Immune Responses to the Cleavage-Associated Neoantigens of Fibrinogen in Man

IDENTIFICATION AND CHARACTERIZATION OF HUMORAL ANTIBODIES SPECIFIC FOR CLEAVAGE FRAGMENTS

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ABSTRACT Cleavage of human fibrinogen and fibrin by plasmin is associated with modification of native antigenic expression and the exposure of cleavage-associated neoantigenic sites on the derivative molecular fragments. In this study, the presence of humoral antibodies in man to cleavage-associated neoantigens has been demonstrated by primary antigen binding radioimmunochemical assays. Specific binding of radioiodinated human fibrinogen D fragment by serum immunoglobulins was demonstrated in 52 of 59 random normal human sera and was independent of immunoglobulin concentration. Binding was mediated by $F(ab')_2$ fragments of IgG, and specificity for neoantigens was indicated by the capacity of the D fragment but not native fibrinogen to competitively inhibit the antibody. The population distribution of antibody to these cleavage-associated neoantigens indicated the presence of a major group of individuals (77%) with a mean antigen binding capacity of 11.8 pmol/ml serum. Two minor populations with: (*a*) low or undetectable binding capacities (< 6.0 pmol/ml serum) and (*b*) exhibiting markedly elevated binding capacities (> 18.0 pmol/ml serum) were delineated. Independent of these features, sera could also be readily separated into two groups that differed with respect to relative antibody affinity. The antibodies in most sera exhibited marked heterogeneity of binding affinity, whereas a small group of sera contained antibodies exhibiting relative homogeneity of binding affinity. Specific antibody was rather equally distributed between the major immunoglobulin classes, and in no serum was the antibody restricted to a single immunoglobulin class.

Antibodies capable of binding fibrinogen fragments X, Y, and D and fibrin D fragment were detected in most sera. The quantity of antibody differed for different fragments with $X > Y \simeq D >$ fibrin D. The presence of antibody capable of binding any single fragment was statistically correlated with the presence of antibody capable of binding other cleavage fragments. No antibody to the E fragment was detected. Antibody to cleavage fragments was not demonstrable in sera containing fibrinogen or fibrin cleavage fragments. Demonstration of this humoral immune response to the products of the fibrinolytic systems provides a new interface between the coagulation and immune system.

INTRODUCTION

The participation of protein molecules in biologic events may be associated with specific structural and conformational modification through processes intrinsic to activation or catabolism. Such structural alterations influence the antigenic expression of molecules, and may produce molecular species which are antigenically distinct from the native form of the precursor or native molecule (1-5). When such structural changes are associated only with deletion of native antigenic expressions, immunologic tolerance of the host to the native form of the molecule should preclude specific immunologic responses to the antigenically deficient form. On the other hand, when new antigenic expressions are generated, these neoantigens may be recognized by the immune system, and elicit

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an immune response by the autologous host (6-9). Molecular derivatives may circulate transiently, be generated at low concentrations, or may not be tolerogenic. These circumstances may preclude effective induction and maintenance of specific immunological tolerance to neoantigenic determinants (10).

The fibrinolytic pathway provides a model system to assess the immunologic response of man to the in vivo generation of neoantigens. The cleavage of fibrinogen or fibrin by plasmin leads to a series of defined fragments (11-13), which exhibit structural and conformational modifications as well as the expression of specific cleavage-associated neoantigens not associated with the parent molecules (14-16). Two independent cryptic neoantigens have been demonstrated in this system utilizing heterologous immune responses; one is present in the C-terminal aspects (D region) (14, 15) and the second in the N-terminal region (E region) (16). Cleavage fragments expressing these neoantigens are not present in the plasma of healthy adults at concentrations greater than 20 nM (15-17). In a number of pathological conditions leading to the activation of plasminogen, cleavage fragments are generated (18-22), and physical exercise (23) as well as forms of stress (24) may also lead to increased levels of cleavage fragments. Because of the potential pathophysiologic manifestations of immune responses to the derivatives of fibrinogen and fibrin, we have examined the endogenous immune responses of normal individuals to the plasmin cleavage fragments of fibrinogen and fibrin.

METHODS

Cleavage fragments of fibrinogen and fibrin. Fibrinogen and fibrin cleavage fragments, produced by plasmin, were isolated by molecular exclusion chromatography on 6% beaded agarose (A-1.5, 200–400 mesh, Bio-Rad Laboratories, Richmond, Calif.) followed by anion exchange chromatography on DEAE-cellulose for fibrinogen fragment D (fg-D) [1] and fg-E. The characteristics of fragments as prepared in this laboratory have been previously described (15–17), and each isolated fragment appeared to be homogeneous based on sedimentation velocity, molecular exclusion chromatography, immunoelectrophoresis, and polyacrylamide gel electrophoresis. Radioiodination with ^{125}I was performed according to the modified chloramine T method of McConahey and Dixon (25) with minimal concentrations of the oxidizing agent consistent with iodination. Subsequently, the fragments were reisolated by molecular exclusion chromatography on a 1.5 × 30-cm column of A-1.5 (Bio-Rad) in 1 M NaCl, 0.01 M Tris-HCl, pH 8.0, at a flow rate of 6 ml/h. Each cleavage fragment yielded a single symmetrical peak indicative of apparent homogeneity and each was recovered at its characteristic elution volume well beyond the void volume of the column to preclude the presence of aggregated protein. Trichloroacetic acid precipitability of the fragments was greater than 95% and specific activities ranged from 2 to 4 mCi/mg. Molar concentrations of the fragments were calculated from estimated molecular weights for fibrinogen, fg-X, fg-Y, fg-D, and fg-E of 340,000, 270,000, 165,000, 80,000, and 50,000, respectively; and protein concentrations were determined by micro-Kjeldahl nitrogen analysis.

Immunochemical assay. A double antibody radioimmunoassay (14) was utilized for demonstration and quantitation of antibody in human sera to the cleavage fragments. This system consisted of: (*a*) the radiolabeled fragment diluted to a 33 nM concentration with 0.1 M sodium borate, 0.1 M NaCl, pH 8.4; (*b*) borate buffer or in some experiments unlabeled inhibiting antigens; and (*c*) serum, at a dilution of not less than 1/5. Each component was added as a 0.2-ml aliquot. The system was thoroughly mixed and incubated at 4°C for 18 h. The precipitating antibody (anti-immunoglobulin), 0.4 ml, consisted of a mixture of goat antisera to human IgG, IgA, and IgM, and contained sufficient antibody to precipitate all detectable immunoglobulin present in 1/5 dilutions of several normal serum pools, as well as from several individual sera containing high concentrations of each immunoglobulin class. The precipitating capacity of the antisera was evaluated by quantitative precipitin techniques using ^{125}I-labeled immunoglobulins. Specificity of the mixture of antisera for only immunoglobulins was indicated by immunoelectrophoresis and Ouchterlony gel diffusion. After an additional 18-h incubation, all samples were centrifuged for 20 min at 1,800 *g*, and 0.5 ml of the supernate was removed and counted for γ emission at 22.5–55 keV. In each analysis, controls were included to permit correction for the trichloroacetic acid precipitability of the radioactivity. Results were expressed either: (*a*) in terms of the precent binding observed with a particular dilution of a serum or, alternatively, (*b*) as the antigen binding capacity of a particular serum (ABC) based on the dilution required to achieve 25% binding and then calculating the quantity of fragment bound per milliliter of undiluted serum (26). The coefficient of variation observed for the determination of the serum ABC was 8.6%, and there was no statistically significant difference in the ABC of plasma versus serum samples. Competitive inhibition slopes, observed upon the introduction of competing antigens in compartment two, were calculated as previously described (17).

Statistical analyses. Analyses were performed according to Goldstein (27). Comparison of sample means were performed by two-tailed *t* test and included testing of variance estimates by *F* test. Correlation coefficients were derived by linear regression analysis on a Texas Instruments (Houston, Tex.) SR-51 calculator and strength of correlation was estimated from two-tailed *t* test as suggested by Goldstein (27). Statistical tables of Fisher and Yates (28) were used for analysis. The population distribution of ABC values was determined by probit analysis as described by Newmann (29).

Sera. Normal sera utilized in this study were drawn from adults between the age of 18 and 40. These individuals were considered healthy on the basis of medical history, physical examination, and routine laboratory survey, complete blood count, and urinalysis. Blood was permitted to clot spontaneously, and the serum, obtained by centrifugation, was stored at − 20°C.

Fractionation of sera. Gamma globulin fractions were prepared by precipitation from serum diluted with an equal volume of 0.14 M sodium chloride, 0.01 M sodium phosphate, pH 7.3, (PBS) by addition of saturated ammonium

[1] *Abbreviations used in this paper:* ABC, antigen binding capacity of a particular serum; Fb-D, fibrin fragment D; fg, fragments derived from fibrinogen, e.g., fg-D is fibrinogen D fragment; PBS, 0.14 M sodium chloride, 0.01 M sodium phosphate, pH 7.3.

sulfate at 4°C to a final 50% saturation. The precipitate was recovered by centrifugation, dissolved in PBS, and reprecipitated at 50% saturation with ammonium sulfate two additional times. The precipitated gamma globulin fraction was exhaustively dialyzed against PBS. 7S fractions were prepared by molecular exclusion chromatography on a 2.5 × 85-cm column of Sephadex G-200 in PBS and the second peak containing IgG was retained for analysis. $F(ab')_2$ fragments were prepared from the 7S IgG fraction by pepsin cleavage for 18 h at 37°C in 0.05 M sodium chloride, 0.07 M sodium acetate, pH 5.0, at a pepsin: protein ratio of 1:100 (30). After exhaustive dialysis, the extent of cleavage and identity of the isolated $F(ab')_2$ fragments was confirmed by analytical acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate as described by Weber and Osborn (31).

Antisera. Antisera specific for IgG, IgM, or IgA were prepared by immunization of goats with purified immunoglobulins. Each antiserum was rendered specific for the appropriate immunoglobulin heavy chain by absorption with other immunoglobulin classes. Serum immunoglobulin levels were quantitated by single radial diffusion (32) utilizing a reference serum (Meloy Laboratories Inc., Springfield, Va.) as a standard. Antisera to human C3 and α_1-antitrypsin were prepared in goats and rabbits, respectively, by utilizing the purified proteins (33, 34) as immunogens. All antisera gave single precipitin lines by gel diffusion and immunoelectrophoresis and exhibited identity with reference antigens and antisera by Ouchterlony gel double diffusion.

Assay of fibrinogen degradation products. Fibrinogen-related antigens in serum were quantitated by radial diffusion techniques (15). Sera containing fibrinogen-related antigens utilized in this study were collected from patients with diseases commonly associated with circulating fibrinogen/fibrin degradation products including: meningococcemia, metastatic carcinoma, gram-negative septacemia, and obstetrical complications. The presence of fibrinogen degradation products in these sera was also confirmed by a latex agglutination test (Thrombo-Welcotest, Burroughs Wellcome & Co., Greenville, N. C.).

RESULTS

Recognition of the binding of cleavage fragments by normal human sera. The presence of humoral antibody with specificity for fibrinogen cleavage fragments was examined with primary binding radioimmunochemical assays. Fg-D was used as the prototype cleavage fragment. In the system employed, dilutions of test sera were incubated with ^{125}I-fg-D. Soluble complexes of antibody and ^{125}I-fg-D were then precipitated by polyvalent antiserum to human immunoglobulins. Binding of ^{125}I-fg-D was observed with 52 of 59 (88%) of normal sera. Characteristic binding profiles for five selected sera are illustrated in Fig. 1; and dependence of binding on serum dilution is evident. At $\frac{1}{5}$ dilutions of the sera, binding of ^{125}I-fg-D varied from 42 to 18%; and with dilution of each serum, binding is proportional to the $\log_{10}$ concentrations of serum. Differences in the binding characteristics of individual sera are indicated not only by the variations in the absolute binding capacity but also by differences in the slopes of the binding profiles upon dilution of the sera.

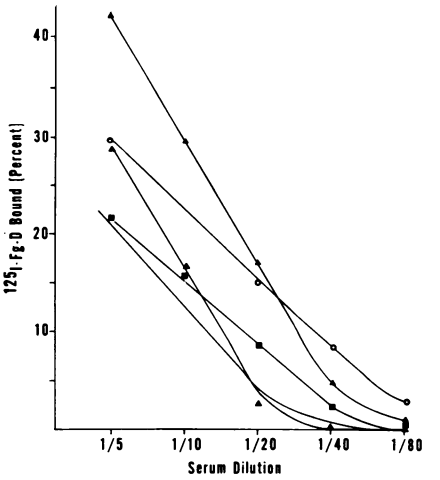

FIGURE 1 Binding of ^{125}I-fg-D by normal human sera. The radiolabeled fg-D ligand was present at 33 nM concentration and dilutions of each serum were introduced. After precipitation with the anti-immunoglobulin antiserum, the proportion of the free and bound ligand was calculated.

Specificity of the serum interaction with the D fragment. Evidence for the specificity of the observed binding was derived by several approaches. First, if binding of the fg-D depended on nonspecific coprecipitation, there should be a positive correlation between the quantity of immune complexes formed and binding of ^{125}I-fg-D. Sera from individuals with relatively high levels of immunoglobulin and relatively greater quantities of precipitate should exhibit relatively greater binding of ^{125}I-fg-D. The results of analysis of 40 normal sera indicate no significant correlation exists betwen the ABC for ^{125}I-fg-D and the immunoglobulin levels of the sera (Table I). Correlation coefficients of less than 0.07 were calculated for the association between the observed binding and the concentration of IgG, IgM, IgA, or total immunoglobulin.

Second, the possibility that low concentrations of fg-D might bind indiscriminantly to all immune complexes is negated by the absence of significant binding by unre-

TABLE I

Correlations between ABC for the Fg-D and Serum Immunoglobulin Concentration

Immunoglobulins*	Correlation coefficient‡
IgG	0.070
IgA	0.005
IgM	0.002
IgG + IgA + IgM	0.030

* Levels quantitated by radial immunodiffusion.

‡ Based on linear regression analysis of data from 40 sera.

TABLE II

Immunoglobulin Dependence of Binding of Fg-D

Serum	Precipitating antibody	Fg-D bound
		%*
1	Anti-α_1-antitrypsin	0.0
	Anti-C3	0.0
	Anti-α_1 + anti-C3	0.0
	Anti-immunoglobulin	20.8
2	Anti-α_1-antitrypsin	0.0
	Anti-C3	0.0
	Anti-α_1 + anti-C3	0.0
	Anti-immunoglobulin	14.9
3	Anti-α_1-antitrypsin	0.0
	Anti-C3	0.0
	Anti-α_1 + anti-C3	0.0
	Anti-immunoglobulin	10.6

* Percent of ^{125}I-fg-D bound by a 1/20 dilution of serum in standard assay.

lated immune complexes (Table II). Immune complexes were generated by the addition of antiserum to the third component of complement and/or α_1-antitrypsin. Although these reactions produced similar quantities of precipitate as obtained with the anti-immunoglobulin serum, coprecipitation of ^{125}I-fg-D is not observed.

Third, the binding of fg-D does not appear to reflect denaturation during radioiodination. As shown in Table III, addition of unlabeled fg-D, at a concentration of 1.0 μM, produces marked or complete inhibition of the binding of the ^{125}I-fg-D by antibody present in positive sera. Specificity for cleavage-associated neoantigens is indicated by the addition of fibrinogen at the same concentration which does not significantly inhibit binding of ^{125}I-fg-D.

TABLE III

Specific Inhibition of the Binding of Fg-D

Serum*	^{125}I-Fg-D bound	Inhibition by competing antigen	
		Fg-D‡	Fibrinogen‡
	%	%	
1	21.4	100.0	2.4
2	15.2	100.0	0.0
3	20.3	100.0	3.6
4	13.8	100.0	4.8
5	19.0	100.0	0.0
6	16.8	100.0	0.0
7	25.0	98.0	1.8
8	7.5	88.8	7.9

* At 1/10 dilution.
‡ Added at a 1 μM concentration.

TABLE IV

Association between the Binding of Fg-D and Serum Immunoglobulin Fractions

Serum	Fg-D bound by unfractionated serum*	50% ammonium sulfate precipitate‡	7S fraction‡	F(ab')2 fraction‡
	%			
1	20.8	100.0	93.6	87.3
2	32.0	100.0	78.2	75.2
3	25.9	95.2	83.0	72.4
4	21.8	94.8	72.0	60.0
5	0	0	0	0

* Percent of ^{125}I-fg-D bound by a 1/10 dilution of each serum.
‡ Each fraction was concentrated to the initial serum volume and the percent of ^{125}I-fg-D bound by the fractions are expressed as percent of initial ABC of the unfractionated serum.

Association of serum binding of cleavage fragments with immunoglobulin fractions. The specificity of the precipitating antibody employed in the assays for immunoglobulins implicates serum immunoglobulins in the binding of fg-D. Confirmatory evidence was provided by isolation of immunoglobulins from five individual sera (Table IV). Four of the sera, at a 1/10 dilution, bound 20–30% of the ^{125}I-fg-D whereas no binding is observed with the fifth serum. Fg-D binding of the four positive sera was completely recovered in the gamma globulin fraction prepared by precipitation at 50% saturation with ammonium sulfate. When this fraction of each serum was subjected to molecular exclusion chromatography on Sephadex G-200, 70-90% of the initial binding activity was recovered in the 7S peak coinciding with IgG and monomeric IgA, and no activity could be detected in other column fractions. F(ab')$_2$ fragments of IgG retained an average of 73.8% of the ^{125}I-fg-D binding capacity of four selected sera, further supporting the participation of the antigen binding region of immunoglobulin molecules in the binding of the cleavage fragments. A fifth serum and its equivalent fractions were negative.

Distribution of serum binding capacities. The quantitative distribution of antibody in a population of normal sera was analyzed. Based on the dilution of serum required to achieve 25% binding of ^{125}I-fg-D, the ABC was determined and expressed as picomoles of fg-D bound per milliliter of serum. The population distribution obtained with 59 sera (Fig. 2) presents evidence for the occurrence of three distinct populations. The range of 6–18 pmol with a mean of 11.8 pmol is defined as a normal population by probit analysis according to the method of Neumann (29). A second population is suggested by those sera with no demonstrable antibody. Of the eight sera constituting this population, five exhibited no detectable binding of fg-D even at a $\frac{1}{5}$ dilution. The other three sera exhibited slight binding but

TABLE V

Distribution by Immunoglobulin Class of Antibodies to Cleavage-Associated Neoantigens of Fg-D

Serum	Polyvalent IgG, IgM, IgA	IgG*	IgA*	IgM*	Sum‡ IgG + IgA + IgM
	%	%	%	%	%
1	100	0.0	50.3	55.9	106.2
2	100	33.2	29.3	40.3	103.0
3	100	37.6	33.3	15.0	85.9
4	100	34.3	41.3	28.7	104.3
5	100	33.5	33.8	25.0	92.3
6	100	36.7	50.5	0.0	87.2
7	100	67.8	0.0	42.6	110.4
8	100	52.0	47.7	0.0	97.7
9	100	0.0	56.0	45.0	101.0
Mean		32.8	38.0	28.0	98.7
SD		±21.8	±16.9	±19.9	±8.0

* Expressed as relative percent of the fg-D bound by the complete anti-immunoglobulin serum.

‡ Arithmetic sum of the percent fg-D bound by each of the three immunoglobulin classes.

at levels which were insufficient to quantitate; binding by these three sera varied from 6 to 18% at a $\frac{1}{5}$ dilution. Whether the former sera are absolutely devoid of antibody to fg-D or have levels of antibody that can not be demonstrated with the assay system is not known. The third population consists of those sera exhibiting relatively elevated binding of fg-D. Sera with ABC greater than 38 pmol/ml deviate from the mean of the major normal population by more than five standard deviations. Of the four sera with ABC > 38 pmol/ml, two were males and two females, and their ages varied from 23-36. Determination of the ABC at the 33% rather than 25% binding level does not alter the relative distribution of sera within these populations.

Immunoglobulin classes participating in the binding of the D fragment. The classes of immunoglobulins that participate in the binding of fg-D were determined by utilizing precipitating antibodies specific for immunoglobulin class. The quantity of fg-D bound by an individual human serum upon precipitation with a mixture of anti-IgG, anti-IgM, and anti-IgA was assigned a value of 100%. The binding observed with antibody to each individual class of immunoglobulin was calculated relative to the total binding. Nine sera were analyzed (Table V); and the arithmetic sum of antibody by the individual classes of immunoglobulin agree well with the total antibody. In no serum was the antibody confined to a single immunoglobulin class; and in the majority of sera, antibody was found in all three major classes. Four of the ten sera contained antibody of only two immunoglobulin classes; and an example lacking antibody of each class is found.

Clonal heterogeneity of antibody to fg-D. The clonal heterogeneity of the antibody responses to fg-D were analyzed by reference to the slope of competitive inhibition assays (35, 36). The addition of serial concentrations of fg-D competitively inhibits the binding of the ^{125}I-fg-D, and the inhibition profiles of two representative sera are shown in Fig. 3. These two sera nave markedly different competitive inhibition slopes. The steeper slope

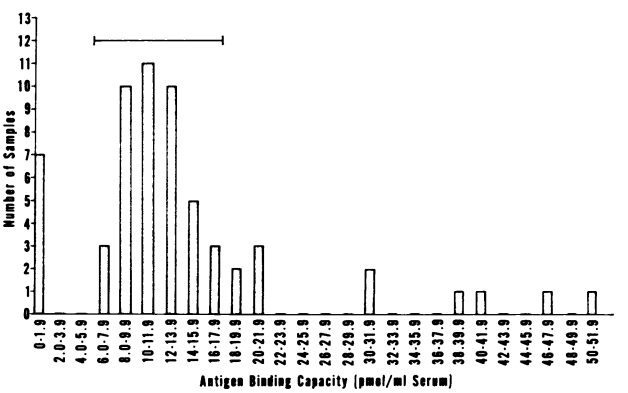

FIGURE 2 The distribution of the ABC for fg-D of 59 normal human sera. The ABC for each was calculated from the concentration of serum required for 25% binding of radiolabeled fg-D. The bracketed area indicates the range of the normal population as established by probit analysis (29).

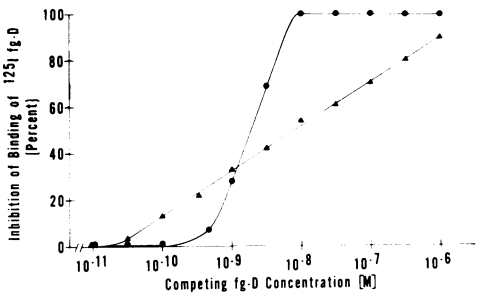

FIGURE 3 Representative competitive inhibition by fg-D of binding of the ^{125}I-fg-D by serum antibody. The percent inhibition at each concentration of competing fg-D was calculated relative to the binding of the ^{125}I-fg-D ligand in the absence of competing antigen. The slope of the linear portion of the plot reflects the relative degree of heterogeneity of binding affinity. Shallow slopes indicate marked heterogeneity whereas steep slopes indicate restricted heterogeneity (35, 36).

is indicative of relative clonal homogeneity by reference to binding affinity, whereas the shallower slope indicates significant heterogeneity evident by differences between clones of antibodies in respect to binding affinity (35, 36). The patterns generated by these two sera are representative of the two patterns observed among 10 sera which have been analyzed (Table VI). Three of ten sera are characterized by competitive inhibition slopes of 0.740–0.770, indicative of a relatively homogeneous response, while 7 of 10 sera exhibit shallow slopes, 0.120–0.180, indicative of marked clonal heterogeneity of specific antibodies.

Autoantibody response to other fibrinogen and fibrin derivatives. The results summarized in Table VII indicate the relative capacity of antibodies in 19 sera to bind various fibrinogen and fibrin cleavage derivatives. Binding of the fragments fg-X, fg-Y, fg-D, and fibrin fragment D (fb-D) is observed by the majority of the sera whereas no binding of fg-E is detected. All sera tested bound fg-X, and the mean ABC, 36.8±29.2 pmol bound/ml, is significantly higher than for fg-Y, fg-D, and fb-D (all $P < 0.01$ by two-tailed t test). Fibrinogen fragments fg-Y and fg-D are bound by a similar number of the sera, 17 of 19 and 16 of 19, respectively, and the mean ABC of these fragments are not statistically different ($P > 0.1$). Fb-D is bound by fewer sera, 14 of 19, and the mean ABC is significantly lower than for fg-X ($P < 0.01$) and fg-D ($P < 0.05$) but equivocal for fg-Y ($P < 0.1$). With the exception of serum no. 17, sera which failed to bind fb-D were capable of binding fibrinogen derivatives fg-Y and fg-D. Of particular note is serum no. 9, which had relatively high binding capacities for all cleavage fragments, and sera nos. 17-19, which exhibit low or negative binding capacities for all fragments with respect to the mean ABC for each individual fragment. A significant statistical association between the capacity of antibodies in any particular serum to bind the various fragments is suggested by linear regression analysis. Correlation coefficients of greater than 0.80 were ob-

TABLE VI

Analysis of Relative Clonal Heterogeneity of Antibody to Cleavage-Associated Neoantigens of Fg-D

Serum	Competitive inhibition slope*
1	0.155
2	0.120
3	0.180
4	0.165
5	0.150
6	0.160
7	0.140
8	0.770
9	0.760
10	0.740

* Slope value are inversely proportional to a function of the degree of clonal heterogeneity (35, 36).

TABLE VII

Quantitative Distribution of Antibody to Cleavage-Associated Neoantigens of Different Fibrinogen and Fibrin Derivatives

	ABC				
Serum	Fg-X	Fg-Y	Fg-D	Fg-E	Fb-D
	*pmol/ml serum**				
1	63.3	15.1	17.2	0.0	14.2
2	16.3	2.9	3.4	0.0	6.3
3	28.5	12.4	27.0	0.0	9.6
4	13.0	2.9	4.8	0.0	3.4
5	35.5	17.2	14.8	0.0	4.3
6	21.9	7.5	11.6	0.0	5.4
7	31.7	17.2	9.5	0.0	4.4
8	27.2	8.9	15.5	0.0	0.0
9	145.5	43.1	58.5	0.0	25.7
10	29.9	5.5	12.3	0.0	3.7
11	20.8	4.8	7.1	0.0	0.0
12	24.9	3.0	4.9	0.0	0.0
13	45.7	11.4	10.5	0.0	8.4
14	41.6	13.5	12.0	0.0	0.0
15	52.5	11.5	21.8	0.0	9.2
16	28.5	3.2	4.7	0.0	2.9
17	30.5	0.0	0.0	0.0	0.0
18	19.0	4.5	0.0	0.0	5.9
19	23.3	0.0	0.0	0.0	3.6
Mean	36.8	9.7	12.4	0.0	5.6
SD	±29.2	±9.8	±13.4	0	±6.2

* Based on the concentration of serum required to bind 25% of the ^{125}I-labeled fragment.

served in all cases. The strength of correlation was $P < 0.01$ by a two-tailed t test of significance as summarized in Table VIII. Although there are individual differences in specificities of the antibodies found in individual sera, indicative of antigenic differences between these fragments and differences in specificity or affinity of antibody, there is a significant trend toward *relative* binding of different fragments to a comparable degree.

Neutralization of autoantibody to cleavage fragments in vivo. The presence of antibodies to the cleavage fragments in vivo was explored in sera from individuals in which spontaneous fibrinolysis had occurred in vivo, and cleavage fragments could be detected in the serum samples. The results indicated in Table IX, namely the absence of binding of fg-D, are consistent with neutralization of antibodies by the cleavage fragments generated in vivo.

TABLE VIII

*Correlations between the Binding of Various Cleavage Fragments by Serum Antibodies**

Fragments compared	Correlation coefficient (r)	Strength of association (P)
Fg-X vs. fg-D	0.890	<0.001
Fg-X vs. fg-Y	0.880	<0.001
Fg-X vs. fb-D	0.854	<0.001
Fg-Y vs. fg-D	0.912	<0.001
Fg-D vs. fb-D	0.826	<0.001
Fg-Y vs. fb-D	0.801	<0.01

* Based on linear regression analyses of the 19 sera in Table VII. Strength of association determined by two-tailed t test.

TABLE IX

Association between the Presence of Circulating Fibrinogen or Fibrin Cleavage Fragments and Serum to Cleavage-Associated Neoantigens of Fg-D

Serum	Cleavage fragments	ABC
	*μg fibrinogen-related antigen/ml**	*pmol/ml*
1	565	<2.0
2	80	<2.0
3	140	<2.0
4	85	<2.0
5	86	<2.0
6	117	<2.0
7	240	<2.0

* Estimated by quantitative single radial diffusion analysis (15). Normal serum contains <3 μg/ml by this technique.

DISCUSSION

Sera from normal adults contains antibodies specific for the cleavage fragments of fibrinogen. The participation of specific antibody is indicated by (*a*) the specificity of the precipitation phase of the assay system for immunoglobulin (*b*) the association of binding with the isolated immunoglobulin fractions, and (*c*) the retention of binding activity by isolated $F(ab')_2$ fragments. Antibody specificity is demonstrated by the capacity to bind fg-X, fg-Y, fg-D, and fb-D but not intact fibrinogen or fg-E. In concert, these observations are consistent with an immune response to a cleavage-associated neoantigen in the D region of fibrinogen, a determinant(s) not expressed by the parent fibrinogen molecule but generated by plasmin cleavage. In this respect, the presence of antibodies to cleavage-associated neoantigens of fibrinogen is analogous to the presence of antibodies with specificity for pepsin- or papain-cleaved IgG in the sera of most adults (6-8) and the recognized specificity of rheumatoid factor for IgG (37) that is modified by physiological interaction with specific antigen (38). The ubiquitous nature of the antibody response to the cleavage fragments of fibrinogen is also similar to the response of man to antigens of myelin in which 88% of normal adults possess antibody with this specificity (39).

In characterizing the immune response to the cleavage fragments of fibrinogen, heterogeneity and the presence of distinct subpopulations were noted with respect to antibody concentration, affinity, and class. Within the panel of 59 sera, three distinct subpopulations in terms of ABC were recognized. The normal population exhibited a mean ABC of 11.8 pmol of fg-D bound/ml of serum. On the assumption that one molecule of IgG is bound to each fg-D molecule at the 25% binding level, a mean concentration of 2 μg/ml of specific antibody is present in this population. The other two populations consisted of individuals with either low or elevated levels of specific antibody. Whether these populations, hyporesponsive or hyperresponsive, respectively, arise from differences in genetically determined immune responsiveness (40) or, alternatively, reflect neutralization of antibody by low levels of antigens in the case of hyporesponders and stimulation of antibody synthesis by exposure to antigen in the case of hyperresponders remains conjectural.

The heterogeneity of antibody affinity observed in most sera and the participation of multiple heavy-chain classes of antibody is consistent with a polyclonal immune response to the cleavage fragments. Although cross-reactions among monoclonal antibody responses to individual cleavage fragments could be hypothetically considered, these observations are most readily explained by the presence of multiple clones of antibody molecules varying in the structure of both their variable and constant regions.

In comparing the antibody content and specificity of the sera, there is a significant correlation between the qualitative capacity to bind fg-D and the capacity to bind other cleavage fragments containing a D region, namely, fg-X and fg-Y and fb-D. It might be hypothesized that antibody molecules regardless of the particular fragments serving to elicite the immune responses may also bind other cleavage fragments, but to a quantitatively different extent. In this case, such differences in the apparent antibody concentration for each fragment might reflect differences in the local conformation and the structure of the particular neoantigenic determinant(s) associated with each fragment (41). Alternatively, subsets of antibody specific for neoantigens unique to each individual fragment may exist so that the differences in the mean ABC for each fragment may reflect true differences in antibody concentrations. Regardless of whether differences in the ABC for the various fragments reflects antibody specificity or affinity, the series of fg-X > fg-Y $\simeq$ fg-D exhibits an interesting parallel with the cleavage process. Fg-X is the first major derivative resulting from the cleavage of fibrinogen by plasma (12), and more antibody reactive with this fragment is present. The process need not proceed beyond the generation of fg-X so that less fg-Y and fg-D may be generated (42). The mean ABC of sera for fg-Y and fg-D are comparable, and this is compatible with similar immunogenicity upon simultaneous generation in equal quantities from the immediate cleavage of fg-X (12).

The cleavage-associated neoantigenic determinant(s) responsible for eliciting this immune response in man is in many respects similar to, but is clearly not identical with, the fg-D_{neo} determinants recognized by the heterologous immune response of rabbits. The capacity of antibody to react with all fragments containing a D region is observed with both responses, but rabbit anti-fg-D_{neo} exhibits an equal reaction with all fragments on a molar basis (15). Although differences in the ABC of human sera for the various fragments may be explained in at least two independent ways (see above), this differential reaction establishes that the determinants recognized by the autologous and heterologous immune responses cannot be identical. The failure to detect antibody to fg-E in man may reflect a tolerance to those fg-E_{neo} determinants recognized by rabbits or a lack of appropriate exposure to this determinant. In this respect fg-X exhibits only minimal expression of fg-E_{neo} relative to the other cleavage fragments containing an E region (41).

Although normal plasma has been shown to contain early fibrin(ogen) catabolic fragments (43), these derivatives do not express the neoantigenic determinants recognized by the rabbit and do not apparently neutralize human antibodies to the intermediate or late cleavage products. These early derivatives may contain moderately degraded alpha chains of fibrinogen, but extensive cleavage of the beta chain appears to be confined to pathological circumstances. In this respect expression of the determinants recognized by both the autologous and heterologous responses may be contingent upon cleavage of the beta chain.

In sera containing intermediate or terminal cleavage fragments, antibodies to the fragments were not detected. Although this observation is consistent with the neutralization of specific antibodies by these fragments, formation of immune complexes remains to be directly established. In some diseases such as cryoglobulinemia and glomerulonephritis, immunoglobulins are associated with fibrinogen-related material (44-46). The possibility must be considered that this association may, in part, reflect specific immune complex formation. The interaction of the antibody with the cleavage fragments may have antithetical effects. It may be beneficial in neutralizing the physiologic activities of the cleavage fragments (47, 48) or in accelerating their clearance from the circulation. Such responses may also possess pathogenetic potential in leading to localized immunological reactions at the sites of fibrinolysis and also to the formation of circulating immune complexes and the diseases mediated by their deposition. Although the physiological and pathogenetic implications remain to be established, the existence of this response provides another example of the complex interactions of mediation systems such as the coagulation, fibrinolytic, and immune systems.

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