Involvement of the α Chain in Fibrin Clot Formation. Effect of Monoclonal Antibodies†

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ABSTRACT: Murine monoclonal antibodies 9C3, 7B1, and 9E9 have been obtained using native human fibrinogen as the antigen. The antibodies reacted with the epitopes in the COOH-terminal domain of the Aα chain. Fragmentation of the Aα chain with plasmin, and, as in the case of the 9E9 epitope, with V8 protease, followed by isolation of the smallest reacting peptides, allowed the localization of the epitopes for 9C3, 7B1, and 9E9 to the amino acid sequences of α240–268, α425–440, and α541–574, respectively. All three monoclonal antibodies strongly inhibited the rate of fibrin polymer assembly from monomers, both in the purified system and in the human plasma. The mechanism of this strong inhibition implied a rapid formation of fibrin protofibrils, followed by capping with IgG molecules of protofibrils containing approximately ten monomers. These observations demonstrated that certain regions in the COOH terminus of the α chain may play an important role in the assembly of a fibrin clot, presumably being involved in lateral aggregation of protofibrils.

The intact fibrinogen molecule consists of three pairs of Aα, Bβ, and γ chains and has a dimeric structure that is functionally bivalent. Of the three constituent chains of fibrinogen, the Aα chain is the largest, comprising 610 amino acids and lacking carbohydrates (Doolittle, 1984). The NH_2-terminal one-third of the Aα chain is contained within the D and E domains and interconnecting segments, whereas the COOH-terminal two-thirds resides outside the plasmin-resistant core of fibrinogen (Doolittle et al., 1979). The COOH terminus of the Aα chain forms a loose structure that is polar and exposed on the surface of the fibrinogen molecule, which has been implicated in a number of the biological functions. The covalent cross-links introduced by factor XIIIa between the α chains of fibrin have been localized to this region (Doolittle et al., 1977; Fretto & McKee, 1978; Cottrell et al., 1979); α_2-antiplasmin (Sakata & Aoki, 1980), fibronectin (Mosher, 1975), von Willebrand factor (Hada et al., 1986), and thrombospondin (Tuszynski et al., 1985) are also cross-linked to fibrin in this region. Two Arg-Gly-Asp sequences, which have been postulated to mediate the interaction of fibrinogen with platelet membrane receptors, are located in the Aα chain (Gartner & Bennet, 1985; Plow et al., 1985). In addition, the initial sites of plasmic cleavage of fibrinogen reside within the COOH terminus of the Aα chain (Mosesson et al., 1972; Takagi & Doolittle, 1975; Cottrell & Doolittle, 1979).

Fibrinogen exposed to thrombin is converted to a fibrin monomer which spontaneously polymerizes forming protofibrils; these intermediate species assemble both linearly and laterally into a fibrin clot. Many investigators observed a slow rate of polymerization and decreased coagulability of thrombin-treated derivatives of fibrinogen lacking parts of the COOH terminus of the Aα chain. Recently, Hasegawa and Sasaki (1990) compared several coagulation-related properties of fibrinogen band I (having all intact chains) and band II (with cleaved Aα COOH and Bβ NH_2 termini) and proposed that the Aα COOH terminus plays an essential role in the lateral polymerization of fibrin. However, there is a lack of direct studies documenting the involvement of the Aα chain in clot formation.

Results presented in this work, together with previous observations, indicated that the COOH-terminal region of the Aα chain may play a role in the polymerization properties of fibrin. Three murine monoclonal antibodies reacting with the epitopes in the COOH terminus of the Aα chain have been characterized in this work and tested as to the effect on the assembly of a fibrin clot.

Experimental Procedures

Purification of Fibrinogen and Its Peptide Fragments. Human fibrinogen (band I) was isolated from normal human citrated plasma by precipitation with ammonium sulfate (0.85 M) and glycine (2.1 M), followed by gel filtration on Sepharose CL-4B (Pharmacia, Piscataway, NJ) and another ammonium sulfate (0.65 M) precipitation, as described previously (Pandya & Budzynski, 1984).

After reduction and carboxymethylamidation of the protein, the Aα, Bβ, and γ chains of fibrinogen were purified by ion-exchange chromatography on a CM-52 cellulose column (25 × 1.5 cm). Citrate-free fibrinogen (500 mg), in 0.3 M NaCl, was reduced with 70 mg of dithiothreitol and carboxymethylamidated with 500 mg of iodoacetamide, according to Murano and colleagues (Murano et al., 1971).

Analytical Procedures. Protein concentration was determined either by the microbiuret method (Itzhaki & Gill, 1969) or by spectrophotometry at 280 nm using an absorption coefficient of 1.5 and 1.35 for fibrinogen and IgG, respectively. Protein concentration of the Aα, Bβ, and γ chain solutions was determined from absorption coefficients 1.18, 1.74, and 2.04, respectively, in 0.1 M NaOH.

The amino acid sequence from the NH_2 terminus was determined by Edman degradation in an automatic gas-phase sequencer (Model 470A, Applied Biosystems, Foster City, CA). The PTH derivatives were directly identified and quantified by HPLC (Hewick et al., 1981).

SDS-polyacrylamide slab gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970). Completed gels

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were transferred electrophoretically (6V/cm for 15 h at 4 °C) onto a nitrocellulose paper for staining with a specific antibody, according to Towbin and colleagues (Towbin et al., 1979).

Preparation of Monoclonal Antibodies to Human Fibrinogen. BALB-c mice, 3–4 months old, were immunized with purified human fibrinogen. The first injection (50 μg) was given intraperitoneally with complete Freund's adjuvant and the second (50 μg) 30 days later, intravenously in saline. Three days later, spleen cells were fused with P3/NS1/1-Ag4-1, a nonsecretory variant of mouse plasmocytoma cells (Kohler & Milstein, 1975). The culture fluids of growing hybrids were tested for the presence of anti-fibrinogen antibody. The positive hybridoma clones were recloned three times at limiting dilutions of the cells and screened each time for antigen binding affinity. Three hybrid cultures, appearing to be the most productive clones, were propagated in the ascites of mice primed with pristane. Monoclonicity was confirmed by recloning at limiting dilutions and typing the immunoglobin light and heavy chains. Conditioned media from hybridoma cell lines or ascites fluids were screened for anti-fibrinogen antibodies and anti-Aα chain antibodies with a direct ELISA on polystyrene microtiter plates (Dynatech Laboratories Inc., Alexandria, VA) using ABTS (Engvall, 1980).

Characterization of Monoclonal Antibodies. Immunoglobulins of three clones, 9C3, 7B1, and 9E9, were isolated from the clarified ascites fluid by affinity chromatography on a protein A–Sepharose column, which was washed sequentially with 0.14 M phosphate buffer, pH 8.0, 0.1 M citrate buffer, pH 6.0, and 0.1 M citrate buffer, pH 3.0. The heavy and light chain subclasses for the monoclonal antibodies were determined using a screening/isotyping kit (Screentype, Boehringer-Mannheim Biochemicals, Indianapolis, IN) for murine monoclonal antibodies.

Determination of specificity of monoclonal antibodies to the fibrinogen chains was performed using an ELISA test on 96-well microtiter plates (Immulon, Dynatech, Chantilly, VA) coated separately with either fibrinogen or the Aα, Bβ, or γ chains, respectively. Monoclonal antibodies were diluted to 1 μg/mL with PBS containing 1% BSA, pH 7.4; 100 μg of each monoclonal antibody was added to four wells coated with each antigen. Reaction for the monoclonal antibody was developed after incubation with goat anti-mouse IgG conjugated with horseradish peroxidase. The plate was washed and then incubated with ABTS (0.4 mg/mL) in 0.1 M sodium citrate, pH 3.0, containing H_2O_2 (0.6 mg/mL), for 10 min and read at 405 nm (Model 250 EIA Reader, Bio-Rad Laboratories, Richmond, CA). A semiquantitative determination of the interaction of monoclonal antibodies wih fibrinogen fragments was done directly on nitrocellulose paper, using a dot technique (Hawkes et al., 1982).

The expression of the 9C3, 7B1, and 9E9 epitopes by the isolated short-peptide fragments of the Aα chain was analyzed by the equilibrium competitive radioimmunoassay in which ^{125}I-fibrinogen was used as a ligand. Assays were performed in duplicate for each concentration of competing antigen by adding to each microfuge tube ^{125}I-fibrinogen (100 μL) and competing antigen (100 μL) at varying concentrations from 0 to 10^{-5} M, followed by 100 μL of the monoclonal antibody (9C3, 7B1, or 9E9). ^{125}I-Fibrinogen and unlabeled degradation products were dissolved in 0.1 M borate buffer, pH 8.3, containing 0.1% Triton X-100, heparin (100 units/mL), and normal mouse serum (1:30 in borate buffer). Serial dilutions of monoclonal antibodies were made in the same buffer. The ^{125}I-fibrinogen concentration was 5×10^{-10} M in all experiments. The tubes were incubated at 4 °C overnight to achieve equilibrium. Free ^{125}I-fibrinogen was separated from antibody-bound ^{125}I-fibrinogen by goat anti-mouse IgG antiserum (100 μL). After centrifugation, the supernate was discarded, the precipitate was washed, and the radioactivity in the precipitate was counted. When the concentration of a multivalent antigen, such as the dimeric fibrinogen molecule, exceeds the K_d value of the antigen–antibody complex, each antigen molecule is likely to bind at most a single antibody molecule. Consequently, multiple binding of antibodies will be negligible (Scatchard, 1949).

The dissociation constant for the binding of monoclonal antibodies to the native human fibrinogen was measured using the assay procedure described for competitive inhibition RIA. Purified monoclonal antibody (100 μL), ^{125}I-fibrinogen (100 μL), and the RIA buffer (100 μL) were mixed and equilibrated. The concentration of monoclonal antibodies was 1×10^{-9} M throughout, and the ^{125}I-fibrinogen concentrations ranged from 1.5×10^{-5} to 5×10^{-10} M in the binding experiments. For the control assay to determine a nonspecific binding of ^{125}I-fibrinogen, monoclonal antibodies were replaced with 100 μL of the RIA buffer. The binding data for monoclonal antibodies were analyzed by the Scatchard method (Scatchard, 1949). The data were plotted as r/c versus r, where r = bound antigen/total antibody and c = free antigen, expressed in molar concentration. The K_d value for binding of monoclonal antibodies was determined from the binding data by the method of Klotz (1982).

Identification of Epitopes in Fibrinogen. Fibrinogen (band I) dissolved in 0.14 M NaCl buffered with 0.01 M phosphate, pH 7.3 (20 mg/mL), was digested with plasmin (3 units/g) at 37 °C for 20 min to produce peptide fragments containing epitopes for 9C3, 7B1, and 9E9. Hydrolysis was stopped by adding inhibitors to a final concentration of 1 mM PMSF, 0.2 M EACA, and Trasylol (50 units/mL) and heating at 80 °C for 20 min. After removal of heat-denatured high molecular weights products by centrifugation at 3000 rpm for 20 min, supernates were analyzed in SDS–PAGE followed by immunoblotting (Towbin et al., 1979). Immunoreactive supernates were fractionated by ultrafiltration on Amicon membranes PM30 and PM10.

Monoclonal antibodies 9C3, 7B1, and 9E9 were coupled to CNBr–activated Sepharose 4B (Pharmacia, Piscataway, NJ) in 0.1 M $NaHCO_3$ and 0.5 M NaCl, pH 8.0, following the instruction of the manufacturer. The amount of protein bound per gram of dry resin was 10 mg, and the coupling efficiency, as judged by absorbance of the protein in the supernates, was greater than 95%.

Peptides expressing 9C3 and 7B1 epitopes were isolated from the digest's fractions containing fragments with M_r below 10000 by affinity chromatography using respective monoclonal antibodies immobilized on Sepharose 4B. The peptides were further purified by HPLC using an analytical C18 reverse-phase column (C18 μBondapak, Waters Associates, Milford, MA). A linear gradient from 0.1% TFA to 80% acetonitrile in 0.1% TFA was used at 1 mL/min. Peptides reacting with antibodies 9C3 and 7B1 were recycled on the same column, sequenced, and used as competitive inhibitors in the RIA system. The shortest plasmic fragment isolated from a 20-min digest of fibrinogen, containing 9E9 epitope, had M_r 25000 (not shown). This fragment was isolated by affinity chromatography on immobilized 9E9 antibody and further digested with V-8 protease (1:40, enzyme to peptide ratio) at 37 °C for 6 h. The digest was fractionated by HPLC on a C18 reverse-phase column. The pepide reacting with 9E9 antibody in a dot assay (Hawkes et al., 1982) was recycled, sequenced,

and used in the RIA studies. The molecular weight of peptides containing 9C3, 7B1, and 9E9 epitopes was determined by SDS–PAGE.

Inhibition of fibrin polymerization was measured in three systems. In the first system, 0.1 mL of human fibrinogen, 1.5 mg/mL, was mixed with 0.8 mL of 0.05 M Tris-HCl buffer and 0.1 M NaCl, pH 7.4, containing various concentrations of monoclonal antibodies. After a 10-min incubation, 0.1 mL of human thrombin, 5 units/mL, was added, and the increase in turbidity was recorded at 350 nm for 10 min (Budzynski et al., 1979). For the second system, fibrin monomer was prepared according to the method of Belitser and colleagues (Belitser et al., 1968). Polymerization was measured spectrophotometrically at 350 nm for 10 min as described (Budzynski et al., 1979). Each reaction mixture contained 0.1 mL of 1.5 mg/mL fibrin monomer and 0.9 mL of the antibody to be tested which was dialyzed in 0.05 M Tris-HCl and 0.1 M NaCl, pH 7.55. To measure the rate of polymerization of the fibrin monomer alone (100% of the maximum rate plotted on the ordinate), 0.1 mL of the fibrin monomer was added to 0.9 mL of buffer. In the third system, normal human citrated plasma was used as a substrate; 0.06 mL of plasma, containing 2.5 mg/mL fibrinogen, was mixed was 0.84 mL of 0.05 M Tris-HCl buffer, 0.1 M NaCl, pH 7.4, and 0.1 mL of human thrombin, 5 units/mL. The maximum rate of polymerization was calculated from the slope of the steepest part of the curve.

Results

Immunochemical Characterization of Monoclonal Antibodies 9C3, 7B1, and 9E9. Monoclonal antibodies against the COOH terminus of the Aα chain were unusually strong inhibitors of thrombin-induced fibrinogen clotting. To understand this phenomenon, the antibodies, 9C3, 7B1, and 9E9, have been thoroughly characterized and epitopes determined.

Immunization of mice with native human fibrinogen resulted in a predominant antibody response to the Aα chain. Three cell clones, 9C3, 7B1, and 9E9, all IgG1 immunoglobulins containing the κ light chain, were selected for further studies on the basis of strong interaction with fibrinogen, the Aα chain, and the fibrin clot formation process. The three antibodies did not react in ELISA with the Bβ and γ chains of fibrinogen. Monoclonal antibodies 7B1 and 9E9 reacted only with fibrinogen whereas 9C3 also reacted slightly with fragment X. No reaction was observed with fragments Y, D, or E. These results demonstrated that the epitopes recognized by these antibodies resided in the COOH terminus of the Aα chain that was rapidly cleaved by plasmin.

A radioimmunoassay was developed using monoclonal antibodies and radioiodinated fibrinogen. Antibodies 9C3, 7B1, and 9E9 bound ^{125}I-fibrinogen in the range of 1–1000 ng, showing similar binding affinity. Scatchard analysis yielded a value of K_d equal to 5.70×10^{-9} M, 8.33×10^{-9} M, and 4.25×10^{9} M for the 9C3, 7B1, and 9E9 antibodies, respectively.

The susceptibility of the epitopes recognized by 9C3, 7B1, and 9E9 antibodies to proteolytic degradation by different proteases was tested. Chymotrypsin and trypsin destroyed all epitopes during 10 min of digestion while plasmin, after a 20-min incubation, produced short fragments with epitopes recognized by 9C3 and 7B1 antibodies and an intermediate product with M_r of 25 000 containing th 9E9 epitope.

In order to purify peptide fragments containing 9C3, 7B1, and 9E9 epitopes, monoclonal antibodies 9C3, 7B1, and 9E9 were immobilized on CNBr-activated Sepharose 4B and used for affinity chromatography. When peptides with M_r below 10 000 were applied to the immobilized 9C3 antibodies, a significant amount of the material was recovered in a peak eluted with 0.5 M acetic acid. This peptide material, subjected to further purification on HPLC, showed the presence of a single component eluting at 18.35 min (Figure 1A). The amino acid sequence of this peptide corresponded to fragment α240–268 (Table I). Since the analyzed peptide was a plasmic degradation product, lysine or arginine was the expected COOH-terminal amino acid, but neither was detected. It was inferred, therefore, from the approximate M_r of 3000 for the peptide that Arg 268 would terminate the sequence. This peptide, tested in a radioimmunoassay system, consisting of ^{125}I-fibrinogen and 9C3 antibody, inhibited binding of the ligand by the antibody; however, the binding affinity was lower

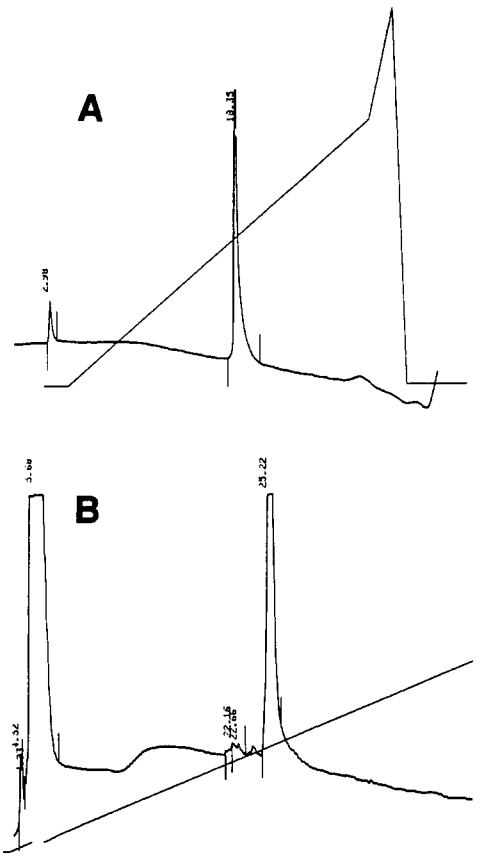

FIGURE 1: Separation of the Aα chain peptide fragments containing epitopes of the monoclonal antibodies 9C3 and 7B1, respectively. Antigenically active peptides were isolated from the plasmic digest of fibrinogen by affinity chromatography using 9C3 or 7B1 antibodies immobilized on Sepharose 4B. Peptides were further purified by HPLC using an analytical C18 reverse-phase column. HPLC profiles show the purity of the peptides containing epitopes of monoclonal antibodies 9C3 (A) and 7B1 (B). Peptides obtained in this way were directly applied on the filter and subjected to Edman degradation in an automatic gas-phase sequencer.

Table I: Amino Acid Sequencing of the Peptides Containing Epitopes Recognized by Monoclonal Antibodies 9C3, 7B1, and 9E9[a]

epitope	sequence	location
9C3	Met-Glu-Leu-Glu-Arg-Pro-Gly-Gly-Asn-Glu-Ile-Thr-Arg-Gly-Gly-Ser-Thr-Ser-Tyr-Gly-Thr-Gly-Ser-Glu-Thr-Glu-Ser-Pro-Arg	α240–268
7B1	Thr-Gly-Lys-Glu-Lys-Val-Thr-Ser-Gly-Ser-Thr-Thr-Thr-Thr-Arg-Arg	α425–440
9E9	Ser-Ser-Ser-His-His-Pro-Gly-Ile-Ala-Glu-Phe-Pro-Ser-Arg-Gly-Lys-Ser-Ser-Ser-Tyr-Ser-Lys-Gln-Phe-Thr-Ser-Ser-Thr-Ser-Tyr-Asn-Arg-Gly-Asp	α541–574

[a] Peptides were purified by affinity chromatography and HPLC on a C18 reverse-phase column and sequenced by Edman degradation in an automatic gas-phase sequencer.

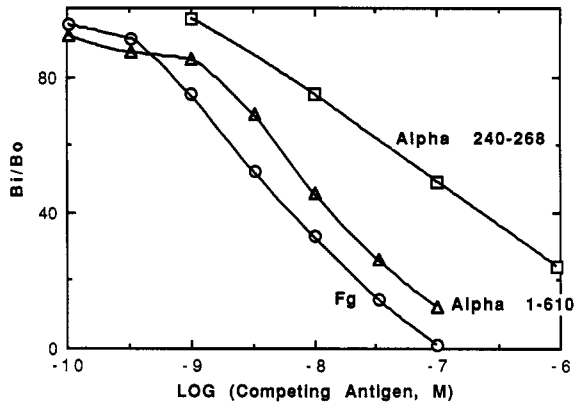

FIGURE 2: Competitive inhibition binding curves for monoclonal antibody 9C3 and ^{125}I-fibrinogen used as a ligand in the presence of unlabeled fibrinogen (Fg), the Aα chain, and its fragment. B_i is the percent of ^{125}I-fibrinogen bound in the presence of inhibitor and B_0 is the percent of ^{125}I-fibrinogen bound in the absence of any inhibitor. All samples were incubated at 4 °C for 16 h. The data have been normalized so that binding in the absence of any competitive inhibitor corresponds to 100% of ^{125}I-fibrinogen bound.

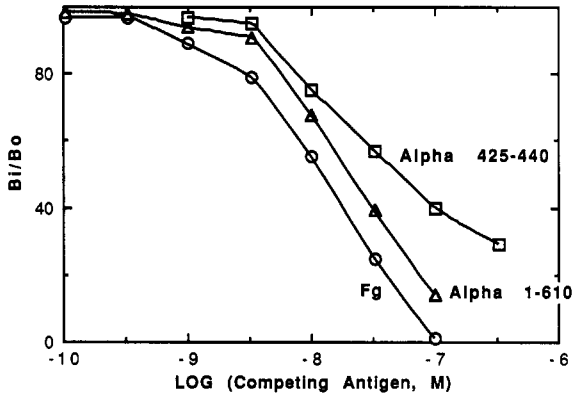

FIGURE 3: Competitive inhibition binding curves for monoclonal antibody 7B1 and ^{125}I-fibrinogen as a ligand in the presence of unlabeled fibrinogen (Fg), the Aα chain, and its fragment (see legend to Figure 2).

than that of the intact fibrinogen or the Aα chain (Figure 2).

The same approach was used to isolate the peptide fragment containing an epitope reacting with monoclonal antibody 7B1. After the fractions eluted from the column containing immobilized immunoglobulin 7B1 were recycled, the major peak eluting at 25.22 min (Figure 1B) contained a peptide with an α425–440 sequence (Table I). This peptide, tested in the radioimmunoassay system, containing ^{125}I-fibrinogen and 7B1 antibody, caused a significant inhibition, demonstrating expression of the 7B1 epitope albeit with a decreased binding affinity. The latter is evidenced by a more shallow slope of the competitive inhibition curve (Figure 3). The plasmic intermediate fragment (M_r 25 000) of the Aα chain, reacting with 9E9, was purified by affinity chromatography using the monoclonal antibody immobilized on Sepharose 4B. Digestion of this fragment with V-8 protease did not destroy the 9E9 epitope. The peptide reacting with 9E9 was purified by HPLC on a C18 column and was eluted as a single component at 21.18 min. Its M_r was 3300 in SDS–PAGE, and its amino acid sequence corresponded to fragment α541–574 (Table I). This peptide produced a partial inhibition of binding of ^{125}I-fibrinogen to 9E9, indicating the presence of the analyzed epitope (Figure 4).

Interference of Monoclonal Antibodies with Fibrin Clot Formation. The rate of assembly of fibrin monomers into polymers was calculated from the turbidity change. It was found that certain antibodies had no effect on this process in

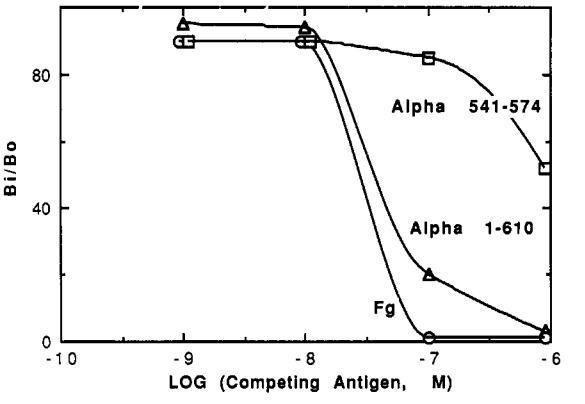

FIGURE 4: Competitive inhibition binding curves for monoclonal antibody 9E9 and ^{125}I-fibrinogen used as a ligand in the presence of unlabeled fibrinogen (Fg), the Aα chain, and its fragment (see legend to Figure 2).

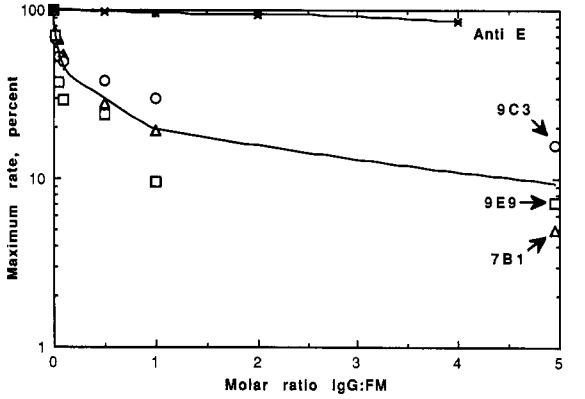

FIGURE 5: Inhibition of fibrin monomer polymerization by monoclonal antibodies. Experiments were done in the presence of increasing amounts of antibodies. As a control, a polyclonal antibody to fragment E was used that was noninhibitory. FM = fibrin monomer.

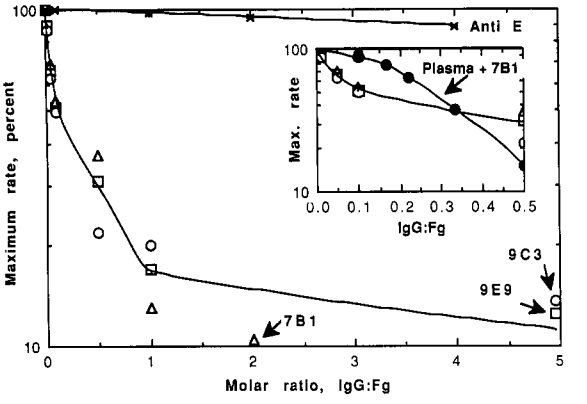

FIGURE 6: Effect of monoclonal antibodies on fibrinogen clotting. Purified fibrinogen (0.15 mg/mL) or human plasma was mixed with increasing concentrations of monoclonal antibodies, and thrombin (5 units/mL) was added. The increase of turbidity was recorded at 350 nm for 10 min. Fg = fibrinogen.

spite of the demonstrated binding to fibrinogen and fibrin. Thus, rabbit polyclonal anti-E, IgG-type antibodies raised against fragment E_3 as antigen (Figures 5 and 6) and murine monoclonal anti-α603–610, IgM-type antibodies (not shown) did not inhibit the polymerization reaction. Anti-D polyclonal or monoclonal antibodies inhibited the polymerization reaction. A decrease of the maximum reaction rate to 50% occurred at a molar ratio between 1 and 3, depending on the kind of antibody. In contrast, 9C3, 7B1, and 9E9 antibodies strongly inhibited the polymerization rate in both fibrin monomer (Figure 5) and fibrinogen–thrombin (Figure 6) systems. It

appeared that the inhibitory effect was slightly more pronounced with antibody 7B1 and slightly less with 9C3, yet the curves shown in Figures 5 and 6 were computed as average values for all three antibodies. A decrease of the maximum reaction rate of 50% occurred at a molar ratio of 0.10 and 0.12 for IgG:FM and IgG:Fg, respectively. This meant that one molecule of IgG was interacting with a fibrin oligomer containing about five fibrin monomer molecules. The same inhibitory effect was evident in the presence of plasma proteins, as shown, for example, for antibody 7B1 in Figure 6 (inset).

DISCUSSION

Fibrinogen is a multifunctional protein possessing diverse biological activities in the coagulation and hemostatic systems and broadly participating in extravascular inflammatory responses (Girmann et al., 1976; Belew et al., 1978; Marguerie & Plow, 1983). Biological activity of the fibrinogen molecule is associated with its native structure, but the mechanisms by which this protein expresses its functions are still poorly understood. Like many other proteins, fibrinogen may exist in more than one conformational state depending on the posttranslational modifications, the degree of sialylation of oligosaccharides, and the presence of calcium ions. Immunochemical analysis of fibrinogen was particularly relevant, permitting examination of conformation in solution and in systems approaching physiological conditions (Plow et al., 1983).

Recent studies have shown that antigenic sites of a protein most likely are located on a highly mobile region of the amino acid backbone. Such regions are frequently present at the surface of the protein and correspond to the most accessible parts of the molecule. Therefore, the study of the antibody binding sites provides a unique way of probing the surface structure of proteins, and such an approach has been successfully applied to the determinations of local conformation in several proteins (Gariepy et al., 1986; Van Regenmontel, 1987; Berlinev et al., 1988). In this study, monoclonal antibodies to native fibrinogen were successfully produced and used in probing the molecular structure of this protein. In good agreement with previously published data describing the predominant role of the Aα chain in eliciting monoclonal antibodies (Erhlich et al., 1983; Thurlow et al., 1987), most of our clones recognized the epitopes on the Aα chain. Three antibodies reacted with epitopes on the unfolded fibrinogen Aα chain and also caused a significant inhibition of fibrin clot formation. Purification of short peptides from digests of fibrinogen by affinity chromatography on immobilized monoclonal antibodies allowed identification of peptide regions of the Aα chain involved in binding of antibodies 9C3, 7B1, and 9E9. These three antibodies recognized the epitopes located in residues 240–268, 425–440, and 541–574, respectively, of the Aα chain (Table I). As peptides are invariably largely unfolded, the peptide containing the native folding represents less than one among the 10^4 or 10^5 molecules having other conformations (Leach, 1983; Cierniewski & Budzynski, 1987). Hence, the epitopes recognized by antibodies 9C3 and 7B1 must be formed by amino acid residues which are close in the sequence so that the expression of the epitopes is not affected so much by conformational transition occurring in the peptide in solution. The effect of such conformational changes on the expression of the 9E9 epitope by a short peptide was more profound. The hydrophobicity of the Aα chain revealed that 9C3, 7B1, and 9E9 epitopes are situated in highly hydrophilic regions (Figure 7). It was postulated that the COOH-terminal region of the Aα chain may represent a flexible free-swimming domain of the molecule existing mostly as a random

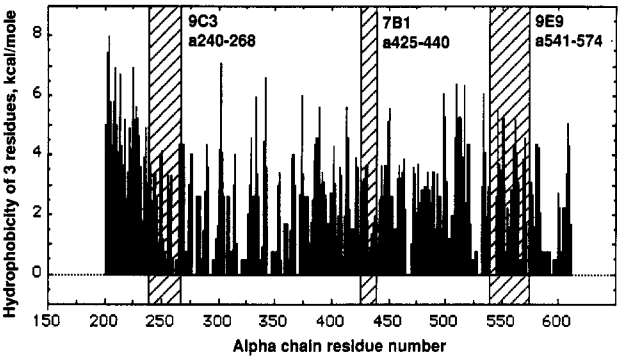

FIGURE 7: Hydrophobicity profile of α200–610. The ordinate shows the mean hydrophobicity of three amino acid residues. The shaded areas show the location of epitopes for antibodies 9C3, 7B1, and 9E9 in hydrophilic regions.

coil (Doolittle, 1984). This concept was supported by the presence of several early plasmic attack sites and the absence of α-helical structure within this region. A number of immunochemical and physicochemical studies demonstrated that although the COOH-terminal segment of the Aα chain is surface-oriented, it also contains ordered structures when these regions are integrated into the native molecule (Huseby et al., 1970; Lotter & Timpl, 1975; Medved et al., 1983; Cierniewski & Budzynski, 1987). The presence of such ordered and less mobile structures in this chain is compatible with the characteristic hydrophobicity pattern depicted for the Aα chain (Figure 7). Clusters of nonpolar amino acid residues, occurring within the polypeptide chain in this region, suggest their interiorization in the fibrinogen structure. However, peptide segments between these clusters may be free to undergo conformational transitions. Such properties and location of the Aα chain on the surface of the native molecule explain its significant and predominant role in the immunogenicity of fibrinogen and its biological activity (Bang et al., 1962; Cierniewski et al., 1984; Weisel et al., 1985).

A participation of the COOH terminus of the Aα chain in clot formation, due to the presence of lateral binding sites, is implied by the following observations: (1) This region of the Aα chain contains specific donor and acceptor NH_2 groups involved in the covalent stabilization of fibrin catalyzed by factor XIIIa. Formation of ϵ-(γ-glutamyl)lysine bonds must be preceded by interactions of loosely spaced COOH termini of the α chains around protofibrils, thereby bringing to close vicinity donor and acceptor residues. (2) The low molecular weight fraction of fibrinogen lacking almost half of the COOH terminus of the Aα chain forms a transparent, fine clot, indicating impaired lateral aggregation of protofibrils (Bang et al., 1962; Holm et al., 1985). (3) The clotting time of intact fibrinogen, as compared to that of fibrinogen derivatives lacking the COOH terminus of the Aα chain, is more affected by the synthetic peptide Gly-His-Arg-Pro postulated to be involved in lateral aggregation (Hasegawa & Sasaki, 1990). (4) Monoclonal antibodies 9C3, 7B1, and 9E9 recognizing epitopes present in the Aα chain have strong anticlotting activity.

The unexpectly strong inhibition of fibrin polymerization by antibodies 9C3, 7B1, and 9E9 (Figures 5 and 6) seems to result from a novel mechanism. Considering an inhibitor of polymerization that can occupy two binding sites on a fibrin monomer, for example, fragment DD (Budzynski et al., 1979), a 50% inhibition of the maximum reaction rate occurs at the molar ratio of the reactants of 1:1. A 50% inhibition of the maximum polymerization rate by the monoclonal antibodies 9C3, 7B1, and 9E9 was found at an approximate molar ratio

of IgG to fibrin monomer or fibrinogen of 0.1. Extrapolating the data to a total inhibition (=0% of the maximum rate), one can calculate that in this condition one molecule of antibody binds to five molecules of fibrin monomer. Since a protofibril consists of two strands of fibrin oligomers, one should double the number so that on the average the product of fibrin polymerization in the presence of antibodies 9C3, 7B1, and 9E9 would have two staggered linear fibrin oligomers, containing a total of ten monomers, with two IgG molecules attached at each end. This reasoning implies that a double-stranded protofibril is a rapidly forming intermediate that can be stabilized by inhibitors of fibrin polymerization. In support, such structures have been observed using fragment D_1 as an inhibitor of fibrin polymerization (Knoll et al., 1984). However, it cannot be definitely concluded from this work whether some segments of the COOH terminus of the α chain constitute an integral part of polymerization sites located in the structural D domain. The clusters of polar amino acid residues coinciding with the determined amino acid sequence of the epitopes may be localized in the vicinity of fibrin polymerization sites. Since the IgG molecules used had a large size (M_r 160 000), steric hindrance caused by the monoclonal antibodies must have been considerable. Nevertheless, the inhibitory effect of these antibodies was an order of magnitude stronger than that of anti-D antibodies. Thus, the COOH terminus of the α chain appears to be involved in the process of fibrin clot formation.

References

Bang, N. U., Fletcher, A. P., Alkjaersig, N., & Sherry, S. (1962) *J. Clin. Invest. 41*, 935–948.

Belew, M., Gerdin, B., Porath, J., & Saldeen, T. (1978) *Thromb. Res. 13*, 983–994.

Belitser, V. A., Varetskaja, T. V., & Malneva, G. V. (1968) *Biochim. Biophys. Acta 154*, 367–375.

Berlinev, S., Niiya, K., Roberts, J. R., Houghten, R. A., & Ruggeri, Z. M. (1988) *J. Biol. Chem. 263*, 7500–7505.

Budzynski, A. Z., Olexa, S. A., & Brizuela, B. S. (1979) *Biochim. Biophys. Acta 584*, 284–287.

Cierniewski, C. S., & Budzynski, A. Z. (1987) *J. Biol. Chem. 262*, 13896–13901.

Cierniewski, C. S., Plow, E. F., & Edgington, T. S. (1984) *Eur. J. Biochem. 141*, 489–495.

Cottrell, B., & Doolittle, R. F. (1979) *Biochem. Biophys. Res. Commun. 71*, 754–761.

Cottrell, B. A., Strong, D. D., Watt, K. W. K., & Doolittle, R. F. (1979) *Biochemistry 18*, 5405–5410.

Doolittle, R. F. (1984) *Annu. Rev. Biochem. 53*, 195–209.

Doolittle, R. F., Cassman, K. G., Cottrell, B. A., & Friezner, S. J. (1977) *Biochemistry 16*, 1715–1719.

Doolittle, R. F., Watt, K. W. K., Cottrell, B. A., Strong, D. D., & Riley, M. (1979) *Nature 280*, 464–468.

Ehrlich, P. H., Sobel, J. H., Moustafa, Z. A., & Canfield, R. E. (1983) *Biochemistry 22*, 4184–4192.

Engvall, E. (1980) *Methods Enzymol. 70*, 418–439.

Fretto, L. J., & McKee, P. A. (1978) *J. Biol. Chem. 253*, 6614–6622.

Gariepy, J., Mietzner, T. A., & Schoolnik, G. K. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 8888–8892.

Gartner, T. K., & Bennet, J. S. (1985) *J. Biol. Chem. 260*, 11891–11894.

Girmann, G., Pees, H., Schwarze, G., & Scheurlen, P. G. (1976) *Nature 259*, 399–401.

Hada, M., Kaminski, M., Bockenstedt, P., & McDonagh, J. (1986) *Blood 68*, 95–101.

Hasegawa, N., & Sasaki, S. (1990) *Thromb. Res. 57*, 183–195.

Hawkes, R., Niday, E., & Gordon, J. (1982) *Anal. Biochem. 119*, 142–147.

Hewick, R. M., Hunkapiller, M. W., Hood, L. E., & Dreyer, W. J. (1981) *J. Biol. Chem. 256*, 1990–1997.

Holm, B., Brosstad, F., Kierulf, P., & Godal, H. C. (1985) *Thromb. Res. 39*, 595–606.

Huseby, R. M., Mosseson, M. W., & Murray, M. (1970) *Physiol. Chem. Phys. 2*, 374–384.

Itzhaki, R. F., & Gill, D. M. (1969) *Anal. Biochem. 9*, 401–410.

Klotz, I. M. (1982) *Science 217*, 1247–1249.

Knoll, D., Hantgan, R., Williams, J., McDonagh, J., & Hermans, J. (1984) *Biochemistry 23*, 3708–3715.

Kohler, G., & Milstein, C. (1975) *Nature 236*, 495–497.

Laemmli, U. K. (1970) *Nature 227*, 680–685.

Leach, S. J. (1983) *Biopolymers 22*, 425–440.

Lotter, H., & Timpl, R. (1975) *Eur. J. Biochem. 60*, 221–226.

Marguerie, G. A., & Plow, E. F. (1983) *Ann. N.Y. Acad. Sci. 408*, 556–567.

Medved, L. V., Gorkun, O. V., & Privalov, P. L. (1983) *FEBS Lett. 160*, 231–235.

Mosher, D. F. (1975) *J. Biol. Chem. 250*, 6614–6621.

Mosseson, M. W., Umfleet, R. A., Finlayson, J. S., & Galanakis, D. J. (1972) *J. Biol. Chem. 247*, 5210–5219.

Murano, G., Wiman, B., Blomback, M., & Blomback, B. (1971) *FEBS Lett. 14*, 37–41.

Nagy, J. A., Meinwald, Y. C., & Scheraga, H. A. (1982) *Biochemistry 21*, 1794–1806.

Pandya, B. V., & Budzynski, A. Z. (1984) *Biochemistry 23*, 460–470.

Plow, E. F., Edgington, T. S., & Cierniewski, C. S. (1983) *Ann. N.Y. Acad. Sci. 408*, 44–49.

Plow, E. F., Pierschbacher, M. D., Ruoslahti, E., Marguerie, G. A., & Ginsberg, M. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 8057–8061.

Sakata, Y., & Aoki, N. (1980) *J. Clin. Invest. 65*, 290–297.

Scatchard, G. (1949) *Ann. N.Y. Acad. Sci. 51*, 660–672.

Takagi, T., & Doolittle, R. F. (1975) *Biochemistry 14*, 5149–5156.

Thurlow, P. J., Connellan, J. M., & Kenneally, D. A. (1987) *Thromb. Res. 47*, 427–441.

Towbin, H., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350–4354.

Tuszynski, G. P., Srivastava, S., Switalska, H., Holt, J. C., Cierniewski, C. S., & Niewiarowski, S. (1985) *J. Biol. Chem. 260*, 12240–12245.

Van Regenmontel, M. H. V. (1987) *Trends Biochem. Sci. 12*, 237–240.

Weisel, J. W., Stauffacher, C. V., Bullitt, E., & Cohen, C. (1985) *Science 230*, 1388–1391.