Fibrinopeptide A and the Phosphate Content of Fibrinogen in Venous Thromboembolism and Disseminated Intravascular Coagulation


Concentrations of plasma fibrinopeptide A (FPA) were measured by radioimmunoassay in 50 patients with venous thromboembolism or disseminated intravascular coagulation or both. A consistent discrepancy was observed in values obtained with two anti-FPA antisera. Analysis of extracts from plasma of these patients by high-performance liquid chromatography (HPLC) revealed the presence of a phosphorylated and an unphosphorylated form of the A peptide. Differences in concentrations of FPA measured with the two antisera could be accounted for by their different reactivity with phosphorylated FPA (FPA-P). The differences were abolished by treatment with alkaline phosphatase. A good correlation was observed between the FPA-P content of free A-peptide material and of fibrinogen in plasma as determined by HPLC (r = .88, P < .001, n = 11). In patients with elevated FPA levels, the mean FPA-P content of fibrinogen was significantly higher (P < .002, n = 13) than in patients with normal FPA levels (n = 8) and in healthy controls (n = 14). Phosphorus in fibrinogen did not correlate with fibrinogen degradation products or fibrinogen levels and became normal on adequate anticoagulation. Therefore, blood-clotting activation may lead to a high phosphate content of fibrinogen and of free FPA in plasma.

Ant-FPA antiserum. Antiserum R 6216 has been described previously, except that rabbits were immunized with synthetic (Serva, Heidelberg, FRG) instead of native FPA coupled to human serum albumin (Serva; 5x crystallized) by the carbodiimide method. Cross-reactivity of these sera with purified human fibrinogen, prepared as described (a gift from Drs. F. Haverkate and W. Nieuwenhuizen, Gaubius Institute—TNIO, Leiden, The Netherlands), was determined according to Canfield et al. (1985).

Collection and processing of blood. After clean venipuncture, 4.5 mL of venous blood was collected in siliconized Vacutainer tubes, containing 0.5 mL of an anticoagulant mixture of 500 units of heparin (Kabi Vitrum, Stockholm), 500 U of Trasylol (Bayer, Leverkusen, FRG), and 19 mg trisodium citrate 0.2 aq. The tubes were shaken, placed in melting ice, and centrifuged for ten minutes at 4 °C and 2,000 g, the whole procedure being completed within 30 minutes after venipuncture. Plasma proteins were immediately precipitated by mixing (in duplicate) 0.8 mL of plasma with 0.8 mL of 40% ice-cold PEG (PEG 6000; Merck, Darmstadt, FRG) in phosphate-buffered saline (0.14 mol/L NaCl, 0.010 mol/L Na2HPO4, pH 7.4). After incubation for ten to 20 minutes in an ice bath, these mixtures were centrifuged at 4 °C for 30 minutes at 2,000 g. The supernatants were heated for two minutes in a boiling water bath. Thereafter, tubes were centrifuged at room temperature for ten minutes at 2,000 g; then 0.6-mL aliquots of supernatants were stored at −30 °C or used directly for assay. When synthetic FPA was added to five citrated plasmas in six concentrations, ranging from 6.25 to 200 ng/mL, the mean recovery was 103% (94% to 116%) as determined by radioimmunoassay with R 6216.

Radioimmunoassay of FPA. The immunoassay was performed as described, except that PEG precipitation instead of dialysis was used to separate low-molecular-weight FPA-immunoreactive material from fibrinogen. A mixture of PEG, citrate, heparin, and Trasylol in assay buffer (0.1 mol/L NaCl, 0.05 mol/L Tris, pH 8.5, 0.25% ovalbumin, Serva) in concentrations equivalent to 1:1 diluted sample supernatant was used to prepare the standard curve. Values
in specimens from 70 healthy controls (aged 18 to 68 years) ranged from undetectable to 2 ng/mL. The value 2 ng/mL was therefore chosen as the upper limit of the normal range, with a 95% to 99% confidence interval.

**Material from patients.** Fifty patients with clinical signs of venous thromboembolism or disseminated intravascular coagulation (DIC) or both were studied. The presence of deep venous thrombosis was established by venography in 24 patients and suggested by abnormal impedance plethysmography in three. One patient (No. 3, Table 1) had a thrombophlebitis migrans (no venography performed). In ten patients, the appearance of multiple segmental or lobular perfusion defects in two or more views on a technically adequate perfusion scan, in the presence of a normal chest x-ray, suggested pulmonary embolism. A Xenon-133 ventilation scan, performed in two of these patients, supported the diagnosis of pulmonary embolism.

In 12 patients, a diagnosis of diffuse intravascular coagulation was made. All but two of these patients had elevated FDP/fdp. The ethanol-gelation test was positive in six. In addition, eight patients showed a low platelet count, and a low fibrinogen concentration was present in four of them. DIC was attributed to disseminated neoplasia in nine, and in the other three, to severe acute hemolytic anemia of unknown etiology, chronic myeloid leukemia, and an autoimmune disorder (No. 9, Table 1). Blood was only obtained after informed consent from the patient, and our study was approved by a local ethical committee.

**Purification of fibrinopeptides.** One gram of fibrinogen (Kabi; grade L) was dissolved in 100 mL of distilled water and allowed to

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**Table 1. Two Groups of Patients in Whom the FPA-P Content of Fibrinogen was Assessed**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Sex</th>
<th>Diagnosis</th>
<th>FDP/fdp (µg/mL)</th>
<th>Platelets (10^9/L)</th>
<th>Serum Alkaline Phosphatase (U/L)</th>
<th>FPA (ng/mL)*</th>
<th>Fibrinogen (g/L)</th>
<th>FPA-P/Total (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>With venous thromboembolism or DIC</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. A.S.</td>
<td>41</td>
<td>F</td>
<td>Metastasized ovarian carcinoma, Denver shunt, DIC</td>
<td>20.0</td>
<td>64</td>
<td>89</td>
<td>23.4</td>
<td>10.2</td>
<td>4.5</td>
</tr>
<tr>
<td>2. G.K.</td>
<td>74</td>
<td>F</td>
<td>Metastasized rectal carcinoma, DIC</td>
<td>320.0</td>
<td>74</td>
<td>—</td>
<td>21.1</td>
<td>5.7</td>
<td>0.9</td>
</tr>
<tr>
<td>3. J.B.</td>
<td>64</td>
<td>F</td>
<td>Metastasized endometrical carcinoma, thrombophlebitis migrans</td>
<td>&lt;5.0</td>
<td>225</td>
<td>84</td>
<td>8.8</td>
<td>7.0</td>
<td>3.9</td>
</tr>
<tr>
<td>4. A.O.</td>
<td>63</td>
<td>M</td>
<td>Metastasized pancreatic carcinoma, thrombophlebitis migrans, DIC</td>
<td>320.0</td>
<td>235</td>
<td>980</td>
<td>11.8</td>
<td>8.8</td>
<td>3.9</td>
</tr>
<tr>
<td>5. J.V.</td>
<td>33</td>
<td>M</td>
<td>Deep venous thrombosis, alcohol abuse, no concurrent disorder</td>
<td>≤40.0</td>
<td>704</td>
<td>—</td>
<td>11.0</td>
<td>7.9</td>
<td>6.2</td>
</tr>
<tr>
<td>6. M.R.</td>
<td>68</td>
<td>F</td>
<td>Metastasized ovarian carcinoma, diabetes mellitus, pulmonary embolism</td>
<td>≤1.25</td>
<td>210</td>
<td>123</td>
<td>22.0</td>
<td>9.3</td>
<td>4.9</td>
</tr>
<tr>
<td>7. C.Z.</td>
<td>84</td>
<td>M</td>
<td>Metastasized rectal carcinoma, pulmonary embolism</td>
<td>≤1.25</td>
<td>120</td>
<td>258</td>
<td>8.8</td>
<td>6.1</td>
<td>4.0</td>
</tr>
<tr>
<td>8. H.V.</td>
<td>38</td>
<td>F</td>
<td>Metastasized ovarian carcinoma, pulmonary embolism</td>
<td>≤1.25</td>
<td>330</td>
<td>265</td>
<td>5.1</td>
<td>2.1</td>
<td>4.0</td>
</tr>
<tr>
<td>9. J.W.</td>
<td>27</td>
<td>F</td>
<td>Autoimmune disorder (juvenile rheumatoid arthritis, SLE), DIC</td>
<td>≤1.25</td>
<td>19</td>
<td>345</td>
<td>4.5</td>
<td>1.7</td>
<td>1.1</td>
</tr>
<tr>
<td>10. M.P.</td>
<td>59</td>
<td>M</td>
<td>Deep venous thrombosis, no concurrent disorder</td>
<td>≤1.25</td>
<td>174</td>
<td>44</td>
<td>4.7</td>
<td>3.7</td>
<td>3.5</td>
</tr>
<tr>
<td>11. F.R.</td>
<td>68</td>
<td>M</td>
<td>Non-Hodgkin lymphoma, thrombophlebitis migrans</td>
<td>10.0</td>
<td>155</td>
<td>104</td>
<td>3.6</td>
<td>2.1</td>
<td>2.5</td>
</tr>
<tr>
<td>12. H.W.</td>
<td>73</td>
<td>M</td>
<td>Metastasized prostatic carcinoma, DIC</td>
<td>&lt;2.5</td>
<td>33</td>
<td>218</td>
<td>27.2</td>
<td>4.8</td>
<td>2.2</td>
</tr>
<tr>
<td>13. H.D.</td>
<td>64</td>
<td>F</td>
<td>Metastasized ovarian carcinoma, pulmonary embolism</td>
<td>&lt;2.5</td>
<td>303</td>
<td>175</td>
<td>6.8</td>
<td>2.6</td>
<td>5.1</td>
</tr>
<tr>
<td><strong>Without venous thromboembolism or DIC</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14. A.H.</td>
<td>71</td>
<td>M</td>
<td>Metastasized nasopharyngeal carcinoma</td>
<td>&lt;2.5</td>
<td>246</td>
<td>—</td>
<td>1.6</td>
<td>0.8</td>
<td>3.9</td>
</tr>
<tr>
<td>15. M.S.</td>
<td>41</td>
<td>F</td>
<td>Metastasized breast carcinoma</td>
<td>&lt;2.5</td>
<td>214</td>
<td>63</td>
<td>1.6</td>
<td>0.1</td>
<td>—</td>
</tr>
<tr>
<td>16. P.O.</td>
<td>71</td>
<td>F</td>
<td>Metastasized carcinoid tumor</td>
<td>&lt;2.5</td>
<td>234</td>
<td>220</td>
<td>1.2</td>
<td>0.6</td>
<td>3.9</td>
</tr>
<tr>
<td>17. R.B.</td>
<td>30</td>
<td>M</td>
<td>Metastasized melanoma</td>
<td>≤1.25</td>
<td>185</td>
<td>51</td>
<td>0.0</td>
<td>0.0</td>
<td>1.5</td>
</tr>
<tr>
<td>18. C.L.</td>
<td>44</td>
<td>F</td>
<td>Metastasized breast carcinoma</td>
<td>≤1.25</td>
<td>295</td>
<td>349</td>
<td>1.9</td>
<td>0.8</td>
<td>4.1</td>
</tr>
<tr>
<td>19. A.K.</td>
<td>27</td>
<td>F</td>
<td>Acute myelomonocytic leukemia</td>
<td>≤1.25</td>
<td>70</td>
<td>71</td>
<td>1.6</td>
<td>1.0</td>
<td>2.7</td>
</tr>
<tr>
<td>20. M.H.</td>
<td>59</td>
<td>F</td>
<td>Metastasized breast carcinoma</td>
<td>≤1.25</td>
<td>228</td>
<td>66</td>
<td>0.0</td>
<td>1.5</td>
<td>3.3</td>
</tr>
<tr>
<td>21. J.D.</td>
<td>70</td>
<td>F</td>
<td>Vulva carcinoma</td>
<td>&lt;5.0</td>
<td>350</td>
<td>100</td>
<td>1.6</td>
<td>0.4</td>
<td>6.7</td>
</tr>
</tbody>
</table>

Normal values

| <10 | 150-300 | 35-110 | <2 | <2 | 2-4 | 16 | 42 |

FPA levels and the content of FPA-P in fibrinogen in patients of group I differ significantly (P < .002, Wilcoxon-White, two-sample ranks test) from those of group II. Differences in platelet counts, fibrinogen concentrations, FDP levels, and serum concentrations of alkaline phosphatase are not significant (P > .1). FPA levels from the two groups together, as determined with R 44656, correlate significantly with the content of phosphopeptide in fibrinogen (y = 1.4 x + 32.8, n = 21, r = .76, P < .001). The correlation found by using the FPA concentrations measured with R 6216 was less (y = 2.5 x + 35.2, n = 21, r = .53, P = .05). No correlation was found by either linear regression or ranks analysis between the content of FPA-P in fibrinogen and FDP levels, platelet counts, or concentrations of fibrinogen or serum alkaline phosphatase.

*Values denote concentrations of free A peptide in plasma as determined by radioimmunoassay with either R 44656 or R 6216.

†Mean ± 2 SD, as determined in healthy controls (n = 14).
clot for two to 24 hours at 37°C by adding 1 unit of human thrombin per milliliter (500 U/mL, Sigma, St Louis). The fibrin supernatant was mixed with an equal volume of ice-cold ethanol (Merck) and incubated for 20 minutes in an ice bath. Supernatants were heated for two minutes at 100°C, mixed once more with an equal volume of ethanol, and recentrifuged (30 minutes, 15,000 rpm, 4°C). This supernatant (about 400 mL) was evaporated to dryness by rotary film evaporation at 60°C and taken up in 4 mL 1% acetic acid. This volume was reduced to about 1 mL by blowing a stream of nitrogen over the sample at 40°C. The material was then spun for four minutes at 8,000 g. Next, 0.4 mL of the supernatant was chromatographed on a Biogel-P6 column, 60 × 0.9 cm (Bio-Rad, Richmond, Calif), equilibrated with 1% acetic acid (vol/vol), 1 mmol/L NaH₂PO₄, pH 2.4. Immuno reactive material eluting from this column was lyophilized and dissolved in 10% (vol/vol) acetonitrile (Rathburn Chemicals, Edinburgh, Scotland; HPLC, grade S), 0.1% (vol/vol) phosphoric acid (Merck), pH 2.1.

Of this eluate, 75 to 100 µL (about 4 mg of A-peptide immunoreactivity per milliliter) was chromatographed on an octadecylsilica-C18 column (for conditions, see later). Alkaline phosphatase treatment of concentrated fibrin clot supernatant was performed as described for patients’ samples (see later).

**Amino acid analysis.** After hydrolysis in 6 mol/L HCl in vacuo for 24 hours at 110°C, fractions were analyzed by standard techniques on a Chromatron-500 amino acid analyzer (Kontron, Zurich). ¹⁴

**HPLC analysis of plasma-FPA immunoreactivity.** Blood used for HPLC analysis was collected and centrifuged as described. Plasma was mixed with an equal volume of ice-cold ethanol and, after a 20-minute incubation period in an ice bath, spun for 30 minutes at 2,000 g at 4°C. Supernatants were decanted, incubated for another five to ten minutes in an ice bath, and recentrifuged. Supernatants were then heated for two minutes in a boiling water bath, centrifuged for ten minutes at 2,000 g at room temperature, then stored in plastic tubes at −70°C until further analysis. Disposable 6-mL octadecylsilica-(C18) columns (J.T. Baker Chemical Co, Philipsburg, NJ) were washed with 6 mL methanol and 18 mL of 0.1% phosphoric acid before the addition of samples. A single ethanolic supernatant, usually 5 mL, was diluted tenfold in 0.1% phosphoric acid, pH 2.1, and applied on this column. After several washes with 0.1% phosphoric acid, peptide material was eluted with (5 × 0.5 mL) 50% acetonitrile in 0.1% phosphoric acid. The eluate was concentrated, under a stream of nitrogen at 40°C, to 150 to 200 µL. Thereafter, samples were spun for two minutes at 8,000 g, and 0.1 mL supernatant was injected for HPLC analysis.

To test the effect of alkaline phosphatase treatment on the FPA immunoreactivity in these supernatants, duplicate samples were diluted in 5 mL 0.1% phosphoric acid and passed once more through a C18 column. Columns were washed with 0.1% phosphoric acid and then with distilled water, followed by elution of peptide material with 20% acetonitrile in 0.1 mol/L NaCl, 0.05 mol/L Tris, pH 8.0. Eluted material was pooled and concentrated under a stream of nitrogen to about 2 mL. Aliquots of 800 µL were then mixed with 2.2 mL of 0.1 mol/L NaCl, 0.05 mol/L Tris, pH 8.5. The mixture was incubated for two hours at 37°C with or without 10 units calf intestine alkaline phosphatase per milliliter (Boehringer, Mannheim, FRG; 1,500 U/0.74 mg/0.146 mL). The reaction was stopped by mixing these 3-mL aliquots with 30 mL 0.1% phosphoric acid. To remove the enzyme, samples were again passed through a C18 column, equilibrated with 0.1% phosphoric acid; peptides were eluted with 50% acetonitrile in 0.1% phosphoric acid.

Reversed-phase HPLC was performed on a µ-Bondapack C18 column (0.39 × 30.0 cm) from Millipore Waters (Milford, Mass.), by using a Perkin-Elmer series 2 liquid chromatograph, equipped with a Rheodyne injector (0.1-mL loop). Gradient elution was achieved with a mobile phase consisting of acetonitrile and 0.1% phosphoric acid, pH 2.1. The concentration of acetonitrile was initially 10% and was programmed to increase ten minutes after application of the sample at the rate of 1.25% per minute. Chromatography was conducted at room temperature with a flow rate of 2 mL/min. Fractions of 0.6 mL were collected and then tested for FPA immunoreactivity. Tubes for the standard curves contained 0.1-mL aliquots of 1% acetonitrile in 0.1% phosphoric acid, 0.2 mL assay buffer containing various amounts of synthetic FPA, 0.1 mL tracer, and 0.1 mL diluted antiserum. Assay tubes for “fraction testing” contained 0.1-mL aliquots (10% to 30% acetonitrile in 0.1% phosphoric acid), 0.2 mL assay buffer, 0.1 mL tracer, and 0.1 mL diluted antiserum. Standard curves obtained with samples containing acetonitrile were identical with those obtained with 0.1-mL aliquots of diluted PEG (cf. standard radioimmunoassays) in a total assay volume of 0.5 mL.

**Extent of phosphorylation of fibrinogen.** Blood (containing 13 mmol/L trisodium citrate), collected on ice, was centrifuged within 30 minutes of venipuncture. Supernatant plasma (ten minutes, 2,000 g, 4°C) was rendered platelet-poor (two minutes, 8,000 g, 4°C) and stored in plastic tubes at −70°C. To determine the fibrinopeptide A-P content of plasma fibrinogen by HPLC analysis, by using an ammonium acetate buffer,¹⁵ in combination with radioimmunoassays with R 44656, plasma samples were rapidly thawed at 37°C and incubated for 30 minutes at 37°C with 20 units of human thrombin per milliliter (Sigma; 3,000 U/mg). Clot supernatants were mixed with β-glycerophosphate (Sigma) 100 mmol/L and precipitated with ethanol as described. Supernatant (100 µL) was added to 5 mL 0.1% phosphoric acid, pH 2.1, and analyzed by HPLC as described. FPA release from fibrinogen by thrombin was complete as determined by comparing the theoretical concentrations of A peptide with the measured concentrations. Recovery of FPA from disposable extraction columns and HPLC was >98%. The mean percentage of fibrinogen, phosphorylated at position Αα Ser3, obtained from total plasma fibrinogen by this procedure, in healthy controls (n = 14, aged 18 to 55 years) was 29% (19% to 42%), in dogs (n = 2, four determinations) 68% (60% to 72%), and in full-term newborn infants (n = 3) 58% (54% to 65%). ²⁰

**Statistical methods.** FPA values were not normally distributed; therefore, Spearman’s rank test was used to determine the correlation between values measured with the two anti-FPA sera (Fig 1). The significance of the differences between values measured with the two antisera was tested by Wilcoxon’s signed rank test (Fig 1). Comparisons between patients with venous thromboembolism and control patients or normal volunteers were made with the Wilcoxon-White two-sample ranks test (Table I). The possibility of a correlation between the phosphopeptide content of fibrinogen and the FPA levels, the fibrinogen concentration, the platelet count, the FDPs, and the alkaline phosphatase levels was tested by both linear regression and rank analysis, the latter by using Spearman’s rank test as well as the Kendall rank correlation coefficient test (Table I). Comparisons between results obtained before and during anticoagulation were analyzed by using Student’s t test for paired data.

**RESULTS**

FPA in plasma from patients with venous thromboembolism or DIC or both. When antiserum R 44656 was used in the radioimmunoassay, elevated concentrations of FPA were observed in plasma from 40 out of 50 patients with venous thromboembolism or DIC or both. With antiserum R 6216, FPA concentrations were increased in 35 of these patients. The values found with the two antisera differed significantly (P < .0005). R 44656 reacted apparently almost invariably
With more material in PEG supernatants from plasma than did R 6216 (Fig 1). Administration of heparin resulted in all patients with venous thromboembolism, in which the effect of this drug was evaluated, for both antisera in a prompt decline of plasma-FPA immunoreactivity (Fig 2).

**HPLC of fibrinopeptides.** In view of the dissimilar reactivity of two anti-FPA sera with the immunoreactive material in clinical blood samples, it was thought that plasma-FPA immunoreactivity was heterogeneous. To test this hypothesis, native fibrinopeptides and immunoreactive material in plasma were analyzed by HPLC. Figure 3A shows a chromatogram of a fibrin clot supernatant, obtained by allowing purified human fibrinogen to clot with thrombin for two hours at 37°C. Four major peaks were eluted with the acetonitrile gradient. Peaks I and II had an identical composition, corresponding to that of FPA, whereas the amino acid composition of peaks III and IV corresponded to fibrinopeptide B and its degradation product Bβ1-13, respectively. These results were in agreement with those of Koehn et al.16 who identified peak I as the unphosphorylated and peak II as the phosphorylated forms of FPA. Peak I exhibited similar FPA immunoreactivity with our two sera. Peak II, in contrast, reacted a hundredfold less with R 6216 as compared with R 44656 (Fig 3A and B). Incubation of peak II with alkaline phosphatase had no effect on the reactivity of R 44656, but abolished the difference in reactivity between the two sera. Treatment of concentrated fibrin clot supernatant with alkaline phosphatase resulted in a complete disappearance of peak II and a corresponding relative increase in peak I, whereas peaks III and IV were unchanged (Fig 4). These results suggested that R 6216 was directed to antigenic sites of the peptide that became exposed on removal of the serine-bound phosphate. This finding provided a potential explanation for the dissimilar reactivity of the two sera with material from patients.

**Fig 1.** Plasma FPA levels as measured with two anti-FPA sera (R 44656 and R 6216) in 70 normal individuals depicted within a square, 50 patients with venous thromboembolism or DIC or both (□). The broken line indicates the hypothetical ideal relationship between the results obtained with the two sera. The observed correlation for the thrombosis patients was: r = .91, P < .001 (Spearman's rank test). Differences between values measured with the two sera were highly significant (P < .0005 Wilcoxon's signed rank test).

**Fig 2.** Effect of a bolus injection of heparin (5,000 to 7,500 units) on plasma-FPA immunoreactivity in eight patients with venous thromboembolism. The horizontal broken line denotes the upper limit of the normal range. Time between blood sampling is indicated on the abscissa: ○, values before heparin; □, data with R 44656; □, data with R 6216. Values measured with R 44656 or R 6216 declined after administration of heparin, suggesting that material detected with the two sera is a result of thrombin activity.

**HPLC of plasma-FPA immunoreactivity from patients with venous thromboembolism or DIC or both.** To investigate whether FPA immunoreactivity in plasma from patients consisted of a mixture of phosphorylated and unphosphorylated FPA, plasma samples were analyzed by HPLC. For practical reasons, ethanol instead of PEG supernatants were used. This was legitimate because experiments indicated that ethanol and PEG supernatants yielded identical HPLC patterns (not shown).

Figure 5A shows a chromatogram of a plasma extract from a patient with DIC. Two major peaks of FPA immunoreactivity were eluted from the column; the first was recognized equally well by both sera. The second peak reacted (almost) exclusively with antiserum R 44656 and disappeared completely if samples were pretreated with alkaline phosphatase (Fig 5B). HPLC-purified native fibrinopeptide A as well as synthetic FPA were added as internal standards to patients' samples and were eluted at exactly the same position as the first peak, whereas FPA-P purified by HPLC behaved identically to peak II. FPA immunoreactivity containing fractions from Fig 5A were pooled, concentrated, and rerun with an ammonium acetate buffer as described by Kehl et al.15 This experiment provided an additional argument that peak II represented FPA-P and peak I, FPA because, as expected, the order of elution of these compounds reversed under these conditions (Fig 5C).

HPLC analysis of plasma-FPA immunoreactivity was performed in 14 patients with venous thromboembolism. A phosphorylated form and an unphosphorylated form of the A
peptide were always observed. The ratio between these two forms varied considerably from one individual to another. As no dephosphorylation of FPA-P added immediately after collection of blood was observed and addition of even high concentrations of phosphatase inhibitors, such as β-glycerophosphate (up to 0.1 mol/L), either to the anticoagulant mixture or to plasma before precipitation with ethanol, had no effect on the FPA-P-FPA ratio (data not shown), the presently observed variation appears to reflect the situation in vivo.

Phosphate content of fibrinogen in patients with venous thromboembolism or DIC or both. In several patients with high FPA levels, well over 30% of the A peptide was in its phosphorylated form (cf. Fig 5). If this was a reflection of a high FPA-P content of fibrinogen, a high phosphorus content in fibrinogen might be related to coagulation activation in vivo. Therefore, the FPA-P content in fibrinogen was determined by HPLC in patients with venous thromboembolism or DIC or both (n = 13), selected from the 50 patients depicted in Fig 1 on the basis of elevated FPA levels, as well as in control patients (n = 8) without venous thromboembolism or DIC and without elevated FPA levels (Table 1). The mean phosphorus in fibrinogen from patients with venous thromboembolism or DIC or both was always significantly higher (p < 0.01) than in control patients. The mean phosphorus in fibrinogen from patients with venous thromboembolism or DIC or both was 2.16 ± 0.25 mg/mg fibrinogen, compared to 0.76 ± 0.10 mg/mg fibrinogen in control patients. This difference was significant (p < 0.001).

Fig 3. (A) Analysis of a fibrin clot supernatant by HPLC. Seventy-five microliters of concentrated clot supernatant, containing about 400 μg of FPA immunoreactivity, was injected and then eluted with an acetonitrile gradient as indicated. Fractions of 0.6 ml were collected for radioimmunoassay and amino acid analysis. I. FPA; II. FPA-P; III. FPB; IV. βF 1–13. v-v, R 44656. o-o, R 6216. (B) Reactivity with synthetic FPA, native FPA, and phosphorylated FPA of two anti-FPA sera. We tested 100-μL volumes, containing various concentrations of fibrinopeptide A or AP, determined by amino acid analysis. On the ordinate: the observed inhibition of binding of [125I]-Tyr FPA. Triangles depict values with antiserum R 44656; circles, values with R 6216. v, synthetic FPA; v, native FPA (peak I, Fig 3A); v, native FPA-P (peak II, Fig 3A).

Fig 4. HPLC analysis of fibrin clot supernatant before and after treatment with alkaline phosphatase. Fibrinogen was clotted for 24 hours with 1 unit of thrombin per milliliter. The supernatant was passed through a disposable C18 column. The eluate, containing 0.1 mg of A-peptide immunoreactivity per milliliter, was incubated for two hours at 37 °C in 0.1 mol/L NaCl, 0.05 Tris, pH 8.5, in the absence (A) or presence (B) of alkaline phosphatase (10 U/ml). HPLC conditions were identical with those in Fig 3. One hundred microliters (~13 μg A-peptide immunoreactivity) were injected. Absorbance was monitored at 205 nm and 0.6-ml fractions were tested with R 6216 (o-o) and R 44656 (v-v).

Fig 5. (A) HPLC analysis of plasma-FPA immunoreactivity from a 76-year-old patient, V., with a metastasized small cell lung carcinoma and signs of DIC. FDP/fdp, 80 μg/mL; fibrinogen, 1.5 mg/mL; platelets, 8 × 10⁵/L; factor II, 54%; factor V, 90%; factor VII, 68%; and factor X, 70%. FPA immunoreactivity as measured in PEG supernatant: R 44656, 8.7 ng/mL; R 6216, 3.3 ng/mL. (B) Effect of pretreatment with alkaline phosphatase. Control incubation without the enzyme had no effect (not shown). (C) FPA immunoreactivity-containing fractions from A and the control were rerun in an ammonium acetate buffer. FPA immunoreactivity was measured in each fraction with antiserum R 44656 (v-v) and R 6216 (o-o).
with a standard deviation of the slope of 0.125 and of the intercept of 7.9. The non-zero intercept is not significantly different from zero.

boembolism and elevated FPA levels (group I) was significantly higher ($P < .002$) than in control patients (group II) and healthy individuals. The FPA-P content in fibrinogen correlated significantly ($P < .001$) with FPA levels, but not with the level of fibrin or fibrinogen degradation products, the plasma fibrinogen concentration, the platelet count, or the serum alkaline phosphatase concentration. In 11 patients from group I, the FPA-P total FPA ratio was analyzed in fibrinogen as well as in the population of free FPA present in the circulation. An excellent correlation ($r = .88, P < .001$) was observed between the two analyses (Fig 6).

Differences in FPA levels measured in group I with R 44656 and R 6216 in plasma PEG supernatants in general reflected the extent of phosphorylation of fibrinogen. In several patients, however, the FPA content in fibrinogen, as calculated from the data on free FPA in plasma PEG supernatants, was lower than actually observed by HPLC analysis. Differences in FPA concentrations determined with the two antisera in plasma from control patients (group II) were no reflection of the FPA-P content in fibninogen, which is not surprising because values below 2 ng/mL plasma could not be measured accurately enough.

Nine patients from group I received anticoagulant treatment. Its effect on the FPA-P content in fibrinogen was studied in six. Adequate anticoagulation (ie, a doubling in the activated partial thromboplastin time [APTT], or thrombostest values <10%) was achieved in four (No. 5, 6, 8, 13). The FPA-P content in fibrinogen in these four patients was significantly lower ($P < .0025$), when FPA concentrations had become normal on therapy with heparin and oral anticoagulants, than before (mean before 56% [41% to 66%], during 32% [20% to 41%]). On-line studies were performed in three of these patients and are shown in Fig 7. From the two remaining inadequately anticoagulated patients, receiving only low-dose heparin (No. 2) or only oral anticoagulants (No. 3), one sample was obtained during therapy (nine and 14 days after initiation of therapy) showing no normalization of the phosphopeptide content in fibrinogen (before 72% and 51%; during 72% and 48%) or of the FPA level (determined only in No. 2, before R 44656 21.1 ng/mL; R 6216 5.6 ng/mL; during R 44656 15.0 ng/mL; R 6216 4.4 ng/mL).

DISCUSSION

Nossel et al reported that epitopic differences in anti-FPA antibody specificities may account for differences in measurable FPA levels in plasma samples due to fibrinogen breakdown in vitro, eg, during dialysis of plasma samples. Generation, in vitro, of a fragment of the α-chain of fibrinogen (AαI-23) containing the FPA moiety was thought to explain the discrepancies seen in values measured with two anti-FPA sera because similar discrepancies were observed in dialsates of streptokinase-treated plasma samples. Rapid removal of fibrinogen from samples of plasma, by precipitation with ethanol and centrifugation, effectively inhibited this proteolysis of fibrinogen in vitro and resulted in the observation of similar FPA levels in patients with two anti-FPA sera.

In the present study, fibrinogenolysis in vitro was inhibited by precipitating plasma samples with polyethylene glycol within 30 minutes of venipuncture, followed by heating of the supernatant in a water bath at 100°C. Despite these precautions, discrepancies between values measured with two anti-FPA sera (R 44656 and R 6216, developed in our laboratory) were observed that could not be explained by assuming the presence of (plasmin-induced?) fibrinogen fragments. The
following arguments substantiate this view. (1) The discrepancies remained on treatment with thrombin (not shown). (2) Such fibrinogen fragments are anticipated to react better with R 6216 than with R 44656. (3) The dissimilar reactivity of these antisera with patient’s material was abolished by treatment with alkaline phosphatase (Table 2). (4) HPLC analysis, combined with treatment with alkaline phosphatase, of purified peptides and patients’ samples indicated that the phosphorylated form of FPA accounted for the observed dissimilar reactivity of the two sera.

Our results obtained with these two anti-FPA sera show for the first time that, in patients with venous thromboembolism, FPA circulates in both its unphosphorylated and its phosphorylated forms. This observation may be relevant for the sensitivity of FPA immunoassays because, as reported in this article, polyclonal rabbit-anti-FPA sera, prepared according to routine protocols, may react differently with these two forms of the peptide. A difference in t\(\frac{1}{2}\) of these two forms of the peptide seems unlikely in view of the prompt decline of FPA values with both antisera on intravenous heparin infusion (Fig 2).

As to the high FPA-P content in fibrinogen in patients with venous thromboembolism and elevated FPA levels (Table 1), the present study provides no evidence that these results are representative for the larger group of patients depicted in Fig 1. There can be no doubt, however, that highly phosphorylated fibrinogen can appear in the circulation in patients with venous thromboembolism. The appearance of this fibrinogen correlated with the presence of increased concentrations of FPA (Table 1 and Fig 7). That this correlation was not perfect can possibly be accounted for, at least partially, by the different t\(\frac{1}{2}\)s of fibrinogen (in the order of days) and fibrinopeptide A (in the order of minutes). This difference can manifest itself during anticoagulant therapy (Fig 7). Although adequate anticoagulation (as judged by APTT or thrombostest values) eventually led to a normalization of both FPA levels as well as the extent of phosphorylation of fibrinogen, this effect occurred at a slower pace than anticipated in one of the patients with a coexistent malignancy (patient 6, Fig 7). This observation appears to be in agreement with what has been observed by others for FPA levels in such patients.

An interesting question is why the percentage of phosphorylated FPA in fibrinogen as well as free A-peptide material in samples of clinical blood may be as high as 80% of the total A-peptide material. Shainoff et al hypothesized that, in newly synthesized fibrinogen, the serine in FPA was phosphorylated and an increased fibrinogen turnover would lead to the appearance in plasma of fibrinogen with a high phosphate content. Our results fit within this hypothesis because coagulation activation may lead to an increase in the fibrinogen turnover rate. Regardless of which factor(s) will govern the extent of phosphorylation of plasma fibrinogen, we can now identify two situations in humans in which an increased FPA-P content, and therefore increased phosphate content, of plasma fibrinogen is found: in infants at birth (especially preterm) and in some patients with signs of thrombin activity in vivo.

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Fibrinopeptide A and the phosphate content of fibrinogen in venous thromboembolism and disseminated intravascular coagulation

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