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Bacteroides intermedius Binds Fibrinogen

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The binding of *Bacteroides intermedius* VPI 8944 to human fibrinogen has been characterized. The binding is time dependent, at least partially reversible, saturable, and specific. On an average, a maximum of 3,500 fibrinogen molecules bind per bacterial cell, with a dissociation constant of 1.7×10^{-11} M. These bacteria also exhibit a fibrinogenolytic activity which can be partially inhibited by protease inhibitors. Bacteria release fibrinogenolytic activity into the surrounding medium without loss of binding activity, but more pronounced fibrinogen breakdown occurs when ^{125}I-labeled fibrinogen is associated with the bacteria, suggesting that fibrinogen is degraded at the cell surface. Fibrinogen binding by *B. intermedius* might represent a mechanism of bacterial tissue adherence.

Oral surfaces are colonized with a high degree of specificity, suggesting that well-developed recognition systems are involved in the attachment of bacteria to these surfaces (5). Bacteria attach to the root surface and to other bacteria in the process of colonizing the periodontal pocket (16, 26). The ability of bacteria to colonize the tissue lining the wall of the periodontal pocket remains largely unexplored. Eucaryotic cells recognize a number of adhesive proteins, including fibronectin and laminin, which mediate the adhesion of these cells to the surrounding tissue (1, 35). It is possible that bacteria also use the same molecules to attach to host tissues. Some pathogenic strains of staphylococci, streptococci, *Treponema pallidum*, and *Escherichia coli* bind fibronectin (4, 20, 21, 28), and some streptococci and *E. coli* bind laminin (29, 33). The observed ability of bacteria to bind host-matrix proteins appears to represent a mechanism of bacterial tissue adherence and may be an initial step in bacterial colonization of host tissue.

The main role of the soluble plasma protein fibrinogen is in blood clot formation through its conversion to insoluble fibrin which forms the matrix of the clot. However, fibrinogen may also interact with both procaryotic and eucaryotic cells (3). Specific sites on the fibrinogen molecule bind to specific receptors on staphylococci, streptococci, and platelets (7, 8, 15). Bacteria that are able to specifically bind fibrinogen might be able to utilize this molecule or its polymeric form, fibrin, to adhere to tissue.

Black-pigmented *Bacteroides* species appear to be associated with all forms of periodontal disease (17). *Bacteroides intermedius* has been associated with bleeding gingivitis, acute necrotizing ulcerative gingivitis (ANUG), and some forms of periodontitis (12, 18, 27). Active bleeding or fibrin deposition (pseudomembrane formation) are characteristic features of bleeding gingivitis and ANUG, respectively (13). We have observed that some oral and nonoral strains of *B. intermedius* bind fibrinogen. The ability to bind fibrinogen might explain the association of *B. intermedius* with bleeding gingivitis and ANUG. In the present study, we characterized the binding of an oral strain of *B. intermedius* (VPI 8944) to human fibrinogen.

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MATERIALS AND METHODS

Chemicals. Human fibrinogen (Kabi, Stockholm, Sweden) was found to contain a small amount of fibronectin and hence was further purified by successive passage through Sepharose columns (approximate 10-ml-bed volume, 1 mg of protein per ml of gel) substituted with gelatin and antifibronectin immunoglobulin G (IgG). The fibrinogen obtained was shown to be free of fibronectin as determined by immunological methods. Fibrinogen was labeled with ^{125}I by the chloramine-T method (9), and the specific activity of labeled fibrinogen was estimated to be 1.3×10^6 cpm/μg.

Egg albumin (ovalbumin), α-acid glycoprotein (orosomucoid), bovine serum albumin, human IgG, and fetuin were purchased from Sigma Chemical Co. (St. Louis, Mo.). Fibronectin purified from human plasma (36) was supplied by Gunnar Fröman, University of Alabama, Birmingham. Na^{125}I (specific activity, 15 mCi/μg) was purchased from Amersham Corp. (Arlington Heights, Ill.), and Percoll was purchased from Pharmacia (Uppsala, Sweden). All other chemicals were of reagent grade and obtained from commercial sources.

Bacteria. Two oral strains of *B. intermedius*, VPI 8944 and VPI 9145, derived from ANUG and dental plaque, respectively (11), and two nonoral strains of *B. intermedius*, VPI 9466 and ATCC 25611, derived from a transtracheal aspirate (H. M. Wexler, personal communication) and an empyema (11), respectively, were used in this study. These bacteria were obtained from the American Type Culture Collection, Rockville, Md. In addition, a strain of *Bacteroides gingivalis* (W) and a nonoral strain of *Bacteroides fragilis* were isolated in our laboratories and used for purposes of comparison.

Bacteria were grown anaerobically at 37°C in complete basal anaerobic broth as described by Syed and Loesche (34). Bacteria were grown to early logarithmic phase (approximately 10^8 cells per ml), harvested by centrifugation (13,000 × *g*; 20 min), washed three times with phosphate-buffered saline (0.13 M sodium chloride, 10 mM phosphate buffer, 0.02% sodium azide [pH 7.4]) (PBS), and resuspended in the same buffer. Bacteria were counted by using a Petroff-Hausser chamber, and the cell density was adjusted to 1×10^{10} to 2×10^{10} cells per ml. Bacterial suspensions

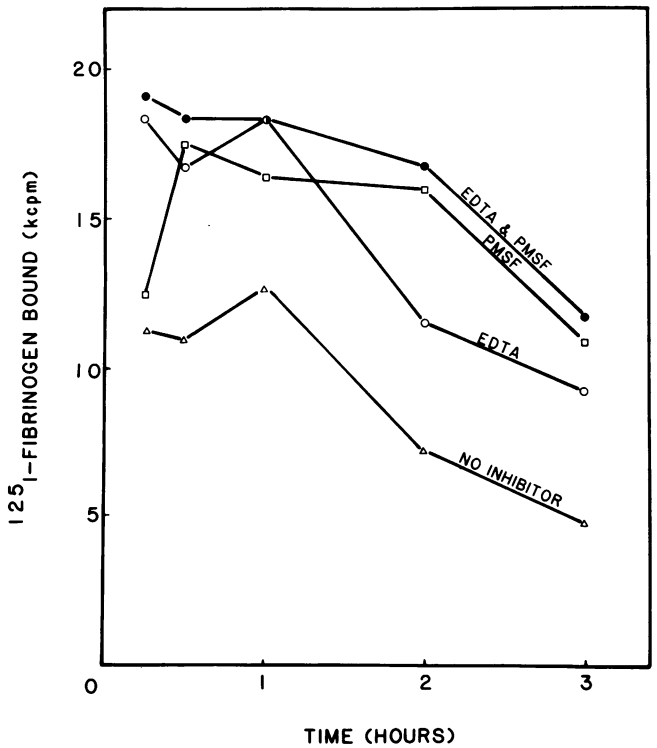

FIG. 1. Time dependence of binding of ^{125}I-fibrinogen to *B. intermedius* VPI 8944 in the absence of protease inhibitors (△) and in the presence of 10 mM EDTA (○), 2 mM PMSF (□), and 2 mM PMSF plus 10 mM EDTA (●). Bacteria and ^{125}I-fibrinogen were incubated with or without protease inhibitors for the indicated times and the amount of ^{125}I-fibrinogen bound to bacteria was determined as described in the text. Each value is the average of triplicate assays.

retained full binding activity for several weeks when stored at 4°C.

Binding of ^{125}I-fibrinogen to bacteria. The binding of ^{125}I-fibrinogen to bacteria was quantitated essentially as described by Rydén et al. (21) for binding of fibronectin to staphylococci. Briefly, 1×10^9 to 2×10^9 bacteria were incubated with 2.5×10^4 to 5×10^4 cpm of radiolabeled fibrinogen in PBS containing 0.1% bovine serum albumin in a total volume of 0.5 ml. The tubes containing the incubation mixtures were rotated end-over-end at 20°C for 30 min unless otherwise stated. Subsequently, 400 μl of the incubation mixture was added to 0.5 ml of PBS layered on top of 3 ml of a Percoll solution in PBS (density, 1.020 g/ml). The samples were centrifuged at 1,300 × *g* for 15 min in a swinging bucket rotor, the supernatants were aspirated, and the radioactivity associated with pelleted bacteria was quantitated in a gamma counter (LKB-Wallac, Turku, Finland). Radioactivity recovered from incubation mixtures containing no bacteria was considered to be background and was subtracted from that obtained for incubations containing bacteria. In experiments assessing saturability of binding, a laboratory strain of *B. fragilis* which does not bind fibrinogen was used as a control for unspecific binding of fibrinogen to bacteria. Unspecific binding never exceeded 2% of the fibrinogen bound to *B. intermedius* 8944 over the range of fibrinogen concentrations tested. All samples were analyzed in duplicate or triplicate. Plastic tubes used in experiments were precoated with albumin to minimize unspecific binding of bacteria and proteins to the walls of the tubes.

The reversibility of ^{125}I-fibrinogen binding to *B. intermedius* VPI 8944 was determined as follows. Bacteria (10^9 cells) were incubated in the presence of ^{125}I-fibrinogen (5×10^4 cpm) for 15 min, after which unlabeled fibrinogen was added. The amount of ^{125}I-fibrinogen that bound to bacteria was determined as described above.

Gel electrophoresis. Electrophoresis in polyacrylamide gel was performed by the method of Studier (31). The running gel consisted of either a linear 5 to 15% acrylamide gradient or 7.5% acrylamide overlaid with a stacking gel (4.8% acrylamide). Samples consisted of 10^4 cpm of radiolabeled protein or 2 to 25 μg of unlabeled protein. Protein samples were applied to the gel after reduction by boiling in the presence of dithiothreitol and sodium dodecyl sulfate and alkylation by the addition of iodoacetamide. After electrophoresis, gels were stained with Coomassie brilliant blue R, destained, dried, and subjected to autoradiography with X-Omat AR film (Eastman Kodak Co., Rochester, N.Y.).

Preparation of rabbit anti-human fibrinogen IgG. Antisera against purified human fibrinogen were prepared essentially as described by Johansson and Höök (10) for production of antisera to human fibronectin. Briefly, antisera were produced in New Zealand white rabbits by an initial intramuscular injection of 100 μg of fibrinogen in 0.5 ml of azide-free PBS and emulsified in the same volume of complete Freund adjuvant (Difco Laboratories, Detroit, Mich.), followed by two injections of fibrinogen emulsified in incomplete adjuvant at 10-day intervals. Rabbits were bled 10 days after the last injection and subsequently were bled four times at 10-day intervals. The IgG fraction of the serum was purified on a protein A-Sepharose column (Pharmacia), eluted with 3 M $MgCl_2$, dialyzed to PBS, and stored frozen in small aliquots.

RESULTS

When *B. intermedius* strains were incubated with 2.4×10^4 cpm of ^{125}I-labeled fibrinogen (specific activity, 1.3×10^6 cpm/μg), three strains (VPI 8944, VPI 9145, and ATCC 25611) bound appreciable but different amounts of fibrinogen, ranging from 15 to 35% of available fibrinogen per 10^9 cells. This corresponds to 2.7 to 6.4 ng of protein bound per 10^9 cells. *B. intermedius* VPI 9466 bound a small amount of fibrinogen compared with the other three strains: 10^9 cells bound only 2 to 4% (corresponding to 0.3 to 0.7 ng) of available fibrinogen. *B. gingivalis* (W) and a laboratory strain of *B. fragilis* failed to bind fibrinogen under these experimental conditions. *B. intermedius* VPI 8944 was chosen for more detailed characterization of the fibrinogen-binding reaction because its binding capacity is intermediate between strains VPI 9145 and ATCC 25611, it is an oral strain of *B. intermedius*, and it had been isolated originally from a patient with periodontal pathology (ANUG).

When bacteria were incubated with ^{125}I-fibrinogen for increasing periods, a rapid binding of ^{125}I-fibrinogen to the cells was noted. The amount of ^{125}I-fibrinogen bound to the bacteria reached a maximum within 30 min. Prolonged incubations resulted in decreasing amounts of ^{125}I-labeled material associated with the bacterial cells (Fig. 1). Addition to the system of a serine protease inhibitor, phenylmethylsulfonyl fluoride (PMSF), or the chelator EDTA, which inhibits most metalloproteases, reduced the rate of loss of bacteria-associated ^{125}I-fibrinogen. This suggests the involvement of proteolytic enzymes. In the presence of *N*-ethylmaleimide and 4-aminophenylmercuric acetate, which inhibit glutamyl and thiol proteases, respectively, initial amounts of bound fibrinogen were lowered but no effect on

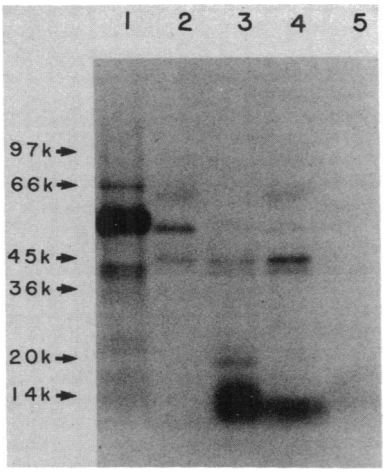

FIG. 2. Distribution of ^{125}I-fibrinogen and its degradation products between cell pellets and supernatant fractions after incubation with *B. intermedius* VPI 8944. Autoradiogram of a sodium dodecyl sulfate 5 to 15% gradient gel with approximately 10^4 cpm of ^{125}I-labeled protein applied per lane. Lanes: 1, ^{125}I-fibrinogen; 2 and 3, supernatant fraction and cell pellet, respectively, after 20-min incubation; 4 and 5, supernatant fraction and cell pellet, respectively, after 4-h incubation of bacteria and ^{125}I-fibrinogen. 97k, 97,000-Molecular-weight marker.

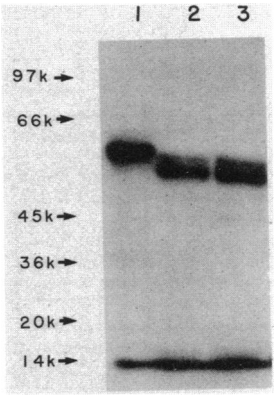

FIG. 3. Degradation of ^{125}I-fibrinogen by cell-free incubation medium from which *B. intermedius* VPI 8944 had been removed. Autoradiogram of a sodium dodecyl sulfate 7.5% polyacrylamide gel. Bacteria (10^9 cells) were placed in PBS-albumin for 30 min at 20°C and centrifuged, and then the supernatant fraction was incubated with ^{125}I-fibrinogen for 30 min at 20°C. ^{125}I-fibrinogen (lane 1) incubated with the bacterial supernatant in the presence (lane 2) or absence (lane 3) of 2 mM PMSF. 97k, 97,000-Molecular-weight marker.

the decay of the bacteria-associated ^{125}I-fibrinogen was seen. When bacterial suspensions were heat treated in an attempt to inactivate proteases, binding activity was lost.

The observed decrease in cell-associated ^{125}I-fibrinogen with time could be caused by degradation of bound fibrinogen, degradation of the fibrinogen-binding region on the bacteria, or both of these processes. These possibilities were examined further. The data presented in Fig. 2 show that fibrinogen was degraded by the bacteria and suggest that ^{125}I-fibrinogen is degraded progressively as the length of incubation increases. Albumin added to the incubation mixture as a carrier was not degraded to any noticeable extent. To examine whether the fibrinogenolytic activity is released from cells or is cell associated, we incubated bacteria in PBS-albumin and then removed them from this solution by centrifugation. As can be seen in Fig. 3, the PBS-albumin solution from which bacteria had been removed contained fibrinogenolytic activity, although the extent of ^{125}I-fibrinogen degradation was not as marked as when bacteria were present in the incubation mixture. These observations suggest that fibrinogenolytic activity may be located at the surface of the bacteria.

In apparent support of this conclusion is the finding that ^{125}I-fibrinogen recovered from the bacteria is more degraded than that remaining in the incubation medium (c.f. lanes 3 and 2 [Fig. 2] and lanes 5 and 4 [Fig. 2]). To examine whether there is also a degradation of the fibrinogen-binding component on the bacteria, we incubated cells for different lengths of time in the presence or absence of protease inhibitors before the addition of ^{125}I-fibrinogen. The results (Fig. 4) indicate that bacteria preincubated under binding conditions for up to 3 h bind the same amount of ^{125}I-fibrinogen as nonpreincubated controls. Apparently, autodegradation of the fibrinogen-binding components on the bacterial surface does not occur. Because binding of fibrinogen to bacteria is a rapid reaction, all subsequent experiments were performed for 30 min in the presence of PMSF and EDTA.

The binding of ^{125}I-fibrinogen to strain VPI 8944 appears to be specific in the sense that binding was not significantly reduced by the presence of large molar excesses (10^4-fold) of egg albumin, α-acid glycoprotein, human IgG, or fetuin (Table 1). The addition of unlabeled fibrinogen to the incubation mixture effectively blocked the binding of the ^{125}I-fibrinogen to the bacteria (Fig. 5). Fibronectin appears to be an efficient inhibitor of fibrinogen binding. Strain VPI 8944 did not bind fibronectin; however, fibronectin binds fibrinogen (3) and may compete with the bacteria for binding sites on the ^{125}I-fibrinogen. To further examine the specificity of the interaction between *B. intermedius* VPI 8944 and fibrinogen, we compared the ability of preimmune and immune (anti-human fibrinogen) IgG to block the binding of ^{125}I-fibrinogen to bacteria (Fig. 6). Preincubation of ^{125}I-

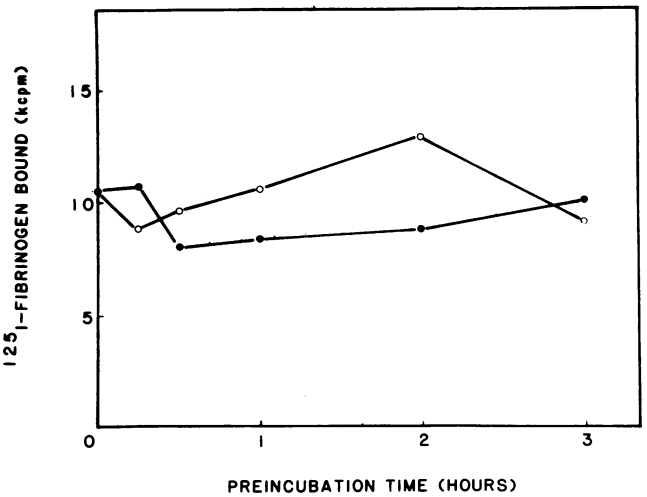

FIG. 4. Retention of ^{125}I-fibrinogen-binding ability of *B. intermedius* VPI 8944. Bacteria were preincubated in PBS-albumin in the presence (●) or absence (○) of 2 mM PMSF and 10 mM EDTA for indicated periods of time. Subsequently, the ability of bacteria to bind ^{125}I-fibrinogen was determined as described in the text. Each value is the average of triplicate assays.

TABLE 1. Specificity of binding of fibrinogen to *B. intermedius* VPI 8944[a]

Competing protein	Relative ^{125}I-fibrinogen bound to bacteria (%)
None	100
Fibrinogen	14.2 ± 1.91
Egg albumin	93.9 ± 2.34
α-Acid glycoprotein	100.6 ± 7.40
Human IgG	87.1 ± 4.38
Fetuin	97.1 ± 5.03
Fibronectin	61.6 ± 2.51

[a] Bacteria (10^9 cells) were incubated in the presence of competing protein (100 μg/ml) for 15 min, after which ^{125}I-fibrinogen (5×10^4 cpm) was added to the incubation mixture. The amount of ^{125}I-fibrinogen that bound to the bacteria was determined as described in the text. Data are presented as mean percents ± standard deviation of four replicate samples.

fibrinogen with preimmune rabbit IgG did not block ^{125}I-brinogen binding to bacteria. In contrast, preincubation of ^{125}I-fibrinogen with rabbit anti-human fibrinogen IgG strongly inhibited ^{125}I-fibrinogen binding in a concentration-dependent fashion. These data suggest that bacteria recognize a specific region on the fibrinogen molecule which is blocked by antifibrinogen IgG.

The binding of fibrinogen to strain VPI 8944 appears to be at least partially reversible because bound ^{125}I-fibrinogen can be displaced from bacteria by incubation with a large excess of unlabeled fibrinogen. The addition of 100 μg of unlabeled fibrinogen per ml to the incubation mixture resulted in 38% displacement of bound ^{125}I-fibrinogen from bacteria whereas 500 μg of unlabeled fibrinogen per ml displaced 55% of bound ^{125}I-fibrinogen. Complete reversal of ^{125}I-fibrinogen binding was not achieved. The effect of incubating strain VPI 8944 with increasing amounts of ^{125}I-fibrinogen is shown in Fig. 7. With increasing concentrations of ^{125}I-fibrinogen in the incubation mixture, the amount of ^{125}I-fibrinogen bound to the bacteria increased to a maximum of 2.00 μg per 1.03×10^9 bacteria at a fibrinogen concentration of 50 μg per sample. Further increases in the ^{125}I-fibrinogen concentration did not result in more binding of fibrinogen to bacteria. These results are compatible with specific binding of fibrinogen to a limited number of binding sites on the cell surface of the bacteria and, in addition, unspecific binding of fibrinogen that increases as a function of the amount of radioactivity added. Assuming a molecular weight of 3.4×10^5 for fibrinogen (3) and, further, that ^{125}I-fibrinogen occupies specific binding sites on the bacteria and that these sites are completely occupied at saturation, the average number of fibrinogen-binding sites per cell is 3,500. A Scatchard anal-

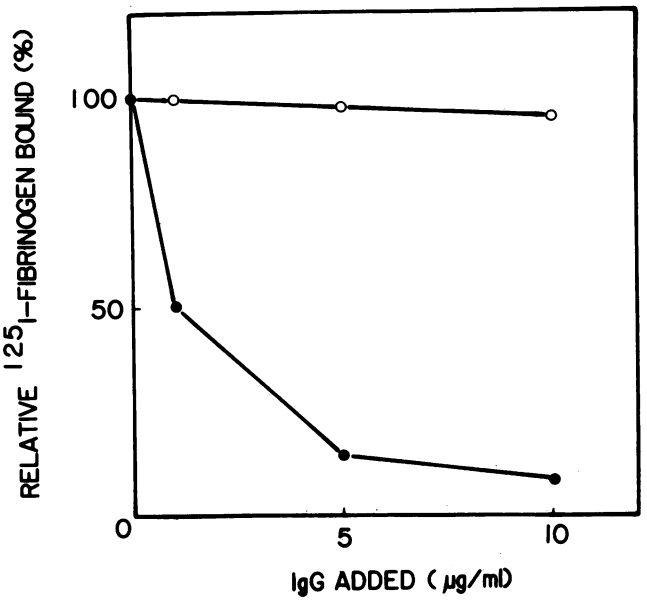

FIG. 6. Inhibition of binding of ^{125}I-fibrinogen to *B. intermedius* VPI 8944 by antifibrinogen antibodies. ^{125}I-fibrinogen (5×10^4 cpm) was preincubated with preimmune rabbit IgG (○) or rabbit anti-human fibrinogen IgG (●) for 15 min before the addition of bacteria (10^9 cells) to the assay mixture. After 30 min of incubation, the amount of ^{125}I-fibrinogen bound to bacteria was determined as described in the text. Each value is the average of triplicate assays.

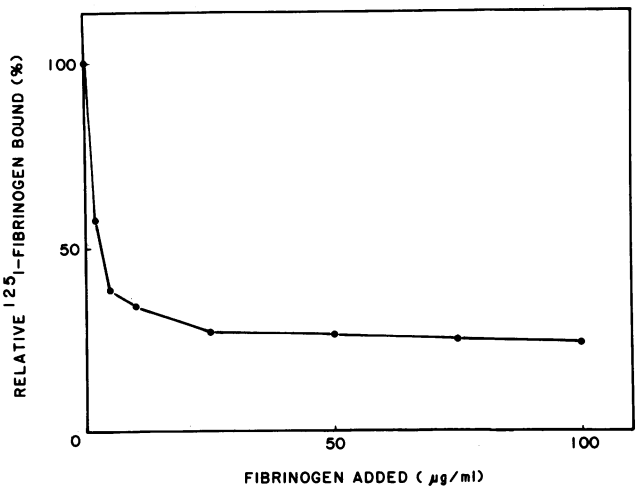

FIG. 5. Inhibition of binding of ^{125}I-fibrinogen to bacteria by unlabeled fibrinogen. Bacteria were incubated with indicated concentrations of unlabeled fibrinogen for 15 min. ^{125}I-fibrinogen (5×10^4 cpm) was added to each mixture, incubation was continued for 30 min, and the amount of labeled protein bound to bacteria was determined as described in the text. Each value is the average of triplicate assays.

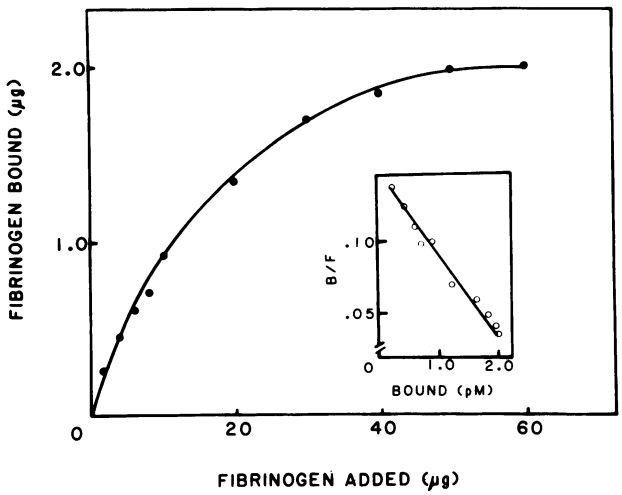

FIG. 7. Saturability of binding of fibrinogen to *B. intermedius* VPI 8944. Bacteria (1.03×10^9) were incubated with increasing amounts of ^{125}I-fibrinogen (specific activity, 1.94×10^4 cpm/μg) for 30 min. The amount of ^{125}I-fibrinogen bound to bacteria was determined as described in the text. Insert shows Scatchard analysis of the data. Each value is the average of triplicate assays.

ysis (23) (insert, Fig. 7) of the data presented in the saturation curve suggests that one type of binding site is involved in the interaction between cells of strain VPI 8944 and fibrinogen. By using the slope of the Scatchard plot, it is possible to calculate an apparent K_d of 1.7×10^{-11} for the reaction.

DISCUSSION

Fibrinogen binding to both streptococci and staphylococci has previously been described (8, 14). Fibrinogen binds to a wide variety of streptococcal strains from groups A, B, C, and G (14, 15, 24), but the binding has not been characterized. The binding of fibrinogen to staphylococci has been characterized previously (7, 8, 30), and it appears that it is at least partially reversible and saturable. Binding of fibrinogen to *Staphylococcus aureus* is rapid, and the affinity of fibrinogen for these cells is high. The region of the fibrinogen molecule that binds to these bacteria is located at the carboxy terminus of the γ-chain in a 15-residue peptide.

We examined the ability of four *B. intermedius* strains to bind human fibrinogen. Three of the strains bound appreciable amounts of fibrinogen, whereas one (VPI 9466) bound only a small amount. Such strain variability in binding of proteins to bacteria has been described previously for binding of streptococci to fibrinogen (14) and for binding of streptococci and staphylococci to fibronectin (21, 28). The failure of laboratory strains of *B. fragilis* and *B. gingivalis* (W) to bind fibrinogen under the same conditions suggests that fibrinogen binding is a property of only some *Bacteroides* species or subspecies. The time required to achieve maximal binding of proteins to bacteria is quite variable, ranging from minutes (8, 28) to hours (32, 33). Although it is difficult to assess the amount of time required for maximal binding of fibrinogen to *B. intermedius* because of the concomitant breakdown of fibrinogen by these bacteria, the three strains that bound appreciable amounts of fibrinogen reached maximal levels of binding within 30 min to 1 h of incubation.

The binding of ^{125}I-labeled fibrinogen to *B. intermedius* VPI 8944 appears to be specific and at least partially reversible. The partial reversibility of the binding may reflect specific versus unspecific binding of fibrinogen to these cells, or different classes of fibrinogen-binding sites, reversible and irreversible, might be present on the bacterial cell surface. Alternatively, if the kinetics of displacement are different from the kinetics of binding, then displacement may not be complete after short incubations of unlabeled fibrinogen with ^{125}I-fibrinogen-interacted bacteria. Data obtained from prolonged incubation of unlabeled fibrinogen with ^{125}I-fibrinogen-interacted bacteria would be difficult to interpret because of the fibrinogen breakdown that occurs even in the presence of PMSF and EDTA. We have calculated that there are approximately 3,500 fibrinogen-binding sites per bacterial cell and that the K_d for the reaction is approximately 10^{-11} M, suggesting a strong affinity of fibrinogen for the bacteria.

A recent finding of periodontal research is that microorganisms can be found in the gingival tissues in severe forms of periodontal disease (6, 19, 22). It has been suggested that invasiveness is an important aspect in the virulence of periodontopathic bacteria (25), and one report has appeared that suggests that *B. intermedius* has the capacity to invade gingival connective tissue (2). For bacteria to invade tissue and disseminate to distant sites, mechanisms allowing bacteria to detach from the original site of colonization must exist. Oral black-pigmented *Bacteroides* species possess fibrinogenolytic or fibrinolytic activity (37), and it may be that this activity represents a mechanism of detachment from host tissue. Fibrinolysis by *B. intermedius* has been reported to be weak, variable, and fairly nonspecific (25). *B. intermedius* VPI 8944 appears to possess an active fibrinogenolytic activity which shows some specificity since it does not degrade bovine serum albumin present in the incubation mixture. It is not clear whether fibrinogen breakdown occurs at the cell surface after binding or whether fibrinogen is broken down into smaller fragments which then bind to the bacteria. The more extensive degradation of bacteria-associated ^{125}I-fibrinogen as compared with fibrinogen degradation in the incubation medium supports the hypothesis that degradation occurs at the cell surface. The continual decrease in the amount of ^{125}I-fibrinogen that is bound with time is also compatible with progressive cleavage of the molecule at the cell surface after it is initially bound. The time-dependent release of fibrinogenolytic activity from the bacteria without loss of binding activity suggests that these activities are independent. After brief and prolonged periods of preincubation in buffer, these bacteria bind the same amount of ^{125}I-fibrinogen, suggesting that they do not autodegrade their fibrinogen-binding sites.

In summary, some *B. intermedius* strains appear to possess the ability to reversibly bind as well as degrade the plasma protein fibrinogen. This ability may render them able to attach to and detach from a fibrinous substratum. Presence of such a substratum may modulate tissue colonization by providing specific attachment sites only for those bacteria possessing the ability to bind fibrinogen. The data presented in this study support the hypothesis that the composition and distribution of host proteins along the tissue wall of the gingival sulcus or periodontal pocket is a major determinant of the composition of the subgingival microflora.

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