Scanning Microscopy Supplement 3, 1989 (Pages 109-115)
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

STRUCTURAL FEATURES OF BORRELIA BURGDORFERI - THE LYME DISEASE SPIROCHETE: SILVER STAINING FOR NUCLEIC ACIDS

C. F. Garon*, D. W. Dorward, and M. D. Corwin

Department of Health and Human Services, Public Health Service, National Institutes of Health, National Institute of Allergy and Infectious Diseases, Laboratory of Pathobiology, Rocky Mountain Laboratories, Hamilton, Montana 59840

Abstract

Borrelia burgdorferi - the Lyme disease spirochete - was grown in modified Kelly medium and characterized by transmission and by scanning electron microscopy. Using silver staining procedures which preferentially bind to nuclear components of eukaryotic cells, signal could be detected by backscattered electron imaging throughout the length of the prokaryotic spirochete. Interestingly, however, the highest levels of backscattered signal were observed in naturally elaborated membrane blebs that were visible attached to cell surfaces and free in the medium. These membrane vesicles could be enriched by filtration through nitrocellulose or Anopore membranes and by differential centrifugation. The possibility of contaminating cellular DNA coating the membrane vesicles was ruled out by exhaustive digestion with pancreatic DNAase I. Intact DNA was demonstrated both by lysing blebs directly on the surface of microscope grids and by extracting molecules from purified bleb preparation with detergents and solvents. Both linear and circular DNA molecules could be identified in purified membrane blebs. A simple, one-step, alternative silver staining procedure is described which appears to effectively label the protein-nucleic acid complexes contained in the membrane vesicles of the human pathogen B. burgdorferi, and may provide an important method to track and to define the biological function of these structures.

Key Words: Silver staining, backscattered electron imaging, nucleic acids, spirochetes

*Address for correspondence:
C. F. Garon
Laboratory of Pathobiology
Rocky Mountain Laboratories
Hamilton, MT 59840
Phone: (406) 363-3211

Introduction

Lyme borreliosis is now the most common arthropod-borne disease in the United States. While originally thought to be confined to limited areas, the disease has now been found in at least 35 states and 6 continents. Early treatment, which is critical to the prevention of more serious consequences, is often delayed by the vagaries of clinical presentation and available diagnostic procedures. Borrelia burgdorferi, the causative agent (7) has been isolated from humans, mammals, birds and arthropods and is cultivable in the laboratory. Careful structural analysis of the DNA content of several early passage isolates has revealed a unique mixture of terminally cross-linked, linear and covalently closed, circular DNA molecules ranging in size from a few to fifty kilobases (kb) in length (3, 18). No molecules longer than 900 kb could be detected. Often DNA patterns appear to differ among isolates (2, 18). Given 1) the number of molecules in this apparent chromosome pool, 2) the presence of genes for major surface proteins located on relatively small linear DNA molecules (4), 3) the uniqueness of the DNA profile among isolates, and 4) the structural features of these molecules, which related them to human telomeres, we wondered if this pool of DNA molecules might function as a collection of mini-chromosomes. This view has been supported recently by the description of a similarly segmented arrangement of DNA molecules in Borrelia duttonii - an agent of relapsing fever (14). Furthermore, we wondered if these molecules were partitioned in any particular fashion throughout the cell.

These questions led us to attempt several silver staining procedures (11, 19) which had been shown previously to bind preferentially (6, 16, 19, 20, 21, 23), although not exclusively (8, 9), to the nuclear components of eukaryotic cells. We wondered how similarly stained B. burgdorferi cells would compare with the published images of darkly stained nuclei (11, 19, 20, 21, 23) and nucleoli (1, 16, 22) when viewed with backscattered electron detectors (10, 17). Finally, we present a simple, one-step, alternative silver staining procedure, originally developed by Moreno et al. (16) for thin section staining, which appears to work well in our system and which has led to the description of a new DNA containing compartment.

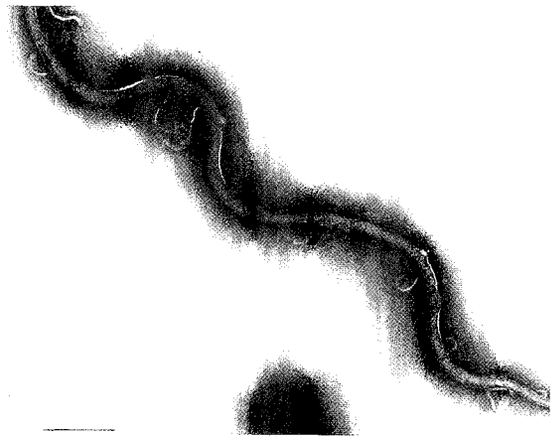


Figure 1. Electron photomicrograph of Borrelia burgdorferi cell negatively stained with 3% ammonium molybdate, pH 6.5. Numerous outer membrane blebs are shown along its length. Bar = 0.5 μm.

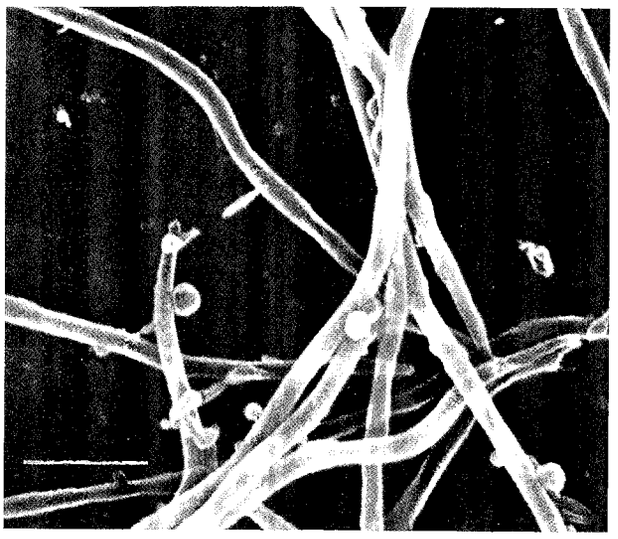


Figure 2. Outer membrane blebs confirmed by scanning electron microscopy of gold coated cells. Bar = 1.0 μm.

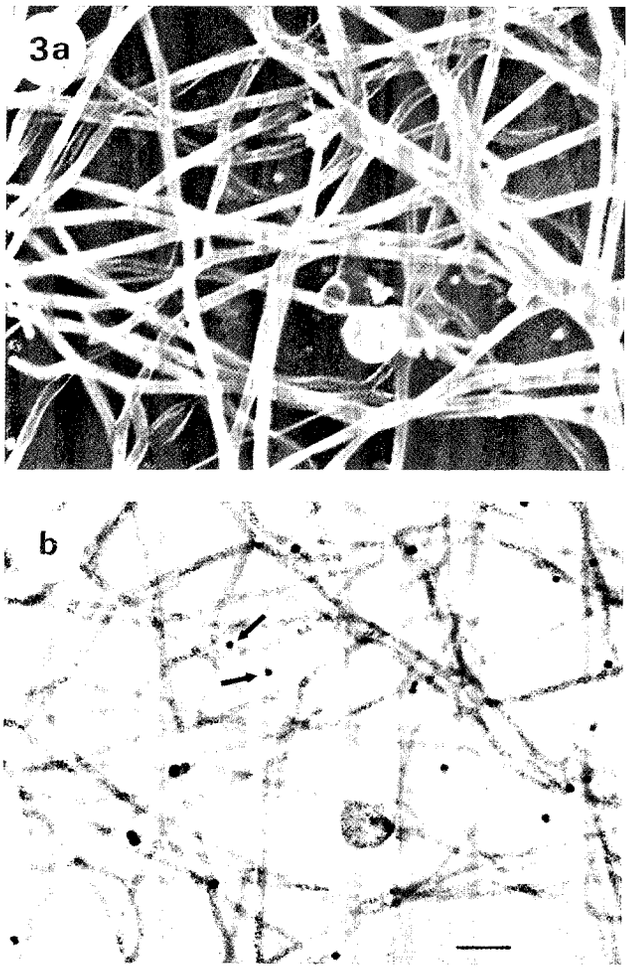


Figure 3. Silver staining of Borrelia burgdorferi cells by the Becker and Sogard method as seen by secondary (a) and backscatter (b) electron imaging. While some backscattered signal is detectable along the length of the spirochetes, the highest level of signal (darkest spots marked with arrows) is seen in membrane blebs both attached to cells and free. Bar = 1.0 μm.

Materials and Methods

Borrelia burgdorferi strain SH-2-82 was originally obtained as a tick isolate and was used throughout these studies. The early passage strain was grown in 500 ml bottles of modified Kelly medium at 34°C as previously described (18). Membrane blebs were purified from late log phase cultures by a modification of a procedure described by Dorward and Judd (12). Spirochetes were removed from the culture by centrifugation at 13,000 xg for 30 min at 25°C in a GSA rotor (Sorvall, Norwalk, Conn.). The supernatants were filtered through 2.0 μm nitrocellulose (Millipore, San Francisco, Calif.) or Anopore (Anotec Separations, New York, N.Y.) membranes to remove residual cells. Blebs were recovered from resulting filtrates by centrifugation at 208,000 xg for 90 min at 25°C in a 60Ti rotor (Beckman, Palo Alto, Calif.). Bleb pellets were rinsed and resuspended in Dulbecco's phosphate buffered saline (PBS) at pH 7.2.

Twelve millimeter glass cover slips were cleaned with a sulfuric acid-potassium dichromate solution, rinsed exhaustively with tap water followed by a final rinse with deionized water. Cleaned cover slips were placed on edge in a porcelain rack and immersed for 1 h in a 0.015% solution of poly-D-lysine hydrobromide mol. wt. 30,000-70,000 (Sigma #P-7886). Racks containing the cover slips were rinsed several times in deionized water and dried overnight at 37°C. The polylysine coated cover slips were placed in a multiwell tissue culture plate (Falcon #3008) and 0.4-1.0 ml of bacterial suspension was added either directly from liquid culture or resuspended in PBS. The suspension was allowed to adsorb to the cover

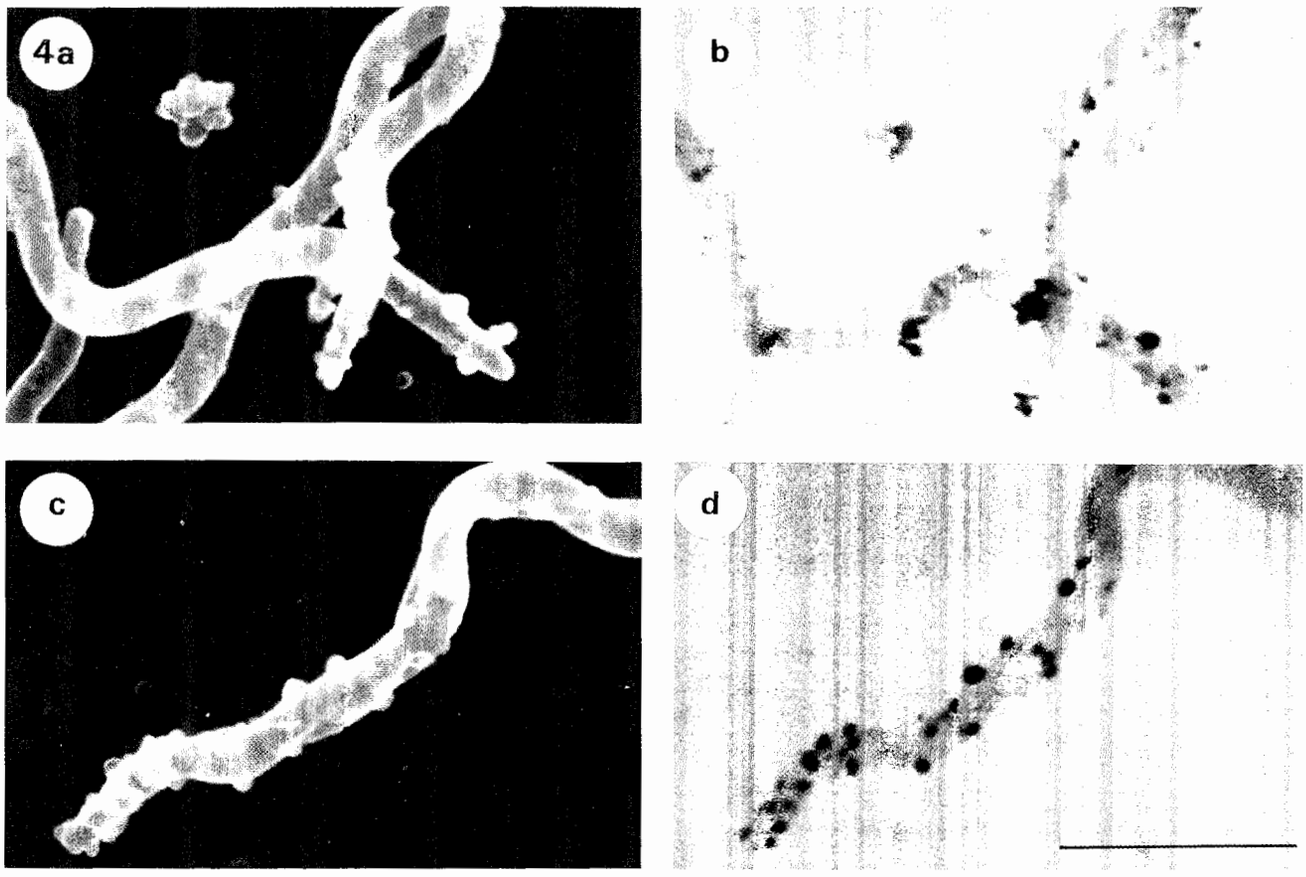


Figure 4. A comparison of Borrelia burgdorferi cells silver stained by the Becker and Sogard method (a+b) or by the one-step Moreno method (c+d). Panels a and c are secondary electron images. Panels b and d are backscattered electron images. Darkly staining areas in BEI appear to correspond to membrane blebs seen on intact organisms by SEI. Bar = 1.0 µm.

slips for 1 h at room temperature. Solutions were rapidly withdrawn from the wells and replaced with a second solution using Pasteur pipettes to avoid air drying. Rinsed cover slips were fixed in 0.5% glutaraldehyde in this manner. After fixation and multiple rinses in PBS and then deionized water, the cover slips were transferred to a porcelain rack. The rack with cover slips was kept in deionized water until staining.

The silver staining method of Becker and Sogard (6) was performed as follows: Fresh stain was prepared by adding 5 ml of 5% silver nitrate solution, dropwise with shaking, to 100 ml of 3% hexamethylenetetramine. Fifty milliliters of this solution was mixed with an equal volume of deionized water and 4 ml of 5% sodium borate solution. The coated cover slips prepared above and held vertically in a porcelain rack were submerged into the silver stain solution, capped and agitated at 120 rpm in a rotary shaker for 40 min at 50°C. The rack was removed from the silver stain and transferred to an identical container of deionized water. After three washes in fresh deionized water, the rack was placed into a fresh container of silver stain, reincubated for 40 min at 50°C, rinsed as before and subjected to a third silver staining. After final rinsing, racks containing specimens were immersed into a 5% sodium thiosulfate solution for 10 min, rinsed in deionized water and prepared for further processing.

The silver staining method of Moreno et al. (16) was performed as follows: A silver nitrate staining mixture consisting of equal parts of 2% gelatin in 1% formic acid and a 50% silver nitrate solution was prepared immediately before use. Deionized water was drawn away from the cover slip with a Pasteur pipette and replaced with the silver staining solution for 5 min. The staining solution was removed and the specimen rinsed 3 times with deionized water before a 5% sodium thiosulfate solution was added. After 10 min, this solution was removed and the sample rinsed three times in deionized water. Subsequently all cover slips were transferred to a special holder, put through a graded series of ethanols and critical point dried directly from CO_2. Specimens were attached to carbon SEM stubs with double sided tape, coated with a thin film of carbon in a vacuum evaporator and examined.

Parlodion-coated grids were prepared as previously described (13). Intact cells or purified blebs were adsorbed to coated grids, washed briefly in 0.25 M ammonium acetate and stained for 30 sec with ammonium molybdate (3%, pH 6.5). The Kleinschmidt technique for DNA microscopy was used as previously described (13).

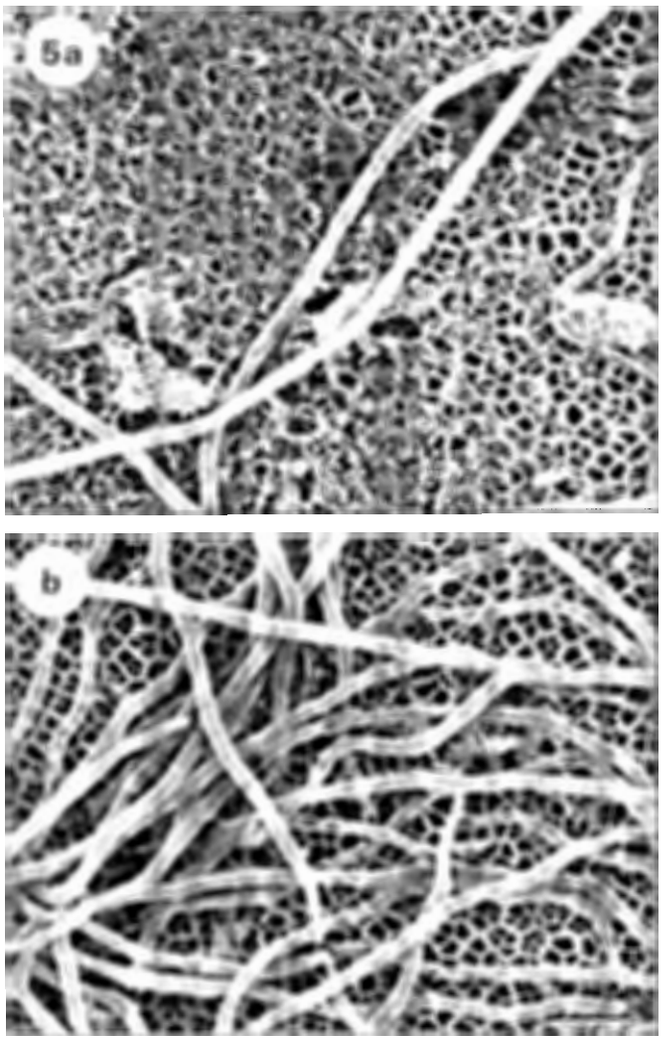


Figure 5. Surfaces of Anopore filter membranes following filtration of culture medium (a) and PBS washed cells (b). Bar = 1.0 μm.

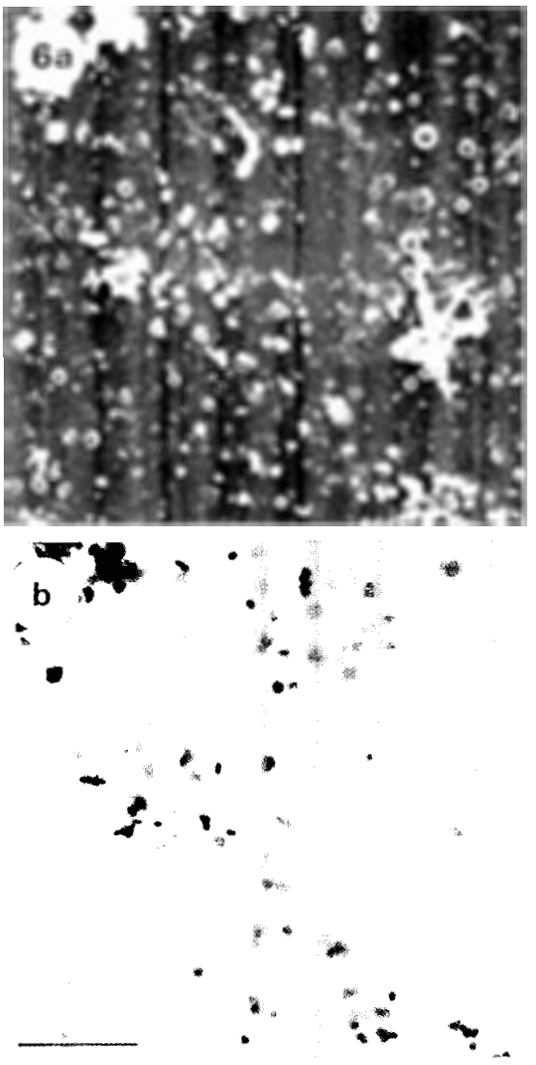


Figure 6. Silver stained purified blebs as seen by secondary (a) and backscatter (b) electron imaging. Bar = 1. 0 μm.

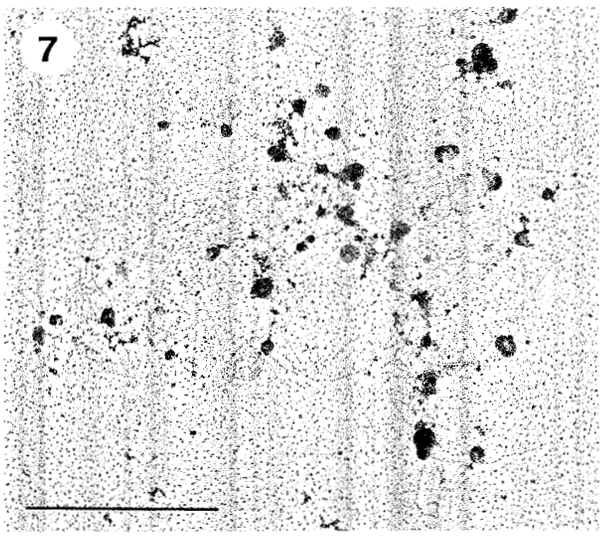
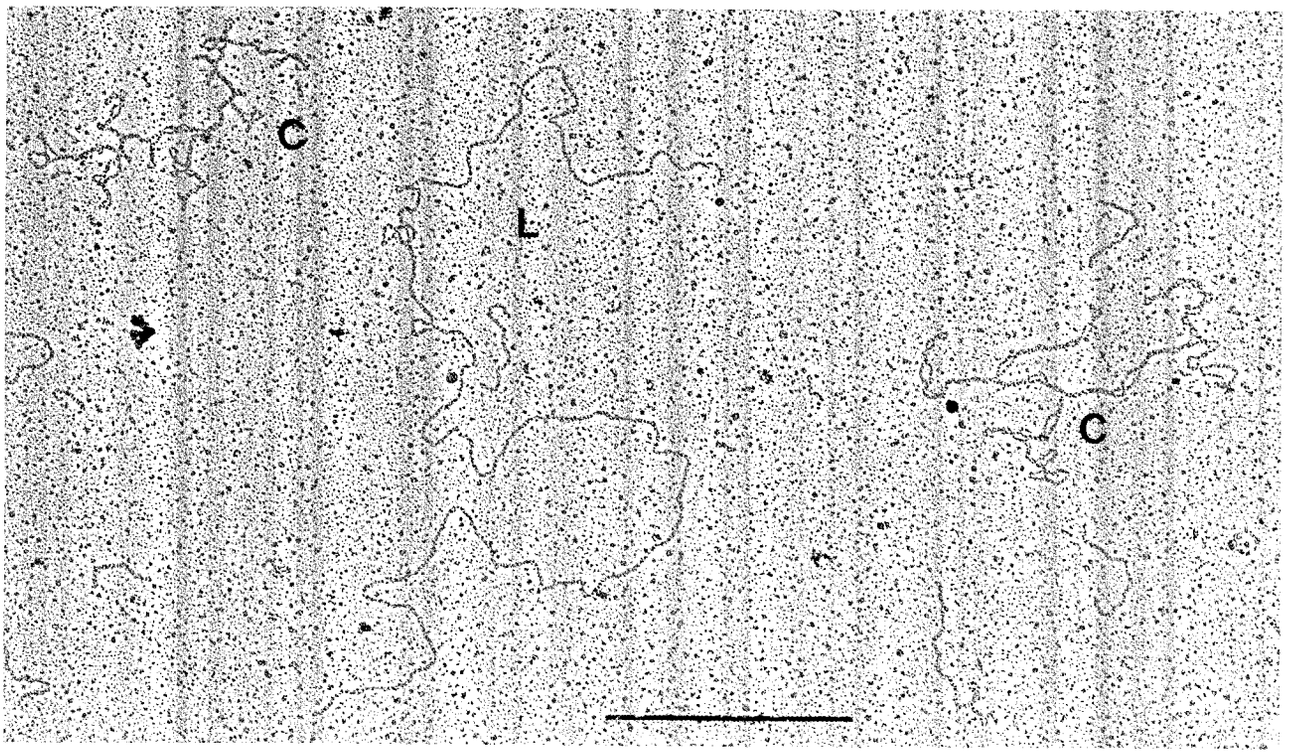


Figure 7. Purified blebs were incubated for 10 min in 1 μg/ml of pancreatic DNAase I prior to mounting for electron microscopy in the presence of urea and formamide as previously described (13). Nucleic acids strands are observed emerging from osmotically lysed membrane blebs. Grids were subsequently rotary shadowed with platinum-palladium. Bar = 1.0 μm.



Purified blebs were incubated with 1.0 µg/ml of pancreatic DNAase I (2.5 units/µg) (Worthington Biochemicals, Freehold, N.J.) for 10 min at 25°C. DNA was then purified from identical samples by lysis with sodium dodecylsulfate, digestion with proteinase K, and extraction by phenol-chloroform as previously described (15).

Figure 8. Purified blebs were treated with an excess of pancreatic DNAase I prior to conventional DNA extraction procedures involving proteinase K, phenol extraction and alcohol precipitation. Both linear (L) and covalently-closed circular (C) molecules were observed. Molecules were mounted by the Kleinschmidt aqueous technique and rotary shadowed with platinum-palladium. Bar = 1.0 µm.

Results

Early passage strains of Borrelia burgdorferi when viewed by negative staining show membrane vesicles along the length of the organism (5) and free in the medium (Fig. 1). While the size of these membrane structures varies considerably, their numbers remain high even into late stages of growth. The presence of these structures could be demonstrated by scanning electron microscopy of gold coated samples as well (Fig. 2). Unattached membrane blebs are also visible in these preparations. Silver staining using the method of Becker and Sogard (6 Fig. 3) showed not only a mottled staining pattern along the length of the spirochete, but a strong backscattered signal at the sites of membrane blebs both attached to and free from cells. Not all detectable membrane blebs, however, gave a backscattered electron signal.

Given the long incubation times required for silver staining by the Becker and Sogard method (6), we wondered if better preservation of fragile structures might not be obtained by use of a shorter, more gentle procedure. We, therefore, adapted a method proposed by Moreno et al. (16) for the silver staining of thin sections of plant and animal tissue and compared the results obtained with those of the previous method (Fig. 4). While both methods show strong backscattered signal in the regions identifiable as membrane blebs, more defined signal with less background was produced by the simpler, one-step method. It is not clear whether these results are due to altered specificity of staining or to different sample stabilities.

Membrane blebs could be cleanly separated from whole cells by filtration. Careful analysis by both scanning (Fig. 5) and transmission electron microscopy of membrane filtering surfaces, inner membrane areas, filter membrane bottoms and filtrates revealed no leakage of intact organisms into the fluid filtrates provided the membrane remained intact. Given the nature of the complex, rich medium used in the culture of B. burgdorferi, a single rinse in PBS appeared, as might be expected, to facilitate the filtration process. While cellulose nitrate filters work equally well for purifying blebs, their irregular surfaces make scanning for intact organisms and blebs more difficult.

Filtrates were analyzed using the one-step silver staining method, an example of which is presented in

Fig. 6. The impression, when examined by transmission electron microscopy after negative staining, that some blebs appeared "full" and resistant to stain penetration while others looked "empty" and collapsed was supported by backscattered electron imaging data. In these experiments not every detectable membrane vesicle gave a backscattered signal. Estimates (acquired by superimposing images containing the secondary electron and backscattered electron signals) of silver stained to non-staining blebs ranged from 25-50% for preparations of purified blebs to a higher number (70-80%) when blebs were attached to intact cells. Although difficult to quantitate, both "empty" and "full" blebs could be observed (Fig. 7) when purified (and exhaustively DNAase treated) blebs were directly lysed on the surface of a grid using a modification of the Kleinschmidt spreading procedure (13). DNA strands could be detected which appeared to emerge from residual, bleb-like structures. The granular background (Figs. 7, 8) is the result of cytochrome C used in preparing the monolayer and is typical of this method of mounting for microscopy. Detergent lysed, deproteinized bleb fractions mounted by the conventional Kleinschmidt aqueous technique showed both linear and supercoiled DNA molecules (Fig. 8) consistent with forms seen when the DNA from intact cells is similarly analyzed. The presence of covalently closed, supercoiled molecules showing no evidence of nuclease damage is evidence for the level of protection afforded these DNA molecules by blebs during exhaustive nuclease digestion (control experiments show complete degradation of exogenously added DNA). It is possible, therefore, that DNA molecules could survive to function in a bleb-mediated, genetic exchange system. Experiments are underway to explore this possibility more directly.

Conclusions

The application of a simple one-step silver staining technique to the ultrastructural analysis of Borrelia burgdorferi has permitted a potentially important observation about a human pathogen and has provided a valuable technique to study its significance. Although the function of naturally elaborated membrane blebs has not been defined in this organism, the observation that intact DNA is tightly packaged within some of these structures suggest that these vesicles may play a role in the protection and transfer of genetic markers. While these experiments have added little to the debate about the specificity of silver staining procedures beyond the observation that the stain binds to components of the DNA-protein complex (probably acidic scaffolding proteins) contained in membrane blebs, it is our hope that experiments in progress in our laboratory will provide purified reagents derived from B. burgdorferi which might be useful for defining silver staining specificity as well.

Acknowledgements

We thank Tom Schwan for Borrelia burgdorferi strain Sh-2-82, for helpful discussions and for critical review of this manuscript. We thank Lori Lubke for technical assistance and Betty Kester for manuscript preparation.

References

1. Allen, TD, Jack EM, Harrison JC, Claugher D., Harris R. (1986). Human metaphase chromosome preparation for scanning electron microscopy - a consideration of inherent problems. In: Science of Biological Specimen Preparation 1985. Scanning Electron Microscopy, AMF O'Hare, Chicago, IL, 299-307.

2. Barbour, AG. (1988). Plasmid analysis of Borrelia burgdorferi, the Lyme borreliosis agent. J. Clin. Microbiol. 26, 475-478.

3. Barbour, AG, Garon CF. (1987). Linear plasmids of the bacterium Borrelia burgdorferi have covalently closed ends. Science 237, 409-411.

4. Barbour AG, Garon CF. (1988). The genes encoding major surface proteins of Borrelia burgdorferi are located on a plasmid. Annals NY Acad. Sci. 539, 144-153.

5. Barbour AG, Hayes SF. (1986). Biology of borrelia species. Microbiol. Rev. 50, 381-400.

6. Becker RP, Sogard M. (1979). Visualization of surface structures in cells tissues by backscattered electron imaging. Scanning Electron Microsc. 1979; II: 835-870.

7. Burgdorfer W, Barbour AG, Hayes SF, Benach JL, Brunwaldt E, Davis JP. (1982). Lyme disease - a tick-borne spirochetosis? Science, 216, 1317-1319.

8. Burr FA. (1973). Staining of a protein polysaccharide model with silver methenamine. J. Histochem. Cytochem, 21, 386-388.

9. Carter HW. (1980). Backscattered electron imaging: theory applications. Micron., 11, 259-260.

10. De Bruijn WC, Van Mourik W, Bosveld IJ. (1974). Cell border demarcation in the scanning electron microscope by silver stains. J. Cell Sci., 16, 221-239.

11. Denee PB, Abraham JL, Willard PA. (1974). Histochemical stains for the scanning electron microscope - qualitative and semiquantitative aspects of specific silver stains. Scanning Electron Microsc., 1974: 259-266.

12. Dorward DW, Judd RC. (1988). Characterization of naturally-evolved outer membrane blebs of Neisseria gonorrhoeae. In: Gonococci and Meningococci. Kluwer Academic Publishers, Dordrecht, The Netherlands, 349-356.

13. Garon CF. (1986). Electron microscopy of nucleic acids. In: Ultrastructure Techniques for Microorganisms. Plenum Publishing Corp., New York, NY, 161-181.

14. Hayes LJ, Wright DJM, Archard LC. (1988). Segmented arrangement of Borrelia duttonii DNA and location of variant surface antigen genes. J. Gen. Microbiol. 134, 1785-1793.

15. Maniatis T, Fritsch EF, Sambrook J. (1982). Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 458-459.

16. Moreno FJ, Villamarin A, Garcia-Herdugo G, Lopez-Campos JL. (1988). Silver staining of the nucleolar organizer regions (NOR_s) in semithin Lowicryl sections. Stain Technology, 63, 27-31.

17. Ogura K, Hasegawa Y. (1980). Application of backscattered electron image to biological specimens. J. Electron Microsc., 29, 68-71.

18. Schwan TG, Burgdorfer W, Garon CF. (1988). Changes in infectivity and plasmid profile of the Lyme

disease spirochete, Borrelia burgdorferi, as a result of in vitro cultivation. Infect. Immun., 56, 1831-1836.

19. Soligo D, De Harven E, Nava MT, Lambertenghi-Deliliers G. (1986). Immuno-cytochemistry with backscattered electrons. In: The Science Of Biological Specimen Preparation for Microscopy and Microanalysis 1985. Scanning Electron Microscopy, AMF O'Hare, Chicago, Il, 289-297.

20. Soligo D, Lampen N, De Harven E. (1981). Backscattered electron imaging of human leukocytes. Scanning Electron Microsc., 1981; II: 95-103.

21. Soligo D, Pozzoli E, Nava MT, Polli N, Lambertenghi-Deliliers G, De Harven E. (1983). Cytochemical methods for the backscattered electron imaging mode of scanning electron microscopy: Further applications to the study of human leukemic cells. Scanning Electron Microsc. 1983; IV: 1795-1802.

22. Thiebaut F, Rigaut JP, Feren K, Reith A. (1984). Smooth-surfaced control and transformed C3H/10T- cells differ in cytology: A study by secondary electron, backscattered electron and image analysis. Scanning Electron Microsc., 1984; III: 1249-1255.

23. Vogel G, Becker RP, Swift H. (1976). Backscattered electron imaging of nuclei in rat testis. Scanning Electron Microsc., 1976; II: 409-416.

Discussion with Reviewer

J. S. Hanker: Do you think that Kelly's medium could be useful for culturing, and the Moreno stain for staining spirochetes associated with periodontitis? Very little has been done for culturing or staining these pathogens.

Authors: Members of the family Spirochaetaceae differ widely in their growth requirements. Some are obligately anaerobic while others are microaerophilic. Requirements for carbon and energy sources vary as well. Some, like Treponema pallidum, the causative agent of syphilis, have never been successfully cultivated in pure culture on artificial medium. This variation makes it difficult to predict how a "new" organism will respond to attempted laboratory cultivation. Given, however, the cost, the complexity, the lability and the general dissatisfaction with modified Kelly medium (generation times for Borrelia burgdorferi, are on the order of 12-20 hrs) simpler formulations developed for less fastidious Spirochaetaceae might be tested first. Staining with the Moreno procedure would, however, be very interesting to try.

T. D. Allen: The significance of membranous "bags" of DNA shed from the surface of an organism is intriguing. As these animals are cultured readily would it be possible to either radio label or incorporate BrdU into one population, isolate the membrane blebs and add them to a second population to check for a mechanism of genetic transmission?

Authors: The experiments you describe are in progress in our laboratory at the present time. The experiments described in Fig. 5 were set up with that approach in mind. We have shown it is possible to filter purify membrane blebs (with encased DNA) away from intact organisms directly from culture medium (Fig. 5a). Since these structures have not been exposed to osmotically damaging conditions in the course of purification we have used these directly in genetic exchange assays.

In addition to the radioisotope labelling experiment you suggest, we are presently testing the "biological equivalent" of that experiment as well. Using low passage strains that have shown a complex DNA profile and infectiousness in laboratory animals (18) as bleb donor and high passage, non-infectious laboratory stains showing a simpler DNA profile as whole cell acceptor, we have set out to ask whether or not functional genetic transmission takes place. Since selectable genetic markers (such as antibiotic resistance) are not available in this organism, more conventional methods of molecular genetic analysis are not yet possible.