Interaction of Human Fibrinogen With Staphylococci: Presence of a Binding Region on Normal and Abnormal Fibrinogen Variants and Fibrinogen Derivatives

By Jack Hawiger, Dianne K. Hammond, Sheila Timmons, and Andrei Z. Budzynski

Human fibrinogen interacts with pathogenic staphylococci, causing their clumping through an unexplained mechanism. We attempted to characterize the interaction between the staphylococcal fibrinogen receptor(s) and the binding region on the fibrinogen molecule by employing binding studies and the clumping reaction. Binding studies revealed that the Aα chain of human fibrinogen possesses a high affinity for staphylococci, the affinity of the Bβ chain is somewhat less, and the γ chain has no apparent affinity. The binding region seems located on the C-terminal portion of the Aα and Bβ chains because (1) its function is gradually destroyed by progressive degradation with plasmin and (2) neither purified fragment E nor the N-terminal disulfide knot binds to staphylococci. These findings were supported by experiments with abnormal variants of fibrinogen. Fibrinogen Detroit, with a defect in the N-terminal part of the Aα chain, retained full clumping reactivity toward the staphylococcal receptor, as did fibrinogen Paris I, which manifests a defect in the C-terminal portion of the γ chain. After reduction and carboxymethylation, fibrinogen retained its ability to bind to staphylococci; however, the clumping reactivity was lost, suggesting that disulfide bonds contribute not to the binding properties of fibrinogen but only to its ability to clump. The clumping reaction occurred in the presence of diisopropylfluorophosphate at a concentration that inactivated staphylococagulase and thrombin, indicating that the clumping reaction does not depend on the enzymatic activity of a serine protease type. Other factors, such as pH, chelators, polyanions, and detergents, were examined for their influence on the clumping reaction. The existence of a binding region(s) for staphylococci provides a basis for their use in measurement of normal and abnormal variants of human fibrinogen and its derivatives.

PLASMA PROTEINS able to interact with bacteria are usually thought to be immunoglobulins and components of the classic and alternate complement system. The fact that fibrinogen, a clottable protein of human plasma, interacts with staphylococci has remained unexplained for several decades. The interaction between staphylococci and fibrinogen, as depicted in Fig. 1, is almost immediate and results in the formation of clumps visible to the naked eye, which bear a striking similarity to the agglutination of bacteria by antibodies. Indeed, the observation that staphylococci are clumped not only in...
human plasma but also, to a much lesser degree, in serum, which is essentially free of fibrinogen, has led some to believe that clumping of staphylococci is caused by antibodies. However, accumulating knowledge on the structure of fibrinogen and on the enzymatic degradation of the fibrin clot provided a sound basis for explaining the clumping of staphylococci in human serum as a phenomenon caused by soluble fibrin monomer complexes. Subsequently, the staphylococcal clumping test for measurement of fibrin degradation products was developed and its correlation with immunologic techniques established.

The purpose of this study was to determine the mechanism of interaction between human fibrinogen and staphylococci by means of binding studies and clumping reaction with $^{125}\text{I}$-fibrinogen, its subunits, and its degradation products. The molecular basis of the interaction between fibrinogen and staphylococci is supported by experiments with abnormal variants of fibrinogen with distinct defects involving different regions of the molecule. The evolving mechanism of interaction between the staphylococcal receptor and fibrinogen takes into account established structural features of this plasma protein. It also provides a groundwork for the use of staphylococci for detection of normal and abnormal variants of human fibrinogen and for measurement of its derivatives. Furthermore, the proposed mechanism may serve as a model of the interaction between fibrinogen and its receptors present on microbial agents. A preliminary report of some of these experiments has appeared elsewhere.

MATERIALS AND METHODS

Preparation of $^{125}\text{I}$-fibrinogen. Human fibrinogen (Grade L; Kabi, Stockholm, Sweden), 95%, clottable after addition of thrombin (bovine; Parke-Davis, Detroit, Mich.), was dialyzed against 0.145 M NaCl containing 0.013 M sodium citrate and 0.0012 M phosphate buffer, pH 7.6. It was then labeled with $^{125}\text{I}$ by the iodine monochloride method of McFarlane using Na $^{125}\text{I}$ (5 mCi, carrier-free and reducing agent free; Amersham-Searle, Arlington Heights, Ill.) with substitution...
not exceeding 0.5 atom of iodine per molecule of fibrinogen. The labeled protein was dialyzed against 0.145 M NaCl containing 0.013 M sodium citrate and 0.0012 M phosphate buffer, pH 7.6, to remove free 125I, more than 95% of the radioactivity was clottable with thrombin. 125I-fibrinogen was kept at 4°C and used immediately for experiments.

Reduction and carboxymethylation of 125I-fibrinogen. The disulfide bonds of 125I-human fibrinogen were reduced and carboxymethylated according to the procedure of Crestfield et al. Briefly, 125I-fibrinogen was incubated in a N₂ atmosphere in a 0.05 M Tris-HCl buffer, pH 8.6, containing 8 M urea (Schwarz-Mann, Orangeburg, N.Y.), 0.2%, ethylenediaminetetraacetic acid (EDTA), and 0.12 M β-mercaptoethanol (Sigma, St. Louis, Mo.). After 5 min in a boiling water bath, the mixture was cooled in a light-protected bottle, and 0.1 M iodoacetic acid (Calbiochem, La Jolla, Calif.), which had been recrystallized from petroleum ether and dissolved in 0.1 N NaOH, was added. After 15 min the mixture was dialyzed against water and 3 M urea. In some experiments reduction and carboxymethylation were performed in 5 M guanidine HCl according to the procedure of Cottrell and Doolittle.

Cleavage of fibrinogen with cyanogen bromide (CNBr). According to the procedure of Blömbäck et al., 125I-fibrinogen was dissolved in 70%, formic acid (Matheson, Coleman & Bell, Norwood, Ohio) and treated with 0.12 M CNBr (Eastman, Rochester, N.Y.) in a glass-stoppered light-protected bottle under an exhaust hood. After evaporation through a trapping solution of 1 N NaOH, the material was lyophilized. The completeness of CNBr cleavage was documented by amino acid analysis, which demonstrated the absence of methionyl residues. Isolation of N-terminal disulfide knot (NDSK) was done by chromatography on Sephadex G-100 and G-200 gels according to Blömbäck et al.

Amino acid analysis. Duplicate samples of fibrinogen and CNBr-cleaved fibrinogen were hydrolyzed in 6 N HCl at 110°C in vacuo for 20 hr. Quantitative analysis was performed by ion exchange chromatography employing a Beckman Spinco Model 120B automatic amino acid analyzer. Half cysteine was determined by the method of Moore.

Determination of 125I-fibrinogen with plasmin. The system for digestion of 125I-fibrinogen was 4.5 ml 0.5%, human fibrinogen in 0.05 M Tris-HCl buffer, pH 7.4, 0.3 ml 0.9% NaCl, and 0.2 ml 0.1%, plasin (pig plasmin containing 2.2 casein units/mg; Lysofibrin, Novo Laboratories, Copenhagen, Denmark) at 0°C. Immediately after the plasmin solution was added and while the incubation mixture remained at 0°C, the first sample was removed for analysis. The digestion mixture was then incubated at 37°C, and the next samples were removed at subsequent time intervals for determination of binding to staphylococci and clumping titer and for analysis by electrophoresis on polyacrylamide gels. Proteolysis was terminated by addition of e-aminocaproic acid (EACA) in a concentration of about 100 times the plasminase activity present in the incubation mixture. The system for determination of plasmin degradation products was similar to that for digestion of 125I-fibrinogen except that plasmin, EACA, and fibrinogen were incubated at 37°C, and the mixture was cooled in a light-protected bottle, and 0.1 M iodoacetic acid (Calbiochem, La Jolla, Calif.), which had been recrystallized from petroleum ether and dissolved in 0.1 N NaOH, was added. After 15 min the mixture was dialyzed against water and 3 M urea. In some experiments reduction and carboxymethylation were performed in 5 M guanidine HCl according to the procedure of Cottrell and Doolittle.

Preparation of 125I-fibrinogen degradation products. Fragments X (stage 1) and X (stage 2) were purified from stage 1 and stage 2 plasmin digests of fibrinogen by Sephadex G-200 gel chromatography. Fragment D (stage 2) was prepared from stage 2 and fragments D (stage 3) and E were prepared from stage 3 by Pevikon (C-870, Mercer, N.Y.) block electrophoresis. Fragment D-D derived from crosslinked fibrin was isolated according to the method of Marder et al. The relative purity of plasmin degradation products was at least 98%, as calculated from densitometric scans of Coomassie blue stained sodium dodecyl sulfate (SDS) polyacrylamide gels after electrophoresis. The fragments were dialyzed against 0.145 M NaCl containing 0.013 M sodium citrate and 0.0012 M phosphate buffer, pH 7.6, and labeled with 125I by the iodine monochloride method as described above. Concentration of fragments was determined on the basis of the following extinction coefficients (ε): 280 nm: fragment X, 15; fragment D, 20; fragment E, 12.

Determination of thrombin and reptilase times. Thrombin and reptilase clotting time determinations were performed according to the procedure of Blömbäck using bovine thrombin (Parke-Davis, Detroit, Mich.) in final concentrations of 1 NIH unit/ml and Reptilase-R (Bothrops Atrox; Abbott Laboratories, Chicago, Ill.) in a final concentration of 1 mg/ml.

Abnormal variants of human fibrinogen. Fibrinogen Detroit was kindly provided by Dr. E.
Mammen, Detroit, Michigan, and fibrinogen Paris I by Dr. Doris Ménaché, Clichy, France. Plasma samples containing abnormal fibrinogen were lyophilized. Tested samples contained 0.02 M e-aminocaproic acid (Lederle, Pearl River, N.Y.) or Kunitz inhibitor (Iniprol, Choay), 500 U/ml blood.

Preparation of staphylococci with clumping factor. Staphylococcus aureus Newman D2-C variant producing clumping factor but not producing staphylocoagulase was grown in Brain Heart Infusion Broth (Difco, Detroit, Mich.) for 18 hr at 37°C on a shaker at 100 oscillations/min. The volume of medium was one-fifth that of the flask in order to provide appropriate aeration. At the end of the incubation period, a small sample was subcultured on a blood agar plate to evaluate contamination with other bacteria. Staphylococci were centrifuged at 120,000 g, suspended in 0.25 volume saline, and killed by heating at 70°C for 90 min. The effectiveness of killing was verified by subculturing a small sample on a blood agar plate. The bacteria were then diluted and washed twice with saline, once with sterile distilled water, suspended in 0.2 volume distilled water, and lyophilized on the same day. If results of sterility testing revealed no viable bacteria, the lyophilized preparation containing staphylococci with clumping factor was stored at 4°C under dry conditions and used within several months. Staphylococcus epidermidis Zak not producing clumping factor was prepared under the same conditions and used as a control in binding studies.

Determination of the staphylococcal clumping titer. Twofold serial dilutions of the tested material were made in saline buffered with 0.05 M Tris-HCl buffer, pH 7.4, unless otherwise stated. The last tube contained diluent alone and served as the negative control. A suspension of 10 mg lyophilized staphylococci containing clumping factor was freshly made in 1 ml of 0.05 M Tris-HCl buffer, pH 7.4, containing 0.01% bovine albumin (30% solution: Hyland, Travenol, Costa Mesa, Calif.). The mixture was vortexed for 2 min to obtain a smooth suspension, and 0.05 ml was added to 0.1 ml of each dilution of tested material, starting with the last tube containing diluent alone. After being shaken for 2 min, 0.5 ml saline buffered with 0.05 M Tris-HCl, pH 7.4, was added, and the tubes were observed within 20 min for the presence of clumps. The reciprocal value of the highest dilution giving a positive clumping reaction was taken as the staphylococcal clumping titer; the titer could also be expressed as a logarithm (base 2) for statistical analysis.

Binding of 125I-fibrinogen and its polypeptide subunits and fragments to staphylococci. Because polypeptide chain subunits of human fibrinogen are poorly soluble in an aqueous solution at neutral pH, it was necessary to perform most of the binding experiments in a buffered solution of 3 M urea, which has been shown not to inhibit the staphylococeal clumping reaction. Therefore since a high reactivity of staphylococcal preparations toward fibrinogen was needed, only those batches of staphylococci that were clumped in the presence of 2 μg or less of human fibrinogen were used for binding experiments. 125I-fibrinogen or its derivatives were dissolved in 3 M urea buffered with Tris-HCl buffer, pH 7.4. The concentration of protein was 0.5 mg/ml in experiments employing fibrinogen and reduced and carboxymethylated fibrinogen. In experiments with isolated fragments their concentration was 0.2 mg/ml. Then 25 mg of lyophilized staphylococci or its radioiodinated derivatives were added. The mixture was gently vortexed and placed on a shaker at room temperature for 20 min. Then the suspension was centrifuged at 12,000 g for 10 min. The supernatant was collected and added to another 25 mg of lyophilized staphylococci in a Corex centrifuge tube. This procedure was repeated twice. After the third incubation, the final supernatant was collected, analyzed for radioactivity in a Gamma Counter (Nuclear Chicago, Searle, Atlanta, Ga.), and subjected to electrophoresis in SDS polyacrylamide gels.

Gel electrophoresis. Polyacrylamide gel electrophoresis (PAGE) was performed according to previously described methods. Throughout these experiments 7.5% and 10% gels (0.5 x 7 cm) containing 0.1% SDS were used. Each sample was run in triplicate with approximately 10-20 μg of protein applied to each gel. Electrophoresis was performed at 20-25°C at a constant current of 8.3 mA per gel for approximately 4-6 hr. One gel was stained in Coomassie brilliant blue for 4 hr and then destained 18-24 hr in a solution containing 7% methanol and 14% acetic acid that was changed frequently. Another gel was sliced in a gel slicer (Specialized Medical Instruments, Baltimore, Md.), and distribution of radioactivity was compared with the protein pattern. A third gel was stained for carbohydrate-containing protein bands with the periodic acid-Schiff base reagents according to the method of Zacharius et al. 21

Experiments with diisopropylfluorophosphate (DFP). DFP was kindly provided by Dr. Tadashi Inagami, Dept. of Biochemistry, Vanderbilt University. DFP was dissolved in anhydrous iso-
propanol. In preliminary experiments concentrations of DFP inhibitory to 1 NIH unit of bovine thrombin (Parke-Davis, Detroit, Mich.) were determined as $10^{-5} M$. Then aliquots of clumping factor were pretreated with DFP at concentrations of $10^{-4}$ and $10^{-3} M$; anhydrous isopropanol was used as a control. Serial dilutions of fibrinogen were prepared and the clumping titer determined with pretreated and untreated clumping factor.

**Determination of conditions affecting the staphylococcal clumping reaction.** To determine the effect of pH, acetate-borate-cacodylate buffer, 0.1 M, was used with a pH range of 3.0-11.0. Serial dilutions of human fibrinogen were prepared at each pH value and the clumping titer was determined. Then the pH was readjusted to 7.0 using 1 N NaOH or 1 N HCl and the titer was determined again. The results varied within two dilutions, which is an acceptable range for a method employing a twofold dilution technique.

The effect of the chelator EDTA was assessed by adding a solution of EDTA, in final concentrations $5 \times 10^{-5}$ to $5 \times 10^{-2} M$ and adjusted to pH 7.4 with NaOH, to fibrinogen serially diluted in Tris-saline, pH 7.4. Then the staphylococcal clumping titer was determined. The effect of negatively charged polymers was tested by adding a solution of the polymer, buffered with Tris-saline, pH 7.4, to serially diluted fibrinogen and then determining the staphylococcal clumping titer. The following negatively charged polymers were used: heparin (Connaught Laboratories, Toronto, Canada), chondroitin sulfate (Mann Research Laboratories, New York, N.Y.), and sodium poly-anethole sulfonate (Liquoid; Roche, Nutley, N.J.).

**RESULTS**

**Demonstration of binding of normal and modified human fibrinogen to staphylococci.** Data shown in Table 1 indicate that human fibrinogen possesses a high affinity toward staphylococci; over 90% of the fibrinogen molecules were bound as judged by radioactivity counts, and clumping of staphylococci, as measured by serial dilutions, gave a high titer. Since the number of staphylococci containing cell wall receptor (clumping factor) added to human fibrinogen had been established as $5 \times 10^9$ cells added to each dilution, it was possible to determine the number of human fibrinogen molecules necessary to bind and clump staphylococci. It was determined that the smallest amount of fibrinogen causing clumping in our system, employing the test tube method, was 0.5 $\mu g$/ml, which, when calculated for the number of staphylococci added, corresponds to 20 molecules of fibrinogen per staphylococcal cell. In the slide test method, eight times more sensitive, allowing the detection of clumping with 0.05 $\mu g$/ml human fibrinogen, only two molecules of fibrinogen per staphylococcus were necessary to cause staphylococcal clumping.

**Reduction and carboxymethylation of fibrinogen, causing it to separate into**

<table>
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<tr>
<th>Binding (%)</th>
<th>Clumping (titer)</th>
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<tr>
<td>91.7</td>
<td>1024</td>
</tr>
<tr>
<td>60.0</td>
<td>2</td>
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*Material in 3 M urea was added to 25 mg staphylococcal clumping factor, incubated with shaking, and centrifuged at 12,000 g for 10 min. After three absorptions the supernatant was examined for residual radioactivity and evaluated by SDS-PAGE.*

*Serial dilutions of the tested material were mixed with a suspension of staphylococci containing clumping factor. Indicated titer was the highest dilution giving a positive reaction.*

*125I-Fibrinogen was treated with a mixture of urea, EDTA, and $\beta$-mercaptoethanol in 0.04 M Tris-HCl buffer (pH 8.6) under N2 atmosphere. After 5 min in a boiling water bath iodoacetic acid in 0.1 N NaOH was added; after 15 min material was dialyzed.*
polypeptide chain subunits (Aα, Bβ, and γ), decreased the binding from 92% to 60% and diminished the clumping reaction to a negligible level.

Characterization of the binding region for the staphylococcal receptor on polypeptide chain subunits of human fibrinogen. Since 60% of reduced and carboxymethylated 125I-human fibrinogen could bind to staphylococci, the question arose as to which of the three polypeptide chain subunits possesses a binding region for the staphylococcal receptor. This question was studied by analysis of polypeptide chain subunits of 125I-fibrinogen in SDS polyacrylamide gels before and after binding to staphylococci. Subsequently, it was deduced from protein and radioactivity patterns which subunit was bound to staphylococci due to the presence of the binding region. Distribution of 125I among the chains of human fibrinogen was as follows: Aα chain, 20%; Bβ chain, 52%; γ chain, 28%.

Radioactivity in SDS polyacrylamide gel, before and after binding to staphylococci, was compared individually for each chain. The value obtained for each chain before binding to staphylococci was accepted as 100%, and a decrease in radioactivity after binding represented the amount bound to staphylococci. As shown in Fig. 2, the Aα polypeptide chain of human fibrinogen bound almost completely to staphylococci. The Bβ chain showed 65% of binding, and the γ chain demonstrated negligible binding, 20% ± 10%. Since the nonspecific association of radiolabeled material with staphylococci (background binding) amounted to 10% of the total radioactivity added to the incubation mixture (see below, the experiment with strain Zak), the values obtained for the γ chain were within the limits of the experimental error of the method. The values obtained for binding of the Aα and Bβ chains were compatible with the value of total binding obtained for reduced and carboxymethylated 125I-fibrinogen (Table 1), which indicated that approximately 60% of the subunit polypeptide chains were bound to staphylococci, leaving 40% of the subunit polypeptide chains unbound. Since the γ chain comprises 27% of the human fibrinogen molecule and the unbound fraction of the Bβ chain constitutes 11% of the fibrinogen molecule, they represent together 38% of the molecule, which is very close to the 40% of unbound material determined for reduced and carboxymethylated fibrinogen.

The same pattern of binding was obtained with polypeptide chain subunits of human fibrinogen reduced and carboxymethylated in 5 M guanidine.

Fig. 2. Binding of polypeptide chain subunits of 125I-fibrinogen to staphylococci. (For details see text.)
FIBRINOGEN-STAPHYLOCOCCI INTERACTION

Fig. 3. Binding and clumping of digestion products of $^{125}$I-fibrinogen during plasminolysis. Dotted bars represent binding; open bars represent clumping. At specified time intervals proteolysis was terminated with e-aminocaproic acid (0.2 M final concentration) and samples were taken for analysis in SDS-PAGE using 7.5% gels and for determination of binding and clumping activities. At time 0, the band at the cathode represents fibrinogen; at the 3-mm interval corresponding to stage 1, fragment X is present at the cathodic end and a few lower molecular weight species are visible. At 30 mm, corresponding to stage 2, fragments Y, D, and E are visible, in addition to fragment X. Finally, at 240 mm, corresponding to stage 3, predominantly fragments D and E are seen. Samples of digest were analyzed for binding and clumping as described in the notes to Table 1. Clumping titer on the ordinate expressed as a base 2 logarithm.

Specificity of the binding phenomena of the staphylococcal receptor was verified by control experiments in which the strain Zak of S. epidermidis was employed. This strain does not possess the staphylococcal receptor for fibrinogen and thereby does not give a clumping reaction. Binding experiments done with clumping negative staphylococci revealed that at least 90% of each chain remained unbound in the supernatant after addition of the strain Zak, indicating essentially no binding of any polypeptide chain subunit of human fibrinogen.

Effect of plasmin digestion on the binding region for the staphylococcal receptor of human fibrinogen. Figure 3 shows the course of digestion of $^{125}$I-fibrinogen by plasmin. By using $^{125}$I-fibrinogen it was possible to follow the digestion process not only by measuring the clumping activity but also by radioactivity counting to determine the ability of the digest to bind to staphylococci due to the presence of the binding region on degradation products. After 3 min of digestion, which corresponded to the first stage of digestion, characterized primarily by the appearance of fragment X, the binding to staphylococci was observed. Finally, after 240 min of digestion (stage 3), in which almost exclusively fragments D and E were present, negligible binding to staphylococci (6%) and no ability to clump could be demonstrated in the digest.

Binding region for the staphylococcal receptor on isolated fragments of human fibrinogen: comparison with clumping activity. An analysis of binding and clumping of staphylococci by isolated fragments of the fibrinogen molecule is given in Table 2. In agreement with the analysis of plasmin digest, binding of staphylococci was observed with species of high molecular weight, such as fragments X (stage 1) and X (stage 2). Early fragment D (stage 2) showed low bind-
Table 2. Binding and Clumping of Staphylococci by Isolated Fragments of Human Fibrinogen

<table>
<thead>
<tr>
<th>Fragment</th>
<th>Binding (%)</th>
<th>Clumping (Titer)</th>
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<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td></td>
<td>Reduction</td>
<td>Carboxymethylation</td>
</tr>
<tr>
<td>Fragment X (stage 1)</td>
<td>49</td>
<td>45</td>
</tr>
<tr>
<td>Fragment X (stage 2)</td>
<td>39</td>
<td>32</td>
</tr>
<tr>
<td>Fragment D (stage 2)</td>
<td>21</td>
<td>10</td>
</tr>
<tr>
<td>Fragment D-D</td>
<td>31</td>
<td>13</td>
</tr>
<tr>
<td>Fragment D (stage 3)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Fragment E (stage 3)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>N-terminal disulfide knot</td>
<td>0</td>
<td>0</td>
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</table>

Details of binding and clumping determinations are described in the notes to Table 1 and in Materials and Methods.

ing, and late fragments D (stage 3) and E demonstrated no binding at all. Along with the evidence presented in Fig. 3, it can be seen from Table 2 that the ability to clump staphylococci paralleled the degree of binding of nonreduced fragments. The highest values were observed in the case of fragment X (stage 1). It can be seen further that reduction of isolated fragments X affected their binding capacity only slightly but abolished their clumping ability, a phenomenon also noted in the case of reduced fibrinogen (Table 1). Plasmin-resistant fragment E did not show binding or clumping of staphylococci. Likewise, isolated N-terminal disulfide knot (NDSK) did not possess the binding site for staphylococci. The fragment D-D obtained from crosslinked fibrin showed slightly higher binding than that of fragment D (stage 2) derived from fibrinogen but no ability to clump staphylococci.

Characteristics of the interaction between staphylococci and human fibrinogen. The staphylococcal receptor (clumping factor) is distinct from staphylocoagulase, another product of staphylococci, which activates prothrombin and thus clots fibrinogen. However, the possibility that the staphylococcal receptor acts through enzymatic cleavage of fibrinogen in a manner analogous to staphylocoagulase has not been ruled out. Since staphylocoagulase (or staphylothrom-
bin) is inhibited by DFP,\textsuperscript{24} this inhibitor was used at $1 \times 10^{-5} M$, which abolished the enzymatic activity of staphylothrombin as well as thrombin used as a control. DFP did not inhibit the staphylococcal clumping reaction with human fibrinogen, indicating that the staphylococcal clumping reaction does not involve the activity of serine protease.

**Effect of pH.** Figure 4 shows the effect of pH on the interaction of human fibrinogen. Optimal pH for the reaction was between 6 and 8, with a peak at pH 7. Conditions below pH 6 and above pH 9 were distinctly inhibitory. However, this inhibitory effect of pH could be totally reversed by readjusting the pH to 7, as shown by the dotted line. After readjustment, results varied within two dilutions at some pH values, the acceptable range for a method employing the two-fold dilution technique.

**Divalent ions and charged polymers.** The role of divalent ions in the interaction between staphylococci and human fibrinogen was assessed by adding the chelator EDTA in concentrations of $5 \times 10^{-3}$ to $5 \times 10^{-2} M$ to the reaction mixture buffered to pH 7.4 with Tris-HCl buffer. The titer of the staphylococcal clumping reaction remained unchanged in comparison with the control, indicating that divalent ions are not essential for the interaction of staphylococci with human fibrinogen.

The effect of negatively charged polymers was examined in regard to the possibility that the interaction between staphylococci and fibrinogen could be inhibited by polyanions. The results indicated that heparin (15 and 150 USP units/ml), chondroitin sulfate (1 and 10 mg/ml), and sodium polyanethole sulfonate (1 and 10 mg/ml) were not inhibitory.

**Role of hydrogen bonding and hydrophobic interactions.** The interaction between staphylococci and human fibrinogen resulting in clumping of staphylococci was examined in regard to the effect of compounds known to break hydrogen bonds and hydrophobic interactions. Whereas the reaction occurred in $3 M$ urea, $8 M$ urea caused a fourfold decrease in titer. Either $5 M$ guanidine hydrochloride or $0.02 M$ SDS caused complete disappearance of clumps. These results indicate that hydrophobic interactions play a role in this phenomenon.

**Interaction of staphylococci with abnormal variants of human fibrinogen.** Having obtained results indicating that the binding region of staphylococci is located on those portions of the Aα and Bβ chains of human fibrinogen that do not involve the N-terminal region (both N-terminal disulfide knot and fragment E were negative), we examined the interaction of staphylococci with abnormal variants of human fibrinogen. It was reasoned that if the N-terminal part of the

<table>
<thead>
<tr>
<th>Fibrinogen Studied</th>
<th>Staphylococcal Clumping Titer*</th>
<th>Thrombin Clotting Time (sec)†</th>
<th>Reptilase Clotting Time (sec)‡</th>
</tr>
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<tbody>
<tr>
<td>Normal (Control)</td>
<td>5120</td>
<td>14</td>
<td>21</td>
</tr>
<tr>
<td>Detroit</td>
<td>5120</td>
<td>&gt;999</td>
<td>&gt;999</td>
</tr>
<tr>
<td>Paris I</td>
<td>5120</td>
<td>&gt;999</td>
<td>&gt;999</td>
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*Serial dilutions of the tested plasma were mixed with a standardized suspension of staphylococci containing clumping factor. Indicated titer was the highest dilution giving a positive reaction.
†Time obtained after adding 1 NIH unit of thrombin.
‡Time obtained after adding 1 mg of reptilase.
fibrinogen molecule were not involved in the interaction with staphylococci, then fibrinogen variants with a molecular defect in the N-terminal portion, e.g., fibrinogen Detroit,25 should be able to interact with staphylococci. Likewise, if the γ chain had no binding region for staphylococci, then fibrinogen variants with a defect in the γ chain, e.g., fibrinogen Paris I,26,27 should be able to interact with staphylococci. As can be seen from Table 3, fibrinogen Detroit and fibrinogen Paris I interacted with staphylococci as measured by the staphylococcal clumping test. Titers obtained with these abnormal variants were identical with the normal control fibrinogen, whereas the clottability with thrombin and reptilase of these variants was greatly impaired. Thus in spite of the molecular defects involving the N-terminal portion of the Aα chain and the γ chain, the binding region for staphylococci remained intact on abnormal fibrinogen variants. This observation supports the evidence presented above that the N-terminal portion of the Aα chain and the γ chain are not involved in the interaction with the staphylococcal receptor.

**DISCUSSION**

*Interaction of staphylococci with normal human fibrinogen.* From the studies described here and those reported earlier,8,9,10 it is possible to determine how staphylococci interact with human fibrinogen. Examination of the interaction of human fibrinogen with staphylococci by means of binding studies and clumping titer determinations revealed four major points: (1) Human fibrinogen is effective, even in extremely small amounts, in causing the clumping of staphylococci. Two molecules of fibrinogen for each staphylococcus are sufficient to cause staphylococci to clump. (2) Under conditions employed in this study the binding of over 90% of the fibrinogen molecules to staphylococci was observed. The remainder represents either nonfibrinogen proteins present in the preparation used or fibrinogen molecules devoid of binding properties. (3) The binding region for the staphylococcal receptor is localized on the Aα and Bβ polypeptide chains of human fibrinogen beyond the N-terminal disulfide knot. (4) The interaction between human fibrinogen and staphylococci appears not to involve a serine protease type of enzymatic activity, since it proceeds in the presence of DFP, which inhibits staphylococcal, another product of staphylococci inducing, in complex with prothrombin, the enzymatic attack on fibrinogen.

Furthermore, elution of fibrinogen bound to staphylococci revealed an essentially unchanged mobility in SDS-PAGE, indicating a lack of significant proteolytic degradation during the interaction with staphylococci.

In order to examine the mechanism of this interaction, data from experiments with modified and degraded fibrinogen will be discussed.

*Interaction of staphylococci with modified fibrinogen.* Dissociation of fibrinogen into polypeptide chain subunits by reduction and carboxymethylation resulted in a loss of the ability of fibrinogen to clump staphylococci, indicating that there is little intrinsic activity in separated polypeptide chains to clump staphylococci. This finding is in accord with the previous observation of Stemberger and Hormann.28 However, by using 125I-fibrinogen we were able to demonstrate that two-thirds of the polypeptide chains were bound to staphylococci and that those bound were Aα and Bβ chains.
The failure of separated chains to cause clumping of staphylococci, while retaining in full their ability to bind staphylococci, indicates that most probably it is the reduction of disulfide bonds in the fibrinogen molecule that destroys the ability to clump staphylococci, i.e., to link at least two staphylococci together. Since disulfide bonds maintain the dimeric structure of fibrinogen, it is logical to postulate that the dimeric structure of fibrinogen is not needed for binding but contributes to clumping of staphylococci. Consequently, the clumping phenomenon, like agglutination of bacteria by antibodies, would require “divalent” fibrinogen to cause clumping, which means that there should be at least one binding site in each “half molecule” as a prerequisite for clumping. Reduced and carboxymethylated $\alpha$ and $\beta$ polypeptide chains, which can bind but do not clump staphylococci, can therefore possess only a single binding region each.

**Localization of the binding region for staphylococci based on their interaction with fragments of fibrinogen.** The binding region for staphylococci appears to be located on the C-terminal part of the $\alpha$ chain and $\beta$ chain of the native molecule of human fibrinogen. The following facts support this contention: During plasmin digestion of human fibrinogen we observed rapid reduction in binding as well as in clumping reactivity. The C-terminal portion of the $\alpha$ chain is most susceptible to plasmin digestion, and in addition to a very early splitting of the 27-residue peptide a sizable part of the $\alpha$ chain, representing altogether fragments corresponding to molecular weights of 40,000-50,000 daltons, is removed. Since this degradation is accompanied by a 2.5-fold decrease in binding and an eightfold decrease in clumping, we can deduce that the binding region on the $\alpha$ chain is located in the carboxyl terminal portion, probably within the last 400 residues of the amino acid sequence ending with the carboxyl-terminal valine.

The contribution of the $\beta$ chain to the binding region for staphylococci on human fibrinogen is less clear. The $\beta$ chain interacted with staphylococci to a lesser degree than $\alpha$ chain, suggesting that the $\beta$ chain has a lower affinity for staphylococci. The N-terminal portion of the $\beta$ chain, which is split off during early plasmin digestion, cannot account for binding to staphylococci; this portion of the $\beta$ chain is present in the N-terminal disulfide knot that does not exhibit binding or clumping. On the basis of binding and clumping reactivity of fragment D (stage 2), isolated from an early stage of plasmic digestion, it appears that the interaction of fragment D (stage 2) with staphylococci is due to the preserved C-terminal portion of the $\beta$ chain, as reported by Takagi and Doolittle. However, further digestion with plasmin, resulting in the smaller fragment D (stage 3), is accompanied by the complete loss of its ability to interact with staphylococci.

Although the binding assay we used is not so highly accurate that we would feel comfortable attaching a value to the relatively small amount of bound radioactivity (21%) associated with fragment D (stage 2), there is little doubt that the changes occurring in fragment D, in regard to the interaction with staphylococci, are important for analysis of the binding region of the $\beta$ chain. Since the binding region remains only in the $\beta$ chain, at least two possibilities can be considered for the explanation of the loss of this function and warrant...
further investigation, (1) enzymatic degradation of the Bβ chain as suggested by Mosesson and Finlayson, and (2) changes in the structure of fragment D that may occur as the result of the plasmic cleavage of the carboxyl-terminal portion of the γ chain remnant. It thus appears that like the antibody molecule that agglutinates bacteria through the contribution of light and heavy chains to the antigenic binding site in the Fab region the fibrinogen molecule and its derivatives attain the best binding and clumping capacity when both chains, Aα and Bβ, are present.

Significance of the interaction of human fibrinogen with staphylococci. The very high sensitivity of the interaction between staphylococci and fibrinogen is expressed in the fact that two molecules of fibrinogen per staphylococcal organism are sufficient to cause detectable clumping, making staphylococcal clumping eminently suitable for detection of fibrinogen and its derivatives in biologic fluids other than blood and within cells such as granulocytes and platelets. The localization of the binding site beyond the N-terminal region of the fibrinogen molecule makes it possible to measure soluble fibrin monomers separated from plasma by gel chromatography. Furthermore, localization of the binding region beyond the N-terminal disulfide knot on the Aα and Bβ chains makes it possible to apply the staphylococcal clumping phenomenon for measurement of abnormal variants of human fibrinogen. Defects in abnormal fibrinogen molecules are manifested by slow release of fibrinopeptides or slow polymerization. In the case of fibrinogen Detroit, this is attributed to changes in the N-terminal region of the Aα chain, identified as molecular substitution, and in the case of fibrinogen Paris I, this is attributed to changes in the γ chain. Neither fibrinogen Detroit nor fibrinogen Paris I has an impaired ability to interact with staphylococci. The latter finding is consistent with the lack of involvement of the γ chain in the interaction with staphylococci. In addition to the abnormal variants of fibrinogen examined in our laboratory, fibrinogen New York also shows an unimpaired ability to interact with staphylococci.

The molecular basis of staphylococcal clumping is primarily attributable to the presence of binding regions on human fibrinogen and to the dimeric structure of the molecule. The binding region for staphylococci on human fibrinogen represents a very unique structural and functional feature of the fibrinogen molecule. It offers an attractive model to explain the interaction of human fibrinogen with other microorganisms such as streptococci. It is striking that both staphylococci and streptococci have a special predilection to seed on intravascular surfaces covering heart valves and vascular prostheses. Fibrinogen, by the virtue of binding to bacterial cells, may facilitate formation and spread of infected microemboli in the vascular bed.

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Interaction of human fibrinogen with staphylococci: presence of a binding region on normal and abnormal fibrinogen variants and fibrinogen derivatives

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