Bacteroides gingivalis and Bacteroides intermedius Recognize Different Sites on Human Fibrinogen

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Bacteroides (Porphyromonas) gingivalis and Bacteroides (Porphyromonas) intermedius have been implicated in the etiology of human periodontal diseases. These organisms are able to bind and degrade human fibrinogen, and these interactions may play a role in the pathogenesis of periodontal disease. In attempts to map the bacterial binding sites along the fibrinogen molecule, we have found that strains of B. gingivalis and B. intermedius, respectively, recognize spatially distant and distinct sites on the fibrinogen molecule. Isolated reduced and alkylated α-, β-, and γ-fibrinogen chains inhibited binding of 125I-fibrinogen to both Bacteroides species in a concentration-dependent manner. Plasmin fragments D and to some extent fragment E, however, produced a concentration-dependent inhibition of 125I-fibrinogen binding to B. intermedius strains but did not affect binding of 125I-fibrinogen to B. gingivalis strains. Radiolabeled fibrinogen chains and fragments were compared with 125I-fibrinogen with respect to specificity and reversibility of binding to bacteria. According to these criteria, γ-chain most closely resembled the native fibrinogen molecule in behavior toward B. gingivalis strains and fragments D most closely resembled fibrinogen in behavior toward B. intermedius strains. The ability of anti-human fibrinogen immunoglobulin G (IgG) to inhibit binding of 125I-fibrinogen to B. intermedius strains was greatly reduced by absorbing the IgG with fragments D. Absorbing the IgG with fragments D had no effect on the ability of the antibody to inhibit binding of 125I-fibrinogen to B. gingivalis strains. A purified staphylococcal fibrinogen-binding protein blocked binding of 125I-fibrinogen to B. intermedius strains but not to B. gingivalis strains.

Bacteroides (Porphyromonas) gingivalis and Bacteroides (Porphyromonas) intermedius are metabolically diverse species of gram-negative anaerobic bacilli implicated in the etiology of human periodontal diseases (14, 25, 33, 35). These diseases are characterized by gingival inflammation and loss of connective tissue and bone from around the roots of the teeth (13). B. gingivalis and B. intermedius are able to specifically recognize and bind the plasma protein fibrinogen with high affinity; in addition, they are able to proteolytically cleave fibrinogen (22, 23). The bacterial components responsible for these interactions have not been identified, and the role of these interactions in the pathogenesis of periodontitis is unknown. Fibrinogen binding and cleaving activities may be important virulence factors for these bacteria. These interactions may promote adherence of bacteria to periodontal tissues, prevent elimination of bacteria by immune mechanisms, or contribute to tissue destruction by interfering with fibrin formation or lysing blood clots.

When we initially examined the interactions of these bacteria with fibrinogen (22, 23), we found that the characteristics of binding of both Bacteroides species to fibrinogen were very similar: the binding is rapid, reversible, saturable, and of high affinity (Kd ≈ 10 to 30 nM); however, degradation of fibrinogen appeared to involve a serine protease in B. intermedius and a thiol-dependent protease in B. gingivalis. Determination of the site or sites on human fibrinogen recognized by B. gingivalis and B. intermedius is important for several reasons. Knowledge of the recognition site would contribute to our understanding of the mechanism of the interaction and might clarify the role of these bacterial receptors and enzymes in pathogenesis. In addition, similarities or differences between B. gingivalis and B. intermedius recognition sites might be exploited in the design of future diagnostic and therapeutic agents to detect and block specific interactions of these bacteria with fibrinogen.

Several recent studies have appeared which suggest that binding sites for some eucaryotic cells on large protein ligands, such as fibronectin and laminin, are contained in relatively small regions of the ligand molecule. Small synthetic peptides containing the sequence arginyl-glycyl-glutamate (RGD) inhibit binding of some eucaryotic cell receptors to fibronectin (27) and other matrix proteins. CDPGYIGSR peptide has been reported to inhibit attachment of HT-1080 cells to laminin (12). Fibrinogen is a dimeric protein, Mf 340,000, with the general structure αβγβγγ, in which the six polypeptide chains are held together by a complicated set of disulfide bonds (7, 34). A schematic representation of the fibrinogen molecule is shown in Fig. 1. The insoluble polymeric form, fibrin, forms the matrix of blood clots (7). Fibrinogen has been shown to bind specifically to some connective tissue matrix components, including fibronectin (17, 28, 29) and hyaluronic acid (24). Fibrinogen also binds specifically to a number of eucaryotic and eucaryotic cells, including strains of Staphylococcus aureus (16, 30), streptococci (20), and Candida albicans (3), rat hepatocytes (1), cultured endothelial cells (6), and human platelets (15, 21).

Studies attempting to identify the site(s) on human fibrinogen recognized by bacteria and mammalian cells have focused on S. aureus and human platelets (15, 21, 30). These studies used the strategy of examining the ability of progressively smaller fibrinogen fragments and, in some cases,
antibodies to fibrinogen fragments to inhibit interaction of the cells with the native fibrinogen molecule. A pentadecapeptide, which retained the ability to block fibrinogen binding to S. aureus and platelets, was recovered from a staphylococcal protease digest of a 27-residue CNBr peptide that contained the carboxy terminus of the fibrinogen γ chain (30). This pentadecapeptide, which represents the 15-residue carboxy terminus of the γ chain (Fig. 1), has been synthesized and reported to contain a cell-binding site for both S. aureus and human platelets (7, 30). More recent studies have appeared which suggest that long-range intramolecular interactions are needed to stabilize the conformation assumed by fibrinogen chains in the native fibrinogen molecule (4, 5, 26). Data presented in one of these studies (5) suggest that binding sites that have a strong dependence on native conformation will likely be destroyed as the secondary and tertiary structure of the native molecule is disrupted in the generation of chains or fragments.

In attempting to determine the site(s) on human fibrinogen recognized by strains of B. gingivalis and B. intermedius, we have followed the approach of examining 125I-fibrinogen (125I-Fbg) binding by bacteria in the presence of fibrinogen chains and plasmin-generated fibrinogen fragments described above. Our results indicate that B. gingivalis and B. intermedius recognize different sites on the fibrinogen molecule, and we have tentatively identified the regions of the fibrinogen molecule where these Bacteroides species interact.

**MATERIALS AND METHODS**

**Materials.** Fibronecctin-free human fibrinogen (L grade; Kabi, Stockholm, Sweden) and rabbit anti-human fibrinogen (anti-Fbg) immunoglobulin G (IgG) were prepared as previously described (23). Bovine serum albumin, plasmin, ovalbumin, human IgG, fetuin, and lysostaphin were purchased from Sigma Chemical Co. (St. Louis, Mo.). Na125I (specific activity, 15 mCi/μg) was purchased from Amersham Corp. (Arlington Heights, Ill.), and Sepharose CL-4B was purchased from Pharmacia Fine Chemicals (Piscataway, N.J.). All other chemicals were of reagent grade and purchased from commercial sources.

**Preparation of staphylococcal fibrinogen receptor.** A fibrinogen receptor from S. aureus Newman was prepared by using an approach similar to the one described by Froman et al. (10) for purification of a fibronectin receptor from the same organism. Bacterial cell walls were solubilized by digestion with lysostaphin in the presence of protease inhibitors, including 1 mM phenylmethylsulfonyl fluoride and 10 mM N-ethylmaleimide, followed by heat treatment at 88°C for 20 min and centrifugation (1,350 × g, 20 min). The supernatant fraction (designated lysate) was supplemented with 1 mM phenylmethylsulfonyl fluoride and 10 mM EDTA, and the pH was adjusted to 7.4. The lysate of staphylococcal cells was chromatographed on a column of human fibrinogen-Sepharose CL-4B (3 by 20 cm; approximately 3 mg of fibrinogen per ml of gel) equilibrated with Tris-buffered saline (0.14 M sodium chloride, 50 mM Tris hydrochloride [pH 7.5]). Loosely bound components of the lysate were eluted with Tris-buffered saline supplemented with 0.36 M NaCl (final concentration of NaCl, 0.5 M), and material bound to the column was eluted with 2 M guanidine hydrochloride in phosphate-buffered saline (PBS; 0.13 M sodium chloride, 10 mM phosphate buffer, 0.02% sodium azide [pH 7.4]). The eluted material was extensively dialyzed to dis-

**FIG. 1.** Schematic drawing of the fibrinogen molecule adapted from Doolittle (7). The molecule is dimeric and composed of two of each of three polypeptide chains, α (66,000), β (54,000), and γ (48,000). The overall molecular weight is 340,000. The amino-terminal ends of all six chains are held together by disulfide bonds, two between the γ chains and one between the α chains, and form the central domain or disulfide knot region. Fibrinopeptides A and B are located at the amino-terminal ends of the α and β chains (αn and βn). The coiled coils are approximately 112 amino acid residues long and connect the central domain to the terminal domains. The binding site for S. aureus is reported to be located in the 15 amino acid residues that make up the carboxy terminus of the γ chain (γc) (30). Although there are many regions of the molecule that are sensitive to plasmin, cleavage at the protease-sensitive sites indicated generates fragments D and E. Fragments D and E correspond approximately to the terminal and central domains, respectively, except that parts of the coiled coil between the protease-sensitive site and the proximal or distal disulfide ring are part of the degradation products (indicated by boxes).
tilled water, lyophilized, and rechromatographed under the same conditions. This procedure yielded a single peak that was found to contain a single band, $M_r$ 55,000 to 57,000, on 5 to 15% sodium dodecyl sulfate (SDS)-polyacrylamide gels (see below) when stained with Coomassie blue R (L. M. Switalski and M. Hook, manuscript in preparation).

**Preparation of fibrinogen chains.** The $\alpha$, $\beta$, and $\gamma$ chains of human fibrinogen were prepared by reduction and carboxymethylation of the native molecule as described by Gollwitzer et al. (11). Reduced, alkylated chains were separated by ion-exchange chromatography, using carboxymethyl-Sepharose (Pharmacia) as described by Doolittle et al. (8). Reduced, alkylated fibrinogen as well as purified $\alpha$, $\beta$, and $\gamma$ fibrinogen chains were dialyzed to distilled water and lyophilized. Before use, lyophilized chains were dissolved in 8 M urea and exhaustively dialyzed to PBS. The purity of the isolated chains was assessed by SDS-polyacrylamide gel electrophoresis (PAGE) according to the method of Studier (31). The resolving gel consisted of a linear 5 to 15% acrylamide gradient overlaid with a 4.8% acrylamide stacking gel. Samples were dissolved in sample buffer containing 4% (wt/vol) SDS. Fibrinogen chains prepared in our laboratory were compared for purity and function with samples of purified $\alpha$, $\beta$, and $\gamma$ chains of human fibrinogen, obtained as a generous gift from Russell Doolittle, University of California, La Jolla.

**Preparation of fibrinogen fragments D and E.** Fibrinogen was digested with plasmin to produce fragments D and E according to the method of Doolittle et al. (8). The plasmin digest was dialyzed to 10 mM sodium bicarbonate (pH 8.9) and passed over a Mono Q fast protein liquid chromatography column (Pharmacia) equilibrated with the same buffer. Separation of fragments D and E was achieved by eluting the column with the same buffer containing a linear sodium chloride gradient (0 to 1 M; 20 ml of buffer). Protein-containing fractions eluted from the Mono Q column with the salt gradient were dialyzed to PBS and analyzed by SDS-PAGE on 7.5% acrylamide gels overlaid with a 4.8% stacking gel as described above. Fractions containing fragments D contained components that ranged in $M_r$ from approximately 75,000 to 100,000 and consisted mainly of two bands of equal staining intensity. We have identified these bands as fragments D$_1$ (late D) and D$_2$ (early D) on the basis of their relative molecular weights (19). These Mono Q-purified fragments D contained only a trace amount of material below $M_r$ 75,000. When the fractions containing fragment E ($M_r$ 50,000) were analyzed by SDS-PAGE, only one band was detected by Coomassie blue R staining. Fibrinogen fragments D and E prepared in our laboratory were compared for purity and function with samples of fibrinogen fragments D and E obtained as a generous gift from Gerald Fuller, University of Alabama at Birmingham.

**$^{125}$I labeling of proteins.** Fibrinogen, fibrinogen fragments D and E, and reduced, alkylated $\alpha$, $\beta$, and $\gamma$ fibrinogen chains were labeled with $^{125}$I by the chloramine T method (18) as previously described (23). The specific activities of labeled proteins (in counts per minute per microgram) were as follows: $^{125}$I-Fbg, $3.9 \times 10^6$; $^{125}$I-$\alpha$ chain ($^{125}$I-$\alpha$), $1.5 \times 10^6$; $^{125}$I-$\beta$ chain ($^{125}$I-$\beta$), $9.5 \times 10^5$; $^{125}$I-$\gamma$ chain ($^{125}$I-$\gamma$), $2.3 \times 10^6$; $^{125}$I-fragments D ($^{125}$I-D), $3.7 \times 10^5$; and $^{125}$I-fragment E ($^{125}$I-E), $1.2 \times 10^5$. Structural integrity of labeled fibrinogen, fibrinogen fragments, and their reduced alkylated products was determined by SDS-PAGE, using 7.5% gels as described above, followed by autoradiography. Autoradiograms of gels of labeled proteins were compared with Coomassie blue R-stained gels of unlabeled fibrinogen, fibrinogen fragments, and their reduced, alkylated products. In no case was any difference observed. Functional integrity of labeled proteins was assessed by mock labeling fibrinogen and fibrinogen fragments with nonradioactive iodine and comparing the behavior of mock-labeled and unlabeled proteins in the ability to block binding of $^{125}$I-Fbg to bacteria.

**Bacteria.** B. gingivalis W and W12 and B. intermedius VPI 8944 and VPI 9145 were obtained from stock cultures maintained in the Anaerobic Core Facility of the University of Alabama School of Dentistry. B. gingivalis ATCC 33277 and B. intermedius ATCC 25261 were obtained from the American Type Culture Collection, Rockville, Md. Bacteria were grown anaerobically at 37°C to early logarithmic phase (approximately 5 x 10$^6$ cells/ml) in complete basal anaerobic broth (32), harvested by centrifugation, washed three times with PBS, and resuspended in PBS at a cell density of 1 x 10$^7$ to 2 x 10$^10$ cells/ml as previously described (22). Bacterial suspensions were stored for several weeks at 4°C before use in binding studies to reduce proteolytic activity (22).

**Binding of bacteria to labeled proteins.** Bacteria (1 x 10$^8$ to 3 x 10$^9$ cells) were incubated with 2.5 x 10$^4$ to 5.0 x 10$^5$ cpm of radiolabeled protein in albumin-coated tubes containing 0.1% (wt/vol) bovine serum albumin, plus other additives as required, in a total volume of 0.5 ml. Tubes containing the incubation mixtures were rotated on a hematology mixer at 22°C for 15 min unless otherwise stated. This incubation time was chosen because there was little change in the amount of $^{125}$I-Fbg bound to bacteria between 15 and 30 min of incubation when cells stored at 4°C for several weeks were used in binding assays and protease inhibitors were included. Subsequently, 400 µl of the incubation mixture was placed in an albumin-coated tube and rapidly diluted with 3 ml of ice-cold PBS containing 0.02% (wt/vol) bovine serum albumin. The samples were centrifuged at 1,300 x g for 15 min in a swinging-bucket rotor, the supernatants were aspirated, and the radioactivity associated with the pelleted bacteria was measured 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. All samples were analyzed in triplicate unless otherwise noted. Plastic tubes holding incubation mixtures were precoated with bovine serum albumin to minimize unspecific binding of bacteria and proteins to the walls of the tubes. Incubations of labeled proteins with B. gingivalis strains were performed in the presence of 20 mM (final concentration) N-ethylmaleimide, and incubations of labeled proteins with B. intermedius strains were performed in the presence of 2 mM (final concentration) phenylmethylsulfonyl fluoride. At these concentrations of protease inhibitors, proteolysis of the native molecule and of prepared chains and fragments was completely blocked during the incubation periods, as confirmed by SDS-PAGE and autoradiography.

**Specificity of binding of $^{125}$I-protein to bacteria.** Bacteria were incubated with competing protein for 15 min before addition of $^{125}$I-protein, after which incubation was continued for an additional 15 min. The amount of radioactive material that bound to bacteria in the presence of the competing protein was determined as described above.

**Reversibility of binding of $^{125}$I-protein to bacteria.** Bacteria were incubated with $^{125}$I-protein for 15 min, after which an unlabeled displacing protein was added and incubation continued for an additional 15 min. The amount of $^{125}$I-protein that remained bound to bacteria after addition of displacing protein was determined as described above.
Preparation of anti-Fbg IgG absorbed with fibrinogen fragments D. Rabbit anti-Fbg IgG was prepared as previously described (23). After dialysis to PBS, the antibody (540 µg/ml) was absorbed with Mono Q-purified fragments D (100 µg/ml, final concentration) overnight at 4°C. The resulting solution was centrifuged at 50,000 × g for 3 h to remove precipitated protein. The supernatant fraction was passed over a protein A-Sepharose column (Pharmacia), which was eluted with 0.5 M NaCl followed by 3.0 M MgCl2. The fractions eluting with salt were dialyzed to PBS and analyzed by SDS-PAGE on 7.5% acrylamide gels. The runthrough from the protein A-Sepharose column contained some fragments D, but the bulk of the fragments D eluted in the 0.5 M NaCl fraction. IgG was eluted by 3.0 M MgCl2, and did not contain any fragments D as assessed by SDS-PAGE.

Effect of S. aureus fibrinogen receptor on binding of Bacteroides species to 125I-Fbg. Various concentrations of the purified staphylococcal receptor were incubated with 125I-Fbg for 15 min, after which either B. gingivalis or B. intermedius cells were added and incubation was continued for an additional 15 min. The 125I-Fbg bound to bacteria in the presence of the staphylococcal receptor was determined as described above.

RESULTS

Effect of fibrinogen fragments on binding of 125I-Fbg to bacteria. Fibrinogen binding by B. gingivalis and B. intermedius is specific in the sense that unrelated proteins do not inhibit binding of fibrinogen to bacteria (Fig. 2 and 3, columns 1; 22, 23). To identify sites in the fibrinogen molecule to which bacteria bind, we decided to take the approach of generating large fragments of fibrinogen and then testing each fragment for the ability to block binding of 125I-Fbg to bacteria. In this manner, we expected to identify fragments of fibrinogen that contained bacterial binding sites. The fibrinogen molecule was disassembled in two ways: it was chemically cleaved by reduction and carboxymethylation to generate the α, β, and γ chains, and it was enzymatically cleaved by plasmin to generate fragments D and E (Fig. 1). Reduced and alkylated fibrinogen (data not shown) and, somewhat surprisingly, purified α, β, and γ chains all caused a concentration-dependent inhibition of binding of 125I-Fbg to all strains of bacteria used. Fragments D and, to a much smaller extent, fragment E produced a concentration-dependent inhibition of 125I-Fbg binding to B. intermedius strains only. Figure 4 illustrates representative data for one strain of B. intermedius (VPI 8944) and one strain of B. gingivalis (W12). Fragments D strongly blocked binding of 125I-Fbg to all B. intermedius strains used and failed to block 125I-Fbg binding to any of the B. gingivalis strains. These data suggest that cleavage of native fibrinogen into plasmin fragments D and E destroys the binding site for B. gingivalis strains and that Mono Q-purified fragments D contain a site recognized by B. intermedius strains. Furthermore, both Bacteroides species recognized all three polypeptide chains. To further explore the nature of the inhibition of 125I-Fbg binding to bacteria produced by these fibrinogen fragments, we prepared iodinated chains and fragments and examined their interactions with bacteria.

Effect of iodination on behavior of fibrinogen fragments in binding inhibition assays. The fibrinogen fragments described...
above were iodinated both with $^{125}$I and with nonradioactive iodine to assess the effects of iodination on protein function. Mock-labeled fragments were compared with unlabeled fragments for the ability to block binding of $^{125}$I-Fbg to bacteria. Nonradioactive iodinated $\alpha$, $\beta$, and $\gamma$ chains and fragments D were indistinguishable from the corresponding unlabeled chains or fragments in their behavior in binding inhibition studies of the type shown in Fig. 4 (data not shown). We concluded from these experiments that iodination by the chloramine T method (18) did not functionally alter $\alpha$, $\beta$, or $\gamma$ chain or fragments D in this system. In contrast, unlabeled fragment E reduced the binding of $^{125}$I-Fbg to all $B$. intermedia strains tested, whereas nonradioactive iodinated fragment E did not. Fragment E labeled poorly by the chloramine T method (18), and we were unable to produce high-specific-activity (>10$^6$ cpm/µg) $^{125}$I-E by using the iodination method of Bolton and Hunter (2). Finally, $^{125}$I-E did not bind to $B$. intermedia strains. The reasons for the failure of both iodination methods to produce functional, high-specific-activity $^{125}$I-E are not clear. Since iodination appeared to alter the ability of fragment E to inhibit binding of $^{125}$I-Fbg to $B$. intermedia strains, no experiments involving fragment E-bacterium interactions were pursued.

$B$. intermedia strains bound substantial amounts of $^{125}$I-$\alpha$, $^{125}$I-$\beta$, $^{125}$I-$\gamma$, and $^{125}$I-D, whereas $B$. gingivalis strains bound substantial amounts of $^{125}$I-$\alpha$, $^{125}$I-$\beta$, and $^{125}$I-$\gamma$ (Table 1). $B$. gingivalis strains did not bind $^{125}$I-D. It was somewhat surprising that all of the reduced, alkylated chains bound to all of the bacteria. These data can be explained in several
ways. First, bacteria may nonspecifically bind many proteins. Second, if fragment binding is specific, then either one bacterial component (receptor) recognizes a common site present in different fibrinogen chains or, third, bacteria possess multiple receptors that recognize distinct sites in different fibrinogen chains. The next series of experiments was performed to compare the binding observed for fibrinogen chains and fragments D with that observed for the native fibrinogen molecule in terms of specificity and reversibility.

**Specificity and reversibility of binding of fibrinogen fragments to bacteria.** The inhibition of binding of $^{125}$I-Fbg, $^{125}$I-$\alpha$, $^{125}$I-$\beta$, or $^{125}$I-$\gamma$ to *B. gingivalis* strains by either unlabeled fibrinogen, ovalbumin, fetuin, human IgG, or the unlabeled chain under consideration is shown in Fig. 2. At the protein concentration used (100 $\mu$g/ml), unlabeled fibrinogen but no other competing protein inhibited binding of $^{125}$I-Fbg to bacteria (Fig. 2, column 1). Although some strain variation was apparent, fibrinogen blocked binding of all three strains to $^{125}$I-$\gamma$, and unlabeled $\gamma$ chain blocked binding of $^{125}$I-$\gamma$ to bacteria. Moreover, $\gamma$ chain blocked binding of $^{125}$I-Fbg to all strains of *B. gingivalis* (see Fig. 4 for strain W12; data not shown for other strains). These data suggest that $\gamma$-chain binding by *B. gingivalis* strains is specific and support the presence of a binding site for *B. gingivalis* on the fibrinogen $\gamma$ chain. Figure 3 shows the specificity of binding of $^{125}$I-Fbg, $^{125}$I-$\alpha$, $^{125}$I-$\beta$, $^{125}$I-$\gamma$, and $^{125}$I-D to *B. intermedius* strains in the presence of the same competing proteins that were used in examining the specificity of binding to the *B. gingivalis* strains. As with *B. gingivalis* strains, unlabeled fibrinogen inhibited binding of $^{125}$I-Fbg to all three *B. intermedius* strains, whereas other competing proteins did not. Although there was some strain variation in recognition of fibrinogen chains, fibrinogen blocked binding of $^{125}$I-D to all three strains of *B. intermedius*, and unlabeled fragments D blocked binding of $^{125}$I-D to all strains of *B. intermedius*. Unlabeled fragments D blocked $^{125}$I-Fbg binding to all *B. intermedius* strains (see Fig. 4 for strain VPI 8944; data not shown for other strains). These data suggest that binding of fragments D by *B. intermedius* strains is specific and indicate that a binding site on fibrinogen for *B. intermedius* is located in fragments D.

**Table 1. $^{125}$I-protein binding to bacteria**

<table>
<thead>
<tr>
<th>$^{125}$I-protein</th>
<th>B. gingivalis</th>
<th>B. intermedius</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>ATCC 33277</td>
<td>VPI 8944</td>
</tr>
<tr>
<td>Fbg</td>
<td>21,000 ± 530</td>
<td>9,070 ± 99</td>
</tr>
<tr>
<td>$\alpha$</td>
<td>11,200 ± 192</td>
<td>5,140 ± 112</td>
</tr>
<tr>
<td>$\beta$</td>
<td>16,300 ± 368</td>
<td>6,120 ± 456</td>
</tr>
<tr>
<td>$\gamma$</td>
<td>5,700 ± 512</td>
<td>4,520 ± 209</td>
</tr>
<tr>
<td>D</td>
<td>86 ± 12</td>
<td>2,980 ± 152</td>
</tr>
<tr>
<td></td>
<td>W12</td>
<td>VPI 9145</td>
</tr>
<tr>
<td>Fbg</td>
<td>23,500 ± 580</td>
<td>4,730 ± 139</td>
</tr>
<tr>
<td>$\alpha$</td>
<td>18,700 ± 660</td>
<td>5,440 ± 188</td>
</tr>
<tr>
<td>$\beta$</td>
<td>20,500 ± 462</td>
<td>5,150 ± 108</td>
</tr>
<tr>
<td>$\gamma$</td>
<td>11,200 ± 520</td>
<td>2,630 ± 101</td>
</tr>
<tr>
<td>D</td>
<td>92 ± 35</td>
<td>1,200 ± 94</td>
</tr>
</tbody>
</table>

* Counts per minute added varied from 2.7 x 10$^4$ to 3.5 x 10$^4$.

$^{125}$I-Fbg and $^{125}$I-$\gamma$. In addition, $\gamma$ chain could displace some $^{125}$I-$\gamma$ in two of the three strains. Under the conditions of the experiment, only a small amount of $^{125}$I-$\alpha$ and $^{125}$I-$\beta$ could be displaced from the bacterial cells. For all three strains of *B. gingivalis* studied, bound $^{125}$I-Fbg was displaced (30 to 50%) by unlabeled $\gamma$ chain but not by unlabeled $\alpha$ or $\beta$ chain (data not shown). The results from these experiments suggest that fibrinogen and $\gamma$ chain may compete for the same site on *B. gingivalis* strains and, like the specificity data, are compatible with a binding site on fibrinogen for *B. gingivalis* being located on the $\gamma$ chain. Figure 6 represents the same type of experiments using *B. intermedius* strains as target bacteria, and hence fragments D were added as binding and displacing ligands. For all strains of *B. intermedius* examined, unlabeled fibrinogen could displace a large proportion of bound $^{125}$I-Fbg from bacteria, and unlabeled fibrinogen and fragments D could displace similar and substantial amounts of bound $^{125}$I-D from bacteria. Some bound $^{125}$I-$\alpha$ and $^{125}$I-$\gamma$ could be displaced from these bacteria; however, bound $^{125}$I-$\beta$ could not be displaced. For all three strains, unlabeled fragments D displaced a large amount (50 to 70%) of $^{125}$I-Fbg (data not shown). These data suggest that fibrinogen and fibrinogen fragments D may compete for the same binding site on *B. intermedius* strains.

These specificity and reversibility data should be interpreted with caution for several reasons. Only one concentration (100 $\mu$g/ml) of competing and displacing ligands was used in this study. Although this concentration represented a large molar excess relative to that of the radiolabeled ligand, it may have been insufficient in some cases, particularly if the affinity of the labeled ligand for the bacteria was much greater than that of a competing or displacing component. In addition, the displacement studies were performed under conditions that gave maximal binding and displacement of fibrinogen from bacteria and no proteolysis of fibrinogen. If a fibrinogen chain or fragment bound bacteria with substantially different on or off rates, then displacement may not have been detected. Under these experimental conditions, chain and fragment interactions with bacteria that were similar kinetically with those of the native fibrinogen molecule would be most readily detected.

The results of some of the experiments performed in this part of our study are difficult to interpret. Attempts to displace $^{125}$I-$\alpha$ and $^{125}$I-$\beta$ with unlabeled ligand resulted in an increased binding of radioactivity to bacteria. Aggregation of labeled and unlabeled ligand followed by cell binding of complexes could account for these results. Chains or fragments whose binding is not blocked or displaced by intact fibrinogen may contain new and different cell-binding sites that are hidden in the native molecule but become exposed as the fragments are generated. Also, in some of the experiments, binding of a $^{125}$I-labeled chain to bacteria was not...
FIG. 5. Reversibility of binding of $^{125}$I-Fbg, $^{125}$I-$\alpha$, $^{125}$I-$\beta$, and $^{125}$I-$\gamma$ to B. gingivalis. Bacteria ($10^9$) were incubated with $5 \times 10^4$ cpm of either $^{125}$I-Fbg (F), $^{125}$I-$\alpha$ (a), $^{125}$I-$\beta$ (b), or $^{125}$I-$\gamma$ (g). After a 15-min incubation, a 100-µg/ml concentration of either unlabeled fibrinogen (□) or unlabeled like chain (II) was added as a displacing protein, and incubation was continued for an additional 15 min. Binding of $^{125}$I-proteins to bacteria in the absence of displacing protein was arbitrarily set at zero (0%), i.e., no change in baseline amount of $^{125}$I-protein bound. Error bars indicate standard deviations of triplicate assays. The standard deviations for the $^{125}$I-Fbg, $^{125}$I-$\alpha$, $^{125}$I-$\beta$, and $^{125}$I-$\gamma$ controls (not shown) ranged from ±2.5 to 5.1, ±0.8 to 20.7, ±0.9 to 9.8, and ±1.65 to 9.64%, respectively. Bars that fall below the horizontal line indicate the percent displacement produced by addition of the displacing protein; bars that fall above the horizontal line indicate increased binding of $^{125}$I-protein to bacteria caused by addition of the displacing protein.

FIG. 6. Reversibility of binding of $^{125}$I-Fbg, $^{125}$I-$\alpha$, $^{125}$I-$\beta$, $^{125}$I-$\gamma$, and $^{125}$I-D to B. intermedius. Bacteria ($10^9$) were incubated with $5 \times 10^4$ cpm of either $^{125}$I-Fbg (F), $^{125}$I-$\alpha$ (a), $^{125}$I-$\beta$ (b), $^{125}$I-$\gamma$ (g), or $^{125}$I-D (D). After a 15-min incubation, a 100-µg/ml concentration of either unlabeled fibrinogen (□) or unlabeled like chain or fragments D (D) was added as a displacing protein, and incubation was continued for an additional 15 min. Binding of $^{125}$I-proteins to bacteria in the absence of displacing protein was arbitrarily set at zero (0%), i.e., no change in baseline amount of $^{125}$I-protein bound. Error bars indicate standard deviations of triplicate assays. The standard deviations for $^{125}$I-Fbg, $^{125}$I-$\alpha$, $^{125}$I-$\beta$, $^{125}$I-$\gamma$, and $^{125}$I-D controls (not shown) ranged from ±1.2 to 3.0, ±2.4 to 4.8, ±5.2 to 11.8, ±1.8 to 7.3, and ±4.8 to 24.0%, respectively. Bars that fall below the horizontal line indicate the percent displacement produced by addition of the displacing protein; bars that fall above the horizontal line indicate increased binding of $^{125}$I-protein to bacteria caused by addition of the displacing protein.
determined as control values; error after rabbit anti-Fbg IgG absorbed either site binding described experiments rabbit that differences strains intermedius for site dent bacteria. should gingivalis 125I-Fbg of also 125I-D to Fbg and the D binding sites on strain intermedius of tion 125I-Fbg and been best increasing inhibition of receptor (16, 125I-Fbg intermedius strains resulted in 7. FIG. Effect of anti-Fbg IgG on binding of fibrinogen fragments to bacteria. Previous results from our laboratory have shown that rabbit anti-Fbg IgG inhibits in a concentration-dependent manner the binding of 125I-Fbg to B. gingivalis and B. intermedius strains (22, 23). The data presented in Fig. 7 show the effect of absorption with fragments D, which should remove antibodies to the terminal domains of fibrinogen, on the ability of anti-Fbg IgG to inhibit binding of 125I-D and 125I-Fbg to B. intermedius VPI 8944 and of 125I-Fbg to B. gingivalis W12. Absorption of anti-Fbg IgG with fragments D removed virtually all of the antibody that inhibited 125I-D binding to the B. intermedius strain (Fig. 7a). It also removed most of the antibody that inhibited binding of 125I-Fbg to these bacteria (Fig. 7b). In contrast, absorption of anti-Fbg IgG with fragments D had little effect on the ability of the antibody to block binding of 125I-Fbg to the B. gingivalis strain (Fig. 7c). These data suggest that the B. intermedius strain binds fibrinogen in the D domain and that the B. gingivalis strain binds fibrinogen at a site remote from the D domains.

Effect of S. aureus fibrinogen receptor on binding of 125I-Fbg to bacteria. Other bacteria have been reported to bind 125I-Fbg (16, 20, 30). The interaction with fibrinogen has been best characterized for S. aureus. To compare the binding sites on fibrinogen for S. aureus with those for B. gingivalis and B. intermedius, we examined the effect of increasing amounts of a purified S. aureus fibrinogen receptor on binding of 125I-Fbg to S. aureus, B. gingivalis, and B. intermedius strains. Addition of the S. aureus fibrinogen receptor to incubation mixtures containing S. aureus or B. intermedius strains resulted in a concentration-dependent inhibition of 125I-Fbg binding (Fig. 8). In contrast, addition of S. aureus fibrinogen receptor to incubation mixtures containing B. gingivalis strains had no effect on 125I-Fbg binding. These results suggest that S. aureus receptor binds directly to, or sterically blocks accessibility of, the site on fibrinogen recognized by B. intermedius strains. Since 125I-Fbg binding to B. gingivalis strains was unaffected by the presence of S. aureus receptor in the incubation mixture, we conclude that B. gingivalis strains bind a site on fibrinogen remote from the site recognized by S. aureus. Experiments with the S. aureus receptor as a potential inhibitor of fibrinogen binding to bacteria have been extended to include several other B. gingivalis and B. intermedius strains for which other binding data are incomplete. These other strains

FIG. 7. Inhibition of binding of 125I-proteins to bacteria by rabbit anti-Fbg IgG. Preimmune rabbit IgG (□), rabbit anti-Fbg IgG (▲), or rabbit anti-Fbg IgG absorbed with fragments D (○) was incubated with either 125I-D or 125I-Fbg in the presence of protease inhibitors for 30 min, after which bacteria (10⁷) were added and incubation was continued for an additional 15 min. Radioactivity associated with bacteria was determined as described in the text. Data are presented as mean percent 125I-protein bound to bacteria in the presence of IgG relative to control values; error bars represent standard deviations of triplicate assays.

FIG. 8. Inhibition of 125I-Fbg binding to B. gingivalis ATCC 35277, W, and W12. B. intermedius ATCC 25261, VPI 8944, and VPI 9145, and S. aureus Newman by purified S. aureus fibrinogen receptor. 125I-Fbg (5.0 × 10⁶ cpm) was incubated for 15 min in the presence of purified staphylococcal receptor, after which either S. aureus (5 × 10⁶ cells), B. intermedius (2 × 10⁶ cells), or B. gingivalis (2 × 10⁶ cells) was added to the incubation mixture. Incubation was continued for an additional 15 min, and then radioactivity associated with bacteria was determined as described in the text. Each value is the mean of duplicate samples; error bars were used only when the difference in the duplicates was greater than 10% of the value of the largest measurement.
(data not shown) follow the same pattern for effect of S. aureus receptor on 125I-Fbg binding as the strains shown in Fig. 8.

In summary, our data suggest that the B. gingivalis and B. intermedius strains so far examined recognize distinct and spatially distant sites on the human fibrinogen molecule and that fibrinogen-binding sites for these bacteria may be species specific.

DISCUSSION

Two general approaches have been used in attempts to locate cell-binding sites on the fibrinogen molecule (15, 16, 30). The first approach relies on the assumption that small fragments of large ligands, like fibrinogen, can be generated that retain the binding site for cells. Fibrinogen fragments have been generated both chemically (by reduction and alkylation, treatment with CNBr, or both) and enzymatically (by digestion with plasmin or other proteases) and then tested for the ability to inhibit the interaction between cells and the native fibrinogen molecule (16, 30). This approach has been technically problematic for several groups, including ours, because many of these fibrinogen fragments, particularly the reduced, alkylated α chains and some of the CNBr peptides, show a strong tendency to aggregate and are very insoluble in aqueous solvents (15). These properties make binding studies difficult to perform and may account for a portion of the unexpected results obtained in some of our experiments, such as increased binding of iodinated chains to bacteria after addition of unlabeled chains.

Previous studies suggest that some epitopes present in native fibrinogen are destroyed and new epitopes are created when fibrinogen is chemically or enzymatically cleaved (4, 9). Similar changes may also occur among receptor-binding sites. Nagy et al. (26) have determined that amino acid residues 20 to 28 in the β chain (Bβ 20–28) of bovine fibrinogen constitute an epitope in an antigenic determinant of bovine fibrinogen. Their calculations show that only a small percentage (less than 0.00006) of the synthetic peptide Bβ 20–28 is in the native conformation when this peptide is in solution. Recognition of a binding site in a protein by a cellular receptor or other interacting molecule may rely largely on the primary structure of the ligand in some cases, whereas in other cases recognition may rely largely on the conformation of the polypeptide chain (5, 27). In cases where conformation is more important than primary structure for recognition, attempts to identify receptor-binding sites by testing the activity of peptides generated by disassembly methods that destroy native ligand conformations (i.e., reduction and alkylation) have a low probability of success.

To overcome technical difficulties of working directly with fibrinogen chains and fragments in solution, Hawiger et al. (15) have used a second approach, in which antibodies raised against isolated fibrinogen fragments are tested as potential inhibitors of binding of native ligand to cells. This approach is based on the assumption that antibodies generated against small ligand fragments will react with the same fragments in their native conformation in the parent molecule and block binding of cells to the ligand at or near the cell-binding site. A potential problem with this immunological approach is the possibility that antibodies, as they bind to the ligand molecule, may induce long-range conformational changes that affect the structure of a distant receptor-binding site. Consequently, an antibody may inhibit cell binding of a ligand by interacting with the ligand molecule at a substantial distance from the actual cell-binding site.

In this study, we have attempted to locate binding sites on fibrinogen for B. gingivalis and B. intermedius strains by using several different approaches. In our initial attempts to inhibit fibrinogen binding to bacterial cells by using isolated fibrinogen chains and fragments, we found that all chains exhibited inhibitory activity toward all bacteria and that fragments D inhibited binding by B. intermedius strains only. The data supporting a binding site for B. intermedius on fragments D are unambiguous, and 125I-D binding is specific and kinetically similar to binding of intact fibrinogen by these bacteria. This is not surprising, since fragment D1 retains many of the biological properties attributed to the terminal domains and probably much of the conformation of the terminal domains of native fibrinogen (5, 7). The interaction of the bacteria with all of the fibrinogen chains is surprising and somewhat more difficult to interpret. In a preliminary characterization of the binding of different fibrinogen chains to strains of the two Bacteroides species, we found that all chains bound to all bacterial cells. Further analysis revealed a substantial variation among different strains in the specificity and reversibility of their interactions with isolated fibrinogen chains. The reasons for these strain differences are not clear; however, little is known about the amount of strain-to-strain variability in outer membrane proteins of these Bacteroides species, and it is possible that the presence of capsular material could alter binding of cells to host tissue components (25). Considering the magnitude of the disruption of the conformation of the native molecule when α, β, and γ chains are generated, it is not surprising that the studies performed failed to detect which chain or chains within the D fragments contain a cell-binding site for B. intermedius strains. Although the results are somewhat more ambiguous than those for the fragments D-B. intermedius interaction, our data support the presence of a binding site for B. gingivalis on the fibrinogen γ chain.

Despite the complexity of our data, certain conclusions can be drawn. First, B. gingivalis and B. intermedius preferentially bind fibrinogen at different sites. In the case of B. gingivalis, γ-chain binding mimics most closely the binding of native fibrinogen to bacteria. Since B. gingivalis recognizes intact γ chain but not fragment D1 or E, which contain between them almost the entire γ chain (D1 contains approximately the carboxy-terminal three-fourths of the γ chain, and E contains approximately the amino-terminal one-fourth), we hypothesize that B. gingivalis binds fibrinogen at the plasmin-sensitive site of the coiled-coil region between the D and E domains. With respect to B. intermedius strains, binding of fragments D mimics binding of native fibrinogen to bacteria, and it appears that a fibrinogen-binding site for B. intermedius is contained in fragments D. These conclusions are summarized in Fig. 9. That B. gingivalis and B. intermedius species recognize different sites on the fibrinogen molecule was further demonstrated by the different effects that fragments D-adsorbed anti-Fbg IgG and the S. aureus fibrinogen receptor had on the binding of 125I-Fbg to different strains of the two species. The results of this study and of others (4, 5, 26) highlight the difficulties involved in trying to find specific sites on large complex protein ligands, particularly sites whose activity is highly conformationally dependent, in ligand fragments that may contain the appropriate primary sequences but whose tertiary or quaternary structures may be different from those they assume in the native molecule.

The finding that B. gingivalis and B. intermedius bind fibrinogen at spatially distant and distinct sites may have clinical significance. The difference in fibrinogen recognition
by these organisms might be exploited to develop new antimicrobial agents with specificity at the species level. In addition to being therapeutically useful, these agents may elucidate the role of a suspected virulence factor or a particular bacterial species in the pathogenesis and etiology of human periodontal diseases.

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the staphylococcal clumping receptor. Biochemistry 26:6152–6156.