Integrin $\alpha_{IIb}\beta_3$ mediates binding of the Lyme disease agent *Borrelia burgdorferi* to human platelets

JENIFER COBURN*, JOHN M. LEONG*†, AND JOHN K. ERBAN‡

*Division of Rheumatology and Immunology, Box 406, and ‡Division of Hematology and Oncology, Box 245, Tufts-New England Medical Center, 750 Washington Street, Boston, MA 02111

ABSTRACT   Lyme disease is a chronic, multisystemic infection caused by the tick-borne spirochete *Borrelia burgdorferi*. Attachment of the spirochete to host cells via specific receptors is likely to be important in the establishment of infection. *B. burgdorferi* have previously been shown to bind to a variety of mammalian cells *in vitro*. Here we demonstrate that binding of *B. burgdorferi* to human platelets is mediated by the integrin $\alpha_{IIb}\beta_3$ (glycoprotein IIb–IIIa), a critical receptor in thrombosis and hemostasis. Functional expression of this receptor requires platelet activation, and binding of the spirochete was observed only to activated platelets. Binding was inhibited by a synthetic Arg-Gly-Asp peptide that blocks ligand interaction with many integrins and by a synthetic peptide based on the γ chain of fibrinogen that blocks binding to $\alpha_{IIb}\beta_3$. In addition, attachment of the spirochete to platelets was inhibited by monoclonal antibodies directed against $\alpha_{IIb}\beta_3$ that are known to block ligand–receptor interaction. No inhibition was seen with control peptides or with antibodies directed against other platelet receptors. *B. burgdorferi* bound efficiently to purified $\alpha_{IIb}\beta_3$ but did not bind to platelets deficient in this integrin. Efficient platelet binding was displayed by a cloned, infectious *B. burgdorferi* strain, whereas a cloned noninfectious strain did not bind to platelets. Binding to integrins may be important for the ability of *B. burgdorferi* to establish infection in the diverse tissues affected by Lyme disease.

Lyme disease is a chronic, multisystemic infection caused by the tick-borne spirochete *Borrelia burgdorferi* (1). After several days of localized skin infection at the site of the tick bite, the spirochete disseminates to multiple tissues, including the synovium, central nervous system, and heart (1–3). In untreated patients, *B. burgdorferi* can go on to establish chronic infection, most commonly affecting the joints, central nervous system, and skin. *B. burgdorferi* is able to survive in the face of a specific immune response and has been recovered from some patients years after the initial infection (4). It therefore seems likely that the spirochete attains an immunologically protected niche, possibly within one or more of these tissues.

Most pathogenic bacteria express multiple mechanisms for successful attachment to host cell surfaces. Recognition of specific receptors on host cells is likely to be an important step in the colonization and long-term infection of multiple tissues by *B. burgdorferi*. The Lyme disease spirochete binds to a variety of cultured mammalian cells (5–7), but the bacterial and host cell surface structures that mediate binding have yet to be characterized. It was recently reported that *B. burgdorferi* binds to human and rodent platelets (8). Platelet binding by bacterial pathogens is thought to facilitate the establishment of certain infections—e.g., endocarditis caused by streptococci and staphylococci (9–11). In this report, we identify a receptor on human platelets that mediates binding of *B. burgdorferi*.

MATERIALS AND METHODS

Reagents. Peptides were synthesized at the Tufts Protein Chemistry Facility and were from R. Isberg, Tufts University, Boston. Maltose-binding protein–Inv479, a hybrid protein containing the cell-binding domain of the *Yersinia pseudotuberculosis* invasin protein, was prepared as described (12). Antibodies used for immunoblot analysis were as follows: monoclonal anti-α_{IIb} or anti-β_3 from AMAC, Westbrook, ME; polyclonal anti-$\alpha_5\beta_1$ from Telios Pharmaceuticals, San Diego; polyclonal anti-P-selectin from B. Furie and B. C. Furie, New England Medical Center. Monoclonal antibodies (mAbs) known to block receptor function and tested in our binding assays were as follows: anti-P-selectin mAb GE12 from B. Furie and B. C. Furie; anti-α_2 mAb CLB-thromb/4, clone CLB-150, anti-α_6 mAb GoH3, clone CLB-701, and anti-β_3 mAb CLB-thromb/1, clone CLB-37, from CLB Reagentia, Amsterdam, The Netherlands; anti-α_3 mAb P2E6, anti-α_5 mAb B2G2, and anti-β_1 mAb A2B2 from C. Damsky, University of California, San Francisco; anti-$\alpha_5\beta_1$ mAb VD1 from G. Tran Van Nhieu and R. Isberg, Tufts University; anti-$\alpha_{IIb}\beta_3$ complex mAb CD41a (clone P2) from AMAC. mAb 9G11 is directed against the cell-binding domain of the *Y. pseudotuberculosis* invasin protein (13).

B. burgdorferi Strains. N40 clone D10/E9 has been described (14). The bacteria used in this study were recovered from a mouse infected with strain N40 clone D10/E9. A high-passage, noninfectious isolate of HB19 was cloned once on soft agar (15) and designated clone 1. Both strains are derived from North American isolates, and their protein profiles are virtually indistinguishable by two-dimensional gel electrophoresis. Bacteria were cultured at 34°C in MKP medium (16)/6% human serum. Late-logarithmic-phase bacteria were harvested by centrifugation and washed three times in ≈250 vol of phosphate-buffered saline (PBS)/0.2% bovine serum albumin (BSA), resuspended in MKP medium without serum (MKP-S) containing 20% (vol/vol) glycerol, and stored at −70°C. Radiolabeled *B. burgdorferi* were cultured in the same medium supplemented with [^{35}S]methionine at 10 μCi/ml (1 Ci = 37 GBq), then washed, and stored as described above.

Platelets. Blood from human volunteers was drawn into citrate anticoagulant after obtaining informed consent. Gel-filtered human platelets were prepared as described (17). For most experiments platelets were activated for 20 min at room temperature (RT) with thrombin at 0.2 unit/ml (Sigma). Activation by 100 μM thrombin receptor peptide SFLLR (18)

Abbreviations: mAb, monoclonal antibody; GT, Glanzmann thrombasthenia; BSA, bovine serum albumin; RT, room temperature. One-letter code is used for amino acids.
†To whom reprint requests should be addressed.

(B. Furie and B. C. Furie) or 20 μM ADP plus 100 μM epinephrine and apyrase at 0.1 unit/ml gave similar results.

Flow Cytometry. Resting and thrombin-activated platelets were fixed in 3.7% formalin in PBS, pH 7.4 overnight at 4°C, washed once in PBS/1% BSA, and resuspended in the same buffer to 7 × 10^7 platelets per ml. Aliquots (100 μl) were incubated with purified mAbs directed against $\alpha_{IIb}\beta_3$ (AMAC, clone P2) or β_3 (AMAC, clone SZ1) at 1.33 μg/ml, or with anti-P-selectin hybridoma supernatant AC1.2 (B. Furie and B. C. Furie) diluted 1:30. As controls, samples were also treated with irrelevant antibodies or with no primary antibody. After incubation at room temperature for 4 hr platelets were pelleted and washed once in PBS/1% BSA, resuspended in the same buffer, and incubated with fluorescein-conjugated Fab developed in goat against mouse IgG (Tago). After 30 min at room temperature, platelets were washed twice, resuspended in PBS/1% BSA, and analyzed using a Coulter EPICS 541 fluorescence-activated cell sorter.

Assays of *B. burgdorferi*-Binding. *Solid-phase assay:* Aliquots of frozen radiolabeled bacteria were thawed, pelleted, resuspended in MKP-S, and enumerated using dark-field microscopy. Volumes were adjusted to give $\approx$1.3 × 10^9 spirochetes per ml. Thorough dispersion of the spirochetes was verified microscopically at the start of each assay. 10^7 activated platelets per well were added to Nunc Break-Apart 96-well plates and centrifuged at RT for 15 min at 540 × *g*. Platelet monolayers were incubated with 35 μl of MKP-S diluted 1:3 with 10 mM Hepes, pH 7.8/10 mM glucose (M/3) or with test reagents in the same medium. After 1 hr at RT, $\approx$2 × 10^7 ^{35}S-labeled *B. burgdorferi* were added in 15 μl of MKP-S. After 30 min at RT, wells were washed three times with PBS and dried. Bound bacteria were quantitated by liquid scintillation counting. For some experiments, wells were coated with MBP-Inv479 before addition of platelets, as the anti-β_3 mAb CLB-thromb/1 caused detachment of the platelet monolayers. Precoating with MBP-Inv479 did not affect binding of *B. burgdorferi*.

Suspension assay: Activated platelets were preincubated with test reagents for 10 min at RT. Unlabeled bacteria were thawed, pelleted, resuspended in MKP-S, and mixed with platelets at a ratio of $\approx$10 spirochetes per platelet. After 20 min at RT, binding in coded replicate samples was scored by an independent observer using dark-field microscopy at ×400 magnification; at least 20 fields were examined for each sample.

Purification of $\alpha_{IIb}\beta_3$. $\alpha_{IIb}\beta_3$ was purified by Arg-Gly-Asp (RGD)-Sepharose affinity chromatography (19) from outdated pheresis platelets purchased from the American Red Cross. The peptide GRGDSPK (Tufts Protein Chemistry Facility) was coupled to cyanogen bromide-activated Sepharose (Sigma) according to manufacturer's directions. Protein concentrations were estimated by the Bradford microassay (Bio-Rad). To assay *B. burgdorferi* binding, wells were coated overnight at 4°C with purified $\alpha_{IIb}\beta_3$ at 7–70 μg/ml or MBP-Inv479 or human fibrinogen (KabiVitrum, Stockholm) at 150 μg/ml in Tyrode's buffer (17)/0.1% octylglucoside. Wells were blocked with 1% BSA/Tyrode's buffer for 4 hr at 4°C. "Uncoated" wells were treated with buffer and blocked in parallel.

RESULTS

The platelet-binding activities of two cloned North American *B. burgdorferi* strains, one infectious and one noninfectious, were studied extensively. Strain N40 clone D10/E9 is a low-passage infectious strain (14), whereas strain HB19 clone 1 has been extensively passaged *in vitro* and is no longer infectious. Strain N40 bound to activated platelets immobilized in microtiter wells 30- to 50-fold more efficiently than did strain HB19 (Fig. 1). Strain N40, but not strain HB19, also

bound to activated platelets in suspension (Fig. 2). Binding was predominantly associated with one end of the spirochete (Fig. 2*A*, curved arrow), as has been noted with cultured tick cells (20). Neither N40 nor HB19 bound to resting platelets (data not shown).

Platelet activation is a complex process that includes the acquisition of increased cellular adhesive properties. At least two receptors become functionally expressed on the cell surface upon platelet activation: P-selectin, a calcium-dependent lectin (21, 22), and $\alpha_{IIb}\beta_3$ (glycoprotein IIb-IIIa) (23–25). $\alpha_{IIb}\beta_3$, the primary integrin involved in hemostasis and thrombosis, binds fibrinogen, fibronectin, vitronectin, thrombospondin, and von Willebrand factor (26). Integrins are divalent cation-dependent heterodimeric cell surface receptors that mediate a variety of adhesive functions. Several integrin ligands contain the amino acid sequence RGD, and synthetic peptides containing RGD can block ligand binding to many of these receptors (27).

A number of reagents that inhibit receptor–ligand interactions were tested to identify the receptor(s) involved in the attachment of strain N40 to activated platelets. Binding was inhibited by EDTA and by a peptide containing RGD but was not inhibited by a blocking antibody directed against P-selectin (Table 1, Fig. 1), suggesting that an integrin is involved. In addition to $\alpha_{IIb}\beta_3$, platelets express the integrins $\alpha_2\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, and $\alpha_v\beta_3$ (28). mAbs that block ligand binding to specific integrins were therefore tested to determine which receptor(s) bind(s) N40. Antibodies directed against the β_3 subunit and the $\alpha_{IIb}\beta_3$ complex inhibited the binding of N40 to platelets (Table 1; Fig. 3). No inhibition was observed with antibodies directed against $\alpha_2\beta_1$, $\alpha_5\beta_1$, or $\alpha_6\beta_1$. Strain N40 binding to platelets was also inhibited by a synthetic peptide (γ peptide), the sequence of which corresponds to the carboxyl-terminal dodecapeptide of the γ chain of fibrinogen and which inhibits ligand binding to $\alpha_{IIb}\beta_3$ (Table 1) (29, 30).

The clotting disorder Glanzmann thrombasthenia (GT) is characterized by a lack of functional $\alpha_{IIb}\beta_3$ (31, 32). Platelets from a GT patient were analyzed and tested for the ability to bind *B. burgdorferi*. The GT platelets displayed typical GT aggregation profiles (33). α_{IIb} and β_3 were undetectable by either immunoblot or flow cytometry. In contrast, P-selectin and $\alpha_5\beta_1$ appeared normal by both criteria (Fig. 4*A*; data not shown). Upon activation, surface expression of P-selectin by both the normal and GT platelets increased, as determined by flow cytometry, and both platelet types acquired the ability to bind HL-60 cells, an interaction that requires surface expression of P-selectin (21, 22, 43). Binding of N40 to GT
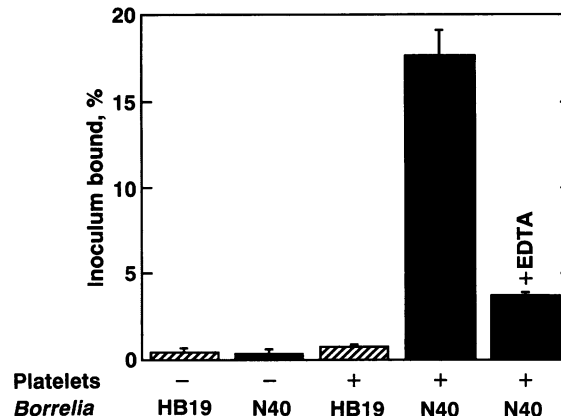

FIG. 1. Binding of *B. burgdorferi* to platelets in microtiter wells. Quantitation of binding of ^{35}S-labeled *B. burgdorferi* N40 or HB19 to platelet monolayers (+) or empty wells (−) was performed as described. The EDTA concentration used was 5 mM. Data shown are the means ± SEMs of four determinations.

Microbiology: Coburn *et al.*

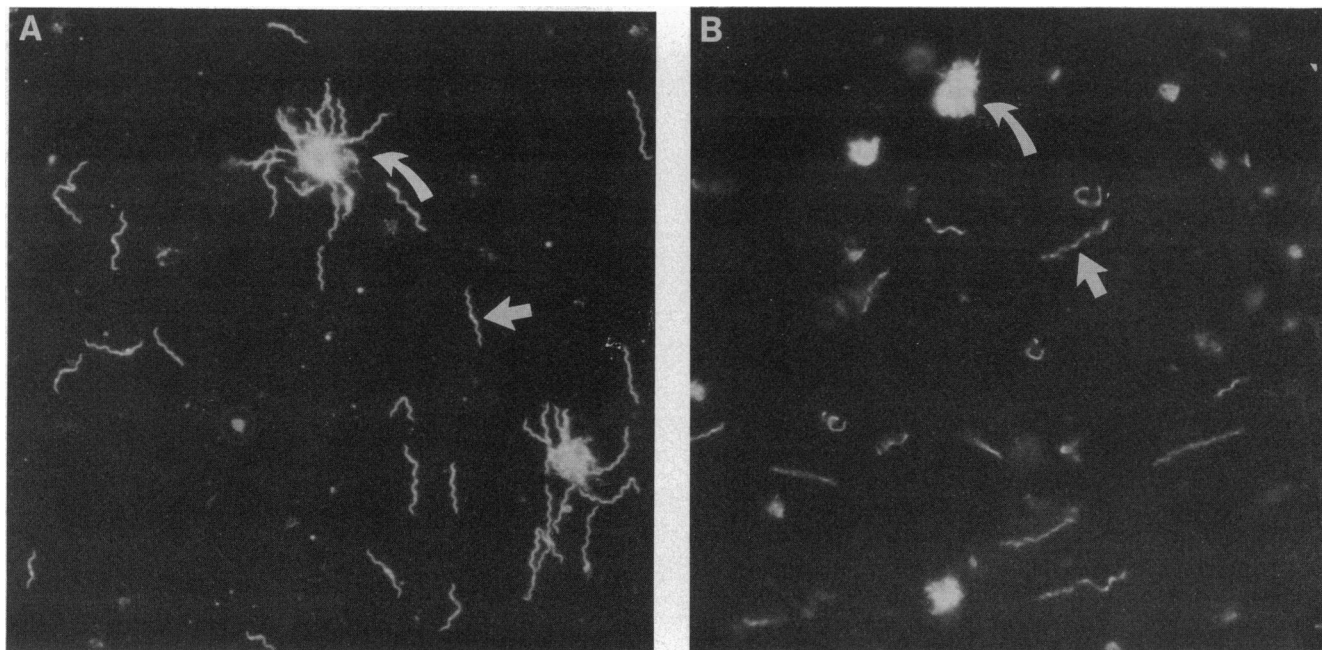*Proc. Natl. Acad. Sci. USA 90 (1993)* 7061

FIG. 2. Binding of *B. burgdorferi* to platelets in suspension. *B. burgdorferi* (straight arrows) were incubated with thrombin-activated platelets (curved arrows) at a ratio of ≈10 spirochetes per platelet. Binding was visualized using dark-field microscopy. (*A*) Binding of several bacteria to each platelet with infectious strain N40. (*B*) No binding was seen with noninfectious strain HB19. (×300.)

platelets either in suspension (data not shown) or immobilized in microtiter wells was reduced to a level similar to that obtained with normal platelets in the presence of either EDTA or anti-β_3 mAb (Fig. 4*B*). The inability of N40 to bind GT platelets provides genetic evidence that $\alpha_{IIb}\beta_3$ is necessary for binding of *B. burgdorferi* to platelets.

To determine whether $\alpha_{IIb}\beta_3$ is also sufficient for binding, the receptor was purified from human platelets to near homo-

Table 1. Inhibition of *B. burgdorferi* binding to platelets

Addition	Binding
Reagent	
None	++
EDTA ≥ 1 mM	0
Peptide	
RGD peptide (2 mg/ml)	+/0
RGE peptide (2 mg/ml)	++
γ peptide of fibrinogen (1 mg/ml)	0
Control peptide (3.3 mg/ml)	
Blocking mAb	
Anti-P-selectin	++
Anti-α_2	++
Anti-α_3	++
Anti-α_5	++
Anti-α_6	++
Anti-β_1	++
Anti-$\alpha_5\beta_1$ complex	++
Anti-β_3	0
Anti-$\alpha_{IIb}\beta_3$ complex	0

Activated platelets were preincubated with the reagent indicated before addition of *B. burgdorferi* N40. Binding in coded replicate samples was scored by an independent observer using dark-field microscopy at ×400. 0, No binding; +/0, <1 spirochete was seen bound to a platelet in 10 fields; ++, ≥2 bacteria were bound to each of ≥5 platelets in every field. Peptides were as follows: GRGDSP (RGD), GRGESP (RGE), HHLGGAKQAGDV (γ peptide), DV-LDLGVNAGTDLD (control peptide). The anti-P-selectin, anti-α_3, anti-α_5, and anti-β_1 mAbs used were hybridoma supernatants diluted 1:3. Purified monoclonal anti-$\alpha_{IIb}\beta_3$, anti-α_2, and anti-α_6 were used at 12 μg/ml; anti-β_3 was at 6 μg/ml; and anti-$\alpha_5\beta_1$ was used at up to 25 μg/ml.

geneity by RGD-Sepharose affinity chromatography (19). Microtiter wells coated with the purified receptor bound N40 but not HB19 (Fig. 5). Binding of strain N40 to purified $\alpha_{IIb}\beta_3$ was inhibited by anti-$\alpha_{IIb}\beta_3$ mAb but was not inhibited by anti-$\alpha_5\beta_1$ mAb. A number of $\alpha_{IIb}\beta_3$ ligands are potentially associated with the receptor on intact platelets, raising the possibility that N40 binds $\alpha_{IIb}\beta_3$ indirectly through one of these adhesive proteins. However, there was no significant binding to wells coated with fibrinogen (Fig. 5), vitronectin, fibronectin, collagen, von Willebrand factor, or thrombospondin (data not shown). These results indicate that $\alpha_{IIb}\beta_3$ is the only platelet-associated protein required for binding *B. burgdorferi*.

DISCUSSION

Several lines of evidence indicate that the integrin $\alpha_{IIb}\beta_3$ mediates the attachment of *B. burgdorferi* to human platelets. (*i*) Strain N40 bound to activated but not to resting platelets. (*ii*) Binding was inhibited by an RGD-containing peptide, the
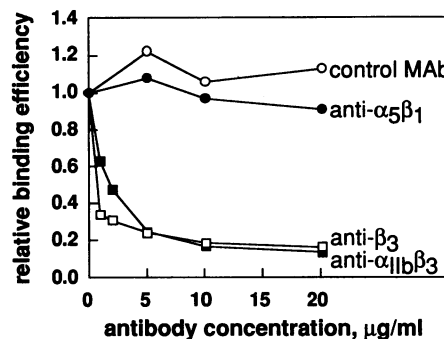

FIG. 3. Inhibition of *B. burgdorferi* binding to platelets by mAbs. Before the addition of ^{35}S-labeled strain N40, platelet-monolayers were preincubated with mAbs that block ligand binding to platelet integrins. Results shown were obtained by using anti-$\alpha_{IIb}\beta_3$ (■), anti-β_3 (□), and anti-$\alpha_5\beta_1$ (●). Anti-invasin mAb 9G11 was used as a control (○). Relative binding efficiency equals the percentage of inoculum bound for each test point divided by the percentage of inoculum bound without antibody. Each point represents the mean of four determinations. Results obtained with anti-α_2 and anti-α_6 mAbs were similar to those shown for $\alpha_5\beta_1$ and the control.
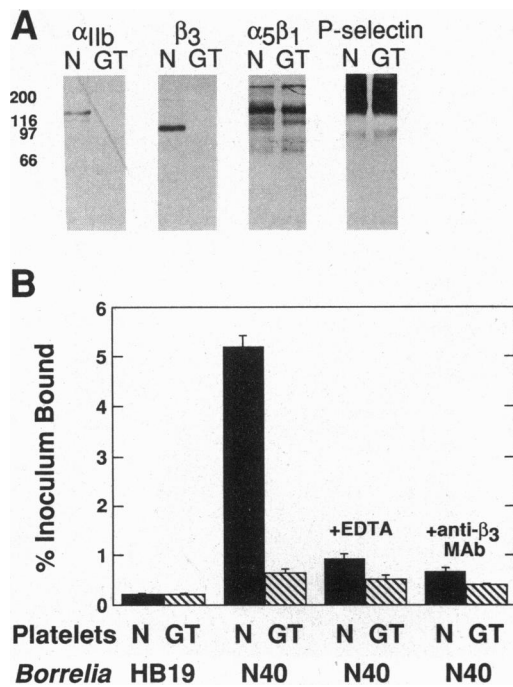
FIG. 4. Dependence of binding of *B. burgdorferi* on $\alpha_{IIb}\beta_3$. Platelets were prepared in parallel from a normal control (N) and a patient with GT. (*A*) Immunoblots of total platelet protein. Similar amounts of N and GT platelet lysates were fractionated by SDS/PAGE under nonreducing conditions and transferred to poly(vinylidene difluoride) membranes. Membrane strips were probed with anti-α_{IIb} or anti-β_3 mAbs at 2 μg/ml or with anti-$\alpha_5\beta_1$ or anti-P-selectin antiserum diluted 1:3000. (*B*) Binding of ^{35}S-labeled *B. burgdorferi* to GT and N platelets. The EDTA concentration was 5 mM; the anti-β_3 mAb concentration was 10 μg/ml.

fibrinogen γ peptide, and mAbs directed against $\alpha_{IIb}\beta_3$. (*iii*) Platelets that lack this receptor did not bind *B. burgdorferi*. (*iv*) *B. burgdorferi* N40 efficiently bound to purified $\alpha_{IIb}\beta_3$ immobilized in microtiter wells. Our results do not eliminate the possibility that other platelet receptors also bind *B. burgdorferi*. For example, $\alpha_v\beta_3$ shares a common subunit with $\alpha_{IIb}\beta_3$ and binds many of the same ligands. Nevertheless, the observations that binding is activation-dependent and inhibited by reagents that specifically block $\alpha_{IIb}\beta_3$ indicate that $\alpha_{IIb}\beta_3$ plays the major role in the attachment of *B. burgdorferi* to platelets.
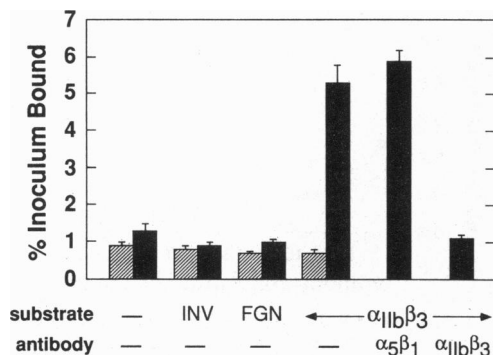

FIG. 5. *B. burgdorferi* binding to purified $\alpha_{IIb}\beta_3$. Wells were coated with purified $\alpha_{IIb}\beta_3$ at 70 μg/ml or MBP-Inv479 (INV) or human fibrinogen (FGN) each at 70 μg/ml. $\alpha_{IIb}\beta_3$-coated wells were tested for the ability to bind strain N40 (solid bars) after preincubation with no addition, or with anti-$\alpha_5\beta_1$ or anti-$\alpha_{IIb}\beta_3$ mAbs at 10 μg/ml. HB19 (hatched bars) was not tested in the presence of antibodies.

It is not yet known whether $\alpha_{IIb}\beta_3$ binds a bacterial protein directly. *B. burgdorferi* require the presence of serum for *in vitro* cultivation, raising the possibility that during growth the bacteria might acquire a serum protein that mediates indirect binding to $\alpha_{IIb}\beta_3$. Indirect attachment to host-cell receptors is not uncommon among pathogenic bacteria. Fibronectin may play a role in adherence of mycobacteria, streptococci, staphylococci, and treponemes (34–38). In immunoblotting, immunofluorescence, and agglutination experiments using antibodies directed against the known mammalian $\alpha_{IIb}\beta_3$ ligands, we have obtained no evidence supporting a role for any of these proteins in the binding of *B. burgdorferi* to $\alpha_{IIb}\beta_3$ (data not shown). Furthermore, N40 grown for several generations in the absence of serum retains full platelet-binding activity. Nevertheless, long-term cultivation of N40 in the absence of serum remains elusive, and the possibility of a role for a serum protein in the binding of *B. burgdorferi* to platelets has not been eliminated.

In many pathogenic bacteria, adherence is an important virulence trait that is lost in avirulent strains (39–41). The platelet-binding activity displayed by the infectious *B. burgdorferi* strain N40 was absent in the high-passage, noninfectious strain HB19. We have recently found that two other high-passage, noninfectious *B. burgdorferi* strains do not bind platelets. In contrast, uncloned, low-passage, infectious HB19 and low-passage skin isolates from four patients with Lyme disease bind platelets efficiently (J.C. and J.M.L., unpublished work). One prominent feature of Lyme disease is vascular injury (2, 42), and damage to vascular endothelium results in localized aggregation of activated platelets. Binding of *B. burgdorferi* to activated platelets may, therefore, facilitate tissue colonization by concentrating spirochetes at these regions. Alternatively, binding of *B. burgdorferi* to platelets at the site of inoculation in the skin might provide a foothold for the establishment of infection—e.g., a niche where the bacteria can replicate while protected from the host defense system by a surrounding thrombus. In support of this hypothesis, in the mouse model it appears that dissemination of *B. burgdorferi* from the site of the tick bite is delayed for several days (3). Alternatively, attachment to platelets may be more important in the natural vector–reservoir transmission cycle of *B. burgdorferi*, for example, by increasing the local concentration of spirochetes available for ingestion by an uninfected tick. It is less likely that binding to circulating platelets contributes to the hematogenous dissemination of the spirochete, as activated platelets are not abundant in the circulation.

$\alpha_{IIb}\beta_3$ cannot be the only mammalian receptor for *B. burgdorferi*, as the spirochete binds to a number of cell types that do not express this receptor (5–7). We have found that epithelial and endothelial cells, which do not express $\alpha_{IIb}\beta_3$, bind to N40 more efficiently than to HB19 (unpublished work). These observations parallel our results with platelets and suggest that integrins on other cell types may also mediate binding of *B. burgdorferi*. It is, therefore, possible that the ligand used by the spirochete to bind $\alpha_{IIb}\beta_3$ plays an important role in the pathogenesis of Lyme disease by promoting attachment to a number of cell types in addition to platelets. A comprehensive understanding of the interactions between *B. burgdorferi* and mammalian cells will provide insight into how the spirochete is able to invade and colonize multiple tissues and cause the diverse manifestations of Lyme disease.

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