Use of All-trans Retinoic Acid in the Treatment of Acute Promyelocytic Leukemia

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A. Introduction

Acute promyelocytic leukemia (APL) is considered to be a distinct entity among the acute myeloid leukemias (AMLs). Hemorrhagic diathesis often occurs and results in a rapid fatal outcome. The bleeding episodes are usually attributed to thrombocytopenia and/or disseminated intravascular coagulation (DIC), which is thought to result from the release of a procoagulant factor from the promyelocyte granules [1].

The use of daunorubicin in the induction therapy and improvement in the supportive therapy has greatly raised the rate of complete remission (CR) in APL [2, 3]. However, the increased mortality during induction therapy is higher in APL than in other forms of AML [3, 4] and there are still cases which continue to be refractory to induction chemotherapy. DIC remains a common lethal complication.

Induction of differentiation may be an alternative approach to the treatment of APL. Retinoic acid (RA), an analog of vitamin A, is one of the many agents which can induce differentiation and terminal cell division of leukemic cells in vitro [5]. At the present time, several cases of APL treated with 13-*cis* RA have been reported with encouraging results [6–9]. In this paper, we report the in vitro studies and therapeutic trials of 24 APL patients using all-*trans* RA.

B. Materials and Methods

I. Patients

The diagnosis of APL was made according to the criteria of the French-American-British (FAB) cooperative study group [10]. Every patient presenting to our hospitals since early 1986 with a diagnosis of APL was included in this study. The clinical characteristics of the 24 patients with APL are shown in Table 1. There were 11 females and 13 males, with a mean age of 35.5 years (range = 5–69 years). The total white blood cell counts ranged from 0.5×10^9/liter to 15.8×10^9/liter, including 20 cases (83.3%) with less than 3×10^9/liter, 3 cases (12.5%) with between 3×10^9 and 10×10^9/liter, and 1 case (4.1%) with more than 10×10^9/liter. The hemoglobin concentrations ranged from 41 g/liter to 121 g/liter including 8 cases (33.3%) with less than 60 g/liter, 12 cases (50%) between 60 and 90 g/liter and 4 cases (16.7%) with more than 90 g/liter. Platelet counts ranged from 10×10^9/liter to 337×10^9/liter including 15 cases (62.5%) with less than 50×10^9/liter, 7 cases (29.2%) between 50×10^9 and 100×10^9/liter and 2 cases (8.3%) with more than 100×10^9/liter. The percentage of promyelocytes in the marrow ranged from 15.6% to 94%, with 22 patients having more than 30% and the remaining 2 patients having between 15.6% and 30%. Of these 24 studied patients, 16 had never been treated. The

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Table 1. Data of 24 patients with APL

Case	Sex	Age (years)	Previous therapy/ duration	PB WBC ($\times 10^9$/liter)	BM Prom (%)	Present therapy (mg/m^2/day)	PR (day)	CR (day)	BM Prom	Further therapy	Duration of CR (months)
1.	F	5	HOAP/7d HOP/9d H/20d	1.16	78	RA 80	31	68	1.5	(1)	8+
2.	M	6	HOP/26d	1.7	73.5	RA 100	18	28	2.5	(3)	–
3.	F	28	HOA/62d	1.8	89	RA 60	21	34	2.0	(3)	4[a]
4.	M	8	HOAP/10d OH/21d CR/3 months Relapse	2.2	33	RA 80	18	43	3.6	(2)	5[a]
5.	F	38	HOP, HOAP, H CR/1 month Relapse	7.7	15.5	RA 45	17	20	4.5	(1)	5+
6.	F	54	HOP, HOAP CR/30 months Relapse	4.0	28.5	RA 45	22	22	2.0	(1)	4[a]
7.	M	54	H/10d	1.4	94	RA 45	29	38	1.0	(1)	2[a]
8.	F	69	H/5d	0.5	48	RA 45	22	44	4.0	(3)	1+
9.	M	61	Untreated	1.0	74	RA 45–50	29	36	2.5	(2)	11+
10.	M	31	Untreated	1.6	76	RA 50	35	35	1.5	(2)	5[a]
11.	M	37	Untreated	1.4	65	RA 45	26	43	3.5	(1)	10+
12.	F	18	Untreated	0.9	81.5	RA 45–50	21	40	2.5	(4)	8+
13.	F	35	Untreated	2.2	70	RA 50	20	39	2.0	(2)	4[a]
14.	M	45	Untreated	2.1	84.5	RA 45	36	119	1.0	(4)	5+
15.	F	57	Untreated	1.9	88.5	RA 45	23	51	2.5	(4)	5+
16.	F	20	Untreated	0.9	85.5	RA 50	22	46	2.0	(1)	8+
17.	M	32	Untreated	1.1	78	RA 45	29	39	3.0	(4)	4[a]
18.	M	36	Untreated	1.1	89.7	RA 50	35	52	2.0	(4)	4+
19.	F	53	Untreated	15.8	75.5	RA 45	23	39	0	(3)	5+
20.	M	30	Untreated	6.5	90	RA 45	46	46	2.5	(4)	3+
21.	F	36	Untreated	1.7	91	RA 50	36	50	1.0	(3)	1+
22.	M	21	Untreated	1.7	90	RA 45	28	56	1.0	(4)	2+
23.	M	45	Untreated	1.4	78	RA 45	25	45	3.0	(4)	1+
24.	M	34	Untreated	1.1	30	RA 45 + ara-C 20	60	98	1.5	(4)	3[a]

PB, peripheral blood count; BM, bone marrow; Prom, promyelocyte; PR (day), time to partial remission; CR (day), time to complete remission; –, lost to follow-up; +, "greater than", still under follow-up; d, days; (1), RA (20–30 mg/m^2/day); (2), RA (20–30 mg/m^2/day) + ara-C (10 mg every 12 h) or H (0.5 mg/m^2/day); (3), ara-C (10 mg every 12 h); (4), consolidated with HOAP, maintained by 6-mercaptopurine, methotrexate, or cyclophosphamide

[a] Relapse following CR

other eight (case #1–#8) had previously been treated with chemotherapy (HOAP[1], HOP, OH, COH, H). Of the eight treated patients, three were in relapse after 1–30 months of CR and five were resistant to or could not tolerate the chemotherapy (5–62 days of treatment). Twenty-two of the patients showed mild to moderate hemorrhagic manifestations (purpura, gingivorrhagia, gastrointestinal bleeding) but no laboratory evidence of DIC prior to treatment with RA except for a positive plasma protamin sulfate paracoagulation ($3P^+$) in three of the cases.

II. Marrow Preparation and Culture

A modification of the method of Flynn et al. [6] for short-term suspension culture was used. Marrow cells were aspirated from the iliac crest, layered onto Ficoll-Hypaque (specific gravity 1.077), and centrifuged at 800 *g* for 15 min. Interface cells were collected, washed with McCoy's 5A medium, and resuspended at a concentration of 5×10^5 cells/ml in McCoy's 5A medium containing 15% fetal calf serum. All-*trans* RA (Shanghai No. 6 Pharmaceutical Factory, Shanghai) was dissolved in absolute ethanol to a concentration of 1 m*M* and further diluted with the medium so that the final ethanol concentration in the cultures was 0.1% and the final RA concentration 1 μ*M*. Controls were cultured in medium alone. (It had been previously demonstrated that 0.1% ethanol had no effect on cell growth and on differentiation of HL-60 cells [11].) All cultures were incubated at 37° C in a 5% CO_2 atmosphere for up to 7 days. Cell density was determined by hemacytometer and cell viability by the trypan blue dye exclusion method. Aliquots of cells were removed for morphological examination on the 2nd, 4th, and 6th day of culture.

[1] H, Harringtonin (0.02–0.07 mg/kg/day); O, oncovin (0.02–0.03 mg/kg/day); A, cytosine arabinoside ara-C); P, prednisone; C, cyclophosphamide

III. Morphological Studies

Differential counts were performed on cell smears stained with Wright's solution. Chloroacetate esterase and alpha-naphthyl acetate esterase stains were performed using standard techniques [12]. Samples from four cases were prepared for transmission electron microscopic study. The nitroblue tetrazolium (NBT) reduction assay was performed as described by Francis et al. [13]. The percentage of cells containing intracellular blue-black deposits was determined in 200 cells on Wright's stained slide preparations.

IV. Colony Formation Assay

Blast cell colonies were grown as described by Minden et al. [14]. Conditioned medium was prepared from leukocytes (10^6 cells/ml) incubated at 37° C for 7 days in McCoy's 5A medium with 10% fetal calf serum and 1% (v/v) phytohemagglutinin-P (PHA-P) (DIFCO) and stored at 4° C until used. The preparation was termed PHA-LCM. Bone marrow cells were plated at 1×10^6 cells/ml using McCoy's 5A medium supplemented with 0.3% agar, 20% fetal calf serum, and 25% (v/v) PHA-LCM. GM-CFU was determined according to the technique of Pike and Robinson [15] for colony growth in agar. Briefly, 2×10^5 marrow cells were plated in 35-mm tissue culture dishes over a feeder layer of 1×10^6 leukocytes from healthy donors. The plates were incubated at 37° C in a humidified 5% CO_2 atmosphere. L-CFU colonies (more than 20 cells) were scored on day 8 and GM-CFU colonies (more than 40 cells) on day 10.

V. Treatment of Patients

The 24 patients in this series received all-*trans* RA (45–100 mg/m^2/day) as the remission induction therapy. Informed consent was obtained from all patients (or their parents). Peripheral blood counts, bone marrow aspiration, and co-

agulation parameters (in 21 cases) including thrombin time, prothrombin time, plasma protamin sulfate paracoagulation test, euglobulin lysis test, and fibrinogen levels were determined before the start of therapy and at regular intervals thereafter. CR is defined as less than 5% blasts plus promyelocytes in a normal cellular marrow with a normal peripheral blood count and an absence of the signs and symptoms of leukemia on physical examination [16]. Partial remission (PR) is defined as less than 5% blasts plus promyelocytes in a normal cellular marrow but with a clinically moderate anemia. Blood transfusion and antibiotics were given as supportive treatment when necessary.

VI. Continuation Therapy Following Complete Remission

Twenty-three patients were followed after attaining CR. Further therapy was as follows: (1) Maintained by RA, 20–30 mg/m^2/day (six cases), (2) maintained by RA, 20–30 mg/m^2/day plus low-dose ara-C (10 mg i.m. every 12 h) or low-dose Harringtonin (0.5 mg/m^2 i.v. daily) in rotation (four cases), (3) maintained by low-dose ara-C, 10 mg i.m. every 12 h (five cases), (4) consolidated by chemotherapy (HOAP) and maintained by 6-mercaptopurine (2 mg/kg daily p.o.) and methotrexate (10 mg/m^2, i.v. weekly), or cyclophosphamide (200 mg/m^2, i.v. weekly) (nine cases).

C. Results

I. In Vitro Studies

Leukemic bone marrow cells derived from 15 patients and incubated for 7 days in suspension culture, with or without all-*trans* RA (1 μ*M*), showed little change in cell density. Viability of both control and RA-treated cells was consistently greater than 75%.

Leukemic promyelocytes from 14 patients showed morphologic and functional maturation when cultured in the presence of RA (Table 2), (Fig. 1 A, B). The

Table 2. Response of promyelocytes to RA in suspension culture

Case No.	Blasts (%)		Promyelocytes (%)		Myelocytes (%)		Mature[a] (%)		NBT (%)	
	Control	RA-treated	Control	RA-treated	Control	RA-treated	Control	RA-treated	Control	RA-treated
1.	3	2	68	7	15	34	4	48	ND	ND
3.	0	0	86	1	6	26	7	73	3.5	52
4.	0	1	47	4	14	48	18	36	ND	ND
9.	0	0	95	5	2	25	1	63	3	54
10.	0	0	98	2	0	24	2	74	5	38
11.	2	1	78	2	5	26	11	64	ND	ND
12.	2	1	81	4	9	27	6	62	2	39
13.	0	0	86	9	8	38	6	53	ND	ND
14.	0	0	86	3	11	29	3	68	ND	ND
16.	0	0	93	12	6	36	1	52	0	35.5
18.	1.5	0	77	1	20.5	42.5	1	55	12	43
19.	0	0	92	4	0	53	2	39	ND	ND
20.	0	0	90	8	8	38	2	52	ND	ND
21.	0	0	91.5	20	3.5	62	2	15	4	33
24.	0	0	84	80	2.5	4	6.5	7	ND	ND

Control, RA not added to the culture; NBT, nitroblue tetrazolium; ND, not done

[a] Metamyelocytes + bands + polymorphonuclear leukocytes

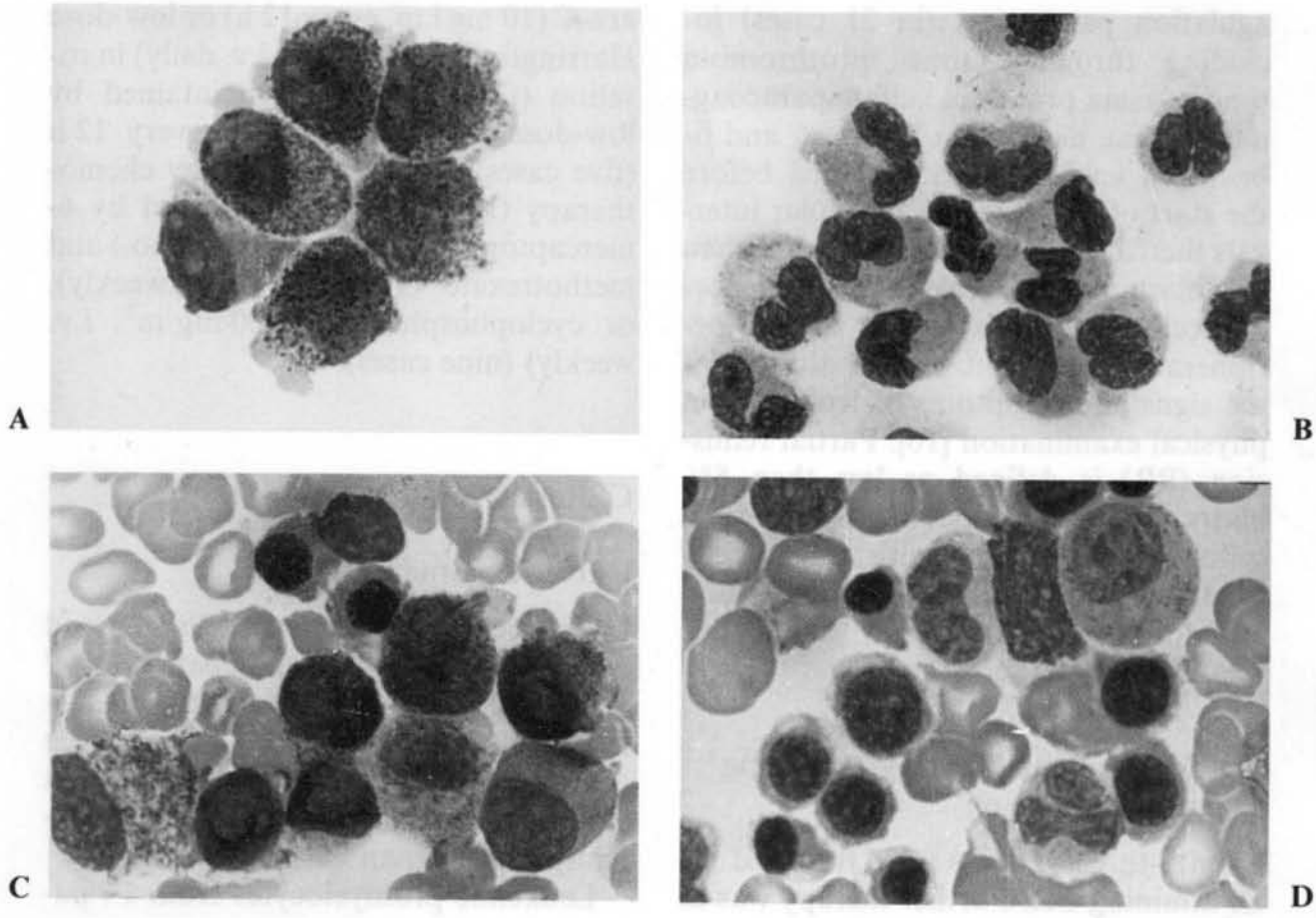

Fig. 1 A–D. Morphological maturation of leukemic cells of case 10 in vitro and in vivo. **A** Cells cultured without RA, consisting of promyelocytes with characteristic cytoplasmic granules, × 1000. **B** Cells cultured with RA, showing maturation of granulocytes, × 1000. **C** Bone marrow before RA treatment. The predominance of promyelocytes (76%) indicates typical APL. **D** Bone marrow after 5 weeks of RA treatment. Promyelocyte level under 2%, and restoration of normal hemopoiesis without an aplasia phase are consistent with differentiation induction

percentage of promyelocytes in the control group versus the RA-treated group was $83.5\% \pm 12.8\%$ [2] and $5.9\% \pm 5.0\%$ [2] respectively. The percentage of mature cells (metamyelocytes + bands + PMNs) was $4.7\% \pm 4.5\%$ [2] and $53.9\% \pm 15.4\%$ [2] respectively. The rate of NBT reduction in RA-treated cells was $42.0\% \pm 7.5\%$ [2], significantly higher than that of the control group $(4.2\% \pm 3.5\%)$ [2].

To examine the progression of cellular differentiation, we incubated cells from four patients with 1 μM RA for various time intervals. After 48 h, morphologically recognizable changes in the promyelocytes could be observed. The nucleus became larger and fewer primary granules were observed in the cytoplasm. On the 4th day of culture, these cells gave rise to myelocytes which contained specific, or secondary, granules. The nuclear chromatin was more condensed and the nucleoli were either vague or no longer visible. There was an elevated population of metamyelocytes which had indented or horseshoe-shaped nuclei and cytoplasm filled with both primary and secondary granules appearing by day 6, as well as some band and fully mature granulocytes. When the cultures were continued for 7–8 days, the relative number of mature granulocytes increased.

Cytochemical analysis showed that in the control cells chloroacetate esterase activity varied from mildly to moderately

[2] Results represent the data from the patients studied and are expressed as mean % ± standard deviation.

positive, while in the RA-treated cultures intensely positive granules were seen, either diffusely scattered or accumulated in some portion of the cytoplasm. The majority of control cells showed weak nonspecific esterase activity while RA-treated cells had a stronger reaction.

Transmission electron microscopic examination of four cultures confirmed that in the presence of RA the cells had been differentiated to mature granulocytes. Condensation of the heterochromatin became evident and the nucleus had often been changed to a bean-shaped or even a segmented form. Neutrophilic granules were smaller and diffusely scattered throughout the cytoplasm. Azurophilic granules were markedly decreased.

II. Clinical Studies

Twenty-four patients were treated with all-*trans* RA as a single agent. All achieved both PR and CR except the one patient (# 24) whose cells were not inducible when cultured with RA in vitro. Subsequent bone marrow examination of this patient revealed a continuing proliferation of leukemic promyelocytes. When ara-C (10 mg) was added intramuscularly every 12 h, the patient achieved CR in 98 days (Table 1).

In the 12 patients studied who responded to the induction differentiation effect of RA, L-CFU growth was predominant (163.3 ± 129.0 colonies) and GM-CFU suppressed (0.63 ± 1.3 colonies) prior to treatment. GM-CFU reached normal levels (100.2 ± 55.1 colonies) with little or no growth of L-CFU after CR was achieved.

III. Pattern of Clinical Response to *trans*-Retinoic Acid

Systematic observation of the peripheral blood counts during RA treatment of the previously untreated patients revealed some specific patterns of change. There was a progressive rise in the total white blood cell count which started with initiation of treatment and which reached a peak between 7 and 14 days. After this, the white blood cell count fell with the progressive maturation of granulocytes. Increase in platelets was most prominent after 3 weeks. Elevation of the hemoglobin concentration appeared reluctant and slow. Bone marrow aspirate revealed that hypercellularity existed throughout the RA treatment. Partial remission could be expected within 1 month (Fig. 1 C, D). Therapy with oral all-*trans* RA was accompanied by mild toxicity that consisted of dryness of the lips and skin (100%), headache (25%), nausea or vomiting (20.8%), moderate bone or joint pain (12.5%), and mild exfoliation (8.3%). Two patients had elevated SGPT. All of these side effects were well tolerated or alleviated when the dosage of oral RA was reduced.

IV. Disseminated Intravascular Coagulation

Coagulation parameters, including thrombin time, prothrombin time, plasma protamin sulfate paracoagulation test (3P), euglobulin lysis test, and fibrinogen levels, were measured simultaneously, in 21 patients, at the beginning of RA therapy and throughout the course of treatment. Of these patients, 18 who were normal in coagulation parameters prior to the start of RA therapy showed no changes during treatment. The other three patients who had been previously treated and who were 3P(+) became negative 7–10 days after RA. Therefore, DIC or other hemorrhagic complications did not occur when patients with APL were induced to remission with RA.

V. Duration of Clinical Remission

Twenty-three patients were followed after induction of CR (Table 1). Of the six patients maintained on RA alone, four were still in remission for a period of 5–10 months. Two patients relapsed in 2 and 4 months. Among the four patients

maintained on RA with either low-dose ara-C or low-dose Harringtonin in rotation, three relapsed within a period of 4–5 months. Of the five patients maintained on low-dose ara-C alone, one case (# 2) was lost to follow-up, one relapsed in 4 months, and the other three remained in CR for 1^+ to 5^+ months. Of the remaining nine patients who were consolidated by chemotherapeutic regimens and maintained on 6-mercaptopurine, methotrexate, or cyclophosphamide, two relapsed and seven have been in CR from 1^+ to 8^+ months. The new population of APL promyelocytes at relapse differed morphologically from those present at the start of treatment and were resistant to all-*trans* RA induction of differentiation in vitro.

D. Discussion

Recent approaches in treatment of leukemia include the use of "differentiation-inducing agents" such as RA, vitamin D_3, or low-dose ara-C [17–19]. Numerous studies both in vitro and in vivo have revealed that RA is a potent inducer of myeloid differentiation, both in the promyelocytic cell line HL-60 as well as in fresh promyelocytes from patients with APL, and at a concentration that was pharmacologically obtainable in man [11, 20]. 13-*cis* RA and all-*trans* RA were equally effective in induction of differentiation in vitro [5]. Our studies confirm that in vitro leukemic promyelocytes could be induced by all-*trans* RA to differentiate toward mature granulocytes. One exception was that the cells from patient # 24 were resistant to RA induction. The morphological characteristics of these RA-resistant cells revealed a scanty cytoplasm with less-prominent coarse granulation. The differences in sensitivity to RA may be due to the heterogeneous entities of APL [21, 22].

In 1983, Flynn et al. [6] described the first case of APL treated with 13-*cis*-RA. Unfortunately, this patient died from disseminated candidiasis although there was a marked elevation in his peripheral granulocyte count after 2 weeks of treatment. Nilsson [7] reported a 30-year-old woman with APL in relapse for 10 months; she was treated by 13-*cis* RA (1 mg/kg) and began to respond after 1 month, and normal blood and bone marrow pictures continued for 11 months. Daenen et al. [8] reported a 33-year-old patient with refractory APL complicated by fibrinolysis and aspergillus pneumonia. He was treated with 13-*cis* RA (80 mg/day) alone, and attained a CR after 7 weeks. Recently, Fontana et al. [9] reported one case of refractory APL treated with 13-*cis* RA (100 mg/m^2) which resulted in CR after 13 days. In vitro studies of this patient's leukemic blasts showed differentiation in the presence of RA. Sampi et al. [23] reported a 58-year-old Japanese man who also had relapsed APL and failed to respond to etretinate (a form of retinoid) and dactinomycin, although the leukemic cells were sensitive to all-*trans* RA (10^{-6}–10^{-7} *M*) in vitro. We have treated our patients with all-*trans* RA and found that all-*trans* RA was not only effective in patients who had been refractory to chemotherapy, but also effective in those with "de novo" APL. Moreover, we were able to find predictive value in the in vitro differentiation studies. The single patient who was resistant to RA induction failed to show marrow improvement when treated with RA as the sole agent.

According to most authors, the main disquieting problem of APL is death during induction treatment [3, 4, 24], especially because of intracerebral hemorrhage. DIC is the most common complication of APL. Its severity and frequency are often aggravated by chemotherapy despite the use of heparin. In this study we report no aggravation of hemorrhagic manifestation or appearance of coagulation parameter abnormalities suggesting DIC during the course of RA treatment. This would be one of the striking advantages over aggressive chemotherapy which could destroy the leukemic cells and cause the release of procoagulant

factors from the azurophilic granules into the circulation. It is possible that the leukemic cells are not destroyed during treatment of APL with RA, but that they have differentiated, undergone terminal cell division, and lost the capacity to release these coagulant factors during this process. The fact that there was no decrease, but rather an increase, of marrow cellularity during induction therapy supports this possibility.

The role of all-*trans* RA in the maintenance of remission is undetermined. Two cases of APL, reported by Daenen et al. [8] and Fontana et al. [9], relapsed in 6 and 12 months respectively. In our series, the patients were further treated with four different regimens after CR was induced, but it is too early to conclude which of these is the most effective. From the data obtained from both our clinical survey and the cytogenetic studies showing the persistence of abnormal clones (unpublished data), we suggest that intensive chemotherapy after CR may be beneficial.

The knowledge about the side effects of oral RA is mainly from the dermatological literature. Our data are compatible with other reports on the toxicity of oral all-*trans* RA [25]. The toxicity of 13-*cis* RA has been shown to be relatively lower than all-*trans* RA [25], but in our experience the side effects were well tolerated by the patients, some of whom have been taking RA for more than 10 months with no severe untoward effects.

Based on these observations, we conclude that all-*trans* RA is an effective agent for obtaining CR in APL. How to maintain and prolong the duration of the CR, however, requires further study.

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