Development of a Radioimmunoassay for the Fibrinogen-Derived Peptide Bβ1-42

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The peptide Bβ1-42 is the initial cleavage product of plasmin-mediated proteolysis of the NH2-terminal region of fibrinogen or fibrin I, while β15-42 is the major fragment released by plasmin degradation of fibrin II. Numerous studies have described the measurement of plasma Bβ1-42 levels as an index of plasmin activity. Previous assays were indirect and included quantitation of thrombin-increasable fibrinopeptide B immunoreactivity (TIFPB) or measurement of fibrinogen and its subsequent proteolysis by thrombin. The studies have described the determinant of thrombosis. Specific and quantitative analyses directed against B91-42 which does not cross-react with B/91-42. We report on a new antiserum (R142) of 5-42 with an antiserum (132) which cross-reacted with B91-42. To circumvent this problem, we have developed an antiserum (R142) which, like antiserum 132, cross-reacts with Bβ1-42. However, Bβ1-42/β15-42 immunoreactivity with R142 is stable in the presence of carboxypeptidase activity. Thus, these studies confirm the utility of measurement of plasma Bβ1-42 as an index of plasmin activity. Further, in vitro studies of Bβ1-42 generation by recombinant tissue-plasminogen activator suggest that radioimmunoassays for the plasmin-derived fibrinopeptides may provide a method for investigating the fibrinogenolytic activity of thrombolytic agents.

Unlike the Aα and γ-chains, the NH2-terminal region of the Bβ-chain of fibrinogen contains cleavage sites for both thrombin (Bβ14-15 bond) and plasmin (Bβ42-43 bond). The measurement of the products released by these cleavages, fibrinopeptidase-B and Bβ1-42, respectively, reflects the balance between thrombin and plasmin action on fibrin I. Methods to detect circulating levels of fibrinopeptide B have been described, but to date only indirect methods to measure Bβ1-42 have been available.

In this study, several β-chain fragments from the NH2-terminal region of human fibrinogen were purified. The peptides Bβ1-42 and β15-42 were selected as immunogens because they are most characteristic of the plasmin-derived fragments from this region. Each peptide was coupled to bovine serum albumin by carbonyldiimidazole, and polyclonal antibodies were produced in rabbits. These antisera were then characterized using radioimmunoassay techniques and their specificity explored using a panel of purified human β-chain fragments. Radioimmunoassays employing these antisera provide useful tools for in vitro and in vivo study of plasmin-mediated proteolysis of fibrinogen and fibrin.

MATERIALS AND METHODS

Preparation of Fibrinogen and Plasmin

Human fibrinogen (Grade L, Kabi, Sweden) was purified as previously described. Plasminogen was prepared from fresh frozen plasma by affinity chromatography, further purified by ion-exchange chromatography, and activated with insolubilized urokinase as described previously.

Purification of Immunogens

N-DSK was prepared by digesting human fibrinogen (Grade I, Kabi) with cyanogen bromide followed by gel filtration over a G-100
As described previously, the radioimmunoassay for Bf1-42 was gel filtered over a Sephadex G-50 superfine (Pharmacia) column (2.5 x 150 cm) in 10% acetic acid. The region containing Bf1-42 was identified using high performance liquid chromatography (HPLC) and its composition confirmed by amino acid analysis. The Bf1-42 pool was lyophilized and further purified using semipreparative high performance liquid chromatography, as described elsewhere.

Purification of β-chain Fragments

Bf1-118 was prepared as previously described. Bf22-42 was purified by exhaustive plasmin proteolysis of purified Bf1-42. The Bf1-42 was dissolved in Tris-buffered saline, pH 7.4, and digested with human plasmin (1 CU/ml Bf1-42) for 3 hours at 37 °C. The reaction was stopped by the addition of acetic acid (final concentration 10%) and the digest was gel filtered over a Sephadex G-50 superfine (Pharmacia) column (2.5 x 150 cm) in 10% acetic acid. The region containing Bf1-42 was identified using high performance liquid chromatography (HPLC) and its composition confirmed by amino acid analysis. The Bf1-42 pool was lyophilized and further purified using semipreparative high performance liquid chromatography, as described elsewhere.

High Performance Liquid Chromatography (HPLC)

Analytical and semipreparative HPLC were performed using a Waters Associates liquid chromatograph equipped with a Model 660 solvent programmer, two Model 6000A solvent delivery systems, a Waters automatic injector Model 710A, and a Waters data module. Peptides were monitored using a Model 450 variable wave length absorbance detector set at 220 nm. The column employed for all analyses was a µ-Bondapak C18 (3.9 mm i.d. x 30 cm) from Waters Associates, which was maintained at 37 °C using a water bath and thermal jacket. Peptide samples were eluted from the µ-C18 column either isocratically or using gradients containing various concentrations of acetonitrile in the mobile phase. Buffers used in the chromatography included 0.1% phosphoric acid (HPLC grade, Fisher Scientific Co, Fair Lawn, NJ), pH 2.1 (buffer A), and 0.1% phosphoric acid containing 50% acetonitrile, pH 2.1 (buffer B). Milligram quantities of Bf1-42 and Bf15-42 were purified using a larger, semipreparative µ-Bondapak C18 column (7.8 mm i.d. x 30 cm) also from Waters Associates. The peptides were eluted with acetonitrile gradients produced by a buffer system prepared with 0.1% Trifluoroacetic acid (Pierce Chemical Co, Rockford, Ill), pH 2.1 (buffer A), and 0.1% Trifluoroacetic acid containing 50% acetonitrile, pH 2.1 (buffer B) and were detected at 230 nm. All buffers were prefiltered through a preparative µ-Bondapak C18 Porasil B column (7.8 mm i.d. x 61 cm) from Waters Associates and degassed prior to use.

Amino Acid Analysis

Fractions to be analyzed were hydrolyzed in 6N HCl in vacuo for 24 hours at 110 °C and analyzed using standard techniques on a Beckman Model 121 MB amino acid analyzer.

Immunization

Purified Bf1-42 and Bf15-42 were conjugated to 2 x crystallized bovine serum albumin (Sigma Chemical Co, St. Louis, Mo) using N-N-carboxylydiamide (Pierce). In a 5 ml reaction vial, 3 mg of peptide was suspended in 3 ml of anhydrous dimethylformamide (Pierce) containing 10 mg carbonylimidazole and the mixture was bubbled with nitrogen for 15 minutes. Five mg of BSA was added and the reaction was continued with stirring and nitrogen bubbling for 30 minutes at 23 °C. The reaction mixture was then desalted on a Sephadex G-25 (Pharmacia) column (2.5 x 40 cm) in 10% acetic acid and lyophilized. The immunogens were resuspended in phosphate buffer saline (0.9% NaCl containing 0.01 mol/L phosphate, pH 7.4) to a concentration of 2 mg/ml and vortexed thoroughly with 3 parts Freund’s Complete Adjuvant (Calbiochem Behring Corp, La Jolla, Calif) until a thick emulsion formed.

White male New Zealand rabbits were immunized with 200 µg of this conjugated peptide using the multiple intraderal injection technique. Ten weeks after the primary injection, each rabbit was boosted with 100 µg of the immunogen given intramuscularly into the haunches. Blood was collected at 8 weeks and every 2 weeks thereafter. To detect an immune response, Bf1-42 and Bf15-42 were radioiodinated at residue Bf341 (tyrosine) with 125I (Amersham Corp, Arlington Heights, Ill) to a specific activity of 30-50 µCi/µg by the chloramine-T method, and specific binding of these tracers was determined.

Radioimmunoassays

TIFPB, fibrinopeptide A (FPA), and fibrinopeptide B were assayed as previously described. For assay of Bf1-42 or Bf15-42, dilutions of standard, test samples, antiserum, and tracer were made in 0.01 mol/L phosphate buffered saline, pH 7.4, with 1% (v/v) normal rabbit serum, 0.1 mg/ml lysosome (Worthington), and 350 K1 units/ml aprotinin (Mobay Chemical Corp, New York, NY). Each assay tube contained 650 µL final volume consisting of the following, in order of addition: 500 µL of standard concentration of peptide to be measured or a dilution of test sample, 100 µL of antiserum (sufficient to bind 25% to 35% of the total bindable counts), and 50 µL of tracer (about 10,000 cpm). After 24 hours incubation at 4 °C, tracer bound to antibody was separated by a second antibody technique. Sheep antirabbit IgG, in quantities capable of binding all of the rabbit IgG, and diluted in 0.01 mol/L phosphate-buffered saline, pH 7.4, supplemented with 20 mg/ml polyethylene glycol 6,000 (Fisher Scientific Co, Springfield, NJ) was added. The tubes were then incubated for 30 minutes at 23 °C, diluted with 2 ml of 0.9% NaCl and centrifuged at 4,000 g for 20 minutes at 4 °C. The supernatant fluid was aspirated and the pellet was counted for 1 minute on a Rackgamma counter (#1270, LKB Instruments, Rockville, Md).

Kinetic Studies

Because of the specificity of the Bf1-42 antiserum (see below), kinetic data describing plasmin-mediated cleavage of the Bf42-43 bond of fibrinogen and subsequent thrombin proteolysis of the Bf14-15 bond of Bf1-42 could then be estimated. Fibrinogen (0.1 to 12 µmol/L) was digested with plasmin (0.1 CU/mL) at 37 °C and the initial velocity of Bf1-42 cleavage calculated using appearance of Bf1-42 immunoreactivity during the first 5 minutes of the reaction. Less than 10% of total substrate was digested during this
time. Purified Bβ1-42 (1.0 to 100 μmol/L) was then digested with thrombin (1.0 U/mL) at 37 °C and initial velocity calculated by measuring disappearance of Bβ1-42 immunoreactivity during the first 5 minutes of the reaction. Again, less than 10% of the substrate had been consumed during this time.

**Blood Collection and Processing**

Blood from normal individuals was donated by laboratory personnel and house officers who were in apparent good health. Samples were collected from an antecubital vein using a 21-gauge butterfly needle. For Bβ1-42 and TIFPB measurements, 4.5 mL blood was collected into a plastic syringe and transferred into a plastic tube containing 0.5 ml of anticoagulant solution consisting of 1,000 U/ml Trasylol, 1,400 U/ml heparin, 0.01 mol/L adenosine, and 0.02 mol/L theophylline in HEPES-buffered saline, pH 7.4. For in vitro recovery experiments, blood was collected into a plastic tube containing 0.5 ml of anticoagulant employed. The reaction was terminated by adding 10 μL of a stock solution of 1.25 x 10^2 and 1.25 x 10^3 units/pmol of Bβ1-42 were incubated at 23 °C for 60 minutes with a final carboxypeptidase B concentration of 1.0 x 10^{-4} units/pmol of Bβ1-42. Under the same conditions the experiment was repeated with a carboxypeptidase B concentration of 1.0 x 10^{-3} units/pmol of Bβ1-42.

**Generation of Bβ1-42 in Vitro**

Recombinant human tissue plasminogen activator (rt-PA; Genentech, San Francisco, Calif) diluted in Tris-buffered saline, pH 7.5, with 0.1 mg/mL Tween 20 (Fisher), was incubated at 37 °C with 2 mL citrated plasma. At intervals, aliquots were removed, aprotinin (100 U/mL) was added, and the samples were precipitated with 50% ethanol, processed as described above and assayed for Bβ1-42 (using antiserum R142) and for FPA. The experiment was repeated first using citrated plasma that had been preincubated with 0.1 U/mL of a purified fraction of reptilase (a gift of Dr B. Blomback) for 10 minutes at 23 °C before the addition of rt-PA, and then with citrated plasma to which reptilase and rt-PA were added simultaneously. The reptilase produced a visible clot within 90 seconds.

**RESULTS**

**Characterization of the Antisera**

Purified Bβ1-42 and β15-42 were conjugated to bovine serum albumin and used as immunogens in rabbits. Two of four rabbits receiving the Bβ1-42 immunogen responded. One antiserum, designated R142, showed the highest titer and was employed in this study. Fig 1A shows the reactivity of this antiserum with the various β-chain competitors. There was complete cross-reactivity between Bβ1-42 and Bβ21 while FPB, β15-42, and the other β-chain competitors (β12-42, β12-19, β22-42) did not react.

In contrast, although eight rabbits received the β15-42 immunogen, all produced low titer antisera after repeated booster immunizations and four died of unknown causes during a four-month period. Only one antiserum, designated R154, exhibited a sufficient titer to be employed in a radioimmunoassay. Competitive binding studies using this antiserum and 125I-β15-42 as tracer are shown in Fig 1B. There was complete cross-reactivity among Bβ1-42, Bβ15-42, and β22-42 while FPB and Bβ21 exhibited little reactivity.

**Plasmin Cleavage of the β-chain of Fibrinogen**

In a previous report the sequence of plasmin cleavage of the NH²-terminal region of the Bβ-chain of fibrinogen was studied using HPLC methods. Because of the specificity of
antiserum R142, these results could now be confirmed using radioimmunoassay, as well as HPLC techniques, to monitor Bβ1-42 release. Immunoreactivity, as measured using antiserum R142, reached a maximum between 40 and 60 minutes reflecting quantitative peptide release. HPLC was employed to separate and quantify the β-chain fragments. Bβ1-42 levels, as measured using HPLC, increased in parallel with immunoreactivity during the early stages of the digestion, reached a maximum, and then began to decline. As reported previously,13 increasing levels of Bβ1-21 were seen following the initial Bβ1-42 release. The radioimmunoassay utilizing antiserum R142, which cross-reacts completely with Bβ1-42 and Bβ1-21, measured both of these peptides.

### Kinetic Studies

Figure 2 illustrates thrombin proteolysis of purified Bβ1-42. The disappearance of Bβ1-42 immunoreactivity, measured using antiserum R142, was mirrored by the subsequent appearance of FPB immunoreactivity quantitated with R22 antiserum. HPLC measurements of these two peptides were in good agreement with the immunochemical data. The estimated kinetic constants for plasmin-mediated cleavage of the Bβ2-43 bond of the intact fibrinogen molecule and thrombin proteolysis of the Bβ14-15 bond of Bβ1-42 are listed in Table 1.

### Recovery of Bβ1-42 in Blood and Plasma

The recovery of various concentrations of Bβ1-42 (50, 25, 12.5, 6.3, and 3.2 pmol/mL) added to blood and measured directly (employing antiserum R142) was compared with TIFPB determination. The Bβ1-42 assay gave a mean recovery of 114% as compared to 83% with the TIFPB assay.

### Similar Results

Similar results were obtained when Bβ1-42 was added to plasma with a mean recovery of 107% when quantitated as Bβ1-42 and 74% when measured as TIFPB. A comparison of the Bβ1-42 and TIFPB levels in 37 stored patient samples is illustrated in Fig 3. The coefficient of covariance is 0.91.

### Bβ1-42 Concentration in Normals

The median Bβ1-42 level in 15 normal individuals was 1.2 pmol/mL with a 10% to 90% range of 1.1 to 1.7 pmol/mL. This correlates favorably with an estimated normal range for TIFPB of 1 to 4 pmol/mL.11 In contrast, employing antiserum 132, which does not distinguish between Bβ1-42 and Bβ15-42, normal levels of 0.4 pmol/mL were reported.12 To explore this discrepancy, the stability of Bβ1-42 immunoreactivity in plasma was studied.

### Stability of Bβ1-42 Immunoreactivity

Bβ1-42 immunoreactivity was stable in plasma over a 60-minute incubation at 23°C when measured with antiserum R154 (Fig 4A). In contrast, when antiserum 132 was employed, more than 50% immunoreactivity was lost. Since previous studies had shown loss of fibrinopeptide B immunoreactivity in blood due to cleavage of arginine (Bβ14) by carboxypeptidase-like activity,22 the effect of porcine pancreas carboxypeptidase B on Bβ1-42 immunoreactivity was determined in a buffer system. Treatment of Bβ1-42 with carboxypeptidase B resulted in a loss of immu-

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### Table 1. Comparison of Kinetic Constants

<table>
<thead>
<tr>
<th>Bond hydrolyzed (substrate)</th>
<th>$K_m$ (M)</th>
<th>$K_{m} \times 10^{10}$ [CU/Ls]$^{-1}$</th>
<th>$K_{m}/K_{m} \times 10^{6}$ [IU/Ls]$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrolysis by plasmin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bβ42-43 (human fibrinogen)</td>
<td>0.7</td>
<td>4.0</td>
<td>571</td>
</tr>
<tr>
<td>Bβ21-22 (Bβ1-42)</td>
<td>18</td>
<td>2.0</td>
<td>11</td>
</tr>
<tr>
<td>Bβ14-15 (Bβ1-21)</td>
<td>28</td>
<td>1.2</td>
<td>4</td>
</tr>
<tr>
<td>Hydrolysis by thrombin</td>
<td>$K_m$ (M)</td>
<td>$K_{m} \times 10^{10}$ [NIH units/Ls]$^{-1}$</td>
<td>$K_{m}/K_{m} \times 10^{6}$ [NIH units/Ls]$^{-1}$</td>
</tr>
<tr>
<td>Bond hydrolyzed (substrate)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bβ14-15 (bovine fibrinogen)</td>
<td>11.3</td>
<td>115</td>
<td>1020</td>
</tr>
<tr>
<td>Bβ14-15 (human fibrinogen)</td>
<td>16.0</td>
<td>165</td>
<td>1050</td>
</tr>
<tr>
<td>Bβ14-15 (Bβ1-42)</td>
<td>28</td>
<td>0.004</td>
<td>0.01</td>
</tr>
</tbody>
</table>
Fig 4. (A) Stability of Bβ1-42 immunoreactivity in plasma: Bβ1-42 (30 pmol/mL) was incubated in plasma at 23 °C. At intervals aliquots were removed, ethanol was added, and Bβ1-42 immunoreactivity was measured with antisera R142 (○), R154 (△), and 132 (□). (B) The effect of carboxypeptidase B on Bβ1-42 immunoreactivity with antiseraum 132: Bβ1-42 (20 pmol/mL) was incubated in buffer, pH 7.4, at 23 °C without carboxypeptidase (○) or with 1.25 x 10^-4 units/pmol (△) or 1.25 x 10^-3 units/pmol (□) carboxypeptidase B. At intervals, aliquots were removed, o-phenanthroline (0.01 mol/L) was added, and residual immunoreactivity measured.

Immunoreactivity similar to that occurring in plasma when the Bβ1-42 was measured with antisera 132 (Fig 4B). There was no loss of immunoreactivity when antisera R142 or R154 were employed. To determine whether this finding was a result of cleavage of the COOH-terminal arginine (Bβ42), native Bβ1-42 was treated with carboxypeptidase B and the digestion monitored by high performance liquid chromatography. Following 30 minutes of carboxypeptidase treatment (1.0 x 10^-4 units/pmol Bβ1-42), two major peaks were identified and the corresponding peptides characterized by their amino acid content. One represented residual Bβ1-42 while the other (Table 2) showed loss of an arginine residue and thus was identified as Bβ1-41. Under the same conditions but with a higher carboxypeptidase B concentration, two peptide peaks were again seen. The major peak gave the amino acid analysis shown in Table 2, with loss of an arginine and tyrosine residue and thus was identified as Bβ1-40. The other peak represented a smaller peptide but amino acid analysis was not performed.

The immunoreactivity of the carboxypeptidase B digestion products was studied using antisera R142 and 132. With antisera R142 there was complete cross-reactivity between Bβ1-42 and the carboxypeptidase digestion products (Fig 5A). In contrast, when antisera 132 was employed, Bβ1-41 and Bβ1-40 exhibited a two-fold loss of immunoreactivity as compared to Bβ1-42 (Fig 5B).

Immunochemic Analysis of Plasmin Proteolysis of Fibrinogen

Plasmin cleavage of the Bβ42-43 bond of fibrinogen and subsequent thrombin proteolysis of the Bβ14-15 bond of Bβ1-42 were assessed immunochemically using radioimmu-

Table 2. Amino Acid Analysis of Bβ1-42 After Carboxypeptidase B Digestion

<table>
<thead>
<tr>
<th></th>
<th>Bβ1-42 From Sequence Data</th>
<th>Bβ1-42 Before CPB Treatment</th>
<th>CPB-Treated Bβ1-42 After HPLC Separation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CPB Treatment</td>
<td>1.25 x 10^-4 U/pmol</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>4</td>
<td>4.2</td>
<td>4.2</td>
</tr>
<tr>
<td>Glutamic acid*</td>
<td>5</td>
<td>5.1</td>
<td>5.3</td>
</tr>
<tr>
<td>Proline</td>
<td>6</td>
<td>5.8</td>
<td>6.3</td>
</tr>
<tr>
<td>Glycine</td>
<td>6</td>
<td>5.6</td>
<td>6.3</td>
</tr>
<tr>
<td>Serine</td>
<td>3</td>
<td>2.8</td>
<td>2.9</td>
</tr>
<tr>
<td>Alanine</td>
<td>3</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td>Valine</td>
<td>1</td>
<td>1.1</td>
<td>1.0</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>1</td>
<td>1.1</td>
<td>1.0</td>
</tr>
<tr>
<td>Leucine</td>
<td>2</td>
<td>2.2</td>
<td>2.1</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>2</td>
<td>2.0</td>
<td>2.1</td>
</tr>
<tr>
<td>Histidine</td>
<td>1</td>
<td>1.0</td>
<td>1.1</td>
</tr>
<tr>
<td>Lysine</td>
<td>2</td>
<td>2.1</td>
<td>1.8</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>1</td>
<td>0.9</td>
<td>0.8</td>
</tr>
<tr>
<td>Arginine</td>
<td>5</td>
<td>4.9</td>
<td>4.1</td>
</tr>
</tbody>
</table>

CPB, carboxypeptidase B; HPLC, high performance liquid chromatography.

*One glutamic acid residue represents a pyroglutamic acid residue.
no assays employing antisera R142 and R154 (Fig 6). Proteolysis of fibrinogen by urokinase-activated plasminogen resulted in quantitative release of B31-42 after 60 minutes when either assay was employed. The addition of thrombin to the reaction mixture at this point produced no change in immunoreactivity when determined with antiserum R154 (Fig 6A), whereas progressive loss of immunoreactivity occurred when antiserum R142 was utilized, reflecting thrombin cleavage of B31-42 to FPB and B315-42 (Fig 6B).

Generation of B31-42 in Vitro

When rt-PA was incubated with citrated human plasma, there was progressive generation of B31-42 immunoreactivity (Fig 7A) with concentrations of rt-PA of 50 ng/mL plasma or higher. However, even with the highest concentration of rt-PA employed, this represented <1% of total releasable B31-42 (plasma fibrinogen concentration was 7 μmol/L). The plasma FPA concentration of 0.5 pmol/mL did not change throughout the incubation period. The experiment was then performed in plasma pretreated with reptilase (Fig 7B). Here fibrin I, rather than fibrinogen, was the substrate for plasmin action as was confirmed by demonstrating quantitative FPA release within 5 minutes of reptilase addition. Reptilase alone produced no significant B31-42 cleavage but the addition of rt-PA at concentrations of 0.5 and 5 ng/mL, which resulted in no B31-42 release in citrated plasma, produced slow generation of B31-42 for 20 minutes followed by rapid B31-42 release which was still increasing at 60 minutes. With 5 ng rt-PA/mL, less B31-42 was released, but it was still more than that generated by 375 ng/mL in citrated plasma. To determine whether the 20-minute period of slow B31-42 cleavage was due to mechanical limitation of rt-PA access to the fibrin I clot, a subsequent experiment was done in which rt-PA and reptilase were added to plasma simultaneously. This yielded results similar to those shown in Fig 7B.

DISCUSSION

Since there is much evidence suggesting that the balance between thrombin and plasmin proteolysis of fibrinogen is an important determinant of normal hemostasis and thrombosis,1 the development of radioimmunoassays for measurement of specific plasmin-derived fragments from the NH2-terminal region of the B3-chain of fibrinogen permits a direct and quantitative approach to explore this hypothesis. Previous studies of plasmin proteolysis of this region indicated a sequence of events in which B31-42 was the initial cleavage product. Once released from the parent fibrinogen molecule, B31-42 underwent secondary plasmin attack but the kinetic data indicated that these latter cleavages were unlikely to be physiologically important. Whereas B31-42 was the principal species released from fibrinogen or fibrin I by plasmin action, B315-42 was the major fragment cleaved by plasmin proteolysis of fibrin II.123 With the concept that determination of B31-42 levels would reflect plasmin action on fibrinogen or fibrin I while B315-42 levels would reflect proteolysis of fibrin II, we set out to develop specific radioimmunoassays to quantitate these peptides.

Antiserum R142, produced by immunizing with B31-42, has an almost absolute requirement for the presence of an
intact Bβ14-15 bond so that peptides lacking this bond are very poor competitors. This is illustrated in Fig 2, where thrombin cleavage of the Bβ14-15 bond of the Bβ1-42 molecule results in progressive loss of Bβ1-42 immunoreactivity which is mirrored by an increase in FPB immunoreactivity. These results are confirmed by HPLC quantitation of the peptides. Thus, the radioimmunoassays for Bβ1-42 and FPB provide an index of plasmin and thrombin activity, respectively, on the NH2-terminal region of the Bβ-chain of fibrinogen. Recently, Kudryk et al24 have reported on the preparation and use of a monoclonal antibody to the N-DSK fragment of human fibrinogen that may have an epitope in or around the thrombin-susceptible Bβ14-15 bond such that its specificity appears to be similar to that of antiserum R142. That Bβ1-42 quantitated in the assay employing antiserum R142 provides a good index of fragment X formation was recently confirmed by a study correlating plasmin-mediated Bβ1-42 cleavage from fibrinogen with peptides released from the COOH-terminal domain of the Aα chain. The loss of Aα-chain segments 540–554 and 259–276 coincided with proteolysis of Bβ1-42 measured with this assay. These data indicate that, during fragment X formation, there is simultaneous plasmin-mediated cleavage of the COOH-terminal Aα-chain and NH2-terminal region of the Bβ-chain of fibrinogen.25

Because of the unique specificity of the radioimmunoassay employing antiserum R142, the kinetic data describing thrombin and plasmin proteolysis of Bβ1-42 could be estimated. The stability of Bβ1-42 in the presence of plasmin or thrombin activity in the blood can be explained by these data, which are summarized in Table 1. The Bβ242-43 bond of fibrinogen is the preferential plasmin cleavage site on the NH2-terminal region of Bβ-chain31,32 with the resultant release of Bβ1-42. Once released, this peptide is a relatively poor substrate for subsequent thrombin or plasmin attack. The selectivity constant (Kse = Kcat/Km) for plasmin cleavage of the Bβ21-22 bond of Bβ1-42 is 140-fold less than that for proteolysis of the Bβ42-43 bond of fibrinogen, suggesting that this hydrolysis is unlikely to be of physiologic importance. Similarly, the selectivity constant for thrombin cleavage of the Bβ314-15 bond of Bβ1-42 is 102-fold less than that for proteolysis of the same bond in the intact fibrinogen molecule.

In contrast to the unique specificity of antiserum R142, antiserum R154, obtained by immunizing with β15-42, cross-reacts with several peptides derived from the NH2-terminal region of the Bβ-chain (Fig 1B). Almost no competition was seen using Bβ1-21, while parallel curves were generated with fragments Bβ1-42, Bβ22-42, and β15-42, suggesting that the epitope for this antiserum is nearer to the COOH-terminal region of the peptide. Thus, its specificity is similar to that of antiserum 13215 which has been employed in the development of a commercially available radioimmunoassay for quantitating fibrinogen/fibrin fragments containing the β15-42 sequence. Clinical studies utilizing this assay have been reported.26,27

The assay employing antiserum R142 gave consistently better recovery of Bβ1-42 added to blood or plasma than the indirect TIFPB assay. However, a comparison of these two methods for quantitation of Bβ1-42 in patient samples yielded a good correlation over a wide range of levels (Fig 3), indicating that TIFPB determination does indeed reflect Bβ1-42, thus validating the results of previous studies which employed this indirect assay.11

The discrepancy between the plasma levels of Bβ1-42 in normal individuals as measured in the assay employing antiserum R142 and those reported using antiserum 132,12 which does not distinguish between β15-42 and Bβ1-42, is best explained by rapid removal of Arg (Bβ42) by plasma carboxypeptidase. This results in at least a 50% loss of Bβ1-42 immunoreactivity when quantitated with the latter antiserum (Fig 4). The finding that the carboxypeptidase digestion products, Bβ1-41 and Bβ1-40, exhibited two-fold less immunoreactivity than Bβ1-42 with antiserum 132 (Fig 5B), indicates that Arg (Bβ42) is an important determinant of the antigenic site. That removal of Tyr (Bβ41), in addition to Arg (Bβ42), resulted in no further loss of immunoreactivity suggests that conformational changes produced by removal of Arg (Bβ42) may be partly responsible for this loss. Although antiserum R154 also cross-reacted completely with Bβ1-42 and β15-42, Bβ1-42 immunoreactivity with this antiserum was stable in plasma and unaffected by in vitro carboxypeptidase treatment. This indicates that the antigenic determinant, although in the COOH-terminal region, is not dependent on Arg (Bβ42). Finally, since the epitope for antiserum R142 is in the region of the Bβ14-15 bond, immunoreactivity measured with this antiserum was unaffected by COOH-terminal degradation of the Bβ1-42 molecule (Fig 5A).

Radioimmunoassays employing antiserum R142 and R154, when used in combination, provide a method for quantitating Bβ1-42 and β15-42 release from a variety of substrates. As illustrated in Fig 5, quantitative release of Bβ1-42, mediated by plasmin action on fibrinogen, was obtained using either radioimmunoassay. If thrombin was then added, there was rapid loss of Bβ1-42 immunoreactivity when antiserum R142 was utilized (Fig 5B), reflecting the requirements of this antiserum for an intact Bβ14-15 bond. In contrast, there was no loss of immunoreactivity when antiserum R154 was employed (Fig 5A). Therefore, in terms of plasmin proteolysis of the parent fibrinogen molecule, results obtained utilizing antiserum R142 reflect plasmin activity only on a substrate that has an intact Bβ14-15 bond, i.e., fibrinogen or fibrin I. Since antiserum R154 has no such requirement, the result obtained with this assay reflects global plasmin activity regardless of whether the substrate is fibrinogen, fibrin I, or fibrin II.

The in vitro generation of Bβ1-42 was studied by the addition of rt-PA to plasma. The activation of plasminogen by tissue-type plasminogen activator is enhanced in the presence of fibrin.28,30 The clot-selective properties of this agent have made it an attractive candidate for lytic therapy since it can produce clot lysis without depleting circulating fibrinogen, in contrast to the generalized lytic state produced by streptokinase or urokinase. Specific and quantitative assays for peptides released by plasmin proteolysis of the
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NH2-terminal region of fibrinogen and fibrin provide a method for differentiating the fibrinogenolytic effects of rt-PA from its fibrinolytic activity.

That the Bβ1-42 level is a sensitive index of plasmin-mediated proteolysis of fibrinogen is illustrated by progressive Bβ1-42 generation, without change in the FPA level, on addition of rt-PA to citrated plasma (Fig 7A). With the highest concentration of rt-PA employed there was cleavage of <1% of the total Bβ1-42 content from the parent fibrinogen molecule, consistent with the clot-selective properties of this agent. However, the finding that Bβ1-42 release was dose-dependent suggests that high plasma concentrations of rt-PA may produce significant fibrinogenolysis. In fact, this has been the reported experience with the administration of rt-PA for the treatment of venous thrombosis.31 That low doses of rt-PA (0.5 and 5 ng/mL plasma) did not generate Bβ1-42 may be due to the presence of plasma inhibitors to t-PA and/or plasmin. The enhanced plasminogen activation by rt-PA in the presence of fibrin I is demonstrated by the significant Bβ1-42 release in reptilase-treated plasma with concentrations of rt-PA which had no effect in citrated plasma (Fig 7B). The period of slow Bβ1-42 release over the initial 20 minutes is unlikely due to mechanical limitation of rt-PA access into the fibrin clot since the simultaneous addition of rt-PA and reptilase produced similar results. A possible explanation relates to the finding that tissue plasminogen activator can induce plasminogen binding to fibrin I by catalyzing plasmin-mediated modification of the fibrin substrate and exposing new binding sites for Glu-plasminogen.34

In summary then, radioimmunoassays have been developed that can quantitate the products of plasmin cleavage of the NH2-terminal region of the Bβ-chain of fibrinogen. The kinetic studies employing the specific Bβ1-42 assay explain the stability of Bβ1-42 in plasma, thus confirming the utility of measurement of this peptide. Because of carboxypeptidase-mediated degradation, the specificity of antisera to Bβ1-42 must be extensively explored prior to development of clinical assays for this peptide. Antiserum R154, because of its cross-reactivity with the carboxypeptidase-mediated degradation products, may prove useful in the development of a radioimmunoassay for global plasmin activity. Assays employing these antisera provide a direct and quantitative method to further explore the hypothesis that the balance between thrombin and plasmin proteolysis of fibrinogen is an important determinant of normal hemostasis and thrombosis. Further, they may prove useful in monitoring patients undergoing thrombolytic therapy.

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REFERENCES

Development of a radioimmunoassay for the fibrinogen-derived peptide B beta 1-42

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