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Characterization of the Physiological Requirements for the Bactericidal Effects of a Monoclonal Antibody to OspB of *Borrelia burgdorferi* by Confocal Microscopy

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A confocal microscopy study was undertaken to characterize the bactericidal effects of the Fab fragments of CB2, an immunoglobulin G1κ murine monoclonal antibody, to an epitope in the carboxy region of the outer surface protein B (OspB) of *Borrelia burgdorferi*. Simultaneous direct labeling of both fixed and live spirochetes with fluorochrome-labeled Fab-CB2 and 11G1, and an immunoglobulin Mκ monoclonal antibody to OspA, showed that OspA and OspB seem to colocalize in dead spirochetes but do not appear to be physically associated when the organisms are alive. A polar bleb composed of a Fab-CB2-OspB complex, followed by incorporation of 11G1-OspA, precedes the formation of a spheroplast. The spheroplasts contain both OspA and OspB and are a terminal stage in the bactericidal process induced by Fab-CB2. Outer membrane destabilization by Fab-CB2, but not cell wall or cytoplasmic membrane alterations, was demonstrated experimentally by the sequential treatment of spirochetes with Fab-CB2 and monoclonal antibodies to flagellin and DnaK. The action of Fab-CB2 is epitope specific, as another monoclonal antibody to an epitope in the amino terminus of OspB was not bactericidal. The bactericidal effect of Fab-CB2 is not dependent on the induction of spirochetal proteases but is dependent on the presence of Ca^{2+} and Mg^{2+}. Supplementation of Ca^{2+}- and Mg^{2+}-free medium with these cations restored the bactericidal effects of Fab-CB2. The mechanism by which a Fab fragment of an antibody destroys a bacterium directly may represent a novel form of antibody-organism interaction.

Borrelia burgdorferi sensu lato, the etiological agent of Lyme disease, has an abundant number of lipoproteins with covalently attached lipids which collectively have been termed outer surface proteins (Osps) (5, 8, 9). Many of these lipoproteins can be found in the outer membrane (OM) as well as in internal sites, notably the periplasm and the cell membrane-cell wall complex (5, 8, 9, 12, 19, 30, 31). The functions of these lipoproteins in *B. burgdorferi* are not known. By analogy with other bacterial lipoproteins of known function, the *Borrelia* analogs could be involved in maintaining the OM structure, in transport systems, in lytic mechanisms, in conferring hydrophobicity to the cell surface, and in other functions as well (10, 41, 42).

The first two Osp to be studied (OspA and OspB) are highly abundant lipoproteins that are strongly immunogenic (5). In particular, OspA has been studied extensively and proposed as a vaccine for human use. Immunization with lipidated recombinant OspA can lead to protection of laboratory animals against challenge with infectious organisms (21, 36, 40) and also can lead to spirochetal death in the midguts of ticks feeding on vaccinated animals (22).

Attempts at inhibiting spirochetal adhesion to cells by blocking OspB with monoclonal antibody (MAb) CB2 showed that the antibody alone destroyed the spirochetes in a system devoid of complement (17, 23). Later studies showed that the Fab fragments of CB2 were also bactericidal (18). Identical results were obtained with H6831, another MAb to OspB (32, 34, 35). Both MAbs, CB2 and H6831, require the presence of a lysine residue at position 253 in the carboxy terminus of OspB for recognition of the antigen and hence for their lytic function. The epitopes for both MAbs probably are identical as well. Selective pressure from these antibodies allows for the proliferation of escape variants or OspB mutants which lack Lys-253, or have other amino acids at this position, and which in general are less pathogenic than the wild-type parent strains (3, 18, 33).

There are several unique features about the direct action of CB2 and H6831 on *B. burgdorferi*. That univalent Fab fragments can destroy these organisms precludes agglutination and complement dependency as mechanisms for cell death. The epitope for both MAbs is located in a variable and hydrophilic region of OspB, and there may be strong selective pressure for change in this vulnerable domain. Other complement-independent bactericidal MAbs to epitopes in OspA and OspB have been identified (29), but the action of their Fab fragments was not studied. Whole immunoglobulins of human MAbs to p39 were also bactericidal in the absence of complement (39). Collectively, the data suggest that *B. burgdorferi* is extremely susceptible to the direct action of antibodies and that this is a major mechanism of host defense (21, 27, 36, 40).

Paradoxically, antibodies to OspA and OspB do not develop until the later stages of Lyme disease (14) and are not induced in mice through tick transmission of *B. burgdorferi* or by low numbers of spirochetes inoculated by needle (6). Recent attempts to explain this paradox have shown that OspA (and also OspB, since these lipoproteins are encoded by a two-gene operon under the same promoter in a linear plasmid [8]) is

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expressed in the midgut of unfed ticks and in culture, but at the time of transmission and in the vertebrate host expression of OspA and OspB is downregulated, at least until the late stages of the disease (13, 38). Furthermore, the formation of antibodies to OspA in late-stage Lyme disease has been associated with the development of arthritis (28). In this regard, bactericidal antibodies to OspB may have in vivo relevance in late-stage disease and in vaccines for the elimination of spirochetes from ticks feeding on immune individuals. More importantly, however, the mechanism by which an antibody directly destroys a bacterium represents a novel form of host response. In this study, confocal microscopy was used to study the physical associations of the MAb-CB2 antibody-OspB antigen complex and to study some of the physiological requirements for the bactericidal action of this antibody.

MATERIALS AND METHODS

Bacteria and antibodies. The B31 strain of *B. burgdorferi* sensu stricto was grown in serum-free BSK medium at 33°C. Monoclonal antibodies CB2, an immunoglobulin G1κ (IgG1κ) to OspB (3, 16–18), 11G1, an IgMκ to OspA (7), CB1, an IgG3κ to flagellin (15), CB312, an IgG2aκ to DnaK (16), and H4610, an IgG2aκ to OspB (3), were purified from murine ascites with protein A-Sepharose CL-4B columns (Pharmacia LKB Biotechnology, Piscataway, N.J.). 11G1, an IgM antibody, was purified from ascites by precipitation with saturated ammonium sulfate, resuspension of the precipitate in phosphate-buffered saline (PBS), dialysis, and further purification of the immunoglobulin by agarose affinity chromatography (2) (Bioaffinity Systems, Inc., Roscoe, Ill.) according to the manufacturer's protocol. Reactivity of all MAbs was verified by immunoblotting.

Fab fragments of the MAbs were prepared by papain digestion followed by the elimination of the Fc fragments by protein affinity chromatography (Immuno-Pure Fab Preparation kit; Pierce, Rockford, Ill.). Purity of the Fab preparations was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Fluorochrome labeling of MAbs. Both Fab fragments and intact immunoglobulin molecules were labeled with rhodamine and fluorescein by using established methods (24). Briefly, for rhodamine, 1 ml of MAb (2 mg of Fab or whole molecule per ml) in 0.1 M sodium carbonate (pH 9.0) was incubated with 50 μl of 1 mg of tetramethylrhodamine isothiocyanate (TRITC; Aldrich Chemicals, Milwaukee, Wis.) per ml in dimethyl sulfoxide for 8 h at 4°C followed by the addition of xylene cyanol to 0.1% and glycerol to 5%. Excess fluorochrome was separated by repeated passages through a column of Sephadex G-25 (Pharmacia). The final product was resuspended in PBS supplemented with 1% bovine serum albumin (BSA). The method for fluorescein labeling using dichlorotriazinylamino-fluorescein (DTAF; Sigma, St. Louis, Mo.) was identical to that used for rhodamine except that the mixture was incubated with stirring for 10 min at room temperature followed by incubation at 4°C for 2 h. The ratios of fluorochrome to protein were estimated by absorbances at 495 nm for TRITC, at 575 nm for DTAF, and at 280 nm for protein. Optimal ratios for TRITC ranged between 0.3 and 0.7, and those for DTAF ranged between 0.3 and 1.0.

Direct and indirect immunofluorescence assays. For slide assays, spirochetes were separated from the medium by centrifugation (7,000 × *g* at room temperature for 8 min) and washed twice in PBS supplemented with 2% BSA and 5 mM $MgCl_2$. The spirochetes were adhered to slides, allowed to dry overnight at 4°C, and stored at −70°C until used. For direct immunofluorescence, the conjugated antibody was incubated for 1 h at 37°C in moist chambers, and the slides were washed in two changes of PBS. For indirect immunofluorescence, the slides were incubated with both primary MAbs and secondary antibodies (affinity-purified TRITC or fluorescein isothiocyanate-conjugated goat anti-mouse IgG (γ-chain specific) or IgM (μ-chain specific) for 1 h each at 37°C with two washes in PBS following each incubation. Slides were mounted with SlowFade (Molecular Probes, Inc., Eugene, Oreg.).

A previously described technique (5) was followed for tube assays. Briefly, washed spirochetes resuspended in medium, or unwashed spirochetes directly in medium, both from rapidly growing cultures, were incubated for 1 h at either 4 or 33°C with the labeled antibody or with the primary antibody (for indirect immunofluorescence), followed by one wash in large volumes of PBS-BSA-$MgCl_2$ after each incubation. Both wet and dry mounts of labeled spirochetes were used for the experiments. All conjugates were purchased from Organon-Teknika, Durham, N.C.

Confocal microscopy. A Nikon Diaphot inverted microscope equipped with epifluorescence (Nikon, Melville, N.Y.) and with a Noram Odyssey laser confocal system (Noram, Middleton, Wis.) was used. Image capture was done with Image software from Universal Imaging, Westchester, Pa.

Reagents. Protease inhibitors (leupeptin [1.6 μg/ml], pepstatin [1.6 μg/ml], aprotinin [0.7 μg/ml], phenylmethylsulfonyl fluoride [34.8 μg/ml], and EDTA [5 mM] [Sigma]) were also added to the BSK medium singly or in combination for incubation with spirochetes. Hanks' balanced salt solution (HBSS) without calcium and magnesium (Hw/o) and complete HBSS (CH; Gibco BRL Products, Gaithersburg, Md.) were used to determine the effect of these ions on the bactericidal properties of CB2.

A BacLight viability kit (Molecular Probes) was used to determine bacterial mortality as recommended by the manufacturer.

RESULTS

CB2-Fab leads to the formation of spheroplasts. In previous studies, spirochetal death due to the action of bactericidal MAbs was measured visually and metabolically (17, 18, 32–35). For these studies, the bactericidal effect of MAb CB2 to OspB was measured with a vital stain, BacLight, which allowed for rapid evaluation of bacterial death by direct enumeration of differentially stained organisms. Incubation of *B. burgdorferi* with 2 μg of the Fab fragments of CB2 per ml resulted in nearly complete mortality of the spirochetes within 3 h as determined by the use of BacLight (Fig. 1A). The staining pattern of a control (untreated) culture with the vital stain is shown in Fig. 1B. The method used to determine whether OspB and the CB2-OspB complex were physically associated with OspA used the DTAF-labeled Fab fragment of CB2 (Fig. 2) and the TRITC-labeled 11G1 (whole molecule of IgM MAb to OspA) for examination with confocal microscopy. These two similar lipoproteins are cotranscribed by the same operon in a linear plasmid, and their structural similarities suggest similar functions and locations (12). In this procedure, monovalent antibodies which cannot cross-link antigens (Fab fragments) do not cluster, whereas intact Ig molecules cross-link their respective antigens and cluster them.

The simultaneous use of whole molecules (bivalent) as well as Fab fragments (monovalent) of the MAbs (each with a different fluorochrome label) will detect whether capping with a bivalent MAb "drags" other proteins which may be physically associated into visible clusters. Through image integration of both fluorochromes, confocal microscopy can provide visual information on the physical association of the antigens.

Spirochetes fixed to a slide showed a diffuse pattern of antibody binding for both OspA and OspB with full integration of the double image, suggesting that in the dead organism, these two lipoproteins overlap fully (Fig. 3A). This was a constant finding for all fixed (dead) spirochetes. However, when live spirochetes were labeled in a tube assay for 1 h, the pattern of antibody binding was different. OspA and OspB did not appear to colocalize in the live organism, as indicated by the discrete, patchy staining (Fig. 3B), suggesting that the two lipoproteins are intercalated or distributed as opposing helices along the length of the organism. Quantitation of this observation was not possible since this staining pattern is transient in the live organisms, and with increasing incubation time, the diffuse antibody distribution (as organisms die) became predominant. Confirmation of the lack of colocalization of OspA and OspB in live *B. burgdorferi* will require other experiments. Nonetheless, a disruption of this architecture, noted with increasing time of incubation with the MAbs, could have an effect on the structural integrity of the spirochetes. The polar bleb detected with the Fab-CB2-OspB complex contained no OspA (Fig. 3B). Since in this experiment the organisms were labeled with Fab-CB2 while alive, it is unlikely that the formation of a polar bleb containing the Fab-CB2-OspB complex could be due to a capping mechanism. Rather, perhaps the polar bleb formed as a result of the planar waveform motion of *B. burgdorferi*, where a wave begins at one end and propagates toward the other end of the cell (25). With increasing time of incubation, the 11G1-OspA complex, in addition to the Fab CB2-OspB complex, began to be seen in the polar bleb, coinciding with a loss of wave amplitude and preceding the formation of spheroplasts (Fig. 3C).

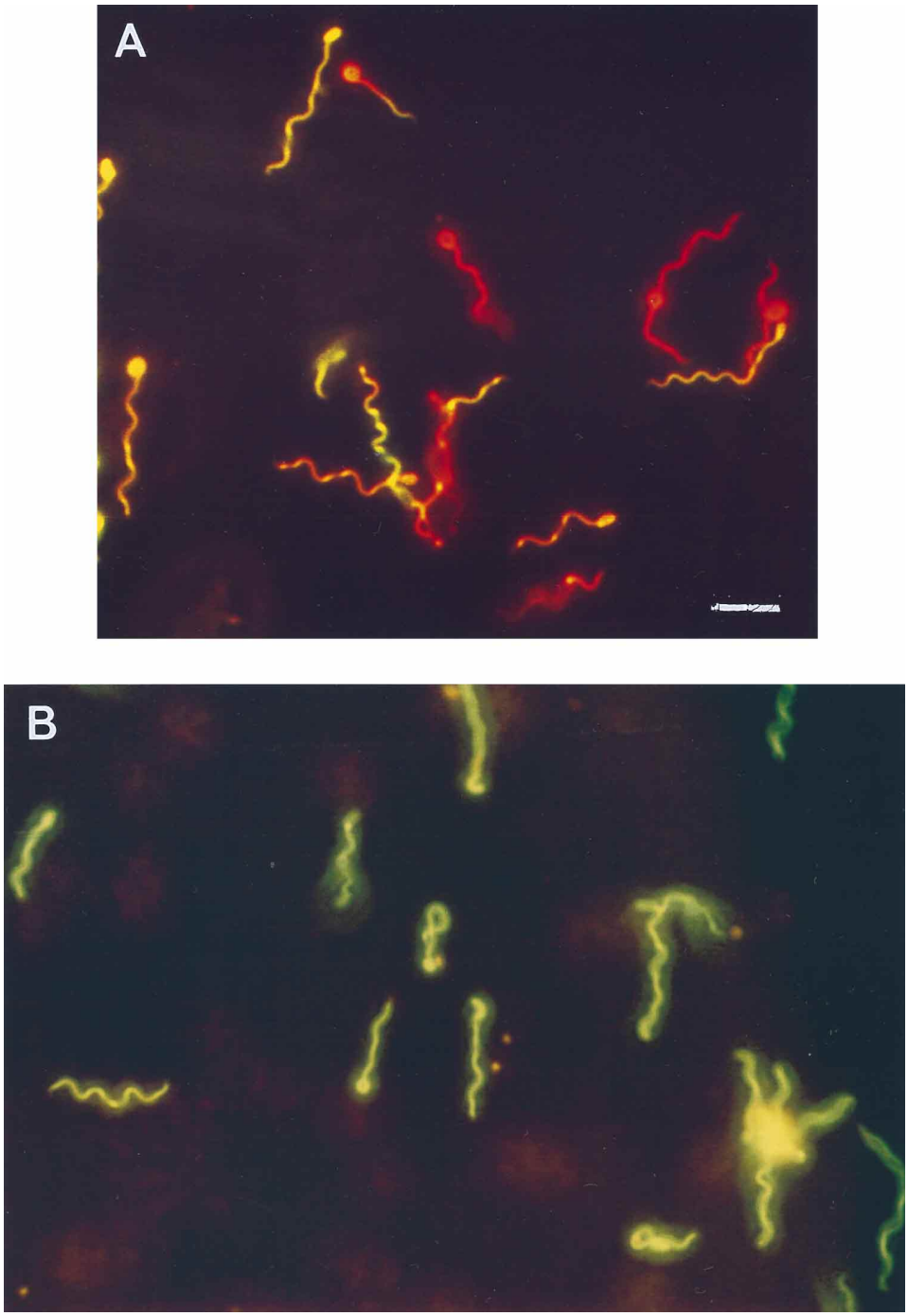

FIG. 1. (A) Vital stain (BacLight) of *B. burgdorferi* following a 1-h incubation with 2 μg of Fab-CB2 per ml. Dead spirochetes appear red. (B) Vital stain (BacLight) of an untreated control culture of *B. burgdorferi*. Live spirochetes appear green. Bar = 10 μm.

Significant amounts of both OspA and OspB remained associated with the spheroplast after a 3-h incubation with the labeled MAbs (Fig. 3D), but the discrete pattern of patching was lost in these forms. Rather, both molecules colocalized as in the dead spirochete (Fig. 3A). The spheroplasts aggregated spontaneously in experiments using Fab-CB2 alone (Fig. 4), indicating that the aggregation was not due to antibody-mediated agglutination. Identical aggregated forms were obtained by treatment of spirochetes with Tris-buffered lysozyme–EDTA, also without a decrease in the concentration of OspA and OspB (11).

Fab-CB2 destabilizes the OM of *B. burgdorferi*. Anti-flagellin MAb CB1 did not bind to live organisms with an intact OM, but it penetrated into the periplasmic space where the endoflagella are located when the organisms were permeabilized by fixation with methanol (Fig. 3E). Coincubation of Fab-CB2-DTAF with CB1-TRITC (anti-flagellin whole molecule) with *B. burgdorferi* for 1 h showed a left-handed helical distribution for OspB and a right-handed helical distribution for flagellin around the cell body axis (Fig. 3F). This same distribution of the periplasmic flagella in *B. burgdorferi* was noted in a recent study (26). The areas of fluorochrome overlap may represent

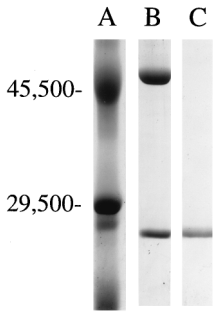

FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the whole molecule and Fab fragments of CB2 used in this study. Lane A, M_r markers; lane B, whole CB2 molecule; lane C, Fab fragment of CB2.

periplasmic OspB (Fig. 3F). Sequential double-label experiments were done to assess the integrity of spirochetal membranes after treatment with MAb CB2. Live spirochetes were incubated with 2 μg of Fab CB2-DTAF per ml for 1 h followed by a wash and a 1-h incubation with 2 μg of CB1-TRITC per ml. Prior incubation with Fab CB2-DTAF for 1 h allowed for the staining of the endoflagella of the spirochetes with CB1-TRITC (Fig. 5). This experiment indicates that Fab-CB2 altered the integrity of the OM by permitting the staining of the periplasmic flagella. Controls of live spirochetes incubated with CB1-TRITC alone were not stained (data not shown). An identical sequential experiment was conducted with anti-DnaK CB312-TRITC following a 1-h incubation with Fab CB2-DTAF, but the antigen was not detected by this assay (data not shown). DnaK is a cytoplasmic marker, and so this experiment suggests that the integrity of the cytoplasmic membrane or the cell wall or both was not compromised by Fab-CB2 under these conditions.

Bactericidal effect of CB2 is epitope specific, requires bivalent cations, but does not require the activation of spirochetal proteases. Although the bactericidal effect of Fab-CB2 is associated with the destabilization of the OM, the physiological requirements for this effect are not known. This series of experiments examined the effect of an MAb to a different epitope in OspB. MAb H4610, to an epitope in the amino-terminal region of OspB (3), at whole-molecule concentrations 5- and 10-fold greater than those of Fab-CB2, did not affect the long-term growth of *B. burgdorferi* or the viability of these organisms, even though this antibody can bind live as well as fixed *B. burgdorferi* (Fig. 6).

The possibility that the formation of a Fab-CB2-OspB complex could result in an influx of ions into the periplasm leading to the loss or destabilization of the OM was studied by determining the effect of Ca^{2+} and Mg^{2+} in this process. These experiments were done by incubating spirochetes in CH and in Hw/o, with 2 μg of Fab-CB2 per ml. HBSS does not support the growth of *B. burgdorferi*. Two measurements were taken: total number of organisms following incubation with Fab-CB2 at 33°C (Fig. 7A) and mortality of remaining spirochetes (Fig. 7B). Both cations were required for the bactericidal effect of Fab-CB2, as indicated by the lack of spirochetal death in Hw/o (Fig. 7). However, the bactericidal effect could be partially restored by supplementing the Hw/o with various concentrations of the cations up to 1.4 mM $CaCl_2$ and 2 mM $MgCl_2$, which are the concentrations in CH. Restoration of the bactericidal effect was more prominent with Ca^{2+} supplementation (Fig. 8A) than with Mg^{2+} alone (Fig. 8B). Supplementation with both cations restored the bactericidal effect of Fab-CB2 (Fig. 8C). The binding of Fab-CB2 to *B. burgdorferi* was not affected by the absence of these bivalent cations, as determined by direct immunofluorescence assays carried out in Hw/o.

The possibility that activation of a spirochetal protease(s) is a mechanism for the bactericidal action of Fab-CB2 is unlikely since protease inhibitors singly or in combination at the concentrations listed in Materials and Methods failed to alter the bactericidal effect of Fab-CB2 (data not shown). For these experiments, the spirochetes were incubated for 1 h with the protease inhibitors in BSK at 33°C, and the spirochetes were assessed for viability with BacLight both before and after the addition of Fab-CB2. EDTA alone at the concentration used (5 mM) was toxic to the spirochetes, which precluded its further use in these experiments.

DISCUSSION

These studies indicate that Fab-CB2 kills *B. burgdorferi* by an irreversible destabilization of the OM, followed by spheroplast formation or lysis. This bactericidal action is epitope specific, as a MAb to another epitope in OspB did not harm the organisms. A requirement for bivalent cations for the bactericidal action but not for the binding of Fab-CB2 was also demonstrated.

Disruption of the OM of *B. burgdorferi* by CB2 (17) and by H6831 (35) has been shown by transmission electron microscopy. It was of interest to study this process in the context of the roles of OspA and OspB and their physical association in both the live and injured organisms. The physical association of OspA and OspB in both live and dead spirochetes was determined by confocal microscopy through the simultaneous use of Fab-CB2-DTAF (anti-OspB) and 11G1-TRITC (anti-OspA). There was a differential distribution of both lipoproteins that was dependent on the viability of the organisms at the time of staining. In the fixed organisms, the two lipoproteins colocalized completely (Fig. 3A), suggesting a loss of the discrete locations that was seen in the live organisms (Fig. 3B). The locations of OspA and OspB in the live organisms suggest that these lipoproteins are intercalated or distributed in opposing helices along the length of the spirochete, but as mentioned earlier, confirmation of this distribution in the live spirochete will require further studies.

A polar bleb containing the Fab-CB2-OspB complex was formed in the initial stages of the bactericidal process. Given the constraints of this experiment, we cannot preclude that the polar bleb has a more extensive composition indicative of physical associations of OspB with other unidentified molecules. However, OspA was clearly not part of the initial constituents of this polar bleb. Since Fab fragments of CB2 were used, it is unlikely that the polar bleb formed as a result of capping. Capping could have been expected from the use of 11G1 whole molecule (anti-OspA). If OspA and OspB were physically associated, the 11G1-OspA complex would have dragged the Fab-CB2-OspB complex along. With increasing incubation time with both MAbs, an 11G1-OspA complex appeared in the polar bleb. This is interpreted as a sequential passive mechanism of molecular aggregation as the OM is shed (5), possibly driven by the planar wave motion of *B. burgdorferi* (25). It is important to emphasize that CB2 does not recognize OspA and that 11G1 does not recognize OspB by enzyme-linked immunosorbent assay or immunoblotting (7, 16). In fact, CB2 has been used to purify native OspB to homogeneity by affinity chromatography (3).

The spheroplasts that form as an end stage to the bactericidal action of Fab-CB2 are morphologically similar to the spheroplasts induced by treatment with Tris-buffered ly-

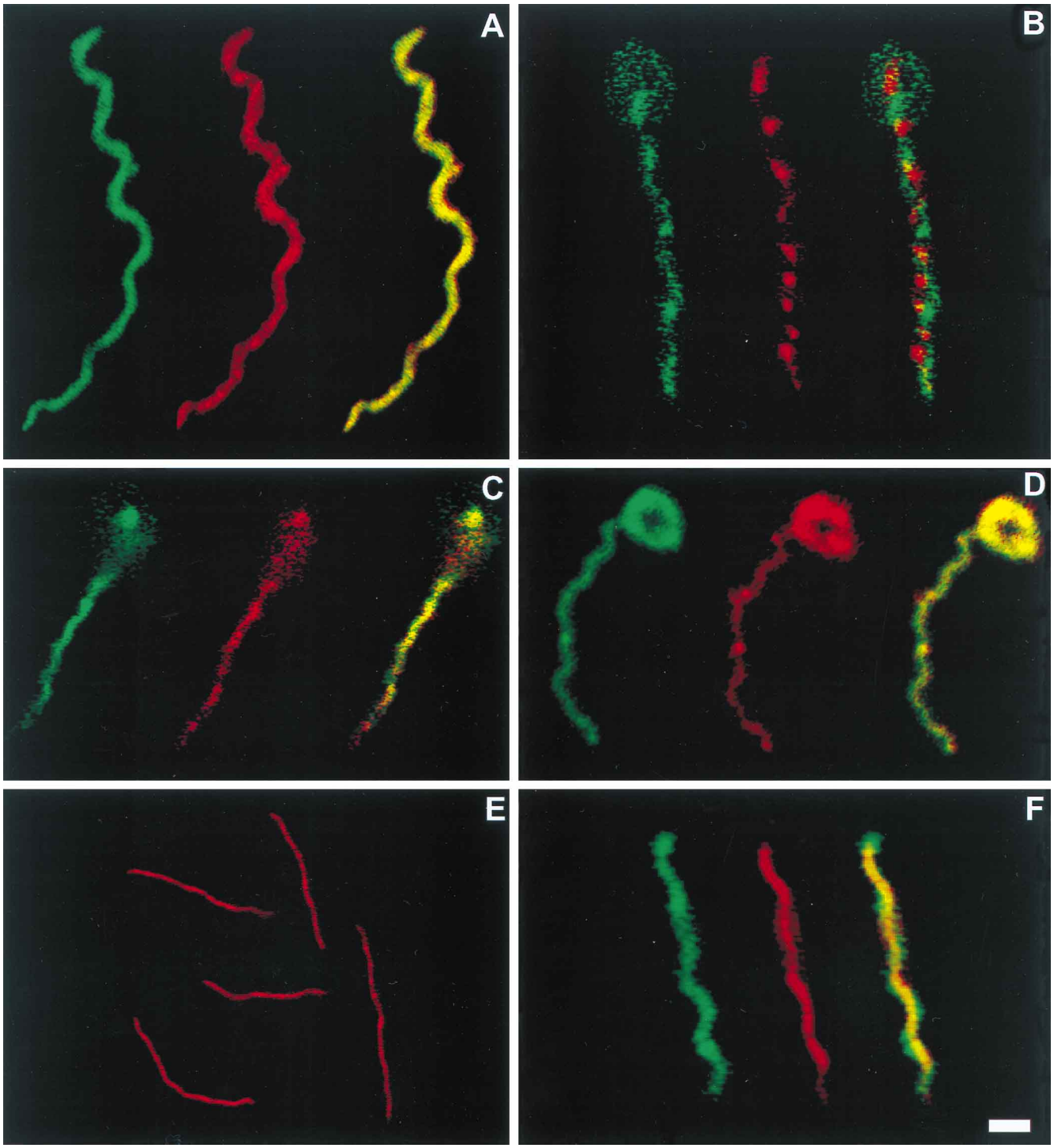

FIG. 3. (A) Direct immunofluorescence of dead *B. burgdorferi* (slide assay) with Fab fragment of CB2-DTAF (left), direct immunofluorescence with anti-OspA 11G1-TRITC (center), and confocal integration of both fluorochrome probes (right). (B) Direct immunofluorescence of live *B. burgdorferi* (tube assay) incubated for 1 h with Fab fragment of CB2-DTAF (left), direct immunofluorescence with anti-OspA 11G1-TRITC (center), and confocal integration of both fluorochrome probes (right). This assay showed that OspA and OspB do not appear to be physically associated, and the polar bleb contains only the Fab-CB2-OspB complex. (C) Direct immunofluorescence of live *B. burgdorferi* (tube assay) incubated for 3 h with Fab fragment of CB2-DTAF (left), direct immunofluorescence with anti-OspA 11G1-TRITC (center), and confocal integration of both fluorochrome probes (right). The polar bleb contains both OspA and OspB antibody complexes. (D) Formation of spheroplasts. Stains are as in panel A. (E) Direct immunofluorescence of anti-flagellin CB1-TRITC in methanol-fixed *B. burgdorferi*. (F) Direct immunofluorescence of live *B. burgdorferi* (tube assay) with Fab-CB2-DTAF (left), indirect immunofluorescence with anti-flagellin CB1-TRITC (center), and confocal integration of both fluorochromes probes (right). Bar = 2 μm.

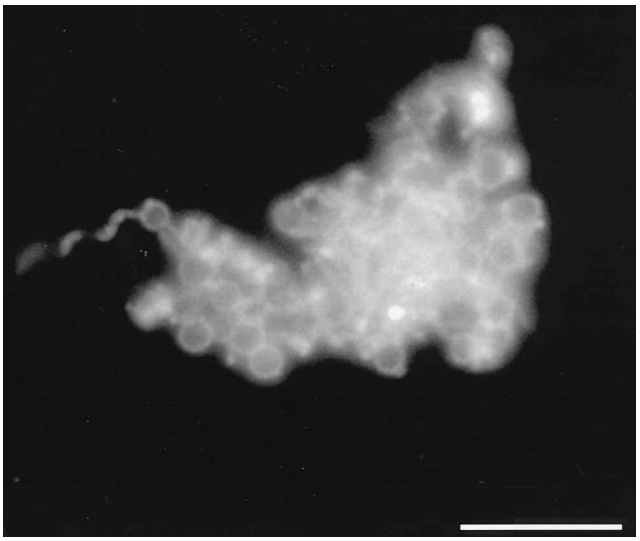

FIG. 4. Aggregations of spheroplasts following a 4-h incubation of spirochetes with Fab-CB2-DTAF. Bar = 7 μm.

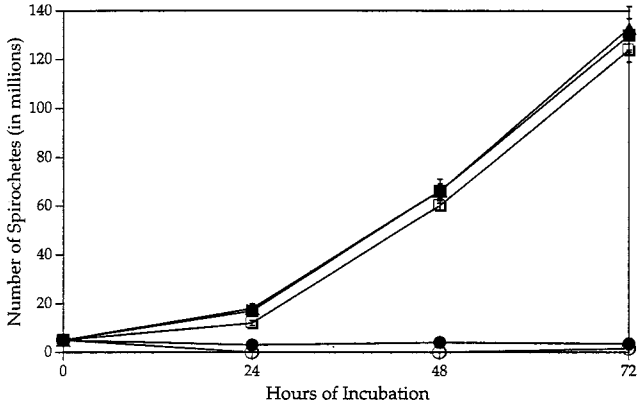

FIG. 6. Effects of various concentrations of anti-OspB MAb H4610 on the growth of *B. burgdorferi*. ▲, control (BSK only); ■, 10 μg of MAb H4610 per ml; □, 20 μg of MAb H4610 per ml; ●, 2 μg of Fab-CB2 per ml; ○, 4 μg of Fab-CB2 per ml.

sozyme–EDTA (11). The formation of spheroplasts ultimately leads to cell death; in our studies, only mutants grow after treatment of spirochetes with CB2 (17, 18). The spontaneous aggregation of spheroplasts seen in our experiments (Fig. 4), as well as in a previous study (11), could be responsible for the actual decrease in numbers of organisms in experiments where enumeration has been used to measure cell death. Another parallel finding is the presence of colocalized OspA and OspB in the spheroplasts. The content of OspA and OspB in spheroplasts induced by treatment with Fab-CB2 was visually undiminished (Fig. 3D) and in agreement with the electrophoretic analyses of spheroplasts induced by Tris-buffered lysozyme–EDTA (11). In both studies, the presence of OspA and OspB (11) and of OspA-OspB-MAb complexes in the spheroplasts also supports the existence of the periplasmic or cell membrane location of these lipoproteins since spheroplasts lack an OM (11, 19, 30, 31).

In the initial stages, Fab-CB2 leads to the physical perturbation of the OM. This has been visualized by transmission electron microscopy (18, 35) and detected experimentally by the ability to stain periplasmic flagella following incubation of the spirochetes with Fab-CB2 (Fig. 3E and F and 5). The initial stage of the bactericidal action would not be possible unless there was surface accessibility of OspB for at least the epitopes for CB2 and H6831, but it is also clear that other mechanisms need to be operational to account for the irreversibility of the MAb's action. As has been pointed out, it is unlikely that the Fab fragments, much less the Fab-OspB complex, could be internalized (35). Thus, binding of the MAbs to their surface epitopes could initiate a signal for the activation of macromolecules in the OM itself or in the periplasm and at the cell wall that could further mediate the irreversible changes. This model assumes an exposed epitope with the lipidated amino terminus anchored (by the covalently bound lipids) to the periplasmic leaflet of the cytoplasmic membrane (9, 12, 19, 30, 31). This model further assumes that the molecule spans the periplasmic space, from the cytoplasmic membrane to the OM. An alternative hypothesis drawn from biochemical and freeze-fracture

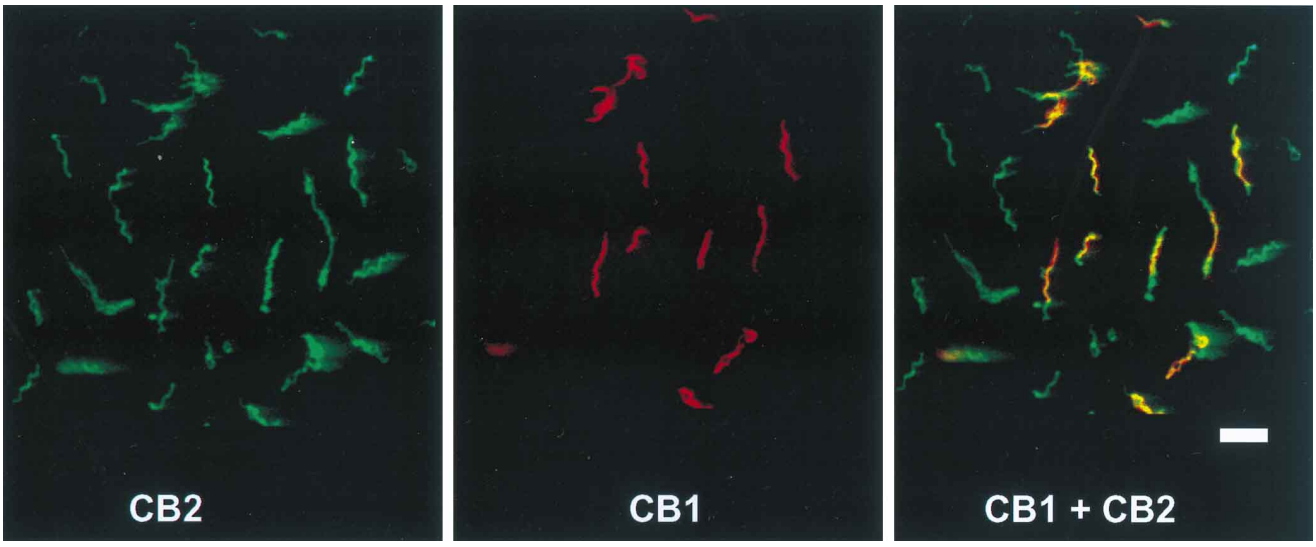

FIG. 5. Sequential staining of live *B. burgdorferi* with Fab-CB2-DTAF for 1 h in a tube assay, followed by anti-flagellin CB1-TRITC for another hour, with subsequent confocal integration of both fluorochrome probes. Bar = 0.5 μm.

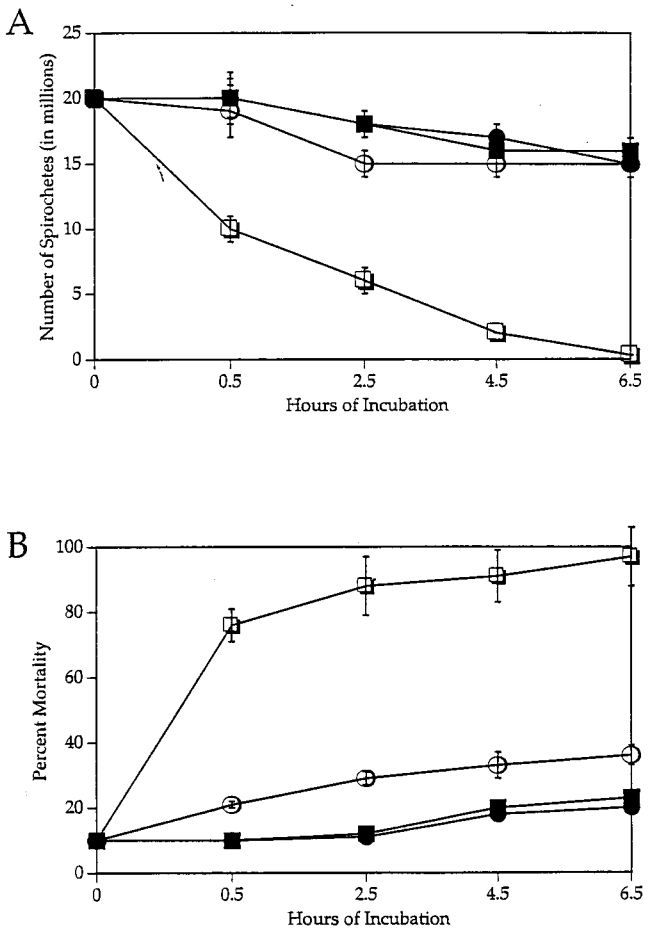

FIG. 7. Effects of Ca^{2+} and Mg^{2+} on the bactericidal action of Fab-CB2 on total numbers of *B. burgdorferi* following incubation with Fab-CB2 (A) and percent mortality (B). ■, CH; □, CH with 2 μg of Fab-CB2 per ml; ●, Hw/o; ○, Hw/o with 2 μg of Fab-CB2 per ml.

electron microscopy studies (31) would have a small number of OspB molecules on the bacterial surface, with the remaining molecules anchored to the cytoplasmic membrane. This model would require an interaction between the OM-exposed OspB and a transmembrane second messenger in order to transfer the signal to initiate the lytic cascade.

The bactericidal action of CB2 (and of H6831 [35]) is epitope specific and requires the presence of a Lys residue at position 253. MAb H4610, with an epitope within amino acid residues 17 to 118 of OspB, failed to injure spirochetes at whole-molecule concentrations 5 and 10 times greater than those used for Fab-CB2 (Fig. 6). The binding of Fab-CB2 to this epitope could cause a conformational change in OspB, which could, in turn, facilitate its interaction with a second messenger.

The similarities between the morphological changes that spirochetes undergo in response to the anti-OspB MAb by H6831 and to antibiotics have been noted (35). This may reflect a common pathway for spirochetal death or simply that different killing pathways result in similar morphological characteristics. While antibiotic-induced changes of spirochetes indeed resemble the changes induced by CB2 and H6831, including the formation of spherical structures (4, 20, 37, 43), the changes with the MAbs occur more rapidly. Morphological changes in *B. hermsii* as a result of treatment with benzylpenicillin required 10 h of incubation (4); 24 h was required for *B. burgdorferi* with penicillin and vancomycin (20), and 5 days was required with benzylpenicillin (37).

Fab-CB2-OspB complexes could create physical openings in the OM allowing for rapid infusion of electrolytes, increasing the osmolarity of the periplasm and triggering bivalent cation-dependent cascades. The requirement for bivalent cations (Mg^{2+} and Ca^{2+}) for the bactericidal action of CB2 was demonstrated by the inability of Fab-CB2 to kill the spirochetes in their absence. It is of interest that the lack of these cations did not prevent the binding of Fab-CB2 but clearly interfered, or at least delayed, its bactericidal action (Fig. 7 and 8). The inability of HBSS to support the growth and integrity of *B. burgdorferi* beyond 6 h precluded extending these experiments for longer time intervals, but the results clearly indicated that the cations are required for the destabilization of the OM. In this regard, daptomycin, an acidic lipopeptide antibiotic, has an absolute requirement for Ca^{2+} to induce the collapse of the membrane potential of *Staphylococcus aureus* (1).

The possibility that the Fab-CB2-OspB complex could induce the development of spirochetal proteases was explored by

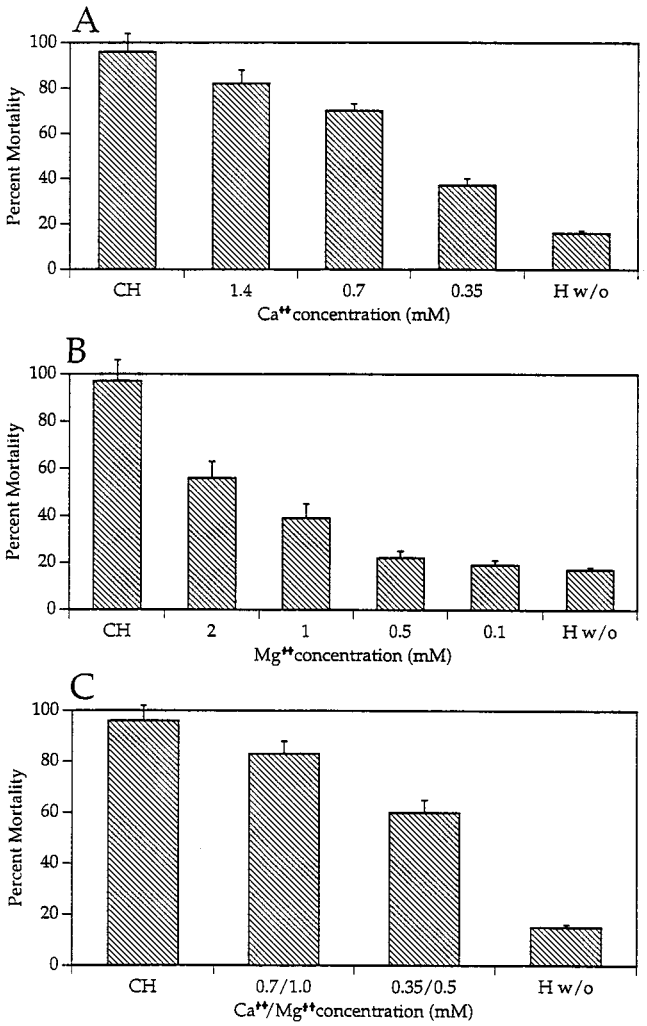

FIG. 8. Restoration of the bactericidal activity of Fab-CB2 after supplementation of Hw/o with various concentrations of $CaCl_2$ and $MgCl_2$. *B. burgdorferi* was incubated with 2 μg of Fab-CB2 per ml at 33°C for 4 h. CH contains 1.4 mM $CaCl_2$, 1 mM $MgCl_2$, and 1 mM $MgSO_4$.

incubation of spirochetes with protease inhibitors. With the exception of EDTA, which damaged the organisms, none of the protease inhibitors used singly or in combination affected the bactericidal effects of Fab-CB2. From these studies, we conclude that Fab-CB2 destabilizes the OM of *B. burgdorferi*, with subsequent formation of spheroplasts, through an epitope-specific, bivalent cation-dependent mechanism.

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