Monoclonal Antibody to the Region of Fibronectin Involved in Cross-Linking to Human Fibrin†

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ABSTRACT: A cross-link-containing fragment (αXLCNBr), derived from the α-polymer component of human fibrin following CNBr digestion, has been isolated and characterized. NH_2-terminal sequence studies of three αXLCNBr derivatives, each prepared by a different method, indicate that the Aα-chain regions comprised of residues 241–476 (CNBr VIII) and 518–584 (CNBr X) are the major constituents of the cross-linked fragments examined. Evidence for at least two additional sequences suggests that Aα 208–235 (CNBr V) and Aα 585–610 (CNBr XI) may have auxiliary roles in α-polymer formation. When αXLCNBr was used as an immunogen for the production of murine hybridoma cell lines, two groups of antibodies were obtained. The majority of supernatants from primary hybridoma cultures did not discriminate between fibrinogen and αXLCNBr and appeared to contain antibodies directed against determinants within either CNBr VIII or CNBr X [see Ehrlich, P. H., Sobel, J. H., Moustafa, Z. A., & Canfield, R. E. (1983) *Biochemistry* (following paper in this issue)]. Several supernatants from primary hybridoma cultures, however, did exhibit significant binding toward the α-polymer derivative in the absence of demonstrable immunoreactivity toward either highly purified fibrinogen or its Aα-chain peptides, CNBr VIII and CNBr X. The monoclonal antibody produced by the first of these lines to be cloned (F-101) has been identified as an anti-fibronectin immunoglobulin G (IgG) based on data obtained from dose–response studies in which a variety of antigen preparations were tested for their ability to displace bound ^{125}I-labeled rabbit anti-mouse IgG in a two-step competitive immunoradiometric assay. When timed plasmin digests of [^{3}H]putrescine-labeled plasma fibronectin were subjected to Western blotting and the transfer was reacted with the monoclonal antibody (F-101) in an enzyme-linked immunosorbant assay (using horseradish peroxidase conjugated goat anti-mouse Ig for detection), coincident immunoreactivity and radioactivity were localized in a M_r 30 000 fragment(s). Collectively, these data provide confirmatory evidence for a factor XIIIa mediated covalent interaction between fibronectin and the α chains of fibrin and characterize a monoclonal antibody whose determinant is harbored within a cross-linking domain of fibronectin.

The transition from fibrinogen to insoluble fibrin occurs in several stages. Following the release of fibrinopeptide A by thrombin, desAA fibrinogen[1] molecules (fibrin monomers) polymerize in an end to end fashion, forming a structure commonly referred to as the fibrin I protofibril (Blombäck et al., 1978; Hantgan et al., 1980). This alignment appears to be sufficient for the introduction of factor XIIIa mediated ε-(γ-glutamyl)lysine cross-links between the γ chains of adjacent fibrin monomers (Chen & Doolittle, 1970). During later stages of fibrin assembly, protofibrils associate laterally, leading to the formation of the thick fibers referred to as fibrin II. Within the structure of these thickened fibrils, fibrin molecules are oriented so as to facilitate the introduction of intermolecular ε-(γ-glutamyl)lysine cross-links between neighboring α chains (McDonagh et al., 1971). This process, by which α polymers are formed, involves specific residues that are located within the COOH-terminal half of the Aα chain (Doolittle et al., 1977; Fretto & McKee, 1978; Cottrell et al., 1979) and appears to impart considerable stability to the expanding fibrin network since cross-linked fibrin II is characterized both by reduced solubility and by decresed susceptibility to lysis by plasmin (Gaffney & Whitaker, 1979; Francis et al., 1980). Fibronectin has also been reported to engage in ε-(γ-glutamyl)lysine cross-linking to the α chain of fibrin (Mosher, 1975), but the location of attachment sites and the extent to which this reaction occurs under physiological conditions have not been extensively studied.

Radioimmunoassays, developed to monitor the action of thrombin (Nossel et al., 1971) or plasmin (Nossel et al., 1979) on fibrinogen, have been widely employed to measure events occurring during the early stages of clot formation. Far less progress has been made in developing assays that recognize the introduction of factor XIIIa catalyzed cross-links, which are specific molecular hallmarks for the later stages of fibrin formation. In previous studies, we have described the utility of polyclonal immunoassays to quantify CNBr peptides from the regions involved in γ-chain dimerization (Lahiri et al., 1981) as well as those that participate in α-chain polymerization (Sobel et al., 1982). In this report, we describe the preparation and characterization of a cross-link-containing immunogen, designated αXLCNBr, derived from the α polymer of human fibrin. We also detail the immunochemical characterization of a monoclonal antibody, generated against this immunogen, which reacts with fibronectin and appears to recognize a determinant located within the region of fibronectin that is involved in cross-linking to fibrin. An accompanying report (Ehrlich et al., 1983) describes the char-

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[1] Abbreviations: desAA fibrinogen, fibrinogen lacking fibrinopeptide A regions on both Aα chains; CNBr, cyanogen bromide; αXLCNBr, cross-link-containing CNBr derivative of α polymer; CNBr I–CNBr XI, the 11 CNBr peptides of the Aα chain of human fibrinogen, designated by roman numerals to indicate their relative position from the Aα NH_2 terminus (see Figure 3); DEAE, diethylaminoethyl; NaN_3, sodium azide; Tris, tris(hydroxymethyl)aminomethane; $NaDodSO_4$, sodium dodecyl sulfate; TBS, Tris-buffered saline (0.05 M Tris–0.14 M NaCl, pH 7.4); TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; PBS, phosphate-buffered saline (0.01 M phosphate–0.14 M NaCl, pH 7.4); IRMA, immunoradiometric assay; GAM $F(ab')_2$, goat anti-mouse $F(ab')_2$; BSA, bovine serum albumin; RAM IgG, rabbit anti-mouse immunoglobulin G; ELISA, enzyme-linked immunosorbant assay; HRP-GAM Ig, horseradish peroxidase conjugated goat anti-mouse immunoglobulin; HPLC, high-performance liquid chromatography; PTH, phenylthiohydantoin.

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acterization of two other monoclonal antibodies also raised against this same immunogen. The binding sites for these two immunoglobulins appear to reside within regions of the fibrinogen Aα chain that harbor acceptor and donor activity during α-chain polymerization.

Materials and Methods

Citrated fresh frozen plasma, obtained from normal donors (New York Blood Center, New York, NY), and commercially available human fibrinogen (grade L, Kabi, Stockholm, Sweden) were used for the preparation of cross-linked fibrin. For some immunochemical studies, Kabi fibrinogen was further purified by a two-step procedure that included filtration on lysine–Sepharose and subsequent ion-exchange chromatography on DEAE-cellulose using a concave gradient of increasing phosphate–Tris and decreasing pH for elution. Details of this methodology have been reported elsewhere (Koehn & Canfield, 1981). The Aα-chain peptides, CNBr VIII and CNBr X, were isolated from fibrinogen as previously described (Sobel et al., 1982). Citrated fresh frozen plasma, obtained by plasmapheresis (Presbyterian Hospital, New York, NY), was used for the preparation of fibronectin (see below), factor XIII (Lorand & Gotoh, 1970), and plasminogen (Robbins & Summaria, 1970; McClintock et al., 1974). Sepharose CL-4B and all Sephadexes were from Pharmacia. DEAE-cellulose (DE52) was from Whatman. All chemicals were of reagent grade unless otherwise indicated.

Preparation of Cross-Linked Fibrins. Two units of fresh frozen plasma or 1 g of Kabi fibrinogen was clotted under conditions designed to promote maximal cross-linking. Methods for this procedure and subsequent processing steps have been previously described (Sobel et al., 1982). When the fibrin originating from whole plasma was to be employed for α-polymer isolation, a 4 M urea–1% acetic acid wash (28 °C, 24 h) was included to remove the bulk of incompletely cross-linked soluble fragments as well as plasma proteins that may have adhered to the fibrin surface. Prior to lyophilization, all preparations were exhaustively dialyzed against distilled water containing 0.02% NaN_3.

Preparation and Isolation of α Polymers. Cross-linked fibrin, originating from 2 units of fresh frozen plasma, was dissolved in 50–100 mL of 8.4 M guanidinium chloride (Heico)–0.15 M Tris, pH 8.6, and reduced and alkylated according to general procedures detailed elsewhere (Sobel et al., 1982). In some instances, carboxymethylation was conducted with ^{14}C- or ^{3}H-labeled iodoacetic acid (New England Nuclear), both of which were recrystallized (in the presence of unlabeled iodoacetic acid) from petroleum ether prior to use.

Fibrin chains were isolated directly from the reaction mixture by gel filtration on Sepharose CL-4B in 6 M guanidinium chloride, pH 2.9; 20-mL aliquots were processed at a time. The column effluent was monitored for 280-nm absorbance and for radioactivity in a Packard Tricarb Model 3255 scintillation counter using Ultrafluor (National Diagnostics) or Scinti Verse (Fisher) as diluents. Fractions representing α polymers of differing M_r were pooled (Figure 1A), exhaustively dialyzed against 1% formic acid, and lyophilized.

Preparation and Isolation of αXLCNBr Fragments. α polymer or nonreduced fibrin preparations were suspended in 70% formic acid at a protein concentration of 1–2% and stirred for 1 h at 28 °C prior to the addition of CNBr. Digest conditions were similar to those described elsewhere (Sobel et al., 1982).

CNBr-digested α polymer was gel filtered on Sephadex G-150 equilibrated in 10% acetic acid. The effluent was monitored for 280-nm absorbance and radioactivity. Fractions containing high molecular weight material were pooled and lyophilized (Figure 1B).

When CNBr-digested fibrin was used as a source of αXLCNBr, high molecular weight fragments were isolated and then purified free of disulfide-containing contaminants according to conditions described by Doolittle et al. (1977) in their studies of α-polymer derivatives. Column effluents were monitored for 280-nm absorbance and for radioactivity, where indicated. Fractions were pooled as shown in Figure 2 for whole plasma fibrin (panels A and B) and Kabi fibrin (panels C and D), respectively.

Preparation of Fibronectin and Its Derivatives. Plasma fibronectin was purified by sequential affinity chromatography on gelatin- and arginine-substituted Sepharoses (Vuento & Vaheri, 1979). In some cases, ion-exchange chromatography on DEAE-cellulose, using a linear phosphate–Tris gradient at constant pH for elution (Mosesson & Umfleet, 1970), was employed in place of the arginine–Sepharose step. Both protocols yielded a similar final product whose behavior on $NaDodSO_4$–polyacrylamide gel electrophoresis and whose amino acid composition were in keeping with reported findings for purified plasma fibronectin (Vuento & Vaheri, 1979). Fibrinogen contamination, measured immunologically (Sobel et al., 1982), was less than 1%.

[^{3}H]Putrescine labeling of fibronectin (10 mg) was conducted in 10 mL of 0.01 M Tris–0.14 M NaCl–0.02 M calcium chloride, pH 7.4, with purified preparations of fibronectin, factor XIII, and human thrombin (kindly supplied by Dr. John Fenton; 2000 NIH units/mg). The final concentration of reagents in the reaction mixture was as follows: fibronectin, 5.9×10^{-6} M; cold putrescine, 5.0×10^{-4} M; [^{3}H]putrescine (putrescine dihydrochloride, [2,3-^{3}H(N)]putrescine, 39 Ci/mmol; New England Nuclear), 2.4×10^{-7} M; factor XIII, 77 μg/mL; thrombin, 1 unit/mL. [^{3}H]Putrescine (100 μCi) was diluted with an equal volume of TBS, and the pH of this solution was adjusted to neutrality with solid Tris prior to its addition to the reaction mixture. Following an 18-h incubation at 28 °C with occasional stirring, the putrescine-labeled fibronectin was isolated by affinity chromatography on gelatin–Sepharose (Mosher et al., 1980). The material was exhaustively dialyzed at 4 °C (2 h, distilled water; 18 h, 0.1 M ammonium bicarbonate, pH 7.5) and then lyophilized. Under these conditions, 0.90–1.04 mol of putrescine was incorporated per mol of fibronectin subunit (M_r 220 000).

Fibronectin and its [^{3}H]putrescine-labeled derivative were subjected to timed plasmin proteolysis at neutral pH and 37 °C using a protein concentration of 0.05–0.1% and a final enzyme:substrate ratio of 0.075 casein unit/mg. Plasmin, prepared by streptokinase (Lederle) activation of plasminogen (McClintock et al., 1974), was used for the digestions which were terminated by the addition of aprotinin (100 units/mL; purchased as Trasylol from FBA Pharmaceuticals) and subsequent lyophilization.

NH_2-Terminal Analysis of αXLCNBr Fragments. Quantitites ranging from 2 to 8 mg of each αXLCNBr preparation were subjected to automated sequencing using general conditions detailed elsewhere (Sobel et al., 1982).

Amino Acid Analysis. Amino acid compositions were determined from duplicate 24-h hydrolysates obtained as previously described (Sobel et al., 1982).

Cyanoethylation of αXLCNBr Fragments. Quantitation of ϵ-(γ-glutamyl)lysine cross-links was performed on 10–50-μg aliquots of trypsin-treated αXLCNBr. TPCK–trypsin (Worthington) was employed for the digestions which were

carried out for 5 h at 37 °C with an enzyme:substrate ratio of 2% (w/w).

Acrylonitrile-mediated cyanoethylation was conducted according to a modification (Fretto & McKee, 1978) of methods originally described by Pisano et al. (1969). Cyanoethylated lysine derivatives not involved in cross-linking were effectively resolved from free lysine with the elution conditions used for amino acid analysis in this laboratory.

NaDodSO$_4$–Polyacrylamide Gel Electrophoresis. Electrophoresis was performed under reducing and nonreducing conditions in the presence of 0.1% sodium dodecyl sulfate (Weber & Osborn, 1969). Commercially available standards were employed for molecular weight estimates (BDH, Pharmacia). αXLCNBr fragments were run in a Buchler cylindrical gel apparatus using gels with a total acrylamide concentration of either 4% or 5%. For the characterization of fibronectin and its derivatives, and for all "Western blot" applications (see below), electrophoresis was performed under reducing conditions in an LKB Model 2001 unit using vertical slab gels with a total acrylamide concentration of either 5% or 7.5%. Reagents specially purified for electrophoresis (acrylamide, sodium dodecyl sulfate; obtained from BDH) were employed for slab gel studies.

Production of Hybrid Cells and Isolation of Monoclonal Antibodies. The αXLCNBr preparation used for initial immunizations originated from whole plasma fibrin and corresponds to the material designated as pool 1 in Figure 2B. These fragments, which were virtually insoluble in physiologic buffers following lyophilization, were suspended in PBS and mixed at length to obtain as even and fine a suspension as possible. Approximately 100 μg of this material, in Freund's complete adjuvant, was injected intraperitoneally into BALB/c mice, and a second dose was administered 1 month later. Following a $2^1/_2$-month rest period, the animals were boosted with the more soluble αXLCNBr derivatives corresponding to material designated pool 2 in Figure 2B; a 30-μg dose, in PBS, was injected intravenously 3 days prior to fusion.

The procedures employed for the fusion betwen αXLCNBr-sensitized splenocytes and cells from the myeloma line P3-NSI/4-AG-4-1 as well as general methods used for the selection and cloning of resulting hybridoma colonies were similar to those described elsewhere (Ehrlich et al., 1982).

Hybrid colonies were screened for antibody-producing cell lines, prior to and following cloning, by a solid-phase microtiter plate IRMA similar to that described by Pierres et al. (1979); ^{125}I-labeled GAM F(ab')$_2$ was employed to enable the detection of all immunoglobulin classes. The test antigens that were included in these initial evaluations were αXLCNBr (pool 2, Figure 2B), CNBr VIII (native as well as reduced and carboxymethylated), CNBr X, Kabi fibrinogen, and purified Kabi fibrinogen; all were plated at a concentration of 5–10 μg/50 μL in PBS.

Cloned hybridoma cells were grown in 150-cm^2 flasks, up to 200 mL, in medium supplemented with heat-inactivated horse serum or fetal calf serum (20% v/v). Following dialysis against PBS–0.02% NaN_3 (w/v) for 24 h at 4 °C, the culture supernatant was used directly as a source of monoclonal antibody for immunochemical study. Alternatively, when the cells were confluent, the supernatant was removed by centrifugation, and the cells were transferred to the same volume of Dulbecco's modified Eagle's medium but with 0.1 mg/mL BSA replacing the serum. After a 24-h incubation, the serum-free supernatant was harvested from the cell culture, dialyzed, and then used directly as a source of monoclonal antibody for immunochemical study.

Development of a Two Step Competitive IRMA for the Immunochemical Characterization of the Monoclonal Antibody F-101. Poly(vinyl chloride) microtiter plates (Dynatech) were coated with 50 μL of a standardized solution (20 μg/mL in PBS) of αXLCNBr (pool 2, Figure 2B). Following an overnight incubation at 4 °C, the wells were rinsed 3 times with distilled water. Unreacted sites on the wells' surface were blocked by a $1^1/_2$-h incubation at 28 °C in PBS containing 5% BSA (crystallized, Sigma). Following this and all subsequent steps, the plates were again washed 3 times with distilled water. Standards quantified by amino acid analysis, or unknowns (100 μL), were mixed, in plastic tubes, together with an equal volume of the monoclonal antibody (F-101). All dilutions of antigen and immunglobulin were made in PBS containing 1% BSA. F-101 was used at an initial dilution of 1:50 based on titration studies in which direct binding to αXLCNBr-coated wells was assessed; at this dilution, the binding of F-101 to αXLCNBr was 12 times above background. Following a $1^1/_2$-h incubation at 28 °C, 50 μL of the antigen–antibody solution was added to the αXLCNBr-coated solid phase, and incubation was continued for an additional $1^1/_2$-h period. Fifty microliters of ^{125}I-RAM IgG (New England Nuclear), diluted to contain 50000 cpm/50 μL, was introduced next, and after an overnight incubation at 4 °C, the wells were cut out and counted in a Packard Model 800C auto-γ counter. Results were expressed as picomoles per milliliter of standard solution with data plotted according to the logistic transformation model described by Rodbard & Lewald (1970).

Binding Site Localization of the Monoclonal Antibody F-101. [^{3}H]Putrescine-labeled fibronectin and its plasmin-digested derivatives were subjected to NaDodSO$_4$–polyacrylamide gel electrophoresis under reducing conditons on 7.5% gels (see above). Samples were run in triplicate with approximately 0.1–0.3 nmol of material applied per lane. The separated components within one lane were stained with Coomassie Blue while those within a second lane were subjected to Western blotting. In this procedure, protein material within the gel was electrophoretically transferred onto nitrocellulose paper (0.45 μm, Schleicher & Schuell) by using an ElectroBlot apparatus (E.C. Appartus Corp.); 7.5% gels were transferred for 2 h (200 mA) at 4 °C with buffers and blocking and washing reagents as specified by Burnette (1981). Underivatized fibronectin and its plasmin-digested products were treated in parallel as controls.

Localized bands of immunoreactivity on the transfer were detected by an ELISA in which HRP-conjugated GAM Ig (New England Nuclear) was employed for the visualization (Towbin et al., 1979) of antigen-bound F-101. The blot was intially incubated for 18 h at 28 °C in a 15–25-mL volume of F-101 diluted 1:10 in 0.01 M Tris–0.14 M NaCl–5% BSA (fraction V, Sigma), pH 7.4. It was subsequently reacted for $3^1/_2$ h at 28 °C with an equal volume of HRP-GAM Ig diluted 1:250 in this same buffer. Both steps were conducted in sealed plastic bags (Dazey Corp.) which were rotated end over end throughout the entire incubation period. Conditions employed for the enzymatic reaction between hybridoma-bound HRP-GAM Ig and the substrate *o*-dianisidine were those described by Towbin et al. (1979).

Fragments that contained incorporated [^{3}H]putrescine (reflecting their origin from a region of transglutaminase acceptor activity) were localized within the third electrophoresed sample as follows: 2-mm gel slices were solubilized in 0.5 mL of 30% hydrogen peroxide (18 h, 50 °C) and then diluted in 10 mL of Hydrofluor (National Diagnostics). After

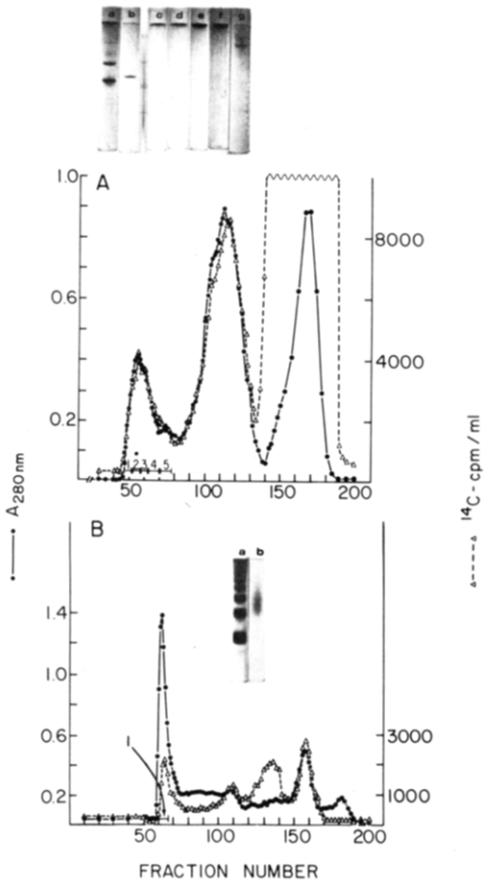


FIGURE 1: Isolation of αXLCNBr from purified α polymer. (A) Isolation of purified α polymers (originating from fibrin formed by clotting whole plasma) by Sepharose CL-4B chromatography. 119 mg (6.9 mg/mL) of reduced, ^{14}C-carboxymethylated fibrin was applied to the column (4.0 × 144 cm) and eluted as described in the text. 10.8-mL fractions were collected at a flow rate of 45 mL/h. Five α-polymer pools were obtained, as indicated, and pool 2 was taken for further study. γ-Dimer, β-chain, and α-chain remnants were localized within fractions 90–140. The large peak of 280-nm absorbance and radioactivity (fractions 150–190) contained excess β-mercaptoethanol and iodoacetic acid. (Gel insert; 5% gels, reducing conditions) Gels c, d, e, and f correspond to high molecular weight α polymers isolated from pools 1, 2, 3, and 4, respectively. Gel g shows the lower molecular weight oligomers confined to pool 5. Load material (gel a) and purified Aα chain (gel b) are included for reference. (B) Isolation of αXLCNBr from CNBr-digested α polymers by Sephadex G-150 chromatography. 71 mg (9.4 mg/mL) of CNBr α polymer (Figure 1A, pool 2) was applied to the column (2.5 × 145 cm) and eluted as described in the text. 4-mL fractions were collected at a flow rate of 18 mL/h. Pool 1, as indicated, was taken for biochemical characterization. (Gel insert; 4% gels, reducing conditions) Gel b corresponds to material from pool 1. Gel a, included for reference, is a high molecular weight standard comprised of multimers of a M_r 56 000 unit.

an 18-h equilibration at 4 °C, [^{3}H]putrescine radioactivity was determined.

Results

When highly cross-linked α polymer or the fibrin from which it originated was treated with CNBr, high molecular weight fragments could be separated by gel filtration as illustrated in Figures 1 and 2. Reduction, alkylation, and rechromatography of the material within the void volume of the column effluent shown in Figure 2A resulted in the removal of smaller fragments that contained the radioactivity introduced by S-carboxymethylation (Figure 2B). Similar results were obtained when Kabi fibrinogen was used as a source of cross-linked fibrin (Figure 2C,D).

Table I: Amino Acid Composition of αXLCNBr Fragments Obtained from Preparations of α Polymer (Figure 1) and Native Fibrins (Figure 2)[a]

	pool 1 αXLCNBr		
	Figure 1B	Figure 2B	Figure 2D
CM-cysteine[b]	1.2	1.0	1.2
aspartic acid[c]	8.6	8.6	9.2
threonine[d]	11.4	12.3	11.4
serine[d]	15.0	16.4	14.6
glutamic acid[c]	11.2	11.2	11.4
proline	8.3	8.0	7.4
glycine	13.0	14.3	13.5
alanine	3.9	3.4	3.9
valine	5.0	4.5	5.1
methionine[e]	0.3[f]	*f, g*	1.1
isoleucine	2.4	1.9	2.3
leucine	4.1	3.3	3.9
tyrosine	2.1	1.9	2.1
phenylalanine	3.0	2.7	2,5
histidine	2.5	2.5	2.6
lysine	4.9	5.2	5.3
arginine	5.7	5.7	5.6
tryptophan	ND[i]	ND	ND
cross-linked lysyl residues[h]	ND	1.2	1.2

[a] Expressed as mole percent. [b] Determined as (carboxymethyl)cysteine following reduction and alkylation. [c] Aspartic acid and glutamic acid include asparagine and glutamine, respectively. [d] Corrected by assuming 10% destruction. [e] Determined as the sum of homoserine and homoserine lactone. [f] Homoserine and/or homoserine lactone was not resolved in this analysis. [g] Values determined for similar preparations were 0.8–1.0 mol %. [h] Determined as free lysine in 24-h hydrolysates following cyanoethylation. [i] ND, not determined.

The properties of each of the three CNBr-derived high molecular weight pools, containing the fragments referred to here as αXLCNBr (Figures 1B, 2B, and 2D), were subsequently compared. Because frequent references to the CNBr peptides of the Aα chain of human fibrinogen are made in presenting and discussing the data described in this report, we have included Figure 3 for purposes of clarity; it shows the 11 Aα-CNBr peptides drawn to scale and aligned, based on the sequence studies of Watt et al. (1979), to indicate their relative position from the NH_2 terminus of the molecule. Putative cross-linking acceptor sites have been identified within CNBr VIII (Cottrell et al., 1979), and donor activity has been described for CNBr X (Doolittle et al., 1977; Fretto & McKee, 1978).

When αXLCNBr fragments were subjected to NaDodSO$_4$–polyacrylamide gel electrophoresis, considerable heterogeneity of size (M_r 100 000–300 000) was observed as shown in Figure 1B for the CNBr derivatives isolated from purified α polymer. The amino acid compositions obtained for each αXLCNBr preparation are listed in Table I.

Calculation of the number of α-chain cross-links, based on cyanoethylation data included in Table I, indicated that 2.8 and 3.0 mol of ε-(γ-glutamyl)lysine bonds per mol of CNBr VIII + CNBr X were present in the Kabi and whole plasma fibrin derivatives, respectively. CNBr VIII, treated in parallel as a control, yielded a value of 0.4 mol of cross-links/mol of peptide. Quantitation of ε-(γ-glutamyl)lysine bonds could not be carried out for the αXLCNBr preparation obtained from α polymer (Figure 1B, pool 1) due to insufficient material.

Results obtained following automated Edman degradation of the three αXLCNBr fragments are illustrated in Figure 4 and detailed in the figure caption.

The smaller αXLCNBr derivatives, shown in both panel B and panel D of Figure 2 as pool 2, migrated as M_r 80 000–

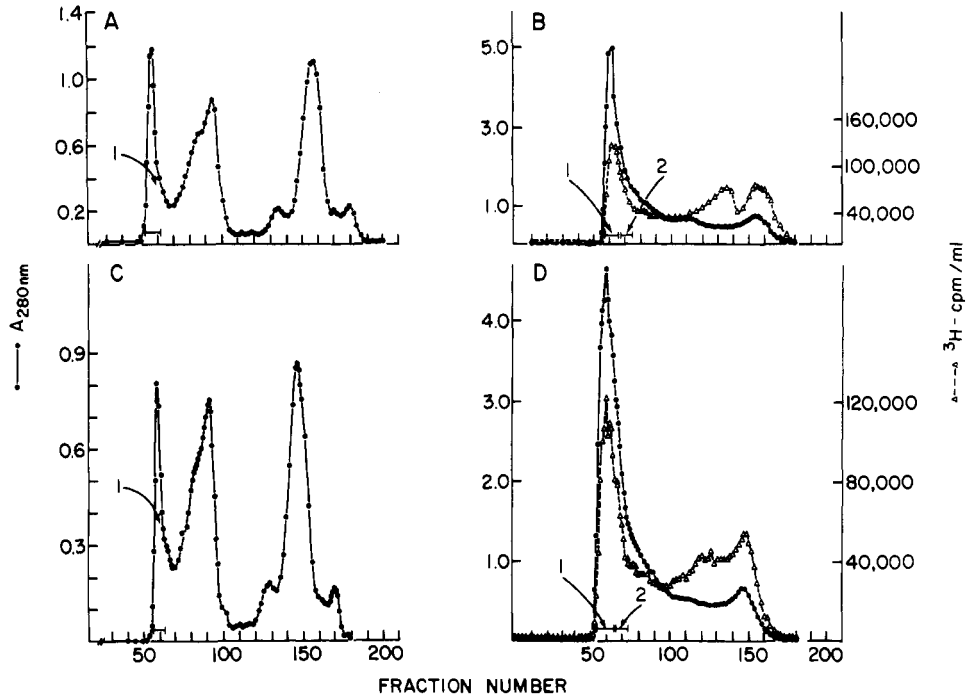


FIGURE 2: Isolation of αXLCNBr from nonreduced fibrin preparations. (Panels A and B) Isolation of αXLCNBr from fibrin prepared by clotting whole plasma. (A) 83 mg (11.8 mg/mL) of CNBr–fibrin was applied to a column of Sephadex G-150 (2.5 × 145 cm) equilibrated in 10% acetic acid. 4-mL fractions were collected at a flow rate of 20 mL/h. Pool 1 was taken for further study. (B) 226 mg (26.6 mg/mL) of ^{3}H-carboxymethylated pool 1 material (Figure 2A, pool 1) was applied to a column of Sephadex G-150 (2.5 × 148 cm) and eluted as described for panel A. Pool 1, as indicated, was taken for biochemical characterization. Material from this pool was used for immunization. Pool 2 was also characterized (see text for details) and served as the standard αXLCNBr preparation for all immunologic studies. (Panels C and D) Isolation of αXLCNBr from fibrin prepared by clotting Kabi fibrinogen. (C) 87 mg (12 mg/mL) of CNBr–fibrin was applied to a column of Sephadex G-150 (2.5 × 140 cm) and eluted as described for panel A. Pool 1 was taken for further study. (D) 338 mg (33.8 mg/mL) of ^{3}H-carboxymethylated pool 1 material (Figure 2C, pool 1) was applied to a column of Sephadex G-150 (2.5 × 147 cm) and eluted as described for panel B. Pool 1, as indicated, was taken for biochemical characterization.

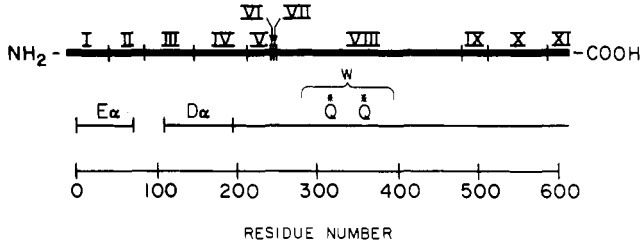


FIGURE 3: CNBr peptides of the the Aα chain of human fibrinogen. The 11 CNBr peptides are designated by roman numerals to indicate their relative distance from the NH_2 terminus of the Aα chain. The 10 methionine residues, indicated by vertical bars, are positioned according to residue number on the basis of the reported sequence data for the 610 amino acids that comprise the Aα chain (Watt et al., 1979). The following structural features of the α chain, discussed here and/or in the accompanying paper (Ehrlich et al., 1983), are included for reference: Eα and Dα, the α-chain components contained within plasmin-generated fragments E and D, respectively; Q*, transglutaminase-sensitive glutamine residues; W, the α-chain region that harbors a series of tryptophan-rich 13-residue repeats.

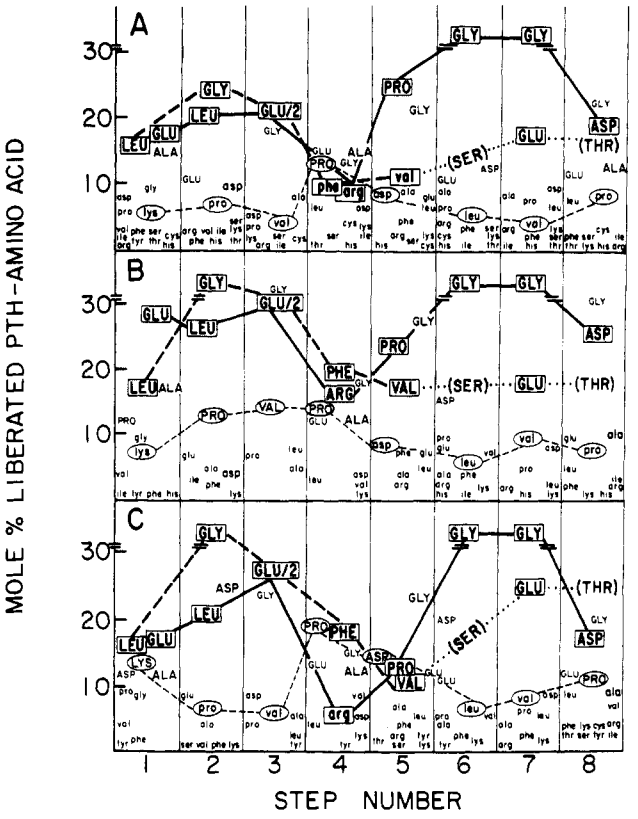
130000 fragments when subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis. While characterization by amino acid analysis, cross-link content, and Edman degradation indicated no apparent difference between these derivatives and the largest αXLCNBr fragments, the smaller molecules were more readily solubilized in aqueous solutions following lyophilization. Suspensions of the larger derivatives were used for immunization while the lower molecular weight αXLCNBr materials were employed for all immunologic characterization studies.

Following fusion of αXLCNBr-sensitized splenocytes with murine myeloma cells, many hybrid cells were obtained after approximately 2 weeks of growth in selective media. Cultures from each of 55 wells were taken for initial studies while those remaining were pooled, grown to a level of approximately 5 × 10^6 cells, and then frozen for studies to be pursued at a later time. Of these 55 colonies, 60% produced antibodies that bound to the cross-linked derivative. Five of the supernatants from primary hybridoma cultures exhibited apparent specificity for αXLCNBr as judged by their inability to bind to non-cross-linked CNBr fragments of the Aα chain. Cells from the well exhibiting the highest antibody titer were cloned, and the resulting immunoglobulin product (designated F-101) was characterized.

Table II: Immunochemical Characterization of F-101 As Determined by IRMA

antigen tested	ED_{50} (pmol/mL)	slope
plasma fibronectin	4.2	$2.21^{\pm 0.46}$
αXLCNBr (Figure 2B, pool 2)	36.1	$1.93^{\pm 0.45}$
Kabi fibrinogen	42.8	$1.70^{\pm 0.36}$
purified Kabi fibrinogen	12615	$(1.93^{\pm 1.87})$

Data shown in Figure 5 and Table II indicate that an immunoassay could be developed in which αXLCNBr in solution effectively competed with αXLCNBr attached to microtiter plates for F-101 binding. Displacement of bound ^{125}I-RAM IgG (identifying F-101 as an IgG immunoglobulin) occurred over a 100-fold dose range with an ED_{50} of 36 pmol/mL. All αXLCNBr fragments, whether isolated from Kabi fibrin (Figure 2, pool 2) or originating from purified α polymers (Figure 1A, pools 1–4), behaved in a comparable fashion (data not shown). When Kabi fibrinogen was subjected to similar dose–response studies, it competed as effectively as αXLCNBr for F-101 binding. By contrast, when Kabi fibrinogen was further purified and then tested in the IRMA, approximately 300 times as much material was needed to achieve the ED_{50}



CNBr VIII	GLU	LEU	GLU	ARG	PRO	GLY	GLY	ASN
CNBr X	LEU	GLY	GLU	PHE	VAL	SER	GLU	THR
CNBr V	LYS	PRO	VAL	PRO	ASN	LEU	VAL	PRO
CNBr XI	ALA	ASP	GLU	ALA	GLY	SER	GLU	ALA

FIGURE 4: NH_2-terminal sequences of αXLCNBr fragments. Panels A, B, and C graphically summarize the composition of residues released at each of the first eight steps (abscissa) following automated Edman degradation of αXLCNBr fragments. Yields were determined by amino acid analysis of back-hydrolyzed thiazolinones and are expressed as the mole percent of total amino acids recovered (ordinate). An elevated Gly background as well as servere destruction of Ser and Thr is expected with this methodology. Ser and Thr were identified by HPLC using 313-nm absorbance detection to monitor the unique byproducts generated by these two PTH-amino acids (Hunkapiller & Hood, 1978). Since this method was only semiquantitative, Ser and Thr, when present, are indicated enclosed in parentheses. Residues present at background level (<10%) are indicated in lower case letters while those representing significant contribution to the NH_2-terminal structure of αXLCNBr appear in capital letters. Panels A, B, and C illustrate results obtained for the pool 1 αXLCNBr fragments shown in Figure 1B, Figure 2B, and Figure 2D, respectively. The amino acids predominating at each step were compared with the reported sequence of the 11 Aα-chain CNBr peptides (Watt et al., 1979). Residues were boxed and connected by lines where yields were consistent with the known structure. Processed in this way, the data obtained for all three fragments were most compatible with the presence of CNBr VIII (heavy solid lines) and CNBr X (heavy dashed lines) as major components within αXLCNBr. The nanomole recovery of Glu obtained at step 3 in panels A, B, and C, while actually twice the value indicated, has been expressed as Glu/2 to emphasize that both CNBr VIII and CNBr X contribute a Glu residue at this step. Hence, the best estimate of yield from each is half the amount recovered. A third pattern, best seen in panel B (light dashed line), was consistent with a contribution from CNBr V as well. The reported sequences for the first eight residues of CNBr VIII, CNBr X, and CNBr V are indicated at the bottom of Figure 4. The NH_2-terminal structure of CNBr XI is also included since the sequence data suggest involvement of this Aα-chain region as well (see Discussion).

levels observed both for the cross-linked polymer derivatives and for Kabi fibrinogen that had not been subjected to additional purification steps. When cross-linked fibrin was prepared from the highly purified fibrinogen and then digested with CNBr, no significant immunoreactivity was observed

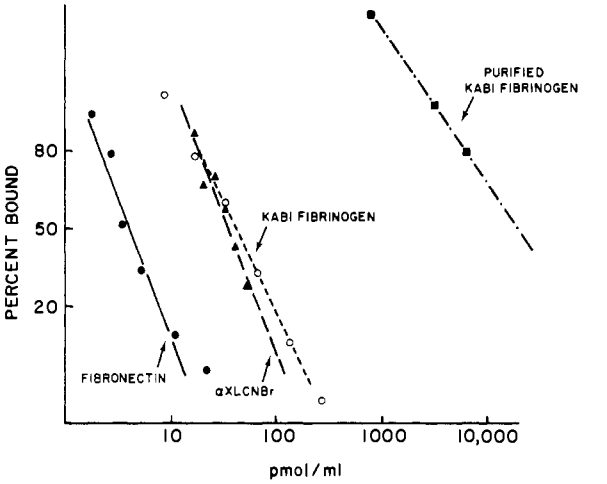


FIGURE 5: Immunochemical characterization of the monoclonal antibody F-101 by IRMA. αXLCNBr (Figure 2B, pool 2), Kabi fibrinogen, purified Kabi fibrinogen, and plasma fibronectin were tested for their ability to compete for F-101 binding with αXLCNBr attached to the solid phase. ^{125}I-RAM IgG displacement was measured (see text for details). Note that an acceptable computer fit could not be obtained for the purified Kabi fibrinogen dose–response curve due to an insufficient number of data points. (This is reflected in the wide confidence interval derived for the computed slope, indicated in Table II.) Molar concentrations were determined by using the following molecular weights: αXLCNBr, M_r 130000; fibrinogen, M_r 340000; fibronectin subunit, M_r 220000.

either in the soluble portion of the digest (as detected by IRMA) or in the digest precipitate (as detected by combined Western blotting and ELISA). These data (not shown) established that contaminating fibrin, if present at all, was not responsible for the strong F-101 immunoreactivity observed in Kabi fibrinogen. As shown in Figure 5, when fibronectin was tested in dose–response studies using the F-101 IRMA, its observed ED_{50} was 4.2 pmol/mL or approximately one-tenth the dose needed for comparable displacement by αXLCNBr. No significant difference was seen in the slopes of the fibronectin, Kabi fibrinogen, and αXLCNBr dose–response curves, as indicated in Table II.

Figure 6 illustrates results of studies in which [^{3}H]putrescine derivatives of fibronectin were subjected to Western blotting in order to localize the F-101 antigenic determinant. Plasmin proteolysis of labeled as well as native fibronectin produced similar patterns of released fragments as detected by Coomassie staining; in each case, a discrete band of M_r 30000 peptide material was observed. When the electrophoresed bands were assessed for F-101 immunoreactivity and for incorporated [^{3}H]putrescine, both the antibody binding site and the cross-linking acceptor region also appeared localized within a M_r 30000 fragment(s).

Discussion

These studies were initiated to learn more about the process of α-chain cross-linking that occurs in later stages of thrombus formation. Specifically, we sought to determine whether structural markers unique to α polymer could be defined and, if so, whether antigens bearing these markers might be of use in generating fibrin-specific antibodies. This report details the characteristics of the cross-linked derivative, αXLCNBr, used for immunization and describes the features of one monoclonal antibody, fortuitously discovered, that appears to react with a portion of fibronectin involved in cross-linking to the α chain of fibrin. The following paper (Ehrlich et al., 1983) describes two additional antibodies raised against the same immunogen; these recognize defined segments within CNBr VIII and CNBr

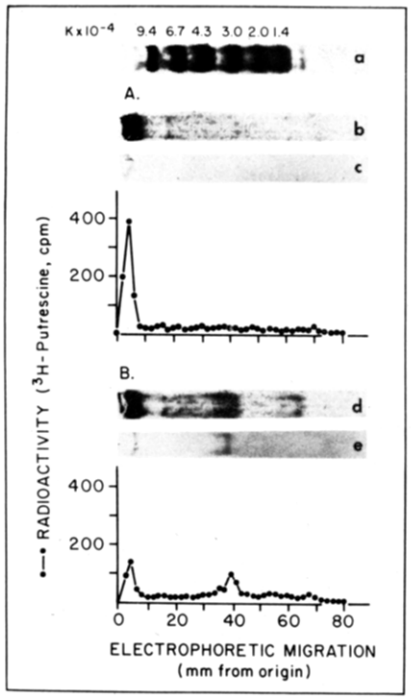


FIGURE 6: Localization of F-101's antigenic determinant within a transglutaminase-sensitive region of fibronectin. (A) [^{3}H]Putrescine-labeled fibronectin (13 μg; 59 pmol) was subjected to NaDodSO$_4$–polyacrylamide gel electrophoresis under reducing conditions, and the separated components were transferred onto nitrocellulose paper as described in the text. Lane c shows results obtained when the blot was subjected to an ELISA, using F-101. The migration of putrescine-associated radioactivity, determined for a duplicate electrophoresed sample (see Materials and Methods), is indicated directly beneath lane c. Lane b is the Coomassie-stained gel obtained following electrophoresis of unlabeled fibronectin (67 μg; 305 pmol) treated in parallel as a control. Lane a is a standard mixture of proteins, included for molecular weight reference. (B) A 5-h plasmin digest of [^{3}H]putrescine-labeled fibronectin (13 μg) was treated as described for panel A. Lane e shows results obtained when the blot was subjected to an ELISA using F-101; the migration of putrescine-associated radioactivity is indicated directly beneath it. Lane d is the Coomassie-stained gel obtained following electrophoresis of plasmin-digested unlabeled fibronectin (67 μg) treated in parallel as a control.

X (see Figure 3) which are Aα-chain regions that play significant roles in the process of polymer formation (Doolittle et al., 1977; Fretto & McKee, 1978).

In developing methods for the isolation of an immunogen that contained α-chain cross-linking regions, we chose to work with highly cross-linked fibrin preparations so that all possible contributing elements would be included. α polymers were chromatographically separated, and the largest species were selected for further study (Figure 1A). CNBr, rather than plasmin, was used to generate α-polymer fragments (Figure 1B) since the latter is known to produce peptides with considerable heterogeneity. The possibility that prior degradation of Aα chains in different starting fibrinogen preparations might lead to incomplete cross-linking was also explored by comparing αXLCNBr fragments derived from fresh frozen plasma with those isolated from Kabi fibrinogen (Figure 2). In these studies, nonreduced native fibrin preparations were treated directly with CNBr in an effort to achieve higher yields of αXLCNBr than those obtained from purified α-polymer preparations. The large fragments isolated in initial chromatographic steps (Figure 2A,C; pool 1) were reduced and carboxymethylated to alter the size of possible coeluting disulfide-rich CNBr fibrin derivatives (e.g., N-DSK, Ho-1DSK). This would also, we felt, eliminate regions that might have become covalently attached to the half-cystines within CNBr VIII (Aα 442 and 472) via disulfide interchange occurring under the influence of the reducing agents employed for the stabilization of activated factor XIII during clot formation. As shown in Figure 2B,D, the reduction and carboxymethylation step did lead to the elimination of some radiolabeled material in the subsequent gel filtration step.

All three αXLCNBr preparations (Figure 1B and Figure 2B,D; pool 1) exhibited strinkingly similar features with respect to size, degree of cross-linking, amino acid composition, and NH_2-terminal structure. Each was comprised of a heterogeneous group of large fragments whose apparent molecular weights were in the range of 100 000–300 000. Table I lists the amino acid compositions obtained for the three derivatives. The analyses indicate that each αXLCNBr fragment included a high proportion of hydrophilic residues consistent with their origin from the COOH-terminal half of the Aα chain (Lottspeich & Henschen, 1978; Henschen et al., 1978, 1979; Watt et al., 1979). While these data were also largely in agreement with the composition expected (Watt et al., 1979) if αXLCNBr's mass were comprised predominantly of CNBr VIII and CNBr X in an equimolar ratio, the increased levels of *S*-(carboxymethyl)cysteine, Ala, Ile, and Leu, as well as the decreased levels of Ser and Gly, indicated that elements other than CNBr VIII and CNBr X were included in each cross-linked derivative.

Results obtained from NH_2-terminal analyses of the three αXLCNBr preparations, shown in Figure 4, confirmed this view. While the interpretation of sequence data for multichained fragments must be viewed with caution, the comparison of the predominant PTH-amino acids recovered at each step with the known sequence of the Aα chain (Watt et al., 1979) provided convincing evidence for the identification of the major structural elements within αXLCNBr. Despite the variety of starting materials and routes of isolation employed for the purification of αXLCNBr, all three preparations exhibited nearly identical results following eight steps of Edman degradation. In each case, CNBr VIII (Aα 241–476) and CNBr X (Aα 518–584) represented the major components; the sequences identifying these two Aα-chain peptides were present in an approximately equimolar ratio and accounted for 45–50% of the amino acids recovered. All three fragments also exhibited significantly increased levels of Pro at step 4. Examination of the pattern of PTH residues observed at higher than background amounts at the other seven steps of the degradations suggested to us that CNBr V (Aα 208–235) was also included in the structure of αXLCNBr; the sequence identifying CNBr V (see, for example, panel B, Figure 4) was present at approximately half the level seen for CNBr VIII and CNBr X. When the remaining residues at each step were again evaluated for the possibility of additional Aα-chain contributions, elevated levels of Ala were observed at steps 1 and 4 in all three αXLCNBr preparations as well as at step 8 in the preparation shown in panel A of Figure 4. The presence of Ala at positions 1, 4, and 8 is unique to CNBr XI (Aα 585–610), suggesting that the α-chain COOH terminus may also be involved in the formation of highly cross-linked polymers. While incomplete CNBr cleavage could account for our sequence data, this seems unlikely on the basis of the fact that homoserine and homoserine lactone were the only methionine derivatives observed in the amino acid analyses. Similarly, although residual plasmin activity (occurring either before or during the long incubation period required for maximum α-chain cross-linking) could contribute to the observed NH_2-terminal heterogeneity, the reproducibility of the patterns seen in more than 10 sequencer runs of at least 8

different αXLCNBr preparations argues against this possibility.

Little is known about the detailed structure of α-polymer lattices except that multiple α chains can be involved in highly cross-linked preparations. The biochemical bindings reported here, which indicate that the regions represented by CNBr VIII and CNBr X are the major cross-linking constituents in the generation of α polymer, are consistent with earlier results obtained by Doolittle et al. (1977), Fretto & McKee (1978), and Cottrell et al. (1979). They are also supported by recently published immunological studies (Sobel et al., 1982). Moreover, there is sufficient resemblance to the sequences of CNBr V and CNBr XI in our data to suggest an auxiliary role in fibrin formation for these Aα-chain regions. This latter conclusion, based on evidence from multiple-step sequencer runs, supports observations made by others (Fretto & McKee, 1978) in which results of NH_2-terminal residue analyses were used to implicate CNBr V and CNBr XI in α-chain cross-linking. A recent report (Corcoran et al., 1980) suggests a donor function for the region represented by CNBr IX (Aα 477–517), but our data would not appear to support this proposal.

It should be emphasized that our findings regarding the involvement of a variety of α-chain components in polymer formation are derived from studies of cross-link-containing fragments that were isolated from late-stage clots; 3.0 and 2.8 ε-(γ-glutamyl)lysine bonds per mole of CNBr VIII + CNBr X were present in αXLCNBr isolated from whole plasma and Kabi fibrins, respectively. While it is generally accepted that each α chain contributes two cross-links per mole of plasma fibrin formed in vitro, it has also been suggested that a higher degree of α-chain cross-linking may occur (Pisano et al., 1971). Alternatively, mechanisms occurring in vivo may produce polymers cross-linked by less than two ε-(γ-glutamyl)lysine bonds per α chain as suggested by the data reported by Fretto & McKee (1978).

The formation of ε-(γ-glutamyl)lysine cross-links between fibronectin and the α chains of fibrin (Mosher, 1975) is thought to occur via an acceptor glutamine residue localized within the NH_2-terminal region of fibronectin (Jilek & Hörmann, 1977; Mosher et al., 1980; Furie & Rifkin, 1980). Structural studies have identified a pyroglutamic acid at the NH_2-terminus of the fibronectin molecule (Mosesson et al., 1975; Furie & Rifkin, 1980). In view of these features, a cross-linked contribution to αXLCNBr by the NH_2-terminal CNBr fragment of fibronectin would not be expected to contribute to our Edman degradation data but does account for the compositional findings obtained for each αXLCNBr fragment. Immunologic data using the monoclonal antibody F-101 confirmed that α-polymer–fibronectin hybrids were indeed present in the fibrin preparations used to isolate αXLCNBr.

The murine antibody producing cell line F-101 was originally selected for study toward the goal of isolating a fibrin-specific antibody. Initially, F-101 appeared to discriminate between cross-linked (αXLCNBr) and non-cross-linked forms (purified fibrinogen, CNBr VIII, and CNBr X) of the Aα-chain regions involved in polymer formation. As shown in Figure 5, it was possible to develop a radioimmunoassay for F-101 by employing a soluble form of αXLCNBr as the competitor. Evidence that we were in fact not dealing with a fibrin-specific antibody began to emerge when it was discovered that fibrinogen, purified from Kabi starting material, was approximately 300-fold less reactive than its parent preparation in the F-101 assay. A variety of plasma proteins are expected to be present as contaminants of commercial fibrinogen preparations by virtue of their coprecipitation during fractionation procedures designed to isolate the clotting protein (Mosher, 1980). Among these, fibronectin is known for its ability to cross-link with fibrin. Since F-101's determinant had to be covalently bound to α polymer in order for it to survive as an immunogenic component within αXLCNBr, given the chemical cleavages used in the preparation of this derivative, we considered fibronectin to be a likely candidate for the reaction with F-101. As illustrated in Figure 5, the relative ED_{50} levels seen for the fibronectin, Kabi fibrinogen, and αXLCNBr dose–response curves confirmed that the antibody, produced by clone F-101, had indeed been generated against a determinant within fibronectin. The parallelism in slope observed for all three competitors (see Table II) indicated that this epitope was the source of the F-101 immunoreactivity observed in both Kabi fibrinogen and αXLCNBr. While dose–response studies comparing the inhibitory effect of reduced, carboxymethylated, and native forms of fibronectin on the binding of F-101 to αXLCNBr (a reduced, carboxymethylated derivative) were not included here, results of Western blotting experiments shown in Figure 6 (panel A) indicate that immunoreactivity of the F-101 epitope is unaffected by disruption of the fibronectin molecule's disulfide bonds.

Several quantitative inferences can be drawn from the immunoassay data shown in Figure 5. Since the molecular weights of plasma fibronectin and fibrinogen are similar, it would appear that the fibronectin dimer (M_r 440000) is present as approximately a 5% contaminant in Kabi fibrinogen. This value is in keeping with the levels of fibronectin contamination reported for other commercially available fibrinogen preparations (Coller, 1980) and should be considered when these materials are used in experiments designed to study clotting. If one assumes that the F-101 epitope in αXLCNBr is fully cross-reactive and that the ratio of circulating fibrinogen:fibronectin is approximately 10:1, then the data shown in Figure 5 also suggest that fibronectin's contribution to the total mass of a plasma–fibrin clot is about 5%; this value is consistent with one of 4.4% reported by Mosher (1980).

In subsequent studies, we sought to determine whether the monoclonal antibody F-101 had been generated to an epitope adjacent to the cross-linking region of fibronectin. According to published reports, fibronectin acceptor glutamine activity can be localized within a M_r 27000 CNBr derivative and in a plasmin fragment of approximately the same molecular weight (Jilek & Hörmann, 1977). When CNBr- and plasmin-treated fibronectins were tested in the F-101 IRMA, both digests exhibited significant immunoreactivity. We elected to work with plasmin-treated material in our Western blotting studies since these digests were fully soluble and consistently retained more than 50% of their original immunoreactivity even after exposure to plasmin for 24 h. Results shown in Figure 6, for a 5-h plasmin digest of [^{3}H]putrescine-labeled fibronectin, strongly suggest that a M_r 30000 fragment originating from a region of acceptor cross-linking activity within intact fibronectin also contained the antigenic determinant recognized by F-101. These findings support reported data localizing fibronectin's transglutaminase acceptor activity to a M_r 27000 plasmin fragment (Jilek & Hörmann, 1977), which is presumed to originate from the NH_2 terminus of the molecule (Furie & Rifkin, 1980; Mosher et al., 1980), and demonstrate the specificity of F-101 for this cross-linking domain of fibronectin.

There have been several recent reports describing the isolation and characterization of anti-fibronectin monoclonal antibodies with specificity for the cell-binding (Pierschbacher et al., 1981) and heparin-binding (Smith & Furcht, 1982) regions of the molecule. The addition of the monoclonal antibody F-101 to this group of monospecific reagents provides a useful probe for addressing the as yet poorly defined role played by fibronectin in thrombus formation. For example, while it is evident from our studies with αXLCNBr that fibronectin becomes incorporated into α polymer under conditions designed to promote maximal cross-linking, little is known about how extensively this process occurs in vivo. It is also unclear how the formation of fibronectin–fibrin cross-links affects the stability of a clot. While the donor lysine residue involved in cross-linking with fibronectin is apparently included in one of the strutural elements comprising αXLCNBr, the precise location of its attachment site within the COOH-terminal half of the α chain remains to be determined. The use of the monoclonal antibody F-101, in conjunction with the α-chain-specific immunoglobulins described in the following paper (Ehrlich et al., 1983), can be applied to explore issues such as these that deal with the formation and fate of fibronectin–fibrin polymers.

Registry No. Factor XIIIa, 9067-75-8.

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