Identification of a Region of Human Fibrinogen Interacting with Staphylococcal Clumping Factor†

J. Hawiger,* S. Timmons, D. D. Strong, B. A. Cottrell, M. Riley, and R. F. Doolittle

ABSTRACT: Human fibrinogen possesses cell agglutinating properties and interacts with a cell wall component of certain staphylococci, inducing their clumping. This property of fibrinogen is related to its binding to staphylococcal cell wall sites through an unidentified portion of the fibrinogen molecule. On an average, 2130 fibrinogen molecules bind per one cell, with a dissociation constant of 9.9×10^{-9}. The localization of the binding region for the staphylococcal clumping factor on human fibrinogen was investigated by employing isolated portions of fibrinogen and its chains and by using monovalent antibody fragments specific for distinct structural domains of fibrinogen. The binding of ^{125}I-labeled fibrinogen to staphylococci and the concomitant clumping reaction were blocked by Fab antibody against fibrinogen and against fragment D whereas antibody Fab fragments against fragment E and against the middle section of the α chain (residues α241–476) were without effect. The blocking effect of Fab antibody against fragment D was completely reversed by purified fragment D_1 (M_r 90000) but not by purified fragment D_3 (M_r 80000). Furthermore, isolated fragment D_1 inhibited the binding of ^{125}I-labeled fibrinogen to staphylococci and the concomitant clumping reaction whereas fragment D_3 was inactive. Direct binding of ^{125}I-labeled fragment D_1 to staphylococci was 6-fold greater than the binding of ^{125}I-labeled fragment D_3. Amino-terminal sequences of both fragments were identical, thus indicating that the main difference was due to the apparent proteolytic loss of 109 residues at the carboxy-terminal end of the γ chain. Isolated γ chains, but not α and β chains, reversed the blocking effect of antibody Fab on the clumping reactivity of fibrinogen. Moreover, isolated γ-chain multimeric suspension effected a positive clumping reaction which was abolished after treatment with plasmin and staphylococcal protease, α-chain reactivity was 150-fold weaker, and β chain was inactive. α-chain fragments, representing the middle and carboxy-terminal zones overlapping the plasmin-sensitive region (residues α220–229 and α241–424), did not interfere with the blocking effect of Fab antibody against fibrinogen and against fragment D, whereas residues α165–441 containing a portion of fragment D domain partially reversed the blocking effect of Fab antibodies. It is concluded that the carboxy-terminal segment of the human fibrinogen γ chain has the main binding site for the staphylococcal clumping factor.

Although the main function of plasma fibrinogen is its enzymatic transformation into a fibrin clot upon the action of thrombin (Doolittle, 1973), it also participates in a number of important nonenzymatic cell surface interactions. These interactions include staphylococci, streptococci, platelets, and macrophages (Much, 1908; Duthie, 1955; Hawiger et al., 1975; Mustard et al., 1978; Hawiger et al., 1979; Colvin & Dvorak, 1975). The affinity of fibrinogen toward a cell wall component of staphylococci seems to be very high; indeed, clumping of the staphylococci can be observed when only 20 molecules of human fibrinogen per one staphylococcus are present (Hawiger et al., 1978).

Recent establishment of the complete covalent structure of human fibrinogen including identification of about 50 plasmin attack points (Henschen & Lottspeich, 1977a; Watt et al., 1978; Doolittle et al., 1979) prompted us to undertake a more extensive study of the localization of the fibrinogen site which interacts with the staphylococcal clumping factor. This report describes our analysis of the structural and functional attributes of the fibrinogen molecule in regard to its interaction with the clumping factor of staphylococci. The results of this investigation may also be of relevance for other instances of fibrinogen interactions with cell surfaces.

Materials and Methods

Human fibrinogen was prepared from blood plasma (San Diego Blood Bank) according to a previously described cold ethanol precipitation procedure (Doolittle et al., 1976). Alternatively, commercially available human fibrinogen (grade L, Kabi, Stockholm, Sweden) was used in some experiments.

Preparation and Purification of Polypeptide Chains of Human Fibrinogen. Human fibrinogen was dissolved in 6 M guanidinium chloride–0.2 M Tris,[1] pH 8.2, containing 0.01 M dithiothreitol (Sigma), reduced and alkylated, and chromatographed on CM-cellulose as previously described (Cottrell & Doolittle, 1976; Doolittle et al., 1977a).

Preparation of Polypeptide Chain Fragments. The α-chain fragment containing residues 241–476 (αCNI) was obtained after cyanogen bromide cleavage of α chains (Doolittle et al., 1977a). The α-chain fragment containing residues 165–441 (α-chain 277) was isolated after cleavage of cysteine bonds in the α chain by cyanylation with 2-nitro-5-(thiocyano)benzoic acid (Degani & Patchornik, 1974); the α-chain fragment containing residues 241–424 (αPLI) was obtained after plasmin digestion of fragment αCNI (Strong et al., 1979). An α-chain fragment containing residues 220–229 was synthesized in a solid phase system according to established Merrifield procedures (Stewart & Young, 1969).

Preparation of Fibrinogen Fragments. Human fibrinogen fragments D and E were prepared by plasmin digestion of

† From the Departments of Pathology and Medicine, Vanderbilt University, Nashville, Tennessee 37232, and the Department of Chemistry, University of California at San Diego, La Jolla, California 92037. *Received August 11, 1981.* This work was supported by research grants from the National Institutes of Health, U.S. Public Health Service: HL-25,935, HL-08,338, HE-12,759, HE-18,576, and GM-17,702.

* Address correspondence to this author at the Department of Pathology, Vanderbilt University.

[1] Abbreviations: Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; CM, carboxymethyl; DEAE, diethylaminoethyl; CNBr, cyanogen bromide; $NaDodSO_4$, sodium dodecyl sulfate; TATG, (thioacetyl)thioglycolic acid; IgG, immunoglobulin G; EDTA, ethylenediaminetetraacetic acid.

0006-2960/82/0421-1407$01.25/0

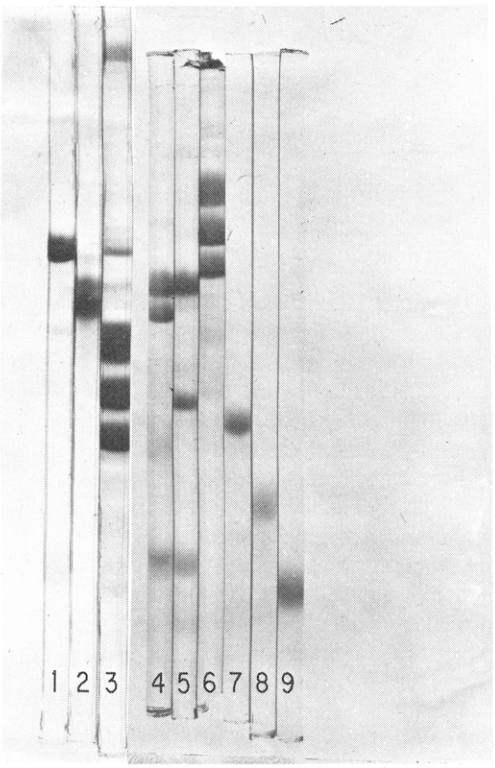

FIGURE 1: Sodium dodecyl sulfate gel (5% in 1–3 and 7.5% in 4–9) electrophoresis of isolated fragments D_1 and D_3 of human fibrinogen: (1) fragment D_1 (nonreduced), (2) fragment D_3 (nonreduced), (3) fibrinogen (reduced), (4) fragment D_1 (reduced), (5) fragment D_3 (reduced), (6) fibrinogen (reduced), (7) chymotrypsin, (8) myoglobin, and (9) cytochrome *c*. Gels were stained with Coomassie Blue.

fibrinogen as described previously (Takagi & Doolittle, 1975a; Doolittle et al., 1977b). Some modifications have been introduced, including the presence of calcium during the digestion (Haverkate & Timan, 1977). In brief, plasmin (Kabi, Stockholm, Sweden) in 50% glycerol (10 C.T.A. units/mL) was mixed with human fibrinogen (10 mg/mL) in 0.15 M NaCl–0.05 M Tris buffer (pH 7.5) in a ratio of 1:15 (v/v). Digestion was carried out at 37 °C for 6 h in the presence of 0.002 M $CaCl_2$ and then for 3 h under dialysis conditions against the starting buffer for DEAE chromatography, except with 0.002 M $CaCl_2$. The digest was chromatographed on DEAE-cellulose by using a modified system based on the original procedure of Nussenzweig et al. (1961). Separated peaks were pooled, dialyzed, and freeze-dried. $NaDodSO_4$–polyacrylamide (5.0%) gel electrophoresis of obtained fragments indicated that fragment D_1 had an apparent M_r of 90000 and fragment D_3 had an apparent M_r of 80000 (Figure 1). The subunit chain composition of fragments D_1 and D_3 after reduction revealed the following apparent M_r values: for fragment D_1, α chain 10000–12000, β chain 42000, and γ chain 38000; for fragment D_3, α chain 10000, β chain 42000, and γ chain 25000. A full characterization of these two species of fragment D will appear elsewhere.[2] Fragment E had an apparent M_r of about 50000.

Amino-Terminal Group Analysis. The amino-terminal sequences of different fragments were determined with the TATG acetylation procedure introduced by Mross & Doolittle (1971) as modified by Takagi & Doolittle (1974).

Labeling of Fibrinogen and Its Fragments with ^{125}I. This was done by using the monochloride method of McFarlane (1963) as described previously (Hawiger et al., 1978). The specific radioactivity of labeled fibrinogen was an average 5 $\times 10^7$ cpm/mg of protein, and 95% radioactivity was clottable with thrombin. The clumping reactivity of ^{125}I-labeled fibrinogen was the same as that of unlabeled protein.

Preparation of Antibody Fab Fragments against Fibrinogen and Its Fragments. Rabbits were immunized against purified human fibrinogen, fragment D, fragment E, and a cyanogen bromide fragment from α chain designated αCNI (residues α241–476). Antigens were suspended in complete Freund's adjuvant and administered both subcutaneously and intramuscularly over the course of 6–8 weeks. The rabbits were completely exsanguinated and the sera stored at −20 °C.

In the case of fibrinogen, fragment D, and αCNI, monospecific antibodies were prepared by the method of Shainoff & Braun (1973). Antibodies to fragment E could not be prepared by this method because the antigen survives the low pH treatment and recombines with the antibody after neutralization. Antibodies to fragments E and D were prepared by affinity chromatography on a Sepharose 4B–fibrinogen column prepared according to the procedure described by Heene & Mathias (1973). In each preparation, 10 mL of antiserum was passed over a column containing 10 mL of packed Sepharose with 100 mg of immobilized fibrinogen. The columns were washed extensively at that point with phosphate-buffered saline, and then the antibodies were eluted with 8 M urea. The eluted protein was dialyzed extensively against phosphate-buffered saline before precipitation with ammonium sulfate. The precipitates were stored at −20 °C until needed. The specificity of antibodies was examined. Anti-fragment D antibodies were not reactive with isolated fragment E used in these experiments, and anti-fragment E antibodies were not reactive with isolated fragments D in the double immunodiffusion system of Ouchterlony (1958).

Fab fragments were prepared according to the general procedures described by Porter (1959). The purified antibodies were suspended in 0.05 M sodium acetate buffer, pH 5.5, containing 0.01 M cysteine and 0.001 M EDTA. Papain was added to a final concentration of 0.1 mg/mL, and the mixture was incubated for 2 h at 37 °C. Digestion was stopped by the addition of iodoacetamide and raising the pH to 8.5. Digests were dialyzed against phosphate-buffered saline and stored frozen. Antibody Fab fragments were separated from undigested IgG and from the Fc fragment by absorbing the mixture of antibody fragments with protein A positive staphylococci (IgGsorb, The Enzyme Center, Inc., Boston, MA). A 10% (w/v) cell suspension of IgGsorb was sonicated until homogeneity. One milliliter of this suspension was combined with 1 mg of purified papain-digested IgG and incubated at room temperature for 30 min with occasional stirring. After centrifugation at 10000*g* for 5 min, the supernatant containing Fab fragments was collected and stored at −20 °C. Concentration of Fab fragments was determined spectrophotometrically at A_{280}.

Preparation of Staphylococci Possessing and Lacking Clumping Factor. Staphylococcus aureus Newman D_2C variant, clumping factor positive, was prepared as a nonviable, freeze-dried preparation as previously described (Hawiger et al., 1978). *Staphylococcus epidermis* Zak, clumping factor negative, was prepared in a manner analogous to *S. aureus* Newman D_2C.

Staphylococcal Clumping Assay To Measure Interaction of Fibrinogen and Its Fragments with Staphylococci. The assay system consisted of measurement of clumping (agglutination) of staphylococci in serially diluted solutions of fi-

[2] B. A. Cottrell, D. D. Strong, and R. F. Doolittle, unpublished experiments.

brinogen or its fragments (Hawiger et al., 1978). Each assay was run with both preparations of staphylococci to check for nonspecific clumping or agglutination. None of the material assayed in this study gave a positive clumping reaction when tested with Zak cells (lacking a clumping factor). Results were expressed as a titer representing the highest dilutions giving positive clumping reaction. If only one concentration of fibrinogen was used and comparison was made of the inhibitory effect of antibody fragments, results were graded from 4 (strongest reaction) to 0 (negative reaction).

Binding Assay. Following our previous investigation (Hawiger et al., 1978), a steady-state binding system was designed which allowed us to determine simultaneously the basic parameters of binding and clumping reactions. For these studies, ^{125}I-labeled human fibrinogen and its fragments (containing tyrosine) were used. Unless otherwise stated, dilutions of ligand were made in 0.3 M NaCl buffered with 0.005 M sodium phosphate to pH 7.0 and containing 0.5% bovine serum albumin. A smooth suspension of freeze-dried staphylococci with clumping factor (10 mg/mL) was prepared in the same buffer and was added in $^1/_{10}$ volume in regard to the ligand. In parallel, a similarly prepared suspension of staphylococci without clumping factor (Zak strain) was run. After 2-min shaking, the mixture was incubated for 3 min, and the titer of the clumping reaction was recorded. The incubation mixtures were applied on top of an Apiezon Oil C/*n*-butyl phthalate [1:9 (v/v)] mixture (0.5 mL) in conical Eppendorf tubes which were immediately spun down at 8000*g* for 5 min. Samples of the supernatant were collected for measurement of unbound radiolabeled ligand. The remaining content of the tube was frozen in dry ice–acetone mixture, and the tips of the tubes containing immobilized pellets were separated with the hot blade of a utility knife. Pellets were transferred to a counting vial, and the radioactivity bound to cells was measured in a Searle automated γ counter. The amount of radioactivity present in the system with staphylococcal cells without clumping factor as well as background radioactivity was subtracted from the values obtained in the system containing staphylococcal clumping factor.

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. Throughout these experiments, 5% or 7.5% gels containing 0.1% $NaDodSO_4$ were used. Electrophoresis was performed according to Weber & Osborn (1969), as described previously (Doolittle et al., 1977b; Hawiger et al., 1978). The material bound to staphylococci was analyzed in pellets obtained after the oil centrifugation step. Pellets were suspended in 1% $NaDodSO_4$ in 8 M urea with 0.1% 2-mercaptoethanol, and the material was applied to the $NaDodSO_4$–polyacrylamide gel electrophoresis system.

Results

Affinity of Human Fibrinogen for Staphylococci. In the initial experiments, we measured the binding of ^{125}I-labeled human fibrinogen to staphylococci, in order to determine the number of fibrinogen molecules bound at equilibrium to staphylococcal cell wall sites. In the range of fibrinogen concentrations used in these experiments, binding reached equilibrium within 1–2 min, and in most experiments 5-min incubations were performed. We found that correction was necessary for nonspecific binding of ^{125}I-labeled fibrinogen to staphylococci. In subsequent experiments, parallel determinations were performed by using staphylococci which gave positive clumping reaction with fibrinogen and staphylococci lacking a cell wall component and giving negative clumping reaction. We corrected each value for ^{125}I-labeled fibrinogen bound by subtracting the value obtained in a duplicate incubation mixture containing clumping negative staphylococci. The magnitude of correction for nonspecific binding varied from 10% to 20% of total ^{125}I-labeled fibrinogen bound. A similar degree of nonspecific binding was observed when ^{125}I-labeled fibrinogen was added to staphylococci with a clumping factor in the presence of 20–50-fold molar excess of unlabeled fibrinogen.

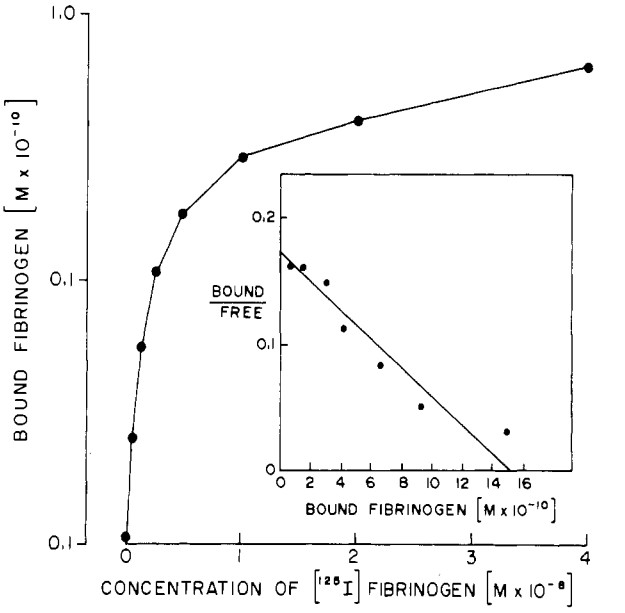

FIGURE 2: Steady-state binding of ^{125}I-labeled human fibrinogen to staphylococci. Reaction mixtures contained 5 × 10^8 freeze-dried bacterial cells and varying amounts of ^{125}I-labeled fibrinogen. Bound ^{125}I-labeled fibrinogen was determined as described under Materials and Methods. Each point represents "specific" binding computed after subtracting binding to clumping factor negative bacteria (Zak strain) from total binding to clumping positive strain Newman D_2C. The time of incubation was 5 min, and all studies were performed at room temperature (t = 22 °C). (Inset) Scatchard plot of specific binding of ^{125}I-labeled fibrinogen to staphylococci. Correlation coefficient for the plot is 0.998.

For determination of the number of fibrinogen molecules bound at equilibrium to staphylococci and the affinity constant, binding data were expressed in a Scatchard plot (Scatchard, 1949). From a number of similar experiments, we determined that staphylococci bind (M ± SD) 2130 ± 48 molecules of fibrinogen per cell with a dissociation constant (K_d) of 9.6 × 10^{-9} ± 0.02 × 10^{-9} (see Figure 2).

It has been previously observed that degradation of fibrinogen with plasmin rapidly destroys the clumping reactivity of human fibrinogen (Hawiger et al., 1970). We measured the binding of fibrinogen at different stages of limited digestion with plasmin and compared the binding with the ability of digested fibrinogen to clump staphylococci. Binding decreased rapidly during the first 3–10 min and essentially paralleled a decrease in the clumping titer (from 512 to 64). Between 10 and 240 min of plasmin action, the binding remained within the same reduced range, whereas the clumping titer was negative (results not shown). Thus, the decrease in the clumping activity of fibrinogen closely paralleled a decline in binding.

Inhibition of the Interaction between Human Fibrinogen and Staphylococcal Clumping Factor by Fab Antibody Fragments Directed against Different Regions of the Fibrinogen Molecule. The following Fab antibody fragments were tested for their inhibitory effect on the interaction with staphylococci: anti-human fibrinogen, anti-α241–476 (directed against the middle zone of the α chain), anti-fragment D, and anti-fragment E. In addition, Fab fragments prepared from

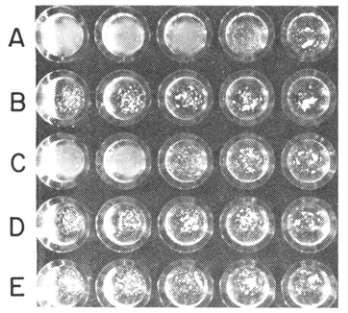

FIGURE 3: Effect of Fab antibody fragments on reactivity of fibrinogen with staphylococci. Fibrinogen (1.9 μg) was incubated for 30 min at 37 °C with Fab antibody fragments in different dilutions and tested for clumping reactivity with staphylococci on microtiter plates. (A) Anti-fibrinogen Fab antibody + fibrinogen; (B) anti-fragment E Fab antibody + fibrinogen; (C) anti-fragment D Fab antibody + fibrinogen; (D) anti-α-chain fragment α241–476 + fibrinogen; (E) nonimmune Fab + fibrinogen. Wells contain dilutions of Fab fragments from undiluted to 1:16. Note inhibition of clumping reaction in (A) and (C) and normal reaction in (B), (D), and (E).

Table I: Effect of Antibody Fab Fragments and of Isolated Fibrinogen Fragments D_1 and D_3 on Binding of ^{125}I-Labeled Fibrinogen to Staphylococci and on Clumping Reaction

system[a]	^{125}I-labeled fibrinogen binding (% inhibition)	clumping reaction
nonimmune Fab + ^{125}I-labeled fibrinogen	0	4
anti-fibrinogen Fab + ^{125}I-labeled fibrinogen	83	0
anti-fragment D Fab + ^{125}I-labeled fibrinogen	79	0
anti-fragment E Fab + ^{125}I-labeled fibrinogen	0	4
buffer + staphylococci + ^{125}I-labeled fibrinogen	0	4
fragment D_1 + staphylococci + ^{125}I-labeled fibrinogen	77	2
fragment D_3 + staphylococci + ^{125}I-labeled fibrinogen	0	4

[a] Antibody Fab fragments (150 μg) were preincubated with ^{125}I-labeled fibrinogen (4 μg) for 30 min at 37 °C. The incubation mixture was added to staphylococci and assayed for binding to staphylococci in a steady-state system (see Materials and Methods) and for clumping reactivity. Purified fragments D_1 (360 μg) and D_3 (300 μg) were added to staphylococci prior to the addition of ^{125}I-labeled fibrinogen. The cells were incubated for 2 min with constant shaking and assayed as above.

the IgG fraction of nonimmunized rabbit serum were tested (nonimmune Fab). Only anti-fibrinogen and anti-fragment D antibodies blocked the clumping of staphylococci by 1.9 μg of fibrinogen (Figure 3). Maximal inhibitory effect of Fab fragments was observed at a concentration of 80 μg, which corresponds approximately to 140 monovalent Fab fragments per half-fibrinogen molecule. When Fab antibody against fragment D was adsorbed with and eluted from fragment D prior to use in blocking experiments, the ratio of Fab fragments sufficient to block the interaction of fibrinogen with staphylococci was no more, and probably less, than 11 specific Fab fragments per half-molecule. Inhibition of clumping reactivity by antibody fragments was paralleled by their blocking of binding of ^{125}I-labeled fibrinogen to staphylococci (Table I).

Interaction of Fragment D of Fibrinogen with Staphylococci. The striking inhibition of the interaction of fibrinogen with staphylococci by Fab antibody fragments directed against fragment D prompted us to investigate two species of fragment D: fragment D_1 (M_r 90 000) and fragment D_3 (M_r 80 000)

Table II: Amino-Terminal Amino Acids Recovered from Three-Step Degradation of Fragments D_1 and D_3

fragment	TATG degradation cycle: first residue	second residue	third residue
D_1	Asx	Asx	
	Asx	Asx	Glx
		Arg	Lys
D_3	Asx	Asx	
	Asx	Asx	Glx
		Arg	Lys

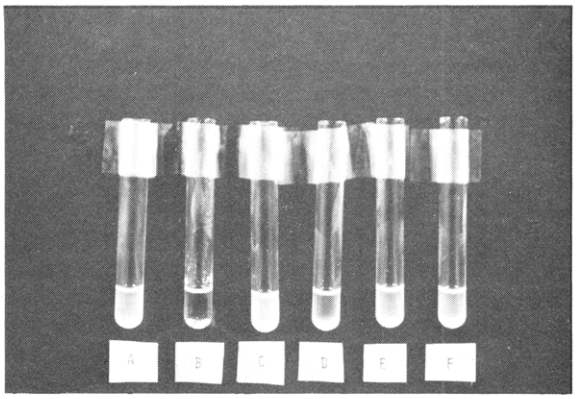

FIGURE 4: Reversal of immunoinhibition of clumping reactivity of fibrinogen by isolated fragments D_1 and D_3 as compared to α-chain fragments. Isolated fragments D_1 and D_3 in concentration of 100 μg were preincubated with anti-fragment D Fab antibody (10 μg); isolated α-chain fragments (50 μg) were preincubated with anti-fibrinogen Fab antibody (20 μg) for 30 min at 37 °C, and then fibrinogen (3 μg) was added for an additional 30-min incubation. The mixture was then assayed for clumping reactivity of fibrinogen with staphylococci. (A) Anti-fragment D antibody preincubated with buffer; (B) anti-fragment D antibody preincubated with isolated fragment D_1; (C) anti-fragment D antibody preincubated with fragment D_3; (D) anti-fibrinogen antibody preincubated with α165–441 fragment; (E) anti-fibrinogen antibody preincubated with α241–424 fragment; (F) anti-fibrinogen antibody preincubated with α220–229 fragment. Note positive clumping reaction only in (B) where fragment D_1 reversed the inhibitory effect of anti-fragment D antibody on fibrinogen.

(see Materials and Methods). The apparent differences in M_r between fragments D_1 and D_3 are primarily related to the loss of a sizable portion of γ chain (an approximate decrease in apparent M_r of 13 000; see Figure 1). Amino-terminal analysis (Table II) showed that fragments D_1 and D_3 have identical amino-terminal profiles, corresponding to Asp-105 of the α chain, Asp-134 of the β chain, and Ser-86 of the γ chain. Thus, proteolytic cleavage and loss of a sizable portion of the γ chain of approximately M_r 13 000 occurred at its carboxy-terminal region during the transition from fragment D_1 to fragment D_3.

Both fragments were incubated with anti-fragment D Fab antibody to see whether they would neutralize those Fab fragments that block the interaction of fibrinogen with staphylococci. Fragment D_1 fully reversed the blocking effect of anti-fragment D Fab on fibrinogen with regard to its ability to clump staphylococci (Figure 4). Fragment D_3 remained without measurable effect; i.e., it did not reverse the immunoinhibition of fibrinogen by anti-fragment D Fab antibody. Furthermore, it was possible to inhibit directly the binding of ^{125}I-labeled fibrinogen (1.9 μg/L) to staphylococci and the concomitant clumping reaction by adding unlabeled fragment D_1 to staphylococci (Table I). Fragment D_3 was without an effect. Experiments done with several isolated fragments of α chain and shown in Figure 4 indicated that fragments de-

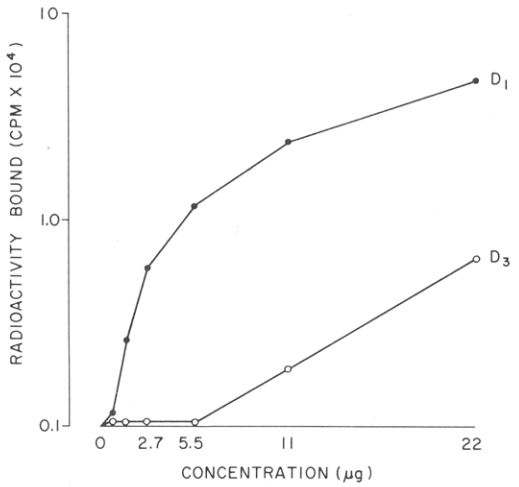

FIGURE 5: Steady-state binding of ^{125}I-labeled fragment D_1 (●) and ^{125}I-labeled fragment D_3 (○). Bound radioactivity was corrected for ^{125}I-labeled fragments bound to clumping negative staphylococci, and conditions of incubation were as described in the legend to Figure 2.

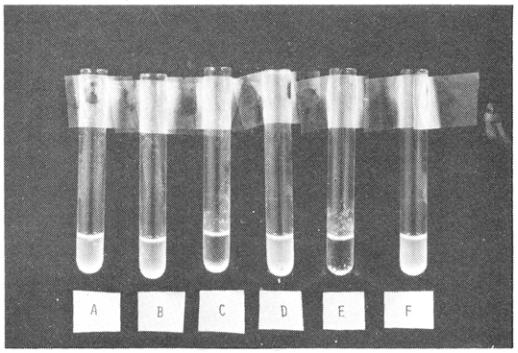

FIGURE 6: Reversal of immunoinhibition of clumping activity of fibrinogen by isolated chains. Anti-fragment D (9.0 μg) was used to inhibit the fibrinogen (3 μg) interaction with staphylococci; α, β, and γ chains in concentration of 100 μg were incubated with anti-D Fab for 30 min at 37 °C and then anti-D Fab was incubated with fibrinogen for 30 min at 37 °C. The mixture was then assayed for clumping activity of fibrinogen. (A) Anti-D Fab plus α chain; (B) anti-D Fab plus β chain; (C) anti-D Fab plus γ chain; (D) anti-D Fab plus buffer; (E) no anti-D Fab added to fibrinogen; (F) staphylococci in buffer without fibrinogen. Note positive clumping reaction in tube C where the γ chain reversed the inhibitory effect of anti-fragment D antibody on fibrinogen and in tube E where fibrinogen was not incubated with antibody.

rived from those portions of the α chain that are localized beyond the D domain (α241–424 and α220–229) were without an effect on anti-fibrinogen Fab antibody inhibition of fibrinogen interaction with staphylococci. A fragment which encompasses part of the α-chain sequence present in fragment D (α165–441) has some effect on anti-fibrinogen Fab antibody, partially reversing the blocking effect on the interaction of fibrinogen with staphylococci.

Direct binding studies were also performed by using fragments D_1 and D_3 labeled with ^{125}I (Figure 5). Assuming uniform labeling of both fragments and M_r values of 90 000 and 80 000, we calculated that approximately 6 times more fragment D_1 was bound to staphylococci (4.7×10^4 cpm per 5×10^8 cells) as compared to fragment D_3 (6.6×10^3 cpm per 5×10^8 cells). Material bound to staphylococci was solubilized and analyzed in a $NaDodSO_4$–polyacrylamide gel electrophoresis system. The main radioactive fractions bound to staphylococci corresponded to M_r of unbound ^{125}I-labeled fragment D_1 subunits. The radioactivity associated with fragment D_3 was not detectable in the solubilized pellet by using this system. Neither fragment induced positive clumping of staphylococci before or after labeling with ^{125}I. α-chain fragments (α241–424 and α165–441) were also labeled with ^{125}I. None of these fragments showed any specific binding to staphylococci, nor did the unlabeled fragment inhibit the binding of ^{125}I-labeled fibrinogen to the staphylococcal clumping factor. The smallest fragment (α220–229) was also tested in the binding system in 100–1000-fold molar excess, and it did not have any measurable effect on the binding of ^{125}I-labeled fibrinogen to staphylococci (results not shown).

Interactions of Polypeptide Chains of Fibrinogen with Staphylococci. Since the main differences between fragment D_1 and fragment D_3 were attributed to a sizable portion of the γ chain at the carboxy-terminal end (see Figure 1), we examined whether the γ chain would reverse the blocking effect of anti-fragment D Fab antibody toward fibrinogen interacting with staphylococci. The addition of γ chain to anti-fragment D Fab antibody neutralized those antibody molecules that were blocking the fibrinogen region interacting with the staphylococcal clumping factor, thus resulting in a positive clumping reaction (tube C in Figure 6). It should be noted that the mixture of the γ chain and anti-fragment D Fab antibody did not give a positive clumping reaction prior to the addition of fibrinogen. The addition of α and β chains to anti-fragment D Fab antibody did not reverse its blocking effect on fibrinogen (tubes A and B in Figure 6).

The question then arose whether γ chains could interact directly with staphylococci. Instead of solubilizing chains of fibrinogen in urea or other unfolding agents, isolated chains were dispersed in 0.05 M ammonium bicarbonate, pH 7.8, and sonicated to assure uniform suspension. The chains were prepared in equimolar concentrations, and serial dilutions of chain suspensions were tested for positive clumping reaction with staphylococci. In five experiments, the γ chain gave positive clumping reaction at concentrations of 140 nM, the β chain was negative at 85 μM, and the α chain was active at 21 μM (Figure 7). Human fibrinogen was used for a control and produced positive clumping at a concentration of

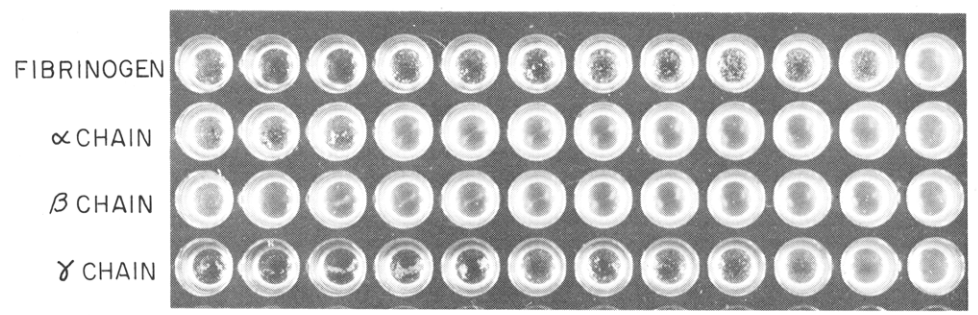

FIGURE 7: Direct interaction of polypeptide chains of fibrinogen with staphylococci. Chains (85 μM) were suspended in 0.05 M ammonium bicarbonate, pH 7.8, and after 1 h sonicated to prepare a smooth suspension of multimers. Twofold dilutions of each chain suspension were made and tested for staphylococcal clumping reaction. Note positive clumping reaction in wells containing fibrinogen and γ-chain multimers.

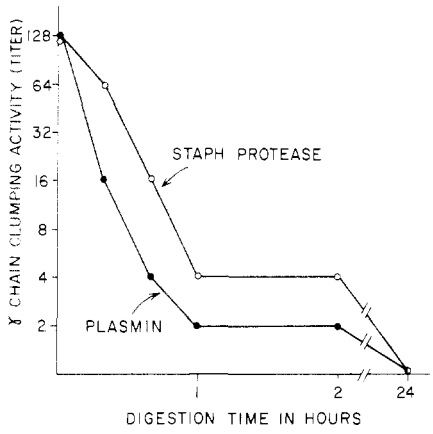

FIGURE 8: Effect of enzymatic degradation of γ-chain multimers on their reactivity toward staphylococcal clumping reaction. The γ chain (4 mg/mL) was dispersed in 0.05 M ammonium bicarbonate buffer, pH 7.8, sonicated, and assayed for clumping activity in 2-fold dilutions by using a standard staphylococcal clumping system before addition of plasmin (0.12 unit per 1 mg of γ chain) and staphylococcal protease (66 μg per 1 mg of γ chain) and at different time intervals; (●) plasmin-treated γ chain; (○) staphylococcal protease treated γ chain.

60 nM. It should be noted that all three chains of fibrinogen were in a suspended multimeric form. Chromatography of the γ chain on Sephadex G-100 showed that the bulk of the material with clumping activity toward staphylococci appeared in the void volume of the column, thus indicating that the molecular weight of multimers was greater than 100000. The γ-chain multimers effecting a positive clumping reaction with staphylococci were susceptible to plasmin digestion (0.12 unit per 1 mg of γ chain) and to staphylococcal protease (66 μg per 1.0 mg of γ chain) which reduced clumping reactivity of γ-chain multimers within 1 h to very low values (Figure 8).

Discussion

Our original aim in studying the interaction of human fibrinogen with the staphylococcal clumping factor was to identify the binding region on the fibrinogen molecule. Fibrinogen is composed of three pairs of nonidentical polypeptide chains (α, β, and γ) forming a polydomainal structure containing one central domain (fragment E) and two major terminal domains (fragment D) (e.g., Doolittle, 1977). These domains possess distinct antigenic determinants which allow the use of monospecific antibodies for selective blocking.[3] Therefore, our basic strategy toward identification of the binding region(s) on fibrinogen for the staphylococcal clumping factor employed isolated fragments, and monospecific antibody Fab fragments directed against fibrinogen and its different domains.

Localization of the Binding Region on the Fragment D. Several lines of evidence indicate that fragment D bears a binding region for the staphylococcal clumping factor. First, Fab antibody obtained after immunization with purified fragment D (generated during the early stage of digestion) blocked the binding of ^{125}I-labeled fibrinogen to staphylococci and the concomitant clumping reaction. A more potent Fab antibody to fragment D, prepared by specific immunoabsorption on a fragment D affinity column, showed a similar effect. Second, neutralization of anti-fragment D by fragment D_1 and not by fragment D_3 suggested that the feature of fragment D which recognizes the staphylococcal cell wall receptor resides in fragment D_1 but is missing in fragment D_3. Third, fragment D_1 but not fragment D_3 blocked directly binding of ^{125}I-labeled fibrinogen to staphylococci and the concomitant clumping reaction. Fourth, ^{125}I-labeled fragment D_1 showed a 6-fold greater binding to staphylococci over ^{125}I-labeled fragment D_3.

The major change occurring during the plasmin-induced transition of fragment D_1 into fragment D_3 involved degradation of the γ chain, a result in keeping with the observation of others (e.g., Haverkate & Timan, 1977). Since the amino-terminal end of the γ chain remained unchanged, the main degradative attack by plasmin obviously removed a sizable portion of the γ chain at its carboxy-terminal zone, encompassing 109 residues. Nevertheless, there was also the possibility of a change at the carboxy-terminal segment of the α chain of fragment D (the amino-terminal residues remained the same). Fortunately, we had on hand a series of α-chain fragments overlapping the plasmin-sensitive region of α chain which could undergo change during the transition from fragment D_1 into fragment D_3. In direct binding experiments, the three α-chain fragments did not show any significant binding, nor did they inhibit binding of ^{125}I-labeled fibrinogen to staphylococci. The only observed effect of α-chain-derived fragments was some partial reversal of the blocking activity of anti-fragment D Fab and anti-fibrinogen Fab antibodies by fragment α165–441. It is possible that the γ-chain site involved in the interaction of fragment D with the staphylococcal clumping factor resides in close proximity to the γ chain, and a portion of the antibody is directed toward the antigenic determinant formed jointly by these two chain segments.

The γ Chain Bears a Major Site Reacting with the Staphylococcal Clumping Factor. Suspensions of dispersed chains in the form of multimers in ammonium bicarbonate buffer, pH 7.8, appeared to be suitable for measuring their clumping activity with remarkable consistency. The γ chain was especially active toward the staphylococcal clumping factor, and it caused clumping of staphylococci at concentrations of 140 nM, which was of the same order as that of human fibrinogen (60 nM). α chains showed 150-fold lower reactivity toward staphylococci than the γ chain. The possibility of cross-contamination of the α chain with the γ chain exists. However, this seemed unlikely when one considers that the β chain, which elutes between α and γ chains during isolation, remained inactive.

Altogether, the results presented here differ from our previous experiments in which a mixture of chains was solubilized in 3 M urea and did not show clumping activity, although a strong association between α chains and staphylococci was observed (Hawiger et al., 1975, 1978). In fact, the unfolding effect of urea strikingly enhanced the reactivity of the α chain and its fragments toward staphylococci.[4] However, when some of these soluble α-chain fragments were retested under physiological buffer conditions in the absence of urea, they did not show a significant direct reactivity toward the staphylococcal cell wall receptor. In contrast, γ-chain reactivity toward staphylococci was abolished in 3 M urea, while it was remarkably high in urea-free buffer. It should be noted that a relatively high reactivity of a mixture of carboxymethylated chains toward staphylococci was observed by Stemberger & Horman (1974), although when isolated α, β, and γ chains were tested, they did not exhibit reactivity toward staphylococci. Since the chains in that study were twice dissolved in 8 M urea and then dialyzed, this procedure could affect γ-chain reactivity toward the staphylococcal receptor. Whatever

[3] D. D. Strong and R. F. Doolittle, unpublished experiments.

[4] J. Hawiger, S. Timmons, B. Cottrell, and R. F. Doolittle, unpublished experiments.

the mechanism of the urea effect on separated fibrinogen chains (unfolding, prevention of multimer formation), the previously made observations by one of us (J.H.) employing the system with 3 M urea (Hawiger et al., 1975, 1978) should be reinterpreted in light of our current findings obtained in the more physiologic urea-free system.

Structure–Function Relationship of Fibrinogen in Regard to Its Interactions with the Staphylococcal Clumping Factor. As stated before the clumping of staphylococci by fibrinogen is an example of a cell agglutination reaction. As such, this reaction requires that the agglutinin, i.e., fibrinogen, be bivalent in order to effect the binding of two cells together. Localization of the binding region on fragment D is compatible with the rule of bivalency, since two fragments D are recognized within one fibrinogen molecule (Marder, 1970; Pizzo et al., 1973; Takagi & Doolittle, 1975b). The breakdown of the bivalent fibrinogen molecule into two monovalent fragments D should abolish the clumping reaction but preserve the ability to bind the staphylococcal receptor. Indeed, fragment D_1 did not clump staphylococci but showed significant binding. Theoretically, fragment E, representing the central domain of the fibrinogen molecule, has structural features fulfilling the criteria of bivalency. However, we were unable to find any evidence linking fragment E to the clumping activity of fibrinogen, thus confirming our earlier observations performed in a buffer system without urea (Hawiger et al., 1970).

Analysis of our results, in light of a three-dimensional representation of the fibrinogen molecule offered elsewhere (Doolittle, 1977), indicates that the prominent polar appendages corresponding to the carboxy-terminal halves of the α chains do not appear to be involved in the interaction with staphylococci. On the other hand, the carboxy-terminal segments of the γ chains, depicted in that model at the extremities of the tridomainal structure (Chen & Doolittle, 1971), would make major sites for bridging one macroparticle to another. Moreover, although the β and γ chains are homologous over a good deal of their lengths (Henschen & Lottspeich, 1977b; Watt et al., 1978), they are distinctly different at their carboxy termini (Takagi & Doolittle, 1975b), thus explaining the lack of β-chain involvement.

In summary, the localization of the binding region for the staphylococcal clumping factor to the carboxy-terminal segment of the γ chain provides solid structural support for the cell agglutinating function of human fibrinogen in the case of staphylococci and conceivably other cells involved in hemostatic and nonhemostatic interactions.

References

Chen, R., & Doolittle, R. F. (1971) *Biochemistry 10*, 4486.

Colvin, R. B., & Dvorak, H. R. (1975) *J. Exp. Med. 142*, 1377.

Cottrell, B. A., & Doolittle, R. F. (1976) *Biochem. Biophys. Res. Commun. 71*, 754.

Degani, Y., & Patchornik, A. (1974) *Biochemistry 13*, 1.

Doolittle, R. F. (1973) *Adv. Protein Chem. 27*, 1.

Doolittle, R. F. (1977) *Horiz. Biochem. Biophys. 3*, 164–191.

Doolittle, R. F., Schubert, D., & Schwartz, S. A. (1976) *Arch. Biochem. Biophys. 118*, 456.

Doolittle, R. F., Cassman, K. G., Cottrell, B. A., Friezner, S. J., Hucko, J. T., & Takagi, T. (1977a) *Biochemistry 16*, 1703.

Doolittle, R. F., Cassman, K. G., Cottrell, B. A., Friezner, S. J., & Takagi, T. (1977b) *Biochemistry 16*, 1710.

Doolittle, R. F., Watt, K. W. K., Cottrell, B. A., Strong, D. D., & Riley, M. (1979) *Nature (London) 280*, 464.

Duthie, E. S. (1955) *J. Gen. Microbiol. 13*, 383.

Haverkate, P., & Timan, G. (1977) *Thromb. Res. 10*, 803.

Hawiger, J., Niewiarowski, S., Gurewich, V., & Thomas, D. P. (1970) *J. Lab. Clin. Med. 75*, 93.

Hawiger, J., Hammond, D. K., & Timmons, S. (1975) *Nature (London) 258*, 643.

Hawiger, J., Hammond, D. K., Timmons, S., & Budzynski, A. Z. (1978) *Blood 51*, 799.

Hawiger, M. M., Timmons, S., & Hawiger, J. (1979) *Clin. Res. 27*, 345A.

Heene, D. L., & Mathias, F. R. (1973) *Thromb. Res. 2*, 137.

Henschen, A., & Lottspeich, F. (1977a) *Hoppe-Seyler's Z. Physiol. Chem. 358*, 935.

Henschen, A., & Lottspeich, F. (1977b) *Thromb. Res. 11*, 869.

Marder, V. J. (1970) *Thromb. Diath. Haemorrh., Suppl. No. 39*, 187.

McFarlane, A. S. (1963) *J. Clin. Invest. 42*, 346.

Mross, G. A., & Doolittle, R. F. (1971) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 30*, 1241.

Much, H. (1908) *Biochem. J. 14*, 143.

Mustard, J. F., Packham, M. A., Kinlough-Rathbone, R. L., Perry, D. W., & Regoeczi, E. (1978) *Blood 52*, 453.

Nussenzweig, V., Seligman, M., Pelmont, J., & Grabar, P. (1961) *Ann. Inst. Pasteur, Paris 100*, 377.

Ouchterlony, O. (1958) *Prog. Allergy 3*, 1–78.

Pizzo, S. W., Schwartz, M. L., Hill, R. L., & McKee, P. A. (1973) *J. Biol. Chem. 248*, 4574.

Porter, R. R. (1959) *Biochem. J. 73*, 119.

Scatchard, G. (1949) *Ann. N.Y. Acad. Sci. 51*, 660.

Shainoff, J. R., & Braun, W. E. (1973) *Anal. Biochem. 55*, 206.

Stemberger, A., & Horman, H. (1974) *Thromb. Res. 4*, 753.

Stewart, J. M., & Young, J. D. (1969) *Solid Phase Peptide Synthesis*, W. H. Freeman, San Francisco, CA.

Strong, D. D., Watt, K. W. K., Cottrell, B. A., & Doolittle, R. F. (1979) *Biochemistry 18*, 5399.

Takagi, T., & Doolittle, R. F. (1974) *Biochemistry 13*, 750.

Takagi, T., & Doolittle, R. F. (1975a) *Biochemistry 14*, 940.

Takagi, T., & Doolittle, R. F. (1975b) *Biochim. Biophys. Acta 386*, 617.

Watt, K. W. K., Takagi, T., & Doolittle, R. F. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 1731.

Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406.