Monoclonal Antibodies to α-Chain Regions of Human Fibrinogen That Participate in Polymer Formation†

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ABSTRACT: Monoclonal antibodies have been generated against a cross-link-containing derivative of α polymer (αXLCNBr), isolated following CNBr digestion of fibrin [Sobel, J. H., Ehrlich, P. H., Birken, S., Saffran, A. J., & Canfield, R. E. (1983) *Biochemistry* (preceding paper in this issue)]. One cloned cell line (F-102) was chosen for characterization based on its apparent specificity for the Aα-chain region Aα 518–584 (CNBr X). A second line (F-103) was selected because of its anti-Aα 241–476 (CNBr VIII) properties. These two regions of the Aα chain have previously been implicated as major contributors to the cross-linking process that leads to α-polymer formation. Radioimmunoassays have been developed, employing the immunoglobulins produced by clones F-102 and F-103. These assays have been applied, in conjunction with high-performance liquid chromatography purified tryptic and chymotryptic derivatives of CNBr VIII and CNBr X, to localize the respective determinants involved in antibody binding. In each case, virtually full immunoreactivity was exhibited by both the CNBr fragment and a single tryptic or chymotryptic peptide originating from it. These findings indicate that sequence-specific, rather than conformational, determinants were operative in the generation of antibodies F-102 and F-103. The epitope recognized by F-102 was localized to the region of Aα 540–554, while the F-103 binding site resided within Aα 259–276. When these radioimmunoassays were applied to study the relative immunoreactivity exhibited by a variety of fibrinogen derivatives, the results obtained support earlier suggestions that the COOH-terminal portion of the Aα chain contains regions of random conformation.

The stabilization of aggregated fibrin monomers occurs via the introduction of ε-(γ-glutamyl)lysine cross-links between neighboring γ chains and α chains. While this process as it relates to γ-dimer formation is well-defined, the mechanism of α-polymer generation remains poorly understood. Reciprocal cross-links, involving γ chains of adjacent fibrin monomers, appear to form relatively rapidly, and the sites of donor and acceptor activity have been localized to the sixth and fourteenth residues, respectively, from the COOH terminus of the γ chain (Chen & Doolittle, 1971). By contrast, the cross-linking process between α chains is thought to occur at several locations with the potential of forming polymers that link many fibrin monomers together. While it is established that these latter reactions occur very slowly and that cross-linking activity is confined to the COOH-terminal two-thirds of the α chain, the precise location of all the bonds involved as well as the sequence of their formation remains unknown (Doolittle et al., 1977; Fretto & McKee, 1978; Cottrell et al., 1979; Sobel et al., 1982). Just as the details of α polymerization are as yet poorly defined, the manner in which these polymers are degraded by plasmin or other enzymes during fibrinolysis is also not established. A recently proposed scheme for the breakdown of cross-linked fibrin accounts for the fate of the γ-chain cross-links represented within D dimer complexes, but no detailed structural information is available concerning the fate of α-chain cross-linking regions during thrombus degradation (Francis et al., 1980). The ability of monoclonal antibodies to probe specific regions within large proteins makes them potentially useful as tools with which to search for cross-linked α-chain fragments in physiological systems.

Since the introduction of ε-(γ-glutamyl)lysine bonds into γ chains and α chains reflects events that occur late in fibrin formation, the capability of quantifying circulating fibrin degradation products that contain these cross-links could provide a valuable test for thrombosis. Immunoassays specific for α-chain cross-links might be especially valuable since the presence of such fragments in the circulation would imply that the parent fibrin molecule had been in residence within a thrombus for an extended period of time and was not derived from a rapid turnover state; i.e., such assays might have the capability of allowing one to distinguish among the various clinically documented disorders involving intravascular coagulation.

In order to study these problems, we have begun to generate murine hybridoma lines that produce antibodies which react with a cross-link-containing derivative of α polymers, designated αXLCNBr.[1] This fragment, which was employed for immunization, was isolated following CNBr cleavage of human fibrin. The details of its preparation, as well as its biochemical characteristics, appear in the preceding paper (Sobel et al., 1983). We describe here the immunochemical characterization of two monoclonal antibodies (F-102 and F-103) generated against αXLCNBr; the determinant recognized by antibody F-102 has been localized within a putative cross-linking donor region of the α chain while antibody F-103 appears to recognize a determinant within the acceptor region. While neither

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[1] Abbreviations: CNBr, cyanogen bromide; 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-chain NH_2 terminus [see Figure 3 of Sobel et al. (1983)]; X-T or VIII-T, trypsin-treated CNBr X or CNBr VIII; VIII-C, chymotrypsin-treated CNBr VIII; αXLCNBr, cross-link-containing CNBr derivative of the α polymer; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; DEAE, diethylaminoethyl; $NaDodSO_4$, sodium dodecyl sulfate; PMSF, phenylmethanesulfonyl fluoride; HPLC, high-performance liquid chromatography; AUFS, absorbance units full scale; PBS, phosphate-buffered saline (0.01 M phosphate–0.14 M NaCl, pH 7.4); NaN_3, sodium azide; Tris, tris(hydroxymethyl)aminomethane; BSA, bovine serum albumin; IgG, immunoglobulin G; RIA, radioimmunoassay; GAM, goat anti-mouse; RAM, rabbit anti-mouse; SAR, sheep anti-rabbit; EDTA, disodium ethylenediaminetetraacetate.

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antibody can distinguish between fibrinogen and cross-linked fibrin, the immunochemical specificity of these reagents for epitopes defined in this report allows one to measure localized segments within the COOH-terminal portion of the α chain toward the goal of clarifying the α-chain interactions that occur during fibrin formation and to search for these regions among the products of fibrinolysis.

Materials and Methods

Methods for the preparation of αXLCNBr as well as those describing the production, isolation, and screening of murine hybridoma lines generated against αXLCNBr have been detailed in the preceding paper (Sobel et al., 1983). Procedures for the preparation of the Aα-chain peptides, CNBr VIII and CNBr X, and for the purification of Kabi fibrinogen (grade L, Stockholm, Sweden) have also been previously described (Koehn & Canfield, 1981; Sobel et al., 1982). Plasmin-digested fibrinogen was prepared according to general procedures reported in the accompanying publication (Sobel et al., 1983), and the presence of end-stage fragments, D and E, was confirmed by $NaDodSO_4$–polyacrylamide gel electrophoresis using 7.5% gels. All other methods not specified here are detailed in two other recent publications from this laboratory (Sobel et al., 1982, 1983). TPCK–trypsin and chymotrypsin were purchased from Worthington, papain was obtained from Millipore, and human plasmin was prepared from purified plasminogen isolated from fresh human plasma. Sephadex G-50 was obtained from Pharmacia, DEAE-cellulose from Whatman, and DEAE Affi-Gel Blue from Bio-Rad. All chemicals were reagent grade unless otherwise indicated.

Preparation of Proteolytic Fragments of CNBr VIII and CNBr X. Reduced, S-carboxymethylated CNBr VIII and native CNBr X were each digested with trypsin at a protein concentration of 0.5% in 0.2 M ammonium bicarbonate, pH 8.5. Enzyme aliquots were added at 0 and 45 min in a final enzyme:substrate ratio of 1:50. Proteolysis was inhibited either by the addition of PMSF (0.1 mM) or by acidification, following which the digests were lyophilized. The identical protocol was used for the digestion of CNBr VIII with chymotrypsin.

Peptide Purification and Mapping by HPLC. Trypsin-digested CNBr VIII was initially gel filtered on Sephadex G-50 (fine) equilibrated in 0.08 M ammonium acetate, pH 5.4. The column effluent was monitored for 230-nm absorbance, and fractions were pooled, lyophilized, and resuspended in 0.5% acetic acid. Peptides recovered within pool 4 (Figure 5A) were subjected to further purification on HPLC. Trypsin-digested CNBr X and chymotrypsin-treated CNBr VIII were subjected to HPLC without any preliminary purification steps.

Details for each HPLC run appear in the figure captions. The following general methods are applicable in each case. Reverse-phase HPLC was performed on a μBondapak C_{18} column (0.39 × 30 cm, 20 μm; Waters) by using a Waters Associates system equipped with a 710A automatic injector, two M-6000A pumps, a 720 system controller, 450 (variable) and 440 (fixed) wavelength detectors, and a 730 data module. Gradient or isocratic elution was achieved by using a two-component mobile phase comprised of an initial solvent, A (0.1% orthophosphoric acid), and a limit solvent, B (50% acetonitrile in 0.1% orthophosphoric acid); both reagents were HPLC grade (Fisher). Lyophilized peptide material was resuspended in solvent A (2–10 mg/mL), and chromatography was conducted at 40 °C by using a flow rate of 1 mL/min. Absorbance was monitored at both 220 and 280 nm. One-milliliter fractions were collected, immediately neutralized with 0.1 mL of 0.2 N sodium hydroxide, and then lyophilized either as individual components or as pools. The dried material was resuspended in distilled water, and aliquots were taken for amino acid analysis and immunochemical application (see below).

Isolation of Anti-CNBr X (F-102) and Anti-CNBr VIII (F-103) Monoclonal Antibody Preparations. Two hybrid cultures that appeared to be directed against determinants within CNBr X (antibody F-102) and CNBr VIII (antibody F-103), respectively, based on results obtained in the initial screening assay, were cloned twice by limiting dilution. The supernatant harvested after 2 weeks of growth in medium containing either fetal calf serum or horse serum (20% v/v) was exhaustively dialyzed against PBS–0.02% NaN_3 (w/v) for 72 h at 4 °C and then used directly as a source of monoclonal antibody for immunochemical studies.

Preparation of Purified IgG and Fab Forms of the Monoclonal Antibody F-103. The supernatant (750 mL) harvested from F-103 cells grown in serum-free medium was exhaustively dialyzed (72 h, 4 °C) against 0.02 M Tris–0.05 M NaCl, pH 7.8, and then chromatographed on DEAE Affi-Gel Blue (2.5 × 5 cm) which had been equilibrated in this same buffer. The column was washed with 0.02 M Tris–0.05 M NaCl, pH 7.8, until the 280-nm absorbance returned to the base-line level. Bound components, predominantly BSA, were subsequently eluted with 1.5 M NaCl. The early-eluting and wash fractions were pooled, dialyzed against PBS containing 0.02% NaN_3 (48 h, 4 °C), and then lyophilized. This material was resuspended in water ($^1/_{50}$th of the original pool volume), exhaustively dialyzed against PBS–NaN_3, and then stored frozen as a source of purified F-103 IgG.

F-103 Fab was prepared from F-103 IgG by papain digestion and then isolated free of Fc fragments by ion-exchange chromatography on DEAE-cellulose. Both steps were conducted according to a reported procedure (Mage, 1980).

Radiolabeling. ^{125}I-CNBr VIII and ^{125}I-CNBr X were prepared by using either Chloramine-T or the oxidizing agent Iodogen (1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril; Pierce). In the latter case, CNBr VIII or CNBr X (10 μg) was mixed with 1 mCi of $Na^{125}I$ (Amersham) and insolubilized Iodogen (1 μg). Iodination was carried out according to a reported procedure (Fraker & Speck, 1978) except that PBS was substituted for the borate–saline, pH 8.2, buffer system described in the original protocol.

Liquid-Phase RIA. Duplicate point assays were conducted in plastic tubes by using a fixed volume of 0.5 mL. All dilutions of antigen and immunoglobulin were made in PBS containing 0.1% ovalbumin (crystallized, Sigma), 10 mM EDTA, 350 kallikrein inactivator units (KIU)/mL aprotinin (purchased as Trasylol; FBA Pharmaceuticals), and 0.01 unit/mL hirudin (Sigma). When fibrinogen and plasmin-digested fibrinogen were used as competitors in immunochemical characterization studies of F-102 and F-103, the final concentration of hirudin in the assay buffer was increased to 0.5 unit/mL. Preparations of each monoclonal antibody were used at the dilutions shown, in titration studies, to bind approximately 20% of the total counts in the absence of unlabeled antigen. Tracers were diluted so that 20000–30000 cpm were added per assay tube. Standard, known competitor or unknown (100 μL), monoclonal antibody (100 μL), and tracer (50 μL) were mixed with assay buffer (250 μL) and incubated initially for 2 h at 37 °C and then for 18 h at 4 °C. Antibody-bound counts were precipitated by the successive addition of carrier normal mouse serum and GAM (or RAM) IgG at dilutions previously shown to complex maximum levels of

Table I: Immunochemical Characterization of F-102

antigen	assay conditions: Ab preparation	assay conditions: tracer	ED_{50} (pmol/mL)	molar cross-reactivity	slope	K_a ($\times 10^8$ M^{-1})
CNBr X	F-102 in calf serum[a]	Chloramine-T	$298^{\pm 42}$	1.00	$1.71^{\pm 0.38}$	$2.3^{\pm 1.3}$
CNBr X-T	F-102 in calf serum	Chloramine-T	$275^{\pm 46}$	0.92	$1.26^{\pm 0.20}$	
Aα 540-554	F-102 in calf serum	Chloramine-T	$383^{\pm 69}$	0.78	$1.19^{\pm 0.25}$	
CNBr X	F-102 in calf serum	Iodogen	$171^{\pm 40}$	1.00	$1.64^{\pm 0.41}$	$2.1^{\pm 1.7}$
fibrinogen	F-102 in calf serum	Iodogen	$79^{\pm 19}$	2.16	$1.51^{\pm 0.40}$	
plasmin-treated fibrinogen	F-102 in calf serum	Iodogen	$141^{\pm 46}$	1.21	$1.01^{\pm 0.24}$	
αXLCNBr	F-102 in calf serum	Iodogen	$113^{\pm 29}$	1.51	$1.51^{\pm 0.43}$	

[a] F-102 in medium containing 20% fetal calf serum.

Table II: Amino Acid Composition of CNBr X Tryptic Peptides Isolated by HPLC (Figure 2) and Used for the Localization of F-102's Binding Site[a]

	T-1, fraction 32	T-2,[g] fraction 28	T-3,[h] fraction 29	T-4, T-9,[i] fraction 4	T-5, fraction 9	T-6, fraction 23	T-7, fraction 13	T-8, fraction 7
aspartic acid[b]	0.3	1.1 (1)	1.5	0.1		1.0 (1)	0.9 (1)	
threonine[c]	1.1 (1)	1.9 (2)	2.3			1.6 (2)	0.7 (1)	
serine	2.3 (2)	3.3 (2)	5.9 (4)	0.3	3.5 (4)	2.3 (2)[k]	1.7 (2)	1.1 (1)
glutamic acid[b]	2.8 (3)	1.9 (1)	3.3 (2)	0.1	0.2	1.0 (1)	0.8 (1)	0.2
proline		0.7	1.9 (2)					
glycine	1.0 (1)	2.2 (2)	2.2 (1)	1.1 (1)	0.2	0.3	0.9 (1)	0.6
alanine	0.1	0.4	0.9 (1)	0.1		0.2	0.2	
valine	0.8 (1)							
methionine[d]				0.9 (1)	0.2			0.3
isoleucine	0.1	1.2 (1)	1.3 (1)				0.4	0.1
leucine	0.9 (1)	0.2	0.2			0.6		
tyrosine	0.1		0.4		0.8 (1)	0.6 (1)		0.8 (1)
phenylalanine	1.0 (1)	1.3 (1)	2.1 (1)			0.7 (1)	0.7 (1)	
histidine	0.1	0.7	1.6 (2)					
lysine	0.2	0.9 (1)	0.9	0.9 (1)	1.1 (1)	0.1	0.8 (1)	1.0 (1)
arginine	0.9 (1)	0.5	1.4 (1)	0.1		0.7 (1)	0.2	
yield[e] (%)	76	97	59	98 (T-4), 101 (T-9)[j]	78	52	69	54[j]
Aα-chain residue no.[f]	518-528	529-539	540-554	555-556 (T-4), 584 (T-9)	557-562	563-572	573-580	581-583

[a] Listed as residues per molecule; 45-93% of the indicated peak fractions were hydrolyzed for 24 h. [b] Aspartic acid and glutamic acid include asparagine and glutamine, respectively. [c] Corrected by assuming 10% destruction. [d] Determined as the sum of homoserine and homoserine lactone. [e] Based on summed recoveries for all fractions within the peak. [f] Based on data reported by Watt et al. (1979a,b). [g] The residues indicated in parentheses define the composition of T-2; fraction 28 contained 27.6 nmol of T-2 and 8.7 nmol of T-3. [h] The residues indicated in parentheses define the composition of T-3; fraction 29 contained 16.9 nmol of T-2 and 15.6 nmol of T-3. [i] Fraction 4 contained 41.4 nmol of T-4 and 37.5 nmol of T-9 (free Met). [j] Approximately 30% of the total T-9 recovery was in the form of partially cleaved T-8 + T-9, localized in fractions 8 and 9; approximately 75% of the total T-8 recovery was in the form of partially cleaved T-7 + T-8 and T-8 + T-9, localized in fractions 8 and 9. [k] Reported Ser levels, based on sequence data (Watt et al., 1979a), are three residues per T-6.

monoclonal antibody in checkerboard titration studies. Incubation with a second antibody was conducted for 10 min at 37 °C and then for an additional 2 h at room temperature. Bound radioactivity was pelleted by centrifugation, and following aspiration of the supernatants, the tubes were counted directly in a Packard Model 5266 γ counter. Data were processed on an IBM 4341 computer using a four-parameter curve-fitting program based on the logistic model described by Rodbard & Lewald (1970). Results were expressed as picomoles per milliliter of standard solution where the dose was quantified by amino acid analysis. Antibody affinities were derived from the RIA data by using a program based on the model described by Scatchard (1949).

Determination of IgG Subclass. IgG subclass identification was performed by using commercially obtained preparations of RAM IgG_1, IgG_{2a}, IgG_{2b}, and IgG_3 (Bionetics) in the above-described RIA system which was modified as follows: Equal volumes (100 μL) of buffer, 4% normal rabbit serum, tracer (20000–30000 cpm/100 μL), and monoclonal antibody were mixed and incubated initially for 1 h at 37 °C and then for 30 min at room temperature. Control tubes for each subclass to be tested contained no monoclonal antibody. Following the addition of subclass-specific antiserum (100 μL of a 1:25 dilution), the tubes were successively incubated for 1 h at 37 °C and then for 18 h at 4 °C. Bound counts were precipitated by the addition of SAR IgG (1 mL of a 1:10 dilution) and a 4-h incubation at 4 °C.

Results

Immunochemical Characterization of the Anti-CNBr X Monoclonal Antibody, F-102. F-102 was selected as the product of a CNBr X directed line based on results obtained in the initial screening assay to which all hybrid cell cultures were subjected. The particular culture chosen for further study, in this case, exhibited strong reactivity toward αXLCNBr and CNBr X in the absence of any significant binding to CNBr VIII. As shown in Figure 1, it was possible to develop an RIA using the monoclonal antibody F-102 in which CNBr X was detected over a 1000-fold dose range with an ED_{50} of 300 pmol/mL. Subclass characterization studies indicated that F-102 was an IgG_1 immunoglobulin. Scatchard analysis yielded a value of $K_a = 2.3 \times 10^8$ M^{-1} for the binding of F-102 to the peptide antigen CNBr X.

In initial studies designed to localize F-102's antigenic determinant, the observation that trypsin-treated CNBr X (X-T) and intact CNBr X behaved identically in RIA dose–response studies suggested that a unique tryptic peptide might harbor the binding site recognized by F-102 (Table I). Figure 2, in conjunction with the amino acid composition data listed in Table II, characterizes the peptide map obtained when tryp-

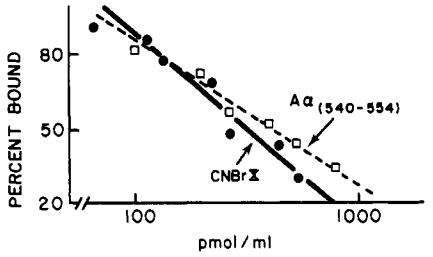

FIGURE 1: Immunochemical characterization of the monoclonal antibody F-102. CNBr X and Aα 540–554 (Figure 2B, fraction 29) were tested for their ability to displace hybridoma-bound ^{125}I-CNBr X, as described in the text. ^{125}I-CNBr X was prepared by using Chloramine-T. F-102, in medium containing fetal calf serum, was employed at an initial dilution of 1:3. Control tubes containing growth medium but no monoclonal antibody failed to exhibit any significant displacement of bound ^{125}I-CNBr X.

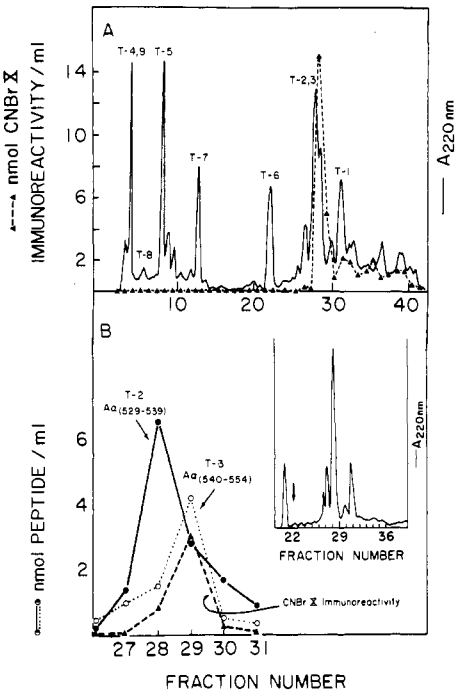

FIGURE 2: Localization of F-102's antigenic determinant by employing CNBr X tryptic fingerprints obtained by HPLC. (A) Trypsin-digested CNBr X (386 μg; 54.6 nmol) was applied in 200 μL of solvent A and eluted with a linear gradient (0–70% solvent B) developed over a 1-h period. AUFS (220 nm) = 1.0. Aliquots representing 0.5–10% of each fraction were assayed for CNBr X immunoreactivity (as described in the text). Note that the immunoreactivity profile has been drawn to compensate for the approximate 25-s delay between detection and elution of peptide material. Aliquots representing 45–93% of each fraction were subjected to amino acid analysis (see Table II). Peptides (T-1–T-9) are numbered to indicate their relative position from the NH_2 terminus of CNBr X based on the known primary structure of the Aα chain (Watt et al., 1979a). (B) *Inset*: Trypsin-treated CNBr X (74 μg; 10.3 nmol) was applied in 45 μL and chromatographed by using combined gradient and isocratic elution. A linear gradient was initially developed (0–26% solvent B) over a period of 22 min following which a constant acetonitrile concentration (26% solvent B) was maintained, as indicated by the arrow. AUFS (220 nm) = 0.4. Only the absorbance profile obtained during isocratic elution is shown since the earlier part of the chromatogram is identical with that indicated in panel A. Aliquots representing 2–10% of each fraction were assayed for CNBr X immunoreactivity, and aliquots representing 60–70% of each fraction were subjected to amino acid analysis. The peptide elution and immunoreactivity profiles obtained for the region of fractions 27–31 are illustrated in the left-hand portion of panel B, with the adjustments noted in the caption to panel A. Peptides are identified by number and inclusive Aα-chain residues, also as indicated in the caption to panel A.

sin-digested CNBr X was subjected to HPLC. Peptides were identified by amino acid analysis and have been referred to

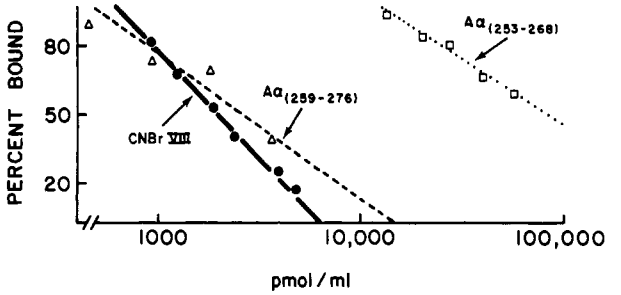

FIGURE 3: Immunochemical characterization of the monoclonal antibody F-103. CNBr VIII, Aα 259–276 (Figure 4C, fraction 8), and Aα 253–268 (Figure 5B, pool C) were tested for their ability to displace hybridoma-bound ^{125}I-CNBr VIII, as described in the text. ^{125}I-CNBr VIII was prepared by using Chloramine-T. F-103, in medium containing fetal calf serum, was employed in undiluted form. Control tubes containing growth medium but no monoclonal antibody failed to exhibit any significant displacement of bound ^{125}I-CNBr VIII. Purified bovine fibrinogen, when tested as a competitor in the F-103 assay, exhibited no cross-reactivity even at 60 times the ED_{50} molar concentration observed for human fibrinogen (data not shown).

by a number indicating their relative position from the NH_2 terminus of CNBr X based on reported sequence data (Watt et al., 1979a). As shown in Figure 2A for a fingerprint obtained by using gradient elution, of the nine peptides expected, five (T-1 and T-5–T-8) were identified within individual elution fractions, and two (T-4 and T-9) were found to coelute in a sixth fraction. T-2 and T-3 were poorly resolved, but, as shown in Figure 2B, a more effective separation was achieved by adjusting chromatographic conditions to include a period of isocratic elution. When HPLC effluent fractions were monitored for CNBr X immunoreactivity by RIA, the results obtained indicated that T-3, corresponding to Aα 540–554, harbored the antigenic determinant recognized by antibody F-102. As shown in Figure 2B, the immunoreactivity profile and the profile of T-3 elution, as determined by amino acid analysis, were coincident.

When the isolated tryptic peptide T-3 (Figure 2B, fraction 29) was used as a competitor in the CNBr X RIA, the dose–response curve obtained exhibited essentially the same slope over the same dose range as that observed for both intact and trypsin-digested CNBr X; the ED_{50} molar cross-reactivity of Aα 540–554 was 0.78 (Figure 1, Table I).

Immunochemical Characterization of the Anti-CNBr VIII Monoclonal Antibody, F-103. F-103 was selected as the product of a CNBr VIII directed cell line based on the observation that its parent cell culture exhibited strong reactivity toward αXLCNBr and CNBr VIII in the absence of any significant binding to CNBr X. As shown in Figure 3, it was possible to develop an RIA using the monoclonal antibody F-103 in which CNBr VIII was detected over a 1000-fold dose range with an ED_{50} of approximately 1900 pmol/mL. The use of Iodogen rather than Chloramine-T in the preparation of ^{125}I-CNBr VIII resulted in a significant increase in assay sensitivity (ED_{50} = 400 pmol/mL), as shown in Figure 7. F-103 was identified as an IgG_{2a} immunoglobulin on the basis of subclass characterization studies. Scatchard analysis yielded a value of $K_a = 2.2 \times 10^7$ M^{-1} for the binding of F-103 to the peptide antigen CNBr VIII.

In initial experiments designed to localize F-103's antigenic determinant, CNBr VIII as well as trypsin-treated CNBr VIII (VIII-T) and chymotrypsin-treated CNBr VIII (VIII-C) was subjected to RIA dose–response studies. Results obtained indicated ED_{50} molar cross-reactivities of 0.03 and 1.25 for the tryptic and chymotryptic digests, respectively (Table III). These data suggested that F-103's antigenic determinant might

Table III: Immunochemical Characterization of F-103

antigen	assay conditions: Ab preparation	assay conditions: tracer	ED_{50} (pmol/mL)	molar cross-reactivity	slope	K_a ($\times 10^7$ M^{-1})
CNBr VIII	F-103 in calf serum[a]	Chloramine-T	$1992^{\pm 185}$	1.00	$1.97^{\pm 0.32}$	$2.2^{\pm 4.8}$
CNBr VIII-C	F-103 in calf serum	Chloramine-T	$1560^{\pm 455}$	1.25	$1.82^{\pm 0.97}$	
Aα 259–276	F-103 in calf serum	Chloramine-T	$2839^{\pm 1042}$	0.69	$1.34^{\pm 0.69}$	
CNBr VIII-T	F-103 in calf serum	Chloramine-T	$64041^{\pm 9926}$	0.03	$1.08^{\pm 0.18}$	
Aα 253–268	F-103 in calf serum	Chloramine-T	$94101^{\pm 16468}$	0.02	$0.97^{\pm 0.21}$	
CNBr VIII	F-103 in horse serum[b]	Iodogen	$401^{\pm 94}$	1.00	$1.65^{\pm 0.76}$	$3.5^{\pm 3.7}$
fibrinogen	F-103 in horse serum	Iodogen	$98^{\pm 116}$	4.09	$1.32^{\pm 0.80}$	
plasmin-treated fibrinogen	F-103 in horse serum	Iodogen	$207^{\pm 229}$	1.94	$1.23^{\pm 1.00}$	
αXLCNBr	F-103 in horse serum	Iodogen	$50^{\pm 8}$	8.02	$2.34^{\pm 0.81}$	
CNBr VIII	F-103 IgG	Iodogen	$347^{\pm 126}$	1.00	$1.03^{\pm 0.26}$	$2.5^{\pm 1.8}$
fibrinogen	F-103 IgG	Iodogen	$354^{\pm 230}$	0.98	$0.60^{\pm 0.17}$	
CNBr VIII	F-103 Fab	Iodogen	$279^{\pm 167}$	1.00	$0.92^{\pm 0.31}$	$1.7^{\pm 1.5}$
fibrinogen	F-103 Fab	Iodogen	$332^{\pm 335}$	0.84	$0.57^{\pm 0.23}$	

[a] F-103 in medium containing 20% fetal calf serum. [b] F-103 in medium containing 20% horse serum.

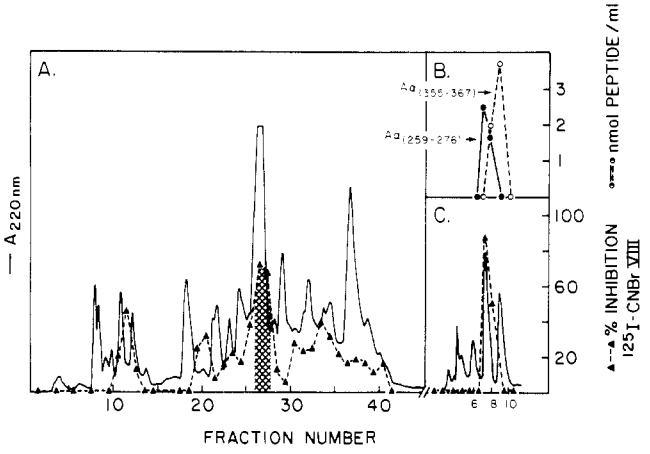

FIGURE 4: Localization of F-103's antigenic determinant by employing CNBr VIII chymotryptic peptides obtained by HPLC. (A) Chymotrypsin-treated CNBr VIII (840 μg; 41 nmol) was applied in 200 μL of solvent A and eluted with a linear gradient (0–70% solvent B) developed over a 1-h period. AUFS (220 nm) = 2.0. Aliquots representing 4% of each fraction were assayed for CNBr VIII immunoreactivity as described in the text. Note that the immunoreactivity profile (▲) has been drawn to compensate for the approximate 25-s delay between detection and elution of peptide material. Fractions within the crosshatched peak were pooled and subjected to further purification. (B and C) Approximately 60% of the pooled material indicated in panel A was applied in 200 μL of solvent A and eluted isocratically (31% solvent B). AUFS (220 nm) = 1.0. Aliquots of the column effluent (C) were subjected to RIA and amino acid analysis using 15% and 70% of each fraction, respectively. The immunoreactivity profile indicated in panel C (▲) and the composition of eluting peptides shown in panel B have been drawn with the adjustments noted in the caption to panel A. The actual amino acid composition data obtained are listed in the first two columns of Table IV. Peptides are designated by inclusive Aα-chain residues and/or by peptide number according to their relative position from the NH_2 terminus of CNBr VIII based on the known primary structure of this CNBr fragment (Strong et al., 1979; Watt et al., 1979a).

Table IV: Amino Acid Composition of CNBr VIII Peptides Isolated by HPLC (Figures 4C and 5B) and Used for the Localization of F-103's Antigenic Determinant[a]

	Figure 4C: fraction 8	Figure 4C: fraction 10	Figure 5B: pool C, T-3
aspartic acid[b]	1.7 (1)	0.4	0.3
threonine[c]	2.4 (2)	2.0 (2)	2.9 (3)
serine[c]	4.6 (5)	4.8 (5)	3.8 (4)
glutamic acid[b]	1.6 (2)	2.0 (2)	2.1 (2)
proline	1.8 (2)		1.0 (1)
glycine	4.6 (3)	2.4 (2)	3.7 (4)
alanine	1.1 (1)	0.3	0.2
valine		0.8 (1)	0.2
isoleucine			
leucine	0.2	0.1	0.1
tyrosine			0.7 (1)
phenylalanine			
histidine	0.1		0.1
lysine	0.1	0.1	0.1
arginine	1.0 (1)		1.0 (1)
tryptophan[d]	+	+	–
Aα-chain residue no.[e]	259–276	355–367	253–268

[a] Listed as residues per molecule; 70% of the peak fractions, 8 and 10, and 5% of the material recovered in pool C were hydrolyzed for 24 h. [b] Aspartic acid and glutamic acid include asparagine and glutamine, respectively. [c] Corrected by assuming 10% destruction. [d] Qualitative identification based on 280-nm absorbance monitored during HPLC. [e] Data taken from Watt et al. (1979a).

be harbored fully intact within a unique chymotryptic peptide of CNBr VIII and in a partially preserved form within this region's tryptic overlap.

As shown in Figure 4A, when chymotrypsin-digested CNBr VIII was subjected to HPLC and the effluent fractions were monitored by RIA, a significant proportion of the applied F-103 immunoreactivity was recovered as a discrete peak that eluted coincident with a region of high 220-nm absorbance. When this peptide material was subjected to further purification on HPLC, two major components were resolved (Figure 4C). These were identified by amino acid analysis (Table IV, first two columns) as C-2 (Aα 259–276) and C-7 (Aα 355–367). Chymotryptic peptides are referred to by a number indicating their relative position from the NH_2 terminus of CNBr VIII, assuming that the principal cleavage sites involve aromatic residues (Strong et al., 1979). The coincident profiles observed for F-103 immunoreactivity (Figure 4C) and C-2 elution (Figure 4B) indicated that the 18-residue chymotryptic peptide Aα 259–276 harbored the antigenic determinant recognized by F-103.

Figure 5A illustrates the results obtained when pools derived from a gel-filtered tryptic digest of CNBr VIII were tested in the F-103 RIA. Pool 4 was the only one to exhibit significant immunoreactivity. When material contained in this pool was subjected to HPLC using gradient elution conditions, at least 10 major components could be resolved as shown in Figure 5B. When the pooled fractions indicated in Figure 5B were assayed, significant F-103 immunoreactivity was localized uniquely to pool C. The amino acid composition of material from pool C (Table IV, last column) identified the tryptic peptide which we refer to as T-3 on the basis of its relative position from the NH_2 terminus of CNBr VIII. We conclude that the 14-residue Aα-chain region Aα 253–268, represented

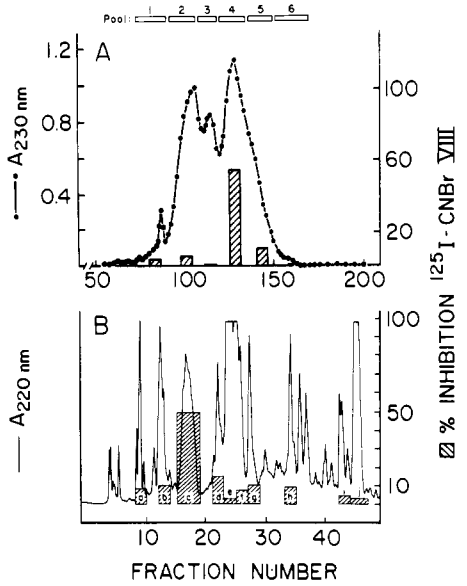

FIGURE 5: Localization of F-103's antigenic determinant (partial) by employing CNBr VIII tryptic peptides isolated by gel filtration and purified by HPLC. (A) Isolation of CNBr VIII tryptic peptides by gel filtration on Sephadex G-50. 40 mg (1.66 μmol) of trypsin-treated CNBr VIII was applied (5 mL) on a 2.5 × 198 cm column; 5.5-mL fractions were collected at a flow rate of 60 mL/h. Fractions were pooled as indicated by the horizontal bars. Aliquots representing 10% of each pool were assayed for immunoreactivity as described in the text. Pool 4 was taken for further study. (B) HPLC purification of a CNBr VIII tryptic peptide that contains F-103 immunoreactivity. 2.09 mg of pool 4 (Figure 5A) was applied in 125 μL of solvent A and eluted with a linear gradient (0–70% solvent B) developed over a 1-h period. AUFS (220 nm) = 2.0. Fractions were pooled as indicated (a–j). Aliquots of each pool were assayed for immunoreactivity and subjected to amino acid analysis by using 10% and 5% of the total material within each pool, respectively. The amino acid composition data obtained for material within pool C are listed in the last column of Table IV.

by the CNBr VIII peptide T-3, contributed elements to the binding of antibody F-103.

As illustrated in Figure 3, when C-2 (Figure 4C, fraction 8) and T-3 (Figure 5B, pool C) were used as competitors in the CNBr VIII RIA, the individual peptides exhibited patterns of immunoreactivity similar to those originally found for their respective whole digests. The ED_{50} molar cross-reactivity for Aα 259–276, compared to CNBr VIII, was 0.69, while that calculated for Aα 253–268 was 0.02 (Table III). The binding site for antibody F-103 would appear to span the trypsin-sensitive bond located between residues 268 and 269.

Binding of Monoclonal Antibodies F-102 and F-103 to Fibrinogen, Fragments of Fibrinogen, and αXLCNBr. Two issues were addressed by employing F-102 and F-103 in studies of binding to their epitopes in different size molecules. First, the immunoreactivity of the different molecules was compared with the immunoreactivity of the short peptide (which probably lacks any stable conformation) that encompasses the epitope. Second, it was determined whether each antibody could bind simultaneously to the epitope on both α chains of a fibrinogen molecule by comparing affinity constants for the whole antibody and its Fab fragment. These data are summarized in Tables I and III. When ^{125}I-labeled CNBr X was used, all competitors tested in the F-102 assay (CNBr X, Aα 540–554, fibrinogen, plasmin-treated fibrinogen, and αXLCNBr) exhibited significant cross-reactivity (Figures 1 and 6 and Table I). The data for antibody F-103 (Figures 3 and 7 and Table III) indicate that the affinity for the epitope was also not changed significantly when it was contained within an intact molecular fragment thereof. While a higher affinity of F-103

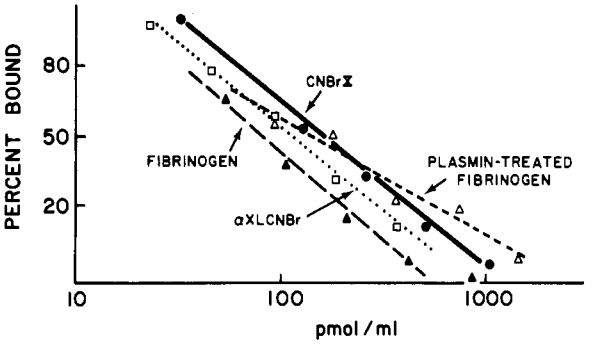

FIGURE 6: Immunoreactivity of fibrinogen and its derivatives toward the monoclonal antibody F-102. CNBr X, fibrinogen, plasmin-treated fibrinogen, and αXLCNBr were tested for their ability to displace hybridoma-bound ^{125}I-CNBr X as described in the text. ^{125}I-CNBr X was prepared by using Iodogen. F-102 in medium containing fetal calf serum was employed at an initial dilution of 1:3. Growth medium alone failed to cause any significant displacement in hybridoma-bound ^{125}I-CNBr X, and purified bovine fibrinogen, when tested as a competitor in the F-102 assay, exhibited no cross-reactivity even at 125 times the ED_{50} molar concentration observed for human fibrinogen (data not shown). Molar concentrations were based on the following molecular weights: CNBr X, M_r 6000; fibrinogen and plasmin-treated fibrinogen, M_r 340 000; αXLCNBr, M_r 130 000.

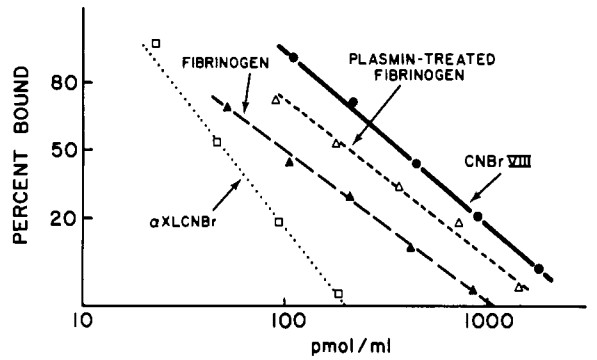

FIGURE 7: Immunoreactivity of fibrinogen and its derivatives toward the monoclonal antibody F-103. CNBr VIII, fibrinogen, plasmin-treated fibrinogen, and αXLCNBr were tested for their ability to displace hybridoma-bound ^{125}I-CNBr VIII, as described in the text. ^{125}I-CNBr VIII was prepared by using Iodogen. F-103 in medium containing horse serum was employed at an initial dilution of 1:3. In the absence of monoclonal antibody, the growth medium did not produce any significant displacement in hybridoma-bound ^{125}I-CNBr VIII. Molar concentrations were based on the following molecular weights: CNBr VIII, M_r 24 000; fibrinogen and plasmin-treated fibrinogen, M_r 340 000; αXLCNBr, M_r 130 000.

for fibrinogen (over the affinity for CNBr VIII) was detected in the dialyzed cell supernatant, purified IgG and Fab forms of the antibody F-103 showed a slightly lower reactivity with fibrinogen than CNBr VIII (Table III) (when it is assumed that fibrinogen has 2 mol of each epitope). Thus, bivalent binding to fibrinogen is not important for either antibody since both antibodies F-102 and F-103 bind fibrinogen with equal or lower affinity than the CNBr peptide, and the Fab fragment of F-103 has approximately equal affinity for fibrinogen and CNBr VIII.

Discussion

Concepts that correlate fibrinogen structure with its function in thrombus formation have been aided by three types of investigation, namely, the elucidation of the primary structure of the α, β, and γ chains (Henschen & Lottspeich, 1977; Lottspeich & Henschen, 1978a,b; Henschen et al., 1978, 1979; Watt et al., 1979a,b), the identification of three cystine-rich globular domains following plasmin cleavage (in a ratio of two

D fragments to one E fragment per mole of fibrinogen) (Marder et al., 1969), and high-resolution electron microscopy studies indicating an alignment of these cystine-rich regions in the fibrinogen molecule (Hall & Slayter, 1959; Fowler & Erikson, 1979). Additional electron microscopy studies of protofibril structures have led to a model of fibrin assembly that accounts for (1) interactions between the E domain and the D domains of neighboring fibrin monomers following removal of fibrinopeptide A by thrombin, (2) the end to end growth of the protofibril, and (3) the interactions between regions within adjacent fibrin monomers that permit the introduction of γ-chain cross-links (Fowler et al., 1981). These models also account for the coiled-coil regions within fibrinogen that were proposed as connections between the D and E domains of the molecule (Doolittle et al., 1979). The only portion of the fibrinogen molecule that remains relatively ill-defined in these models is the COOH-terminal two-thirds of the α chain. This region, into which α-chain cross-links are introduced during α-polymer formation, plays a central role in thrombus stabilization (Gaffney & Whitaker, 1979). For this reason, as noted earlier, it could serve a a focal point for the development of diagnostic methods to detect circulating α-polymer degradation products.

The studies described in this and the preceding paper, while designed toward the practical goal of isolating a fibrin-specific monoclonal antibody, provided a vehicle for exploring the relationship between the primary structure of the α-chain cross-lining region and the multiple interactions that occur among α chains during fibrin formation. In selecting an immunogen that would best reflect all possible α-chain associations in their stabilized form (i.e., cross-linked), we chose to work with the largest α-polymer derivative isolated from highly cross-linked fibrin following CNBr digestion. As detailed in the preceding paper (Sobel et al., 1983), biochemical characterization of the fragment αXLCNBr indicated that its α-chain constituents included Aα 241–476 (CNBr VIII) and Aα 518–584 (CNBr X) as well as Aα 208–235 (CNBr V) and possibly Aα 585–610 (CNBr XI). Two of the many monoclonal antibodies generated against αXLCNBr were isolated and immunochemically characterized. These studies, in which we took advantage of the high resolving power of HPLC to provide a battery of peptides with which to define the respective determinants recognized by the antibodies, provide data from which structural inferences about the α-chain cross-linking region can be drawn.

Antibody F-102 bound the Aα-chain derivative CNBr X with an affinity of 2.3×10^8 M^{-1}, and the particular epitope recognized was identified as residing in the CNBr X tryptic peptide corresponding to Aα 540–554. As shown in Figure 2A and Table I, we were able to obtain a fully characterized tryptic fingerprint of CNBr X by starting with only 386 μg (54.6 nmol) of digested material. Of the nine peptides expected, based on the known primary structure of this region of the Aα chain (Lottspeich & Henschen, 1978b; Watt et al., 1979a), six were recovered in yields ranging from 76 to 100% while three were recovered in yields ranging from 52 to 59%. The amino acid compositions obtained for each of the CNBr X tryptic fragments were in keeping with reported sequence data, the single discrepancy being a lower Ser level (two residues per mole) observed for our T-6 preparation compared to the three residues per mole expected for this peptide. The disparity in our results can be explained in light of the known lability of serine during acid hydrolysis and the fact that quantitation in these studies did not include extrapolation following timed hydrolysis.

When consecutive fractions from the CNBr X fingerprint were assayed for their ability to displace hybridoma-bound CNBr X radioactivity, a single peak of immunoreactivity was found. As suggested by data shown in Figure 2A and confirmed by results found with the improved resolution fingerprint shown in the inset to Figure 2B, the peak of F-102 immunoreactivity was coincident with the elution of the CNBr X tryptic peptide Aα 540–554.

By similar procedures, antibody F-103 was found to bind CNBr VIII with an affinity of 2.2×10^7 M^{-1}. In pursuing the identification of the antigenic determinant recognized by this antibody, we were aware that HPLC fingerprints derived from proteolytic digests of CNBr VIII would be far more complex than the nine-component map described above for trypsin-treated CNBr X. We elected, therefore, to purify and characterize only those peptides which eluted within immunoreactive regions of the HPLC column effluent, as localized by the F-103 RIA. As shown in Figure 4A, the fingerprint obtained following HPLC of chymotrypsin-digested CNBr VIII contained a variety of peptides, many of which were poorly resolved under the gradient elution conditions used. While several immunoreactive regions were localized when this column effluent was assayed with F-103, only the one corresponding to fractions 26–29 contained a major proportion (41.3%) of the total CNBr VIII activity applied to the column. Purification of this material, as shown in Figure 4C, resolved two predominant chymotryptic peptides whose respective compositions were in keeping with the presence of Aα 25–276 (C-2) and Aα 355–367 (C-7) based on the known primary structure of CNBr VIII (Strong et al., 1979; Watt et al., 1979a,b; Henschen et al., 1979). The amino acid compositions obtained for these peptide preparations are listed in the first two columns of Table IV. Except for slightly higher Asp and Gly levels (one and three residues per mole expected, respectively) in the case of C-2, the analyses are in excellent agreement with those anticipated. As shown in Figure 4B,C, F-103 immunoreactivity was associated uniquely with the peptide C-2 and was present at a level that approximated the amount expected based on yields obtained by amino acid analysis of C-2-eluting material in fractions 8–10. We conclude that the antigenic determinant recognized by the CNBr VIII directed monoclonal antibody F-103 was localized within the region Aα 259–276.

Studies designed to isolate and characterize the entire tryptic peptide that overlapped the fully reactive chymotryptic fragment C-2 provided a further definition of the residues involved in the antigenic determinant recognized by antibody F-103. As shown in Figure 5, a combination of gel filtration, HPLC, and RIA techniques was used to recover a single immunoreactive tryptic peptide, Aα 253–268 (T-3), from among the 19 fragments expected based on the known sequence of CNBr VIII. The composition of this material, listed in the last column of Table IV, is entirely consistent with the reported primary structure of this region of the Aα chain (Strong et al., 1979; Watt et al., 1979a; Henschen et al., 1979). When Aα 253–268 was subjected to dose–response studies using F-103, it exhibited only 3% of the immunoreactivity found for the chymotryptic representative of this same region, Aα 259–276. These data indicate that most of F-103's binding site was localized within residues Aα 269–276.

It is apparent that the determinants recognized by both monoclonal antibodies, F-102 and F-103, are sequence specific rather than conformational since the small peptides (tryptic, chymotryptic) bound approximately as well as larger fibrinogen-derived fragments (CNBr) and even as well as the

fibrinogen molecule itself. The epitopes of other monoclonal antibodies directed against proteins or peptides have been determined mainly by correlating variations in immunoreactivity with variations in primary structure (Berzofsky et al., 1982; East et al., 1982; Smith-Gill et al., 1982; Hou-Yu et al., 1982).

It is relatively uncommon to find antibodies to proteins with such high cross-reactivity to small peptides (Sachs et al., 1972; Furie et al., 1975; Atassi, 1977) although it remains to be determined if there are any monoclonal antibodies against αXLCNBr that do not have linear determinants. Therefore, we infer that the cross-reactivities have implications for the structures of the immunogen and fibrinogen. It is possible that we have selected for antibodies to linear determinants by the use of the immunogen αXLCNBr. This molecule may be highly denatured because much of the fibrin polymer is cleaved from it (thus eliminating possible long-range interactions) and because of the harsh conditions of CNBr digestion. Nevertheless, the use of these antibodies as tools in the study of the structure of the α chain in various states is still of value since the interpretation of the data can be independent of the method used to elicit the antibodies.

Little is known about the secondary and tertiary structure of the COOH-terminal two-thirds of the Aα chain. It is generally considered to be a region of random conformation based on its extreme susceptibility to proteases (Doolittle et al., 1979) and its apparent lack of defined structure in immunoelectron microscopy studies (Price et al., 1981). Several elements of the primary structure of the COOH-terminal portion of the Aα chain suggest, however, that it may be more organized than currently thought. Within the region of Aα 208–476 (i.e., from the NH_2 terminus of CNBr V to the COOH terminus of CNBr VIII), there are eight tryptophan residues. Those at Aα 302, 315, 341, 354, and 367 occur with striking periodicity, separating repeating stretches of 12 hydrophilic amino acids. This predominantly hydrophilic portion of the Aα chain is followed by a major segment, Aα 391–610, which contains hydrophobic residues representing approximately 15% of the total amino acids in this region. These features of the primary structure suggest that a degree of ordered conformation might exist in the distal portion of the Aα chain in order to sequester the hydrophobic side chains from the aqueous environment, and this inference is supported by recent electron microscopy studies of fibrinogen, conducted by Erickson & Fowler (1982). A newly visualized fourth globular domain lying adjacent to the central E domain is believed to represent the extreme COOH-terminal Aα-chain region. In such a model, the long extension of the α chain from the D region would be comprised of the hydrophilic portion of the α chain, and the two COOH-terminal regions might form a globular protein domain. Such a possibility is amenable to studies with monoclonal antibodies to specific sequences that probe local conformation and also to immunoelectron microscopic localization.

Following the theoretical and experimental foundation laid by Schechter, Anfinsen, and their colleagues (Sachs et al., 1972; Furie et al., 1975), the high cross-reactivity observed in our studies with both fibrinogen and a peptide of less than 20 residues seems to indicate that the regions of the Aα chain to which antibodies F-102 and F-103 bind are in the state of random conformation. It is possible that there is some secondary and tertiary structure in the short peptide, but at the very least, the epitopes appear to assume a structure that is only dependent on a short linear sequence of amino acids. This is consistent with one of the views of the structure of the COOH-terminal region of the Aα chain (as noted above). However, both models may be reconciled with the experimental results if it is assumed that both random and highly structured regions exist.

Several other applications of antibodies F-102 and F-103 become feasible since their epitopes are now known. Immunoelectron microscopy should be valuable in determining the exact position of the epitopes in a model of fibrinogen, similar to experiments reported by Price et al. (1981), except that the position of the antibody probe will be known with more precision. In addition, the presence or absence of these α-chain regions in various degradation products such as fragment X can be characterized by these monoclonal antibodies.

The hybridoma antibodies F-102 and F-103 do not cross-react with bovine fibrinogen. When the sequences for the last 200 residues of the Aα chain in bovine and human fibrinogen are aligned to maximize homology, a deletion involving the region Aα 540–554 exists in bovine fibrinogen (Chung et al., 1983). Therefore, they may be useful in experiments in which human and bovine fibrinogen are polymerized together in order to obtain more information on the structure of fibrin. The non-cross-reactivity with bovine fibrinogen may have been selected by the hybridoma technology that was employed; fetal calf serum in the cell culture medium (that may have included fragments of bovine fibrin) may mask an antibody that reacts with regions of the same structure on human fibrin. This may be a general problem with hybridoma antibodies to human blood proteins, and a possible solution is the use of serum-free media such as ones described by Chang et al. (1980) and Murakami et al. (1982).

Acknowledgments

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Urea–DNA Glycosylase in Mammalian Cells†

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ABSTRACT: Urea–DNA glycosylase, an enzyme presumed to be active in the repair of DNA damage caused by oxidizing agents, has been identified previously in *Escherichia coli*. This enzyme has now been shown to be present in cell extracts of calf thymus and human fibroblasts. It catalyzes the release of free urea from a double-stranded polydeoxyribonucleotide containing thymine residues fragmented by $KMnO_4$ and NaOH treatment. The calf thymus enzyme has been 400-fold purified and largely separated from previously identified mammalian DNA glycosylases. It has a molecular weight of about 25 000 and requires no cofactors. The identity of the enzymatically released product as unsubstituted urea has been verified by its susceptibility to urease.

The nitrogenous bases of DNA are susceptible to damage by oxidizing agents, and the lesions introduced by treatment with such agents resemble those observed after exposure to ionizing radiation (Howgate et al., 1968; Scholes, 1976; Teoule et al., 1977). This damage may be corrected through an excision-repair process initiated by a DNA glycosylase catalyzing the release of a degraded base residue. Extensive studies on DNA glycosylases have been performed in *Escherichia coli* [reviewed by Lindahl (1982)], and in several cases, similar activities have been demonstrated in mammalian cells. On the other hand, an enzyme such as the pyrimidine dimer–DNA glycosylase of phage T4 infected *E. coli* does not appear to have a counterpart either in uninfected *E. coli* or in mammalian cells (Demple & Linn, 1980). Two different DNA glycosylases which act on fragmented base residues have been detected. One activity liberates a substituted formamidopyrimidine, that is, a purine residue with an opened imidazole ring (Chetsanga & Lindahl, 1979). This formamidopyrimidine–DNA glycosylase has been found in mammalian cells (Margison & Pegg, 1981). Another enzyme catalyzes the release of free urea from an oxidized polydeoxyribonucleotide containing fragmented thymine residues (Breimer & Lindahl, 1980). Here it is shown that an analogous urea–DNA glycosylase is present in bovine and human cells.

Experimental Procedures

Materials. Phosphocellulose P11 was obtained from Whatman and Ultrogel AcA-54 from LKB Products. Single-stranded DNA–cellulose was made according to Alberts & Herrick (1971). Urease (crystalline, 79 units/mg) was purchased from Sigma.

Polydeoxyribonucleotide Substrates. The preparation of a polydeoxyribonucleotide substrate to measure urea–DNA glycosylase activity has been described (Breimer & Lindahl, 1980). Briefly, a poly(dA,[^{14}C]dT) copolymer containing 97% dAMP residues was synthesized with calf thymus terminal transferase and treated with $KMnO_4$ to convert the thymine moieties to a mixture of N-substituted urea and urea moieties.

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