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THE MECHANISM OF COMPLEMENT-INDEPENDENT BACTERICIDAL ANTIBODIES AGAINST BORRELIA

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Abstract of the Dissertation

The Mechanism of Complement-Independent Bactericidal Antibodies Against *Borrelia*

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This dissertation examines the mechanism of complement-independent bactericidal antibodies to *Borrelia* in consideration of the possibility that the mechanism could be the result of the contributions of the antibody, the antibody:pathogen interface, and the uniqueness of the *Borrelia* organisms. To investigate contributions of the antibody, a single chain variable fragment (scFv) of CB515 (IgM against relapsing fever *Borrelia*) was constructed and shown to retain the bactericidal activity of its parent antibody. The CB515 scFv is species- and serotype-specific, damages the outer membrane, and can eliminate an entire serotype population, demonstrating that residues within the variable region are critical for the mechanism whereas the constant region is dispensable. In investigating events at the antibody:pathogen interface, CB2 (IgG1

against the OspB of *Borrelia burgdorferi*) was shown to create outer membrane projections/blebs that are released from the surface rapidly and breaks (58 – 145 nm) that may be transient. The action of CB2 results in the creation of openings (2.8 – 4.4 nm) in the outer membrane that increase its permeability, killing the spirochetes by osmotic lysis. The bactericidal effect of CB2 was not transferable to other bacteria expressing OspB, suggesting the requirement of factors unique to *Borrelia* for the mechanism. Investigation of factors unique to *Borrelia* and required for this mechanism revealed that a porin, P66, co-precipitated with OspB but was also shown to be dispensable for the bactericidal effect of CB2. However, the mechanism of complement-independent bactericidal antibodies may be dependent on the high fluidity of the outer membrane of *Borrelia*, a characteristic that is not shared among many bacteria. These factors in *Borrelia*, as well as factors in the antibody variable region, are absolutely critical for the bactericidal function of the antibody as its effects are not transferable to another Gram-negative bacterium. This body of work is significant in that it provides a bactericidal mechanism for a specific class of antibodies that diverges from the traditional dogma. Never before has a bactericidal function been observed to reside in the antibody variable region as the constant region is typically thought to provide effector functions to these molecules. That the mechanism of these antibodies relies on the remodeling and removal of membrane lipids and pore formation and that both of these features are dependent on properties specific to the *Borrelia* organisms is unique.

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INTRODUCTION

Spirochetes of the *Borrelia* genus have undergone intense study and evaluation since the discovery of *B. burgdorferi*, the etiologic agent of Lyme disease, over 25 years ago (37, 56). These organisms are responsible for Lyme disease and relapsing fever and represent quite a fascinating and unique group of pathogens in relation to other microorganisms. It is the unique and novel features of the organisms themselves as well as the specific makeup of the host response to these invading pathogens that has fueled an area of microbiological research which has consistently accelerated since its inception.

Of particular interest is the host immune response to *Borrelia*, which has proven to be unique in its constituents as well as in the manner in which these pathogens are eliminated from the infected host. The *Borrelia* organisms are extracellular pathogens and, as such, humoral immunity is of exceptional importance in their clearance. Specifically, antibodies can eliminate these pathogens (204). Through the study of the antibody response to *Borrelia*, it was discovered that there are antibodies which function to kill the organisms directly in the absence of complement (77, 80, 204, 298). The mere presence of these antibodies and their significance in an *in vivo* setting challenges traditional antibody dogma and warrants the study of their mechanism of action. While some of the requirements for the function of these antibodies are known, there is not enough known about them to elucidate a specific mechanism of action.

For this dissertation, a deeper examination of the mechanism of these complement-independent bactericidal antibodies was carried out. It is our hypothesis that the mechanism of action of these antibodies requires a specific interplay in which the antibody variable region, specific antigen, and properties of the *Borrelia* organisms themselves all contribute and play crucial roles. The importance of the variable region, mode of death of the spirochetes, and the specificity of the effects of these antibodies are the primary focus of this research. Throughout, two complement-independent bactericidal antibodies were considered for this dissertation. These monoclonal

antibodies are CB515 (IgM) and CB2 (IgG1), which are directed against, and lethal to, an uncultivable species of relapsing fever *Borrelia* and *B. burgdorferi*, respectively.

The background that follows (Chapter 1) contains the topics (please see Table of Contents, p. v) which pertain to the microbiology and immune response to *Borrelia* as well as B cells and antibodies with a specific focus on complement-independent antibodies.

CHAPTER 1 - BACKGROUND

1.1 BIOLOGY OF SPIROCHETES

Borrelia spirochetes are responsible for Lyme disease and relapsing fever. These tick-transmitted organisms are found worldwide and are distinct from one another but also share similarities. The *B. burgdorferi* sensu lato complex comprises the genospecies that cause Lyme disease which includes *B. burgdorferi* sensu stricto (37, 56), *B. garinii* (20), *B. afzelii* (65), and several other genospecies, *B. japonica* (184), *B. andersoni* (8, 9), *B. lusitaniae* (207), *B. valaisiana* (355), *B. bissettii* (276), *B. takunii* (126), and *B. turdi* (126), whose role in human disease has not been directly proven (Table 1.1) (356). Similarly, relapsing fever is caused by several different species including the tick-transmitted *B. hermsii*, *B. turicatae*, *B. parkeri*, *B. mazzotti*, *B. venezuelensis*, *B. duttonii*, *B. crocidurae*, *B. persica*, *B. hispanica*, *B. latyschewii*, and *B. caucasia* and the louse-transmitted *B. recurrentis* (Table 1.1) (60, 92, 286). Although endemic to certain geographical locations such as the northeast and western states of the U.S. for Lyme disease and relapsing fever, respectively, the presence of a great number of species that may cause the diseases allows for the worldwide distribution and significance of both diseases.

1.1.1 Growth and Morphology

As their name suggests, the *Borrelia* spirochetes have an obvious spiral morphology. This, as well as their movement, is entirely due to the presence of bundles of periplasmic flagella. The spirochetes have a Gram-negative-like morphology with an

inner membrane (which surrounds the protoplasmic cylinder), thin peptidoglycan cell wall, periplasmic space, and outer membrane. However, there are key differences in the outer envelope of the *Borrelia* spirochetes compared to typical Gram-negative organisms. As mentioned, there are bundles of periplasmic flagella that are never exposed to the extracellular environment unless the integrity of the outer membrane has been compromised. *Extensive damage such as this is characterisitic of the effects exerted by complement-independent bactericidal antibodies such as CB515 and CB2*. The periplasmic flagella underlie the outer membrane and exist between this barrier and the cell wall. As a result, the cell wall in *Borrelia* is more closely associated with the inner membrane in contrast to typical Gram-negative bacteria where the cell wall underlies the outer membrane. Additionally, the *Borrelia* spirochetes do not have a typical lipopolysaccharide (LPS) that contains lipid A as a major constituent of their outer membrane; rather, a number of different glycolipids, some which contain cholesterol, take the place of LPS (36, 282, 309, 361). The *B. burgdorferi* glycolipids have been identified and are cholesteryl 6-O-acyl-β-d-galactopyranoside (referred to as *B. burgdorferi* glycolipid I [Bb GL-I] or acylated cholesterol galactopyranoside [ACGal]), 1,2-di-O-acyl-3-O-α-d-galactopyranosyl-sn-glycerol (referred to as *B. burgdorferi* glycolipid II [Bb GL-II]), cholesteryl-b-D-galactopyranoside (referred to as CGal), and mono-a-D-galactosyl-diacyl-glycerol (referred to as MGalD), as well as less-studied cholesterol glucosides (36, 308, 309, 341)**.** Furthermore, the outer membrane of *Borrelia* contains free cholesterol and cholesterol esters. Altogether, this accounts for an outer membrane that is made up of 40% cholesterol molecules and cholesterol-containing glycolipids (341). *The vast presence of cholesterol may contribute to the unusually high fluidity of the outer membrane of Borrelia (25, 87) which, in turn, could explain why the Borrelia spirochetes (and in particular their outer membrane) are so susceptible to the effects of complement-independent bactericidal antibodies.*

1.1.2 Different Antigens of *Borrelia*

Recently, the unique glycolipids of *Borrelia* have come into prominence as important antigens especially in the late stages of disease (308, 341), but major protein antigens of *Borrelia* are the surface-exposed lipoproteins present in the outer membrane

(49). In *B. burgdorferi* these are referred to as **o**uter **s**urface **p**roteins (Osps) (reviewed in 22, 38, 42, 66, 123, 168) but there are other lipoproteins which may serve antigenic roles as well. The most prominent antigens of relapsing fever *Borrelia* are collectively referred to as **v**ariable **m**ajor **p**roteins (Vmps) but can be divided into two groups of lipoproteins based on their molecular weight: the **v**ariable **s**mall **p**roteins (Vsps) of 19-22kDa, and **v**ariable **l**arge **p**roteins (Vlps) of 35-40kDa (reviewed in 22). As their name suggests, the Vmps are the antigens responsible for antigenic variation in relapsing fever which will be discussed in detail in the following section. The lipoproteins associated with organisms of both diseases contain a lipid modification at the amino-terminal cysteine comprised of three palmitic acids covalently linked to a diacylglycerylcysteine, a modification of the invariant cysteine to generate tripalmitoyl-S-glyceryl-cysteine (91). Polypeptides targeting for these modifications initially contain a lipoprotein consensus sequence, [L, V, I]$_{-3}$[A, S, T, G]$_{-2}$[G, A]$_{-1}$C$_{+1}$, recognized by signal peptidase II. Once recognized, the sequence is cleaved to yield an amino terminal cysteine which undergoes the lipid modification (91). *The lipoproteins are of significance as antigens (specifically the Vsps and OspB) as they are the known targets of complement-independent bactericidal antibodies such as CB515 and CB2 (78, 82).*

Differential Osp expression has been well-studied and certain Osps appear to be important in the tick transmission of *B. burgdorferi* (313). In an unfed tick, spirochetes are present in very low numbers in the tick midgut (23^{o}C, pH ~7.5) and predominantly express OspA and OspB (313, 344). Upon feeding, mammalian blood enters the midgut and dramatically changes the environment (35^{o}C, pH < 7.0). The new environment allows for the spirochetes to replicate to greater numbers. As the tick feeds, spirochetes migrate to the salivary glands where they now predominantly express OspC in place of OspA/B (313, 344). While in the salivary glands, spirochetes can be transmitted to the mammal via tick saliva. The tick must feed for approximately 48 hours for efficient transmission of *B. burgdorferi*. Tick-transmission of relapsing fever *Borrelia* occurs similarly via the soft-bodied *Ornithodoros* ticks. However, these ticks feed for very short periods of time (less than half an hour) and can efficiently transmit the infecting spirochetes in this time frame.

The functions of the Osps and Vmps, other than that of antigenic variation and persistence, are debated but several pieces of evidence suggest functions beyond antigenic variation. OspA and OspB appear to be critical for spirochete adherence in the tick gut and their downregulation in during transmission from the tick to mammalian host supports this evidence. However, adherence to the tick gut does not explain the appearance of *B. burgdorferi* that express OspA and OspB in late/chronic Lyme disease. OspA has been shown to bind plasmin(ogen) (125), which can explain the expression of OspA in late infection. *That OspB, a known target two 2 complement-independent bactericidal antibodies (CB2 and H6831) (77, 297), is only briefly expressed during infection where it would be vulnerable to the antibodies demonstrates the extraordinary susceptibility of these molecules to the lethal antibodies.* OspC and several OspE/F related proteins (Erps) also have the ability to bind plasmin(ogen) (51, 203). Some evidence suggests that OspA and other lipoproteins of *B. burgdorferi* are involved in shielding other antigens from the immune response, most notably integral membrane protein P66 (55). OspA seems to specifically provide this "antigenic shielding" role in the tick during incoming blood meal (32). OspC was shown to bind a particular tick salivary protein, termed Salp15 (284), and the fact that OspC is upregulated during spirochete migration to the tick salivary glands supports this evidence. OspC was also shown to retain binding to Salp15 in early murine infection and this interaction increased the persistence of *B. burgdorferi* (284). Bound Salp15 inhibited the antibody-mediated killing of *B. burgdorferi* (284) and complement-mediated killing of serum-sensitive *B. burgdorferi* (310) *in vitro* and may perform these functions in an infected host. Additionally, Salp15 has been shown to inhibit CD4 T cell activation (11) and cytokine expression by binding to DC-SIGN (167).

Less work has been done on the potential functions of Vmps of relapsing fever but different Vmps have been shown to provide specific tissue tropisms for *B. turicatate* (63, 269). One Vmp serotype (serotype A) exhibited central nervous system (CNS) invasion early in infection whereas the other (serotype B) exhibited a preference for joint infiltration (63). Furthermore, serotype B achieved higher levels in the blood and joints (269), whereas serotype A achieved higher levels in the leptomeninges and brain (61, 316). This is of interest as antibodies are linked to the production of different relapsing

fever serotypes (discussed in 1.4.1). In this scenario, the host antibody response may eliminate one relapsing fever serotype with a preference for a certain tissue, allowing for the production of an alternate serotype and thus preference for a different tissue. This becomes apparent as residual brain infection with *B. turicatae* serotype A occurred in about 20% of immunocompetent C57BL/6 mice (62). This suggests that the other 80% of mice were infected with *B. turicatae* that were forced to seroconvert due to the host antibody response, and thus lost their neurotropism. *These findings indirectly demonstrate a role for host serum antibodies in relapsing fever Borrelia tissue preference and demonstrate the power and versatility of complement-dependent and independent antibodies.*

1.2 PATHOGENESIS AND PERSISTENCE

Lyme disease and relapsing fever are similar spirochetal infections that are acquired through tick-transmission. The organisms of these respective diseases, however, differ in the tick vector responsible for their transmission. *Borrelia* that cause Lyme disease are transmitted by hard-bodied ticks of the *Ixodes* genus whereas relapsing fever *Borrelia* are commonly transmitted by soft-bodied ticks of the genus *Ornithodoros* (56, 57, 59, 97, 98, 271). Once transmitted from the bite of an infected tick to a mammalian host, acute manifestations of both diseases can include fatigue, fever, headache, arthralgia, or myalgia. Both diseases have a tropism for similar tissues, as well, which include the joints, heart, and nervous systems. Lyme disease differs in that it is often marked by a redish, bullseye-like skin lesion at the site of the tick bite called erythema migrans. In relapsing fever, acute manifestations evolve more rapidly and tend to be more severe, sometimes resulting in death, as a result of the high density bacteremia (spirochetemia) caused by these spirochetes (327).

1.2.1 Antigenic Variation in *Borrelia*

The spirochetemia in relapsing fever is characterized by an initial large peak that occurs after spirochetes disseminate from dermal tissue to the blood (Figure 1.1). This peak is rapidly cleared by antibodies and it is followed by a series of smaller peaks that develop as a result of antigenic variation of these spirochetes (26, 74, 340). The antigenic

variation is such that each peak is predominantly composed of spirochetes of one serotype, with different serotypes present at much lower densities. As the immune system develops a response to the major serotype, spirochetes of that serotype are cleared, allowing for organisms expressing another serotype to thrive causing a subsequent peak (26, reviewed in 177, 340). In this manner antibodies are intimately associated with the cycle of antigenic variation in relapsing fever.

The power and lethality of complement-independent bactericidal antibodies like CB515 suggests that they may be major players in forcing antigenic seroconversion, which has been observed experimentally with this antibody (82, 204, 205) . The antigenic switch occurs at the genetic level via transfer of silent variable major protein (*vmp*) genes that become expressed one at a time and are interchangeable (reviewed in 21, 58, 239, 273). Once translated, the expressed variable major protein (Vmp) antigen becomes the dominant surface protein of the spirochete, producing a serotype. The insertion of a previously silent (archival) *vmp* gene into the expression plasmid depends on recombination between upstream and downstream homology sequences (UHS and DHS, respectively) that flank the genes (93). Frequency of antigenic switch depends upon identity between UHS sequences and placement of DHS sequences on different *vmp* genes indicating a programmed method of antigenic variation (24). Due to this programmed antigenic variation there is a hierarchy of switching that occurs, with certain Vmps favoring expression in early infection and others favoring expression in later infection. This allows for the antigenic variation to be semi-predictable, and so, even though there may be many antigenically variable serotypes, the relapse cycle is self-limited (24).

Borrelia burgdorferi has an antigen similar to the Vmps of relapsing fever spirochetes called the **V**mp-**l**ike **s**equence, **E**xpressed (VlsE) antigen (374). This antigen is highly polymorphic and is thought to contribute to the persistence of *B. burgdorferi* in mammalian hosts by providing a mechanism of antigenic variation, however the mechanism of antigenic variation of VlsE differs from that of the Vmps of relapsing fever (261). Instead of the replacement of an already existing VlsE with a completely different VlsE (as is the case with Vmps) on the surface of *B. burgdoferi*, a number of different

small cassettes in the central region of VlsE are replaced resulting in segmental antigenic variation (261, 374). Since different cassettes and different numbers of cassettes can be replaced during this antigenic variation it results in the appearance of countless variants suggesting that *B. burgdorferi* antigenic variation, unlike that of relapsing fever, is not self-limited. This mechanism combined with the high frequency of antigenic switches in VlsE results in the presence of thousands of different VlsE variants at any one time during infection, as opposed to one predominant serotype in the case of relapsing fever (261). There does not seem to be a programmed hierarchy of switching in VlsE antigenic variation, as well (86). The clear differences in these antigenic variation systems may reflect the differences of relapsing fever and Lyme disease pathogenesis as relapsing fever spirochetes are prominent in the blood during the infection cycle whereas Lyme disease spirochetes have a very brief spirochetemia. Furthermore, VlsE is present on the surface of *B. burgdorferi* among many other antigens (OspC, CRASPs, etc) and in this respect (as well as the fact that thousands of VlsE variants can be present at once) would not be expected to produce the appearance of a true serotype, as is the case in the early stages of relapsing fever. These other antigens are capable of eliciting strong immune responses and their presence along with VlsE on the surface of *B. burgdorferi* can lead one to believe that the VlsE system is irrelevant as host antibodies to the other antigens would be expected to eliminate the spirochetes. However, *B. burgdorferi* lipoproteins present on the surface with VlsE, such as OspA, OspB and OspC, have been observed to be downregulated in response to immune pressure resulting in the appearance of escape mutants (77, 159, 213, 297). Therefore, despite the absence of serotype production and immune response specific to one antigen, VlsE variation in conjunction with production of Osp escape mutants may comprise a more unique antigenic variation system than that of relapsing fever. Whether or not the antigenic variation of VlsE plays a crucial role in the persistence of *B. burgdorferi* in human infection has not been directly proven. However, human sera reacts with different epitopes on VlsE (206) and, more importantly, reacts with epitopes in the variable regions of VlsE (231). The *vls* locus also appears to be critical for murine infection (19).

1.2.2 Increased Persistence of *Borrelia* by Enhanced Dissemination

In addition to antigenic variation the *Borrelia* spirochetes have also evolved other methods to increase their persistence in mammalian hosts. Most notably, spirochetes that cause Lyme disease and relapsing fever have the ability to evade the effects of host complement, but this will be discussed in greater detail in section 1.3.2. Just as important is the ability of *B. burgdorferi* to bind host plasmin(ogen) (79, 189). Plasminogen (PLG) is a zymogen present in circulating mammalian plasma which is cleaved to become the active enzyme plasmin by the urokinase-type PLG activator (uPA) or tissue-type PLG activator. Active plasmin traditionally cleaves fibrin in a function to clear blood clots. *B. burgdorferi* binds host PLG via lysine residues and exogenous uPA (which would be present in the host) has the ability to cleave PLG to plasmin on the surface of *B. burgdorferi* (79). The presence of active plasmin on *B. burgdorferi* allows the spirochete to persist by increasing its dissemination since it can allow for increased endothelial cell penetration, and can "clear a path" for the spirochete by degrading fibronectin, laminin, and vitronectin of the extracellular matrix (ECM) (75, 76, 79). Both of the above strategies are those that can allow for increased persistence in the early stage of infection, allowing for *B. burgdorferi* to have the opportunity to disseminate to its target tissues, the joints, heart, and nervous system where it may fly "under the radar" of the immune response and complement-independent bactericidal antibodies. As the spirochetes disseminate to different tissues, it is possible that alternative persistence mechanisms are employed.

In addition to the Osps many other lipoproteins are expressed by on the surface of *B. burgdorferi*. Among these are the decorin-binding proteins (Dbps), which allow for the binding to decorin, a major ECM component (212). Two tissues with a high decorin content, the joints and skin (both of which are *B. burgdorferi* targets) were shown to be protective infection sites for *B. burgdorferi* in wild type infected mice and passively immunized SCID mice and this protection was lost when decorin was disrupted (212). According to this evidence it is thought that *B. burgdorferi* may bind host decorin via DbpA and use it to mask itself from the immune system in the disseminated stage of infection (212).

1.3 IMMUNITY TO BORRELIA INFECTIONS

1.3.1 Complement

The complement system comprises a set of serum proteins and proteases important in innate immunity and in bridging the innate and acquired immune systems (Figure 1.2) (173). These proteins comprise one arm of humoral immunity with antibodies representing the other and both are important in the defense against extracelluar pathogens. The classical, alternative, and mannan-binding lectin complement pathways differ in their initial steps and method of recruitment but produce similar results that include opsonization, inflammatory cell recruitment, and the formation of the membrane attack complex (MAC – lytic complement). Complement gets its name from the first pathway discovered, the classical pathway, as its lytic effects were said to complement the binding activity of antibodies (173).

1.3.2 Complement-Dependent Antibody-Mediated Killing of *Borrelia*

Complement-dependent antibody-mediated killing of *B. burgdorferi* via the classical pathway has been documented *in vitro* (190). This complement-dependent killing was rather unusual as fragment antigen binding (Fab) fragments were able to mediate complement killing. As the fragment crystallizable (Fc) is the complement-activating domain, this complement-dependent effect was presumably the result of Fab binding which in turn altered steric conformation to allow for proper MAC deposition (191, 192). Many monoclonal antibodies (MAbs) and antisera to *B. burgdorferi* have also been shown to be dependent on complement for bactericidal activity *in vitro* (15, 220, 223, 252, 253, 263, 288, 295, 326). These numerous *in vitro* accounts underscore a role for antibody-mediated killing dependent on complement in immunity against *Borrelia*, as would be expected in the host response to an extracellular bacterium.

1.3.3 Complement Evasion by *Borrelia*

Despite the above evidence, recent studies have shown that *Borrelia* develops resistance to certain forms of complement-dependent killing, particularly by evasion of the alternative complement pathway (Figure 1.2) (reviewed in 199, 351). In Lyme

disease, this evasion is mediated by the binding of the **c**omplement **r**egulator-**a**cquiring **s**urface **p**roteins (CRASPs) and proteins of the OspE/F Erp family, present on the spirochete surface, to host Factor H and Factor H-like protein-1 (FHL-1) (4, 53, 156, 158, 166, 195, 196, 198, 294). Complement evasion is made possible by Factor H and FHL-1, which are host alternative complement pathway regulators, acting as cofactors for Factor I. This in turn promotes the degradation of C3b and C3 convertase and thus inhibits the lytic effects of complement. The CRASPs bind to the C-terminus of Factor H or FHL-1, orienting them in such a way as to allow for the continuation of their regulatory function, contributing to complement evasion by *Borrelia* (156, 195, 197). This binding interaction is quite intricate as it seems to depend on the existence of a discontinuous binding site, in which charge-charge and hydrophobic interactions both contribute, and is stabilized by C-terminal CRASP residues (84, 85). There has been some debate as to whether CRASP-1 has a significant role in complement evasion, as it was shown that patient serum did not react against CRASP-1 in immunoblots (230). Moreover, upregulation of the CRASP-1 gene did not occur in *B. burgdorferi* recovered from infected mice (230). However, patient serum has shown reactivity to this protein in its native form, suggesting that it is expressed in human infection and relevant to complement evasion *in vivo* (294, 354).

Proteins of the OspE/F Erp family also function in a similar manner, although their binding may depend on the presence of coiled-coil domains, with contributing C-terminal lysine residues (3, 158, 196, 233). These complement evasion proteins have been shown to possess the ability to bind Factor H from different animal hosts, presumably contributing to the host range of Lyme disease *Borrelia* (166, 338). Similar alternative complement evasion occurs in relapsing fever infection, as well (164, 232). This is mediated by Factor H binding protein A1 (FhbA1) and FhbA2, which are functionally similar to the CRASP and OspE/F proteins. Their binding is contingent on N- and C-terminal residues and may depend on loop or coiled-coil domains (163, 165). *Since Borrelia has been shown to be resistant to the effects of complement via the alternative pathway, the other major constituents of humoral immunity, antibodies, certainly acquire greater importance in the host response.*

Interestingly, relapsing fever spirochetes *B. recurrentis* and *B. duttonii* were shown to bind C4b-binding protein (C4BP), the functional homolog to Factor H in the classical complement pathway (Figure 1.2) (240). This binding was achieved in a manner similar to the binding of Factor H by *Borrelia*. This suggests that relapsing fever *Borrelia* may be capable of evading destruction via the classical complement pathway as well. *If this is the case, evasion of complement deposition may contribute to the survival of relapse populations in the infection, and more importantly, may explain the importance of complement-independent, bactericidal antibodies crucial for immunity to relapsing fever (see section 1.4.3)* (23, 80, reviewed in 81, 82, 257).

1.3.4 The Role of B cells in the Host Response to *Borrelia*

Antibodies are small, flexible proteins of great specificity and are considered to be among the most powerful weapons in defense of extracellular pathogens or pathogens traveling in the extracellular milieu. They are secreted by mature B cells and perform effector functions that are mediated by their Fc portion. These can include opsonization, neutralization, and complement recruitment, which can lead to the formation of the MAC, enabling the antibody to become bactericidal in concert with complement (173).

B cells can be divided into two main sets, B1 and B2, each with two subsets (Figure 1.3) (173, 226). B1 B cells, which contain the B1a and B1b subsets, are T cell-independent, self-renewing B cells found mainly in the pleural and peritoneal cavities (173, 226). These B cells are responsible for "natural IgM" (264), but can be driven to expand and secrete specific IgM, which is important in the early responses to some infections (5, 6, 33, 144, 243). Mature B2 B cells, which contain follicular (FO) and marginal zone (MZ) B cell subsets, account for the majority of B cells found within a host. Follicular B cells are the textbook examples of B cells, which require T cell help, generally of the T helper-2 (T_{H2}) phenotype (173, 291). Marginal zone B cells are T cell-independent, IgM-secreting B cells found in the the MZ at the border of the white pulp in the spleen and do not reciruculate (143, 173, 200-202, 265, 272). In infection with Lyme disease and relapsing fever *Borrelia*, there are both T cell-dependent and T cell-independent antibody responses. This introduction will consider both mechanisms and the contexts in which they operate.

1.3.5 T Cell Dependent B cell Responses to *Borrelia*

In Lyme disease FO B cells are considered to be of particular importance in immunity, as a T_{H2} response is associated with resolution of infection and disease (182, 228, 291). This is apparently based upon the cytokines elicited by *B. burgdorferi* infection in BALB/c and C3H/HeN (or C3H/HeJ) mice (228). In C3H mice, where a severe, non-resolving arthritis phenotype is always seen, IFN-γ is the predominant cytokine elicited. This is consistent with a T_{H1}-type cellular immunity where inflammation would play a large role and contribute to Lyme arthritis. Furthermore, neutralization of IFN-γ attenuates disease (172, 228, 337). In contrast, BALB/c mice, which are associated with much milder disease that eventually resolves, have a predominant IL-4 response to *B. burgdorferi*, consistent with a T_{H2}-type humoral immunity, as IL-4 is a cytokine important for inducing antibody class switch in B cells (228, 325, 337). Very early in infection (2 days), BALB/c mice elicit a T_{H1} response which evolves into a T_{H2} response (14 days) until arthritis resolves (30 days), clearly indicating the importance of FO B cells (182, 228). Moreover, transfer of *B. burgdorferi*-reactive T_{H2} cells protected naïve mice, and depletion of CD4 T cells in BALB/c and C3H/HeN mice increased arthritis severity as well as spirochete numbers in joints and skin (187, 285). Adoptive transfer of T cells to SCID mice led to mild improvement in Lyme arthritis and carditis (29). In infected C3H mice, the IgG2a antibody subclass predominates, characteristic of a T_{H1} response, in contrast to BALB/c where IgG1 is predominant, the characteristic antibody subclass elicited in a T_{H2} response (172, 186, 325, 337). Treatment of C3H mice with rIL-4 early in infection led to a decrease in joint swelling and spirochete numbers (185). Moreover, rIL-4 also led to a decrease in IFN-γ, IgG2a, and IgG3 and an increase in T_{H2}-associated IgG1 *in vitro* (185). IL-$4^{-/-}$ BALB/c mice demonstrated the development of a T_{H1} response with increased carditis severity, in contrast to IL-$4^{+/+}$ mice (300). Further demonstration of T_H humoral immunity in response to Lyme disease was seen as rOspA immunization of C3H/HeJ mice elicited an OspA T cell epitope which induced the production of OspA-reactive IgG (45). Co-incubation of hamster T and B cells in vitro, 2 weeks after vaccination with *B.*

burgdorferi, produced bactericidal antibodies (175). A possible factor in promotion of a T_{H2} response is IL-6, as IL-6-deficient mice developed arthritis quickly with reduced IgG2b, consistent with a lowered T_{H2} response (12). A role for T cells in *B. burgdorferi* immunity is further underscored by the induced migration of T cells across human umbilical vein endothelial cells (HUVEC) in response to *B. burgdorferi*, *in vitro* (94, 134, 135). That the T_{H2} response develops in mouse strains that are more resistant to infection such as BALB/c and C57BL/6, is an indication of the strong protective role of antibodies that are associated with this response. *It is possible that some complement-independent bactericidal antibodies are produced by FO B cells in the T cell-dependent response as two of these monoclonal antibodies, CB2 and H6831, are of IgG1 and IgG2a classes, respectively (77, 297).*

1.3.6 T Cell Independent B Cell Responses to *Borrelia*

Though T_{H2}-type immunity was shown to be important in helping the B cell antibody response in Lyme disease infection, there is evidence suggesting that T cells are not necessary to battle *B. burgdorferi* infection successfully. Initial evidence was observed in CB-17 SCID mice, as presensitized B cells alone conferred partial protection against *B. burgdorferi* (302). Further evidence was shown in BALB/c, C3H/HeN, and C57BL/6 mice previously immunized with *B. burgdorferi* soluble sonicate antigen and then challenged at different times. Both T_{H1} and T_{H2}-type antibodies were elicited early in the response after immunization and infection, however, without production of the T_{H2}-associated IL-4 (124). In comparison to controls, there was no difference in arthritis and remission in CD40 ligand (CD40L)-deficient mice, which would be defective in T cell help, as CD40L is a T cell costimulatory molecule responsible for antigen presenting cell activation. Sera from these mice passively protected SCID mice, suggesting that T cell help, in this setting, may be expendable (112). Sera from class II transactivator (CIITA)-deficient mice (defective in antigen presentation) were also shown to be protective in C3H and SCID mice (111). T cell deficient (deficient in all T cells) mice were shown to have no effect on the resolution of Lyme arthritis and carditis, and sera from these mice protected naïve mice, but it did not resolve arthritis when passively

administered to SCID mice (235). These experiments indicate that T cell independent antibody responses to *B. burgdorferi* infection can occur.

T cell independent antibody responses also occur in relapsing fever infection and seem to be the predominant method for the clearance of spirochetemia in this disease. Neonatally thymectomized mice, nude mice (deficient in αβ T cells), and mice deficient in all T cells cleared *B. turicatae* with similar efficiency to normal mice, providing the first piece of evidence that T cells are not needed for an efficient immune response against relapsing fever (258). B cells were shown to be responsible for immunity, and IgM was the predominant antibody class produced even when T cells were present (258).

Furthermore, nude mice had no defect in clearing *B. hermsii* infection, as well (23). A robust, early IgM response was shown to be responsible for relapsing fever clearance *in vivo* (80), and mice incapable of secreting IgM were unable to clear infection, suggesting that IgM is critical and other antibody classes are not, as would be the case in a T cell independent response (5). Additionally, activation induced deaminase deficient mice ($AID^{-/-}$) cleared *B. hermsii* without a problem, suggesting a T cell independent response as there was no antibody class switch (6). Mice of SCID and $Rag^{-/-}$ phenotypes could not clear *B. hermsii* infection, pointing to the importance of lymphocytes and antibodies in relapsing fever clearance. It was further shown that IgM induction by B1b B cells, a subset known to function independently of T cell help, contributed to the resolution of relapsing fever caused by *B. hermsii* (5). Mice deficient in T cells ($TCR^{-/-}$) and both T and FO B cells ($IL\text{-}7^{-/-}$) exhibited efficient *B. hermsii* clearance suggesting the importance of other B cell subsets, such as B1 and MZ B cells. Those mice with a depleted B1 cell population (X-linked immunodeficiency [*xid*] mice) exhibited longer, more severe spirochetemia, and were even driven to expand their already depleted B1b B cells after *B. hermsii* resolution, clearly suggesting an important role for this B cell subset in relapsing fever immunity (5). The immunity afforded by this subset of B cells in relapsing fever infection was shown to be long-lasting, as primed B1b B cells from convalescent mice conferred protection to $Rag^{-/-}$ mice upon reconstitution and induced the rapid production of *B. hermsii*-specific IgM (6). This certainly suggested a role for B1b B cells in a T cell-independent, IgM memory response. These findings

also seemed to implicate B1b B cells as the most important B cell subset in the early host response to *B. hermsii*, as splenectomized mice (deficient in MZ B cells) also cleared infection. However, these mice could not clear spirochetemia when a low-passage, virulent *B. hermsii* strain was used, indicating that B1b B cells are not the only important subset in this host response (5). Indeed, another T cell-independent B cell subset, MZ B cells, was shown to be important in relapsing fever immunity, and may work cooperatively with B1b B cells (35). Marginal zone B cells become activated after association with virulent *B. hermsii*, and loss of activation is coincident with IgM production and spirochete burden reduction. Mice depleted of MZ B cells, by anti-LFA-1 and anti-α4β1 integrin MAb treatment, had reduced anti-*B. hermsii* IgM and increased pathogen burden. *A very early IgM response to relapsing fever, from which CB515 is derived (82), was observed to be sufficient for clearance in the absence of complement (80), tempting speculation that B1b and MZ B cells are responsible for production of the complement-independent bactericidal antibodies in a T cell –independent host response.*

CD1d (a nonclassical MHC molecule predominant on MZ B cells and important in the recognition of lipid antigens) was shown to have a role in spirochete reduction and anti-*B. hermsii* antibody production from MZ B cells. $CD1d^{-/-}$ mice had similar results to MZ B cell depleted mice in terms of spirochete burden and were defective in MZ B cell IgM secretion (35). This may suggest a new function for CD1d, since traditionally CD1d has been known to be involved in lipid antigen presentation to natural killer T cells, which in turn promote IgM and IgG1 production, provide T_H cytokines, and have immune regulatory function (reviewed in 40, reviewed in 127, reviewed in 353, 368). As MZ B cells in $CD1d^{-/-}$ mice retained an activated state longer than those from wild type mice, the involvement of CD1d in immune regulation of MZ B cells is suggested during the host response to *B. hermsii* (35). It could be that CD1d is providing an autocrine signal to MZ B cells (without T cell assistance) promoting the production of *Borrelia*-specific IgM in response to *B. hermsii* antigens (35). Recently, MyD88, a toll-like receptor adaptor molecule, was shown to contribute to the kinetics of the IgM response against *B. hermsii* (47). $MyD88^{-/-}$ mice displayed a very slight delay in IgM response that was overcome by 6 d.p.i . Despite this delay, antibodies from these mice could clear spirochetemia when transferred to SCID, so the precise role that MyD88 may play in this

is still unclear (47). These results collectively give strong support to the idea that antibody-mediated immunity against relapsing fever infection is largely T cell independent, and that rapid production of IgM by B1b and MZ B cells is a necessity for an efficient host response and immunological memory to these pathogens.

The mitogenic properties of *Borrelia* lipoproteins are also suggestive of T cell independent responses and may be indicative of what is occurring in the T cell independent responses against Lyme disease and relapsing fever. *Borrelia burgdorferi* sonicate and membrane blebs stimulated proliferation and differentiation of B cells into antibody-secreting cells (IgM) in the absence of accessory cells and concomitant with IL-6 production *in vitro* (307, 362). These results were replicated *in vivo*, and OspA and OspB were specifically shown to be capable of B cell mitogenic stimulation (224, 369). OspA and OspB were capable of inducing IgM production *in vitro* and both proteins were shown to have equal activity, suggesting that this may be a property of all *Borrelia* lipoproteins (224). These effects were also seen in human B cells, as *B. burgdorferi* sonicate or OspA were mitogenic to these cells and stimulated IL-6 *in vitro* (343). Another *B. burgdorferi* antigen consisting of lipoprotein and glycolipid induced proliferation of B cells and antibody secretion *in vitro* (161). This indicates the involvement of many different *Borrelia* antigens in B cell mitogenic stimulation. *The inherent mitogenic properties of Borrelia lipoproteins may drive the development of a T cell-independent antibody response in these infections.*

1.3.7 The Protective Role of Antibodies in Experimental Models

Bactericidal antibodies are of extreme importance in the host response to *Borrelia* as antisera have been shown to be bactericidal, and a number of bactericidal MAbs, directed against OspA, OspB, and OspC in *B. burgdorferi*, and different Vmps in relapsing fever *Borrelia*, have been discovered and investigated (15, 23, 77, 82, 223, 252, 295, 297). Bactericidal antibodies of IgG1, IgG2a, and IgG2b isotypes against OspA appear to be dependent upon complement for their action and are produced extensively in response to IL-6 *in vitro*. In addition, IL-6 also causes an expansion in B cell number, seen previously upon mitogenic B cell stimulation (discussed in section 3.6) (253). Treatment with IL-4 or IFN-γ inhibit the production of these antibodies *in vitro* with anti-

IL-4 or anti-IFN-γ restoring antibody production (251, 252). This seems to contrast with what was discussed in section 3.5, however, these *in vitro* cultured B cells have already undergone class switch as they produce IgG. Thus, one may not expect IL-4 to have an effect, while IL-6 may be important for *in vitro* B cell proliferation and stimulation of antibody secretion. Interestingly, B cells treated with anti-IFN-γ cause severe destructive arthritis *in vivo*, perhaps suggesting an overwhelming antibody response that may promote pathology (250). Exogenous macrophages were required in conjunction with IL-6 to cause increased anti-OspA bactericidal antibody production, suggesting that macrophages may process *Borrelia* antigens and elicit IL-6 for antibody production (253). However, macrophages were not required for the IL-6 stimulated production of anti-OspC bactericidal IgG2b *in vitro* (288). These findings indicate some of the important factors required for antibody production/suppression in *Borrelia* infection, particularly those which rely on complement-mediated lysis.

The first demonstration of the vast importance of antibodies in defense against *Borrelia* infection *in vivo* was the finding that passive transfer of immune sera is protective against these infections. Passive transfer of rabbit immune serum to hamsters conferred sufficient protection against *B. burgdorferi*, and was the first demonstration of the protective capacity of antibodies in this infection (178). Subsequently, antibodies have been shown to be the primary mediators of this protection, as immune serum was shown to protect SCID mice, which are deficient in both B and T cells (29-31, 301, 376). Indeed, this fact has been studied extensively, and the protective capacity of sera against specific Ags, and monoclonal antibodies (MAbs) derived from these immune sera was assessed (28, 30, 31, 117, 229, 277, 301, 306, 376). Anti-OspC serum was shown to be effective therapeutically in eliminating spirochetes. It also induced arthritis and carditis resolution in SCID mice (375, 376). Anti-OspB serum was effective in growth inhibition of *B. burgdorferi* as well as protection of C3H/HeJ mice (277, 375, 376). Passive transfer of MAbs, reactive against arthritis-related protein (Arp) and decorin-binding protein A (DbpA), to SCID mice was also shown to induce arthritis and carditis remission (31). Only anti-DbpA was protective, however, and neither sera caused reduction in tissue spirochetes, when administered after infection, pointing to the importance of antibodies reactive to other antigens (31). Anti-OspA IgG2a and IgG2b

conferred protection in SCID mice. Immunoglobulin G2 from 3 week immune serum protected hamsters, and anti-OspC IgG2a protected C3H/HeJ mice, underscoring the importance of this antibody class in protection against *B. burgdorferi* (229, 301, 306). The importance of serum IgG in Lyme arthritis was further demonstrated as the presence of the neonatal Fc receptor (FcRn), an important receptor in recycling IgG and extending its serum half-life, was shown to contribute to the resolution of arthritis (89). This was achieved by demonstrating that $FcRn^{-/-}$ mice infected with *B. burgdorferi* had decreased bactericidal IgG and increased ankle swelling (89). Transfer of immune serum to *B. burgdorferi* infected SCID mice has also been shown to mediate arthritis remission (29-31).

The importance of antibodies in *Borrelia* immunity can also be seen in the success of active immunization studies specific for *B. burgdorferi* infection. These studies made use of OspA (by itself or delivered on bacterial or viral surfaces, delivered orally or by syringe), truncated OspA, OspC, Omp66, and DbpA, all of which elicited protective antibodies as the main mediators of protection (46, 104, 110, 113-115, 193, 222, 255, 303, 322, 323, 349).

Antibodies are also of critical importance to immunity against the various tick- and louse-borne relapsing fever species of *Borrelia* and play a critical role in clearance of these pathogens from the blood. The protective capacity of passively transferred immune sera was demonstrated several times in this infection, the first demonstration dating as far back as eighty years prior to this same discovery in Lyme disease (64, 262, 340). Subsequently, serotype-specific IgM antibodies were shown to mediate passive protection of BALB/c and SCID mice against infection with different relapsing fever *Borrelia* spp. (23, 82, 370). Antibodies are also involved in the mechanism of antigenic variation utilized by these spirochetes (340). Initially, it was observed that fluorescent antiserum to *B. hermsii* was serotype-specific (73). This was expanded by the finding that the antibody response in relapsing fever develops specific to the dominant serotype of a spirochetemia peak, eliminating this population, and allowing for a different serotype, previously present in very low numbers, to thrive. In this manner the antibody/relapse cycle perpetuates in the infection (340). These serotype-specific

antibody responses occur due to the fact that the majority of relapsing fever spirochetes at any one time are comprised of those that predominantly express one Vmp, the major antigens of this disease (26, 27). *The high specificity of antibodies involved in relapsing fever serotype elimination suggests that it may be the complement-independent bactericidal antibodies that are responsible. There is direct in vivo evidence that demonstrates this as well (80).*

An antibody class response profile has been observed in *B. duttonii* infection of BALB/c mice (370). A rapid antibody response was seen as IgM first emerged, followed by IgG2a and IgG3, after which the subsequent emergence of IgG1 and IgG2b occurred. It was assessed that IgG3 and IgM were the most important antibody classes in this response, as they were both shown to be passively protective against *B. duttonii* infection (249, 370).

The protection afforded by antibodies in *Borrelia* infection also includes phagocytosis of opsonized spirochetes (39, 119, 221, 244-247, 289, 290, 328). The general consensus is that spirochetes cannot survive the bacterial-killing weaponry of professional phagocytes as they have no known defense mechanisms against phagocytosis. The predominant mechanism of spirochete ingestion appears to be coiling phagocytosis (290). This is a mechanism in which the phagocyte forms one pseudopod and coils around the pathogen until it is brought to the cell within a phagosome (162). Destruction of *B. burgdorferi* by this mechanism occurs without the involvement of lysosomes and is influenced by NO and O_2 radical formation (241, 290). The Rho GTPase, CDC42Hs, Wiskott-Aldrich Syndrome protein, Arp2/3 (actin polymerization regulators), and f-actin are important in the formation of pseudopods for *B. burgdorferi* removal (218). Opsonization of *B. burgdorferi* by antibodies plays an important role as rate of coiling phagocytosis, NO and O_2 radical synthesis, and spirochete killing were all enhanced when spirochetes were opsonized (241, 290). In this aspect of the host response, antibodies are important as they bridge the innate and acquired immune systems by opsonizing spirochetes for removal via Fc receptors on phagocytes. Considering that *Borrelia* can evade complement deposition, opsonization by antibodies followed by recognition of the Fc fragment may be the best method of phagocytic

removal in *Borrelia* infection. *That the action of complement-independent bactericidal antibodies appears to be rapid (109, 205) indicates that these antibodies are not involved in opsonization of intact organisms.*

1.3.8 Antibodies in the Clinical Human Borrelioses

The *Borrelia* spirochetes are fastidious organisms with a long cell division period. For this reason, bacterial cultures have never been used for diagnosis of active infections. Since a robust antibody response is the hallmark of the host response to *Borrelia*, serology (antibody reactivity to certain antigens) is the main method for diagnosis of Lyme disease. In studies prior to the discovery of *B. burgdorferi*, immunoglobulins and their complexes were shown to be useful markers supplementing the clinical diagnosis of Lyme disease (reviewed in 140, 153-155, 334, 335). Antibodies were absolutely critical in the discovery of *B. burgdorferi*, as reactivity of patient sera to spirochetes and spirochetal lysate isolated from *Ixodes scapularis* ticks provided the first evidence linking this spirochete to the disease (56). Due to the success of antibodies in these applications, studies began investigating their use in the serodiagnosis of Lyme disease. Serodiagnostic techniques for Lyme disease have undergone quite an evolution over the past 27 years. These have included indirect immunofluorence assays (IFA) with whole spirochetes (296, 363), whole lysate enzyme-linked immunosorbent assays (ELISAs) (296, 363), whole lysate ELISAs in conjunction with immunoblots (two-tiered serodiagnosis) (96, 176, 208, 267, 329), and the C_6 Lyme test, which utilizes a peptide derived from invariant region 6 (IR_6) of the VlsE antigen in an ELISA (211, 215, 216, 374). The two-tiered test remains the preferred method for the laboratory diagnosis of Lyme disease recommended by the CDC (176, 208, 348) and is based on the immunoblot banding criteria developed by testing well-defined patient populations (101, 108). However, the C_6 Lyme test has come into prominence recently as it is very specific, sensitive and rapid, allowing for the omission of a confirmatory test (17, 107, 174, 206, 214, 216, 225, 242). These tests underscore the tight relationship between the observations of basic science and the clinical applicability of such observations. Antibodies are important in the diagnosis of relapsing fever as well, as the glycerophosphodiester phosphodiesterase (GlpQ) of different agents of relapsing fever

elicits antibodies important for serology (275, 312, 314). *That serodiagnosis is the main method of diagnosis for Borrelia infection underscores the scope and importance of the antibody response in this infection and adds one more important role to the antibody repertoire of function.*

Intrathecal antibodies, produced locally, have been shown to be induced in the neuroborreliosis of Lyme disease (reviewed in 128, 129, 149, 152, 180, 209, 227, 254, 331, 365). Accumulation of B cells in cerebrospinal fluid (CSF) correlated with the intrathecal antibody response suggesting compartmentalization of the B cell response in the nervous tissue (18, 43). Immunoglobulin G, IgM, and IgA classes with reactivity against OspA, OspB, and OspC have all been associated with neuroborreliosis in CSF (18, 311). In patients where neuroborreliosis occurred early in disease, an intrathecal IgM response to OspC was observed, consistent with the antigenic profile of *B. burgdorferi* (311). Intrathecal antibodies are markers of neurologic disease in patients, and have been used effectively for diagnosis and staging of the infection. While intrathecal antibodies are not unique to *Borrelia* infections of the CNS, these are particularly prominent in neuroborreliosis.

In initial observations of chronic Lyme arthritis, serum IgM was seen in disease and IgG was associated with remission (335). Elevated IgA immune complexes were seen in ~25% of patients with Lyme arthritis in one study, but did not seem to be associated with disease (14). Recently it was shown that IgG is in abundance in chronic Lyme arthritis synovial fluid, and appears to be present due to antigen-driven selection (137).

Autoimmunity has long been sought as a major reason for the pathogenesis of chronic infections. In particular, the chronic course of the spirochetoses, whether syphilis or the borrelioses, has prompted investigations into the possible role of autoimmunity to explain the long term infectious process. Antibodies elicited by *Borrelia* infection have raised the possibility of autoimmunity in the neurological and, more recently, the arthritic manifestations of Lyme disease (reviewed in 130, reviewed in 318). Manifestations of acute and chronic neuroborreliosis include cranial neuropathy resulting in facial palsy (Bell's palsy) and axonopathy in peripheral neurologic Lyme disease and central nervous

system involvement including encephalopathy (reviewed in 130, reviewed in 145, reviewed in 146, 147-150, reviewed in 318, reviewed in 330). *B. burgdorferi* is rarely seen in nervous system tissue biopsies and has never been seen in peripheral nerve tissue biopsies in the mouse model suggesting that autoimmunity may contribute to this manifestation (reviewed in 105, 318). Neurologic Lyme patients contained an abundance of anti-*B. burgdorferi* IgM, auto-reactive to human peripheral nerve axons (319). Furthermore, a murine monoclonal IgG (H9724) specific for *B. burgdorferi* flagellin$_{213-224}$ was reactive against human nerve axons, specifically, heat shock protein (HSP)-60 of neuroblastoma cells (116, 317, 319, 373). Patient sera were also reactive against HSP-60, indicating this host protein as a primary autoimmune target of antibodies in neurologic Lyme disease (317). H9724 prevented axonal formation suggesting that auto-reactive antibodies, not Borrelia, may prevent axonal formation (320). Auto-reactive antibodies have also been observed in the central nervous manifestations of Lyme disease (304), as IgM cross reacted with gangliosides and non-protein antigenic fractions of *B. burgdorferi* (reviewed in 130, 131, 361). Intrathecal IgG, IgM, and IgA from patient cerebrospinal fluid were also autoreactive, as they bound myelin basic protein and different neurofilament proteins (179). In addition, anti-OspA antibodies have been shown to be cross-reactive with neurons in the brain, spinal cord, and dorsal root ganglia, implicating autoantibodies induced by *B. burgdorferi* as potential contributors to the pathology of the disease (2).

Treatment-resistant Lyme arthritis (TRLA) is a condition observed in about 10% of Lyme arthritis patients, characterized by continuous joint inflammation that does not resolve upon therapy, and, moreover, is not associated with active *B. burgdorferi* infection (reviewed in 142, reviewed in 333, reviewed in 357). T_{H1}-cell cross-recognition of OspA$_{165-173}$ and human lymphoid/myeloid adhesion molecule LFA-1 has previously been associated with TRLA, making a strong case for autoimmunity in the pathology of this condition (141, 142, 332, 347). Subsequent to this discovery, the involvement of auto-reactive antibodies was suggested to contribute to this condition (142). Antisera reactivity to OspA and OspB has consistently been observed in patients with TRLA (71, 181). Indeed, through experiments that made use of single chain variable fragments (scFvs) derived from IgG of patient synovium, antibody cross-reactivity between OspA

and host cytokeratin (CK)-10 was observed (136, 137). Thus, there seems to be a role for autoantibodies in the pathology of TRLA that has not yet been completely elucidated. This role would certainly explain the many enigmatic observations that have been made in the natural human infection as well as in the experimental murine setting. *Nonetheless, the high specificity of complement-independent bactericidal antibodies (78, 82, 204, 205, 297) suggests that they would not be involved in the human tissue cross-reactivity seen in Borrelia-induced autoimmunity.*

1.4 UNIQUE PROPERTIES OF ANTIBODIES IN BORRELIA INFECTIONS

1.4.1 Immune Pressure and Escape Mutants

In addition to their traditional roles in host defense, antibodies are the effectors in antigenic variation of *Borrelia*. The specificity of antibodies is such that only certain epitopes are recognized (serotype specificity) and organisms bearing those epitopes are eliminated, allowing other serotypes to have unrestricted growth. Thus, antibodies appear to impose such stress on *Borrelia* that they cause the appearance of surface antigen escape mutants (77, 78, 159, 213, 297). In C3H/HeN mice, it was seen at 17 d.p.i. that anti-OspC antibodies emerge coincident with the appearance of OspC variants (213). Moreover, the variants reexpressed OspC in culture and naïve mice. In SCID mice, OspC was persistently expressed by *B. burgdorferi*, however, passively administered anti-OspC MAb eliminated these spirochetes which then reemerged when MAb was taken away (213). OspC mRNA was also greatly decreased under immune pressure *in vivo*, and BBF01 and vlsE were concomitantly upregulated (217). In SCID mice OspA transcription was regulated by the presence of mouse serum, IgG, or IgM, suggesting that Osp expression is greatly influenced by host antibodies (159). Furthermore, constitutive expression of OspC by the *B. burgdorferi* flagellar gene (FlaB) promoter resulted in clearance of spirochetes *in vivo* (SCID mice), when anti-OspC MAb was administered (367). *This indicates that differential Osp expression in response to the great pressure imposed by host antibodies is an important persistence mechanism of B. burgdorferi during infection (367).*

1.4.2 Antibody Interactions in the Tick Vector

Antibodies against *Borrelia* also have an effect on spirochetes in the tick vector. This was clearly demonstrated in the work that led to the creation of a Lyme disease OspA subunit vaccine. It was observed early on that OspA elicited protective antibodies, but exactly how the antibodies prevented infection became apparent when the effect of the antibody was shown to occur in the tick vector stage (114, 118). Outer surface protein A is highly expressed by spirochetes in the tick gut (as it is in culture) and downregulated upon blood feeding by the tick. Increased expression of OspC was noted more or less simultaneously with incoming host blood (313). Antibodies to OspA blocked transmission of spirochetes to mammalian hosts by acting within a narrow window of time, when blood meal, where host antibodies would be present, is taken up by the ticks (99). As possible variation in surface Osps became a question, it was shown that OspA antiserum was more efficient at spirochete killing in the presence of complement *in vitro*, allowing for destruction of escape variants (263, 326). However, complement is likely inactivated in the tick and was shown to be dispensable for efficient spirochete blockage, as anti-OspA antibodies were not required to kill *B. burgdorferi* in the tick gut to exert their transmission-blocking effects (100, 138, 287). The fact that antibodies are the sole purveyors in blocking *Borrelia* transmission from the tick is direct evidence of their power and versatility. The protection afforded by antibodies in response to the OspA vaccine was quite novel at the time of its discovery, and was remarkably effective. In clinical trials it was 72% and 92% effective with and without adjuvant, respectively (321, 336). A number of concerns regarding the vaccine resulted in its removal from the market (1, 88, 141, 293). Studies are currently underway for the design of a second generation OspA vaccine that eliminates the N-terminal portion of the protein implicated in autoimmunity (193, 255, 303, 364). It has been noted recently that B cell inhibitory proteins exist within *Ixodes ricinus* and *Hyalomma asiaticum asiaticum* tick vectors, but what this means to spirochete transmission is not quite clear (151, 372). *The method of protection of antibodies to OspA underscores the importance of interactions that may occur at the tick-host interface, and widens the potential roles that antibodies may play in immunity. It may very well be the complement-independent*

bactericidal antibodies that are critical for the effectiveness of the OspA vaccine as it was protective even in complement-deficient mice (138, 287).

1.4.3 The Presence and Importance of Complement-Independent Bactericidal Antibodies

The array of functions performed by antibodies was expanded with the discovery of Borrelia-specific antibodies that had complete bactericidal capability in the absence of complement. Initial indications of this unique capability were seen *in vivo* as complement was shown to be dispensable for efficient immunity against relapsing fever and Lyme disease *Borrelia*. Mice deficient in C5, required for the lytic effects of complement, had no defect in removal of relapsing fever agent *B. turicatae* as compared to controls (257). The irrelevance of complement in the host response to relapsing fever was further demonstrated as C5, C3, and C1q-deficient (defective for complement opsonization) mice efficiently cleared the spirochetemia caused by an uncultivable relapsing fever *Borrelia* originally isolated from patients (7, 80, 82). In *B. burgdorferi* murine infection, depletion of complement with cobra venom factor suggested that complement may not be necessary for an efficient host response against the spirochete, however, at times during the infection complement was needed (305). Further *in vivo* studies with C5-deficient mice conclusively showed that lytic complement was unnecessary for efficient immunity against *B. burgdorferi* (44).

The first anti-Borrelia complement-independent MAb, CB2 (IgG1), was specific to OspB in B. burgdorferi and shown to exert bactericidal effects in vitro (77). The CB2:OspB interaction was shown to be dependent on lysine 253 in OspB, without which binding does not occur and bactericidal activity is abrogated (78). It is of note that a characteristic of known complement-independent bactericidal antibodies is high binding specificity (204, 205). Importantly, the Fab fragment of CB2 was shown to be bactericidal in the absence of complement in vitro, proving that agglutination did not contribute to activity, as these molecules are monomeric. The damage imparted to B. burgdorferi after CB2 binding included destabilization and destruction of the outer membrane, likely resulting in lysis of the spirochete (77, 109). Another complement-independent, bactericidal MAb specific for OspB, H6831(IgG2a), was discovered and

shown to be functionally identical to CB2, even dependent upon the same residue, lysine 253, for binding and activity (297). Other *B. burgdorferi*-specific, complement-independent, bactericidal antibodies with reactivity to OspA, OspB, and P39 have also been discovered, but have not been investigated insofar as to completely characterize them and their bactericidal ability (15, 23, 223, 315).

Complement-independent, bactericidal MAbs against relapsing fever *Borrelia* spp. have also been discovered and investigated, and seem to function similarly to those *B. burgdorferi*-specific MAbs previously discussed (23, 82, 297). Two such MAbs are CB515 and H4825, of IgM and IgG2a classes, respectively (82, 297). *One of these MAbs, CB515, was shown to have in vivo significance, as it was derived from a polyclonal complement-independent IgM response to relapsing fever and was shown to passively protect B cell deficient, C5-deficient, and wild type C57BL/6 mice (80, 82). This IgM was reactive against a Vsp and caused similar damage to that caused by CB2, H6831, and H4825. This included the disruption of the spirochetal outer membrane allowing for exposure of periplasmic flagella and the induction of severe membrane blebbing, resulting in death of the pathogen. (82).* It is tempting to speculate that the *in vivo* complement-independent IgM response against relapsing fever from which CB515 is derived, and other complement-independent MAbs, may be derived from a T cell independent, B1b/MZ B cell response, shown to be critical for resolution of relapsing fever spirochetemia. As B1b and MZ B cells are both IgM secreting cells and as both these B cell subsets and complement-independent IgM antibodies were shown to be critical for spirochetemia clearance in relapsing fever, they seem to correlate. However, production of complement-independent IgM antibodies from these particular cells has not been directly observed experimentally (5, 6, 35). Therefore, whether or not these B cells are the source of the complement-independent IgM antibodies has not yet been conclusively determined.

The fact that these antibodies are capable of bactericidal activity is unique, as MAC formation via classical complement recruitment has traditionally been thought of as the mechanism whereby antibodies can exert a bactericidal effect without cellular assistance. However, complement-dependent antibodies against *Borrelia* exist (discussed

in section 2.1), and generally it is thought that complement-dependent and complement-independent antibodies work in concert to make possible efficient immunity against *Borrelia* infection (15, 44, 223, 305). Where have the complement-independent antibodies come from, and are they present in immunity against any infection? The answers to these questions are not known, however, there may be some clues as to why these antibodies exist in the *Borrelia* immune response, and are particularly prominent and effective in the response to relapsing fever. As discussed before, *Borrelia* have the ability to evade complement-mediated destruction via the alternative pathway (discussed in section 2.2). Due to the fact that they are complement-resistant, this may increase the importance of complement-independent antibodies. However, this does not rule out possibility of *B. burgdorferi* destruction induced by complement via the classical pathway, on which complement-dependent antibodies rely (190-192). The recent finding that relapsing fever *Borrelia* are also able to bind C4BP, suggests that these spirochetes are capable of evasion of the classical complement pathway (240). Interestingly, this may render complement completely ineffective against relapsing fever *Borrelia*, allowing complement-independent, bactericidal antibodies to come into prominence in immunity against this infection. This may explain why complement-independent, bactericidal antibodies are so critical for relapsing fever clearance. These connections, however, have not been made experimentally and are, thus far, speculation.

There have been studies that investigated the function of some of these complement-independent bactericidal antibodies but the mechanism of action has remained elusive. Polar blebbing was shown to be induced in *B. burgdorferi* upon CB2 Fab:OspB immune complex formation (109). This was followed by OspA colocalization, and spheroplast induction causing outer membrane destabilization and, ultimately, lysis of the spirochetes (109). Bactericidal function of the CB2 Fab was also shown to be dependent on the presence of Mg^{2+} and Ca^{2+}, but how these ions may be specifically involved in the process remains unclear (109). Upon binding, CB2 also causes a change in OspB that is reflected in a difference in the susceptibility of the antigen to certain proteases (183). Specific structural changes, in the form of a disordered secondary structure, were observed in OspB upon H6831 Fab binding, after investigation of the immune complex crystal structure (34). The fact that OspB undergoes changes after

complement-independent, bactericidal MAb binding suggests that these antibodies may induce a process in *Borrelia* upon binding. In fact, upregulation of *blyA* and *blyB* phage holins system genes, of circular plasmid (cp) 32 in *B. burgdorferi*, was observed upon CB2 Fab binding to OspB (10). It is not known if complete phages were assembled upon binding. However, the BlyA protein is known to be membrane-interactive and, upon *blyA* and *blyB* upregulation, phage induction has been shown to occur (95). Interestingly, phage release was associated with spontaneous lysis of *B. hermsii* (25), which contain homologous holins genes to *B. burgdorferi* (339). Moreover, phage production in *Borrelia* has been shown to be induced in response to environmental stress (157, 256). This lends support to the idea that complement-independent MAb binding to *Borrelia* may result in phage formation, possibly contributing to the bactericidal effect of this interaction.

Other possibilities include the utilization of the antibody-catalyzed water-oxidation pathway (ACWOP) (358). This is a process proposed to be universal among all antibodies regardless of specificity. In this process antibodies can utilize singlet oxygen ($^1O_2^*$) and water in order to produce reactive oxygen species such as hydrogen peroxide (H_2O_2), dihydrogen trioxide (H_2O_3), and ozone (O_3) (358, 360). Production of such species would allow the antibodies to become intrinsically bactericidal (358-360). It is proposed that there is an "active site" located in all antibodies within the interfacial region of the constant and variable regions, which catalyzes this reaction (377). This is proposed as residues within both the constant and variable regions of Fab fragments were modified after exposure to UV radiation or H_2O_2. While some of these modifications were inconsistent, a residue within the constant region, Trp^{L163}, was consistently and extensively modified when exposed to UV or H_2O_2. Modification of Trp^{L163} occurred in two different Fabs, and this residue is highly conserved among murine antibodies, suggesting that it is important in the utilization of this reaction (377). In a host, it is thought that this reaction may be allowed to begin as $^1O_2^*$ is released from phagocytes due to oxidative burst (16). It is unlikely that these *Borrelia* complement-independent MAbs utilize this as their method of killing, as a source of $^1O_2^*$, such as a phagocyte, was not included in their *in vitro* bactericidal assays. Irrelevant MAb controls used in these experiments did not affect *Borrelia* either, further suggesting that conditions for this

pathway probably did not exist in these experiments (77, 82, 223, 297). It has recently been shown that antibodies can catalyze the antibody-catalyzed water-oxidation pathway through utilization of riboflavin (vitamin B2) (259). This may possibly be a mechanism utilized by the anti-*Borrelia* complement-independent MAbs, as riboflavin is a component of the Barbour-Stoenner-Kelly (BSK)-H medium in which the *in vitro* bactericidal assays were conducted. However, as stated previously (section 2.1), there are antibodies against *Borrelia* that are not bactericidal in the absence of complement and the presence of a source of riboflavin (serum or BSK-H) (15, 220, 223, 252, 253, 263, 288, 295). Thus utilization of riboflavin by antibodies from these sources seems unlikely, and casts doubt as to whether these particular antibodies utilize this pathway. Since Trp^{L163} of the constant region seems to be essential for utilization of this pathway, investigation of an scFv derived from an anti-*Borrelia* complement-independent, bactericidal MAb should indisputably indicate whether or not this pathway accounts for the unique bactericidal capability of these MAbs. An scFv would be an ideal vehicle for this investigation as these molecules contain no constant region and are wholly composed of the antibody variable region (reviewed in 160, 171).

It is entirely possible that the complement-independent MAbs to Borrelia utilize a completely different and previously overlooked method, to become bactericidal. As Fabs of some of these MAbs have been shown to exert bactericidal activity (77, 297), it appears that the answer resides in their variable regions. Are the variable regions of these MAbs bactericidal on their own? It is possible that a feature of the variable regions, not present in complement-dependent antibodies, allows these MAbs to have an innate bactericidal quality. *An scFv would be useful in testing this theory, as well, since constant regions would not be present.* If these MAbs are innately bactericidal, maybe they work in concert with changes that occur in their antigens and the borreliae upon binding, as discussed before. The question has, as yet, remained unanswered.

1.5 HYPOTHESIS AND RATIONALE

For this dissertation, the bactericidal mechanism of complement-independent antibodies to *Borrelia* was examined. This project was undertaken with the hypothesis that the mechanism is dependent upon critical aspects of the antibodies, unique events at

the antibody:pathogen interface, and factors within the *Borrelia* organisms themselves. As such, this project and its results were broken down into three sections which consider these important aspects. Part 1 (**Chapter 2**) examines the antibody side of the mechanism and demonstrates that the bactericidal function resides in the variable region. In this chapter we specifically consider a recombinant single chain variable fragment (scFv) construct of CB515 against relapsing fever to determine if the variable region is sufficient for bactericidal action. CB515 is of particular interest here as this complement-independent antibody has shown *in vivo* bactericidal significance (82). Part 2 (**Chapter 3**) delves into the specific effects of complement-independent antibodies at the antibody:pathogen interface and posits the formation of openings in the outer membrane as an explanation for the observed bactericidal effects of these antibodies. Here, we consider that complement-independent antibodies directly damage the outer membrane, removing it in pieces until pores are formed. In this chapter CB2 (against *B. burgdorferi* OspB) is predominantly examined as critical *in vitro* growth experiments could not be accomplished with the species of relapsing fever to which CB515 is specific. Finally, part 3 (**Chapter 4**) considers the pathogen side of the mechanism and makes a strong case for the existence of critical factors in the *Borrelia* organisms themselves that are not present in typical Gram-negative bacteria and without which the bactericidal mechanism of complement-independent antibodies cannot occur. Here, the differences in the outer envelopes of *Borrelia* and other Gram-negative bacteria are considered, specifically investigating membrane fluidity as a characteristic of *Borrelia* that makes them uniquely susceptible to the action of complement-independent bactericidal antibodies. Taken as a whole, these results will make a strong case for a mechanism effected by antibodies that has not previously been considered but yet retains familiar aspects of immunology and microbiology while challenging the traditional dogma of antibody function.

1.6 FIGURES AND TABLES

Table 1.1: Different Species of *Borrelia* Found Around the World. Shown are the tick-borne agents of Lyme disease and relapsing fever as well as their geographical locations. Relapsing fever agents are divided into two groups, old world and new world, based on their geographical distribution.

Lyme Disease Agent	Tick Vector	Geographical Location
B. burgdorferi	*Ixodes scapularis*	North America, Europe
B. garinii	*Ixodes ricinus*	Asia, Europe
B. afzelii	*Ixodes ricinus*	Asia, Europe
B. andersoni	*Ixodes dentatus*	North America
B. japonica	*Ixodes tanukii*	Japan
B. lusitaniae	*Ixodes ricinus*	Southern Europe
B. valaisiana	*Ixodes ricinus*	Europe, Ireland, Britain

Relapsing Fever Agent (New World)	Tick Vector	Geographical Location
B. hermsii	*Ornithodoros hermsi*	Western U.S.
B. turicatae	*Ornithodoros turicata*	Southwestern U.S., Mexico
B. parkeri	*Ornithodoros parkeri*	Western U.S.
B. mazzotti	*Ornithodoros talaje*	Central America
B. venezuelensis	*Ornithodoros rudis*	Central America

Relapsing Fever Agent (Old World)	Tick Vector	Geographical Location
B. duttonii	*Ornithodoros moubata*	Sub-Sahara Africa
B. crocidurae	*Ornithodoros erraticus*	North Africa, Middle East
B. persica	*Ornithodoros tholozani*	Middle East, Central Asia
B. hispanica	*Ornithodoros marocanus*	Iberian peninsula, North Africa
B. latyschewii	*Ornithodoros tartakowskyi*	Iran, Iraq, Eastern Europe
B. caucasia	*Ornithodoros asperus*	Iraq, Eastern Europe

Figure 1.1: *Borrelia* Infection. Shown is an image from a 5 mm paraffin thin section of murine liver during peak spirochetemia with silver-stained *Borrelia*. The presence of a great number of spirochetes in the liver sinusoids demonstrates the impressive high density spirochetemia that is characteristic of relapsing fever *Borrelia*.

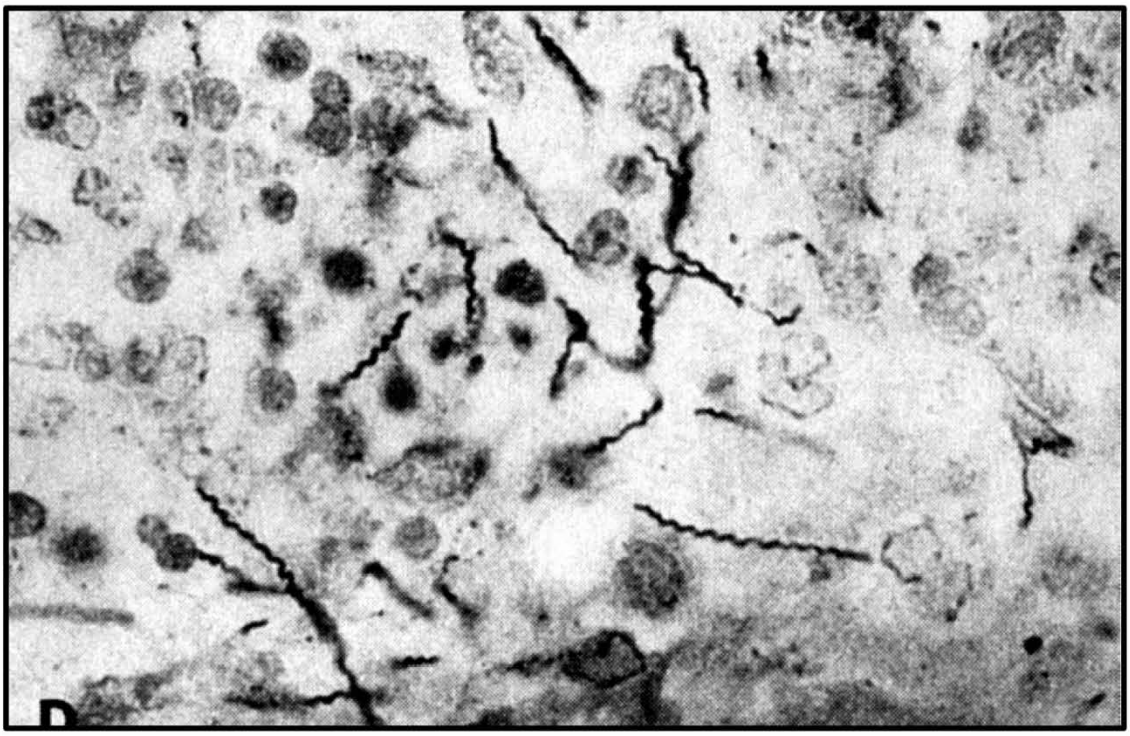

D

Figure 1.2: The Complement System and Borrelia. Complement deposition can occur through three pathways, the classical, alternative, and mannan-binding (MB) lectin pathways, which differ in their initial steps and method of recruitment. In the classical pathway, the C1 complex (C1q with bound C1r and C1s) binds the antibodies of already formed Ab:Ag immune complexes. After being cleaved by C1r, C1s cleaves C2 and C4 to C2a and C4b. Components C2a and C4b can associate to form C3 convertase allowing for complement activation. *B. recurrentis* and *B. duttonii* have been shown to bind complement regulator C4b binding protein (C4BP) which inhibits association of C2a and C4b. C4b is subsequently cleaved by Factor I (not shown), and complement activation is prevented. It is possible that relapsing fever *Borrelia* evade the classical pathway in this way. In the alternative pathway, a series of hydrolysis and cleavage events leads to the production of C3b which can bind covalently to the pathogen membrane. Factor B normally binds C3b forming a C3 convertase that allows for complement activation. However, *Borrelia* bind complement regulator Factor H via CRASPs, OspE/F, or Fhb1 which inhibits the association of C3b and Factor B. Factor H catalyzes the cleavage of C3b by Factor I and complement activation is prevented. In the MB lectin pathway, carbohydrate recognition domains of the mannan-binding lectin bind appropriately spaced sugar residues, such as mannose, on the pathogen surface. If complement activation occurs (C3 and C5 convertases formed) complement effector functions, common to all 3 pathways, can occur. The effector functions include the following: 1. recruitment of inflammatory cells 2. Opsonization (coating) of pathogens for enhanced removal by phagocytes. 3. Formation of the membrane attack complex (MAC). The MAC forms a lytic pore in the pathogen membrane and has traditionally been thought of as the sole method whereby antibodies can exert a bactericidal effect in the absence of phagocytic cells.

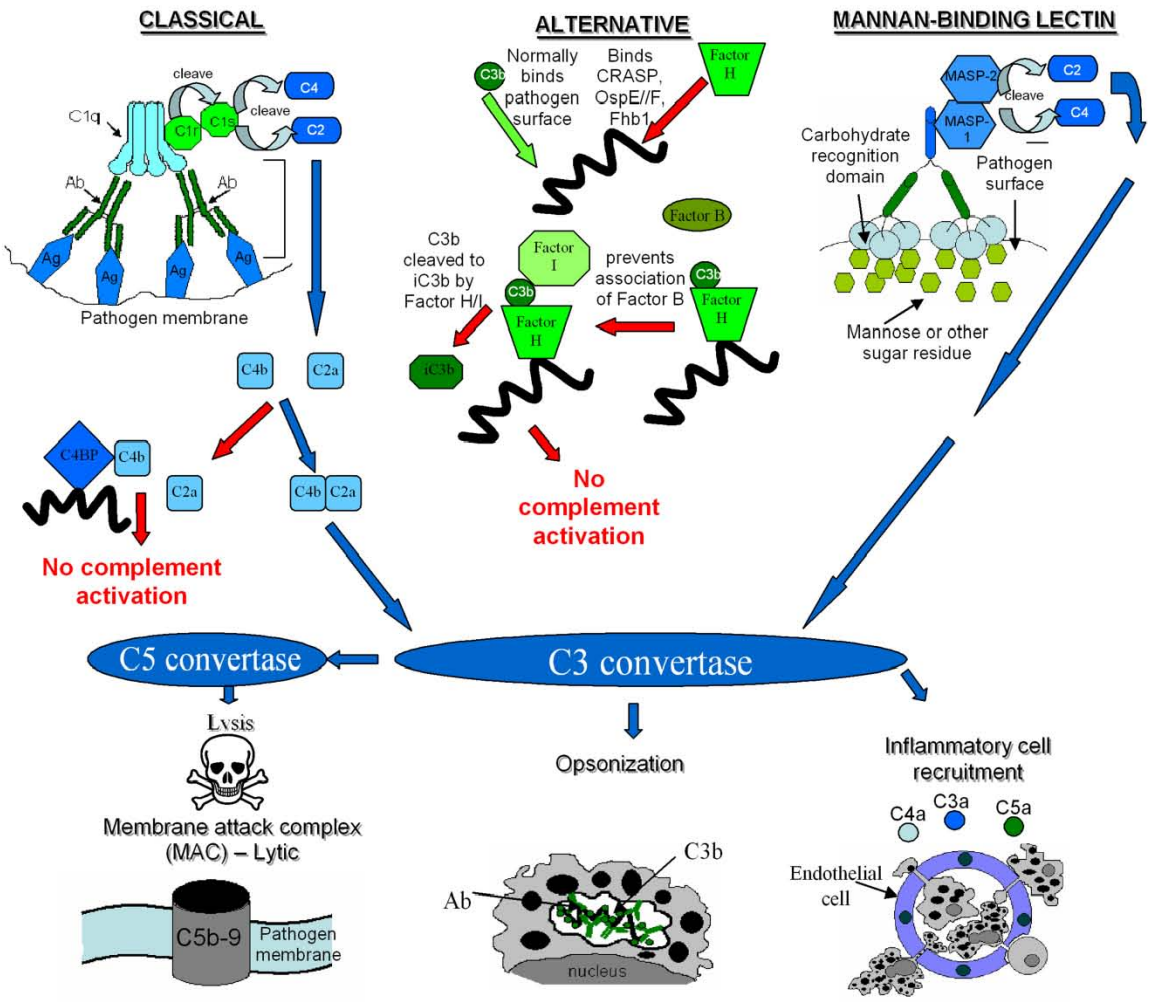
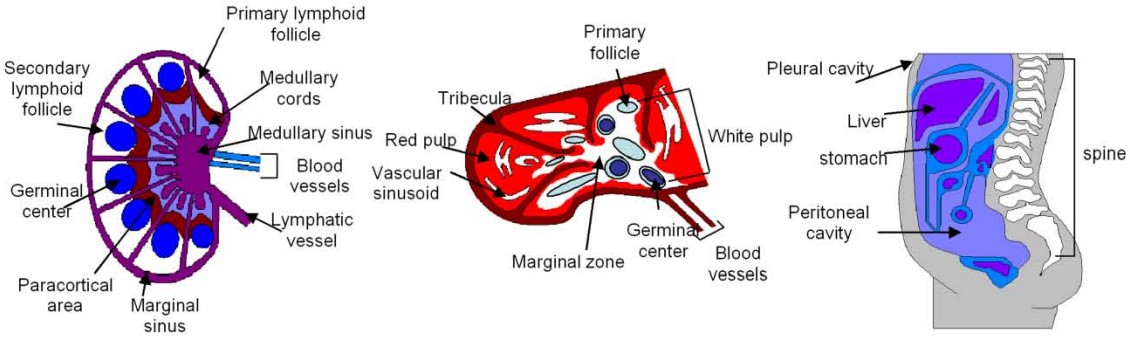

CLASSICAL
cleave
C4
C1q
C1r
C1s
cleave
C2
Ab
Ab
Ag
Ag
Ag
Ag
Pathogen membrane
C4b
C2a
C4BP
C4b
C2a
C4b
C2a
No complement activation
ALTERNATIVE
Normally binds pathogen surface
C3b
Binds CRASP, OspE//F, Fhb1
Factor H
Factor B
C3b cleaved to iC3b by Factor H/I
Factor I
C3b
Factor H
prevents association of Factor B
C3b
Factor H
iC3b
No complement activation
MANNAN-BINDING LECTIN
MASP-2
C2
cleave
MASP-1
C4
Carbohydrate recognition domain
Pathogen surface
Mannose or other sugar residue
C5 convertase
C3 convertase
Lysis
Membrane attack complex (MAC) – Lytic
C5b-9
Pathogen membrane
Opsonization
C3b
Ab
nucleus
Inflammatory cell recruitment
C4a
C3a
C5a
Endothelial cell

Figure 1.3: Different B cell Subsets. Follicular (FO) B cells are commonly located in secondary lymphoid organs such as the spleen and lymph nodes (depicted). FO B cells can be found within the primary and secondary lymphoid follicles in lymph nodes. Within the secondary lymphoid follicles are regions of intense B cell proliferation called germinal centers in which T cell dependent B cell responses occur (class switching, somatic hypermutation, affinity maturation). FO B cells elicited by a T_{H2} response are known to be important in the host response against Lyme disease infection (*B. burgdorferi*), however there is evidence that this immunity occurs in the absence of T cells, as well. Marginal zone (MZ) B cells are located in the marginal zone at the border of the white pulp in the spleen as indicated in the diagram. B1 B cells are commonly found in body cavities such as the pleural and peritoneal cavities depicted in the diagram. Both MZ and B1b B cells have been shown to be important in the host response to relapsing fever *Borrelia* (*B. hermsii*) and function in the absence of T cell help. These cells produce serotype-specific IgM which is critical for the clearance of relapsing fever spirochetemia. Notable characteristics of each B cell subset are listed in the figure.



Secondary lymphoid organ (lymph node)

Primary source of FO B cells

Spleen

Primary source of MZ B cells

Pleural and peritoneal cavities

Primary source of B1 B cells

Produced after birth	Produced after birth	Produced in fetus
Recirculate	Do not recirculate	Do not recirculate
Derived from bone marrow	Derived from bone marrow	Self-renewing
Diverse Ab repertoire	Restricted Ab repertoire but indications of diversity have been demonstrated	Partially restricted Ab repertoire but indications of diversity have been demonstrated
Predominantly secrete IgG, but can secrete all Ab classes	Predominantly secrete IgM	Predominantly secrete IgM
Mainly respond to protein Ags	Some response to protein Ags	Some response to protein Ags
Some response to carbohydrate Ags	Respond to carbohydrate Ags	Respond to carbohydrate Ags
Require T cell help	Do not require T cell help	Do not require T cell help
High somatic hypermutation	Little-no somatic hypermutation	Little-no somatic hypermutation
High memory	Little memory, but indications of memory have been demonstrated	Little memory, but indications of memory have been demonstrated
Little spontaneous Ab production	Little spontaneous Ab production	High spontaneous Ab production
Important in host response against Lyme disease infection	Important in clearance of relapsing fever spirochetemia	Important in clearance of relapsing fever spirochetemia

CHAPTER 2 – THE ANTIBODY SIDE - The Bactericidal Action of a Complement-Independent Antibody against Relapsing Fever Borrelia Resides in Its Variable Region

2.1 CHAPTER SUMMARY

A single chain variable fragment (scFv) of CB515, a complement-independent bactericidal IgM against a relapsing fever *Borrelia*, was constructed to investigate the region wherein the unique bactericidal function resides. Monomeric CB515 scFv (26 kDa) was capable of binding its antigen on whole organisms and by immunoblot. This binding was shown to be species and serotype-specific to the 19 kDa variable small protein (Vsp), recognized by its parent monoclonal IgM. The monomeric scFv, however, exhibited a lower level of binding compared to the pentameric CB515 IgM. A dose-dependent bactericidal effect of the CB515 scFv was detected by direct enumeration of spirochetes. Spirochetes treated with the CB515 scFv prior to inoculation into mice grew into escape mutants (seroconverted), whereas spirochetes treated with an irrelevant scFv developed as the original infecting serotype. The bactericidal effect, as seen at the ultrastructural level, was due to disruption of the outer membrane and severe membrane blebbing eventually resulting in death. These results indicate that the variable region of CB515 is responsible for the bactericidal activity and that the constant region of the antibody is dispensable.

2.2 INTRODUCTION

Traditional dogma dictates that antibodies can only exert a microbicidal effect with the assistance of complement (and, thus, formation of the membrane attack complex) or through recruitment of phagocytic cells following antigen binding (173). However, several monoclonal antibodies against different species of *Borrelia* can produce a bactericidal effect in the absence of immune cells or complement (77, 82, 297). Since antibodies can range from bivalent to decavalent molecules, agglutination may make it appear as though a bactericidal effect has occurred through grouping/clumping of organisms. Several monovalent Fab fragments of these antibodies still exert their

bactericidal effects, ruling out this possibility (77, 297). The effector functions of antibodies (complement recruitment, etc) reside in their constant regions, thus, it is reasonable to assume that the bactericidal function of complement-independent antibodies also resides within this region. The variable regions of antibodies are traditionally thought to only provide antigen binding but it is possible that the bactericidal function of complement-independent antibodies resides within this region as it is highly polymorphic and the complement-independent bactericidal effect is not shared by all antibodies. Therefore it was of interest to determine the antibody region wherein this unique bactericidal function may reside. This was achieved by constructing and conducting studies with an scFv, an antibody fragment which consists only of the variable region. The fragment was derived from CB515 against relapsing fever for several reasons. This antibody has shown *in vivo* significance making it interesting in that respect and is an IgM which may be associated with a unique, very early, T cell-independent immune response to relapsing fever.

2.3 MATERIALS AND METHODS

Mice and Bacteria

A patient-derived, virulent strain of relapsing fever *Borrelia* was used in the CB515 scFv and relapsing fever studies (7). To obtain spirochetes for *in vitro* assays, 6-8 week old C3H/HeN mice (Charles River) were inoculated with stocks of the strain frozen at -80^{o}C and sacrificed during the first peak of spirochetemia. Blood was centrifuged and washed with fresh Barbour-Stoenner-Kelly-H (BSK-H) (serum-free) medium (Sigma-Aldrich). The escape mutant of this strain was generated as described in the results section. *B. hermsii*, *B. crocidurae*, and *B. burgdorferi* were grown in BSK-H medium at 33^{o}C to stationary phase. Animal procedures were done by protocols approved by the institutional review board.

Cloning of the Single Chain Variable Fragment of CB515

RNA from the V_H and V_L chains of MAb CB515 from hybridoma cells (82) was reverse transcribed to cDNA using consensus primers from the Ig-Prime kit (Novagen, San Diego, CA) and AMV reverse transcriptase (Roche). V_H and V_L chain cDNAs were amplified (Ig-Prime consensus primers) and cloned into Perfectly Blunt vectors (Novagen) and transformed into clone-competent NovaBlue singles *E. coli* (Novagen). After screening, plasmid DNA was isolated (Promega, Madison, WI), concentrated, and sequenced. V_H and V_L chains were amplified from clones and joined by a linker encoding for $(Gly_4Ser)_3$ by overlap extension PCR using the forward primer G G C G G C G G C G G C T C C G G A G G T G G T G G A T C C G A C A T T G T G A T G A C C (for partial linker sequence and V_L) and the reverse primer C C G G A G C C G C C G C C G C C A G A A C C A C C A C C A C C T G C A G A G A C A G T G A C C A G (for partial linker sequence and V_H). The linker-joined CB515 scFv gene was amplified using the forward primer C C A G C C T G C A G G G G C C C A G G T T C A G C and the reverse primer T G G T G G T G C T C G A G C C G T T T T A T T T C C A and was cloned into a pET26b expression plasmid (Novagen) and inserted into DH5α *E. coli* (Novagen) for sequencing. After sequencing, the pET26b plasmid was inserted into Rosetta 2 (DE3) *E. coli* (Novagen) for expression. From the pET26b plasmid, the CB515 scFv gene encodes for a 6x HisTag at its C-terminus and a PelB signal sequence at its N-terminus, intended to direct the scFv polypeptide to the *E. coli* periplasm for folding and disulfide bond formation during expression. Sequences for the heavy and light chain variable regions, and completed CB515 scFv were deposited with Genbank under the accession numbers EU073761, EU073762, and EU073760.

CB515 scFv Expression and Purification

Rosetta 2 *E. coli* were grown at 37°C until an OD_{600} of ~0.6 was reached. Cultures were then induced with 100 μM IPTG for 3 hours and harvested. Harvested *E. coli* were resuspended in periplasmic extraction buffer (20 mM Tris-HCl, 20% sucrose, 5 mM EDTA, 75 μg/ml lysozyme, pH 8.0) and incubated on ice for 40 minutes after which 20 mM $MgCl_2$ was added. Soluble fractions were concentrated in Centriprep YM-10

centrifugal filter units (Millipore), pooled, and diluted 5-fold in binding buffer (20 mM $NaPO_4$, 0.5 M NaCl, 25 mM imidazole, pH 7.4). The sample was applied to a 1 ml HisTrap HP Ni^+ column (GE Healthcare Amersham Biosciences) using an Akta FPLC (GE Heatlthcare Amersham Biosciences). *E. coli* contaminants were eluted from the column using 101 mM imidazole and the CB515 scFv was eluted in a pure form using 500 mM imidazole.

MAb Purification

CB515 hybridoma supernatant was obtained as previously described (82). The antibody was purified in 2 steps using a HiTrap IgM affinity HP column (GE Healthcare Amersham Biosciences) according to the manufacturer's instructions and a Superdex 200 16/60 size exclusion column on an Akta FPLC. The CB515 IgM eluted in a pure form in the void volume.

Size Exclusion Chromatography and Homology-Based Fv Modeling

Affinity-purified CB515 scFv was applied to a Superdex 200 16/60 size-exclusion column (GE Healthcare Amersham Biosciences) using an Akta FPLC. The scFv was eluted using 96 ml PBS, and this volume was compared to a standard curve of proteins of known MW to determine the quaternary structure and MW of the scFv. The Web Antibody Modeling (WAM) service (http://antibody.bath.ac.uk/) provided by the University of Bath, UK, was used to generate a 3-D molecular model of the CB515 Fv. Sequences were aligned according to the WAM specifications and compared to a library of known, crystallized V regions to generate the structure.

CB515 scFv/Relapsing Fever Immunoblots

12.5% SDS-PAGE gels were run with 37.5 μg of whole *Borrelia* lysate per well, transferred to nitrocellulose and exposed to CB515 scFv or monoclonal CB515 at a concentration of 1 μg/ml for 1 hour. The CB515 scFv was detected with a 2^o anti-HisTag mouse IgG (Novagen) followed by a 3^o anti-mouse IgG infrared conjugate (Rockland Immunochemicals). A 2^o anti-IgM infrared conjugate (Rockland, 1:1000) was used to

detect CB515. Reactivity of the immunoblot was observed by scanning in an Odyssey infrared scanner (LI-COR Biosciences) in the 800 channel.

Monoclonal CB515 – CB515 scFv Comparison ELISAs

To compare the level of CB515 scFv Ag binding to that of its parent antibody, monoclonal CB515, indirect ELISAs were performed. The wells of a 96-well ELISA plate were coated with relapsing fever *Borrelia* lysate at a concentration of 5μg/ml in carbonate buffer (pH 9.6) at 4^{o}C overnight. The wells were then blocked with 0.5% BSA for 30 minutes at 37^{o}C. Different concentrations of biotinylated CB515 scFv, irrelevant scFv (synovial scFv 2-2, a gift from Brigitte Huber and Srimoyee Ghosh of Tufts University, Boston, MA (136)), or monoclonal CB515 IgM were added to the wells in triplicate and incubated for 1 hour at 37^{o}C. The CB515 scFv was added at the following concentrations: 7.5 μg/ml (282 nM), 5 μg/ml (188 nM), 1 μg/ml (38 nM), 0.5 μg/ml (19 nM), 0.25 μg/ml (9.0 nM), and 0.015 μg/ml (0.6 nM). Concentrations of monoclonal CB515 IgM that were equimolar to these scFv concentrations, 182 μg/ml (187.7 nM), 36.4 μg/ml (38 nM), 18.2 μg/ml (189 nM), 9.1 μg/ml (9.0 nM), 0.546 μg/ml (0.6 nM), were used in order to compare level of binding of equal amounts of CB515 scFv and monoclonal CB515. Level of binding was detected with alkaline phosphatase conjugated streptavidin and the ELISA was developed at 37^{o}C for 1.5 hours.

CB515 *In Vitro* Bactericidal Assays and TEM Analysis

Relapsing fever *Borrelia* were harvested from the plasma of C3H/HeN mice as described. Spirochetes from a pooled sample in BSK-H medium were aliquoted into separate tubes to a final volume of 100 μl. The spirochetes were exposed to either the CB515 scFv or an irrelevant scFv at the concentrations indicated in the results section. ScFv elute buffer and BSK-H medium alone also served as negative controls. After 15 and 75 minutes or 2 hours of incubation at 33^{o}C, spirochetes were enumerated directly by dark field microscopy. Following the 75 minute time points, spirochetes were prepared for negative-stain TEM analysis as described in (205) . Relapsing fever *Borrelia* analyzed by ultrathin section TEM were exposed to 200 μg/ml of an irrelevant scFv or the CB515 scFv for 75 min at 33^{o}C and prepared into thin sections as previously

described (77). Images were captured using a BioTwinG^2 TEM (FEI) and digital camera (AMT) at an accelerating voltage of 80 kV.

Fluorescent Microscopic Imaging

For CB515 scFv binding experiments, spirochetes were treated with several concentrations of CB515 scFv for 1 hour at 37^{o}C followed by anti-HisTag (Novagen) 2^o mouse IgG. Controls included an irrelevant scFv and the parent MAb CB515. The irrelevant scFv, which has a protein A tag, was detected with mouse anti-protein A IgG (Sigma). For both, a 3^o FITC-labeled anti-mouse IgG (Sigma) was used. MAb CB515 was detected with a FITC-labeled anti-mouse IgM (Sigma). Fluorescence was observed using an Eclipse E600 fluorescence microscope (Nikon).

2.4 RESULTS

A Single Chain Variable Fragment (scFv) of CB515 was Contructed and Inserted into a pET26b Expression Plasmid

RNA was extracted from CB515 hybridoma cells and probed with primers from an IGPrime primer kit to locate CB515 heavy and light chains. Variable heavy and variable light chain cDNA were then cloned into respective blunt vectors and transformed into clone-competent NovaBlue singles hosts. These clones were sequenced and the variable heavy and light chains were amplified and joined by a linker encoding for $(Gly_3Ser)_4$ by overlap extension PCR. The gene for the CB515 scFv was cloned into a pET26b expression plasmid and inserted into DH5α cells. These clones were screened and confirmed after which the CB515 scFv gene in pET26b was inserted into Rosetta 2 *E. coli* cells for expression. The CB515 scFv gene contains a 6x HisTag at its C-terminus to allow for affinity purification and to facilitate detection in experiments. The CB515 scFv gene also contains a PelB leader signal sequence at its N-terminus intended to direct the newly formed scFv polypeptide to the periplasm in an actively expressing *E. coli* cell. The oxidizing environment of the periplasm is more favorable for disulfide bond formation and promotes proper protein folding (Figure 2.1).

Expression Conditions for the CB515 scFv

We tested whether the PelB system was working as intended by performing osmotic lysis. This was achieved by exposing Rosetta 2 *E. coli* to conditions sufficient for the preparation of spheroplasts. Soluble contents from these preparations were electrophoresed, transferred to immunoblot, and probed with an anti-HisTag antibody. Compared to the soluble fraction of whole *E. coli* lysate, osmotic lysis yielded comparable levels of soluble CB515 scFv as determined by immunoblot (Figure 2.2). Since the CB515 scFv could be readily obtained from the Rosetta 2 *E. coli* periplasm and since this increases sample purity by excluding cyotosolic contents, periplasmic extraction was chosen as our preferred method to obtain the CB515 scFv.

Optimal conditions for expression of soluble CB515 scFv were determined by testing different growth temperatures and IPTG concentrations for the Rosetta 2 *E. coli.* Cultures were grown at 25^{o}C or 37^{o}C until an OD_{600} of ~0.6 was reached. Cultures were then induced for 3 hours with 33 μM, 50 μM, 75 μM, 100 μM, or 400 μM IPTG, or grown uninduced, harvested by centrifugation and protein was obtained from the periplasm via spheroplast preparation. Soluble fractions were run on SDS-PAGE, transferred to nitrocellulose, and probed with an anti-HisTag antibody to assess soluble scFv expression. Growth of *E. coli* at 37^{o}C and induction with 100 μM IPTG for three hours were the optimal conditions for high yield production of soluble CB515 scFv (Figure 2.2B – C).

The CB515 scFv was Purified to Homogeneity by Affinity Chromatography

To purify the CB515 scFv for further studies, Ni^{+} chelate chromatography was used so as to exploit the 6X HisTag placed on the recombinant scFv. A linear gradient of imidazole was initially used for elution employing imidazole concentrations in the range of 25 – 500 mM. Soluble proteins from Rosetta 2 *E. coli* periplasm were run through a 5ml HisTrap HP column using an AKTA FPLC. Two defined peaks of absorbance at 280 nm were observed at two different imidazole concentrations representing an impure solution of CB515 scFv and a CB515 scFv solution of increased purity (Figure 2.3A). Based upon the linear gradient elution profile, a step elution method, based on these

imidazole concentrations was devised. The step elution was designed to allow for complete elution of contaminants first, followed by elution of pure CB515 scFv. In the step elution method CB515 scFv was purified in a 1ml HisTrap HP column using 101mM imidazole to elute *E. coli* contaminants and 500mM imidazole to elute pure CB515 scFv and optimize yield of the scFv by "chasing off" protein due to the high imidazole concentration (Figure 2.3B). The step elution method resulted in the successful purification (>95%) of the CB515 scFv (Figure 2.3C). Typical yield for the CB515 scFv was > 1 mg per affinity purification.

The CB515 scFv has a Monomeric Quaternary Structure

CB515 scFv was analyzed by size exclusion chromatography to determine the quaternary structure of the fragment. The 15 amino acid linker that joins the V_H and V_L chains is designed for production of a monomeric scFv (Figure 2.1) (160, 169, 194). However, scFvs intended to be monomers are known to form non-covalent multimers for a number of reasons. Production of high concentrations of scFvs in the bacterial periplasm and existence of scFvs in solution at high concentrations (>1 mg/ml) are two factors that promote scFv multimerization (13, 169, 278). Since these are conditions that would occur during hyperexpression of the scFv in *E. coli*, we analyzed affinity-purified CB515 scFv by size-exclusion chromatography to determine if the scFv is monomeric, as intended, or multimeric. Elution properties corresponded to an scFv monomer as compared to protein standards (Figure 2.4). Specifically, the CB515 scFv eluted at 96.30 ml, corresponding to a monomeric scFv of ~13 kDa. The predicted MW of the CB515 scFv in a monomeric form, however, is 26 kDa.

As our observed MW was lower than that which was predicted, we chose to obtain an accurate MW by analyzing the CB515 scFv with matrix-assisted laser desorption/ionization in a time-of-flight mass spectrometer (MALDI-TOF). After affinity-purified CB515 scFv was analyzed by MALDI-TOF it was determined that its MW was, in fact, 26.645 kDa, indeed the size of a monomer and much closer to the predicted MW. The MW observed by size exclusion chromatography was probably the apparent MW of the scFv, a reflection of CB515 scFv protein folding, and not necessarily MW based on amino acid content.

Homology-Based 3-D Modeling of the CB515 Fv Region

A 3-D molecular model of the CB515 Fv region (scFv without linker sequence) was generated by homology-based modeling using the Web Antibody Modelling (WAM) service provided by the University of Bath, UK (Figure 2.5). The nucleotide sequences of the variable heavy and variable light chains of CB515 were aligned according to WAM specifications and the sequence was compared to a library of known, crystallized Fv regions to generate an accurate 3-D model. The model provides the predicted scFv structure, particularly demonstrating the correct orientation of complementarity-determining regions (CDRs) forming the accessible antigen binding site.

The CB515 scFv Binds its Antigen in Immunoblot

Affinity-purified CB515 scFv was capable of binding the antigen that it recognizes in immunoblots. The species of relapsing fever *Borrelia* that CB515 recognizes is an uncultivable, patient-derived strain, that has to be maintained by mouse-to-mouse passage (7, 80, 82). In order to obtain spirochetal lysate for the binding assay, C3H/HeN mice were inoculated intradermally with relapsing fever *Borrelia*. Spirochetemia was monitored daily until the first peak of spirochetemia was observed, at which point mice were sacrificed, blood was obtained by cardiac puncture, and spirochetes were harvested from plasma. It was important to harvest spirochetes of the first peak of spirochetemia as this population will predominantly consist of the serotype recognized by CB515 and future relapse populations will not. Lysate from these spirochetes was blotted to nitrocellulose and probed with either monoclonal CB515 IgM as a positive control or CB515 scFv at a concentration of 1μg/ml. The CB515 scFv was shown to bind the same antigen as its parent antibody in this assay (Figure 2.6).

The CB515 scFv is Species and Serotype-Specific

To determine its binding specificity, the CB515 scFv was tested for binding to an escape mutant of the infecting relapsing fever organisms, as well as, *B. hermsii*, *B. crocidurae*, and *B. burgdorferi* to determine its specificity (Figure 2.7). To generate an escape mutant of this strain, spirochetes harvested from C3H/HeN plasma were adjusted to a concentration of 2 X 10^4/ml and used to inoculate B cell deficient B6.129S2-IgH6

mice (Jackson laboratories). Monoclonal CB515 (50 μg) was administered intraperitoneally daily, from days 0 – 5 in order to eliminate the predominant relapsing fever serotype and promote the generation of an escape mutant. Since B cell-deficient mice were used, the relapse population, generated by our passive immunization regimen, was allowed to grow unrestricted due to the absence of host antibodies. In immunoblot the CB515 scFv only recognized the 19 kDa Vsp present in the original infecting relapsing fever serotype, and did not recognize the escape mutant or the other species, indicating that it is species and serotype-specific (Figure 2.7).

The CB515 scFv Binds its Native Antigen on Relapsing Fever *Borrelia*

To determine if the CB515 scFv had the ability to bind its native antigen in relapsing fever *Borrelia,* an indirect immunofluorescence assay (IFA) was performed. After titration of reagents, fixed spirochetes were exposed to either 56 μg of CB515 scFv, 56 μg of irrelevant scFv (negative control), or 10 μg of CB515 IgM (positive control) for 1 hour. Binding was detected with FITC-labeled anti-mouse IgM and IgG in a Zeiss Eclipse E600 fluorescence microscope. Fluorescent spirochetes were observed in samples exposed to the CB515 scFv (Figure 2.8A – G), or its parent MAb, CB515 (Figure 2.8I), but not in those exposed to an irrelevant scFv (Figure 2.8H).

The CB515 scFv Exhibits a Lower Level of Binding Compared to its Parent Antibody

Since the CB515 scFv is a monomeric antibody fragment derived from a pentameric IgM with high avidity, it was a concern that it might exhibit a lower level of binding. This information would also prove useful in planning experiments to test for the bactericidal activity of the CB515 scFv. Different concentrations of CB515 scFv, irrelevant scFv, or monoclonal CB515 were added to wells of an ELISA plate coated with relapsing fever *Borrelia* lysate and incubated for 1 hour at 37^{o}C. The CB515 scFv was added at the following concentrations; 7.5 μg/ml (282 nM) 5 μg/ml (188 nM), 1 μg/ml (38 nM), 0.5 μg/ml (19 nM), 0.25 μg/ml (9.0 nM), and 0.015 μg/ml (0.6 nM). Concentrations of monoclonal CB515 IgM that were equimolar to these scFv concentrations; 182 μg/ml (188 nM), 36.4 μg/ml (38 nM), 18.2 μg/ml (19 nM), 9.1 μg/ml

(9.0 nM), 0.546 μg/ml (0.6 nM), were used in order to compare level of binding of equal amounts of CB515 scFv and monoclonal CB515. Level of binding was detected with alkaline phosphatase conjugated antibodies and the ELISA was developed at 37^{o}C for 1.5 hours. These experiments showed that the CB515 scFv exhibits a lower level of binding to its antigen than does monoclonal CB515. This conclusion is drawn since equimolar concentrations of scFv exhibited lower levels of absorbance at 405 nm, in comparison to monoclonal CB515 (Figure 2.9).

The CB515 scFv Retains the Bactericidal Activity of its Parent Monoclonal Antibody

We next wanted to determine whether or not the CB515 scFv could retain the bactericidal function of its parent antibody. To test this, relapsing fever *Borrelia* harvested from mouse plasma were exposed to 100, 200, or 300 μg/ml CB515 scFv, or an irrelevant scFv *in vitro*. Spirochetes, at an initial concentration of 8.3 X 10^{7} spirochetes/ml, were incubated at 33^{o}C and enumerated under dark field microscopy after 15 and 75 minutes of incubation to determine bactericidal effect (Figure 2.10). A significant bactericidal effect was observed when spirochetes were exposed to either 200 or 300 μg/ml of CB515 scFv as compared to an irrelevant scFv at both time points. At the highest concentration a 50% reduction in spirochete numbers was observed after 15 minutes (Figure 2.10A). After 75 minutes of incubation, 200 μg/ml CB515 scFv caused a 50% reduction in spirochete numbers (Figure 2.10B). Additionally, spirochetes were also exposed to buffer or BSK-H medium without scFv in triplicate and no effect was seen (data not shown). The dose-dependence of this bactericial effect was investigated by incubating spirochetes, at an initial concentration of 7.2 X 10^{6} spirochetes/ml, with different concentrations of the CB515 scFv ranging from 100 – 300 μg/ml, with increasing 10 μg/ml intervals, for 2 hours (Figure 2.10C). As with previous experiments, the CB515 scFv exhibited a significant bactericidal effect at all concentrations as compared to 300 μg/ml of an irrelevant scFv. These experiments demonstrated a dose-dependent bactericidal effect of the antibody fragment. It is, however, important to note that in these experiments darkfield microscopy does not distinguish between live or dead microorganisms, so it is possible that many spirochetes counted were dead or in the

process of dying. Since this was a concern, we investigated the bactericidal efficiency of the CB515 scFv even further. Relapsing fever *Borrelia* were exposed to 300 μg/ml of either an irrelevant scFv or the CB515 scFv for 2 hours at 33^{o}C to determine their capacity for *in vivo* infection. Naïve mice were inoculated with spirochetes from this experiment (10^5 spirochetes per mouse) and spirochete recovery, which would be indicative of the killing efficiency of the CB515 scFv, was evaluated. The spirochetes caused murine infection in both cases, and were therefore harvested at peak spirochetemia, lysed, electorphoresed and Coomassie-stained or probed with the CB515 scFv in an immunoblot to determine serotype identity (Figure 2.11). Spirochetes exposed to an irrelevant scFv grew as the original infecting serotype whereas those exposed to the CB515 scFv were composed of escape mutants not recognized by the CB515 scFv (Figure 2.11). This experiment demonstrated that incubation with the CB515 scFv prior to infecting mice completely eliminated those organsims to which the CB515 scFv is specific, suggesting a killing efficiency of 100%.

The CB515 scFv Causes Membrane Damage to Relapsing Fever *Borrelia*

To assess ultrastructural damage that may have occurred to spirochetes and to confirm the bactericidal effect observed with the CB515 scFv, relapsing fever *Borrelia* exposed to 100, 200, and 300 μg/ml CB515 scFv, or irrelevant scFv at the same concentrations, for 75 minutes were examined by negative-stained TEM. *Borrelia* exposed to the irrelevant scFv or to medium alone appeared intact and unaffected, showing characteristic morphology and membrane structure (Figure 2.12A – B). *Borrelia* exposed to the CB515 scFv, however, exhibited visible damage. When exposed to 100 μg/ml CB515 scFv, periplasmic flagella were exposed to the extracellular environment, indicating damage to the spirochetal outer membrane (Figure 2.12C – D). Damage was more extensive when spirochetes were exposed to 200 μg/ml CB515 scFv and included membrane disruption and blebbing (Figure 2.12E – H). The most dramatic effect was observed using 300 μg/ml CB515 scFv where there was even more exposure of periplasmic flagella and severe membrane blebbing (Figure 2.12I – P). In some cases complete spirochete degradation appeared to be taking place (Figure 2.12I – K, M, O, P). These results confirm the dose-dependent bactericidal effect observed in the previous

experiments and demonstrate that this effect is achieved through a process that involves disruption of the outer membrane of *Borrelia* by the CB515 scFv identical to the damage caused by its parent antibody (82). The damaged spirochetes presumably represent a large population of spirochetes counted in the *in vitro* bactericidal assays as those experiments only considered spirochetes that had disappeared as affected by the scFv and not those that may have been in the process of dying or already dead but not removed. In this respect, the TEM analysis complements the *in vitro* bactericidal assays and demonstrates the magnitude of the power of the CB515 scFv.

The damage imparted by the CB515 scFv was also analyzed by ultrathin section TEM (Figure 2.13). Relapsing fever *Borrelia* that were exposed to an irrelevant scFv appeared normal with an obvious intact outer membrane (Figure 2.13A – F). Those spirochetes exposed to 200 μg/ml of the CB515 scFv exhibited damage similar to that observed in the negative stain preparations but from a different vantage point. A disrupted outer membrane was seen in CB515 scFv treated samples, as evidenced by large gaps in the outer membrane and the release of periplasmic flagella (Figure 2.13 G – L). Distention of the outer membrane was also observed in CB515 scFv treated spirochetes representing the large blebs seen in negative stain samples and shows that the blebbing is the result of outer membrane distention rather than involving both membranes (Figure 2.13H – L). Outer membrane blebs were observed to be released from the spirochete surface as well (Figure 2.13G).

An additional control for the morphological damage analysis was the escape mutant of relapsing fever *Borrelia* generated in B cell-deficient mice by monoclonal CB515 passive vaccination. These spirochetes were exposed to 300 μg/ml CB515 scFv and analyzed for damage (Figure 2.14). As indicated by the lack of binding in Figure 2.7, the CB515 scFv did not cause any damage to the relapsing fever escape mutant as this mutant does not express the antigen recognized by CB515 (Figure 2.14).

2.5 DISCUSSION

In investigating the significance of the antibody variable region, we have shown that an unmodified scFv retains the bactericidal effect of its parent IgM. This is

important for several reasons. Unmodified scFvs are not, by themselves, bactericidal, rather they exert their effects through the introduction of specific modifications that can include antimicrobial peptides and other toxic molecules (41, 50, 103, 160, 170, 188, 210, 270, 342, 350, 366, 371). The CB515 scFv does not contain this type of modification, but rather is a direct clone of the variable region of its parent antibody. A bactericidal effect of this nature has never been observed to occur solely within the antibody variable region, the domain which is traditionally considered to be important only for antigen binding. That the constant region, traditionally thought of as the antibody effector domain, is not required for this effect to occur is a significant finding.

Antibodies typically do not kill microorganisms without the assistance of complement or phagocytic cells that ingest opsonized pathogens. Bactericidal antibodies of both IgG and IgM classes have been characterized for *in vitro* and *in vivo* effects against *Borrelia* (77, 80, 82, 297); yet, the mechanism by which these antibodies kill the spirochetes is not known. The bactericidal effect could be a function of the uniqueness of these antibodies and their variable regions that target equally unique and vital antigens on the surface of the *Borrelia*. Our results show that the complement-independent bactericidal function of CB515 resides within the variable region of the antibody. The monomeric scFv (26kDa) derived from this IgM MAb exerted a dose-dependent bactericidal effect against relapsing fever *Borrelia*. The CB515 scFv binds the 19 kDa Vsp from this bacterium and is not cross-reactive, indicating species and serotype-specificity. Further, we have shown that the scFv binds its native antigen on whole relapsing fever *Borrelia* in an IFA. The bactericidal effect is due to outer membrane damage, evidenced by the release of periplasmic flagella and the formation and release of membrane blebs and may involve more. These results demonstrate that the CB515 scFv is capable of attacking the spirochetes by itself; thus, confirming that the bactericidal effect of CB515 resides within the variable region. The TEM analysis confirmed the results obtained from the *in vitro* killing assays. This killing activity is also significant in demonstrating the uniqueness of the variable region–antigen relationship that is not shared with other types of microbicidal antibodies.

Non-traditional mechanisms utilized by directly antimicrobial antibodies are known (68). They range from antibody-catalyzed water oxidation reactions (discussed in 1.4.3) (358, 360, 377) to interference with iron uptake (120, 139). The antibody-catalyzed water oxidation pathway is a mechanism that was originally considered to be an explanation for the effects of complement-independent bactericidal antibodies to *Borrelia*. This is a process proposed to be universal among all antibodies, regardless of specificity or binding and involves the use of singlet oxygen and water to produce reactive oxygen species resulting in a bactericidal effect (358, 360). Residues within both the constant and variable regions are important for the active site that drives this reaction, but Trp^{L163} of the constant region appears to be of critical importance (377). Since the CB515 scFv contains no constant region and retains the bactericidal function of its parent antibody, utilization of the antibody-catalyzed water oxidation pathway can be ruled out, pointing to a unique and specific mechanism for CB515 and functionally related antibodies.

Fungicidal antibodies as well as their derivative scFvs can destroy yeast (248, 274) and interfere with other biological functions of *Cryptococcus neoformans* and *Candida albicans* (69, 248, 292). Many of these antibodies exhibit their fungicidal activity by mimicking a killer toxin and are broadly specific for many species of yeast (248, 274). Other anti-fungal antibodies are bacteriostatic to *Crytococcus neoformans* (292), block C*andida albicans* hyphae formation (69), interfere with adherence and germination of *C. albicans*, or are directly candidacidal (248). The fungicidal antibodies, however, appear to have a very specific mechanism that contrasts with the effects exerted by complement-independent bactericidal antibodies to *Borrelia*. Additionally, the morphological differences between yeast and spirochetes, namely the thick chitin cell wall of yeast, suggest the utilization of microbicidal mechanisms that are functionally distinct from one another.

Bactericidal assays using 100 μg/ml (0.11 μM) of monoclonal CB515 resulted in a 77% reduction in spirochete numbers in previous studies (82). The scFv, in an identical assay, required 200 μg/ml (7.4 μM) to achieve a 50% reduction, which would correspond to a sizable decrease in activity compared to the parent IgM. This is not surprising as the

difference in bactericidal effect can be attributed to a higher number of binding sites on the pentameric CB515 in contrast to the monomeric CB515 scFv. In fact, in ELISAs that compared the binding level of the CB515 scFv to its parent IgM, the CB515 scFv exhibited a lower level of binding, likely due to the difference in quaternary structure (Figure 2.9). In addition, recent studies have shown that the constant regions of antibodies directed to infectious organisms induce structural changes in the variable regions that affect binding affinity and fine specificity (83, 236, 345, 346). Although the bactericidal effect was retained in the variable regions, the killing efficiency was reduced compared to the parent antibody. Thus, it is possible that the absence of the constant regions could account for some of the decrease in bactericidal activity.

We believe that the bactericidal activity of the CB515 scFv and related antibodies to *Borrelia* may be due to a synergistic effect in which the variable region of the antibody and the antigen play integral parts in creating the effect. As the CB515 scFv is so specific, it would appear that there is something about the combination of the variable region with this unique *Borrelia* antigen that is extraordinarily lethal. In fact, high specificity is shared by all the known bactericidal antibodies to either Lyme disease or relapsing fever *Borrelia* (77, 78, 81, 82, 297). Bactericidal antibodies to *B. burgdorferi* are specific to one amino acid of OspB (78, 297), a lipoprotein that is downregulated as spirochetes are transmitted from tick to mammal (313). It is of note that this lipoprotein, regardless of its physiological role to the spirochete, is not exposed to immune surveillance as antibodies directed against it would be lethal to the spirochetes. Perhaps the profound effects of these bactericidal antibodies were a factor in driving the evolution of antigenic variation in relapsing fever, and the differential expression of antigens in the Lyme disease *Borrelia*, owing to their specificity and lethality. As the variable region of CB515 and functionally related antibodies are so efficient at eliminating spirochetes, one would assume that antigenic variation would be a necessity for relapsing fever *Borrelia* to persist and prolong infection.

2.6 FIGURES

Figure 2.1: CB515 scFv design. A. Amino acid sequence of the CB515 scFv. In light blue, a PelB leader sequence is represented, which was placed at the N-terminus of the scFv to direct it to the *E. coli* periplasm for proper folding and disulfide bond formation following polypeptide expression. PelB is cleaved once the polypeptide reaches the periplasm and so is not part of the scFv after expression. In green, the variable heavy chain is shown, followed by a 15 amino acid, glycine-serine linker sequence in black. This is a common scFv linker whose 15 amino acid length promotes the production of monomeric scFvs. In blue, the variable light chain is shown, followed by a 6X histidine tag (in olive green), added to the C-terminus to detect the scFv in immunoblots and to facilitate affinity purification using Ni^{+}. Complementarity-determining regions (CDRs) of the variable heavy and light chains are in boldface and underlined. These sequences collectively form the antigen binding site by creating an antibody surface that is complementary to that of the antigen. **B.** Illustration of a whole antibody compared with its derivatives. **Left:** Whole IgG molecule containing Fc and Fab portions as well as constant (C, blue) and variable (V, green) regions. **Center:** An Fab fragment is essentially just 1 arm of the whole antibody, composed of V and C regions. **Right:** A single chain variable fragment (scFv) is composed of V heavy (H) and light (L) chains, joined by a linker (in black). It is this region of the antibody that contains the antigen combining site.

A

PelB ____ V_H ____

MKYLLPTAAAGLLLLAAQPAGAQVQLQQSGPELV

KPGASVKMSCKAS***GYTFTDYV***ISWVKQRTGQGLEWI

GE***IYPGSGST***YYNEKKGKATLTADKSSNTAYMQLSSL

Linker

TSEDSAVYFC***ARSVWWGFAY***WGQGTLVTVSAGGG

V_L

GSGGGGSGGGGSDIVMTQSQKFMSTSVGDRVSITC

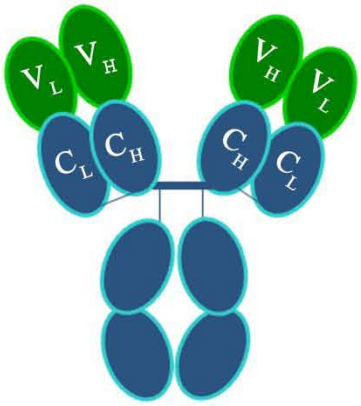
KAS***QNVGTA***VAWYQQKPGQSPKLLIY***SAS***NRYTGVP

DRFTGSGSGTDFTLTISNMQSEDLADYFC***QQYSSYL***

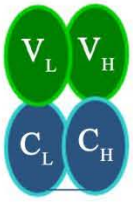
6xHis

YTFGGGTKLEIKRLEHHHHHH

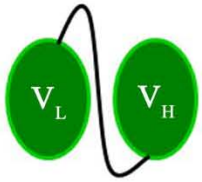
B



Whole Immunoglobulin



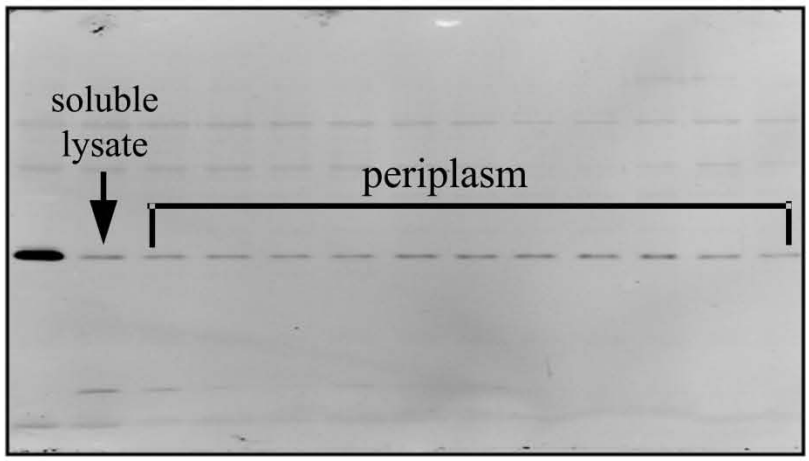
Fab Fragment



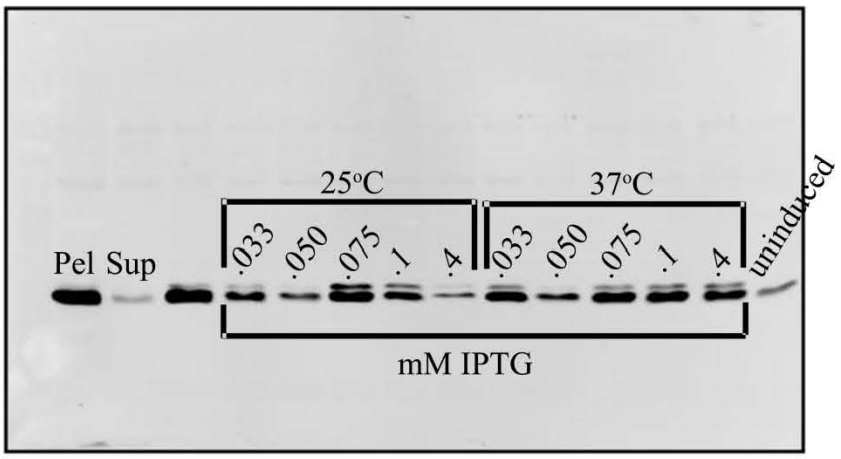
scFv

Figure 2.2: Expression of soluble CB515 scFv and periplasmic extraction. A. The CB515 scFv was obtained from the periplasm of harvested *E. coli* using conditions sufficient for the creation of *E. coli* spheroplasts. The soluble portions from these preparations were then run on SDS-PAGE, blotted to nitrocellulose and probed with an anti-HisTag antibody to evaluate level of soluble CB515 scFv obtained from the periplasm. This was compared with whole cell sonication in which the soluble fraction was analyzed by immunoblot (soluble lysate). Comparable amounts of soluble CB515 scFv can be obtained from the periplasm and soluble lysate. Lane 1 is a loading control. **B.** Rosetta 2 *E. coli* harboring the CB515 scFv gene were exposed to 11 different growth and induction conditions to determine those that are sufficient for optimal CB515 scFv production. 25^{o}C or 37^{o}C refers to growth temperature and IPTG concentrations are specified. Soluble CB515 scFv was obtained from the periplasm and detected in immunoblot with an anti-HisTag antibody to detect differences in expression. Growth at 37^{o}C generally yielded more protein. Pel, Sup, and lane 3 are loading controls. **C**. The optimal IPTG concentration was narrowed down to 0.1 mM, and growth at 25^{o}C or 37^{o}C was monitored a second time to see which yielded greater expression. Growth at 37^{o}C with 0.1 mM IPTG were the optimal expression conditions for production of soluble CB515 scFv extracted from the periplasm. Lanes 1 and 2 are loading controls.

A


soluble
lysate
periplasm

B


25°C
37°C
Pel Sup
.033
.050
.075
.1
.4
.033
.050
.075
.1
.4
uninduced
mM IPTG

C

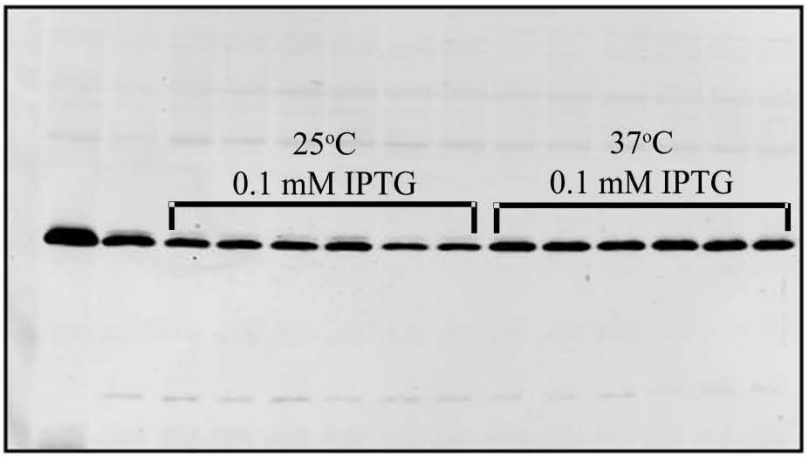

25°C
0.1 mM IPTG
37°C
0.1 mM IPTG

Figure 2.3: Affinity purification of the CB515 scFv. **A**. Crude protein containing CB515 scFv was extracted from Rosetta 2 *E. coli* periplasm and purified using a linear gradient of imidazole. UV_{280} absorbance is shown, and two distinct peaks, representing *E. coli* proteins (more contaminants) and CB515 scFv with some *E. coli* proteins (less contaminants), can be seen. **B**. CB515 scFv was obtained as described in A, and purified with a step elution method utilizing different imidazole concentrations based on the elution profile shown in A. *E. coli* proteins were eluted with 101 mM imidazole, and the scFv was completely eluted using 500 mM imidazole. Imidazole concentrations of each step are specified. **C**. After purification with the step elution method, peak fractions were run on SDS-PAGE and Coomassie-stained to assess purity. The CB515 scFv is eluted with 101 mM imidazole along with *E. coli* contaminants but is predominantly eluted in a homogenous form using 500 mM imidazole.

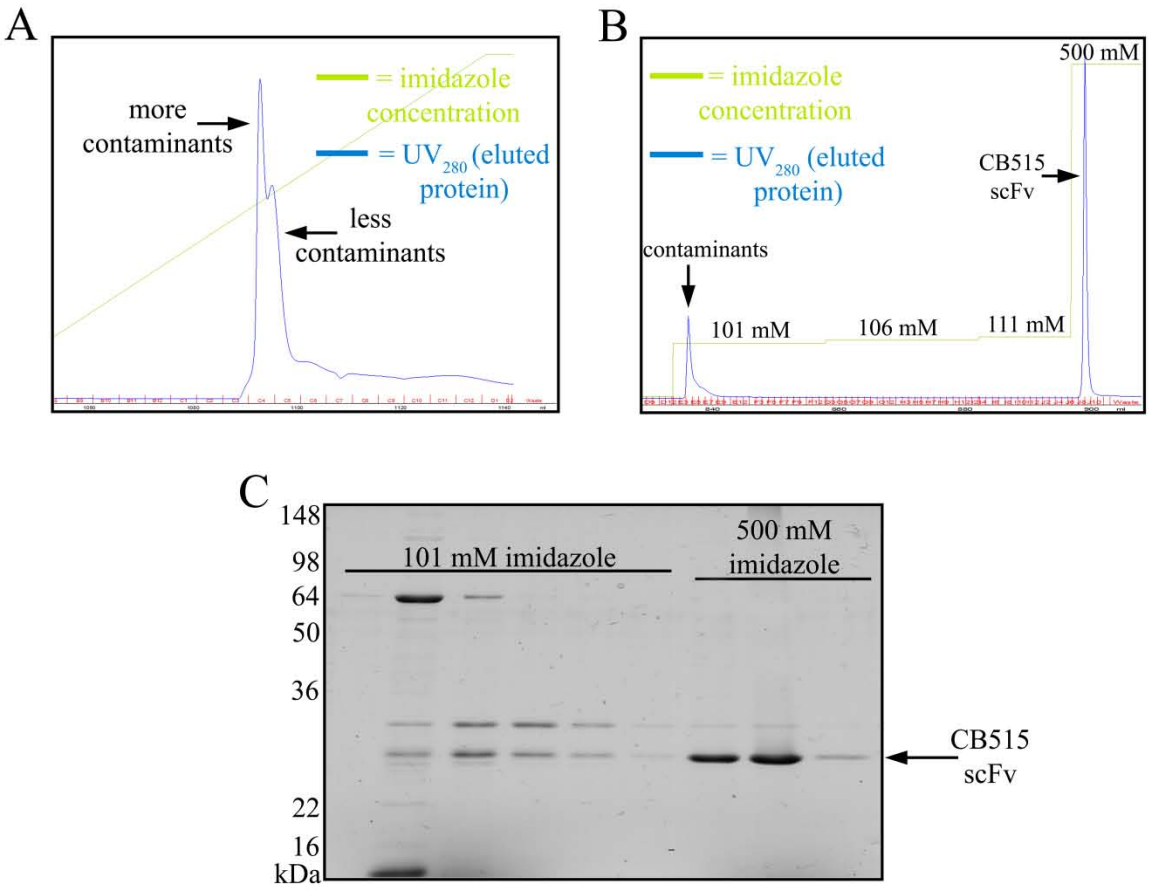
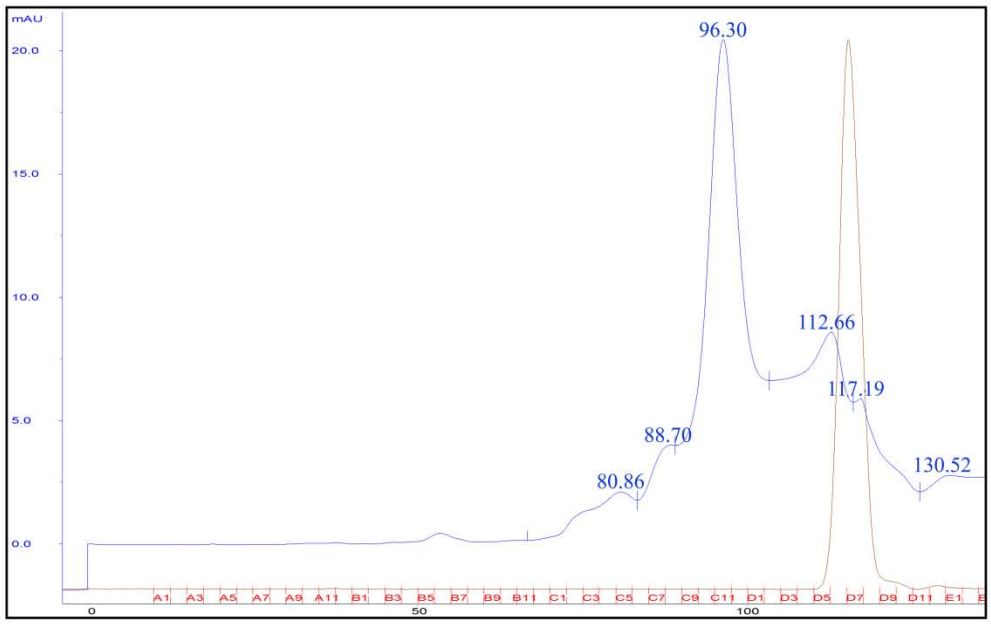

A
more contaminants
= imidazole concentration
= UV280 (eluted protein)
less contaminants
B
= imidazole concentration
= UV280 (eluted protein)
500 mM
CB515 scFv
contaminants
101 mM
106 mM
111 mM
C
148
98
64
50
36
22
16
kDa
101 mM imidazole
500 mM imidazole
CB515 scFv

Figure 2.4: Size exclusion chromatography and quaternary structure determination of the CB515 scFv. Affinity-purified fractions containing the CB515 scFv were run through a Superdex 200 16/60 size exclusion column and eluted with GIBCO PBS to assess whether the scFv is monomeric or multimeric. **A.** UV_{280} absorbance from size exclusion chromatography is shown. Volume of 1x PBS needed for peak elution (labeled above each peak) corresponds to the size of proteins eluted in a particular peak. 96.30 ml is the predominant peak and so was further analyzed. **B.** Fractions from 96.30 ml peak were analyzed by immunoblot. The CB515 scFv elutes with 96.30 ml PBS, suggestive of a monomeric scFv as compared to known standards. MALDI-TOF mass spectrometry analysis revealed the molecular weight of the monomeric scFv to be 26.645 kDa.

A


mAU
20.0
15.0
10.0
5.0
0.0
96.30
112.66
117.19
88.70
80.86
130.52
0
50
100

B

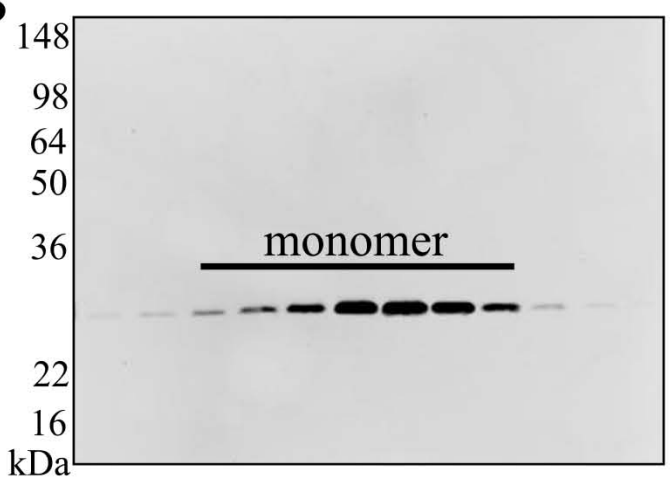

148
98
64
50
36
22
16
kDa
monomer

Figure 2.5: Three dimensional structure of the CB515 Fv region. A molecular model of the CB515 Fv was constructed based on homology of the protein sequence to a library of known, crystallized variable regions using the web antibody modelling (WAM) service provided by the University of Bath (UK). Variable heavy and light chains are in light red and blue, respectively. CDRs of the heavy chain are in dark red, and CDRs of the light chain are in dark blue, illustrating the antigen binding site.

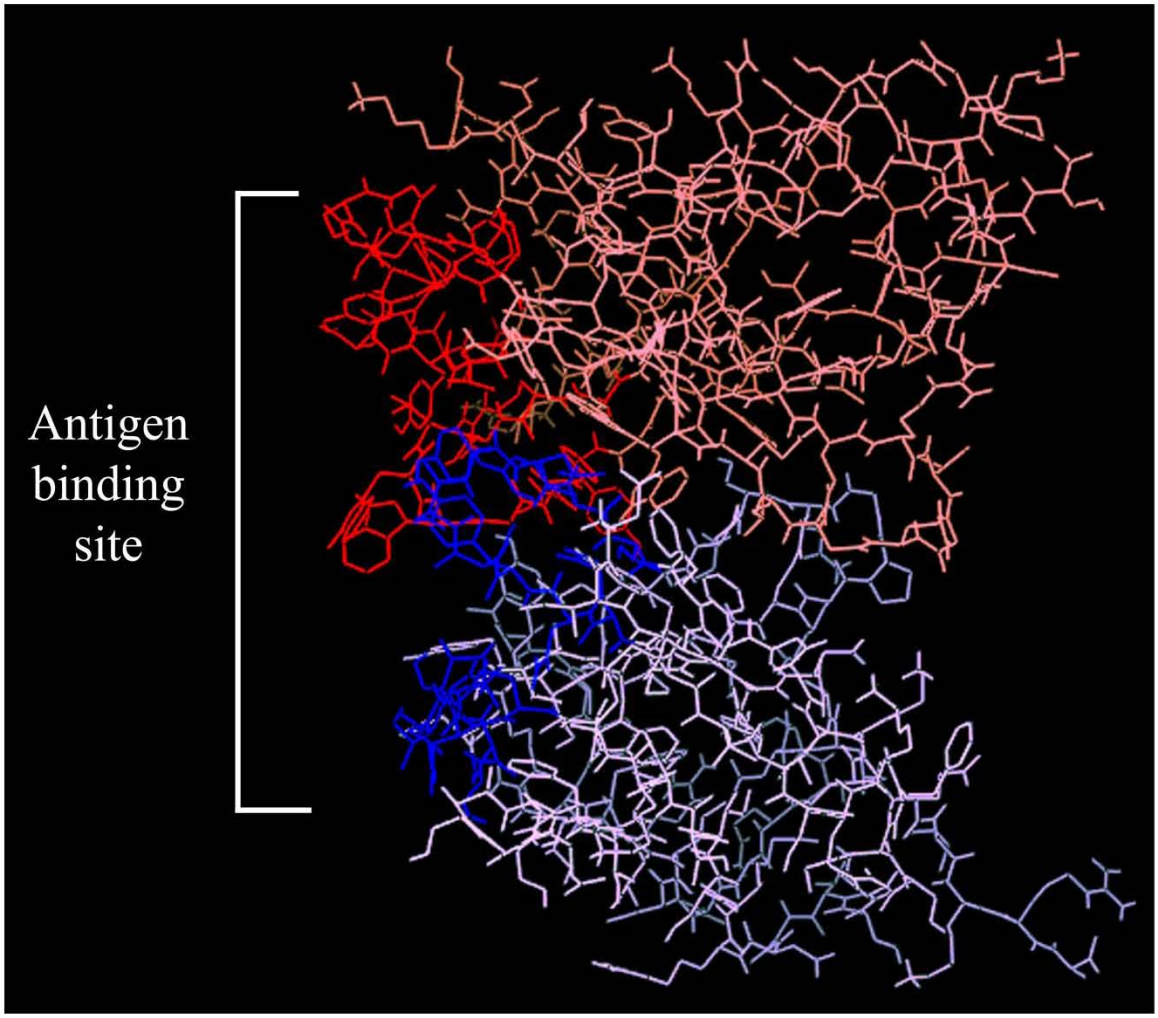

Antigen
binding
site

Figure 2.6: The CB515 scFv binds its antigen in immunoblot. Blots containing whole cell lysate of relapsing fever *Borrelia* were probed with either 1μg/ml of **A.** monoclonal CB515 as a positive control to locate antigen or **B.** CB515 scFv to determine if the scFv can recognize and bind its antigen. The CB515 scFv recognizes and binds to an antigen corresponding to the same band recognized by monoclonal CB515. This indicates that the CB515 scFv is capable of binding the antigen to which its parent antibody is specific in immunoblot.

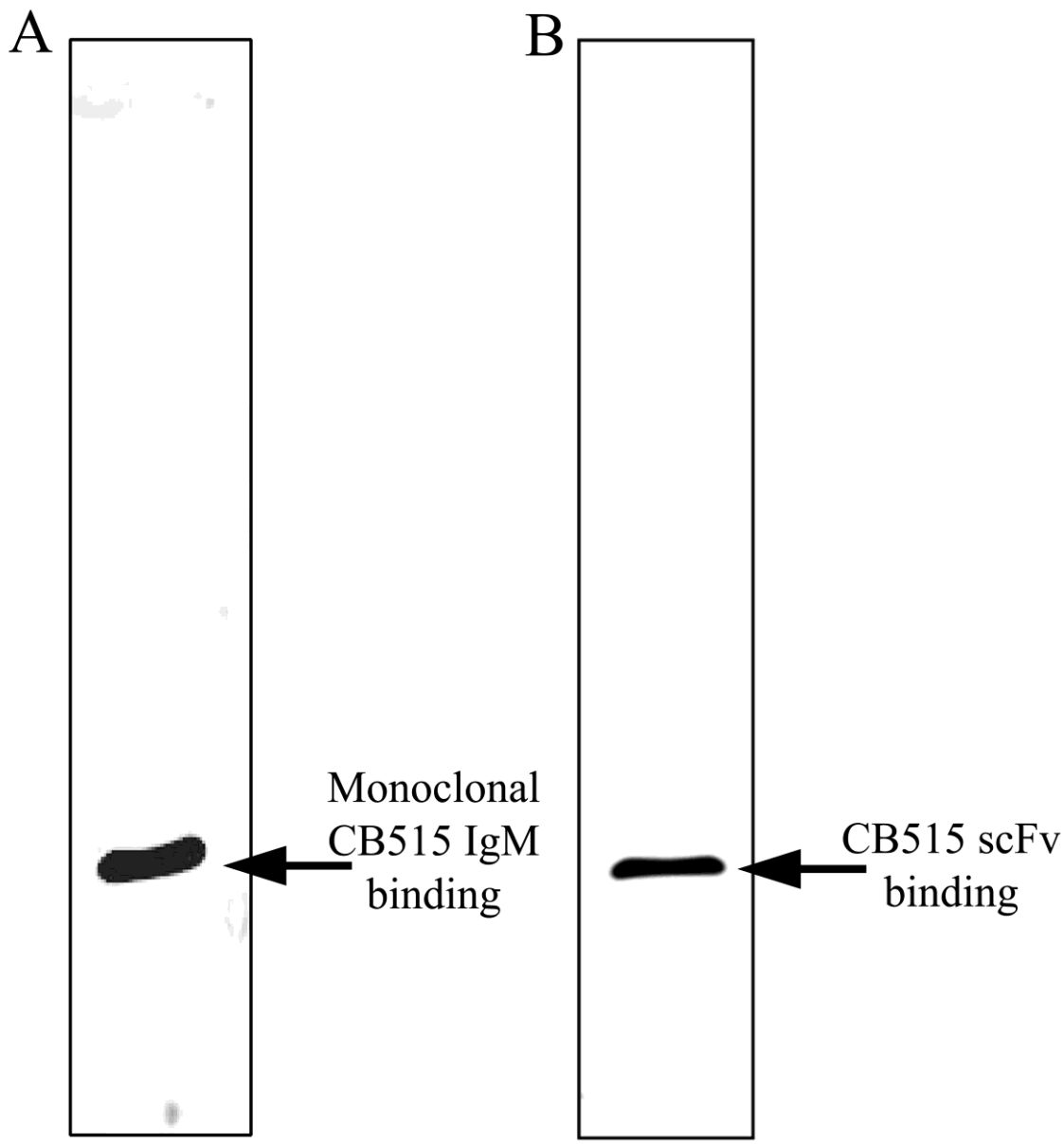

A
B
Monoclonal
CB515 IgM
binding
CB515 scFv
binding

Figure 2.7: The CB515 scFv is species and serotype-specific. **A.** Coomassie blue-stained SDS-PAGE gel of *Borrelia* lysates: original infecting serotype (1), an escape mutant of this same species (2), *B. hermsii* (3), *B. crocidurae* (4), and *B. burgdorferi* (5). A 19 kDa band can be clearly seen in the lysate of the original infecting serotype (arrow, lane 1) representing the Vsp recognized by CB515. Of note is the absence of this antigen in the escape mutant lysate (arrow, lane 2). **B.** Corresponding immunoblot using CB515 scFv as a probe. The CB515 scFv only binds the 19 kDa Vsp band in lane 1, confirming that the scFv is species-specific and serotype-specific.

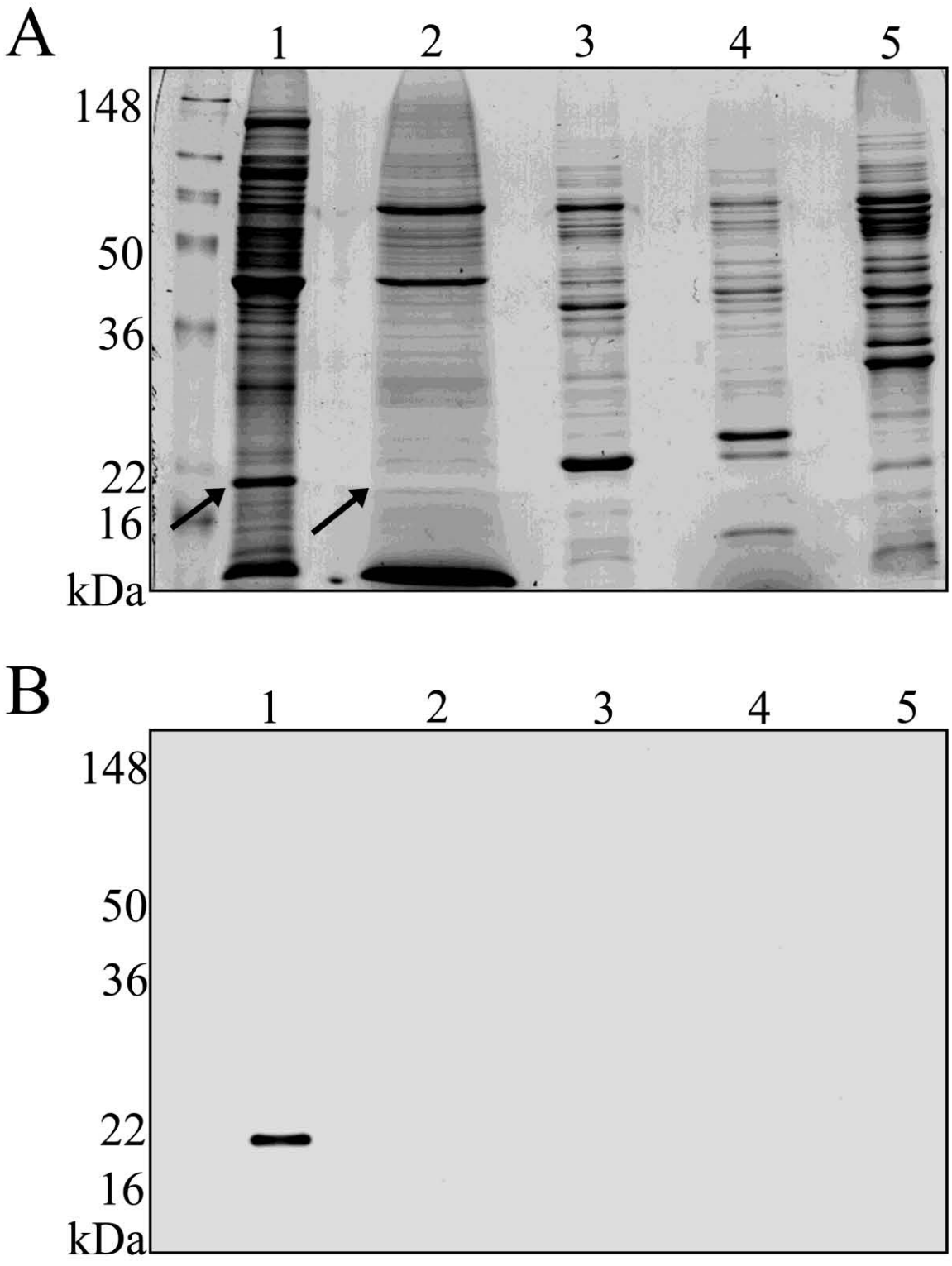

A
1
2
3
4
5
148
50
36
22
16
kDa
B
1
2
3
4
5
148
50
36
22
16
kDa

Figure 2.8: The CB515 scFv binds to whole cells of relapsing fever *Borrelia*. A – G. Spirochetes were fixed and treated with 55 μg of the CB515 scFv detected by a goat-anti mouse IgG FITC conjugate and viewed at 40X (A – D) and 100X (E – G). Numerous fluorescence micrographs clearly indicate that the CB515 scFv can bind its native antigen on whole relapsing fever *Borrelia*. **H.** When treated with identical concentrations of an irrelevant scFv, spirochete fluorescence was not detected. **I.** As a positive control 10 μg of monoclonal CB515 was used showing fluorescence identical to that of the scFv in A – G. All size bars = 20 μm.

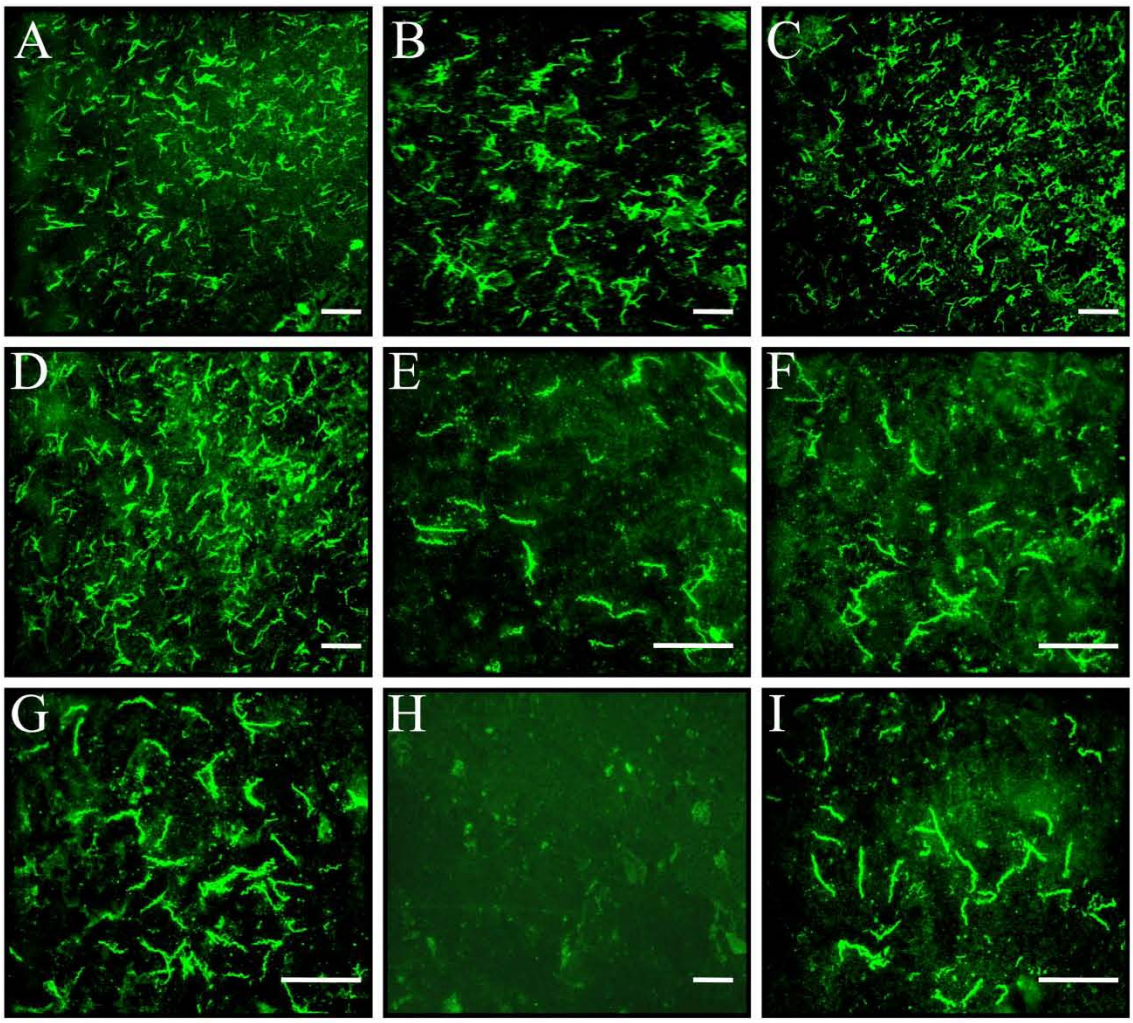

A
B
C
D
E
F
G
H
I

Figure 2.9: The CB515 scFv exhibits a lower level of antigen binding compared to equimolar concentrations of monoclonal CB515. Biotinylated CB515 scFv or monoclonal CB515 was added to ELISA wells coated with whole spirochetal lysate at the following concentrations: 0.6, 9.0, 19, 38, 188, or 282 nM. The ELISAs were developed with alkaline phosphatase-conjugated streptavidin. Monoclonal CB515 exhibits a higher level of binding than does the CB515 scFv. It takes significantly higher molar concentrations of the CB515 scFv to equal the binding exhibited by its parent antibody, such as 188 nM scFv to equal the level of binding of 9.0 nM monoclonal CB515. The difference in binding is most likely due to the pentameric nature of monoclonal CB515 which presumably accounts for a greater avidity (synergism of the strength of individual interactions when they work as a group) compared to the CB515 scFv monomer. Results are from 3 independent experiments all performed in triplicate, ANOVA, $p < 0.001$.

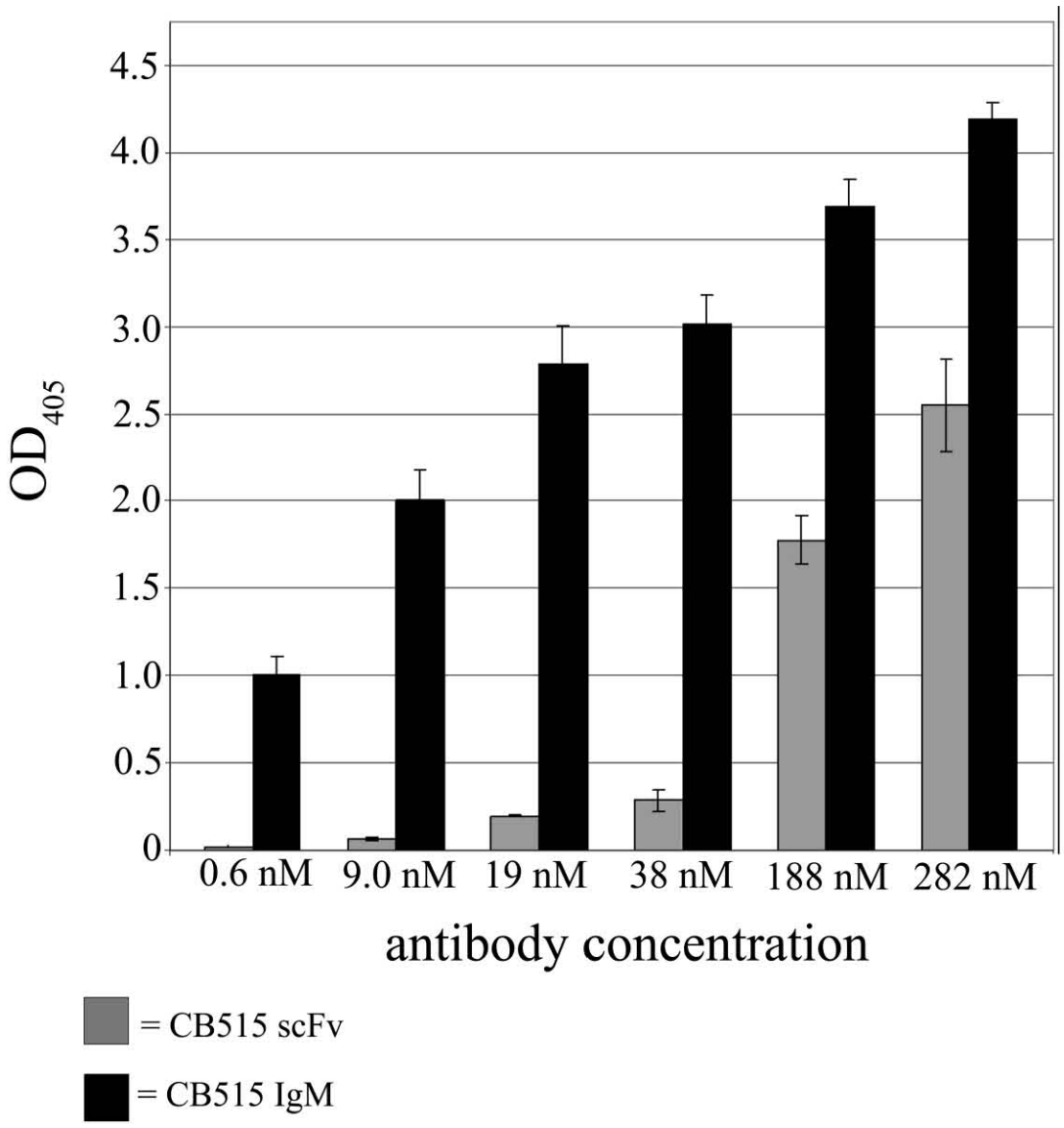
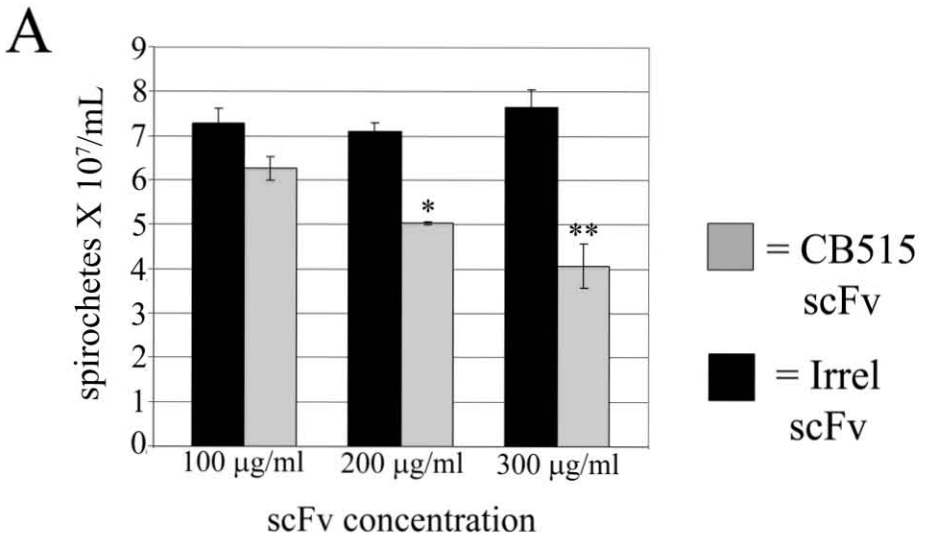
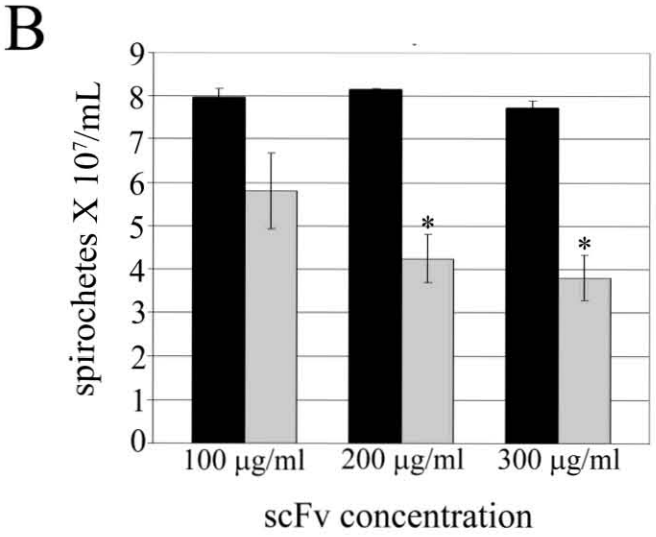

4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
OD405
0.6 nM
9.0 nM
19 nM
38 nM
188 nM
282 nM
antibody concentration
= CB515 scFv
= CB515 IgM

Figure 2.10: CB515 scFv retains the bactericidal activity of its parent antibody. *In vitro* bactericidal assays using 100, 200, or 300 μg/ml of CB515 scFv (gray bars) compared to an irrelevant scFv (black bars) (A – C). **A.** Results after 15 minutes of treatment with the CB515 scFv or an irrelevant scFv at 33°C. A significant bactericidal effect can be observed using 200 and 300 μg/ml compared to negative controls. **B.** Results after 75 minutes of scFv treatment at 33°C. Increased spirochete death is observed for all CB515 scFv concentrations compared to spirochete death levels in A. The results of A and B are suggestive of a dose-dependent bactericidal effect for the CB515 scFv. Results are from 3 independent experiments performed in triplicate, ANOVA, * = $p < 0.01$, ** = $p < 0.001$. **C.** *In vitro* bactericidal assay using 100 - 300 μg/ml of CB515 scFv (circles) or 300 μg/ml of irrelevant scFv (square) for 2 hours at 33°C. The CB515 scFv is bactericidal at all concentrations tested and exhibits a clear dose-dependent effect. Results are from 3 independent experiments performed in triplicate. All CB515 scFv concentrations result in significantly lower spirochete numbers than treatment with an irrelevant scFv, ANOVA, $p < 0.001$.


A
spirochetes X 10^7/mL
9
8
7
6
5
4
3
2
1
0
*
**
100 µg/ml
200 µg/ml
300 µg/ml
scFv concentration
= CB515 scFv
= Irrel scFv


B
spirochetes X 10^7/mL
9
8
7
6
5
4
3
2
1
0
*
*
100 µg/ml
200 µg/ml
300 µg/ml
scFv concentration

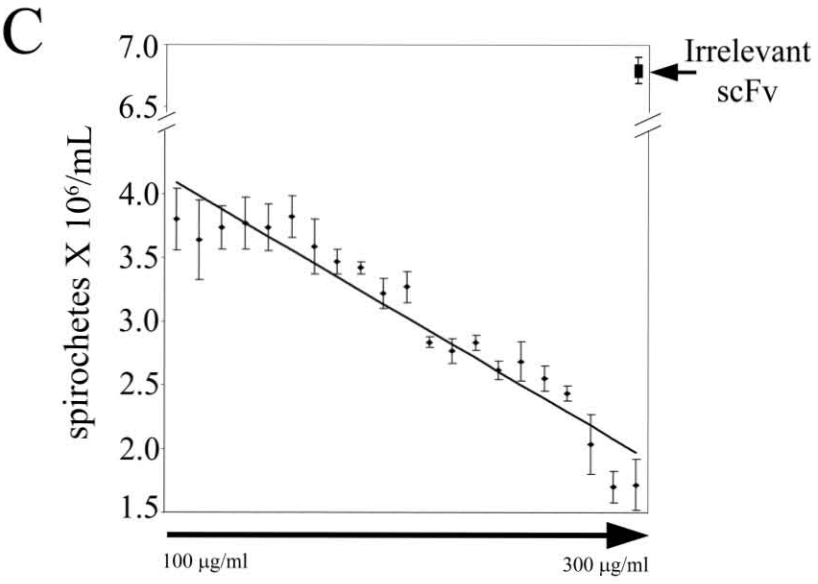
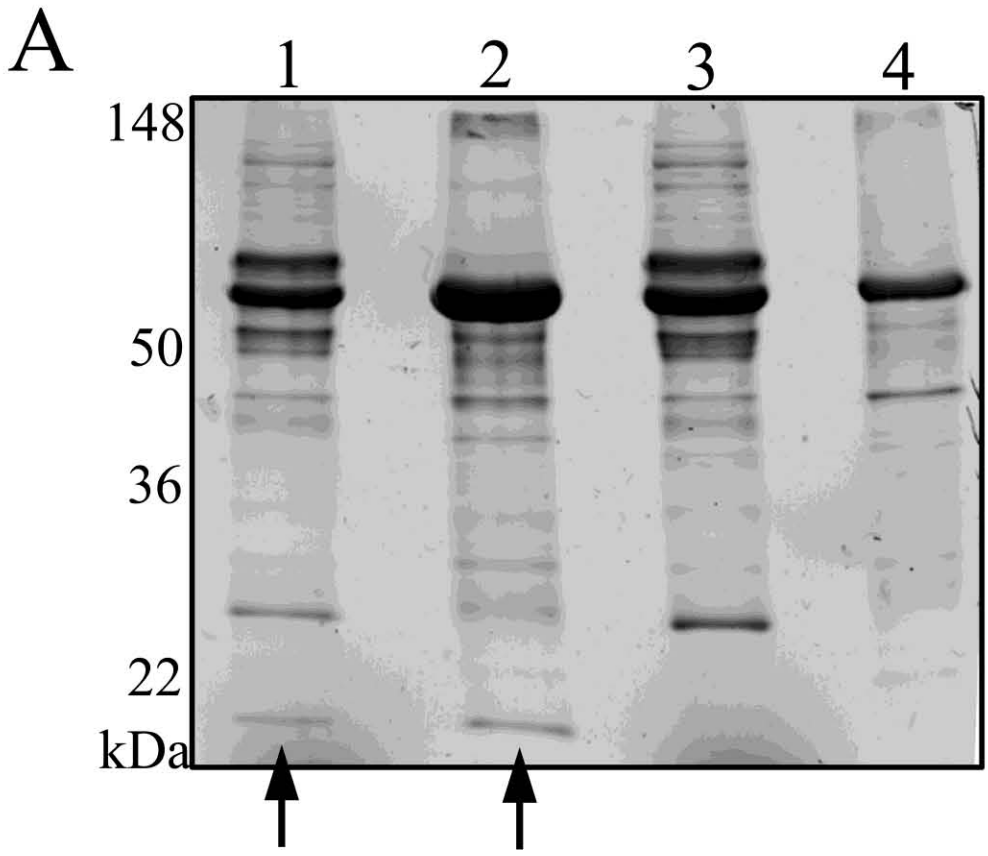

C
spirochetes X 10^6/mL
7.0
6.5
4.0
3.5
3.0
2.5
2.0
1.5
Irrelevant scFv
100 µg/ml
300 µg/ml

Figure 11: The CB515 scFv eliminates an entire serotype population. Spirochetes were exposed to 300 μg/ml of an irrelevant scFv or the CB515 scFv for 2 hours and subsequently used to inoculate naïve mice to determine their capacity for *in vivo* infection. Mice became infected in all cases thus, spirochetes were harvested, lysed, and separated by SDS-PAGE to determine serotype identity as a marker for CB515 scFv killing efficiency. **A.** Coomassie-stained gel containing lysates from spirochetes exposed to an irrelevant scFv (lanes 1 and 2) or the CB515 scFv (lanes 3 and 4) prior to murine infection. Of note is the absence of the 19 kDa antigen of CB515. **B.** Immunoblot corresponding to the gel in A, showing the killing efficiency of the CB515 scFv. The blot was probed with the CB515 scFv to identify serotypes. Those spirochetes exposed to an irrelevant scFv (lanes 1 and 2) still exist as the original infecting serotype whereas those exposed to the CB515 scFv (lanes 3 and 4) are composed of escape mutants, demonstrating that the CB515 scFv completely eliminates those spirochetes to which it is specific (100% killing efficiency).


A
1
2
3
4
148
50
36
22
kDa

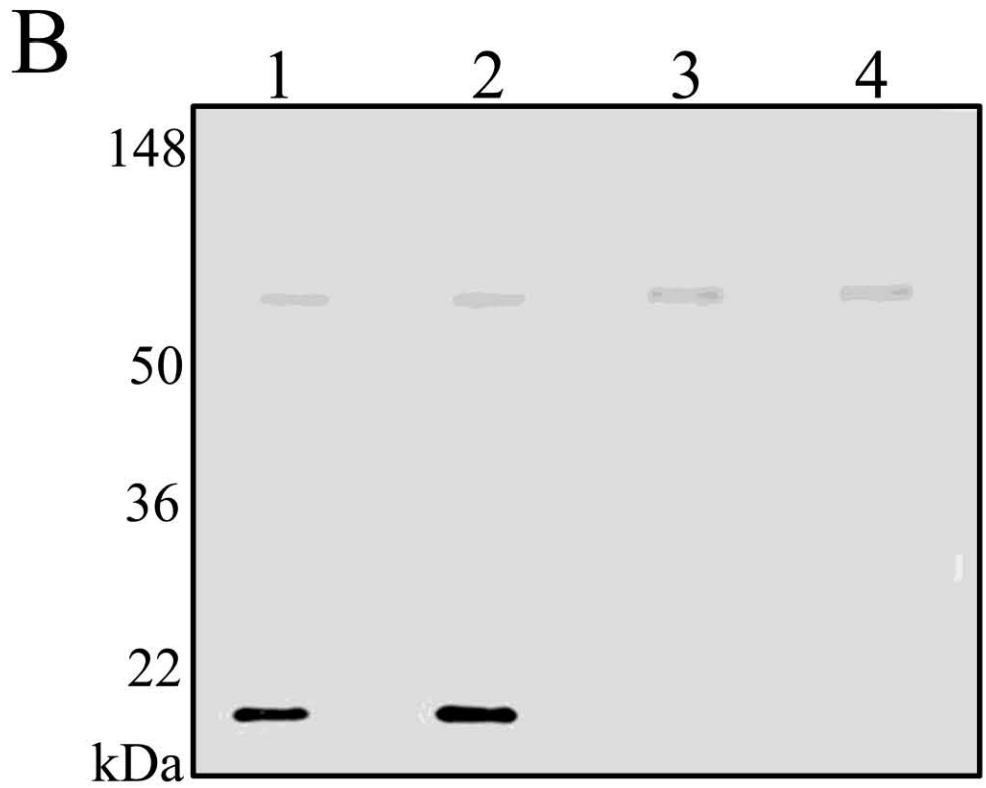

B
1
2
3
4
148
50
36
22
kDa

Figure 2.12: CB515 scFv causes ultrastructural damage to relapsing fever *Borrelia*. **A – B.** Negative controls (300 μg/ml irrelevant scFv) show spirochetes with an intact outer membrane and classic morphological features. **C – D.** After treatment with 100 μg/ml CB515 scFv, exposure of periplasmic flagella and minor blebbing can be observed indicating slight damage. **E – H.** Treatment with 200 μg/ml CB515 scFv causes considerable damage to the outer membrane as is evidenced by increased exposure of periplasmic flagella (F, G) and the formation of large membrane blebs (F, G, pronounced in E). **I – P.** Treatment with 300 μg/ml CB515 scFv causes the most damage to spirochetes, with even more exposure of periplasmic flagella (J – M, O – P), more severe membrane blebs that have been released from the spirochete surface (pronounced in K – L, N, P), and increased degradation of spirochetes (seen clearly in I – J, M, O – P). All size bars = 500 nm.

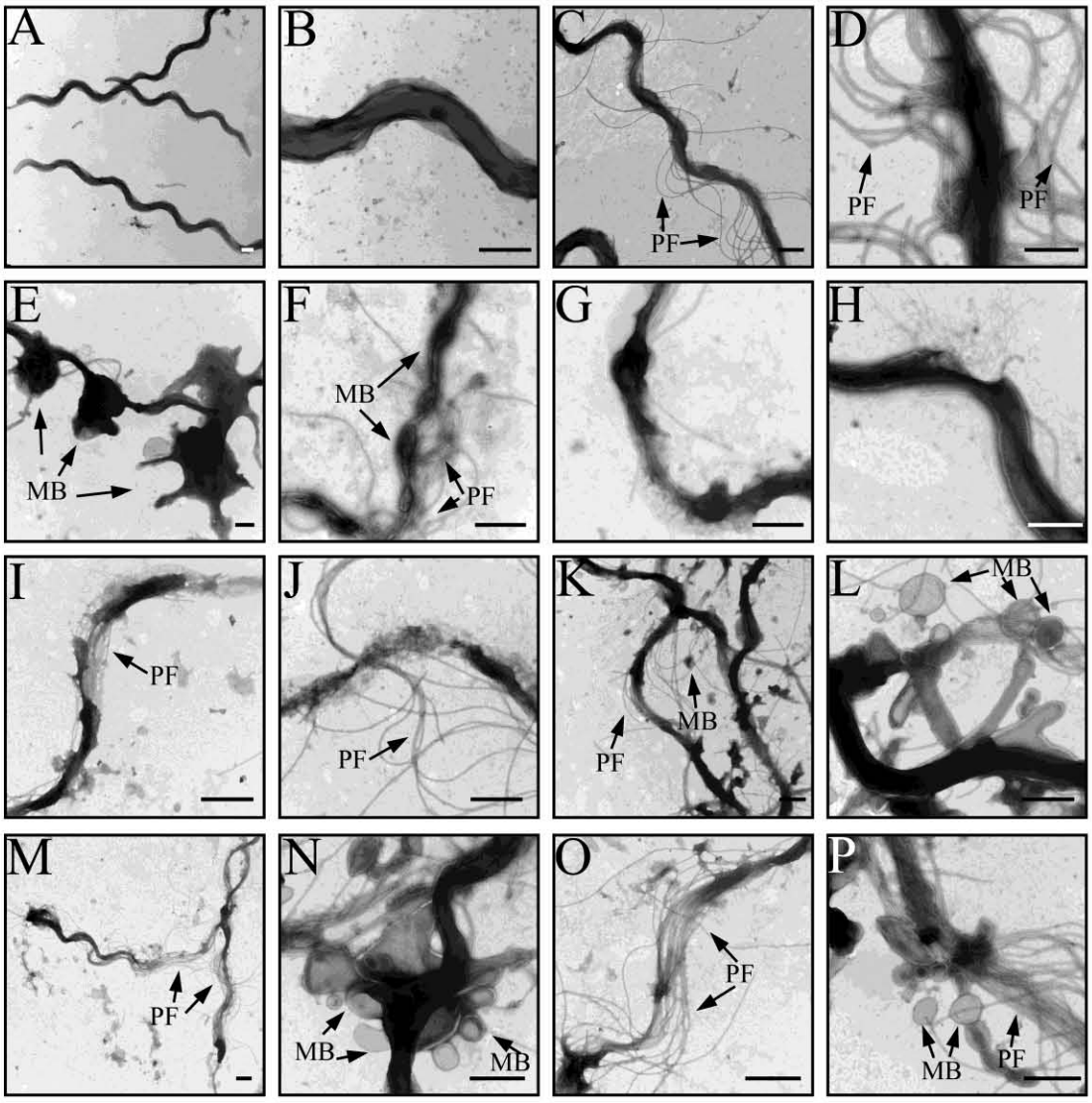

A
B
C
PF
D
PF
PF
E
MB
MB
F
MB
PF
G
H
I
PF
J
PF
K
MB
PF
L
MB
M
PF
N
MB
MB
O
PF
P
MB
PF

Figure 2.13: Examination of fine spirochete ultrastructure in thin sections reveals extensive damage to the pathogen upon CB515 scFv treatment. Relapsing fever *Borrelia* were treated with 200 μg/ml of **A – E.** an irrelevant scFv, or **F – L.** the CB515 scFv for 75 minutes, fixed, embedded, sectioned, stained and examined by TEM. As in Figure 2.12, spirochetes exposed to an irrelevant scFv are never damaged and possess a normal, intact outer membrane (A – E). After treatment with the CB515 scFv all spirochetes are generally damaged with a more ragged appearance with features including distension of the outer membrane (double arrows) signifying membrane blebbing, release of membrane blebs (BL) from the spirochete surface, and exposure of periplasmic flagella (PF) indicating complete outer membrane rupture (F – L). All size bars = 500 nm.

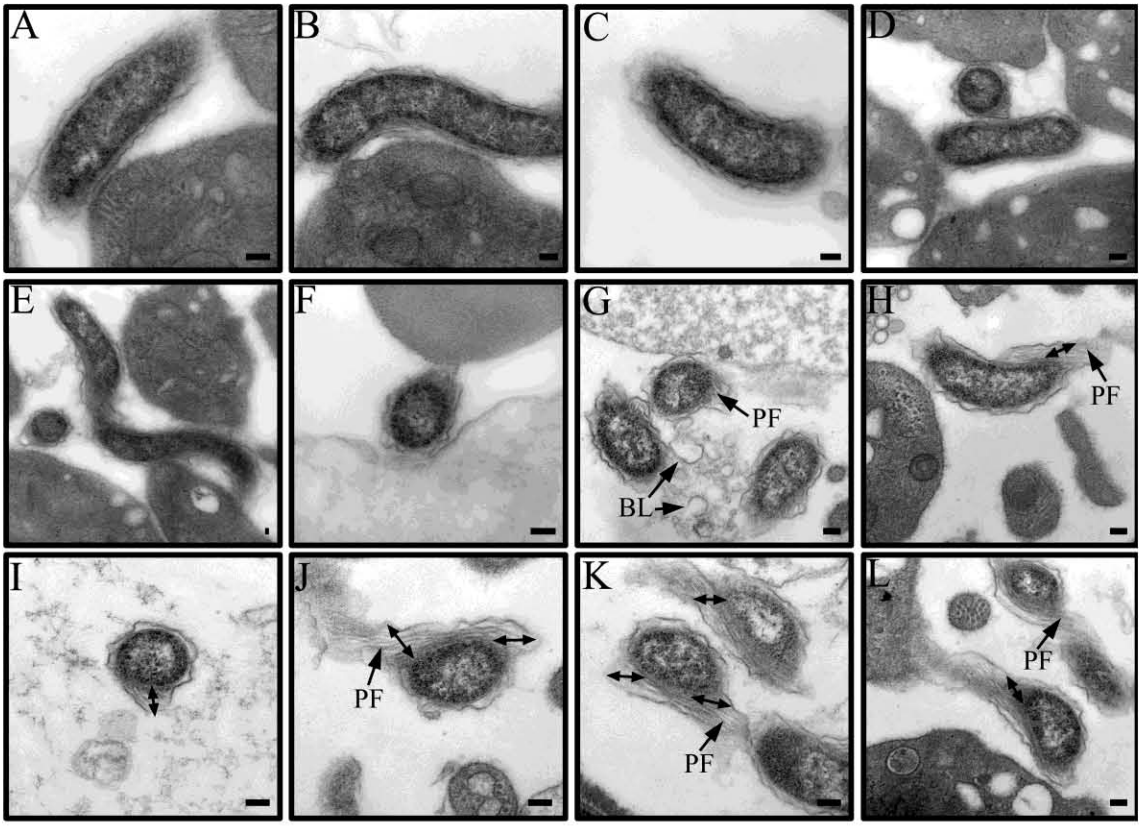
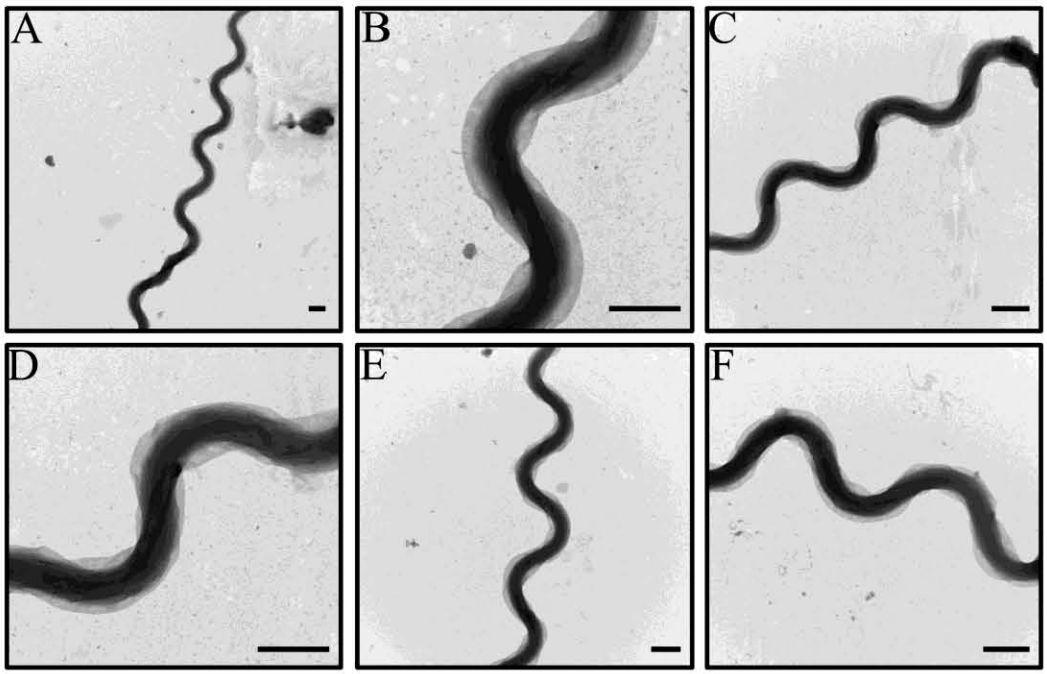

A
B
C
D
E
F
G
PF
BL
H
PF
I
J
PF
K
PF
L
PF

Figure 2.14: The CB515 scFv has no bactericidal activity against a relapsing fever escape mutant. A – F. Relapsing fever Borrelia escape mutants were created by passive immunization with CB515 as described in section 3.1.7, treated with the CB515 scFv for 75 minutes, and examined by negative-stain TEM. As indicated by its species and serotype binding specificity (Figure 2.7), the CB515 scFv exhibits no bactericidal effect against an antigenic variant of its originally targeted serotype. All size bars = 500 nm.


A
B
C
D
E
F

CHAPTER 3 - THE ANTIBODY:PATHOGEN INTERFACE - The Bactericidal Mechanism of Complement-Independent Antibodies to *Borrelia* is Osmolytic and Relies on Direct Pore Formation

3.1 CHAPTER SUMMARY

CB2, a complement-independent bactericidal IgG1 against the OspB of *B. burgdorferi*, increased the permeability of the outer membrane upon binding, resulting in its osmotic lysis by the creation of openings in the membrane of 2.8 – 4.4 nm as determined through osmoprotection experiments. Cryo-electron microscopy and tomography demonstrated that CB2 causes the formation of outer membrane projections and large breaks which may precede the general increase in permeability of the outer membrane. The projections/blebs created in the outer membrane are of a base diameter which correlates to that of the large breaks, suggesting that the projections bleb off resulting in the breaks. CB2 is directly involved in the creation of the outer membrane projections/blebs and removes them from the spirochete surface while bound to OspB. Once the outer membrane is permanently ruptured by CB2, the inner membrane undergoes osmotic lysis resulting in death of the organisms.

3.2 INTRODUCTION

Complement-independent antibodies against *Borrelia* have been observed to cause death to the spirochetes against which they are directed (77, 82, 297). It is known that these antibodies require divalent cations such as Mg^{2+} and Ca^{2+} to exert their effects (109) and, once bound, the antibodies cause slight structural changes in their antigens (34, 183). With respect to the antibodies themselves the bactericidal function resides in the variable region (chapter 2, (205)). Beyond this, however, little is known regarding their actual mechanism of action. Through transmission electron microscopy complement-independent antibodies have been indirectly shown to damage the outer membrane of *Borrelia* (77, 82, 205, 297) but the precise nature of this damage also remains elusive. Complement, through formation of the membrane attack complex, and antimicrobial peptides exert their microbicidal effects by creating pores in the outer

membrane of pathogens, rendering them susceptible to osmotic lysis (52, 173, 204). Since these well-known immune components exert their effects through pore formation we reasoned that complement-independent bactericidal antibodies could, in their own way, also form openings in the outer membrane. Through a series of osmoprotection experiments, cryo-electron tomography, and immunogold TEM we sought to investigate this possibility as the mechanism of action for complement-independent bactericidal antibodies. For these studies we used CB2 against the OspB of *B. burgdorferi*. CB2 was specifically chosen as many of the critical experiments for this chapter involved *in vitro* growth assays which would not be possible to conduct with CB515 and the uncultivable species of relapsing fever *Borrelia* against which it is directed.

3.3 MATERIALS AND METHODS

Osmoprotection Experiments

B. burgdorferi strain B31 was harvested from mid-log phase culture and resuspended in BSK-H medium (Sigma) supplemented with raffinose (Fisher), inulin (Fisher), dextran 6000 MW (Fluka), dextran 81500 MW (Sigma), or dextran T500 (Pharmacia). CB2 or an irrelevant IgG (Sigma) were added to the spirochetes at 2 μg/ml and CB2 alone was also added to spirochetes as a positive control for 15 minutes, after which spirochetes were enumerated or prepared for negative-stain TEM analysis. Negative-stain preparation and analysis were performed as previously described (205). For experiments where *B. burgdorferi* was cultured in the presence of the antibodies and sugars for 4 days, the starting concentration was 3.2×10^6 or 5×10^6 spirochetes/ml for dextran T500 and sucrose (JT Baker), respectively, and antibodies were added at 2 μg/ml. Spirochetes were grown at 33°C and enumerated under dark-field microscopy daily.

For experiments which investigated the growth/recovery of spirochetes in the presence of osmoprotectants following CB2 treatment, *B. burgdorferi* were harvested and introduced into media containing dextran T500 or sucrose as described above. CB2 or an irrelevant IgG was added to spirochetes in sugar medium at a concentration of 2 μg/ml or CB2 was added to spirochetes in the absence of the sugars. Spirochetes were incubated with the Abs for 30 minutes at 33°C after which 1.6×10^5 spirochetes from each

preparation were inoculated into fresh media containing the same sugars but lacking antibodies. Cultures were incubated at 33°C for up to 10 days and spirochetes were enumerated at days 5, 6, 8, 9, and 10 to assess the growth/recovery of *B. burgdorferi* following CB2 exposure in the presence of particular osmoprotectants.

MAb Purification

CB1, CB2, CB10, and CB312 hybridoma supernatants were obtained as previously described (77). The antibodies were purified by affinity chromatography on HiTrap 1 ml Protein G HP columns (GE Healthcare/Amersham) using an Akta FPLC according to the manufacturer's instructions.

Cryo-Electron Microscopy Sample Preparation

A thin carbon coat was evaporated onto Quantifoil electron microscopy (EM) specimen grids (R3.5/1; Quantifoil Microtools, Jena, Germany); then the grids were treated with a 10-nm colloidal gold solution to provide fiducial markers for alignment of the tomographic tilt series (268). Approximately 4-5 µl of cell suspension in culture medium were applied to freshly glow-discharged grids, excess medium was blotted with filter paper, and the grids were immediately plunge-frozen into liquid ethane at liquid-nitrogen temperature (102). Grids were stored under liquid nitrogen until EM examination. Specimens were made for control cells (unexposed to antibodies or exposed to an irrelevant IgG), and cells exposed to CB2 at 2 and 4 µg/mL for 2 and 10 min.

Cryo-Electron Microscopy and Tomographic Reconstruction

Images were recorded at -178°C, using a JEOL JEM 4000FX equipped with a Gatan GIF2002 energy filter and 2K x 2K CCD camera, with a pixel resolution of 1.8 nm. The microscope was operated at 400 kV acceleration voltage, in zero-loss energy-filtered mode. Single-axis tilt series were collected with a 1° increment and 120° angular range. The thickness of the ice layer was ~350 nm, as measured by electron energy-loss spectroscopy (106). This was sufficient to avoid flattening of the cells, as evidenced by the cross-sections from the tomograms. The total electron dose for a tilt series was ~100

$e^-/Å^2$, with the higher dose used with thicker specimens. The calculated resolution (90) in the x-y plane (parallel to grid plane) was 8 nm. The calculated z (depth) resolution (280) was 12 nm, due to the elongation factor from the "missing wedge" caused by the limited tilt range. The underfocus value, 15 μm, was chosen to maximize the transfer of information at the expected resolution limit, in order to optimize the signal-to-noise ratio (234). All image processing was done using SPIDER (122), and the reconstructions were computed by weighted back-projection (279). Three independent tomographic reconstructions were computed for different fields, each containing all or parts of 1-2 cells treated with 4 μg/ml CB2 antibody. In addition, single images were recorded from over 100 individual cells under the six experimental conditions.

Post-Reconstruction Image Processing and Visualization

Tomograms were processed for segmentation by low pass filtering to 7.6 nm resolution (the first zero in the phase contrast function) and binning to 3.6 nm pixel size. The images were de-noised for segmentation by moderate anisotropic diffusion filtering (121). Membrane surfaces were rendered in AMIRA (Visage Imaging Inc.) by two-dimensional region growing of the membranes and supplemented by hand tracing. From the contours a three-dimensional mesh was generated which was smoothed by the surface smoothing function of AMIRA to remove jagged edges.

CB2-Induced Antigen Release Immunoblot Assays

Experiments that measured the release of antigens from the spirochete surface into the surrounding medium were performed by immunoblot. Spirochetes were treated with CB2 in the presence of dextran T500 for 15, 60, or 120 minutes, centrifuged, and the supernatants were run on 12.5% SDS-PAGE, blotted to nitrocellulose, and probed with CB2 or CB10 to detect OspB and OspA, respectively. Dextran T500 was used to prevent spirochete lysis upon CB2 exposure, ensuring that what was measured in the supernatant was released from the spirochete surface and not due to lysis. Blots were also probed with CB312 to detect Dnak, which would be indicative of spirochete lysis.

Immunogold-Detected CB2 TEM Experiments

Experiments that detected localization of CB2 on the spirochete surface and release membrane blebs were completed by treating *B. burgdorferi* with CB2 for 15 minutes at 33^{o}C and applying the samples to polyvinyl formal-coated grids (Ernest F. Fullam). Once on the grids, spirochetes were fixed in 1% glutaraldehyde (Sigma) in PBS and blocked for 30 minutes at room temperature in 1% BSA (Sigma). Grids were then placed in a 1% BSA solution containing goat anti-mouse IgG gold-labeled antibodies at a dilution of 1:30 for 1 hour at room temperature. Subsequently, grids were prepared for analysis by negative-stain TEM according to that described in section 2.7.

3.4 RESULTS

Complement-Independent Bactericidal Antibodies Directly Damage the Outer Membrane of *Borrelia* Rendering the Inner Membrane Susceptible to Osmotic Lysis

Based on our previous work and that of others (Figures 2.12 and 2.13) (77, 82, 109, 205), we had a good indication that the mechanism of complement-independent bactericidal antibodies to *Borrelia* had to be membrane interactive. To address specifically the mechanism of bactericidal action of these antibodies, we asked if their effects occur at both the inner and outer membranes or just the outer membrane. It was thought that either the antibodies acted directly at both membranes, directly at the outer membrane with signals transmitted to trigger inner membrane destruction, or directly at the outer membrane resulting in indirect osmotic lysis of the inner membrane following outer membrane disruption. To investigate this, a well-known inner membrane osmoprotectant, sucrose, was used. Sucrose was also chosen as it has been shown to maintain *B. burgdorferi* spheroplasts stably for several days (54). If the spirochetes exhibited an inhibition of death when treated by the CB515 scFv in the presence of sucrose, it would indicate that the CB515 scFv causes osmotic lysis. When enumerated by dark field microscopy, relapsing fever *Borrelia* appeared to survive when exposed to the CB515 scFv in the presence of sucrose for 60 minutes as compared to controls and the CB515 scFv alone (Figure 3.1A). This is of interest, of course, since we have confirmed that exposure to the CB515 scFv results in a decrease in spirochete numbers

(205), and so it appears that when *Borrelia* are in the presence of sucrose they are protected osmotically.

A similar result was obtained when *B. burgdorferi* was exposed to CB2, a complement-independent bactericidal IgG against OspB, in the presence of sucrose for 15 minutes (Figure 3.1B). This demonstrates that this phenomenon is shared among complement-independent bactericidal antibodies to *Borrelia*. These protective effects were truly due to sucrose controlling osmotic pressure in the extracellular environment as glycerol, a sugar alcohol known to diffuse through the inner membrane, could not replicate this protection (Figure 3.1C). Sugars larger than sucrose (raffinose and inulin) and thus not expected to diffuse through the inner membrane provided osmotic protection identical to that of sucrose (Figure 3.1C).

Sucrose-protected spirochetes were also observed at the ultrastructural level via negative-stain TEM analysis (Figure 3.2). Relapsing fever *Borrelia* exposed to the CB515 scFv (Figure 3.2E – H) or *B. burgdorferi* exposed to CB2 (Figure 3.2M – P) in the presence of sucrose for 15 minutes were devoid of an outer membrane as was evidenced by the exposure of periplasmic flagella and flattening of the spiral morphology. The protoplasmic cylinder, however, remained intact indicating that the inner membrane was unaffected as opposed to when sucrose is absent (Figure 3.2C –D, K – L). These results suggest that complement-independent bactericidal antibodies against *Borrelia* have direct effects at the outer membrane and that destruction of the inner membrane occurs via osmotic lysis, an indirect result of outer membrane destruction by these antibodies.

Since the CB515 scFv and CB2 appear to function in an identical manner, many of the following experiments utilized CB2 instead of the CB515 scFv. This decision was made due to the fact that CB2 is directed against OspB of *B. burgdorferi* strain B31, a high-passage *Borrelia* strain that grows readily in culture as opposed to the uncultivable relapsing fever *Borrelia* to which the CB515 scFv is specific.

Destruction of the *Borrelia* Outer Membrane Occurs By Formation of Openings and Osmotic Lysis

A standout characteristic of exposure to complement-independent bactericidal antibodies is the formation of blebs in the outer membrane of *Borrelia* (Figures 2.12 and 2.13) (77, 82, 109, 205, 297). This consistent observation led to the idea that outer membrane blebbing could result in the formation of openings or pores, which would cause osmotic lysis. To investigate this, we chose dextran T500 and sucrose (of 28 nm and 0.92 nm molecular diameter, respectively) for potential osmoprotection in a 4 day growth inhibition assay in the presence of CB2 and several control antibodies (Figure 3.4). Control antibodies used were an irrelevant IgG or anti-*B. burgdorferi* IgGs (which are not complement-independent bactericidal antibodies) against cytosolic Dnak (CB312), periplasmic flagella (CB1), and outer membrane OspA (CB10). Interestingly, OspA is co-transcribed with OspB and both are very similar in their primary structure and isoelectric points (38, 42).

We first confirmed that dextran T500 provides osmotic protection to spirochetes treated with CB2 for 15 minutes (Figure 3.3). For the growth inhibition assay, control cultures grew normally whereas spirochetes with CB2 decreased in numbers and did not grow. Spirochetes cultured with CB2 and dextran T500 did not grow but did not decrease in numbers (Figure 3.4A). In contrast, spirochetes cultured with CB2 and sucrose were not protected, decreasing to similar numbers as those cultured with CB2 alone (Figure 3.4B). That dextran T500 inhibited lysis is an indication that *B. burgdorferi* were protected osmotically from injury to the outer membrane by the action of CB2. The decrease in spirochete numbers in the presence of sucrose contrasts with results seen prior (Figure 3.1), where the presence of sucrose resulted in the apparent survival of spirochetes after 15 or 60 minutes of incubation. However, these results make sense, as over an extended period of time, spirochetes devoid of an outer membrane in the presence of an inner membrane osmoprotectant would be expected to progress to spheroplasts, which would be overlooked during enumeration (54). In fact, formation of *Borrelia* spheroplasts has been observed as a step in the bactericidal process induced by CB2 (109). Since spirochetes were killed by CB2 in the presence of sucrose but not

dextran T500, it appears that the osmotic protection that prevents lysis is size-dependent, suggesting the presence of openings or pores of a defined size in the outer membrane.

To further analyze the protective effects of dextran T500, *B. burgdorferi* were pre-exposed to CB2 alone or in the presence of dextran T500 for 30 minutes and cultured without antibody to assess post-exposure growth. Spirochetes from the CB2 and dextran T500 preparation had no defect in growth whereas spirochetes from the CB2 alone preparation grew at significantly lower levels (Figure 3.4C). This shows that osmotic protection with dextran T500 allows for normal growth of spirochetes. An identical experiment using sucrose resulted in spirochete growth at lower levels which were comparable to exposure to CB2 alone, demonstrating lack of protection (Figure 3.4D). These experiments suggest that when spirochetes are exposed to CB2 in the presence of dextran T500 they remain intact and capable of normal growth/recovery in contrast to spirochetes exposed to CB2 alone. The fact that inocula equal in spirochete numbers gave rise to such a difference in spirochete numbers during growth probably signifies that many spirochetes counted in the CB2 alone and CB2/sucrose preparations were dead or in the process of dying as dark-field counting does not take this into account. This was also noticed in killing assays that made use of the CB515 scFv and relapsing fever *Borrelia* (205).

To gain further insight into the protection afforded by dextran T500, *B. burgdorferi* incubated with CB2 in the presence of this sugar were analyzed by negative-stain TEM (Figure 3.5). Those spirochetes protected by dextran T500 appeared to have the characteristic membrane blebs, however, exposure of periplasmic flagella, another characteristic consistently observed after incubation with these antibodies and a key indicator of outer membrane disruption, was absent (Figure 3.5I – L). This suggests that the outer membrane was affected by CB2 but not ruptured as a result of the osmotic protection provided by the presence of dextran T500. These images lend support to the hypothesis that CB2 and related antibodies form openings in the outer membrane which would allow periplasmic osmotic changes to occur resulting in lysis of the membrane.

It is possible that spirochete survival may have been resultant of a defect in CB2 binding due to the presence of the large sugar, dextran T500, in the medium. Even though spirochetes looked affected by CB2 in the presence of dextran T500 when

analyzed by dark-field microscopy and TEM, we were still concerned that there may have been a binding defect. In order to investigate this possibility, we conducted an indirect immunofluorescence assay (IFA) in the presence of dextran T500 (Figure 3.6). Live *B. burgdorferi* were exposed to CB2 or an irrelevant IgG in the presence or absence of dextran T500 for 1 hour. CB2 binding was detected with a FITC conjugated antibody and fluorescence was observed. Spirochetes that were exposed to CB2 in the presence of dextran T500 (Figure 3.6C and E) exhibited similar fluorescence to those exposed to CB2 alone (Figure 3.6D and F), indicating that the presence of dextran T500 does not affect CB2 binding. This clearly demonstrates that the difference in the effect of CB2 in the presence of dextran T500 compared to CB2 alone is not due to a defect in antigen binding.

CB2-Induced Osmotic Lysis of the Outer Membrane is the Result of the Formation of Membrane Openings of 2.8 – 4.4 nm in Diameter

The size of the putative openings in the outer membrane that may be responsible for osmotic lysis was determined by using sugars of several molecular sizes. The sizes correspond to the molecular diameters of the sugars, therefore, a sugar of a molecular diameter larger than the openings will stay in the external medium and control osmotic pressure, protecting the outer membrane from lysis. Conversely, a sugar of a molecular diameter smaller than that of the openings will diffuse through and afford no protection to the membrane (219, 352). As shown earlier, sucrose (molecular diameter = 0.92 nm) did not provide osmotic protection to the outer membrane whereas dextran T500 (molecular diameter = 28 nm) did, giving us a wide range of sizes, 0.92 nm – 28 nm, to consider for the putative openings. The sugars (and their molecular diameters) used were: raffinose (1.14 nm), inulin (2.8 nm), dextran 6000 MW (4.4 nm), dextran 81500 MW (12 nm) and dextran T500 (28 nm). After a 15 minute incubation with CB2 and the sugars, spirochetes were examined for outer membrane rupture by negative-stain TEM (Figure 3.5), as this is a critical time point where spirochetes decrease in numbers sharply (Figures 2.10, 3.1, 3.3) (205). Exposure of periplasmic flagella was the key indicator of outer membrane rupture in this experiment. Dextran 81500 MW and dextran 6000 MW served as osmoprotectants to the *Borrelia* outer membrane (Figure 3.7G – L) producing

results identical to dextran T500 (Figure 3.7D – F). Periplasmic flagella exposure began to occur with inulin (Figure 3.7M – O), and was readily apparent with raffinose (Figure 3.7P – R). Thus, we were able to determine that CB2 causes the formation of outer membrane openings in the range of 2.8 – 4.4 nm in diameter based on the molecular diameters of the sugars used and show that these openings result in the osmotic rupture of the outer membrane.

Openings in the Outer Membrane of *B. burgdorferi* Increase in Size During Prolonged Exposure to CB2

To determine whether the pores were of a fixed size or could increase in size over time, we performed osmoprotection studies for 4 days. Raffinose and inulin afforded no protection against CB2 as in the prior experiment (Figure 3.8A – B). Spirochetes were partially protected by dextran 6000 MW as their numbers after 1 day of CB2 exposure were two times higher than those exposed to CB2 alone (Figures 3.8C and 3.9A). A similar trend was observed when *B. burgdorferi* was cultured with CB2 and dextran 81500 MW where spirochetes did not decrease in numbers until the second day (Figures 3.8D and 3.9B – C). Although spirochetes decreased by day two, their numbers were still six times greater than those exposed to CB2 alone (Figure 3.8C). Spirochetes decreased further by the third day but numbers were still three times greater than those exposed to CB2 alone (data not shown). It is important to note that dextrans do not slow the effects of CB2 but rather, osmotic protection is lost in these cases. This is indicative of the presence of membrane openings larger than is suggested by the molecular diameters of these sugars such that the openings in the outer membrane increase in size with increasing incubation time with CB2, and the sugars lose the ability to osmoprotect.

CB2 Rapidly Creates Large Breaks in the Outer Membrane of *B. burgdorferi*

To observe openings formed in the outer membrane at an early time point we analyzed the effects of CB2 against *B. burgdorferi* using cyro-electron microscopy and tomography (Figures 3.10 – 3.12). This is a high resolution technique that allows for three-dimensional visualization of surface structures in great detail without the use of fixatives or dehydration steps. Instead, specimens are instantly frozen, preserving

structures in their native state. Since specimens are immediately frozen following experiments, this allowed us to observe the effects of CB2 at strict 2 and 10 minute time points. Micrographs from this experiment showed distention of the outer membrane of *Borrelia* after exposure to CB2 (Figures 3.10B, E, G and 3.11E, F) which we have observed previously in negative-stain TEM as membrane blebs (Figures 2.12, 2.13, 3.5, and 3.7) (77, 82, 205). We have previously observed disruption of the outer membrane indirectly as determined by the exposure of periplasmic flagella (Figures 2.12, 2.13, 3.2, 3.5, and 3.7) (77, 82, 205); however, the cryo-TEM images clearly show disruptions which appear as breaks in the membrane (Figures 3.10C – H, 3.11A – D, and 3.12A – F). When measured at their base, these disruptions were of varying size ranging from 58 - 145 nm.

Tomograms from this experiment also showed the formation of thin, branch-like outer membrane projections (Figure 3.11C – D) seen also in negative-stain TEM images (Figure 3.11E – F). The diameters of the projections (128 nm, 144 nm) correlate with the diameters of the outer membrane breaks observed here, suggesting that the projections are the cause of the breaks. In fact, Figure 3.10E clearly shows an outer membrane projection/bleb being released from the surface simultaneously resulting in the creation of an break. Due to the large size of these breaks and the nature of flash-freezing the spirochetes, it is possible that the breaks are transient after forming from the thin projections. In this manner CB2 creates the large breaks, which we believe are a precursor to the smaller openings that we have observed through osmoprotection (2.8 – 4.4 nm). After the large breaks are formed, lipid redistribution or sealing of the outer membrane would account for the appearance of the smaller openings (237), which are critical for osmotic lysis of the spirochetes. Tomograms of CB2-treated spirochetes provided a three dimensional view of the effects of CB2 on the organisms and clearly show the presence of breaks and blebs in the outer membrane all around the *Borrelia* cell (Figure 3.12). It is also interesting to note the differences in effects on the outer membrane around the cell. At certain angles around the cell the outer membrane appears smooth with various breaks (Figure 3.12A – C) and at other angles it has a more ragged appearance (Figure 3.12D – F), indicating that its stability has been greatly affected.

CB2 is Directly Involved in the Formation of Outer Membrane Projections on *B. burgdorferi*

The presence of a fringe/shadowing effect in CB2-treated spirochetes was observed at various areas of the outer face of the outer membrane in many cryo-TEM images (Figures 3.10D, E, 3.11A, C, and 3.12C – E). Since the fringe/shadowing effect was only observed in CB2-treated samples and never in controls, this indicates that the fringe/shadowing may represent bound antibody to the outer membrane of *Borrelia*. In fact, the fringe/shadowing was observed to be comprised of globular particles when tomograms of the outer membrane were analyzed. When measured directly in the tomograms, the particles that comprise the fringe are 16 – 25 nm, a range consistent with the size of intact IgG (data not shown). If the fringe/shadowing is, in fact, bound CB2, it is of note that appears localized on outer membrane blebs and projections in many images (Figures 3.10D, E, 3.11C, and 3.12F), suggesting the direct involvement of CB2 in the creation of these structures. To link the fringe/shadowing to bound CB2 and to prove that CB2 is directly involved in the creation of these membrane effects we performed labeling experiments with negative stain immunogold TEM (Figure 3.13). Live *B. burgdorferi* were treated with CB2 for 15 minutes, fixed for TEM analysis, and labeled with anti-IgG gold conjugated 2^{o} antibodies. CB2-treated spirochetes exhibited the outer membrane projections previously seen and gold-labeled CB2 was seen bound to these outer membrane projections in numerous images (Figure 3.13C – F). That CB2 was seen bound to the outer membrane projections is an indication that CB2 is directly involved in their creation. The concentration of gold-labeled CB2 on outer membrane projections correlates with the localization of the fringe/shadowing on these projections in cryo-TEM images and lends support to the idea that the fringe/shadowing represents bound CB2.

CB2 Removes OspB from the Surface of *B. burgdorferi* in Outer Membrane Blebs

The evidence that the creation of projections/blebs that result in breaks in the outer membrane is a critical step in the bactericidal mechanism of CB2 led us to demonstrate that the removal of membrane projections/blebs containing OspB by CB2 into the extracellular environment is another critical event. Thus, we wanted to determine if CB2 treatment causes a release of OspB-containing membrane blebs from *B.*

burgdorferi into the external medium. We investigated this by treating spirochetes with CB2 for 15 minutes, 1 hour, or 2 hours, centrifuging to separate out cells, resolving the supernatant by SDS-PAGE, blotting to nitrocellulose, probing with CB2 for OspB and finally detecting with an anti-mouse IgG-infrared conjugated antibody. CB2 treatment was conducted in the presence of the osmoprotective dextran T500 as we wished to avoid lysis of the cells allowing us to purely analyze material released from the surface of *B. burgdorferi*. Supernatants from *B. burgdorferi* samples treated with CB2 exhibited more OspB at all time points (Figure 3.14A). The majority of OspB detected appeared to be released into the supernatant by 15 minutes of treatment with CB2 as after 1 or 2 hours there did not appear to be a significant increase in the OspB released. Levels of OspA in the supernatant were also analyzed as this antigen is co-transcribed from a shared operon with OspB, and the two antigens have been shown to colocalize upon CB2 treatment (109). Similarly to OspB, there was an increase in OspA supernatant levels at all time points suggesting that, like OspB, this antigen is released from the spirochete surface upon CB2 treatment as well (Figure 3.14B). As a control, supernatant Dnak levels were tested with CB312 to ensure that the antigens released into the supernatant are not the result of spirochete lysis. The supernatants from all time points had no detectable levels of Dnak in the supernatant showing that dextran T500 osmotically protected the spirochetes from lysis and proving that OspA and OspB detected in the supernatant upon CB2 treatment are released from the spirochete surface and are not the result of lysis (Figure 3.14C).

To further prove that OspB is released from the spirochete surface upon CB2 treatment and to show that CB2 is directly involved in this action, we performed more morphological studies with immunogold detected CB2 (Figure 3.15). Spirochetes were exposed to CB2 for 15 minutes and the immunogold labeling was performed as before. TEM images clearly showed bound gold-labeled CB2 on membrane blebs at the spirochete surface and also showed gold-labeled CB2 bound to many small membrane pieces (blebs) released from the spirochete surface (Figure 3.15C – L). CB2 was consistently observed bound to small membrane pieces (indicated by a lighter gray ring around the gold particles) but was also observed to be bound to larger membrane blebs (Figure 3.15E – F, H – L), some of which were removed from the spirochete surface

(Figure 3.15E, H, K – L). Not only is this visual evidence that CB2 directly removes outer membrane pieces from *B. burgdorferi*, but it is further evidence that the blebs contain OspB, which are thus removed from *Borrelia* by CB2.

3.5 DISCUSSION

As stated in 3.4, antibodies are not thought to exert a bactericidal effect against microorganisms without the assistance of complement and formation of the membrane attack complex. Monoclonal antibodies and polyclonal IgM against Lyme disease and relapsing fever spirochetes are unique in that they are directly bactericidal in the absence of complement. Although some of the bactericidal requirements are known, the actual mechanism of complement-independent antibodies has not been worked out. Outer membrane damage is apparent during exposure to bactericidal antibodies observed through the release of periplasmic flagella (77, 82, 109, 297), although the precise nature of this damage remains unknown. We now show that complement-independent bactericidal antibodies to *Borrelia* exert their effects through formation of openings in the outer membrane followed by osmotic lysis of the organisms. The openings may be the result of outer membrane projections and blebs breaking off of the spirochete surface, as the diameters of outer membrane projections correlate with that of the observed openings. Thus, complement-independent antibodies may create openings in the outer membrane of *Borrelia* due to membrane lipid loss or membrane micellization. Complement-independent bactericidal antibodies are directly involved with the creation of these effects at the antibody-pathogen interface as cryo-electron tomography and immunogold TEM have demonstrated the localization of CB2 on outer membrane projections and blebs of *B. burgdorferi*.

Based on our osmoprotection experiments, CB2 creates openings in the outer membrane of 2.8 – 4.4 nm in diameter. These may not be pores per se, which would involve the formation of fixed-size protein channels, but rather, they may be openings in the outer membrane itself that result in altered (increased) permeability of this barrier as a result of CB2 binding and outer membrane blebbing. Longer CB2 treatment times (2 – 4 days) in the presence of dextran 6000 MW and dextran 81500 MW resulted in a loss of protection. This is indicative of outer membrane openings that gradually increase in size

over time, which is likely the result of continual membrane blebbing or micellization caused by CB2. This is a fundamentally different mechanism from the pores that are formed during the assembly of the membrane attack complex following complement recruitment as those effects rely on the creation of a protein-lined membrane channel on the pathogen surface. That longer treatment with CB2 results in the appearance of larger membrane openings in an indication that CB2 recycles back to the spirochete surface, a turnover effect observed previously with CB2 and purified OspB (183). A current model for the function of cationic antimicrobial peptides called the "carpet" mechanism, where the peptides coat the membrane and insert causing micellization, leading to the formation of transient, toroidal pores that can increase permeability and result in osmotic lysis, is reminiscent of the increasingly larger pores created by CB2 (52). If lysis is prevented, blebbing would continue causing larger membrane openings and thus increased permeability over time to the point where the membrane would disintegrate (52). Based on our experiments, we believe that the outer membrane blebbing caused by CB2 and related antibodies increases the permeability of the membrane in a manner similar to the "carpet" mechanism, allowing for osmotic changes and lysis of this barrier. The range of 2.8 – 4.4 nm appears to be the critical size for openings responsible for increased permeability and susceptibility to osmotic lysis as this size was observed at a time in the reaction when the majority of spirochetes are killed (Figures 3.3, 3.4E – H, and 3.7B – C). That spirochetes can persist in culture during constant CB2 exposure and recover to normal growth following exposure in the presence of dextran T500 but not when smaller sugars are used is an additional indication of CB2-induced osmotic lysis of the outer membrane due to openings. Furthermore, this shows that the outer membrane is absolutely required for persistence of *Borrelia*.

Cryo-electron microscopy and tomography has allowed us to visualize the very early effects of CB2 on the spirochetes in a two minute exposure and has demonstrated previously overlooked characteristics of the outer membrane "frozen in time" due to the flash-freezing preparation of the organisms. The thin, branch-like membrane projections are one of these characteristics. Another is the large outer membrane breaks. That the diameters of the projections at their base are similar to those of the breaks suggests that the breaks are the result of the projections blebbing or breaking off the bacterial surface.

Additionally, the membrane breaks are pointed in an outward orientation (Figure 3.10C, E, F, H), reminiscent of the projections. In addition, Figure 3.10E shows a clear picture of a membrane bleb/projection being released from the spirochete surface right where an opening of 115 nm resides. There is, however, a discrepancy between the size of the openings deduced from the osmoprotection studies and the size of the breaks visualized in cryo-electron microscopy and tomography. From the osmoprotection studies, the size of the openings was deduced to be approximately 2.8 – 4.4 nm, which can also increase in size during prolonged incubation with the antibody in the presence of the osmoprotective sugar. The breaks in cryo-electron microscopy and tomography images are larger (58 – 145 nm) after a 2 minute incubation with CB2. We have considered two possibilities to explain this discrepancy. The first possibility considers that the outer membrane projections seen in cryo-electron tomography form very rapidly after exposure to CB2, break off, and leave temporary breaks of 58 – 145 nm. These large breaks are transient and energetically unfavorable for the membrane and may then be sealed by the remaining lipids in the outer membrane (237, 238). As this process continues and more membrane breaks off and is lost, the sealing will continue until the lipids begin to run out and the openings will become of a smaller, semi-fixed or ambient size (2.8 - 4.4 nm), thus resulting in an outer membrane with increased permeability. The other possibility is that the osmoprotective sugars do not allow for the larger openings to occur. Only after lengthier antibody and sugar exposure (1 – 2 days) do the openings increase in size.

In line with the idea that CB2 removes membrane pieces from the spirochete surface thus resulting in breaks and openings, we have shown evidence that CB2 is directly involved in removing OspB from the spirochete surface in membrane blebs (Figures 3.14 and 3.15). This was shown by treating spirochetes with CB2 for several time points in the presence of dextran T500 (to prevent spirochete lysis), centrifuging, and analyzing the supernatant for release of specific antigens from the spirochete surface. At as early as 15 minutes of exposure to CB2, significant quantities of OspB and OspA were released into the supernatant proving that CB2 alters the outer membrane of *B. burgdorferi* by removing antigens. The nature of antigen removal by CB2 was clarified by negative-stain immunogold TEM analysis of CB2-treated spirochetes. Gold-labeled CB2 was consistently observed to be released from the spirochete surface, bound to small

and large pieces of released membrane (blebs) (Figure 3.15C – L). This is clear visual evidence that CB2 directly removes OspB in membrane blebs which likely creates the breaks in the outer membrane. In addition to being bound to released blebs, CB2 was frequently seen bound to the outer membrane of *B. burgdorferi*, and bound to projections (Figure 3.15C – F, I – J) and blebs that were still on the spirochete surface. These results fit nicely into the model which proposes that complement-independent antibodies exert their bactericidal effects through the creation of outer membrane projections and blebs which break off resulting openings in the outer membrane that eventually kill the spirochetes by osmotic lysis.

A previous observation with CB2 and a related antibody, H6831, demonstrated structural changes in OspB upon binding (34, 183). It is possible that the changes effected in OspB are a trigger for the membrane effects that we have observed. A conformational change in OspB upon CB2 binding could result in the further insertion of OspB into the outer membrane, thereby causing membrane instability, blebbing and, consequently, formation of outer membrane openings that increase permeability. Membrane insertion is what triggers membrane micellization in the antimicrobial peptide "carpet" mechanism, as well (52).

Through the use of small sugars, namely inulin, raffinose, sucrose, and glycerol, we have also demonstrated that the inner membrane suffers the fate of osmotic lysis following rupture of the outer membrane (Figures 3.1 and 3.2), and is the terminal step in this bactericidal process. Although inulin, raffinose, and sucrose did not appear to be protective in extended CB2 growth inhibition experiments, where CB2 treatment times ranged from 1 – 4 days (Figures 3.5 and 3.8), at shorter time points (15 minutes) they provided clear protection against the effects of CB2 (Figure 3.1). The nature of this protection was clarified by examination of the spirochetal morphology after treatment with CB2 or the CB515 scFv for 15 minutes in the presence of these sugars. Negative-stain TEM images showed spirochetes devoid of an outer membrane in these conditions, evidenced by the complete exposure of periplamic flagella and flattened morphology of the spirochetes (Figures 3.2E – H, M – P, and 3.7I – S). However, the protoplasmic cylinders of the organisms remained intact in contrast to its damaged and ragged

appearance during exposure to CB2 or the CB515 scFv without sugars (Figures 2.12E – P, 3.2C – D, 3.5E – H, and 3.7B – C) . Based on this, it was determined that inulin, raffinose, and sucrose provide osmotic protection to the inner membrane of *Borrelia*, which apparently undergoes osmotic lysis following rupture of the outer membrane resulting in death of the organisms. That these sugars could not protect spirochetes at longer time points (1 day or longer) suggests that spirochetes had progressed to spheroplasts by this time, as clusters of spheroplasts were consistently observed in these samples under dark-field microscopy (data not shown). The formation of spheroplasts upon prolonged CB2 treatment in the presence of the smaller sugars is indeed an additional indication that the protection afforded by these small sugars at earlier time points occurs at the inner membrane.

3.6 FIGURES

Figure 3.1: Relapsing fever and Lyme disease spirochetes are protected osmotically from CB2-induced injury by sugars of different MWs. A. Relapsing fever *Borrelia* were exposed to 300 μg/ml of an irrelevant scFv or the CB515 scFv alone or in the presence of the inner membrane osmoprotectant, sucrose for 75 minutes at 33^{o}C and enumerated. As seen previously, the CB515 scFv exerted a bactericidal effect against the spirochetes. This effect is abolished, however, in the presence of sucrose, which is osmotically protecting the spirochetes from CB2-induced damage. Spirochete numbers are significantly lower when treated with CB515 scFv alone compared to when sucrose is present. Results are from 3 independent experiments all performed in triplicate, ANOVA, $p < 0.001$. **B.** The experiment described in A was replicated for CB2 and *B. burgdorferi* to see if this characteristic is shared among different species of *Borrelia*. Sucrose exhibited the same protective effect for *B. burgdorferi* treated with CB2. Spirochete numbers are significantly lower when treated with CB2 alone compared to when sucrose is present. Results are from 3 independent experiments all performed in triplicate, ANOVA, $p < 0.001$. **C.** *B. burgdorferi* were treated with CB2 alone or in the presence of inulin, raffinose, sucrose, or glycerol for 15 minutes and enumerated. Inulin and raffinose, which are larger than sucrose, provide osmotic protection identical to that of sucrose. Glycerol, which is smaller and known to diffuse across the membrane does not protect spirochetes, confirming that the protection exhibited by sucrose, raffinose, and inulin are due to control of osmolarity in the surrounding medium. Results are from 3 independent experiments all performed in triplicate, ANOVA, *** $p < 0.001$.

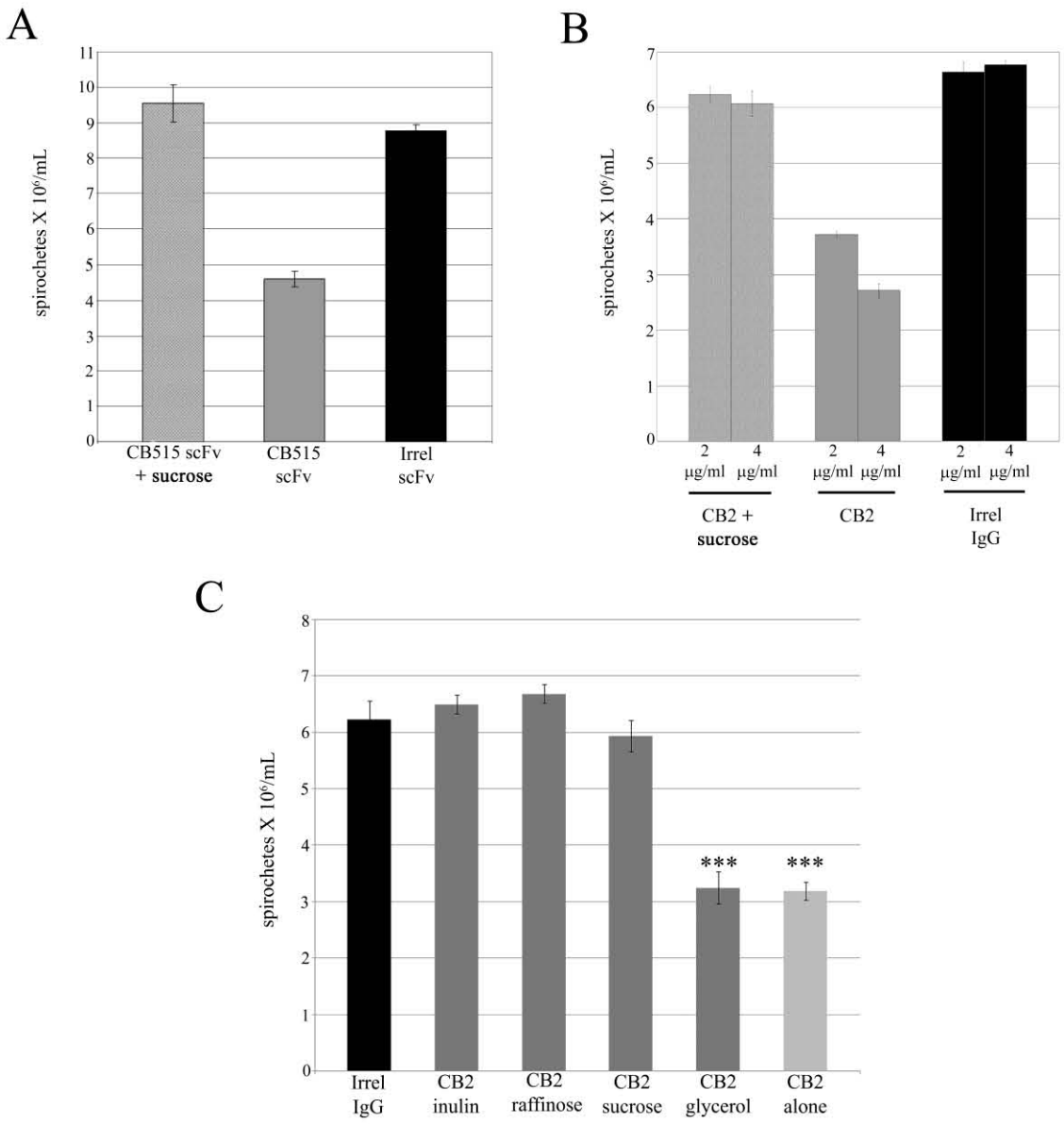
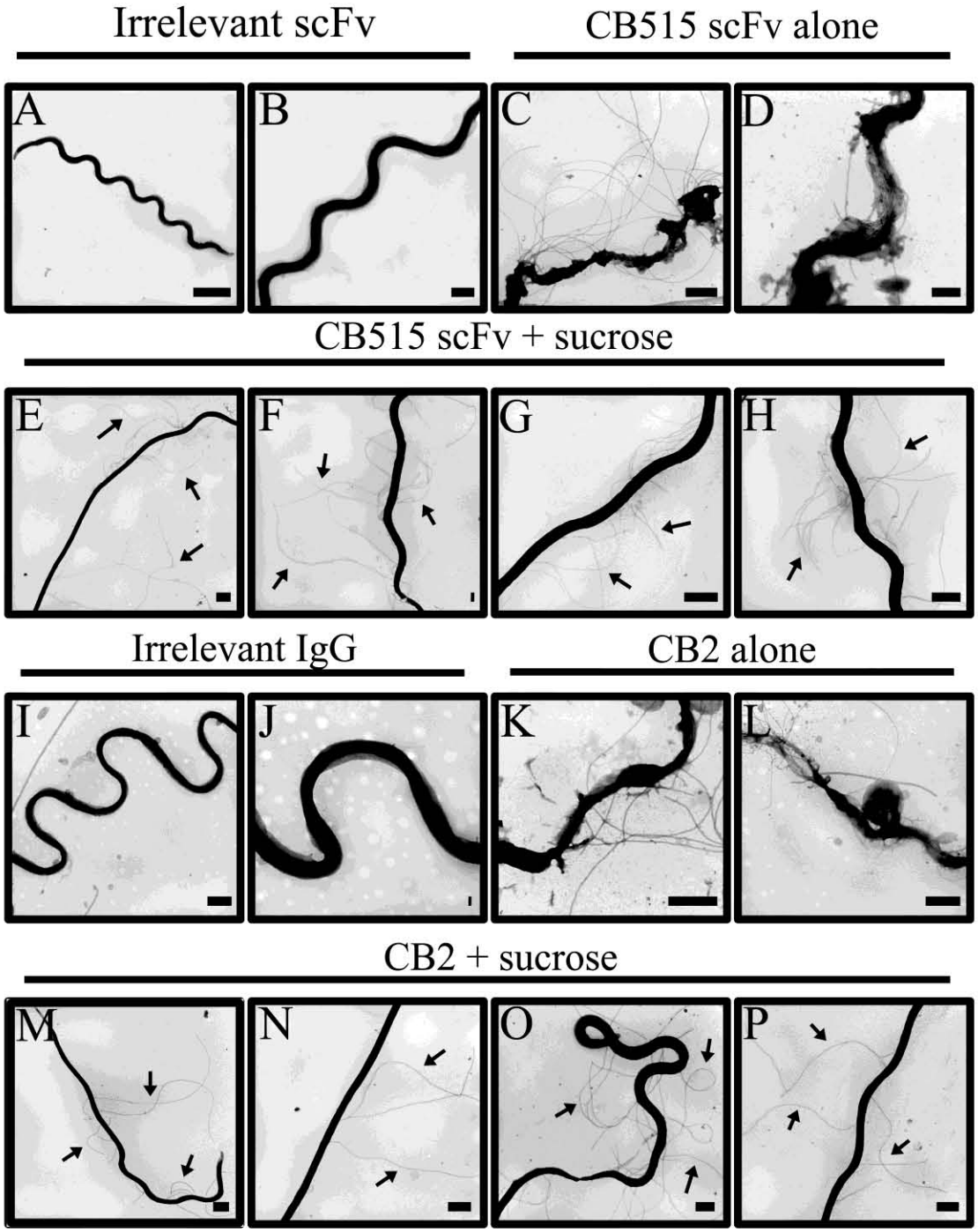

A
spirochetes X 10⁶/mL
CB515 scFv + sucrose
CB515 scFv
Irrel scFv
B
spirochetes X 10⁶/mL
2 µg/ml
4 µg/ml
CB2 + sucrose
CB2
Irrel IgG
C
spirochetes X 10⁶/mL
***
***
Irrel IgG
CB2 inulin
CB2 raffinose
CB2 sucrose
CB2 glycerol
CB2 alone

Figure 3.2: Sucrose protects the inner membrane of *Borrelia* from osmotic lysis during treatment with the CB515 scFv or CB2. Relapsing fever *Borrelia* were treated with **A – B.** an irrelevant scFv, **C – D.** the CB515 scFv, or **E – H.** the CB515 scFv in the presence of sucrose. *B. burgdorferi* were treated with **I – J.** an irrelevant IgG, **K – L.** CB2 alone, or **M – P.** CB2 in the presence of sucrose. Relapsing fever *Borrelia* and *B. burgdorferi* are not completely damaged by the CB515 scFv or CB2, respectively, to due osmotic protection of the inner membrane by sucrose (E – H, M – P). The exposure of periplasmic flagella (arrows) indicates extensive outer membrane rupture whereas the intact appearance of the protoplasmic cylinder clearly indicates an inner membrane that has not been damaged. This is evidence that following direct outer membrane rupture by complement-independent antibodies the inner membrane undergoes osmotic lysis as well as an indication that the mechanisms of these two antibodies are functionally identical. Size bars = 500 nm, 10 μm (A), or 10 nm (J).


Irrelevant scFv
CB515 scFv alone
A
B
C
D
CB515 scFv + sucrose
E
F
G
H
Irrelevant IgG
CB2 alone
I
J
K
L
CB2 + sucrose
M
N
O
P

<u>Figure 3.3</u>: Dextran T500 osmotically protects *B. burgdorferi* from the effects of CB2. *B. burgdorferi* was incubated with CB2 or an irrelevant IgG in the presence of the large sugar dextran T500 or CB2 alone for 15 minutes and counted under dark-field microscopy. As with sucrose, those spirochetes exposed to CB2 in the presence of dextran T500 appeared to survive illustrating osmotic protection. Treatment with CB2 alone resulted in significantly lower spirochete numbers. Results are from 3 indpendent experiments all performed in triplicate, ANOVA, $p < 0.001$.

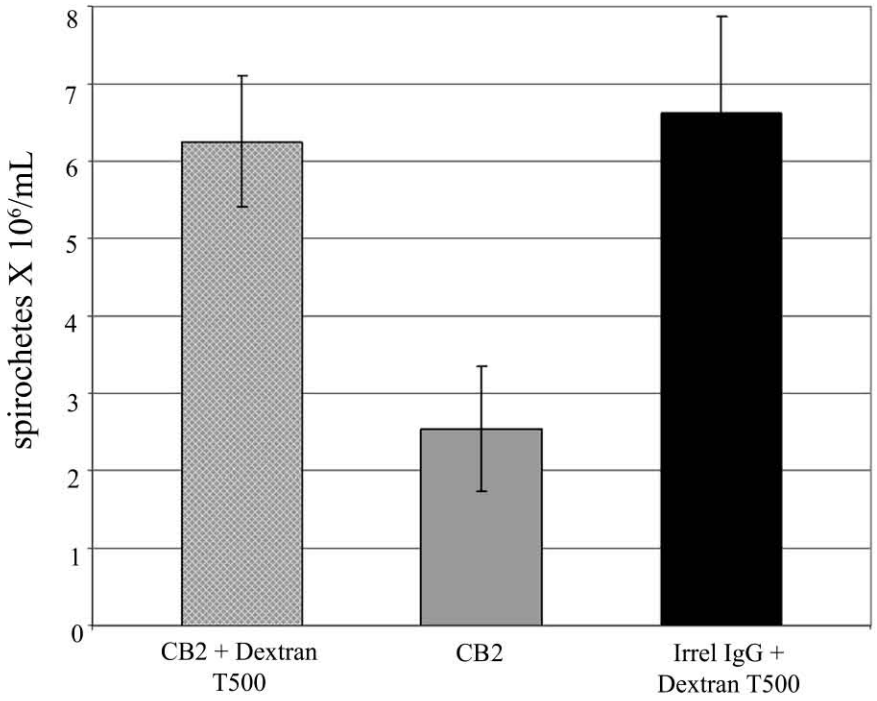

spirochetes X 10^6/mL
8
7
6
5
4
3
2
1
0
CB2 + Dextran T500
CB2
Irrel IgG + Dextran T500

Figure 3.4: *B. burgdorferi* persists during exposure to CB2 with dextran T500 and recovers to normal growth following CB2 exposure with dextran T500. A. Spirochetes exposed to CB2 with dextran T500 did not decrease in numbers in contrast to CB2 alone instead remaining constant. Results are from 3 independent experiments, ANOVA, *** $p < 0.001$, * $p < 0.05$. **B.** Spirochetes exposed to CB2 with sucrose decreased to numbers comparable to incubation with CB2 alone by 1 day of exposure which were both significantly lower than controls. Results are from 3 independent experiments, ANOVA $p < 0.001$. **C.** Following 30 minutes of CB2 treatment in the presence of dextran T500, *B. burgdorferi* grows normally in culture in contrast to treatment with CB2 alone which shows significantly lower spirochete levels. Results are from 3 independent experiments, ANOVA $p < 0.001$. **D.** Following 30 minutes of CB2 treatment in the presence of sucrose, *B. burgdorferi* grows at significantly lower levels than normal. These results suggest that spirochetes survive due to an osmotically protected outer membrane in the presence of dextran T500 distinguishing this protection from that provided by sucrose. Results are from 3 independent experiments, ANOVA, $p < 0.001$.

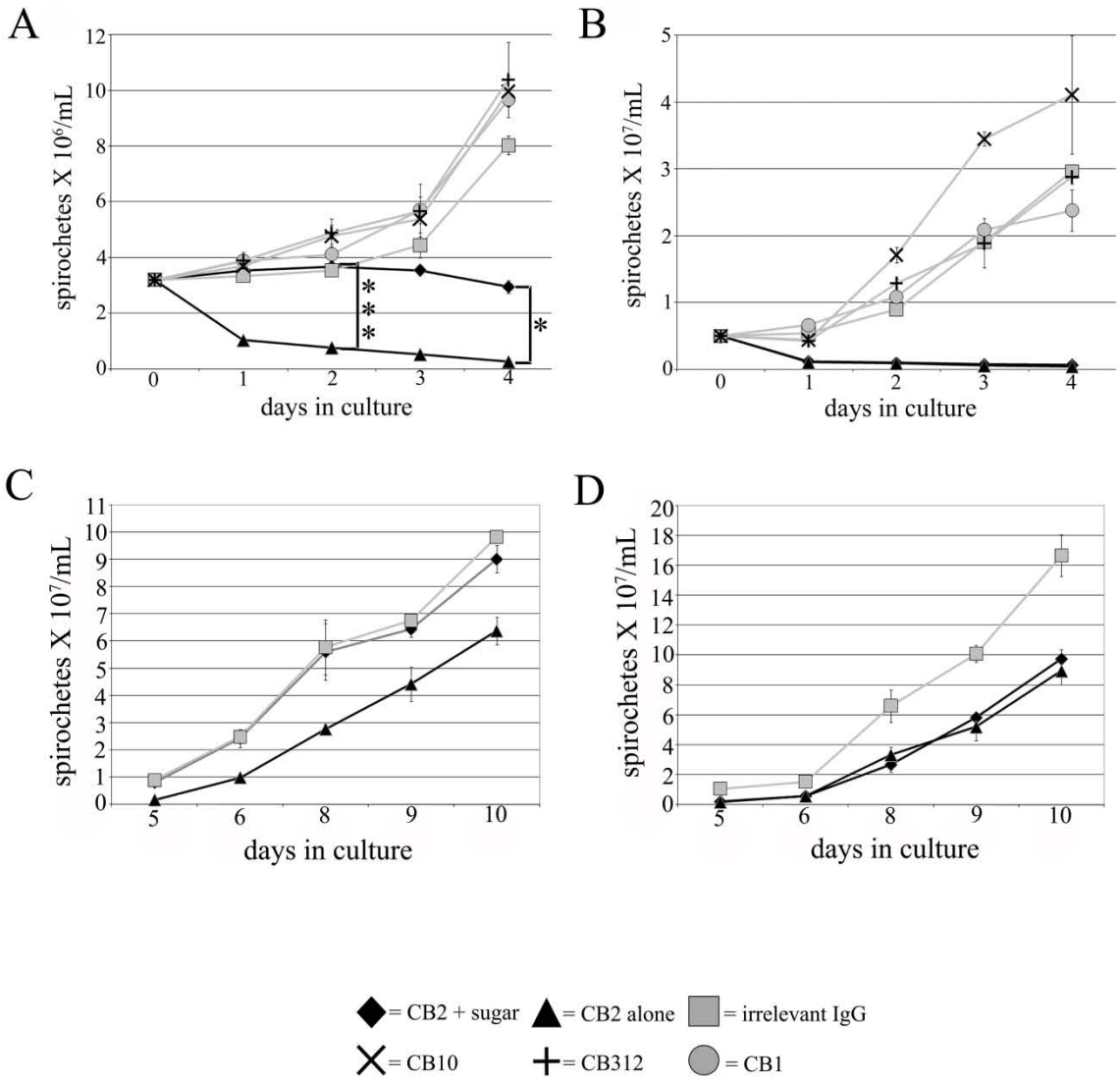

A
spirochetes X 10^6/mL
days in culture
***
*
B
spirochetes X 10^7/mL
days in culture
C
spirochetes X 10^7/mL
days in culture
D
spirochetes X 10^7/mL
days in culture
= CB2 + sugar
= CB2 alone
= irrelevant IgG
= CB10
= CB312
= CB1

Figure 3.5: Dextran T500 protects the outer membrane of *B. burgdorferi* from osmotic rupture. *B. burgdorferi* were treated with CB2 in the presence of dextran T500 for 15 minutes and analyzed by negative-stain TEM. **A – D.** *B. burgdorferi* exposed to 2 μg/ml of an irrelevant IgG. **E – H.** *B. burgdorferi* exposed to 2 μg/ml of CB2. **I – J.** *B. burgdorferi* exposed to 2 μg/ml of CB2 in the presence of dextran T500. These spirochetes exhibited membrane blebs but showed no exposure of periplasmic flagella indicating an intact outer membrane due to osmotic protection. Size bars = 500 nm or 10 μm (B).

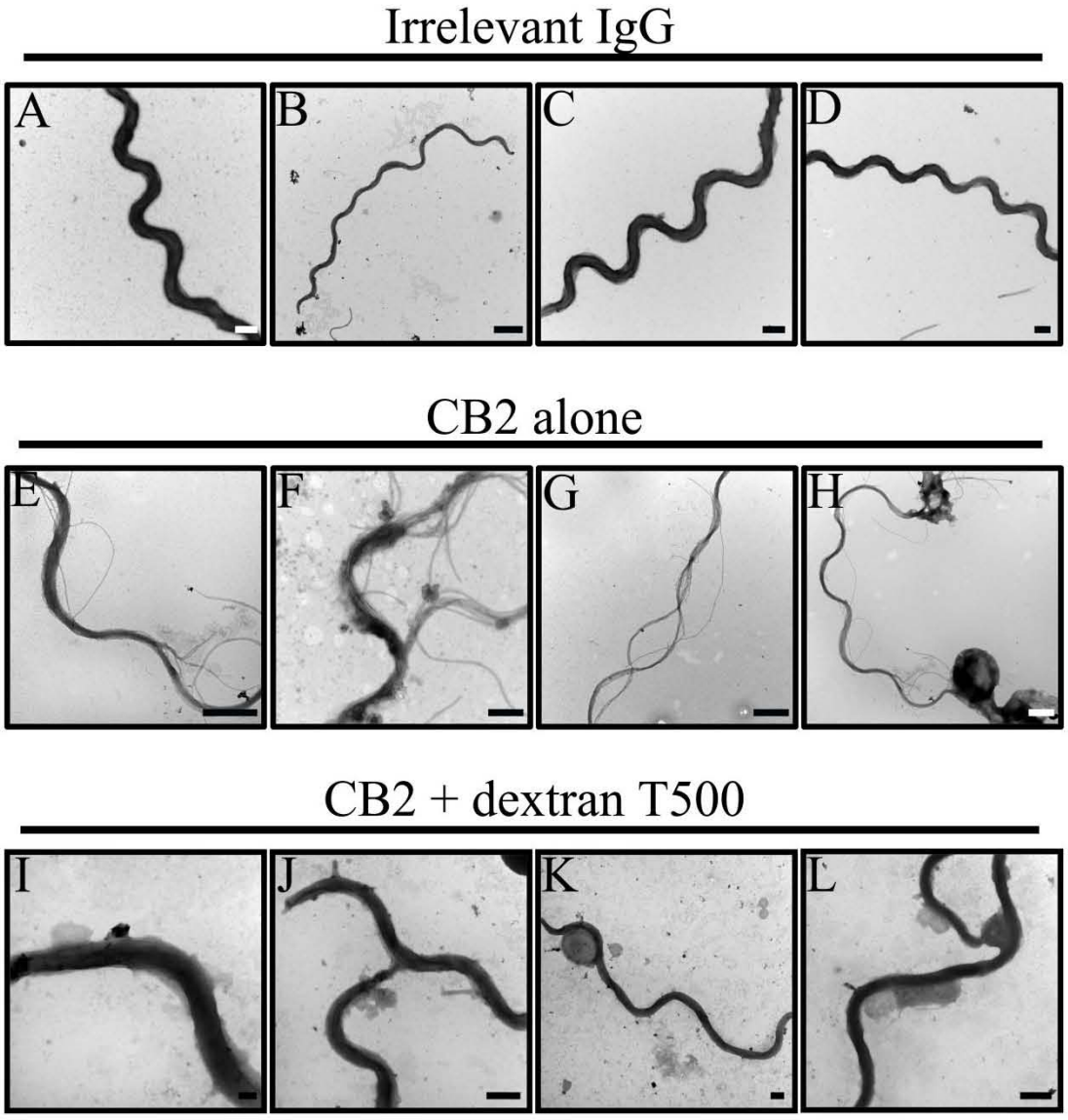

Irrelevant IgG
A
B
C
D
CB2 alone
E
F
G
H
CB2 + dextran T500
I
J
K
L

Figure 3.6: Dextran T500 does not hinder CB2 antigen binding. Live spirochetes were exposed to 10 μg of **A – B.** an irrelevant IgG in the presence of dextran T500, **C, E.** CB2 in the presence of dextran T500, or **D, F.** CB2 alone. The primary antibodies were detected with FITC-conjugated antibodies. CB2 in the presence of dextran T500 exhibited fluorescence similar to that of CB2 alone indicating that dextran T500 does not affect CB2 binding. Size bars = 10 μm.

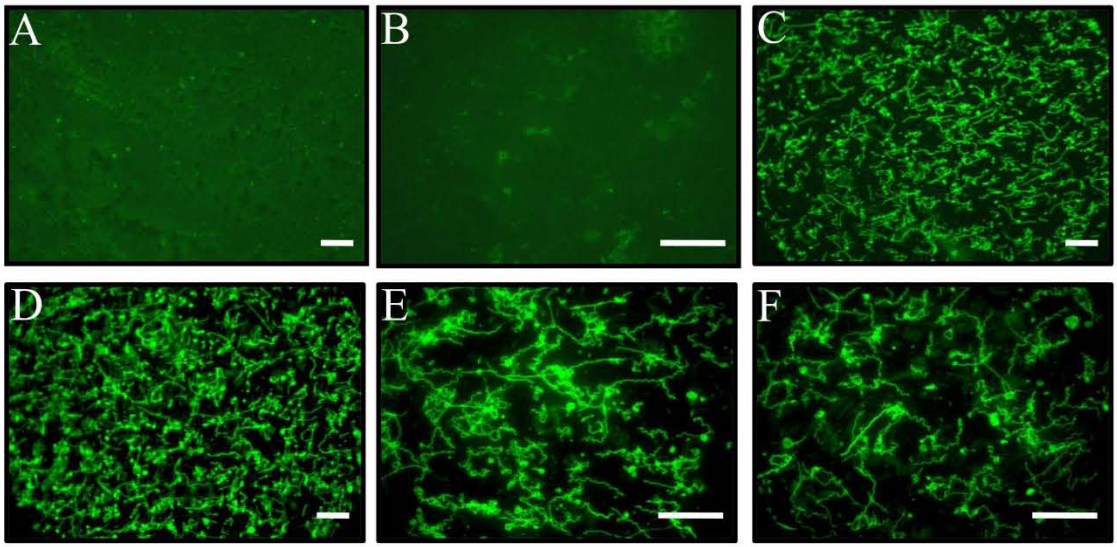

A
B
C
D
E
F

Figure 3.7: CB2 causes the formation of openings in the *B. burgdorferi* outer membrane of 2.8 – 4.4 nm. **A.** Spirochetes are intact and unaffected after treatment with an irrelevant IgG. **B – C.** Exposure to CB2 alone completely disrupts *B. burgdorferi*. The outer membrane of *B. burgdorferi* is not ruptured when exposed to CB2 with dextran T500 (**D – F**), dextran 81500 MW (**G – I**), or dextran 6000 MW (**J – L**) due to osmotic protection. The exposure of periplasmic flagella (arrows) in those spirochetes exposed to CB2 with inulin (**M – O**) or raffinose (**P – R**) indicates a ruptured outer membrane. These results suggest that CB2 induces the formation of openings in the outer membrane of 2.8 – 4.4 nm leading to osmotic lysis. Size bars = 500 nm.

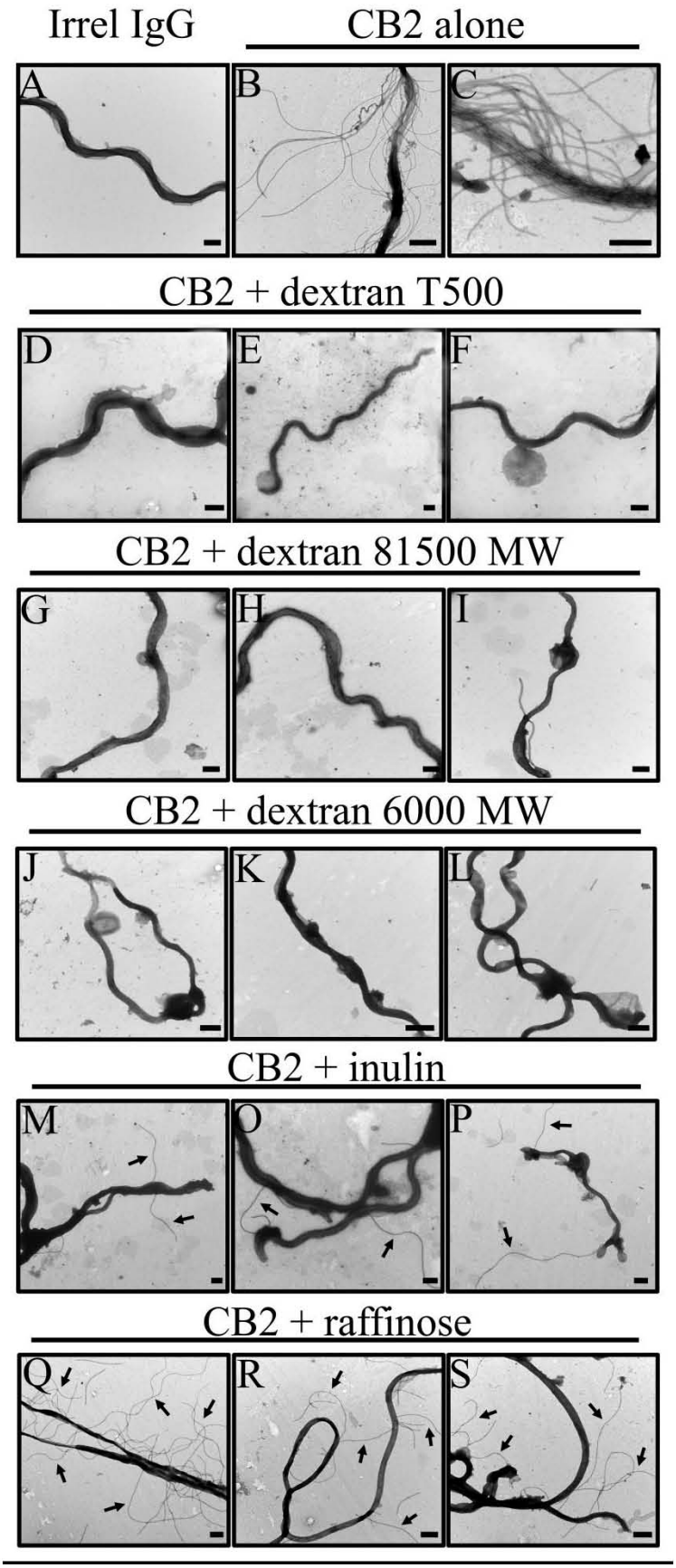

Irrel IgG
CB2 alone
A
B
C
CB2 + dextran T500
D
E
F
CB2 + dextran 81500 MW
G
H
I
CB2 + dextran 6000 MW
J
K
L
CB2 + inulin
M
O
P
CB2 + raffinose
Q
R
S

Figure 3.8: CB2 causes the formation of pores that increase in size during prolonged exposure. *B. burgdorferi* were treated with an irrelevant IgG, CB2 alone or CB2 in the presence of **A.** raffinose, **B.** inulin, **C.** dextran 6000 MW, or **D.** dextran 81500 MW consistently for 4 days. Raffinose and inulin provide no osmotic protection for the spirochetes after 1 day of incubation (A – B). Dextran 6000 MW partially protects the spirochetes after 1 day of incubation but loses this protection at 2 days (C, see figure 3.9 also). Dextran 81500 MW fully protects spirochetes after 1 day of exposure and partially protects after 2 days but eventually loses this capacity for protection (D, see figure 3.9 also). This suggests that membrane pores eventually reach a size that is greater than the diameters of dextran 6000 and dextran 81500. Rather that forming pores of a static size, it is more likely that CB2 creates openings that gradually increase the permeability of the outer membrane, much like the "carpet" mechanism of cationic antimicrobial peptides. In A, B, and C spirochete numbers were significantly lower than in controls when treated with CB2 alone or CB2 with the respective sugar. Results are from 3 independent experiments, ANOVA, $p < 0.001$. In D spirochete numbers were significantly lower than controls or treatment with CB2 and dextran 81500 MW when treated with CB2 alone. Results are from 3 independent experiments, ANOVA, *** $p < 0.001$.

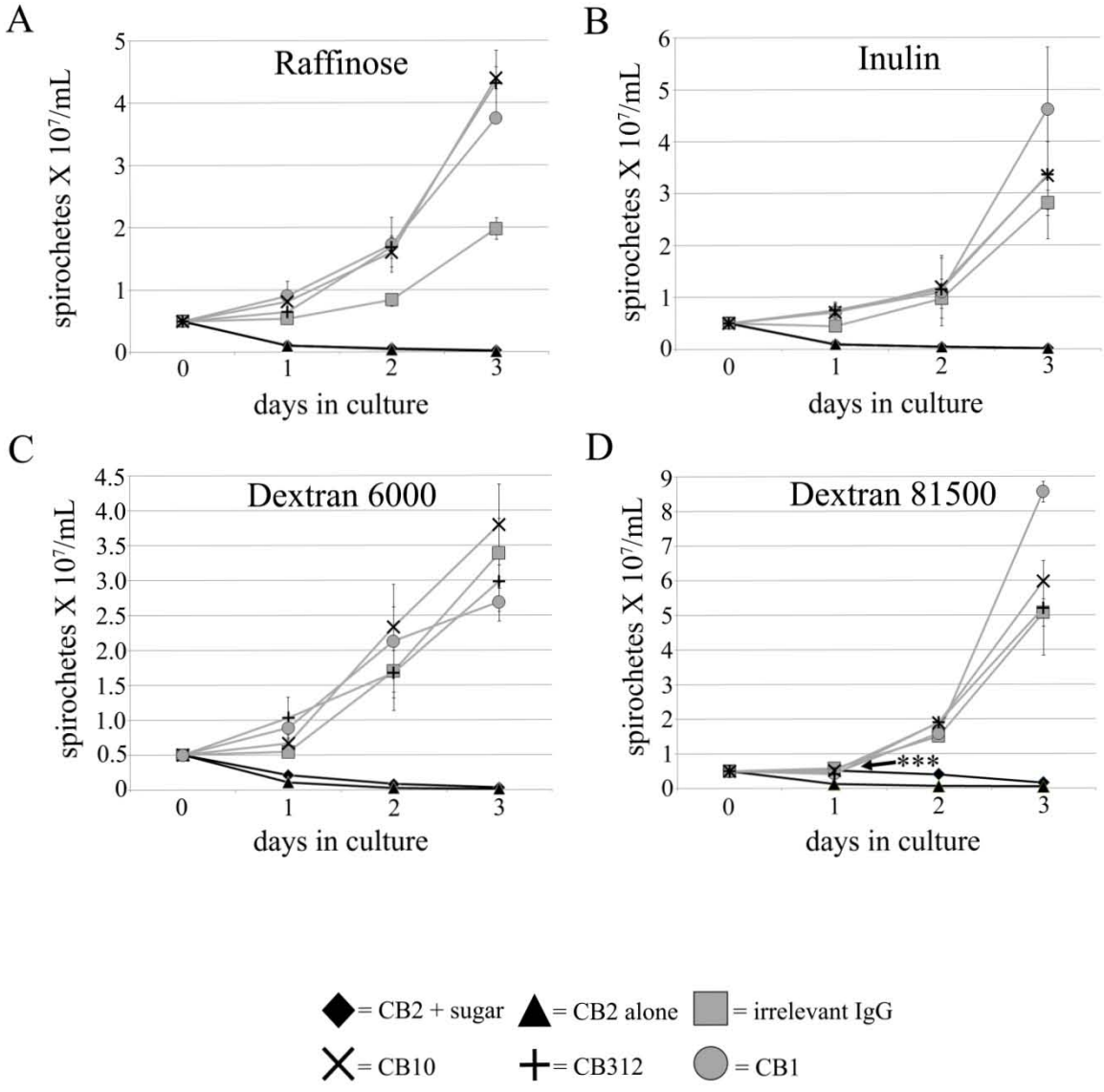
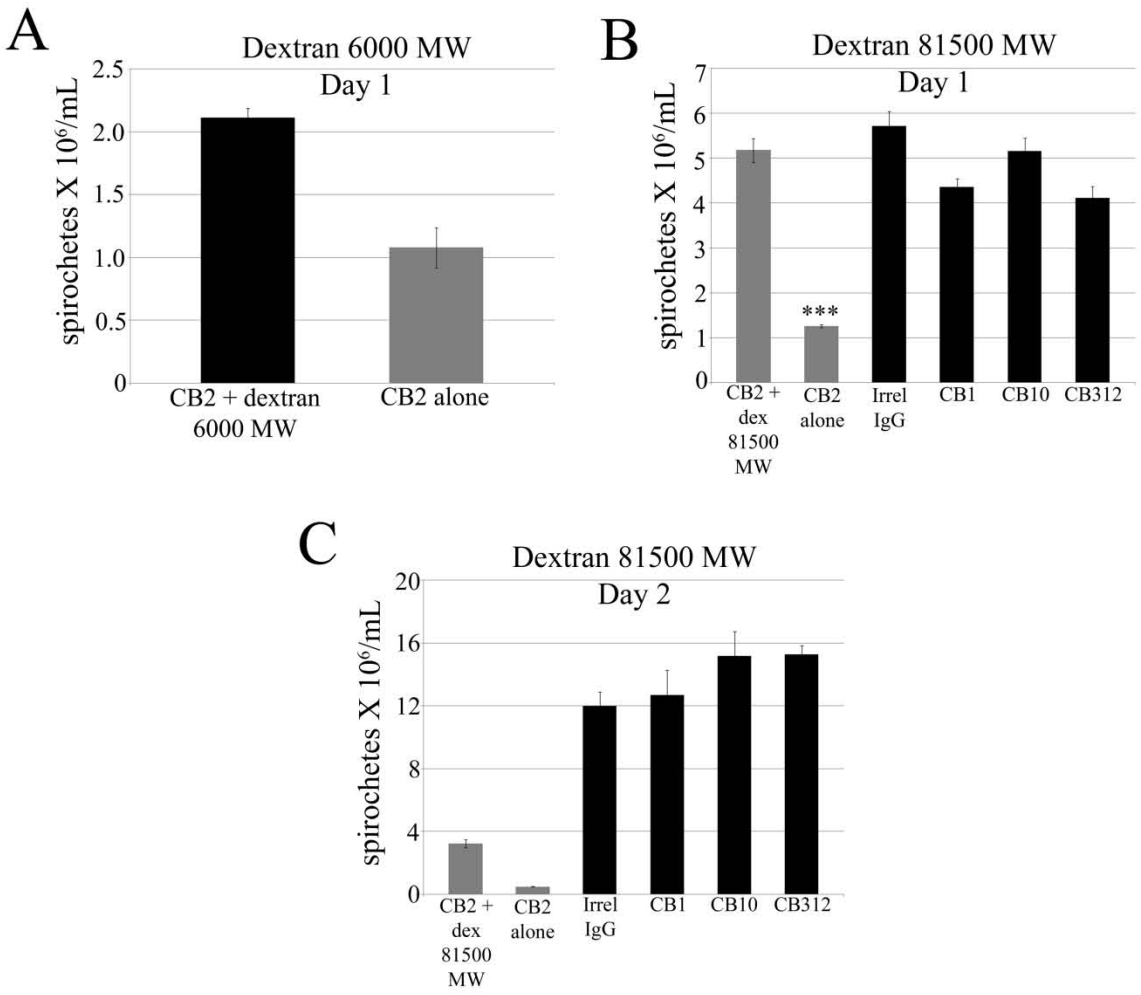
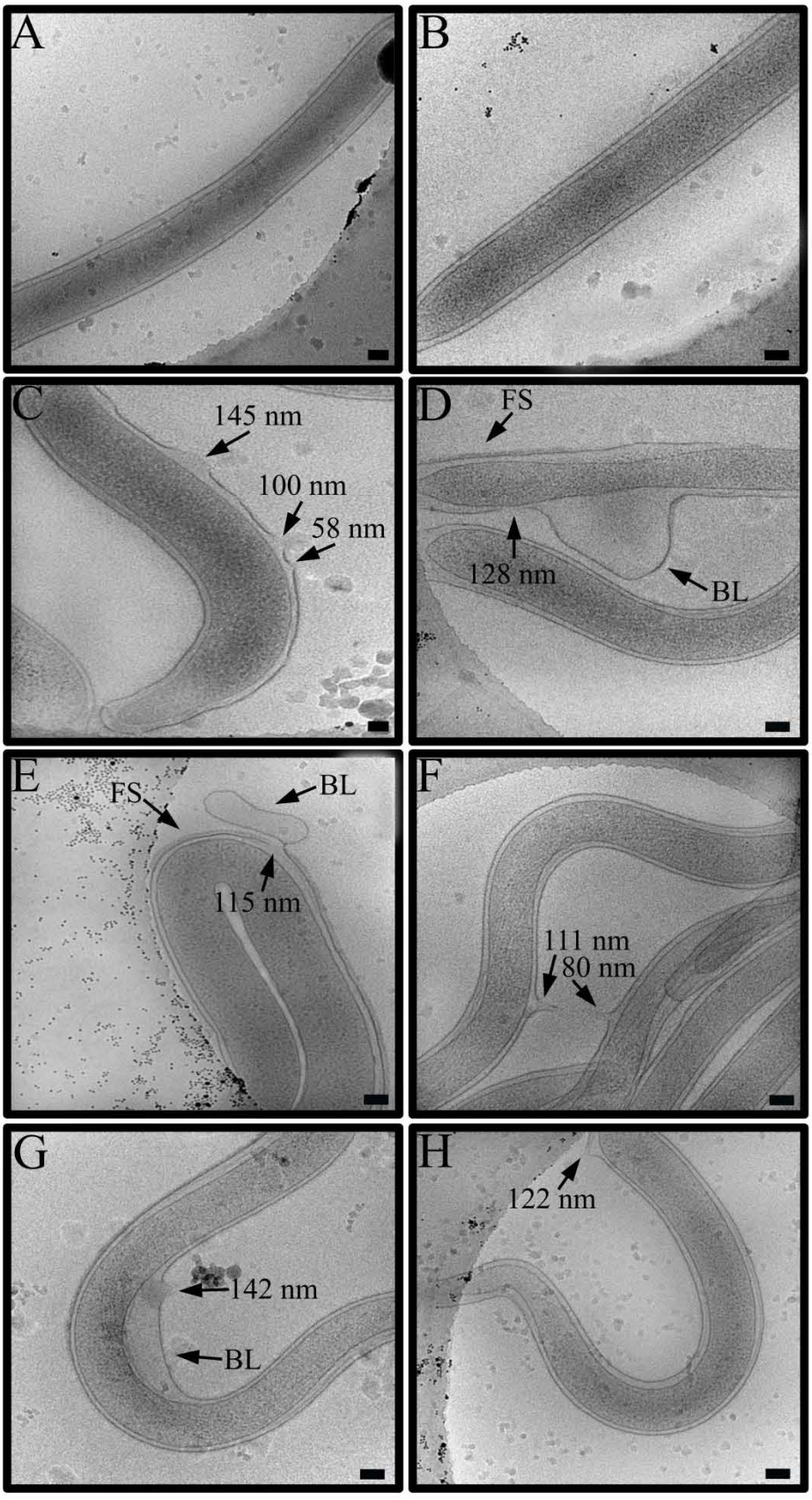

A
Raffinose
spirochetes X 10^7/mL
days in culture
B
Inulin
spirochetes X 10^7/mL
days in culture
C
Dextran 6000
spirochetes X 10^7/mL
days in culture
D
Dextran 81500
spirochetes X 10^7/mL
***
days in culture
= CB2 + sugar
= CB2 alone
= irrelevant IgG
= CB10
= CB312
= CB1

Figure 3.9: CB2 creates openings in the outer membrane that can increase in size. Spirochete numbers after exposure to CB2 in the presence of **A.** dextran 6000 MW for 1 day, **B.** dextran 81500 MW for 1 day, or **C.** dextran 81500 MW for 2 days. These graphs contain the same information as those in figure 3.8, but in a format that better illustrates the partial protection provided by dextran 6000 MW and dextran 81500 MW. The difference in spirochete numbers in A and C is not statistically significant however it is in B. All results are from 3 independent experiments, ANOVA, *** $p < 0.001$.


A
Dextran 6000 MW
Day 1
spirochetes X 10^6/mL
2.5
2.0
1.5
1.0
0.5
0
CB2 + dextran 6000 MW
CB2 alone
B
Dextran 81500 MW
Day 1
spirochetes X 10^6/mL
7
6
5
4
3
2
1
0
***
CB2 + dex 81500 MW
CB2 alone
Irrel IgG
CB1
CB10
CB312
C
Dextran 81500 MW
Day 2
spirochetes X 10^6/mL
20
16
12
8
4
0
CB2 + dex 81500 MW
CB2 alone
Irrel IgG
CB1
CB10
CB312

<u>Figure 3.10</u>: CB2 creates openings in the outer membrane of *B. burgdorferi*. A – B. Cryo-electron microscopic images of intact spirochetes exposed to an irrelevant IgG**. C – H.** Cryo-electron microscopic images of spirochetes exposed to CB2 show outer membrane disruptions which appear as "breaks" in the membrane (arrows, with diameters of the breaks indicated) and outer membrane blebs (BL). Notice in E that a membrane bleb/projection appears to be pinching off where an opening in the membrane (115 nm) now resides, suggesting that blebbing induced by CB2 causes the openings. CB2 treated cells also displayed a fringe or shadow in several regions on the outer face of the outer membrane (signified by arrows labeled FS in D and F) not seen in controls suggesting that it may be bound antibody. Size bars = 100 nm.


A
B
C
145 nm
100 nm
58 nm
D
FS
128 nm
BL
E
FS
BL
115 nm
F
111 nm
80 nm
G
142 nm
BL
H
122 nm

<u>Figure 3.11</u>: CB2 creates openings in the outer membrane around the entire *Borrelia* cell. A – F. Tomographic images of *B. burgdorferi* treated with CB2 for 2 minutes captured at different angles around the entire organism. These images reveal the differering appearance of the outer membrane around the cell showing several breaks (arrows with diameters indicated) and blebs (BL) in different areas and a fringe/shadow presumably representing bound CB2. Notice that at certain angles the outer membrane appears very smooth aside from the breaks (A – C) and at other angles it has a more ragged appearance (D – F). Size bars = 100 nm.

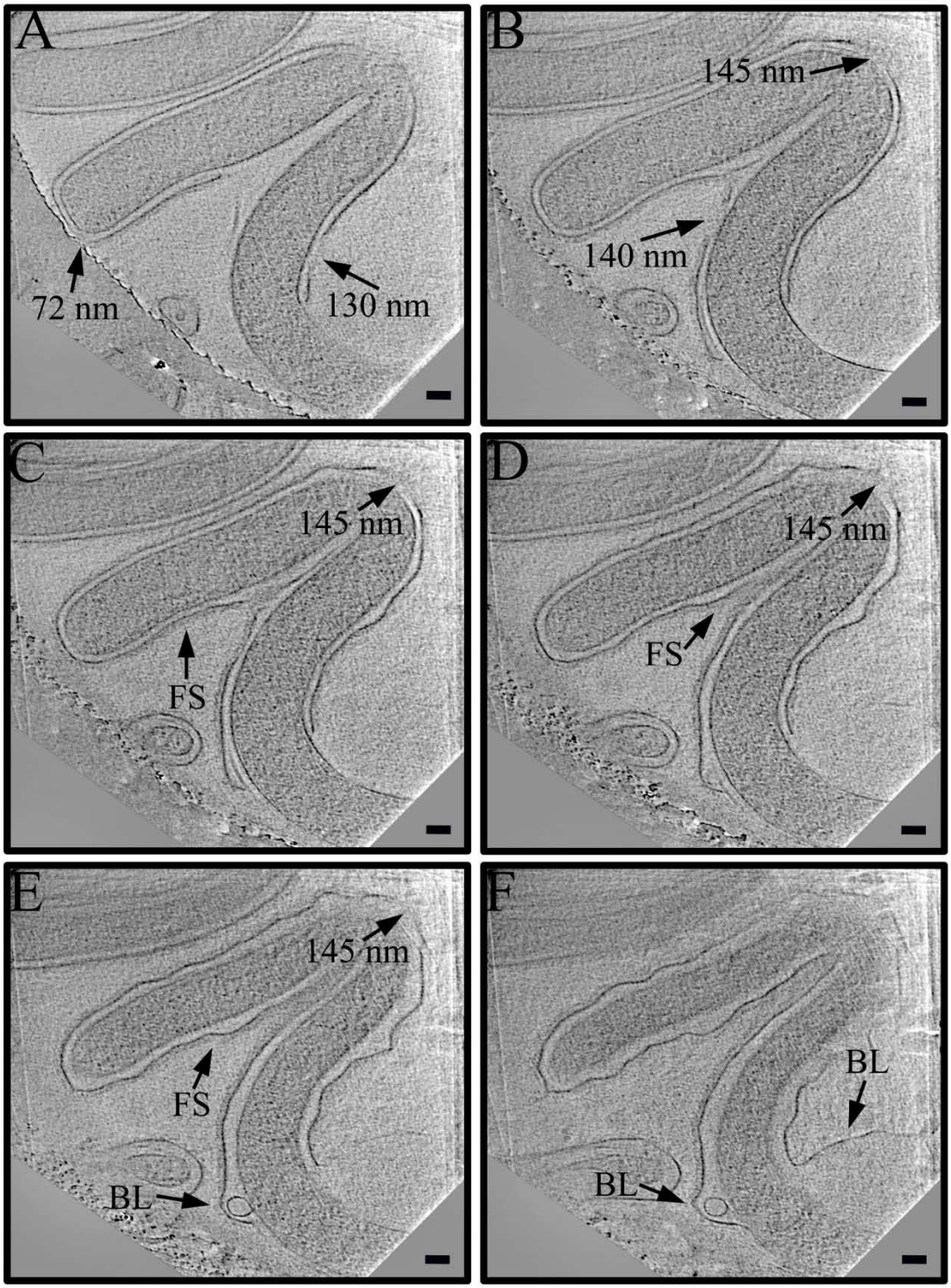

A
72 nm
130 nm
B
145 nm
140 nm
C
145 nm
FS
D
145 nm
FS
E
145 nm
FS
BL
F
BL
BL

Figure 3.12: The early events of exposure to CB2 include the formation of outer membrane projections as well as breaks. A – D. Cryo-electron tomography shows the 3-D structure of CB2-induced breaks as well as thin outer membrane projections all around the organism. **A, C.** Individual 3.6 nm thick slices from two tomographic volumes. **B, D.** Surface rendered models of the outer membrane (yellow) and flagella (green) in the corresponding tomograms. The diameters at the base of the outer membrane projections correlate with those of the outer membrane breaks here and in figures 3.10 and 3.11, suggesting that the outer membrane projections precede the breaks and break or bleb off to form them (as seen in Figure 3.10E). In A – D size bars = 100 nm. **E – F.** Negative-stain TEM images of *B. burgdorferi* treated with CB2 for 15 minutes. The thin, branch-like projections in the outer membrane (arrows) are a common feature of exposure to CB2 also seen here. Size bars = 500 nm.

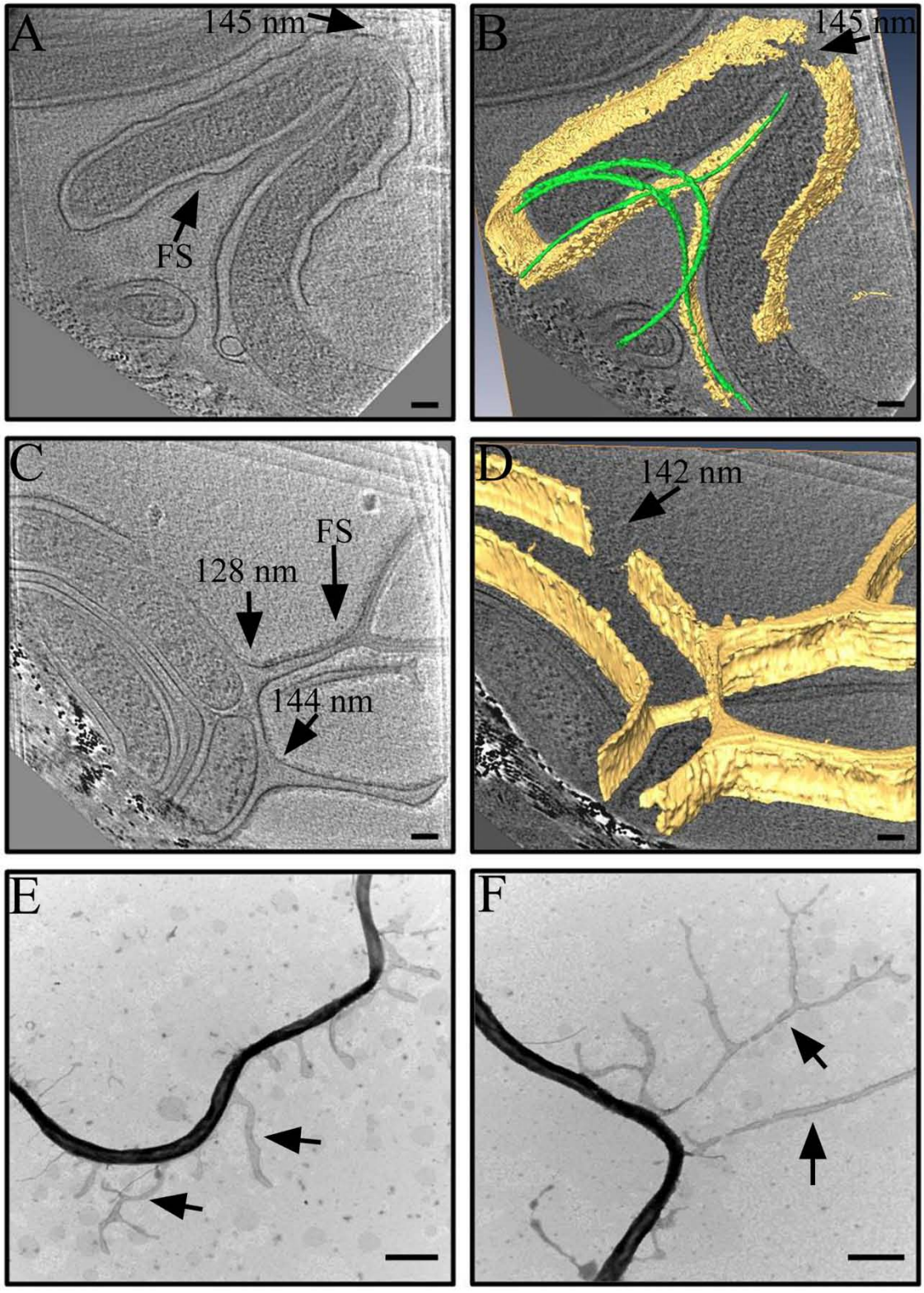
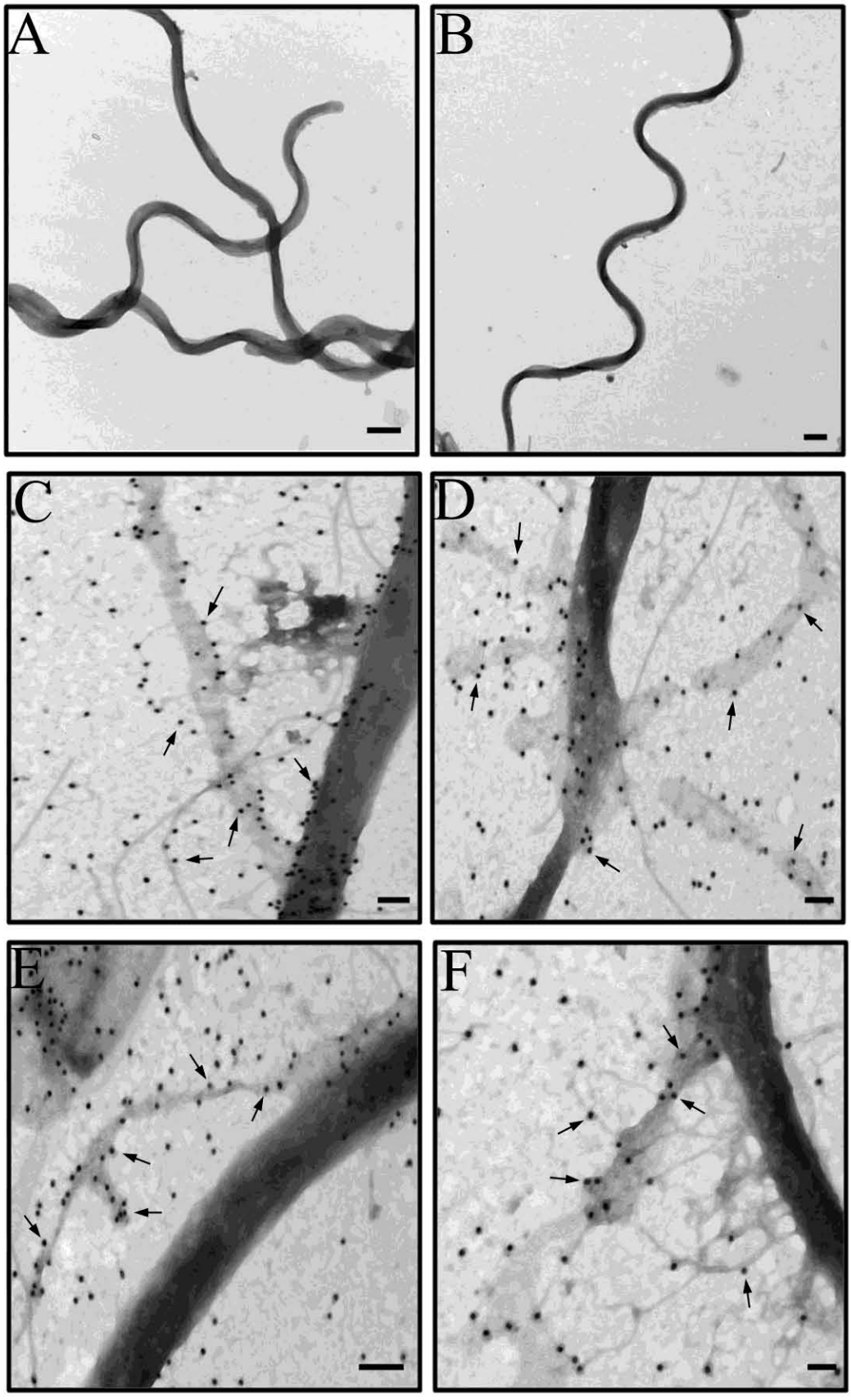
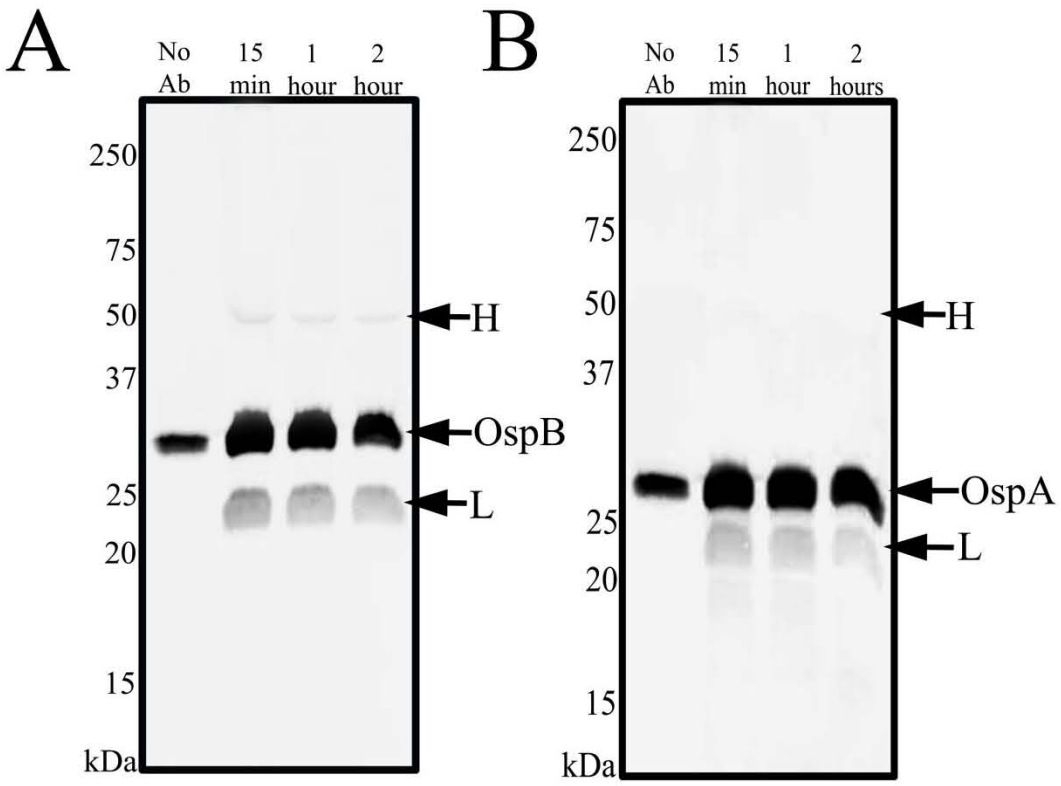

A
145 nm
FS
B
145 nm
C
FS
128 nm
144 nm
D
142 nm
E
F

Figure 3.13: CB2 is directly involved in the creation of the outer membrane projections. *B. burgdorferi* were treated with **A – B.** an irrelevant IgG or **C – F.** CB2 for 15 minutes, fixed, treated with gold-labeled anti-IgG, and analyzed by negative-stain TEM. The outer membrane projections, seen in cryo-TEM images of Figure 3.12 coated in a fringe/shadow, are visible in CB2-treated samples here and similarly coated with gold-labeled CB2. This implies that CB2 is directly involved in creating these projections and suggests that the fringe/shadow seen in cryo images is bound CB2. A – B, Size bars = 500 nm. C – F, Size bars = 100 nm.


A
B
C
D
E
F

<u>Figure 3.14</u>: CB2 treatment causes antigens to be released from the surface of *B. burgdorferi*. Spirochetes were treated with CB2 for 15 minutes, 1 hour or 2 hours with dextran T500 and centrifuged. Supernatants from each time point were run on SDS-PAGE, blotted, and probed with **A.** CB2 to detect OspB, **B.** CB10 to detect OspA, or **C.** CB312 to detect Dnak. After all CB2 treatments times there was significantly more OspA and OspB in the supernatants compared to controls indicating that CB2 causes the release of these antigens from the spirochete surface most likely due to outer membrane fluidity and blebbing. No Dnak was detected in the supernatants indicating that the increased OspA and OspB levels are not the result of total spirochete lysis. The heavy (H) and light (L) chains of CB2 can be seen in the supernatants on all blots, as well.


A
No Ab
15 min
1 hour
2 hour
250
75
50
37
25
20
15
kDa
H
OspB
L
B
No Ab
15 min
1 hour
2 hours
250
75
50
37
25
20
15
kDa
H
OspA
L

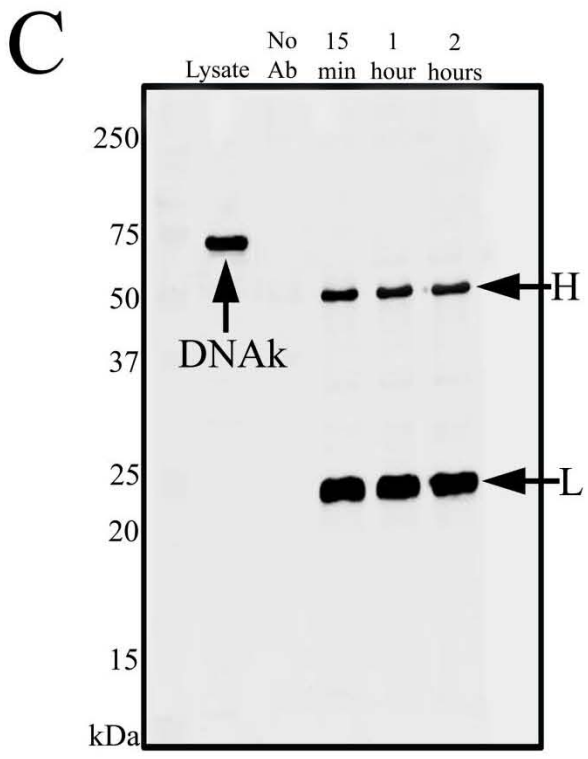
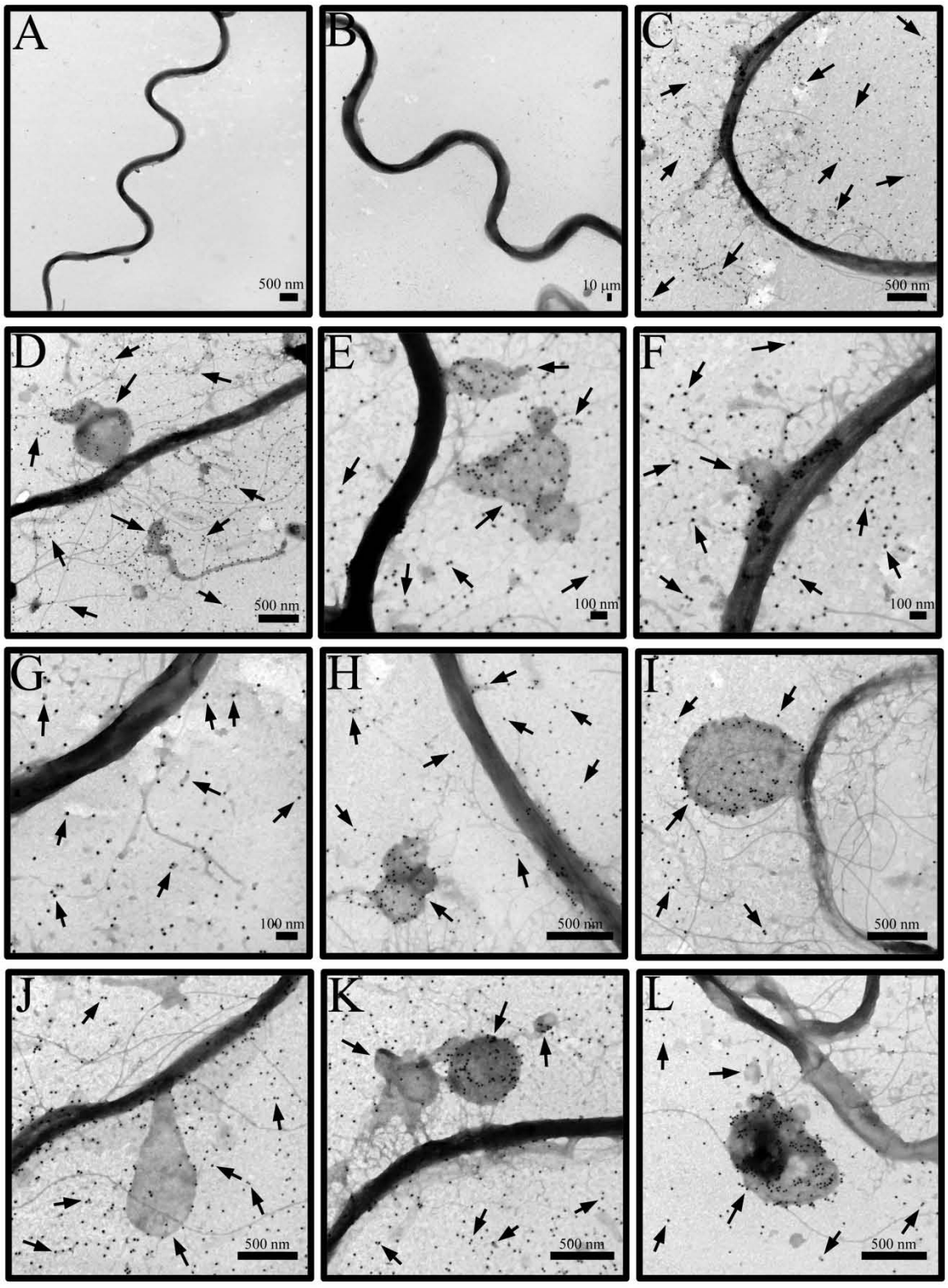

C
Lysate
No Ab
15 min
1 hour
2 hours
250
75
50
37
25
20
15
kDa
DNAk
H
L

Figure 3.15: CB2 directly removes OspB from the surface of *B. burgdorferi* in membrane blebs. Spirochetes were prepared for analysis of CB2 localization by immunogold TEM as described in Figure 3.13. **A – B.** Spirochetes treated with an irrelevant IgG show no damage or antibody binding. **C – L.** B. burgdorferi treated with CB2 are extensively damaged and show bound CB2 in a number of different areas. A and B provide wide shots of spirochetes showing bound CB2 and also many gold particles representing CB2 released from the spirochete surface. E – L provide higher magnification images showing that the all of the CB2 released from the spirochete surface is actually bound to small membrane pieces or blebs (arrows, seen most clearly in E – G). The membrane pieces/blebs are distinguished from the general background of the image due to their darker gray color. Also seen in many of these images are many molecules of CB2 bound to larger membrane blebs on the surface (arrows, E – F, I – J) and released from the surface (arrows, E, H, K – L) of *Borrelia*. All arrows point out membrane pieces/blebs with one or more dots representing gold-labeled CB2 bound to them. These images provide clear evidence that CB2 directly removes membrane pieces from the surface of *B. burgdorferi* which contain OspB. This result is highly suggestive of a mechanism involving membrane bleb formation and removal resulting in pore formation. Size bars are indicated in the images.


A
500 nm
B
10 µm
C
500 nm
D
500 nm
E
100 nm
F
100 nm
G
100 nm
H
500 nm
I
500 nm
J
500 nm
K
500 nm
L
500 nm

CHAPTER 4 - THE PATHOGEN SIDE – The Effects of Complement-Independent Bactericidal Antibodies are Specific to *Borrelia* and Rely on Outer Membrane Fluidity

4.1 CHAPTER SUMMARY

The specificity of the bactericidal effect of complement-independent antibodies to *Borrelia* and factors within the organisms critical for these effects were investigated. For specificity of the effect, OspB, the *B. burgdorferi* antigen recognized by CB2, was expressed on the surface of *E. coli*. The bactericidal effect of CB2 was not transferable to *E. coli* expressing rOspB on its outer membrane despite binding. This raised the possibility that factors specific to *Borrelia*, and not present in other bacteria, are critically required for the mechanism of complement-independent bactericidal antibodies. The porin, P66, co-precipitated with OspB upon immunoprecipitation with CB2 suggesting that this protein may be important for the bactericidal effect. However, through use of *B. burgdorferi* (*Δp66*) mutants, P66 was shown to be dispensable for the bactericidal mechanism of CB2. Interestingly, the high fluidity of the outer membrane of *B. burgdorferi* was observed to be a critical factor for the bactericidal mechanism of CB2.

4.2 INTRODUCTION

Some antibodies against *Borrelia* exhibit the unusual property of exerting a bactericidal effect in the absence of complement. That the constant region is dispensable for this action makes the action of these antibodies unique considering that this region contains the effector functions for antibodies. The variable region alone can exert the bactericidal effect which certainly underscores the importance and uniqueness of the antibodies themselves in creating the effects but there may be other factors required. Considering that *B. burgdorferi* has a 910-kb linear chromosome, over 20 linear and circular plasmids (encoding many genes with no known functions) (123), is distinct in morphology compared to other bacteria (25, 177), and contains many unique antigens (specifically, cholesterol-containing glycolipids in place of LPS (36, 309, 341)) it is possible that there are factors specific to the *Borrelia* organisms which contribute to the

bactericidal mechanism of these antibodies. In this chapter critical factors within the pathogens were investigated through the use of recombinant *E. coli* expressing a *Borrelia* antigen (OspB), immunoprecipitations, and manipulation of the membrane fluidity with different temperatures.

4.3 MATERIALS AND METHODS

rOspB Construction and Expression

The *ospB* (BBA16) construct used in this study was made identically to that described in (183), with the exception that this full-length *ospB* contained the consensus sequence required by signal peptidase II, allowing for the production of lipidated OspB. Briefly, *ospB* amplified using the forward primer GCACACTTCATATGAGATTATTA ATAGGATTT and the reverse primer CTAGCTCGAGTTATTTTAAAGCGTTTTT, which introduced NdeI and XhoI sites 5' and 3' of *ospB*, respectively, and the gene was cloned into a pET30a vector (Novagen). The construct was introduced into DH5α *E. coli* for sequencing. rOspB was expressed in BL21(DE3) *E. coli* by growing the cells at 37^{o}C to an OD_{600} of 0.6 followed by IPTG induction (1 mM) for 3 hours.

rOspB Surface Exposure

To assess if rOspB was expressed on the *E. coli* surface, 10 μg of CB2 or an irrelevant IgG (Sigma) were added to live, unfixed *E. coli* following expression and incubated at 4^{o}C for 1 hour in 1X PBS (Gibco). The bacteria were washed, resuspended in PBS/goat anti-mouse IgG FITC conjugate (Sigma, 1:1000), and incubated at 4^{o}C in the dark for 1 hour. *E. coli* were again washed, added to the wells of a Teflon-coated IFA slide, dried and fixed in 100% methanol. Slow-fade mounting medium was placed on the slides and they were viewed in a Nikon Eclipse E400 microscope. For proteinase K degradation assays, the protease was added to live *E. coli* at 100 or 250 μg/ml following expression of rOspB and allowed to incubate for 1 hour at room temperature. HALT protease inhibitor cocktail (Pierce) was added to stop the reaction and the samples were electrophoresed, blotted and probed with CB2.

rOspB – *E. coli* Bactericidal Assays

CB2, CB10, or an irrelevant IgG (Sigma) were added to *E. coli* at 4, 10, or 50 μg/ml, following expression, and incubated in 1X HBSS (Gibco) for 3 hours at 37°C. Bactericidal effects were determined by OD_{600} measurements or negative-stain TEM analysis. Additionally, the growth/recovery of *E. coli* was assessed by exposure to CB2 at 5, 15, or 30 μg/ml or an irrelevant IgG or CB10 at 30 μg/ml for 3 hours in 1X HBSS. *E. coli* were diluted 1:10 in LB and incubated at 37°C for 4 hours. The OD_{600} was measured hourly.

Immunoprecipitations

10 μg of CB2 was added to live *B. burgdorferi* for 1 hour at 4°C. Spirochetes were then resuspended in 1X RIPA buffer (Pierce) with HALT protease inhibitor cocktail (Pierce) and incubated for 1 hour at 37°C. Protein G agarose beads (Pierce) were added and allowed to incubate overnight at 4°C. Agarose beads were washed 4 times with 1X PBS (Gibco) and resuspended in 1X Laemmli buffer. Samples were electrophoresed and stained with 0.1% Coomassie or blotted and probed with CB2, antisera to *B. burgdorferi*, or antisera to P66 (a gift from Jennifer Coburn, Tufts-New England Medical Center, Boston, MA).

Temperature difference immunoprecipitation experiments were conducted similarly to that described above with the exception that all steps of the procedure were either conducted at 4°C or 33°C to assess any effect that temperature may have on CB2 binding to OspB.

P66 Knockout

The *p66* gene (BB0603) was amplified from *B. burgdorferi* strain B31 using the forward primer ATGAAAAGCCATATTT and the reverse primer GCTTCCGCTGTAGGCTA. The gene was then cloned into a pGEM T Easy Vector (Promega) and screened by PCR and restriction digestion. A kanamycin resistance cassette was amplified from plasmid pJLB12a (a gift from Patricia Rosa, Rocky Mountain Laboratories, NIAID, Hamiliton,

MT) using the forward primer ACCGGTGTCTGTCGCCTCTTG and the reverse primer ACCGGTGGCGAATGAGCTAGC introducing an *AgeI* site. The cassette was cut with *AgeI* and purified via QiaQuick gel extraction (Qiagen). *p66* in pGEM T Easy was cut with *AgeI* and the kanamycin cassette was ligated, creating an insertional inactivation of *p66* at 1168 bp. The ampicillin resistance gene of pGEM T Easy was removed with *BspHI*. The plasmid was purified and concentrated using Wizard Plus SV Midiprep (Promega). *B. burgdorferi* B31 were made electrocompetent and were transformed with DNA according to (299) and cultured without antibiotic for 24 hours at 33^{o}C. Selection was done on BSK plates supplemented with 0.7% agarose and 250 μg/ml kanamycin after incubation at 33^{o}C with 5% CO_2 for 2 – 3 weeks. Isolated colonies were inoculated into BSK-H with kanamycin, incubated at 33^{o}C for 6 – 9 days and screened by PCR. Positive colonies were shown to include the full panel of wild type *B. burgdorferi* plasmids by PCR screening.

Δp66 Bactericidal Assay

B. burgdorferi B31 WT or *Δp66* in serum-free BSK-H were exposed to CB2 or an irrelevant IgG (Sigma) at the concentrations indicated for 15 minutes at 33^{o}C and enumerated under dark-field microscopy.

CB2 – Temperature Difference Experiments

To assess the effect of temperature and membrane fluidity on the bactericidal action of CB2, *B. burgdorferi* were kept at either 33^{o}C or 4^{o}C and treated with CB2 at these temperatures for 15, 60, or 120 minutes. Effects were determined by dark-field spirochete enumeration and negative-stain TEM analysis of spirochete ultrastructure as described in section 2.1.

4.4 RESULTS

The Bactericidal Effects of CB2 are Specific to the *Borrelia* Organisms

We have previously shown that the bactericidal action of complement-independent antibodies resides in the variable region (Chapter 3.1, Figures 2.10 – 2.13) (205). However, properties of the antigen and of the organisms themselves may be critical for

the bactericidal effect as well. To investigate the specificity of the bactericidal effect, we analyzed the effect of CB2 against recombinant *E. coli* expressing OspB. The strain of *E. coli* used was constructed to express the full-length, lipidated OspB to which CB2 is specific. Upon induction with 1 mM IPTG for 3 hours, *E. coli* expressed large quantities of rOspB (data not shown). We first tested if the bacteria could express this *Borrelia* antigen on their surface. Through immunofluorescence using live, unfixed *E. coli* we have shown that the *E. coli* express rOspB on their outer membrane (Figure 4.1A). Furthermore, this proves that CB2 is capable of binding to rOspB on *E. coli*. Proteinase K degradation assays showed cleavage of rOspB, further demonstrating its surface exposure on *E. coli* (Figure 4.1B). When tested for activity, CB2 did not cause a decrease in *E. coli* numbers after 3 hours even when tested at concentrations up to 50 μg/ml (Figure 4.2A). *E. coli* were completely capable of normal growth after CB2 exposure as well, compared to controls which included CB10 (anti-OspA) and an irrelevant IgG (Figure 4.2B). Additionally, when analyzed by negative-stain TEM, *E. coli* exhibited no ultrastructural damage after CB2 exposure (Figure 4.3D – F). *Since CB2 had no effect against E. coli despite binding rOspB, these results collectively demonstrate that there are unique factors present in the outer membrane of Borrelia that contribute to the effects of complement-independent bactericidal antibodies and that are not transferable to other organisms.*

P66, a Porin That Co-Precipitates with OspB, is Not Critical for the Bactericidal Mechanism of CB2

In searching for additional molecules in *Borrelia* required for the bactericidal action of CB2, several immunoprecipitations with CB2 demonstrated one protein band that consistently co-precipitated with OspB (Figure 4.4A). This protein had a spirochetal origin as it was recognized by antisera to *B. burgdorferi* (Figure 4.4A). Mass spectrometry analysis revealed the protein to be P66, a porin/adhesin in *B. burgdorferi* (72, 324) and this was confirmed through the use of antisera to P66 in immunoblot (Figure 4.4B). To determine whether or not P66 is critical for the bactericidal mechanism, we analyzed the effect of CB2 against a knockout strain of *B. burgdorferi* (*Δp66*) (Figure 4.4C). There was no difference in the effect exerted by CB2 against

Δp66 compared to wild type *B. burgdorferi* indicating that while P66 co-precipitates with OspB, it does not appear to be critical for the bactericidal mechanism induced by CB2 (Figure 4.5A).

Spirochetal Periplasmic Flagellar Movement is Not Critical for the Bactericidal Mechanism of CB2

As CB2 exerted no bactericidal effect against recombinant *E. coli* expressing OspB (Figures 4.2 and 4.3), it made sense to consider differences in the outer membranes of *B. burgdorferi* and *E. coli*, as a particular factor unique to *Borrelia* may be critical for the bactericidal mechanism of CB2. One key difference is the presence of bundles of periplasmic flagella which underlie the outer membrane of *B. burgdorferi*. It was thought that perhaps the movement of these periplasmic flagella may be involved in the effects exerted by CB2 as it is conceiveable that periplasmic flagellar movement could contribute to rupture of the outer membrane by "flipping off" pieces of the membrane. This possibility was investigated by treating spirochetes with the proton motive force inhibitor, carbonyl cyanide-m-chlorophenylhydrazone (CCCP), and analyzing the effect of CB2 on immobile spirochetes (Figure 4.5B). After treatment for 1 hour, CCCP had rendered *B. burgdorferi* completely immobile. The immobile spirochetes, however, did not exhibit a difference in the bactericidal effects induced by CB2 suggesting that periplasmic flagellar movement is not critical for this mechanism (Figure 4.5B).

Membrane Fluidity is a Characteristic of the Outer Membrane of *B. burgdorferi* that is Required for the Mechanism of CB2

Another key difference in the outer membranes of *B. burgdorferi* and *E. coli* is the unusually high fluidity of the outer membrane of *Borrelia* (25, 70, 87, 281). We believe that the high membrane fluidity of *B. burgdorferi* could contribute to many of the membrane effects we have observed, such as projection/bleb formation, membrane removal, and pore formation. In this manner, we believe the specific fluid makeup of the outer membrane of *Borrelia* relative to other organisms like *E. coli* is critically required for many of the effects exerted by CB2 to take place. For this reason we sought to investigate the effects of membrane fluidity on the bactericidal mechanism of CB2. We

achieved altering the fluidity of the *Borrelia* outer membrane by cooling spirochetes down to 4^{o}C, as this temperature is below the membrane's liquid crystalline to gel phase-transition temperature (48, 87, 132, 133, 281) and has been shown to greatly decrease the permeability and fluidity of the outer membrane of *Borrelia* and other spirochetes in the past (48, 87, 281). Thus we conducted bactericidal activity experiments with CB2 at two different temperatures, 33^{o}C and 4^{o}C to see if membrane fluidity plays a role in the mechanism of action of CB2. We first wanted to make sure that the lower temperature of 4^{o}C does not affect the level of CB2 binding to OspB. This was done by conducting immunoprecipitations with CB2 at 4 and 33^{o}C for 15 minutes, 1 hour, or 2 hours and then measuring the amount of OspB pulled down by immunoblot densitometry (Figure 4.6).

The temperature of 4^{o}C did not inhibit CB2 binding as similar amounts of OspB were pulled down at this temperature relative to 33^{o}C at all time points (Figure 4.6). When exposed to CB2 for 15 minutes, spirochetes kept at 4^{o}C did not decrease in numbers relative to those exposed to an irrelevant IgG and in contrast to those treated with CB2 at 33^{o}C (Figure 4.7A). After 1 hour of incubation with CB2, spirochetes at 4^{o}C decreased in numbers by about 2 X 10^{6}, but were about 75% greater than the numbers of spirochetes exposed to CB2 at 33^{o}C (Figure 4.7B). After progression to 2 hours of exposure to CB2, spirochetes kept at 4^{o}C decreased in numbers by 50% relative to the control but were still significantly greater (by about 50% again) than the numbers of spirochetes treated with CB2 at 33^{o}C (Figure 4.7C). Morphological examination of *B. burgdorferi* treated with CB2 at 4^{o}C revealed a slower progression of damage as well (Figure 4.8). In particular, spirochetes treated with CB2 at 4^{o}C for 15 minutes appeared unaffected aside from a few small membrane blebs (Figure 4.8A – B). Hindrance of membrane bleb creation is suggestive of a decrease in membrane fluidity at 4^{o}C. That cooling spirochetes to 4^{o}C slowed the kinetics of killing by CB2 suggests that outer membrane fluidity may be an important factor in the bactericidal mechanism of CB2 as it greatly influences the effect of CB2 at the spirochete surface. Above all, these results indicate that the inherent fluidity of the outer membrane of *Borrelia* may be a characteristic that contributes to the effects and kinetics of complement-independent bactericidal antibodies.

4.5 DISCUSSION

In 2.1 and a previous publication (205), we showed that the bactericidal effect of complement-independent antibodies resides in the variable region. In addition to this, we have demonstrated here that the bactericidal effect of the antibodies is not transferable to another Gram-negative bacterium since CB2 had no bactericidal effect against *E. coli* expressing full-length, lipidated rOspB. Thus, there appears to be unique factors present in *Borrelia* that are just as critical as the variable region and antigen for completion of this mechanism. One potential factor, as the only outer membrane protein candidate that co-precipitated with OspB, P66, was not required for the bactericidal activity of CB2.

There was no bactericidal activity against *E. coli* expressing rOspB on their surface. Recombinant *E. coli* did not decrease in numbers, grew normally, and were not damaged after exposure to CB2. Since CB2 bound rOspB on these bacteria but had no other apparent effects, it seems there must be unique accessory factors in the outer membrane of *Borrelia* not found in *E. coli*. Whatever these factors may be, they are absolutely required for the effects of complement-independent bactericidal antibodies such as CB2. P66, which co-precipitated with OspB, appeared to be the ideal candidate accessory protein as this integral membrane protein is localized to the outer membrane and is a porin (324). It was conceivable that a porin could be required to effect osmotic changes by allowing the passage of ions or could be in an open conformation after CB2 binding allowing for the influx of water and, thus, osmotic lysis. However, CB2 has no defect in its bactericidal activity against *Δp66* indicating that P66 is not critical for the bactericidal mechanism. It is possible that P66 co-precipitated with OspB because of natural colocalization, as is the case with P66 and OspA (55). In fact, CB10, an IgG directed against OspA is not bactericidal in the absence of complement, and was used as a control for all the studies with CB2. OspA and OspB are similar, highly cationic lipoproteins that are co-transcribed (38, 42). Based on this, it appears that the openings responsible for lysis of the outer membrane are created by the binding of CB2, and may be the result of a critical role for OspB, but not OspA, in the fluidity (70) and increased blebbing of the spirochetal outer membrane.

It was also thought that perhaps periplasmic flagella, unique to the spirochetes, are a contributing factor to the effects of complement-independent bactericidal antibodies. When periplasmic flagellar movement was inhibited with CCCP, however, there was no effect on the bactericidal activity of CB2 indicating that flagellar movement is not critical for the effects of CB2.

This unique bactericidal effect is the result of a lethal association of the variable region of the antibody, its targeted antigen, and of unique properties within the organism itself. Since susceptibility to the bactericidal effect is not transferrable to *E. coli* and since there is no obvious accessory protein required for this mechanism, what makes the *Borrelia* outer membrane so susceptible to the effects of complement-independent bactericidal antibodies? One feature that needs to be considered in the context of outer membrane fluidity in *Borrelia* is the absence of LPS and the presence of cholesterol and cholesterol-containing glycolipids (36, 282, 309, 361). Since the outer membrane of *Borrelia* contains cholesterol, a characteristic that is not common for prokaryotes, there is the possibility that lipid raft domains could contribute to increased blebbing and membrane permeability following antibody binding. Whatever the case, it has become abundantly clear that the combination of the variable region, antigen, and the *Borrelia* organisms create a specific effect that is extraordinarily lethal and is triggered by increased outer membrane permeability and osmotic lysis as the mechanism.

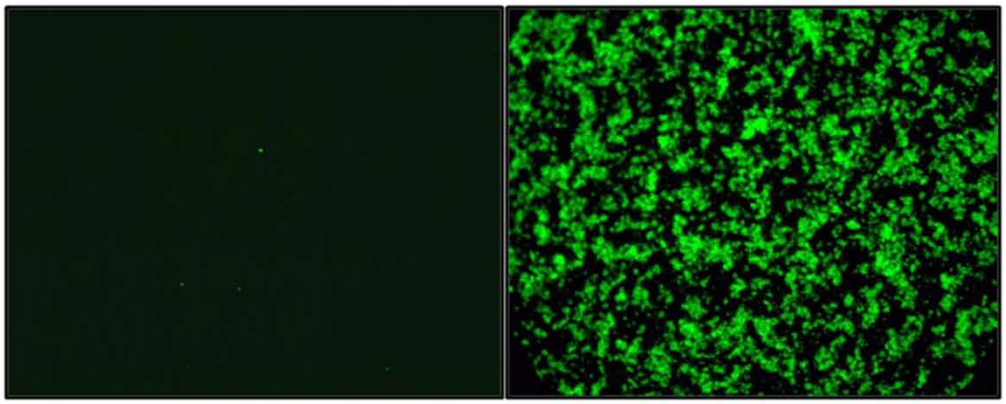
It was of interest to investigate the role of the unusually high outer membrane fluidity in the bactericidal mechanism of CB2 as this is a characteristic that is not shared by many bacteria (25, 70, 87, 260, 281). Moreover, the direct creation of membrane projections/blebs by CB2 is a consistent effect exerted by all complement-independent bactericidal antibodies to *Borrelia* and most likely requires a very fluid membrane. Analysis of the role of membrane fluidity was achieved by conducting CB2 bactericidal assays at either 33^{o}C, the optimal growth temperature of *B. burgdorferi* where normal membrane fluidity would be expected, or 4^{o}C, a temperature below the liquid crystalline to gel phase-transition temperature of the membrane which was previously shown to greatly decrease the fluidity and permeability of the outer membrane of *B. burgdorferi* (87). The bactericidal kinetics of CB2 were slowed at the temperature of 4^{o}C supporting

the idea that the fluidity of the outer membrane is a characteristic of *Borrelia* that influences the mechanism and activity of CB2. As CB2 binding is not inhibited at 4^{o}C (Figure 4.6) and specific gene induction in *B. burgdorferi* does not occur until later CB2 treatment time points (10), the slower antibody kinetics may be due to a decrease in outer membrane fluidity at this temperature. While we cannot completely rule out a different mechanism, such as alteration of a metabolic process, we believe the slower kinetics of CB2 at 4^{o}C reflects decreased membrane fluidity. When examined at the ultrastructural level there were no apparent membrane blebs and/or morphological defects when spirochetes were treated with CB2 for 15 minutes at 4^{o}C. The fact that spirochetes did not exhibit the characteristic membrane blebs until treatment with CB2 reached 1 hour suggests that the temperature of 4^{o}C decreases the membrane fluidity of *B. burgdorferi*. This also suggests that the high fluidity of the outer membrane of *Borrelia* is a critical factor not only in the creation of membrane projections/blebs by CB2 but in the overall bactericidal mechanism. Since fluidity of the outer membrane has such a profound effect on the bactericidal kinetics of CB2 it stands to reason that perhaps membrane cholesterol is the specific factor in *Borrelia* that is critically required for the mechanism of complement-independent bactericidal antibodies. Cholesterol is present in the outer membrane of all *Borrelia* in the form of major glycolipids but is also found as free cholesterol and cholesterol esters (36, 266, 309, 341). Cholesterol compounds account for 40% of the outer membrane of *B. burgdorferi* (341) thus it is likely that cholesterol is responsible for the great inherent fluidity of the outer membrane of *Borrelia* which in turn is absolutely critical for the observed membrane effects of CB2. It may be that cholesterol and cholesterol glycolipids in *Borrelia* provide a role that contrasts with the role LPS provides for Gram-negative bacteria. Instead of strengthening the outer membrane and transforming it into a strong permeability barrier (as is the case with LPS) (67, 260, 283), cholesterol and cholesterol glycolipids may provide an increased fluidity to the outer membrane allowing for it to be uniquely flexible yet unfortunately susceptible to the specific action of complement-independent bactericidal antibodies.

4.6 FIGURES

Figure 4.1: Recombinant OspB is expressed on the surface of transformed *E. coli*. *E. coli* transformed with pET30a containing rOspB were induced to express the antigen and its surface exposure was tested by treating live bacteria with **A.** CB2 and FITC-labeled antibodies for fluorescence microscopy or **B.** proteinase K (100 or 250 μg/ml) to observe OspB degradation. CB2 binds to live *E. coli* in contrast to an irrelevant IgG (A) and proteinase K degrades OspB on live *E. coli* indicating that rOspB is expressed on the surface of *E. coli*.

A

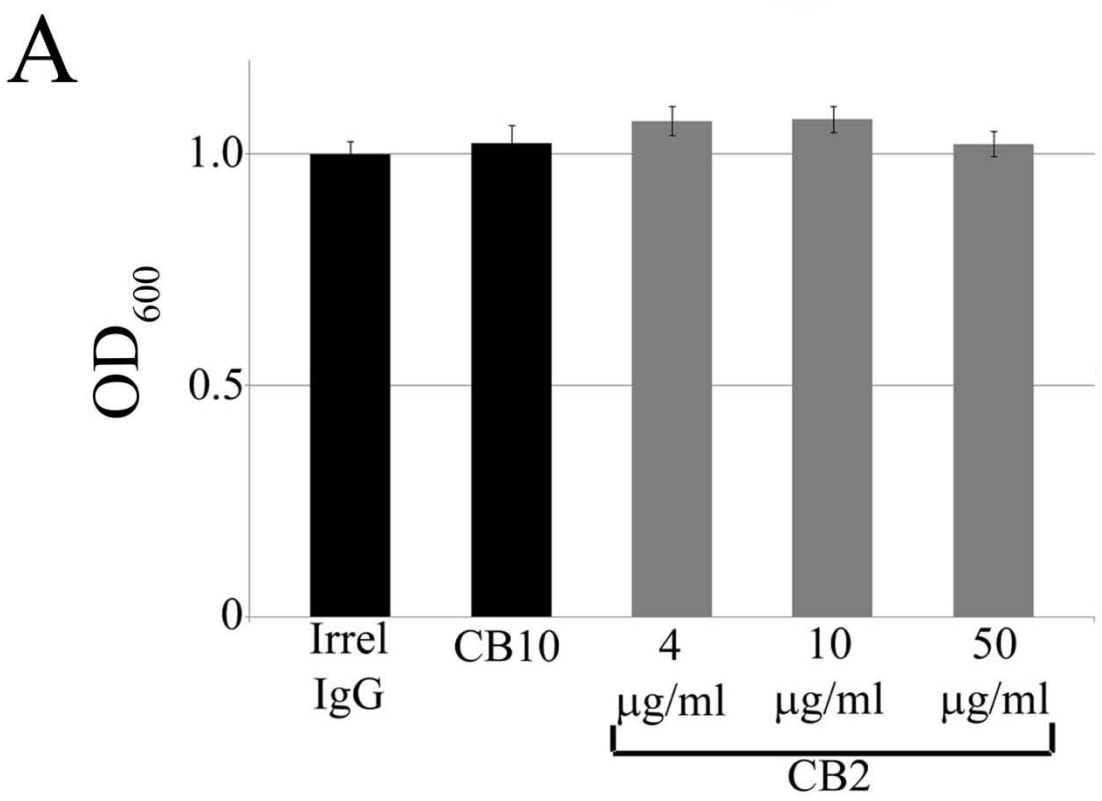


Irrel IgG CB2

B

0 μg/ml 100 μg/ml 250 μg/ml

<u>Figure 4.2</u>: CB2 requires unique factors in *Borrelia* to create its bactericidal effects. **A.** *E.* coli induced to express rOspB were treated with different concentrations of CB2 or control antibodies for 3 hours and optical density was determined. CB2 has no bactericidal effect against recombinant *E. coli* expressing OspB. **B.** *E. coli* induced to express rOspB were cultured with different concentrations of CB2 or control antibodies for up to 4 hours and the optical densities were determined. *E. coli* have no defect in growth following CB2 treatment (◇= 5 μg/ml CB2, □= 15 μg/ml CB2, △= 30 μg/ml CB2, **✚** = irrel IgG, **X** = CB10). Since CB2 has no bactericidal effects against *E. coli* despite binding we conclude that these effects are not transferable from *Borrelia* to *E. coli* by OspB alone.


A
1.0
0.5
0
OD600
Irrel IgG
CB10
4 µg/ml
10 µg/ml
50 µg/ml
CB2

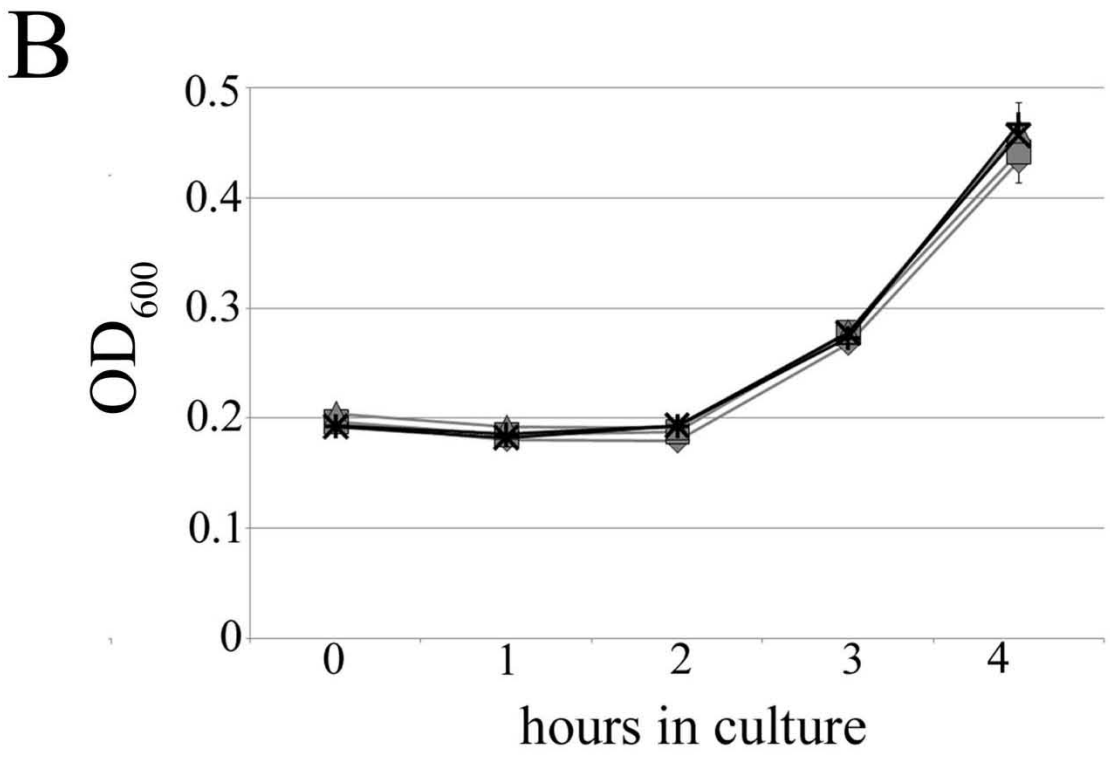

B
0.5
0.4
0.3
0.2
0.1
0
OD600
0
1
2
3
4
hours in culture

Figure 4.3: CB2 does not cause damage to the fine ultrastructure of *E. coli*. *E. coli* expressing surface rOspB were treated with **A – C.** an irrelevant IgG, or **D – F.** CB2 for 3 hours and examined by negative stain TEM. There is no evidence of damage to recombinant *E. coli* after CB2 exposure. The results of this figure and Figure 4.2 collectively demonstrate that despite surface exposure of rOspB, the *E. coli* are not affected by CB2 suggesting the critical requirement of factors unique to *Borrelia* to create the bactericidal effects of CB2. Size bars = 500 nm.

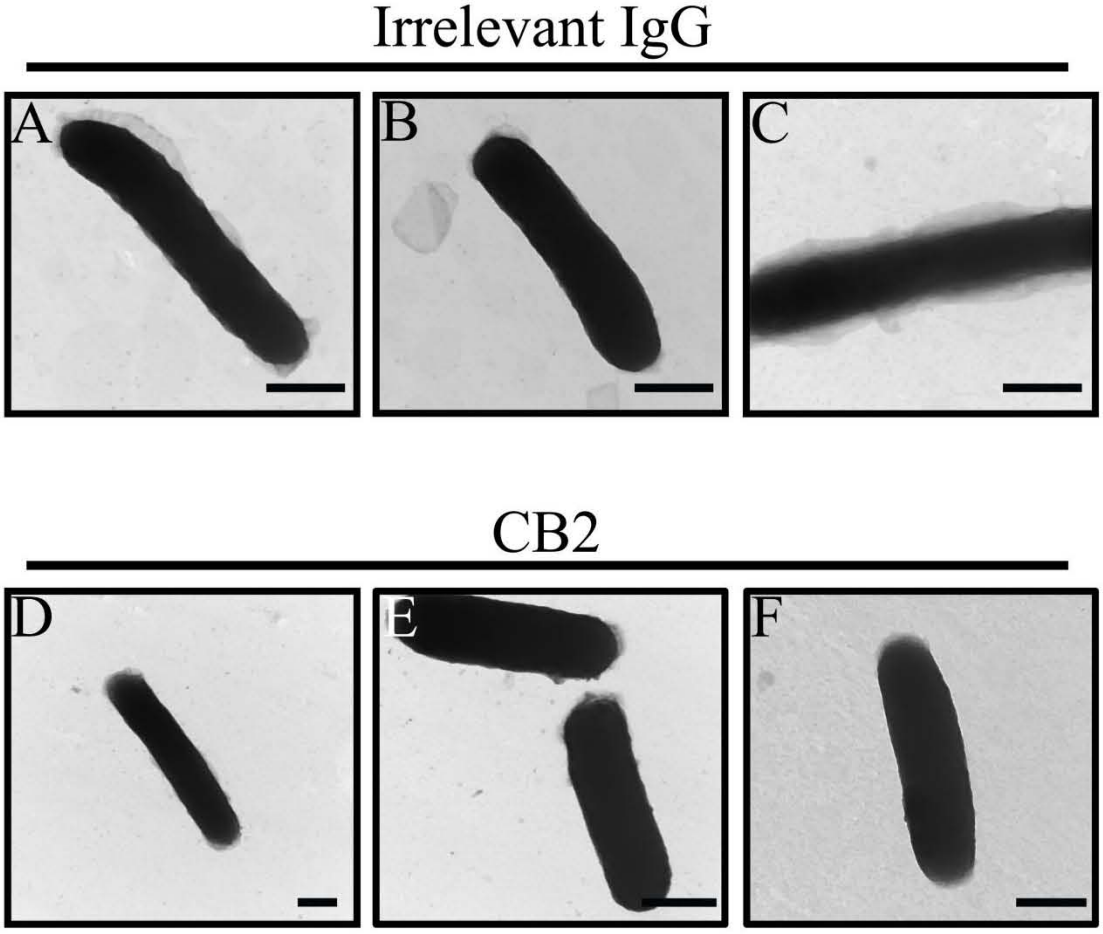
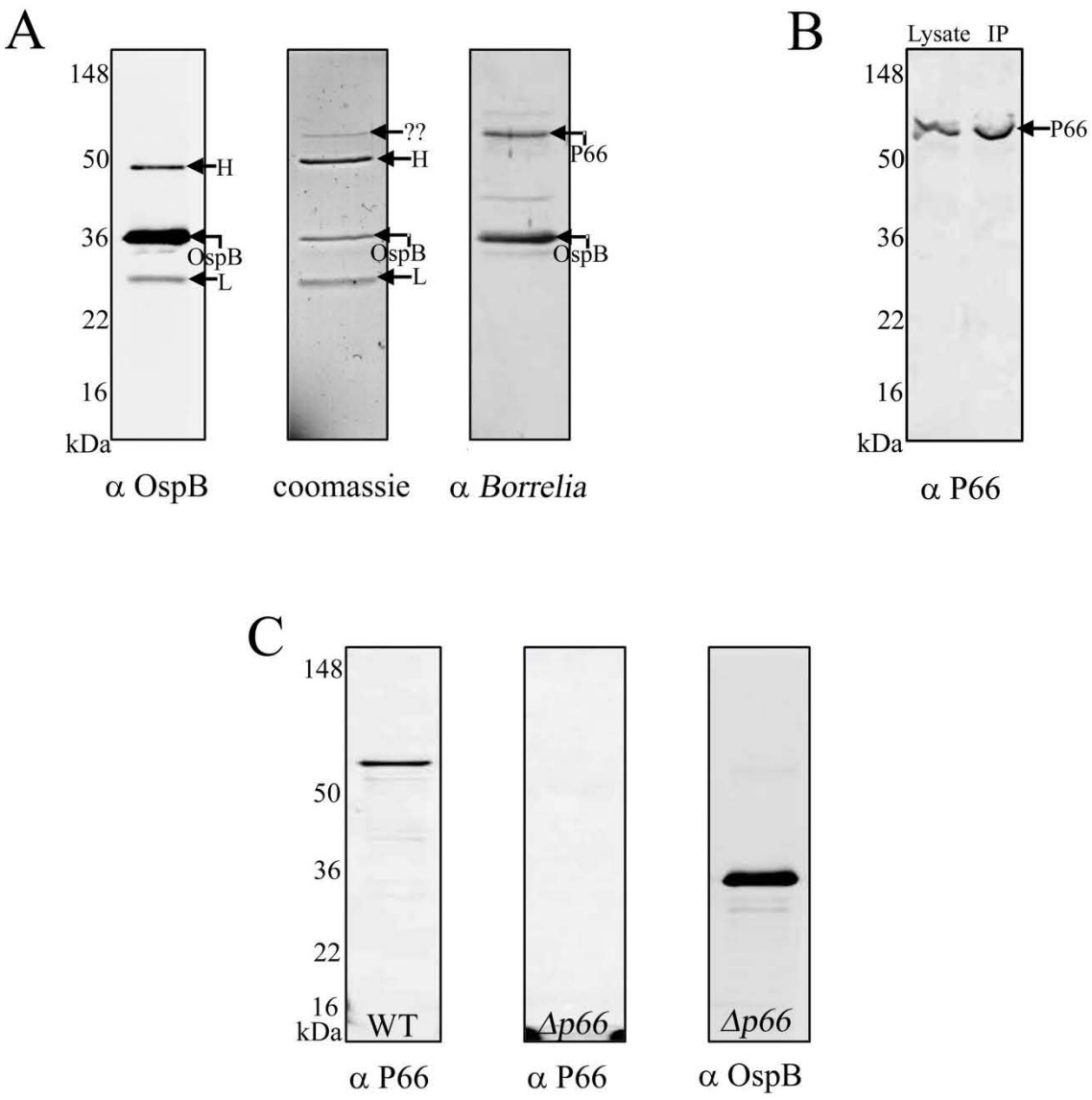
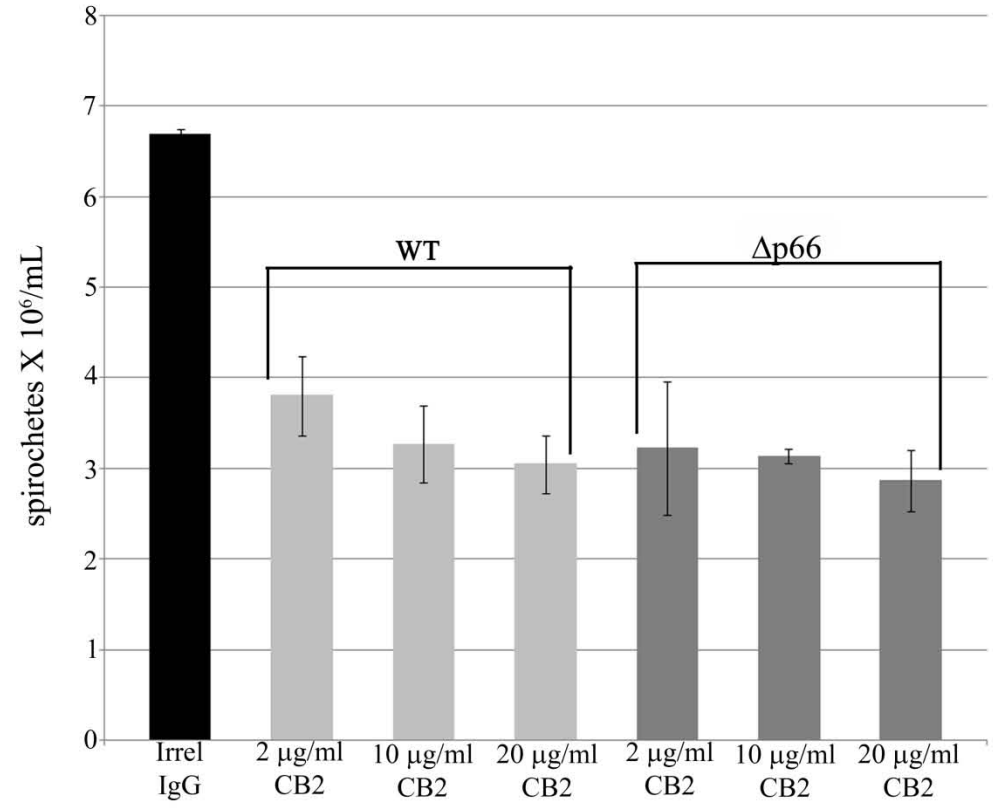

Irrelevant IgG
A
B
C
CB2
D
E
F

Figure 4.4: The porin, P66, is a candidate accessory protein for the bactericidal mechanism of CB2. **A.** Immunoblots and Coomassie-stained gel of OspB immuoprecipitation showing the co-precipitant later identified by mass spectrometry as P66. H and L refer to the heavy and light chains of CB2. **B.** Immunoblot showing specific recognition of P66 in *B. burgdorferi* lysate and in the OspB immunoprecipitate confirming mass spectrometry results that identified P66 as the co-precipitant. **C.** Immunoblots showing the absence of P66 from *Δp66* mutant and the presence of OspB recognized by CB2.

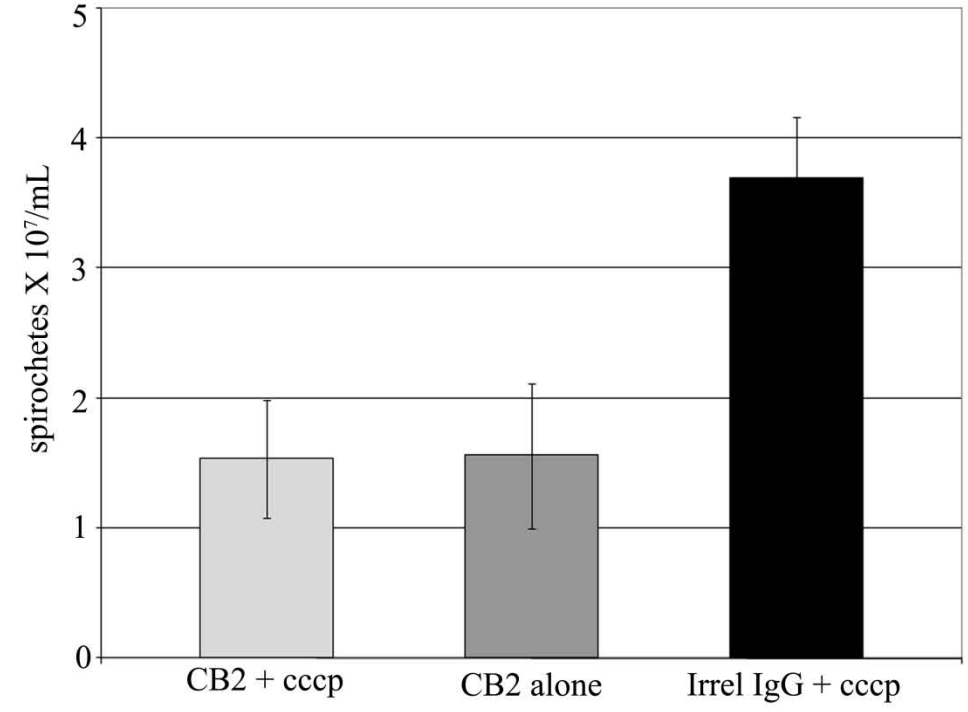

A
148
50
36
22
16
kDa
H
OspB
L
??
H
OspB
L
P66
OspB
α OspB
coomassie
α Borrelia
B
Lysate
IP
148
50
36
22
16
kDa
P66
α P66
C
148
50
36
22
16
kDa
WT
Δp66
Δp66
α P66
α P66
α OspB

<u>Figure 4.5</u>: P66 and periplasmic flagellar movement are not critical for the bactericidal function of CB2. A. Killing assay showing that CB2 has identical effects when used against WT or *Δp66* demonstrating that P66 is not critical for the bactericidal mechanism of CB2. **B.** Killing assay where *B. burgdorferi* were immobilized with CCCP or not and treated with CB2. CB2 has identical bactericidal effects against moving or immobile spirochetes indicating that periplasmic flagellar movement is not critical for the bactericidal action of CB2.

A


spirochetes X 10^6/mL
8
7
6
5
4
3
2
1
0
WT
Δp66
Irrel IgG
2 µg/ml CB2
10 µg/ml CB2
20 µg/ml CB2
2 µg/ml CB2
10 µg/ml CB2
20 µg/ml CB2

B


spirochetes X 10^7/mL
5
4
3
2
1
0
CB2 + cccp
CB2 alone
Irrel IgG + cccp

<u>Figure 4.6</u>: Lower temperatures do not affect the binding of CB2 to OspB. Immunoprecipitation of OspB with CB2 was completed using the CB2 binding times of **A.** 15 minutes, **B.** 1 hour, or **C.** 2 hours at 33^{o}C and 4^{o}C to determine any difference in the level of CB2 binding. These immunoblots show that the quantity of OspB pulled down at 4^{o}C was not different from that pulled down at 33^{o}C at all time points indicating that the lower temperature of 4^{o}C does not cause a decrease in CB2 binding. Numbers below each OspB band are trace quantity of the protein as determined by densitometry. H and L refer to the heavy and light chains of CB2.

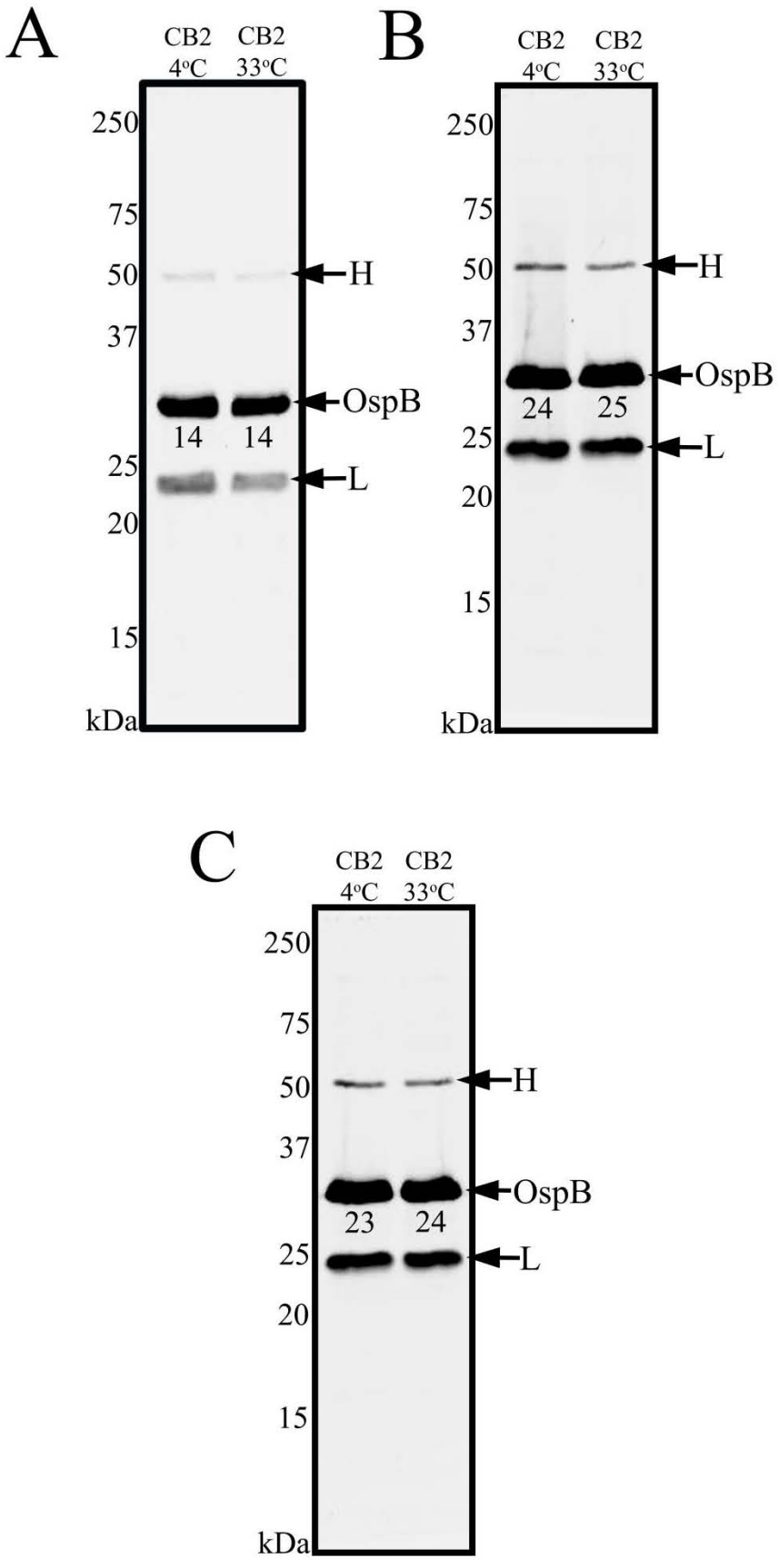

A
CB2 4°C
CB2 33°C
250
75
50
37
25
20
15
kDa
H
OspB
14
14
L
B
CB2 4°C
CB2 33°C
250
75
50
37
25
20
15
kDa
H
OspB
24
25
L
C
CB2 4°C
CB2 33°C
250
75
50
37
25
20
15
kDa
H
OspB
23
24
L

Figure 4.7: CB2 killing is significantly reduced at 4oC. CB2 killing assays were carried out at 33^{o}C and 4^{o}C for **A.** 15 minutes, **B.** 1 hour, and **C.** 2 hours to determine if outer membrane fluidity has an effect on spirochete death. 4^{o}C significantly slowed the bactericidal kinetics of CB2. That the bactericidal activity of CB2 was slowed at 4^{o}C suggests that it may be lower membrane fluidity that slows the activity. ANOVA, *** $p < 0.001$.

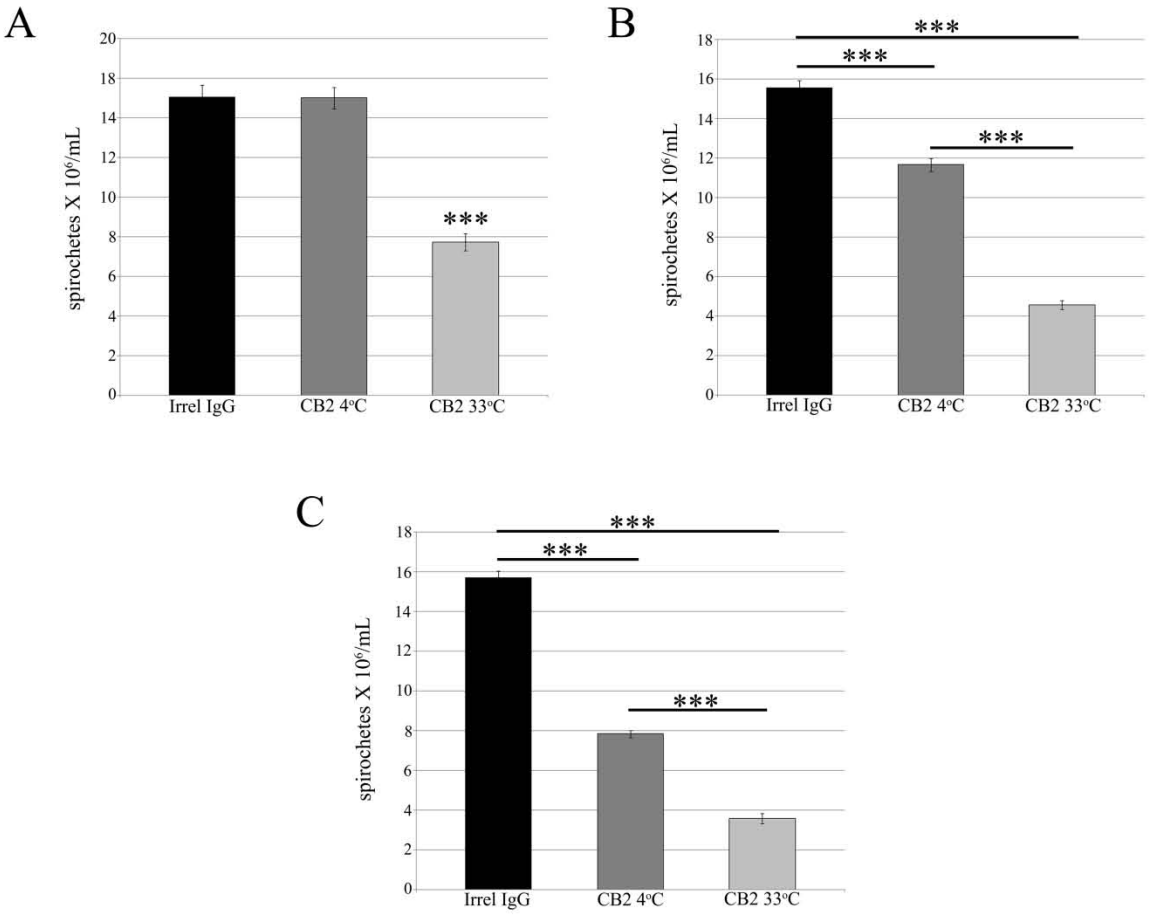
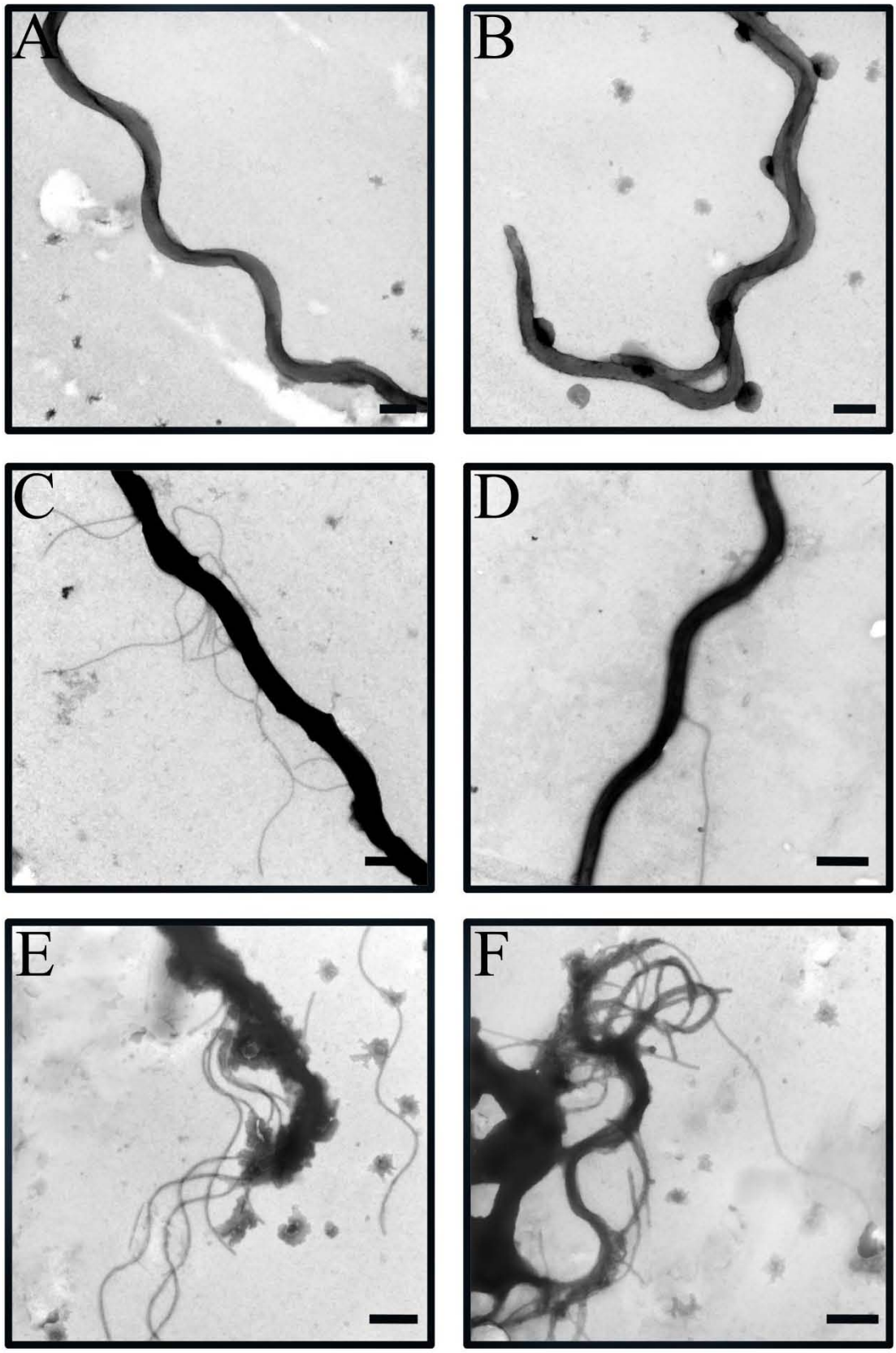

A
spirochetes X 10^6/mL
20
18
16
14
12
10
8
6
4
2
0
***
Irrel IgG
CB2 4°C
CB2 33°C
B
spirochetes X 10^6/mL
18
16
14
12
10
8
6
4
2
0
***
***
***
Irrel IgG
CB2 4°C
CB2 33°C
C
spirochetes X 10^6/mL
18
16
14
12
10
8
6
4
2
0
***
***
***
Irrel IgG
CB2 4°C
CB2 33°C

Figure 4.8: Lower temperatures slow the progression of damage caused by CB2. *B. burgdorferi* were treated with CB2 for 15 minutes (**A – B**), 1 hour (**C – D**), or 2 hours (**E – F**) at 4°C and analyzed by negative stain TEM. Incubation at 4°C greatly slows the progression of damage imparted by CB2. Spirochetes treated with CB2 for 15 minutes at 4°C appear intact with only very few membrane blebs (A – B). The lack of membrane blebs after treatment with CB2 for this time suggests a decreased outer membrane fluidity due to 4°C that may be responsible for slowing the effects of CB2.


A
B
C
D
E
F

CHAPTER 5 - PROPOSED MECHANISM FOR COMPLEMENT-INDEPENDENT BACTERICIDAL ANTIBODIES TO BORRELIA

The results of this Dissertation provide answers to important questions regarding the bactericidal mechanism of complement-independent antibodies to *Borrelia* and consider all aspects of the interaction including important properties of the antibody, specific ciritical events required for the observed effects at the antibody-pathogen interface, and important and critically required properties within the *Borrelia* organisms that are missing from other microbes. Since we have unlocked these pieces of information we provide evidence as to the manner in which complement-independent bactericidal antibodies operate. Thus, we propose the following model (Figure 5.1) for the mechanism of complement-independent bactericidal antibodies to *Borrelia* spp:

1. The antibody (CB515 or CB2) binds its antigen (a 19 kDa Vsp in relapsing fever spirochetes and OspB in *B. burgdorferi*) on the spirochete surface. The variable region of the antibody is sufficient and absolutely required for all events of this mechanism; therefore, the constant region is dispensable, and the unique variable region is absolutely required for the effects exerted by these antibodies.

2. Due to the unusually fluid nature of the outer membrane, the antibodies create thin membrane projections and blebs on the spirochete surface.

3. Likely due to the fluid outer membrane again, the antibody-bound projections and blebs break off of the spirochete surface causing loss of membrane lipids and concomitant release of outer membrane antigens such as OspB.

4. After the antibody-bound projections and blebs break off the surface, large transient breaks (58 – 145 nm) are formed, leaving the outer membrane in an energetically unfavorable state.

5. Redistribution of membrane lipids seals the gaping breaks in the outer membrane.

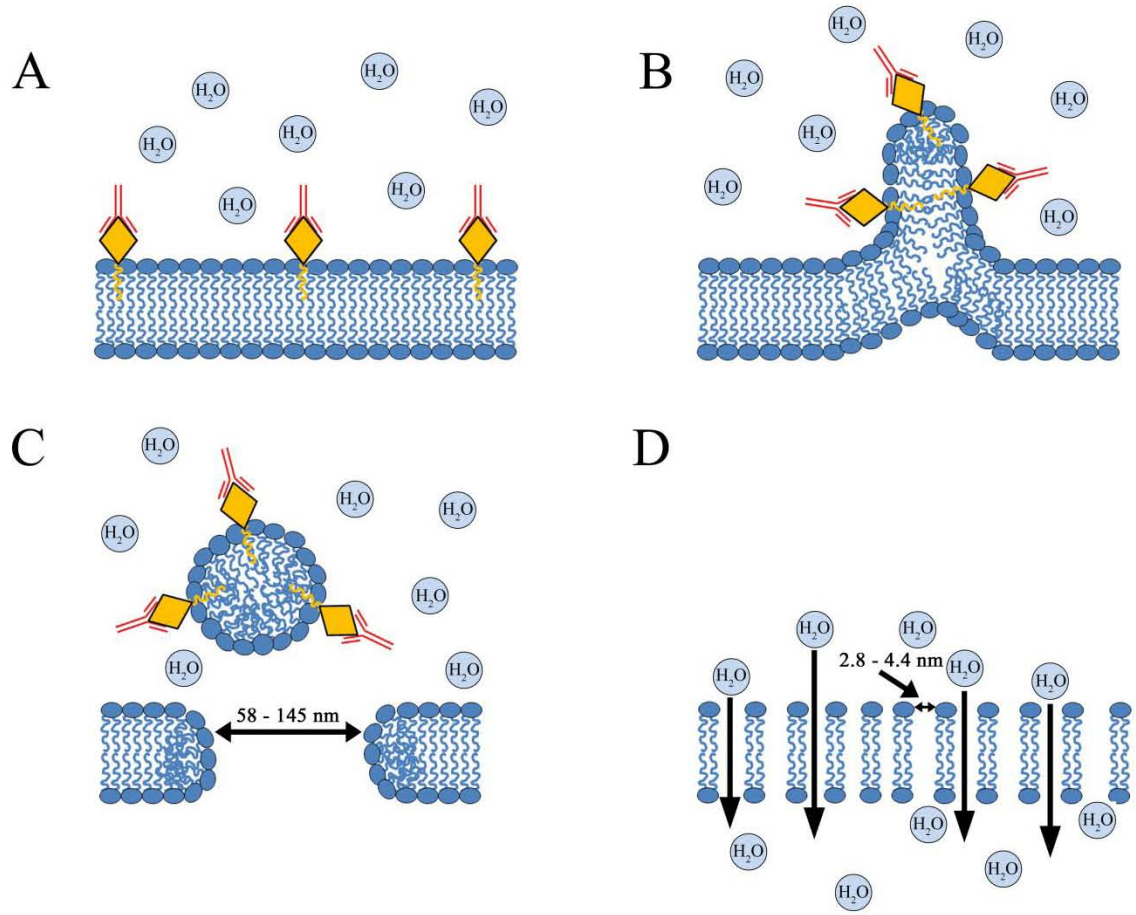
6. As outer membrane projections and blebs continue to be formed, pieces of membrane are increasingly lost, resulting in increased permeability of the outer membrane due to

smaller ambient openings (2.8 – 4.4 nm) across the entire membrane as surrounding membrane lipids become scarcer.

7. The openings of 2.8 – 4.4 nm result in the osmotic lysis of both the outer and inner membranes and thus cause the death of the spirochete.

5.1 FIGURES

Figure 5.1: Proposed mechanism for complement-independent bactericidal antibodies against *Borrelia*. This is a proposed mechanism for the bactericidal action of all complement-independent antibodies against *Borrelia* but this figure is illustrated for CB2 and *B. burgdorferi*. It is also important to remember that the only antibody region required for these actions is the variable region. **A.** CB2 binds to OspB (dependent on Lys 253) on the surface of *B. burgdorferi*. Water (H_2O) is shown surrounding the cell. **B.** Critical residues in the antibody variable region as well as the high fluidity of the spirochetal outer membrane are required for the creation of outer membrane projections and blebs. **C.** The projections/blebs break off, and thus CB2 removes outer membrane pieces which contain OspB. As pieces of membrane are lost, the results are transient large breaks in the outer membrane of 58 – 145 nm in diameter that are rapidly sealed due to hydrophobic effects. **D.** Outer membrane blebbing and transient break formation with sealing continues until so many lipids are lost that there are openings of an ambient size all around the membrane (2.8 – 4.4 nm), thus increasing its permeability. The openings responsible for the increased permeability of the outer membrane allow for the rapid influx of water as the osmolarity of the periplasm and cytosol are greater than that of the extracellular environment, **E.** thus rendering the spirochete susceptible to osmotic lysis.


A
B
C
D
E
58 - 145 nm
2.8 - 4.4 nm
= OspB
= CB2

CHAPTER 6 - DISSERTATION SUMMARY

The vast majority of this dissertation includes an already published review, experimental study, and a second experimental study that has been accepted for publication:

- **LaRocca, T. J., and J. L. Benach.** 2008. The important and diverse roles of antibodies in the host response to *Borrelia* infections. Curr Top Microbiol Immunol **319:**63-103.
- **LaRocca, T. J., L. I. Katona, D. G. Thanassi, and J. L. Benach.** 2008. Bactericidal Action of a Complement-Independent Antibody against Relapsing Fever Borrelia Resides in Its Variable Region. J Immunol **180:**6222-8.
- **LaRocca, T. J., D. J. Holthausen, C. Hsieh, C. Renken, C. A. Mannella, and J. L. Benach.** 2009. The Bactericidal Effect of a Complement-Independent Antibody is Osmolytic and Specific to *Borrelia*. Proc Nat Acad Sci. Accepted for publication.

The accomplishments of this dissertation are now summarized:

- An scFv derived from a monoclonal complement-independent IgM to relapsing fever *Borrelia* (CB515) was constructed, expressed, and purified to homogeneity
- The CB515 scFv was found to be monomeric and of a MW of 26 kDa
- The CB515 scFv bound its antigen in immunoblot and in its native form on whole relapsing fever spirochetes
- The binding of the CB515 scFv was shown to be species- and serotype-specific
- The monomeric CB515 scFv exhibited a lower level of binding in comparison to its parent pentameric IgM
- The CB515 scFv replicated the bactericidal effect exhibited by its parent antibody, proving that the constant regions are dispensable and the bactericidal function resides in the variable region

- The CB515 scFv was so effective that treatment resulted in the elimination of the entire serotype population to which it is specific
- Negative-stain and thin section TEM analysis showed that the CB515 scFv causes extensive damage to the spirochetes, identical to its parent antibody
- Treatment with CB2 or the CB515 scFv resulted in osmotic lysis of the inner membrane following outer membrane rupture
- CB2 treatment resulted in the formation of membrane projections and breaks in the outer membrane of 58 – 145 nm in diameter at very early time points as determined by cryo-electron tomography
- The diameters of the membrane projections correlate with the breaks suggesting that the projections are released to form the breaks
- CB2 treatment resulted in an increase in outer membrane permeability due to the formation of openings between 2.8 – 4.4 nm in diameter, resulting in osmotic lysis of the spirochetes
- CB2 was shown to be directly involved in the creation of outer membrane projections/blebs in *B. burgdorferi*
- CB2 directly removed OspB in membrane blebs from the surface of *B. burgdorferi*, likely resulting in the creation of breaks/openings
- CB2 caused a related antigen, OspA, to be released from the spirochete surface
- The antigen of CB2, OspB, was expressed on the surface of *E. coli*
- The bactericidal effect of CB2 was not transferrable to *E. coli*
- The porin, P66, co-precipitated with OspB but was shown to be dispensable for the bactericidal mechanism of CB2
- The bactericidal kinetics of CB2 were greatly slowed at 4^{o}C suggesting that the high fluidity of the outer membrane of *B. burgdorferi* may be critically required for the bactericidal mechanism

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