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Superoxide Dismutase Induces Differentiation of Friend Erythroleukemia Cells

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Friend erythroleukemia cells (FELC) served as a model system for cell differentiation because these cells can be triggered to differentiate by a variety of chemical agents. Treatment with the classical inducer of differentiation, hexamethylene bisacetamide (HMBA), stimulated superoxide dismutase (SOD) activity, which increased in parallel with HMBA-induced differentiation. Furthermore, FELC were shown to differentiate in response to the addition of liposomes containing SOD. Oxidative treatment with liposomes containing D-amino acid oxidase or xanthine oxidase, cumene peroxide, or potassium superoxide also induced differentiation, whereas antioxidants such as α-tocopherol, butylated hydroxytoluene, or β-carotene did not induce differentiation. Also, HMBA induction of differentiation was suppressed by treatment with antioxidants.

Aerobic cells univalently reduce a small percent of the oxygen they consume, to produce superoxide free radicals ($\cdot O_2^-$). The steady-state concentration of $\cdot O_2^-$ in cells is limited both by its spontaneous dismutation and by the enzyme superoxide dismutase (SOD), which removes ($\cdot O_2^-$) and produces O_2 and H_2O_2 (Halliwell, 1981; Chance et al., 1979; Foreman and Fischer, 1981). A wide variety of chemical and environmental factors are known to induce SOD activity; however, all of these stimuli can be linked to an augmentation of $\cdot O_2^-$ generation (Crapo and Turney, 1974; Mason, 1982; Halliwell, 1981). Alterations in SOD activity are also frequently observed during cell state transitions of various types. For example, differentiation in the slime mold *Physarum polycephalum* occurs with a concomitant 46-fold increase in SOD (Allen et al., 1985). A non-differentiating strain of *Physarum* fails to exhibit this increase when maintained under identical culture conditions. SOD activity also increases during the development of other phylogenetically diverse organisms, including acellular slime molds (Lott et al., 1981), insects (Fernandez-Souza and Michelson, 1976; Massie et al., 1980; Nickla et al., 1983), amphibians (Barja Quiroga and Gutierrez, 1984), and mammals (Mavelli et al., 1978; Frank et al., 1978; Autor et al., 1976; van Hien et al., 1974; Gerdin et al., 1985; Frank and Groseclose, 1974).

In contrast to the changes in SOD observed in developing tissues, dedifferentiated tumor cells exhibit lower levels of SOD activity than do their normal, fully differentiated counterparts (Oberley et al., 1980; Bize et al., 1980; Sykes et al., 1978). Oberley (1982) postulated that the decreased SOD activity plays a causal role in a cellular transformation and that experimental increases in SOD activity should induce differentiation in tumor cells. Supportive of this hypothesis was the recent demonstration that addition of SOD to a non-differentiating strain of *Physarum* induces differentiation in the organism (Allen et al., 1988). It was of interest to further examine the possible functional significance of low SOD activity on the state of differentiation in mammalian tumor cells.

Friend erythroleukemia cells (FELC) provide an excellent model for studies of differentiation in tumor cells. Undifferentiated cells contain no hemoglobin, whereas induction of differentiation by any of several known chemical inducers stimulates hemoglobin production (Friend et al., 1971; Marks and Rifkind, 1978). Additionally, the ease of determining the state of differentiation with microscopy permits quantification of the effectiveness of inducers of differentiation. To find out if SOD activity plays any physiological role in the differentiation of Friend erythroleukemia cells, we determined the activity of the enzyme in cultures of cells induced to differentiate with hexamethylene bisacetamide (HMBA). We also examined the effects of liposomal augmentation of SOD on the state of differentiation in these cells. Additionally, the effects of other antioxidants and of several oxidants were examined. The results of this study indicate that augmentation of SOD activity via liposomes acts as a stimulus to differentiation in the Friend erythroleukemia cell system; however, the results presented here also indicate that oxidation rather than antioxidation is the probable cause of differentiation in Friend erythroleukemia cells.

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TABLE 1. Effect of hexamethylene bisacetamide (HMBA) ± antioxidants on Friend erythroleukemia cell (FELC) differentiation

Drug treatment	Differentiation (%)
HMBA, 5 mM	52.2 ± 4.3
HMBA + α-tocopherol	13.5 ± 7.5
HMBA + BHT	14.5 ± 1.5

FELC were pretreated with antioxidant liposomes and then treated for 96 hr with HMBA. The percentage of benzidine-positive cells was determined. BHT, butylated hydroxytoluene.

TABLE 2. Effect of hexamethylene bisacetamide (HMBA) on superoxide dismutase (SOD) activity

Treatment HMBA (hr)	Total/mg protein	Mn SOD/mg protein
0	4.67 ± 0.01	1.43 ± 0.04
72	9.60 ± 0.90	5.37 ± 1.11
144	11.60 ± 3.02	7.23 ± 1.40

Friend erythroleukemia cells (FELC) were treated with HMBA (5 mM) for 0, 72, or 144 hr. After pelleting, the cells total and Mn SOD activity were determined, as described in the text.

MATERIALS AND METHODS

HMBA, SOD, and other chemicals were obtained from Sigma Chemical Co., St. Louis, MO. SOD for the liposomes was purchased from Boehringer (Indianapolis, IN). All culture supplies were obtained from Gibco, Grand Island, NY.

Preparations of cell suspensions

FELC clone DS19-10 was kindly provided by Dr. Shigeru Sassa, The Rockefeller University, New York, NY. Cells were cultured in minimal essential medium (MEM) containing Earle's salts supplemented with heat-inactivated fetal bovine serum (5% v/v), penicillin (100 U/ml), streptomycin (100 μg/ml), non-essential amino acids (3×), and MEM vitamins (3×). Cultures were maintained at 37°C with 5% CO_2 in humidified air. Cultures were diluted twice weekly to maintain the cells in a logarithmic phase of growth. Cell doubling time was approximately 11 hr.

Cells in log phase were treated with oxidant or antioxidant-containing liposomes for 3 hr at room temperature in Ca^{2+}-, Mg^{2+}-free MEM. Cells were then washed twice in Ca^{2+}-, Mg^{2+}-free MEM and seeded at 100,000 cells/ml in 25 mm^2 culture flasks. At the indicated times after seeding, viability was assessed with trypan blue exclusion, and the cells were stained for hemoglobin using 3′,3′-diaminobenzidine (Pincus et al., 1984). Most experiments were repeated three to four times with duplicate flasks, for each condition.

Procedure for construction of liposomes

Liposomes were made by a modification of the procedure suggested by Szoka and Papahadjopoulos (1978). The buffer employed for liposome construction contained the following components: a) 137 mM NaCl, b) 2.6 mM KCl, c) 6.4 mM Na_2HPO_4, d) 1.4 mM KH_2PO_4, e) The pH was 7.4 when these components were mixed. No further adjustment was made.

One hundred milligrams of SOD (5,000 U/mg; Boehringer), 90 μg FAD, and 100 mg D-amino acid oxidase (0.12 U/mg) or 0.93 mg xanthine oxidase (1.5 U/mg) were dissolved in 14 ml buffer and set on ice; 93 mg dipalmitoyl phosphatidycholine (DPPC; Sigma) and 9.8 mg stearylamine (Sigma) were then dissolved in 30 ml chloroform. After all of the materials were completely dissolved, the enzyme in buffer was added to the chloroform and lipid, shaken, and placed on ice for about 10 min; 35 ml ethyl ether was added to a clean tube and set on ice. After chilling, the chloroform enzyme mixture was sonicated for 30 sec and allowed to stand 1 min. The mixture was sonicated again. When the material had a white, even consistency, it was added to a 500 ml round-bottom flask. The chilled ether was added to it. The flask was placed on a rotary evaporator, and the organic phase was removed at 44–45°C. The resulting liposome suspension was left under a vacuum at room temperature for 24 hr before use.

Water-soluble suspensions of lipid-soluble antioxidants

Suspensions of α-tocopherol and butylated hydroxytoluene (BHT) were prepared by a modification of the procedure for liposome preparation. One gram tocopherol or BHT and 0.3 g stearylamine was dissolved in 10 ml ethyl ether; to this mixture was added 30 ml chilled buffer. The mixture was sonicated several times and then placed in a rotary evaporator to remove the organic phase. The evaporation was performed without the use of the water bath, since heating resulted in aggregation of undissolved particles of antioxidant. A suspension of β-carotene was prepared by dissolving 1 g β-carotene and 0.06 g stearylamine in 10 ml chloroform. Sixty milliliters buffer were added to this, and the mixture was sonicated several times. The chloroform was removed by heating at 30°C for 1 hr in a rotary evaporator. All of the suspensions were left under vacuum for an additional 24 hr before use. The suspensions were stored under N_2, in darkness, at 4°C prior to use. In most cases, cells were treated with the antioxidant suspensions within a few hours of their preparation.

Superoxide dismutase (SOD) assays

SOD activity was determined according to the method of Misra and Fridovich (1977a), as previously described (Nations et al., 1987). The assay was standardized to the cytochrome C assay. SOD activity in samples was in some cases determined by a modification of the cytochrome C method of Crapo et al. (1978), as previously described by Sohal et al. (1984).

RESULTS

Effects of HMBA in the presence or absence of antioxidants

HMBA (5 mM) treatment resulted in 52% differentiation at 96 hr. In the presence of antioxidants, such as α-tocopherol or BHT, the HMBA-induced differentiation was suppressed, as shown in Table 1. HMBA treatment also caused a time-dependent increase in total and Mn SOD activity from 0 to 144 hr, as shown in Table 2.

Effect of SOD-containing liposomes

Log-phase FELC were treated either with empty liposomes or liposomes containing bovine erythrocyte

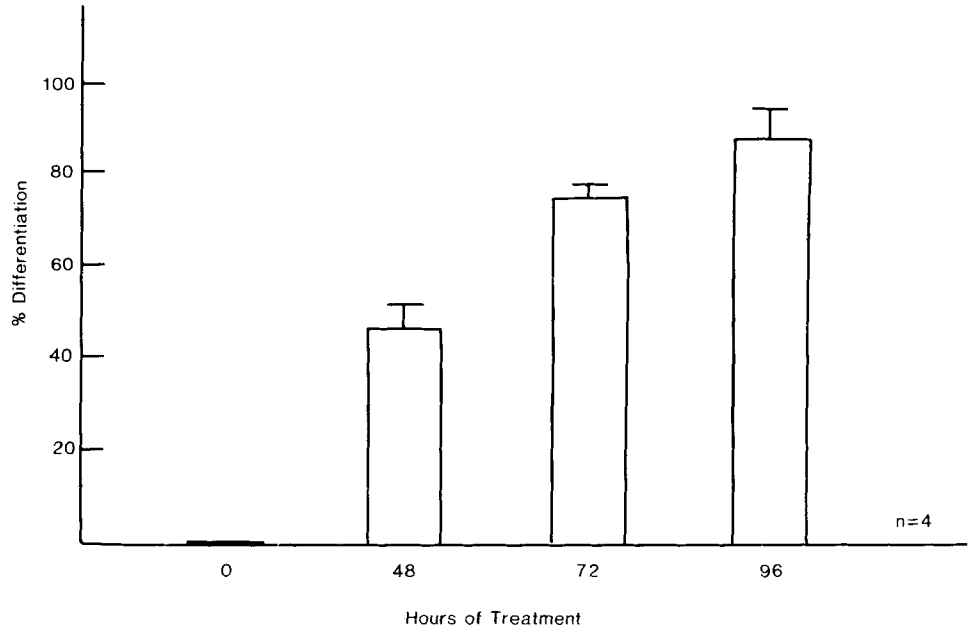

Fig. 1. The effect of superoxide dismutase (SOD)-containing liposomes on Friend erythroleukemia cell differentiation. Each bar represents the percentage of benzidine-positive cells counted in four experiments after treatment with SOD liposomes for 0, 48, 72, or 96 hr.

SOD for 3 hr at room temperature. After washing, cells were transferred to MEM supplemented with fetal bovine serum. Cell differentiation was determined after 48, 72, or 96 hr. Cells treated with "concentrated" SOD-containing liposomes were 46% differentiated at 48 hr, 75% at 72 hr, and 86% at 96 hr (viability was > 50%), whereas FELC treated with empty liposomes or untreated FELC exhibited no differentiation by 96 hr (Fig. 1). At a 1:10 dilution of SOD-containing liposomes, the cell viability was > 90%; there were 5.5% differentiated FELC at 48 hr, 55% at 72 hr, and 48% at 96 hr. Light microscopy depicts the effect of SOD-treatment in Figure 2. Total SOD activity, as determined at 96 hr, increased from 4.40 U/mg protein in cells treated with empty liposomes to 128.07 U/mg protein in cells treated with concentrated SOD-containing liposomes; in the cells treated with a 1:10 dilution of liposomes, total SOD activity was 20.41 U/mg protein (Table 3). As a further control, SOD (1,500 units or roughly 1,500 U/mg cell protein) was added in the external medium to FELC. No cell differentiation occurred at 96 hr.

Effects of oxidant treatments

Although treatment with hydrogen peroxide did not result in an appreciable effect upon FELC differentiation (data not shown), treatment with a more stable peroxide compound, cumene peroxide, did induce FELC differentiation at 96 hr (Fig. 3). Concentrations higher than 100 μM were toxic to the cells. Potassium superoxide also induced differentiation in a concentration-dependent manner when added to the culture medium. Liposomes containing xanthine oxidase also induced differentiation (29.5%) when incubated with FELC, as did liposomes containing D-amino acid oxidase. Cells that were pretreated with buthionine sulfoximine (BSO) to deplete GSH and then treated with HMBA differentiated to a greater degree than cells treated with HMBA alone (Fig. 4).

Effects of antioxidant treatment

In contrast, antioxidants such as α-tocopherol, β-carotene, or butylated hydroxytoluene presented to FELC in liposomes did not induce appreciable cell differentiation (Table 4).

DISCUSSION

The results of the present study reveal that SOD activity increases during HMBA-induced differentiation in Friend erythroleukemia cells. Liposomal augmentation of SOD activity stimulated differentiation in the absence of any other inducer of differentiation, but this effect was more pronounced if GSH synthesis was inhibited prior to treatment of the cells with the SOD-containing liposomes. Inhibition of GSH synthesis without subsequent augmentation of SOD activity could not induce a significant level of differentiation. Oxidants such as cumene hydroperoxide and potassium superoxide also induced differentiation. Conversely, antioxidants failed to stimulate differentiation, and tocopherol and BHT severely inhibited induction of differentiation by HMBA. These results strongly implicate increased cellular oxidation as the primary stimulus of differentiation in Friend erythroleukemia cells.

Paradoxically, the effects of SOD reported here are probably not the result of antioxidation. Supportive of this view is the failure of other antioxidants to induce significant differentiation in the cells. Although small increases in SOD activity would be expected to de-

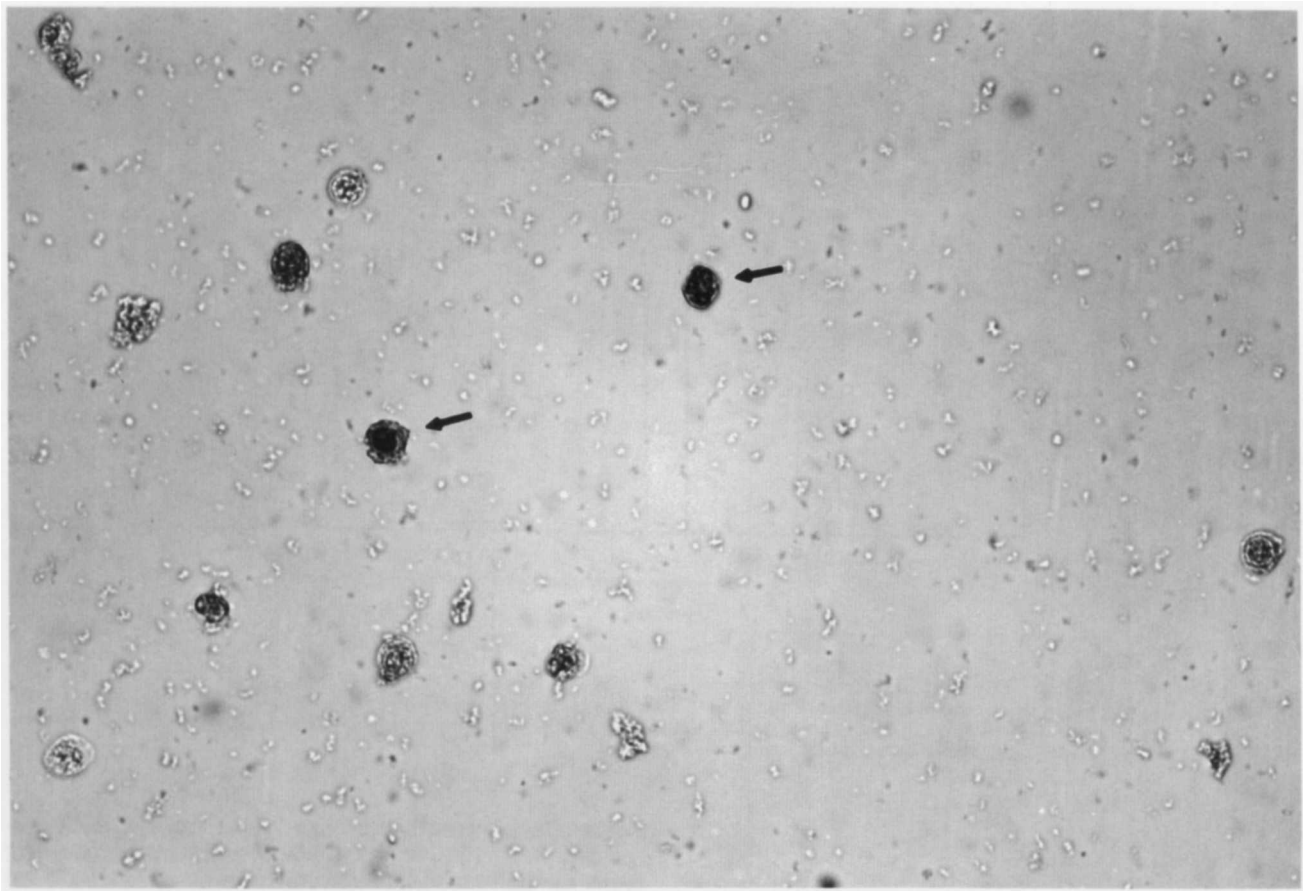

Fig. 2. Superoxide dismutase (SOD)-treated cells. Cells were pre-treated with SOD-containing liposomes for 3 hr. After washing, cells were seeded at 100,000/ml and incubated for 96 hr. Dark-stained cells (arrows) represent benzidine-positive cells. ×250

TABLE 3. Superoxide dismutase (SOD) activity in Friend erythroleukemia cells (FELC) treated with liposomes

Treatment (U/mg protein)	Total SOD
Empty liposomes	4.40 ± 0.13
SOD liposomes (concentrated)	128.07 ± 5.19
SOD liposomes (1:10 dilution)	20.41 ± 2.19

FELC were treated with 3 ml each of empty liposomes, with SOD liposomes that were undiluted, or with SOD liposomes that were diluted with Ca^{2+}-, Mg^{2+}-free MEM (1:10). Cells were then washed twice with MEM and seeded at 1×10^5 cells/ml in culture flasks. At 96 hr of incubation, cells were pelleted for SOD determinations.

crease the cellular susceptibility to oxidation, large increases in SOD activity have already been associated with increased cellular oxidation. In the slime mold, differentiation is associated with increases in SOD activity of up to 46-fold (Allen et al., 1985). Concomitant with this increase in SOD activity are increases in H_2O_2 and lipid peroxidation. As discussed above, augmentation of SOD activity has been observed to stimulate differentiation in a strain of slime mold that is usually incapable of differentiation. However, augmentation of SOD activity in *Physarum* also increased H_2O_2 concentration.

In agreement with the results of the present study, oxidants, but not antioxidants, were found to stimulate differentiation in the non-differentiating strain of *Physarum* (Allen et al., 1988). Furthermore, over-production of SOD in HeLa cells by an inserted SOD gene has been reported to result in massive peroxidative damage (Elroy-Stein et al., 1986). Although SOD is an antioxidant enzyme, it produces H_2O_2: $\cdot O_2^- + \cdot O_2^- + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$. However, in view of the relatively short half-life of $\cdot O_2^-$ (high spontaneous dismutation rate), it would be expected that H_2O_2 concentration would depend more on the rate of $\cdot O_2^-$ generation than on the rate of dismutation. Thus, the mechanism by which augmentation of SOD activity also increases cellular oxidation is not clear. It is probable that some oxidative enzymes are protected from auto-inactivation when the concentration of SOD is sufficiently increased (Lynch and Fridovich, 1978, 1979). Additionally, in vitro some oxidation reactions are inhibited by $\cdot O_2^-$, and its removal permits this type of reaction to proceed (Martin et al., 1987; Misra and Fridovich, 1977a,b). It is possible that similar reactions occur in living cells.

Pre-treatment of cells with BSO, to decrease GSH concentration, was observed to augment the effects of SOD on differentiation. This effect is similar to one previously observed in *Physarum* (Allen et al., 1988). The result is consistent with the hypothesis that increased susceptibility to oxidation due to loss of GSH

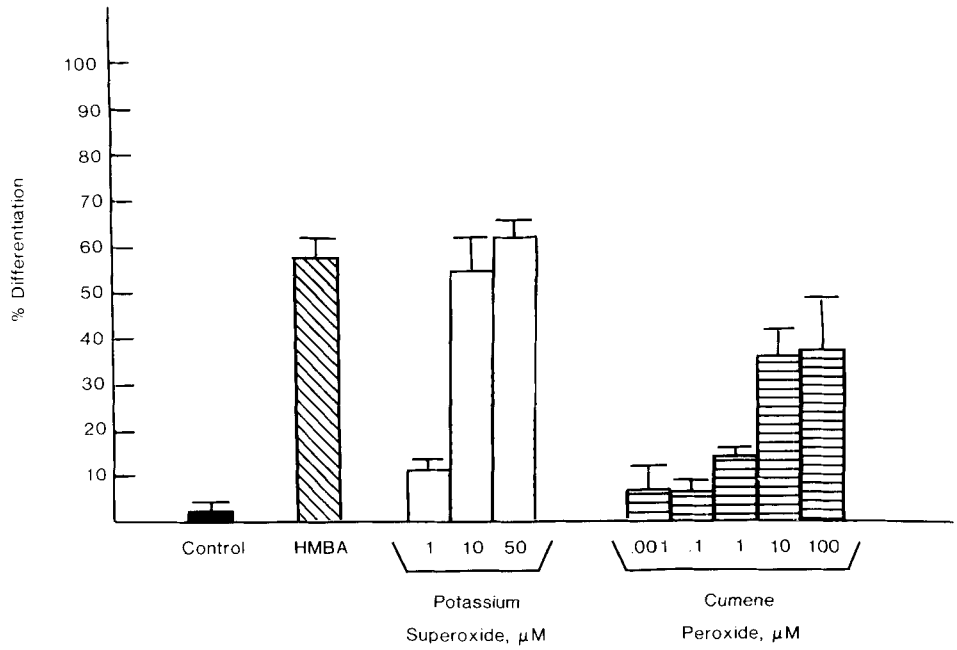

Fig. 3. Effects of hexamethylene bisacetamide (HMBA), potassium superoxide, or cumene peroxide on Friend erythroleukemia cells (FELC) differentiation. Cells were treated for 96 hr with HMBA (5 mM), potassium superoxide (1, 10, 50 μM), or cumene peroxide (0.001, 0.1, 10, or 100 μm), and differentiation was then determined.

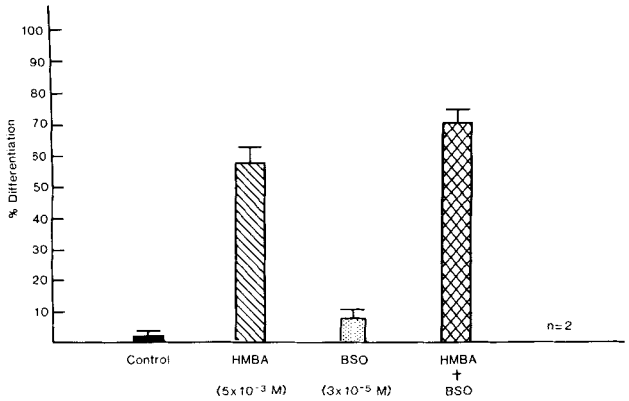

Fig. 4. Effects of buthionine sulfoximine pre-treatment on hexamethylene bisacetamide (HMBA)-induced differentiation. Friend erythroleukemia cells (FELC) were pre-treated for 3 days with buthionine sulfoximine (BSO). Cells were then washed and seeded at 100,000/ml in the presence or absence of HMBA (5 mM) for 96 hr. Control cells were not treated.

favors differentiation when an appropriate stimulus is applied. On the other hand, inhibition of GSH synthesis alone did not induce differentiation even though the cells would have been less protected against oxidation. GSH oxidants have previously been reported to augment the effects of other chemical inducers of differentiation in Friend cells although GSH oxidants alone fail to induce differentiation. (Maresca et al., 1979).

The above suggest that secondary effects of GSH, not related to antioxidation, may also influence the differentiation process. However, the underlying nature of these effects remains obscure. Sohal et al. (1986) postulated that changes in cellular redox status resulting from an increased generation of oxidants stimulated the release of ionic stores from cellular compartments and that the resulting changes in ionic distribution were the underlying cause of some developmental changes in gene expression. Supportive of this view is the observation that treatment of cells with lipid solvents such as dimethylformamide (DMF) can induce differentiation without corresponding changes in SOD activity (Speier and Newburger, 1986). Treatment of cells with DMF would make membranes more permeable to ions, even in the absence of changes in the rate of oxidant generation. Further study will be required to determine whether changes in cellular ion distribution play a role in the differentiation of Friend erythroleukemia cells.

It is noteworthy that differentiation has been induced in several types of tumor cells by addition of tocopherol to their growth medium (Prasad et al., 1980, 1981, 1982). It is possible that different types of tumor cells respond to different stimuli. However, in our opinion, the most probable reason for these conflicting results is the growth conditions employed. Studies in which tocopherol has been successfully employed to induce differentiation have examined cells grown attached to a support surface. The present study was conducted with cells grown in suspension. In surface culture, changes in membrane fluidity resulting from addition of various substances to membranes are transmitted to the nucleus via the cellular cytoskeleton and can be translated into altered gene expression (Ben-Ze'ev et al., 1980, 1981; Oliver, 1982). Conversely, gene expression in suspended cells is nearly unaffected by

TABLE 4. Effects of antioxidants on Friend erythroleukemia cell (FELC) differentiation

Anti-oxidant	Differentiation (%)
α-Tocopherol	8.8 ± 5.2
Butylated hydroxytoluene (BHT)	0
β-Carotene	6.0 ± 2.0

Antioxidant liposomes were diluted 1:10 prior to pre-incubation with FELC for 3 hr. After washing, the cells were seeded at 1×10^5 cells/ml in culture flasks. At 96 hr the percentage of benzidine-positive cells was determined.

plasma membrane alterations (for discussion: Scott, 1984; Oliver, 1982).

Whether the effects of SOD activity on the state of differentiation of Friend cells results from changes in the steady-state concentration of $\cdot O_2^-$ or from indirect effects that favor oxidation cannot be decisively determined on the basis of this study, albeit our results favor the latter hypothesis. A recent study just reported by Paoletti and Mocali (1988) also documents changes in SOD during induced differentiation of Friend cells. Of central importance is that the results of this study support Oberley's hypothesis that SOD can induce differentiation in tumor cells. Implicit to this hypothesis is the tenet that loss of SOD activity may be in some way be causal to cellular transformation. These results also support the hypothesis of Sohal et al. (1986) that cellular oxidation acts as a stimulus for differentiation.

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