INFECTION AND IMMUNITY, Dec. 1995, p. 4857–4861 Vol. 63, No. 12
0019-9567/95/$04.00+0

Borrelia burgdorferi Vesicle Production Occurs via a Mechanism Independent of Immunoglobulin M Involvement

RUSSELL J. SHOBERG† AND D. DENÉE THOMAS*

Department of Periodontics, University of Texas Health Science Center at San Antonio, San Antonio, Texas 78284-7894

Received 13 April 1995/Returned for modification 26 June 1995/Accepted 20 September 1995

Borrelia burgdorferi **produces extracellular vesicles containing various borrelial protein antigens when propagated in vitro in culture media. Commonly observed components of borrelial vesicle preparations are borrelial surface antigens, bovine serum albumin, and the heavy chains of rabbit immunoglobulin G and immunoglobulin M. This study employed ultracentrifugation to harvest borrelial vesicles and analyzed these preparations by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western immunoblotting. We demonstrated that the rabbit μ heavy-chain band observed was devoid of OspA or at most levels below those detectable by immunoblot. We also demonstrated the recovery of borrelial vesicles at relative centrifugal forces as low as 25,000 × *g*, compared with the force of >200,000 × *g* normally employed. Further, the μ heavy-chain band was recovered from uninoculated growth media processed at 25,000 × *g*, suggesting that it behaves as a particle rather than as a soluble molecule under these conditions. Lastly, vesicles were demonstrated to be present in preparations harvested from growth media supplemented with fetal calf serum, suggesting that vesicle production by *B. burgdorferi* can occur in the absence of immunoglobulins.**

Borrelia burgdorferi sensu lato, the etiologic agent of Lyme disease, has been described as capable of producing extracellular vesicles (blebs) when grown in laboratory culture media (2, 7). The mechanism for production of these vesicles is presently unknown, but researchers from several laboratories have described their existence and utilized vesicles both as a model for studying the spirochete and, in some cases, as a tool for studying proposed mechanisms in the pathogenesis of Lyme disease (6, 7, 10, 12, 13, 17). In addition, one of these reports proposed the production of vesicles in vivo, via antigen capture analysis of specimens acquired from mammals infected both naturally and experimentally with *B. burgdorferi* (6).

One study of borrelial vesicles has demonstrated the presence of DNA molecules within the vesicle (7). The presence of outer surface lipoproteins (Osps) A and B in isolated vesicles has been described (6). Another study, examining the in situ membrane structure of vesicles, has demonstrated that while all vesicles appear to exhibit an outer membrane, some vesicles are observed to contain a cytoplasmic membrane as well (12). Vesicles have also been described as containing a low-molecular-weight lipoprotein of undefined function (10) and are proposed to contain or express the borrelial adhesin for human endothelial cells (13) as well as a mitogenic factor for murine B lymphocytes (17), the latter probably being OspA (15).

One intriguing report defined a major protein observed in stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and Western immunoblot profiles as being a multimolecular complex of rabbit μ heavy chain (Hμ) and OspA and OspB (5). This major band was referred to as "the 83-kDa band" (5), and in a previous report by the same group it was designated the "major extracellular protein" (or MEP) (6). We wish to emphasize, as did the previous authors, that the 83-kDa band described by Dorward et al. (5) is distinct from the 83-kDa antigen described by LeFebvre et al. (11).

We have used isolated vesicles as a tool to examine borrelial adherence to cultured human umbilical vein endothelial (HUVE) cells because OspA is present in vesicle preparations (13) and we had additional evidence suggesting the involvement of OspA in borrelia-HUVE cell attachment (4). We observed the immunoglobulin M (IgM)-containing 83-kDa band in vesicles prepared from cultures in which the growth medium had been supplemented with either normal rabbit serum (NRS) or normal human serum (13). In that paper we reported, as did Dorward et al. (6), that the 83-kDa band was observed in mock vesicles prepared from uninoculated culture medium as well.

We considered the 83-kDa band interesting in the context of borrelial attachment in the HUVE cell model system because of the following observations: (i) we were able to detect a small amount of 83-kDa antigen adsorbed to HUVE cells, (ii) an even smaller amount of human IgM myeloma protein was observed to be adsorbed to HUVE cells, and (iii) we were unable to detect adsorption of mock vesicles to HUVE cells, although these mock vesicles contained the 83-kDa antigen (13). These data suggested that vesicle function (assayed as HUVE cell adsorption) was independent of the 83-kDa antigen band (i.e., Hμ or IgM).

In the present study, we have assessed the relationship between this 83-kDa band and borrelial vesicles via Western immunoblotting. We have also investigated the behavior of IgM during the preparation of vesicles (as assessed by the presence of Hμ). Lastly, we have investigated the dependence of the still-undescribed vesicle production mechanism on the presence of immunoglobulins in the growth medium. The data presented here support our conclusions that (i) the Hμ band observed on SDS-PAGE analyses of vesicles is devoid of OspA; (ii) in the absence of borrelial components, IgM can be removed from suspension in BSK II broth medium at very low relative centrifugal forces (RCFs), suggesting behavior as a particle rather than as a soluble molecule; and (iii) vesicles are

* Corresponding author. Mailing address: Department of Periodontics, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Dr., San Antonio, TX 78284-7894. Phone: (210) 567-3586. Fax: (210) 567-6858. Electronic mail address: thomasd@uthscsa.edu.

† Present address: IDEXX Laboratories, Inc., Westbrook, ME 04092.

produced by *B. burgdorferi* in the absence of immunoglobulins. These data, considered together, strongly suggest that the Hμ band observed in borrelial vesicle preparations is an artifact of the preparation methodology and is unlikely to function in any yet-to-be-defined relationships between vesicles and the physiology of *B. burgdorferi*, or in any role of the vesicles in the pathogenesis of Lyme disease.

(Portions of this research were presented at the 94th General Meeting of the American Society for Microbiology, Las Vegas, Nev., 23 to 27 May 1994.)

MATERIALS AND METHODS

Bacteria and media. *B. burgdorferi* sensu stricto HB19, a North American isolate from human blood (14), was propagated in BSK II liquid medium (1) supplemented with either 6% NRS (GIBCO Laboratories, Grand Island, N.Y.) or 6% fetal calf serum (FCS) (GIBCO) at 34°C with static incubation. All cultures used in this study were tested at fewer than 10 passages from initial isolation.

Preparation of borrelial extracts and vesicles. Washed whole cell extracts (WCE) were prepared from late-exponential-phase cultures by centrifugation at 13,000 × *g* for 30 min at room temperature (RT). The supernatant was removed, and the cell pellet was washed three times at RT with phosphate-buffered saline (pH 7.35) plus 5 mM $MgCl_2$ (PBSM). Protein concentration was determined by the modified Bradford assay as recommended by the vendor of the assay kit (Bio-Rad Laboratories, Richmond, Calif.) with bovine serum albumin (BSA) as a standard, with storage at −20°C.

Vesicles were recovered from culture supernatants as described previously (13). Briefly, late-exponential-phase cultures were clarified at 13,000 × *g* for 30 min at RT to remove spirochetes. The supernatants were filtered through 2-μm-pore-size polycarbonate membranes (Poretics Corp., Livermore, Calif.) with positive pressure, and the filtrates were ultracentrifuged in polycarbonate bottles (Beckman Instruments, Inc., Fullerton, Calif.) at 208,000 × *g* in a Ti55.2 rotor (Beckman) for 90 min at 27°C. The resultant pellet was washed once with PBSM and subjected to ultracentrifugation as described above, except at 4°C. Washed vesicles were resuspended in PBSM and stored at −20°C. Protein concentration was determined as described in the preceding paragraph. Mock vesicles were prepared in an identical fashion except that uninoculated culture medium which had been incubated for 1 week at 34°C served as the starting material.

For some experiments, vesicles were prepared from 2-μm-pore-size-membrane-clarified filtrates by centrifugation at various RCFs. RCFs were estimated by using the following formula from the guide provided with the Sorvall rotor: $\text{RCF} = 11.17 \times r \text{ (in centimeters)} \times \left(\frac{\text{rpm}}{1{,}000}\right)^2$, where rpm is revolutions per minute. Vesicles were prepared at 25,000, 38,000, 50,000, 100,000, and 208,000 × *g* with an SS34 rotor (r = 10.18 cm; Ivan Sorvall, Inc., Norwalk, Conn.) or a Ti55.2 ultracentrifuge rotor (r = 7.45 cm; Beckman) as appropriate. In all cases, centrifugation was for 90 min at 25 to 27°C and pellets were washed once with PBSM with subsequent resuspension in PBSM and storage at −20°C.

In one experiment, vesicles were also prepared at 0 × *g* by incubating a 2-μm-pore-size-membrane-clarified supernatant from uninoculated medium for 90 min at RT in a sterilized polycarbonate ultracentrifuge tube. Following incubation, the liquid was decanted and replaced with PBSM and a second 90-min incubation was performed at 4°C. Finally, the wash fluid was decanted and a small amount of PBSM was added to the tube to recover any adsorbed materials. This final volume was recovered and the protein concentration was determined, with the 0 × *g* sample being stored at −20°C.

SDS-PAGE and Western immunoblotting. Samples were prepared by being boiled for 5 min in a double-strength Laemmli-type sample buffer consisting of 25% (wt/vol) glycerol, 0.125 M Tris-HCl (pH 6.8), 2.5% (wt/vol) SDS, 0.63% (wt/vol) bromophenol blue, and 4.9 mM dithiothreitol (0.75 mg/ml) and resolved over discontinuous SDS–10% polyacrylamide gels (13). Separation was at a constant current of 20 to 25 mA per gel, and gels were cooled with a recirculating-tap-water heat exchanger. Ten to 40 μg of vesicle proteins or 2.5 μg of WCE proteins was resolved per lane (see the figure legend for each figure). The apparent excess of vesicle proteins was intentional, an attempt to visually normalize the amounts of OspA and/or OspB between vesicles and WCE. The low relative percentages of OspA and OspB in vesicles have been described previously (17). Molecular weight standards were purchased and used according to the recommendation of the vendor (Bio-Rad or Pharmacia Inc., Piscataway, N.J.). Resolved proteins were detected by Coomassie blue staining or Western immunoblotting.

Western immunoblotting analysis was performed as described previously (13). Briefly, proteins were electrotransferred to nitrocellulose membranes (BA83; Schleicher and Schuell, Inc., Keene, N.H.) by the method of Towbin, et al. (16) at a constant current of 300 mA for 3 h at RT. After transfer, membranes were washed briefly in PBSM and blocked with 5% (wt/vol) low-fat dry milk in PBSM for 30 min at RT. Primary screening with antibodies or antisera was performed overnight at RT, and then three 15-min washes with PBSM were done at RT. Secondary screening with horseradish peroxidase (HRP)-conjugated anti-immunoglobulin antisera diluted in PBSM was performed for 1 to 2 h at RT, and this was followed by three 15-min washes in PBSM at RT. Colorimetric development with imidazole, 4-chloro-1-naphthol, and H_2O_2 as substrates (all from Sigma Chemical Co., St. Louis, Mo.) was performed at RT. Color development was terminated by extensive washing in deionized water and air drying of the membrane at RT.

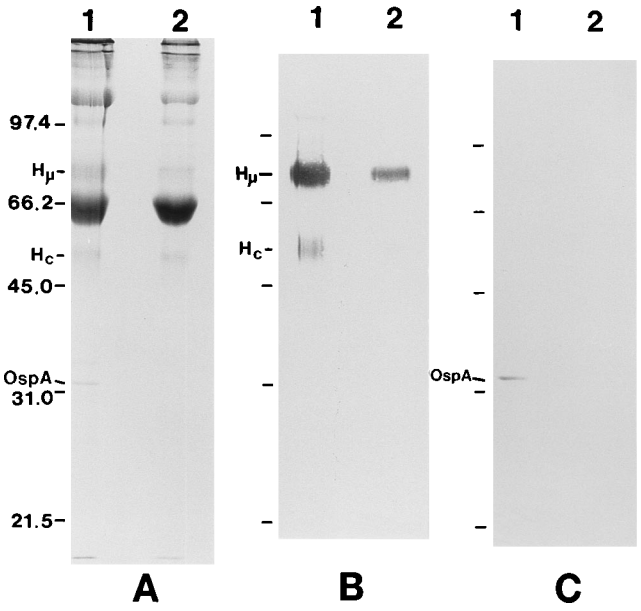

FIG. 1. Analysis of *B. burgdorferi* vesicles prepared from NRS-supplemented growth medium. Twenty micrograms of vesicle proteins was resolved by SDS–10% PAGE and analyzed by Coomassie blue staining (A) and Western immunoblotting with anti-rabbit IgM-HRP (B) or anti-OspA monoclonal antibodies 9B3D and H5332 followed by anti-mouse IgG-HRP as a secondary antiserum (C). Lanes 1 contain *B. burgdorferi* HB19 vesicles, and lanes 2 contain a mock vesicle preparation. Positions of OspA, Hc, and Hμ, as well as molecular weight standards (Bio-Rad) (in thousands), are indicated to the left of each panel.

Antibodies and antisera. The anti-OspA monoclonal antibodies H5332 and 9B3D have been described previously (references 3 and 4, respectively); they were used on blots as hybridoma culture supernatants diluted in PBSM. Goat anti-rabbit IgM-HRP and goat anti-mouse IgG-HRP conjugated secondary antisera were obtained from Cappel Research Products (Durham, N.C.), and a goat polyclonal anti-rabbit IgG-HRP conjugate was purchased from Boehringer Mannheim Biochemicals (Indianapolis, Ind.). All secondary antisera were diluted from 1:500 to 1:1,000 in PBSM before use.

RESULTS

Composition of the 83-kDa band. Western immunoblotting was used to resolve vesicle proteins and to probe the 83-kDa band for the presence or absence of OspA. Figure 1 shows the Coomassie blue staining profile of vesicle proteins (panel A) and the immunoreactive protein profiles of vesicle proteins probed with a polyclonal anti-rabbit IgM-HRP conjugate (panel B) and with the anti-OspA monoclonal antibodies H5332 and 9B3D (panel C). Figure 1A shows that both vesicles prepared from strain HB19 and a mock vesicle preparation (lanes 1 and 2, respectively) contain a relatively small number of stainable proteins, notably, major bands at >110 kDa, the 83-kDa band, the ~66-kDa BSA band, and the ~50-kDa Hc band of rabbit IgG. Also, unique to the HB19 vesicle preparation are the relatively faintly stained OspB and OspA bands, at 34 and ~31 kDa, respectively (Fig. 1A, lane 1). This sort of profile has been reported previously by other authors (5, 6) as well as by us (13). When identically prepared blots were probed with the anti-IgM conjugate, a densely reactive signal

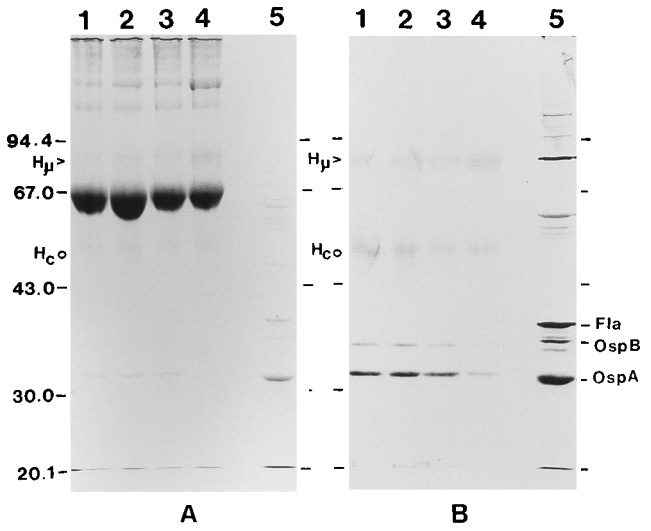

FIG. 2. Analysis of *B. burgdorferi* HB19 vesicles and washed WCE from NRS-supplemented growth medium processed at various RCFs. Ten micrograms of vesicle proteins was resolved by SDS–10% PAGE and analyzed by Coomassie blue staining (A) or Western immunoblotting with anti-*B. burgdorferi* HB19 polyclonal antiserum followed by anti-rabbit IgG and anti-rabbit IgM-HRP conjugates as secondary antisera (B). Vesicles were harvested at 25,000 (lanes 1), 38,000 (lanes 2), 50,000 (lanes 3), and 208,000 (lanes 4) × *g*. For comparative purposes, 3 μg of *B. burgdorferi* HB19 WCE proteins prepared at 13,000 × *g* was resolved (lanes 5). Positions of some major *B. burgdorferi* antigens, Hc, and Hμ, as well as molecular weight standards (Pharmacia) (in thousands), are indicated at the margins of each panel. Fla, flagellin.

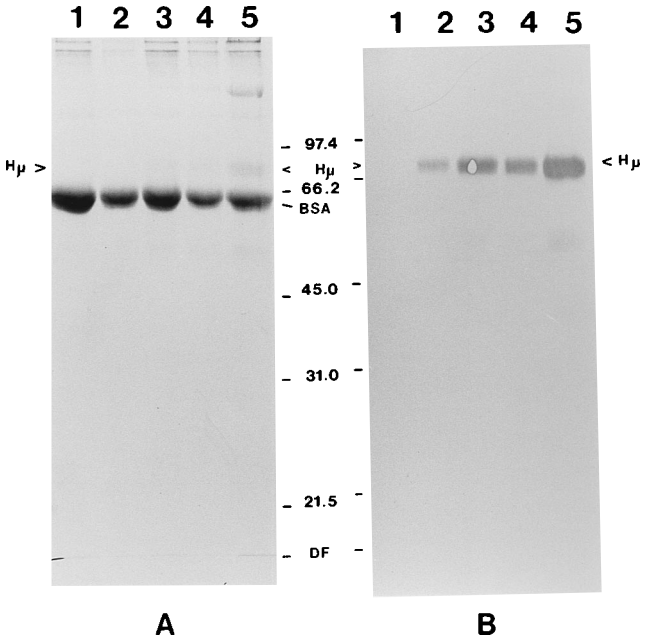

FIG. 3. Analysis of a mock vesicle preparation harvested from NRS-supplemented growth medium at various RCFs. Ten micrograms of vesicle proteins were resolved by SDS–10% PAGE and analyzed by Coomassie blue staining (A) or Western immunoblotting with anti-rabbit IgM-HRP (B). Vesicles were harvested at 0 (lanes 1), 25,000 (lanes 2), 50,000 (lanes 3), 100,000 (lanes 4), or 208,000 (lanes 5) × *g*. The positions of BSA, Hμ, and molecular weight standards (Bio-Rad) (in thousands) are indicated in the margins of the panels. DF, dye front.

corresponding to the 83-kDa band described by Dorward et al. (5) was observed at ~83 kDa in both types of preparation. We also observed faintly reactive bands at ~100 and ~50 kDa in the HB19 vesicle lane. When an identically prepared blot was probed first with the anti-OspA monoclonal antibodies and then with an anti-mouse IgG-HRP conjugate, a faint (relative to the predominant 83-kDa band) signal was observed only at ~31 kDa, demonstrating the presence of OspA in the HB19 vesicle lane. Predictably, no anti-OspA reactivity was observed in the mock-vesicle lane.

These data demonstrate that the 83-kDa band contains Hμ, which substantiates our belief that we were studying the same vesicle-associated band as Dorward et al. were (5) (Fig. 1B). Also, we present direct evidence that while OspA is vesicle associated, it is not observed at the 83-kDa position with the very sensitive Western immunoblot technique (Fig. 1C, lane 1). These data are in disagreement with those presented by Dorward et al. (5).

Vesicle preparation at various RCFs. The standard method used in other laboratories to prepare vesicles from *B. burgdorferi* involves ultracentrifugation of filtered culture supernatants at a very high force, typically greater than 200,000 × *g* (5–7, 13, 17). Since investigators studying vesicles in another bacterial system have reported the use of much lower centrifugal forces (e.g., 27,000 × *g*) to harvest vesicles (8), it seemed worthwhile to test the potential of using lower RCFs to prepare borrelial vesicles.

Figure 2 shows the results of such an experiment. Figure 2A demonstrates the Coomassie blue-stainable protein profiles of vesicles prepared at 25,000, 38,000, 50,000, and the standard 208,000 × *g* (lanes 1 to 4, respectively), with WCE of *B. burgdorferi* HB19 (lane 5) being used as an immunoreactivity control standard to identify defined borrelial antigens. Figure 2B demonstrates the immunoreactive bands observed when an identically prepared blot was probed with a rabbit anti-HB19 WCE polyclonal antiserum and then with anti-rabbit IgG-HRP and anti-rabbit IgM-HRP.

These data demonstrate that borrelial vesicles can be prepared at RCFs as low as 25,000 × *g*. This is evidenced by the presence of stainable OspA (Fig. 2A, lanes 1 to 4) and Western immunoblot-reactive OspA and OspB (Fig. 2B, lanes 1 to 4). All of these vesicle preparations lacked Western immunoblot-detectable levels of flagellin (Fig. 2B; compare lanes 1 to 4 with lane 5), in agreement with the description of vesicles by Dorward et al. (6) and our own criteria for a clean vesicle preparation (13). The lack of detectable flagellin in the vesicle preparations supports the conclusion that the OspA and OspB detected are vesicle associated and not due to minor levels of contamination with spirochetes.

The stainable protein profile also demonstrated that a large amount of BSA and a smaller amount of Hμ were being harvested at these comparatively low RCFs (Fig. 2A). We observed Hμ reactivity upon Western blotting in vesicle preparations at even the lowest RCF tested (Fig. 2B, lane 1). This suggested that either (i) IgM was a component of the vesicle or (ii) it was behaving as a particle and being removed from solution at 25,000 × *g*.

To test the latter possibility, we used a variety of lower RCFs to prepare mock vesicles from uninoculated culture medium that had been supplemented with 6% NRS. Figure 3 represents the data from this experiment. Figure 3A demonstrates that a relatively large amount of BSA is recovered from supernatants centrifuged at RCFs from as low as 25,000 × *g* and up to 208,000 × *g* (lanes 2 to 5). In fact, BSA was even observed to be recovered from a tube of mock vesicles which had not been centrifuged (Fig. 3A, lane 1), suggesting that BSA was adsorbing to the polycarbonate tube via reversible interactions,

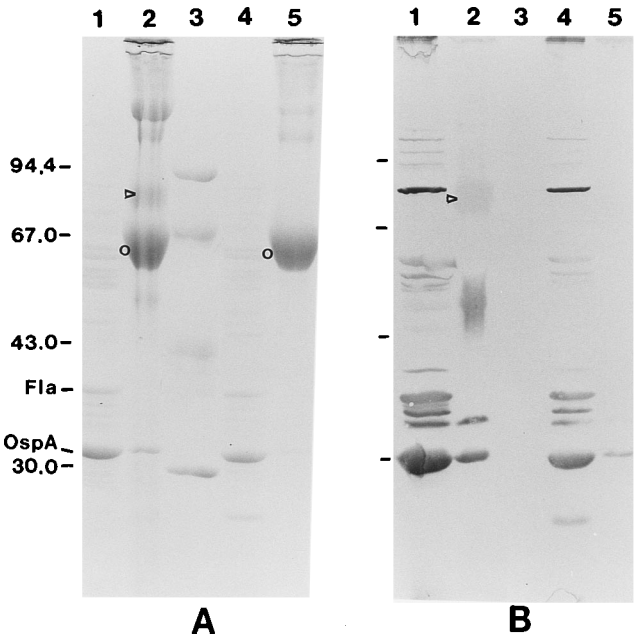

FIG. 4. Analysis of *B. burgdorferi* vesicles and WCE prepared from growth medium supplemented with 6% NRS or 6% FCS. Forty micrograms of vesicle proteins or 2.5 μg of WCE proteins was resolved by SDS–10% PAGE and analyzed by Coomassie blue staining (A) or Western immunoblotting with anti-*B. burgdorferi* HB19 antiserum followed by anti-rabbit IgG and anti-rabbit IgM-HRP conjugates as secondary antisera (B). Lanes (the sample is listed, with the type of serum supplement in parentheses): 1, WCE (NRS); 2, vesicles (NRS); 3, molecular weight standards; 4, WCE (FCS); and 5, vesicles (FCS). The positions of two major borrelial antigens (flagellin [Fla] and OspA) and molecular weight standards (Pharmacia) (in thousands) are indicated on the left. The positions of rabbit Hμ (▷) and BSA (○) are indicated on each panel.

allowing for its removal with PBSM. Western blot analysis with anti-rabbit IgM-HRP demonstrated that Hμ could be recovered from supernatants centrifuged at an RCF as low as 25,000 × *g* (Fig. 3B, lanes 2 to 5). We concluded from this experiment that IgM was sedimenting out of the culture supernatant at what would be considered a relatively low RCF, suggesting that under the conditions used to grow borrelias, it behaves as a particle rather than as a soluble molecule as previously assumed.

Preparation of vesicles from media devoid of IgM. In order to assess the role or requirement of rabbit IgM in the vesicle production process, we attempted to prepare vesicles from borrelias grown in broth media that had been supplemented with either NRS or FCS, both at 6% (vol/vol). Figure 4 demonstrates the data from a representative experiment. Figure 4A shows the Coomassie blue-stainable proteins observed in WCE and vesicles prepared from HB19 grown in BSK II medium supplemented with NRS (lanes 1 and 2, respectively) or with FCS (lanes 4 and 5, respectively). Figure 4B shows the Western immunoblot-reactive bands observed when an identically prepared blot was probed first with an anti-HB19 WCE polyclonal antiserum and then with anti-rabbit IgM-HRP and anti-rabbit IgG-HRP.

We observed typical stained profiles in the WCE lanes for both sets of culture conditions tested, namely, stainable flagellin and OspA (Fig. 4A, lanes 1 and 4). The stained vesicle preparations again showed a relatively sparsely stained profile, with major bands observed at >110, 83, ~66 (the BSA band), and ~31 (the OspA band) kDa (lanes 2 and 4).

The corresponding Western blot demonstrated the presence of OspA in both the NRS- and FCS-propagated vesicles (Fig. 4B, lanes 2 and 5), as well as the presence of OspB in the NRS-propagated vesicles (lane 2). Also, the NRS-propagated vesicles demonstrated Hμ and Hc, confirming their presence in vesicle preparations from NRS-supplemented media. The presence of immunoblot-detectable OspA with the concomitant absence of flagellin in the FCS-propagated vesicle preparation confirmed that vesicles were being produced in the absence of immunoglobulins. This last observation supports our conclusion that vesicle production occurs via an immunoglobulin-independent mechanism.

DISCUSSION

We initiated the present study because of observations during a previous study which suggested that the HUVE cell adsorption of borrelial vesicle proteins was independent of the putative IgM-containing 83-kDa antigen band (13). We were interested in determining (i) the role (if any) of the IgM molecule in the formation of vesicles and (ii) why an apparently similar band was observed in mock vesicle preparations which had no measurable activity in our HUVE cell adsorption assay system (13).

We addressed the problem by preparing vesicles from borrelias propagated in BSK II medium supplemented with NRS (the normal in vitro growth conditions) or FCS, normally devoid of significant levels of immunoglobulin contamination. We also addressed the presence of IgM in vesicles by preparing vesicles by a standard protocol by altering the variable of final RCF. Vesicle preparations were analyzed by SDS–10% PAGE with Coomassie blue staining and Western immunoblotting. The latter analyses used monoclonal antibodies with defined specificities or specific polyclonal antisera to identify specific antigens present (or absent) in vesicles and, in some cases, washed whole borrelia cells.

We have presented data in this report which give direct evidence that the previously described 83-kDa band (5) lacks immunoblot-detectable OspA (Fig. 1C). In the previous report, Dorward et al. used a variety of indirect evidence to conclude that their 83-kDa band also contained minor levels of OspA and OspB, including observations that (i) antivesicle antisera and anti-MEP (the 83-kDa band) antisera both reacted with OspA and OspB and other borrelial antigens on a Western blot (6) and (ii) an anti-rabbit IgM-colloidal gold conjugate coprecipitated OspA and OspB in immunoprecipitation experiments (5).

With regard to the former line of evidence that an anti-MEP antiserum reacted with OspA and OspB, the immunogen used by Dorward et al. was electrophoretically purified MEP (6). In retrospect, once the 83-kDa band was identified as being rabbit Hμ (5), one would not have expected to see an anti-rabbit IgM response in the immunized rabbits. The anti-OspA and -B reactivities observed by Dorward et al. may be explained by the fact that their immunogen was electrophoretically purified from an SDS-PAGE gel by a previously published method (9) which utilized samples prepared with β-mercaptoethanol as the reducing agent (6). An unusual characteristic of OspA is that upon Western blot analysis of samples reduced with β-mercaptoethanol, smearing trails of reactivity greater than 31 kDa can be observed, while similar samples prepared with dithiothreitol as a reducing reagent do not exhibit the multiple bands of reactivity (3). This undefined phenomenon of variable OspA reduction may explain the presence of trace levels of Osps being found at other positions in an SDS-PAGE gel prepared with samples reduced with β-mercaptoethanol.

With regard to the second line of evidence presented by

Dorward et al., that immunoprecipitation experiments with anti-rabbit IgM-colloidal gold yielded OspA and OspB, their report indicates that they recovered their antigen-antibody complexes at 38,500 × *g*. We have presented evidence from the present study demonstrating that both vesicles (Fig. 2) and IgM in the absence of borrelial vesicles (Fig. 3) can be precipitated from BSK II medium at RCFs as low as 25,000 × *g*, which could explain the previous investigators' conclusion that IgM and Osps were being coprecipitated by the formation of the IgM–anti-IgM conjugate complex.

We attempted to segregate the IgM molecule from vesicles which we presumed would behave as particles in the culture medium by differential centrifugation of clarified culture supernatants. We hypothesized that at lower RCFs, we would be able to selectively remove vesicles from suspension while soluble molecules (e.g., BSA and IgM) would remain in solution. We also sought to develop an alternative method for vesicle preparation that would not require the use of an ultracentrifuge in order to facilitate preparation of larger amounts of vesicles. Figure 2 demonstrates that ultracentrifugation is not required to precipitate vesicles from culture supernatants. We were able to remove vesicles with RCFs as low as 25,000 × *g* (Fig. 2, lane 1), suggesting that vesicle preparation can be done in a high-speed rotor such as a Sorvall SS34. Surprisingly, we observed that IgM was also precipitated at similarly low RCFs (Fig. 2, lane 1; Fig. 3, lane 2). This observation suggested to us that the IgM was behaving as a particle rather than as a solute under the conditions found in BSK II medium. The finding that IgM was still precipitated at low RCFs in the absence of borrelial vesicles (Fig. 3) gives further support to our proposal that it is behaving as a particle and not as a constituent of the borrelial vesicles. These data are considered to be strong evidence that the presence of IgM (i.e., Hμ or the 83-kDa band) in borrelial vesicles is an artifact of the techniques currently used to isolate vesicles from the culture medium.

Lastly, we present data which demonstrate that *B. burgdorferi* is able to produce vesicles in the absence of immunoglobulins and, further, that these vesicles can be prepared by standard methods. We propagated *B. burgdorferi* HB19 in media supplemented with FCS and successfully prepared vesicles from these cultures (Fig. 4, lane 5). These data support our proposal that vesicle formation is independent of the presence of immunoglobulins in the growth medium. To ensure that trace amounts of immunoglobulin (i.e., rabbit IgM) were not being carried over into the culture used to prepare the FCS-grown vesicles used in analysis, the initial stock of HB19 growing in NRS-supplemented media was transferred three times at dilutions of 1:10 into fresh BSK II medium supplemented with 6% FCS. Therefore, the contribution of any contaminating rabbit IgM would be, at most, at a level of 0.06%, while the FCS in that same culture was at 6%. Therefore, we strongly feel that the vesicles prepared from this final FCS-supplemented culture were produced by borrelias which had been growing in the FCS-supplemented media and were not carried over from the NRS-supplemented starter culture 3 passages distant.

Editor: A. O'Brien

Taking together the data presented in this report, we conclude that (i) the presence of IgM in borrelial vesicle preparations is an artifact of the techniques used to isolate vesicles, (ii) *B. burgdorferi* is able to produce vesicles in the absence of IgM, and (iii) borrelial vesicles can be prepared at RCFs substantially lower than previously described, facilitating their production for use in future studies.

ACKNOWLEDGMENTS

We thank Alan G. Barbour for providing the H5332 antibody and for constructive discussions.

This research was supported by Public Health Service grant AI26804 from NIAID to D.D.T.

REFERENCES

1. **Barbour, A. G.** 1984. Isolation and cultivation of Lyme disease spirochetes. Yale J. Biol. Med. **57:**71–75.
2. **Barbour, A. G., and S. F. Hayes.** 1986. Biology of *Borrelia* species. Microbiol. Rev. **50:**381–400.
3. **Barbour, A. G., S. L. Tessier, and W. J. Todd.** 1983. Lyme disease spirochetes and ixodid tick spirochetes share a common surface antigenic determinant defined by a monoclonal antibody. Infect. Immun. **41:**795–804.
4. **Comstock, L. E., E. Fikrig, R. J. Shoberg, R. A. Flavell, and D. D. Thomas.** 1993. A monoclonal antibody to OspA inhibits association of *Borrelia burgdorferi* with human endothelial cells. Infect. Immun. **61:**423–431.
5. **Dorward, D. W., E. D. Huguenel, G. Davis, and C. F. Garon.** 1992. Interactions between extracellular *Borrelia burgdorferi* proteins and non-*Borrelia*-directed immunoglobulin M antibodies. Infect. Immun. **60:**838–844.
6. **Dorward, D. W., T. G. Schwan, and C. F. Garon.** 1991. Immune capture and detection of *Borrelia burgdorferi* antigens in urine, blood, or tissues from infected ticks, mice, dogs, and humans. J. Clin. Microbiol. **29:**1162–1170.
7. **Garon, C. F., D. W. Dorward, and M. D. Corwin.** 1989. Structural features of *Borrelia burgdorferi*—the Lyme disease spirochete: silver staining for nucleic acids. Scanning Microsc. **3**(Suppl.)**:**109–115.
8. **Grenier, D., and D. Mayrand.** 1987. Functional characterization of extracellular vesicles produced by *Bacteroides gingivalis*. Infect. Immun. **55:**111–117.
9. **Judd, R. C.** 1982. ^{125}I-peptide mapping of protein III isolated from four strains of *Neisseria gonorrhoeae*. Infect. Immun. **37:**622–631.
10. **Katona, L. I., G. Beck, and G. S. Habicht.** 1992. Purification and immunological characterization of a major low-molecular-weight lipoprotein from *Borrelia burgdorferi*. Infect. Immun. **60:**4995–5003.
11. **LeFebvre, R. B., C.-G. Perng, and R. C. Johnson.** 1990. The 83-kilodalton antigen of *Borrelia burgdorferi* which stimulates immunoglobulin M (IgM) and IgG responses in infected hosts is expressed by a chromosomal gene. J. Clin. Microbiol. **28:**1673–1675.
12. **Radolf, J. D., K. W. Bourell, D. R. Akins, J. R. Brusca, and M. V. Norgard.** 1994. Analysis of *Borrelia burgdorferi* membrane architecture by freeze-fracture electron microscopy. J. Bacteriol. **176:**21–31.
13. **Shoberg, R. J., and D. D. Thomas.** 1993. Specific adherence of *Borrelia burgdorferi* extracellular vesicles to human endothelial cells in culture. Infect. Immun. **61:**3892–3900.
14. **Steere, A. C., R. L. Grodzicki, A. N. Kornblatt, J. E. Craft, A. G. Barbour, W. Burgdorfer, G. P. Schmid, E. Johnson, and S. E. Malawista.** 1983. The spirochetal etiology of Lyme disease. N. Engl. J. Med. **308:**733–740.
15. **Tai, K.-F., Y. Ma, and J. J. Weis.** 1994. Normal human B lymphocytes and mononuclear cells respond to the mitogenic and cytokine-stimulatory activities of *Borrelia burgdorferi* and its lipoprotein OspA. Infect. Immun. **62:**520–528.
16. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.
17. **Whitmire, W. M., and C. F. Garon.** 1993. Specific and nonspecific responses of murine B cells to membrane blebs of *Borrelia burgdorferi*. Infect. Immun. **61:**1460–1467.