Scanning Microscopy Vol. 13, No. 1, 1999 (Pages 123-131) 0891-703599$5.00+.25
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

USE OF RECOMBINANT CYTOADHESIVE MOLECULES AND SCANNING ELECTRON MICROSCOPY TO ASSESS THE INTERACTION OF *BORRELIA BURGDORFERI*, THE LYME DISEASE SPIROCHETE, WITH SPECIFIC CELLULAR RECEPTOR SITES

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(Received for publication April 7, 1998 and in revised form October 28, 1998)

Abstract

Cytoadhesive receptors expressed on cell surfaces are essential for cell-cell interaction and adhesion to the extracellular matrix. Many microorganisms have evolved the ability to use these surface receptors, called integrins, to bind and then to invade host cells. A broadly applicable method is described in which scanning electron microscopy (SEM) is used to assess this binding potential under carefully controlled conditions. The rapid assay which uses commercially available, genetically engineered cytoadhesive molecules and site specific competitive inhibitors in a convenient filter/cell culture insert format may be used to yield either qualitative and/or quantitative data. Furthermore, the specimens appear suitable for high resolution SEM examination as well. The efficient use of the fibronectin, but not the vitronectin or laminin, cellular binding receptor by *Borrelia burgdorferi*, the causative agent of Lyme disease, is demonstrated using the SEM assay method. Gentle disruption and washing of bound spirochetes with N-lauroylsarcosine followed by detergent elution and polyacrylamide gel electrophoresis of bound polypeptides revealed a unique, limited subset of components. Borrelial major outer surface protein A (OspA) was most prominent among them. Pretreatment of fibronectin coated membranes with recombinant OspA protein blocked binding of spirochetes as efficiently as did the disintegrin molecule flavoridin.

Key Words: Scanning electron microscopy, cytoadhesive molecules, *Borrelia burgdorferi*, Lyme disease, outer surface protein A (OspA), fibronectin, laminin, vitronectin, cellular receptor binding sites.

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Introduction

Lyme borreliosis is the most prevalent tick-borne disease of the northern hemisphere with many highly endemic areas in Europe and the United States [3, 5]. The disease is a multi-system spirochetosis with dermatologic, neurologic, and rheumatologic manifestations [1, 6, 12, 31]. Central to the disease process, in the absence of a demonstrated toxin, is the interaction between the microorganism and host cells. Georgilis *et al*. [14] have demonstrated, using laser confocal microscopy, that intracellular spirochetes may be protected to some degree from the effects of certain antibiotic treatment protocols. Dorward *et al*. [11] have shown by a combination of video, transmission and scanning electron microscopy that *Borrelia burgdorferi* attach and invade human B-cells sometimes killing and sometimes sparing the cells. Although the use of integrin binding sites has been convincingly demonstrated with a number of microbial pathogens [2, 4, 9, 10, 13, 15-17, 19-21, 23, 25, 26, 29, 30, 33, 36], conflicting data have appeared in the literature as to the specifics of integrin binding by the Lyme disease spirochete [19, 32, 34]. Made possible largely by the availability of recombinant protein molecules made up of multiple copies of a specific integrin binding site [8, 37], we report here on the development of a simple, sensitive and specific scanning electron microscopy (SEM) method for assessing integrin receptor binding used by microorganisms to gain entrance into eukaryotic cells. Although we have focused on the cellular integrin receptor usage by *Borrelia burgdorferi*, the method is useful in many contexts and provides a versatile and convenient SEM mounting protocol as well as a useful method to assess the binding characteristics of many types of both eukaryotic and prokaryotic cells.

Materials and Methods

Borrelia burgdorferi, strain B31, a prototype strain (American Type Culture Collection, Rockville, MD), was used throughout these experiments. Spirochetes were grown in 500 ml bottles of Barbour-Stoenner-Kelly II (BSK II) medium without antibiotics (Sigma, St. Louis, MO) at

34°C as previously described [24]. Cultures were centrifuged at 3,000 x g for 2 min at 4°C and gently resuspended, and repelleted in Tyrode's buffer. The resuspended spirochetes were applied to the well of a sterile Falcon Cell Culture Insert containing integrin pretreated Cyclopore, polyethylene terephthalate, track etched, 3.0 micron pore size membrane (VWR Scientific, Seattle, WA; Cat # 62406-169) and allowed to settle for 5 min at room temperature before the culture medium was gently drawn through the filter. Filter pretreatment involved adding 50-100 µl of various dilutions of ProNectin F (Protein Polymer Technologies, San Diego, CA; Cat # PF 1001, supplied as powder with proprietary diluent), laminin (Sigma Chemical Co., St. Louis, MO; Cat # L-6274), vitronectin (Sigma; Cat # V-8379), and poly-D-lysine (Sigma; Cat # P-7886) in phosphate buffered saline to the filter well for 5 min at room temperature before aspirating the solution gently through the membrane. It is important that, as solutions are incubated and drawn through the filter membrane, the samples are never allowed to become dry. The following detergents (purchased from Calbiochem, La Jolla, CA) were used as a 1% solution: CHAPS (3-cholamidopropyldimethyl-ammonio-1-propanesulfonate) (Cat # 220201), deoxycholic acid (Cat # 264103), n-octyl-b-D-glucopyranoside (Cat # 494459), n-octyl-b-D-thioglycopyranoside (Cat # 494461), ZWITTERGENT (Cat # 693017). Also used in these experiments as 1% solutions were n-lauroylsarcosine, sodium salt (Sigma; Cat # L5777) and BRIJ 58 (ICI Specialty Chemicals Division, Wilmington, DE; Cat # 1936B). Recombinant *Borrelia burgdorferi* OspA protein was a generous gift from John Dunn (Brookhaven National Laboratory, NY). Following filter pretreatment, spirochete binding and detergent washing, a graded series of ethanol was added to the vessel containing the filter membrane and incubated for 5 min each at room temperature with each solution as follows: 25%, 50%, 75%, 95%, 100%, 100%, 100%. Filters were cut from the cell culture insert vessel, critical point dried from liquid CO_2, mounted on an SEM stub, and sputter coated with 8-10 nm chromium (VCR Group Inc., South San Francisco, CA; Model IBS/TM200S Ion Beam Sputterer). Stubs were examined in a Hitachi (Tokyo, Japan) S4500 Field Emission Scanning Electron Microscope at 10 kV accelerating voltage. Single dimension polyacrylamide gel electrophoresis (PAGE) was performed following detergent treatment as previously described [22] and adherent polypeptides were visualized using Silver Stain Plus (Bio-Rad Laboratories, Richmond, CA).

Results

Viable, log phase *Borrelia burgdorferi* was tested by SEM for the ability to bind to untreated filter membranes.

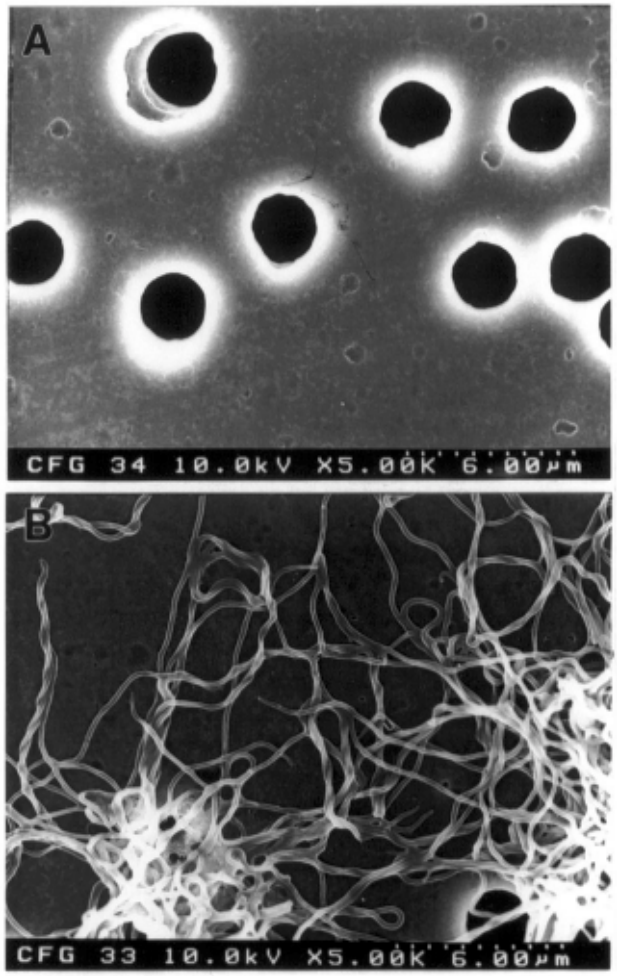
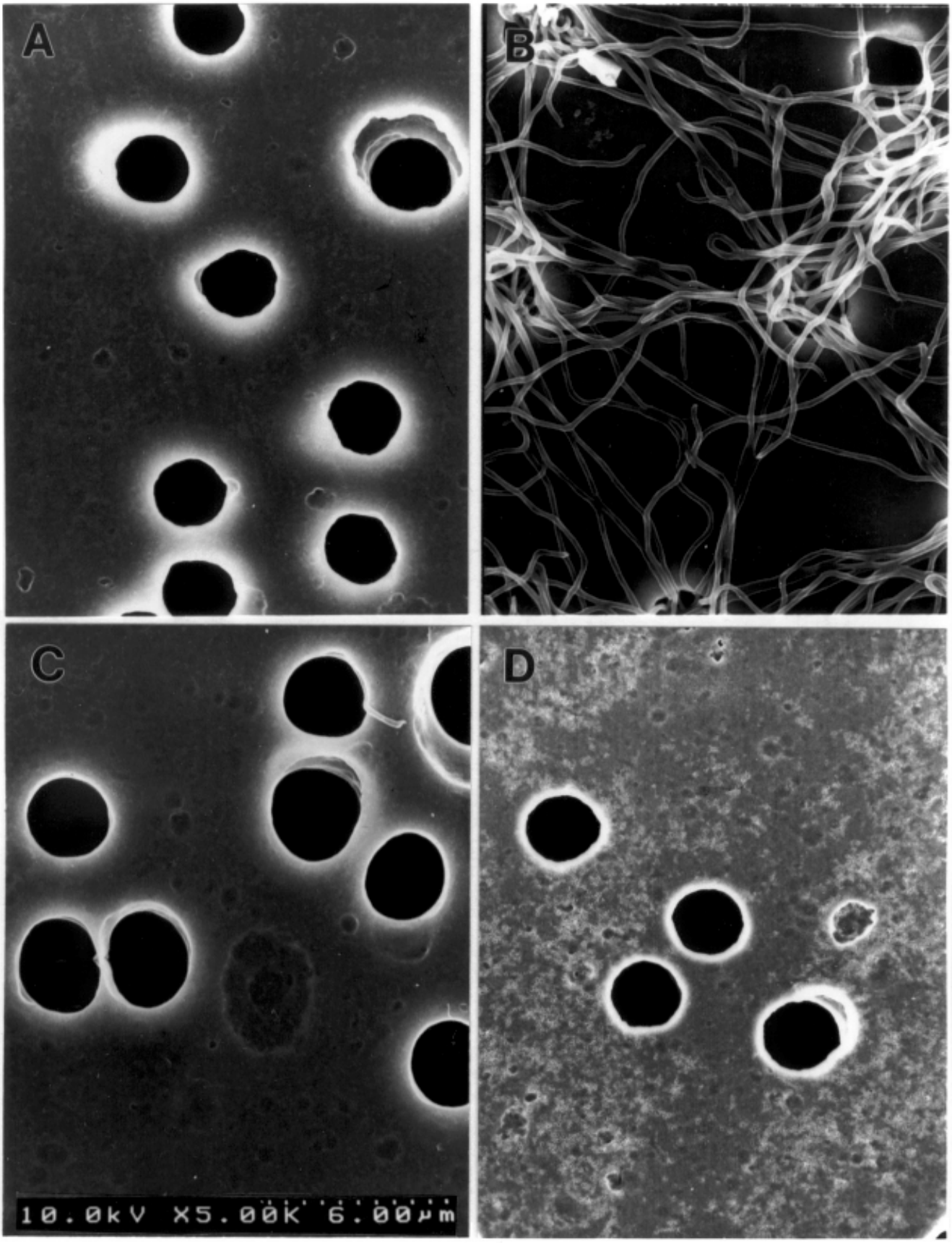


Figure 1. SEM analysis of the binding of *Borrelia burgdorferi* spirochetes to the surface of 3.0 µm pore size, track-etched, cell culture membrane either untreated (Panel **A**) or with standard polylysine/glutaraldehyde treatment (Panel **B**).

Figure 2 (*on facing page*). SEM analysis of spirochete binding to membranes which are: Panel **A**: untreated; Panel **B**: pretreated with 0.01 µg/ml ProNectin F; Panel **C**: pretreated with 0.001 µg/ml ProNectin F; or Panel **D**: pretreated with 0.01 µg/ml of ProNectin F followed by 0.01 µg/ml flavoridin, an RGD-specific fibronectin disintegrin.

Figure 1 shows electron micrographs of a typical field of filter membrane surface untreated (Panel A) before the addition of spirochetes compared with the standard laboratory method of mounting microorganisms onto filter surfaces by pre-treating with polylysine followed by glutaraldehyde fixation shown in Panel B. While virtually no binding was detectable in untreated filters, polylysine pre-


A
B
C
D
10.0kV X5.00K 6.00µm

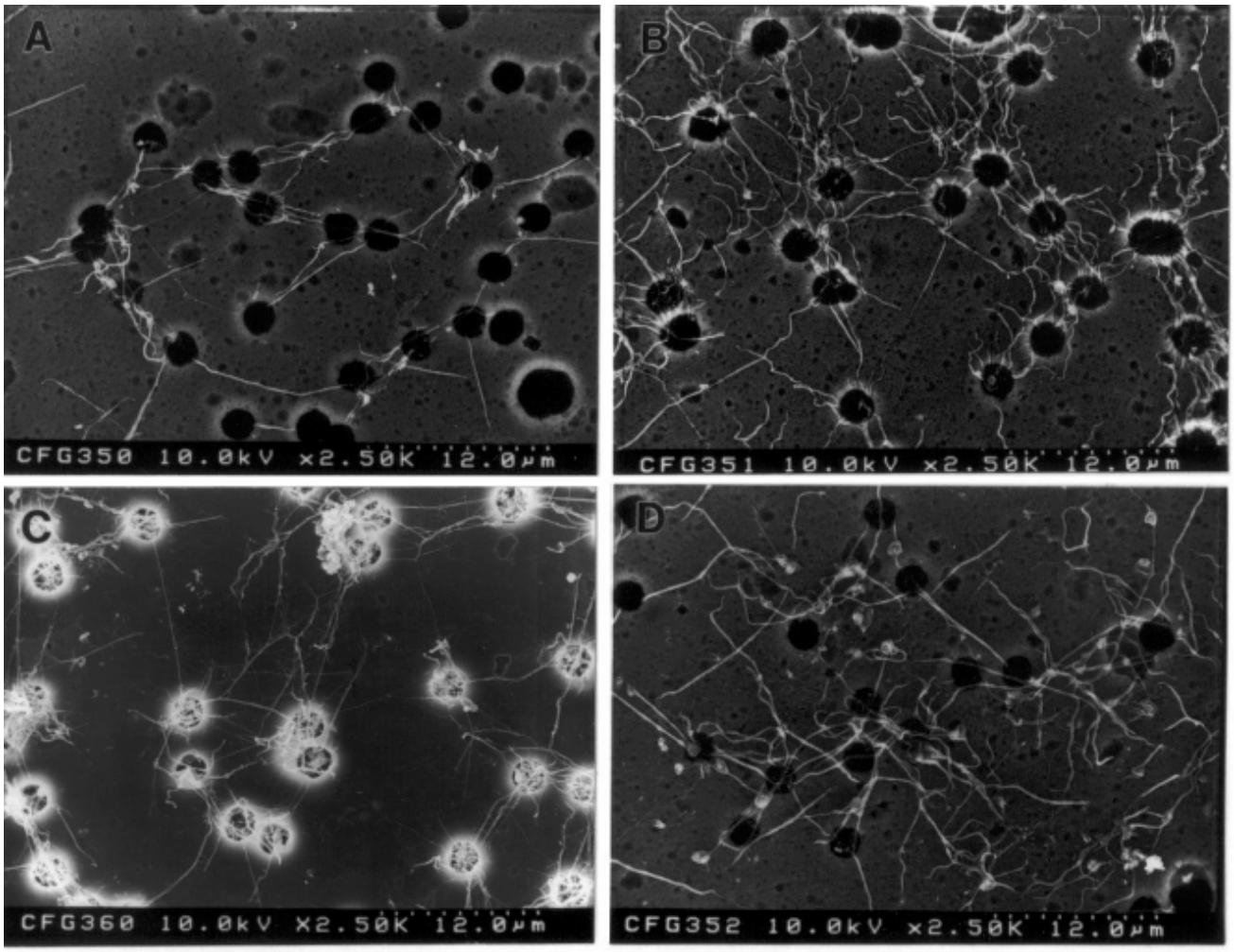


Figure 3. SEM analysis of spirochete binding to ProNectin F treated membrane filters that have subsequently been washed with a 1% solution of: n-octyl-b-D-thioglycopyranoside (Panel **A**); ZWITTERGENT (Panel **B**); or n-lauroylsarcosine (Panel **C**) before critical point drying and sputter coating. (Panel **D**) shows ProNectin F treated membranes without subsequent detergent treatment.

treatment allows numerous, intact spirochetes to bind.

Figure 2 demonstrates the binding efficiency of filter membranes pretreated at room temperature for 10 min with ProNectin F as described in materials and methods (Panel B). Furthermore, the availability of purified, arginine-glycine-aspartic acid (RGD)-specific disintegrin molecules allowed us to document, by competitive inhibition, the specificity of the fibronectin binding reaction [7, 28]. Flavoridin, a fibronectin disintegrin, was added to a ProNectin F-coated filter prior to spirochete binding (Fig. 2, panel D). In contrast to the ProNectin F-treated filter shown in panel B, little or no spirochete binding was observed following flavoridin treatment of the ProNectin F-coated filters. These results provide strong evidence that the interaction involved in holding spirochetes onto filter membranes in these experiments is not due to random sticking or trapping, but rather likely involves the well characterized RGD binding site of fibronectin. Neither untreated (Panel A) nor ProNectin F treatment at concentrations below 0.01 µg/ml (Panel C) allowed effective binding of *Borrelia burgdorferi* to filters. Interestingly, neither prior nor post fixation of spirochetes with glutaraldehyde (1%) appears to affect the binding patterns seen in Figure 2.

Similar binding assays with other integrin molecules [18] and a dose response assessment of each is summarized in Table 1. Only ProNectin F pretreatment at concentrations at or above 0.01 µg/ml and the highly non-specific, standard laboratory procedure involving polylysine and glutaraldehyde produced detectable filter binding of spirochetes in these assays.

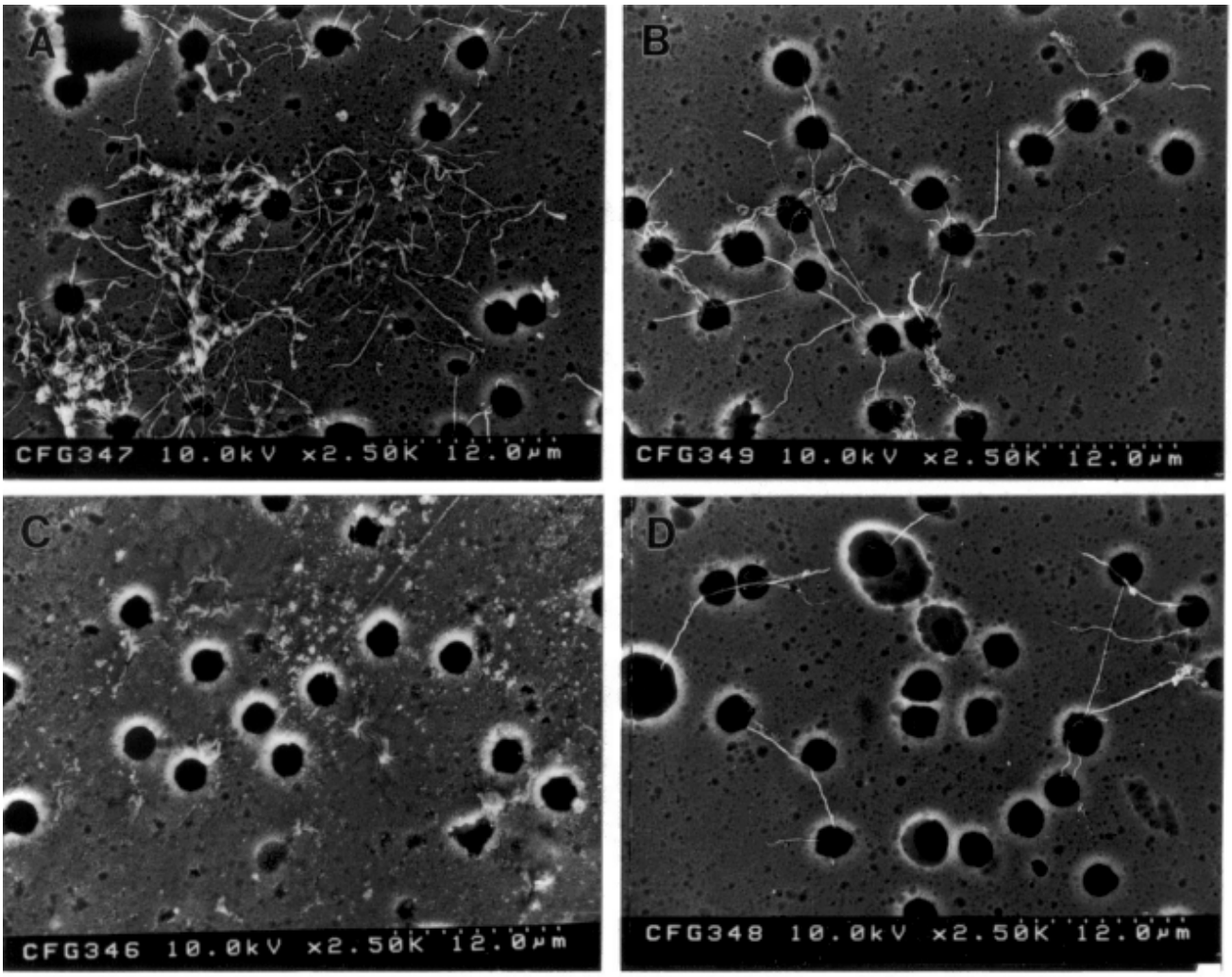


Figure 4. SEM analysis of spirochete binding to ProNectin F treated membrane filters that have subsequently been washed with a 1% solution of: CHAPS (Panel **A**); n-octyl-b-D-glucopyranoside (Panel **B**); BRIJ 58 (Panel **C**); or deoxycholate (Panel **D**) prior to further processing for microscopy.

Once bound to ProNectin F pretreated filter membranes, we wondered if it would be possible to gently disrupt the spirochetes *in situ*, removing those components not involved in binding, while retaining those polypeptides that may be specifically involved. Several detergents, chosen initially for their previous use in disrupting microbial outer membranes, were used to gently wash filters containing bound spirochetes and filters were subsequently screened, as before, by SEM. The microscopy results are shown in Figures 3 and 4. While some detergents, did not appear to disrupt spirochetes significantly (Fig. 3, panel B; Fig. 4, panel A) when compared to the untreated control (Fig. 3, panel D), others appeared to have visible effects on the structure of the bound spirochetes (Fig. 3, panels A and C; Fig. 4, panels B, C and D). Treatment with BRIJ 58 (Panel C) appeared to remove all recognizable structure from the filter, perhaps disrupting the fibronectin binding sites. Attractive detergent candidates, such as those shown in Figures 3 and 4, were tested further for the presence of unique, filter bound protein.

Similarly prepared, detergent washed, filters were eluted by boiling in sodium lauryl sulfate (SDS) and analyzed by polyacrylamide gel electrophoresis [22]. In this experiment ProNectin F bound polypeptides were compared with those which might be bound non-specifically to un-pretreated filters. The results of this comparison are shown in Figure 5. While the positions of molecular weight standard markers are indicated at the left, lanes a and b, lanes c and d, lanes e and f and lanes g and h contain deoxycholic acid, filter eluate and control; BRIJ 58,

Table 1. SEM filter binding assay.

Treatment	Binding
Control	-
Polylysine	+
Pronectin F	
100	+
10	+
1	+
0.1	+
0.01	+
0.01+flavoridin	-
0.001	-
Laminin	
1	-
0.1	-
0.01	-
0.001	-
Vitronectin	
1	-
0.1	-
0.01	-
0.001	-

All concentrations in µg/ml of supplied diluent.

filter eluate and control; n-lauroylsarcosine, filter eluate and control; and CHAPS, filter eluate and control, respectively. ProNectin F and control filters washed with deoxycholic acid, BRIJ 58, or CHAPS showed a similar subset of proteins adhering to the filter membranes indicating that these detergents were inefficient in the removal of proteins that were non-specifically bound to filters. The n-lauroylsarcosine treated sample (lane e) showed a limited subset of borrelial proteins which appear to bind specifically to the ProNectin F coated filter membranes, but which are not bound to filters in the absence of ProNectin F pretreatment (lane f). Furthermore, the major protein detected resides in an area of the gel (lane e) very near the 31-32kD major outer surface protein of *Borrelia burgdorferi*, designated OspA.

To determine if major outer surface protein, OspA, is involved in the ProNectin F binding seen in Figure 5 (lane e), highly purified, recombinant OspA protein was added to the ProNectin F coated filters prior to spirochete binding. The results of that competition experiment are shown in Figure 6. In contrast to the binding of spirochetes to ProNectin F coated filter membrane seen (Panel A), pretreatment of ProNectin F coated membranes with an

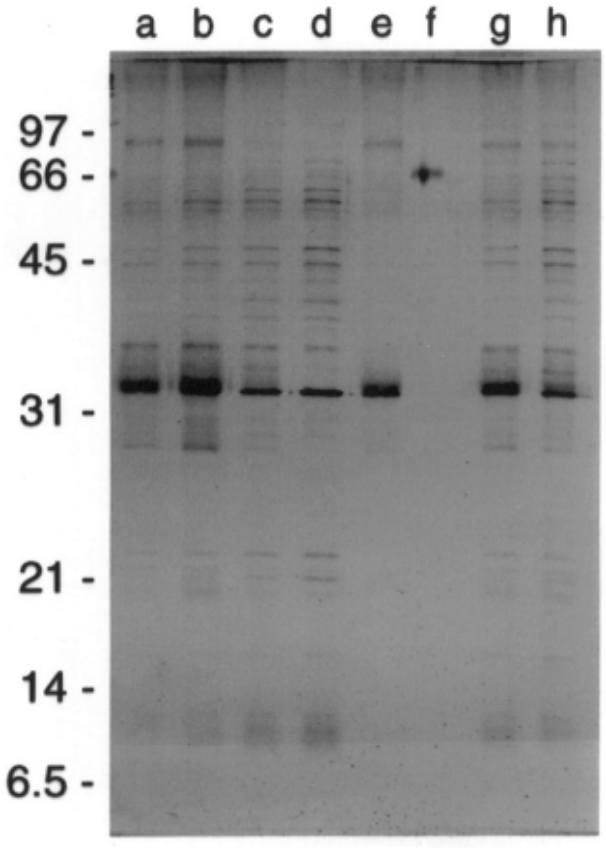


Figure 5. SDS-PAGE analysis of borrelial, silver staining proteins which remain associated with ProNectin F treated membranes following extensive washing with one of several detergents at room temperature. Borrelial proteins which remained bound to filters following detergent washes, were subsequently eluted from the membrane with hot SDS/mercaptoethanol and analyzed by acrylamide gel electrophoresis [22]. Lane pairs represent ProNectin F treated and untreated membranes respectively for each detergent used as follows: lanes **a** and **b** - deoxycholic acid; lanes **c** and **d** - BRIJ 58; lanes **e** and **f** - n-lauroylsarcosine; lanes **g** and **h** - CHAPS. The positions of molecular weight standard markers are indicated to the left of the gel in kD.

equal concentration of recombinant *Borrelia burgdorferi* OspA protein, appears to effectively inhibit binding of the spirochetes to the filters (Panel B). These data would support the involvement of the major outer surface protein, OspA, in specific ProNectin F binding.

Discussion

Fibronectins are high molecular weight glycoproteins found in many extracellular matrices and in blood plasma. They have been shown to promote cell adhesion and affect

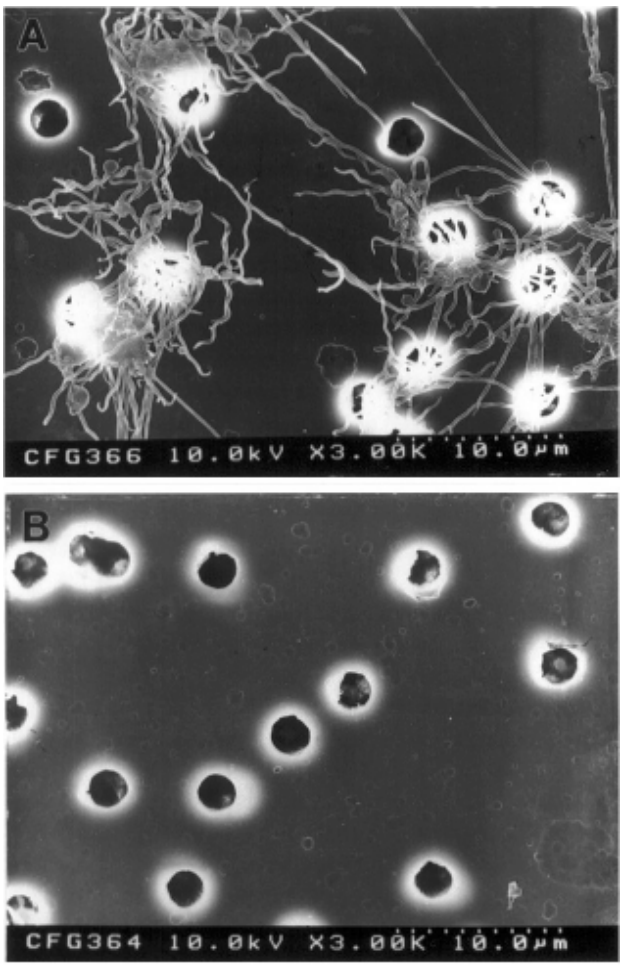


Figure 6. SEM analysis of spirochete binding to ProNectin F treated filters either without (panel **A**) or with (Panel **B**) added recombinant OspA protein as a potential competitive inhibitor of spirochete binding.

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cell morphology, migration, differentiation and cytoskeletal organization. Fibronectin and other extracellular matrix proteins are ligands for cell surface receptors known as integrins. Great interest has been focused recently on these proteins, not only because of their well documented role in the normal structure and function of eukaryotic cells, but also because they have been shown recently to be absent or non-functional in some transformed cells and because they are often used by pathogenic microorganisms to gain entry into cells during infection [35]. Furthermore, recent research has suggested that integrins can participate in bi-directional signal-transduction processes. Integrins can convey signals from the extracellular matrix to the cell interior, and intracellular events can influence the affinity of integrins for their ligands. On a slightly more mundane level, some data would suggest that integrin proteins might substitute for expensive, serum-based components in tissue culture medium [27]. Many integrins have been available only as partially purified products and when used in sensitive laboratory assays often cause ambiguous experimental results due to low levels of non-specific binding. However, some integrins are now becoming available as highly specific, genetically engineered bioproducts virtually free of interfering contaminants. One such product is ProNectin F (see materials and methods), a commercially available, recombinant protein polymer which integrates 13 identical copies of the specific tripeptide receptor site arranged between repeated copies of an unrelated (and non-binding) spacer sequence. This RGD cell attachment epitope polymer is available as a 100,000 MW polypeptide which binds readily to surfaces which might be used for electron microscopy and is both active and stable over long periods of time. Furthermore, specific RGD competitive inhibitors or disintegrins are available which can be used to verify specific binding [38]. We have developed, and present here, a convenient and broadly applicable SEM method, which takes advantage of the specificity of this bioproduct to assess the presence of the cytoadhesive receptor on the surface of *Borrelia burgdorferi.* Although conflicting information appears in the literature concerning the use of various integrin receptors by the Lyme disease spirochete, our data would suggest that *Borrelia burgdorferi* actively binds to the recombinant fibronectin binding site of ProNectin F, perhaps using its major outer surface protein, OspA. Additional research will be required to define this potentially important interaction more fully.

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Discussion with Reviewers

R.G. Richards: Having an FESEM why did the authors not image the filters and spirochetes at accelerating voltages lower than 5kV which would have prevented the charging around the holes and the excess charging of the spirochetes themselves? The lower voltages also would have allowed any detail in the surfaces of the spirochete to be seen.

Authors: We, like many microscopy laboratories around the world, often find ourselves struggling to obtain biological structural information seen only at the very highest resolution our instruments will deliver. However, in these initial experiments, high resolution was not the issue. Since expensive, field emission equipment is not always available, we purposely attempted to design an assay useful to a broad range of biologists where specific microbe-integrin interaction could be sensitively assessed using virtually any available scanning microscopy equipment. Were high resolution images needed to view spirochete surfaces or integrin interaction, we might have used the recording parameters you suggested. However, we are convinced that useful biological data does not always reside in the highest resolution images. Furthermore, "optimizing scanning electron microscopy" can legitimately involve innovative experimental design where the need for the highest resolution is not necessarily required.