Immunobiology of Fibrinogen

EMERGENCE OF NEOANTIGENIC EXPRESSIONS DURING PHYSIOLOGIC CLEAVAGE IN VITRO AND IN VIVO

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ABSTRACT Physiological degradation of fibrinogen by plasmin leads to a recognized series of intermediate and stable terminal cleavage fragments and is associated with complex modulation and progressive loss of native antigenic expressions. Early in association with progressive plasmin cleavage, a stable cleavage-associated neoantigen, present in the D-fragment region of the molecule, is exposed in vitro and can be recognized by competitive inhibition radioimmunoassay with specific antiserum. It is demonstrated that there is an approximate equimolar expression of the cleavage-associated neoantigen, fg-D_{neo}, on the X-, Y-, and D-fragments and no recognizable ($< 10^{-3}$) expression by fibrinogen or by the E-fragment. The X-fragment contains two D regions in respect to total D-fragment-associated antigenic expressions but unitary expression of fg-D_{neo} is observed. The Y-fragment appears to contain one D-fragment region in respect to total D-fragment-associated antigens and exhibits close to unitary expression of fg-D_{neo}. Terminal cleavage digests containing the D- and E-fragments exhibit more than 10-fold greater native fibrinogen antigenic expression than the sum of the constituent fragments. This suggests the presence of a non-covalently associated native complex of the D- and E-fragments, and implies contiguity of the D- and E-fragments in the native fibrinogen molecule. The cleavage-associated neoantigen, fg-D_{neo}, is also generated in vivo and is generically demonstrable in the plasma of patients with various forms of in vivo fibrinolysis.

These studies offer a precise immunochemical system, based upon defined molecular events, for the investigation of physiological and pathophysiological cleavage of fibrinogen. By contrast with other approaches to the assay of in vitro or in vivo cleavage of fibrinogen, assay of the cleavage-associated neoantigen fg-D_{neo} is specific, sensitive, directly yields the molar concentration of all cleavage fragments except E, and is directly applicable to *plasma*.

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INTRODUCTION

The cleavage of fibrin by plasmin has been viewed as the terminal physiologic event in blood coagulation, serving as a scavenger process to preserve the integrity of the vascular system. More recently it has become evident that physiologic and pathogenetic significance must be attributed to the products of this enzymatic degradation (1–5). The appearance of non-coagulable fibrinogen-related antigenic material in serum is observed in association with diverse disease states in man and apparently reflects fibrinolysis secondary to in vivo coagulation (6–8). Catabolism of fibrinogen also may occur through direct enzymatic degradation with release of fibrinogen cleavage fragments.

Degradation of fibrinogen by plasmin in vitro is limited, and approximately 70% of the original molecule is retained in the form of two terminal protein fragments (9, 10). With limited plasmin cleavage of fibrinogen or fibrin, discrete intermediate protein fragments can be recognized (11). Immunologic studies of the cleavage fragments have been limited to relatively qualitative techniques (11, 12), and antigenic expressions specific for cleavage fragments, but not expressed by the parent molecule, had not been demonstrated.

We have recently reported the emergence of a specific neoantigen on the D-fragment (fg-D) [1] of fibrino-

[1] *Abbreviations used in this paper:* fg-X, fibrinogen fragment X; fg-Y, fibrinogen fragment Y; fg-D, fibrinogen fragment D; fg-E, fibrinogen fragment E; putative fg-D:E core, the mixture of fg-D and fg-E yielded by terminal plasmin cleavage of fibrinogen; fg-D_{neo}, cleavage-associated neoantigen of fibrinogen; V_o, void volume.

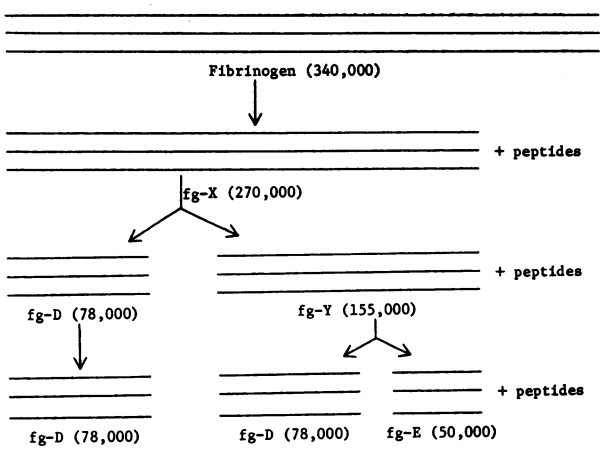

FIGURE 1 Schematic model of sequential enzymatic degradation of fibrinogen by plasmin, adapted from that proposed by Marder, Shulman, and Carroll (17). Intermediate fragments, fg-X and fg-Y, are generated and subsequently cleaved to produce terminal fragments, fg-D and fg-E. More recent studies have suggested several additional stages in cleavage prior to fg-X (18, 19).

gen following physiologic cleavage by plasmin (13). In this study the qualitative and quantitative expression of the cleavage-associated neoantigen(s) in each of the characterized fragments generated during in vitro plasmin cleavage of fibrinogen is described; and the pathophysiologic emergence of this cleavage-associated neoantigen in vivo is demonstrated. The susceptibility of molecules to antigenic modulation in vivo, with emergence of sterically hindered or cryptic sites, is germane not only as a molecular marker for the investigation of pathogenetic mechanisms and clinical disease but may also be utilized in fundamental studies of molecular conformation and immunochemistry (14).

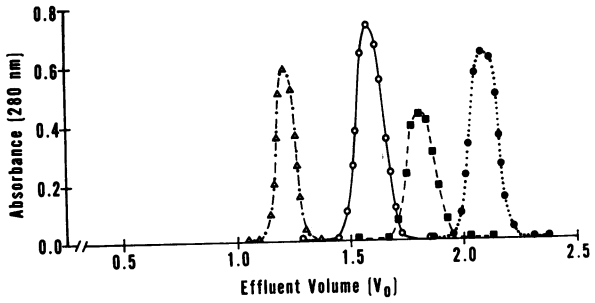

FIGURE 2 Molecular exclusion chromatography of fibrinogen and the purified cleavage fragments on Bio-Rad A 1.5 agarose. From the order of elution and identification in immunoelectrophoresis (see Fig. 3), the peaks correspond to fg (△-·-·-△), fg-X (○——○), fg-Y (■ - - - ■), and putative fg-D:E core (● · · · ●). The void volume (V_o) of the 2.5 × 90 cm column was 165 ml.

METHODS

Fibrinogen and plasmin cleavage fragments. Human fibrinogen was isolated from plasma collected in acid citrate-dextrose buffer by differential ethanol precipitation (15). This preparation was further purified by precipitation with ammonium sulfate at 26% saturation in the cold and clarified by ultracentrifugation in 5% sucrose. Protein was 97–98% coagulable by thrombin (Bovine Thrombin, Parke-Davis, Detroit, Mich.) assayed by Kjeldahl nitrogen. Further purification to remove plasminogen and Factor XIII was performed on DEAE cellulose (16). Purified fibrinogen (2 mg/ml) was enzymatically cleaved with urokinase-activated purified human plasmin[2] or streptokinase-activated human plasmin (Merck, Sharp & Dohme, West Point, Pa.), in 0.14 M NaCl, 0.01 M sodium phosphate, pH 7.3 (PBS, phosphate-buffered saline) at 37°C using 0.4 μg urokinase-activated plasmin or 1 MSD unit streptokinase-activated plasmin per mg fibrinogen. Optimal cleavage times for recovery of each of the various fragments (Fig. 1) were determined by analytical molecular exclusion chromatographic patterns on a 1.5 × 30 cm column of Bio-Rad A-1.5 beaded agarose[3] in 1.0 M NaCl, 0.01 M epsilon aminocaproic acid, 0.01 M Tris-NCl, pH 8.0. Fibrinogen is slightly included, but following plasmin cleavage, four additional fractions are clearly resolved. By reference to size (17), through sequence of elution from molecular exclusion columns, these conform to the described emergence of progressively smaller fragments namely: fg-X, fg-Y, and fg-D plus fg-E (putative fg-D:E core [20]), and small peptides. Optimal yields of each fragment were observed at the following incubation periods: fg-X = 1 h; fg-Y = 6 h; fg-D and fg-E = 16 h. Initial preparative isolation of each of the fragments was performed by molecular exclusion chromatography on a 2.5 × 90 cm column of agarose A-1.5. Fg-X, fg-Y, and putative fg-D:E core fractions were recycled on A-1.5 agarose to achieve purity. The 2.09 void volume (V_o) peak, twice isolated was employed as putative fg-D:E core. Fg-D and fg-E were directly isolated and partially purified from the 2.09 V_o fraction by DEAE cellulose chromatography using a combined salt-pH gradient (21), and each was separately recycled on DEAE for purification. Fibrinogen and each of the cleavage fragments were free of detectable serum protein contaminants by Ouchterlony gel diffusion and immunoelectrophoresis using goat anti-human serum. The identity, homogeneity, and purity of fibrinogen and each fragment was affirmed by reference to analytical molecular exclusion chromatography (17) and immunoelectrophoresis (11). Specific elution volumes, by reference to V_o, were: fg = 1.22, fg-X = 1.60, fg-Y = 1.81, and putative fg-D:E core = 2.09 (Fig. 2). Contamination with discrete fragments was not observed although detectable at the 2–3% level. Immunoelectrophoretic behavior was qualitatively typical (11) for each fragment (Fig. 3). Sensitivity for each fragment by immunoelectrophoresis was fg-X = 25 μg/ml, fg-Y = 50 μg/ml, fg-D = 50 μg/ml, and fg-E = 50 μg/ml. Analysis at 1 mg/ml excludes discrete contamination by individual fragments at a 5% level of confidence. All isolated fragments were free of other detectable fragments by both immunoelectrophoretic analysis and analytical molecular exclusion chromatography and appeared homogeneous by these same criteria.

Antisera. Antiserum to fibrinogen (anti-fg) was prepared by immunization of two New Zealand White rabbits

[2] Kindly supplied by Cutter Laboratories, Berkeley, Calif.
[3] Bio-Rad Labs, Richmond, Calif.

with 200 μg fibrinogen emulsified in complete Freund's adjuvant for the initial immunization in the rear footpads followed by biweekly subcutaneous boosters of the same antigen in incomplete adjuvant. Serum was harvested weekly after 3 wk, and the antisera assayed by Ouchterlony gel double diffusion. Both rabbits responded equally well and antisera 6 wk following primary immunization was pooled. Although the antisera appeared monospecific for fibrinogen antigens, pools of anti-fg were absorbed with 1/50 volume of human serum, devoid of detectable fibrinogen-related antigens by radial immunodiffusion, to remove any undetected trace antibodies to serum proteins.

Antisera to fg-D (anti-fg-D) were prepared by immunization of four New Zealand White rabbits with 50 μg of fg-D in complete Freund's adjuvant, first into the rear footpads then subcutaneously in incomplete adjuvant on a weekly schedule. Antisera were harvested weekly after 3 wk. Antisera forming precipitins with fg-D but with no detectable antibodies to normal serum proteins or to fg-E by Ouchterlony gel double diffusion and immunoelectrophoresis were employed.

Antisera to the cleavage-associated neoantigen, anti-fg-D_{neo}, were prepared from anti-fg-D by absorption with purified fibrinogen. Anti-fg-D was mixed with an equal volume of purified fibrinogen at 2 mg/ml in PBS; following incubation at 4°C for 20 h, the solution was centrifuged for 20 min at 2,000 *g*. The supernatant antiserum was serially diluted and assayed for anti-fg-D_{neo} antibody by its capacity to bind [^{125}I]fg-D in the double antibody radioimmunoassay (13, 14). Specificity was established by systematic competitive inhibition by fg-D but not by fibrinogen. Three of the four rabbits made satisfactory anti-fg-D_{neo} responses varying in titer from 1:40 to 1:200 for 66% binding of [^{125}I]fg-D at 1.0×10^{-9} M. Antiserum (R6127-4), 5 wk following primary immunization, was employed in this study.

Immunoelectrophoresis and gel diffusion. Immunoelectrophoretic analysis was performed in 1% agar as previously described (14). 10 μl samples at 1 mg/ml were electrophoresed at 5.5 V/cm for 75 min and precipitins developed with 100 μl of rabbit anti-fg. Ouchterlony gel double diffusion was performed as previously described (13).

Radioimmunoassay. Quantitative radioimmunochemical studies were performed in a four compartment double antibody system as previously described (13). [^{125}I]fg-D or [^{125}I]fibrinogen was introduced at 1×10^{-9} M (specific activity approximately 56 μCi/μg). Competing antigens were added in terms of molar concentration. Protein concentration was determined on the basis of $E^{1\%}_{280}$ for each fragment (11), or by micro-Kjeldahl nitrogen determination. Results were expressed as percent [^{125}I]fibrinogen or [^{125}I]fg-D bound. Precision was within 4% and an intra-run standard error of 1% was usually achieved.

Radial immunodiffusion assay of fibrinogen and serum cleavage products. Quantitative immunochemical assay of plasma fibrinogen and of the noncoagulable cleavage fragments remaining in the serum following in vitro coagulation of plasma at 37°C with thrombin at 20 U/ml was performed by an adaptation (22) of the radial diffusion technique of Mancini, Vaerman, Carbonara, and Heremans (23) using goat anti-fg antisera. This assay is generically similar to other reported radial immunodiffusion assays (24, 25). 1% agarose incorporating anti-fibrinogen in 0.10 M NaCl, 0.01 M disodium ethylenediamine tetraacetic acid, 0.01 M epsilon aminocaproic acid, 0.03 M sodium phosphate, pH 8.0, is poured to a 3 mm depth in plastic plates. 5 μl samples are applied in triplicate to each 3 mm sample well. Total

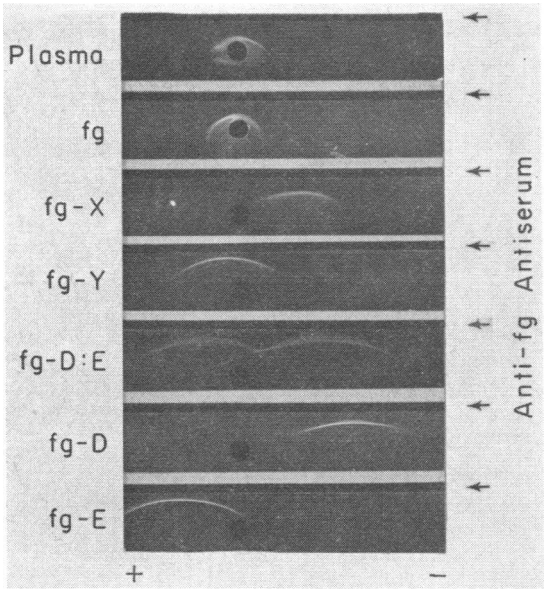

FIGURE 3 Immunoelectrophoretic patterns of fibrinogen and the purified cleavage fragments at 1 mg/ml. Each fragment appears homogeneous and forms a precipitin arc characteristic of the particular fragment. Reduced mobility of constituent fg-D and fg-E are noted for fg-D:E as compared with fg-D and fg-E isolated from the same preparation.

plasma fibrinogen was determined utilizing diluted plasma. Residual fibrinogen antigen in the serum, following coagulation of plasma with 20 NIH units thrombin/ml for approximately 1 min at 37°C, was also assayed for total fibrinogen cleavage products. Assays were standardized with highly purified fibrinogen quantitated by Kjeldahl nitrogen, and incorporated a secondary standard plasma in which the fibrinogen had been quantitated by reference to the primary standard and also by clottable nitrogen (26). Assays were developed for 24 h at 22°C and sensitivity was to 0.5 mg/100 ml fibrinogen. Normal values determined on 100 healthy individuals were: plasma fibrinogen = 147–370 mg/100 ml, percent coagulable = 99.98 (97–100%), serum cleavage products (noncoagulable fibrinogen antigen) = 0.06 (0–3) mg/100 ml. All assays were performed in duplicate and standard error was within 5%. The results of these assays are generally comparable to other fibrinogen-related antigen assays (25, 27, 28).

Blood collection. Blood from normal volunteers and patients with evidence of in vivo fibrinolysis was collected by venipuncture into 1/70 vol 0.25 M tripotassium ethylenediamine tetraacetic acid, pH 7.4, 0.70 M epsilon aminocaproic acid and cooled to 4°C. Following sedimentation of erythrocytes, plasma was withdrawn and centrifuged for 10 min at 2,000 *g*. Specimens were portioned at −76°C for storage.

Control plasmas were acquired from healthy volunteers; plasma fibrinogen was normal and serum cleavage products were undetectable (< 0.5%) by radial immunodiffusion. Plasmas were also collected from patients with: (*a*) diseases frequently associated with disseminated intravascular coagulation, and (*b*) direct evidence of in vivo fibrinolysis (29). The primary diagnostic criterion for in vivo fibrinolysis was the presence of serum cleavage products of > 5.0 mg/100 ml. All patients also met at least three of four secondary criteria suggesting associated disseminated intra-

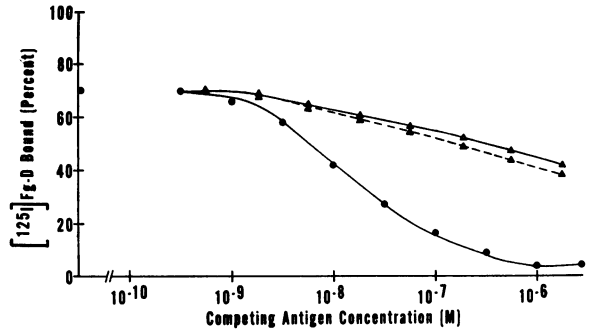

FIGURE 4 The expression of fg-D antigens is assessed by competitive inhibition of anti-fg-D binding of [^{125}I]fg-D by fg-D (●——●), isolated fibrinogen (△——△), and fresh plasma fibrinogen (▲ - - - ▲).

vascular coagulation: (*1*) platelets $< 140{,}000/mm^3$; (*2*) prolonged thrombin time (2 SD greater than control); (*3*) prothrombin time > 17 s; and (*4*) reduced plasma fibrinogen < 147 mg/100 ml (29).

RESULTS

Consideration of the experimental data is facilitated by reference to Fig. 1 in which the sequential enzymatic degradation of fibrinogen by plasmin is schematically illustrated. This scheme, proposed by Marder, Shulman, and Carroll (17), is reasonably consistent with published data from a number of laboratories. Plasmin cleavage generates intermediate fragments fg-X and fg-Y and culminates in the generation of two relatively stable and antigenically independent fragments, referred to as fg-D and fg-E (30). More recent evidence has indicated the existence of large intermediate fragments preceding the appearance of fg-X (18, 19).

The diverse antigenic expressions of the D region of the fibrinogen molecule were explored by quantitative and qualitatively discriminating radioimmunochemical assays (13). Exploration of total fg-D-associated antigenic expressions employed anti-fg-D antiserum, which at a 1:200 dilution bound greater than 98% of [^{125}I]-fg-D at 1.0×10^{-9} M. Nonspecific binding was less than 2%. Competitive inhibition studies, illustrated in Fig. 4, represent the average of assays performed with two preparations of [^{125}I]fg-D, fg-D, fibrinogen, and plasma. Anti-fg-D was used at a concentration sufficient to bind 70% of the [^{125}I]fg-D; and systematic competitive inhibition was observed with fg-D. A semilogarithmic relationship was observed between the concentration of competing fg-D and [^{125}I]fg-D bound with maximum inhibition achieved at 1.0×10^{-6} M fg-D. By contrast, both isolated fibrinogen and fresh plasma fibrinogen exhibited only partial competition with the labeled antigen; and 50% relative inhibition could not be attained even in the presence of $> 10^3$ molar excess of fibrinogen. The differential binding of these antigens by anti-fg-D implicates the presence of two generic populations of antibodies; namely, (*a*) those directed to native fibrinogen antigens present on both fibrinogen and fg-D, and (*b*) those specific for neoantigenic determinants unique to the fg-D fragment. The partial competition observed in Fig. 4 with fibrinogen appears to represent binding of antibodies to native fibrinogen antigens expressed on fg-D, and complete inhibition of [^{125}I]fg-D binding attained with fg-D indicates competitive binding of both populations of antibodies.

The existence of these two independent subpopulations of antibodies was further substantiated by differential absorption. Absorption of anti-fg-D with excess fibrinogen removed approximately 75% of antibodies reactive with [^{125}I]fg-D (Fig. 5). 50% relative binding was attained at 1:1260 dilution of the unabsorbed anti-fg-D while equivalent binding was observed at 1:316 dilution of the absorbed antiserum. Concomitant with absorption of antibody to native fibrinogen determinants, a difference in the binding affinity was observed as indicated by a decrease in the slope of the binding profile. The long linear semilogarithmic relationship observed with the absorbed antiserum also suggests that residual antibody specifically reactive with fg-D was relatively homogeneous.

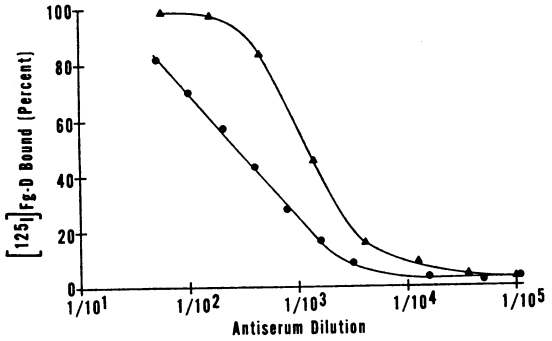

FIGURE 5 Comparison of [^{125}I]fg-D binding by anti-fg-D (▲——▲), and the same anti-fg-D following absorption with fibrinogen at 1 mg/ml (●——●).

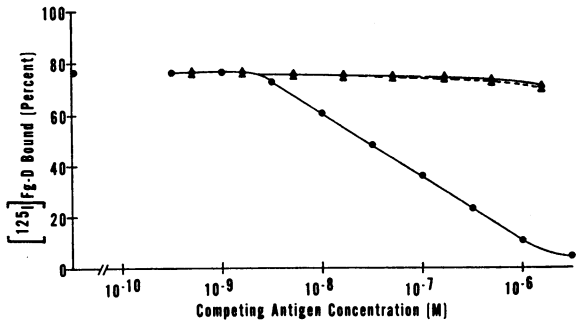

FIGURE 6 Competitive inhibition of anti-fg-D_{neo} binding of [^{125}I]fg-D by fg-D (●——●), isolated fibrinogen (△——△), and fresh plasma fibrinogen (▲ - - - ▲).

TABLE I

Expression of Native Fibrinogen Antigens by Cleavage Fragments of Fibrinogen

Molecule or fragment	Estimated molecular weight	Relative binding affinity*	50% competitive inhibition	Fibrinogen antigen per molecule‡
			M	
Fibrinogen	340,000 (17)	*0.314*	*1.45×10^{-9}*	*1.000*
fg-X	270,000 (17)	0.326	3.98×10^{-9}	0.365
fg-Y	155,000 (11)	0.258	1.88×10^{-8}	0.077
fg-D:E	130,000§	0.304	5.75×10^{-8}	0.025
fg-D	78,000 (31)	0.173	$> 10^{-6}$	<0.001
fg-E	50,000 (11)	0.129	$> 10^{-6}$	<0.001

* Expressed as the slope of competitive inhibition of [^{125}I] fibrinogen binding by anti-fg.

‡ Molar concentration of unknown for 50% competitive inhibition/molar concentration of fibrinogen for 50% competitive inhibition.

§ Estimated from the sum of the molecular weights of fg-D plus fg-E.

Fg-D and fibrinogen were clearly discriminated by competitive inhibition using absorbed anti-fg-D (Fig. 6). Fg-D exhibits semilogarithmic competitive inhibition from 3.3×10^{-9} to 1.0×10^{-6} M. Isolated fibrinogen and plasma fibrinogen exhibit negligible binding of antibody; at 1.65×10^{-6} M fibrinogen less than 7% competitive inhibition is observed. Thus, absorption of anti-fg-D with fibrinogen, results in an antiserum (anti-fg-D_{neo}) which specifically recognizes antigenic sites on the fg-D fragment of fibrinogen not exposed on the parent fibrinogen molecule, and permits specific assay of the cleavage-associated neoantigenic expression, fg-D_{neo}.

The demonstration of a cleavage-associated neoantigen suggests that two events may contribute to the composite antigenic expressions of the fibrinogen cleavage products: (*a*) the loss and modulation of native fibrinogen antigens; and (*b*) the emergence and modulation of unique cleavage-associated neoantigens. These two events can be assessed independently. Employing anti-fg and [^{125}I]fibrinogen in the competitive inhibition radioimmunoassay, it is possible to qualitatively assess loss or modulation of native fibrinogen antigens during cleavage. Systematic and sequential loss of native fibrinogen antigens closely parallels the cleavage process (Table I). Significant loss of native antigenic expression is associated with the initial cleavage of fibrinogen to fg-X. Although fg-X represents 80% of the parent molecule it expresses only 36% of the native antigens recognized by this antifibrinogen antiserum. A similar disproportionate loss of native antigenic expression relative to molecular weight is noted during subsequent cleavage with expression of native antigens as fibrinogen> fg-X> fg-Y> fg-D:E> fg-D> fg-E. The competitive inhibition slope, a reflection of antibody binding affinity, differs significantly with each fragment and indicates qualitative differences in the antigenic expression of each fragment. It is noteworthy that the sum of the native antigenic expressions of isolated fg-D and isolated fg-E is markedly deficient relative to putative fg-D:E core, indicative of marked loss of native antigenic expression upon separation of fg-D and fg-E. This data independently suggests the existence of the fg-D:E core as a complex, indicates that some native conformational antigens of intact fibrinogen are dependent on the association of fg-D and fg-E, and also implies conformational contiguity of these fragments in the parent molecule.

The expression of fg-D-associated antigens during progressive plasmin cleavage of fibrinogen was also investigated by competitive inhibition assays employing [^{125}I]fg-D. Both anti-fg-D and anti-fg-D_{neo} were utilized in order to assess expression of (*a*) the composite of total fg-D antigens, and (*b*) the cleavage-associated neoantigen fg-D_{neo} at various stages of cleavage. It is evident from Fig. *7A* that fg-X, fg-Y, and putative fg-D:E core possess binding properties and thus fg-D related antigenic expressions similar to fg-D when assayed with anti-fg-D; and these fragments are clearly distinguishable from intact fibrinogen indicating that not all of these antigens are exposed on the parent molecule. Antigenic differences between the cleavage fragments are observed. Utilizing 50% relative inhibition for quantitation, fg-X has nearly twice the quantity of fg-D associated antigens as fg-D, and fg-Y and fg-D are equal. At high concentrations fg-X and fg-Y do not inhibit [^{125}I]fg-D binding to the degree observed with fg-D suggesting minor quantitative antigenic de-

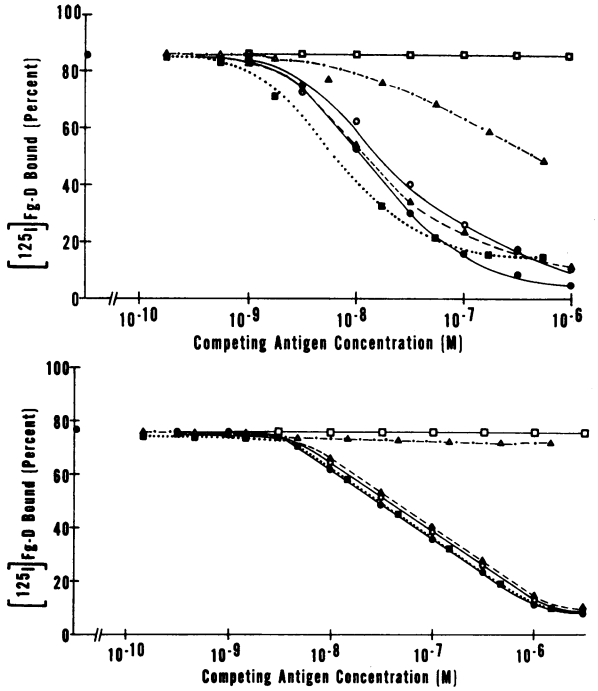

FIGURE 7 The expression of fg-D antigens of fibrinogen and cleavage fragment is assessed by competitive inhibition of binding of [^{125}I]fg-D by fibrinogen (△-·-·-△), fg-X (■ · · · ■), fg-Y (▲ - - - ▲), fg-D:E (○——○), fg-D (●——●), and fg-E (□——□). (*A*) The composite of native antigens and neoantigens are assayed using anti-fg-D. (*B*) The cleavage-associated neoantigenic expression, fg-D_{neo}, is assayed using anti-fg-D_{neo}.

ficiency of the D regions of fg-X and fg-Y in respect to fg-D. The putative fg-D:E core exhibits consistent qualitative and quantitative antigenic deficiency at all concentrations. This suggests that antigenic determinants are hindered and fg-D present in putative fg-D:E core is not entirely free. The fg-E fragment does not competitively bind anti-fg-D antibodies, confirming the complete segregation of antigenic determinants between the two terminal cleavage products.

Competitive inhibition by fibrinogen cleavage fragments in the anti-fg-D_{neo} system provides more precise and discriminating results (Fig. 7*B*). Parallel inhibition profiles are observed with fg-X, fg-Y, putative fg-D:E core, and fg-D indicating that the cleavage-associated neoantigenic expression, fg-D_{neo}, is exposed in the initial cleavage by plasmin of fibrinogen to fg-X. The neoantigen is qualitatively identical throughout the subsequent cleavages as indicated by the parallel competitive inhibition slopes, and complete inhibition observed with each fragment. Minimal quantitative differences in fg-D_{neo} expression on each of the fragments are indicated by minor lateral displacement of the competitive inhibition plots. These results are in contradistinction with those obtained with the unabsorbed anti-fg-D in Fig. 7*A* where qualitative as well as quantitative differences in binding are observed. The data indicate that native antigenic expressions of fibrinogen, present on fg-D, are modulated during sequential cleavage, but the cleavage-associated neoantigenic site is stable.

Quantitative differences noted in Fig. 7*B* are further considered in Table II. The slope of each inhibition profile, an indication of the binding affinity of specific antibody molecules for the neoantigen site, confirms that expression of fg-D_{neo} is qualitatively identical on each fragment except fg-E. The concentration of given fragments required for 50% inhibition reflects quantitative differences in fg-D_{neo} expression on these molecular fragments. The integer relationship between fg-D

TABLE II

Expression of the Cleavage-Associated Neoantigen, Fg-D_{neo}, by Cleavage Fragments of Fibrinogen

Molecule or fragment	Estimated molecular weight	Relative binding affinity*	50% competitive inhibition	Fg-D_{neo} per molecule‡
			M	
Fibrinogen	340,000 (17)	0.013	$> 10^{-5}$	0.00
fg-X	270,000 (17)	0.258	8.70×10^{-8}	0.99
fg-Y	155,000 (11)	0.267	1.07×10^{-7}	0.81
fg-D:E	130,000§	0.264	9.89×10^{-8}	0.87
fg-D	78,000 (31)	*0.260*	*8.61×10^{-8}*	*1.00*
fg-E	50,000 (11)	0.000	$> 10^{-5}$	0.00

* Expressed as the slope of competitive inhibition of ^{125}I fg-D binding by anti-fg-D_{neo}.
‡ Molar concentration of unknown for 50% competitive inhibition/molar concentration of fg-D for 50% competitive inhibition.
§ Estimated from the sum of the molecular weights of fg-D plus fg-E.

and fg-X appears quite significant, and indicates that only one neoantigenic site is exposed on the fg-X fragment, though two fg-D regions have been proposed (11) and were supported by quantitative assay of total fg-D related antigens expressed on fg-X. The number of fg-D_{neo} sites calculated from fg-Y and putative fg-D:E core differ slightly from unity although expression of the fg-D_{neo} expression appears qualitatively identical to that observed with standard fg-D. Such variations from integer values could arise from minor differences in the estimated molecular weights of fg-Y and putative fg-D:E core or due to complete steric hindrance of the fg-D_{neo} site on a small percent of molecules.

The possibility that the cleavage of fibrinogen, or fibrin, in vivo would also lead to expression of fg-D_{neo} was evaluated, and the results are illustrated in Fig. 8. Utilizing a concentration of anti-fg-D_{neo} sufficient to bind 41% of the [^{125}I]fg-D, the plasmas from two normal individuals (C100 and C101) demonstrated no recognizable fg-D_{neo} activity. These plasmas were also devoid of serum fibrinogen or fibrin cleavage products by radial diffusion assay. By contrast, when control plasma C100 was exposed to plasmin (4 μg plasmin/ml plasma at 37°C for 3 h), fg-D_{neo} expression was readily generated as indicated by the competitive inhibition of [^{125}I]fg-D binding. Plasmas from two patients with metastatic carcinoma and moderate levels of serum cleavage products by radial immunodiffusion ($\times$ 100 = 5 mg/100 ml; $\times$ 101 = 8 mg/100 ml) were also assayed (8, 32, 33). Both produced semilogarithmic inhibition parallel to that produced by the in vitro cleaved plasma sample, indicating the appearance in vivo of fg-D_{neo} indistinguishable from that generated in vitro. The lateral displacement of the curves reflects the quantity of neoantigen present and correlates in a general fashion with the levels of cleavage products detected by radial diffusion. Although the relative distribution of each type of cleavage fragment cannot be determined independently, the molar content of the sum of fg-X, fg-Y, fg-D:E, and fg-D can be precisely calculated.

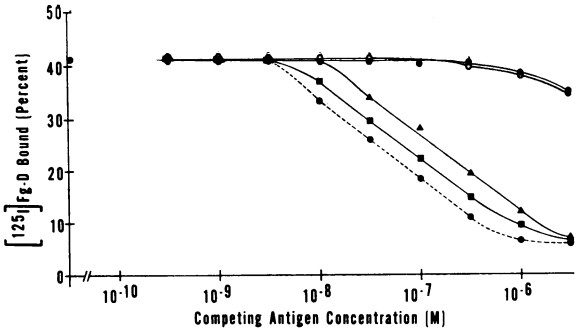

FIGURE 8 Generation of the cleavage-associated neoantigen in vivo. Normal and pathologic plasmas were tested by competitive inhibition of anti-fg-D_{neo} binding of [^{125}I]fg-D. Normal plasmas C100 (●——●) and C101 (○——○). Plasmin treated C100 (● - - - ●). X100 (▲——▲) and X101 (■——■) are patient plasmas positive for serum cleavage products by radial immunodiffusion.

Further evidence for the generation of fg-D_{neo} in vivo is given in Table III. Plasmas were selected from patients with diseases associated with disseminated intravascular coagulation and the presence of circulating fibrin or fibrinogen cleavage fragments, e.g., metastatic carcinoma (8, 32, 33), abruptio placentae (6, 34), consumptive coagulopathy (disseminated intravascular coagulation) (8, 35, 36), meningococcal meningitis (37, 38), and endotoxic shock (39, 40). Serum cleavage fragments, primary evidence for in vivo fibrinolysis, were present in all (17–300 mg/100 ml). Normal plasma samples contained no detectable (< 0.5 mg/100 ml) serum cleavage fragments. All patient plasmas were positive for fg-D_{neo}, whereas normal plasmas were negative. The fg-D_{neo} titer of plasma indicates wide variations in the concentrations of the cleavage-associated

TABLE III

The Presence of Cleavage-Associated Neoantigen, Fg-D_{neo}, in Plasma from Patients with In Vivo Fibrinolysis

Plasma	Diagnosis	Cleavage-associated neoantigen	
		Titer	fg-D_{neo} concentration
			μM
X102	Metastatic carcinoma of the prostate	40	1.20
X103	Abruptio placentae with fatal defibrinogenation	1000	30.00
X104	Sepsis	56	1.68
X105	Metastatic melanoma	162	4.80
X106	Peritonitis (*Escherichia coli, proteus*), sepsis, carcinoma of the prostate	15	0.45
C100–112	Normal	0	0.00

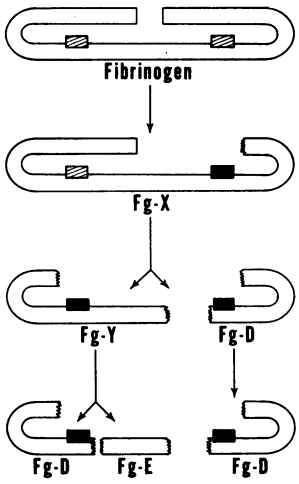

FIGURE 9 Proposed model for the sequential emergence of fg-D_{neo} expressions during progressive cleavage of fibrinogen by plasmin. The shaded regions indicate latent sterically hindered fg-D_{neo} sites; the solid regions represent fully expressed cleavage-associated neoantigens.

neoantigen in these diverse clinical states. In all cases fg-D_{neo} was detectable at relatively high dilution of the plasmas, indicating considerable sensitivity of the fg-D_{neo} radioimmunoassay. Since each cleavage fragment, except fg-E, expresses the same quantity of neoantigen, results can be expressed in molar concentrations by relating them to standard inhibition produced by fg-D. These data clearly demonstrate the emergence of the cleavage-associated neoantigen, fg-D_{neo}, in vivo, and they further suggest that this neoantigenic marker may prove valuable in a direct investigative assay of plasma for in vivo fibrinolysis.

DISCUSSION

Although several models can be conceived to explain the emergence of neoantigenic expressions, the cleavage-associated neoantigen of fibrinogen, fg-D_{neo}, appears to arise through steric exposure of cryptic amino acid sequences in native fibrinogen (14). The quantitative and qualitative identity of the neoantigen on the various cleavage fragments as described in this study further supports this model. With the marked modulation of native antigenic expressions, it seems unlikely that an antigenic expression determined by conformation would remain entirely constant during cleavage. According to the steric model (14) the sequence of events illustrated in Fig. 9 is proposed and is consistent with the expression of the cleavage-associated neoantigen observed in this study. Although the conformation of native fibrinogen remains highly debatable (41, 42), it is known that the molecule is dissociable into three pairs of polypeptide chains (43); thus, two identical latent neoantigenic determinants could be present in native fibrinogen. The results of this study suggest that the action of plasmin leading to the fg-X fragment is asymmetrical or culminates in an asymmetric conformation with exposure of only one of the two latent fg-D_{neo} sites. The presence of two D regions in the X-fragment is supported by quantitation of total fg-D associated antigens in fg-X. Continued proteolysis of fg-X leads to the complete exposure of the second fg-D_{neo} site with one neoantigenic expression on fg-Y and the second on the free fg-D fragment. Again, quantitation of total fg-D-associated antigenic expressions supports the presence of only one D-fragment region in the fg-Y. Cleavage of fg-Y further segregates the neoantigen with the remaining expression appearing on the second fg-D and none on fg-E.

The cleavage-associated neoantigen is expressed in a qualitatively similar fashion on each of the cleavage fragments; and only minor differences in quantitative expression are noted. The similarity of binding inhibition by each fragment strongly supports expression of the complete neoantigen on each fragment; thus quantitative deviations from integer values reflect either: (*a*) inaccurate estimates of the molecular weights of fragments; or (*b*) heterogeneity of a given fragment with a subpopulation devoid of the neoantigenic site. Greatest variation from unitary expression of the neoantigen is exhibited by fg-Y. A unit value is derived with a molecular weight of approximately 200,000 daltons; and Mills and Karpatkin (44) have estimated the molecular weight of an intermediate fragment, presumably fg-Y, to be 205,000 daltons by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Our data thus supports the latter molecular weight estimate of this cleavage fragment.

The modulation and loss of native antigenic determinants of fibrinogen during cleavage is disproportionate in respect to the loss of mass and suggests that many of the native determinants are conformationally dependent or associated with small peptides. This is consistent with the demonstration of substantial differences in helical content of fg-D, fg-E, and fibrinogen which might reflect changes in conformation with cleavage (45). The existence of a non-covalently associated fg-D:E core, rather than a simple mixture of these two fragments in free solution, is indicated by the much greater native fibrinogen antigenic expression of fg-D:E as compared with either constituent fragment alone or the sum of native antigenic expressions of the two fragments. Total fg-D antigens are also incompletely expressed by fg-D:E. Such behavior supports the existence of a noncovalently associated bimolecular core, indicates that it is a native molecular conformation, and implies that these two fragments are integrally related in the intact fibrinogen molecule through conformation or by the presence of hypothetical small anti-

genic peptides which are lost during the dissociation of the fg-D : E core (46).

The emergence of fg-D_{neo} in vivo is indicated by the assay of plasmas from patients with various forms of in vivo fibrinolysis. The expression of fg-D_{neo} in vivo is qualitatively identical to the neoantigen generated by incubation of plasmin with normal human plasma. While other physiologic enzymes such as the leukocyte proteases are also capable of cleaving fibrinogen and may be of pathophysiological significance, the expression of the neoantigen on these degradation products differs from the expression of neoantigen on plasmin-generated fragments (14). The present study is compatible with plasmin as the major enzyme in the production of cleavage products in disease-related conditions.

The assay system for cleavage, based on the detection of fg-D_{neo}, appears to be highly sensitive and offers distinct advantages over currently employed clinical and investigative assay systems (6–8, 38, 39, 47). Specifically: (*a*) Assays based on the detection of fg-D_{neo} may be performed directly on *plasma*. Most other currently employed immunologic techniques assay noncoagulable fibrinogen antigen following spontaneous or thrombin-induced coagulation. Such systems may exclude a significant portion of fg-X, which is slowly coagulable (48) and may exclude other fragments which are specifically coincorporated or nonspecifically bound to the fibrin polymer (49). (*b*) Results from the fg-D_{neo} assay may be realistically dealt with in quantitative molar terms since each circulating fragment (fg-X, fg-Y, fg-D : E core, and fg-D) possesses one neoantigenic expression. Other assay systems which employ anti-fg in the detection of cleavage fragments must relate results to the fibrinogen molecule. It is apparent from Table I that an anti-fg antiserum may exhibit differential sensitivity to the various fragments such that quantitative results do not reflect absolute quantity. Different anti-fg antisera give different results and preferentially bind different fragments to variable degrees (50). The loss and segregation of antigenic determinants during plasmin cleavage of fibrinogen or fibrin precludes equal sensitivity to all fibrinogen-related antigens. (*c*) With a firm molecular basis for fg-D_{neo} expression, interpretation, and assessment of associated pathophysiologic mechanisms may be more effectively achieved.

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