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Molecular Events Responsible for Modulation of Neoantigenic Expression: The Cleavage-Associated Neoantigen of Fibrinogen

(blood coagulation/fibrinogen cleavage products/fibrinolysis/molecular conformation)

EDWARD PLOW AND THOMAS S. EDGINGTON

Department of Experimental Pathology, Scripps Clinic and Research Foundation, La Jolla, California 92037

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ABSTRACT **Molecular events responsible for modulation of neoantigenic expressions of a defined molecule have been explored in relation to three hypothetical molecular models (see below). Fibrinogen and its cleavage-associated neoantigen have been used as a prototype system. Physicochemical and enzymatic factors influencing neoantigenic expression were evaluated. The cleavage-associated neoantigen was not only exposed by plasmin and enzymes of similar specificity, but also in a qualitatively and quantitatively deficient fashion by enzymes of differing specificities. Denaturation of fibrinogen via reduction or pH alteration did not induce the neoantigen, but oxidation of the native fibrinogen molecule did elicit this neoantigenic expression. The neoantigen, once exposed on the D-fragment, was relatively stable to physical and chemical denaturation. These results are inconsistent with proposed *cleavage site specific* and *neoconformational* determinant models and are consistent with a *steric* model, which postulates that the cleavage-associated neoantigenic determinant is buried in native fibrinogen but is exposed in certain altered molecular species. The importance of molecular conformation in the exposure of antigenic expressions of a molecule and in modulation of the binding affinity of a neoantigen for specific antibody is demonstrated.**

The antigenic expressions of a protein molecule appear to be determined at all levels of molecular organization (1, 2). Although antigenic determinants may be dependent upon genetically-coded amino-acid sequences, tertiary conformation and quaternary organization, which can be modified at a more peripheral level of biological organization, may be equally effective in determination or modulation of antigenic expression. The deletion of select native antigens and the appearance of neoantigenic determinants on altered molecules occurs with aggregation of bovine serum albumin (3) and after enzymatic cleavage of immunoglobulin G (IgG) (4) or bovine serum albumin (5) by pepsin. The cleavage of fibrinogen by plasmin, a physiological mechanism (6), generates two terminal cleavage fragments that are pathobiologically significant, fg-D and fg-E, and are noncovalently associated with one another (7, 8). We have recently reported the emergence of a neoantigenic expression(s) after plasmin cleavage of fibrinogen (9). This cleavage-associated neoantigen (fg-D_{neo}) was localized in the fg-D fragment and has now been demonstrated after *in vivo*, as well as in *in vitro*, cleavage of fibrinogen (10).

Abbreviations: fg-D, fibrinogen fragment D; fg-E, fibrinogen fragment E; fg-D_{neo}, cleavage-associated neoantigen of fibrinogen.

This system provides a model in which the emergence and modification of neoantigenic expressions can be studied. Similar considerations may apply to neoantigens associated with neoplasia and some immunologically mediated diseases arising from modification of molecular species on the surfaces of normal cells. In consideration of the emergence of a neoantigenic expression via enzymatic molecular cleavage, three hypothetical schematic models are considered here (Fig. 1). All have conformational elements, but also present distinct conceptual differences. (*a*) The neoantigen may be associated with amino acids directly at the site of cleavage—*cleavage site specific*. (*b*) The neoantigen may be a new, conformationally determined, antigenic site induced by changes in internal ionic and hydrophobic forces that are secondary to enzymatic cleavages—*neoconformational*. (*c*) The neoantigenic expression may be present in the intact molecule at a sterically hindered site, and may become exposed after enzymatic cleavage—*steric*. By observation of the effects of diverse enzymes and denaturants on fibrinogen and the fg-D_{neo}, some of the molecular events associated with expression of the cleavage-associated neoantigen of fibrinogen can be deduced. These data strongly implicate the *steric* model in fg-D_{neo} expression, and by analogy suggest that other neoantigenic expressions may be similarly modulated.

MATERIALS AND METHODS

Human fibrinogen was isolated by the method of Doolittle (11) and further purified by ammonium sulfate precipitation at 26% saturation and 4°C and ion-exchange chromatography on DEAE-cellulose (12). Fg-D was produced by enzymatic digestion of fibrinogen at 37°C for 18 hr with urokinase-activated plasmin (Cutter Laboratories, Berkeley, Calif.). Separation from the other terminal digestion products (fg-E and small peptides) and purification was accomplished by repeated DEAE-cellulose chromatography (7). The relative purity of fg-D and fg-E was confirmed by polyacrylamide gel electrophoresis (13).

Antisera to fibrinogen and fg-D were prepared in rabbits (9). Antiserum to fg-D_{neo} was prepared by absorption of a single pool of selected antiserum to fg-D with fibrinogen for the removal of antibodies to native fibrinogen antigens that are present on fg-D (9).

The antigenic expression of fg-D_{neo} was assayed radioimmunochemically by use of a four-compartment, double-antibody system as described (9). This competitive inhibition

system measures the ability of various fibrinogen products to compete with [^{125}I] labeled fg-D (65 Ci/mol) for binding by antiserum to fg-D_{neo}. [^{125}I]fg-D was provided at a concentration of 1.0 nM and competing antigens were introduced at indicated concentrations. Results, corrected for trichloroacetic acid-precipitability of radioactivity, were expressed as percent of [^{125}I]fg-D bound.

Enzymatic digestion of fibrinogen by plasmin (EC 3.4.4.14), trypsin (EC 3.4.4.4), chymotrypsin (EC 3.4.4.5), and papain (EC 3.4.4.10) were performed at 1:50 molar ratios of enzyme to substrate in physiologically buffered saline (pH 7.3). Leukocytes, isolated according to the method of Ohlsson (14) from 300 ml of normal human blood, were suspended in saline at a concentration of 1×10^7 cells per ml, and lysed by repeated freezing and thawing. Activities of the leukocyte proteases were assessed at pH 8.5 and 7.0 by the addition of 1 ml of the lysate to 16 mg of fibrinogen. Enzymatic digestions were incubated for 18 hr at 37°C; digests were immediately assayed by radioimmunoassay at 4°C.

4-ml aliquots of fibrinogen at a concentration of 4 mg/ml or fg-D at 1 mg/ml were used in physical and chemical denaturation studies. The effect of acid or base was assayed by exposure to pH 4.0 or 9.5 for 1 hr. Exposure to oxidizing or reducing agents (chloramine T or cysteine, respectively) in 0.14 M NaCl–0.01 M phosphate (pH 7.3) or 0.2 M 2-mercaptoethanol in 0.5 M Tris·HCl (pH 8.2) was also for 1 hr and was followed by exhaustive dialysis against physiological saline at 4°C.

Immunoelectrophoretic analyses were performed on standard 7.5 × 2.5 cm microslides coated with 1% Difco Special Agar in 25 mM Veronal buffer (pH 8.8). A standard sample volume of 5 μl was electrophoresed for 75 min at about 6 V/cm. Precipitin lines were developed at 20°C with 100 μl of antiserum to fibrinogen, prepared in goats. Ouchterlony double-diffusion analysis was performed in petri dishes containing 1% agarose in 0.1 M NaCl–0.03 M sodium phosphate–0.01 M EDTA (pH 7.4). 50 μl of a 1:5 dilution of each enzymatic digest was allowed to diffuse against an equal volume of antiserum to fibrinogen. Thrombin times were determined at 37°C; 1 unit of bovine topical thrombin (in 0.1 ml) was added to 0.1 ml of each fibrinogen sample.

RESULTS

The three hypothetical molecular models (Fig. 1) proposed for the genesis of neoantigenic expressions were evaluated relative to the emergence of the fg-D_{neo} expression after enzymatic cleavage and chemical modification of fibrinogen. All enzymes used in this study significantly influenced molecular integrity and physiological function of the fibrinogen substrate, as demonstrated by: (*a*) immunoelectrophoretic fragmentation and heterogeneity (Fig. 2); (*b*) acquired antigenic deficiency of some products (Table 1); and (*c*) loss of physiological coagulability by thrombin (Table 1).

Standard fg-D_{neo} expression is provided by purified fg-D in the competitive inhibition assays illustrated in Fig. 3. Plasmin and trypsin, enzymes of similar specificity, yielded similar fragments (Fig. 2) and the digests gave parallel semilogarithmic inhibition plots, indicating the emergence of a neoantigen(s) qualitatively identical to fg-D_{neo} (Fig. 3).

Leukocyte proteases, which may be physiologically significant in fibrin and fibrinogen degradation, yielded a group of fibrinogen fragments that were immunoelectrophoretically

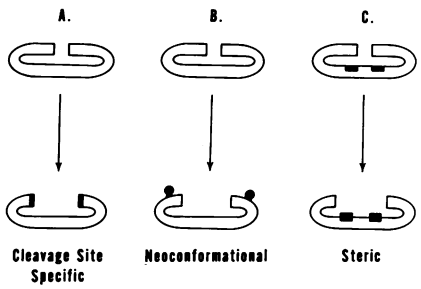

FIG. 1. Schematic representations of three hypothetical molecular models to explain emergence of a neoantigen after enzymatic cleavage. (*A*) *Cleavage site specific*, The neoantigen is associated with amino acids directly at the site of cleavage; (*B*) *Neoconformational*, The neoantigen is created by a new local conformation unrelated to the cleavage site; (*C*) *Steric*, The neoantigen is present within the intact molecule but is sterically hindered and becomes exposed on the cleaved molecule. While these models are conceptually distinct, all are modulated by molecular conformation.

distinct from those of plasmin digestion (Fig. 2). Quantitative antigenic deficiency was well demonstrated by the 4.8-fold increased concentration of leukocyte protease-cleaved fragments of fibrinogen required for 20% inhibition of [^{125}I]fg-D binding, as compared with fg-D. Qualitative differences were also

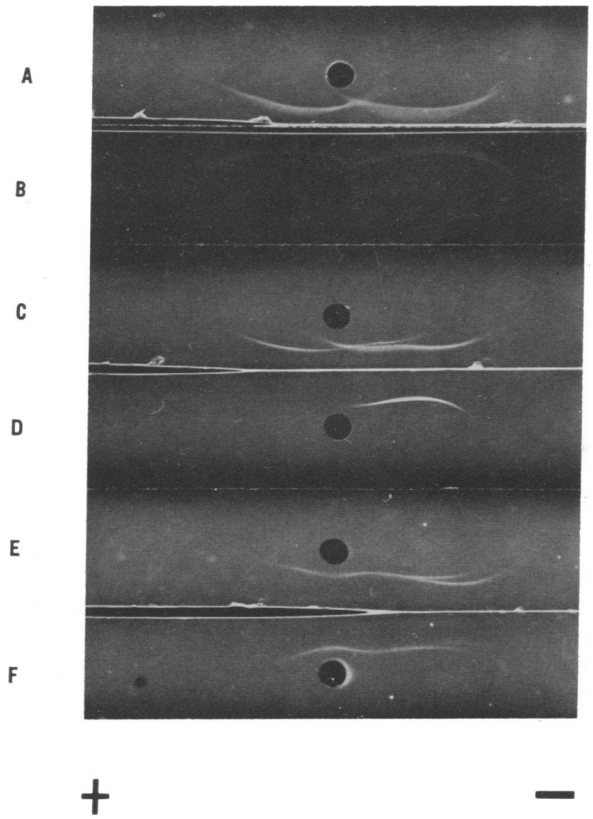

FIG. 2. Immunoelectrophoresis of enzymatic digests of fibrinogen. Fragments characteristic of fg-D and fg-E are clearly recognized in both plasmin (*A*) and trypsin (*B*) digests. Fragmentation is clearly produced by chymotrypsin (*C*), papain (*D*), and leukocyte proteases at pH 8.5 (*E*) and pH 7.0 (*F*), but the immunoelectrophoretic characteristics are distinct from those of the plasmin digest and reflect the differing specificities of these enzymes.

TABLE 1. *Effects of enzymatic cleavage on coagulability and immunochemical integrity of fibrinogen*

Enzyme	Thrombin time (Sec) 1 hr	Thrombin time (Sec) 18 hr	Ouchterlony analysis* Identity with fibrinogen	Ouchterlony analysis* Identity with plasmin digest
Control (saline)	11.2	18.9	+	−
Plasmin	>180	>180	−	+
Trypsin	>180	>180	−	+
Chymotrypsin	>180	>180	+	−
Papain	14.1	>180	+	−
Leukocyte proteases at pH 8.5	>180	>180	+	−
Leukocyte proteases at pH 7.0	19.8	>180	+	−

* By the use of antiserum to fibrinogen.

suggested by a slight change in the inhibition slope (Fig. 3). Chymotrypsin and papain cleavage of fibrinogen were associated with individually distinctive patterns of fragmentation (Fig. 2); and neoantigenic expression was both qualitatively and quantitatively deficient (Fig. 3). The markedly reduced slope of inhibition by fragments produced by digestion with chymotrypsin was paralleled by a 17.4-fold decrease of neoantigenic expression. Minimal fg-D_{neo} expression was evoked by papain cleavage and the neoantigen-like determinants were qualitatively quite distinct from those present on fg-D. The different specificities of these proteolytic enzymes strongly suggest that different amino acids are present at exposed cleavage sites. The qualitative emergence of fg-D_{neo}-related expressions after digestion with enzymes of differing specificities is inconsistent with the *cleavage site specific* model. These results also detract from a strict *neoconformational* model, since it is doubtful that similar new conformational determinants would be created. However, if fg-D_{neo} is present in native fibrinogen, but sterically hindered, various cleavage patterns may not only expose the neoantigen but may influence the degree of binding of the antibody.

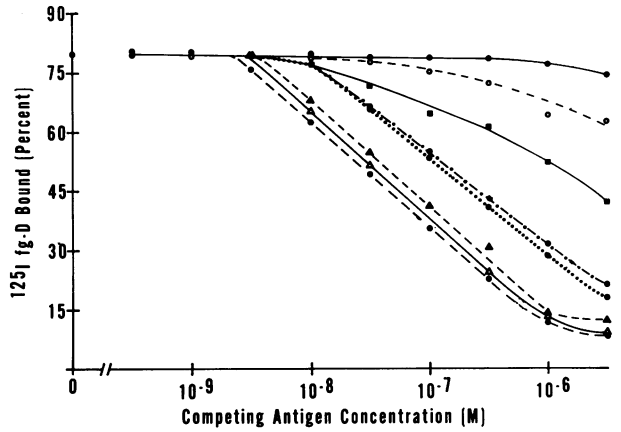

FIG. 3. Competitive inhibition radioimmunoassay for fg-D_{neo}; reference antigen is fg-D. Enzymatic digests of fibrinogen were directly assayed for qualitative and quantitative fg-D_{neo} expression (see *Methods*). Concentration is that of original fibrinogen and is not corrected for the 2 mol of fg-D_{neo}/mol of fibrinogen. Plasmin (△——△) and trypsin (▲- - -▲) digests yielded inhibition patterns quite similar to the standard semilogarithmic profile obtained with isolated fg-D (●- - -●). Digests produced by leukocyte proteases at both pH 7.0 (●···●) and pH 8.5 (●-·-●) possessed the neoantigen but were quantitatively deficient and appeared qualitatively slightly deficient, as expressed by a slightly reduced slope of the inhibition line. Chymotrypsin (■——■) and papain (○- - -○) digests exhibited marked qualitative differences in the neoantigenic expression. Inhibition by fibrinogen (●——●) was not significant.

TABLE 2. *Effect of denaturants on thrombin-induced coagulability of fibrinogen*

Denaturant	Thrombin time (sec)
Control	12.2
Acid (pH 4.0)	14.2
Base (pH 9.5)	>180
Cysteine (0.1 M)	40.4
2-Mercaptoethanol (0.2 M)	>180
Chloramine T (0.14 M)	>180

The *steric* model was further explored by subjection of fibrinogen to various chemical denaturants. The influence of these agents on the conformation of native fibrinogen was monitored by changes in thrombin-induced coagulability (Table 2). Oxidation by chloramine T, reduction by 2-mercaptoethanol, and exposure to an alkaline environment yielded unclottable species. Milder reduction by cysteine reduced coagulability. Fibrinogen was precipitated at pH 4.0, but regained solubility and coagulability upon return to neutrality.

In spite of chemically induced alterations of fibrinogen conformation, fg-D_{neo} expressions were observed only after oxidation of fibrinogen by chloramine T (Fig. 4). Fg-D_{neo} associated with chloramine T oxidation was quantitatively and qualitatively similar to purified fg-D, although no fragmentation or alteration in mobility was observed on analysis by immunoelectrophoresis (Fig. 4, *insert*).

Neoantigenic expression by the intact fibrinogen molecule, after oxidation, was confirmed in further experiments. A selective influence of varying degrees of oxidation on fg-D_{neo} expression was observed (Fig. 5); and the effects of chloramine T oxidation could be reproduced by simple oxidation with hydrogen peroxide.

Further evidence to support a *steric* model, in which fg-D_{neo} presumably represents one or more cryptic amino acid sequences, was provided by studies of fg-D_{neo} stability (Fig. 6). Neither reduction nor changes in pH, shown to influence molecular function, and undoubtedly to interfere with disulfide bond-dependent conformation, had any appreciable effect on neoantigen expression by fg-D. Chloramine T oxidation of fg-D moderately reduced the quantitative expression or exposure of fg-D_{neo}, and is consistent with the previously observed effects of excessive oxidation on fibrinogen. It is apparent that the neoantigen is rather stable. Such stability does not support a conformationally created neoantigenic site.

DISCUSSION

Of the three hypothetical models proposed for neoantigenic expression, the molecular properties of fg-D_{neo} suggest a

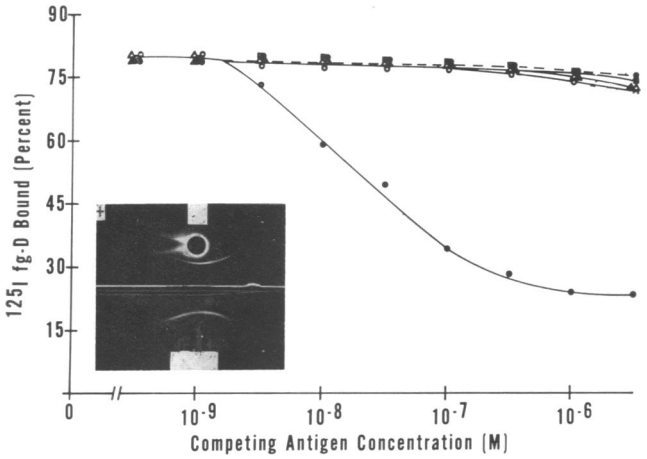

FIG. 4. The effects of physical and chemical denaturants on fg-D_{neo} expression by native fibrinogen. Treatment of fibrinogen with 0.14 M chloramine T (▲——▲) led to direct expression of this neoantigen by the intact molecule. Reduction by 0.1 M cysteine (O——O) and 0.2 M 2-mercaptoethanol (●- - -●), and exposure to pH 4.0 (■——■) and pH 9.5 (▲——▲) failed to expose fg-D_{neo}. *Insert*, Immunoelectrophoretic comparison of plasma fibrinogen (*up*) and fibrinogen oxidized with chloramine T (*down*). Conditions of the electrophoresis are identical to those described in Fig. 2. Although chloramine T-treated fibrinogen expresses fg-D_{neo}, there is no recognizable cleavage of fibrinogen.

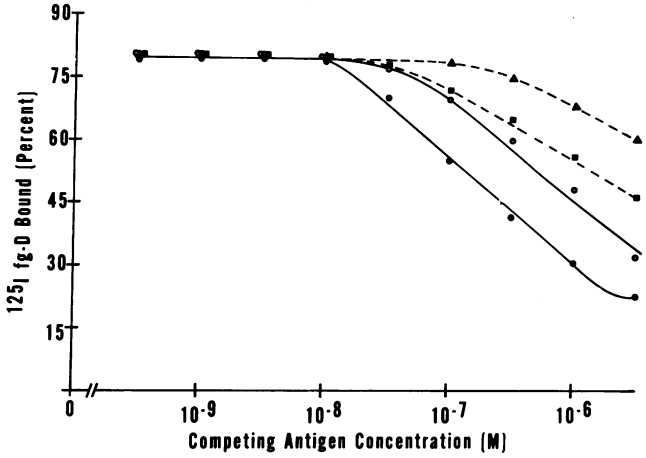

FIG. 5. Oxidation-induced neoantigenic expression by native fibrinogen. Fibrinogen oxidized with chloramine T exhibited fg-D_{neo}, although a distinct concentration effect was noted that was maximal at 0.14 M (O——O), and less well expressed at 14 mM (●——●). Very little neoantigenic expression was evident at a concentration of 1.4 M chloramine T (▲- - -▲). Oxidation with 1% hydrogen peroxide (■- - - -■) also led to fg-D_{neo} expression by intact fibrinogen.

steric basis for expression. This neoantigen appears to be associated with one or more specific determinants, buried in native fibrinogen, which become exposed in certain altered molecular species. The *steric* model of expression of the fg-D_{neo} is consistent with (*a*) the ability of enzymes of different specificities to elicit the neoantigen, (*b*) the presence of the neoantigen on altered but uncleaved fibrinogen, and (*c*) the high stability to physical and chemical denaturants. Other possible modes of expression appear to be inconsistent with these results. A *cleavage site specific* neoantigen apparently can be dismissed since proteolytic enzymes of various specificities and phenomena not associated with cleavage (e.g., oxidation) generate the neoantigen. A *neoconformational* model also does not seem tenable in the fibrinogen system. It is not only doubtful that oxidation and various proteolytic processes would generate a similar neoconformational determinant, but it is unlikely that such a determinant would exhibit such marked stability to physical and chemical denaturants.

The importance of steric exposure is also indicated in other neoantigenic systems. The subunits of hemocyanin possess unique antigenic expressions that are exposed upon dissociation of the native molecule (15). Steric exposure of neoantigens may be implicated in some tumor-specific antigenic systems; the carcinoembryonic antigen appears to possess an antigenic site that is exposed only at low ionic strength (16). Cleavage fragments of IgG possess a unique determinant(s), which is elicted by enzymes of differing specificities (4, 17). In other neoantigenic systems *cleavage site specific* and *neoconformational* models may be applicable. Mildly denatured aggregated bovine serum albumin possesses determinants not expressed in native or denatured albumin (3) suggesting *neoconformational* genesis. *Cleavage site specific* neoantigens are suggested on the conversion of procarboxypeptidase to carboxypeptidase. Activated forms of the enzyme, which differ in N-terminal portions created by differential cleavage of the proenzyme, can be distinguished immunochemically (18).

Although amino-acid sequence appears to be of primary importance for determination of fg-D_{neo}, it is apparent that molecular conformation plays a significant role in modulating antibody binding to this neoantigen. The effect of conformation on expression varies from complete hindrance of antibody binding in native fibrinogen to complete exposure on fg-D. The qualitative differences in antibody binding observed with papain and chymotrypsin digests could be achieved by exposure of the neoantigen to antibody under conditions of variable conformationally-modulated exposure. To generate quantitative differences in binding as observed with the fibrinogen digests by leukocyte proteases, the number of frag-

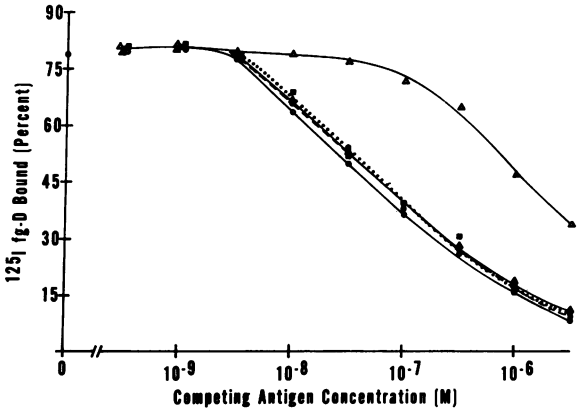

FIG. 6. Stability of the fg-D_{neo} expression on fg-D to physical and chemical denaturation. Reduction with 0.1 M cysteine (▲——▲) and 0.2 M 2-mercaptoethanol (●- - -●) failed to influence neoantigenic expression. Similarly, there was no effect upon exposure to pH 4.0 (●· · · · ·●) or pH 9.5 (O——O). Oxidation with 0.14 M chloramine T (△——△) appeared to partially degrade or modify the fg-D_{neo} expression on fg-D.

ments expressing fg-D_{neo} must be greatly reduced. A differential cleavage of fibrinogen by these proteases and by plasmin is consistent with this explanation (Fig. 2) and is indicated in the immunoelectrophoretic patterns.

Oxidation appears to significantly and progressively influence the steric exposure of fg-D_{neo}. The effect of excessive oxidation may be explained by either denaturation of the neoantigenic site or by reconformation of the fibrinogen or fg-D molecule with progressive hindrance of this site. Although oxidative changes might yield a more hydrophilic molecular species, the presence of polar amino acids in the fg-D_{neo} determinant cannot be directly implied. Brown (19) has demonstrated that fragments from oxidized ribonuclease, containing largely nonpolar amino acids, most strongly inhibit antibody binding to the oxidized protein.

Although this study has been concerned with the fibrinogen system as a molecular model, several of these observations are specifically applicable to the study of the pathophysiology of fibrinogen. Assay of fg-D_{neo} in plasma may provide a specific indicator of *in vivo* fibrinogenolysis or fibrinolysis (10). Although the possible pathophysiological role of the fg-D_{neo} site has not yet been investigated, it is of interest that enzymatic cleavage fragments of fibrinogen that possess the greatest anti-coagulative activity (20, 21) also express fg-D_{neo} to the greatest degree. Papain cleavage fragments, which exhibit only slight fg-D_{neo} expression, also fail to inhibit thromboplastin generation and only weakly inhibit fibrin polymerization (20). The relationship between the neoantigenic sites and physiologic sites might well be explored. Antibodies reactive with papain-cleaved human IgG have been demonstrated in normal and pathological human sera (22). The observation of spontaneous immune responses to neoantigenic expressions of IgG suggests that antibodies to fibrinogen neoantigens may also be found *in vivo* if sought.

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