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Role of Immunoglobulin G in Killing of *Borrelia burgdorferi* by the Classical Complement Pathway

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The antibody and complement requirements for killing of *Borrelia burgdorferi* 297 by normal human serum (NHS) and NHS plus immunoglobulin G (IgG) were examined. *B. burgdorferi* activated both the alternative and classical complement pathways in NHS. In NHS chelated with 10 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid plus 4 mM $MgCl_2$ (Mg-EGTA) to block classical pathway activation, consumption (activation) of total hemolytic complement, complement component 3 (C3), and C9 by *B. burgdorferi* was observed. Furthermore, challenge of unchelated NHS with 297 cells resulted in the consumption of C4, in addition to an increase in C3 and C9 consumption over that observed in chelated serum. In spite of complement activation, *B. burgdorferi* was resistant to the nonspecific bactericidal activity of NHS. The addition of human anti-*B. burgdorferi* IgG to NHS, however, resulted in the complete killing of 297 cells. Bactericidal activity of this serum was abrogated if NHS was immunochemically depleted of C1, indicating that killing was mediated by the classical pathway. The manifestation of bactericidal activity was accompanied by a large increase in total complement and C3 consumption over that observed in NHS alone. Under similar conditions, only a minimal increase in C9 consumption was observed. No increase in total complement consumption was observed if NHS plus anti-*B. burgdorferi* IgG was treated with Mg-EGTA prior to challenge. The results of these experiments demonstrate that *B. burgdorferi* is resistant to the nonspecific bactericidal activity of NHS, in spite of classical and alternative complement pathway activation. *B. burgdorferi* is sensitive to serum, however, in the presence of IgG, which mediates bacterial killing through the classical complement pathway.

Lyme disease is a complex, multisystemic illness caused by the spirochete *Borrelia burgdorferi* (7, 44). Relatively little, however, is known about the immunological factors that influence the expression of the disease. An important aspect of the disease is the ability of the bacterium to survive in the host for months to years after the initial onset of disease (39, 43). The disease may manifest itself in many organ systems, although isolation of the bacteria has proven difficult owing to the small number of bacteria present in any one organ. Interestingly, *B. burgdorferi* has been isolated, albeit not exclusively, from experimentally infected animals in sites regarded to be immunologically privileged (22, 36). While *B. burgdorferi* appears to elicit an immune response, the spirochete appears to be able to circumvent destruction by the host immune system. Novel *B. burgdorferi*-reactive immunoglobulin M (IgM) as well as IgG species continue to appear months to years after initial infection in chronically ill patients (9). Furthermore, decreased levels of serum complement component 3 (C3) and C4 have been observed, although it is not clear whether activation of complement is due directly to the bacteria or to some secondary manifestation of the disease (45).

In the case of a number of gram-negative bacterial infections, resistance to complement-mediated killing in serum is an important factor in determining the ability of the infecting organism to survive in the host (29, 40). In general, serum-resistant organisms are more pathogenic than those which are serum sensitive, since the bactericidal activity of normal human serum (NHS) generally prevents the dissemination of serum-sensitive organisms. Furthermore, the presence of natural or immune antibodies may significantly affect serum bactericidal activity and, ultimately, the manifestations of the disease (40, 47). Thus, the bactericidal activity of antibody and complement in human serum plays an essential role in the host defense against gram-negative bacteria. To more fully understand the immunological aspects of Lyme disease, we undertook an analysis of the possible mechanisms employed by *B. burgdorferi* to survive for prolonged periods of time in an infected host.

In this study we examined the susceptibility of *B. burgdorferi* to the bactericidal activity of NHS and immune human serum, the antibody and complement requirements for bacterial killing, and the possible mechanism by which this process takes place.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *B. burgdorferi* 297, which was isolated from the cerebral spinal fluid of a patient with Lyme disease, was originally obtained from Allen Steere, Yale University, New Haven, Conn. (44). Strain 297 was maintained in Barbour-Stoenner-Kelly (BSK) medium (2) at 30°C in a standard air incubator and was subcultured every 5 days.

Serum. Blood from two or more healthy donors (with no known previous history of spirochetal infection) was obtained by venipuncture and allowed to clot for 30 min at room temperature and then for 2 h at 4°C. After centrifugation at 2,000 × *g*, the serum was removed, fractionated into small volumes, and stored at −80°C until needed. All normal serum samples were screened for *Borrelia*-reactive antibodies by immunofluorescence. Sera were considered seronegative if the immunofluorescence titer was <1:16.

IgG was obtained by fractionation of NHS (Lyme disease titer, <1:16; as determined by immunofluorescence) or im-

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mune human serum from a patient with chronic Lyme disease (titer, >1:8,092) on a QAE-Sephadex column (1 by 30 cm; A-50; Pharmacia Fine Chemicals, Piscataway, N.J.). IgG preparations contained 7.1 mg of IgG per ml from NHS and 6.8 mg of IgG per ml from immune human serum, as determined by radial immune diffusion (Calbiochem-Behring, La Jolla, Calif.). The IgG preparations were free of detectable IgM, IgA, C1, C3, and C4, as determined by double immunodiffusion against goat anti-human IgM, IgA, and C1 (Cappel Laboratories, Cochranville, Pa.); C3 (Sigma Chemical Co., St. Louis, Mo.); and C4 (Calbiochem-Behring).

Treatment of serum. For some experiments, NHS was inactivated by heating it at 56°C for 60 min. To selectively block classical pathway activation, NHS was treated with 4 mM $MgCl_2$–10 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (10 mM Mg-EGTA) (11). To block activation of both the classical and alternative pathways, NHS was treated with 10 mM EDTA (10).

Complement component-depleted human serum. NHS was immunochemically depleted of C1 by the modification of a previously described procedure (32). Briefly, C1 was precipitated from NHS by treatment with 38.5% polyethylene glycol 6000 and incubated at 4°C for 30 min (33). The precipitate was pelleted by centrifugation, and the supernatant was dialyzed against 2 liters (three changes in 24 h) of Veronal-buffered saline (VBS). The dialysate was passed over an IgG-Sepharose column (16) and concentrated to the original serum volume by ultrafiltration. (PM-10 membrane; Amicon Corp., Danvers, Mass.). C1-deficient (C1D) human serum was fractionated into small volumes and stored at −80°C until it was needed. The alternative complement pathway integrity of C1D human serum was assessed by using a rabbit erythrocyte assay (34). Total hemolytic complement activity was determined by using a sheep erythrocyte assay (31). By the sheep erythrocyte assay, no classical pathway activity in C1D human serum was detected. The minimal amount of total complement activity detectable by our system was 5 CH_{50} U/ml (CH_{50}, 50% complement hemolysis; by comparison, NHS contained an average of 60 to 70 CH_{50} U/ml). Greater than 87% of the original alternative complement pathway activity remained after C1 depletion. No C1 activity was detected in C1D human serum, when the serum was assayed as described below. The addition of 100 U of functionally pure human C1 (Diamedix Corp., Miami, Fla.) per ml to C1D human serum resulted in the restoration of approximately 60% of the original C1 titer and 79% of the original CH_{50}. While this procedure undoubtedly precipitated other complement components, the addition of purified C1 restored the CH_{50} value to an acceptable level for our purposes. Nevertheless, to compensate for this lower activity, the concentration of C1D human serum was increased from 20 to 40% for use in the serum bactericidal assay.

Serum bactericidal assay. The serum bactericidal assay was modified slightly from a previously described method (21). For these experiments, overnight cultures of strain 297 cells grown in BSK medium were centrifuged at 4,300 × *g* for 20 min at 23°C. The cell pellet was washed once with Eagle minimal essential medium with Hanks salts (pH 7.4; Sigma), and the cell concentration was determined in a Petroff-Hausser counting chamber. The cells were adjusted to the desired concentration in minimal essential medium. The serum bactericidal assay was carried out in plastic tubes (12 by 75 mm; Becton Dickinson Labware, Oxnard, Calif. [Div. Becton Dickinson and Co.]). NHS was serially diluted in minimal essential medium, and 10^7 297 cells and 20 μg of lysozyme (Sigma) were added. In some cases, 5% (vol/vol) (135 μg) human nonimmune IgG or immune IgG was added. The reaction mixtures were then incubated at 37°C for 2 h with periodic shaking. The addition of lysozyme alone to cell preparations had no apparent effect on cell viability. It did, however, facilitate differentiation between viable and nonviable cells by enhancing morphological changes in nonviable cells. Afterward, the percent cell survival was determined by removing 10-μl volumes for examination by dark-field microscopy and by counting triplicate samples (>100 cells per sample). Cells were considered killed when loss of motility, extensive surface blebbing, loss of refractility, or all three conditions were observed. Results were compared with those for control tubes which contained minimal essential medium in place of NHS. Bactericidal activity was determined by calculating the percentage of viable cells in each test sample. In addition, 0.1-ml samples were removed from each tube, inoculated into 5 ml of BSK medium, and incubated at 30°C for 5 days; the number of viable cells was then counted. The growth, or lack of growth, in the 5-day-old cultures was compared with results obtained by dark-field microscopy.

Complement consumption assay. Complement activation assays were performed as described previously (42). NHS was mixed with 10 mM Mg-EGTA or 10 mM EDTA, to block complement activation as described above, or an equivalent volume of saline as a control. An equal volume of the treated or untreated NHS was then mixed with an equal volume of the challenge substance: 10^9 strain 297 cells per ml, 10^9 sensitized sheep erythrocytes per ml, 10 mg of zymosan per ml, or an equivalent amount of saline (final volume, 0.5 ml). In these experiments, no exogenous lysozyme was used. The reaction mixtures were incubated at 37°C for 60 min with frequent mixing. Subsequently, the challenge substances were pelleted by centrifugation at 20,000 × *g* for 10 min at 4°C. A 250-μl volume of challenged serum supernatant was removed from each sample, and the chelator was saturated with 10 mM $CaCl_2$. Total complement, C3, C4, and C9 activities were then assayed as described below.

Complement assays. Total hemolytic complement titrations were performed on serum before and after chelation and challenge to determine nominal and residual complement activities. Briefly, sensitized sheep erythrocytes (5 × 10^8 cells per ml) were mixed with untreated or chelated NHS or challenged sera supernatants and incubated at 37°C for 60 min with frequent mixing. Unhemolyzed erythrocytes were removed by centrifugation, and the amount of released hemoglobin was measured spectrophotometrically at an A_{541}. Assays for individual complement component consumption were performed by using serum depleted of the component being measured and challenged sera supernatants, as described previously (14). Hemolytic titrations were performed by using the appropriate complement-cellular intermediate (EAC) at 10^8 cells per ml. EAC 4, EAC 1,4, and EAC 1 to 7 (Diamedix) were used for determining C1, C3, and C9 consumptions, respectively. The C1 titer was measured in C1D human serum only. C4 consumption was measured by using C4-deficient guinea pig serum (Diamedix) and sensitized sheep erythrocytes, as described elsewhere (13). The hemoglobin released into the supernatant after titration of the challenged sera supernatants was measured spectrophotometrically at an A_{415}, and the functional complement units in each sample were calculated.

Immunofluorescence assay. C3 and immunoglobulin binding to strain 297 cell surfaces were assessed by using

fluorescein isothiocyanate-conjugated goat anti-human C3 (Sigma) and anti-human IgG, IgM, and IgA (Cappel). Strain 297 cells were incubated in treated or untreated serum at 37°C for 25 min, pelleted by centrifugation at 20,000 × *g*, and washed three times in VBS. The pellet was suspended in fluorescein isothiocyanate-conjugated antiserum diluted 1:200 in VBS containing 1% bovine serum albumin. After incubation at 37°C for 60 min, the stained cells were washed twice in VBS, smeared on a microscope slide, and observed under UV light. Binding of C3 or immunoglobulin to the cell surface of *B. burgdorferi* was scored positive if the observed fluorescence was greater than that observed for the control sample.

RESULTS

Susceptibility to NHS and NHS-IgG. The susceptibility of *B. burgdorferi* to NHS was determined by incubating strain 297 cells at NHS concentrations ranging from 1 to 90% for 2 h at 37°C in an in vitro bactericidal assay. The amount of cell killing was determined by dark-field microscopy. Under these conditions, 297 cells were resistant to the nonspecific bactericidal activity of NHS at all tested concentrations. On average, greater than 85% of the cells counted at each serum concentration were viable. The deviation from this mean was less than 5% over the entire test range. No significant change in the bactericidal activity of NHS was observed when the incubation period was increased to 4 h or when the cell concentration was decreased from 10^7 to 10^5 cells per ml.

In contrast, the addition of 5% purified human anti-*B. burgdorferi* IgG to NHS resulted in cell killing. Greater than 70% of the cells counted were killed at NHS concentrations as low as 5%, in a manner that was dependent on the serum dosage, when anti-*B. burgdorferi* IgG was present (Fig. 1). Five percent anti-*B. burgdorferi* IgG was found to be the minimal amount of IgG required to kill 100% of the cells in 20% NHS in 2 h (data not shown). To determine whether IgG alone was bactericidal, 1, 5, 10, or 25% anti-*B. burgdorferi* IgG was added to 297 cells in test buffer alone. The results indicate that anti-*B. burgdorferi* IgG alone has no detectable effect on cell viability, although some cell clumping at 10 and 25% IgG was observed. The borreliacidal activity of

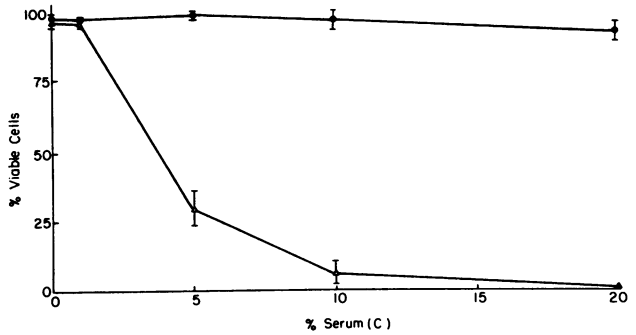

FIG. 1. Bactericidal activity of NHS or NHS-IgG for strain 297. Bacteria were incubated in increasing concentrations of NHS or NHS–anti-*B. burgdorferi* IgG for 2 h at 37°C. Serum bactericidal activity was evaluated by calculating the percentage of viable cells at each serum concentration, as determined by dark-field microscopy. Values for percent viability are expressed relative to those for the control tube and represent the mean ± standard deviation of three experiments run in triplicate. Symbols: ●, NHS; △, NHS–anti-*B. burgdorferi* IgG.

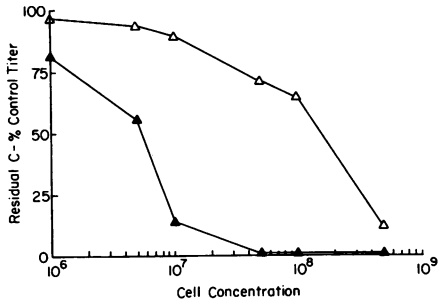

FIG. 2. Consumption of total hemolytic complement in NHS and NHS-IgG challenged with strain 297. Increasing concentrations of bacteria were incubated in 20% NHS or 20% NHS–anti-*B. burgdorferi* IgG for 60 min at 37°C. Residual complement activity was determined by the sheep erythrocyte assay, as described in the text. Values for percent consumption are expressed relative to those for the control tube and represent the mean of duplicate samples of a representative experiment. Symbols: △, NHS; ▲, NHS-IgG.

NHS–anti-*B. burgdorferi* IgG was abrogated if NHS was heat inactivated prior to the assay. In these samples, as in those with anti-*B. burgdorferi* IgG only, the number of surviving organisms was found to be equivalent to that seen in samples incubated in NHS alone.

Effect of IgG on activation of complement. To determine the importance of complement to the bactericidal activity of NHS-IgG, we examined the relative contribution of IgG to complement activation. Complement consumption in NHS challenged with strain 297 cells was consistently observed; approximately 80% of total hemolytic complement was depleted by 5 × 10^8 cells (Fig. 2). The addition of anti-*B. burgdorferi* IgG to NHS, however, resulted in a large increase in total complement consumption by 297 cells over that observed in NHS. In this case, 80% of total hemolytic complement consumption was observed with 10^7 cells. The effect of anti-*B. burgdorferi* IgG on complement consumption in NHS was not observed if an equivalent amount of nonimmune IgG was used, or if NHS was chelated with Mg-EGTA to block classical pathway activation. Differences in the consumption of the individual complement components C3 and C9 were also observed. Consumption of C3 paralleled that observed for total complement consumption (Fig. 3A). The dose-response curves for C3 consumption rapidly became skewed. At the three highest cell concentrations tested, 100% depletion of C3 was observed in NHS–anti-*B. burgdorferi* IgG challenged with 297 cells. In contrast, only 15 to 40% consumption of C3 was observed in NHS tested at similar cell concentrations. The difference in C9 consumption was less pronounced. Although the consumption of C9 varied from approximately 10 to 30% at a given cell concentration, the dose-response curves paralleled each other relatively closely over the entire cell concentration range (Fig. 3B).

Role of complement in killing by NHS and anti-*B. burgdorferi* IgG. Results of an examination of the contribution of the classical and alternative complement pathways toward killing of strain 297 cells by NHS–IgG are shown in Fig. 4. C1D human serum, which had no classical pathway activity, lacked bactericidal activity in the presence and absence of anti-*B. burgdorferi* IgG. When 100 U of purified C1 per ml was added to C1D human serum plus anti-*B. burgdorferi* IgG, however, the reaction mixture became highly bactericidal; greater than 95% of the cells were killed. C1D human serum was not bactericidal when classical pathway activity

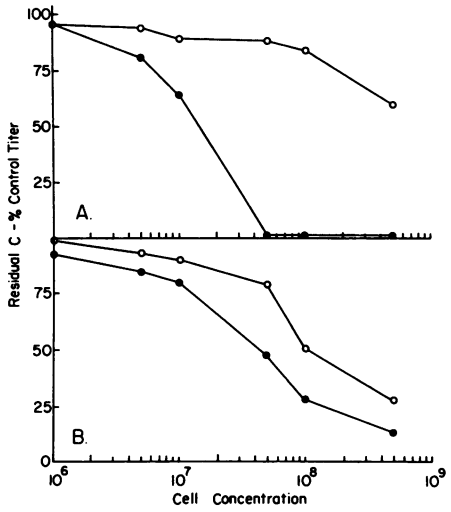

FIG. 3. Consumption of C3 (A) and C9 (B) in NHS or NHS-IgG incubated with strain 297. Increasing concentrations of 297 cells were incubated in 20% NHS or NHS supplemented with 5% anti-*B. burgdorferi* IgG for 60 min at 37°C. Residual C3 and C9 activities were determined by hemolytic titration, as described in the text. Values for percent consumption are expressed relative to those for the control tube and represent the mean of duplicate samples of a representative experiment. Symbols: ○, NHS; ●, NHS–anti-*B. burgdorferi* IgG.

was restored with purified C1 in the absence of anti-*B. burgdorferi* IgG.

Complement activation by *B. burgdorferi* in NHS. Two series of experiments were performed to evaluate the activation of the alternative and classical pathways by strain 297 cells in NHS alone. In the first series of experiments, residual complement in untreated NHS and NHS treated to inactivate the classical pathway (Mg-EGTA) or the alternative and classical pathways (EDTA) was measured by the sheep erythrocyte assay, after challenge with 297 cells or complement activators with known activity. Hemolytic complement activity was not detected in untreated serum after challenge with any activator (Fig. 5). No residual hemolytic activity was detected in Mg-EGTA-chelated NHS after challenge with zymosan, a known activator of the alternative pathway. The residual complement titer in Mg-EGTA-chelated serum challenged with 297 cells was decreased by 40% compared with the control level. Serum treated with EDTA showed no significant decrease in hemolytic titer after incubation with any agent. To preclude the possibility that NHS contained *B. burgdorferi*-reactive antibodies that were not detected by the immunofluorescence assay or enzyme-linked immunosorbent assay, NHS was preabsorbed with 2×10^9 strain 297 cells per ml. Preabsorption of untreated or chelated serum prior to challenge resulted in titers that were not significantly different from those of nonabsorbed sera (data not shown).

In the second series of experiments, consumption of the individual complement components C3, C4, and C9 was examined. Untreated NHS or NHS chelated with Mg-EGTA or EDTA was incubated with 5×10^8 297 cells per ml at 37°C for 60 min. The consumption of C3, C4, or C9 was quantitated by hemolytic assay with complement-cellular intermediates. A significant consumption of C4, a component that is exclusive to the classical pathway, was observed only in untreated serum (Fig. 6). Consumption of C3, a component of both complement pathways, paralleled total hemolytic complement consumption; 65% of total C3 was consumed in untreated NHS, but only 30% consumption was observed in serum in which only the alternative pathway was functional. Consumption of C9, an essential component of the C5b-C9 membrane attack complex, was also examined. C9 consumption paralleled total complement consumption in a manner similar to that seen for C3 consumption; 100% of the available C9 was consumed in untreated serum versus 70% in Mg-EGTA-chelated serum.

Deposition of C3 onto *B. burgdorferi* in NHS. Strain 297 cells were examined by fluorescence microscopy after incubation with untreated and chelated NHS. Bacterial cells incubated in NHS or NHS treated with 10 mM Mg-EGTA were strongly fluorescent when incubated subsequently with

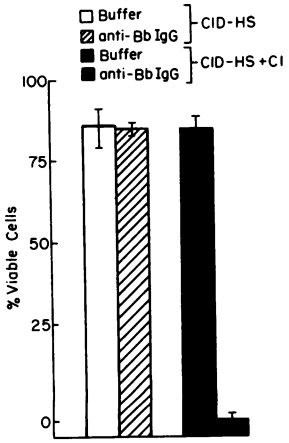

FIG. 4. Classical pathway-mediated bactericidal activity of C1D human serum (C1D-HS) for *B. burgdorferi*. Cells were incubated in 40% C1D human serum or C1D human serum reconstituted with 100 U of purified human C1 per ml. The bactericidal activity of C1D human serum or repleted serum in the presence or absence of 5% anti-*B. burgdorferi* IgG was then evaluated by dark-field microscopy as described in the legend to Fig. 1. Values represent the mean ± standard deviation of two experiments run in triplicate.

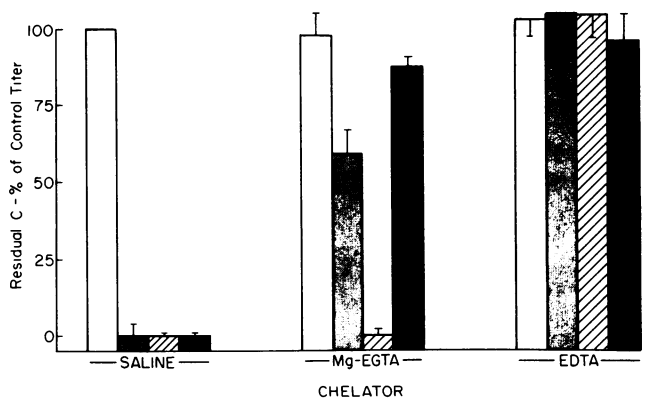

FIG. 5. Consumption of total hemolytic complement in chelated (with 10 mM EDTA or 10 mM Mg-EGTA) and unchelated NHS incubated with various challenge agents. Residual complement activity was measured after incubation of 20% treated or 20% untreated NHS with saline, SHEA (classical pathway activator), zymosan (alternative pathway activator), or *B. burgdorferi* 297. Values for complement consumption are expressed as the mean ± standard deviation of six experiments. Symbols: ▭, saline; ▬, strain 297; ▨, zymosan; ▬, SHEA.

anti-human C3 (Fig. 7), but not when incubated with anti-human IgG, IgM, or IgA. The fluorescent staining of cells by anti-C3 was not observed if NHS was heat inactivated or chelated with 10 mM EDTA. C3 binding in both untreated and Mg-EGTA-chelated NHS was stable, as washing of the cells with VBS containing 0.05% sodium dodecyl sulfate failed to significantly reduce the amount of fluorescence.

DISCUSSION

It is well established that the deposition of a stable membrane attack complex (MAC), which is composed of the terminal components of the complement system (C5b-C9), on the outer membrane of a serum-sensitive bacterium is required for complement to exert its bactericidal effect (25, 26, 29). By destroying membrane integrity, complement-mediated damage allows the entrance of the extracellular enzyme lysozyme into the bacterial periplasm, facilitating bacteriolysis. Lysozyme is not required for the bactericidal activity of complement alone (5, 17, 18, 40), and its role in the serum bactericidal reaction has not been fully elucidated (48).

While gram-negative bacteria may be either sensitive or resistant to the bactericidal action of serum, essentially all gram-positive bacteria resist direct killing (23). The thick peptidoglycan layer of the cell wall inhibits effective MAC penetration, indicating that the surface and structural architecture of the bacterium is a primary factor in the ability of the cell to resist complement-mediated killing. Only a minimal amount of information is available about the surface composition of *Borrelia* spp. The structure of *B. burgdorferi* in general appears to be similar to that of other gram-negative bacteria, in that the protoplasmic cylinder is surrounded by a trilaminar cytoplasmic membrane-type outer membrane (19). However, this structure differs from that of traditional gram-negative bacterial outer membranes because of its extreme fluidity (3). Morphological examination of *B. burgdorferi* exposed to human immune serum indicated that the resulting blebbing and distortion of this outer membrane was similar to that observed in the immune lysis of *Leptospira interrogans* (20; S. Kochi, unpublished data). While the presence of a lipopolysaccharide component in the outer membrane of *B. burgdorferi* has not been consistently demonstrated (46), lipopolysaccharidelike activity has been observed in phenol-water extracts of *B. burgdorferi* cells (4). The lipopolysaccharide was chemically similar to that of other gram-negative bacteria that possess a rough-type oligosaccharide side chain. Endotoxinlike activity with respect to pyrogenicity, mitogenicity, and cytotoxicity was observed. The activity of this lipopolysaccharide, however, was 10-fold less active, on a per weight basis, than that of *Escherichia coli* in the *Limulus* amebocyte assay.

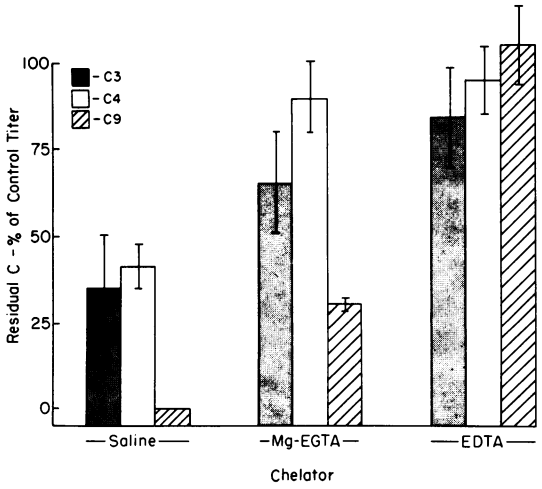

FIG. 6. Consumption of complement components C3, C4, and C9 in chelated (10 mM EDTA or Mg–10 mM EGTA) and unchelated NHS by *B. burgdorferi*. Bacteria were incubated in 20% treated or 20% untreated NHS for 60 min at 37°C; and the residual C3, C4, and C9 hemolytic activities in test sera supernatants were assayed. Values for complement component consumption are expressed relative to those for the control tube. All values represent the mean ± standard deviation of three experiments.

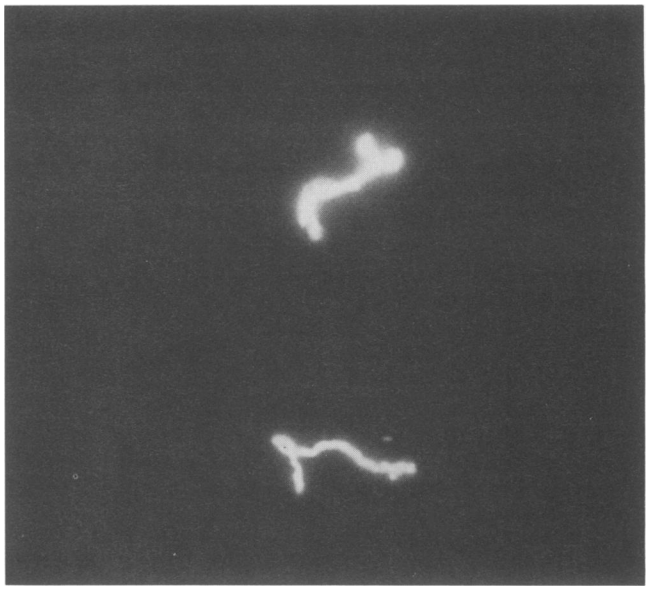

FIG. 7. Photomicrograph of *B. burgdorferi* stained by immunofluorescence with fluorescein isothiocyanate-conjugated anti-human C3 antiserum. Cells were incubated in 10% NHS, as described in the text. Fluorescence was evenly distributed over the bacterial cell surface. Cells incubated in Mg-EGTA-chelated NHS fluoresced similarly, while cells incubated in EDTA-chelated or heat-inactivated NHS were dark. Magnification, ×780.

In this study, the susceptibility of *B. burgdorferi* 297 to the bactericidal activity of NHS and NHS-IgG was examined. *B. burgdorferi* was resistant to killing by NHS alone at concentrations as high as 90%. Killing of *B. burgdorferi* by NHS did not appear to be delayed, as has been observed for *Treponema pallidum* (35), since results obtained by dark-field microscopy correlated with results obtained in BSK growth medium 5 days later. No change in the serum sensitivity of 297 cells to NHS was detected if the bacterial cell concentration was decreased 100-fold, suggesting that saturation of the bactericidal system was unlikely. In contrast, the addition of 5% anti-*B. burgdorferi* IgG to NHS resulted in significant cell killing at NHS concentrations as low as 5% in a dose-dependent manner. IgG was necessary but not sufficient for killing of strain 297. Heat inactivation of NHS at 56°C for 45 min prior to the addition of IgG abolished all bactericidal activity, suggesting that complement is a necessary requirement for killing. The heterogeneity of the IgG response makes it difficult to determine the bactericidal immunoglobulin binding site. This situation is further complicated by the relative lack of information about the *B. burgdorferi* surface structure and difficulties in isolating and purifying the spirochetal outer membrane. Research into this

aspect of the bactericidal reaction is currently being conducted. It should be noted that in this study we evaluated the bactericidal activity of IgG only. This aspect was emphasized because of the continuing expansion of the IgG response during the chronic stage of the illness (9) and the importance of IgG in immune protection.

An interesting result was our observation that *B. burgdorferi* was not killed by NHS, in spite of the activation of both the alternative and classical pathways. The results of the total complement and complement component hemolytic assays indicate that strain 297 activates complement, and that consumption does not represent inactivation of a specific complement component. Furthermore, results of these studies indicate that activation is associated with consumption of late components. The results also indicate that strain 297 activates the classical pathway in the absence of detectable *B. burgdorferi*-reactive antibodies. In spite of our effort to exclude putative antibody, however, we could not conclude that *B. burgdorferi* activated the classical pathway by directly interacting with C1. Our observation that *B. burgdorferi* activated the classical pathway in the absence of detectable antibody is not without precedent. Such activation has been observed by both bacteria (1, 15, 30) and other microorganisms (8).

Activation of complement via the classical complement pathway was found to be essential for killing by NHS and IgG. The removal of C1 from NHS resulted in the abolition of borreliacidal activity by NHS–anti-*B. burgdorferi* IgG. Bactericidal activity was restored if C1D human serum was reconstituted with purified human C1. The addition of anti-*B. burgdorferi* IgG to C1D human serum alone did not result in the manifestation of any significant bactericidal activity. This was consistent with our observation that anti-*B. burgdorferi* IgG did not enhance total hemolytic complement consumption in Mg-EGTA-chelated NHS, and indicates that only the classical pathway is responsible for mediating bactericidal activity in the presence of IgG.

The addition of IgG to NHS resulted in a decrease of 1.5 log units in the number of strain 297 cells required for the consumption of approximately 80% of the total hemolytic complement when compared with that of NHS alone. The rapid divergence of the dose-response curves for consumption of C3 suggests that immune antibody has a direct effect on C3 activation. It has been demonstrated that complement activation by immune antibody leads to an increase in C3 consumption, most likely because of the propensity of C3b to bind to antibody molecules (6, 12). Thus, the difference in C3 consumption between the two sera could be a result of this fact. Alternatively, IgG could act by increasing C1 activation, prompting an increase in C3 consumption. Results of immunofluorescence studies indicate that C3 consumption does not occur exclusively in the fluid phase, and that C3 is stably deposited on the cell surface. These results have been confirmed by deposition studies in which ^{125}I-labeled C3 was used, and indicated that C3 is deposited in a serum dose-dependent manner (S. K. Kochi, R. C. Johnson, and A. P. Dalmasso, manuscript in preparation). Thus, the C3b that was bound to the bacterial surface is ineffective in promoting bacteriolysis in the absence of antibody. As a result, we conclude that serum resistance is not due to a block in C3 utilization.

The consumption of fluid-phase C9 parallels the formation of the MAC on the surface of serum-sensitive organisms. In the case of some serum-resistant organisms, however, interference by bacterial surface structures with the attachment, formation and insertion of the MAC appears to result in the consumption of C9 without significant deposition (24, 38). The difference in C3 consumption between NHS- and NHS–anti-*B. burgdorferi*-challenged sera did not appear to manifest itself in a commensurate increase in C9 consumption. Furthermore, the similarity between the dose-response curves suggests that the effect of bactericidal antibody was not simply to increase C9 activation. This suggests that the manner in which the MAC binds to the cell surface, rather than a quantitative difference in the number of attack complexes deposited, could be a determining factor in serum resistance by *B. burgdorferi*. Studies are currently being conducted to examine this possibility.

Our results suggest that the antibody and complement requirements for killing of *B. burgdorferi* appear to be similar to those for killing of other serum-resistant bacteria that require antibody for killing (37, 40). Several mechanisms have been postulated to explain how specific antibody enhances the bactericidal activity of complement. In the case of serum-resistant *Neisseria gonorrhoeae*, immune antibody appears to change the molecular configuration of the MAC, allowing more favorable binding (28, 31). In the case of other resistant bacteria, antibody appears to function by facilitating the clustering of attack complexes (24) by relieving steric hindrance between surface components and the attack complex (27, 31) or by influencing the kinetics of terminal component deposition (19). Our evidence for similar mechanisms that operate in this system, however, is indirect and based only on the depletion of serum components. These observations may not accurately reflect that which occurs at the cell surface. Further studies are being conducted to examine the effects of immune antibody on terminal component deposition and stability.

To our knowledge, this is the first report in which the interaction of *B. burgdorferi* with normal and immune serum components has been examined. Our results are similar to those observed with another pathogenic spirochete, *L. interrogans* (21); both organisms require the presence of immune antibodies for serum-mediated killing. Complement activation has also been observed with *Borrelia hermsii* (S. K. Kochi, unpublished data) and *Borrelia turicatae* (K. Newman, Ph.D. thesis, University of Minnesota, Minneapolis, 1984). It is conceivable that the ability of *B. burgdorferi* to resist complement-mediated killing by NHS is an invasive mechanism used by these spirochetes to establish infection. The dissemination of *B. burgdorferi* to sites that are not readily accessible to antibody could preclude antibody from interacting with complement, resulting in an inability by the host to kill the bacteria.

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