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Characterization of *Borrelia burgdorferi* Binding to Mammalian Cells and Extracellular Matrix

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Abstract

Lyme disease *Borreliae* produces outer surface adhesins to confer bacterial attachment to the extracellular matrix (ECM) components on the surface of mammalian cells. Here, we describe protocols to characterize the activity and specificity of these adhesins by flow cytometry or measurement of the binding of radiolabeled spirochetes to immobilized ECM or mammalian cells.

Keywords

Lyme disease; *Borrelia burgdorferi*; Adhesins; Flow cytometry; Radiolabeling; Extracellular matrix; Mammalian cells

1 Introduction

Lyme disease, caused by the spirochete *Borrelia burgdorferi* sensu lato, is the most common vector-borne pathogen in the United States, Europe, and parts of Asia [1, 2]. Following the bite of a *B. burgdorferi*-infected tick, spirochetes can spread via the bloodstream to distal tissues, such as the heart, joints, and brain, leading to carditis, arthritis, and neuroborreliosis, respectively [2]. *B. burgdorferi* produces numerous outer surface proteins, termed adhesins [3, 4], that promote spirochetal binding to host cells or extracellular matrix (ECM) components [5–8], thus promoting tissue colonization [9–13]. Candidate Lyme disease *Borreliae* adhesins often have been initially identified by demonstrating that purified recombinant versions of these proteins bind to cultured cells and/or ECM components [5–8, 14–17]. However, the recombinant derivatives do not uniformly reflect the behavior of these proteins when localized on the surface of spirochetes [18]. Therefore, ectopic production of putative adhesins on high-passage, noninfectious, and non-adhesive *B. burgdorferi* strains to assess gain-of-function [7, 19, 20] or deletion of genes encoding putative adhesins in fully infectious spirochete strains to assess loss-of-function [8, 21, 22] have been utilized as strategies to evaluate these adhesins.

In this chapter, we describe protocols routinely used in our laboratories to measure binding of *B. burgdorferi* to ECM components or mammalian cells by flow cytometry or quantitation of radiolabeled spirochetes. These assays permit the characterization of the role of specific adhesins in promoting spirochetal attachment to mammalian cells or ECMs.

2 Materials

Prepare all solutions using ultrapure water and analytical grade reagents.

2.1 Flow Cytometry

1. BSK medium without serum: Dissolve 5 g of Neopeptone, 2 g of Yeastolate, 6 g of HEPES, 5 g of glucose, 0.7 g of sodium citrate, 0.8 g of sodium pyruvate, 0.4 g of N-acetyl-glucos-amine, 2.2 g of sodium bicarbonate, and 9.8 g of 10 × CMRL1066 in 1 L of water and adjust pH to 7.6. Filter sterilize the medium with a 0.22 µm filter and store at −20 °C. This medium can be used for up to 6 months.
2. BSK medium with serum: Add 50 g of BSA (bovine serum albumin) (*see Note 1*), 200 milliliters (mL) of 7% porcine skin gelatin in water, and 60 mL of rabbit serum (Sigma) to 900 mL BSK medium without serum from the **step 1** in Subheading 2.1. Filter sterilize the medium using a 0.22 µm filter and store at 4 °C. This medium can be used for up to 1 month.
3. HBSC-DB buffer: Add 5.95 g of HEPES, 6.13 g of sodium chloride, 0.095 g of manganese chloride, 0.027 g of calcium chloride, 1 g of dextrose, and 2 g of BSA in 1 L of water and adjust pH to 7.8. Filter sterilize the buffer using a 0.22 µm filter and store at 4 °C.
4. HBSC-DB buffer with formaldehyde: Add 1 mL of formaldehyde in 1 L of HBSC-DB buffer.
5. Phosphate-buffered saline (PBS): Add 0.136 g of potassium phosphate monobasic, 0.425 g of sodium phosphate dibasic, and 9.06 g of sodium chloride in 1 L of water and adjust pH to 7.4.
6. GAG lyases stock solution: dissolve each of the GAG lyases (Heparitinase (Sigma), Chondroitinase AC (Sigma), or Chondroitinase B (Sigma)) into RPMI 1640 medium mentioned supplemented with 1% BSA, aprotinin (0.01 Unit per mL), and PMSF (165 µg per mL). The stock concentration of those GAG lyase is 5 Units per mL of medium.

2.2 Radiolabeled B. burgdorferi Binding Assay

1. Carbonate coating solution: Add 3.7 g of sodium bicarbonate and 0.64 sodium carbonate in 1 L of water and adjust pH to 9.5.
2. PBS: Add 0.136 g of potassium phosphate monobasic, 0.057 g of sodium phosphate dibasic, and 9.06 g of sodium chloride in 1 L of water and adjust pH to 7.4.
3. PBS with 0.05% BSA: Add 0.5 g of BSA in 1 L of PBS.
4. PBST: Add 500 µL of Tween 20 in 1 L of PBS.
5. Blocking solution: Add 2.5 g of BSA in 25 mL of PBS.

6. GHS solution: Add 1.8 g of glucose, 2.38 g of HEPES, and 2.92 g of sodium chloride in 1 L of water and adjust pH to 7.0.
7. Collagen solution: Add 1 g of calf skin collagen (Sigma) in 1 L of 0.1 N acetic acid in water.
8. BSK without serum with glycerol: Add 20 mL of glycerol in 80 mL of BSK without serum.
9. GAG lyase stock solution: *see step 6* in Subheading 2.1.

3 Methods

3.1 Flow Cytometry to Determine *B. burgdorferi* Binding to Mammalian Cells

3.1.1 Preparation of Bacteria

1. Grow 10 mL of *B. burgdorferi* to mid-logarithmic phase in BSK medium with serum at 33 °C to a concentration of approximately 1×10^8 bacteria per mL.
2. Centrifuge 10 mL of culture at $8000 \times g$ at room temperature in a tabletop centrifuge for 10 min.
3. Remove the supernatant and suspend bacterial pellet in 5 mL of PBS with 0.05% of BSA to wash the bacteria.
4. Centrifuge culture at $8000 \times g$ at room temperature in a tabletop centrifuge for 10 min. Repeat **steps 3** and **4** in Subheading 3.1.1 two more times for a total of three washes (*see Note 2*).
5. Suspend bacteria in 5 mL of HBSC-DB solution at a final concentration of approximately 2×10^8 bacteria per mL (*see Note 3*).

3.1.2 Preparation of Suspensions of Cultured Mammalian Cells (See Note 4)

1. Grow monolayers of the mammalian cells of interest to near-confluence in media appropriate for the particular mammalian cell line (approximately 1×10^7 cells in a T25 flask).
2. Lift the mammalian cells with 1.5 mL of Trypsin-EDTA (0.25%) solution with phenol red.
3. Centrifuge 1.5 mL of the mammalian cells at $100 \times g$ for 5 min at room temperature.
4. Remove the supernatant and suspend mammalian cells in 2 mL of PBS.
5. Centrifuge the mammalian cells at $100 \times g$ for 5 min at room temperature.
6. Repeat **steps 3** and **4** in Subheading 3.1.1 to wash mammalian cells for a total of three times.
7. Suspend the mammalian cells into 2 mL of HBSC-DB buffer to a final concentration approximately 5×10^6 cells per mL.

3.1.3 Incubation of Bacteria and Mammalian Cells

1. Mix 20 μL of bacteria (4×10^6 cells) from Subheading 3.1.1 with 20 μL of mammalian cells (1×10^5 cells) from Subheading 3.1.2. Manually flick the tubes to mix (see **Note 2**).
2. Incubate the bacteria-mammalian cell mixture on a laboratory rocker set at a speed of 50 rpm at 37 °C for 1 h.

3.1.4 Fixation and Detection

1. Dilute 1 μL of FITC-conjugated mouse anti-*B. burgdorferi* antibody in 47 μL of PBS. Add 10 μL of the diluted anti-*B. burgdorferi* antibody to the 40 μL of bacteria-mammalian cell mixture from Subheading 3.1.3. The final dilution ratio of the antibody in the bacteria-mammalian cell mixture will be 1:240 (see **Note 5**).
2. Incubate the mixture on a laboratory rocker at room temperature with speed of 50 rpm for 1 h.
3. Add 900 μL of HBSC-DB buffer with formaldehyde and incubate at room temperature for 30 min to fix the bacteria-mammalian cell mixture.
4. Analyze the bacteria-mammalian cell mixture with flow cytometry to quantitatively determine the ability of *B. burgdorferi* to bind to mammalian cells (see Fig. 1 as representative results of flow cytometry).
5. If unable to immediately analyze the bacteria-mammalian cell mixture by flow cytometry, it can be stored at 4 °C until needed (see **Note 6**).

3.1.5 Inhibition of *B. burgdorferi* Binding to Mammalian Cells by Mixing Purified ECMs with Bacteria (See Note 7)

1. Prepare stock solution of soluble ECMs (6.25 mg per mL) in HBSC-DB buffer.
2. Add 10 μL of the inhibitor stock solution to the bacteria from the Subheading 3.1.1, and incubate the bacteria-inhibitor mixture at room temperature for 1 h. Then, proceed to Subheading 3.1.3.

3.1.6 Inhibition of *B. burgdorferi* Binding to Mammalian Cells by Enzymatically Removing Specific Glycosaminoglycans (GAGs) (See Note 8)

1. Suspend the mammalian cells from Subheading 3.1.2 in RPMI 1640 medium with HEPES and no glutamine in a volume that results in a final concentration of 5×10^6 cells per mL.
2. Add GAG lyases stock solution to the suspension of mammalian cells from **step 1** in Subheading 3.1.6 for a final concentration of GAG lyases in the mixture of 0.5 Unit per mL.
3. Gently mix and incubate the lyase-mammalian cell mixture on a laboratory rocker with speed of 50 rpm at 37 °C for 1 h.

4. Centrifuge mammalian cells at $100 \times g$ for 5 min at room temperature.
5. Remove the supernatant and then suspend mammalian cells in 2 mL of HBSC-DB buffer.
6. Repeat **steps 4 and 5** in Subheading 3.1.6 to wash mammalian cells for a total of three times.
7. Suspend the mammalian cells into HBSC-DB buffer at a final concentration of 5×10^6 cells per mL. Then, proceed to Subheading 3.1.3.

3.2 Detecting the Radiolabeled *B. burgdorferi* Bound to Mammalian Cells or ECMs

1. Separately sterilize plastic break-apart ELISA plates and plate-sealers by exposure to UV light in a biosafety cabinet at room temperature for 2 h (*see Note 9*). After sterilization, apply plate sealers to the plates and store at room temperature.

3.2.1 Preparation of the Wells on Break-Apart ELISA Plates

3.2.2 Coating of ELISA Plate Wells with ECM Components

1. To prepare ECM-coated wells in ELISA plates, dissolve 10 μ g of the ECM component into 1 mL of carbonate coating solution (*see Notes 10 and 11*). Add 100 μ L of this ECM solution to each of the wells in a plate from Subheading 3.2.1.
2. Incubate the plate at 4 °C overnight.
3. Wash the wells with PBST three times to remove unbound ECM components.
4. Block the wells by adding 100 μ L of blocking solution and incubate for 1 h at room temperature.
5. Wash the wells with PBST three times. Add 100 μ L of GHS solution to each well and incubate at room temperature for 1 h.

3.2.3 Preparing Microtiter Wells with Mammalian Cell Monolayers (See Note 4)

1. Add 50 μ L of collagen solution to each well of a plate from Subheading 3.2.1. Tap the plates lightly to ensure the solution is coating the entire bottom of the wells and incubate the plate at 4 °C overnight.
2. Wash the wells with PBST three times.
3. Grow monolayers of the mammalian cells of interest to nearconfluence in media appropriate for the particular mammalian cell line (approximately 1×10^7 cells in a T25 flask).
4. Lift the mammalian cells with 1.5 mL of 0.25% of Trypsin-EDTA solution.
5. Centrifuge the mammalian cells at $100 \times g$ for 5 min at room temperature.
6. Remove the supernatant and suspend the mammalian cells in 2 mL of PBS.

7. Repeat **steps 5** and **6** Subheading 3.2.3 to wash mammalian cells for a total of three times.
8. Suspend the mammalian cells into the medium appropriate for the growth of those mammalian cells in a volume that results in a final concentration of 1×10^7 cells per mL. Add 100 μ L to each well of the ELISA plate from **step 2** in Subheading 3.2.3 (see **Note 11**).
9. Visually check the growth of the mammalian cell monolayers on the next day. The monolayers are ready to be used when they are more than 80% confluent.

3.2.4 Preparation of Radiolabeled *B. burgdorferi*

1. Seed 5×10^6 bacteria in 20 mL of BSK medium with serum supplemented with 6 μ Ci per mL of ^{35}S -Methionine (11 mCi per mL) and grow at 33 °C until the culture reaches approximately 1×10^8 bacteria per mL (see **Note 3**).
2. Centrifuge 10 mL of the bacteria at $8000 \times g$ in a tabletop centrifuge at room temperature for 10 min.
3. Remove the supernatant and then add 5 mL of BSK medium without serum to wash the bacteria.
4. Repeat **steps 2** and **3** in Subheading 3.2.4 to wash bacteria for a total of three times (see **Note 2**).
5. Suspend the bacterial pellet in 2 mL of BSK without serum with glycerol. The final concentration should be approximately 1×10^9 bacteria per mL.
6. Prepare 1 mL of aliquots and store at -80 °C.

3.2.5 Incubation of Radiolabeled Bacteria with ECM Components or Mammalian Cells

1. Warm BSK without serum at 37 °C for 10 min. Quickly thaw one vial of radiolabeled bacteria (described in Subheading 3.2.4) by warming the tube in hand until all traces of ice have just disappeared.
2. Centrifuge 1 mL of bacteria at $8000 \times g$ in a tabletop centrifuge at room temperature for 10 min.
3. Remove the supernatant and suspend bacterial pellet in 1 mL of BSK without serum.
4. Repeat **steps 2** and **3** in Subheading 3.2.5 to wash bacteria for a total of three times (see **Note 2**).
5. Suspend the bacteria in 2.5 mL of BSK without serum to a final concentration of approximately 4×10^8 bacteria per mL (see **Note 12**).
6. Incubate the suspension at room temperature for 2 h (see **Note 3**).
7. Dilute the bacteria 4 $\times$ in GHS solution so the concentration of bacteria should be approximately 1×10^8 bacteria per mL.

8. To measure binding of bacteria to ECM components or mammalian cells, respectively, add 50 μL of the bacterial suspension (approximately 5×10^6 bacteria) to each break-apart ELISA plate well from Subheading 3.2.2 (plates coated with ECM components) or Subheading 3.2.3 (plates with mammalian cell monolayers). Centrifuge the plate at room temperature at $1000 \times g$ in a tabletop centrifuge for 5 min to enhance the bacterial attachment to ECM components or mammalian cells.
9. Incubate the plates on a laboratory rocker with speed of 50 rpm at room temperature for 1 h.
10. Use pipette to remove supernatant and unbound bacteria, and wash the plate three times with PBS (*see Note 13*). Put the plate in a biosafety hood to dry for approximately 30 min.

3.2.6 Radioactive Counting

1. Break up the plates from Subheading 3.2.5 into individual wells and place each well into a 7 mL glass scintillation vial (*see Note 14*). Include four clean empty wells for background counts.
2. In addition, add 200 μL of bacteria suspension from **step 7** in Subheading 3.2.5 (approximately 2×10^7 bacteria) into a QIAprep mini-spin column with a filter.
3. Centrifuge it at $13,500 \times g$ for 5 min at room temperature.
4. Remove the flow-through and add 500 μL of PBS to wash the column by centrifuging the column at $13,500 \times g$ for 5 min at room temperature.
5. Place the column into a 7 mL glass scintillation vial.
6. Add 2.5 mL of Opti-Fluor O scintillation fluid to each vial. Check to make sure the wells are totally covered with the fluid.
7. Cap the vials tightly and number the tops.
8. Quantify the radiation activity of ^{35}S by the scintillation counter.
9. Multiply the values obtained from the column from **step 5** in Subheading 3.2.6 by four to calculate the “total radioactive count” of the bacteria prior to being added into the wells. Calculate the percentage of bacteria bound by ECMs or mammalian cells by normalizing the values obtained from the wells with bacteria/ECM components or bacteria/mammalian cells to the “total radioactive count” (*see Fig. 2 as a representative results of the percentage of radiolabeled bacteria bound by mammalian cells or ECM components*).

3.2.7 Inhibition of *B. burgdorferi* Binding to ECM Components or Mammalian Cells by Adding Soluble ECM Components as Inhibitors (See Note 7)

1. Prepare the stock solution of soluble ECM components (6.25 mg per mL) as inhibitors in PBS.

2. Add 7 μ L of the inhibitor stock solution to the radiolabeled bacteria from Subheading 3.2.4. Immediately after that, proceed to Subheading 3.2.5.

3.2.8 Inhibition of *B. burgdorferi* Binding to Mammalian Cells by Enzymatically Removing Specific Glycosaminoglycans (GAGs) (See Note 8)

1. Remove the medium from the mammalian cells in Subheading 3.2.3 and then add 200 μ L of BSK without serum.
2. Repeat **step 1** in Subheading 3.2.8 to wash mammalian cells a total of three times.
3. Add GAG lyases stock solution to the RPMI 1640 medium supplemented with 1% BSA, aprotinin (0.01 Unit per mL), and PMSF (165 μ g per mL) for a final concentration of GAG lyases as 0.5 Unit per mL.
4. Add 35 μ L of diluted GAG lyase to each well from the **step 3** in Subheading 3.2.8.
5. Incubate the mammalian cell-GAG lyase mixture at 37 °C for 1 h. Proceed to Subheading 3.2.5.

4 Notes

1. We routinely pretest the ability of many sources and lots of BSA for the ability to support *B. burgdorferi* growth. Recently, we have observed that *B. burgdorferi* grows well in media prepared with specific lots of BSA from EMD Millipore.
2. Do not vortex spirochetes to avoid disrupting the outer membrane and exposing the cytoplasmic or periplasmic proteins.
3. Evaluate the viability of bacteria under dark-field microscopy. Most of the bacteria should appear intact and highly motile.
4. The identification of receptors that mediate spirochetal adhesion has utilized mammalian cells that are deficient in or that ectopically produce candidate receptors [21, 23]. For example, Chinese Hamster Ovary (CHO) cells produce both heparan sulfate and chondroitin sulfate. The mutant cell lines CHO-*pgsA*-745, -*pgsB*-761, -*pgsB*-618, -*pgsB*-650, -*pgsD*-677, and -*pgsF*-17 cells produce reduced amounts of the various GAGs [24]. In addition, human embryonic kidney (HEK) 293 cells do not produce integrin β_3 , and transfection of the genes encoding the α_v plus β_3 integrin subunits into HEK293 cells results in a cell line (denoted “835 cells” or “293-15D cells”) that produces functional integrin $\alpha_v\beta_3$ [25].
5. Include one sample without adding FITC-conjugated antibody as an unstained sample to serve as a negative control during detection by flow cytometry.
6. We were able to utilize flow cytometry to detect fluorescence of stained samples within 1 week of storage at 4 °C.

7. The purified ECMs serve as inhibitors that bind to bacteria and block bacterial binding to mammalian cells.
8. If GAGs mediate bacterial binding to mammalian cells, the binding of bacteria to mammalian cells treated with GAG lyases will be reduced.
9. The plate should be rotated 90 degrees every 30 min when UV-sterilizing so that all parts of the well are sterilized.
10. The ECM components coated on the wells should be able to be detected by an antibody against that ECM component. If they cannot be detected, a different coating solution (e.g., PBS) can be used.
11. To quantify any nonspecific binding of spirochetes to uncoated wells, an empty well incubated with either carbonate coating solution (for Subheading 3.2.2) or the culture medium (for Subheading 3.2.3) should be included during the assessment of binding to ECM components or mammalian cells, respectively.
12. Suspend bacteria until no aggregate clumps of bacteria can be observed under dark field microscopy.
13. Wash wells gently because some mammalian cell monolayers can be easily removed by rigorous washing.
14. Plastic scintillation vials have been observed to block the detection of the radiation signals from ^{35}S . Therefore, glass scintillation vials (ThermoFisher) are suggested.

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CHO cells

Fig. 1.

B. burgdorferi adhesin Lmp1 promotes spirochetal binding to Chinese Hamster Ovary (CHO) cells determined by flow cytometry. The histogram shows the fluorescence intensity of the flow cytometry analysis from *B. burgdorferi* strains bound by CHO cells. The *B. burgdorferi* strains include wild-type *B. burgdorferi* strain B31-A3 (WT), a strain deficient for the adhesin Lmp1 (Δ lmp1), and the lmp1-deficient mutant complemented to ectopically express *Imp1* (lmp1-com). CHO cells without *B. burgdorferi* (no bacteria) were used as a negative control. (Reproduced from ref. 8 with permission from John Wiley & Sons Ltd.)

Fig. 2.

B. burgdorferi adhesin Lmp1 facilitates radiolabeled spirochetes binding to chondroitin-6-sulfate. *B. burgdorferi* strains B31-A3 (WT), Δ lmp1, or lmp1-com were radiolabeled with [³⁵S] methionine and incubated with either chondroitin-6-sulfate (top panel) or BSA (bottom panel) as a negative control on the microtiter plate wells. After washing, radioactivity of the bound bacteria was measured using a liquid scintillation counter. Each bar represents the mean of six independent determinations $\pm$ SEM. Significant ($p < 0.05$) differences in binding relative to wild-type *B. burgdorferi* (“*”) are indicated. (Reproduced from ref. 8 with permission from John Wiley & Sons Ltd.)