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Complement-Mediated Serum Sensitivity among Spirochetes That Cause Lyme Disease

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***Borrelia burgdorferi*-related isolates were tested for their sensitivity to normal human serum (NHS) and their ability to activate complement. By dark-field microscopy, electron microscopy, and subsurface plating, it was shown that exposure of a *Borrelia garinii* isolate to 10% or more NHS resulted in immobilization, blebbing, and killing of the spirochetes. These effects were mediated by complement, since they were not seen after heat treatment of NHS, in the presence of EDTA, or in an agammaglobulinemic serum. All seven *B. garinii* type 5 or 6 and all four VS116/M19 strains were serum sensitive, whereas all eight *Borrelia afzelii*, five of eight *B. garinii* type 4, and three of seven *B. burgdorferi* sensu stricto isolates were serum resistant. The other isolates were partially serum sensitive. Four serum-sensitive *B. garinii* isolates had been isolated from human cerebrospinal fluid. Most likely, activation of both the alternative pathway and the classical pathway of complement was involved, since bactericidal activity was diminished in properdin-deficient sera as well as in a C1q-depleted serum and in a C4-deficient serum. Bactericidal activity could be restored when a serum lacking C1q or C4 was mixed with a properdin-deficient serum. Isolates with various genetic backgrounds were equally able to activate C3 as measured by enzyme-linked immunosorbent assay. In the presence of Mg-EGTA, C3 was activated by all isolates after exposure to ≥10% NHS. This study shows that *B. burgdorferi*-related spirochetes can be either serum sensitive or serum resistant in vitro and that this characteristic is associated with their genetic background.**

Lyme borreliosis (LB) is a multisystem disorder caused by the tick-borne spirochetes *Borrelia burgdorferi* sensu stricto, *Borrelia garinii*, and *Borrelia afzelii*. Erythema migrans (EM), a skin lesion surrounding the site of the initial tick bite, is often the presenting symptom of LB (54). Subsequently, spirochetes can disseminate to other skin sites, resulting in multiple EM lesions and finally acrodermatitis chronica atrophicans (ACA), or to various organs, such as the heart, the joints, the eyes, or the central nervous system (54). Dissemination of *Borrelia* from the skin to distant sites is generally considered to occur hematogenously. After needle or tick-borne inoculation of mice with *B. burgdorferi*, all inoculated mice had positive blood cultures up to 6 weeks postinoculation (p.i.), while after 1 year only 1 of 10 mice was positive (6). Spirochetes have been cultured in low frequency from blood from rabbits 2 weeks p.i. (26) and from rhesus monkeys 1 to 6 weeks p.i. (41), although in another study blood cultures from such animals were negative (37). Culture of spirochetes from human blood has been successful in a few cases (7, 34, 58) but is usually negative (35). These findings suggest that spirochetes are present in the circulation in the early stage of infection but that they disappear in later stages of the disease. Whether the humoral immune system plays a role in the prevention of dissemination of spirochetes has been a focus of extensive research. It has been shown that *B. burgdorferi* is able to activate both the alternative and the classical pathways of complement activation but that it is not susceptible to the bactericidal activity of serum, mediated by complement (24). In contrast, immune sera from hamsters (30), rats (38), and humans (21) were bactericidal for *B. burgdorferi*. These findings are compatible with the assumption that early hematogenous dissemination of spirochetes occurs, while new phases of spirochetemia are prevented after antibodies have been elicited.

It should be noted that in the studies mentioned above only *B. burgdorferi* sensu stricto isolates were tested, whereas nowadays at least eight genospecies of *B. burgdorferi*-related spirochetes have been recognized (42). Of these genospecies, *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii* have been cultured from patients with clinical LB. Up to now, spirochetes belonging to the other five genospecies have never been cultured from patients, so that their pathogenicity for humans remains unproven. Studies based on culturing of spirochetes from various sites from patients (4, 15, 57, 62) or on serology (1, 2) suggest that infection by *B. burgdorferi* sensu stricto or *B. garinii* is associated with dissemination to extracutaneous sites and that infection due to *B. afzelii* is mainly associated with long-standing EM and ACA, although *B. afzelii* has been cultured from a small number of patients with neuroborreliosis (13).

In a recent study, it was shown that two *Borrelia* isolates differ in their ability to activate complement and to resist killing by serum bactericidal activity (SBA) (10). Since differences in susceptibility to SBA among the various *Borrelia* isolates might well lead to differences among their abilities to disseminate, we compared SBAs against *Borrelia* isolates cultured from different sites in LB patients and from ticks. We also investigated which activation pathway of complement was involved in SBA.

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MATERIALS AND METHODS

***Borrelia* isolates.** Isolate B31 was obtained from the American Type Culture Collection (ATCC 35210). Isolates VS102, VS116, VS130, VS134, VS215, VS219, VS293, VS461, VSBM, VSBP, and VSDA were provided by O. Péter, Sion, Switzerland. Isolate pKo was given by V. Preac-Mursic, Munich, Germany, and isolates M19, M49, and M53 were provided by R. de Boer, Amsterdam, The Netherlands. All isolates used in this study have been described previously (36, 39, 40, 57) and have been passaged fewer than 15 times, except for VS219 (P19) and the high-passage reference isolates B31 and pKo. Characterization of the isolates as belonging to *B. burgdorferi* sensu stricto, *B. garinii*, or *B. afzelii* has been described in other reports (31, 39, 42, 57). Isolates M19, M49, and M53 belong to the M19/VS116 group (36, 44). Isolates were stored at −70°C in 50% glycerol peptone and cultured in modified Kelly's medium (MKM) containing 5% (vol/vol) heat-inactivated normal rabbit serum (43).

Sera. Blood samples were obtained from eight healthy laboratory donors, from one patient with agammaglobulinemia, from six patients with a properdin deficiency (20), and from one patient with a C4 deficiency (23). Samples were allowed to clot for 20 min and centrifuged at 2,000 × *g* for 10 min. All sera were immediately aliquoted and frozen at −70°C. Freeze-thaw cycles were avoided. A serum sample, depleted of C1q by affinity chromatography with immunoglobulin G (25), was kindly provided by M. Daha (Department of Nephrology, University of Leiden, Leiden, The Netherlands). All sera were thawed on ice before use. Some sera contained particles in dark-field microscopy, which diminished the visibility of the spirochetes, and were therefore centrifuged at 14,000 × *g*, 4°C, for 20 min. All sera were tested for the presence of antibodies against *B. burgdorferi* by enzyme-linked immunosorbent assay (ELISA) (27) and reacted negatively. Sera were also tested by Western blotting with *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii* isolates (B31, A87S, and A38S, respectively) (28); none of the sera reacted positively, according to established criteria (18). Sera from healthy donors had initially been selected for their absence of reactivity in Western blots. Hemolysis-in-gel tests in which sensitized sheep erythrocytes or rabbit erythrocytes in the presence of Mg-EGTA were used (56) showed normal complement activity in the sera from healthy donors and confirmed the absence of alternative pathway activity in sera from patients with a properdin deficiency and the absence of classical pathway activity in the C1q-depleted serum and the C4-deficient serum.

Assays for bactericidal activity. *Borrelia* isolates were thawed and grown to a density of 0.5×10^7 to 1×10^7 spirochetes per ml in MKM, as judged by dark-field microscopy. An aliquot of this suspension was added to an aliquot of serum to give a final total volume of 100 μl. For these experiments, a 96-well microtiter plate was used. The plate was sealed and incubated at 33°C. After 1, 3, and 5 h of incubation, an aliquot of 5 μl was drawn from the suspension to assess the mobility and the extent of blebbing of the spirochetes by dark-field microscopy. Mobile spirochetes were judged viable (30). In some experiments, EDTA (final concentration in the assay, 10 mM) or $MgCl_2$ (final concentration, 4 mM) and EGTA (final concentration, 10 mM) were added to the sera, prior to the addition of spirochetes at the start of the assay.

In separate control experiments to ascertain the equivalency of immobilization and viability, spirochetes were plated out onto solid medium as described by Dever et al. (17). In wells of a microtiter plate, 50 μl of a spirochete suspension in MKM (0.5×10^7 to 1×10^7 spirochetes per ml) was added to either 50 μl of normal human serum (NHS), 50 μl of heat-inactivated serum, or 50 μl of MKM and incubated for 3 h at 33°C. After incubation, the percentage of motile spirochetes in each well was assessed as before by dark-field microscopy. The spirochete density in the well to which only MKM had been added was assessed by dark-field microscopy. Subsequently, spirochete suspensions from wells to which either heat-inactivated or normal serum had been added were diluted and aliquots calculated to contain 2,500, 250, and 25 spirochetes were mixed with 1.5 ml of MKM containing 1% SeaPlaque agarose (FMC, Rockland, Maine) at 37°C and were poured onto a solid-bottom agarose (MKM containing 1.5% SeaKem agarose [FMC]). Plates were incubated at 33°C in a sealed plastic bag containing a CO_2 GasPak (Becton Dickinson, Cockeysville, Md.) and inspected weekly for the appearance of colonies up to 6 weeks. The experiment was performed in duplicate by the use of spirochetes from two different culture tubes. Plating was also performed in duplicate. After 6 weeks of incubation, the experiment had to be ended because of contamination of the plates.

Electron microscopy. For transmission electron microscopy, 150 μl of spirochetes in MKM was mixed with 50 μl of NHS. After 3 h of incubation, bacterial suspensions were centrifuged for 20 min at 5,000 × *g* and the spirochetal pellet was resuspended in 10 μl of phosphate-buffered saline (140 mM NaCl, 10 mM Na-phosphate) (PBS). This suspension was brought onto Parafilm, and a Formvar-coated grid was put upside down on the droplet for 5 min. The grid was washed with three droplets of PBS, stained with 3% uranyl-acetate, and washed again with three droplets of PBS and subsequently with three droplets of water to remove salt. Grids were studied with a Philips 201 transmission electron microscope.

SDS-PAGE analysis and Western blotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining of gels were performed as described earlier (57). Monoclonal antibodies (MAbs) L32 1C8, L32 1F7, L32 1G3, and L32 1D11 (62) and H5332 (5) have been described elsewhere. MAb 84C, directed against OspB (53), was provided by D. D. Thomas, San Antonio, Tex. Proteins were blotted from SDS-PAGE gels to nitrocellulose and incubated with MAbs as described elsewhere (57).

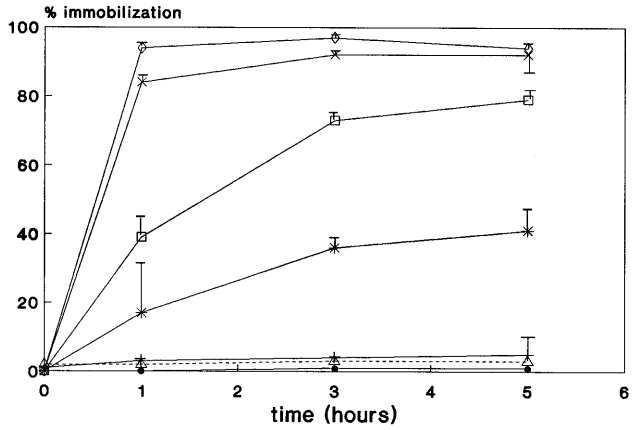

FIG. 1. Relation between immobilization of spirochetes (isolate A87S) and serum concentration. Isolate A87S was incubated with different concentrations of NHS. The percent nonmotile spirochetes was determined at different time points. Bars indicate standard deviations, based on duplicate countings of two independent experiments. ●, 0% NHS; +, 5% NHS; ✱, 10% NHS; □, 15% NHS; ×, 20% NHS; ◇, 25% NHS. For comparison, lack of killing of isolate A38S by NHS is also shown (△, dashed line).

Measurement of complement activation. To assess complement activation, an ELISA based on the technique described by Bredius et al. (11) was used. Spirochetes grown in MKM were harvested by centrifugation at 5,000 × *g* for 20 min and resuspended in PBS. From a spirochete suspension with a density of 2.5×10^7 organisms, as determined by dark-field microscopy, 100 μl was applied as a coating to an ELISA plate at 4°C for 2 h. Subsequently, the wells were emptied and aspecific binding sites were blocked with PiCM buffer (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.3 mM KH_2PO_4, 1.0 mM $MgCl_2$, 0.6 mM $CaCl_2$, 1% [wt/vol] glucose, 2.5% [wt/vol] human serum albumin) by incubation at 4°C for 30 min. Plates were washed once with PBS–0.05% Tween 20 (Sigma) (PBST) and three times with PBS. NHS was diluted in PiCM to final concentrations ranging between 1 and 25%, and 100 μl of the dilutions was added to the wells. Plates were incubated at 37°C for 1 h and washed once with PBST and three times with PBS. Plates were incubated with anti-C3c conjugated to horseradish peroxidase (Dako), diluted 1/3,000 in PBS containing 0.01% gelatin (Merck) and 0.02% Tween 20 at 37°C for 1 h, and washed again once with PBST and three times with PBS. The anti-C3c reagent recognizes the C3c part of native C3, C3b, and C3bi (60). Color development was performed with tetramethylbenzidine. The experiment was performed in triplicate and repeated three times.

To study complement activation in the absence of bivalent cations or in the presence of Mg^{2+} only, serum was diluted in PiEDTA buffer (PiCM containing 10 mM EDTA but no $MgCl_2$ or $CaCl_2$) and PiMEGTA buffer (PiCM containing 4 mM $MgCl_2$, 10 mM EGTA, and no $CaCl_2$), respectively.

In order to compare activation of the alternative pathway of complement by the various isolates, the ELISA was performed four times in triplicate in the presence of Mg^{2+}-EGTA. Isolate A87S was used as a reference. The extinction difference between the isolate under test and A87S was calculated at the serum concentration at which this difference was maximal (varying between 10 and 20% serum between experiments). The paired Student *t* test was used to calculate whether extinctions resulting from C3 activation by the various isolates differed significantly from extinctions resulting from C3 activation by isolate A87S.

RESULTS

Immobilization of spirochetes by NHS. Incubation of a low-passage *B. garinii* isolate, A87S, in 25% NHS, drawn from a healthy laboratory donor who had no antibodies against *Borrelia*, led to an almost complete immobilization of spirochetes after 3 h (Fig. 1). After overnight incubation, the effect was more difficult to judge, since almost all spirochetes had disappeared with time, probably due to lysis. The extent of immobilization was dependent on the serum concentration. Concentrations ranging from 10 to 20% NHS gave partial killing, and no effect after exposure to 5% NHS was noted. Incubation of isolate A87S in 25% heat-inactivated NHS did not result in immobilization of the spirochetes (not shown). In contrast, a

low-passage *B. afzelii* isolate (A38S) was not affected by NHS (Fig. 1).

By dark-field microscopy, large blebs could be seen on immotile spirochetes from isolate A87S after 3 h of incubation with 25% NHS (not shown). By transmission electron microscopy, it was noted that most spirochetes contained one large bleb and several minor blebs at their surface, which were absent before their incubation with NHS (Fig. 2). Spirochetes incubated with 25% heat-inactivated serum were unaffected after 3 h of incubation (not shown). Sera from the other seven healthy seronegative laboratory donors also showed immobilization of more than 90% of isolate A87S spirochetes (Fig. 3; four serum samples not shown). To confirm that antibodies were not responsible for immobilization, isolate A87S was incubated in serum from an agammaglobulinemic patient. This serum was as effective as the sera from healthy controls in immobilizing isolate A87S spirochetes (Fig. 3), confirming that immobilization occurred in the absence of antibodies.

Immobilization is associated with killing of spirochetes. Aliquots of NHS suspensions containing immobilized spirochetes of isolate A87S transferred to fresh medium resulted either in no growth or in markedly delayed growth of spirochetes. Therefore, we assumed that the immobilized, blebbed spirochetes were nonviable. From suspensions of five strains incubated with 50% NHS for 3 h, aliquots containing by calculation 2,500, 250, or 25 spirochetes were plated out by subsurface plating. The number of CFU was determined weekly. Generally, immobilization of spirochetes was associated with killing due to SBA (Table 1). One strain, A40S (*B. afzelii*), failed to grow after subsurface plating. In addition, differences between strains with regard to plating efficiency and growth rate were noted: for both *B. burgdorferi* sensu stricto B31 and A44S, plating efficiency was approximately 100%, while plating efficiencies for isolates A87S (*B. garinii*) and M19 (group VS116/M19) approximated 20 and 4%, respectively (Table 1). Isolates B31 and A44S gave colonies after incubation for 2 weeks. Colonies of isolate A87S, which were more hazy, were seen 1 week later, and no further increase in CFU was seen after 4 weeks. M19 colonies required 5 weeks to grow. It was concluded that the immobilization assay was a suitable method to measure killing of spirochetes and that strain A87S and M19 were serum sensitive.

Serum sensitivity among different *Borrelia* isolates. To investigate whether other *Borrelia* isolates were also killed by NHS, 31 additional isolates, cultured from various LB patients and from ticks, were tested in our immobilization assay. After exposure to 25 or 50% NHS for 3 h, SBAs of NHS against these isolates ranged widely (Table 2). A correlation between the genetic background of the isolates and their susceptibility to SBA was observed. Spirochetes of all eight *B. afzelii* isolates were not at all or only sporadically (less than 15% of the bacteria counted) killed after incubation with 50% NHS; of them, isolate pKo was the most susceptible isolate, showing 10% immotile spirochetes after 3 h of exposure to NHS. Of the 14 *B. garinii* isolates tested, 7 isolates were highly serum sensitive (89 to 100% killing in 50% NHS), 2 isolates were intermediately serum sensitive (A104S and A91C, 46 and 22% killing, respectively), and 5 isolates were serum resistant (<15% killing). Only one of seven *B. burgdorferi* sensu stricto isolates tested was highly serum sensitive, four isolates showed 15 to 35% killing after incubation in 50% NHS, and two isolates, including reference isolate B31, were unaffected by incubation with NHS. Finally, four group VS116/M19 isolates were all highly serum sensitive.

No relation existed between the origin of an isolate and its susceptibility to SBA. Among the 12 highly serum-sensitive isolates, 4 (A77C, VSBM, VSBP, and VSDA) originated from cerebrospinal fluid (CSF) of patients with neuroborreliosis.

Serotypes of serum-sensitive and serum-resistant *B. garinii* isolates. The 14 *B. garinii* isolates included in this study were further characterized by SDS-PAGE, and their reactivity with MAbs was studied. All isolates showed a major OspA protein with a molecular mass of 32 kDa by SDS-PAGE (Fig. 4). Subsequently, typing was performed with various anti-OspA MAbs (62); in this typing scheme, *B. burgdorferi* sensu stricto and *B. afzelii* strains are types 1 and 2, respectively, whereas *B. garinii* strains are subdivided into types 3 to 7. On immunoblots, all *B. garinii* isolates reacted with MAb L32 1C8 (Fig. 5A). Only the five serum-resistant *B. garinii* isolates and the two *B. garinii* isolates with intermediate serum sensitivity reacted with MAb L32 1G3, which reacts only with type 4 strains (Fig. 5B, lanes 10 to 16). Therefore, these seven isolates should be classified as belonging to type 4. None of the isolates reacted with MAbs L32 1F7 (reactive with types 3 and 7) and L32 1D11 (reactive with type 7 only) (data not shown). Serum-sensitive isolates A77S, A77C, VS102, VSBP, and VSDA (Fig. 5C, lanes 3, 4, 6, 8, and 9, respectively) reacted with H5332 and could therefore be classified as type 6 strains. Serum-sensitive strains A87S and VSBM (lanes 5 and 7, respectively) were nonreactive with this MAb and therefore belonged to type 5. All seven type 4 isolates with resistance or intermediate sensitivity to NHS had an OspB at 34 kDa (Fig. 4), reactive with MAb 84C (Fig. 5D). Five of the seven *B. garinii* isolates which were serum sensitive showed an 84C-reactive OspB band at 31 kDa, thus comigrating with OspA. Two strains (VS102 and VSDA) lacked an 84C-reactive OspB band (Fig. 5D, lanes 6 and 9, respectively).

Role of activation of the classical and the alternative pathway of complement. The demonstration of SBA in the absence of antibodies suggested a role for activation of the complement system and in particular for activation of the alternative pathway. For further examination of the complement pathway involved in mediating SBA, isolate A87S was tested for its susceptibility to NHS supplemented with EDTA or Mg-EGTA. Whereas in the presence of EDTA complement activation by the classical as well as by the alternative pathway is completely inhibited, the alternative pathway can still be activated in the presence of Mg-EGTA. In EDTA-supplemented NHS, SBA against isolate A87S was abolished (Fig. 6), whereas in 50% NHS supplemented with Mg-EGTA spirochetes were killed as efficiently as in 50% NHS, indicating that activation of the alternative pathway of complement is imperative for SBA (Fig. 6). Killing was significantly less in 25% NHS plus EGTA than in 25% NHS without EGTA, suggesting a role for activation of the classical pathway of complement. For further examination of the role of the pathways of complement activation, isolate A87S was exposed to sera from patients with a properdin deficiency who had no antibodies against *Borrelia*. In none of the six serum samples was isolate A87S killed (Fig. 7a), indicating that activation of the alternative pathway of the complement system was essential for SBA against this isolate. However, a C1q-depleted serum failed also to immobilize the spirochetes, and a C4-deficient serum gave only partial immobilization of the spirochetes after 3 h (Fig. 7b), indicating that the classical pathway was also involved in killing of the spirochetes. To obtain further evidence that both pathways contributed to the immobilization and blebbing of the spirochetes, strain A87S was incubated for 3 h in a mixture of equal amounts of a C4- or a C1q-deficient serum and a properdin-deficient serum (sera PD1 and PD3, from patients 1 and 3, respectively). Only immotile, blebbed spirochetes were seen after 3 h of incubation in the mixture of the C4-deficient serum

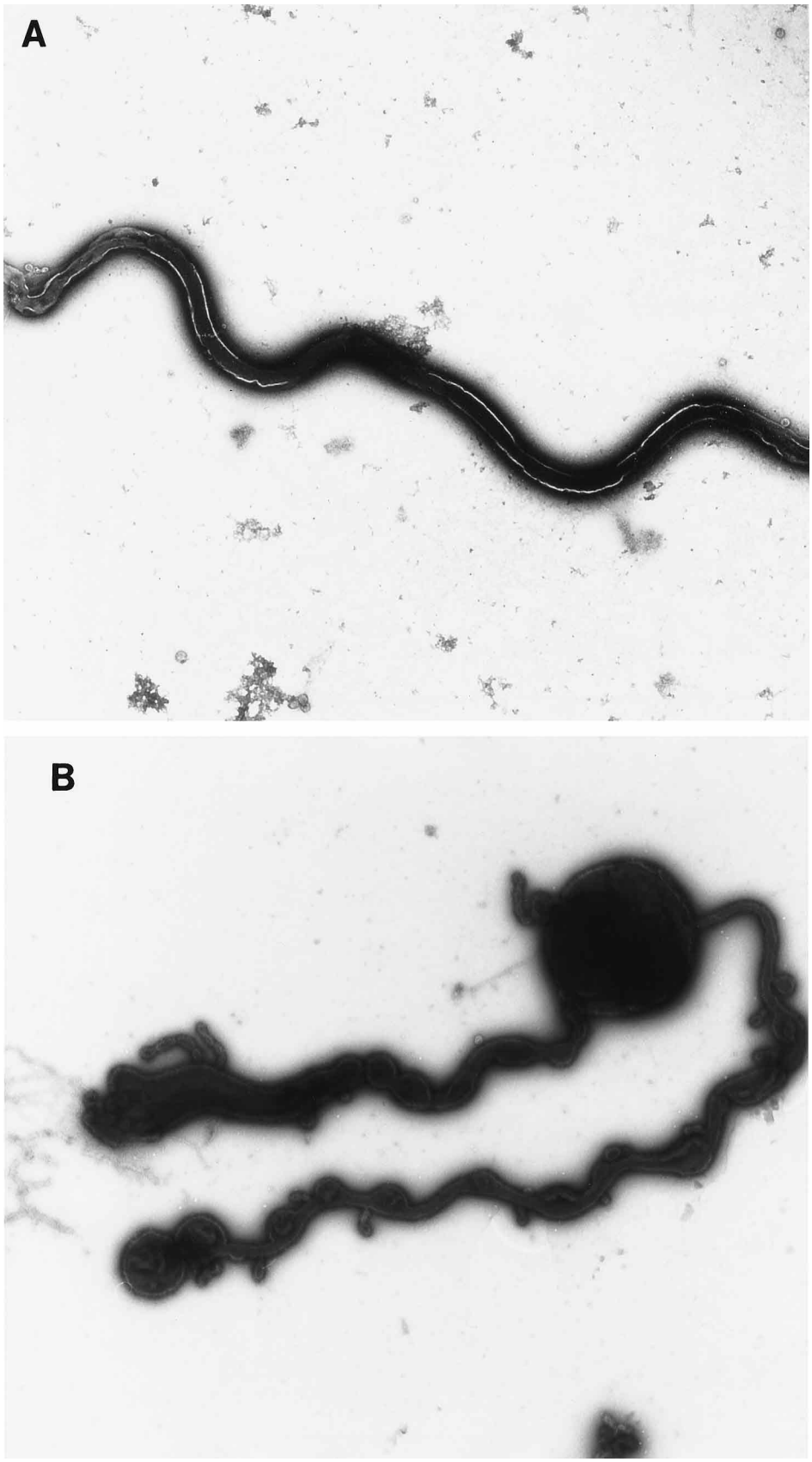

FIG. 2. *B. garinii* spirochete (isolate A87S) before (A) and after (B) 3 h of incubation with 25% NHS, seen under the electron microscope. In panel B, one large bleb and many small blebs can be observed. Magnification, approximately ×37,000.

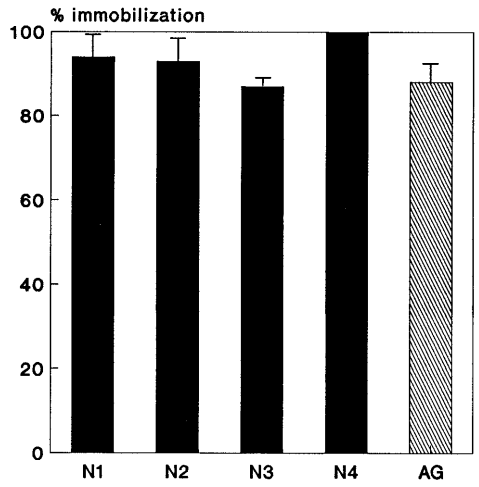

FIG. 3. Immobilization of isolate A87S by four normal serum samples (N1 to N4) and a serum from a patient with agammaglobulinemia (AG). Spirochetes were incubated for 3 h with 25% serum. After that time, the percent nonmotile spirochetes was determined. Standard deviations, based on duplicate countings of two to four independent experiments, are included.

and the serum PD1 or PD3 (Fig. 7b). After incubation for 1 h in the mixture of the C1q-depleted serum and serum PD3, only immotile spirochetes were seen. At 3 h, all spirochetes had completely disappeared. The result of incubation of spirochetes in the mixture of the C1q-depleted serum and properdin-deficient serum PD1 was difficult to interpret, since already at 1 h of incubation most spirochetes had disappeared and only a few moving spirochetes could still be seen. These results show that activation of the alternative pathway of complement is necessary for SBA; however, activation of the classical pathway also has a role.

Direct measurement of C3 activation by different *Borrelia* isolates. We also tested C3 activation by spirochetes by measuring the formation of C3c when spirochetes of seven isolates (Table 3), applied as a coating to ELISA plates, were incubated in various concentrations of NHS at 37°C. In 1% NHS, all seven isolates were equally able to activate C3, and plateau levels of C3c were reached at 3% NHS. Results for two isolates are shown in Fig. 8. Results for the other five strains were identical (not shown). In the presence of 10 mM EDTA, C3 activation by all strains was totally inhibited.

In the presence of Mg-EGTA, C3 activation by spirochetes could not be measured in the presence of 1% NHS but was present at higher concentrations of NHS (>5 to 10%) (Fig. 8). Under such conditions, C3 activation differed among isolates.

Complement-activating properties of isolates were compared to those of reference isolate A87S. It was found that low-passage isolates A38S (*B. afzelii*), A44S (*B. burgdorferi* sensu stricto), and A91C (*B. garinii*), which were serum resistant or intermediately serum sensitive, were less able to activate the alternative pathway of complement compared to isolate A87S (*B. garinii*, serum sensitive) (Table 3). No differences in activation of the alternative pathway of complement were measured among the reference isolate A87S, the high-passage, serum-resistant isolates pKo (*B. afzelii*) and B31 (*B. burgdorferi* sensu stricto), and the serum-susceptible *B. garinii* isolate A77S.

DISCUSSION

The present study shows that *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii* isolates are able to activate complement both by the classical and by the alternative pathway in NHS in the absence of specific antibodies. This phenomenon has already been demonstrated for *B. burgdorferi* sensu stricto strains (24), but the reference *B. burgdorferi* sensu stricto isolate B31 is not killed by exposure to NHS. In contrast, we found that some *B. garinii* isolates and also all group VS116/M19 isolates were readily killed by NHS. Brade et al. (10) found also that two *Borrelia* isolates differed in their ability to activate complement; they reported that a *B. afzelii* isolate (pKo) was susceptible to NHS, in contrast to B31, a *B. burgdorferi* sensu stricto isolate. However, when genetic typing became available for the classification of *Borrelia* strains, the pKo isolate used in this study was identified as a *B. burgdorferi* sensu stricto isolate (9a). More recent work performed by the same authors also showed that *B. afzelii* strains were complement resistant and that some *B. garinii* strains were susceptible (12).

The study of SBA against *Borrelia* is impeded because optimal growth of *Borrelia* is achievable only in liquid medium, while assessment of the number of CFU can be achieved only by the use of solid growth media. In earlier studies, loss of motility and the extent of blebbing have been used as criteria to assess killing of spirochetes (14, 24, 33, 52). Since a solid

TABLE 1. Association between immobilization of spirochetes and growth after subsurface plating[a]

Isolate (sp. or group)	Immobilization at 3 h (%)		CFU at calculated inoculum of:						Killing (%)
			2,500 bacteria		250 bacteria		25 bacteria		
	NHS	HIS	NHS	HIS	NHS	HIS	NHS	HIS	
B31 (*B. burgdorferi* sensu stricto)	2	0	>100	>100	>100	>100	28	31	10
	2	2	>100	>100	>100	>100	32	23	0
A44S (*B. burgdorferi* sensu stricto)	33	2	>100	>100	>100	>100	27	37	27
	33	0	>100	>100	>100	>100	17	68	75
A87S (*B. garinii*)	98	2	43	>100	5	49	0	2	90
	99	3	18	>100	1	32	0	1	97
M19 (VS116/M19)	94	3	11	>100	0	4	0	1	75
	95	1	11	>100	0	10	0	1	89

[a] Spirochetes were incubated for 3 h with 50% NHS or 50% heat-inactivated serum (HIS). Subsequently, the percent immotile spirochetes was determined and aliquots calculated to contain 2,500, 250, or 25 spirochetes were plated out as described in the text. The experiment was performed in duplicate. Plating was performed in duplicate; the mean number of CFU on two plates is shown.

TABLE 2. Bactericidal activity of NHS for different *B. burgdorferi* sensu lato strains[a]

Strain	Passage no.	Genospecies or group	Origin	% Immobilization	
				25% NHS	50% NHS
B31	HP	*B. burgdorferi* ss	Tick	2 ± 2	3 ± 1
A44S	P10	*B. burgdorferi* ss	Skin	9 ± 3	32 ± 2
VS130	P7	*B. burgdorferi* ss	Tick	13 ± 3	23 ± 2
VS134	P8	*B. burgdorferi* ss	Tick	5 ± 2	25 ± 1
VS215	P6	*B. burgdorferi* ss	Tick	53 ± 2	80 ± 1
VS219	P12	*B. burgdorferi* ss	Tick	1 ± 2	0 ± 1
VS293	P12	*B. burgdorferi* ss	Tick	8 ± 3	15 ± 3
A77C	P9	*B. garinii*	CSF	81 ± 2	100 ± 1
A77S	P9	*B. garinii*	Skin	78 ± 4	96 ± 1
A87S	P8	*B. garinii*	Skin	98 ± 3	100 ± 0
VS102	P12	*B. garinii*	Tick	20 ± 3	91 ± 1
VSBM	P6	*B. garinii*	CSF	57 ± 2	89 ± 2
VSBP	P8	*B. garinii*	CSF	92 ± 2	99 ± 1
VSDA	P9	*B. garinii*	CSF	100 ± 1	100 ± 0
A01C	P7	*B. garinii*	CSF	6 ± 2	13 ± 1
A19S	LP	*B. garinii*	Skin	3 ± 1	3 ± 2
A76S	LP	*B. garinii*	Skin	11 ± 1	5 ± 0
A91C	P5	*B. garinii*	CSF	15 ± 1	22 ± 3
A91S	LP	*B. garinii*	CSF	4 ± 2	7 ± 1
A94S	P4	*B. garinii*	Skin	7 ± 1	9 ± 1
A104S	P6	*B. garinii*	Skin	19 ± 4	46 ± 3
A17S	P4	*B. afzelii*	Skin	4 ± 2	4 ± 1
A26S	P6	*B. afzelii*	Skin	1 ± 1	3 ± 1
A38S	P5	*B. afzelii*	Skin	2 ± 4	6 ± 2
A40S	P5	*B. afzelii*	Skin	0 ± 0	1 ± 1
A42S	LP	*B. afzelii*	Skin	1 ± 1	0 ± 0
A67T	P6	*B. afzelii*	Tick	2 ± 0	1 ± 1
pKo	HP	*B. afzelii*	Skin	9 ± 1	10 ± 4
VS461	P8	*B. afzelii*	Tick	0 ± 0	0 ± 0
VS116	P9	VS116/M19	Tick	90 ± 4	100 ± 0
M19	LP	VS116/M19	Tick	75 ± 6	100 ± 0
M49	LP	VS116/M19	Tick	94 ± 1	100 ± 0
M53	LP	VS116/M19	Tick	87 ± 2	100 ± 0

[a] Strains were incubated with 25 or 50% NHS for 3 h; after that time, the percent nonmotile spirochetes was determined by dark-field microscopy. HP, high passage; P, passage; LP, low passage; ss, sensu stricto.

culture medium to grow *B. burgdorferi* has been described elsewhere (17, 47), we used this medium to study whether immobilization in conjunction with the presence of blebbing of NHS-exposed spirochetes was associated with killing. For four isolates, such an association was found, whereas one isolate failed to grow at all on the solid medium. Plating efficiency between the two serum-sensitive and the two serum-resistant strains differed, confirming data reported by Sadziene et al. (48), who found that serum-sensitive B31 mutants had a lower plating efficiency in comparison to the wild-type, serum-resistant parent strain. The inoculated plates have to be incubated for several weeks, the plating efficiency varies highly between strains, and the costs of pouring large numbers of plates are high. Therefore, we used the immobilization assay in most of the studies.

Our experiments suggest that both activation of the classical and that of the alternative pathway are involved in complement-mediated killing of *Borrelia* in the absence of antibodies. Although activation of the classical pathway is generally induced by the binding of antibodies, classical pathway activation by *Escherichia coli* in the absence of antibodies has been described earlier (29, 55). In the present study, low serum concentrations were sufficient for C3 activation, but high concentrations were necessary for bactericidal activity. This may be explained by the inefficiency of the C3-C5 activation step; Bhakdi et al. (8) showed in a liposome model that the ratio of C3 to C5 activation is 50:1.

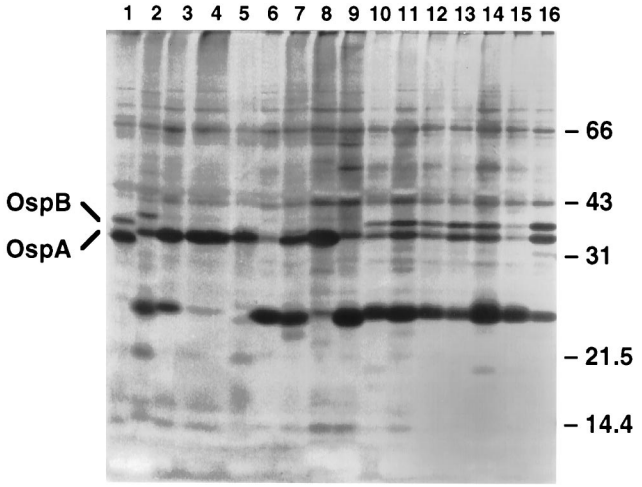

FIG. 4. SDS-PAGE analysis of 16 *Borrelia* isolates. Proteins were separated on an SDS gel containing 13% (wt/vol) acrylamide and stained with silver. Lane 1, isolate B31 (*B. burgdorferi* sensu stricto); lane 2, isolate A40S (*B. afzelii*); lanes 3 to 9, serum-sensitive *B. garinii* isolates A77C, A77S, A87S, VS102, VSBM, VSBP, and VSDA, respectively; lanes 10 to 16, serum-resistant *B. garinii* isolates A01C, A19S, A76S, A91C, A91S, A94S, and A104S, respectively. At the right, the positions of molecular mass markers (in kDa) are shown. At the left, the positions of OspA and OspB are indicated.

It is unclear by which mechanism certain *Borrelia* isolates are killed by NHS in the absence of antibodies whereas other isolates are unaffected. The association between the genetic

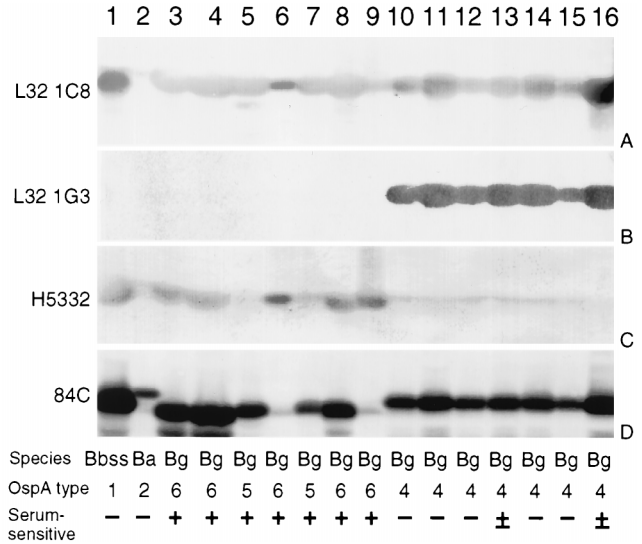

FIG. 5. Reactivity of 16 *Borrelia* isolates with MAbs. Proteins were applied onto an SDS–13% (wt/vol) polyacrylamide gel and blotted to nitrocellulose. For lanes 1 to 16, see the Fig. 4 legend. (A) Reactivity with anti-OspA MAb L32 1C8 (recognizes OspA of all *B. burgdorferi* sensu stricto and all *B. garinii* isolates). (B) Reactivity with MAb L32 1G3 (recognizes OspA of *B. garinii* serotype 4 isolates only). (C) Reactivity with MAb H5332 (reacts with OspA of *B. burgdorferi* sensu stricto and *B. garinii* serotypes 3, 6, and 7). (D) Reactivity of *Borrelia* strains with anti-OspB MAb 84C. Genospecies, OspA type, and serum sensitivity (Table 2) of the strains are indicated in the lower part of the figure.

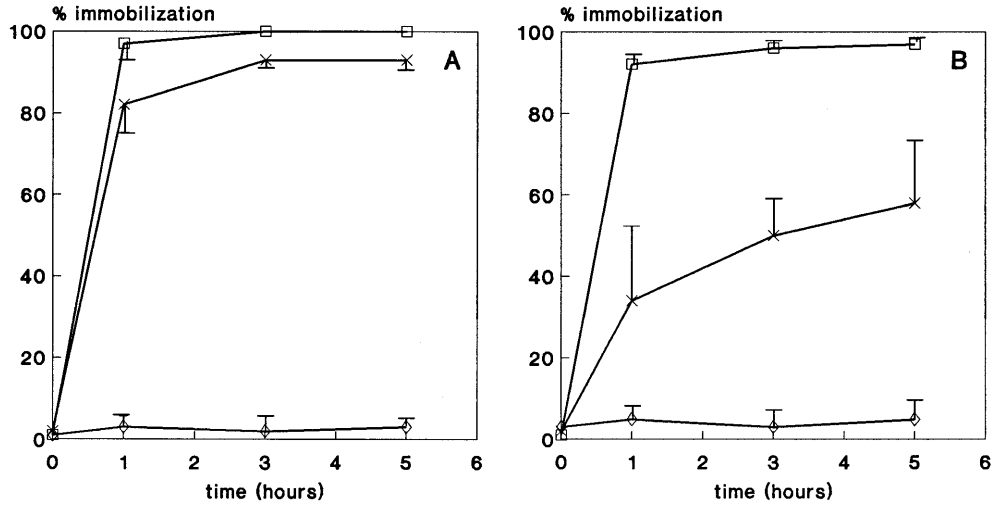

FIG. 6. Immobilization of spirochetes (isolate A87S) by 50% NHS (A) or 25% NHS (B) in modified Kelly's medium (□), with Mg^{2+} (4 mM)–EGTA (10 mM) (×), and with EDTA (10 mM) (◇). The percent nonmotile spirochetes was determined at different time points. Bars indicate standard deviations, based on duplicate countings of two independent experiments.

background of an isolate and its sensitivity to SBA was rather strong in our study. In a recent study, it was shown that a mutant B31 strain, which lacked OspA, -B, -C, and -D, was susceptible to complement whereas the parent strain was resistant (48). Moreover, a mutant *B. burgdorferi* sensu stricto strain lacking OspB also had a lower infectivity in comparison to the wild-type parent (46). In our study, two of seven serum-susceptible *B. garinii* strains lacked an OspB protein, whereas the molecular weight of OspB of the other five was slightly decreased. Therefore, OspB might be truncated in these five strains and the disappearance of the C terminus of OspB might be involved in their serum sensitivity. Alternatively, as in mutants described by Rosa et al. (45), these five strains might contain only one OspA/B hybrid protein which could react with anti-OspA as well as anti-OspB MAbs. It is also possible that certain Osp proteins, expressed in some *B. garinii* and VS116 strains, are not able to generate complement resistance. However, other factors which also vary between the resistant and the susceptible strains may cause differences in serum sensitivity.

Other mechanisms by which killing of gram-negative organisms by complement is prevented are sialylation of lipooligosaccharide in *Neisseria* strains (19, 59) and the presence of long *o*-polysaccharide side chains in *Klebsiella*, *E. coli*, and *Salmonella* strains (32). In the spirochete *Treponema pallidum*, it has been shown that enzymatical removal of sialic acid resulted in enhanced activation of the alternative pathway (22). It is unclear whether *Borrelia* contains sialic acid in its outer membrane. Studies to address this problem are currently under way.

The role of the complement system in host defense mechanisms against LB is still a matter for debate. Antibodies against OspA (47), OspB (16), p39 (52), and other, not-yet-defined proteins (3) are bactericidal for *Borrelia* in the presence of

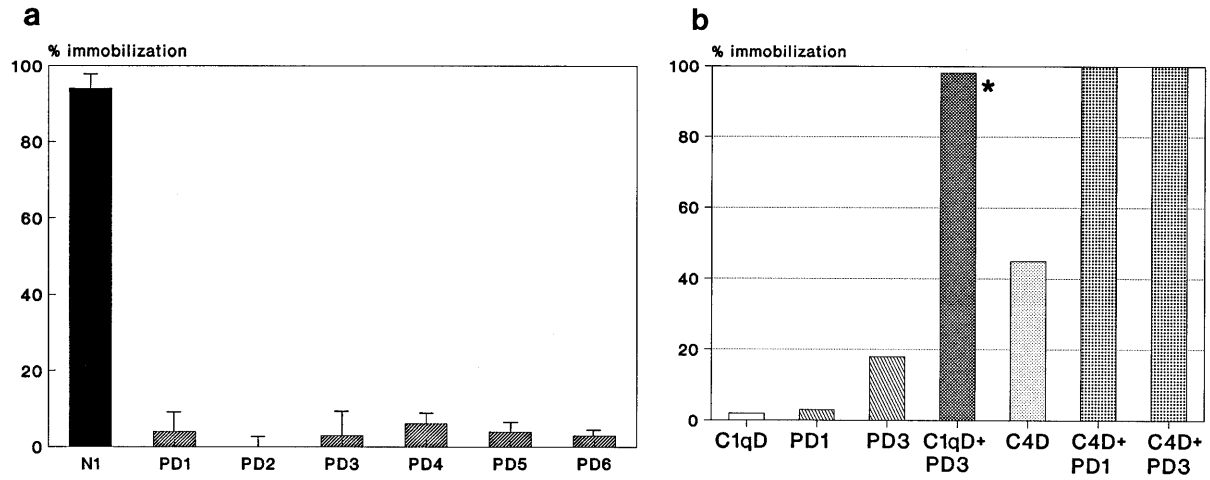

FIG. 7. Involvement of activation of the alternative and the classical pathway in complement-mediated killing of *Borrelia*. (a) Spirochetes (*B. garinii* A87S) in 75 μl of modified Kelly's medium were incubated for 3 h with 25 μl of serum from six patients with a properdin deficiency (PD1 to PD6) and from a healthy control person (N). Subsequently, the percent nonmotile spirochetes was determined. Standard deviations, based on duplicate countings of two to four independent experiments, are included. (b) Spirochetes (*B. garinii* A87S) in 50 μl of modified Kelly's medium were incubated with 50 μl of C1q-depleted serum (C1qD), 50 μl of C4-deficient serum (C4D), and mixtures of 25 μl of either of these sera with 25 μl of a properdin-deficient serum (PD1 or PD3). The results of incubation of spirochetes with 50 μl of properdin-deficient serum (PD1 or PD3) are also shown. *, the percent motile spirochetes was determined after 1 h, since at 3 h all spirochetes had already lysed.

TABLE 3. Differences in extinction between various *Borrelia* strains and *B. garinii* A87S in the C3c ELISA in the presence of Mg^{2+}-EGTA[a]

Strain	Passage no.	Genospecies	E_{450} difference from A87S ± SEM
A87S	P8	*B. garinii*	0.000
A77C	P9	*B. garinii*	+0.025 ± 0.031 (NS)
pKo	HP	*B. afzelii*	−0.064 ± 0.031 (NS)
B31	HP	*B. burgdorferi* ss	−0.009 ± 0.050 (NS)
A44S	P10	*B. burgdorferi* ss	−0.165 ± 0.046 ($P < 0.01$)
A38S	P5	*B. afzelii*	−0.163 ± 0.026 ($P < 0.001$)
A91C	P5	*B. garinii* serotype 4	−0.170 ± 0.036 ($P < 0.001$)

[a] The amount of C3c generated by the spirochetes was assessed by ELISA. The table shows the differences in extinction between the tested strain and reference strain A87S at the serum concentration at which this difference was maximal. The ELISA was performed as described in Materials and Methods. P, passage; HP, high passage; ss, sensu stricto; E_{450}, extinction coefficient at 450 nm; NS, not significant.

complement. Complement is generally required to obtain the bactericidal effect of antibodies (24), but antibodies have been reported to be bactericidal for *Borrelia* in the absence of complement (49). In animal models, complement did not appear to be essential for antibody-mediated protection against LB. C5-deficient mice, which were susceptible to infection with *B. burgdorferi*, could be protected by pretreatment with immune sera or with MAbs against OspA (9). Irradiated hamsters depleted of complement by cobra venom factor (CVF) could be protected against infection with *B. burgdorferi* by immune sera obtained from *B. burgdorferi*-infected hamsters 3 weeks after inoculation (50). Non-CVF-treated hamsters were also protected by passive immunization with sera obtained from animals infected for 1 or 10 weeks, whereas CVF-treated animals were not, suggesting that the presence of complement enhances protection against *B. burgdorferi* infection. It should be noted that in these studies only *B. burgdorferi* sensu stricto isolates were used.

Mice infected with *B. burgdorferi* sensu stricto strains can be bacteremic for weeks (6). This prolonged bacteremia may well be due to serum resistance of those strains. Whether mice develop prolonged bacteremia after infection with serum-susceptible isolates has not been studied. In addition, it should be noted that we investigated only the sensitivity of strains to human serum. Strains may react differently with murine sera. Complement-mediated killing of a *B. burgdorferi* sensu stricto isolate has been reported in the presence of high concentrations (>10%) of normal rat serum (38).

It has not yet been proven whether serum resistance is a virulence factor of certain *Borrelia* strains in human LB. It is noteworthy that *B. afzelii* is known for its ability to persist in patients with ACA (15, 57, 61) and that all *B. afzelii* strains tested were serum resistant. Group VS116 strains, which we found to be serum sensitive, have never been isolated from humans. However, four of seven serum-sensitive *B. garinii* isolates had been isolated from CSF and thus were able to cause disseminated disease. In culture, spirochetes can alter the expression of Osp proteins and may lose infectivity after several passages (51). Although we generally used low-passage isolates, we have not tested whether the isolates we tested were still pathogenic. Therefore, the assessment of the role of the in vitro serum resistance of the various *Borrelia* isolates in their pathogenicity requires further study, including animal models.

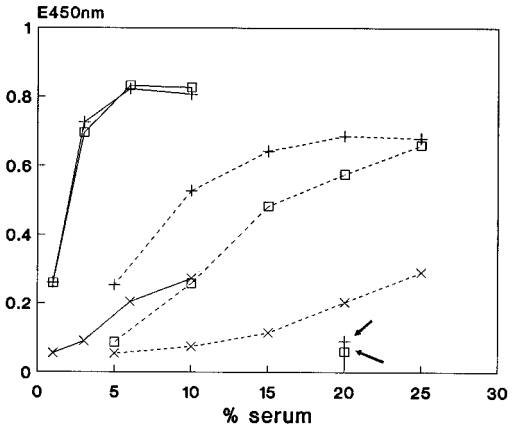

FIG. 8. Activation of C3 by *B. garinii* A87S (+), by *B. afzelii* A38S (□), and in the absence of spirochetes (×). Equal amounts of spirochetes were applied as a coating to ELISA plates and incubated with different concentrations of NHS in PiCM buffer (see text) (solid lines), in PiM-EGTA buffer (dashed lines), or in Pi-EDTA buffer (arrows). After washing, plates were incubated with anti-C3c, washed again, and developed with tetramethylbenzidine. Results of a representative experiment are shown.

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