In Vitro and Preliminary in Vivo Studies of Compounds which Induce the Differentiation of Friend Leukemia Cells*

H. D. Preisler, M. D.

Roswell Park Memorial Institute
Department of Medicine A
Buffalo, New York 14263

The progeny of normal hematopoietic stem cells ultimately become mature functional cells through a process involving both cell replication and differentiation. The proliferative aspect is limited and in fact as full maturation is approached the cells lose their proliferative potentital. Built into the process of differentiation is an inherent limitation of the life span of the maturing cells. In essence, acute myelogenous leukemia (AML) results from a disruption of the normal maturation process. Leukemic stem cells proliferate without normal constraints and their progeny for the most part remain at the stem cell level. This latter phenomenon results in the cells retaining their proliferative potential and endows the cells with a life span which is longer than that of normal cells. The resulting increasing mass of leukemic cells, through an as yet undefined process, interferes with the proliferation and maturation of normal myeloid elements ultimately causing the pancytopenia which is directly responsible for the morbidity and mortality which accompanies AML.

Current approaches to the therapy of AML are directed towards the destruction of the leukemic cells by the administration of cytotoxic chemotherapy. Unfortunately the chemotherapeutic agents are also toxic for normal hematopoietic stem cells and one of the major side effects of this form of therapy is pancytopenia – with attendant morbidity and mortality.

A variety of studies have demonstrated that malignant cells possess the potential to differentiate and that differentiation is frequently associated with a decrease in or a loss of malignant potential (1–4). These observations suggest that it may be possible to treat cancer (including AML) by inducing the differentiation of the malignant cells. Such an approach, even if only moderately effective, might result in the production of mature granulocytes which could alleviate the granulocytopenia associated with the disease.

Friend Leukemia as a Model System

The Friend leukemia is a viral induced murine leukemia (5). The disease is characterized by the proliferation of blast cells with the spleen being the major site of leukemic cell proliferation. Some of the progeny of leukemic cells prolife-

* This research was supported by U. S. P. H. S. grant CA-5834.

rating in the spleen undergo erythroid differentiation while the same leukemic cells growing subcutaneously produce tumors which are devoid of differentiating erythroid cells (6). These tumors have been classifield as "reticulum cell sarcomas" (7).

Several investigators have established long-term suspension culture cell lines from the leukemic mice (8–10). These leukemic cells grow in suspension culture as morphologically undifferentiated blast cells. A small proportion of the tissue culture cells (usually $< 1\ \%$) spontaneously differentiate along the erythroid pathway. It was found that the addition of either dimethylsulfoxide (DMSO) (11) or dimethylformamide (DMF) (12) to the culture media of these cells induced a substantial proportion of the leukemic cells to differentiate along the erythroid pathway. These observations led to the conclusion that the Friend leukemia cells growing in suspension culture represented a class of committed erythroid precursor cells whose normal maturation had been prevented by their neoplastic transformation.

Recent studies in our laboratory have made the question of the nature (multipotential stem cell vs. erythroid progenitor) of the Friend cells growing in culture more complex than had previously been appreciated. We have found that when these tissue culture cells (line 745A) are innoculated subcutaneously into mice, the majority of tumor cells growing at the site of innoculation contain chloroacetate esterase (CAE) and some of the cells are peroxidase positive as well (13). Both enzymes are felt to be characteristic of granulocytic cells and have not been reported to be present in cells of the erythroid series nor in reticulum cell sarcomas. Some of the tumor cells appear morphologically to be undergoing abortive granulocytic maturation. The tissue culture cells themselves are all chloroacetate esterase positive (14). It should be noted that Friend virus infection of mice is associated with a significant increase in granulocyte colony forming units (15). It is not known if this increase is due to neoplastic transformation of granulocytic progenitor cells or if it is merely reactive proliferation of normal progenitor cells.

Thus the question as to the nature of the Friend leukemia cell is more complicated than had hitherto been realized. Are they erythroid progenitors whose maturation has been arrested by the leukemic state and whose genomic regulation has been so distorted by malignant change that it programs for the synthesis of enzymes which are normally not present in erythroid cells? Or are they granulocytic (CAE positive) stem cells which in the in vitro environment have a greater potential for erythroid rather than granuloid differentiation? One interesting aspect of this problem is the fact that while DMSO-induced erythroid differentiation in vitro appears to be associated with a decrease in the degree of CAE positivity of the cells, the majority of cells remain CAE positive and in fact we have found cells which are simultaneously strongly positive for both CAE and heme (benzidine stain). At present wo do not know if the CAE present in cells which are synthesizing heme is being actively synthesized or if CAE synthesis ceases with the onset of erythroid differentiation and we are detecting residual enzyme. In any event, it is clear that the cells which are induced to differentiate along the erythroid pathway are the cells which had previously synthesized an enzyme believed to be characteristic of granulocytic differentiation. Hence if CAE positivity denotes granulocytic differentiation then either the cell can simultaneously differentiate along two different pathways or alternatively the cell must dediffer-

entiate from the granulocytic pathway and then differentiate along the erythroid pathway. At the present time assigning the Friend cell to a particular cell series appears to be essentially a problem of semantics.

The ability to induce erythroid differentiation in vitro and the ability of the tissue culture cells to produce leukemia when innoculated intravenously into DBA 2/J mice suggested that this system could serve as a prototype for determining if agents which induce leukemic cell differentiation in vitro could function as chemotherapeutic agents in vivo.

Cryoprotective Agents as Inducers of Differentiation

Our initial studies demonstrated that both DMSO and DMF were too toxic to permit chemotherapeutic trials in mice. Since both inducers were aproteic polar solvents which were cryoprotective agents as well, we studied the ability of other cryoprotective agents to induce the differentiation of Friend leukemia cells in vitro. These studies ultimately led to the recognition of other inducing agents which were more potent and less toxic than DMSO or DMF (16, 17).

Table I

Inducer	% B+ cells*
Tetramethylurea	70
Dimethylacetamide	70
N-methylacetamide	55
Pyridine N oxide	55
Dimethyl sulfoxide	55
Dimethyl formamide	30
Acetamide	16
Dimethylurea	13
Pyridazine	10
Diethylene glycol	7
Control	0.3

* % of benzidine positive cell after 5 days of culture in the presence of the inducing agent.

Table I lists some of the compounds which we tested and their effects on the proportion of differentiated cells in Friend leukemia cell cultures. Fig. 1 demonstrates that the compounds exhibited various physical structures ranging from linear molecules to aromatic molecules. The agents are all small, polar, freely diffusible compounds. Nash has conducted extensive studies of the physical properties of cryoprotective compounds and has found that the cryoprotective potential of a compound correlates with the ability of the compound to donate free electron pairs (to function as a Lewis base) (18, 19). This same property also correlates with the relative ability of compounds within a family (such as tetramethylurea, dimethylurea, and urea) to induce differentiation (17). While compounds vary widely in their inducing potency, it appears that once begun the biology of the

process of differentiation is the same regardless of the agent used to induce differentiation (17).

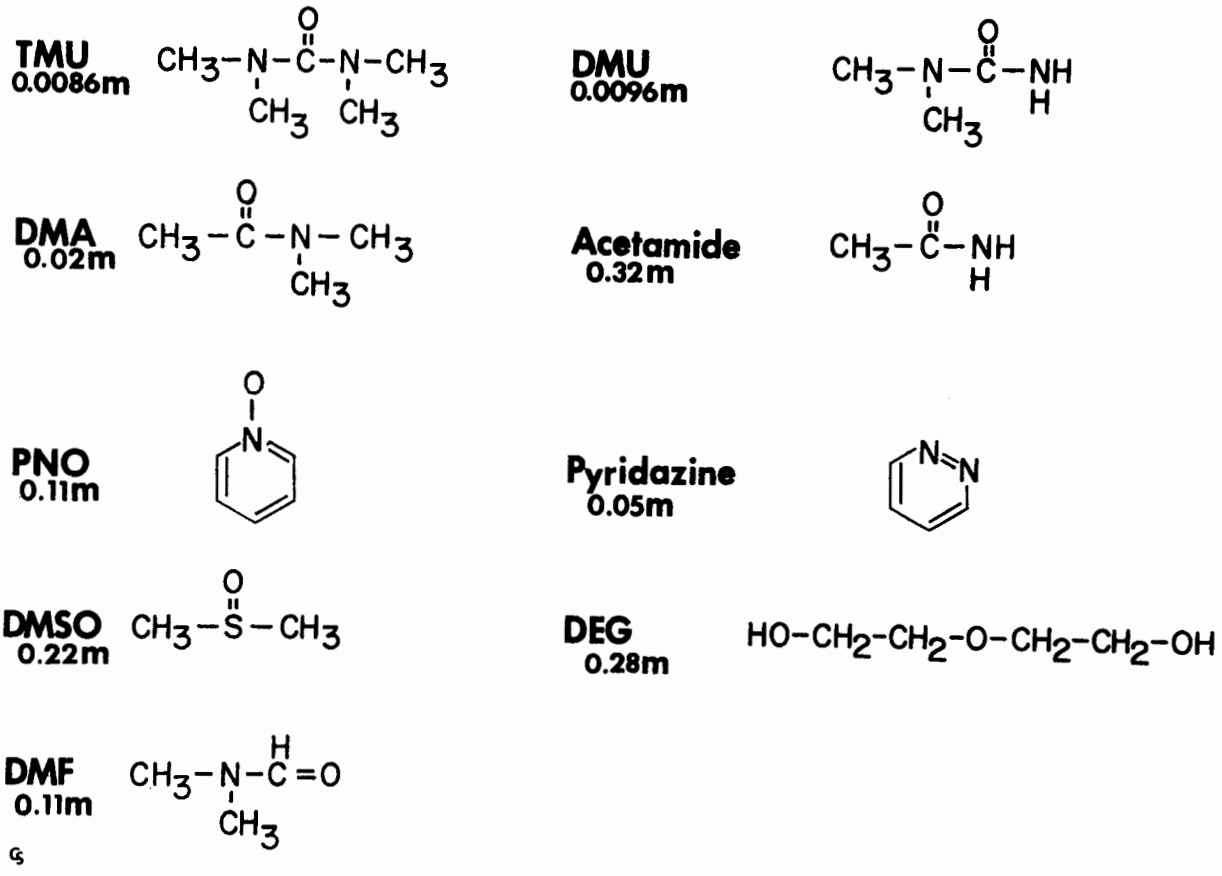

Fig. 1: Physical structure of the inducing agents listed in Table I. Numbers under names of compounds indicate optional inducing concentrations.
This research was supported by U.S.P.H.S. grant CA-5834.

Despite the obvious similarities between the inducing agents and the general similarities in the process of induced differentiation, it should be recognized that not all of the effects of the inducing agents are identical or necessarily related to differentiation per se. For example, when line 745A cells are cultured in the presence of DMSO there is an 80 % decline in the acid soluble ATP pool size by the end of 5 days of culture. This significant fall in ATP pool size is not seen when cells are cultured in the presence of tetramethylurea, a more potent inducer of differentiation than DMSO (20). In this case there is only a 20–25 % decline in pool size. Furthermore BUdR-inhibition of DMSO-induced differentiation does not prevent the decline in ATP pool size. Thus a biological or biochemical phenomenon which accompanies chemical-induced differentiation is not necessarily part of or even related to the process of differentiation.

The situation is further complicated by our observations that cells may be resistant to the inducing effects of one compound and yet be induced to differentiate by a different compound. We have conducted studies with a cell line which is not induced to differentiate by DMSO (cell line 745D). This cell line is responsive, albeit to a low degree, to pyridine N-oxide, N-methylacetamide and DMF (17).

Furthermore, this cell line responds to a significant extent (greater than 20 % of cells induced to differentiate) to butyric acid (14), a compound demonstrated by Leder & Leder to be an effective inducer of differentiation in their Friend leukemia cell line (21). Despite the fact that butyric acid is a much better inducer of 745D differentiation than is DMSO, the former is a less potent inducer of 745A differentiation. From these observations it is apparent that caution must be exercised in interpreting and generalizing from experimental studies since: 1) equally potent inducers of differentiation may exert differing biological and biochemical effects 2) different cell lines may have differing sensitivities to the various inducers of differentiation.

Mechanism of Action of Inducing Agents

The differentiation of FLC in vitro is accompanied by the accumulation of globin mRNA (22, 23). Bromodeoxyuridine (BUdR) which interferes with globin mRNA accumulation inhibits DMSO-induced differentiation (24). Accumulation of globin mRNA during differentiation has been noted in most studies. Therefore any stimulus which induces the differentiation of these cells appears to ultimately act through the process of transcription. There may be however an exception to this statement (25). Paul et. al. have presented evidence for translational control of differentiation of a Friend leukemia line.

Our initial studies of the effects of BUdR on DMSO-induced differentiation led us to postulate the presence of a repressor of differentiation in the leukemic cells with DMSO-induced differentiation resulting either in a decreased affinity of repressor for operon or in a decrease in the level of intracellular inhibitor (24). The recognition of the fact that the inducers were small molecules whose inducing potency appeared to be related to their ability to donate lone pair electrons permitted a broadening of this concept with the actual physical interaction involved in transcription being related to: 1) disruption of water structure with secondary alterations in hydrophobic bonding of regulatory molecules to DNA (such as histones) 2) interaction between inducers and acidic nucleoproteins, or 3) perhaps direct disruption of the internal hydrogen bonding within the DNA helix permitting transcription of the resultant single stranded DNA sequences (16). In support of this possible mode of action are the reports of DMSO stimulates DNA transcription in vitro (26) and the studies of the effects of BUdR and DMSO on chromatin structure (27).

On the other hand, the ability of inducers to cryoprotect erythrocytes suggested that these compounds have significant effects on the cell membrane. Using differential scanning calorimetry (DSC) we have found that the inducing-cryoprotective agents increase the melting temperature of artificial acidic phospholipid vesicles, a finding we interpret as indicative of a decrease in the fluidity of the vesicle membranes. Compounds, such as topical anesthetics, which increase the fluidity of phospholipid membranes in vitro, interfere with the effects of the inducers on the phospholipid vesicles and also inhibit the induction of differentiation of Friend leukemia cells in suspension culture (28). In fact, to date, using DSC and the artificial phospholipid vesicle system we have been able not only to account for our previous observations (such as apparent synergistic induction of differentiation

by the combination of DMSO and DMF and the only additive effects of DMSO and PNO) but also have been able to prospectively predict the effects of agents and combinations on the differentiation of FLC 745A in vitro. These observations suggest that the initial event during the induction of differentiation by cryoprotective agents may occur at the cell surface with transcription being a necessary but secondary phenomenon mediated perhaps by communication between the cell membrane and nucleus.

It is clear that at this time it is not possible to state whether the chemical agents induce differentiation by directly stimulating transcription of those DNA sequences which code for erythroid differentiation or by interacting with the cell membrane and thus indirectly inducing differentiation. It is possible that the same physical properties (low molecular weight, Lewis bascity, etc.) which produce the effects on acidic phospholipid vesicles and provide cryoprotection to erythocytes are also responsible for directly altering the transcription of DNA. To complicate matters further, as noted above, some Friend leukemia cell lines are responsive to some agents and more or less responsive to others. In at least one cell line butyric acid has been found to interfere with DMSO-induced differentiation (21). Conversely, but leading to a similar potential conclusion, the combination of some inducing agents appear to be "synergistic" while others are "additive" (16). Hence it is possible that there is more than one mechanism by which chemical agents induce the differentiation of FLC in vitro or alternatively the various cell lines may differ in the way in which they metabolize the inducers (exclude, activate, or deactivate the compounds) or differ in the composition of their cell membranes and hence while differences in membrane composition result in different inducer sensitivities the acutal changes in the cell membranes produced by the agents may be identical (for example all may decrease membrane fluidity).

Whatever their mechanism of action in vitro, the recognition of a wide variety of inducing agents has made possible the in vivo testing of their possible chemotherapeutic efficacy.

In Vivo Chemotherapy

Our initial studies have employed the intravenous innoculation of FLC lines 745A into syngeneic DBA 2/J mice followed 1 week later by the daily administration of N-methylacetamide. This therapy significantly and consistently inhibited the proliferation of FLC in the spleens of the treated mice and to a somewhat lesser degree the proliferation of leukemic cells in the bone marrows of the mice (29). Survival of the mice has not been consistently prolonged, perhaps a result of the hepatoxicity of the compound. In any event we are now in the position to determine whether the studies of leukemic cell differentiation in vitro are applicable to the treatment of leukemia in vivo.

Future Perspective

The induction of leukemic cell differentiation in vivo may provide a mode of therapy which is significantly different from that currently employed. It now appears feasible to test this proposition in vivo in mice.

References

1. Pierce, G. B. and Wallace, C. (1971) Cancer Res. 31: 127–134.
2. Prasad, K. N. (1972) Cytobios 6: 163–166.
3. De Cosse, J. J., Gossens, C. L., Kuzma, J. F. and Unsworth, B. R. (1973) Science 181: 1057–1058.
4. Ichikawa, Y. (1969) J. Cell. Physiol. 74: 223–234.
5. Friend, C. (1957) J. Exp. Med. 105: 307–318.
6. Friend, C., Preisler, H. D., and Scher, W. (1974) Curr. Top. Develop. Biol. 8: 81–101.
7. Buffet, R. F. and Furth, J. (1959) Cancer Res. 19, 1063–1069.
8. Friend, C., Patuleia, M. C. and Harven, (1966) Natl. Cancer Inst. Monogr. 22: 505–522.
9. Ostertag, W., Melderis, H., Steinheider, G., Kluge, N. and Dube, S. (1972) Nature (New Biol) 239: 231–234.
10. Ikawa, Y., Furusawa, M. and Sugano, H. (1973) in: Unifying Concepts of Leukemia. Bibl. Haemat. No 39, 955–967.
11. Friend, C., Scher, W., Holland, J. G. and Sato, T. (1971) Proc. Nat. Acad. Sci. (USA) 68: 378–382.
12. Scher, W., Preisler, H. D. and Friend, C. (1973) J. Cell Physiol. 81: 63–70.
13. Preisler, H. D., Bjornsson, S., Mori, M. and Barcos, M. (1975). Cell Diff. 4:213–283.
14. Preisler, H. D., Shiraishi, Y., Mori, M., and Sandberg., A. A. Submitted to Cell Diff.
15. Golde, D. W., Faille, A., Sullivan, A., and Friend, C. (1975) in: Proc. Amer. Assoc. Cancer Res. 16:13.
16. Preisler, H. D. and Lyman, G. (1975) Cell Differ. 4: 179–185.
17. Preisler, H. D., Christoff, G. and Taylor, E. (1976). Blood. 47:363–368.
18. Nash, T. (1962) J. Gen. Physiol. 46: 167–175.
19. Nash, T. in: Meryman, H. T. (ed): Cryobiology. New York, Academic Press 1966, Chapter 5, 179–211.
20. Preisler, H. D. and Rustum, Y. (1975) Life Sciences. 17:1287–1290.
21. Leder, A. and Leder, P. (1975) Science. 5:319–322.
22. Ross, J., Ikawa, Y. and Leder, P. (1972) Proc. Nat. Acad. Sci. (USA) 69: 3620–3623.
23. Preisler, H. D., Scher, W. and Friend, C. (1974) Differ, 1: 27–37.
24. Preisler, H. D., Housman, D., Scher, W., and Friend, C. (1973) Proc. Nat. Acad. Sci. (USA) 70: 2956–2959.
25. Harrison, P. R., Gilmour, R. S., Affara, N. A., Conkie, C. and Paul, J. (1974) Cell Differ. 3: 23–30.
26. Travers, A. (1974) Eur. J. Biochem 47: 435–441.
27. Lapeyre, J. and Bekhor, I. (1974) J. Mol. Bio. 89: 137–162.
28. Lyman, G., Preisler, H. D. and Papauadjopoulous, D. submitted to Nature.
29. Preisler, H. D., Bjornsson, S., Mori, M. and Lyman, G. Presented at the American Society of Hematology Meetings, Dallas (1975).