Facilitation of Complement-Dependent Killing of the Lyme Disease Spirochete, *Borrelia burgdorferi*, by Specific Immunoglobulin G Fab Antibody Fragments

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In the absence of specific antibody, *Borrelia burgdorferi* is resistant to the bactericidal action of complement, despite the capacity of the spirochete to activate complement. Complement-mediated killing of *B. burgdorferi* requires the presence of antiborrelial immunoglobulin G (IgG). The effect of bactericidal IgG takes place after formation of the C5 convertase. Therefore, we examined the ability of Fab fragments from bactericidal IgG to mediate killing of *B. burgdorferi* by complement. The complement-activating domain of IgG, the Fc fragment, was not required for killing of borreliae, as monovalent Fab fragments prepared from immune IgG were also able to mediate killing. However, the killing efficiency of the Fab fragments was less than that of intact IgG, suggesting that the bactericidal activity of IgG is enhanced by divalency. IgG Fab-mediated killing occurred without increased complement activation or C3 fluid-phase consumption. Cell killing proceeded via the classical complement pathway, as no killing of Fab fragment-sensitized cells was observed in human serum deficient in C2. These results demonstrate directly that the bactericidal effect of anti-*B. burgdorferi* IgG is independent of the complement-activating properties of the antibody.

The host response to the etiologic agent of Lyme disease, *Borrelia burgdorferi*, is of much current interest (20). Because complement-mediated killing of many gram-negative bacteria is dependent on specific, bactericidal antibody (reviewed in references 5 and 9), we previously studied the role of antibody and complement in killing of *B. burgdorferi* (14, 15). We showed that, in the absence of antibody, *B. burgdorferi* is resistant to the bactericidal activity of human complement, in spite of the ability of *B. burgdorferi* to activate both the classical and alternative complement pathways (14). However, borreliae are killed if immune antibody is present, and killing is mediated through the classical pathway. We recently examined the mechanism by which *B. burgdorferi* evades complement killing (15). We found that antibody does not mediate killing by increasing the rate or extent of C3, C8, or C9 deposition. Instead we showed that immunoglobulin G (IgG) antibody exerts its bactericidal effect by acting after formation of the C5 convertase on the bacterial surface. An examination of the association of C9 with the surface of complement-treated bacteria demonstrated that C9 bound to unsensitized bacteria is more susceptible to proteolysis by trypsin than C9 on antibody-sensitized cells. These results indicated that immune IgG antibody may mediate killing of *B. burgdorferi* by modifying the bacterial cell surface to enhance the insertion of the membrane attack complex (15). This observation suggested to us that the role of antibody might be independent of the Fc fragment, the classical pathway-activating moiety of IgG (4, 17).

In the present study we examined the mechanism by which immune antibody renders *B. burgdorferi* sensitive to complement killing. The ability of IgG Fab fragments prepared from bactericidal IgG to mediate complement activa-

tion and killing of *B. burgdorferi* was compared to that of intact IgG. Our results indicate that pretreatment of borreliae with Fab fragments can mediate complement-induced killing of *B. burgdorferi* without increasing complement activation. The Fab fragments participated in killing of the borreliae in conjunction with activation of the classical complement pathway by the bacteria through an antibody-independent mechanism.

MATERIALS AND METHODS

Bacterial strain and culture conditions. The 297 strain of *B. burgdorferi*, which was isolated from the cerebrospinal fluid of a patient with stage III Lyme disease, was originally obtained from Allen Steere, Tufts University, Boston, Mass. (19). Strain 297 was maintained in Barbour-Stoenner-Kelly medium, as previously described (14).

Sera. Blood was obtained by venipuncture from two or more healthy donors with no history of spirochetal infection; serum was prepared by standard techniques and stored at -80°C until used. All serum samples were screened for borrelia-reactive antibodies by an indirect immunofluorescence assay (1). Serum was considered seronegative if the immunofluorescence titer was $<1:32$. C2-deficient serum was obtained from a patient with systemic lupus erythematosus (16); this serum had no detectable C2 activity, as determined by hemolytic assay. Purified human C2 was obtained from Diamedix Corp. (Miami, Fla.). C2-deficient serum was reconstituted with 2,000 U of C2 per ml.

Complement assays. Sheep erythrocytes were obtained in Alsever's solution (Kroy Medical Supplies, Stillwater, Minn.), washed in saline, and then adjusted to the desired concentration in Veronal buffer containing 0.1 mM CaCl_2 and 1.0 mM MgCl_2 , pH 7.4 (7). Sheep erythrocytes were sensitized with rabbit hemolysin (GIBCO, Grand Island, N.Y.). Standard assays were used for measurement of total hemolytic complement consumption (14). Hemolytic assay

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for determining complement component C3 was performed with sensitized sheep erythrocytes carrying a stable complement intermediate (Diamedix) and immunochemically depleted serum as previously described (14).

Antibody against *B. burgdorferi*. Log-phase cultures of *B. burgdorferi* 297 were washed in phosphate-buffered saline, pH 7.4, and resuspended to approximately 10^9 cells per ml. New Zealand White rabbits weighing from 4 to 5 kg were injected via the marginal ear vein with 1 ml of the cell suspension once a week for 4 weeks. Blood was harvested by cardiac puncture on week 6, and immune serum was collected. IgG was obtained by fractionation of immune rabbit serum on a Zetachrom 60 DEAE anion exchanger (AMF Laboratory Products, Meridan, Conn.), as previously described for the purification of human IgG (15). Antibody titers of immune serum and purified IgG were measured by immunofluorescence assay. Titers of immune serum and IgG were 1:8,092 and 1:4,046, respectively.

Papain digestion of IgG. The Fab fragment of IgG was prepared by digestion of intact IgG with papain. Ten milligrams of lyophilized IgG was dissolved in a 20 mM NaH_2PO_4 , 20 mM cysteine-HCl, 10 mM EDTA buffer, pH 7.0, and mixed with 0.5 ml of immobilized papain (Pierce Chemical Co., Rockford, Ill.) that had been washed and preequilibrated in the same buffer. The suspension was incubated for 5 h at 37°C, and the reaction was terminated by the addition of column buffer (10 mM Tris-HCl buffer, pH 7.5). The immobilized papain beads were removed by centrifugation, and the supernatant was loaded onto an immobilized protein A column (10 by 250 mm; Pierce) at a flow rate of 0.5 ml/min. The void volume containing the Fab fragments was collected and passed over the column two additional times. Two-milliliter fractions were collected and assayed at A_{280} . Fab fragments were eluted with elution buffer (100 mM glycine-HCl, pH 2.8). Peak fractions were pooled, concentrated to the original serum volume by ultrafiltration on an Amicon PM-10 membrane (Amicon Corp., Danvers, Mass.), and dialyzed exhaustively against Veronal buffer. Protein content was quantified by the Bradford protein assay (Bio-Rad Laboratories, Richmond, Calif.).

Serum bactericidal test. The in vitro assay for serum bactericidal activity was conducted as described elsewhere (15). For experiments requiring antibody- or Fab-sensitized cells, bacteria were preincubated for 30 min at 30°C with IgG or IgG Fab. Before use in the bactericidal test the cells were washed in Eagle's minimal essential medium containing Hanks salts (pH 7.4; GIBCO) and 4% (wt/vol) bovine serum albumin (Armour Pharmaceuticals, Kankakee, Mich.).

Western blot analysis. Western immunoblot analysis was performed as previously described (2) with minor modifications. Proteins were separated by electrophoresis on 10% native or denaturing polyacrylamide gels and electrophoretically transferred to a nitrocellulose membrane (Schleicher and Schuell GmbH, Dassel, Germany). Antibodies directed against Fc or Fab fragments of rabbit IgG were purchased from Organon Teknika-Cappel (Durham, N.C.) and Nordic Immunological Labs (Capistrano Beach, Calif.), respectively. Antigens were visualized by using an enzyme immunoassay with a horseradish peroxidase-conjugated second antibody (Organon Teknika-Cappel) and 4-chloro-1-naphthol (Sigma Chemical Co., St. Louis, Mo.) as the chromogenic substrate. The minimum amount of protein antigen detectable with this system was approximately 10 ng.

FIG. 1. Western blot analysis of binding of anti-*B. burgdorferi* IgG Fab and anti-*B. burgdorferi* IgG to *B. burgdorferi* antigenic constituents. Bacteria (10^8) were sonicated and treated with 2-mercaptoethanol prior to electrophoresis in a 10% sodium dodecyl sulfate-polyacrylamide gel. Electrophoresed bands were transferred to nitrocellulose and incubated with anti-*B. burgdorferi* IgG Fab (lane A) or anti-*B. burgdorferi* IgG (lane B). The blots were washed and incubated with horseradish peroxidase-conjugated rabbit anti-human IgG. Blots were examined after development in buffer containing 4-chloro-1-naphthol.

RESULTS

We found that purified IgG isolated from rabbit antiserum against *B. burgdorferi* reacted immunologically with many borrelial proteins, as determined by Western blot analysis (Fig. 1, lane B). This observation is in agreement with previous reports of a heterogeneous anti-*B. burgdorferi* IgG response in patients with Lyme disease (3, 6). The anti-*B. burgdorferi* IgG Fab preparation retained the ability to react with borrelial antigens (Fig. 1, lane A). The Fab preparation was free of Fc fragments as there was no reactivity by Western blot with anti-IgG Fc serum (Fig. 2, lane 1); in contrast, intact IgG reacted strongly with this antiserum (Fig. 2, lane 2). However, immunoreactivity was detected in the IgG Fab preparation with anti-IgG Fab serum (Fig. 2, lane 3). On a native gel this protein migrated with an apparent molecular mass of 50 kDa (result not shown).

Previously we have suggested that anti-*B. burgdorferi* bactericidal IgG causes killing of this organism in the presence of complement through a novel mechanism that consists in antibody facilitating formation of an effective complement membrane attack complex on the bacterial membrane (15). The IgG antibody was bactericidal to *B. burgdorferi* when added after formation of the C5 convertase on the bacterial membrane. Therefore, we postulated that the Fab fragment of anti-*B. burgdorferi* IgG, which should be unable to activate complement, might be bactericidal in the

FIG. 2. Western blot analysis of anti-*B. burgdorferi* Fab IgG fragment preparation. IgG Fab (lanes 1 and 3) and intact IgG (lane 2) were electrophoresed in a 10% sodium dodecyl sulfate-polyacrylamide gel, transferred to nitrocellulose, and then incubated with goat antiserum against rabbit IgG Fc (lanes 1 and 2) or goat antiserum against rabbit IgG Fab (lane 3). The blots were washed and incubated with horseradish peroxidase-conjugated rabbit anti-goat IgG. Blots were examined after development in buffer containing 4-chloro-1-naphthol. Numbers on the left are molecular weights (10^3).

presence of a complement source. Figure 3 shows results of experiments performed to test this hypothesis. We found that Fab fragments were able to mediate complement-dependent killing of *B. burgdorferi* in a dose-dependent manner, although less effectively than intact IgG. On a molar basis, approximately 10 times more IgG Fab than intact IgG was required to produce 50% killing. There was no killing in the absence of antibody against *B. burgdorferi* (Fig. 3). No killing was observed upon incubation of IgG Fab-sensitized borreliae with C2-deficient serum (Fig. 3). As this serum had normal hemolytic alternative pathway activity and an absence of classical pathway activity, this experiment corroborates the essential role of the classical complement pathway in the bactericidal reaction (14, 15). Reconstitution of C2-deficient serum with purified human C2 restored killing by IgG Fab to a level similar to that observed with normal serum (results not shown).

In view of these results it became important to ascertain whether our anti-*B. burgdorferi* IgG Fab preparation activates complement when bound to the bacterium and incubated with normal human serum as a source of complement. We first determined whether anti-*B. burgdorferi* IgG Fab induces consumption of total hemolytic complement. Figure 4 shows that preincubation of borreliae with anti-*B. burg-*

FIG. 3. Killing of IgG- and IgG Fab-sensitized *B. burgdorferi*. Bacteria (10^8 /ml) were presensitized with increasing concentrations of rabbit anti-*B. burgdorferi* IgG and incubated in 20% normal human serum (●) or presensitized with anti-*B. burgdorferi* IgG Fab and then incubated in 20% normal human serum (▲) or 30% C2-deficient human serum (■). Cell killing was determined by dark-field microscopy. Values for percent survival are expressed relative to the control sample and represent the means $\pm$ standard errors for six experiments run in triplicate.

dorferi IgG Fab did not result in significant increment in complement consumption beyond the consumption induced by unsensitized borreliae. In contrast, preincubation of borreliae with anti-*B. burgdorferi* intact IgG induced a significant increase in the consumption of total complement at all cell concentrations tested ($P < 0.01$). We then asked whether anti-*B. burgdorferi* IgG Fab, in comparison with intact IgG, causes consumption of fluid-phase C3 when the Fab is bound to borreliae and incubated with normal human serum. We found that consumption of C3 by borreliae

FIG. 4. Consumption of total hemolytic complement in normal human serum by IgG- or IgG Fab-sensitized *B. burgdorferi*. Bacteria (10^8 /ml) were presensitized with rabbit anti-*B. burgdorferi* IgG (●), anti-*B. burgdorferi* IgG Fab (▲), or buffer (○) for 30 min at room temperature. Bacteria were washed, and then different numbers of bacteria were incubated in 20% normal human serum for 60 min at 37°C. Cells were removed by centrifugation, and residual hemolytic complement in the supernatants was determined by hemolytic assay. Percentages of residual complement are expressed as the means $\pm$ standard errors for two experiments run in duplicate.

sensitized with anti-*B. burgdorferi* Fab (33.4%) was not increased beyond that observed with unsensitized borreliae (49.3%). In contrast, preincubation of borreliae with anti-*B. burgdorferi* intact IgG induced a significant increase in consumption of C3 (85.7%). These experiments demonstrate that purified anti-*B. burgdorferi* IgG Fab causes no complement activation; complement activation by anti-*B. burgdorferi* IgG requires the presence of the Fc fragment.

DISCUSSION

We have previously shown that incubation of *B. burgdorferi* with normal human serum, free of anti-borrelial antibody, results in activation of the classical and alternative complement pathways (14). However, under these conditions killing does not occur unless IgG antibody against *B. burgdorferi* is present (14, 15). We found that the mechanism by which antibody is bactericidal to this microorganism appears to be unrelated to the ability of the antibody to activate complement or to direct the localization of the C3 or C5 convertase to critical areas of the bacterial surface. Instead antibody may facilitate the action of the membrane attack complex so that complement becomes bactericidal (15). Therefore, it was of interest to ascertain whether the Fab fragment of anti-*B. burgdorferi* IgG, which is unable to activate complement, would be bacteriolytic for *B. burgdorferi*. We showed that purified Fab fragments of anti-*B. burgdorferi* IgG, when added to normal human serum as a source of complement, are able to mediate bacteriolysis of *B. burgdorferi*. However, the effectiveness of the Fab fragments in effecting killing of this organism was lower than that of intact IgG. The reason for this difference has not been investigated, but it could be due to a lower binding affinity of Fab compared with that of intact IgG or to a greater efficiency of a bivalent molecule that may produce cross-linking of critical constituents of the bacterial surface. Thus, although bivalency is not an absolute requirement for the bactericidal effect of the antibody, bivalency may be important in facilitating the action of the membrane attack complex.

Previously, we have demonstrated that complement-dependent killing of *B. burgdorferi* requires IgG antibody and is mediated by the classical pathway (14). Now we have found that complement-induced killing of *B. burgdorferi* which occurs when IgG Fab replaces the entire IgG molecule is also dependent on activation of the classical pathway. This was shown by the lack of bacteriolysis when IgG Fab-sensitized borreliae were incubated with human serum deficient in C2; killing was restored by reconstitution of C2-deficient serum with purified C2. Thus, as expected, in causing killing of *B. burgdorferi*, IgG and its Fab fragment share the same pathway of complement activation. We do not understand why the alternative pathway does not participate in the killing mechanism. This is particularly puzzling because we observed previously that killing of the borreliae can be achieved if IgG-sensitized borreliae are incubated with the purified components of the membrane attack complex, C5b through C9 (15). Therefore, failure of the alternative pathway to mediate killing in the presence of the antibody must ultimately be related to inappropriate interaction of the membrane attack complex with the bacterial surface as a consequence of a different localization of the alternative pathway C5 convertase. Thus, the alternative pathway C5 convertase may result in C5b-9 complexes that are lytically inefficient because of deposition of C5b at inappropriate sites not susceptible to the action of the

antibody. Interestingly, in other microbial systems in which IgG Fab-mediated complement killing has been observed, this activity has been attributed to activation of the alternative pathway (8, 13, 18, 21). However, the ability of IgG fragments to augment complement activation appears to depend on the system under investigation, since IgG F(ab)'₂, but not Fab' fragments, are able to mediate killing of *E. coli* O111B4 by the alternative pathway (11).

A number of studies that examined the mechanisms whereby serum-resistant organisms are made serum sensitive by antibody have shown that the function of bactericidal antibody is seldom simply to increase complement activation. In several cases, bactericidal antibody appears to increase the efficiency of the available C5b-9 complexes. Antibody against *Neisseria gonorrhoeae* seems to influence the molecular configuration of C5b-9 deposited on the cell surface, as the conformation of detergent-eluted C5b-9 complexes changed when serum-resistant gonococci were made serum sensitive with immune antibody (12). Bactericidal antibody against *E. coli* O111 enhances the efficiency of the membrane attack complex without increasing the number of C5b-9 complexes bound (11). Although the nature of this increased efficiency is not known, formation of C3b-IgG complexes during the bactericidal reaction appears to be critical for killing (10). As in the studies discussed above, our findings demonstrate that anti-*B. burgdorferi* IgG is bacteriolytic through an effect on the efficacy of C5b-9.

However, the mechanism of the antibody action on *B. burgdorferi* is different from those implicated in antibody action on other gram-negative bacteria investigated (15). We previously demonstrated that identical amounts of C8 and C9 are deposited on *B. burgdorferi* in the presence or absence of anti-*B. burgdorferi* IgG. We also showed that in both situations there was a similar degree of C9 polymerization in the C5b-9 complex. However, killing occurred only in the presence of specific antibody, suggesting that the antibody induces a qualitative modification in the manner that C5b-9 interacts with the borreliae (15). This interpretation was supported by the finding that the C9 molecules on *B. burgdorferi* that had been exposed to bactericidal antibody and complement were significantly less susceptible to cleavage by trypsin than C9 on bacteria that were incubated with complement alone (15). Although several factors can affect trypsin release (12), it was proposed that the presence of antibody on the bacterial surface changed the trypsin accessibility of bound C9 by allowing increased insertion of the C5b-9 complex into the outer membrane (15). Our current results show that IgG Fab fragments derived from bactericidal antibody are able to mediate killing of *B. burgdorferi*, thus directly demonstrating that the bactericidal effect of anti-*B. burgdorferi* IgG is independent of the complement-activating properties of the antibody. These results are compatible with our previous proposal of a novel bactericidal mechanism whereby antibody interacts with *B. burgdorferi* thus reducing steric constraints placed on the membrane attack complex of complement by components of the cell surface.

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