Eur J Cancer Clin Oncol. Vol. 19, No. 11, pp. 1569–1574, 1983.
Printed in Great Britain.

0277-5379/83$3.00+0.00

A Non-clonogenic Assay for the Determination of the Sensitivity of Leukemic Cells to Chemotherapeutic Agents*

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Abstract—*A new method for measuring the effects of chemotherapeutic agents on leukemic cells has been developed. In this method tritated thymidine ([³H]-TdR) is used to determine the surviving proportion of leukemic cells which are stimulated to proliferate by colony-stimulating factor (CSF). The effects of four antileukemia drugs on bone marrow cells from 20 AML patients were studied using this method and the results correlated well with the effects of the drugs on the CFU_c, which were studied simultaneously. Using the liquid culture method drug effects were measured on five bone marrow specimens which failed to clone in the CFU_c assay as well as on three specimens which produced too few colonies/clusters to allow estimation of drug effects.*

INTRODUCTION

TO DATE the most reliable in-vitro assay for predicting the in-vivo sensitivity of human AML cells to chemotherapeutic drugs is the clonogenicity assay [1]. The method for measuring the drug sensitivity of leukemic CFU_c and the correlation between the in-vitro drug sensitivity of AML cells and the outcome of remission induction have been reported from this laboratory [2, 3]. However, the suitability of the clonogenic assay for routine pretreatment screening is limited for several reasons. It is a laborious assay which requires at least 7 days to complete, and the recognition and counting of clusters/colonies is subjective. Additionally, the cells of less than half of AML patients form enough colonies or clusters to permit the measurement of drug sensitivity. In our attempts to develop a simple, rapid assay we reported that the direct effects of chemotherapeutic agents on the synthesis of DNA by the leukemic cell population as a whole did not reflect the effects of the drugs on the clonogenic cells, nor could it predict the outcome of therapy for patients treated with araC + adr [4, 5]. We now report a new method for measuring the effects of chemotherapeutic agents on leukemic cells. In this method the incorporation of tritiated thymidine is used to determine the surviving proportion of leukemic cells which are stimulated to proliferate by colony-stimulating factor.

Accepted 3 May 1983.
*Supported in part by USPHS Grant Nos CA 5834 and CA 28734-01.
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Abbreviations: AML, acute myelogenous leukemia; [³H]-TdR, tritiated thymidine; CSF, colony-stimulating factor; CFU_c, committed colony/cluster-forming cells; FCS, fetal calf serum; araC, arabinosyl cytosine; adr, adriamycin; Acla, aclacinomycine A; PBS, phosphate-buffered saline.

MATERIALS AND METHODS

Specimens

Citrated bone marrow aspirates and/or blood samples were obtained from 26 patients with AML (10 previously untreated and 16 relapsed patients). All donors and patients had signed informed consent forms as approved by the Institute's Human Experimentation Committee. Mononuclear cells were separated using a Ficol-paque density cut (dose, 1.077 g/ml, Pharmacia). The cells were diluted to 5×10^6/ml in RPMI 1640 medium (Gibco) which had been made up with 10% heat-inactivated fetal calf serum (FCS) and contained 100 U/ml penicillin and 100 μg/ml streptomycin. The cell suspension was pre-incubated for 60 min at 37°C in a humidified atmosphere containing 5% CO_2.

Incubation with drug

After preincubation, drugs were added to the cell suspension and incubation continued for

another 60 min. The cells were then washed twice with RPMI 1640 medium, resuspended in Eagle's medium (Gibco), counted using a Coulter Counter model Zbi and diluted to 1×10^6/ml. The drugs studied were cytosine arabinoside (araC, Sigma, at 0.3, 3.0 and 30.0 μg/ml), adriamycin (adr, Adria Laboratories, at 0.3, 0.6, 1.0 μg/μl), *m*-AMSA (Bristol, at 0.3, 0.6 and 1.0 μg/ml), Aclacinomycin A (Acla, at 0.3, 0.6 and 1.0 μg/ml), tritiated thymidine ([^{3}H]-TdR, ICN, 40 μCi/ml, 62 Ci/mmol) and combinations of araC + adr and araC + *m*-AMSA (0.3 μg/ml of each).

Determination of drug effects

The effects of the drugs on the treated cells were determined using two methods. In the one method the drugs' effects on the CFU_c were measured with the clonogenic assay described previously [4]. In short, 1×10^5 cells were plated in 0.3% agar over an underlayer of 0.5% agar containing colony-stimulating factor (CSF) [6]. The cultures were incubated for 7 days in a 37°C, 5% CO_2 humidified incubator. The plates were then fixed with 0.6 ml of 3% glutaraldehyde in PBS and counted in a 'double-blind' fashion.

The second method used for measuring the survival of the drug-treated cells is as follows: the drug-treated cells used for the clonogenic assay were further diluted to 2×10^5 cells/ml (1:5 dilution) in Eagle's medium. One tenth of 1-ml aliquots of this cell suspension were added to microtiter wells containing 0.1 ml Eagle's medium, CSF, FCS and antibiotics. The final contents of each well were: 2×10^4 cells in 0.2 ml Eagle's medium, 10% CSF, 20% FCS and antibiotics. The concentrations of CSF, FCS antibiotics and cells were identical to their concentrations in agar. Six wells were plated for each treatment group. The plates were incubated for 2 days in the same incubator with the plates of the clonogenic assay. After 2 days, 20 μl of [^{3}H]-TdR (2 Ci/mmol, 50 μCi/ml) were added to each well and incubation continued for another day. The plates were then harvested using a Multiple Sample Precipitator (Miller Co. Madison, WI.) and radioactivity incorporated by the cells into macromolecules counted in a Beta counter.

Analysis of results

Survival of drug-treated CFU_c was expressed as percent clonogenicity compared to controls (cell aliquots which were incubated without drug). In the liquid culture method the survival is expressed as percent incorporation of [^{3}H]-TdR (counts/min) compared to untreated control. The relationship between the drug effects as measured by the two methods was determined with the Pearson Parametric Test of Correlation using the Institute's mainframe computer.

RESULTS

In order to determine the relationship between the number of cells plated in each well and the incorporation of [^{3}H]-TdR, a cell dose–response curve was established for bone marrow specimens from two patients. The results shown in Fig. 1 demonstrate a linear relationship between these parameters with the Pearson parametric correlation coefficients $r = 0.983$ and 0.979 for the individual experiments, and $r = 0.969$ for the pooled data.

The incorporation of [^{3}H]-TdR by control cells plated in the absence of CSF ranged from 2 to 60% of the control, with a median of 17% and a mean of 26%, indicating that most of the thymidine was incorporated by cells which were stimulated by CSF.

Effects of drugs on normal bone marrow cells

The effects of four drugs which are used for remission induction therapy in AML patients on bone marrow cells obtained from four normal donors using the liquid culture system are described in Fig. 2 as drug dose–response curves. The concentrations of drugs were selected so as to produce a wide range of effects which would allow a distinction to be made between drug-resistant and drug-sensitive cells. The relationship between the effects of the drugs on normal bone marrow cells as measured by the liquid culture system and by the clonogenicity assay is presented in Fig. 3. The Pearson correlation coefficient for the effects of all drugs at all concentrations on the cells of all donors is 0.818 [$P_{(24)} < 0.001$]. As is evident from Fig. 3, when

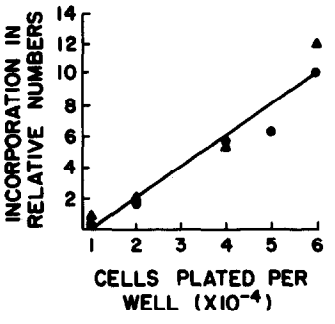

Fig. 1. Relationship between cells plated and incorporation of [^{3}H]-TdR. Cells of two patients were plated in microtiter plate wells with CSF in medium and incubated at 37°C in a humidified atmosphere containing 5% CO_2 in air. After 2 days [^{3}H]-TdR (20 μl of 50 μCi/ml, 2 Ci/mmol) was added and incubation continued for another day, after which the cells were harvested and incorporation counted (see text for details). r = 0.969. Results are expressed as the ratio of counts/min: counts/min of 1×10^4 cells.

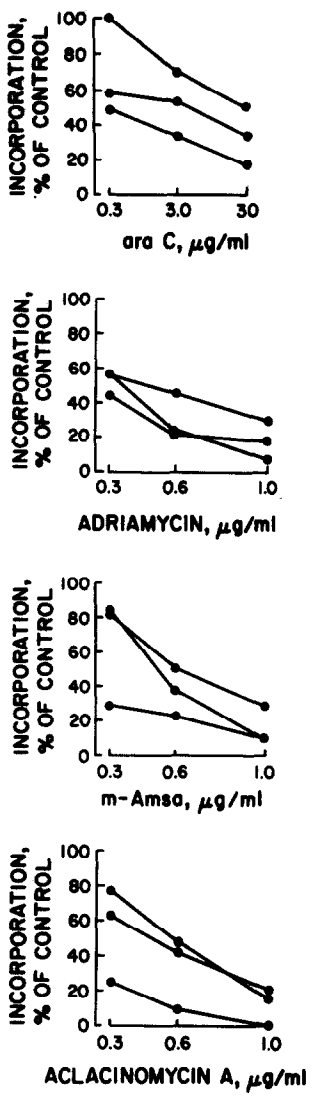

Fig. 2. Effects of incubation with drugs on the incorporation of [^{3}H]-*TdR by normal donor bone marrow cells. Cells were incubated for* 1 hr *with drugs, washed free of drug and cultured as described in Materials and Methods.*

analyzed separately the correlation coefficients for each individual dose-response curve with the added control point (100% for both clonogenicity and incorporation) were greater than 0.87.

Effects of drugs on leukemic cells

The effects of the four antileukemia drugs on leukemic cells are presented in Fig. 4 as dose–response curves. For araC, adr and *m*-AMSA the effects of the drugs on cells from different patients varied from little or no inhibition to marked inhibition of the incorporation of [^{3}H]-TdR. Less variability was observed in the effects of aclacinomycin A.

Of the 20 bone marrow specimens plated in agar, 12 grew $\geqslant$20 colonies/clusters per 1×10^5 cells and were considered evaluable for the purpose of this study. All 12 specimens were also evaluable in the liquid culture system. Figure 5 shows the relationship between the effects of

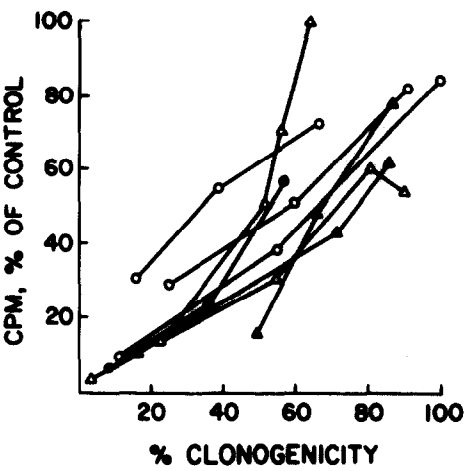

Fig. 3. Relationship between the effects of drugs on normal donor bone marrow cells using the liquid culture and clonogenic assays. ●, *Adr;* ○, m-*AMSA;* ▲, *Acla (all at* 0.3, 0.6 *and* 1.0 μg/ml); *and* △, *araC at* 0.3, 3.0, 30.0 *and* 300.0 μg/ml. *The correlation coefficient for the pooled data is* r = 0.818, [$P_{(24)} = 0.001$].

drugs on leukemic bone marrow cells of one patient as determined by the two methods. Similar comparisons for all 12 patients are summarized in Table 1. The Pearson correlation coefficient was >0.7 for 8 of the specimens, >0.8 in 6 and >0.9 in 2. Of the 6 peripheral blood specimens studied 2 were evaluable. The correlations between the two methods were $r = 0.947$ for one and $r = 0.562$ for the other. Overall, of 14 evaluable AML specimens, the results obtained for 5 showed no significant correlation.

Five bone marrow and 4 blood specimens showed no clonal growth in agar. Table 2 summarizes the effects of drugs on these specimens as well as on 3 marrow specimens which produced <20 clusters/colonies as measured in the liquid culture system. For all but two blood specimens the radioactivity incorporated by the control cells allowed accurate measurement of drug effects. In one sample the control cells showed no incorporation above the blank level (cultured cells exposed to [^{3}H]-TdR seconds before harvesting), which under the conditions described ranged from 45 to 98 counts/min/well in the different experiments. In two experiments the incorporation by the control cells was 72 ± 6 and 238 ± 52 (mean $\pm$ S.E., $n = 6$) counts/min/well above blank. The radioactivity incorporated by the other 'non-growers' ranged from 538 ± 111 to $14{,}006 \pm 412$ counts/min/well, with the overall median for this group of 919 counts/min. The radioactivity incorporated by control cells from specimens which formed colonies/clusters ranged from 302 ± 31 to $10{,}353 \pm 341$ counts/min/well with a median of 4290 counts/min/well. Overall, in 10 of the 12 specimens for which the leukemic cells' drug sensitivity could not be determined

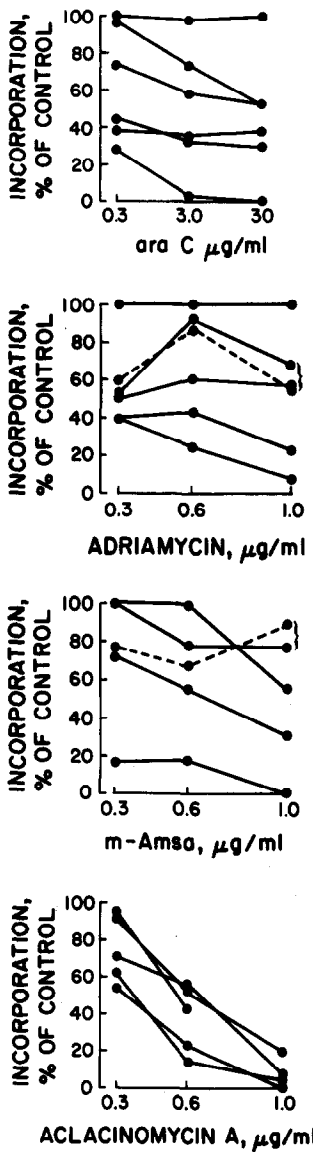

Fig. 4. Effects of drugs on AML cells using the liquid culture system. |————|, %counts/min; |------|, *%clonogenicity;* ═══, %counts/min *and clonogenicity for cells of the same patient. Cells were incubated with drugs, washed, plated and incorporation of* [^{3}H]-*TdR measured as described in Materials and Methods. Note that for some patients there is no drug dose response, as we have previously reported, yet the results of the clonogenicity assay show similar patterns.*

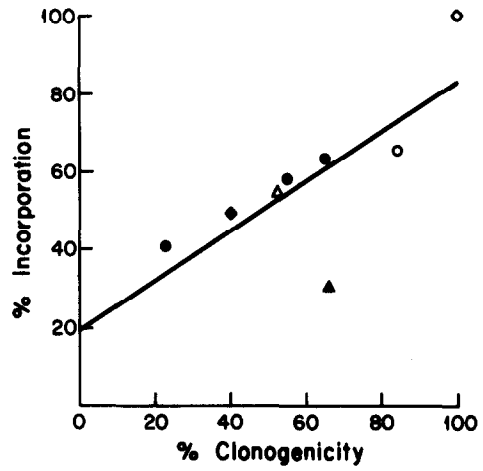

Fig. 5. Relationship between the drug sensitivities of AML cells as measured by the clonogenic assay (% clonogenicity) and by the liquid culture method (% incorporation). ◇, *control;* ●, *adr* 0.3, 0.6 *and* 1.0 μg/ml; ◆, *s.i.;* △, *araC* 30 μg/ml; ○, *araC*, 0.3 μg/ml + *adr* 0.3 μg/ml; △, *araC* 0.3 μg/ml + m-*AMSA* 0.3 μg/ml.

Table 1. Comparison between the liquid culture system and the clonogenic assay

Experiment	Specimens	No. of groups* (n)	Pearson correlation coefficient (r)
1	marrow	10	0.888
2	marrow	8	0.976
3	marrow	8	0.524
4	marrow	11	0.762
5	marrow	10	0.898
6	marrow	10	0.287
7	marrow	4	0.801
8	marrow	14	0.460
9	marrow	8	0.377
10	marrow	5	0.920
11	marrow	5	0.880
12	marrow	8	0.727
13	blood	4	0.561
14	blood	10	0.947

*Each experiment consisted of a control group of cells incubated without drugs and several groups of cells treated with the different drugs at the various concentrations. The number of drugs and concentration was dictated by the availability of cells.

using the clonogenic assay, drug effects were measureable using the liquid culture system.

DISCUSSION

There have been many attempts to develop reliable in-vitro tests for drug sensitivity. Those dependent upon assessments of drug uptake, drug activation or the direct effects of chemotherapeutic agents upon macromolecular synthesis have been of limited usefulness. To date the most promising method for assessing drug sensitivity has been the measurement of the ability of chemotherapeutic agents to kill the leukemic cells which are capable of cloning *in vitro*. It is not clear as to whether the utility of this method derives from the fact that the effects of drugs on a 'relevant' subpopulation of leukemic cells are being measured or if it is simply due to the fact that killing *per se* is being assessed [4]. The liquid culture system reported here has been devised to take into account both possible reasons for the efficacy of the clonogenicity method. In both systems colony-stimulating activity is used to stimulate the proliferation of responsive cells. A

Table 2. Liquid culture drug sensitivity of AML cells which did not clone in agar

Experiment	Specimen	Cluster/colonies per 1×10^5 cells	Control* (counts/min/well)	araC† 30 μg/ml (%)	[^{3}H]-TdR† S.I. (%)	araC 0.3 μg/ml† + adr 0.3 μg/ml (%)
15	marrow	0	238 ± 52	21	100	47
16	marrow	0	919 ± 26	26	49	67
17	marrow	0	869 ± 27	38	51	68
18	marrow	0	12,523 ± 288	71	63	39
19	marrow	0	5380 ± 111	48	52	98
20	blood	0	0			
21	blood	0	72 ± 6	IE‡	IE	IE
22	blood	0	3770 ± 23	67	82	84
23	blood	0	14,006 ± 462	94	100	100
24	marrow	<20	1502 ± 36	49	59	50
25	marrow	<20	2107 ± 84	NA§	NA	NA
26	marrow	<20	1080 ± 86	NA	NA	NA

*Expressed as means ± S.E. of 6 replicate wells.

†Expressed as percent incorporation compared to control.

‡IE = inevaluable.

§NA = not available. These two specimens were studied with adriamycin, *m*-AMSA and aclacinomycine A.

2-day delay between drug exposure and the addition of [^{3}H]-TdR has been used to accomplish two things: to allow any transient inhibitory effects of the chemotherapeutic agents upon macromolecular synthesis to dissipate and to permit enough time to elapse so that irreversibly damaged cells which might be able to go through one division after drug exposure will have done so before the addition of [^{3}H]-TdR. By setting these conditions it is likely that the [^{3}H]-TdR incorporation is a measure of the number of healthy proliferating cells in the culture, and this permits measurement of the total number of leukemic cells which have survived and are proliferating after drug exposure.

A comparison of the effects of chemotherapeutic agents on normal marrow and leukemic cells demonstrated a high level of concordance between the clonogenic and the liquid culture systems. In those few situations where there was not a statistically significant correlation between the results of these two methods it is not possible to determine, at the present time, which method was the most accurate predictor of clinically relevant drug sensitvity. Given the complexities of the cloning method as well as the subjective nature of the method used to assess clonal growth it would not be surprising if in these situations the liquid culture method was more accurate.

Twelve bone marrow and peripheral blood specimens did not produce a sufficient number of colonies/clusters to allow evaluation of drug effects in the cloning system. Of these, only two blood specimens did not incorporate enough [^{3}H]-TdR to allow an accurate measurement of drug effects using the liquid culture system. Using this method, the sensitivity of leukemic cells to antileukemia drugs could be determined in 20 out of 20 bone marrow specimens, in 4 out of 6 blood samples or in 92% of all specimens.

In summary, the liquid culture method for assessing drug sensitivity provides data which are comparable to those obtained by the cloning system used to assess drug sensitivity. The liquid system has the advantage of functioning even when clonal growth is not apparent in the systems which utilize a semi-solid matrix. The liquid system is also simpler to use, experiments are rapidly and objectively evaluated and the data are available in half the time that the cloning systems require. Clinical evaluation of this approach to assessing drug sensitivity should be performed with solid tumor specimens as well as with specimens of leukemia cells.

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