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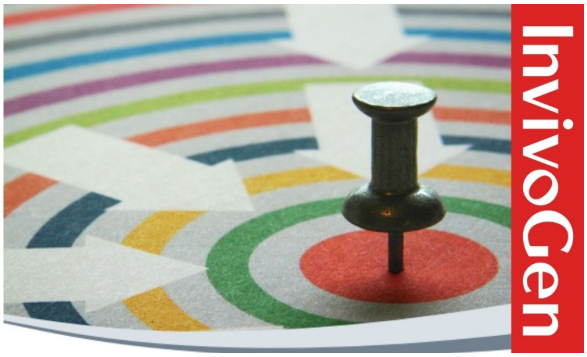

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A NEW CLASS OF INHIBITORS OF LYMPHOCYTE MITOGENESIS: AGENTS THAT INDUCE ERYTHROID DIFFERENTIATION IN FRIEND LEUKEMIA CELLS[1]

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Addition of the polar organic compounds, dimethylsulfoxide, *N,N*-dimethylformamide, *N,N*-dimethylacetamide, and butyric acid, to human lymphocyte cultures stimulated with the tumor-promoting agent, phorbol myristate acetate, results in >90% inhibition of lymphocyte proliferation. Inhibition is achieved at concentrations of the organic compounds reported to be optimal for induction of erythroid differentiation in Friend leukemia cells. Butyric acid is the most potent compound tested. Compounds that are structurally related to butyric acid, but that do not induce erythroid differentiation, do not inhibit lymphocyte mitogenesis. Lymphocyte responses to other mitogens are also suppressed by the polar organic compounds, although higher concentrations are required. These agents are much less inhibitory when added 24 hr after initiation of the cultures, indicating that they may affect an early phase of lymphocyte activation. Compounds that induce erythroid differentiation in Friend leukemia cells constitute a new class of inhibitors of lymphocyte mitogenesis.

Phorbol myristate acetate (PMA)[2] has been reported to be mitogenic for human lymphocytes (1, 2) and co-mitogenic for bovine lymphocytes (3). In studying the mitogenicity of PMA, we noted that relatively low concentrations of dimethylsulfoxide (DMSO) markedly inhibit PMA-induced lymphocyte mitogenesis, but have little effect on DNA synthesis induced by phytohemagglutinin (PHA). DMSO (4), as well as other polar organic compounds, such as butyric acid, *N,N*-dimethylformamide, and *N,N*-dimethylacetamide, have been found to induce erythroid differentiation in cells infected with the Friend leukemia virus (5–8). We report here a direct relationship between the potency of compounds to inhibit lymphocyte mitogenicity and their ability to induce erythroid differentiation in Friend leukemia cells. Although the induction of DNA synthesis by PMA is most sensitive to inhibition by these agents, DNA synthesis induced by other mitogens is also affected.

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[1] This work was supported in part by the Sylvan League Jrs. Fund.
[2] Abbreviations used in this paper: PMA, phorbol myristate acetate; DMSO, dimethylsulfoxide; NAGO, neuraminidase followed by galactose oxidase.

MATERIALS AND METHODS

Materials. PMA was obtained from Sigma Chemical Company, St. Louis, Mo., PHA from *Phaseolus vulgaris* (purified, HA 16) was obtained from Wellcome Research Laboratories, Tuckahoe, N. Y. and concanavalin A (Con A) (twice crystallized) from Miles-Yeda, Ltd., Rehovot, Israel. *Vibrio comma* neuraminidase was obtained from Grand Island Biological Co., Grand Island, N. Y., as a solution containing 500 units/ml and galactose oxidase was obtained from Sigma. DMSO and isobutyric acid were obtained from J. T. Baker Chemical Corporation, Phillipsburg, N. J., *n*-butyric acid, *N*-methylacetamide and *N,N*-dimethylacetamide from Aldrich Chemical Co., Milwaukee, Wis., and *N,N*-dimethylformamide from Mallinckrodt, Inc., St. Louis, Mo. α, β and γ-Amino and β and γ hydroxybutyric acids were obtained from Sigma. RPMI 1640 culture medium and fetal calf serum were obtained from Grand Island Biological Company. ^{3}H-Thymidine (2 Ci/mM), ^{3}H-leucine (51.6 Ci/mM) and ^{3}H-uridine (37 Ci/mM) were obtained from New England Nuclear, Boston, Mass.

Isolation of cells and culture conditions. Human peripheral blood mononuclear cells were obtained from normal subjects by Ficoll-Hypaque density gradient centrifugation as previously described (9). Final cell preparations (1×10^6 cells/ml) were suspended in RPMI 1640 medium containing 5% heat-inactivated fetal calf serum and supplemented with penicillin (100 units/ml) and streptomycin (100 μg/ml). In experiments measuring leucine incorporation, medium containing only 10 μg/ml leucine and dialyzed fetal calf serum were used. Cells were distrubuted (0.2-ml aliquots) in flat bottom microwells (Microtest II, Falcon 3040) and the various additions were made (mitogens and the different polar organic compounds). Sequential treatment of cells with neuraminidase and galactose oxidase (NAGO) was carried out as previously described (9). The cells were then incubated at 37°C in a 95% air, 5% CO_2 atmosphere for 72 hr, and ^{3}H-thymidine incorporation (2 μCi/well) into DNA during 52 to 72 hr of incubation was determined (9). ^{3}H-uridine and ^{3}H-leucine incorporation (2 μCi/well) were also determined during this time interval. Results are expressed as the mean of duplicate cultures. Results from duplicates that varied more than 15% from the mean were not included. Cell morphology was determined by examining Wright-Giemsa stained cell preparations deposited on glass slides by a Shandon cytofuge. Lymphocytes were considered transformed ("blasts") if they were enlarged, had fine nuclear chromatin, and had a distinct basophilic cytoplasm surrounding the nucleus.

RESULTS

Addition of DMSO (100 to 140 mM) to cultures stimulated with PMA inhibits the induction of DNA synthesis by more

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than 80% (Fig. 1-*A*). Inhibition by *N,N*-dimethylformamide is attained by similar concentrations, whereas *N,N*-dimethylacetamide results in >90% inhibition of PMA-stimulated lymphocytes at concentrations of 10 to 25 mM (Fig. 1-*A*). Addition of these compounds to cultures stimulated wth PHA has significantly less inhibitory effect (Fig. 1-*B*). DMSO does not inhibit PHA-induced DNA synthesis at concentrations up to 140 mM. The inhibitory effects of *N,N*-dimethylformamide and *N,N*-dimethylacetamide are evident in PHA-stimulated cell cultures only at concentrations greater than those effective in inhibiting PMA-stimulated cells. For example, *N,N*-dimethylacetamide at concentrations up to 12 mM does not inhibit PHA-induced DNA synthesis (Fig. 1-*B*), but results in >90% inhibition of PMA-induced stimulation (Fig. 1-*A*).

Butyric and propionic acids, also known to induce erythroid differentiation in Friend leukemia cells (5), were tested for their ability to inhibit mitogen-induced DNA synthesis. Butyric acid is a potent inhibitor of PMA-induced DNA synthesis (Fig. 1-*A*) and also inhibits PHA-induced DNA synthesis, but at higher concentrations (Fig. 1-*B*). Propionic acid is also effective in inhibiting PMA-driven responses but has little effect on PHA-induced responses up to a concentration of 4 mM (Fig. 1-*A*, *B*).

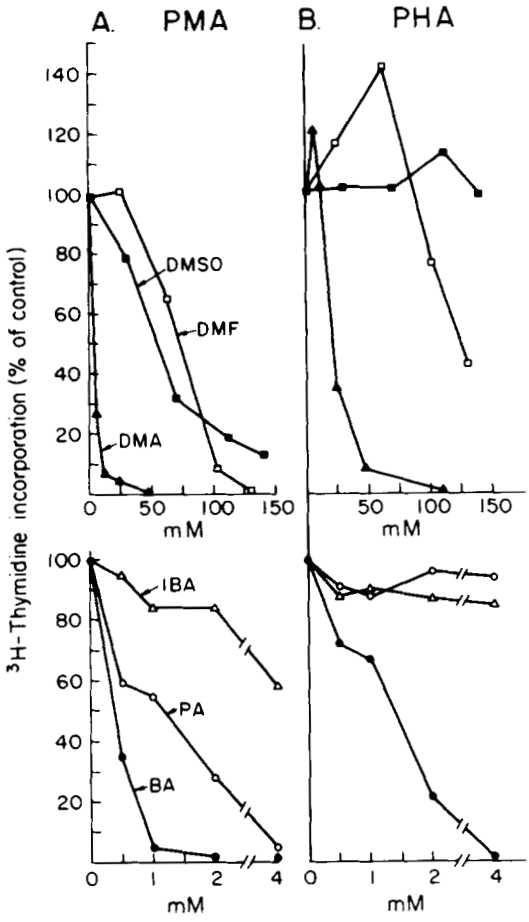

Figure 1. Effect of inducers of erythroid differentiation on the induction of lymphocyte DNA synthesis stimulated by PMA or PHA. Lymphocytes were stimulated with either PMA (10 ng/ml) or PHA (2 μg/ml) and different concentrations of DMSO (■), *N,N*-dimethylformamide (□), *N,N*-dimethylacetamide (▲), butyric acid (●), propionic acid (○), or isobutyric acid (△) were added. ^{3}H-thymidine incorporation is expressed as percentage of control cultures (cultures stimulated with mitogen alone). The incorporation in cpm per culture in the absence of the organic compunds (100% values) were: PMA 78,000 and PHA 135,000.

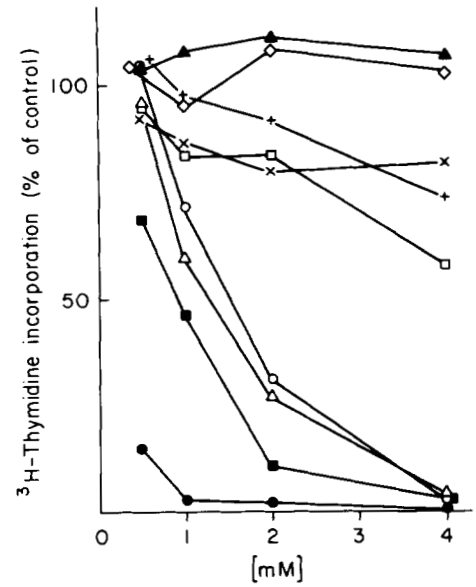

Figure 2. Effect of butyric acid and related compounds on PMA-induced lymphocyte mitogenesis. Cells were stimulated with PMA (10 ng/ml) and different concentrations of butyric acid (●), valeric acid (○), isovaleric acid (△), caproic acid (▲), isobutyric acid (□), propionic acid (■), butylamine (+), β-hydroxybutyric acid (×), γ-aminobutyric acid (◇) were added. ^{3}H-thymidine incorporation is expressed as percentage of control cultures.

Isobutyric acid, reported to be incapable of inducing erythroid differentiation at 1 mM (5) is much less potent in inhibiting PMA-induced DNA synthesis compared to butyric and propionic acids.

An additional group of compounds that are structurally related to butyric acid were tested for their potency to inhibit PMA-induced DNA synthesis. As depicted in Figure 2, there are wide variations in the inhibitory effects of these compounds. From most to least inhibitory, the compounds depicted are: butyric acid>propionic acid>isovaleric acid≥valeric acid>isobutyric acid>butylamine. Isobutyric acid and butylamine are weak inhibitors, inhibiting 85% of PMA-induced DNA synthesis at 10 mM. It is of interest to note that high concentrations of isobutyric acid (30 mM) have been reported to induce erythroid differentiation (10). Caproic acid, β and γ-hydroxybutyric acids and α, β, and γ-aminobutyric acids are essentially noninhibitory up to 10 mM.

The compounds noted in Figures 1 and 2 do not decrease cell viability as assessed by the trypan blue exclusion assay under the conditions and concentrations of inhibitors used in the experiments summarized in these figures. The effect of DMSO and butyric acid on the proliferative capacity of cells stimulated with both PMA and PHA together was also assessed (Table I). Lymphocyte responses to the combination of PMA and PHA are 15 to 20% greater than those to PHA alone. The inhibitory effects of DMSO and butyric acid on responses to the two mitogens together are similar to their effects on cells stimulated with PHA. ^{3}H-Thymidine incorporation into cells stimulated with PHA is slightly enhanced by DMSO at 140 mM (Fig. 1 and Table I). This enhancement is not seen in cells stimulated with PMA and PHA together in the presence of DMSO (Table I).

Addition of the inhibitory polar organic compounds 24 hr after cultures are stimulated with PMA results in significantly less inhibition than when the compounds are added at the same time as the mitogen (Fig. 3). Similarly, much higher concentra-

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TABLE I

Effect of DMSO and butyric acid on lymphocyte responses to PMA and PHA alone and together[a]

Additions	^{3}H-Thymidine Incorporation					
	PMA (10 ng/ml)		PHA (2 μg/ml)		PMA (10 ng/ml) + PHA (2 μg/ml)	
	cpm/Culture	% of Control	cpm/Culture	% of Control	cpm/Culture	% of Control
None	75,915 ±14,564	100	143,242 ±12,646	100	168,112 ±5,508	100
DMSO (140 mM)	8,831 ±2,518	11.8 ±3.2	170,089 ±9,635	120 ±5.7	153,171 ±3,619	92 ±3.9
Butyric acid (1.0 mM)	3,264 ±1,791	6.2 ±3.5	104,343 ±10,367	74.0 ±8.2	105,104 ±9,219	63.3 ±7.1

[a] Results of five experiments expressed as cpm per culture ± S.E.M. and as percent of control culture. Percent of control was determined for each experiment and the results presented as the mean ± S.E.M. of the five studies.

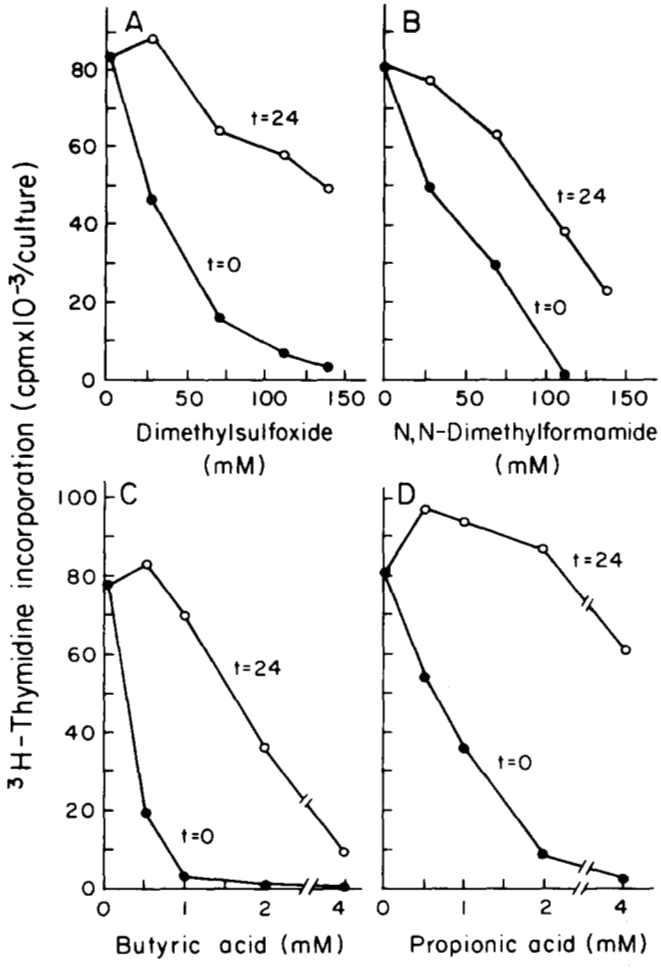

Figure 3. Reduced inhibitory effect of the polar organic compounds when added 24 hr after stimulation with PMA. Cells were treated with PMA (10 ng/ml) and different concentrations of DMSO, *N,N*-dimethylformamide, butyric acid, or propionic acid were added at the initiation of the cultures (t = 0) or 24 hr after addition of PMA (t = 24).

tions of butyric acid are required to inhibit PHA-induced responses when the butyric acid is added 24 hr after PHA stimulation (data not shown). Preincubation of the cells for 24 hr at 37°C in the absence of mitogen, however, does not alter the inhibitory effect of either butyric acid or DMSO.

DMSO (140 mM) uniformly inhibits responses to PMA over a wide range of PMA concentrations capable of activating lymphocytes (Fig. 4). The effects of this concentration of DMSO on cells activated by various concentrations of PHA and Con A, and on cells activated by the mitogenic oxidizing agent, galactose oxidase, were also evaluated. DMSO (140 mM) has little effect on lymphocyte responses to concentrations of PHA and Con A that result in maximal blastogenesis. Responses of cells stimulated with either sub- or supra-optimal concentrations of these lectins appear more sensitive to inhibition by DMSO (Fig. 5). Although lymphocyte responses to PHA, Con A, and NAGO are relatively resistant to DMSO and butyric acid, increasing concentrations of these agents do result in suppression of mitogenesis, as indicated in Figure 1 for PHA-induced responses. Results of a typical experiment assessing the effects of increasing concentrations of DMSO and butyric acid on responses to the different mitogens tested are shown in Table II. The marked sensitivity to suppression of PMA-in-

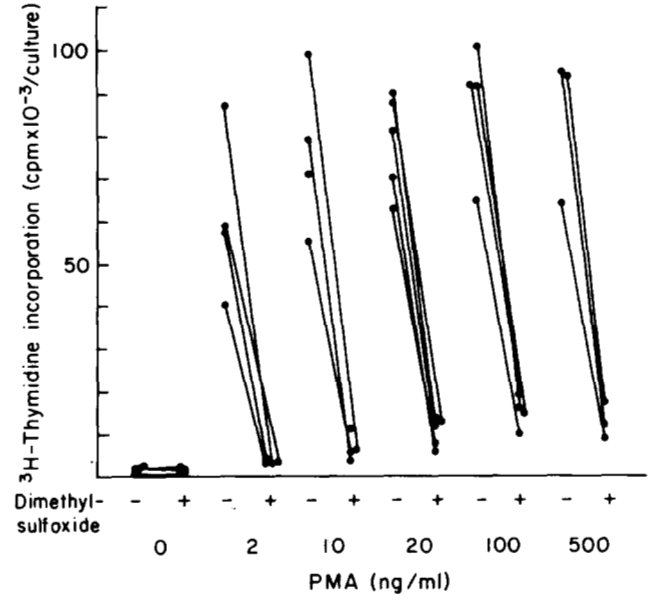

Figure 4. Effect of DMSO on lymphocyte responses to different concentrations of PMA. Connected points without (−) or with (+) DMSO represent results from paired experiments using the same individual's lymphocytes.

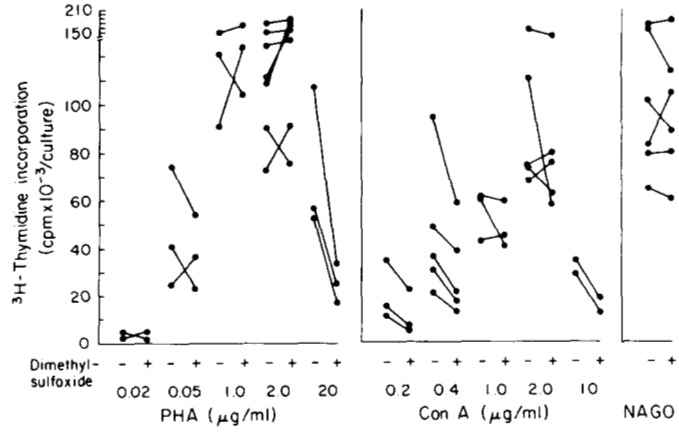

Figure 5. Effect of DMSO on lymphocyte responses to different concentrations of PHA, Con A, and NAGO. Connected points without (−) or with (+) DMSO represent results from paired experiments using the same individual's lymphocytes.

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TABLE II

Effect of DMSO and butyric acid on lymphocyte responses to PMA, PHA, Con A, and NAGO

	^{3}H-Thymidine Incorporation (cpm/Culture)				
	PMA (10 ng/ml)	PHA (2 μg/ml)	Con A (0.4 μg/ml)	Con A (2.0 μg/ml)	NAGO
DMSO *mM*					
0	67,092	121,856	95,508	133,315	164,262
70	19,495	140,872	71,729	118,184	185,821
140	7,029	168,454	59,154	129,136	181,224
210	7,856	157,200	57,464	119,336	164,765
280	1,166	98,010	5,461	34,980	35,515
N-butyric acid *mM*					
0	83,254	127,403	69,203	124,572	204,626
1.0	2,118	93,546	33,242	83,823	103,446
2.0	464	66,834	7,682	37,373	58,622
5.0	302	1,942	369	861	712

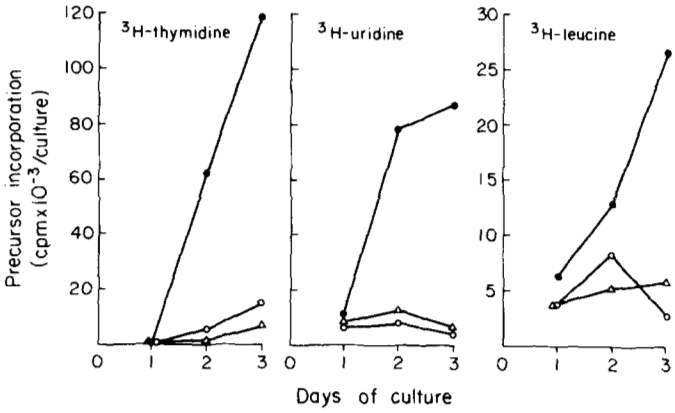

Figure 6. Incorporation of ^{3}H-thymidine, ^{3}H-uridine, and ^{3}H-leucine in cells treated with PMA, 10 ng/ml alone (●); DMSO, 140 mM (○); or butyric acid, 1.0 mM (△). Precursor incorporation was measured over a 5-hr period on days 1 to 3 after initiation of the cultures.

TABLE III

Effect of DMSO and butyric acid on morphologic transformation of lymphocytes induced by PMA and PHA[a]

Additions	PMA (10 ng/ml)		PHA (2 μg/ml)	
	% blasts[b]	^{3}H-thymidine incorporation (*cpm* × 10^{-3})	% blasts	^{3}H-thymidine incorporation (cpm × 10^{-3})
None	58.3 ± 4.8	96.9 ± 21.7	82.5 ± 1.4	176 ± 19.4
DMSO (70 mM)	28.5 ± 2.7	43.1 ± 14.3	83.1 ± 0.8	179 ± 21.9
DMSO (140 mM)	5.3 ± 1.1	15.7 ± 5.1	81.3 ± 1.3	201 ± 9.2
Butyric acid (0.4 mM)	12.0 ± 1.5	18.1 ± 11.9	75.7 ± 2.0	124 ± 8.8
Butyric acid (1.0 mM)	3.3 ± 1.9	1.6 ± 9.8	62.5 ± 4.2	120 ± 8.8

[a] Mean ± S.E.M. of four paired experiments.
[b] A minimum of 200 cells were counted.

duced responses by agents that induce erythroid differentiation is again evident.

In addition to suppression of thymidine incorporation, DMSO and butyric acid inhibit ^{3}H-leucine and ^{3}H-uridine incorporation in cells stimulated with PMA (Fig. 6). Morphologic transformation of PMA-treated cells is also inhibited by DMSO and butyric acid (Table III). Mitotic figures are seen in 4 to 6% of the PMA-induced lymphoblasts, and essentially none (<1%) occur in the DMSO (140 mM) or butyric acid- (1.0 mM) treated cells.

The ability of the various polar organic compounds to inhibit PMA-induced DNA synthesis was compared with their reported ability to induce erythroid differentiation. Table IV indicates a direct relationship between the potency of these agents to inhibit PMA-induced blastogenesis and their capacity to induce erythroid differentiation.

DISCUSSION

Since the observation by Friend *et al.* (4) that DMSO induces erythroid differentiation in Friend erythroleukemia cells, a variety of additional polar organic compounds have been reported to share this effect, and to induce differentiation in other mammalian cells as well (12). DMSO is commonly used as a solvent to dissolve hydrophobic compounds for testing in tissue culture and is also used as a cryo-protective agent. In a study on the use of DMSO as a cryo-protective agent (13), it was shown that high concentrations of DMSO inhibit PHA-induced DNA synthesis. Butyric acid was also shown to inhibit murine lymphocyte responses to PHA, lipopolysaccharide, and Con A at concentrations of 1 to 2 mM (14). While studying the mitogenic properties of PMA, we noted that responses to this mitogen are much more sensitive to inhibition by low concentrations of DMSO than are responses to PHA. After this observation, we systematically evaluated the effects of a variety of polar organic compounds on mitogen-induced DNA synthesis. Our studies indicate that compounds reported to induce erythroid differentiation suppress PMA-induced lymphocyte proliferation. There is a remarkable parallelism between the potency of these compounds to inhibit mitogenesis and to induce erythroid differentiation (Table IV). Moreover, there is a striking similarity in the structural requirements of compounds related to butyric acid for the induction of erythroid differentiation and for the suppression of PMA-induced lymphocyte proliferation (Fig. 2).

Inhibition of PMA-induced blastogenesis does not appear to result from nonspecific cellular injury, since cell viability is preserved at concentrations of the compounds well above those necessary to completely inhibit DNA synthesis. In addition, responses of lymphocytes to PMA and PHA together were not inhibited by concentrations of DMSO and butyric acid that result in marked suppression of PMA-induced responses (Table I).

Addition of the polar organic compounds after 24 hr of cell culture is much less effective in inhibiting DNA synthesis. It is not clear whether these compounds affect early events in the

TABLE IV

Comparison between the inhibitory potency of polar organic compounds on lymphocyte mitogenesis and optimal concentrations for their induction of differentiation

Polar Organic Compound Tested	Concentration (mM) Resulting in 75–90% Inhibition of PMA-Induced Mitogenesis	Optimal Concentration (mM) for Induction of Differentiation
Butyric acid	0.5–1	1.0–2.0 (5),[a] 1.0 (8)
Propionic acid	2–4	2 (5)
N,*N*-dimethylacetamide	15–25	10 (11), 20 (8), 30 (6)
N-methylacetamide	20–30	20 (11), 30 (8), 50 (6)
N,*N*-dimethylformamide	70–110	60 (11), 150 (6)
DMSO	112–140	280 (8), 280 (6), 70–300 (5)

[a] Numbers in parentheses indicate references from which data were abstracted.

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activation process, or whether prolonged exposure of the cells to DMSO or butyric acid is required for maximal effect.

Inhibition of blastogenesis induced by these compounds is selective for cells stimulated with PMA, although responses to other mitogens are also inhibited, but at higher concentrations of the inducers (Table II). The inhibitory effects of these compounds on PHA, Con A, and galactose oxidase-induced responses resemble their effects on PMA-induced responses. They are selectively susceptible to the different polar organic compounds and inhibitory effects are most pronounced when the organic compounds are added during an early phase of mitogenesis. The possibility that the polar organic compounds specifically inhibit PMA binding to cells seems unlikely in view of this inhibition of lymphocyte responses to several other mitogens.

The polar organic compounds have multiple effects on cellular structure and function (15–22). Which of these are relevant to their ability to induce differentiation in animal cells, however, or to inhibit lymphocyte mitogenesis, are unknown. Butyric acid inhibits histone deacetylation in HeLa cells, leading to accumulation of multi-acetylated forms of histones H3 and H4 (17, 18). This alteration in chromatin structure might be associated with gene activation. Butyric acid also induces the synthesis of alkaline phosphatase (23, 24), gonadotrophic hormones (25, 27), and myeloid-associated enzymes (28) in cultured mammalian cells and induces the synthesis of various enzymes related to the synthesis of neurotransmitters in neuroblastoma cells (29, 30). Thus, butyric acid and related compounds might suppress lymphocyte mitogenesis via an action at the chromatin level. This could result in the synthesis of endogenous metabolites that mediate suppression of lymphocyte proliferation. Our observation that butyric acid does not markedly inhibit DNA synthesis in cells stimulated with a combination of PHA and PMA indicates that a free inhibitor does not accumulate in the culture medium. We also found in preliminary studies that dialyzed medium from lymphocyte cultures treated with PMA and butyric acid fails to suppress PMA-induced mitogenesis.

The different organic compounds effective in inducing erythroid differentiation may act by different mechanisms (16). Similarly, different mechanisms may be involved in the suppression of lymphocyte mitogenesis by these compounds. A prominent effect of the cryo-protective agents, such as DMSO, is to decrease fluidity of phospholipid membranes (31). PMA has been reported to have an opposite effect, that is to increase the fluidity of lipid membranes (32). Moreover, PMA has been found to counteract erythroid differentiation induced by polar organic compounds (33). It is possible, therefore, that the inhibitory effect of this class of compounds on mitogenesis results from a reduction in fluidity of the cell membrane. This might counteract the mitogenic effect of PMA and of other mitogens that have also been reported to increase membrane fluidity (34).

Inhibition of lymphocyte proliferation induced by PMA and other mitogens by agents known to induce cellular differentiation provides a new tool for studying mechanisms of mitogenesis as well as for elucidating mechanisms involved in differentiation.

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