Monoclonal Antibodies to Fibrinogen: Modulation of Determinants Expressed in Fibrinogen by γ-Chain Cross-Linking†

George D. Wilner,* M. Susan Mudd, Kun-hwa Hsieh, and David W. Thomas‡

ABSTRACT: Monoclonal antibodies were prepared and characterized by using fibrinogen as immunogen in an attempt to define antigenic differences between fibrinogen and its polymer, fibrin. Monoclonal antibodies specific for human fibrinogen were produced by using spleen cells from BALB/c mice hyperimmunized with purified human fibrinogen. The culture fluids characterized were from three stable subclones derived from the initial cell fusions with the highest fibrinogen-binding activities. Antibodies in these culture fluid supernatants bound fibrinogen with moderate affinities ($K_i \sim 10^7$–10^9 M^{-1}), as determined by analysis of competition immunoassay data using the Sips equation. Two of the antibodies (IgG_1 isotype) bound to determinants which were expressed in plasmic fragment D_1 derived from fibrinogen and fragment D from un-cross-linked fibrin, as well as the parent fibrinogen molecule. However, these determinants were not expressed in plasmic fragment D–D derived from cross-linked fibrin. These D-domain antigenic determinants could be destroyed by further digestion of fragment D_1 with trypsin and with plasmin in the absence of Ca^{2+}. Immunoprecipitation analysis of ^{125}I-labeled fragment D_1 following limited digestion with plasmin in the absence of Ca^{2+} showed that both IgG_1 monoclonal antibodies bound preferentially to fragment D_1 ($M_r \sim 101\,000$) and not to the degraded fragment D species. Since plasmin degradation of fragment D is limited exclusively to the γ-chain remnant, and mainly involves the COOH-terminal portion of this chain, it is surmised that antigenic distinction between fibrinogen and fibrin results from determinants located in this site which become sequestered coincident to γ-chain cross-link formation.

Fibrinogen is a large ($M_r \sim 340\,000$) plasma glycoprotein consisting of three pairs of disulfide-bonded polypeptide chains (Aα, Bβ, and γ). Following limited thrombin proteolysis of the NH_2 termini of the Aα and Bβ chains, releasing small activation peptides known as fibrinopeptides A and B (Blombäck et al., 1966), polymerization sites in the NH_2-terminal or E domains become exposed, allowing these regions to polymerize with complementary sites located in the COOH-terminal or D domains (Blombäck, 1967; Blombäck et al., 1978). Fibrin polymer is rendered insoluble by the action of a transpeptidase, factor XIIIa, which produces isopeptide cross-linking primarily at the COOH termini of the γ chains and secondarily in the hydrophilic protease-sensitive COOH-terminal region of the α chain as well (Lorand et al., 1978; Mâtacic & Loewy, 1968; Pisano et al., 1968; Takagi & Doolittle, 1975). Immunochemical analyses of the events occurring in and around the fibrinogen–fibrin transition have been limited mainly to measurements of specific release products such as the fibrinopeptides themselves and plasmin proteolysis products derived from fibrinogen and cross-linked fibrin [for a review, see Wilner (1978)]. While such measurements have provided important kinetic [e.g., see Bilezikian et al. (1975)] and clinical [e.g., see Nossel (1976)] information regarding the conversion of fibrinogen to fibrin, the demonstration of specific antigenic differences between native fibrinogen and its polymer remains elusive.

The advent of monoclonal antibody technology has permitted immunochemical analysis of highly complex biological systems such as intact cell membranes [e.g., see Hughes & August (1981)] and has been employed to a lesser extent in the immunochemical analysis of proteins in solution [e.g., see Berzofsky et al. (1980)]. The ability of this technique to resolve and identify hitherto unrecognized determinants prompted an examination of whether determinants could be discovered which were uniquely expressed in fibrinogen as compared with fibrin. In the present paper, we describe the preparation and characterization of fibrinogen-specific monoclonal antibodies which recognize unique antigenic determinants in native fibrinogen which are not expressed in cross-linked fibrin.

Materials and Methods

Materials. The following materials were obtained from the sources listed: microtiter plates from Cooke Laboratory Products Div., Dynatech Laboratories, Alexandria, VA; affinity-purified goat anti-mouse immunoglobulins from Gateway Immunosera Co., St. Louis, MO; bovine thrombin from Parke-Davis, Chicago, IL; rabbit antiserum to unfractionated mouse immunoglobulins was a gift from Dr. Judith Kapp, Department of Microbiology; formalin-fixed Cowan strain I *Staphylococcus aureus* was a gift from Dr. Susan Cullen, Department of Microbiology; bovine pancreatic trypsin inhibitor from FBA Pharmaceuticals Co., New York, NY; carboxymethylcellulose (CM-cellulose)[1] (CM 52, preswollen) from Whatman, Inc., Clifton, NJ; trypsin, TPCK treated, from Worthington Biochemical Co., Freehold, NJ; urea (ultrapure) from Schwarz/Mann, Spring Valley, NY; ovalbumin, reagents

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‡ Recipient of Research Career Development Award AI-00352 from NIAID, NIH. Present address: Department of Microbiology, University of Michigan School of Medicine, Ann Arbor, MI 48109.

[1] Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; CM, carboxymethyl; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; Tris, tris(hydroxymethyl)aminomethane; RCM, reduced, carboxymethylated; ELISA, enzyme-linked immunosorbent assay; EDTA, disodium (ethylenedinitrilo)tetraacetate; KIU, kallikrein inactivator units.

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for $NaDodSO_4$–polyacrylamide gel electrophoresis, Tween-20, and NP-40 detergents were all obtained from Sigma Chemical Co., St. Louis, MO; ^{125}I, carrier free, from Amersham, Inc., Arlington Heights, IL. All unspecified chemicals were of reagent grade.

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. Electrophoresis in the presence of $NaDodSO_4$ was carried out as described by Laemmli (1970). Unless specified, 7% resolving gel concentrations were used. Radioautography was performed by directly exposing the dried gel to Kodak SB-5 X-ray film.

Protein Determinations. Concentrations of protein solutions were determined by using a dye-binding assay (Bradford, 1976), following the procedure recommended by the manufacturer (Bio-Rad Laboratories, Richmond, CA). Fibrinogen solutions were used as protein standards and were calibrated by using the extinction coefficient $E^{1\%}_{280nm} = 15.06$ (Doolittle, 1975).

Purified Human Fibrinogen. Fibrinogen was isolated from outdated human ACD plasma by a modification[2] of the glycine precipitation method of Mosesson & Sherry (1966), in which precipitated I-1 was definitively purified by repeated precipitations with $(NH_4)_2SO_4$ such that a final fraction (13–23% saturation) was collected which was >95% clottable and demonstrated the requisite purity on $NaDodSO_4$–polyacrylamide gel electrophoresis under reducing conditions. Trace amounts of plasminogen and fibronectin were removed from the final product by filtration through lysine–Sepharose and gelatin–Sepharose according to established procedures (Deutsch & Mertz, 1970; Engvall & Ruoslahti, 1977). In instances where fibrinogen free from factor XIII was required, urea inactivation of the enzyme was carried out as described by Schwartz et al. (1971). Estimates of residual factor XIII activity were based on $NaDodSO_4$–polyacrylamide gel electrophoresis analysis of γ–γ dimer formation of thrombin-clotted samples.

Fragments D and E. Purified fragments D and E from fibrinogen were prepared essentially as described by Fowler et al. (1980) except that purified human plasmin (generously provided by Dr. Genesio Murano, Bureau of Biologics, Washington, DC) was added in the ratio of 100 units/g of fibrinogen protein and the digestion was carried out for 2 h at 37 °C. Purity of preparations was ascertained by $NaDodSO_4$–polyacrylamide gel electrophoresis. Under these digestion conditions, the resulting proteolytic fragments consisted predominantly of fragments D_1 ($M_r \sim 101\,000$) and E_3 ($M_r \sim 47\,000$) (Budzynski et al., 1974).

Fragment D from Un-Cross-Linked Fibrin. Fibrinogen free from factor XIII activity was dissolved in 0.05 M Tris–0.1 M NaCl buffer, pH 7.5, and was clotted with 0.5 mL of bovine thrombin (1000 units/mL) for 18 h at room temperature. The clot was pressed on filter paper to remove excess fluid, rinsed with water, cut into small pieces with a scissor, and freeze-dried. Digestion and purification of this fragment were similar to those described above for fragments D and E derived from fibrinogen.

Fragment D Dimer. Cross-linked fibrin was prepared and digested with plasmin as described by Olexa & Budzynski (1979). Initial purification procedures for this fragment were identical with those used to isolate fragment D (see above). The partially purified fragment D dimer was then rechromatographed on CM-cellulose in 8 M urea buffer by using loading and gradient elution conditions as described by McDonagh and co-workers (McDonagh et al., 1972). This step was judged helpful in removing small amounts of uncross-linked material which may remain associated with fragment D dimer. The purity of the preparation was ascertained by $NaDodSO_4$–polyacrylamide gel electrophoresis.

RCM Chains of Fibrinogen and Fragment D. These were gifts from Dr. James Koehn, Columbia University, NY. Cyanogen bromide digests of RCM γ chains of fibrinogen were generously provided by Dr. Edward Plow, Scripps Institute, CA.

Enzymatic Digestion of Fragment D. Fragment D_1 was dissolved in 0.05 M Tris–0.1 M NaCl–0.025 M EDTA buffer. Digestion was carried out with either plasmin or trypsin, with stirring using enzyme:substrate ratios, times, and temperatures as specified in the text. The reaction was stopped by the addition of bovine pancreatic trypsin inhibitor (100 KIU/mL). The reaction mixtures were frozen and analyzed either by immunoprecipitation followed by gel electrophoresis or by inhibition radioassays.

Preparation of Monoclonal Antibodies. Male and female BALB/c mice, bred locally at The Jewish Hospital animal care facility, were immunized as follows: Following initial immunization consisting of 100 μg of purified human fibrinogen in complete Freund's adjuvant injected intraperitoneally, hyperimmune responses were induced by intraperitoneal injection of 100 μg of purified human fibrinogen in incomplete Freund's adjuvant at 3–5-week intervals. Following the appearance in serum samples of circulating precipitating antibodies to fibrinogen, as demonstrated on radial immunodiffusion, the animals received a final immunizing boost consisting of 100 μg of fibrinogen in normal saline 7–10 days prior to sacrifice.[3] Somatic cell hybridization using spleen cells harvested from the hyperimmune mice and cloning of hybrids were carried out at the Washington University Hybridoma Facility by using methods and procedures as previously described (Clevinger et al., 1980). Fibrinogen-binding proteins were detected by ELISA (Engvall, 1980) using fibrinogen-coated microtiter plates. Cloned hybrids were grown in culture, and culture media supernatants were used as the source of immunoglobulins, either directly or following $(NH_4)_2SO_4$ concentration (Jonak, 1980).

Isotype Analysis. Isotype analyses of monoclonal antibodies using a previously described radioimmunoassay procedure (Perlmutter et al., 1979) were graciously performed by Dr. Moon Nahm.

^{125}I Radiolabeling. ^{125}I-Labeled antigens and immunoglobulins were prepared by means of the chloramine-T method as previously described (Hunter & Greenwood, 1962).

Competitive Indirect Immunoglobulin-Binding Radioassay. This assay was carried out in a manner identical with the two-step competitive ELISA described by Engvall (1980) except that 100 μL per well of ^{125}I-labeled affinity-purified goat anti-mouse immunoglobulins ($\sim 150\,000$ cpm) was added in lieu of the enzyme-conjugated antibody preparation used to detect bound immunoglobulin. Following 90-min incubation at 37 °C with the radiolabeled second antibody, the plates were washed 3 times with 0.05% Tween-20 in 0.9% NaCl. The microtiter plates were cut with a scissor and the wells counted. Blank values were obtained by running parallel assays with culture media from the unfused plasmacytoma cell line (M5) and were then subtracted from immune-bound counts per minute. Under assay conditions, specific immunoglobulin binding in the absence of inhibitor (B_{max}) was approximately 3000–5000 cpm, while nonspecific binding was 300–500 cpm.

[2] Dr. N. Bang, personal communication.

[3] Dr. T. Edgington, personal communication.

Affinity Constants. Data for these measurements were obtained by using the competitive indirect immunoglobulin binding radioassay described above. For these studies, the abilities of differing concentrations of fibrinogen in solution to inhibit binding of immunoglobulin to fibrinogen-coated plates were measured, and the affinity constants (K_i) of these antibodies for fibrinogen were calculated by using the Sips equation as described by Nahm and co-workers (Nahm et al., 1977).

Immunoprecipitation Studies. Immunoprecipitation of ^{125}I-labeled fibrinogen proteolytic fragments was carried out by incubating 20 μL of hybridoma media supernatant with ~40 μL of radiolabeled protein (~100 000 cpm) in 0.01 M Na_2HPO_4–0.15 M NaCl buffer, pH 7.2, containing 0.01% ovalbumin, for 18 h at 4 °C. Rabbit anti-mouse immunoglobulins (50 μL) were added to each tube, and the mixture was incubated an additional 90 min at 37 °C. Aliquots (200 μL) of 10% suspensions of formalin-fixed Cowan strain I *Staphylococcus aureus* were added, and the tubes were incubated with frequent mixing for 45 min at room temperature. Following centrifugation, the pelleted material was suspended and washed 3 times in 0.01 M Na_2HPO_4–0.15 M NaCl, pH 7.2, buffer containing 1.0% $NaDodSO_4$ and 0.5% NP-40. Elution was carried out by heating the precipitate in nonreducing Laemmli sample buffer (110 °C, 7 min). The eluate was analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis radioautography.

Competition Antibody-Binding Assay. These studies were carried out essentially as described by Nowinski et al. (1980), using a modification of the competitive indirect immunoglobulin-binding radioassay described above. Ammonium sulfate concentrate of monoclonal antibody A5 was ^{125}I radiolabeled without prior additional purification. Radiolabeled A5 was added to microtiter plates whose wells were coated with either fibrinogen or ovalbumin (assay blank). Competition studies were performed by addition of dilutions of unlabeled monoclonal antibody preparations or M5 control. Under assay conditions, specific binding in the absence of inhibitor (B_{max}) was ~2000 cpm.

Results

Monoclonal Antibodies That Bind to Fibrinogen. Antibodies that reacted with fibrinogen were generated by immunizing BALB/c mice with purified human fibrinogen. Spleen cells of the immune mice were fused with mouse myeloma cells, and the cell population was propagated under selective conditions as described under Materials and Methods. Hybridomas that produced antibodies which reacted with fibrinogen were detected by indirect measurement of antibody binding to test antigen-coated microtiter wells by using an enzyme-linked immunoassay system. Control values for this system were obtained with the culture supernatant from the parental mouse myeloma M5 line, and ovalbumin-coated microtiter wells served as negative controls.

Of the 54 initial cell fusions, 3 stable cultures with the highest fibrinogen-binding activities were characterized. Two of these cultures (A5 and A6) secreted a heavy chain of the mouse IgG_1 subclass, while the third culture (B6) produced an IgM antibody. The affinities for fibrinogen of each of these antibodies are measured by application of the Sips equation by using an indirect radioimmune binding assay as described under Materials and Methods (Figure 1). Not unexpectedly, the affinity of the IgM antibody B6 for fibrinogen ($K_i = 4 \times 10^7$ M^{-1}) was lower than that of the two IgG_1 monoclonal antibodies ($K_i = 8.5 \times 10^8$ M^{-1} for A5 and $K_i = 1.6 \times 10^8$ M^{-1} for A6).

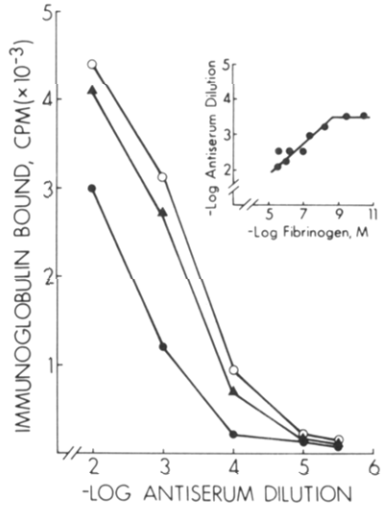

FIGURE 1: Fibrinogen binding by monoclonal antibody A5. The ability of differing concentrations of fibrinogen [(●) 10^{-6}, (▲) 10^{-8}, or (○) 10^{-10} M] to inhibit binding of immunoglobulin to fibrinogen-coated microtiter wells as a function of antibody dilution. Inset shows plot of midpoint values obtained from families of similar fibrinogen inhibition curves as a function of antibody dilution. K_i for antibody A5 was obtained from this plot as described under Materials and Methods.

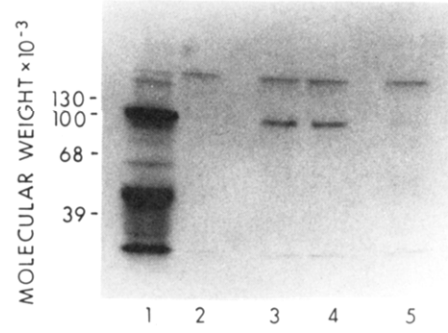

FIGURE 2: Domain specificities of monoclonal antibodies to fibrinogen as determined by immunoprecipitation and $NaDodSO_4$–polyacrylamide gel electrophoresis. ^{125}I-Labeled fibrinogen (9 μg) was digested with purified human plasmin (0.1 unit) for 2 h at 23 °C. The reaction was stopped by addition of bovine pancreatic trypsin inhibitor (100 KIU/mL). Immunoprecipitation and $NaDodSO_4$–polyacrylamide gel electrophoresis were carried out as described under Materials and Methods. Lane 1, total digest; lane 2, M5 supernatant; lane 3, A5 supernatant; lane 4, A6 supernatant; lane 5, B6 supernatant.

Identification of Antigenic Determinants. Incubation of the three monoclonal antibodies with stage 3 (Marder & Budzynski, 1974) plasmin digests of ^{125}I-labeled fibrinogen resulted in precipitation of a single protein band of M_r ~100 000 as determined by $NaDodSO_4$–polyacrylamide gel electrophoresis under nonreducing conditions (Figure 2). This band is consistent in mobility with fibrinogen-derived fragments D. Antibody B6 failed to precipitate any radiolabeled proteins from this digestion mixture. These results indicate that the determinants expressed in fibrinogen which are detectable by the two IgG_1 monoclonal antibodies are localized within the D domain, while those detectable by the IgM antibody are either altered or destroyed as a result of plasmin proteolysis.

These findings in regard to the site specificities of the two IgG_1 monoclonal antibodies were confirmed by means of competition experiments. In these studies, the abilities of purified fibrinogen-derived plasmin fragments D_1 and E_3 were directly compared with fibrinogen in solution with regard to their inhibition of immunoglobulin binding to fibrinogen-coated microtiter plate wells. As shown in Figure 3, fragment D_1 but

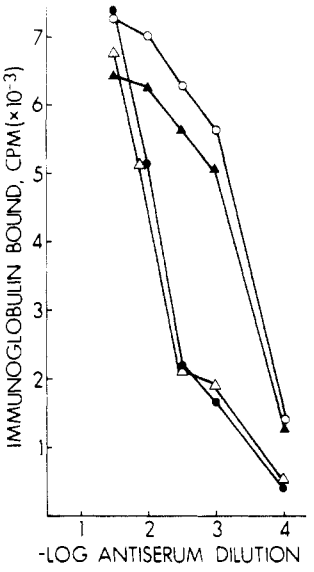

FIGURE 3: Ability of a fixed concentration (10^{-6} M) of different inhibitors to compete with fibrinogen-coated microtiter wells for binding differing dilutions of antibody A5. Inhibitors tested were fibrinogen (▲), fragment D_1 (●), ovalbumin (▲), and fragment E (○). Competitive indirect immunoglobulin-binding radioassays were carried out as described under Materials and Methods.

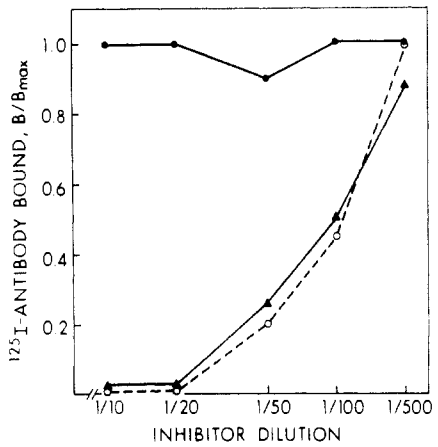

FIGURE 4: Relative abilities of unlabeled monoclonal antibodies A5 (○) and A6 (▲) to inhibit binding of ^{125}I-labeled A5 to fibrinogen-coated microtiter wells, compared with M5 (●) control. The competition antibody-binding assay was carried out as described under Materials and Methods.

not fragment E inhibited the binding of antibody to immobilized fibrinogen, and comparable findings were obtained with antibody A6 (data not shown). Thus, the determinants seen by these two antibodies expressed by intact fibrinogen are likewise expressed in purified fragment D_1 derived by plasmin fibrinogenolysis.

Next, the question as to whether the D-domain determinants detected by the two IgG_1 monoclonal antibodies differed significantly was studied by means of competition experiments using the antibodies themselves as inhibitors. As shown in Figure 4, antibody A6 displaces >90% of the binding of ^{125}I-labeled antibody A5 to microtiter plate wells coated with fibrinogen. These findings indicate that the determinants detected by A5 and A6 are very closely related or possibly identical.

Antibody Binding to Fragment D and D Dimer Derived from Fibrin. For determination of whether D-domain determinants were uniquely expressed in fibrinogen as compared with fibrin, inhibition studies were carried out comparing the abilities of fragment D derived from un-cross-linked fibrin and

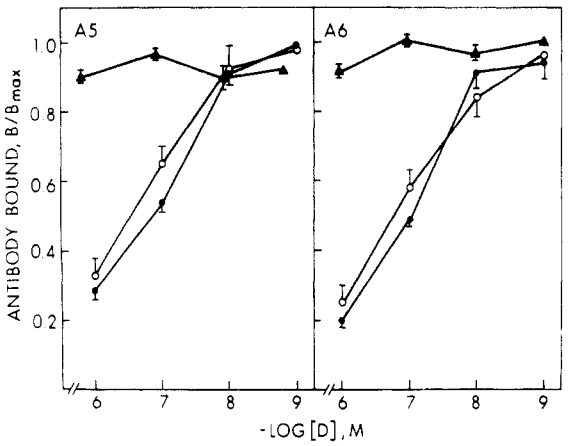

FIGURE 5: Comparative abilities of fragment D_1 derived from fibrinogen (○) and from un-cross-linked fibrin (●) and D dimer (▲) derived from cross-linked fibrin to inhibit binding of monoclonal antibodies A5 and A6 to fragment D_1 coated microtiter wells. Competitive indirect immunoglobulin-binding radioassays were carried out as described under Materials and Methods, using a constant antibody dilution (1:100 for both A5 and A6).

D dimer derived from cross-linked fibrin to inhibit binding of antibodies A5 and A6 to fibrinogen-derived fragment D_1. In the presence of a constant dilution of antibody, no significant differences were noted in the abilities of varying concentrations of fibrin and fibrinogen-derived fragments D to inhibit immunoglobulin binding to fibrinogen-derived fragment D_1 coated microtiter plate wells (Figure 5). By contrast, comparable concentrations of fragment D dimer derived from cross-linked fibrin produced little or no inhibition under similar competition assay conditions (Figure 5). Therefore, the antigenic determinants expressed by the D domain of fibrinogen are similarly available in fragment D derived from un-cross-linked fibrin but are no longer expressed in fragment D dimer derived from cross-linked fibrin.

Effects of Limited Proteolysis on Antigenic Expression. Since fragments D derived from fibrinogenolysis and fibrinolysis and fragment D dimer derived from lysis of cross-linked fibrin differ significantly only by the presence of COOH-terminal D–γ-chain isopeptide cross-links (Schwartz et al., 1971; Pizzo et al., 1973), the D-domain antigenic determinants presumably would involve these cross-linking sites. On the basis of the above considerations, the effects of further limited proteolytic digestion of this region on the expression of these D-domain determinants were studied. As has been shown by other investigators (Pizzo et al., 1973; Purves et al., 1978; Hörmann & Henschen, 1979; Nieuwenhuizen et al., 1981; Olexa & Budzynski, 1981), incubation of fragment D_1 with plasmin in the absence of Ca^{2+} degrades it to lower molecular weight forms (i.e., fragments D_2 and D_3). This degradation is due to the almost exclusive scission of segments from the COOH-terminal region of the γ chain which contains both the cross-linking sites and a sequence involved in fibrin polymerization (Olexa & Budzynski, 1981). Digestion of fragment D_1 by plasmin in the absence of Ca^{2+} results in progressive loss in the ability of this fragment to inhibit binding of antibodies A5 and A6 to D_1-coated microtiter plates (Figure 6). Incubation of antibodies A5 and A6 with plasmin-degraded ^{125}I-labeled fragment D_1 resulted in immunoprecipitation only of the higher molecular weight D fragment (~ 101 000), i.e., D_1, and not the lower molecular weight D variants (Figure 7). Therefore, our findings are consistent with the presumed location of these determinants to sites in proximity to or involving the COOH-terminal portion of the D–γ chain.

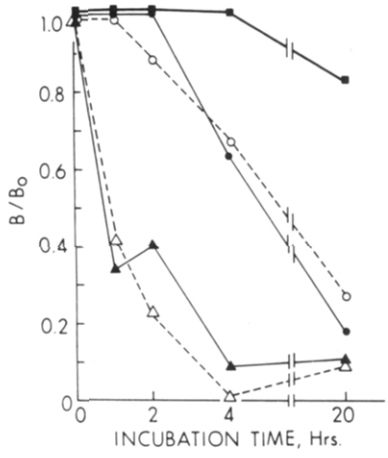

FIGURE 6: Effects of enzymatic digestion on antigenic expression of fragment D_1. Fragment D_1 was digested with plasmin (100 units/g of protein) [(●) A5, (○) A6] or trypsin (1:100 w/w) [(▲) A5, (△) A6] at 37 °C. Control studies were carried out in the absence of enzyme [(■) A5; A6 control not shown]. At the intervals shown, the reaction was stopped by addition of bovine pancreatic trypsin inhibitor. Aliquots (40 mL) of the digestion mixture were assayed at constant antibody dilution (1:100 for both A5 and A6). Concentration of inhibitor in the assay system was 10^{-6} M. Indirect immunoglobulin-binding radioassays using fragment D_1 coated plates were carried out as described under Materials and Methods.

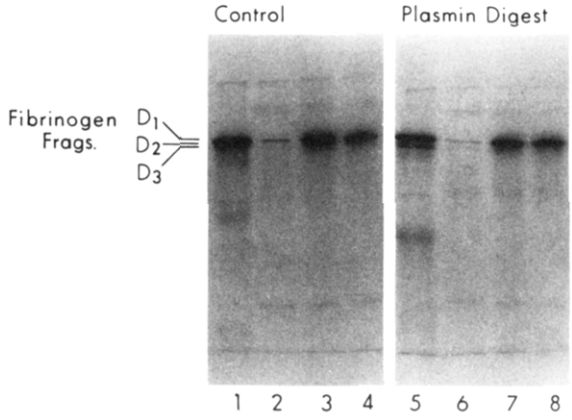

FIGURE 7: Immunoprecipitation of plasmin digests of fragment D_1 by monoclonal antibodies A5 and A6. ^{125}I-Labeled fragment D_1 (12 μg) was digested with plasmin (0.1 unit) for 2 h at 23 °C, and the digests were analyzed by immunoprecipitation and $NaDodSO_4$–polyacrylamide gel electrophoresis, as described under Materials and Methods. For these studies, a 10% resolving gel was used. Lanes 1 and 5, total digest; lanes 2 and 6, M5 supernatant; lanes 3 and 7, A5 supernatant; lanes 4 and 8, A6 supernatant.

Immunochemical Definition of D–γ-Chain Antigenic Sites. In a variety of immunoprecipitation, indirect immune binding, and inhibition studies similar to those described previously, antibodies A5 and A6 failed to bind to RCM chains derived from either fibrinogen or fragment D or to CNBr digests of isolated RCM γ chains derived from fibrinogen. Furthermore, neither plasmic nor tryptic fragments derived by extensive digestion of fragment D_1 inhibited A5 or A6 binding to D_1-coated plates (Figure 6). Since destruction of the secondary structure by either chain reduction or extensive proteolysis results in loss of antigenic expression, antigenic sites recognized by antibodies A5 and A6 may represent conformational rather than sequential antigenic determinants (Sela, 1969).

Discussion

In the present study, the question was raised as to whether immunochemical changes in the surface topography or conformation of fibrinogen would be detectable which would coincide with the fibrin transition. Our data demonstrate clearly the existence of unique antigenic determinants in the intact fibrinogen molecule whose expression is markedly altered coincident to cross-linked fibrin formation. These determinants are completely contained within high molecular weight fragments D (fragment D_1) derived from plasmin digestion of either fibrinogen or un-cross-linked fibrin and are destroyed following further plasmin degradation of fragment D_1 in the absence of Ca^{2+}. Recent studies by Olexa & Budzynski (1981) have confirmed the findings of Pizzo et al. (1973) and Hörmann & Henschen (1979) that plasmin digestion of fragment D under these conditions results in exclusive cleavage of the γ-chain remnant, leaving the α- and β-chain segments intact. Thus, loss of immunoreactivity coincident with further D–γ-chain cleavage and modulation of antigenic expression by the cross-linking process permits definition of these fibrinogen-specific antigenic determinants within the COOH-terminal M_r 13 000 segment of the fibrinogen γ chain. However, identification of the precise sequence which comprises these determinants has not proven feasible, since antigenic expression is lost coincident to loss of secondary structure. On the basis of these negative data, the assumption can be made that these antigenic determinants are conformational rather than sequential and require intact disulfide bridging in the D domain for their expression.

Attempts by other investigators using polyclonal antibodies to define specific antigenic differences between intact fibrinogen and fibrin have not been successful, suggesting great similarities in the surface structure between the protein in solution and its polymeric form. However, the existence of unique γ-chain antigenic determinants which mark the fibrinogen–fibrin transition has been suggested in immunochemical studies of fibrin(ogen) proteolytic fragments. Plow, Edgington, and co-workers (Edgington & Plow, 1975; Fair et al., 1981; Plow & Edgington, 1973) have demonstrated determinants in the γ-chain remnants of plasmin-derived fragments D which are differentially expressed in fibrinogen and fibrin. In addition, studies by Budzynski et al. (1979), Cierniewski et al. (1981), and Lahiri et al. (1981) have shown that the formation of γ-chain cross-links may effect antigenic changes sufficient to render immunochemical distinction between fibrinogen-derived fragment D and fragment D–D cross-linked fibrin. It is likely that the γ-chain determinants described here are related to those expressed in plasmin-derived fragments. However, by virtue of the resolution afforded through hybridoma technology, these γ-chain determinants, representing a small minority of the determinants detectable by polyclonal antibodies, become more clearly manifested.

The failure of these D-domain determinants to be expressed in cross-linked fragment D dimer suggests that the COOH-terminal region of the fibrinogen γ chain becomes conformationally altered or sequestered coincident to the cross-linking process. This hypothesis is supported by recent observations by Fowler and associates (Fowler et al., 1981), who have studied fibrinogen dimers cross-linked by factor XIIIa. These investigators have shown that the γ-chain cross-link site is at the tips of the outer (D-domain) nodules of the trinodular fibrinogen molecule and that there is little bending at this junction. Thus, γ-chain cross-linking produces axial dimers, and the immunochemical data presented here suggest that the hydrophilic γ-chain cross-linking site itself becomes internalized coincident to γ-chain dimerization.

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