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Effects of Trichostatins on Differentiation of Murine Erythroleukemia Cells

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ABSTRACT

The fungistatic antibiotics trichostatins (TS) A and C were isolated from culture broth of *Streptomyces platensis* No. 145 and were found to be potent inducers of differentiation in murine erythroleukemia (Friend and RV133) cells at concentrations of 1.5×10^{-6} M for TSA and 5×10^{-7} M for TSC. Differentiation induced by TS was cooperatively enhanced by UV irradiation but not by treatment with dimethyl sulfoxide. This enhanced activity was completely inhibited by adding cycloheximide to the culture medium 2 h after exposure to TS, suggesting that TS are dimethyl sulfoxide-type inducers of erythroid differentiation. No inhibitory effect of TS was observed on macromolecular synthesis in cultured cells.

INTRODUCTION

The murine erythroleukemia cell line established by Friend *et al.* (1) consists of transformed cells in which erythroid differentiation is blocked by infection with Friend leukemia virus (1, 2). Differentiation is reinitiated effectively by various polar compounds such as DMSO[2] (3) and HMBA (4). Relatively weak differentiation is also induced by a variety of agents affecting DNA metabolism, such as UV, mitomycin C and bleomycin (5–7).

The Friend cell line and the inducers of its erythropoiesis have been extensively studied as a model system of cellular differentiation. Nomura and Oishi (8) showed that erythroid differentiation could be induced indirectly by fusing two genetically marked Friend cell lines, each of which had been separately treated with doses of DMSO and UV insufficient for the induction of their independent differentiation. Their results revealed that the commitment to erythroid differentiation in Friend cells is a synergistic result of two fundamentally different cellular reactions, which may be a membrane-mediated reaction triggered by polar compounds and another reaction involving an intracellular potentiator produced as a result of DNA-damaging treatment (9–11).

The characteristics of erythroid differentiation in the Friend cell line would appear to provide an in vitro screening system for characterizing new agents with differentiation-inducing activities. Recently, as a result of such screening, we found that trichostatins A and C (Fig. 1), originally reported as antifungal antibiotics (12, 13), had potent inductive effect on erythroid differentiation at extremely low concentrations (14). In this paper, we describe the characterization and mode of action of trichostatins on erythroid differentiation. A synergistic effect of trichostatins with UV irradiation but not with DMSO was observed by cell fusion experiments which indicated that trichostatins act as DMSO-type inducers. Furthermore, no inhibitory effect on DNA and RNA synthesis was observed with trichostatins. This finding strongly suggested that they were not DNA-damaging agents, such as mitomycin C and actinomycin D, which caused a significant inhibition of DNA and RNA synthesis. The usefulness of these compounds as reagents for analyzing cell differentiation is also discussed.

MATERIALS AND METHODS

Materials. Trichostatins A and C were prepared from culture broth of *Streptomyces platensis* No. 145 according to the procedure described previously (14). Trichostatin A was purified using the same procedure as that for trichostatin C, except that methanol:water (7:3, v/v) was substituted for acetonitrile:water (35:65, v/v) as the solvent system for the final high-performance liquid chromatographic run.

HMBA, thymidine, aminopterin, hypoxanthine, and cycloheximide were purchased from Sigma Chemical Co., ouabain was from Merck, and polyethylene glycol 6000 from J. T. Baker Chemical. Phorbol and TPA were obtained from Chemicals for Cancer Research, Inc. [*methyl*-^{3}H]Thymidine (77.8 Ci/mmol) and [*G*-^{3}H] uridine (6.0 Ci/mmol) were purchased from New England Nuclear. MEM and RPMI 1640 were purchased from Nissui Seiyaku. FCS was purchased from Armour Pharmaceutical Co. All the other chemicals used in this study were of reagent grade.

Cells and Cell Culture Conditions. A murine Friend virus-transformed cell line (clone DS19), a double mutant of DS19 (Tk^-Oua^r) lacking functional thymidine kinase and resistant to ouabain, and a murine Rauscher virus-transformed cell line (RV133) were generously provided by M. Oishi. Friend cell lines were cultured in MEM supplemented with 12% heat-inactivated FCS. The culture medium for RV133 cells was RPMI 1640 supplemented with 12% heat-inactivated FCS. The 0.1 mM hypoxanthine:0.4 μM aminopterin:16 μM thymidine medium used for selection of the fused cells contained glycine (3 μM) in MEM supplemented with 12% FCS. All cell lines were cultured under the same 5% CO_2–95% air atmospheric conditions in a humidified incubator at 37°C, using tissue culture dishes (100 x 20 mm; Corning) or multiwell plates (24 wells; Costar).

Cell Fusion. The wild-type Friend cell line, DS19 (Tk^+Oua^s) and the double mutant of DS19 (Tk^-Oua^r) were used for cell fusion experiments. UV irradiation, fusion, and selection were performed according to the procedure of Nomura and Oishi (8).

Assay of Erythroid Differentiation. Erythroid differentiation was assayed by benzidine staining of hemoglobin accumulated in the cells according to the method of Orkin *et al.* (15).

Assay of DNA and RNA Synthesis. Levels of incorporation of radioactivities from labeled thymidine and uridine into cellular material were measured in order to estimate synthesis of DNA and RNA, respectively.

Confluently grown Friend cells were collected by centrifugation (1500 rpm; 5 min) at room temperature and resuspended in 0.5 ml of medium containing various concentrations of trichostatins A and C. After 30 min of incubation at 37°C, radioactive precursors were added to the medium at a final concentration of 2 μCi/ml and further incubation was performed. These macromolecular syntheses were determined by measuring incorporation into the cold trichloroacetic acid-insoluble fraction. The precipitates obtained by treatment with 10% trichloroacetic acid at 5°C for 1 h were collected on glass fiber filters, and the levels of radioactivity on the dried filters were measured in a 5-ml volume of toluene-based scintillator using a liquid scintillation spectrometer (Aloka Co.).

RESULTS

Effect of Trichostatins on Erythroid Differentiation of Murine Erythroleukemia Cells. When Friend cells were cultured with

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[2] The abbreviations used are: DMSO, dimethyl sulfoxide; TS, trichostatins; TSA and TSC, trichostatin A and C, respectively; HMBA, hexamethylenebisacetamide; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; MEM, Eagle's minimal essential medium; FCS, fetal calf serum.

1–4 ng/ml of trichostatin A for 5 days, the number of benzidine-positive cells indicating hemoglobin accumulation increased as the dosage was raised (Fig. 2). Trichostatin C showed more potent inducing activity within a concentration range of 0.1–0.4 μg/ml. A maximum induction exceeding 70–80% of benzidine-positive cells was obtained with trichostatins A and C at their optimum concentrations of 1.5×10^{-8} and 5×10^{-7} M, respectively. DMSO caused a maximum induction of approximately 50% at 210 mM. Concomitant suppression of the cell growth occurred with differentiation of the cells in the effective dosage range of trichostatins as observed with DMSO. Induction of erythroid differentiation by trichostatins was also observed with another murine erythroleukemia cell line, RV133 (Fig. 3), which showed induction with TPA as well as with DMSO (16).

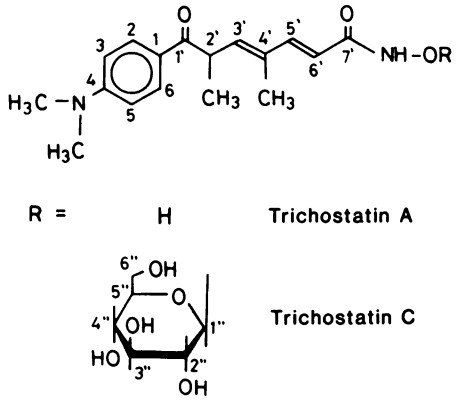

Fig. 1. Structures of trichostatins A and C.

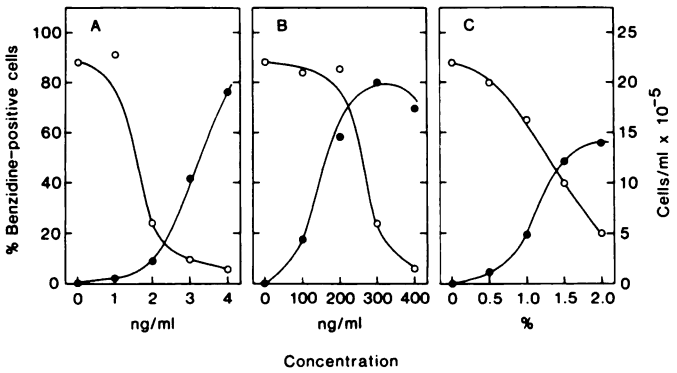

Fig. 2. Effect of trichostatins on induction of Friend cell differentiation. Friend cells were inoculated at an initial density of 10^5 cells /ml and cultured in the presence of various concentrations of trichostatin A (*A*), trichostatin C (*B*), and DMSO (*C*). The percentages of benzidine-positive cells (●) and final cell densities (○) were determined after 5 days of cultivation.

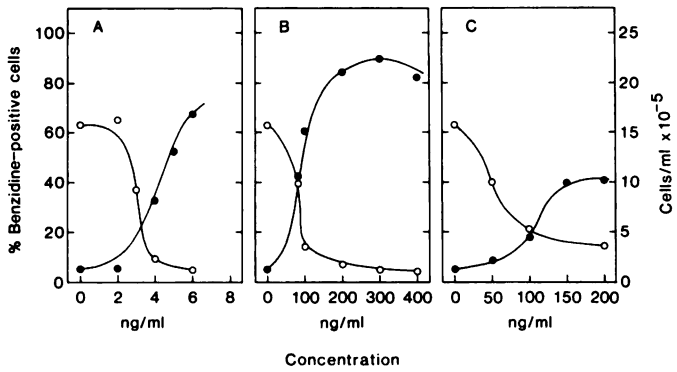

Fig. 3. Effect of trichostatins on induction of RV133 cell differentiation. RV133 cells were inoculated at an initial density of 10^5 cells/ml and cultured in the presence of various concentrations of trichostatin A (*A*), trichostatin C (*B*), and TPA (*C*). The percentages of benzidine-positive cells (●) and final cell densities (○) were determined after 5 days of cultivation.

In order to determine the incubation time with trichostatins necessary for commitment to erythroid differentiation, Friend cells were incubated with 0.2 μg/ml of trichostatin C for various times and then transferred to drug-free medium containing

Table 1 *Effect of length of exposure to inducers on commitment to erythroid differentiation*

Friend cells were cultured in the presence of trichostatin C (0.2 μg/ml), DMSO (1.8%, v/v), or HMBA (2 mM). At the times indicated, the cells were collected by centrifugation, washed once with phosphate-buffered saline and incubated in drug-free medium containing 1.5% methylcellulose for residual lengths of a total of 120 h, at which time they were assayed for benzidine-positive colonies.

Contact period (h)	% benzidine-positive colonies		
	TSC	DMSO	HMBA
0.5	1.0	0.5	0.2
5	1.8	0.5	0.2
10	7.8	0.8	0.1
15	11.8	3.7	6.3
20	18.6	20.6	11.6
30	55.2	28.7	25.5
120[a]	75.5	72.1	80.8

[a] In the case of the contact period of 120 h, each number indicates the percentage of benzidine-positive cells.

Table 2 *Effect of TPA on erythroid differentiation induced by trichostatin C and DMSO*

Various concentrations of TPA were added to cultures of Friend cells together with trichostatin C (0.2 μg/ml) or DMSO (1.5%, v/v), and the extent of differentiation was determined by benzidine staining.

TPA, ng/ml	%B⁺-cells			Viable cell no. (cells/ml × 10^{-5})		
	None	TSC	DMSO	None	TSC	DMSO
0	<0.1	83.3	51.7	27.6	22.1	23.0
10	<0.1	36.0	40.2	28.5	20.0	23.9
20	<0.1	12.5	36.3	22.3	5.9	22.9
50	<0.1	5.3	10.5	26.6	4.8	20.9
100	<0.1	2.9	7.7	25.4	3.9	20.4

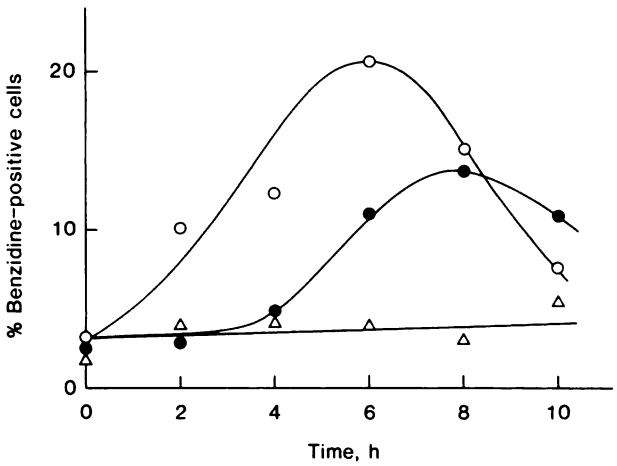

Fig. 4. Erythroid differentiation in fused Friend cells. Trichostatin-treated cells were fused with either UV-irradiated or DMSO-treated cells. Two genetically marked Friend cells, DS19 (Tk^+Oua^s) and DS19 (Tk^-Oua^r), were grown in MEM supplemented with 12% FCS to a cell density of 2×10^6 cells/ml. DS19 (Tk^+Oua^s) cells (5×10^6 cells/ml) were irradiated with UV (20 J/m²) in a Petri dish (60 mm diameter). The cells were then collected, resuspended in the same fresh medium, and further incubated for 24 h at 37°C. Each 1 ml of DS19 (Tk^-Oua^r) cell culture (5×10^5 cells/ml) previously exposed to 0.2 μg/ml trichostatin C (○) or 1.8% (v/v) DMSO (●) for the indicated period was mixed with 5×10^5 cells of the UV-irradiated DS19 (Tk^+Oua^s). Each 1 ml of DS19 (Tk^+Oua^s) cell culture (5×10^5 cells /ml) exposed to 0.2 μg/ml trichostatin C for the indicated period was mixed with 5×10^5 cells of DS19 (Tk^-Oua^r), which had been exposed to 1.8% DMSO for 6 h (△). Cell fusion was then done with polyethylene glycol 6000 according to the method of Nomura and Oishi (8). The fused cells were selected in 0.1 mM hypoxanthine:0.4 μM aminopterine:16 μM thymidine medium containing 1.5 mM ouabain. Benzidine-positive cells were scored on the fifth day after fusion.

Table 3 *Effect of cycloheximide treatment before cell fusion on erythroid differentiation in fused Friend cells*

Friend cells, DS19 (Tk^-Oua^r), were incubated for 2 h with trichostatin C (0.2 μg/ml) or DMSO (1.8%, v/v). After incubation, the cells were collected, washed once with phosphate-buffered saline, resuspended in the same medium, and further incubated for 6 h. Cycloheximide (1 μg/ml) treatment was performed during the first incubation for 2 h or during the second incubation after removal of the inducers for 6 h. The cells were then fused with UV-irradiated cells, selected as described in Fig. 3, and benzidine-positive cells were scored.

Cycloheximide treatment		%B^+-cells	
0–2 h	2–8 h	TSC	DMSO
–[a]	–	26.2	26.8
+	–	23.3	20.9
–	+	4.0	5.3
+	+	4.2	7.6

[a] –, +, absence or presence, respectively, of cycloheximide during the incubation period.

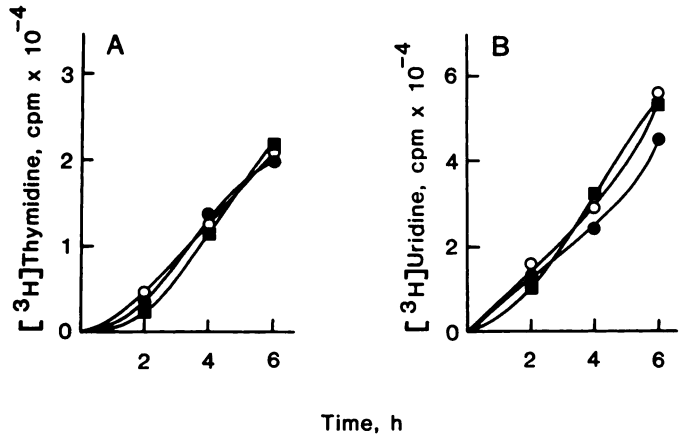

Fig. 5. Effect of trichostatin A on DNA and RNA synthesis of Friend cells. Friend cells (5×10^5 cells/0.5 ml/16 mm/well) were cultured in the absence (○) or presence of 500 (■) or 50 ng/ml (●) of trichostatin A. Incorporation of radioactivity was determined as described in "Materials and Methods." Data are the means of quadruplicate assays. *A*, DNA; *B*, RNA.

1.5% methylcellulose. After cultivation for 120 h after the initial inducer challenge, benzidine-positive colonies were counted. As shown in Table 1, the commitment to erythroid differentiation induced by trichostatin C required approximately 10 h, which was slightly shorter than the time of 15 h required for induction by DMSO and HMBA.

Erythroid differentiation of Friend cells induced by DMSO is inhibited by TPA. Similar inhibition by TPA was also observed with the differentiation induced by trichostatin C (Table 2).

Synergistic Effect of UV Irradiation on Trichostatin-induced Differentiation. Hitherto known inducers of erythroid differentiation in Friend cells can be classified according to their mode of action into two groups, *i.e.*, polar compounds like DMSO and DNA-damaging agents such as UV irradiation and mitomycin C. A synergistic effect between inducers of these different groups can be detected by fusing cells which have been separately treated with doses of each inducer insufficient to induce differentiation (8). Cell fusion experiments were therefore carried out to determine the general mode of induction effected by trichostatins.

Mutant DS19 (Tk^-Oua^r) Friend cells were briefly treated with 0.2 μg/ml of trichostatin C for various lengths of time up to 10 h while DS19 (Tk^+Oua^s) wild-type cells were exposed to UV irradiation (20 J/m²). These dosages were insufficient to cause erythroid differentiation independently. Both types of treated cells were fused, and the fusants were selected by incubation in 0.1 mM hypoxanthine:0.4 μM aminopterine:16 μM thymidine medium containing 1.5 mM ouabain for 5 days. As shown in Fig. 4, considerable proportions of the fused cells became reactive to benzidine. Similar results were obtained when the mutant cells were treated with DMSO (1.8%, v/v) in place of trichostatin. In contrast, no differentiation was observed upon fusion of the trichostatin-treated cells with the DMSO-treated cells. These results indicate that trichostatin C is a DMSO-type inducer and not a DNA-damaging type.

Kaneko *et al.* (9) showed that commitment to erythroid differentiation by DMSO was inhibited by cycloheximide in the second stage of the induction process starting 2 h after DMSO challenge. We therefore performed similar experiments in which cycloheximide was added during or after treatment with trichostatin C for 2 h. As shown in Table 3, the presence of cycloheximide during the first 2 h of incubation with trichostatin caused no inhibition, whereas marked inhibition was observed by adding cycloheximide 2 h after trichostatin challenge. Since these results are identical to those obtained with DMSO, it seems quite likely that trichostatins induce intracellular processes requiring protein synthesis which are probably common to those occurring in DMSO induction.

Effects of Trichostatins on DNA and RNA Synthesis. DNA and RNA synthesis in exponentially growing Friend cells was measured in the presence and absence of trichostatins. As shown in Fig. 5, 500 ng/ml of trichostatin A caused no inhibition of radioactivity incorporation into the cellular material from labeled thymidine and uridine. Trichostatin C also showed no effect on the macromolecular syntheses involving these compounds (data not shown).

DISCUSSION

This work confirmed that TS are extremely potent and effective inducers of erythroid differentiation in murine erythroleukemia cells. Since these compounds showed no significant inhibition of DNA synthesis, they appear to be different from the series of inducers affecting DNA metabolism. The synergistic effect of UV irradiation but not DMSO treatment and the requirement of protein synthesis for TS induction indicate the close similarity of the mode of action of this compound to that of DMSO. Compared with similar types of inducers, the most striking feature of TS is their extremely low dosage required for induction.

TSA and C were reported by Tsuji *et al.* (12) and by Tsuji and Kobayashi (13) as antifungal antibiotics which were active against *Trichophyton* and *Aspergillus*. The minimum inhibitory concentrations of TSA and C against *Trichophyton mentagrophytes* are 3.13 and 25 μg/ml, respectively, which are much higher than those required for induction. TSA is more effective than TSC by one order of magnitude in the fungistatic action but by two orders in the differentiation-inducing activity. This difference may be due to strong cytotoxic activity of TSA to Friend cells, compared with that of TSC. Although their mechanism of fungistatic action and the reason for their narrow range of microbial spectrum have been unknown as yet, we confirmed that they exhibited no inhibitory effects on the incorporation of the precursor of protein, lipid, and glycoprotein into cellular material in Friend cells at least during the first 6 h after challenge of the drugs (data not shown). These preliminary data suggest that TS are not general inhibitors of macromolecular synthesis.

The mechanism of action of inducers classified as DMSO type such as polar-planar compounds (17, 18) and short-chain fatty acids is not clear; however, an alteration in membrane function may be the most likely effect (19–22). The potent

inducing activity of TS suggests that TS can affect the membrane function more specifically than the others.

Trichostatins, which thus appear to be new DMSO-type inducers, are expected to become useful for elucidating the mechanism of erythroid differentiation.

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REFERENCES

1. Friend, C., Patuleia, M. C., and de Harven, E. Erythrocytic maturation *in vitro* of murine (Friend) virus-induced leukemic cells. Natl. Cancer Inst. Monogr., *228:* 505–520, 1966.
2. Marks, P. A., and Rifkind, R. A. Erythroleukemic differentiation. Ann. Rev. Biochem., *47:* 419–448, 1978.
3. Friend, C., Scher, W., Holland, J. G., and Sato, T. Hemoglobin synthesis in murine virus-induced leukemic cells *in vitro*: stimulation of erythroid differentiation by dimethyl sulfoxide. Proc. Natl. Acad. Sci. USA, *68:* 378–382, 1971.
4. Reuben, R. C., Wife, R. L., Breslow, R., Rifkind, R. A., and Marks, P. A. A new group of potent inducers of differentiation in murine erythroleukemia cells. Proc. Natl. Acad. Sci. USA, *73:* 862–866, 1976.
5. Sugano, H., Furusawa, M., Kawaguchi, T., and Ikawa, Y. Enhancement of erythrocytic maturation of Friend virus-induced leukemia cells *in vitro*. Bibl. Heamatol. Basal., *39:* 943–959, 1973.
6. Ebert, P. S., Wars, I., and Bnell, D. N. Erythroid differentiation in cultured Friend leukemia cells treated with metabolic inhibitors. Cancer Res., *36:* 1809–1813, 1976.
7. Scher, W., and Friend, C. Breakage of DNA and alterations in folded genomes by inducers of differentiation in Friend erythroleukemic cells. Cancer Res., *38:* 841–849, 1978.
8. Nomura, S., and Oishi, M. Indirect induction of erythroid differentiation in mouse Friend cells: evidence for two intracellular reactions involved in the differentiation. Proc. Natl. Acad. Sci. USA, *80:* 210–214, 1983.
9. Kaneko, T., Nomura, S., and Oishi, M. Early events leading to erythroid differentiation in mouse Friend cells revealed by cell fusion experiments. Cancer Res., *44:* 1756–1760, 1984.
10. Watanabe, T., Nomura, S., and Oishi, M. Induction of erythroid differentiation by cytoplast fusion in mouse erythroleukemia (Friend) cells. Exp. Cell Res., *159:* 224–234, 1985.
11. Nomura, S., Yamagoe, S., Kamiya, T., and Oishi, M. An intracellular factor that induces erythroid differentiation in mouse erythroleukemia (Friend) cells. Cell, *44:* 663–669, 1986.
12. Tsuji, N., Kobayashi, M., Nagashima, K., Wakisaka, Y., and Koizumi, K. A new antifungal antibiotic, trichostatin. J. Antibiot., *29:* 1–6, 1976.
13. Tsuji, N., and Kobayashi, M. Trichostatin C, a glucopyranosyl hydroxamate. J. Antibiot., *31:* 939–944, 1978.
14. Yoshida, M., Iwamoto, Y., Uozumi, T., and Beppu, T. Trichostatin C, a new inducer of differentiation of Friend leukemic cells. Agric. Biol. Chem., *49:* 563–565, 1985.
15. Orkin, S. H., Harosi, F. I., and Leder, P. Differentiation in erythroleukemic cells and their somatic hybrids. Proc. Natl. Acad. Sci. USA, *72:* 98–102, 1975.
16. Miao, R. M., Fieldsteel, A. H., and Fodge, D. W. Opposing effects of tumour promoters on erythroid differentiation. Nature (Lond.), *274:* 271–272, 1978.
17. Tanaka, M., Levy, J., Terada, M., Bleslow, R., Rifkind, R. A., and Marks, P. A. Induction of erythroid differentiation in murine virus infected erythroleukemia cells by highly polar compounds. Proc. Natl. Acad. Sci. USA, *72:* 1003–1006, 1975.
18. Reuben, R. C., Khanna, P. L., Gazitt, Y., Breslow, R., Rifkind, R. A., and Marks, P. A. Inducers of erythroleukemic differentiation. J. Biol. Chem., *253:* 4214–4218, 1978.
19. Lyman, G. H., and Preisler, H. D. Membrane action of DMSO and other chemical inducers of Friend leukaemic cell differentiation. Nature (Lond.), *262:* 360–363, 1976.
20. Tsiftsoglou, A. S., Bhargava, K. K., Rittmann, L. S., and Sartorelli, A. C. Distribution of the inducer of differentiation bis-acetyl-diaminopentane in murine erythroleukemia cells. J. Cell. Physiol., *106:* 419–424, 1981.
21. Tsiftsoglou, A. S., and Sartorelli, A. C. Changes in surface architecture during murine erythroleukemia cell differentiation as detected by lectin binding and agglutination. Biochem. Biophys. Acta, *649:* 105–112, 1981.
22. Suyama, S., and Goldstein, J. Enzymatic modification of surface carbohydrates of Friend erythroleukemia cells. J. Cell Physiol., *111:* 320–326, 1982.