative and classical pathways which become activated upon the isolation of serum from mice (7, 73), which renders mouse serum largely unusable for in vitro assays. For those reasons, we were unable to directly examine *B. burgdorferi* interactions in vitro using isolated sera from the different complement-deficient mouse strains.

To further explore the role of factor H in the natural course of *B. burgdorferi* infection, individual bacteria were examined during various stages of host vector infection. Bacteria acquired by ticks feeding on infected animals express all known surface proteins capable of binding factor H (60, 84; Bykowski and Stevenson, unpublished). However, none of the examined bacteria acquired by feeding tick larvae had detectable levels of factor H on their surfaces. Our control studies, along with parallel studies utilizing cultured organisms, indicate that *B. burgdorferi* is capable of binding mouse factor H (3, 77; Hellwage, unpublished). Blood meal digestion by ticks occurs intracellularly, and tick midgut cells are not known to secrete any protease which might degrade factor H (1, 8, 76). These data suggest that *B. burgdorferi* CRASPs may bind host components other than factor H, which preclude CRASP-factor H binding. Supporting that hypothesis, the borrelial factor H-binding proteins CRASP-1 (CspA, open reading frame [ORF] BBA68), ErpA (CRASP-5, ORF BBL39/BBP38), ErpC (CRASP-4), and ErpP (CRASP-3, ORF BBN38) all bind additional serum proteins (34a, 37, 54; our unpublished results).

As noted above, no significant differences in bacterial levels were detected between $Bf^{-/-}$ and wild-type mouse tissues at any times postinfection, suggesting that complement activation via the alternative pathway does not play a significant role in controlling *B. burgdorferi* infection. In contrast, C3-deficient mice developed significantly increased bacterial loads in ear

tissues during the first 2 weeks of infection, which may reflect reduced MAC formation and associated lysis and/or a lack of opsonic C3 breakdown products in those tissues (19, 80). However, no differences in bacterial loads were detected in either heart or joint tissue. These data suggest that the classical and/or lectin-binding pathways may affect spirochete numbers in some tissues even before the elicitation of a substantial humoral response. Our study and others have shown that IgM antibodies that bind *B. burgdorferi* are naturally present in uninfected mammals (14), which could account for the observed bacterial increases in $C3^{-/-}$ mice. Some antibodies can effectively kill *B. burgdorferi* even in the absence of complement (25, 26, 72). Other innate and adaptive immune responses may also contribute to the control of *B. burgdorferi* infection and account for the abilities of even C3-deficient mice to control bacterial numbers (17, 22, 38, 52). The borrelial numbers we observed in the complement-deficient mice support previous studies that indicate that the complement system plays a relatively minor role in controlling *B. burgdorferi* in the host (16, 48).

In summary, the results demonstrate that host factor H is not critical for *B. burgdorferi* to efficiently infect the mammalian host. *B. burgdorferi* bacteria can infect and persist at similar levels within multiple tissues of *Cfh*-deficient and wild-type animals. Moreover, *B. burgdorferi* bacteria acquired by feeding ticks from infected hosts did not bind detectable amounts of factor H, despite their production of surface proteins capable of binding that host complement regulator. Thus, *B. burgdorferi* CRASPs may perform additional functions other than factor H binding in vivo, and this spirochetal pathogen must evade complement-mediated killing by some alternative mechanism(s). It is intriguing that mice effectively controlled spirochete infections even in the absence of the key complement component C3, demonstrating that complement-independent mechanisms restrict *B. burgdorferi* proliferation during mammalian infection. Elucidating those mechanisms will provide important insight into the complex pathogenesis of Lyme disease.

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