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Serum-Resistant Strains of \textit{Borrelia burgdorferi} Evade Complement-Mediated Killing by Expressing a CD59-Like Complement Inhibitory Molecule\textsuperscript{1}

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\textit{Borrelia burgdorferi}, the etiological agent of Lyme disease, comprises three genospecies, \textit{Borrelia garinii}, \textit{afzelii}, and \textit{burgdorferi} sensu strictu, that exhibit different pathogenicity and differ in the susceptibility to C-mediated killing. We examined C-sensitive and C-resistant strains of \textit{B. burgdorferi} for deposition of C3 and late C components by fluorescence microscope and flow cytometry. Despite comparable deposition of C3 on the two strains, the resistant strain exhibited reduced staining for C6 and C7, barely detectable C9, and undetectable poly C9. Based on these findings, we searched for a protein that inhibits assembly of C membrane attack complex and documented an anti-human CD59-reactive molecule on the surface of C-resistant spirochetes by flow cytometry and electron microscopy. A molecule of 80 kDa recognized by polyclonal and monoclonal anti-CD59 Abs was identified in the membrane extract of C-resistant strains by SDS-PAGE and Western blot analysis. The molecule was released from the bacterial wall using deoxycholate and trypsin, suggesting its insertion into the bacterial membrane. The CD59-like molecule acts as C inhibitor on \textit{Borrelia} because incubation with F(ab')\textsubscript{2} anti-CD59 renders the serum-resistant strain exquisitely susceptible to C-mediated killing and guinea pig erythrocytes bearing C5b-8, unlike the RBC coated with C5b-7, are protected from reactive lysis by the bacterial extract. Western blot analysis revealed preferential binding of the C inhibitory molecule to C9 and weak interaction with C8β.

differ in C-susceptible and C-resistant strains of *B. burgdorferi* suggests that the ability of surface-bound factor H to control C3 activation is not the only mechanism used by these spirochetes to evade C-dependent destruction. The assembly of MAC on *B. burgdorferi* is another critical step of C activation. Kochi et al. (24) first reported that a C-resistant strain can be made susceptible to C killing following exposure to specific Abs and showed that this change was associated with increased stability of C5b, the first component of this complex, on C-susceptible bacteria. Controversy exists on the relative amounts of the late C components and MAC bound to C-resistant and C-sensitive strains of *Borrelia*. Breitner-Ruddock et al. (16) found lower deposits of C6 and MAC on C-resistant bacteria, whereas Patarakul et al. (18) observed no significant difference in the amount of MAC deposited on the two strains and suggested that bacterial killing was dependent on the binding of MAC to susceptible sites on the bacterial surface.

We have decided to re-examine this issue by evaluating both the amount of C3 and that of the late C components bound to C-resistant and C-sensitive strains of *B. burgdorferi*. Data will be presented showing that the C-resistant strains exhibit reduced amounts of the late C components and almost complete absence of C9 and MAC. In addition, evidence will be provided indicating that the resistant strains express on their surface a protein with antigenic and functional similarity to human CD59 acting at the terminal step of C activation (25–28).

**Materials and Methods**

**Antibodies**

The mouse mAb αE11 recognizing a neoantigen of poly C9 (29) was a gift from T. E. Molines (Institute of Immunology, The National Hospital, University of Oslo, Oslo, Norway). The rat blocking mAb YTH53.3 (30), and the mouse nonblocking mAb HCl1 (31), both directed against human CD59, were kindly provided by S. Meri (Department of Bacteriology and Immunology, Haartman Institute, University of Helsinki, Helsinki, Finland), while the mouse blocking mAb to CD59 BRIC 229 was purchased from International Blood Group Reference Laboratory Research Products (Bristol, U.K.). Rabbit antisera to human vitronectin (32) was obtained as a kind gift from K. Høgason (Medical Department, Lillehammer, Norway). The antisera to human CD59 was raised in goat by repeated s.c. injections of purified urinary CD59 (33). The specificity of the antisera was controlled by ELISA and Western blot. The antisera recognized a 21-kDa protein in the membrane extract of human RBC as well as affinity-purified urinary CD59 and rendered human erythrocytes paroxysmal nocturnal hemoglobinuria-like cells by promoting their complete lysis by the terminal components at the concentration of 40 μg/ml IgG in the reative lysis assay (for experimental details, see *C Components and Hemolytic Assay*). Goat antiserum to C3, C6, C7, C8, and C9 and sheep antisera to rat clusterin strongly cross-reacting with human clusterin were purchased from Quidel (San Diego, CA). The two mAbs anti-OspA (clone H5332) and anti-OspB (clone 84C) were kindly provided by A. G. Barbour (Department of Medicine and Microbiology and Molecular Genetics, University of California, Irvine, CA), whereas mAb anti-OspC (clone L2228b) was a gift from B. Wilske (Max von Pettenkofer Institute for Hygiene and Medical Microbiology, LMU, Munich, Germany). The IgGs were purified from the antiserum by affinity chromatography on protein G-Sepharose columns (Pharmacia Biotech, Milan, Italy) and were biotin labeled using biotinimidoacetoate-m-hydroxysuccinimide ester (Sigma-Aldrich, Milan, Italy) following a previously described procedure (34). F(ab’)_2 were prepared by pepsin (Sigma-Aldrich) digestion of affinity-purified IgG dialyzed against 0.1 M citrate buffer (pH 4.0) at an enzyme/IgG ratio of 5 μg pepsin/mg IgG. After incubation of the mixture at 37°C for 4 h, the digest was further purified by gel filtration on a Superose 12 column using a Fast Protein Liquid Chromatography system (Pharmacia Biotech) and the purity of F(ab’)_2 was checked by SDS-PAGE.

**Nonimmune human serum (NHS)**

Serum samples from at least 10 healthy individuals were screened for the presence of Abs reacting with *B. burgdorferi* by Western blot analysis (35). The negative sera were pooled as the source of C and stored in small aliquots at −80°C.

**Bacterial growth and bactericidal assay**

*B. burgdorferi* strains were grown in Barbour-Steinman-Kelly (BSK) medium at 34°C. C-dependent killing of bacteria incubated with NHS was evaluated by the viable cell count in microtiter plates (36). Briefly, borrelias were grown to log phase in BSK medium, washed, and adjusted to 10⁶ bacteria/ml in MEM (MEM + 0.25% BSA). The spirochetes were suspended either in 10% fresh NHS or in NHS heated at 56°C for 30 min, as a negative control, to a final volume of 200 μl and incubated at 37°C for 120 min. After the addition of 100 μl of BSK medium, double dilutions of each sample were made in duplicate rows. The plates were then sealed with sterile polyester adhesive film (Sigma-Aldrich) and incubated at 34°C for 1 wk. The wells were examined under dark-field microscopy for bacterial growth and the percentage of C-dependent killing was calculated by comparing the first dilution of the NHS sample, that showed no visible growth, with that of the control in MEM made equal to 100.

**Immunofluorescence analysis**

Deposition of C components was evaluated on viable bacteria (2 × 10⁶ cells) of both C-resistant and C-susceptible strains incubated with 10% NHS at 37°C for 30 min to a final volume of 1 ml under gentle agitation. NHS was replaced by 10% NHS heated at 56°C for 30 min in the control sample. The spirochetes were then washed twice with PBS by centrifugation at 12,000 × g at 4°C for 10 min and fixed in 0.5% paraformaldehyde for 10 min. The bacterial pellet was suspended in 200 μl of goat antiserum to C components or mAb αE11, incubated for 1 h at room temperature, washed twice, and finally resuspended in 200 μl of 1/200 FITC-conjugated F(ab’)_2 rabbit anti-goat IgG (Southern Biotechnology Associates, Birmingham, AL) or antimouse IgG (DAKO, Milan, Italy). After washing, the spirochetes were viewed under a fluorescence microscope (Zeiss 250-CF, Jena, Germany).

An essentially similar procedure was followed to analyze borreliae for the expression of CD59 and surface deposition of C components using a FACSCalibur flow cytometry (BD Immunocytometry System, San Jose, CA) equipped with an air-cooled argon ion laser fitted at 488 nm and with a filter setting for FITC (530 nm). Forward and side scatter gates were used to exclude bacterial debris or large aggregates from analysis. Events were acquired in list mode and analyzed with CellQuest software (BD Biosciences, Mountain View, CA).

**Transmission electron microscopy**

Two different approaches were used to study the localization of CD59 on *B. burgdorferi*. In the pre-embedding technique, the immune localization of the CD59-like molecule was analyzed on *B. burgdorferi* (2 × 10⁶ cells) fixed in 2% paraformaldehyde in PBS for 10 min at 4°C, washed twice in PBS, and permeabilized in 0.05% Triton X-100 in PBS for 5 min. After two washings in PBS containing 0.02% glycine and blocking nonspecific binding sites with PBS supplemented with 20% normal rabbit serum, the bacteria were incubated overnight at 4°C either with 1/200 goat IgG anti-CD59 or with the same dilution of preimmune IgG in PBS containing 0.5% BSA (PBSA), washed three times in PBSA, and incubated for 40 min in a 1/50 dilution of 1 nm of colloidal gold-labeled rabbit anti-goat IgG (Biocell, Cardiff, U.K.). After several washings in PBSA, the cells were fixed in 1% glutaraldehyde in PBS, postfixed in 0.5% osmium tetroxide in the same buffer, washed in several changes of milliQ water, and developed with a silver-enhancing kit HQ (Nanoprobes, Yaphank, NY) for 8 min in the dark. Finally, the cells were serially dehydrated in ethanol and embedded, via propylene oxide, in Embed 812/Araldite 502 (Electron Microscopy Sciences, Fort Washington, PA). Gold-colored sections were cut on an ultramicrotome (Pabish Top Ultra 150) and collected on 200-mesh copper grids. The grids were stained with uranyl acetate and lead citrate and viewed on a Philips EM 208 transmission electron microscope (Philips, Eindhoven, The Netherlands). In the whole-mount immunolocalization analysis, viable spirochetes (2 × 10⁶ cells) were incubated for 1 h at room temperature in PBS containing 10% goat serum and biotin-labeled goat IgG anti-human CD59 (14/μg/ml) to a final volume of 200 μl. The bacteria were washed twice, fixed for 10 min in an equal volume of PBS containing 1% glutaraldehyde and 0.1% sodium borohydride, and incubated for 30 min at room temperature with 5 nm of colloidal gold-labeled streptavidin (British Biocell) diluted 1/100 in PBS. After two washings, the bacteria were suspended in distilled water, air dried onto 300 mesh formvar-coated nickel grids, and examined under a Philips EM 208 transmission electron microscope.

**Preparation of bacterial extracts**

A suspension of spirochetes (10¹⁰/ml) was centrifuged at 28,000 × g for 30 min at 4°C and the resulting pellet was suspended in PBS containing both
10 mM n-octyl-β-D-glucopyranoside (n-OCT) (Sigma-Aldrich) and a mixture of protein inhibitors for bacterial extracts (P 8465; Sigma-Aldrich), and incubated overnight at 4°C on a rotator. The suspension was then centrifuged and the supernatant was examined by Western blot for the presence of outer surface lipoproteins. The residual pellet was further extracted with 1% deoxycholate (DOC) overnight at 4°C (37). The extracts were dialyzed against PBS to remove the detergent and the protein content was measured by the Coomassie blue protein assay (Bradford reagent; Sigma-Aldrich).

Bacterial extracts were also obtained by incubating spirochetes (10^7/ml) with trypsin (100 μg/ml), purchased from Sigma-Aldrich, for 60 min at 20°C. The effect of trypsin was neutralized by the addition of 1 mM PMSF. At the end of incubation, the suspension was centrifuged and the bacteria collected in the pellet were analyzed by flow cytometry, while the supernatant was examined by Western blot for the released proteins.

SDS-PAGE and Western blot

Bacterial extracts were subjected to electrophoresis through a 8–15% gradient of acrylamide gel under both reducing and nonreducing conditions. The proteins were blotted onto nitrocellulose membrane (Hybond-C Extra; Amersham Pharmacia Biotech, Milan, Italy) using a semiphor system ( Hoefer Scientific Instruments, San Francisco, CA) and the membranes were blocked with 5% bovine nonfat milk (BM) Sigma-Aldrich) in TBS to block nonspecific binding sites. The CD59-reactive bands and the lipoproteins OspA, OspB, and OspC were detected with goat Abs or rat or mouse IgG (Sigma-Aldrich) or BSA (1 mg/ml) overnight at 4°C followed by 10% NHS for 30 min at 37°C. The bound proteins were allowed to react with DOC extracts from *Borrelia* (100 μg/ml) overnight at 4°C followed by 1/2000 goat IgG anti-CD59 and 1/5000 alkaline phosphatase-labeled rabbit IgG (1 mg/ml; Sigma-Aldrich) as a substrate in 0.1 M glycine buffer (pH 3). The enzymatic reaction was revealed using nitroblue tetrazolium (0.60 mg/ml) and 5-bromo-4-chloro-3-indolyl phosphate (0.30 mg/ml), both purchased from Sigma-Aldrich, and diluted in 0.1 M Tris-HCl (pH 9.5) containing 0.1 M NaCl and 5 mM MgCl2. Rainbow RPN 756 (Amersham Pharmacia Biotech) was used as a mixture of molecular mass markers.

Binding of bacterial extracts to C components

Two different approaches were followed to evaluate the ability of bacterial proteins to interact with C components. Wells of microtiter plates (Corning Costar, Acton, MA) were coated with purified late C components (Qidel) or BSA (1 μg/well) by overnight incubation in 0.1 M sodium bicarbonate buffer (pH 9.6) at 4°C. After washing with PBS containing 0.1% Tween 20, the residual free binding sites were blocked with PBS containing 2% BM (milk PBS) for 1 h at 37°C. The bound proteins were allowed to react with DOC extracts from *Borrelia* (100 μg/ml) overnight at 4°C followed by 1/2000 goat IgG anti-CD59 and 1/5000 alkaline phosphatase-labeled rabbit anti-goat IgG (Sigma-Aldrich). The latter steps were conducted for 1 h at 37°C. The enzymatic reaction was developed using p-nitrophenyl phosphate (1 mg/ml; Sigma-Aldrich) as a substrate in 0.1 M glycine buffer (pH 10.4) containing 1 mM MgCl2 and 1 mM ZnCl2, and read at 405 nm using a Titertek Multiskan ELISA reader (Flow Labs, Milan, Italy).

Binding of bacterial proteins to C components was also evaluated following their electrophoretic separation under nonreducing conditions (1 μg/lane) on 10% acrylamide gels and transfer to nitrocellulose membrane. After blocking the free binding sites with BM in TBS, the membrane was incubated overnight at 4°C with DOC extracts from *Borrelia* (100 μg/ml) in TBS plus 0.1% BM containing the mixture of protein inhibitors. After extensive washing, the bound CD59-like protein was detected using goat IgG anti-CD59 followed by alkaline phosphatase-conjugated secondary Abs.

C components and hemolytic assay

C5b6 was prepared as previously described (38) and purified C7, C8, and C9 were obtained from Quidel. The reactive lysis was performed on guinea pig erythrocytes (GPE) or human erythrocytes following a published procedure (39). Briefly, a suspension of GPE (2 x 10^7 in 50 μl) was incubated with C5b6 (2 U) for 10 min at 37°C, where 1 U represents the volume of complement that lyses 50% of erythrocytes in 30 min at 37°C in the presence of 2 μl of serum containing 10 mM EDTA to a final volume of 250 μl. The mixture was further incubated for 30 min at 37°C following the addition of the remaining late C components (1 ng each). Cell lysis was stopped by adding 1 ml of cold PBS containing 10 mM EDTA and measured spectrophotometrically at 412 nm. A similar approach was followed to test the neutralizing effect of goat IgG anti-C5b9 on human erythrocytes that are highly resistant to lysis induced by human MAC due to the surface expression of CD59, except that 100 U of C5b6 was used instead of 2 U. The intermediates GPEC5b-7 and GPEC5b-8 were prepared by incubating GPE (1 x 10^8 in 100 μl) with 10 U of C5b6 and either C7 (5 ng) or a mixture of C7 and C8 containing 5 ng of each component for 5 min at 37°C.

FIGURE 1. Immunofluorescence analysis of C-sensitive and C-resistant strains of *B. burgdorferi* for deposition of C components. The spirochetes were incubated with 10% NHS for 30 min at 37°C and the bound C components or deposition of MAC were revealed by their reaction with goat-specific Abs or mAb α11 followed by FITC-labeled F(ab')2 rabbit anti-goat IgG or goat anti-mouse IgG. The bacteria were then either dried on slides and examined under a fluorescence microscope (left panels) or analyzed by FACS (right panels). The filled and open histograms (continuous line) represent C-resistant and C-sensitive strains exposed to NHS, respectively. The open histogram (dotted line) represents the C-resistant strain incubated with heat-inactivated serum as a control for nonspecific binding of Abs.

Statistical analysis

The results are expressed as mean ± SD. Statistical significance was determined using Student’s t test to compare two groups of data. Values of p = 0.01 or less were considered to be statistically significant.

Results

Deposition of C3 and late C components on C-resistant and C-sensitive strains of *Borrelia*

In preliminary experiments, we tested 13 strains of *B. burgdorferi* for their susceptibility to killing in the presence of 10% NHS and identified three different groups that were either resistant, or sensitive, or showed intermediate resistance to C bactericidal activity. On the basis of these results, BITs of the genospecies *B. garini* and *Alcaide* of the genospecies *B. burgdorferi sensu stricto* were selected for additional experiments as representative of C-sensitive and C-resistant strains, respectively. *Alcaide* proved to be also resistant to killing in the presence of 50% NHS.

Analysis of the spirochetes incubated with fresh NHS for surface-bound C components under a fluorescence microscope and by flow cytometry (Fig. 1) revealed deposition of similar amount of C3 on both strains, although the C5b bound to C-resistant strains
underwent a more extensive degradation than the C3b on the C-sensitive strain (results not shown). The deposits of C6 and C7 on the C-resistant strain were less intense and staining for C8 was also reduced on the latter strain (data not shown). Alcaide exposed to heat-inactivated NHS, as a control for non-specific binding of the antisera, showed only background staining for most antisera. The only antisera that gave a slightly higher background was anti-C6, and this can presumably be attributed to contaminating Abs directed against borrelliae. Unexpectedly, we found that the resistant strain showed negligible staining for C9, which contrasted with the bright staining observed on the sensitive strain. The markedly reduced deposition of C9 on the surface of Alcaide was associated with impaired assembly of MAC, as revealed by the negative reaction of these spirochetes with mAb aE11.

Detection of the CD59-like molecule on the surface of a C-resistant strain of B. burgdorferi

The finding of markedly reduced deposits of MAC on the membrane of a C-resistant strain of B. burgdorferi led us to suspect that a C regulator expressed on this strain and acting at the level of MAC assembly was responsible for this observation. S protein and clusterin were unlikely to be involved in the protection of spirochetes from MAC attack because these are soluble inhibitors that prevent binding of the trimolecular complex C5b-7 (40), which, in our case, was only slightly reduced in contrast with the negligible binding of C9. The data rather suggested to us that CD59 was the most likely candidate to control MAC assembly on a C-resistant strain. To test this possibility, both strains of B. burgdorferi were incubated with goat anti-CD59 Abs followed by FITC-conjugated anti-goat Abs and finally analyzed by flow cytometry. As shown in Fig. 2, staining of the C-resistant isolate was clearly increased compared with that of the C-sensitive strain, which essentially overlapped the background staining observed with the preimmune goat serum. This finding was confirmed on other C-resistant isolates. To further document the presence of CD59-like molecules on B. burgdorferi, both C-resistant and C-sensitive isolates were gold labeled after incubation with IgG anti-CD59 and analyzed under an electron microscope. Colloidal gold particles were clearly visible on the C-resistant strain Alcaide in numbers that were significantly higher than that of the occasional particles seen on the same strain incubated with preimmune IgG or on the C-sensitive strain BITS treated with anti-CD59 Abs (Fig. 3). It should be pointed out that a proportion of these particles were located intracellularly, but particles were also seen on the surface, although in a limited number (Fig. 3).

Effect of anti-CD59 on serum-mediated killing of B. burgdorferi

The ability of anti-CD59 Abs to bind to the surface of the C-resistant strain led us to test whether blocking the C inhibitor expressed on B. burgdorferi with these Abs rendered the spirochetes susceptible to C killing. To avoid direct C activation by bound anti-CD59 Abs, F(ab')2 were prepared from anti-CD59 IgG, and from preimmune goat IgG, as a control for non-specific activity of the F(ab')2 Abs. C-resistant bacteria were incubated either with F(ab')2 anti-CD59 or with control F(ab')2 before exposure to NHS and the percentage of bacterial killing was evaluated following a 2-h incubation at 37°C. The sensitive strain was also included in this experiment as a control for the serum bactericidal activity. As shown in Fig. 4, ~75% of C-resistant spirochetes were killed by NHS after incubation with F(ab')2 anti-CD59. This killing effect was slightly lower than that observed when the C-sensitive strain BITS was exposed to the same concentration of NHS. No killing of Alcaide was seen in the presence of control F(ab')2.

Characterization of the CD59-like molecule expressed on B. burgdorferi

To investigate the size of the molecule recognized by anti-CD59 Abs on B. burgdorferi and to assess whether this molecule is loosely or tightly bound to the bacterial membrane, proteins were sequentially extracted from the membranes of C-sensitive and C-resistant strains with the mild detergent n-OCT (10 mM) and with 1% DOC. The extracted proteins were analyzed by SDS-PAGE and Western blot for the presence of both CD59 and Osps using monoclonal and polyclonal Abs (Fig. 5). The Osps bands were detected in the n-OCT extracts, whereas DOC was required for the release of the CD59-like molecule, suggesting insertion of this protein into the bacterial membrane. Both a polyclonal and three different mAb anti-CD59 revealed a band of ~80 kDa in the extracts of B. burgdorferi, which was higher than the 19- to 21-kDa bands detected in human urine and RBC. The molecular mass of the CD59-like protein remained unchanged when the bacterial extract was analyzed with polyclonal Abs under reducing conditions (data not shown). Next, we examined other strains of B. burgdorferi for the presence of the CD59-like molecule and found that the 80-kDa band was detectable only in strains that survived in the presence of C and was absent in C-susceptible strains (Fig. 6). We failed to reveal any reaction of the extract obtained from...
C-resistant spirochetes with antisera to S protein and clusterin (data not shown).

Enzymatic release of the CD59-like molecule from *B. burgdorferi*

The finding that the CD59-like protein can only be removed from *B. burgdorferi* using a strong detergent suggests that this molecule, unlike the CD59 of eukaryotic cells (26), is probably inserted into the bacterial membranes. To obtain further information on the type of protein insertion, the C-resistant strain of *B. burgdorferi* was treated with trypsin that was previously shown to render *B. burgdorferi* susceptible to C killing (18). After enzymatic treatment, the spirochetes were examined by FACS for the residual expression of the CD59-like molecule and the culture supernatant was analyzed by Western blot for the presence of the C regulatory molecule. The results presented in Fig. 7 show that treatment of Alcaide with trypsin caused reduced staining for CD59 (upper panel) and release in the supernatant of the anti-CD59-reactive molecule appearing as two bands of 20 and 25 kDa that most likely represent degradation products of the 80-kDa detected on this strain of *B. burgdorferi* (lower panel).

CD59-like molecule binds to C8 and C9 and inhibits reactive lysis

Since human CD59 controls the final step of MAC assembly by binding to C8α and C9 and by inhibiting polymerization of C9 (41, 42), we decided to investigate whether the CD59-like molecule of *B. burgdorferi* also interacts with these terminal components. To show this, we first used an immunoenzymatic assay using solid-phase bound C components and found that the bacterial C regulatory molecule binds selectively to C8 and C9 (Fig. 8A). This finding was further extended by analyzing this interaction by SDS-PAGE and Western blot. Human late C components and BSA, as a control, were separated under nonreducing conditions and transferred to a nitrocellulose membrane. After blocking, the membrane was incubated with the extract of *B. burgdorferi* and the binding of the CD59-like molecule was evaluated using goat anti-CD59 Abs. The results reported in Fig. 8 clearly show that the CD59-like molecule binds to C9 and weakly to the C8 subunit.

To assess whether the CD59-like molecule controls MAC formation, we examined the ability of the extract of *B. burgdorferi* to inhibit the reactive lysis of GPE (39). This was tested by incubating GPE with the DOC extracts from either C-resistant or C-sensitive strains of *B. burgdorferi* for 10 min at 37°C. The RBC were
either washed or left unwashed before the addition of C5b6 followed, 10 min later, by the remaining late C components. As shown in Fig. 9A, the extract of the C-resistant strain inhibited MAC-induced lysis in a dose-related manner. However, this inhibitory effect was lost when the RBC incubated with the bacterial extracts were washed before being tested in the reactive lysis. The extract of the sensitive strain was totally ineffective even at the highest concentration (Fig. 9A), as was the n-OCT extract containing Osps (data not shown). To define the stage of MAC assembly at which the C inhibitor acts, GPC5b-7 and GPC5b-8 were mixed with the bacterial extract before exposure to the remaining late C components to induce cell lysis. The results presented in Fig. 9B clearly show that the bacterial extract was able to inhibit lysis of GPEC5b-8 but had only a marginal effect on GPEC5b-7 that was not significantly different from that observed on GPE.

**Discussion**

Resistance to complement attack is one of the strategies adopted by some strains of *B. burgdorferi* to survive in the host and to disseminate from the area of the tick bite to target tissues. C evasion devised by these spirochetes may be of particular importance in the early phase of infection when the Ab response has not yet developed and C provides an important means of protection. The data obtained in this study clearly show that C-resistant strains of
B. burgdorferi express a CD59-like molecule that inhibits the assembly of MAC on their outer membrane and protects them from C-dependent killing.

Analysis of the different strains of B. burgdorferi for the presence of C components bound to their membrane following exposure to NHS free of specific Abs revealed a striking difference in the level of MAC. This suggests that the assembly of this complex on the outer membrane surface is a critical step of C activation that makes the spirochetes susceptible to C-dependent killing. The negligible amount of MAC observed on the membrane of the resistant strain cannot be attributed to a reduced ability of this strain to activate C because the spirochetes bind an amount of C3 similar to that of a C-susceptible strain. These data confirm previous work by various groups showing that C-susceptible and C-resistant strains of B. burgdorferi activate equally well the C system mainly through the classical pathway, although the alternative pathway may also be involved (16, 43). Thus, it appears that neither the degree of C consumption by these strains nor the level of bound C3 fully explain the C sensitivity of B. burgdorferi.

FACS analysis revealed reduced deposition of the late C components on the C-resistant strain, despite the fact that the level of bound C3 is comparable to that on C-sensitive spirochetes. This observation confirms and extends similar data reported by Breitner-Ruddock et al. (16) and suggests that the resistant strains bear a less efficient C5 convertase that may account for the observed discrepancy between the level of C3 and that of the terminal components. Kochi et al. (24) found that the stability of the C5 convertase on the outer membrane of B. burgdorferi is a critical step in promoting C-mediated killing of these spirochetes and may be related to the membrane sites where the enzymatic complex is assembled. Based on recent reports that factor H, a cofactor of factor I involved in the inactivation of C3b, selectively binds to C-resistant strains (20–23), it is conceivable that C3 inactivation occurs on the surface of resistant B. burgdorferi, leading to deposition of inactive C3. This is supported by the data published by Alitalo et al. (20) and by our present finding that C3b undergoes a more extensive degradation on C-resistant than on C-sensitive strains of Borrelia.

The finding of negligible amounts of C9 deposited on the outer membrane of resistant strain compared with the level of the other late components led us to suspect that the resistant strain controls C activation also at the C9 level. This hypothesis was confirmed by the weak reaction of the resistant strain with the mAb to C9 neoantigen expressed in MAC and absent in native C9. These and similar data reported by Breitner-Ruddock et al. (16) with a different mAb to C9 neoantigen suggest that the assembly of MAC on these spirochetes is impaired. Release of MAC from the surface of resistant spirochetes is unlikely to account for our observation since studies by other groups have shown that MAC can partially be removed by trypsin (18, 44). An alternative explanation for the reduced deposition of MAC, besides the relative inefficiency of the C5 convertase, is that an inhibitor may prevent the assembly of the complex on the bacterial surface. The near normal amount of C7 bound to the C-resistant strain tends to exclude that the uptake of C5b-7 is impaired by the action of an inhibitor released from the spirochetes, as it has recently been shown for virulent strains of Streptococcus pyogenes (45) or as a result of the inhibitory effect of S protein and clusterin acting in the fluid phase (40).

**FIGURE 8.** Identification of the C components interacting with CD59-like molecule expressed on C-resistant strain of B. burgdorferi. A. Late C components or BSA coating the wells of microtiter plates (1 μg/well) were allowed to react with DOC extract from Borrelia (100 μg/ml) and the bound proteins were revealed by their reaction with goat IgG anti-CD59 followed by alkaline phosphatase-labeled secondary Abs. The results are presented as mean ± SD of three different experiments. B. Late C components or BSA were subjected to SDS-PAGE under nonreducing conditions (1 μg/lane), transferred to nitrocellulose membranes, and incubated with DOC extract from Borrelia (100 μg/ml). Bound bacterial proteins were detected using goat anti-CD59 Abs and alkaline phosphatase-labeled secondary Abs. C. Late C components and BSA subjected to SDS-PAGE, as in B, and stained with Coomassie blue.

**FIGURE 9.** Inhibition of reactive lysis by Borrelia extract. A. GPE (2 × 10^7 in 50 μl) were incubated with increasing amounts of membrane proteins extracted with 1% DOC from C-resistant and C-sensitive strains of B. burgdorferi for 10 min at 37°C and either washed or left unwashed before the addition of C5b6 followed 10 min later by the remaining late C components. After incubation at 37°C for 10 min, RBC lysis was measured spectrophotometrically at 412 nm. B. GPE, GPEC5b-7, and GPEC5b-8 were incubated with 1 μg/ml extract from C-resistant strains for 10 min at 37°C and washed or unwashed, in the case of GPEC5b-8 to evaluate the effect of the inhibitor on both C8 and C9, before addition of the required terminal C components of the reactive lysis system. The results are presented as mean ± SD of three different experiments.

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Our inability to detect C9 on the surface of the resistant strain rather suggests that inhibition occurs at a later stage of MAC assembly and possibly involves molecules that prevent polymerization of C9. Evidence supporting this conclusion is provided by the positive reaction of the C-resistant, but not of the C-sensitive, strains of B. burgdorferi with both monoclonal and polyclonal Abs to CD59, as assessed by FACS analysis, immunoelectron microscopy, and Western blot. It is important to emphasize that the CD59-like molecule was observed on all C-resistant strains tested while undetectable on C-sensitive strains.

The molecule recognized by Abs to CD59 proved to be functionally effective in inhibiting the assembly of MAC on the bacterial surface because the C-resistant strain of B. burgdorferi incubated with these Abs became highly susceptible to C-dependent killing. The use of non-C-fixing F(ab')2 Abs in these experiments ruled out the possibility that the bacterial killing was the result of C activation triggered by surface-bound Abs. Further data confirming the ability of the inhibitor to block the lytic effect of MAC were obtained in experiments showing that GPE became resistant to reactive lysis in the presence of extracts obtained from the C-resistant strain. In the reactive lysis system, the cells are exposed to C5b6 and the remaining late components and are lysed by MAC attack proves that the C inhibitor extracted from the C-resistant strain of B. burgdorferi is active on the MAC regulator on B. burgdorferi subunit, it reacts primarily with C8 rather than with C9. Evidence supporting this conclusion is provided by the enzymatic removal of CD59-like protein. The outer membrane proteins OsPA, OsB, and OsC have also been implicated in the protection of spirochete from C attack based on the finding that mutant strains derived from C-resistant strains and lacking these proteins are susceptible to C killing (50). However, these proteins, that can easily be extracted from the outer membrane using the mild detergent n-OCT, failed to protect GPE from MAC-dependent lysis.

In conclusion, our data provide evidence for the ability of some C-resistant strains of B. burgdorferi to evade C-dependent killing by expressing a molecule with a structural and functional similarity to human CD59. The structure of this molecule remains to be defined.

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References


