Eur J Cancer Clin Oncol, Vol. 21, No. 1, pp. 23–30, 1985.
Printed in Great Britain.

0277-5379/85$3.00+0.00

Relationship between Leukemic Cell Retention of Cytosine Arabinoside Triphosphate and the Duration of Remission in Patients with Acute Non-lymphocytic Leukemia*

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Abstract—*Bone marrow cells were obtained from patients with acute non-lymphocytic leukemia immediately prior to the administration of cytosine arabinoside/anthracycline antibiotic remission induction therapy. The ability of the leukemic cells to take up and phosphorylate cytosine arabinoside (araC) and to retain cytosine arabinoside triphosphate (araCTP) was measured and compared to the outcome of remission induction therapy and the duration of remission. While the outcome of remission induction therapy was unrelated to cellular metabolism of araC, the duration of remission was highly correlated with araCTP retention. A comparison of the remission durations of patients treated on successive chemotherapeutic protocols suggests that the benefits of intensive remission consolidation therapy may be limited to patients whose leukemic cell retention of araCTP is low and that aggressive consolidation chemotherapy may reduce the prognostic significance of araCTP retention.*

INTRODUCTION

CYTOSINE arabinoside (araC) is the most commonly employed chemotherapeutic agent used in the treatment of patients with acute non-lymphocytic leukemia (ANLL). It is administered to virtually all patients as part of both remission induction and consolidation/maintenance regimens [1]. While studies by other investigators had failed to identify a relationship between the metabolism of araC by leukemic cells and the outcome of remission induction therapy [2–4], in a previous publication we reported that patients whose leukemic cells retained cytosine arabinoside triphosphate (araCTP) had longer remissions than patients whose cellular retention of araCTP was low [5]. The present report provides follow-up for our previous study and also reports the results of a successor prospective study which also demonstrated that the duration of remission was highly correlated with leukemic cell retention of araCTP.

Accepted 28 June 1984.
*This research was supported by USPHS grants CA-5834, CA-24162, CA-21071 and CA-16056 from the National Cancer Institute, Bethesda, MD, U.S.A.
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Abbreviations: araC, cytosine arabinoside; araCTP, cytosine arabinoside triphosphate; 5-azaC, 5-azacytidine; MeGAG, methylglyoxal bisguanyl hydrazone; adr, adriamycin; 6-TG, 6-thioguanine; ANLL, acute non-lymphocytic leukemia; CR, complete remission; RD, resistant disease; FAB, French-American-British classification of ANLL.

MATERIALS AND METHODS

The patients who were studied were treated by the Leukemia Service either at Roswell Park Memorial Institute or at Buffalo General Hospital during the years 1975–1980. Between 1975 and 1977 patients were treated on protocol 950501 (Fig. 1a). Patients received remission induction therapy consisting of cytosine arabinoside (araC) at 100 mg/m^2/day for 7 days administered by continuous infusion together with either adriamycin (adr) at 30 mg/m^2 by i.v. infusion over 15 min or daunorubicin (DNR) at 45 g/m^2 by i.v. infusion over 15 min on days 1, 2 and 3 administered during the araC infusion. Patients who entered remission with one or two courses of therapy received two courses of consolidation chemotherapy, the first consisting of araC 100 mg/m^2 s.q. twice a day for 5 days with

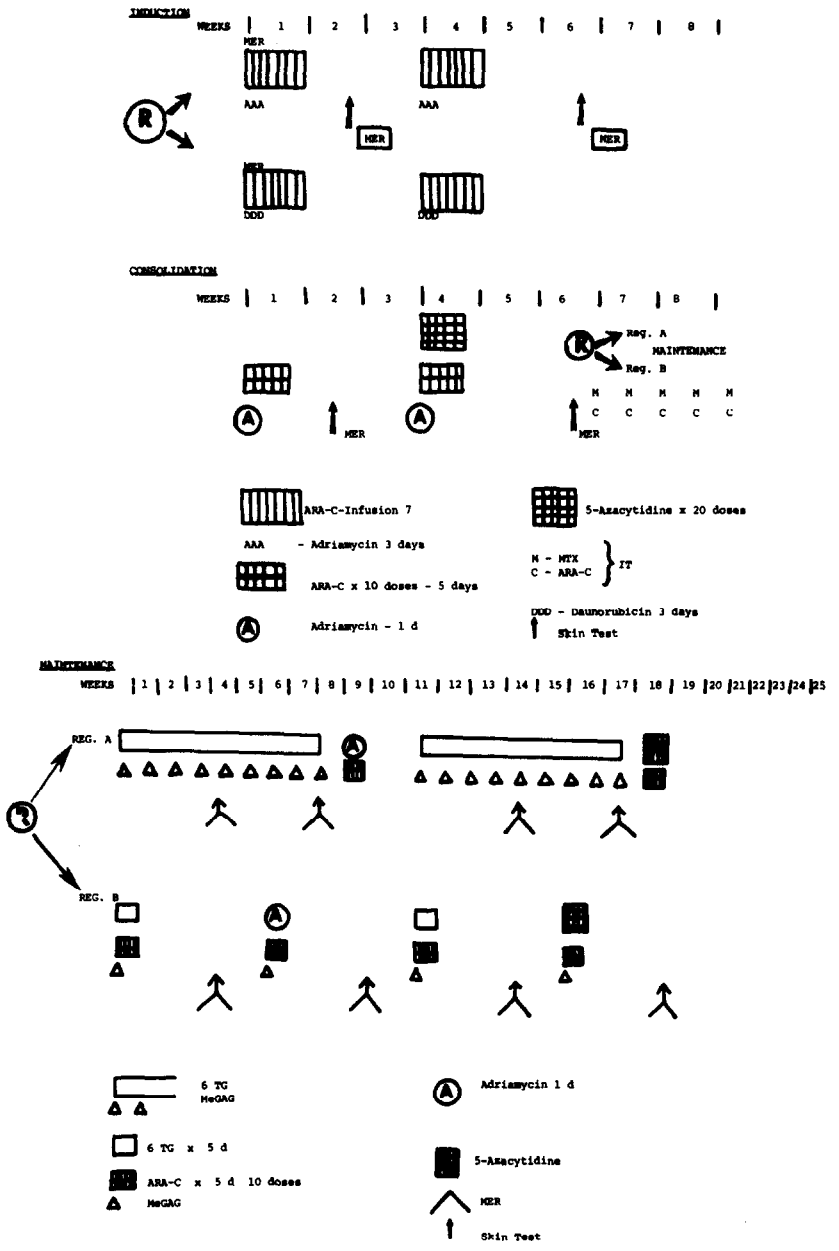

Fig. 1(a)

adr 40 mg/m² i.v. on day 1 and the second consisting of 5 days of araC therapy as before, one dose of adr as before and 5 days of 5-azacytidine (5-azaC) consisting of 120 mg/m²/day by continuous infusion. Patients were then randomized to receive 5 yr of maintenance therapy according to either of two schedules. One consisted of daily 6-thioguanine (6TG) at 100 mg/m²/day p.o. and once weekly methylglyoxalbisguanylhydrazone (MeGAG) at 300 mg/m² i.m. Every 3 months, daily and weekly therapies were stopped and the patient received araC 100 mg/m² s.q. bid × 5 days together with either a single dose of adr (60 mg/m²) or 5 days of 5-azaC at 120 mg/m²/day by continuous infusion. After the peripheral counts recovered the daily and weekly therapy was resumed. The other therapy arm consisted of courses of chemotherapy administered every 6 weeks consisting of 5 days of araC as above and one dose of MeGAG as above, combined either with 6TG at 100 mg/m² bid for 5 days, adr at 40 mg/m² or 5-azaC as above. The schema for this treatment arm is also provided in Fig. 1a. Both maintenance arms provide equivalent remission durations [6]. Protocol 970701 (Fig. 1b) [4] was used to treat patients cared for between 1977 and 1980. Remission induction therapy was identical to that of protocol 950501 save for the fact that

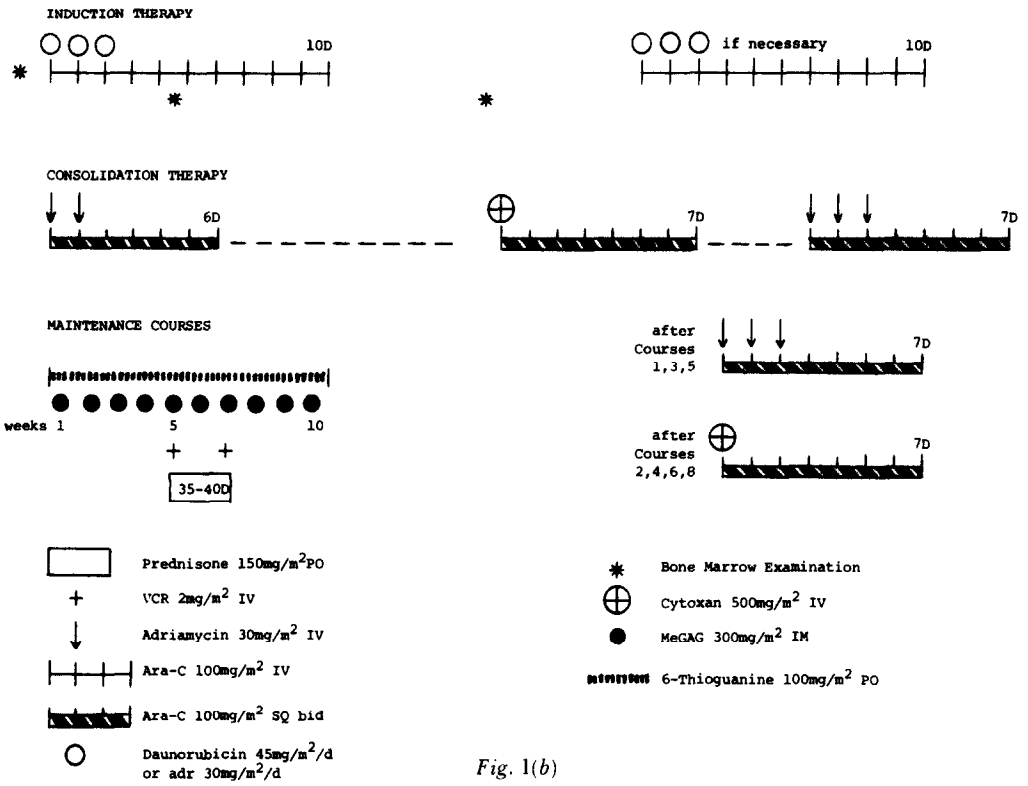

Fig. 1(b)

Fig. 1. Therapeutic protocols. (a) Protocol 950501. Cycles of maintenance therapy were repeated until relapse or discontinued after 5 yr of remission. MER = methanol extracted residue of BCG; 6TG = 6-thioguanine; Megag = methylglyoxalbisguanylhydrazone. (b) Protocol 970701. Therapy was discontinued after 3 yr or when the patient's leukemia recurred.

araC was administered for 10 days. Consolidation chemotherapy consisted of three courses: course No. 1, araC 100 mg/m² s.q. bid for 6 days and adr 40 mg/m² on days 1 and 2; course No. 2, araC 100 mg/m² s.q. bid for 7 days and cyclophosphamide 500 mg/m² i.v. on day 1; course No. 3, araC 100 mg/m² s.q. bid for 7 days and adr 30 mg/m²/day on days 1, 2 and 3. Patients then received 3 yr of maintenance chemotherapy consisting of daily 6TG 100 mg/m²/day p.o. and weekly MeGAG 300 mg/m² i.m. for 10-week courses. During each course, vincristine 2 mg/m² was administered i.v. on days 35 and 40 and prednisone 150 mg/m² p.o. qd on days 35–40. One week after the end of each 10-week course of maintenance therapy araC was administered at 100 mg/m² s.q. bid for 7 days with either adr 30 mg/m²/day on days 1, 2 and 3 or cyclophosphamide 500 mg/m² i.v. on day 1. Upon recovery of peripheral blood counts to normal another 10-week course of maintenance therapy was begun. The schema for this protocol is provided in Fig. 1b [7].

The leukemic subtype was classified according to the French–American–British system [8]. The patients were not selected in any manner save two: (1) in general, patients over 70 yr of age were not treated according to these protocols and hence were usually not studied; and (2) a bone marrow aspirate had to provide at least 2×10^7 cells for the drug sensitivity studies to be done. The latter criterion led to the exclusion of only three otherwise eligible patients. The characteristics of these patients are reported in Table 1. Complete remissions (CR) were defined according to the criteria of the Cancer and Acute Leukemia Group B [9] in that the bone marrow had to contain $\leqslant 5\%$ myeloblasts together with normal hematopoiesis. Peripheral blood counts had to be within normal limits as well. Bone marrow aspirates were

Table 1. Characteristics of patients whose leukemic cells were studied

	Protocol 950501	Protocol 970701
No.	28	63
Age		
mean ± S.D.	43.2 ± 15.4	52.1 ± 17.0
median	42	58
Male	13	36
Female	15	27
FAB		
M_{1-2}	20	39
M_3	1	4
M_4	5	15
M_5	0	0
M_6	2	5

performed every 6 weeks while a patient was in remission. Relapse was defined as a non-therapy-related increase in myeloblasts to >10% in bone marrow aspirations carried out two or more weeks apart or the presence of any bone marrow cells containing Auer rods even in what would otherwise be considered to be a remission marrow.

The relationship between cellular metabolism of araC and the outcome of remission induction therapy was assessed [10–12]. Drug-sensitive disease was considered to be present if the patient's leukemia entered a CR. Documented drug-resistant disease (RD) (or treatment failure due to persistent leukemia) was considered to be present in either of two instances: (1) persistence of a cellular marrow (≥1+ cellularity or aspirate or ≥5% cellularity on bone marrow biopsy) 7 or more days after the end of a course of chemotherapy; or (2) the regrowth of leukemic cells in a bone marrow in which severe marrow hypoplasia (<1+ cellularity on aspirate <5% cellularity on marrow biopsy) had been produced by remission induction therapy. Patients who expired less than 7 days after the end of a course of remission induction therapy and patients who expired with a severely hypocellular marrow ('other failures') were considered to be inevaluable for this study since one could not predict if leukemic cells would have persisted after induction therapy was completed or, in the case of a patient dying with a hypocellular marrow, whether leukemic cells or normal cells would have repopulated the marrow had the patient survived.

Preparation of leukemic cells for study

Five milliliters of bone marrow were aspirated from the posterior iliac crest into a syringe containing 2 ml of 10% sodium citrate. In early studies erythroid cells were lysed by exposure to hypotonic NH_4Cl [5], but recently density cut centrifugation has been used to remove erythrocytes. In brief, the marrow cells were layered over a Ficol-Hypaque Solution (s.g. 1.077) and centrifuged for 10 min at 1000 rpm. The interface cells were recovered, washed and studied. All specimens studied consisted of >5% leukemic cells.

Measurement of leukemic cell metabolism of cytosine arabinoside

The methods utilized in these studies have been described in detail in a prior publication for patients treated on protocol 950501 [5]. This method, however, was modified for patients treated on protocol 970701. Cells obtained from patients treated on protocol 970701 were incubated with a radiolabeled drug together with 1 μg/ml of non-radioactive araC, whereas cells obtained from patients treated according to protocol 950501 were incubated only with radioactive araC. This modification in procedure did not alter the distribution of % araCTP retention values but did result in an apparent increase in the pm of araCTP formed/10^7 cells. Hence the cells of patients treated on protocol 970701 contained 7–10 times as much as araCTP as did the cells of patients treated on protocol 950501 (see Table 2 for a detailed description of these data). All other manipulations were kept as previously described [5].

In brief, 3–10 million leukemic bone marrow cells were suspended in 2–5 ml of RPMI 1640 made 10% (v/v) with dialyzed heat-inactivated fetal calf serum, 2% 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid-2-(*N*-morphidine)-ethanesulfonic containing 20 μCi/ml [5-^{3}H]-araC. The cells were incubated at 37°C in shaking water bath for 30 min. The cells were then washed and resuspended in 5 ml of complete medium free of araC (above medium less araC is complete medium). One aliquot was processed for araCTP extraction while the other was incubated for 4 hr in the araC-free medium, harvested and extracted.

To extract araC and its metabolites the cells were pelleted by centrifugation and extracted with 50–100 μl of 6 N perchloric acid and the supernatant neutralized to pH 7.0 with 2 N potassium hydroxide. The acid-soluble fraction was analyzed for araC anabolites using previous reported methods [5]. 'Zero' time was defined as the time immediately following the initial 30 min incubation of cells with araC. The percentage retention was calculated using the following equation:

$$\frac{\text{araCTP present at 4 hr}}{\text{araCTP present at zero time}} \times 100.$$

Analysis of the data

Cellular metabolism of araC was compared to the outcome of the first course of remission induction therapy and for the final outcome of remission induction therapy regardless of the number of courses administered. The relationship of araC metabolism to the outcome of therapy was assessed using the Mann–Whitney *U* test. The relationship of araC metabolism to the duration of remission was assessed using Cox modeling [13, 14].

RESULTS

Outcome of remission induction therapy

Leukemic cell metabolism of araC *in vitro* did not correlate with the outcome of remission

Table 2. Cellular metabolism of cytosine arabinoside and the outcome of remission induction therapy

	*n**	P950501	*n**	P970701
Entire group				
(1) araCTP, $t=0$†	28	7 ± 1.6 (3.4)‡	63	73.4 ± 8.2 (60.8)
(2) araCTP, $t=4$ hr	28	3.5 ± 9 (.4)	51	18.3 ± 3.3 (9.5)
(3) % retention	22	38.2 ± 5.4 (48)§	51	21.8 ± 2 (17)§
CR patients				
(1) araCTP, $t=0$	20	7.7 ± 2 (4.7)	39	66 ± 8.2 (60.8)
(2) araCTP, $t=4$ hr	20	4.1 ± 1.1 (1.1)	33	18.7 ± 4 (9)
(3) % retention	17	42.3 ± 5.8 (50)	33	20.9 ± 2.2 (17)
RD patients				
(1) araCTP, $t=$ o	3	2.4 ± 1.4 (2.3)	7	52.9 ± 14.1 (43.2)
(2) araCTP, $t=4$ hr	3	0.07 ± .04 (.1)	6	12.7 ± 4.8 (12.4)
(3) % retention	3	3.4 ± 1.4 (3.4)	6	23.9 ± 5.5 (14)
'Other' failures				
(1) araCTP, $t=0$	5	7.1 ± 4.3 (2.9)	16	102 ± 23.8 (63.9)
(2) araCTP, $t=4$ hr	3	38.4 ± 17 (49)	11	21.8 ± 9.5 (18.6)
(3) % retention	3	38.4 ± 17 (49)	11	23.9 ± 5.6 (18.6)

*No. of patients' specimens studied.

†pm/10^7 cells.

‡mean ± S.E. (median).

§Inspection of the population data demonstrates that the change in the incubation conditions for [^{3}H]-araC resulted in an increase in the amount of araCTP which was detectable in the cells at both the $t=0$ and $t=4$ hr time points. This increase in the amount of araCTP was also reflected by the fact that cells from all patients treated on P970701 contained detectable levels of araCTP. If this change was simply a relative shift in araCTP levels then an increase in araCTP levels should be detectable at $t=0$ and $t=4$ hr and the % retention values should not be altered. Nevertheless, the median % araCTP retention appeared to be altered by the change in methodology since the median value for P950501 patients was 48% while it was only 17% for P970701 patients. This difference is probably an artifact which resulted from the method used to calculate median values for the following reasons: six patients treated on P950501 had zero $t=0$ araCTP levels while all P970701 patients had detectable $t=0$ araCTP levels. The calculation of the median araCTP retention values for P950501 patients did not include data for these six patients since their araCTP retentions could not be calculated. The cells of ten P970701 patients had $t=0$ araCTP levels which were $>0 \leqslant 5$ pm/10^7 cells. Seven of these ten patients had % araCTP retentions which were $<18\%$, with the median % araCTP retention for this group being 14%. If, based upon these data, the % araCTP retention values for the six P950501 patients whose araCTP retentions were not calculable is assumed to be $<18\%$, the overall median % araCTP value for P950501 patients becomes 18%, a value indistinguishable from the 17% median value for P970701 patients.

induction therapy (Table 2 provides these data for protocols 950501 and 970701). No relationships were detected between araCTP formation and patient age, sex or leukemic FAB subtype (data not provided). The relationship between the ability of the leukemic marrow cells of 63 patients treated on protocol 970701 to take up and phosphorylate araC and to retain araCTP and the outcome of therapy is illustrated in Figs 2a and b. Median $t=0$ araCTP values in pm/10^7 cells were 60.8, 43.2 and 63.9 for CR and RD patients and for 'other failures' respectively. The araCTP retention values for CR and RD patients and for 'other failures' were 17, 14 and 18.6% respectively. None of these values are statistically different from each other. These data are similar to those for patients treated on protocol 950501 which we have reported in the past [5]. The data provided in Table 2 and Figs 2a and b are for the outcome of the first course of remission induction therapy. A comparison of cellular araC metabolism and the overall outcome of remission induction therapy regardless of the number of courses administered was essentially identical to the data provided for course No. 1.

Remission duration

Follow-up remission duration data were available for 20 patients treated on protocol 950501 and for 39 patients treated on protocol 970701. The duration of remission for both sets of patients was not related to either the $t=0$ araCTP values or to the $t=4$ hr araCTP values. Remission duration, however, was significantly related to the

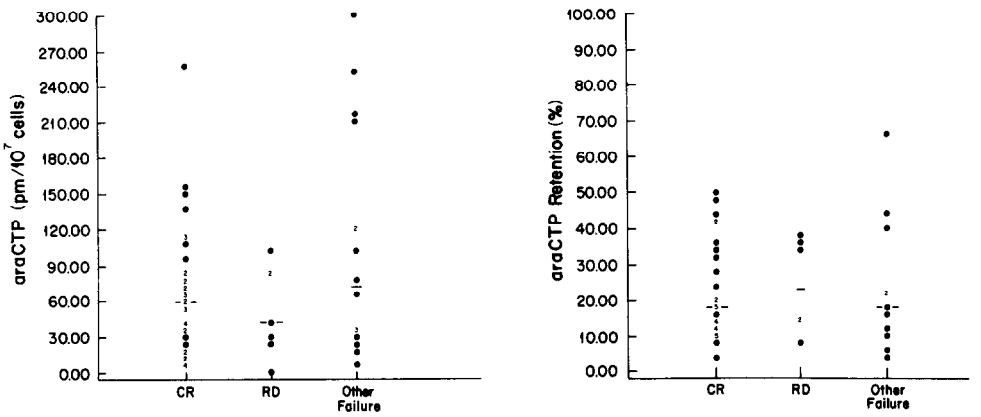

Fig. 2. Relationship between cytosine arabinoside phosphorylation and 4-hr retention and the outcome of remission induction therapy. (a) Outcome of induction therapy vs pm *araCTP formed/*10^7 cells *after* 30 min *of incubation with* [^{3}H]-*araC. (b) Outcome of induction therapy vs % araCTP retention at* 4 hr. *Numbers indicate the number of points which are present at the values indicated by the ordinate.*

% araCTP retention for patients treated with either protocol 950501 or protocol 970701. Figure 3 provides these data. With respect to protocol 950501, the median duration of remission for the 20 patients studied was 53 weeks, with median values of 159 weeks for patients whose araCTP retention values were >20% and 30 weeks for patients whose cellular araCTP retentions were <20% (P <0.002) (Figs 3a and c). Similarly, for patients treated on protocol 970701 the overall median duration of remission was 78 weeks with a median duration of remission of >66 weeks

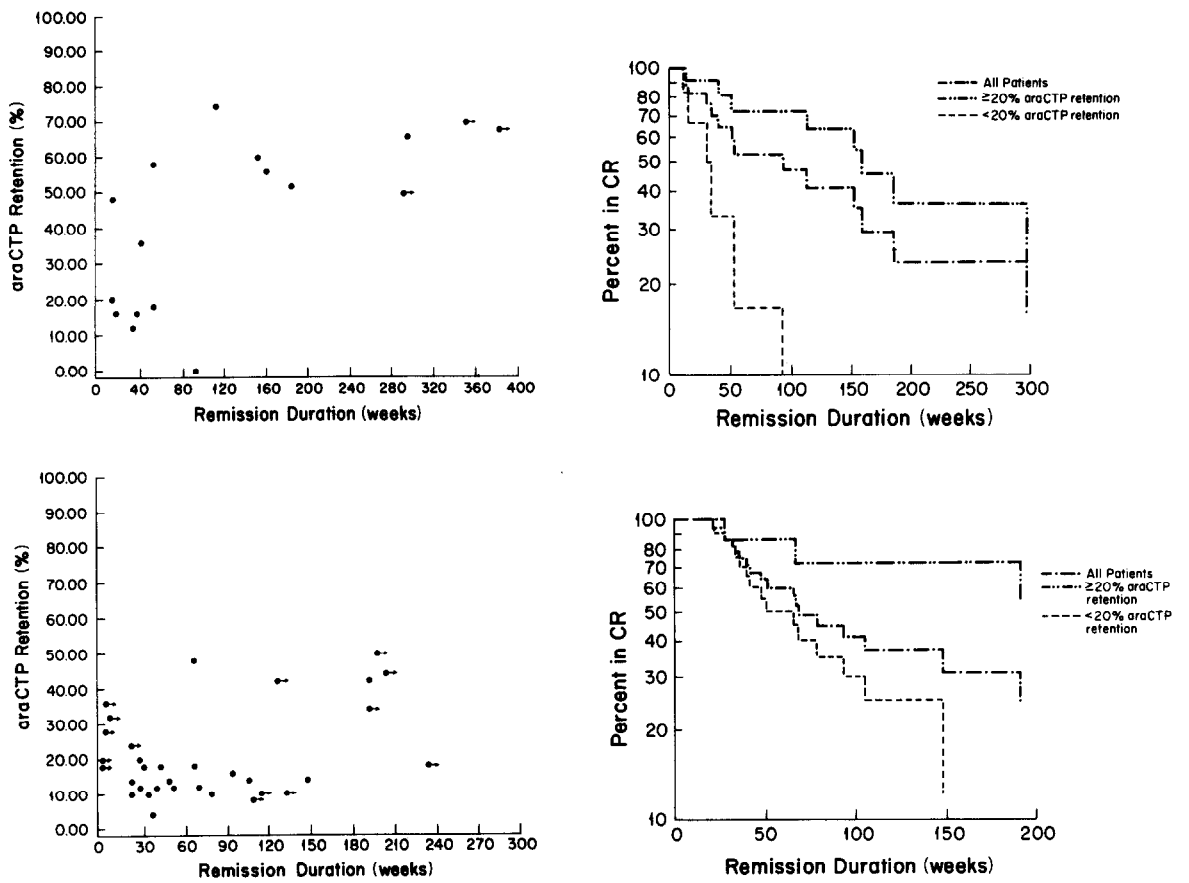

Fig. 3. Relationship between retention of araCTP and duration of remission. (a) Patients treated on protocol 950501. *(b) Patients treated on protocol* 970701. *Arrows indicate patients whose leukemia is still in complete remission. (c) Relationship between remission duration for patients treated on protocol* 950501 *and whether or not the patient's leukemic cell araCTP retention was* <20% *or* ≥20%. *(d) Relationship between remission duration for patients treated on protocol* 970701 *and whether or not the patient's leukemic cell retention of araCTP was* <20% *or* ≥20%.

(median not reached) and 50 weeks for patients whose leukemic cells retained >20% araCTP or <20% araCTP respectively ($P<0.04$) (Figs 3b and d). The apparently lower degree of significance for the relationship between araCTP retention and remission duration for patients treated on protocol 970701 as compared to patients treated on 950501 may be due to the differences in intensity of therapy of these two protocols. The 'dot plots' demonstrate that some patients whose leukemic cells manifested high araCTP retention nevertheless had fairly short remissions while a few patients whose leukemic cell retention of araCTP was poor had fairly long remissions. In the former situation these 'outlyers' probably reflect pharmacokinetic effects *in vivo* whereas the latter is probably a reflection of the therapeutic effects of the other chemotherapeutic agents used in the treatment protocol.

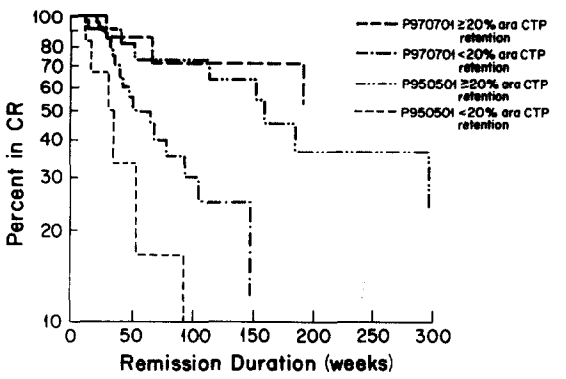

Fig. 4. Effects of intensive consolidation chemotherapy on remission duration. Comparison of remission durations for patients treated on protocols 950501 *and* 970701 *with the patients on each protocol subdivided on the basis of araCTP retention.*

DISCUSSION

The data presented here demonstrate that the duration of remission of patients with ANLL treated with the combination chemotherapy regimens described here was highly correlated with the ability of leukemic marrow cells to phosphorylate araC and retain araCTP. This report updates our initial study [5] and demonstrates that with further follow-up time the differences in remission duration for protocol 950501 patients with high or low retention or araCTP have become more significant and that a similar relationship was evident for patients treated on the successor protocol to protocol 950501, protocol 970701. On the other hand, as in our initial report, the ability of leukemic cells to phosphorylate araC or retain araCTP was not correlated with the outcome of remission induction therapy. Studies of the relationship between araCTP retention and duration of remission should be performed for patients treated on other protocols to determine whether the relationships described here also hold true for other treatment regimens.

While the median duration of remission for patients treated on protocol 970701 was greater than that for patients treated on protocol 950501, these differences are not statistically significantly different, suggesting that intensive consolidation therapy had no effect upon remission duration. If correct, this would be surprising since one would expect that three courses of intensive consolidation chemotherapy would produce greater antileukemic effects than would a treatment regimen which was of lesser intensity. The apparent equivalent therapeutic efficacy is misleading, however, since 2/3 of the patients treated on protocol 950501 were in the high retention category (>20%) while this was true for only 1/3 of protocol 970701 patients. Inspection of the remission duration curves in Figs 3c and d permits a proper appreciation of the significance of this fact since the overall remission duration curves of these protocols are displaced toward the high- or low-retention patients for protocols 950501 and 970701 respectively. Figure 4 compares the remission duration curves for high- and low-retention patients treated on each protocol. It is evident that while intensive consolidation therapy administered to patients in the high-retention groups appeared to have no effect on the relapse rate during the first 3 yr of remission, it appeared to double the remission durations for the low-retention patients. While these differences are not as yet statistically significant ($P = 0.13$), they will probably become so with further follow-up time.

The data described here present an apparent paradox: while araCTP retention was unrelated to the outcome of remission induction therapy it was highly correlated with the duration of remission. The most likely explanation for these observations resides with the differences between the conditions of exposure to araC during remission induction therapy and during consolidation/maintenance therapy. In the former situation, cells were exposed to steady-state araC levels for 10 days while during consolidation/maintenance therapy araC was administered subcutaneously, resulting in only very short durations of therapeutically effective plasma araC levels [15]. It is under the latter conditions of exposure that araCTP retention might be expected to be significant since cells which retain araCTP would have longer effective intracellular araCTP levels than would cells which retain araCTP poorly if effective plasma araC levels were present for short periods of time.

These data demonstrate that the duration of

remission of patients with ANLL treated according to the two protocols described here was highly correlated with the ability of a patient's leukemic marrow cells to retain araCTP. The relationship between araCTP retention and remission duration was noted for patients treated with moderately intensive consolidation therapy or with intensive consolidation therapy but was less dramatic for patients treated according to the more aggressive regimen, suggesting that still more intensive therapy might overcome the adverse prognostic significance of low araCTP retention by leukemic cells. Furthermore, comparison of the patients treated on these sequential protocols suggests that the benefits of intensive consolidation therapy in the first 3 yr of remission may be limited to patients whose leukemic cells rapidly degrade araCTP. Additionally, if the hypothesis presented to explain the relationship between araCTP retention and remission duration is correct, then the administration of araC by continuous infusion during consolidation/maintenance therapy may overcome the adverse prognostic effects of poor leukemic cell retention of araCTP.

Acknowledgements—The authors would like to thank Drs Stein, Doeblin and Bloom for providing the opportunity for studying the leukemic cells of their patients. The authors would also like to acknowledge the Senior Staff and House Staff of the Department of Medical Oncology, without whose assistance this work would not have been possible, and Ms Janet Meyer, Ms Jude Burns and Ms D. Brousse for their excellent secretarial assistance. The excellent technical help of Ms Buscaglia, Ms Wzrozek, Ms Knoof, Ms Jankowsky, Ms Owczarek and Mr Kelly are also acknowledged.

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