Immunochemical Studies of Antisera to Human Fibrinopeptide-B
By Vincent P. Butler, Jr., Dominique A. Weber, Hymie L. Nossel, Doris Tse-Eng, Kalliope S. LaGamma, and Robert E. Canfield

The initial steps in the conversion of human fibrinogen to fibrin are the successive releases by thrombin of the NH₂-terminal peptides of the Aα- and Bβ-chains of fibrinogen. A radioimmunoassay (RIA) for fibrinopeptide-A (FPA; Aα1[Gly]-16[Arg]) has been developed and has been used for measuring FPA concentrations in clinical blood samples. An RIA for fibrinopeptide-B (FPB; Bβ1[Pyr]-42[Arg]) has also been developed. However, unlike the immunologic measurement of FPA, which can be readily and specifically quantified in properly processed plasma samples, the specific immunologic measurement of FPB in clinical blood samples has been more difficult. To date, two causes for the difficulty in specific FPB quantification have been identified. First, larger FPB-containing peptides (e.g., Bβ1[Pyr]-42[Arg], produced by the action of plasmin on fibrinogen) have been detected in plasma samples; such peptides cross-react with anti-FPB serum, thereby contributing to immunoactive plasma FPB concentrations. Second, it has been established that carboxypeptidase-B in human plasma cleaves the COOH-terminal arginine from FPB in vitro, the resulting Bβ1(Pyr)-13(Ala) peptide, desarginy-FPB, shows markedly decreased reactivity with some anti-FPB sera, and hence, immunoactive plasma FPB concentrations may be lowered by carboxypeptidase-B in vivo and during the plasma processing procedure.

Thus, it is apparent that the accurate immunologic assay of FPB in clinical blood samples would be facilitated by its immunochemical distinction from larger FPB-containing peptides and from desarginyl-FPB. Accordingly, the immunochemical specificities of seven rabbit anti-FPB sera have been studied in detail. Evidence has been obtained that, by the appropriate use of anti-FPB sera, FPB can be distinguished from Bβ1(Pyr)-42[Arg] and from desarginy-FPB, thus enabling the immunochemical detection of these latter two β-chain segments and their distinction from FPB in clinical blood samples.

MATERIALS AND METHODS

Materials

Native human FPB and Bβ1(Pyr)-42[Arg] were gifts from Dr. Birger Blomback of the Coagulation Department, Karolinska Institute, Stockholm, Sweden. Synthetic human FPA and a synthetic FPB analogue, which differed from FPB in that its NH₂-terminal amino acid was glutamic acid rather than pyroglutamic acid, were obtained from the Schwarz/Mann Division, Becton, Dickinson & Co., Orangenburg, N.Y. Desarginy-FPB was prepared by treatment of native FPB with a preparation of porcine pancreatic carboxypeptidase-B (COBC, Worthington Biochemical Corp., Freehold, N.J.).

The synthetic FPB analogue was conjugated to bovine serum albumin (BSA) by the glutaraldehyde method, as previously described. The desaminotyrosyl derivative of the synthetic FPB analogue was prepared by the method of Goodfriend and Ball and radiolabeled with [125I] by the chloramine-T method, as previously described.

Human fibrinogen, grade I, was purchased from A.B. Kabi, Stockholm, and was further purified by DEAE-cellulose chromatography. The NH₂-terminal disulfide knot (N-DSK) was prepared by cyanogen bromide treatment of fibrinogen, followed by gel filtration.
solution on Sephadex G-100 and DEAE-cellulose chromatography, as previously described. Reduced, carboxymethylated Bf1(Pyr)-118(Met) was purified by Sephadex G-50 gel filtration of N-DSK, which had been reduced in 10 M urea and alkylated with 1C-iodoacetic acid; the Bf1(Pyr)-118(Met) was the largest component and eluted from Sephadex G-50 near the void volume. The concentrations of purified peptides in stock solutions were determined by subjecting duplicate aliquots to acid hydrolysis, followed by quantitative amino acid analysis employing norelucine as an internal standard. The presence of the predicted quantities of the FPB sequence in fibrinogen, N-DSK, Bf1(Pyr)-118(Met), and Bf1(Pyr)-42(Arg) was established by treating each of these substances with human thrombin (generously provided by Dr. John H. Fenton II, New York State Department of Health, Albany, N.Y.) and determining by radioimmunoassay that the anticipated molar quantity of FPB was released.

Ovalbumin, 3X-crystallized, was purchased from ICN Life Sciences Group, Cleveland, Ohio. Charcoal was obtained as Norit "A" (neutral) from the Amend Drug & Chemical Co., Irvington, N. J. Hirudin was obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks, England.

Anti-FPB Sera

White New Zealand rabbits were immunized with the BSA-FPB conjugate, 1 mg/ml in complete Freund's adjuvant mixture, according to the following schedule: 0.1 ml in each of 2 front toe pads and 0.2 ml in each of 2 rear toe pads, once weekly for 3 wk, followed thereafter by 0.5 ml intramuscularly every 2 wk. Rabbit R-29 did not receive the initial series of toe pad injections, but rather was initially immunized by injection of a total of 60 µg of immunogen in multiple intradermal sites, followed by 0.5 ml intramuscular injections every 2 wk. A single serum specimen from each animal was selected for characterization in this study. Table 1 indicates the duration of immunization and the number of antigen injections that each animal had received prior to the bleeding used in this study.

Association Constants

The affinity of anti-FPB sera for the 125I-labeled FPB derivative was assessed by equilibrium dialysis of increasing concentrations of the 125I-labeled desaminotyrosyl-FPB derivative (83-5300 pg/ml) in 2-ml volumes against equal volumes of appropriate dilutions, ranging from 1:1,500 to 1:20,000, of the 7-anti-FPB sera studied; all studies were carried out in duplicate in a tris-buffered ovalbumin solution containing 0.1 M NaCl, 0.05 M Tris base adjusted to pH 8.5 with concentrated HCl, and 0.1% ovalbumin. After 42 hr of rotation at 4°C at 3 rpm on a rotator (Bellco Glass Inc., Vineland, N.J.), concentrations of the 125I-labeled tracer inside and outside the dialysis sacs were determined in a gamma spectrometer and average intrinsic association constant (Ks) values were calculated, using methods outlined by Eisen. The relative cross-reactivities of various peptides and proteins with anti-FPB sera were determined by assessing their respective abilities to inhibit the binding of the 125I-labeled FPB derivative by anti-FPB serum, as measured by a charcoal separation method. For these studies, dilutions of anti-FPB sera were selected that were capable of binding 41%-58% of the maximal amount of the FPB derivative bound by an excess of anti-FPB serum under similar assay conditions; maximal FPB binding ranged from 55% to 75% (but was less than 65% in only 2 experiments). All inhibition studies were carried out in pH 8.5 Tris-buffered saline, containing 0.1% ovalbumin as follows: to duplicate 400-µl aliquots of buffer, containing various concentrations of inhibitor, were added 50-µl aliquots of 125I-labeled FPB derivative (0.01 µCi/50 µl), followed by 50 µl of an appropriate dilution (1:40 to 1:2000) of anti-FPB serum, containing hirudin (0.1-0.2 U/ml of diluted antiserum, added to inhibit the action of thrombin in rabbit serum). After incubation for 2 hr at 4°C, 0.5 ml of a 1% suspension of charcoal was added with mixing. The reaction tubes were immediately centrifuged for 10 min at 4°C and 6100 g. A 0.5-ml aliquot of the supernatant solution, containing antibody-bound 125I-labeled FPB derivative, was aspirated and counted in a gamma spectrometer. The inhibitory capacity of individual substances tested was expressed as the number of picomoles of that substance required to produce 50% inhibition (I50) of the binding of the 125I-labeled FPB derivative observed in control tubes containing tracer and antiserum without inhibitor. Relative molar inhibitory capacities of individual substances with reference to FPB were calculated by dividing the I50 of that substance by the I50 of native FPB observed in the same experiment; relative immunoreactivities of individual substances with reference to FPB were expressed as the reciprocals of the corresponding relative molar inhibitory capacities. Inasmuch as fibrinogen and N-DSK each contain two FPB sequences per molecule of the parent dimeric protein, the relative molar inhibitory capacities and relative immunoreactivities of these two proteins were expressed on the basis of FPB content rather than on the basis of the molar concentration of the parent protein molecule.

Analysis of Clinical Blood Samples

After obtaining informed consent from patients according to a protocol approved by the Institutional Review Board, clinical blood samples were collected, processed, and assayed as described by Nossel et al. Antiserum R-22 was used in these assays.

<table>
<thead>
<tr>
<th>Table 1. Properties of Anti-FPB Sera</th>
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<tr>
<td><strong>Rabbit</strong></td>
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<tr>
<td>R-22</td>
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<td>R-23</td>
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<td>R-24</td>
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<td>R-28</td>
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<td>R-29</td>
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<td>R-30</td>
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<td>R-31</td>
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*Relative number of moles required to inhibit binding of 125I-labeled FPB derivative by 50%.
RESULTS

Table 1 provides details concerning the duration of immunization and the number of antigen injections received by each rabbit prior to the bleeding used in this study. Also listed in Table 1 are the average intrinsic association constants for each of the seven antisera employed in this study, as determined by equilibrium dialysis using the $^{125}\text{I}$-labeled desaminotyrosyl-FPB derivative as ligand. In every instance, the average intrinsic association constant was in excess of $10^3$.

The relative abilities of FPB and of larger FPB-containing molecules to inhibit the binding, by antibody, of the $^{125}\text{I}$-labeled FPB derivative were studied

by the charcoal separation method. As seen in Fig. 1A, 0.87 pmole of native FPB produced 50% inhibition of the binding of the $^{125}\text{I}$-labeled FPB derivative by antiserum R-28, but greater quantities of the larger FPB-containing molecules were required to produce comparable inhibition of binding. In this experiment, 18 pmole of fibrinogen, 17 pmole of N-DSK, and 22 pmole of Bβ1(Pyr)-118(Met) were required to produce 50% inhibition of binding. The synthetic FPB analogue was almost as effective an inhibitor as native FPB, while fibrinopeptide-A exerted no inhibitory effect. When antiserum R-30 was employed (Fig. 1B), the difference in inhibitory capacity between FPB and the larger FPB-containing molecules was much less

\[\text{Fig. 1. Effect of various quantities of native FPB, synthetic FPB analog, fibrinogen, N-DSK, Bβ1(Pyr)-118(Met), and FPA on the binding of the }^{125}\text{I}-\text{labeled tyrosyl-FPB analog by (A) a 1:1220 dilution of anti-FPB serum R-28, and (B) by a 1:200 dilution of anti-FPB serum R-30.}\]
than with antiserum R-28. Again, the synthetic FPB analogue was an effective inhibitor, while fibrinopeptide-A was ineffective. In Table 1 and in Fig. 2, the immunoreactivity of various compounds with several anti-FPB sera is compared with that of native FPB. In the cases of six of the seven antisera, fibrinogen, N-DSK, and B31(Pyr)-118(Met) were considerably less effective than FPB in inhibiting the binding of the 125I-labeled FPB derivative. In the case of antiserum R-30, the larger FPB-containing molecules exhibited greater relative cross-reactivity.

Similar differences in the immunoreactivities of individual anti-FPB sera were observed when the inhibitory capacities of two shorter NH2-terminal β-chain peptides were compared with that of FPB. As seen in Fig. 3A, 29 times as much Bβ1(Pyr)-42(Arg) and 145 times as much desarginyl-FPB were required to produce as much inhibition of the binding of radiolabeled FPB by antiserum R-28 as that produced by 0.38 pmoles of native FPB; similarly, Bβ1(Pyr)-42(Arg) and desarginyl-FPB were considerably less effective than FPB in inhibiting the binding of tracer by most of the other anti-FPB sera studied (Table 1; Fig. 2). However, when antiserum R-30 was used, Bβ1(Pyr)-42(Arg) and desarginyl-FPB showed considerably more relative immunoreactivity (Fig. 3B), desarginyl-FPB being almost 5 times more immunoreactive than FPB.

As an example of the clinical application of techniques based on the immunoochemical information obtained in this study, data are presented in Fig. 4 and in Table 2 showing FPB immunoreactivity before and after thrombin treatment of plasma extracts obtained serially from two patients, each of whom had a continuous state of intravascular coagulation associated with congenital cavernous hemangiomas.23 On every occasion on which the plasma extracts were tested, a marked increase in FPB immunoreactivity was produced by thrombin treatment and, in both patients, marked decreases in the thrombin-increasable FPB levels occurred following heparin administration, in association with decreases in plasma FPA and serum fibrinogen degradation product levels and in association with an increase in plasma fibrinogen concentrations.

**DISCUSSION**

All seven rabbits immunized with the albumin-FPB conjugate formed antibodies that bound the 125I-labeled tyrosyl-FPB derivative with high affinity (Table 1). The binding of the radiolabeled FPB derivative by antiserum from each of the seven rabbits was specific for FPB in that this binding was inhibited by picomolar quantities of FPB, but not by nanomolar quantities of FPA.

Anti-FPB sera also reacted with human fibrinogen and with other FPB-containing peptides. Although patterns of cross-reactivity varied (Table 1; Fig. 2), larger FPB-containing proteins and peptides were less effective than FPB in inhibiting the binding of the radiolabeled FPB derivative (Table 1; Figs. 1 and 2), indicating that antigenic determinants of the 14 amino acid FPB sequence are conformationally altered and/or sterically inaccessible in the larger FPB-containing molecules.

An early effect of plasmin on the fibrinogen molecule is the cleavage of the bond linking Arg(Bβ42) and Ala(Bβ43),24 plasmin and leukocyte-derived proteases.
are also capable of cleaving other NH$_2$-terminal segments of the B$\beta$-chain.$^{25-28}$ Since B$\beta$1(Pyr)-42(Arg) and smaller plasmin-cleaved peptides interact with anti-FPB antibodies, such peptides may contribute significantly to FPB immunoreactivity in plasma preparations from which fibrinogen has been removed. However, since B$\beta$1(Pyr)-42(Arg) is 6–29-fold less effective than FPB in inhibiting the binding of the $^{125}$I-labeled FPB derivative by anti-FPB sera (Table 1; Figs. 2 and 3), thrombin treatment of B$\beta$1(Pyr)-42(Arg) and of larger FPB-containing peptides results in a significant increase in FPB immunoreactivity, indicating the presence of larger FPB-containing peptides. Such an increase in FPB immunoreactivity following thrombin treatment has enabled us to identify a larger FPB-containing peptide in plasma extracts from patients with in vivo coagulation. Gel filtration studies have provided evidence that the thrombin-increasable FPB immunoreactivity in plasma extracts is B$\beta$1(Pyr)-42(Arg).$^{14}$ In the current study, the thrombin-increasable FPB immunoreactivity was repeatedly responsive to heparin administration (Fig. 4; Table 2).
Fig. 4. Serial measurements of FPB immunoreactivity before (open circles) and after (closed circles) thrombin treatment of plasma extracts from a patient with chronic intravascular coagulation who received intravenous heparin injections as follows: a single dose of 70 U/kg at zero time; a single dose of 35 U/kg at 48 hr, followed by a constant infusion (750 U/hr) from 48 to 72 hr.

Studying of antisera to fibrinopeptide-B

The presence of larger FPB-containing peptides can be established by thrombin treatment of plasma extracts, additional physicochemical separation techniques are required to determine the relative concentrations of FPB and of the larger peptides. It may be possible to quantify the larger Bβ-chain segments in clinical blood samples with antibodies to appropriate segments of the 15(Gly)-42(Ang) sequence of the Bβ-chain and, accordingly, efforts have been instituted to elicit such antibodies.

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The current study, like another study of the reaction of anti-FPB sera with a synthetic desarginyl-FPB homologue, provides evidence that cleavage of the COOH-terminal arginine from FPB by carboxypeptidase-B significantly impairs immunoreactivity with most anti-FPB sera (Table 1; Figs. 2 and 3A). Evidence that carboxypeptidase-B affects FPB added to whole blood in vitro has been presented previously. While the addition of O-phenanthroline may effectively inhibit the in vitro conversion of FPB to desarg-

Table 2. Effect of Heparin and e-Aminocaproic Acid on Thrombin-Increasable FPB Levels and on Other Indices of Fibrinogenolysis in a Patient* With Continuous Intravascular Coagulation

<table>
<thead>
<tr>
<th>Therapy</th>
<th>Normal Range</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of Determinations</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TIFPB (pmole/ml)</td>
<td>22.1</td>
<td>4.6</td>
<td>14.8</td>
<td></td>
</tr>
<tr>
<td>(9-45.4)</td>
<td>(2.7-6.8)</td>
<td>(5.1-21)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FPA (pmole/ml)</td>
<td>9.9</td>
<td>3.2</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>(0.1-1.3)</td>
<td>(1.4-29.3)</td>
<td>(0.5-8.3)</td>
<td>(5.2-13.4)</td>
<td></td>
</tr>
<tr>
<td>FDP (μg/ml)</td>
<td>124</td>
<td>3.8</td>
<td>291</td>
<td></td>
</tr>
<tr>
<td>(&lt;1)</td>
<td>(80-165)</td>
<td>(0-15)</td>
<td>(165-330)</td>
<td></td>
</tr>
<tr>
<td>Fibrinogen (mg/dl)</td>
<td>116</td>
<td>220</td>
<td>139</td>
<td></td>
</tr>
<tr>
<td>(200-400)</td>
<td>(20-190)</td>
<td>(90-260)</td>
<td>(165-330)</td>
<td></td>
</tr>
</tbody>
</table>

Values are the means of the indicated number of determinations; ranges are listed in parentheses beneath mean values.

FFP, fresh frozen plasma; EACA, e-aminocaproic acid; TIFPB, thrombin-increaseable FPB; FDP, fibrinogen degradation products.

*This patient has been described in previous studies, and her continuous intravascular coagulation is associated with a cavernous hemangioma of the right lower extremity.
nyl-FPB, it is likely that a similar conversion may occur in vivo. In order to measure FPB generation in vivo, an essay for desarginyln-FPB has been developed with the use of antiserum R-30 and is being applied to the measurement of this peptide in clinical blood samples.30

Finally, it should be noted that the detailed immunological characterization of the reactions of the seven anti-FPB sera with proteins and peptides containing the NH₂-terminal segment of the Bβ-chain of human fibrinogen (Table 1; Fig. 2) has made possible the selection of antisera that have been useful in the identification not only of FPB but also of Bβ1(Pyr)-42(Arg) and of desarginyln-FPB, thus providing new techniques for analyzing the action of proteolytic enzymes on the NH₂-terminal sequence of the Bβ-chain of fibrinogen in human blood in thrombosis.

REFERENCES

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