Activation of the Coagulation System in Polycythemia Vera

By Angelina Carvalho and Leonard Ellman

Thrombosis is one of the major complications of polycythemia vera. Seventeen patients with polycythemia vera in good hematologic control were evaluated for abnormalities of the coagulation system. Activation of the intrinsic coagulation cascade was suggested by low levels of factor XII, prekallikrein, and kallikrein inhibitors in 12 of 17 patients. The group also demonstrated a significant increase in soluble fibrin complexes using plasma gel filtration on 4% agarose. Fibrin degradation products were normal and antithrombin III levels were slightly elevated. It appears that patients with polycythemia vera have chronic activation of the coagulation system, probably initiated by activation of factor XII. No correlation between the degree of coagulation abnormalities and thromboembolic complications was evident in this group of patients.

MATERIALS AND METHODS

Seventeen subjects with PV confirmed by standard clinical and laboratory criteria10 were evaluated. A group of healthy laboratory individuals was used as control. In addition to the control group, a group of healthy, elderly individuals whose age was comparable to the PV groups was also evaluated. These individuals were having routine physical examinations or elective orthopedic surgery.

Collection of Blood Samples

Nine volumes of venous blood collected with siliconized needles and plastic syringes were added to one volume of 3.8% sodium citrate in plastic tubes. The samples were centrifuged at 4°C for 15 min at 3000 g to obtain plasma for determination of blood clotting factors, antithrombin III, prekallikrein, and kallikrein inhibitors (KI). All samples were frozen at -60°C.

For determination of soluble fibrin complexes (SFC), the blood was collected in 3.8% sodium citrate with epsilon aminocaproic acid (EACA) in a final concentration of $10^{-3}$ M. These
samples were centrifuged at 3000 g for 15 min at 23°C. All samples were frozen at −60°C and thawed only once prior to testing.

For determination of serum fibrin(ogen) degradation products (FDP), whole blood was collected in plastic tubes containing EACA (final concentration 10^{-5} M) and thrombin (20 NIH units/ml) incubated at 37°C for 2 hr and centrifuged at 4°C for 15 min at 2500 g.

Prothrombin time (PT) was measured by the method of Quick using Simplastin (General Diagnostics, Morris Plains, N.J.), as thromboplastin and utilizing the Electra 600 (MLA, Inc., Mt. Vernon, N.Y.).

Activated partial thromboplastin time was measured by a modification of the method of Proctor and Rappaport using rabbit brain phospholipid and micronized silica as reagents (General Diagnostics) and utilizing the Electra 600.

Factor XII was measured by a modification of the activated partial thromboplastin time with the use of congenitally deficient plasma. The results were expressed as a per cent of normal. Standard curves were obtained by using plasma from 36 normal individuals.

Fibrin(ogen) degradation products (FDP) in serum were determined by the staphylococcal clumping test (SCT) and the results are expressed in micrograms per milliliter of fibrinogen equivalents.

Assays of antithrombin III (AT III) and C1 esterase inhibitor (C1 INH) were performed by the radial immunodiffusion method of Mancini et al. Monospecific rabbit antisera against human AT III and human C1 INH were obtained from Behringwerke, A.G., Germany.

Soluble fibrin complexes (SFC) were measured by plasma gel filtration, employing a modification of Fletcher's method. Two milliliters of sample were gel filtered through a 1.5 x 90 cm column containing Sepharose 4B in 0.2 M sodium phosphate and 0.1 M citric acid, pH 7.4 (phosphate citrate buffer), to which 10^{-3} M EACA and 0.05% sodium azide were added. The columns were run at 23°C with a flow rate of 10 ml/hr and an operating pressure of 40 cm H2O. Two-milliliter aliquots were collected. The columns were calibrated with appropriate standards: thyroglobin (MW 650,000), human purified fibrinogen (MW 340,000), rabbit muscle alcohol dehydrogenase (MW 150,000), and bovine serum albumin (MW 69,000). Each standard was filtered separately and the absorbance at 280 nm measured. The void volume (V0) was determined by filtration of Escherichia coli (4 mg/ml). The peak of the absorbance at 280 nm was 46 ml. The total volume (Vt) was determined by filtration of ε-DNP lysine (0.6 mg/ml) and the peak of absorbance at 360 nm was 160 ml. The distribution coefficient (KD) was calculated for each standard using the equation

\[ KD = \frac{(V_e - V_0)}{(V_t - V_0)} \]

where Ve = eluted volume of each standard.

**Gel Filtration of Fibrinogen Derivatives**

Purified human fibrinogen (Kabi, Sweden) 10 mg/ml was dissolved in the same phosphate-citrate buffer and incubated with porcine plasmin (0.1 casein units/ml) at 23°C for 30 min to produce “early” degradation products and for 100 min to produce “late” degradation products. The plasmin digestion of fibrinogen was stopped by the addition of soybean trypsin inhibitor (SBTI) 0.2 mg/ml. These products were identified by immunoelectrophoresis using a rabbit anti-human fibrinogen antiserum (Hyland Lab, Costa Mesa, Calif.) and tested for clottability.

Human purified fibrinogen was also incubated simultaneously with plasmin (0.1 casein units/ml) and highly purified bovine thrombin (1.0 NIH units/ml) for 30 min at 23°C. The reactions were stopped by hirudin (10 NIH units/ml) and SBTI (0.2 mg/ml), respectively.

The “early” and “late” plasmin degradation products and the reaction mixture of fibrinogen digested by both plasmin and thrombin were filtered separately through the same column and the protein content was determined by reading the optical density at 280 nm, using published extinction coefficients. In addition, in each eluted fraction (2 ml) the fibrinogen-reactive material was measured by the SCT and tanned red cell hemagglutination immunoassay (TRCHII). Results were expressed in micrograms per milliliter in terms of a human purified fibrinogen standard (Fig. 1A and B).

**Gel Filtration of Plasma**

Two milliliters of plasma were filtered on the calibrated column as described above.
COAGULATION SYSTEM IN PV

Fig. 1. In each 2-ml fraction the fibrinogen-reactive material was measured by the staphylococcal clumping test (SCT) and the tanned red cell hemagglutination immunoassay (TRCHII). The protein content was determined by reading the optical density at 280 nm.

Assay of Prekallikrein and Kallikrein Inhibitors

The kallikrein system was evaluated by the method of Colman et al.17 The method employs an esterase assay using tosyl arginine methyl ester (TAME). Upon exposure to kaolin the conversion of prekallikrein to kallikrein is catalyzed by activated factor XII or its derivatives. The increase in esterase activity during the first minute results from this conversion and measures the prekallikrein concentration. Conversely, the subsequent decrease in esterase activity is a function of kallikrein inhibitors (KI). Inhibition measured by this procedure is principally due to C1 esterase inhibitor.18 The prekallikrein results are expressed in μmoles TAME hydrolyzed/ml plasma per hour and kallikrein inhibitors are expressed in units. One unit of KI is the amount of inhibitory activity which at 5 min of activation of plasma by kaolin gives 50%, inhibition of the 1-min value.

RESULTS

Hematologic data at the time of the study are summarized in Table 1. All patients were under treatment for periods of 5 mo to 11 yr. With the exception of two samples, which were drawn during hospitalizations for unrelated medical conditions, the samples were obtained from outpatients who were clinically stable. Six of the seventeen patients had a history of thromboembolic complications. In five, the complications developed after hematologic control of the erythrocytosis had been achieved by either phlebotomy, alkylating agents, or radioactive phosphorus; the platelet counts were normal or only slightly elevated at the time of the thromboembolic complication. Total cholesterol and triglyceride levels were determined in the patients with thromboembolism and found to be normal in each of the six cases. Prothrombin and partial thromboplastin times were normal in all patients.
Table 1. Hematologic Data on Patients With Polycythemia Vera

<table>
<thead>
<tr>
<th>Case</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>Hct</th>
<th>WBC per cu mm</th>
<th>Platelets per cu mm</th>
<th>Therapy</th>
<th>Thromboembolic Complications</th>
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<tr>
<td>EM</td>
<td>66 M</td>
<td>43</td>
<td>15,100</td>
<td>326,000</td>
<td>p*</td>
<td>Femoral artery thrombosis</td>
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<tr>
<td>ET</td>
<td>67 F</td>
<td>40</td>
<td>6,700</td>
<td>156,000</td>
<td>p*</td>
<td>Pulmonary embolus</td>
<td></td>
</tr>
<tr>
<td>DR</td>
<td>58 M</td>
<td>52</td>
<td>21,000</td>
<td>485,000</td>
<td>p*</td>
<td>—</td>
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<tr>
<td>AG</td>
<td>60 F</td>
<td>50</td>
<td>12,600</td>
<td>461,000</td>
<td>p*</td>
<td>—</td>
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<tr>
<td>RC</td>
<td>70 M</td>
<td>49</td>
<td>9,700</td>
<td>573,000</td>
<td>p*</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>PD</td>
<td>66 M</td>
<td>50</td>
<td>12,400</td>
<td>605,000</td>
<td>p*</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>LC</td>
<td>62 M</td>
<td>54</td>
<td>13,900</td>
<td>400,000</td>
<td>p*</td>
<td>—</td>
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</tr>
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<td>RW</td>
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<td>56</td>
<td>13,100</td>
<td>413,000</td>
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<td>—</td>
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</tr>
<tr>
<td>SA</td>
<td>50 F</td>
<td>49</td>
<td>15,400</td>
<td>490,000</td>
<td>p*</td>
<td>Acute mesenteric venous occlusion</td>
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<tr>
<td>ET</td>
<td>91 M</td>
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<td>181,000</td>
<td>p*</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>MA</td>
<td>78 F</td>
<td>52</td>
<td>23,000</td>
<td>346,000</td>
<td>p*</td>
<td>Pulmonary embolism</td>
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</tr>
<tr>
<td>LB</td>
<td>56 M</td>
<td>52</td>
<td>22,400</td>
<td>364,000</td>
<td>p*</td>
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</tr>
<tr>
<td>GT</td>
<td>65 M</td>
<td>44</td>
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<td>380,000</td>
<td>p*</td>
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<td>53</td>
<td>24,000</td>
<td>1,040,000</td>
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<td>—</td>
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</tr>
<tr>
<td>GP</td>
<td>50 M</td>
<td>50</td>
<td>6,600</td>
<td>320,000</td>
<td>p*</td>
<td>Cerebral thrombosis</td>
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</tr>
<tr>
<td>AA</td>
<td>54 M</td>
<td>50</td>
<td>12,100</td>
<td>608,000</td>
<td>p*</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>MS</td>
<td>46 M</td>
<td>44</td>
<td>8,200</td>
<td>220,000</td>
<td>Busulfan</td>
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</tr>
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</table>

*p, phlebotomy.

Gel Filtration of Plasma

Filtration of plasma on agarose 4B allows the separation of fibrinogen-like material according to size. In these carefully calibrated columns, fibrinogen elutes as a symmetrical peak at an effluent volume which is constant from assay to assay (Fig. 2A). Early and late plasmin digests of fibrinogen elute distal to the fibrinogen peak as would be expected for molecules of a smaller size (Fig. 2B and 2C). A reaction mixture of fibrinogen, thrombin, and plasmin results in the formation of SFC which, because of their large size, are eluted 12 ml before the fibrinogen peak (Fig. 2D). A representative analysis of a normal individual's plasma (Fig. 3) shows a single symmetric peak of fibrinogen-like material at the same volume as purified fibrinogen. In the example shown in Fig. 3 (B), the SFC of a patient with PV made up 25% of the total fibrinogen-like material in the plasma. The plasma concentration of SFC in 15 normal subjects was 3.2% ± 0.86% of total fibrinogen (mean ± SEM). In eight healthy, elderly patients, age 68 ± 1.8 (mean ± SEM), the plasma SFC concentration was 5.5% ± 1.4%o. The mean per cent of SFC in PV patients was elevated to a level of 27%o ± 1.9%o (p < 0.01) and was increased in every patient with PV (Table 2).

Measurement of Serum Fibrinogen Degradation Products (FDP)

In normal subjects FDP measured by the SCT were 2.75 ± 3 µg/ml. The PV patients showed a comparable low level of FDP (Table 2).
Determination of Hageman Factor

Factor XII levels in PV patients were reduced to 66 ± 6.6 of normal (mean ± SEM) (p < 0.02) (Table 3).

Prekallikrein and Kallikrein Inhibitors

Prekallikrein levels were significantly depressed in PV patients at 78 ± 4.3 μmoles TAMe hydrolyzed/ml/hr (p < 0.01). As a group there was no significant difference between kallikrein inhibitors in PV patients and normals (Table 3), although 12 of the 17 patients had KI levels below normal.

Immunologic Determination of C₁ Esterase Inhibitor (C₁INH)

C₁INH, one of the major kallikrein inhibitors of plasma, was measured by Mancini's method. The mean value for PV patients was significantly lower than normal (p < 0.02) (Table 3).
Table 2. Determination of Fibrinogen Derivatives

<table>
<thead>
<tr>
<th>Group</th>
<th>Age (yr)</th>
<th>SFC* (% fibrinogen equivalents in plasma)</th>
<th>FDPI (μg/ml serum)</th>
</tr>
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<tbody>
<tr>
<td>Patient (17)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EM</td>
<td>28</td>
<td>2.4</td>
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<tr>
<td>ET</td>
<td>29</td>
<td>4.8</td>
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</tr>
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<td>DR</td>
<td>37</td>
<td>9.6</td>
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<td>4.8</td>
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<tr>
<td>RC</td>
<td>24</td>
<td>4.8</td>
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</tr>
<tr>
<td>PD</td>
<td>26</td>
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<td>LC</td>
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<td>9.6</td>
<td></td>
</tr>
<tr>
<td>MA</td>
<td>29</td>
<td>19.2</td>
<td></td>
</tr>
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<td>LB</td>
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<td>2.4</td>
<td></td>
</tr>
<tr>
<td>GT</td>
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<td>FE</td>
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<td>AA</td>
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<tr>
<td>MS</td>
<td>36</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>61 ± 2.9</td>
<td>27 ± 1.9</td>
<td>4.2 ± 1.2</td>
</tr>
<tr>
<td>Normal (15)</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>34 ± 3</td>
<td>3.2 ± 0.86</td>
<td>2.8 ± 0.69</td>
</tr>
<tr>
<td>(8)</td>
<td>65 ± 1.8</td>
<td>5.6 ± 1.4</td>
<td>4.2 ± 0.87</td>
</tr>
</tbody>
</table>

* Soluble fibrin complexes.
† Fibrin degradation products.
| Mean ± SEM.|

Fig. 3. Gel filtration patterns of normal (A) and polycythemia vera patient (B) plasmas. The eluates were assayed by staphylococcal clumping test (SCT).
Table 3. Evaluation of Factor XII and the Kallikrein System

<table>
<thead>
<tr>
<th>Group</th>
<th>Factor XII (% normal)</th>
<th>Prekallikrein (μ mole/TAME/ml/hr)</th>
<th>Kallikrein INH (units/ml)</th>
<th>C1 INH (% normal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient (17)</td>
<td>66 ± 6.6*</td>
<td>78 ± 4.3</td>
<td>0.93 ± 0.07</td>
<td>85 ± 6.3</td>
</tr>
<tr>
<td>Normal (36)</td>
<td>98 ± 6.4</td>
<td>97 ± 4.0</td>
<td>0.99 ± 0.03</td>
<td>118 ± 8.3†</td>
</tr>
</tbody>
</table>

*Mean ± SEM.
†15 individuals.

Immunologic Determination of Antithrombin III (AT III)

AT III, the major inhibitor of thrombin in plasma, was 26.7 ± 1.4 mg/100 ml in the patients with PV and 22.6 ± 0.94 mg/100 ml in 18 normal controls. This difference was not statistically significant.

DISCUSSION

Considerable interest has focused on the role of thrombocytosis and abnormal platelet function in the etiology of thromboembolic complications in PV. Although some investigators have reported an increased incidence of thrombotic complications in patients with elevated platelet counts, others have failed to confirm this association. Abnormal platelet function studies such as decreased platelet factor 3, reduced platelet adhesiveness, and abnormal platelet aggregation are found in almost all patients with PV. In one extensive study, however, no correlation was found between the degree of platelet functional abnormality and thrombotic complications.

Although it is appreciated that patients with PV do not show the hemostatic defects of overt disseminated intravascular coagulation, it is possible that the hypercoagulability of PV is related to low-grade intravascular coagulation. This was initially suggested by Christensen in 1958 when he found a short fibrinogen survival in an asymptomatic patient with PV. Similarly, Blömback and his colleagues found a short fibrinogen survival in three patients with PV; the short fibrinogen survival was corrected by intravenous administration of heparin in the two patients studied. Recently, Martinez et al. found a striking and parallel shortening of both prothrombin survival and fibrinogen survival in patients with myeloproliferative diseases, including three patients with PV. Our study adds further support to the existence of chronic activation of the coagulation system in patients with PV. Twelve of the seventeen patients had activation of the intrinsic clotting pathway characterized by depressed levels of factor XII, prekallikrein, and kallikrein inhibitors.

Activation of the coagulation system eventually results in generation of thrombin which hydrolyzes fibrinogen to form fibrin monomer. This monomer in turn polymerizes to form SFC. A search in plasma for these polymeric fibrin complexes represents a unique approach for the demonstration of intravascular coagulation. SFC cannot be detected in serum because they are clottable by the action of thrombin. Hence, only a search in plasma prepared with epsilon amino caproic acid will allow detection of polymeric complexes of fibrin and fibrinogen. Other techniques to detect fibrin polymerization such as cryofibrinogen and paracoagulation tests are far less sensitive than plasma gel filtration.
Substantial evidence has accumulated that SFC serve as markers for intra-
vascular fibrin deposition. In particular, Fletcher and Alkjaersig and col-
leagues have demonstrated that the determination of SFC in plasma through
gel exclusion chromatography on agarose represents a sensitive and specific
assay for detecting ongoing intravascular thrombosis. SFC determined by this
technique have been found to be elevated in postoperative thrombophlebitis,
acute myocardial infarction, acute cerebral vascular occlusion, in women
taking oral contraceptives, in disseminated intravascular coagulation and
other fibrinolytic states, and in patients with types II and IV hyperlipoprotein-
emia. The nature of SFC in human plasma has recently been studied by Ly
and Jakobson employing plasma gel filtration followed by polyacrylamide gel
electrophoresis. These authors found that SFC contain partially cross-linked
fibrin as a result of the action of factor XIII, but that most of the polymeric
fibrin complexes are held together by noncovalent bonds. Recent in vitro
data employing the technique of plasma gel filtration have demonstrated that SFC
can be generated by addition of estrogens to fibrinogen, an effect similar
to that of thrombin. The present study has demonstrated a consistent, marked
elevation of SFC in plasma of patients with PV, suggesting ongoing intra-
vascular coagulation. However, no correlation was evident between factor XII,
prekallikrein, or SFC levels and the occurrence of thromboembolism in our
group of patients.

Once activation of one of the clotting pathways is initiated, concomitant
triggering of the fibrinolytic system should occur. Our failure to find an eleva-
tion of FDP may reflect an inadequate fibrinolytic response for the degree of
intravascular coagulation. Alternatively, Smith and Bang have demonstrated
in vitro that plasma fibrin degradation products can complex with both fibrino-
gen and fibrin monomer contributing to the formation of SFC. Fibrin degrada-
tion products complexed in this manner would not be “available” to be mea-
sured by the conventional SCT assay performed in serum.

Our results also show a moderate increase in the concentration of anti-
thrombin III, rather than the decrease expected with thrombin formation in
vivo or in vitro. This elevation may represent a reactive response to the in-
creased production of thrombin by a feedback mechanism which increases syn-
thesis or decreases catabolism of antithrombin III.

The exact mechanism leading to activation of the coagulation cascade in PV
is not clear. It is possible that the release of intravascular thromboplastins and
proteases from leukocytes and platelets might activate factor XII and, subse-
quently, prekallikrein. Martinez et al. found that reduction of platelet
counts to the normal range was associated with a normalization of prothrombin
and fibrinogen catabolism in a group of patients with myeloproliferative dis-
orders. In our study, however, no relationship was evident between abnormal
blood counts and laboratory evidence of activation of the coagulation system.
In fact, it was noteworthy that signs of intravascular coagulation persisted in
patients with PV long after hematologic control had been achieved. Our results
suggest that patients with PV have a chronic state of activation of the blood
coagulation system even after normalization of the blood cell counts. Further
investigation is underway to study the effect of coumadin, subcutaneous hep-
arin, and antiplatelet medications on the laboratory evidence of chronic intravascular coagulation in patients with PV.

REFERENCES

Activation of the coagulation system in polycythemia vera

A Carvalho and L Ellman