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Complement evasion by the Lyme disease spirochete *Borrelia burgdorferi* grown in host-derived tissue co-cultures: role of fibronectin in complement-resistance

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Abstract. The effectiveness of complement-mediated killing of *Borrelia burgdorferi*, the causative agent of Lyme disease, in the presence of host-derived tissues was studied. Second and high passage forms of *B. burgdorferi* 297 isolate were grown in a LEW/N rat joint tissue co-culture system and in artificial BSK medium. Guinea pig complement and third week immune serum from hamsters with experimental Lyme disease were added to the cultures. Both high and low passage borrelia grown in BSK medium died and did not revive after 3 weeks incubation in BSK medium. However, 5–12% of tissue co-cultured borrelia survived the first complement-mediated lysis. Repeated re-growth and lysis cycles in tissue co-culture resulted in isolation of an 85% complement-resistant population of *B. burgdorferi*. Joint tissue culture supernatant collected on the third day of tissue culture, and fibronectin (25 μg/ml), also protected spirochetes from complement-mediated lysis in contrast to BSK or fresh co-culture medium. Complement-mediated lysis may not be an effective mechanism in eradication of borrelia, and the chronicity of Lyme disease may be due to resistance of *B. burgdorferi* variants to host immune defense mechanisms in the presence of host-derived tissues.
Key words. Complement; co-culture; Lyme; *Borrelia*; pathogenicity; ESG; arthritis; fibronectin.

Lyme borreliosis is a multisystem inflammatory disease caused by infection with *Borrelia burgdorferi*. The persistent nature of clinical manifestations, prolonged production of antibodies, and isolation of *B. burgdorferi* from patients and experimentally infected animals late in the illness suggest that chronic infection is of major importance in the pathogenesis and treatment of this disease[1]. The persistence of *B. burgdorferi* may be due to resistance to host immune system components in particular antibodies and complement.

In a number of Gram-negative bacterial infections, resistance to complement-mediated killing is an important factor in determining the ability of the etiologic agent to survive in the host[2,3]. Gram-negative bacteria that are complement-resistant are more virulent than complement-sensitive strains such as *Salmonella sp.* and *E. coli*, in which the C5b-9 component fails to insert into hydrophobic domains in the outer membrane due to steric hindrance caused by structural differences in the outer membrane components in the complement-resistant strains[4–8].

It has been shown that *B. burgdorferi* activates both classical and alternative complement pathways in normal serum, and complement-mediated lysis occurs through the classical pathway in the presence of anti-borrelia antibody[9]. Previous studies demonstrated that when BSK medium-cultivated *B. burgdorferi* was incubated with immune serum containing complement from Lyme disease-infected hosts, spirochetes were killed in vitro[10]. However, these in vitro studies on the complement-mediated, antibody-dependent lysis of *B. burgdorferi* were done by using BSK medium-grown spirochetes without the presence of host-derived live tissues in the assay environment.

The influence of host tissues on the biology and pathogenic potential of *B. burgdorferi* has been underestimated in Lyme disease research. Information regarding the biology of *B. burgdorferi*, including expression of surface antigens and interactions between borrelia and host immune system components, were based on artificial medium-grown spirochetes. However, in vitro culture of *B. burgdorferi* in BSK medium results in antigenic changes and loss of infectivity[11]. I have previously reported retention of pathogenicity and infectivity of a *B. burgdorferi* isolate in a LEW/N rat joint derived feeder layer-supported co-culture system[12]. I also observed differential expression of a small group of 22–30 kD range proteins in BSK-grown borrelia cultures compared to tissue co-cultured spirochetes[13].

These differences in biology of *B. burgdorferi* grown in the presence of host-derived tissues compared to BSK-cultivated spirochetes led me to investigate the changes in the self defense mechanisms of borrelia against host immune system components during mammalian tissue-associated growth. The present study was designed to evaluate the effectiveness of complement-mediated lysis on *B. burgdorferi* grown in tissue co-cultures. To determine if the presence of host-derived live tissues in the

assay environment influences the complement action, I investigated the effects of guinea pig complement and immune serum on *B. burgdorferi* grown in tissue co-cultures or in BSK medium. My results demonstrate that escape variants in *B. burgdorferi* populations circumvent complement in the presence of LEW/N rat joint tissue or tissue culture supernatant. The nature of biomolecule(s) found in the tissue monolayers and secreted into the supernatant that might be responsible for protection of *B. burgdorferi* from complement-mediated lysis was also studied. In the course of these investigations, I noted that fibronectin, a major surface-associated glycoprotein which is secreted by fibroblastoid cell cultures and proliferating fibroblasts in chronic inflammation sites, interfered with the complement-mediated lysis of *B. burgdorferi*. Results of this study suggest that the chronicity of Lyme disease may be due to resistance of *B. burgdorferi* variants to host immune factors, especially to circumvention of the complement system in the presence of host tissue-derived biomolecules.

Materials and methods

Borrelia cultures. Isolate of *B. burgdorferi* sensu stricto 297 was obtained from R. C. Johnson (University of Minnesota, Minneapolis). *B. burgdorferi* were cultured in BSK medium[14], or in LEW/N rat derived tissue co-culture system using ESG co-culture medium which contains Dulbecco's Modified Eagle's medium in addition to some ingredients of BSK[12]. Low passage *B. burgdorferi* was cultured twice, and high passage 297 was maintained by weekly passages (<200) in BSK medium at 33 °C to a concentration of 10^7 spirochetes per ml. Five hundred µl samples were then dispensed into 1.5 ml screw cap tubes (Sarstedt, Newton, NC) containing 500 µl of BSK with 30% glycerol (Sigma, St. Louis, MO), sealed, and stored in liquid nitrogen. When needed, a frozen suspension of spirochetes was thawed, and an aliquot was used to inoculate fresh media. Tissue co-culture grown spirochetes attached to the tissue feeder layer were collected with a rubber policeman. Spirochetes were enumerated using phase-contrast microscopy and a Petroff-Hauser counting chamber.

Hamster sera. Six to eight-week-old inbred LHS/Ss WSLH hamsters were obtained from Charles River Breeding Laboratories Inc. (Wilmington, MA). Groups of five or more normal LHS/Ss hamsters were injected subcutaneously with 0.2 ml of 10^6 viable *B. burgdorferi* 297. Three weeks post-injection, hamsters were mildly anesthetized by ether inhalation and bled by intracardiac puncture. After clotting the blood, the serum was separated by centrifugation at 500 × g, pooled and divided into 0.5 ml aliquots, and frozen at −70 °C until used. Pooled normal sera were obtained from non-infected hamsters and used as control.

Borreliacidal assay. *B. burgdorferi* were grown in BSK medium or in tissue co-culture which consists of a confluent fibroblastoid cell monolayer of rat joint tissue in ESG co-culture medium, at 33 °C with 7.5% CO_2 in polystyrene multiwell plates (Falcon, Primaria) until they reached late logarithmic phase. Normal or immune hamster sera were heat inactivated at 56 °C for 45 min and added to BSK medium or to tissue co-culture-grown *B. burgdorferi* cultures, yielding final antiserum dilutions of 1, 1:20, 1:40, 1:80, 1:60, 1:320, 1:640, 1:280 and 1:2560. Twenty µl of sterile guinea pig complement (hemolytic titer 200 CH50 units per ml; Sigma) was added 20 min after incubation with immune serum at 33 °C. Complement or immune serum were omitted in control wells. All assay plates were gently shaken and incubated at 33 °C for 2 to 18 h. Ten µl aliquots were removed in triplicate and added to Petroff-Hauser chambers for enumeration of live and motile spirochetes by phase-contrast microscopy. Spirochetes which exhibited motility were considered viable. I checked whether non-motile *B. burgdorferi* were viable after the borreliacidal assay by the following tests: for BSK-cultured spirochete borreliacidal assay, I harvested *B. burgdorferi* from the remaining assay suspensions by centrifugation at 9500 × g for 15 min and inoculated the resulting pellet into 5 ml of fresh BSK in tightly capped 13 × 100 mm polystyrene culture tubes (Falcon Labware, Lincoln Park, NJ). Cultures were incubated at 33° C for three weeks to observe growth of the survivors. For rat joint tissue-borrelia co-cultures, supernatants of the assayed co-cultures were removed by aspiration after the borreliacidal assay and replaced with one ml of fresh ESG medium without fetal bovine serum (FBS). Cultures were incubated at 33 °C in a CO_2 incubator for three weeks to observe re-growth of complement-resistant *B. burgdorferi*.

Effects of the secondary addition of complement and immune serum during the borreliacidal assay was determined: 20 µl of fresh complement and different concentrations of immune serum were added 8 h after incubation and surviving borrelia were enumerated as described above.

Protective effects of the conditioned co-culture medium during borreliacidal assay. LEW/N rat joint tissue was grown to confluency in 25 cm^2 T-flasks (Nunc) in ESG medium with 6% FBS at 33 °C. Supernatant was removed by aspiration and tissue cultures were fed with fresh ESG without FBS. Culture supernatant (conditioned tissue culture medium) was collected at days 0, 1, 3, 5 and 7. One hundred µl of these conditioned media were added 1:1 (vol/vol) into BSK medium-grown, late logarithmic phase *B. burgdorferi* culture. 1:40 final dilution of immune serum and 20 µl of guinea pig complement was added and motile spirochetes were enumerated 18 h after incubation. The protective role of tissue culture supernatant was determined by compar-

ing the number of motile spirochetes in experimental BSK cultures which contained conditioned medium with the number of motile spirochetes in BSK controls.

Assessment of protective effects of fibronectin during complement-mediated lysis. *B. burgdorferi* were grown in BSK medium at 33 °C until they reached late logarithmic phase. Fibronectin (Sigma, F-4759) (100 µg/ml) was suspended in sterile phosphate buffer solution (PBS, pH 7.5) and vortexed for three min at room temperature. Twenty-five, 50, and 75 µl of fibronectin suspension was added to 200 µl of a BSK culture of *B. burgdorferi* and incubated at 33 °C for 1 h. After incubation, immune hamster serum (1:20 final dilution) and 20 µl of guinea pig complement was added into the cultures. Bovine serum albumin in PBS (100 µg/ml) or sterile PBS was used in control cultures. All cultures were incubated at 33 °C for 18 h and motile borrelia were enumerated as described previously.

Selection of complement mediated lysis escape variants in rat tissue co-culture. High or low passage spirochetes that survived the initial complement lysis in rat tissue co-cultures were collected from the feeder monolayer by scraping with a sterile rubber policeman and confluent rat joint tissue cultures grown in ESG medium in 25 cm^2 T-flasks (Nunc) were inoculated with these survivors (10^4 borrelia per ml). After they reached late logarithmic phase, the borreliacidal assay was repeated for this complement-resistant population. The cycles of complement-mediated lysis and re-growth of the complement-resistant spirochetes in tissue co-cultures were repeated five times by using 1:40 dilution of immune serum and 20 µl of complement to obtain 100% complement-resistant *B. burgdorferi* culture.

Electron microscopy: negative staining. Carbon and formvar-coated electron microscope grids (Fisher, Pittsburg, PA) were sterilized by dipping into 70% ethanol and air dried in a vertical flow hood. Rat joint tissue was grown in ESG with 6% FBS on microscope grids which were placed on the bottom of tissue culture dishes (Nunc, Denmark). After formation of the tissue culture monolayers on microscope grids, tissue culture supernatant was replaced with fresh ESG without FBS and 2×10^4 borrelia were inoculated into the co-culture vessels. Co-cultures were grown at 33 °C with 5% CO_2. Twenty µl of guinea pig complement and 1:40 dilution of immune serum were added after four days of growth and assay grids with attached borrelia were incubated at 33 °C with 7.5% CO_2 for 2 h. Grids were collected with sterile forceps and negatively stained with 1.5% ammonium molybdate for 5 min. General morphology and outer membrane abnormalities during complement-mediated lysis were observed by Jeol transmission electron microscope.

Ruthenium red staining. Rat joint-derived tissue monolayers were grown on Cytodex 3 microcarriers as described elsewhere[12]. Microcarriers covered with joint tissue monolayers with attached *B. burgdorferi* were collected by centrifugation at 500 × g for 15 min, fixed and stained for 1 h with ruthenium red (1500 ppm)-glutaraldehyde (4%) solution in 0.2 M cacodylate buffer (pH 7.3), then post-fixed with OsO_4 (5%)-ruthenium red (1500 ppm) solution in cacodylate buffer for 3 h. Fixed and stained spirochetes were embedded in agar and dehydrated in ethanol series (25, 50, 75, 99, 100, 100, 100%). Dehydrated samples were embedded in Spurr's resin and polymerized in Beem capsules at 70 °C for 18 h, then at ambient tempeature for 1 h. Silver colored sections were examined by Philips transmission electron microscope.

Statistical analysis. Experimental data were tested by analysis of variance. The Fisher least-significance test was applied to examine the mean values in which a significant F ratio indicated reliable differences in the means. Alpha level was 0.05 during the analysis.

Results

Borreliacidal assay; kinetics of death. The borreliacidal activity of guinea pig complement and immune serum was determined for *B. burgdorferi* sensu stricto isolate 297 grown in BSK medium without a host-derived tissue feeder layer and in the rat joint tissue co-culture system. Immune sera from hamsters with experimental Lyme disease were collected 3 weeks post-injection and heat inactivated before the assays. Late logarithmic phase spirochetes (1×10^6 borrelia/ml) grown in BSK or LEW/N rat joint tissue feeder layer-supported co-cultures were incubated with complement and pooled immune serum diluted 20-fold or more for 2 to 18 h. Loss of motility, formation of blebs across the outer membrane of spirochetes and disintegration of the microorganisms were signs of complement-mediated lysis as determined by phase contrast and electron microscopy techniques. These changes occurred rapidly after addition of complement and immune serum in both BSK and tissue co-cultures. A reduction of 85% in the number of motile *B. burgdorferi* was observed after 4 h of incubation in BSK medium-grown spirochete cultures compared wtih normal hamster serum controls. By 6 h of incubation, more than 97% of the borrelia were immobilized. Death rate among *B. burgdorferi* suspended in fresh ESG, borrelia-rat tissue co-culture medium without the feeder layer, was similar to the death rate of BSK-cultured spirochetes during the initial 4 h of borreliacidal assay and more than 97% of these spirochetes died in 10 to 12 h. Spirochetes grown in BSK or suspended in fresh ESG without the feeder layer did not survive complement-mediated lysis 18 h after incubation. Remaining assay suspensions were centrifuged for 15 min and the resulting pellet was inoculated into 5 ml of fresh BSK in tightly capped polystyrene tubes in order to determine the viability of

non-motile spirochetes. There was no re-growth of spirochetes even after three weeks of incubation at 33 °C. However, co-cultured *B. burgdorferi* died at a slower rate compared to BSK-grown and ESG medium-suspended spirochetes and 12% of them survived even after 10 h. Aspiration of borreliacidal assay supernatant from co-cultures and addition of fresh ESG resulted in re-growth of the borreliacidal assay survivors, which all adhered to the rat joint feeder layer. Combined action of complement and immune serum was required since spirochetes in the control cultures without complement or immune serum were 99% motile at 18 h. Normal hamster serum and complement did not kill spirochetes in control cultures (fig. 1).

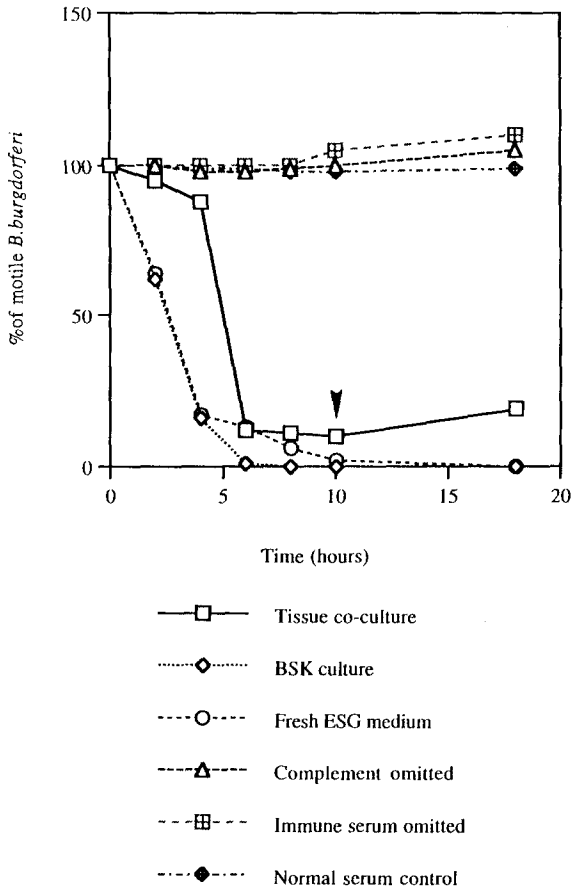

Figure 1. Antibody-dependent, complement-mediated lysis of *B. burgdorferi* 297 grown in BSK medium or in LEW/N rat joint tissue co-culture system with intact joint tissue feeder layer in ESG co-culture medium. Fresh ESG, co-culture medium without the tissue feeder layer was used as a control. BSK medium-grown 297 was collected by centrifugation and resuspended in fresh ESG at a concentration of 10^6 borrelia per ml before addition of complement and immune serum. Immune serum or complement was omitted in control cultures. Normal hamster serum was used as one of the controls in the assay. Sera were derived from 297-infected hamsters 3 weeks post-injection. Final dilutions of the heat-inactivated hamster serum was 1:40 in all experiments. Arrow denotes addition of fresh medium after removing the complement and immune serum from cultures at 10 h. ± SD for each data point was <15% (not shown). Data are from three separate experiments using triplicate cultures for each culture condition tested.

Furthermore, immune serum concentrations required to kill BSK medium-grown spirochetes was higher compared to tissue co-cultured *B. burgdorferi*. Immune serum was diluted 160-fold before borreliacidal activity was lost for BSK cultures; however, tissue co-cultured spirochetes survived even the highest immune serum concentrations (fig. 2).

Kinetics of death in tissue co-culture: comparison of death rates for low passage and high passage *B. burgdorferi*. To investigate the possibility that low passage, pathogenic spirochete populations might contain higher numbers of complement-resistant variants compared to high passage, non-pathogenic *B. burgdorferi*, borreliacidal assays were repeated for these spirochete populations in the tissue co-culture system. Confluent rat joint tissue monolayers were inoculated with low or high BSK-passaged *B. burgdorferi* isolate 297 spirochetes. Twenty µl of guinea pig complement and a 1:40 dilution of the immune serum collected from hamsters with experimental Lyme disease were added when co-cultured borrelia reached the logarithmic growth phase (1×10^7 borrelia/ml), and assay suspensions were incubated at 33 °C with gentle agitation. The number of

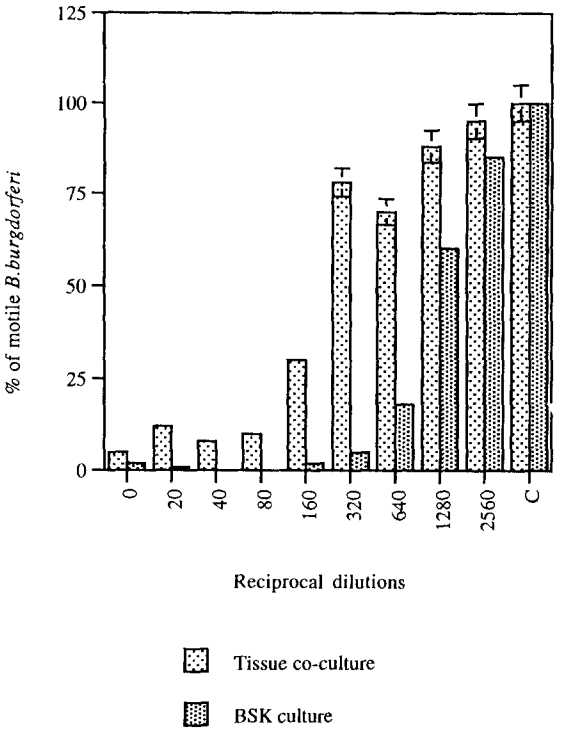

Figure 2. Percentage of survival of *B. burgdorferi* detected in reciprocal dilutions of immune serum collected from hamsters 3 weeks post-injection of *B. burgdorferi* 297. LEW/N rat joint tissue co-cultured and BSK medium-grown spirochetes were tested for complement-mediated lysis. The number of motile spirochetes was determined 6 h after incubation at 33 °C. C: controls in which complement or immune serum was omitted. Substituting normal serum for immune serum in BSK and co-cultures did not affect the number of motile spirochetes detected in the control assay suspension. Data show the mean values y (n = 3) ± SD of a representative experiment repeated three times.

survivors was determined after the borreliacidal action of guinea pig complement and immune serum by direct enumeration. The multiple passage *B. burgdorferi* death rate was higher during complement-mediated killing in tissue co-culture compared to the low passage borrelia; 18% of high passage and 40% of low passage spirochetes were motile after 8 h. Fresh guinea pig complement and immune serum was added to the assay suspensions after eight h in order to maintain high concentrations of complement and immune serum to kill all of the complement-sensitive *B. burgdorferi*. As expected, 2.8% of the high passage and 4% of the low passage *B. burgdorferi* were motile in assay co-cultures even after 18 h. Complement-resistant spirochetes started to re-grow in tissue co-cultures after replacement of assay supernatants with fresh ESG medium.

To investigate the effects of the fresh co-culture medium on the complement-mediated lysis of *B. burgdorferi*, tissue co-cultured spirochetes were harvested by centrifugation and then re-suspended in ESG medium without tissue feeder layer before the borreliacidal test. Unconditioned co-culture medium did not provide any protection against complement-mediated killing; both high and low passage *B. burgdorferi* suspended in fresh ESG died during complement-mediated lysis and did not revive three weeks after incubation in BSK medium (fig. 3).

Inhibition of borreliacidal activity by rat joint tissue culture supernatant. The preceding experiments demonstrated the protective effects of the rat-joint derived tissues during the borreliacidal assay. Borrelia-protective biomolecules, such as fibronectin, might be secreted into the tissue culture supernatant. To investigate the possibility that conditioned medium (post-culture medium) collected from tissue cultures might also protect borrelia from the borreliacidal action of complement and antibody, *B. burgdorferi* were grown in BSK medium (1.7×10^6 spirochetes/ml) and 100 µl of rat joint tissue culture supernatant collected by aspiration at 24 h intervals was added into these cultures prior to the borreliacidal assays. Tissue culture supernatant collected at 0 and 24 h did not protect *B. burgdorferi* from borreliacidal action. However, conditioned medium collected on the seventh day of tissue culture provided the highest level of protection and 8.5×10^4 borrelia/ml, which was 5% of the original population, survived complement-mediated lysis after 18 h incubation (fig. 4). Addition of 100 µg/ml concentrations of bovine serum albumin (BSA) into BSK cultures before the borreliacidal assay did not protect spirochetes from complement-mediated lysis. These experiments clearly demonstrated that biomolecule(s) which might be responsible for protection of borrelia against the complement-mediated, antibody-dependent lysis were secreted by the tissue feeder layer into the culture supernatant. The concentration of the borrelia-protective material(s) gradually

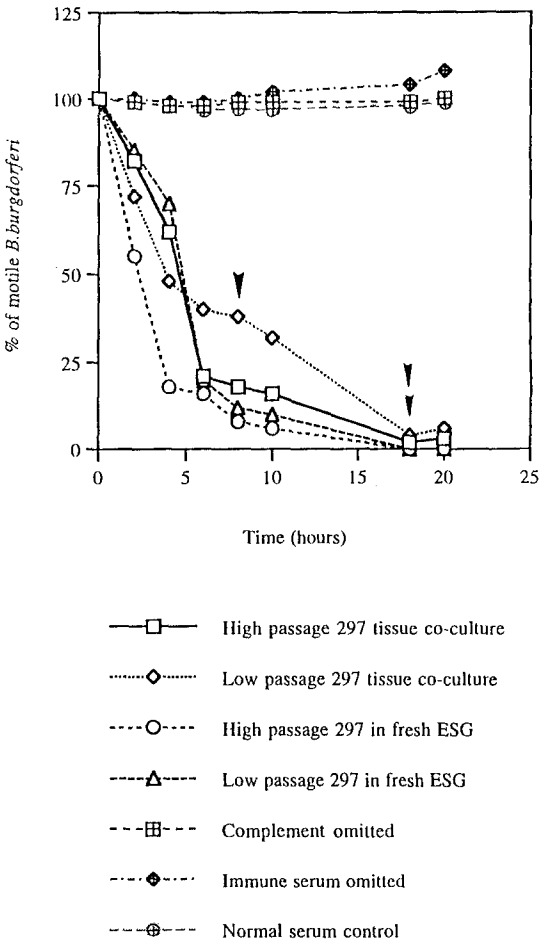

Figure 3. Borreliacidal assay in LEW/N rat joint tissue co-culture. High or low passage *B. burgdorferi* were subjected to complement-mediated lysis. Final dilution of the heat-inactivated hamster serum was 1:40 in all experiments. Single arrow denotes the secondary addition of fresh complement and immune serum after 8 h. Survivors of the first complement-mediated lysis were collected for further lytic cycles. Double arrows denote replacement of complement and immune serum with fresh ESG in both high or low passage *B. burgdorferi* co-cultures after 18 h. In some experiments, high or low passage *B. burgdorferi* were grown in tissue co-culture, harvested by centrifugation and resuspended in fresh ESG, co-culture medium without tissue feeder layer (10^7 borrelia per ml) before addition of guinea pig complement and immune serum. ± SD for each data point was <15 (not shown). Data are from three separate experiments using triplicates for each borrelia culture tested.

increased in the culture supernatant, since the number of survivors of the borreliacidal test was significantly increased by adding the conditioned medium collected after the third day of the tissue culture.

Inhibition of borreliacidal activity by fibronectin. I further investigated the biomolecules secreted from the joint tissue monolayer which might protect borrelia from complement-mediated lysis. Fibronectin was the primary candidate since it was found in fibroblastoid tissue culture supernatant, in the serum, and especially around the inflammation sites. One hundred µg fibronectin were suspended in one ml buffer and 25-fold

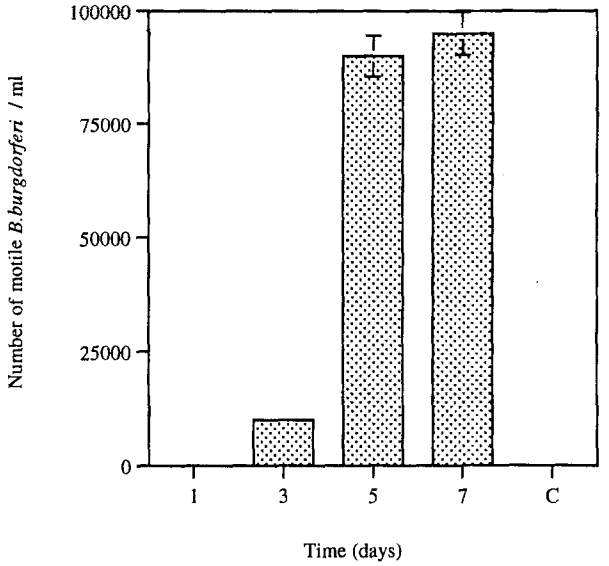

Figure 4. Inhibition of borreliacidal activity of immune serum and complement by rat joint tissue culture supernatant. Borrelia were cultured in BSK medium (1.7×10^6/ml). T (days) denotes rat joint tissue culture supernatant collection time after formation of the tissue monolayer. Equal amounts (100 µl) of tissue supernatant were added into *B. burgdorferi* cultures 1 h before addition of complement and immune serum to examine the protective effect of the conditioned medium during complement-mediated killing. Final dilution of the immune serum was 1:40 and 20 µl of guinea pig complement were used in all experiments and in control cultures (C). Bars denote standard deviations. Experiments were repeated three times with triplicate samples. Control: BSK medium-grown borrelia without tissue culture supernatant.

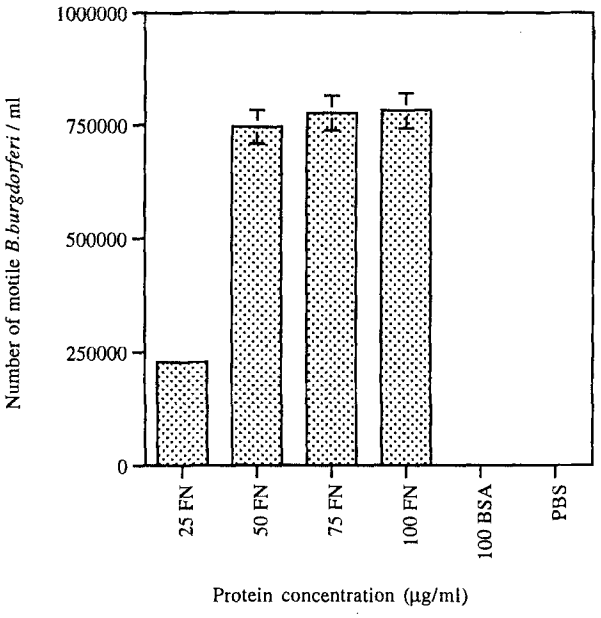

Figure 5. Inhibition of complement-mediated lysis of fibronectin. Borrelia were cultured in BSK medium (3.73×10^6/ml). Dilutions of fibronectin (FN) solution (25-fold) were added to *B. burgdorferi* cultures 1 h prior to addition of complement and immune serum to examine the protective effect of fibronectin. Concentration of bovine serum albumin (BSA) was 100 µg/ml in this assay. Immune serum at 1:40 final dilution and 20 µl of guinea pig complement were used in all experiments. PBS was included in control cultures instead of fibronectin solution. All assay results were determined 18 h after incubation at 33 °C. Data shows the mean values of experiments repeated three times in triplicate.

dilutions of this suspension were added into BSK medium-grown *B. burgdorferi* cultures before the borreliacidal assay. Concentrations of fibronectin higher than 25 µg/ml inhibited complement-mediated lysis of borrelia; the number of motile borrelia was significantly increased compared to the control assays which did not contain fibronectin ($p < 0.0001$). I repeated these experiments using human complement (Sigma, St. Louis, MO) at 37 °C (data not shown) and found that addition of 50 µg/ml fibronectin to BSK resulted in survival of 20% of the original borrelia population during complement-mediated lysis. In contrast to the lysis-inhibitory effect of fibronectin, addition of 100 µg/ml BSA did not have any effect on the borreliacidal action of complement (fig. 5).

Selection of complement-mediated lysis escape variants. During the borreliacidal assays, I observed microscopically that there were spirochetes in the antibody- and complement-treated tissue co-cultures that remained smooth surfaced and motile. The first cycle of complement-mediated lysis in tissue co-cultures resulted in survival of 5–12% of the *B. burgdorferi* population. I reasoned that if the persisting, motile borrelia were complement-resistant variants, upon subsequent passage into fresh, antibody- and complement-containing tissue cultures, the proportion of motile spirochetes would increase further. These variants were collected from the feeder tissue monolayer with a sterile rubber policeman, and joint tissue monolayers grown in ESG co-culture medium in 25 cm^2 T-flasks were inoculated with 1 ml of these complement-resistant survivors (1×10^4/ml). Complement-resistant borrelia co-cultures reached the late logarithmic phase in 5 days and were subjected to a second complement-mediated lysis in multiwell plates covered with rat joint tissue monolayers, which result in 30% survival of *B. burgdorferi* after 18 h. After the fifth re-growth and lysis cycle, an 85% complement-resistant borrelia culture was obtained. However, I could not isolate a 100% complement-resistant *B. burgdorferi* population.

Electron microscopy of *B. burgdorferi*: negative staining. Because of the bactericidal activity of the complement and antibodies noted earlier, I sought to obtain direct evidence of spirochete damage after exposure to complement and immune serum. Rat joint tissue was grown on sterile electron microscope in ESG co-culture medium and inoculated with 2×10^4 spirochetes. Co-cultures were grown at 33 °C for 4 days. After that, guinea pig complement and a 1:40 dilution of immune serum were added to the grids. Assay grids were collected with forceps after 2 h and negatively stained with ammonium molybdate for 5 min. Photomicro-

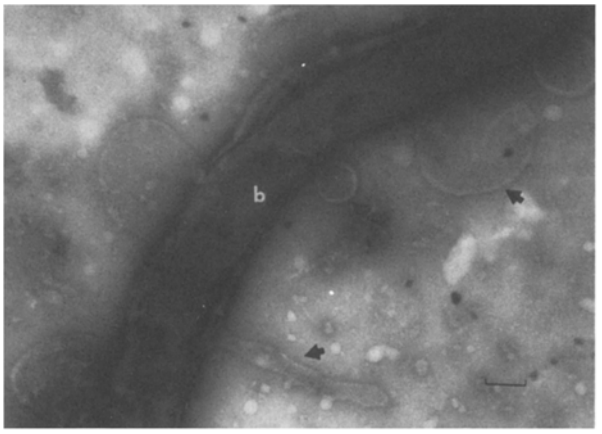

Figure 6. Outer membrane blebs along the membrane of *B. burgdorferi* (B) during complement-mediated lysis 2 h after incubation at 33 °C with guinea pig complement and 1:40 dilution of immune serum. Negative staining with ammonium molybdate. Arrows: outer membrane blebs. Bar: 0.13 μ.

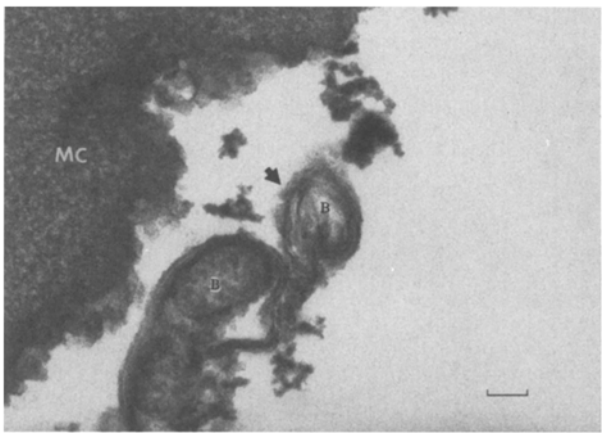

Figure 7. Extensive mucopolysaccharide-rich material accumulation around *B. burgdorferi* (B) in rat joint tissue co-culture control. Arrow: mucopolysaccharide-rich outer coat. Rat tissue feeder layer was grown on cytodex 3 microcarriers (MC) before inoculation of borrelia. Ruthenium red staining. Bar: 0.13 μ.

graphs demonstrated the morphological characteristics of *B. burgdorferi* in rat joint tissue co-cultures and formation of outer membrane blebs during complement-mediated killing. The outer membrane of borrelia was intact in control co-cultures without complement and immune serum, but exposure to complement and immune serum produced refractivity and extensive blebbing along the outer surface of the microorganisms followed by fragmentation and lysis of spirochetes (fig. 6).

Ruthenium red staining. The morphological characteristics of the *B. burgdorferi* isolate 297 grown in tissue co-cultures were investigated by electron microscopy. The ruthenium red staining method, which is specific for acid mucopolysaccharides, or slime layers, was used to demonstrate the differences in the outer membrane of spirochetes grown in tissue co-cultures compared to BSK medium-grown borrelia. Rat joint tissue was grown on Cytodex 3 microcarriers using ESG medium, then inoculated with *B. burgdorferi* after formation of confluent tissue monolayers around the microcarriers, and co-cultures were incubaed for 5 days at 33 °C. Microcarriers covered with tissue monolayers and spirochetes were harvested by centrifugation at 500 × g for 15 min, fixed and stained with a ruthenium red-glutaraldehyde solution. Large amounts of acid mucopolysaccharide-rich material without any substructure surrounding *B. burgdorferi* were found during rat tissue culture-associated growth (fig. 7).

Discussion

The success of microorganisms as pathogens stems partly from their ability to evade recognition or avoid destruction by complement and other natural or acquired defense mechanisms. In vitro studies on the complement-mediated lysis of *B. burgdorferi* were based on experiments using BSK-cultured spirochetes without the presence of host-derived live tissues in the assay environment. However, the presence of host tissues in the growth environment may alter the biological characteristics of borrelia. I previously reported retention of infectivity and pathogenicity of *B. burgdorferi* grown in the presence of host-derived tissues in contrast to BSK medium-grown spirochetes[12]. The *B. burgdorferi*-tissue co-culture system contains a host-derived tissue feeder layer grown in ESG co-culture medium. The growth of *B. burgdorferi* in co-cultures is tissue monolayer-dependent, since spirochetes cannot grow in ESG without the feeder layer. This culture method mimics the host environment without the offensive components of the immune system, and host-derived feeder layer cells provide nutrients and biomaterials to borrelia during their growth. Lysis of *B. burgdorferi* by immune serum and complement was evaluated in co-cultures by adding guinea pig complement and immune serum collected from hamsters with experimental Lyme disease. Approximately 5 to 12% of co-cultured *B. burgdorferi* survived complement-mediated lysis after 18 h in contrast to BSK medium-grown spirochetes which died in 8 h and did not revive 3 weeks after incubation in BSK medium.

In this study, borrelia which survived the first complement-mediated, antibody-dependent lysis were collected, re-grown in tissue co-cultures and subjected to a second lysis. The increase in the number of survivors after repeated re-growth and lysis cycles may suggest that these spirochetes were resistant variants which had the ability to evade complement attack in the presence of host-derived tissues. Both high and low BSK passage cultures of *B. burgdorferi* 297 isolate contained complement-mediated lysis escape variants. The number of

survivors after the complement-mediated lysis was two times higher in low BSK-passaged cultures compared to high passage state borrelia. Recent evidence has shown that the number of complement-resistant spirochetes decreases during multiple passages in BSK medium.

In vitro selection of antibody-resistant mutants of *B. burgdorferi* was demonstrated in complement-free BSK cultures by use of monoclonal antibodies to outer surface protein B (Osp B). Mutants either did not express this protein[15], or the C-terminus, the surface-exposed portion of the protein which might be important in binding monoclonal and polyclonal antibodies, was truncated due to a TAA stop codon at nucleotide 577[16]. Inhibition of borrelia growth in BSK by anti-Osp A antibodies and polyclonal immune serum from Lyme disease patients and rats with experimental Lyme disease was also reported[17]. In this study, less than 1% of the first culture and more than 90% of the third culture were antibody-resistant mutants. Resistant borrelia were not affected by growth-inhibiting antibody and their population increased during subsequent cultures in the presence of monoclonal antibodies. These results demonstrated that even in cloned *B. burgdorferi* cultures, there might be escape variants which could be selected in vitro by using monoclonal anti-borrelia antibodies. However, in these studies, the function of complement during antibody-dependent borreliastatic and borreliacidal action of the immune serum was not investigated, since immune sera were heat inactivated to denature complement before seletion of the antibody-resistant borrelia variants.

In my study, I demonstrated that the host-drived tissue monolayer was necessary to select complement-dependent lysis escape variants of *B. burgdorferi*. Complement-inhibitory or borrelia-protective compound(s) were also found in the supernatant of the rat joint tissue culture since conditioned medium provided protection against complement-mediated lysis of spirochetes. Fresh co-culture medium, ESG, did not prevent the borreliacidal effect without the tissue feeder layer. The extracellular tissue matrix components which are secreted from rat joint-derived feeder layer cells may accumulate on *B. burgdorferi* and interfere with binding of antibodies to spirochete outer membrane which may in turn inhibit insertion of the membrane attack complex to the borrelia surface.

I further investigated the nature of the biomolecule(s) found in the tissue culture supernatant which might be responsible for protection of *B. burgdorferi* from complement-mediated lysis. Addition of fibronectin into BSK cultures of *B. burgdorferi* significantly increased survival of spirochetes during complement-mediated, antibody-dependent lysis. However, even high concentrations of bovine serum albumin did not affect the borreliacidal activity of the complement.

Fibronectin, a major surface-associated glycoprotein, is secreted by fibroblast-rich cell cultures and is also present in human plasma at concentration of 300–400 μg/ml[18–21].

Fibronectin in fibroblast cultures is found both in a soluble form in conditioned medium in large quantities, and in insoluble forms in the extracellular connective tissue matrix and on the cell surface[18,22]. Large amounts of fibronectin are also detected in areas of wound healing and around proliferating fibroblasts in chronic inflammation sites[23]. It has been found that *B. burgdorferi* can recognize and bind fibronectin in vitro. When the extracellular matrix was pretreated with a polyclonal antibody to human plasma fibronectin, *B. burgdorferi* adherence to the matrix of endothelial cell monolayers was reduced 48 to 63%[24]. The results of my study suggest that tissue-derived biomolecules, such as fibronectin, may interfere with the binding of antibodies to the spirochete outer membrane which may in turn inhibit insertion of the membrane attack complex to the borrelia surface. Approximately 88% of *B. burgdorferi* grown in tissue co-cultures died during the initial complement-mediated lysis. However, 12% of the original population of spirochetes survived the complement attack, which might suggest that survivors had the ability to protect themselves by using biomolecules derived from the tissue feeder layers.

The biological importance of the surface coat formed across the invading pathogen might be considerable, possibly affecting increased survival, promotion of membrane integrity, uptake of nutrients and cofactors necessary for growth, induction of autoimmune responses, and decreased immunogenicity. Alderete and Baseman have found loosely or avidly associated host proteins on virulent *Treponema pallidum* surfaces. Virulent treponemes had the ability to adsorb a surface coat of host proteins[25]. These authors suggested that a layer of host proteins acquired immediately after infection may result in decreased immunogenicity through masking of spirochetal antigens. Under these conditions, the spirochete might be protected from specific antibodies and serum proteases, both required for complement-mediated lysis of the bacterium[27,28]. Also, coating of the parasite by host macromolecules may allow for in vivo survival of spirochetes for extended periods of time with further manifestations of the secondary and tertiary stages of the diseases. Therefore, one of the virulence determinants may be the efficient masking of the *B. burgdorferi* surface.

The presence of an outer coat of host proteins as well as a mucoid layer on the *B. burgdorferi* surface during host tissue-associated growth might physically prevent complement components from reaching the outer envelope to cause outer membrane perturbations necessary for lysis. I observed a thick layer of amorphous mucopolysaccharide-rich material accumulation around *B. burgdorferi* during growth in tissue co-cultures by the

ruthenium red staining method. This thick outer layer may contribute to complement resistance by concealing complement binding proteins of *B. burgdorferi* which may result in inhibition of complement-mediated lysis. Complement inhibitory compound(s) were found in the supernatant of the rat joint tissue culture in this study since conditioned medium provided protection against complement-mediated lysis of *B. burgdorferi* and fresh tissue culture medium, ESG, did not prevent the borreliacidal effect without the tissue feeder layer. Soluble forms of extracellular tissue matrix components which are secreted from rat joint-derived feeder layer cells, such as sulphated glycosaminoglycans of fibronectin, may condense on *B. burgdorferi* outer surfaces and interfere with the complement action.

The in vivo function of complement and protective value of antibody-dependent complement activation during Lyme disease progression was investigated by Bockenstedt et al. They demonstrated that the appearance of Lyme disease symptoms was not altered in mice deficient in C5, which prevents C5-mediated events and formation of MAC, and they suggested that C5-mediated complement activation might not be necessary for immune protection during the infection in vivo[28]. Hamsters develop a substantial amount of borreliacidal antibody, but *B. burgdorferi* can still be isolated from hamsters 16 months after infection[29]. In contrast, *B. burgdorferi* cultured in BSK medium can be killed in vitro by antibodies and complement within 2 h. It has been shown that *B. burgdorferi* can be sequestered in tissues that may not be readily accessible to immune factors[30, 31]. Johnson et al. also showed that if immune serum is administered shortly after the infection, it may not eradicate *B. burgdorferi* from hamsters[32].

The chronicity of Lyme disease may be due to resistance of *B. burgdorferi* variants to host immune defense mechanisms, especially to circumvention of the complement cascade or specific modifications of activated components of complement in the presence of host tissue-derived biomolecules. My results are in agreement with in vivo studies which support the contention that activation of complement components and complement-mediated lysis may not be an effective defense mechanism for distintegration of *B. burgdorferi* in the presence of host tissues. Antibody-mediated bactericidal activity of non-specific immune cells such as macrophages might be important rather than complement-dependent lysis. Complement escape variants in a *B. burgdorferi* population which are acquired by a tick bite may survive the initial complement attack and they can multiply and infect tissues located in deeper sections of the host body. The combination of the ability of *B. burgdorferi* to survive inside the host cells such as fibroblasts[33] and the ability of escape variants to evade complement in the presence of host tissues may represent survival tactics of a very successful pathogen. Studies on the biology of *B. burgdorferi* during host tissue-associated growth and phenotypic and genotypic identification of complement-escape variants may help us to define the pathogenicity determinants that play a key role in the chronicity of Lyme disease.

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