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Differentiation in Myelodysplastic, Myeloid Leukaemic and Normal Haemopoietic Cells: A New Approach Exploiting the Synergistic Interaction Between Differentiation Inducers and DNA Synthesis Inhibitors *

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A. Introduction

The normal haemopoietic system behaves as if proliferation and the differentiation transitions on and between the granulocytic, erythroid and other lineages are stochastic events governed by probabilities determined by the levels of growth and differentiation stimuli [1]. Recent studies of myeloid leukaemic and myelodysplastic (AML and MDS) cells suggest that, in contrast to cell lines, there is a shift in the balance of probabilities for proliferation and differentiation rather than a maturation block at any particular stage [2]. This in turn suggests that these cells retain many of the features of normal proliferation and differentiation and that they will be accessible to the same means of altering the proliferation–differentiation balance as in normal cells. We recently proposed that physiological and pharmacological agents which enhance differentiation and maturation in vitro act by two fundamentally different routes: (a) by hastening progression through various differentiation/maturation steps; and (b) by slowing proliferation by inhibition of DNA synthesis. In order to test this thesis we looked for synergistic interaction between these two groups of agents. Combinations of differentiation-inducing agents (retinoic acid and *N*-methylformamide) with DNA synthesis inhibitors (6-mercaptopurine, cytosine arabinoside and aphidicolin) produced a differentiation-inducing effect on normal, MDS and AML cells, equivalent to that of 10-, 100- or even 1000-fold higher concentrations of single agents. Myelotoxic effects in vitro were not synergistic. The use of these highly synergistic combinations should greatly enhance the usefulness of differentiation inducers in the therapy of MDS and AML.

The myelodysplastic syndromes (MDS) [3] are a group of closely related disorders which tend to evolve to acute myeloid leukaemia (AML) [4]. They are characterised by peripheral blood cytopenias, usually in association with a hypercellular bone marrow. This suggests a defective maturation process and there is a detectable maturation delay in vitro [5, 6]. MDS cultures are reminiscent of those in which the proliferation and differentiation of normal cells has been partially uncoupled by supplying them with a source of growth stimulus relatively deficient in differentiation-inducing activity [7].

There is no generally effective therapy available for the MDS [8], but recently there has been an upsurge of interest in using differentiation-inducing agents in MDS and also in refractory AML. Retinoic acid, vitamin D3, butyrate and harringtonine have all been used in limited clinical trials [9–14]. Agents which slow DNA synthesis by a wide variety of different mechanisms have an apparently differentiation-inducing action in vitro and there have been several trials of low dose cytotoxic drug

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therapy with varied results, in some cases suggestive of a cytotoxic action, in others of differentiation induction [15–18].

The first aim of this study was to determine whether DNA synthesis inhibitors have or induce differentiation-inducing activity or whether they act by some other mechanism. One possible mechanism of action is suggested by experimental evidence for the existence of a differentiation-responsive "window" in the cell cycle in or around the S-phase in both granulocyte–macrophage and erythroid cells [19–21]. If DNA synthesis inhibitors act by extending this window or any other mechanism independent of that involved in differentiation induction by other types of agent, DNA synthesis inhibitors and these differentiation inducers would be expected to interact synergistically. In the context of MDS this might be most useful because aggravated cytopenia and marrow hypoplasia is a common complication of therapy with low dose cytosine arabinoside and other DNA synthesis inhibitors [16, 17, 22].

True differentiation-inducing agents have the theoretical advantage that although they inevitably decrease the amplification of the haemopoietic system (i.e. the total number of cells produced from each stem cell committed to the differentiation pathway) they should not impair the stem cell compartment's ability to compensate for this. In contrast, DNA synthesis inhibitors, whatever their mechanism of action in enhancing differentiation, not only reduce the amplification of the system directly by slowing proliferation but also, since they are nonselective, inhibit stem cell division and thus reduce any possibility for compensation. That such a compensatory mechanism may operate in vivo is suggested by the different incidence of hypoplasia in patients receiving DNA synthesis inhibitors and conventional differentiation inducers.

The ability, demonstrated in this report, to enhance the differentiation-inducing effect of retinoic acid and *N*-methylformamide by very low doses of DNA synthesis inhibitors should prove useful in designing new and less toxic differentiation induction therapeutic protocols for the treatment of MDS patients and of AML patients unsuitable for or unresponsive to conventional AML therapy.

B. Materials and Methods

Marrow aspirates were obtained from three healthy volunteers, from three patients with MDS (FAB classification refractory anaemia with excess of blasts (RAEB), RAEB in transformation (RAEB-T), chronic myelomonocytic leukaemia (CMML) [1]) and peripheral blood blasts from one patient with AML (FAB-M4) and another with AML following MDS. With the exception of the last patient none had received cytotoxic therapy.

I. Differentiation Inducers and DNA Synthesis Inhibitors

The following freshly prepared stock solutions were used: 10^{-2} *M* retinoic acid (All *trans*) (Sigma), 0.6 *M* *N*-methylformamide (Aldrich 5×10^{-2} *M* 6-mercaptopurine (Wellcome), 10^{-2} *M* cytosine arabinoside (Upjohn), 10^{-3} *M* aphidicolin (Sigma). Diluents were absolute ethanol, tissue culture medium (McCoy's 5A, Flow), 0.1 *M* NaOH, pyrogen-free water, and propane-1,2-diol, respectively. The concentration of diluent was adjusted to the same level for all drug concentrations. The final concentration of ethanol was 1 : 10 000, of 0.1 *M* NaOH 1 : 500 and of propane-1,2-diol 1 : 1000 (v/v).

II. Semisolid Agar Cultures

A double-layer technique [23] was used. Target marrow cells (10^5 cells < 1.077 g/cm^{-3}) and differentiation-inducing agents were incorporated into 0.3% agar overlayers. Feeder layers contained 10^6 peripheral blood leucocytes/ml and 0.5% agar and in three experiments performed to exclude the possibility of any effect of drugs on feeder layer cells and also to ensure the growth of leukaemic cells, parallel experiments were performed using a cell-free source of gm-CSA, phytohaemagglutinin-stimulated leucocyte-conditioned medium

(5% PHA-LCM). Cultures were incubated for 7 days in 5% CO_2 in air. For morphological studies overlayers were removed and stained for nonspecific and chloroacetate esterase as previously described [7].

III. Suspension Cultures

Marrow cells ($< 1.077\ g/cm^{-3}$) were cultured at low concentration (0.5×10^5 cells/ml) in order to limit contribution of bone marrow derived gm-CSA and thus possible indirect effects; gm-CSA was provided by 5% PHA-LCM.

IV. Nitroblue Tetrazolium Reduction

Oxygen radical generation in response to *Escherichia coli* broth (kindly supplied by Dr. G. W. Smith) was assessed by reduction of NBT to produce blue-black formazan deposits. Suspension culture cells (50 µl containing approximately 10^6 cells) were incubated for 15 min at 37 °C with 20 µl *E. coli* broth and 100 µl NBT (0.1% w/v in Hank's balanced salt solution without calcium, magnesium or phenol red), cytocentrifuged and counterstained (Diffquik).

C. Results

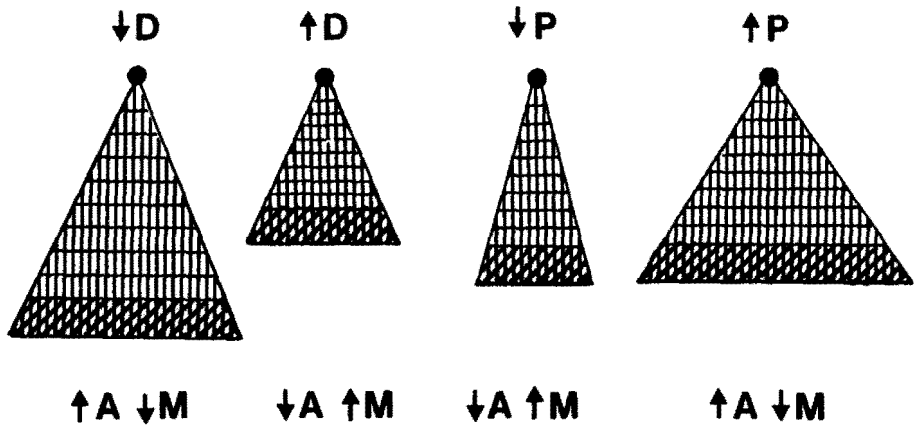
I. Alterations of the Proliferation–Differentiation Balance of Normal Cells

In the normal granulocyte–macrophage pathway proliferation and differentiation can be partially uncoupled and their rates varied independently [7]. This affects both the amplification of the system (since it affects the number of cell divisions occurring before end cell production intervenes) and also the proportion of mature to immature cells observed in in vitro systems and predicted for in vivo steady state conditions. Figure 1 shows the four hypothetical ways in which the proliferation–differentiation balance of normal cells can vary and the effects on the amplification of the system and the proportion of mature cells. Three of the four have been tested experimentally. In a previous study [7] we showed that the effect of reducing the differentiation rate without reducing the proliferation rate is increased amplification and a reduced proportion of mature cells. The converse of this effect is obtained with gm-CSA sources rich in differentiation-inducing activity or with pharmacological inducers of differentiation.

Fig. 1. Since differentiation (*D*) and proliferation (*P*) can be partially uncoupled in normal cells the amplification of the system (*A*) can be varied: (1) by altering the rate at which cells undergo one or more differentiation/maturation transitions (*horizontal lines*); or (2) by altering the proliferation rate (shown here diagramatically as the width of the *triangles*). The proportion of mature cells (*M*) predicted for steady state conditions in vivo (and observed in vitro) also varies

The effects of changes in the proliferation rate cannot be assessed readily with physiological inducers since the proliferation stimulus also conveys a differentiation stimulus. Table 1 shows the effect of reducing the proliferation rate with low levels of DNA synthesis inhibitor. Although the total number of cells produced decreased, both the proportion and the absolute number of mature cells (in this example assessed by NBT reduction) were increased. Modelling experiments based on earlier work [1] showed that this increase in mature cells is not consistent with a system in which differentiation and cell division are completely independent events and the low level of DNA synthesis has merely decreased the rate of cell division (M. F. Leaning and G. E. Francis, unpublished work). It suggests instead some special relationship between proliferation and differ-

Fig. 2. Synergistic interaction between DNA synthesis inhibitors (*vertical hatching*) and differentiation inducers (*horizontal hatching*) used in combination (cross hatching) in day 4–6 suspension cultures of normal marrow cells. The increased percentage NBT-reducing cells was due to an absolute increase in these cells in association with a decrease in the total number of cells formed. RET retinoic acid; N-MF *N*-methylformamide; 6-MP 6-mercaptopurine; ARA-C cytosine arabinoside; APH aphidicolin. The combination of 2 DNA synthesis inhibitors (*black column*) was not synergistic, merely additive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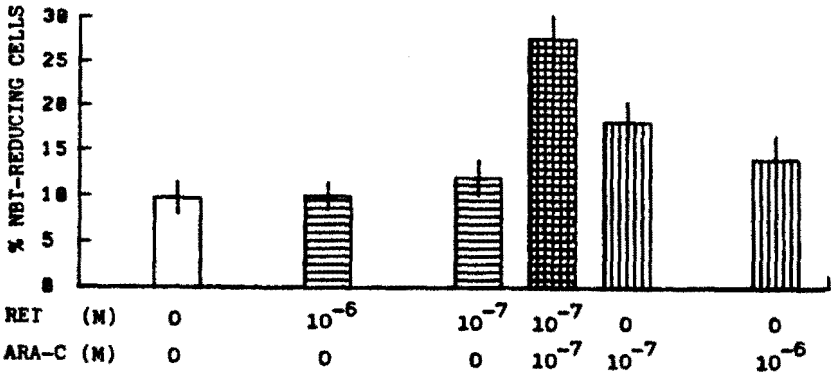
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30
25
20
15
10
5
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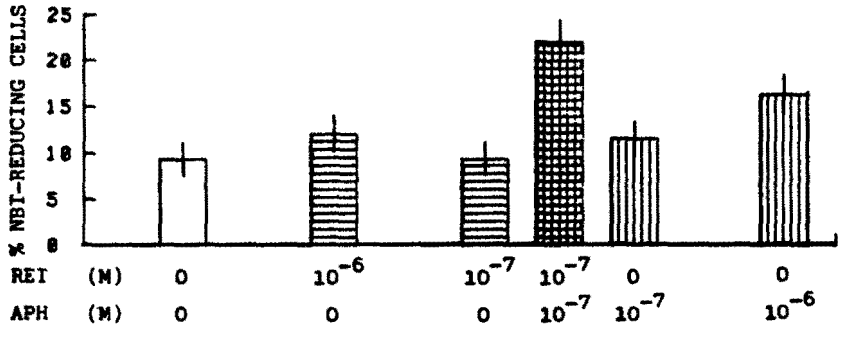
ARA-C (M) 0 0 0 10⁻⁷ 10⁻⁷ 10⁻⁶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25
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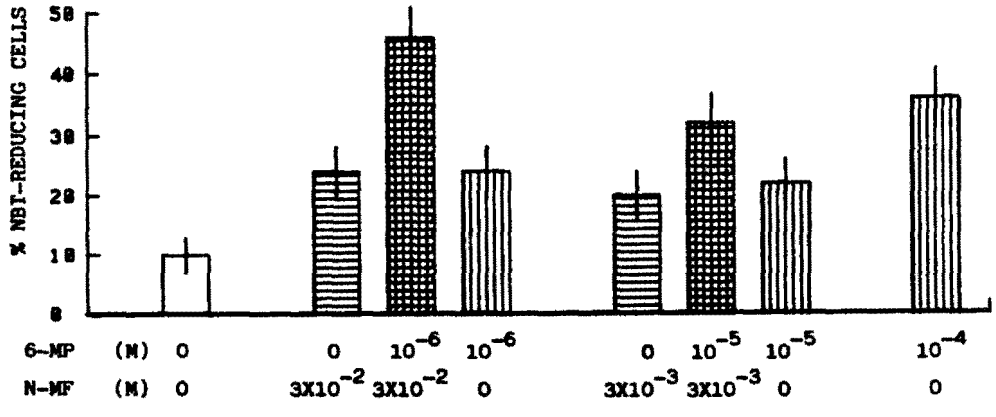
RET (M) 0 10⁻⁶ 10⁻⁷ 10⁻⁷ 0 0
APH (M) 0 0 0 10⁻⁷ 10⁻⁷ 10⁻⁶


% NBT-REDUCING CELLS
50
40
30
20
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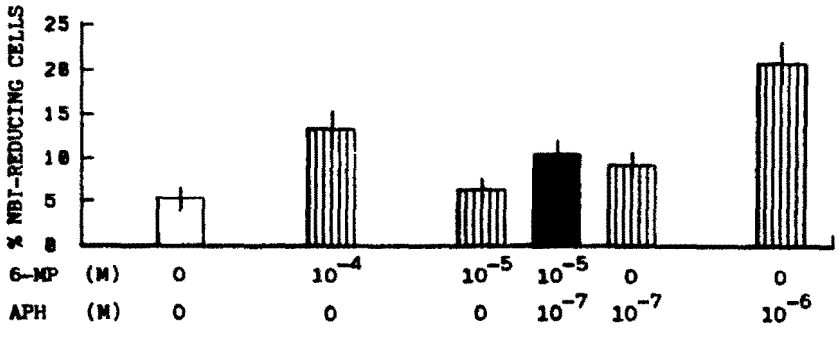
6-MP (M) 0 0 10⁻⁶ 10⁻⁶ 0 10⁻⁵ 10⁻⁵ 10⁻⁴
N-MF (M) 0 3X10⁻² 3X10⁻² 0 3X10⁻³ 3X10⁻³ 0 0


% NBT-REDUCING CELLS
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6-MP (M) 0 10⁻⁴ 10⁻⁵ 10⁻⁵ 0 0
APH (M) 0 0 0 10⁻⁷ 10⁻⁷ 10⁻⁶

Table 1. The effect of DNA-synthesis inhibitors (day 6 suspension cultures of normal marrow cells)[a]

6-Mercaptopurine (*M*)	0	10^{-7}	10^{-6}	10^{-5}	10^{-4}
Nucleated cells (% control)	100	91	77	76	75
NBT-reducing cells (% total)	10	19	24	22	36
NBT-reducing cells (% control)	100	173	183	167	271

[a] Similar results were obtained with cytosine arabinoside, aphidicolin, 6-thioguanine and hydroxyurea

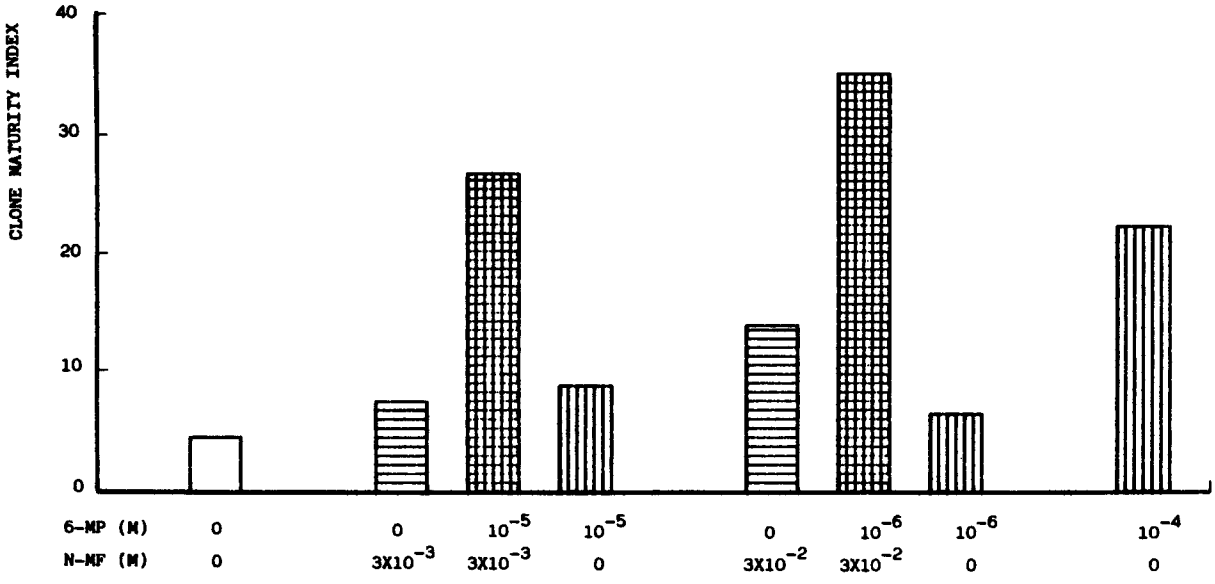

Fig. 3. Clone maturity indices (see text for definition) at day 7 based on differential counts of 90–120 consecutive clones in dual esterase-stained agar gels; marrow from an MDS (CMML) patient. Keys as in Fig. 2

entiation (such as the presence of a differentiation-responsive window already mentioned).

In order to test the thesis that DNA synthesis inhibitors enhance differentiation by a fundamentally different mechanism from that of the differentiation-inducing agents we looked for synergistic interactions between DNA synthesis inhibitors (cytosine arabinoside, 6-mercaptopurine and aphidicolin) and differentiation inducers (retinoic acid and *N*-methylformamide). Figure 2 shows the proportions of NBT reducing cells in day 4–day 6 suspension cultures. In each case there is evidence for synergistic interaction between the DNA synthesis inhibitors and the differentiation inducers. When two DNA synthesis inhibitors were added (6-mercaptopurine and aphidicolin) the effects were not synergistic, but merely additive. Similar results were obtained from dual esterase staining or proliferative capacity (assessed in secondary clonal assays) used to monitor cellular maturity in suspension cultures.

II. Synergistic Differentiation-Inducing Effects on Myelodysplastic and Leukaemic Cells

Figure 3 shows clone maturity indices in semisolid agar cultures at day 7 for a myelodysplastic marrow. In the absence of any differentiation-inducing agent the clone maturity index (defined as the percentage of clones containing mature neutrophils plus the percentage of clones containing mature macrophages divided by the percentage of clones containing blasts) at day 7 of culture was equivalent to that seen at day 4 or 5 for normal marrow cells. There was a highly synergistic interaction between *N*-methylformamide and 6-mercaptopurine such that the clone maturity index was restored to within the normal range for day 7. Figure 4 shows synergistic interactions assessed in suspension cultures by monitoring the percentage of NBT-reducing or the percentage of mature NSE- and CAE-positive cells. The myelotoxic effects assessed by inhibition of colony and cluster formation in day 7 marrow cultures

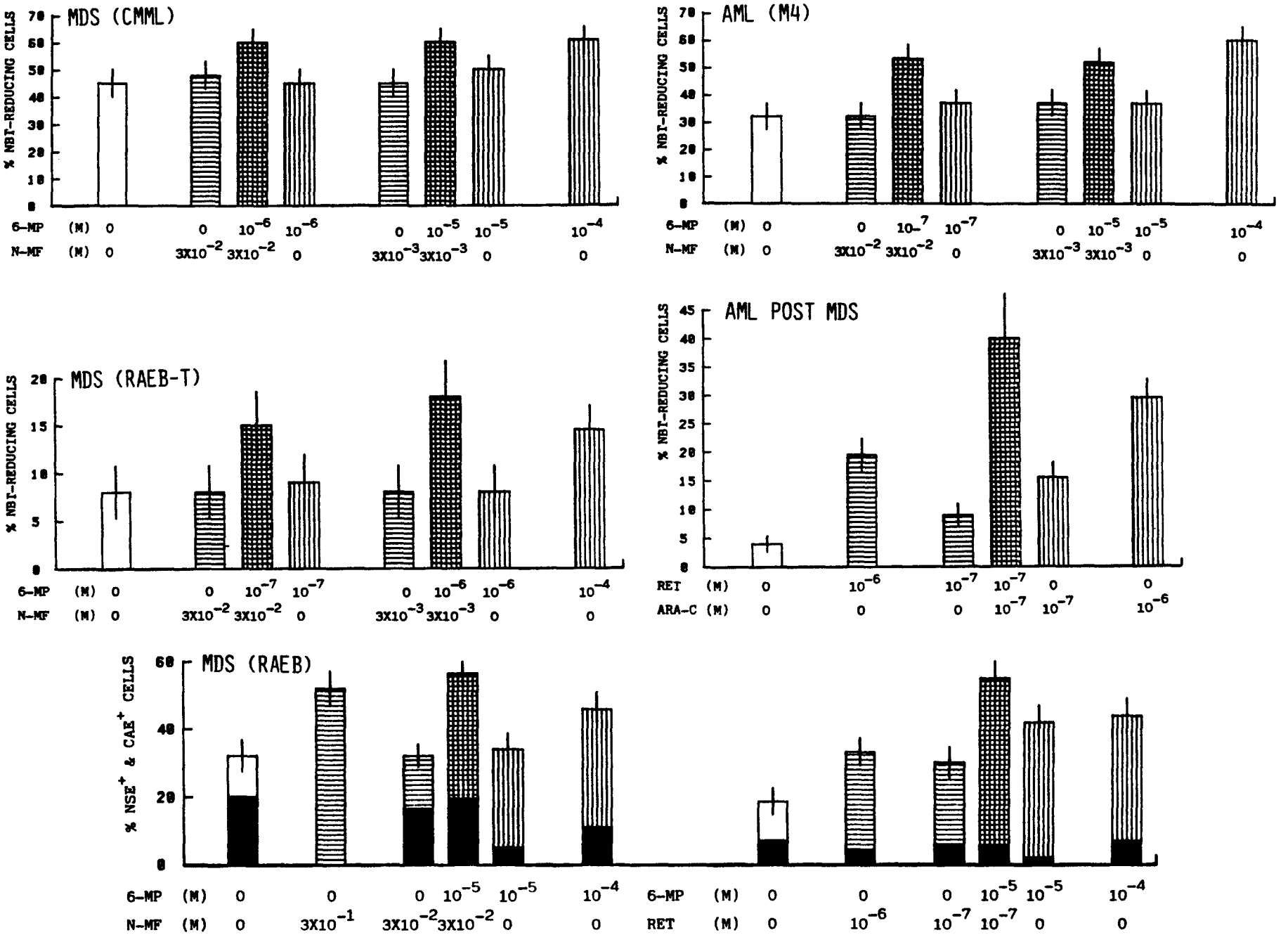

Fig. 4. Synergistic interactions were observed with cells from 3 patients with MDS (FAB classes CMML, RAEB-T, RAEB) and 2 patients with AML (FAB-M4 and post-MDS) in day 4–6 suspension cultures. Cell maturity was assessed by either NBT-reduction or by dual esterase staining for neutrophils (chloroacetate esterase$^+$; CAE$^+$) and macrophages (nonspecific esterase$^+$; NSE$^+$, the filled portion of each column)

did not show evidence of a synergistic interaction (data not shown).

D. Discussion

The results show that DNA synthesis inhibitors enhance differentiation by mechanisms which are distinct from those of the differentiation-inducing agents retinoic acid and N-methylformamide since these two groups of agents show synergistic interactions in differentiation induction. Although maturation appears arrested in acute myeloid leukaemia and differentiation-defective myeloid cell lines are plentiful, cells freshly explanted from patients retain many of the features of the normal proliferation–differentiation balance. The differentiation dose–response curves for these cells do not provide evidence for a maturation block (i.e. a maturational stage through which the cell cannot pass or can only pass with the aid of pharmacological differentiation inducers), but suggest instead that there is a shift in the probability of undergoing one or more differentiation transitions [2]. This implies that the cell is accessible (albeit with reduced sensitivity) to agents which enhance the differentiation of normal cells.

The differentiation-enhancing effect of DNA synthesis inhibitors seems unlikely to be due to any conventional differentiation-inducing activity, since agents inhibiting DNA synthesis by a wide variety of different mechanisms have this effect (e.g. not only the agents tested in this study but also hydroxyurea and 6-thioguanine). The results are, however, consistent with the accumulating evidence for the existence of a differentiation-responsive window in the cell cycle since DNA synthesis inhibitors might enhance differentiation by prolonging this window. The effect might simply be temporal or due to build-up of some receptor or enzyme important in the differentiation process during this period. Whatever the mechanism of interaction the highly synergistic effect observed for myelodysplastic and AML cells suggests that improved differentiation-inducing therapeutic protocols may be devised on this basis.

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