Thrombin Generation in Acute Promyelocytic Leukemia

By Kenneth A. Bauer and Robert D. Rosenberg

Six patients with disseminated intravascular coagulation (DIC) in association with acute promyelocytic leukemia (APL) were studied with sensitive radioimmunoassays that are able to quantify the extent of thrombin generation within the human circulation. The levels of prothrombin activation fragment, F1,2, and thrombin–antithrombin complex (TAT) were obtained at clinical presentation and were then followed serially in several patients during induction chemotherapy. The antileukemic therapy often resulted in a rise in the plasma levels of these molecular species. Simultaneous measurements of fibrinopeptide A resulted in a rise in the plasma levels of these molecular species. Simultaneous measurements of fibrinopeptide A (FPA) were also obtained, and the concentrations of this polypeptide were correlated with the levels of F1,2 and TAT in patients who were not receiving heparin. Nine individuals with other morphological subtypes of acute nonlymphocytic leukemia (ANLL) were investigated and were usually found to have increased levels of F1,2, TAT, and FPA at clinical presentation. However, the magnitude of the elevations was considerably greater and the correlation between TAT and FPA levels was stronger in APL than in ANLL. These studies provide direct evidence that patients with APL, as well as ANLL, generate excessive amounts of thrombin within their vascular system. Furthermore, the data suggest that the concentrations of F1,2, compared with the levels of FPA, may be a more sensitive indicator of hemostatic system hyperactivity in individuals with DIC.

The occurrence of disseminated intravascular coagulation (DIC) in association with acute promyelocytic leukemia (APL) is well documented. Patients with this disorder invariably present with a bleeding diathesis, but thromboembolic events have also been reported in 1% to 2% of cases. Autopsy evidence of diffuse thrombosis with fibrin deposition is more prevalent and is present in at least 15% to 25% of cases.

The most consistent coagulation abnormalities in patients with APL and DIC are thrombocytopenia, prolongations in the prothrombin and thrombin times, increased levels of fibrinogen/fibrin degradation products (FDPs), and hypofibrinogenemia. These laboratory parameters will often become more abnormal upon the initiation of effective cytotoxic chemotherapy for the underlying leukemia, resulting in severe hemorrhagic diatheses. This phenomenon probably stems from the release of substances with procoagulant activity from the leukemic cells into the circulation. However, the efficacy of heparin therapy in treating this coagulopathy remains controversial.

The conversion of prothrombin to thrombin is a key event in the coagulation of blood. This transformation occurs at an appreciable rate only in the presence of factor Xa, factor Va, calcium ions, and a lipid surface (platelets). Under physiologic conditions, this activation results in the release of the inactive F1,2 fragment from the amino terminus of the prothrombin molecule, in conjunction with the generation of the intermediate species, prethrombin 2. Subsequently, prethrombin 2 can be internally scissioned to yield thrombin. Once produced, this serine protease can either proteolyze fibrinogen with the liberation of fibrinopeptide A (FPA), or combine with its major antagonist, the plasma protein antithrombin, via the formation of a stable inactive enzyme–inhibitor complex.

We have developed sensitive and specific radioimmunoassays that are able to accurately quantitate the levels of prothrombin fragment F1,2 and thrombin–antithrombin complex (TAT) within the human circulation. In this article, we describe studies designed to measure these molecular species within the blood of individuals with APL and other subtypes of acute nonlymphocytic leukemia (ANLL) undergoing induction chemotherapy. The plasma concentrations of these two moieties have been correlated with those of FPA and our studies provide direct evidence that excessive thrombin generation is commonly found in association with these hematologic malignancies.

MATERIALS AND METHODS

Patient Selection

Six patients with APL followed at the Beth Israel Hospital and Dana-Farber Cancer Institute were studied during the 36-month period from July 1979 to June 1982. The diagnosis of APL was established by standard clinical, morphological, and histochemical criteria. Five of the six patients accrued into the study were newly diagnosed. Nine patients with ANLL, classified morphologically as having myelocytic, myelomonocytic, or monocytic subtypes, were also studied. Specimen collection and informed consent procedures were approved by the institution’s human experimentation committee.

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Therapy

Induction chemotherapy for patients treated before June 1980 consisted of vincristine (1.5 mg/m² intravenously (IV) days 1, 5), doxorubicin (30 mg/m² days 2, 3), prednisolone (40 mg/m² IV every 12 hours days 1 to 5), and cytarabine (100 mg/m² by continuous infusion IV days 1 to 7). Patients presenting after this date received daunorubicin (45 mg/m² IV days 1, 2, 3) and cytarabine (200 mg/m² IV days 1 to 7). Four of the six patients with APL also received heparin (Panheprin, Abbott Laboratories, North Chicago) during the first several days of antileukemic therapy. Fresh frozen plasma was routinely administered to all patients if the prothrombin time was prolonged by two to three seconds, and platelet transfusions were given if the thrombocyte count fell below 20,000/µL.

Collection of Blood Samples

Specimens were collected prior to the initiation of heparin and antileukemic treatment and at regular intervals thereafter during the period of induction chemotherapy until complete remission was achieved. Venipunctures were performed atraumatically with 21-gauge butterfly infusion sets, using a two-syringe technique. The blood samples for radioimmunoassays were drawn into plastic syringes preloaded with appropriate solutions in the following order.

1. FPA anticoagulant: sodium chloride 8.766 mg/mL, 1,000 U/mL heparin (Panheprin), 1,000 U/mL aprotinin (Trasylol, Mobay Chemical Corp, New York). The ratio of anticoagulant to blood used was 0.1:0.9 (vol/vol).
2. TAT anticoagulant: citric acid 7.3 mg/mL, sodium citrate 22 mg/mL, dextrose 24.5 mg/mL, ethylene-diaminetetraacetic acid (EDTA) 2.234 mg/mL, adenosine 1.602 mg/mL, and hirudin 25 U/mL (Pentapharm, Basel, Switzerland).
3. F₁,₂ anticoagulant: TAT anticoagulant with the substitution of heparin for hirudin at a final concentration of 25 U/mL. The ratio of anticoagulant to blood employed in the latter two assays was 0.2:1.0 (vol/vol). After collection of blood samples, plasma fractions were obtained by centrifugation at 4°C for 15 minutes at 1,600 g and were stored at –80°C prior to use.

Normal values and standard deviations for F₁,₂, TAT, and FPA were estimated utilizing data obtained from control subjects, as previously described. This population consisted of healthy laboratory and medical personnel, who gave a negative history for thrombosis and bleeding and were not taking any medications, including oral contraceptives.

Radioimmunoassays

F₁,₂ and TAT radioimmunoassays were performed for F₁,₂ and TAT, as described in earlier reports from our laboratory. FPA radioimmunoassay. This radioimmunoassay was performed with a kit purchased from IMCO Corp (Stockholm, Sweden). The assay was performed according to the manufacturer's instructions, except that the plasma was treated with bentonite instead of ethanol to remove fibrinogen.

Coagulation Studies

Routine coagulation studies, including prothrombin time, activated partial thromboplastin time, thrombin time, and fibrinogen, were performed by standard laboratory methods. FDPs were determined using the Thrombo-Wellcotest (Wellcome Reagents, Research Triangle Park, NC). The DIC syndrome was identified by characteristic abnormalities of routine clinical tests, such as prolonged thrombin time (five seconds > control), diminished fibrinogen concentration (< 100 mg/dL), elevated FDPs (≥40 µg/mL), or reduced platelet count (<100,000/µL).

Analysis of Data

Estimation of relative immunoreactivity, computation of the slopes of the dose–response curves, as well as determinations of various associated indices were obtained by a least-squares fit of the radioimmunoassay results to a "four parameter" model, as described by Rodbard et al. Statistical analyses of data were performed by standard techniques. In most instances, the means are provided with associated standard deviations.

RESULTS

Samples were obtained from five patients with APL at the time of initial diagnosis. Standard coagulation tests and platelet counts were performed on blood specimens drawn simultaneously with those for the various radioimmunoassays. Prolongation of the prothrombin time, increased levels of FDPs, and hypofibrinogenemia supported the diagnosis of DIC in this population (Table 1). The mean levels of F₁,₂ and TAT in the five APL patients were quite elevated at presentation as compared to normal individuals, 36.9 ± 18.7 nmol/L vs 1.97 ± 0.97 nmol/L and 8.31 ± 4.00 nmol/L vs 2.32 ± 0.36 nmol/L, respectively (Table 2). The ratio of F₁,₂ to TAT ranged from 2.87 to 6.93. Three of these individuals had specimens drawn for measurement of FPA, and all had elevated values. The mean FPA concentration was 31.3 ± 17.8 nmol/L in APL patients vs 0.966 ± 0.514 nmol/L in normals.

Serial determinations of these indices of thrombin generation were made. The results, designated post-

Table 1. Coagulation Studies in Patients With Acute Promyelocytic Leukemia at Initial Diagnosis

<table>
<thead>
<tr>
<th>Patient/Age (yr)/Sex</th>
<th>Prothrombin Time</th>
<th>Partial Thromboplastin Time</th>
<th>Thrombin Time</th>
<th>Fibrinogen (mg/dL)</th>
<th>FDPs (µg/mL)</th>
<th>Platelets (x 10⁹/µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/28/M</td>
<td>20.0/13.9</td>
<td>38.6/31.7</td>
<td>16.6/13.6</td>
<td>57</td>
<td>&gt; 80</td>
<td>42</td>
</tr>
<tr>
<td>2/44/M</td>
<td>20.6/14.4</td>
<td>32.3/33.6</td>
<td>14.8/12.6</td>
<td>160</td>
<td>&gt; 80</td>
<td>22</td>
</tr>
<tr>
<td>3/36/M</td>
<td>16.9/12.4</td>
<td>24.8/31.2</td>
<td>22.6/20.7</td>
<td>120</td>
<td>40</td>
<td>2</td>
</tr>
<tr>
<td>4/46/F</td>
<td>17.8/12.6</td>
<td>25.3/28.4</td>
<td>18.0/19.9</td>
<td>80</td>
<td>160</td>
<td>48</td>
</tr>
<tr>
<td>5/25/M</td>
<td>15.7/11.8</td>
<td>31.0/32.8</td>
<td>ND</td>
<td>90</td>
<td>ND</td>
<td>28</td>
</tr>
<tr>
<td>Normal range</td>
<td>140–430</td>
<td>&lt; 10</td>
<td>150–350</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Each of the above results for the prothrombin time, partial thromboplastin time, and thrombin time represents the value for the individual patient plasma over that for the control plasma in seconds.

ND indicates that the test was not done at initial presentation.
chemotherapy (Table 2), were obtained on either the second or third day following the initiation of antileukemic therapy. Large elevations in F$_{1+2}$ and TAT levels were noted after the initiation of chemotherapy in patients 1 and 2. Patient 5 demonstrated a rise in TAT and FPA levels, but no substantial change in the concentration of F$_{1+2}$. These three individuals were simultaneously receiving intravenous heparin at a dose of 500 to 750 U/h by constant infusion. Therapeutic anticoagulation, as demonstrated by a doubling in the activated partial thromboplastin time, was achieved using 1,000 U/m$^2$/h in only one patient in this series (patient 4). A second blood sample was obtained from the individual after she had received 36 hours of intravenous heparin and the first dose of antileukemic therapy began on day 1 and lasted a total of seven days. Both individuals required two courses of cytotoxic chemotherapy in order to achieve a complete remission, when the two patients were deemed to be in remission based on bone marrow morphological analysis.

In order to examine the interrelationships among FPA, TAT, and F$_{1+2}$, the simultaneous levels of the various species in the blood of individuals with APL have been correlated. Linear regression analysis of the data for FPA vs TAT in four individuals with APL who had not recently received heparin (19 determinations) yields a line described by the equation $y = 3.58x - 7.13$, with an associated correlation coefficient ($r$) of .852. This relationship directly supports the hypothesis that the FPA generated in individuals with APL is produced by the action of thrombin upon the fibrinogen molecule. Similar examination of the results derived for FPA vs TAT in four individuals with APL who were receiving the anticoagulant heparin. The data are best described by the equation $y = 0.617x - 2.47$ ($r = .817$).

We have also determined the relationship between the plasma TAT level and the concentration of F$_{1+2}$ in all six individuals with APL (53 determinations). This analysis also included values of patients who were receiving the anticoagulant heparin. The data are best described by the equation $y = 0.184x + 1.43$ ($r = .837$).

The results suggest that the F$_{1+2}$ assay is 1.6 times more sensitive than the FPA assay in detecting the coagulopathy associated with APL. In contrast, the TAT level would appear to be a less sensitive index of hemostatic system hyperactivity than either of the other two assays.

Similar studies have also been carried out in nine ANLL patients with other histologic subtypes (myelocytic, myelomonocytic, and monocytic). Evidence of DIC by standard laboratory tests was only occasionally

| Table 2. The Levels of F$_{1+2}$, TAT, and FPA in APL Patients |
|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | Presentation    | Postchemotherapy |                 |                 |
| Patient        | F$_{1+2}$ nmol/L | TAT nmol/L       | F$_{1+2}$ to TAT | F$_{1+2}$ nmol/L | TAT nmol/L       | FPA nmol/L       |
| 1              | 22.0            | 7.66 ND          | 2.87            | 84.0            | 12.8 ND          | ND               |
| 2              | 20.8            | 3.84 ND          | 5.42            | 68.5            | 19.0 ND          | ND               |
| 3              | 29.1            | 8.12 ND          | 3.58            | 52.0            | 8.15 19.80 ND    | ND               |
| 4              | 49.3            | 7.11 ND          | 6.93            | 50.2            | 7.51 5.58 ND     | ND               |
| 5              | 63.4            | 14.8 ND          | 4.28            | 54.7            | 18.6 107 ND      | ND               |
| Normal values (mean ± SD): | F$_{1+2}$ 1.970 ± 0.970 nmol/L | TAT 2.320 ± 0.360 nmol/L | FPA 0.966 ± 0.514 nmol/L |
| ND indicates that samples for that assay were not drawn.
observed in these individuals. The mean levels of F$_{1,2}$ and TAT in the nine acute nonlymphocytic leukemia patients were oftentimes elevated at presentation: 10.4 ± 7.29 nmol/L and 3.25 ± 1.20 nmol/L, respectively. Four of these individuals had specimens drawn for measurement of FPA, and the mean value was 4.62 ± 3.97 nmol/L. One patient in this latter group had normal levels of F$_{1,2}$, TAT, and FPA. Plasma concentrations of F$_{1,2}$ and FPA usually rose substantially with the onset of chemotherapy, whereas similar patterns in TAT levels were not consistently observed (data not shown). Linear regression analysis of the data for FPA vs TAT (20 determinations) yields a line described by the equation $y = 1.21x + 4.01$, with an associated correlation coefficient ($r$) of .531.

**DISCUSSION**

Recent communications from this laboratory have described sensitive and specific radioimmunoassays to quantitate the levels of F$_{1,2}$ and TAT within human blood.\textsuperscript{14,22} In this article, we report investigations in which these assays, along with that for FPA, were employed to study the hemostatic mechanisms of patients with APL and ANLL. We have chosen to emphasize our findings in APL because of the well-recognized association of this clinical entity with DIC\textsuperscript{1} and thrombotic phenomena.\textsuperscript{4,5}

The FPA measurement reflects the in vivo enzymatic activity of thrombin upon fibrinogen. In all APL patients at clinical presentation, the levels of this peptide were strikingly elevated, whereas TAT concentrations were increased to a lesser extent. These indices of hemostatic system activity often increased substantially during induction therapy, probably as a consequence of the release of substances with procoagulant activity into the circulation. Excellent correlation was noted between measurements of FPA and TAT. This study thus provides the first direct evidence that the FPA generated in vivo is a consequence of the excessive conversion of prothrombin to thrombin.

The F$_{1,2}$ assay, on the other hand, measures the in vivo cleavage of the prothrombin molecule by factor Xa. Large quantities of F$_{1,2}$ were present in the blood of these individuals at initial presentation and followed patterns very similar to those for FPA and TAT with the onset of chemotherapy. Inasmuch as the immunoreactive site defined by our antibody population is located very close to the region at which prothrombin is cleaved by factor Xa,\textsuperscript{12} these results suggest that the F$_{1,2}$ produced in our patients is a direct result of this enzyme's action upon its substrate zymogen. Furthermore, analysis of the relationship between FPA and F$_{1,2}$ levels leads to the conclusion that the prothrombin fragment assay is a more sensitive parameter than FPA in detecting the coagulopathy associated with APL.

The ratio of F$_{1,2}$ levels to that of TAT in APL patients at presentation ranged from 2.87 to 6.93. Although a delayed rate of in vivo clearance of F$_{1,2}$ vis-à-vis FPA from the human circulation might, in part, account for this observation, based on these data and our earlier studies of whole blood coagulation in vitro,\textsuperscript{14} we suggest that prothrombin activation in this disorder is characterized by the accumulation of a stable precursor, such as prethrombin 2. This phenomenon may be related to an alteration of factor V function, which could result from the proteolysis of factor Va by activated protein C.\textsuperscript{23,25} In order to quantitate protein C activation within the vascular system, we have recently developed a radioimmunoassay for the dodecapeptide, which is liberated when thrombin bound to thrombomodulin activates protein C.\textsuperscript{26} Plasma peptide levels in four individuals with APL were elevated ten- to 30-fold compared with normals, which supports our contention that this mechanism may be responsible for the aforementioned observations.

It is of interest to note that the five APL patients evaluated at initial presentation all had evidence of DIC, as manifested by characteristic abnormalities of routine coagulation tests. The prothrombin time was prolonged (3.9 to 6.1 seconds > control) in all individuals. The activated partial thromboplastin time, however, was minimally elevated in only one patient and was actually shorter than control in the others. The disparity between these two laboratory tests has been noted in other clinical studies of APL.\textsuperscript{7} Reduced levels of several of the coagulation system procoagulants, such as factors II, V, and X, have sporadically been reported in these patients.\textsuperscript{1,4} Deficiencies of these zymogens would be expected to contribute to a lengthening of both the prothrombin time and the partial thromboplastin time. A possible explanation for this paradoxical response in the partial thromboplastin time is the presence of activated intermediates upstream in the intrinsic pathway.\textsuperscript{27} It is also of interest that the prolongations of the thrombin time were small (1.9 to 3.0 seconds > control) in four of the patients despite significant elevations in FDP levels. This might be due to the presence of significant levels of fibrin monomer in the circulation, or it is possible that the proteolysis of fibrinogen by enzymes other than plasmin might produce a different population of FDPs which have little effect on the thrombin time determination.\textsuperscript{28,29}

We subsequently set out to examine the correlations between FPA and TAT levels in patients with other subtypes of ANLL. Myers et al\textsuperscript{30} have previously
demonstrated that FPA levels are elevated in patients with various morphological forms of acute leukemia. They also noted that plasma FPA levels increased significantly during induction chemotherapy and decreased to the normal or near-normal range when clinical remission was achieved. Based on these data, it was suggested that subclinical activation of blood coagulation, as defined by elevation of plasma FPA levels, is common in acute leukemia.

Patients with ANLL not having the promyelocytic variant usually had elevated plasma levels of F$_{1+2}$, TAT, and FPA at clinical presentation. The initiation of antileukemic therapy often resulted in a rise in all three indices of thrombin generation in this population. We have also found a positive correlation coefficient between the levels of FPA and TAT. These observations are thus similar to those in patients with APL and directly support the contention that the FPA generated in these individuals results from the action of thrombin. However, the correlation coefficient between these two parameters in this population is lower than that in APL patients, suggesting that a portion of the FPA immunoreactivity measured in the plasma of these individuals may be due to the proteolysis of fibrinogen by other enzymes in addition to thrombin. It is possible that other proteases elaborated by the neoplastic cells could be released into the circulation. These might directly proteolyze fibrinogen, resulting in peptide fragments larger than FPA that are not removed in the extraction procedure and possess immunoreactivity toward the antisera employed in the assay.28 Elastase is an enzyme found in leukocytes that is able to cleave the A-alpha chain of fibrinogen internal to the thrombin-sensitive Arg$_{16}$-Gly$_{17}$ bond, probably between His$_{24}$ and Cys$_{25}$.31 Utilizing immunologic assays for alpha-1-antitrypsin–human leukocyte elastase complexes, evidence has been provided that this enzyme can be released in excess within the vascular system of patients with acute leukemia or sepsis.32,33

We have also had an opportunity to analyze the effects of heparin therapy in patients with APL, and an interesting interrelationship became apparent from our assays that was not evident from the in vitro coagulation studies. Patient 4 in our study was fully heparinized (1,000 U/m$^2$/hr) and demonstrated a dramatic lowering in her plasma FPA level toward the normal range without any concomitant reduction in the concentrations of F$_{1+2}$ or TAT.

Experimental data obtained in purified systems have demonstrated that the association of factor Xa with the platelet surface or phospholipid micelles protects the enzyme against inhibition by the heparin–antithrombin complex.34–36 Teitel et al37 have recently studied the accessibility of factor Xa to inhibition by the mucopolysaccharide–protease inhibitor complex within plasma and whole blood in vitro, employing the radioimmunoassay for F$_{1+2}$ to detect this serine protease. Their findings indicated that the binding of factor Xa to “activated” platelets, but not to phospholipid micelles, results in the protection of the above enzyme from inactivation by the heparin–antithrombin complex. The magnitude of enzyme protection was strongly correlated with the extent of prothrombin activation at the time of heparin addition.

The findings in patient 4 can be explained by the relationship outlined above. A high rate of in vivo thrombin generation in such an individual leads to the formation of substantial amounts of activated platelet-factor Va-factor Xa interaction product. One would then predict that the sequestered factor Xa hidden within this multimolecular complex should be resistant to the action of heparin–antithrombin and that F$_{1+2}$ levels would not decrease immediately with anticoagulant therapy. Thus, the enzyme might still be able to generate thrombin until the above complex has been cleared from the circulation. On the other hand, the thrombin that is generated would be prevented from acting on fibrinogen by the mucopolysaccharide–protease inhibitor complex, resulting in a fall in FPA concentration. The above situation may represent a general phenomenon in which the duration of adequate heparin therapy is partially determined by the intensity of the thrombotic stimulus via the generation of activated platelet-factor Va-factor Xa complex.

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