

CYTOSINE ARABINOSIDE DOES NOT CAUSE DIFFERENTIATION *IN VITRO* OF CFU-GM IN MARROW FROM NORMAL, MYELODYSPLASTIC OR ANLL SUBJECTS

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Abstract—This study examines the effect of cytosine arabinoside (Ara-C) on CFU-GM progenitor cells grown in methylcellulose culture from normal and myelodysplastic subjects and patients with acute non-lymphoblastic leukaemia. Light density marrow cells were incubated during culture with Ara-C concentrations ranging from 10^{-4} M to 10^{-12} M. After counting, colonies were cytocentrifuged and cells within the colonies examined for alkaline phosphatase positivity and expression of HLA-DR antigen, as indices of differentiation. Monocytes/macrophages were also enumerated in colonies using the monoclonal antibody CD14. In all subjects, 10^{-4} M to 10^{-6} M Ara-C caused significant reduction in CFU-GM colony formation compared with control (no Ara-C). In no instance did colony numbers increase. Ara-C across the dose curve had no effect on myeloid differentiation markers in any of the groups studied. Similarly, percentages of CD14 positive cells in colonies were not altered by exposure to Ara-C. Using this clonogenic model, these data suggest that Ara-C does not induce differentiation of CFU-GM stem cells in normal subjects or patients with myelodysplasia/acute non lymphoblastic leukaemia.

Key words: Myelodysplasia, acute non lymphoblastic leukaemia, CFU-GM stem cell, cytosine arabinoside.

INTRODUCTION

THE MYELODYSPLASTIC syndromes (MDS) are a heterogeneous group of stem cell disorders typified by peripheral cytopenia(s) in association with normocellular or hypercellular bone marrow. The median age of MDS patients at diagnosis varies from 60 years [1] to 75 years, [2] and blood cells are morphologically [3] and functionally abnormal [4–8]. Until ten years ago, treatment strategies were limited with advocates of supportive therapy only [9, 10]. Aggressive chemotherapy only appears worthwhile in younger patients [11]. Low dose cytosine arabinoside (LD Ara-C) has recently been shown to induce response in 42% of patients [12]. There is however considerable debate about the underlying mode of action, with clinical studies suggesting a

cytotoxic effect [13–15]. In-vitro studies, looking at the effect on DNA synthesis and persistence of morphological abnormalities suggest that LD Ara-C works by causing differentiation [16, 17]. This is backed up by recent work where MDS CFU-GM colonies become predominantly monocyteoid in the presence of 10^{-11} M Ara-C [18]. Other workers suggest that there might be a combination of the two effects (T. Hamblin, personal communication). In our experience patients who respond to therapy mostly have hypo/aplastic marrow trephine biopsies 21 days after commencing LD Ara-C therapy, a finding observed by others [15]. In this study we report the effect of Ara-C across a dose curve, encompassing both cytotoxic and potential differentiation concentrations, on marrow CFU-GM colony numbers from patients with different subgroups of myelodysplasia and acute non-lymphoblastic leukaemia. CFU-GM progeny are further scrutinized, using cytochemistry and APAAP immunochemistry for evidence of differentiation.

MATERIALS AND METHODS

Patient groups and marrow sampling

Marrow was aspirated from the posterior iliac crests of

Abbreviations: CFU-GM, colony forming unit granulocytic/macrophage; ANLL, acute non lymphoblastic leukaemia; LD, low dose; APAAP, alkaline phosphatase anti-alkaline phosphatase; RA, refractory anaemia; RARS, refractory anaemia with ring sideroblasts; CMML, chronic myelomonocytic leukaemia; RAEB_t, refractory anaemia with excess of blasts in transformation.

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patients with myelodysplasia and acute non-lymphoblastic leukaemia (*de novo* or secondary to previous myelodysplasia). Patient groups included refractory anaemia 5; refractory anaemia with ring sideroblasts 2; chronic myelomonocytic leukaemia 2; refractory anaemia with excess of blasts 1; RAEB in transformation 5; *de novo* ANLL 8; secondary ANLL 5. Control marrow samples were obtained from 11 patients with iron or B12/folate deficiency whose haemopoiesis could be normalised *in vitro*.

CFU-GM culture system

Light density marrow cells (LDMCs) were obtained using density gradient centrifugation (sp. gr. 1.077). LDMCs were set up in triplicate cultures at a final concentration of 1×10^5 /ml using a previously described semisolid clonogenic system [19]. Briefly, cells were cultured in supplemented Dulbecco's M.E.M. with 0.8% methylcellulose, 20% foetal calf serum and 20% lymphocyte conditioned medium. Colonies (>40 cells) were enumerated after 12–14 days.

Cytosine arabinoside (Ara-C)

Ara-C; mol. wt 243.2 (Upjohn, West Sussex) was dissolved in 0.9% NaCl solution to achieve final concentrations in culture ranging from 10^{-4} M to 10^{-12} M. Ara-C was present throughout the culture period. Saline controls were included for each patient.

Preparation of CFU-GM colonies for staining

Following enumeration of CFU-GM colonies, cells were harvested from the methylcellulose culture system; briefly, volumes of normal saline were added to each well in equal quantity to well contents. After gentle agitation with plastic pipettes, well contents were aspirated and the process repeated to remove any remaining cells. The resulting cell suspensions were spun at 400 g for 5 minutes. Supernatants were discarded and pellets resuspended in up to 1 ml of normal saline. 200 µl volumes were centrifuged using a Shandon cytocentrifuge at 500 rpm for 3 min onto glass slides which were stained freshly, or stored at -20°C for future APAAP or alkaline phosphatase investigation.

Staining techniques

(a) *Cytochemical*. Neutrophil alkaline phosphatase staining (to identify secondary granules) was performed using the method of Rutenberg [20] with fast red counterstain.

(b) *Immunocytochemical*. Two monoclonal antibodies were used: (i) WR18 (Tenovus, Southampton) which identifies HLA-DR antigen; (ii) CD14 (Department of Haematology, Northern General Hospital, Sheffield) which identifies monocytes/macrophages. For CD14, cytopreparations were fixed for 10 min in acetone and incubated with neat antibody for a standard period of 30 min. For WR18, cells were fixed for 90 s in a mixture of methanol, acetone and buffered formalin. WR18 was used neat. Positive cells were identified using an APAAP technique. Cytopreparations were examined by light microscopy and differential counts performed.

RESULTS

Table 1 compares data from normal individuals and those from the various disease categories studied.

Colony numbers were lowest in *de novo* ANLL

TABLE 1. COMPARISON OF COLONY GROWTH IN PATIENTS WITH MDS, PRIMARY AND SECONDARY ANLL AND NORMAL SUBJECTS

| Category of subject | CFU-GM colony numbers |
|---------------------|-----------------------|
| Normals (11) | 200 ± 35 |
| RAEB (1) | 80 ± 21 |
| RAEBt (5) | 51 ± 14 |
| CMML (2) | 105 ± 50 |
| RARS (2) | 111 ± 30 |
| RA (5) | 185 ± 17 |
| 2°ANLL (5) | 172 ± 33 |
| 1°ANLL (8) | 41 ± 18 |

Results expressed as mean values ± 1 standard error for individuals within each category.

CFU-GM colony assays performed in triplicate for each individual.

Results represent colony numbers/ 10^5 cells.

and greatest in CMML as compared with normals. Patients with refractory anaemia and sideroblastic anaemia had near normal capacity to produce CFU-GM colonies (irrespective of blood and neutrophil counts).

Apart from CMML therefore, CFU-GM activity was impaired in MDS patients to a greater or lesser extent.

Table 2 shows the effect of various concentrations of Ara-C on CFU-GM colony formation from patients and control subjects. Results are presented as percentage colony survival compared with no drug control (always 100% for each disease category).

Table 2 demonstrates that Ara-C was considerably detrimental to CFU-GM colony formation. This was particularly the case in the range 10^{-4} M (equivalent of 100 mg/m²) to 10^{-6} M Ara-C, and was seen throughout all the patient categories studied. In the eight patients with *de novo* ANLL, 10^{-4} M Ara-C totally abrogated CFU-GM colony formation. In no instance did CFU-GM colony numbers increase (with any Ara-C concentration).

Figures 1 (a and b) and 2 (a and b) show CD14 and HLA-DR expression respectively in CFU-GM progeny from a patient with RAEB. In both instances plates denoted (a) show cells cultured in the absence of Ara-C, and those denoted (b) show cells cultured in the presence of 10^{-6} M Ara-C. No alteration in the levels of positivity of these parameters was noted across the dose curve. If Ara-C was going to cause differentiation, one would expect a reduction in HLA-DR expression and an increase in NAP expression at around 10^{-6} M Ara-C.

Table 3 quantitates the percentage of positive cells in CFU-GM colonies for the three of the most critical

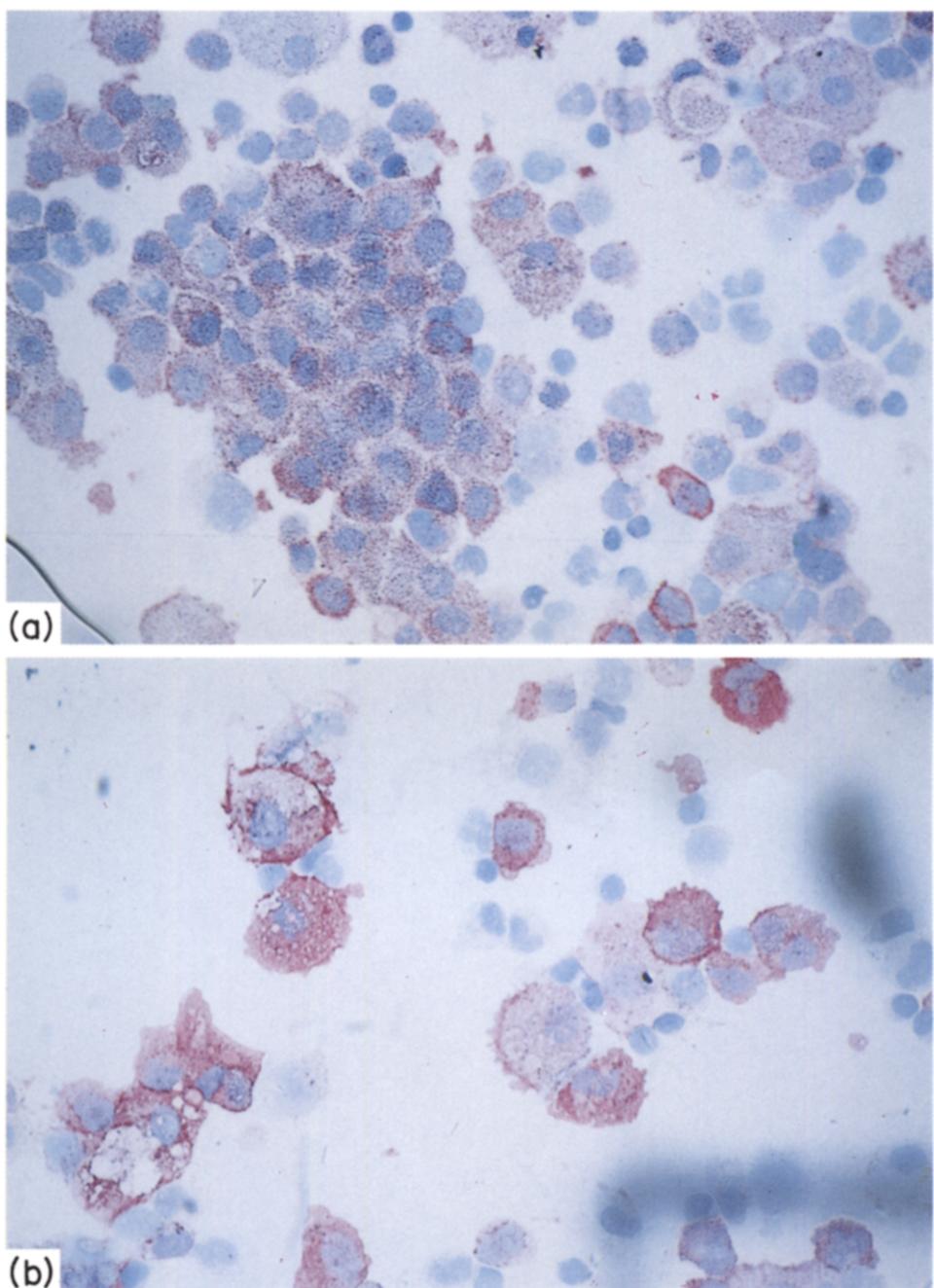


FIG. 1. CD14 expression in CFU-GM stem cell progeny from a patient with refractory anaemia with excess of blasts. (a) cells cultured without Ara-C. (b) cells cultured with 10^{-6} M Ara-C.

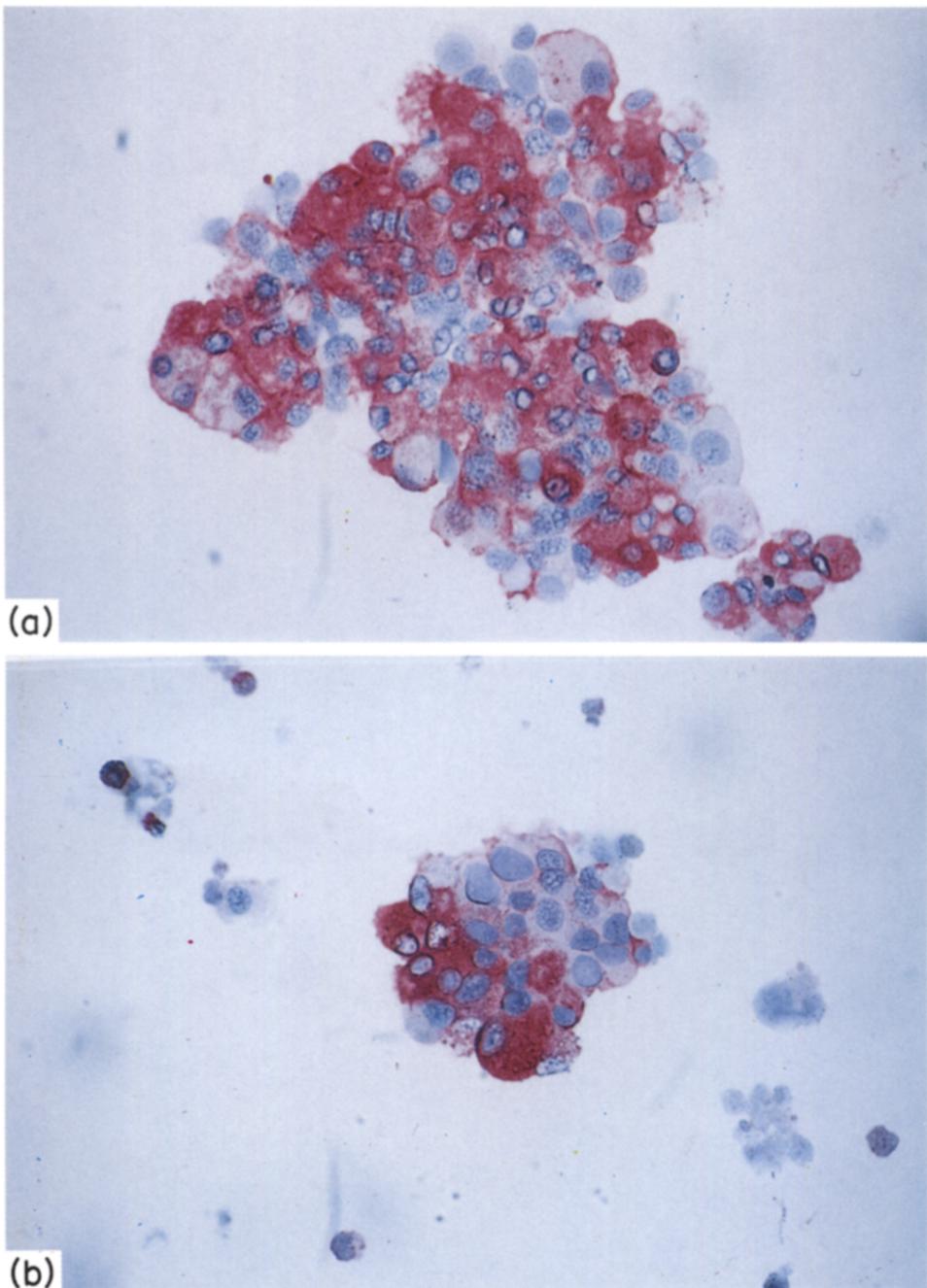


FIG. 2. HLA-DR expression in CFU-GM stem cell progeny from the same patient. (a) cells cultured without Ara-C.
(b) cells cultured with 10^{-6} M Ara-C.

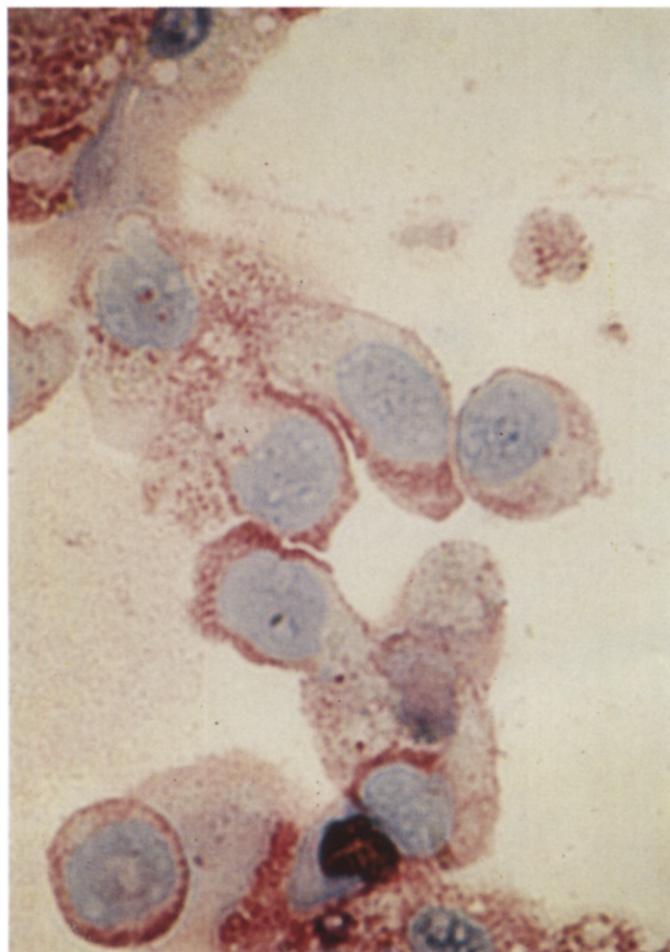


FIG. 3. CD14 negative, HLA-DR positive myeloblasts from a patient with secondary ANLL cultured in the presence of 10^{-6} M Ara-C. This patient ultimately entered complete remission on subcutaneous LD Ara-C (10 mg/M^2 b.d. $\times 21\text{d}$). The cells remain immature despite incubation with 10^{-6} M Ara-C which represents the putative differentiating concentration of Ara-C *in vitro*.

TABLE 2. THE EFFECT OF ARA-C ON CFU-GM COLONY FORMATION IN VARIOUS DISEASE CATEGORIES

| No CFU-GM | Normal | RA | RARS | CMML | RAEB | RAEBt | Secondary ANLL | Primary ANLL |
|---------------------|---------|---------|---------|---------|------|---------|----------------|--------------|
| No Ara-C | 100% | 100% | 100% | 100% | 100% | 100% | 100% | 100% |
| 10 ⁻⁴ M | 26 ± 7 | 32 ± 13 | 10 ± 6 | 32 ± 12 | 50 | 13 ± 5 | 23 ± 7 | 0 ± 0 |
| 10 ⁻⁵ M | 37 ± 4 | 30 ± 2 | — | — | — | — | — | — |
| 10 ⁻⁶ M | 40 ± 9 | 34 ± 12 | 28 ± 4 | 35 ± 7 | 46 | 20 ± 19 | 34 ± 7 | 0 ± 0 |
| 10 ⁻⁷ M | 51 ± 6 | 39 ± 3 | — | — | — | — | — | — |
| 10 ⁻⁸ M | 61 ± 12 | 54 ± 16 | 75 ± 12 | 49 ± 3 | 50 | 38 ± 9 | 62 ± 13 | 45 ± 22 |
| 10 ⁻¹⁰ M | 66 ± 12 | 55 ± 19 | 81 ± 7 | 53 ± 11 | 73 | 73 ± 15 | 72 ± 13 | 73 ± 17 |
| 10 ⁻¹² M | 77 ± 12 | 94 ± 6 | 94 ± 4 | 79 ± 9 | 76 | 91 ± 12 | 89 ± 8 | 118 ± 19 |

Numbers of surviving colonies expressed as per cent of control (no Ara-C present). See Table 1 for colony data in the absence of Ara-C.

Results expressed as mean ± 1 standard error.

Normals $n = 11$, RA $n = 5$, RARS $n = 2$, CMML $n = 2$, RAEB $n = 1$, RAEBt $n = 5$, primary ANLL $n = 8$, secondary ANLL $n = 5$.

parameters studied across the Ara-C dose curve. Two patients were selected who subsequently entered complete remission on low dose subcutaneous Ara-C (10 mg/m² 12-hourly for 21 days). One normal subject is included for comparison. Three interesting phenomena are demonstrated: (a) although the number and size of colonies decreased with Ara-C, the relative proportions of cellular constituents did not alter. In particular, there was no increase in monocyte numbers and no decrease in HLA-DR expression. This suggests that Ara-C may not be causing differentiation and from Tables 1 and 2, the only effect would appear to be cytotoxicity; (b) in the patient with secondary ANLL, there is a hiatus between HLA-DR and CD14 positivity. This is due to the presence of immature myeloblasts (Fig. 3) which are not affected by culture with Ara-C (at any concentration). If Ara-C was going to induce differentiation this immature population would disappear to be replaced by more mature granular precursors. This did not happen, even in a patient who ultimately entered CR on low dose subcutaneous Ara-C; (c) throughout the study we were impressed by the virtual absence of neutrophil alkaline phosphatase in the neutrophils cultured from MDS CFU-GM stem cells. This appears to correlate with low NAP scores in peripheral blood neutrophils and did not increase with *in-vitro* incubation with any concentration of Ara-C. Table 2 is evidence that low dose Ara-C causes CR in MDS patients through a cytotoxic effect and not by inducing differentiation.

DISCUSSION

Cytosine arabinoside can impair DNA replication and thus inhibit cell division [21, 22] and some reports suggest that this action permits subsequent terminal

differentiation in leukaemic cells and cell lines [16, 17]. Francis *et al.* [23] suggest that Ara-C *per se* is neither a differentiation of a cytotoxic agent, but, by reducing cell proliferation, a "window" for differentiation is created.

Other studies suggest that LD Ara-C (10–20 mg/m² b.d.) results in plasma levels concordant with the concentration at which the drug induces differentiation [24]. These however, take no account of the cellular concentrations of the active metabolite Ara-CTP which, when it is incorporated into the DNA strand, is a potent inhibitor of DNA synthesis [22]. In this study, *in-vitro* exposure of CFU-GM stem cells to a wide range of Ara-C concentrations in clonogenic culture, concurrent with cyto- and immuno-chemical analyses of CFU-GM progeny, failed to support a theory of differentiation. The result of this study, encompassing the various myelodysplastic subgroups as well as primary and secondary ANLL and normal subjects, demonstrates that Ara-C is a cytotoxic agent. Other workers in the field would endorse this finding. For example, Weisdorf *et al.* [15] found that marrow cells cultured in liquid with Ara-C showed little evidence of differentiation. Indeed no functional maturation was observed in cells taken from clinically responding patients. Our clinical experience is similar to others [13, 14] in that the use of LD Ara-C in myelodysplastic syndromes usually results in considerable toxicity with worsening pancytopenia before any clinical benefit is observed. In addition we and others [15] observe loss of karyotypic and abnormality post Ara-C therapy. Some workers presume that the period of marrow hypoplasia during treatment allows for the emergence of a haemopoietic clone with greater potential for maturation [25]. The presumption tends to be based on limited work *in vitro* using liquid culture of cell

TABLE 3. IMMUNOCYTOCHEMICAL AND CYTOCHEMICAL DIFFERENTIAL ANALYSIS OF CFU-GM PROGENY

| | No Ara-C | 10^{-4} M | 10^{-6} M | 10^{-8} M | 10^{-10} M | 10^{-12} M |
|---------------------------------------|----------|-------------|-------------|-------------|--------------|--------------|
| Patient 1 (secondary ANLL) | | | | | | |
| HLA-DR | 84% | 80% | 92% | 80% | 87% | 97% |
| CD 14 | 52% | 49% | 50% | 51% | 47% | 51% |
| NAP | 0% | 0% | 0% | 0% | 0% | 0% |
| Patient 2 (refractory anaemia) | | | | | | |
| HLA-DR | 60% | 54% | 57% | 53% | 60% | 52% |
| CD14 | 51% | 57% | 58% | 60% | 53% | 56% |
| NAP | 0% | 0% | 0% | 0% | 0% | 0% |
| Control subject | | | | | | |
| HLA-DR | 48% | 48% | 47% | 49% | 40% | 44% |
| CD-14 | 40% | 45% | 47% | 38% | 48% | 43% |
| NAP | 6% | 7% | 7% | 7% | 7% | 7% |

Cytospin preparations of CFU-GM progeny from two patients who responded to LD Ara-C and one control individual, examined for HLA-DR, CD-14 and NAP positivities.

Results expressed as per cent of 100 cells counted for each permutation.

lines exposed to restricted doses of Ara-C [16]. A self renewing liquid culture system, devoid of the complexity of haemopoietic regularity mechanisms operating in clonogenic CFU-GM culture, seems to present an inadequate model for investigation of the mode of action of Ara-C. Also, investigators have reported differentiation resulting from the exposure to Ara-C of human leukaemic cells in short term primary liquid cultures. We ran assays identical to those of Ishikura [17] and Michaelwicz [26], and could detect no evidence of terminal maturation in cells using our monoclonal and cytochemical criteria. Perhaps the lack of cytotoxicity observed in these assays can be attributed to the fact that inactivation of Ara-C by deamination occurs maximally at cell concentrations of $1 \times 10^6/\text{ml}$ [27], the very concentration favoured in the majority of these liquid culture systems.

It has been suggested that leukaemic cells afford some protection to normal cells [28]. This is not evident from our (unpublished) observations where CFU-GM's growing simultaneously with blast cells from MDS and ANLL patients are equally susceptible to the cytotoxic effects of Ara-C. It seems improbable that a blastic population of cells can alter the sensitivity of the DNA synthetic mechanism of normal cells to Ara-C.

One theory [14], that responding MDS patients have a malignant clone extremely sensitive to Ara-C seems reasonable. Our opinion is that since Ara-C interferes with DNA synthesis, which is an integral feature of cellular kinetics, populations of kinetically normal and abnormal cells are likely to recover from the cytotoxic onslaught differently. The impaired or failed recovery of the malignant clone in responders

to therapy would support this hypothesis. The theory also explains the almost certain period of marrow hypoplasia after successful treatment, as normal cells are not immune to the cytotoxic effects of Ara-C. The fact that chromosomal abnormalities do not persist in patients with complete response is indicative of sensitivity and destruction of the malignant clone.

The next phase of this investigation is underway, and examines the effect of successful LD Ara-C therapy on CFU-GM colony constituents in sequential marrow samples taken before, during and after treatment. Ongoing studies of this nature will permit progressive assessment of the effects of Ara-C both *in vitro* and *in vivo*.

Should a correlation emerge between treatment and culture data, it may become possible to predict those individuals who will respond to Ara-C, and thus avoid the adverse consequences of unnecessary treatment.

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