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Adriamycin and cytosine arabinoside contribute equally to prediction of response in acute myelocytic leukemia with improved confidence level

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Summary. *Samples from 15 patients with acute myeloid leukemia (AML) were studied in a colony-inhibition assay of drug sensitivity. In vitro sensitivity to both Adriamycin (Adr) (1-h incubation) and cytosine arabinoside (AraC) (24-h and continuous incubation) were determined, as were the tritiated thymidine suicide indices (SI) during the 24-h incubation with AraC. The sensitivity of the proportion of cells entering S-phase during the 24-h incubation was also evaluated. Using discriminant analysis a function was derived to separate the patients into two groups, those who failed remission induction therapy, or nonresponders (group 1), and those who entered complete remission (group 2). The only variables that contributed to prediction were the Adr (1 h) and Ara-C (24 h) results. Overall, 87% (13/15) of the patients were correctly grouped using this function, with a confidence level for nine of these 13 patients of* $> 80\%$. *Adr and AraC results contributed equally to the prediction.*

Introduction

Various authors using a colony-inhibition assay to measure drug sensitivity to chemotherapeutic agents have demonstrated that this approach can discriminate between groups of AML patients [11, 12] with reference to sensitivity or resistance to chemotherapy and can thus be of use predictively. However, while standard AML remission induction has involved treatment with at least an anthracycline and AraC, these predictions have relied on results of testing with single agents and have not attempted to estimate the relative importance for prediction of results obtained with each agent. Also, little attention has been paid to the confidence level of the prediction.

In a previous study [6] a function was derived by discriminant analysis, which resulted in 84% of patients correctly classified as sensitive or not to remission induction therapy, but only six of 19 values had a probability of > 0.80 of being selected for the correct group. The standardized discriminant function coefficients for Adr and AraC results indicated that response to Adr in the assay system was considerably more useful in predicting response than AraC data.

Because of individual variations in the pharmacokinetics of these agents, it does not seem likely that this system will allow correct prediction for more than 80%–90% of patients. However, if the predictive assay is to be useful it is desirable that the confidence level be improved upon. One way to achieve this might be to improve the contribution made by AraC to the prediction in our system.

A continuous incubation with AraC incorporated in the agar was used in the previous study, as preliminary experiments had indicated that for this agent the degree of inhibition of colony formation was not constant when the concentration-time product ($c \times t$) was constant; and this continuous exposure paralleled the in vivo administration of AraC as an IV infusion. But without an opportunity for the cells to recover from any cytostatic effects of low-dose AraC, this continuous exposure may give an overestimate of the cytocidal effects of AraC on the population. In an attempt to improve the contribution of AraC results in the discriminant analysis and to improve the confidence level of the results obtained in this study, results from both a continuous and a 24-h exposure to AraC of AML cells from 15 patients were compared, and new functions derived. A 24-h exposure was chosen as this allowed a significant proportion of the AML colony-forming cells to enter S-phase during the incubation period, but also allowed for recovery of cells only blocked from entering S-phase by the AraC.

Materials and methods

Patients. Samples from 15 patients with a diagnosis of AML admitted to the Royal Brisbane Hospital or the Royal Children's Hospital were studied. Patients received induction treatment with an anthracycline and AraC, administered as part of the ADOAP (Adr, vincristine, AraC, and prednisone) regimen [2], or the TAD (thioguanine, AraC, and daunorubicin) regimen [5]. Complete remission was defined as disappearance of evidence of disease, normal bone marrow (blasts $< 5\%$), and normal peripheral blood smear. Patients who did not enter remission were classified according to the criteria of Epstein and Preisler [4]. Type I or type II failures were classified as nonresponders (group 1). Patients who entered complete remission after one or two courses of treatment were classified as responders (group 2).

Sample collection, cell preparation and storage. Heparinized bone marrow and peripheral blood were obtained from patients with AML as part of the diagnostic procedure on presentation or relapse prior to treatment. Peripheral blood mononuclear cells and bone marrow cells were plated in agar to determine cloning efficiency. The remaining cells were suspended in RPMI 1640 with 5% dimethylsulfoxide (DMSO)

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and 15% FCS, frozen and stored in liquid nitrogen. When required they were thawed rapidly to the point of phase transition, then kept on ice, diluted slowly and washed with ice-cold RPMI with 10% FCS.

Colony-forming assay. The agar medium used consisted of 1 part bactoagar (Difco) at 0.8%. The agar was boiled for 2 min then cooled to 37° C and mixed with 1 part hypertonic medium [6] that had been sterilized by 0.45-μm membrane filtration. The hypertonic medium consisted of: FCS, 33%; Dulbecco's modified Eagle's medium (H-16, Gibco), 10 g in 215 ml water supplemented with 0.575 ml penicillin G at 200,000 U/ml and 0.375 streptomycin at 200,000 U/ml, 29%; $NaHCO_3$, 28 mg/ml, 10%; rat erythrocyte lysate, 8.0%; Hepes buffer, 6 mg/ml (pH 7.3), 4.0%; insulin 100 U/ml, 0.80%; L-asparagine, 6.6. mg/ml, 0.40%; hydrocortisone 0.18 mg/ml, 0.020%; and 14% water. Cells were suspended in the agar medium to give a final concentration of 5×10^3, 5×10^4, or 5×10^5/ml. The cell concentration for all experiments was adjusted to result in 30–100 colonies per control plate using results of preliminary platings. Aliquots (1 ml) of this suspension were plated in triplicate in 35 mm petri dishes containing 100 μl giant cell tumor-conditioned medium (GCT.CM) (Gibco). Plates were incubated at 37° C in an environment of 5% O_2, 5% CO_2, 90% N_2, and 100% humidity. Colonies of 40 cells or more were scored at 7 days.

Predictive assay. Peripheral blood or bone marrow cells were incubated with or without Adr at 3.0, 0.9, or 0.3 μg/ml for 1 h or AraC at 4.0, 1.2, 0.4, 0.12, or 0.04 μg/ml for 24 h, washed twice with 5 ml RPMI 1640, and plated. For a continuous exposure to AraC, this agent was incorporated in the agar medium at 55, 16.5, and 5.5 ng/ml. The highest concentration chosen is the calculated c × t value [6] obtained from published pharmacokinetic data. The area under each dose-response curve plotted on a linear scale was calculated [1, 7]. The area expressed as a percentage of no response (AUC % no response) was used as a measure of the magnitude of response. No response is defined as no inhibition of colony formation at the highest drug concentration used. During the 24-h incubation with AraC, cells were also exposed to tritiated thymidine at 20 μCi/ml to determine the SI [12], washed, and plated as above.

Statistical analysis. Discriminant analysis using AUC data from all patients was used to determine discriminant functions, and the posterior probability of selecting the correct group for individual patients on the basis of the derived discriminant functions. All the above statistical analysis was carried out using SPSS programs [10].

Results

The in vitro chemosensitivity of leukemic colony-forming cells from 15 AML patients was determined. Six of these patients were nonresponders (group 1) and nine patients entered complete remission (group 2) as defined under *Patients* in *Materials and methods.* Dose-response curves to Adr (1 h) and AraC (both continuous and 24-h exposure) were obtained for all 15 patients. The area under the dose-response curve (AUC) was calculated and is expressed as a percentage of the area obtained when no colony inhibition is exhibited at the highest drug concentration used (AUC % no response). The SI was also measured over the 24-h incubation period with AraC. The percent of colony-forming cells that entered S-phase and were killed was also calculated and plotted against dose. The area under this curve was calculated and expressed as a percentage of a complete in vitro response. A complete response is defined

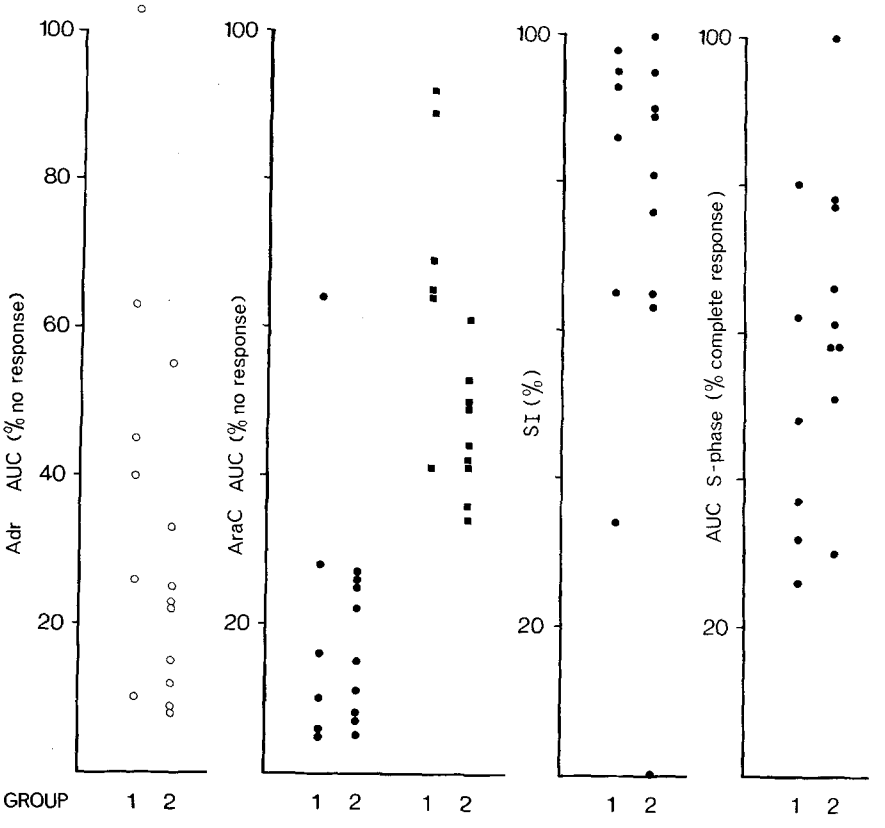

Fig. 1. Separation of group 1 (resistant) from group 2 (sensitive) patients by AUC (% no response) to a 1-h (0.3–3 μg/ml) incubation with Adr (○), and to a continuous (5.5–55 ng/ml) (●) or a 24-h (40–400 ng/ml) (■) incubation with AraC. Results are also shown for separation on the basis of the ^{3}H-thymidine suicide index (SI) (●) and for the AUC calculated for the proportion of cells that enter S-phase during the 24-h incubation and are killed by AraC, expressed as a percentage of complete response (i.e., all cells entering S-phase killed at the lowest dose) (●)

as achieved when all cells entering S-phase are killed at the lowest dose used in vitro (Fig. 1). The response to a 24-h incubation with AraC over a ten-fold higher concentration range was also evaluated (data not shown) but did not result in as great a separation between groups as for the lower range depicted in Fig. 1. Continuous exposure to AraC, the SI, and the response of cells entering S-phase during the 24-h incubation period all failed to result in a good separation of the two groups of patients.

Multivariate discriminant analysis [10] was performed using Adr data plus the results from either the continuous incubation with AraC or the 24-h incubation (Table 1). This procedure allows functions of the data to be derived, together with cut-off score values which separate one group from the other. The confidence level of these predictions can also be calculated and depends on the magnitude of the difference between the score obtained for an individual and the cut-off score. Two discriminant functions were obtained, each of which correctly predicted the outcome for 13 of 15 patients, each function giving 82% (9/11) true-positive and 100% (4/4) true-negative results. The two incorrect predictions obtained in each case were false-positive results.

When the function was derived using continuous AraC results the standardized canonical discriminant function coefficients were 0.98 for Adr and 0.08 for AraC, indicating that the AUC for Adr is much more useful for prediction of response than is the AraC AUC. Using this function five of 13 patients were correctly classified with a degree of confidence above the 80% level, and one patient was incorrectly classified with a degree of confidence > 80%. The function derived using the 24-h incubation data, however, resulted in nine of 13 patients correctly classfied with > 80% confidence. The standardized discriminant function coefficients of 0.76 and 0.71 for Adr and AraC, respectively, indicate that with this method the results obtained for sensitivity to each agent are approximately equal in predictive value.

An analysis of Adr, continuous AraC, and 24-h AraC results by a pooled within-group correlation matrix gave a correlation coefficient of −0.26 for the continuous and 24-h AraC results. This indicates that there is no evidence for a relationship between the two results and suggests that they may reflect different properties.

Table 1. Multivariate discriminant analysis of AUC data obtained for Adr (1 h) and either continous exposure or 24-h exposure to AraC

Group	Adr + AraC continous		Adr + AraC 24 h	
	Score	*P*	Score	*P*
1	3.35	0.99	3.67	1.00
1	−0.20[a]	0.62	1.60	0.93
1	1.76	0.91	1.21	0.87
1	0.35	0.57	0.68	0.71
1	0.74	0.70	−0.18[a]	0.66
1	−1.00[a]	0.83	−0.44[a]	0.76
2	−0.96	0.82	−1.38	0.94
2	−0.20	0.62	−1.11	0.91
2	−0.52	0.71	−1.04	0.90
2	−0.90	0.81	−0.98	0.89
2	−1.04	0.84	−0.96	0.89
2	−0.41	0.68	−0.60	0.81
2	0.07	0.52	−0.46	0.76
2	−0.38	0.67	−0.16	0.65
2	−0.66	0.75	0.14	0.51

Adr + AraC continous $F = -1.49 + 0.047$ Adr $+ 0.0051$ AraC
Cut-off point = 0.19
Adr + AraC 24 h $F = -3.22 + 0.033$ Adr $+ 0.038$ AraC
Cut-off point = 0.18

Score values above cut-off points indicate assignment to group 1 and score values below cut-off points, to group 2
P values indicate the confidence level associated with assignment to group 1 or group 2
[a] Assignment to incorrect group
Group 1, resistant patients; group 2, sensitive patients. For definitions of groups 1 and 2 see *Materials and methods* section

Discussion

A continuous exposure has been suggested as appropriate for determining sensitivity to phase-specific agents in predictive assays [1, 7–9]. In our hands, however, results of a 24-h exposure to AraC separated group 1 from group 2 patients better and resulted in a more reliable function for predicting remission induction in AML. A possible explanation could be that in the presence of low concentrations of AraC, a G1/S block [3, 4] prevents cells both sensitive and resistant to killing by AraC from entering S-phase. Thus, an overestimate of sensitivity to AraC is obtained when this agent is present in the agar medium and cells exposed are not able to recover from any reversible effect of the drug. A study which found that sensitivity to continuous AraC contributed to prediction of remission induction in AML in univariate analysis yielded a median D_{10} value of approximately 0.6 μg/ml [9]. This value is more than 10 times higher than the median D_{10} estimated by us in both this study and a previous study [6]. Reasons for this difference in sensitivity are not apparent but may be related to the different culture systems used.

Whilst a short period of incubation with AraC might not seem appropriate due to the small proportion of cells present entering S-phase, in this study it was found that the number of cells entering S-phase over the 24-h period, in agreement with Preisler [12], and even the colony-inhibition response related to the proportion in S-phase are not useful data for predicting response. The response of the whole population may be a better measure, because it includes cells that may not have entered S-phase during the 24-h incubation but have taken up AraC and subsequently been killed after plating in agar.

The relatively equal contribution of Adr and AraC results in the discriminant function contrasts with results of other studies, in which either the anthracycline [12] or AraC [9] have been found to make the major contribution to prediction. Thus it appears that an assay system has been developed for prediction of response to remission induction which reflects the relative contribution made by each agent to remission induction and results in a higher number (87%) of correct predictions with an acceptable degree of confidence. These results emphasize the importance, in developing predictive assays, of considering not only the number of patients correctly classified but also the confidence level of the prediction.

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