Antigenic Markers on Fragment DD, A Unique Plasmic Derivative of Human Crosslinked Fibrin

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Plasmic degradation of crosslinked human fibrin produces unique degradation products, (DD)E complex and fragment DD, in which the DD moiety contains two fragment D molecules joined together by covalent bonds. In this study, fragment DD antigenic markers were studied using two different antisera: one against intact (DD)E complex, and the other against fragment DD that has been exposed to 3 M urea at pH 5.5. Antisera were absorbed with fibrinogen and purified fibrinogen degradation products. Direct binding and inhibition of binding of labeled antigens with the antiserum against (DD)E complex showed a 50-fold greater reactivity with fragment DD, as compared with fragment D from fibrinogen or noncrosslinked fibrin. Fibrinogen did not inhibit such binding. In this system, 50% inhibition of binding was achieved with 9 μg/ml of fragment DD. Using the antiserum against fragment DD exposed to acid urea, this antigen was 100-fold and 20-fold more reactive than were acid-urea-treated fragments D prepared from fibrinogen and noncrosslinked fibrin, respectively. Acid-urea-treated fibrinogen was 2000-fold less reactive on a molar basis than was acid-urea-treated fragment DD, and unmodified fibrinogen and fragment D moieties were even less reactive. The sensitivity of this system was greater than that using the antiserum against (DD)E complex, with 50% inhibition achieved using only 300 ng/ml of fragment DD exposed to acid urea. Thus, the difference in antibody reaction with crosslinked and noncrosslinked fragment D moieties demonstrated fragment DD antigenic markers that probably result from unique structural features in this degradation product. However, these crosslink-related markers represented only a small proportion of the total antigenicity of fragment DD, which bears striking similarity to that of other fragment D derivatives.

FACCTOR XIII catalyzes the formation of covalent isopeptide bonds between lysine and glutamine residues of contiguous fibrin monomers. Of the three polypeptide chains of fibrinogen, only the α and γ chains are involved. This reaction occurs rapidly between two γ chains near the carboxy terminals and links two molecules together. A similar but slower process of α-chain polymerization binds many fibrin molecules together. The factor-XIII-induced bonds account for the liberation of distinctive products upon degradation by plasmin, as compared with the products derived from fibrinogen and noncrosslinked fibrin. One product, fragment DD, is a molecule of about 189,000 daltons, which consists
essentially of two fragment-D moieties derived from different fibrin molecules, covalently bound by crosslink bonds between the γ-chain remnants. A heterogeneous group of crosslinked α-chain derivatives is also liberated from crosslinked fibrin. It is reasonable to suggest that both types of crosslinked derivatives possess antigenic sites that are not present on the degradation products of fibrinogen or noncrosslinked fibrin. Plasmic digests of crosslinked fibrin contain (DD)E complex, which appears to be an important derivative liberated from fibrin. The complex can be dissociated, for instance in acid urea, but such treatment modifies the conformation of the fragment-DD moiety, increasing its susceptibility to proteolysis by plasmin and its tendency for spontaneous aggregation. In this study, antisera were obtained in chickens and rabbits against a mixture of (DD)E complex and fragment DD and against fragment DD exposed to 3 M urea at pH 5.5. After absorptions with fibrinogen and fragments D and E, these antisera were applied to the study of antigenic markers that are present on various plasmic derivatives of fibrin and fibrinogen. A radioimmunoassay technique was utilized for the quantitative comparison of these derivatives, searching for specific antigenic markers that would distinguish fragment DD from plasmic derivatives of noncrosslinked fibrin and fibrinogen.

MATERIALS AND METHODS

Human fibrinogen (Kabi, grade L, Stockholm, Sweden) dissolved in water (10 mg/ml) was 95% clottable with thrombin (bovine, Parke-Davis, Detroit, Mich.) and contained sufficient plasminogen to cause proteolytic degradation after the addition of streptokinase (100 U/ml final concentration; Varidase, Lederle Laboratories, Pearl River, N.Y.). The reaction was carried out in the presence of 5 mM CaCl₂ and terminated by the addition of soybean trypsin inhibitor (0.1 mg/ml final concentration; Worthington, Freehold, N.J.). Fragment X was purified from stage-I digests and fragment Y from stage-2 digests of fibrinogen by Sephadex G-200 gel filtration. Fragments D and E were purified from stage-2 and stage-3 digests of fibrinogen and noncrosslinked fibrin by block electrophoresis on Pevikon (C-870, Mercer Chemical Corp., New York, N.Y.).

Factor XIII was purified from citrated bovine plasma by the method of Loewy et al. To obtain crosslinked fibrin, 1 g of human fibrinogen was reconstituted in 400 ml 0.05 M Tris-HCl buffer, pH 7.5, containing 0.1 M sodium chloride and supplemented with 1 ml of factor XIII (900 U/ml), 6.5 ml of 1 M calcium chloride (16 mM final concentration), and 0.07 ml of β-mercaptoethanol (2.0 mM final concentration). The clotting was initiated by the addition of 0.4 ml of bovine thrombin (1000 U/ml) to a final concentration 1 U/ml. After incubation at 25°C for 18 hr, the clot was collected on nylon cloth and liquor was removed by compression. The clot was insoluble in 2% acetic acid, and less than 2% of soluble protein appeared in the supernate after overnight incubation. Noncrosslinked fibrin was obtained in a similar way, except that factor XIII, calcium chloride, and β-mercaptoethanol were substituted by 2.7 ml of 0.3 M EDTA. The fibrin clot was shredded and suspended in 100 ml of 0.15 M Tris-HCl buffer, pH 7.8, and incubated at 37°C with human plasmin, 10.8 CTA U/ml, kindly provided by Dr. David L. Aronson, Bureau of Biologics, FDA, Rockville, Md., which was added in 3 0.8-ml aliquots at the start of incubation and after delays of 6 and 12 hr. Total incubation period was 24 hr.

The plasmic digests of crosslinked human fibrin was fractionated in several ways to purify fragment DD species. In this study, fragment DD and (DD)E complex refer to derivatives of crosslinked fibrin that are obtained by techniques which, in our judgment, do not significantly alter the structure of the derivative. The term acid-urea fragment DD means that the fragment has been treated or purified under dissociating conditions in 3 M urea at pH 5.5 that have altered its structure for instance, in a manner that can be seen as increased plasmin susceptibility and aggregability. Purification of (DD)E complex was accomplished by column gel filtration on Sepharose CL-6B (Pharmacia, Piscataway, N.J.); this preparation is referred to as a (DD)E complex, but approximately 50% of it was fragment DD. Fragment DD was purified from the digest by ion-exchange chromatography on DEAE-cellulose.
adjusted to pH 5.5 with 5% acetic acid, as described previously, this preparation, designated as containing 0.1% SDS. Degradation products were assessed for purity by electrophoresis of nonreduced proteins in 7% polyacrylamide gels containing 0.1% SDS.

Fibrinogen and purified plasmic degradation products from fibrinogen, noncrosslinked and crosslinked fibrin were labeled with $^{125}$I by a modification of the chloramine-T method. The labeled proteins contained 0.2–0.6 atoms of iodine per molecule, had a specific radioactivity on the order of 400 mCi/mg, were stored in small aliquots frozen at $-80^\circ$C, and were suitable for binding studies for at least 4 wk. The fragments DD were tested after labeling by polyacrylamide gel electrophoresis in Tris-glycine buffer, pH 8.6. About 96% of $^{125}$I fragment DD was recovered in a DD band; $^{125}$I acid-urea fragment DD was completely aggregated.

White New Zealand rabbits were immunized with 0.2 mg of the immunogen emulsified with an equal volume of complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.) into the right foot pad and repeated after 4 wk into the left foot pad. Every 2 wk, subcutaneous booster injections of approximately 0.1 mg were administered. Seven days later, blood was obtained from the central ear artery, allowed to clot in glass test tubes and serum was prepared. Sera collected between 5 and 20 wk of immunization were pooled for each animal separately. Adult white Leghorn roosters were immunized for 12 wk, with 0.5 mg of immunogen emulsified with an equal volume of complete Freund’s adjuvant, by intracutaneous injections into 10 sites on the back every 14 days. Seven days later, blood was collected from the median wing vein, allowed to clot, and serum collected between 6 and 12 wk was pooled for each bird separately. Antisera were absorbed with normal plasma and with fibrinogen in a repetitive manner until no precipitate was detectable after centrifugation at 12,000 g for 15 min. Antisera against modified fragment DD and against (DD)E complex were then absorbed with fibrinogen and fragments D and E, from a stage-3 digest of fibrinogen, respectively, in the same manner.

The radioimmunoassay (RIA) technique was based on the interference by the test material with the binding of a radiolabeled antigen to the absorbed antiserum developed against the antigen. The radiolabeled antigen bound to the antibody was removed by immunoprecipitation with a specific antiserum against gamma-globulin, using rabbit antiserum against chicken gamma-globulin and goat antiserum against rabbit gamma-globulin (Cappel Laboratories, Cochranville, Pa.). The amount of radiolabeled fragment precipitated by antiserum was inversely proportional to the concentration of nonlabeled fragment in the test sample. Two RIA systems were used: one employing antiserum against (DD)E complex and labeled fragment DD, and the other utilizing antiserum against acid-urea fragment DD and labeled acid-urea fragment DD.

In the experimental procedure, 0.4 ml of sample was mixed with 0.03 ml of $^{125}$I-labeled antigen (approximately 10,000 cpm containing 0.05 ng of the antigen) and 0.05 ml of absorbed antiserum, diluted with buffer containing 0.05 M Tris-HCl, 0.1 M sodium chloride, and 1.0 mg/ml ovalbumin, pH 8.6, and incubated in ice. Then 0.05 ml of antiserum against the corresponding gamma-globulin was added to the mixture in the ice again. After centrifugation at 2000 g at 4$^\circ$C for 15 min, the supernate was decanted, combined with wash (see below), and counted in an automatic gamma counter (Automatic Gamma Counter, Searle, Chicago, Ill.). The precipitate was washed with 0.5 ml of buffer, agitated mechanically (Vortex Genie Mixer, Scientific Products, Evanston, Ill.), and centrifuged at 2000 g for 15 min; the wash was combined with the previously saved decanted supernate, and the radioactivity of the supernate and precipitate was determined.

For each day’s experiment, three values were determined in order to calculate net specific binding: radioactivity that is precipitated nonspecifically with anti-gamma-globulin antiserum, radioactivity that is bound to preimmunization serum and precipitated with anti-gamma-globulin antiserum, and radioactivity that is bound to the postimmunization antiserum and precipitated by the anti-gamma-globulin antiserum.

Binding of the labeled antigen was calculated as the ratio of radioactivity in the precipitate to that of the total mixture (precipitate plus supernate). Net binding of the labeled antigen to antiserum was corrected for nonspecific binding by subtracting the result with preimmunization serum from that obtained with postimmunization serum. This difference was considered as the maximal 100% binding value for subsequent measurements of inhibition of binding by test samples. Inhibition of binding was calculated as the difference in observed binding of labeled antigen in the absence and presence of the test material, both values having been corrected for nonspecific binding by preimmunization serum.
RESULTS

Optimizing the RIA Techniques

The binding between labeled antigen and the absorbed antiserum depended on the following conditions: dilution of antiserum, temperature and duration of incubation, and concentration of the precipitating anti-immunoglobulin antiserum. Figure 1 reflects the amount of labeled antigen–antibody complex that was precipitated by different amounts of heterologous anti-immunoglobulin antiserum using RIA system for the acid-urea fragment DD. In the absence of a second antibody (Fig. 1, NONE), little radioactivity was recovered after centrifugation, indicating that the antigen–antibody complexes were soluble under these conditions. In the presence of antiserum against chicken gamma-globulin, significantly more radioactivity was recovered in the pellet, with maximal immunoprecipitation occurring when the ratio of the second to the first antiserum was 20 or more. The incubation time in ice with the first and second antibody had a marked effect on percent binding, each requiring 3 days to achieve maximal effect. Extension of the incubation beyond this time resulted either in no improvement or in decrease of binding.

Similar results were obtained using the RIA system with antiserum against (DD)E complex, except that maximal binding was 27% at 1:10 antiserum dilution. The binding increased to 37% at a 1:2 dilution. Changes of the labeled antigen concentration over a 50-fold range did not significantly affect the binding. Normal human plasma could be substituted for the buffer in these assays. Binding of fragment DD with absorbed antiserum (1:1000) was only slightly lower when plasma was used as diluent instead of buffer: 37.2% ± 4.7% and 48.4% ± 11.8%, respectively. Binding with preimmunization serum diluted in plasma (4.8% ±

Fig. 1. The effect of heterologous antiserum against gamma-globulin (second antibody) on the degree of precipitation of ³¹P-labeled fragment DD bound to anti-fragment-DD antiserum (first antibody). Absorbed chicken antiserum against acid urea exposed fragment DD was diluted in buffer and plotted on the horizontal axis as the reciprocal of dilution. The experiments were performed on four different days. The values for percent binding are not corrected for nonspecific binding with preimmunization chicken serum. Binding at different dilutions of the first antibody was tested without the addition of the second antibody (□, NONE) and with addition of the second antibody diluted either 1:2 (△, DIL. 1:2) or 1:50 ( ●, DIL. 1:50). Open symbols indicate mean values of percent binding.
2.2%) was also lower than that in the buffer (9.0% ± 0.9%), and net binding obtained in plasma milieu was not significantly different than that observed using the buffer: 31.7% ± 6.2% versus 39.3% ± 11.4%, respectively.

Studies With Antiserum Against (DD)E Complex

The binding of the labeled unmodified fragment DD with antiserum absorbed with fibrinogen and fragments D and E from a plasmic digest of fibrinogen was only 27% at a 1:10 dilution. Gel filtration of the labeled fragment DD on a Sephadex G-200 column slightly improved the binding, but gel filtration on Biogel P-2 was without effect and ion-exchange chromatography on DEAE-cellulose almost completely abolished its binding capacity. Rabbit antiserum bound the labeled antigen better than did chicken antisera; neither absorbed antiserum formed precipitation lines with (DD)E complex or fragment DD, as tested by double immunodiffusion in agarose gel.

The binding of labeled (DD)E complex with absorbed antiserum was about the same as that with labeled fragment DD. However, part of the binding of labeled (DD)E complex with this antiserum may have been the result of some antigenic determinants related to structural changes in the fragment-E, not the DD, moiety. Therefore, subsequent studies utilized only labeled fragment DD as the binding antigen. The binding of this labeled antigen with absorbed antiserum was inhibited equally by acid urea and by unmodified fragment DD and the (DD)E complex.

Table 1. Inhibition of Labeled Fragment DD Binding by Fibrinogen and Fragment D Species

<table>
<thead>
<tr>
<th>Test Material</th>
<th>Anti-Acid-Urea DD + 1251 Acid-Urea DD</th>
<th>Anti-(DD)E + 1251 DD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ng/ml M/M</td>
<td>ng/ml M/M</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>No inhibition at 2.5 x 10^6</td>
<td>No inhibition at 2.5 x 10^6</td>
</tr>
<tr>
<td>Fragment D</td>
<td>21% inhibition at 10^6</td>
<td>2 x 10^5 50</td>
</tr>
<tr>
<td>(stage 3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fragment D</td>
<td>5.3 x 10^6 3.924</td>
<td>2 x 10^5 50</td>
</tr>
<tr>
<td>(non-XL-fibrin)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(DD)E Complex</td>
<td>29% inhibition at 10^6</td>
<td>9 x 10^3 0.8</td>
</tr>
<tr>
<td>Fragment DD</td>
<td>40% inhibition at 10^6</td>
<td>9 x 10^3 1</td>
</tr>
<tr>
<td>(acid urea)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fragment DD</td>
<td>3 x 10^7 1</td>
<td>9 x 10^3 1</td>
</tr>
<tr>
<td>(acid urea)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>1.1 x 10^6 2.025</td>
<td>—</td>
</tr>
<tr>
<td>(acid urea)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fragment D</td>
<td>1.3 x 10^6 97</td>
<td>—</td>
</tr>
<tr>
<td>(stage 3; acid urea)</td>
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<td></td>
</tr>
<tr>
<td>Fragment D</td>
<td>2.5 x 10^7 19</td>
<td>—</td>
</tr>
<tr>
<td>(non-XL-fibrin; acid urea)</td>
<td></td>
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</table>

*The figures represent the concentration of test material that causes 50% inhibition of binding or the inhibition achieved at the indicated concentration. M/M shows the number of molecules of test material relative to that of fragment DD that caused 50% inhibition. Molecular weight values used are: fibrinogen (340,000), fragment D (85,000), DD (190,000), and (DD)E complex (240,000).

†Test system measured inhibition of binding of the indicated labeled antigen.
Fig. 2. Inhibition of binding of 131I-labeled fragment DD with absorbed rabbit antiserum against (DD)E complex (1:25 dilution) by unlabeled fragment DD (○) and by unlabeled fragments D from fibrinogen (▲) and noncrosslinked fibrin (▲). The diluent, citrated normal human plasma with a fibrinogen concentration of 2.5 mg/ml, did not inhibit binding. The data for fragment DD are mean values of five experiments performed on different days; that for fragments D are from two experiments performed on different days using different preparations of labeled fragment DD. Standard deviations are indicated by the vertical bars. The same results as shown for fragment DD were obtained with the (DD)E complex and fragment DD exposed to acid urea (see Table 1).

(Table 1). The inhibition curves were similar, with 50% inhibition at approximately 9 μg/ml. The practical range of fragment DD determination was between 0.1 and 1000 μg/ml, corresponding to 0.5 nM and 5 μM, respectively. Unmodified fragment D from fibrinogen and noncrosslinked fibrin significantly inhibited binding by 50% at a concentration of 2 × 10^2 ng/ml, but this effect was about 50-fold smaller than that with fragment DD (Fig. 2), suggesting that the latter carries fragment-DD antigenic markers.

Studies With Antiserum Against Acid-Urea Fragment DD

Unabsorbed rabbit and chicken antisera against acid-urea fragment DD formed immunoprecipitation lines of identity in agarose gel with fragments DD, D, and fibrinogen, all previously exposed to 3 M urea at pH 5.5.

These lines spurred over those formed with the unmodified counterparts, indicating that the exposure to acid urea revealed new antigenic sites. Most of this precipitation reaction was removed by absorption of the antisera with fibrinogen and fragment D.

Absorbed antisera against acid-urea fragment DD had much higher titer than those raised against the (DD)E complex. Chicken antisera, after absorption, demonstrated higher binding with labeled antigens as compared with rabbit antisera. Binding experiments of absorbed antiserum (1:1000) with labeled acid-urea fragment DD was 48.4% ± 11.8%, using buffer as diluent. Binding with labeled fibrinogen and fragment D (stage 3) from fibrinogen and from noncross-
Fig. 3. Inhibition of binding of $^{125}$I-labeled acid-urea fragment DD with absorbed chicken antiserum against acid-urea fragment DD (1:1000 dilution) by unlabeled plasmic degradation products: acid-urea fragment DD (O), fragment D from noncrosslinked fibrin (Δ), fibrinogen fragments X (■), Y (○), D (stage 2; △), D (stage 3; ▽), and E (□). The diluent was citrated normal human plasma with a fibrinogen concentration of 2.5 mg/ml, which showed no inhibition in this system. Data for fragments X, Y, D (stage 2), D (stage 3), D from noncrosslinked fibrin, and E obtained in the concentration range 3–10,000 ng/ml from single experiments, and in the range $10^4$ to $3 \times 10^6$ ng/ml from two experiments, performed simultaneously with control studies of fragment DD. The same preparation of labeled fragment DD was used for all experiments. Standard deviations are indicated by the vertical bars.

linked fibrin showed only negligible binding at all of the serum dilutions that were tested (1:100 to 1:6400).

The presence of specific antibodies against acid-urea fragment DD was demonstrated by measuring inhibition of binding by acid-urea-exposed fragment DD and other fibrinogen and fibrin derivatives (Fig. 3 and Table I). Approximately 320 ng/ml of fragment DD inhibited 50% of binding. As little as 10 ng/ml clearly inhibited the binding, and practical range of measurement (15%–85% inhibition) was between 20 and 3000 ng/ml, corresponding to 0.1 and 16 nM, respectively. Unmodified fragment DD, purified by ion-exchange chromatography on DEAE-cellulose but not exposed to acid or urea, had a low inhibitory effect (40% at 1 mg/ml) in contrast to the treated counterpart (Table I). The (DD)E complex was even less reactive, with only 29% inhibition at a concentration of 1 mg/ml.

Fibrinogen and fragment D from fibrinogen or noncrosslinked fibrin, all exposed to 3 M urea at pH 5.5, induced 50% inhibition at $1.1 \times 10^6$, $1.3 \times 10^4$, and $2.5 \times 10^3$ ng/ml, respectively (Table I). Although these were significantly greater inhibitory effects than noted for unmodified fragments, inhibition was still substantially less (about 2000-, 100-, and 20-fold lower, respectively) than that observed with acid-urea fragment DD. This comparison suggests that the factor-XIII-induced crosslink bonds retained in fragment DD may contribute to distinct antigenic structures that were recognized by the antiserum against acid-urea fragment DD.

Fragment D and other unmodified fibrinogen degradation products were
compared with acid-urea fragment DD for inhibitory effect (Fig. 3), and as expected from the information in Table 1, acid-urea fragment DD was a more effective inhibitor than any of the unmodified degradation products.

DISCUSSION

It would be expected that factor-XIII-induced isopeptide bonds\textsuperscript{12,13,15,16} result in unique structural regions that are lacking in fibrinogen, its degradation products, and in degradation products derived from noncrosslinked fibrin. Studies on the polypeptide chain structure of fragments D\textsuperscript{26} and DD\textsuperscript{15,16} indicate a virtual identity of the $\alpha$ (13,000 mol wt) and $\beta$ (43,000 mol wt) chain remnants. The difference between these fragments derives from differences in their $\gamma$-chain remnants, which are dimerized in fragment DD by the $\epsilon(\gamma$-glutamyl)$\gamma$lysine isopeptide bonds. These bonds could contribute directly or indirectly to the formation of antigenic markers that can be recognized by the antibodies against either the intact fragment-DD molecule (Fig. 2) or the derivative exposed to acid urea (Fig. 3). In this work, it has been demonstrated that such markers can be distinguished by RIA (Fig. 1) using antibodies evoked in chickens and rabbits against the (DD)E complex from a plasmic digest of crosslinked human fibrin and against acid-urea-treated fragment DD.

The fragment-DD antigenic markers may result from biochemical changes in much the same way as suggested by Plow and colleagues\textsuperscript{3} for neoantigenic expressions of fibrinogen derivatives. An analogous reasoning applied in this work resulted in a conclusion that antibodies were elicited to fragment-DD antigenic markers on the unique crosslinked fibrin derivative. The primary data source for our conclusions was the quantitative differences in the antisera affinity for fragment DD in comparison with fibrinogen and plasmic degradation products of fibrinogen and noncrosslinked fibrin (Table 1). Using absorbed antiserum against either intact or acid-urea-treated derivatives of crosslinked human fibrin, there was a 50-fold and a 20-fold molar difference, respectively, between fragment DD and fragments D from either fibrinogen or noncrosslinked fibrin. Considered in the context of the total antigenicity of these molecules, the factor-XIII-related markers account for only a fraction of the total. This is reflected by the absence of immunoprecipitation in agarose gel using absorbed antisera and by the great decrease in titer of absorbed antisera in the RIA systems, as compared with unabsorbed antisera.

A comparison of fragments DD and D in two RIA systems illustrates similar differential reactivity, although the overall sensitivity was higher using the antigen exposed to acid urea (Fig. 4). Approximately 97- and 50-fold greater molar concentrations of fragment D than fragment DD were required to produce 50% inhibition in the RIA using, respectively, the antigen exposed to acid urea and the unmodified antigen. Of interest, but without obvious explanation as yet, was the observation that acid-urea fragment D from noncrosslinked fibrin more closely approximated the fragment-DD reactivity in the acid-urea system than when the unmodified assay was utilized. Recognition by antisera of proteins exposed to acid urea was more efficient than recognition of antigenic sites on intact molecules, with a difference in sensitivity of 30-fold. The markers on acid-urea fragment DD could well reflect the unfolding of the molecule to expose structures that are located in the
Fig. 4. The comparison of two RIA systems for the determination of fragment DD in the presence of fragments D from fibrinogen-D (stage 3) and noncrosslinked fibrin-D (nXL). The closed symbols indicate fragment concentration necessary to inhibit 50% of the binding. On the left side are data obtained with absorbed chicken antiserum against acid-urea fragment DD and 125I-labeled acid-urea fragment DD. On the right side are results from absorbed rabbit antiserum against the (DD)E complex and 125I-labeled unmodified fragment DD. The type of the antiserum is indicated at the bottom. The symbol "m" indicates a fragment exposed to 3 M urea at pH 5.5.
interior of fibrinogen and its derivatives. It is also likely that such acid-urea modifications of the molecules destroys other antigenic markers, but a differentiation between modified fragments DD and D can still be demonstrated (Table 1, Fig. 4).

When tested in the acid-urea RIA system, unmodified fragment DD showed much lower inhibition than that of acid-urea fragment DD (Table 1). The (DD)E complex was even less reactive. This observation suggested that the structural region encompassing crosslink bonds may not necessarily be fully exposed in the intact fragment-DD moiety.

It was our hope that these RIA systems could be applied to clinical situations, such as the distinction between disseminated intravascular coagulation and primary fibrinogenolysis, since circulating fragment DD should be present in the former conditions but not in the latter. However, such application would require a much higher difference in reactivity between fragments DD and D, since this and other relevant clinical states have high concentrations of circulating fibrinogen derivatives in addition to the crosslinked fibrin fragments. Although we have shown that the assays could be performed even in the presence of enormous concentrations of fibrinogen, further development of the antibody specificity is needed before it can be reliably applied to the relevant clinical situations.

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