Loss of Blast Cell Procoagulant Activity and Improvement of Hemostatic Variables in Patients With Acute Promyelocytic Leukemia Administered All-*trans*-Retinoic Acid

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All-*trans*-retinoic acid (ATRA) induces complete remission (CR) in up to 90% of acute promyelocytic leukemia (APL) patients with rapid amelioration of the bleeding syndrome. Previous studies indicate that ATRA treatment in vitro of the APL NB4 cell line can affect their procoagulant activity (PCA). To assess whether ATRA has this effect also in vivo, we prospectively studied the PCA of bone marrow blasts from APL patients on therapy with ATRA alone or associated with chemotherapy. Samples were obtained before, during, and after ATRA. To characterize the coagulopathy, we measured a series of plasma hemostatic variables before and during the first two weeks of therapy, as follows: (1) markers of hypercoagulability; (2) natural anticoagulants; (3) fibrinolysis proteins; and (4) elastase. The results by enzymatic and immunologic methods show that both total (tissue factor-like) and factor VII-independent (cancer procoagulant-like) blast cell PCAs, present before therapy, were reduced during (69% and 65% decrement, respectively) and virtually undetectable after ATRA. The plasma hemostatic assessment of patients before treatment was elevated hypercoagulability markers, low mean protein C, normal fibrinolysis proteins, and increased elastase. After starting ATRA, hypercoagulability markers were reduced within 4 to 8 days, protein C augmented, the overall fibrinolytic balance was unmodified, and elastase remained elevated. These results were not different either with or without chemotherapy and are consistent with the clinical findings of rapid improvement of the coagulopathy.

ACUTE PROMYELOCYTIC leukemia (APL) is a subtype of acute myelogenous leukemia (AML), which is identified by the French-American-British (FAB) classification as AML-M3, including the hypergranular classical M3 and the microgranular variant (M3v).[1,2] The cytogenetic marker of this leukemia is the balanced reciprocal translocation between chromosomes 15 and 17. Another characteristic of AML-M3 is its frequent association with a life-threatening hemorrhagic diathesis, which is worsened by cytotoxic chemotherapy and is responsible for 10% to 20% of early deaths.[3-6] Improving the hemorrhagic complications is, therefore, important in this disease, whose prognosis is otherwise relatively favorable.[5,6]

Remission induction of APL with all-*trans*-retinoic acid (ATRA), a differentiating agent, induces complete remission (CR) in up to 90% of cases. Most importantly ATRA-induced remission is accompanied by a prompt improvement in the coagulation/bleeding syndrome typical of this disease,[7,8] thus influencing early hemorrhagic deaths.

The coagulation/bleeding syndrome of APL is complex.[9,10] Abnormalities of the blood clotting system compatible with the diagnosis of disseminated intravascular coagulation (DIC) are described,[4,11,12] although fibrinolysis and nonspecific proteolysis can also be activated.[13-15]

Factors associated with the leukemic cells are considered the major pathogenetic determinants of the coagulopathy of acute leukemias.[10] The most extensively studied are the blast cell associated procoagulants: (1) tissue factor (TF), which forms a complex with factor VII (FVII) to activate factors X (FX) and IX (FIX) and occurs in normal and malignant tissues[16,17]; (2) a membrane factor V receptor, which facilitates the assembly of prothrombinase complex, thus accelerating its activity up to 100,000 times[18]; and (3) cancer procoagulant (CP), a cysteine proteinase that directly activates FX, independently from FVII,[19] and described in malignant and fetal tissues.[20,21] Several studies have identified TF[16,22,23] and CP[24,25] in blasts of various AML phenotypes.

Differentiating treatment with ATRA can influence the procoagulant activity (PCA) of cultured APL cells. We have characterized CP in the NB4 cell line, the first isolated human APL line with the t(15;17) translocation,[26] and have shown that CP expression is significantly affected by ATRA in vitro.[27] The same cell line possesses TF, which is also significantly depressed by ATRA.[28] It is not known whether ATRA has the same inhibitory effect on the PCA of APL marrow blasts. In addition, though hypercoagulation and fibrinolysis markers are rapidly corrected by ATRA[29,30] in APL, no studies have followed both the blast cell procoagulants and the pattern of these and other markers (eg, natural anticoagulants, tissue plasminogen activators and inhibitors, elastase) in the same patients. Finally it is not known whether ATRA exerts a comparable effect on hemostatic parameters in the presence of chemotherapy. This is an important question because the combination of ATRA with chemotherapy can prolong disease-free survival and may, therefore, be selected for APL treatment.[7,31]

This study was designed to investigate whether ATRA administered in vivo, with or without chemotherapy, reduced the blast cell PCA and simultaneously affected a series of plasma hemostatic variables. The results show for the first time a reduction of bone marrow cell PCA (both CP and TF) in APL patients given ATRA ± chemotherapy. They also confirm and add information on the improvement of plasma hypercoagulation/fibrinolysis markers in these pa-

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Table 1. Hematologic and Hemostatic Characteristics of APL Patients

Patient No.	Age/Sex	FAB	HgB (g/dL)	Plts ($\times 10^9$/L)	WBCs ($\times 10^9$/L)	Blasts (BM%)	PT (INR)	APTT (Ratio)	Fg (mg/dL)
1	72/M	M3	7.1	16	0.4	100	1.28	0.93	368
2	51/M	M3	12.3	90	1.7	70	1.2	1.06	110
3	59/F	M3	10.6	23	2.3	80	1.42	0.73	90
4	15/F	M3V	12.9	10	38.61	100	3.13	1.15	93
5	27/M	M3V	14.1	13	58.4	100	2.68	0.91	94
6	44/F	M3	6.5	21	1.15	100	1.35	0.94	65
7	14/M	M3	10.6	70	1.07	60	1.39	1.03	335
8	33/F	M3	6.4	25	0.5	100	1.27	0.96	120
9	29/M	M3	13.2	26	2.8	70	1.53	1.05	93

tients and show that a benefit persists when ATRA is associated with chemotherapy.

MATERIALS AND METHODS

Patients

Nine consecutive patients with the diagnosis of APL (five men and four women; median age 33 years, range, 14 to 72 years) admitted to our Department between March 1993 and June 1994 were included. Eight patients had newly diagnosed APL and one patient was in first relapse. According to the FAB classification, seven patients had the classical M3 APL, and two had the M3v. The diagnosis was established by morphologic characteristics, cytochemistry, cytogenetic, and immunophenotyping. Cytogenetic analysis indicated the t(15;17) chromosomal marker in all cases but one. PML-RARα gene rearrangement was present in all nine. Seven patients presented with hemorrhagic manifestations, most commonly, mucosal oozing, spontaneous ecchymoses, petechiae, hematuria, and menorrhagia. None had evidence of concomitant infection.

The characteristics of the patients at presentation, before treatment, are shown in Table 1. Patients with the classical M3 (no. 1 through 3 and 6 through 9) were leukopenic (WBC 0.4 to 2.8 $\times$ 10^9/L), whereas the two M3v (no. 4 and 5) had high WBC count (38.6 and 58.4 $\times$ 10^9/L WBC, respectively). All patients had thrombocytopenia (platelets 13 to 90 $\times$ 10^9/L) and 60% to 100% blast cell bone marrow invasion. Other features included prolongation of the prothrombin time (PT) (INR $\geq$1.3 in all but three patients) and low fibrinogen levels ($\leq$120 mg/dL in all but patients no. 1 and 7). The nine patients were a consecutive series: three were enrolled before and six after we had joined the Italian cooperative protocol GIMEMA No. 0493 for APL induction treatment. Patients no. 1, 2, and 3 received oral ATRA (45 mg/m^2/d) until CR (group A). The other six (no. 4 to 9) received oral ATRA (45 mg/m^2/d) simultaneously with the induction chemotherapy (group A + C), consisting of idarubicin 12 mg/m^2/d on days 2, 4, 6, and 8, according to the GIMEMA No. 0493 protocol; ATRA was continued until CR.

All patients were advised of procedures and attendant risks, in accordance with institutional guidelines, and all patients gave informed consent.

Twenty-two sex- and age-matched healthy individuals acted as a control group for the study of plasma clotting and fibrinolytic parameters.

Samples

Bone marrow samples. Bone marrow samples were obtained from all patients at the onset of the disease and at CR. Additional samples were obtained at an intermediate phase (7 to 10 days after starting ATRA) from three patients of group A and two patients of group A + C. Bone marrow specimens (5 to 10 mL) were collected in 3.8% Na-citrate (1 vol:9 vol) + 250 U/mL sodium heparin. Mononuclear cell fractions were enriched by a Ficoll-Hypaque (Lymphoprep, Nyegaard, Oslo) density gradient system, in which mononuclear cells are at the interface and polymorphonuclear cells and erythrocytes are in the bottom pellet. Mononuclear cells were washed three times with phosphate buffered saline (PBS) pH 7.4. As assessed by May-Grunwald Giemsa staining, they consisted of >95% blasts, at T0; and >95% blasts + maturing myeloid cells, at T1. At T2 both myeloid and nonmyeloid normal precursors were present in this fraction.

For testing cell PCA 30 to 40 $\times$ 10^6 mononuclear cells/ml were extracted in two changes of 20 mmol/L Veronal Buffer, pH 7.8, 2 hours each, at 4°C, as described.[24] This extract was also used for the determination of CP Ag. For the detection of TF Ag 20 $\times$ 10^6 cells/mL were resuspended in 50 mmol/L TRIS, 100 mmol/L NaCl containing 1% Triton X-100, pH 7.5 and were disrupted by three cycles of freezing and thawing. Extracts were done at 4°C for 3 hours.

Blood samples. To measure the plasma levels of hemostatic variables, blood samples were collected from all patients before and every other day for the first two weeks after starting ATRA ± chemotherapy. Samples were collected between 7:30 and 8:00 a.m., before therapy. Blood was drawn in 3.8% Na-citrate (9 vol:1 vol). Plasma was separated by centrifugation at 3,000g for 20 minutes at 4°C within 1 hour of blood collection. Samples were stored at −80°C until assayed (<3 months).

Assay Methods for Cell PCA

PCA assay. PCA was measured on the Veronal buffer extracts of the bone marrow mononuclear cells by the one-stage recalcification assay of normal human plasma (NHP), as described.[19] Briefly, 0.1 mL samples were incubated with 0.1 mL NHP for 1 minute at 37°C, the reaction was started by addition of 0.1 mL 0.025 mol/L $CaCl_2$ and clotting time was recorded. To assess the FVII dependence of PCA, the recalcification assay with human plasma congenitally deficient of FVII (FVII-def plasma, Behringwerke) was done. The coagulation controls of this assay were rabbit brain thromboplastin (RBT, Sigma Chemical Company, St Louis, MO) as a standard FVII-dependent procoagulant and Russell's viper venom (RVV, Sigma) as a standard FX direct activator.

PCA was expressed as seconds or as specific activity = RVV units/mg of total protein. Units were calculated on a calibration curve obtained with different dilutions of RVV (from 10^{-1} to 10^{-6}) as described[24]; 1 unit = activity of 1 mEq/mL of RVV in the one-stage clotting assay. The total protein content of cell extracts was determined by a modified Lowry's method.[32]

Inhibition study. To study the PCA enzymatic characteristics, bone marrow cell preparations were tested in the presence of three cysteine proteinase inhibitors (a property of CP), $HgCl_2$ (Sigma),

Iodoacetic acid (IA; Sigma) and Z-Ala-Ala peptidyl diazomethyl ketone (ZAA-CHN_2, Enzyme System Products, Dublin, CA), and one TF inhibitor, Concanavalin A (Con A, Sigma).[24,25] RVV, a serine proteinase FX activator, papain, a cysteine proteinase FX activator, and RBT, a standard TF, were the experimental controls to calibrate the inhibition study. Samples and standards were incubated for 30 minutes at 25°C with either $HgCl_2$ (0.1 mmol/L), IA (1 mmol/L) or ZAA-CHN_2 (0.2 mmol/L) before the plasma recalcification assay. They were incubated with Con A (200 μg/mL) for 50 minutes at 37°C before testing for PCA.[24,25]

CP Ag. CP was immunologically identified and quantified in Veronal buffer cell extracts by an immunocapture enzyme (ICE) assay, using a pure monoclonal anti-CP IgM, according to Mielicki et al.[33] Briefly, cell extracts were incubated in IgM precoated microtiter wells for 2 hours at 37°C, so that CP Ag was captured by the monoclonal antibody (MoAb). After washing five times with PBS plus 0.05% Tween 20, the activity of the antigen was detected by a three-stage chromogenic assay. In the first stage, bovine FX (Hematologic Technologies Inc, Essex, VT; 7 μg/mL in 10 mmol/L bis-TRIS, pH 7.4) was added as a CP substrate and incubated for 1 hour at 37°C. Thereafter (second stage), bovine prothrombin (Sigma) was added, and after a 30-minute incubation at 37°C, the chromogenic substrate for thrombin (Sar-Pro-Arg-pNA, Sigma) was added (stage three). The absorbance at 405 nm was measured to determine the amount of thrombin generated by FXa, activated by CP. Results were expressed as micrograms of CP per milligram total protein. CP micrograms were calculated on a calibration curve obtained with different concentrations of pure CP (from 0.5 to 10 μg/mL).[27]

TF Ag. TF was immunologically identified and quantified in TRIS/NaCl buffer 1% Triton X-100 cell extracts by an enzyme-linked immunosorbent assay (ELISA) method using the Imubind Tissue Factor kit (American Diagnostica Inc, Greenwich, CT), according to the manufacturer's instructions. Results were expressed as TF pg/10^6 cells, calculated from a calibration curve of standard TF.

Plasma Coagulation Parameters

Routine clotting tests. PT and activated partial thromboplastin time (APTT) were determined by standard procedures using reagents from Ortho Diagnostic System (Milan, Italy). Fibrinogen was measured by the Organon Teknica assay (Organon Teknica Corp, Durham, NC) based on the Clauss technique.

Hypercoagulation markers. TAT complex plasma levels were determined by ELISA, using the Enzygnost TAT kit (Behringwerke, Marburg, Germany). F1 + 2 levels were measured by ELISA, using the Enzygnost F1 + 2 kit (Behringwerke, Marburg, Germany). D-dimer levels were determined by the Ortho Dimertest Latex reagents (Ortho Diagnostic System).

Coagulation inhibitors. Protein C (PC) was measured by an automated functional chomogenic assay, using Coamate Protein C reagents (Chromogenix, Molndahl, Sweden), on an ACL 300 Instrument. Protein S (PS) was measured by an automated functional coagulation assay, using IL Protein S reagents (Instrumentation Laboratory, Milan, Italy), on an ACL 300 Instrument. Antithrombin (AT) was measured by a manual functional chromogenic assay, using Coatest Antithrombin reagents (Chromogenix).

Plasma Fibrinolytic and Proteolytic Parameters

t-PA antigen levels were determined by ELISA, using the Imubind-5 t-PA kit (American Diagnostica Inc). PAI-1 antigen levels were measured by ELISA, using the Imubind plasma PAI-1 kit (American Diagnostica Inc). The Euglobulin Lysis Area (ELA) was measured as an indicator of overall plasma fibrinolytic activity. The euglobulin fraction was prepared with diluted plasma (1:10 with cold bidistilled water) acidified to pH 5.9 with 0.25% acetic acid (vol/vol). Samples were kept on ice for 30 minutes then centrifuged at 3,000*g* for 10 minutes, at 4°C. The resulting precipitate (euglobulin fraction) was resuspended in 0.05 mol/L Tris buffer + 0.01% Tween 20 (pH 8.3) and sampled (30 μL) on fibrin plates. The diameter of the lytic circle was measured after an 18-hour incubation at 37°C and the area (mm^2) was expressed. Fibrin plates were prepared using human fibrinogen (Sigma). t-PA specific activity was tested on the euglobulin fraction by a chromogenic assay (Coa-Set t-PA kit, Ortho Diagnostic System). Elastase, as circulating elastase-α1-proteinase inhibitor complexes, was determined by an immunoassay (PMN Elastase IMAC, Merck, Darmstadt, Germany).

Statistical Analysis

The unpaired Student's *t*-test was used for comparisons of cell extract PCA before and during ATRA treatment and to compare baseline hemostatic variables with those of normal control subjects. The paired Student's *t*-test was used to assess differences between untreated and inhibitor-treated samples in the PCA inhibition study. The two-way analysis of variance was used for inter/intra group differences in hemostatic parameters during treatment.

RESULTS

Treatments A and A + C resulted in 9/9 CR. During the first week of induction regimens, the bleeding symptoms rapidly improved in line with the routine coagulation tests. The platelet number progressively increased to the median level of 50×10^9 and plasma fibrinogen returned to the normal range, with no significant differences between the two treatments (A and A + C). In the second week platelet count was lower in the chemotherapy-receiving subjects, who also needed more platelet concentrates support.

Bone marrow samples' PCA. To determine the effect of ATRA on the promyelocytes' procoagulant properties, PCA was measured on the mononuclear cell fraction from bone marrow specimens of ATRA-treated subjects as follows: (1) before therapy (time 0, T0); (2) after 7 to 10 days ATRA therapy (time 1, T1); and (3) on CR (time 2, T2). At T0 (Fig 1) samples showed PCA in the assays of NHP (total PCA, mean ± SD, 16.3 ± 6.8 RVV unit/mg) and of FVII-def plasma (FVII-independent [CP-like] PCA, 9.1 ± 5.7 RVV units/mg). Both types of activities were significantly decreased after starting ATRA (Fig 1, T1) and became almost undetectable upon CR (Fig 1, T2). To further verify whether the PCA of APL cell extracts shared enzymatic characteristics of known procoagulants, like CP and TF, we tested the sensitivity of four samples (two from group A and two from group A + C) to the three cysteine proteinase inhibitors (a property of CP) and one TF inhibitor at T0 and T1 (Table 2). Treatment with 0.1 mmol/L $HgCl_2$ or 1 mmol/L IA or 0.2 mmol/L ZAA-CHN_2 significantly affected PCA ($P < .01$). The control cysteine proteinase, papain, was sensitive to these inhibitors in the same assay, whereas RVV, the control serine proteinase, was not. The PCA of the same samples was also susceptible ($P < .01$) to the TF inhibitor Con A. RBT, the standard TF, was highly sensitive to Con A in the same assay.

TF and CP Ag. In Fig 2, the patterns of TF and CP Ag of bone marrow samples at the different time of treatment are depicted. Figure 2A shows the TF Ag levels of samples

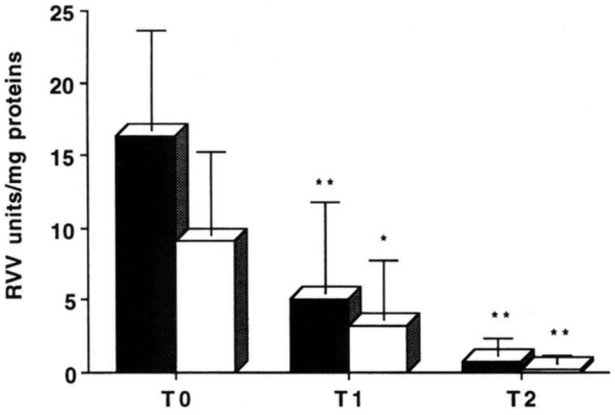

Fig 1. Total PCA (■) and FVII-independent PCA (□) of bone marrow blast extracts of ATRA-treated subjects at diagnosis (T0, n = 9), at an intermediate phase (T1, 7 to 10 days of ATRA treatment, n = 5), and at CR (T2, n = 9). PCA (mean ± SD) was expressed as RVV units per milligram protein. Units were calculated on a calibration curve obtained with different dilutions of RVV (from 10^{-1} to 10^{-6}) as described[9]; 1 unit = activity of 1 mEq/mL of RVV in the one-stage clotting assay. Statistical analysis was done using the unpaired Student's *t*-test. *$P < .05$; **$P < .01$.

from four patients, two from group A (tested at T0, T1, and T2), and two from group A + C (tested at T0 and T2). Figure 2B shows CP Ag of samples from six patients, two from group A (tested at T0, T1, and T2) and four from group A + C (tested at T0 and T2). Results are the mean of two determinations for each sample. At T1 there was a substantial decrease of the two proteins: on the average, 64% reduction of TF and 60% of CP. At T2 both procoagulants were virtually undetectable (96% and 98% reduction, respectively).

Hemostatic parameters. At the onset of the disease all patients had plasma [D-dimer] above the range of normal controls (median: 1.6 μg/mL; range, 0.4 to 3.2 μg/mL) (Fig 3A). The median level dropped to 0.4 μg/mL by day 2 and 4 after starting therapy and reached the normal range by day 6; by the end of the observation period, the majority of subjects were within the control range. Like D-dimer, the patients' plasma [TAT] and [F1 + 2] at presentation were both elevated (Fig 3B and C) (median values were 23.8 ng/mL and 11.0 nmol/L, respectively). Induction treatments progressively reduced the median values of both parameters until day 8. However, they never reached the normal range and remained slightly high during the second week of observation, indicating the persistence of a hypercoagulable state.

To further characterize the hemostatic assessment, the plasma levels of natural anticoagulants, fibrinolysis, and proteolysis proteins were determined. Baseline plasma PC levels, but not PS and AT, were significantly lower than those of control subjects ($P < .001$) (Table 3). Four of the nine patients (one of group A and three of group A + C) had [PC] below the lower limit of the controls. After starting therapy (Fig 4A), [PC] increased from day 1 to day 14 with no differences between the mean values of the two groups. However within-group analysis showed a significant increase of mean [PC] in group A + C on day 14 compared with baseline ($P < .05$). [PS] tended to decrease with treatment (Fig 4B): in group A the mean value on day 14 was significantly lower than basal ($P < .01$) and day 4 values ($P < .05$). However, [PS] constantly remained within the normal range. Variations of [AT] during therapy were not significant (Fig 4C).

The fibrinolytic assessment of the APL patients was based on the plasma levels of t-PA and PAI-1 Ag, the overall fibrinolytic activity (ELA), and t-PA activity. Before therapy, all four parameters of the nine patients appeared similar to controls (Table 3). After starting treatment (Fig 5A and B), the two Ag levels remained stable in group A + C throughout the observation time, but both started to increase from day 4 to day 14 in the patients receiving ATRA alone. By day 14 the mean levels of t-PA and PAI-1 Ag of group A were significantly higher than on day 4; they were also significantly higher than group A + C day 14 ($P < .05$). Thus, the overall fibrinolytic activity (ELA) of group A was not modified, although the proportion due to t-PA specific activity was increased in this group on day 4 compared with baseline ($P < .05$). The overall fibrinolytic balance was not different between the two groups (Fig 5C and D) and values were on average within the normal range on each time point (Fig 5D).

A study of the same fibrinolysis parameters in three of the same patients (one from group A and two from group A + C) during a subsequent phase of consolidation chemotherapy (without ATRA) showed profound depression of PAI-1 release, which corresponded to a significant increment in fibrinolytic activity (data not shown).

The plasma level of elastase-α1-inhibitor complex was the parameter of neutrophil-mediated proteolysis (Table 3). Patient basal levels of circulating elastase (mean ± SD 459 ± 217 μg/L) were significantly higher ($P < .001$) than those of the control group (mean ± SD 77.2 ± 28 μg/L). The absolute amount of this enzyme after 1 and 2 weeks of induction therapy showed no differences between groups (Fig 6A); only within group A + C a significant decrease was observed in the second week. However, the two groups of patients had widely different WBC counts during treat-

Table 2. Sensitivity of APL Blast Extracts' PCA to Cysteine Proteinase and TF Inhibitors

	Inhibitors			
Sample	Iodoacetic Acid 1 mmol/L	HgCl2 0.1 mmol/L	ZAA-CHN2 0.2 mmol/L	Con A 200 μg/mL
T0				
Untreated	92.9 ± 5.1	92.3 ± 4.6	91.2 ± 8.4	109.9 ± 7.1
Treated	132.6 ± 12.1	191.8 ± 16.2	125.7 ± 8.1	139.4 ± 10.3
T1				
Untreated	134.2 ± 2.6	134.2 ± 2.6	134.2 ± 2.6	160.8 ± 16.6
Treated	184.1 ± 4.6	230.3 ± 16.2	156.5 ± 18	182.2 ± 2.6

Samples from four APL patients, two for each group, at diagnosis and at an intermediate phase, were incubated in duplicate with the inhibitor before the plasma recalcification assay. The results (mean ± SD) are expressed as seconds normalized to a blank clotting time of 250 seconds. All the samples are significantly affected by the inhibitors (statistical evaluation was made by a paired Student's *t*-test).

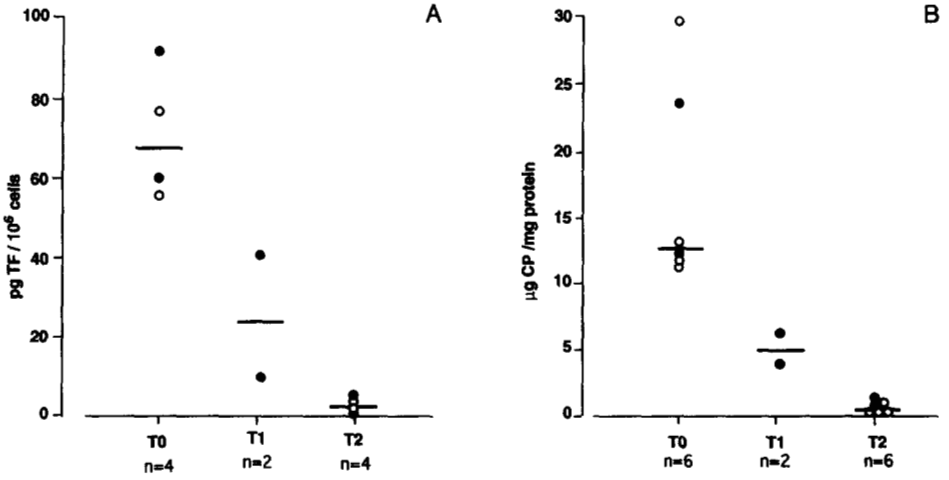

Fig 2. TF antigen (A) and CP antigen (B) levels in cell extract samples of ATRA-treated subjects, as measured at diagnosis (T0), at an intermediate phase (T1, 7 to 10 days of ATRA treatment) and at CR (T2). Black dots represent group A, open dots represent group A + C. Results are the mean of two determinations for each sample; median values at each time point are depicted by the horizontal bars. TF Ag is expressed as TF pg/10⁶ cells. CP Ag levels are micrograms CP per milligram protein, calculated on a calibration curve obtained with different concentrations of pure CP (see Materials and Methods).

ments, group A showing an increment and group A + C a decrement (day 14 WBC count: group A = $10.96 \pm 5.21 \times 10^3$/L; group A + C = $1.14 \pm 1.05 \times 10^3$/L). The concentrations of elastase/WBC ($\mu g/10^3$ WBC) actually decreased in both groups: in group A the day-8 and day-14 values were significantly lower than the basal value ($P < .01$) (Fig 6B).

DISCUSSION

ATRA therapy for remission induction in APL rapidly improves the coagulopathy/bleeding syndrome that causes early deaths in this disease.[4-6] The profound inhibitory effect of ATRA on the expression of two procoagulants in the NB4 cells in vitro prompted us to study whether this mechanism was also active on cell PCA in vivo in APL. This work describes blast cell PCA and a series of hemostatic characteristics in nine APL patients receiving ATRA. Three received oral ATRA alone as remission induction therapy until CR, and six received ATRA combined with induction chemotherapy. ATRA significantly reduced both CP and TF procoagulants in APL patients' bone marrow cells. In addition, laboratory tests of coagulation, fibrinolysis, and proteolysis in the first two weeks of therapy showed treatment-associated reduction of hypercoagulation with improved protein C level, rapid decrease of the D-dimer, with an unmodified plasma fibrinolytic response, and persistently high levels of elastase.

PCA was identified by three different criteria: (1) the clotting activity by the one-stage clotting assay of NHP and FVII-def plasmas; (2) characterization of PCA by testing the sensitivity to specific inhibitors; and (3) immunologic identification of two procoagulants by specific anti-TF and anti-CP MoAbs. These methods have been used in previous studies to characterize cell PCA.[16-22,27,28]

The clotting assay of NHP and FVII-def. plasma provided the first criterion for identifying total PCA (including all possible procoagulants present) and the proportion of FVII-independent PCA. PCA was measured on the mononuclear cell fraction from bone marrow specimens. The myeloid cells were prominent in samples collected at T0 (>95% blast promyelocytes) and at T1 (>95% blasts + maturing cells), while at T2 myeloid and nonmyeloid precursors were represented. However, we did not take into consideration the other cell types in the late samples because we knew that normal bone marrow mononuclear cells do not express any PCA.[24,34] Only blast cells, including those of the lymphoid lineage,[35] constitutively possess different procoagulants. Peripheral blood monocytes can express PCA (TF) too, but only after appropriate stimuli. Therefore, in our conditions (in the absence of any stimulus), the bone marrow PCA reflects the activity of malignant cells.

All the clotting tests were done on cell extracts in Veronal buffer. Because of the limited amount of cells from each bone marrow sample, we gave priority to the Veronal buffer extract preparation, because we have experience with this type of sample and in previous studies have successfully measured both total and FVII-independent PCA.[24,25] The extracts were available for all of the patients at T0 and T2, but at T1 they were missing for four in group A + C because of the particularly poor cell recovery from the bone marrow of chemotherapy-treated subjects at this time. At T0, a large proportion (57%) of PCA was FVII-independent, confirming our previous findings on AML-M3 patients before therapy, in the same experimental conditions.[24,25]

The additional two criteria used to characterize blast cell PCA confirmed at least two procoagulants. The study of sensitivity to inhibitors included Con A, as a known TF inhibitor, and three cysteine proteinase inhibitors known to inhibit CP, ZAA-CHN_2 being very active against purified CP and helpful in previous studies in acute leukemias.[25] APL blast samples were significantly affected by all the inhibitors, as already reported,[24,25] suggesting the presence of CP and TF.

Finally, the third criterion was the immunologic identification of the two proteins, which had to be done on ad hoc prepared samples. The Veronal buffer cell extract was used for the CP ICE, and a TRIS/NaCl 1% Triton cell extract was prepared for the ELISA of TF. Ad hoc experiments had shown this was the most efficient condition for TF Ag recovery. CP Ag was detected by an immunoenzymatic method, which measures the enzymatic activity of the Ag captured by the MoAb; therefore CP Ag is expressed as specific enzy-

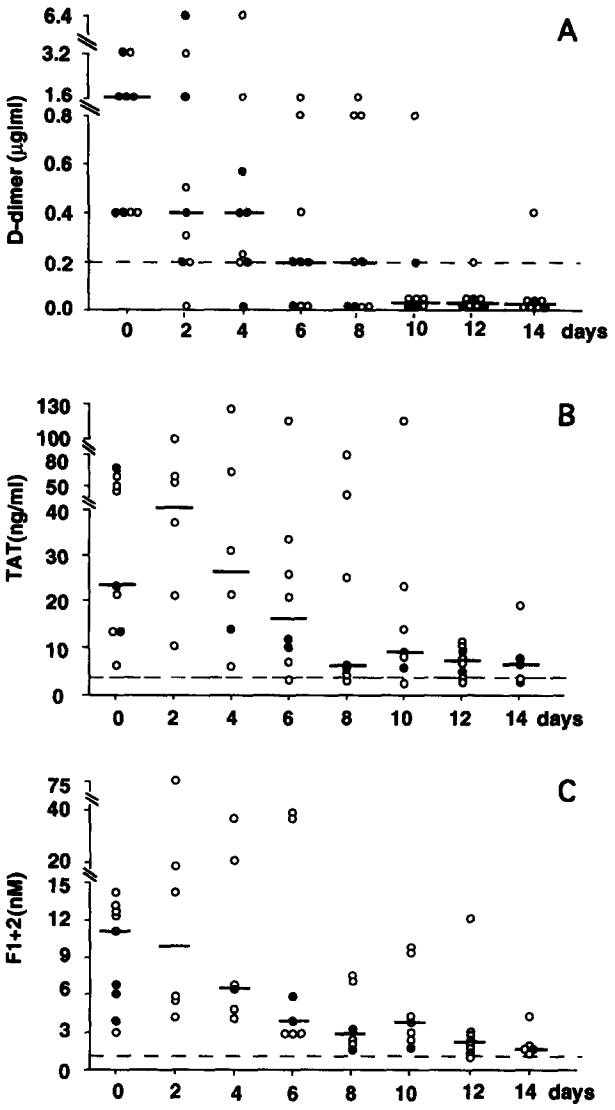

Fig 3. Plasma levels of D-dimer (A), TAT complex (B), and F1 + 2 (C) in patients during the first 2 weeks (day 0 = basal value, before therapy). Determinations were done in duplicate on samples obtained every other day. (●), mean values of samples from group A (on induction therapy with ATRA alone); (○), mean values of samples from group A + C (on induction therapy with ATRA + chemotherapy). The dashed lines indicate the upper limit of the normal control range for each parameter. Median values for the whole group of subjects at each time point are depicted by the horizontal bars.

matic activity (CP µg/mg total protein). TF Ag was detected by a double-antibody ELISA in the Triton solubilized cell samples and the results were expressed per cell number (the protein content was not determined because of interference by the detergent [Triton]).

The two Ag assays were, therefore, profoundly different and did not allow direct comparison of the two procoagulants, which remains an unresolved issue. However our study, which was not designed to evaluate the relative roles of the procoagulants, but primarily to follow the changes in PCA in response to ATRA therapy, does address the main question. In this respect the Ag levels are consistent with the results of the clotting study, indicating a downregulation of PCA by ATRA, and confirm previous in vitro evidence on the NB4 cells.[27,28] It is worth noting the reduction of the two Ag in the two A group subjects at T1 (7 to 10 days treatment), when, in the absence of the chemotherapy-induced hypoplastic bone marrow phase, the myeloid cells could be analyzed. Whether the Ag and the PCA decreases are produced by a direct mechanism or are part of the differentiation process remains to be clarified.

In parallel with the decrease in the cell procoagulant potential the signs of clotting activation in the patients' plasma also progressively decreased. At enrollment, before therapy, the assessment of coagulation confirmed previous findings of hypercoagulation with secondary hyperfibrinolysis[29,30] in APL, ie, elevation of TAT and F1 + 2, the two sensitive markers of thrombin generation, and of D-dimer, the byproduct of plasmin action on cross-linked fibrin. Among coagulation inhibitors, AT was in the normal range, in line with published data,[36,37] whereas PC was abnormally low in 44% of our patients, a figure also close to that reported by others.[37]

After starting ATRA, hypercoagulation markers and D-dimer rapidly dropped within the first week, as reported.[29,30] However, TAT and F1 + 2 were not completely normal in the second week, indicating persistent moderate/low activation of blood coagulation. Interestingly our study shows for the first time that the quenching effect on hypercoagulation markers and D-dimer is also present when ATRA is given in combination with chemotherapy, which on its own worsens the hypercoagulable state. Thus, it appears that the combined regimen leads to a condition different from that of chemotherapy alone.[30] Whether these findings lead to reduced mortality in these patients is a matter to be established

Table 3. Baseline Plasma Levels of Natural Anticoagulants, Fibrinolytic and Proteolytic Parameters of APL Patients

	Patients (n = 9)	Controls (n = 22)	P*
Anticoagulants			
Protein C (%)			
[median (range)]	71 (33-107)	99 (69-145)	.001
Protein S (%)			
[median (range)]	130 (75-156)	123 (71-141)	NS
AT-III (%)			
[median (range)]	103 (85-121)	102 (80-125)	NS
Fibrinolysis			
t-PA Ag (ng/ml)			
[median (range)]	5.7 (2, 5-11)	8.5 (3-18, 5)	NS
PAI-1 Ag (ng/ml)			
[median (range)]	8.5 (4, 5-25, 1)	9 (1, 8-29, 5)	NS
t-PA Act. (U/mg)			
[median (range)]	0.65 (0, 1-1, 5)	0.5 (0, 1-1, 5)	NS
ELA (mm2)			
[median (range)]	106 (9, 6-195)	130 (89-310)	NS
Proteolysis			
Elastase (pg/L)			
[median (range)]	421 (185-800)	78 (35-172)	.001

* Statistical evaluation was made by the nonparametric Mann-Whitney U test.

Abbreviation: NS, not significant.

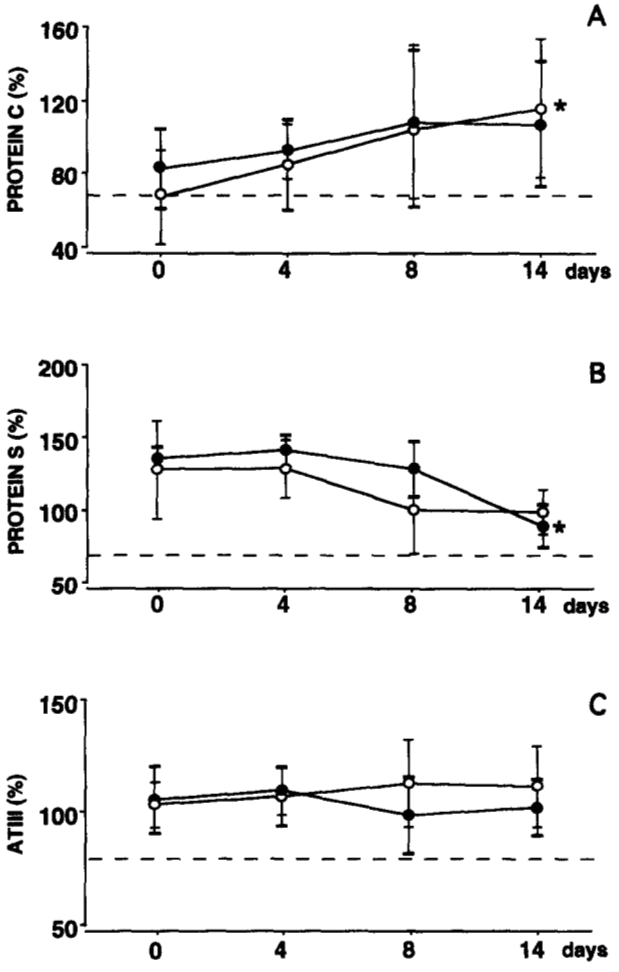

Fig 4. Plasma PC, PS, and AT levels of APL patients during the first 2 weeks of treatment (day 0 = basal value). Results are indicated as mean ± SD for each time point for each separate group; (●), group A; (○), group A + C. The dashed lines are the lower limits of the normal control range. PC (A) increased with the time of treatment in both groups; this became significant for group A + C on day 14 compared with the baseline ($P < .05$). PS (B) decreased, though values remained above the lower limit of the normal range; the decrease was statistically significant ($P < .05$) for group A on day 14 compared with the baseline and day 4 levels. AT (C) showed no significant variations. Statistical analysis was done by two-way analysis of variance.

by large clinical trials. In this study ATRA also had a beneficial effect on the plasma level of the physiologic coagulation inhibitor PC. This original observation provides further evidence of the resolution of DIC. Alternatively, this result might in part be dependent on the known stimulant effect of ATRA on thrombomodulin (TM) expression by both endothelial[38] and leukemic cells.[39] The TM/thrombin complex acts as a potent activator of PC, thus providing an additional mechanism for anticoagulation in this condition.

The fibrinolysis/proteolysis assessment at enrollment showed that the overall fibrinolytic activity (ELA) and t-PA specific activity were not different from normal controls, like the levels of t-PA and PAI-1 Ag, whereas [elastase] was greatly elevated. These findings are in agreement with our previous report of normal fibrinolysis proteins/activity in the plasma of AML-M3 patients at presentation.[40] The elevation of the D-dimer at the onset of the disease, like the signs of increased plasmin activity reported by others (ie, decreased plasminogen and α-2-plasmin inhibitor, increased plasmin/α-2-plasmin inhibitor complex),[30,36] might reflect localized hyperfibrinolysis, taking place on the blast cell[41] or endothelial cell surface.[42] In addition, the elevated basal [elastase], already reported,[43] might further contribute to hyperfibrinolysis by proteolytically degrading the α-2-plasmin inhibitor.[44]

After starting ATRA, both with and without chemotherapy, the overall plasma fibrinolytic activity appeared generally unmodified. In group A, a significant increase of t-PA Ag was paralleled by an increase of PAI-1Ag, with a resulting even fibrinolytic balance. Because ATRA can stimulate the synthesis of plasminogen activators (t-PA and u-PA) and their inhibitors (PAI-1 and PAI-2) by endothelial[45] or leukemic cells,[46] we suggest that the increase of the two Ag is due to a direct action of ATRA on these cell systems in APL patients. ATRA did not affect the two Ag levels in the presence of chemotherapy. We have evidence (data not shown) that chemotherapy without ATRA greatly depresses PAI-1 levels, with a resulting significant increase of plasma fibrinolytic activity (ELA and t-PA). Persistent hyperfibrinolysis (high D-dimer and plasmin/α2-plasmin inhibitor) in the first week of chemotherapy without ATRA was also documented by Kawai et al.[30]

We speculate that the presence of ATRA might prevent the drop in PAI-1 level and the consequent hyperfibrinolysis caused by chemotherapy. In any case, during ATRA therapy, there was no modification of the plasma fibrinolytic balance and the initial signs of reactive hyperfibrinolysis were rapidly quenched. At the onset of APL, the fibrinolytic system may be triggered at a cellular site, where the presence of specific receptors favors the assembly of all of the fibrinolytic components. Thereafter, ATRA-induced PA inhibitor synthesis may downregulate receptor-bound plasminogen activators activity as described in vitro.[46,47] This would result in an unmodified fibrinolytic response. These results may support the clinical finding of thromboembolism, when antifibrinolytic agents are given in the course of ATRA induction therapy.[48] Previous experience with these agents[49] concerns non–ATRA-treated APL patients, so the use of ATRA for APL treatment raises new questions in this field.

Despite the high plasma concentrations of elastase inhibitors, several studies point to a possible in vivo effect of neutrophil elastase on proteins of the coagulation and the fibrinolytic system and on their inhibitors.[50] However, our results question whether this enzyme makes an important contribution to the bleeding disorders of APL. There was, in fact, no relation between [elastase] and the levels of the D-dimer and other hemostatic variables during treatment. The observation that the concentration of elastase corrected for WBC count was significantly reduced by ATRA suggests decreased lysis or releasing activity of the ATRA-differentiated promyelocytes.

In conclusion, this study found that: (1) the procoagulant

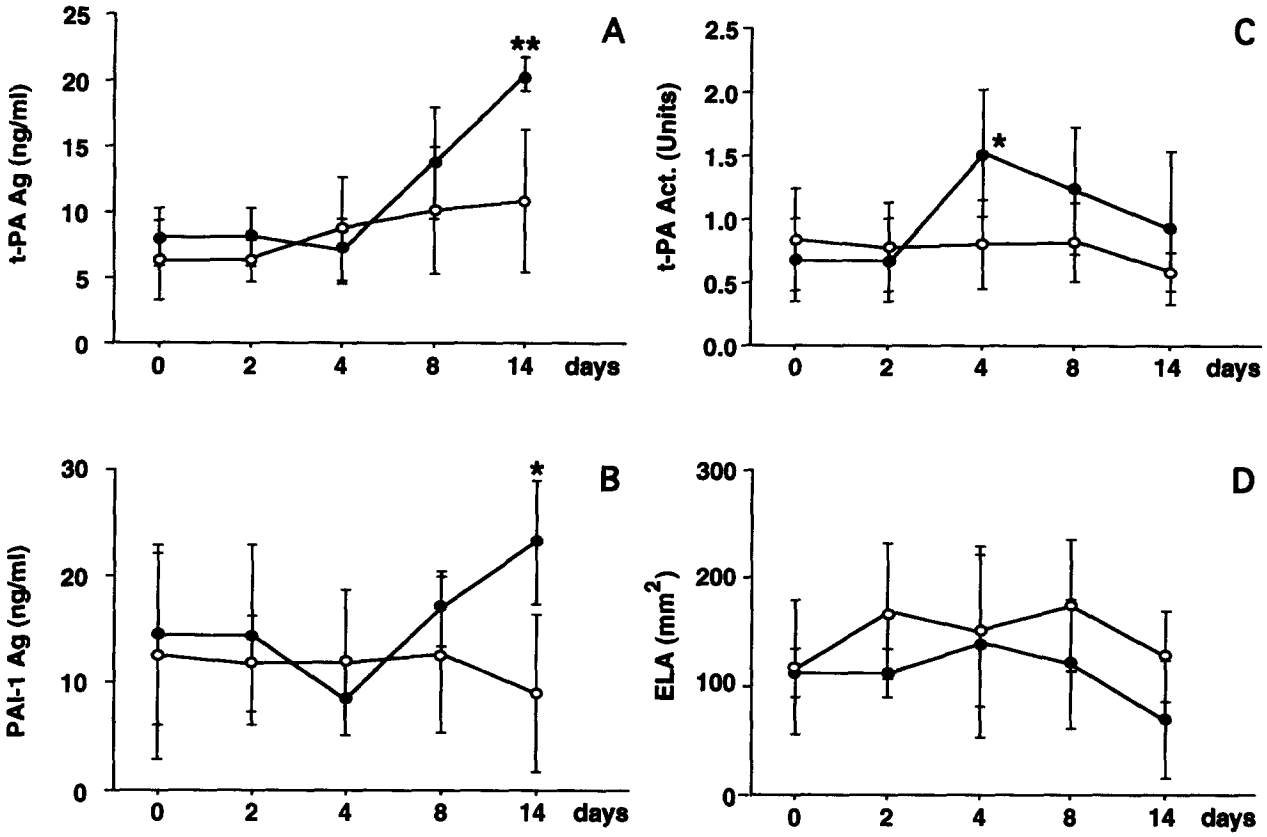

Fig 5. Plasma fibrinolytic parameters of APL patients during the first 2 weeks of treatment (day 0 = basal value). Results (mean ± SD) and statistics as in Fig 4. Black dots represent group A; open dots represent group A + C. t-PA and PAI-1 Ag (A and B) rose significantly in group A (t-PA Ag of day 14 *v* day 4: $P < .01$; PAI-1 Ag of day 14 *v* day 4: $P < .05$) and the differences between the two groups were significant for both Ag on day 14 (t-PA Ag of group A *v* group A + C: $P < .05$; PAI-1 Ag of group A *v* group A + C: $P < .05$). t-PA activity and total fibrinolytic activity (euglobulin lysis area, ELA) (C and D) were not different between the two groups. Only the t-PA activity value of group A on day 4 was significantly greater ($P < .05$) than on day 0.

properties of blast promyelocytes from APL patients appear to be downregulated by ATRA. (2) These results match the resolution of the bleeding symptoms and the quenching effect on hypercoagulation and fibrinolysis markers in the same subjects. (3) ATRA mainly affects the pattern of hypercoagulation, leaving the plasma overall fibrinolytic potential unmodified. Results are comparable when ATRA was given with or without chemotherapy. Because ATRA has independent effects on different cell hemostatic components, including blasts and endothelial cells, it appears that its actions

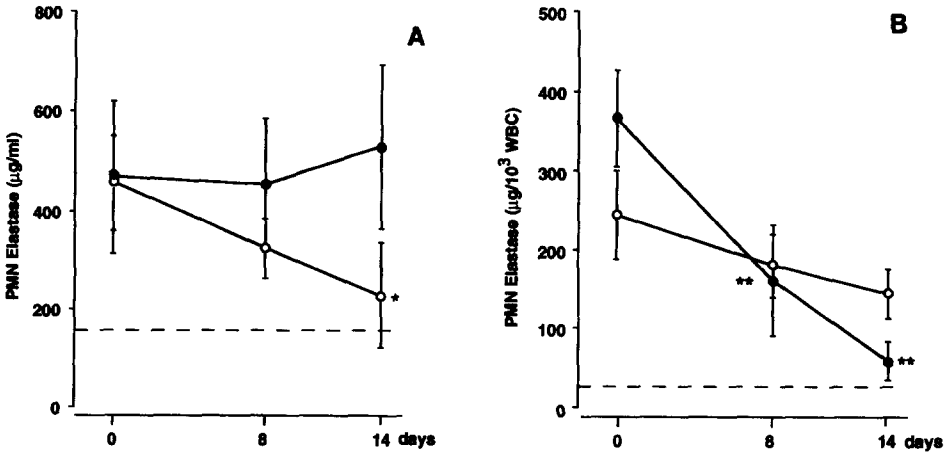

Fig 6. Elastase-α1-proteinase-inhibitor complex (mean ± SD) of APL patients on day 0 (basal value) and on days 8 and 14 of treatment. (●), group A; (○), group A + C. The plasma enzyme concentration (left panel) was not statistically different between the two treatment groups, whereas levels were significantly lower within group A + C on day 14 as compared with day 0. The elastase/10^3 WBC (right panel) was not statistically different between the two groups, but in group A, it was significantly lower than baseline on days 8 and 14. Statistical analysis as in Fig 4.

contribute to the correction of coagulopathy in the early phase of APL.

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