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Hydroxyl radical scavengers inhibit lymphocyte mitogenesis

(free radical scavengers/phorbol myristate acetate/interleukin 1/differentiation/guanylate cyclase)

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ABSTRACT **Agents that are known to be scavengers of hydroxyl radicals inhibit lymphocyte mitogenesis induced by phorbol myristate acetate (PMA) to a greater extent than they inhibit mitogenesis induced by concanavalin A or phytohemagglutinin. These agents include dimethyl sulfoxide, benzoate, thiourea, dimethylurea, tetramethylurea, L-tryptophan, mannitol, and several other alcohols. Their inhibitory effect is not associated with cytotoxicity. The hydroxyl radical scavengers do not inhibit PMA-dependent amino acid transport in T cells or PMA-induced superoxide production by monocytes. Thus, they do not inhibit the primary interaction of PMA with responding cells. Treatment of peripheral blood mononuclear cells with PMA increased cellular guanylate cyclase in most experiments, and dimethyl sulfoxide tended to inhibit this increase. In addition to inhibition of PMA-induced mitogenesis, hydroxyl radical scavengers markedly inhibited the activity of lymphocyte activating factor (interleukin 1). The differential inhibition of lymphocyte mitogenesis induced by different mitogens appears to be related to the differential macrophage requirements of the mitogens. The data suggest that hydroxyl radicals may be involved in mediating the triggering signal for lymphocyte activation. Some of the hydroxyl radical scavengers are inducers of cellular differentiation, and it is possible that their differentiating activity is related to their ability to scavenge free radicals.**

We recently reported that dimethyl sulfoxide (Me_2SO) and other agents that induce cellular differentiation selectively inhibit lymphocyte mitogenesis induced by phorbol myristate acetate (PMA) (1, 2). Because Me_2SO is known to be a scavenger of hydroxyl radicals (·OH) (3), we determined the effect of a variety of additional ·OH scavengers on lymphocyte responses to mitogens. We present data in this report indicating that ·OH radicals may be involved as positive mediators of the triggering signal for lymphocyte mitogenesis.

MATERIALS AND METHODS

Cell Preparations. Human peripheral blood mononuclear cells (PBM) were obtained from normal volunteers by Ficoll/Hypaque density gradient centrifugation of fresh heparinized blood (4). Macrophages were depleted from a level of 20–30% in these preparations to <0.5% by absorption to Sephadex G-10 (Pharmacia) beads as described (5). Macrophages were detected by nonspecific esterase staining (6). Preparations rich in T cells were prepared by a sheep erythrocyte rosetting technique (7). Mouse thymocytes were obtained from 4-week-old BALB/c, C57BL/6J, or C3H/HeJ mice.

Proliferative Responses of Human Cells. The proliferative assays were performed as described (4) in RPMI 1640 medium containing 5% heat-inactivated fetal calf serum and supplemented with penicillin (100 units/ml) and streptomycin (100 μg/ml). Stimulation with PMA (Sigma), concanavalin A (Con A) (Miles-Yeda), or phytohemagglutinin (PHA) (Burroughs Wellcome) was carried out in an atmosphere of 5% CO_2/95% air for 3 days at 37°C. Cells (200,000) were incubated in flat-bottom microwells (Falcon) in a total volume of 200 μl. [^{3}H]Thymidine (New England Nuclear), 1 μCi per well (1 Ci = 3.7×10^{10} becquerels), was added 20 hr before harvesting on a MASH II apparatus (Microbiological Associates, Rockville, MD). The effect of a variety of ·OH scavengers and related compounds on mitogen-induced lymphocyte proliferation was determined. These compounds are listed in Table 1. Me_2SO and the alcohols were obtained from Fisher, dimethylacetamide from Aldrich, ethylene glycol from J. T. Baker, and the remainder of the compounds from Sigma. None of the compounds was cytotoxic as determined by trypan blue exclusion at the highest concentrations tested.

Early Functions. Superoxide anion production by PBM was determined by reduction of cytochrome *c* (Sigma). Cells (6.6×10^7 per ml) were suspended in phosphate-buffered saline (P_i/NaCl) containing glucose (1 mg/ml), and 30 μl was incubated at 37°C for 15 min with 100 μl of cytochrome *c* (4 mg/ml) in a final volume of 200 μl. Specificity of the reaction was determined by adding 30 μl of superoxide dismutase (60 μg/ml) (Sigma). Cells were stimulated with PMA (100 ng/ml final concentration) and the effect of various ·OH scavengers on this reaction was determined. After incubation, cells were diluted with 6 vol of ice-cold buffer and centrifuged, and the cell-free supernates were assayed for cytochrome *c* reduction by determining the absorbance at 550 nm. Superoxide-dependent reduction of cytochrome *c* was assessed by subtracting the A_{550} reading in preparations containing superoxide dismutase from that of preparations without the enzyme.

α-Aminoisobutyric acid transport was determined by incubating T cells (5×10^6 per ml) in Eagle's minimal essential medium (GIBCO) containing 5% fetal calf serum at 37°C with α-amino[*methyl*-^{3}H]isobutyric acid (2 μCi/ml; New England Nuclear) at a final concentration of 0.1 mM, essentially as described by Greene *et al.* (8). Incubations were carried out for various times (usually 5 hr) and terminated by the addition of ice-cold P_i/NaCl containing 10 mM unlabeled α-aminoisobutyric acid. The cell pellets were washed three times and lysed, and radioactivity was determined in a liquid scintillation counter (Packard). Protein determinations were by the method of Lowry *et al.* (9).

Guanylate Cyclase Assay. PBM (5×10^7 per ml) were suspended in P_i/NaCl containing Ca^{2+} (1.0 mM) and Mg^{2+} (0.5 mM) and incubated for 5 min at 37°C. The cells were then

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Abbreviations: Me_2SO, dimethyl sulfoxide; PMA, phorbol myristate acetate; PBM, peripheral blood mononuclear cells; Con A, concanavalin A; PHA, phytohemagglutinin; P_i/NaCl, phosphate-buffered saline; LAF, lymphocyte activating factor.

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cooled in an ice bucket and completely disrupted by sonication for 60 sec. The sonicates were centrifuged at 104,000 × *g* for 60 min and the supernates were assayed for guanylate cyclase activity, essentially by the method of Haddox *et al.* (10). In brief, 50 μl of supernate (containing 25–50 μg of protein) was incubated at 37°C with 100 μl of the following mixture: GTP (1 mM), $MnCl_2$ (2 mM), creatine phosphate (15 mM), creatine kinase (0.2 mg/ml), isobutylmethylxanthine (5 mM), Tris·HCl (50 mM), pH 7.5. The enzymatic reaction was stopped after 20 min by addition of 150 μl of a solution containing sodium acetate (110 mM) and EDTA (11 mM) at pH 4.0, followed by boiling for 3 min. After centrifugation the supernates were assayed for cGMP by using a radioimmunoassay kit (New England Nuclear).

Lymphocyte Activating Factor (LAF). Crude LAF preparations were prepared by incubating human PBM (5×10^6 per ml) in RPMI 1640 medium containing 5% pooled heat-inactivated human AB serum with *Escherichia coli* lipopolysaccharide 0111:B4 (Calbiochem) (24 μg/ml) for 24 hr at 37°C. Cells were removed by centrifugation and supernates were dialyzed against RPMI 1640 medium. BALB/c, C57BL/6J, or C3H/HeJ thymocytes (2×10^6 per ml) were incubated in RPMI 1640 medium with 5% human AB serum in microwells (200 μl per well) for 3 days with various dilutions of the LAF preparations and PHA (2 μg/ml). [^{3}H]Thymidine (1 μCi per well) was added 20 hr before harvesting.

RESULTS

Effect of ·OH Scavengers on PMA-Induced Lymphocyte Mitogenesis. The possibility that some species of activated oxygen radicals might have a positive effect on lymphocyte mitogenesis was suggested by our recent finding that Me_2SO, a highly permeant agent that is known to be a potent scavenger of ·OH, selectively inhibits lymphocyte mitogenesis induced by PMA (1, 2). We therefore determined the effect of this compound and a variety of additional permeant ·OH scavengers on mitogenesis induced by PMA and by the mitogenic lectins Con A and PHA. The concentrations of PMA and the lectins that we used resulted in maximal [^{3}H]thymidine uptake as determined by prior dose–response studies. PMA produces maximal stimulation at 10 ng/ml and does not inhibit mitogenesis up to 500 ng/ml. Me_2SO, benzoate, thiourea, dimethylurea, tetramethylurea, and tryptophan, all reported to be scavengers of ·OH (3, 11–13), all inhibited [^{3}H]thymidine incorporation stimulated by PMA to a greater extent than they inhibited incorporation stimulated by Con A or PHA (Table 1). Inhibition by these agents was dose dependent and at high concentrations responses to Con A and PHA were also inhibited. Urea, an agent that is structurally related to thiourea, dimethylurea, and tetramethylurea, but is not a free radical scavenger, had little effect on mitogen-stimulated [^{3}H]thymidine incorporation. Mannitol, another agent that is known to scavenge ·OH, exhibited a selective inhibitory effect only after preincubation with cells prior to the addition of the mitogens (Table 1). This may be related to the slow permeation of mannitol into the cells. Because aliphatic alcohols have previously been reported to be free radical scavengers (14), we also evaluated the effect of methanol and ethanol as well as that of glycerol and ethylene glycol on lymphocyte mitogenesis. These compounds clearly showed a selective inhibitory effect on PMA-stimulated [^{3}H]thymidine incorporation (Table 1). This selective inhibitory effect was observed over a wide range of PMA concentrations, 10 to 500 ng/ml. The ·OH scavengers used were not cytotoxic, as determined by trypan blue exclusion, at the highest concentrations used in these experiments.

Effect of ·OH Scavengers on Early Cellular Events Stimulated by PMA. PMA is reported to stimulate α-aminoisobutyric acid transport in lymphocytes (15) and the generation of superoxide radicals by macrophages (16). We examined the effect of several ·OH scavengers on these PMA-stimulated events. In contrast to the effect of the scavengers on PMA-induced lymphocyte mitogenesis, Me_2SO, thiourea, and ethanol failed to inhibit PMA-induced amino acid transport in T cells or superoxide generation by PMA (Fig. 1).

Table 1. Effect of ·OH scavengers on [^{3}H]thymidine incorporation induced by different mitogens

·OH scavenger	Conc., mM	[^{3}H]Thymidine incorporation, % of control* PMA (10 ng/ml)	Con A (2 μg/ml)	PHA (2 μg/ml)
Me_2SO	140	38.0	85.5	130
Benzoate	5	11.7	83.3	107
Thiourea	20	39.6	81.4	84.7
Dimethylurea	40	12.5	76.8	86.8
Tetramethylurea	10	3.5	77.1	86.7
Urea†	60	89.8	94.4	97.9
L-Tryptophan	1	52.5	ND	92.5
Mannitol‡	50			
No preincubation		103	70.5	169
15-min preincubation		58.3	84.3	167
60-min preincubation		27.5	73.2	171
Ethanol	220	34.2	ND	105
Methanol	300	30.0	ND	111
Glycerol	200	16.4	ND	110
Ethylene glycol	320	22.0	ND	104

ND, not determined.

* Means of 2–10 experiments for each agent. The standard errors of the means for the percent of control values were not greater than 7.5%. [^{3}H]Thymidine incorporation per culture in these experiments, expressed as mean cpm ± SEM, was: no additions, 1040 ± 330; PMA (10 ng/ml), 49,340 ± 7690; Con A (2 μg/ml), 87,450 ± 10,350; and PHA (2 μg/ml), 112,480 ± 11,290.

† Urea is structurally related to thio-, dimethyl-, and tetramethylurea, but is not an ·OH scavenger.

‡ The effect of mannitol was determined before and after preincubating it with cells prior to the addition of mitogens.

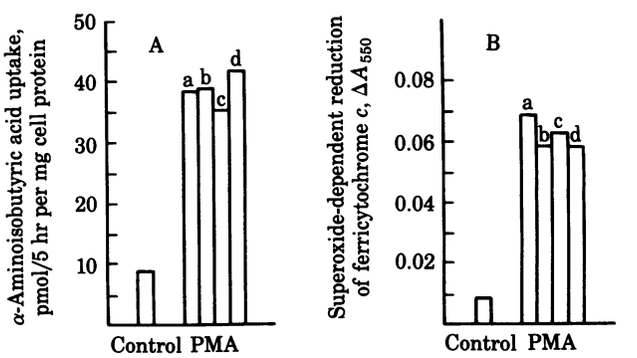

FIG. 1. Effect of several ·OH scavengers on PMA-stimulated amino acid transport by T cells and superoxide production by PBM. (*A*) α-Aminoisobutyric acid transport in control and PMA-stimulated preparations as affected by: a, no additions; b, thiourea (40 mM); c, Me_2SO (140 mM); and d, ethanol (1%). PMA was 10 ng/ml. (*B*) Superoxide anion production as determined by superoxide-dependent reduction of cytochrome *c*. Scavengers were added as indicated in *A*. ΔA_{550} = A_{550} of assay without superoxide dismutase minus A_{550} of assay with the enzyme. PMA was 100 ng/ml. Results are those of a typical experiment.

Table 2. Effect of macrophage depletion on mitogenic responses

Mitogen	Conc., μg/ml	n	[^{3}H]Thymidine incorporation, cpm × 10^{-3} PBM	Macrophage-depleted PBM
PHA	2	2	112 ± 15.9	115 ± 14.8
Con A	2	10	96.6 ± 13.4	72.8 ± 16.0
PMA	0.01	9	51.3 ± 8.3	16.8 ± 3.0

Results are presented as mean ± SEM.

Effect of PMA and Me_2SO on Guanylate Cyclase in PBM. Guanylate cyclase is modulated by oxidative-reductive mechanisms and may be activated by ·OH (10, 17). We therefore measured guanylate cyclase activity in PBM and determined the effect of PMA and the ·OH scavenger Me_2SO. Cell preparations were incubated with PMA for 5 min and guanylate cyclase activity was assayed in the soluble fraction after sonication and high-speed centrifugation. cGMP in zero-time controls (incubation mixture kept at 0°C) varied between 170 and 200 pmol/mg of cell protein, and this value was subtracted from the experimental values. In 9 of 13 experiments, PMA (100 ng/ml) stimulated an increase in guanylate cyclase activity from 258 ± 101 to 751 ± 185 pmol of cGMP per mg of cell protein (mean ± SEM). In the other four experiments no increase was noted. The effect of Me_2SO was determined in six experiments. In three, Me_2SO (140 mM) decreased PMA-stimulated guanylate cyclase activity 50–85%. In two experiments there was no effect of Me_2SO and in one there was a slight increase in activity. In the experiments in which Me_2SO inhibited guanylate cyclase activity, it was effective only when added to whole cells. It did not inhibit guanylate cyclase activity when incubated with soluble extracts prepared from the PMA-treated cells.

Macrophage Dependence of Mitogenic Response. Depletion of macrophages to the level of <0.5% by adsorption on Sephadex G-10 beads had a selective inhibitory effect on responses to different mitogens. Mitogenesis induced by PMA was more inhibited than was mitogenesis induced by Con A or PHA (Table 2). Because addition of ·OH scavengers to unfractionated cell populations had a similar selective effect, we considered the possibility that the scavengers might have an inhibitory effect on the soluble macrophage growth promoter LAF (interleukin 1). We therefore determined the effect of ·OH scavengers on LAF activity, utilizing the mouse thymocyte assay. ·OH scavengers markedly inhibited LAF-mediated potentiation of mitogenesis in mouse thymocytes treated with PHA (Fig. 2). We also determined the effect of the scavengers on PHA-induced mitogenesis of spleen cells obtained from the same mice that provided the thymocytes. Mouse spleen cells, in contrast to thymocytes, respond to PHA without an exogenous source of LAF. PHA-induced mitogenesis in the spleen cell preparations was much less sensitive to the scavengers (Fig. 2). Urea did not appreciably affect LAF action up to a concentration of 60 mM. Similar results were obtained with different murine strains (C57BL/6J and C3H/HeJ). The ·OH scavengers did not significantly affect the lipopolysaccharide-stimulated production of LAF by PBM.

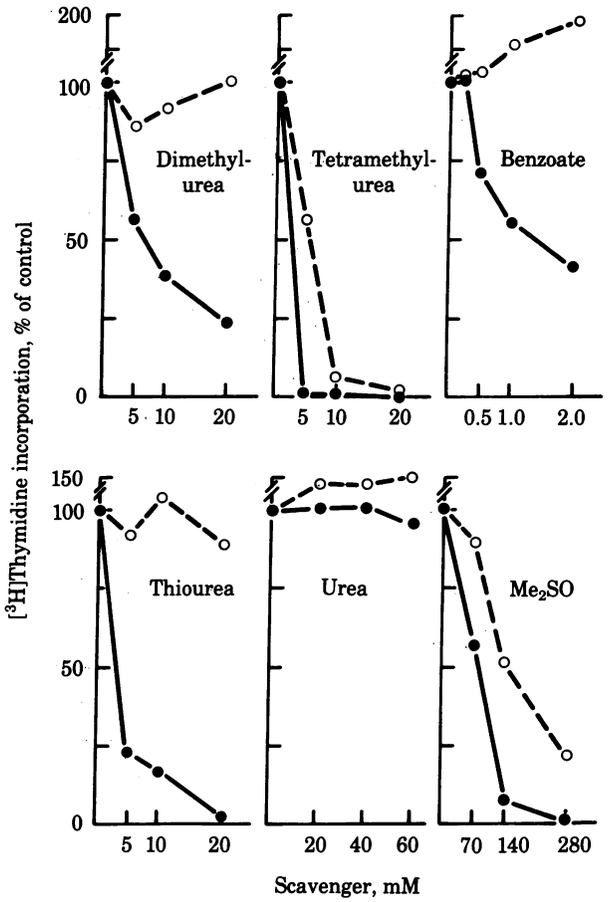

FIG. 2. Effect of various concentrations of the nonscavenger urea and of the ·OH scavengers dimethylurea, tetramethylurea, benzoate, thiourea, and Me_2SO on PHA-treated murine BALB/c thymocyte proliferation in response to LAF (5 μl of LAF preparation per 200-μl culture) (LAF assay) (●) and on PHA-stimulated proliferation of spleen cells from the same mice (○). Results are those of a typical experiment; five additional experiments resulted in similar findings. Control values are the [^{3}H]thymidine uptakes induced by PHA and LAF in the absence of added scavengers. For thymocytes, [^{3}H]thymidine incorporation per culture in this experiment was: no additions, 210 cpm; LAF, 360 cpm; PHA, 2260 cpm; LAF + PHA, 44,330 cpm. For splenocytes, incorporation was: no additions, 210 cpm; PHA, 45,375 cpm.

DISCUSSION

Various studies have indicated that free radicals can induce cellular damage and that this damage can be prevented by free radical scavengers (11). The data presented in this communication, however, suggest that certain species of free radicals have a function in addition to their inhibitory effects. The findings are consistent with the possibility that ·OH radicals are obligatory mediators for the transduction of the mitogenic signal. This possibility is based primarily on the ability of a variety of different compounds, all of which share the property of scavenging ·OH, to inhibit PMA-induced lymphocyte mitogenesis (Table 1). Compounds that scavenge ·OH could also interact with other highly reactive oxy radicals (18), but for the purpose of this discussion we will refer to these agents as ·OH scavengers.

The cellular source of ·OH that might mediate the mitogenic effect of PMA is unknown. They could be produced by the responding lymphocyte or by accessory cells. The poorly permeant ·OH scavenger mannitol did not inhibit PMA-induced mitogenesis unless the cells were preincubated with it. This suggests that free radicals are not generated in one cell type and act on another, but rather they are produced in the responding cells. We recently found that hemin, a compound that, like PMA, can initiate oxidative reactions, is mitogenic for lymphocytes (19). Unpublished experiments indicate that its mitogenic effect is also markedly susceptible to the ·OH scavengers.

·OH could be generated by several cellular mechanisms. PMA activates membrane-bound NAD(P)H oxidase in neutrophils and results in the generation of free radicals (20). Free radicals are also generated by peroxidases involved in the li-

poxygenase and cyclooxygenase pathways of arachidonic acid metabolism (21). Mitogens are known to enhance the release of arachidonic acid (22), the initial substrate for these pathways, and inhibition of the lipoxygenase pathway inhibits lectin-induced lymphocyte proliferation (23, 24). In addition, various intracellular oxidases are capable of generating free radicals.

What cellular biochemical events could be mediated by free radicals? An attractive possibility is the activation of guanylate cyclase by ·OH. Goldberg and his colleagues (10) and others (17) have shown that guanylate cyclase is subject to modulation by oxidative-reductive mechanisms and, in point of fact, is activated by ·OH. A possible role for cGMP in mediating the mitogenic signal has previously been postulated (25), and the mitogenic lectin PHA has been reported to increase guanylate cyclase activity (24). We found that PMA stimulation increased guanylate cyclase activity in most experiments, a finding also recently reported by Coffey and Hadden (26). In addition, we found that the PMA-dependent increase in guanylate cyclase was inhibited in three of six experiments by the ·OH scavenger Me_2SO. Clearly, more studies are required to determine the relationship between ·OH scavenging and guanylate cyclase activity in PBM.

·OH could also inactivate enzymes and thus result in alterations of metabolic pathways. For instance, ·OH has been shown to inactivate cyclooxygenase and peroxidases involved in arachidonic acid metabolism (21).

PMA-induced increases in amino acid transport in purified T cells were not inhibited by ·OH scavengers. Thus it appears that ·OH does not mediate all the cellular events induced by PMA. The fact that Me_2SO, thiourea, and ethanol did not inhibit amino acid transport in lymphocytes or superoxide production by macrophages eliminates the possibility that these agents interfere with mitogenesis by an effect on the interaction of PMA with responding cells.

Because PMA has been shown to mimic some of the effects of LAF (27), we investigated the possibility that LAF activity might also be inhibited by ·OH scavengers. Our data indicate that thymocyte activation by LAF, similar to that by PMA, is markedly inhibited by ·OH scavengers (Fig. 2). It is of interest to note that LAF activity has previously been associated with increased cellular cGMP (28). The extent of ·OH scavenger-mediated inhibition of mitogenic responses to different mitogens might be related to the relative dependency of the mitogens on macrophages or macrophage-generated soluble products. PMA has the most stringent requirement for macrophages among the mitogens tested (Table 2), and it is the most sensitive to inhibition by ·OH scavengers. We previously found that mixed lymphocyte reactions are also highly susceptible to inhibition by Me_2SO (1).

Free radicals may also be involved in proliferation of other cell types via initiation of the cell cycle, independent of external mitogenic stimulation. Some of the ·OH scavengers that inhibit PMA- and LAF-induced mitogenesis (Me_2SO, dimethylurea, and tetramethylurea) are potent inducers of cellular differentiation (29, 30). An intriguing possibility is that the induction of cell differentiation by these agents is related to their ability to scavenge ·OH. Inhibition of the proliferative signal may be a crucial cellular event leading to the induction of differentiation. This inhibition might be achieved by free radical scavengers or by various antimetabolic agents that are also known to induce cellular differentiation (31). Thus, the generation of ·OH may be associated with cell proliferation, and inhibition of ·OH effects, either by ·OH scavengers or by inhibition of ·OH production, may be associated with cell differentiation.

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