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

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Twenty-four patients with acute promyelocytic leukemia (APL) were treated with all-*trans* retinoic acid (45 to 100 mg/m²/day). Of these, eight cases had been either nonresponsive or resistant to previous chemotherapy; the other 16 cases were previously untreated. All patients attained complete remission without developing bone marrow hypoplasia. Bone marrow suspension cultures were studied in 15 of the 24 patients. Fourteen of these patients had morphological maturation in response to the retinoic acid (1 μmol/L). Chloroacetate esterase and α-naphthyl acetate esterase staining as well as electronmicroscopic examination confirmed that retinoic acid-induced cells differentiated to granulocytes with increased functional maturation (as measured by nitroblue tetrazolium reduction, NBT). The single nonresponder to retinoic acid in vitro was resistant to treatment with retinoic acid but attained complete remission after addition of low-dose cytosine arabinoside (ara-C). During the course of therapy, none of the patients showed any abnormalities in the coagulation parameters we measured, suggesting an absence of any subclinical disseminated intravascular coagulation. The only side effects consisted of mild dryness of the lips and skin, with occasional headaches and digestive symptoms. Eight patients have relapsed after 2 to 5 months of complete remission. The others remain in complete remission at 1+ to 11+ months and are still being followed up. We conclude that all-*trans* retinoic acid is an effective inducer for attaining complete remission in APL.

ACUTE PROMYELOCYTIC leukemia (APL) is considered a distinct entity among the acute myeloid leukemias (AML). 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 believed to result from release of a procoagulant factor from the promyelocyte granules.[1]

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

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

MATERIALS AND METHODS

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. Eleven females and 13 males with a mean age of 35.5 years (range 5 to 69 years) were studied. The total WBC counts ranged from 0.5 × 10^9/L to 15.8 × 10^9/L, including 20 cases (83.3%) with <3 × 10^9/L, 3 cases (12.5%) between 3 × 10^9 and 10 × 10^9/L, and 1 case (4.1%) with >10 × 10^9/L. The hemoglobin concentrations ranged from 41 to 121 g/L including 8 cases (33.3%) with <60 g/L, 12 cases (50%) between 60 and 90 g/L, and 4 cases (16.7%) with >90 g/L. Platelet counts ranged from 10 × 10^9/L to 337 × 10^9/L, including 15 cases (62.5%) with <50 × 10^9/L, 7 cases (29.2%) between 50 × 10^9/L and 100 × 10^9/L and 2 cases (8.3%) with >100 × 10^9/L. The percentage of promyelocytes in the marrow ranged from 15.6% to 94%, with 22 patients having >30% and the remaining 2 patients having between 15.6% and 30%. Of the 24 patients, 16 had never been treated. The other 8 (cases 1 through 8) had previously been treated with chemotherapy (HOAP,* HOP, OH, COH, H). Of the 8 treated patients, 3 were in relapse after 1 to 30 months of CR and 5 were resistant to or could not tolerate the chemotherapy (5 to 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.

Marrow preparation and culture. A modification of the method of Flynn and colleagues[6] for short-term suspension culture was used. Marrow cells were aspirated from the iliac crest, layered onto Ficoll-Hypaque (sp gr 1.077), and centrifuged at 800 g for 15 minutes. 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 (FCS). All-*trans* RA (Shanghai No. 6 Pharmaceutical Factory) was dissolved in absolute ethanol to a concentration of 1 mmol/L and further diluted with the medium so that the final ethanol concentration in the cultures was 0.1% and the final RA concentration was 1 μmol/L. Controls were cultured in medium alone. (Ethanol 0.1% had previously been shown to have no effect on cell growth or 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 was determined by trypan blue dye exclusion method. Aliquots of cells were removed for

*Harringtonin (0.02 to 0.07 mg/kg/day); O, oncovin (0.02 to 0.03 mg/kg/day); A, Ara-C; P, prednisone; C, cyclophosphamide.

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Table 1. Data of 24 Patients With APL

Case	Sex	Age	Previous Therapy/Duration	PB WBC ($\times 10^9$/L)	BM Prom (%)	Present Therapy (mg/m^2/day)	PR (day)	CR (day)	BM Prom (%)	Further Therapy	Duration of CR (mo)
1	F	5	HOAP/7 days HOP/9 days H/20 days	1.16	78	RA 80	31	68	1.5	(1)	8*
2	M	6	HOP/26 days	1.7	73.5	RA 100	18	28	2.5	(3)	—†
3	F	28	HOA/62 days	1.8	89	RA 60	21	34	2.0	(3)	4‡
4	M	8	HOAP/10 days OH/21 days CR/3 mo Relapse	2.2	33	RA 80	18	43	3.6	(2)	5‡
5	F	38	HOP,HOAP,H CR/1 mo Relapse	7.7	15.5	RA 45	17	20	4.5	(1)	5*
6	F	54	HOP,HOAP CR/30 mo Relapse	4.0	28.5	RA 45	22	22	2.0	(1)	4‡
7	M	54	H/10 days	1.4	94	RA 45	29	38	1.0	(1)	2‡
8	F	69	H/5 days	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‡
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‡
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‡
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‡

PB, peripheral WBC; BM, bone marrow; Prom, promyelocyte; PR (day), time to partial remission; CR (day), time to complete remission; (1) RA (20 to 30 mg/m^2/day); (2) RA (20 to 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 hours); (4) consolidated with HOAP, maintained by 6-mercaptopurine, methotrexate, or cyclophosphamide.

*"Greater-than," still under follow-up.

†Lost to follow-up.

‡Relapse after CR.

morphological examination on the second, fourth, and sixth day of culture.

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

Colony formation assay. Blast cell colonies were grown as described by Minden and colleagues.[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% FCS and 1% (vol/vol) phytohemagglutinin-P (PHA-P) (Difco, Detroit) 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% FCS, and 25% (vol/vol) PHA-LCM. GM-CFU count was determined according to the technique of Pike and Robinson[15] for colony growth in agar. Marrow cells 2×10^5 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 (>20 cells) were scored on day 8, and GM-CFU colonies (>40 cells) were scored on day 10.

Treatment of patients. The 24 patients in this series received all-*trans* RA (45 to 100 mg/m^2/day) as remission induction therapy. Informed consent was obtained from all patients (or their parents). Peripheral blood counts, bone marrow aspiration, and coagulation 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 <5% blasts plus promyelocytes in a normal cellular marrow with a normal peripheral blood count and an absence of signs and symptoms of leukemia on physical examination.[16] Partial remission (PR) is defined as <5% blasts plus promyelocytes in a normal cellular marrow, but presence of a clinically moderate anemia. Blood trans-

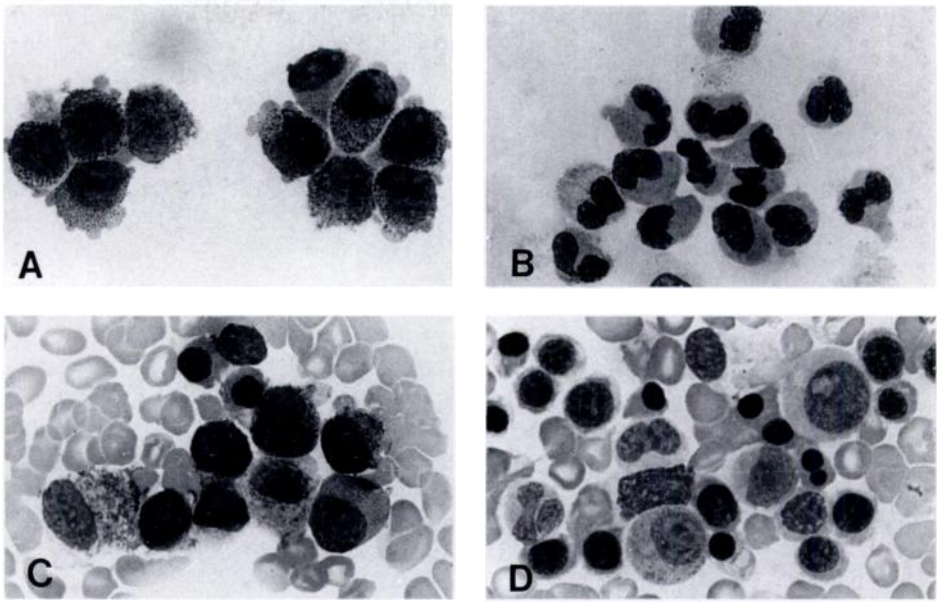

Fig 1. Morphological maturation of leukemic cells of case 10 in vitro and in vivo. (A) Cells cultured without RA, consisting of promyelocytes with characteristic cytoplasmic granules (×1,000). (B) Cells cultured with RA, showing maturation to granulocytes (×1,000). (C) Bone marrow before RA treatment. The predominance of promyelocytes (76%) indicates typical APL. (D) Bone marrow after 5 weeks of RA treatment. Promyelocyte level <2% and restoration of normal hematopoiesis without a phase of aplasia are consistent with differentiation induction.

fusion and antibiotics were given as supportive treatment when necessary.

Continuation therapy following CR. Twenty-three patients were followed up after they attained CR. Further therapy was as follows: (a) maintained by RA, 20 to 30 mg/m^2/day (6 cases); (b) maintained by RA, 20 to 30 mg/m^2/day plus low-dose ara-C [10 mg intramuscularly (IM) every 12 hours] or low-dose harringtonin [0.5 mg/m^2 intravenously (IV) daily] in rotation (4 cases); (c) maintained by low-dose ara-C, 10 mg IM every 12 hours, (5 cases); (d) consolidated by chemotherapy (HOAP) and maintained by 6-mercaptopurine (2 mg/kg daily p.o.) and methotrexate (10 mg/m^2, IV weekly), or cyclophosphamide (200 mg/m^2, IV weekly) (9 cases).

RESULTS

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 μmol/L), showed little change in cell density. Viability of both control and RA-treated cells was consistently >75%.

Leukemic promyelocytes from 14 patients showed morphological and functional maturation when cultured in with RA (Table 2), (Fig 1A and B). The percentage of promyelocytes in the control group *v* the RA-treated group was 83.5% ± 12.8% and 5.9% ± 5.0%, respectively. The percentage of mature cells (metamyelocytes + bands + polymorphonuclear leukocytes, PMNs) was 4.7% ± 4.5% and 53.9% ± 15.4%, respectively. The rate of NBT reduction in RA-treated cells was 42.0% ± 7.5%, significantly higher than that of the control group (4.2% ± 3.5%). (All results represent data from the patients studied and are the mean percentage ± SD.)

To examine the progression of cellular differentiation, we incubated cells from four patients with 1 μmol/L RA for various time intervals. After 48 hours, morphologically

Table 2. Response of Promyelocytes to RA in Suspension Culture

	Blasts (%)		Promyelocytes (%)		Myelocytes (%)		Mature* (%)		NBT (%)	
Case No.	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	3	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.

*Metamyelocytes + bands + polymorphonuclear leukocytes.

recognizable changes in the promyelocytes could be observed. The nucleus became larger, and fewer primary granules were observed in the cytoplasm. On the fourth day of culture, these cells gave rise to myelocytes containing specific, or secondary, granules. The nuclear chromatin was more condensed, and the nucleoli were either vague or no longer visible. An elevated population of metamyelocytes was evident, with indented or horseshoe-shaped nuclei and cytoplasm filled with both primary and secondary granules appearing by day 6 as well as some bands and fully mature granulocytes. When the cultures were continued for 7 to 8 days, the relative number of mature granulocytes increased.

Cytochemical analysis showed that chloroacetate esterase activity varied in the control cells from mildly to moderately positive, whereas in RA-treated cultures intensely positive granules were apparent, either diffusely scattered or accumulated in some portion of the cytoplasm. Most control cells showed weak nonspecific esterase activity; RA-treated cells had a stronger reaction.

Transmission electronmicroscopic 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.

Clinical studies. Twenty-four patients were treated with all-*trans* RA as a single agent. All achieved both PR and CR except for one patient (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 IM every 12 hours, the patient achieved CR in 98 days (Table 1).

In the 12 patients who responded to the induction differentiation effect of RA, L-CFU growth was predominant (163.3 ± 129.0 colonies) and GM-CFU growth was 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.

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. The total WBC count rose progressively starting with initiation of treatment and reaching a peak between 7 and 14 days. The WBC count then 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 RA treatment. PR could be expected within 1 month (Fig 1C and 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 these side effects were well tolerated or alleviated when the dosage of oral RA was reduced.

DIC. 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 before beginning RA therapy showed no changes during treatment. The other three patients who had been previously treated and who were 3P(+) became negative 7 to 10 days after RA. Therefore, DIC or other hemorrhagic complications did not occur when patients with APL were induced to remission with RA.

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 to 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 to 5 months. Of the 5 patients maintained on low-dose ara-C alone, 1 case (case 2) was lost to follow up, 1 relapsed in 4 months, and the other 3 remained in CR for 1+ to 5+ months. Of the remaining 9 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.

DISCUSSION

Recent approaches in treatment of leukemia include 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 is pharmacologically obtainable in humans.[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 owing to the heterogeneous entities of APL.[21,22]

In 1983, Flynn and colleagues[6] described the first case of APL treated with 13-*cis*-RA. Unfortunately, this patient died from disseminated candidiasis, although he had a markedly elevated 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 had normal blood and bone marrow for 11 months. Daenen and co-workers[8] reported a 33-year-old patient with refractory APL complicated by fibrinolysis and *Aspergillus pneumo-*

niae. He was treated with 13-*cis* RA (80 mg/day) alone and attained a CR after 7 weeks. Recently, Fontana and associates[9] reported a case of refractory APL treated with 13-*cis* RA (100 mg/m^2) that resulted in CR after 13 days. In vitro studies of this patient's leukemic blasts showed differentiation in the presence of RA. Sampi and colleagues[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} to 10^{-7} mol/L) in vitro. All-trans RA was not only effective in our patients who had been refractory to chemotherapy, but was 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 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 use of heparin. None of our patients had aggravation of hemorrhagic manifestation or appearance of coagulation parameter abnormalities suggesting DIC during the course of RA treatment. This would be a striking advantage over aggressive chemotherapy—destroying leukemic cells and causing release of procoagulant factors from the azurophilic granules into the circulation. Leukemic cells may not be destroyed during treatment of APL with RA, but instead may differentiate, undergo terminal cell division, and lose the capacity to release these coagulant factors during this process. 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 and colleagues[8] and Fontana and co-workers,[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.

Knowledge about the side effects of oral RA is derived mainly from the dermatologic literature. Our data are compatible with other reports on the toxicity of oral all-*trans* RA.[25] The toxicity of 13-*cis* RA is relatively lower than that of 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 >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. A method of maintaining and prolonging the duration of CR, however, requires further study.

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