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RETINOID INHIBITION OF SUPEROXIDE ANION RADICAL PRODUCTION BY HUMAN POLYMORPHONUCLEAR LEUKOCYTES STIMULATED WITH TUMOR PROMOTERS

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SUMMARY: All-trans retinol, retinyl acetate and retinoic acid were found to be effective inhibitors of $O_2^-\cdot$ production in polymorphonuclear leukocytes stimulated with the tumor promoter phorbol myristate acetate. Retinol similarly inhibited cells stimulated with mezerein or teleocidin B. No-effect concentrations of the protease inhibitor antipain potentiated the inhibitory effect of low levels of retinol. Higher concentrations of retinol and antipain resulted in an additive or less than additive inhibitory effect.

INTRODUCTION

We have hypothesized that tumor promotion by phorbol myristate acetate (PMA) is mediated at least in part by reactive oxygen species derived from phagocytic cells which infiltrate mouse skin as part of the inflammatory response induced by tumor promoters. This hypothesis is based on previous studies (1) which showed that protease inhibitors capable of inhibiting tumor promotion (2) also inhibit the stimulation of phagocytic cells, including macrophages and polymorphonuclear leukocytes (PMN). In addition the degree of stimulation of PMN by phorbol esters correlates with their promoting activity (2). Since promotion by phorbol esters in the mouse skin system is also

Abbreviations: PMA, phorbol myristate acetate; PMN, polymorphonuclear leukocytes.

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inhibited by retinoids (3), we investigated the effect of these agents on the stimulation of PMN by the tumor promoters PMA, mezerein, and teleocidin B, a natural product with suspected tumor-promoting activity (4).

METHODS

Materials: Cytochrome C (horse heart, type III) superoxide dismutase (type I, bovine blood), and all-trans retinol (lot95C-9540), were purchased from Sigma, and PMA from P. Borchert, Univ. of Minn. Antipain was obtained through the U.S.-Japan Cooperative Cancer Research Program. All-trans retinoic acid and retinyl acetate were gifts from M. Sporn, N.I.H. Mezerein was a gift from I.B. Weinstein, N.Y., and teleocidin B a gift from T. Sugimura, Japan.

Isolation of Cells: PMN from human peripheral blood and rat alveolar macrophages were isolated as described previously (1). The purified cells were suspended in balanced salt solution (BSS, 128mM NaCl, 12mM KCl, 1mM $CaCl_2$, 2mM $MgCl_2$, 2mM glucose, 4mM $PO_4^=$), pH 7.4 at a cell concentration of 10^7/ml.

Assays for Measurement of $O_2^-\cdot$ Production and Oxygen Consumption:

The rate of $O_2^-\cdot$ production was measured by the rate of cytochrome C reduction using a Perkin Elmer 552 double beam spectrophotometer as previously described (2). Cytochrome C (100ul, 4mg/ml H_2O), cells (50ul, $1x10^7$/ml BSS), retinoids (10-50ul, 1mg/ml of 10% DMSO, 90% BSS, v:v), were diluted to 650ul with BSS and equilibrated to 37° in a thermostated cell holder before addition of PMA (50ul, lug/ml BSS). The optical density at 550 nm was recorded continuously over time (20 min). The rate of cytochrome C reduction was calculated from the linear portion of the curve as described previously (2). Generation of $O_2^-\cdot$ by xanthine oxidase (5) in the presence of retinoids was also measured spectrophotometrically using samples containing 133ul cytochrome C (4mg/ml H_2O), 100ul acetaldehyde (10^{-2}M in BSS), 30ul xanthine oxidase (1mg/ml PBS) and 10-50ul retinoids (1mg/ml BSS containing 10% DMSO) in a total volume of 1 ml. The reference cuvette contained all components plus superoxide dismutase. Oxygen consumption was measured in the laboratory of M.J. Broekman and A.J. Marcus as described previously (6). Samples consisted of cells (300ul, $1x10^7$/ml), 50ul PMA (10ug/ml) in a total volume of 1.8ml with and without 200ul retinol (10ug/ml).

RESULTS AND DISCUSSION

Retinol and the vitamin A analogs retinyl acetate and retinoic acid are effective inhibitors of $O_2^-\cdot$ production in PMA-stimulated PMN as shown in Table 1. The degree of inhibition is dose-dependent, being greatest at the highest retinoid con-

Table 1. Inhibition of $O_2^-\cdot$ production in PMA-stimulated PMN by retinoids[a]

Retinoid (uM)	$O_2^-\cdot$ Produced nmoles/min	% Reduction in Rate of $O_2^-\cdot$ Formation
Retinol		
0	5.6	-
50	4.5	18
150	3.2	43
250	1.7	70
Retinyl acetate		
0	3.9	-
44	4.4	0
130	3.3	16
270	1.3	67
Retinoic acid		
0	3.6	-
48	2.4	33
240	1.9	47

[a] 71ng PMA/ml, 0.71 x 10^6 cells/ml, 0.57mg cytochrome C/ml

centration employed. DMSO which was used to prepare retinoid stock solutions was less than 0.5% (v:v) in the cell samples examined. Control samples run with DMSO in the absence of retinoids showed that up to 0.5% DMSO does not affect the rate of $O_2^-\cdot$ production of stimulated PMN. Stimulation of PMN by PMA was found to vary for different PMN preparations; however the percent reduction in the rate of $O_2^-\cdot$ formation due to retinoids did not change considerably for different cell batches. Similar to PMN, rat alveolar macrophages exhibited a 68 and 87% decrease in the rate of $O_2^-\cdot$ formation when stimulated with PMA in the presence of 2.6 and 5.2×10^{-4} retinol, respectively, with an initial rate of 4.1 nm $O_2^-\cdot$/min/1.5x10 ^{6} cells in the absence of retinol.

To rule out possible artifacts in the measurement of $O_2^-\cdot$ due to the presence of retinoid, oxygen consumption was measured. As the production of $O_2^-\cdot$ in stimulated PMN is a concomitant of the increase in oxygen consumption (7), any inhibitory effect

on $O_2^-\cdot$ production should also be reflected by a decrease in oxygen consumption. Under the conditions used, PMN stimulated by PMA took up 9.6 nmoles O_2/min compared to 5.8 nmoles O_2/min in the presence of 3.8×10^{-4}M retinol. Experiments carried out using the xanthine oxidase $O_2^-\cdot$ generating system suggest that retinol is not acting as an $O_2^-\cdot$ scavenger. In this system, $O_2^-\cdot$ was produced at a rate of 13 nmoles/min/ml. The presence of 0.3% DMSO (final concentration) decreased the rate of $O_2^-\cdot$ production by 20% as did 1.5×10^{-4}M retinol in the presence of 0.3% DMSO, indicating that the reduction in rate is due to DMSO. Consequently the reduced rates of cytochrome c reduction in the PMN system are probably not due to retinol (or other retinoids) acting as electron acceptor at the expense of ferricytochrome c.

Stimulation of $O_2^-\cdot$ production by mezerein, a tumor promoter of moderate activity (8), and teleocidin B, a suspect tumor promoter (4), is also inhibited by retinol. At 2.2×10^{-8}M mezerein and 1.6×10^{-8}M teleocidin B, 5, 15 and 25×10^{-5}M retinol reduced the rate of PMN-$O_2^-\cdot$ production by 16, 50, and 75%, respectively. Of note is that despite their structural dissimilarity, these three compounds are all inhibited to a similar extent by retinol. A potential mechanism of action by retinol in this system is by altering cell membrane fluidity. Retinoic acid has been shown to alter membrane fluidity in red cell membranes (9) and preliminary data from this laboratory indicate that the fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene-labelled PMN decreases upon stimulation with PMA, suggesting an increase in membrane lipid fluidity upon activation of the oxidase. Thus the inhibition of PMN stimulation observed with retinoids may involve a general effect such as an alteration in the dynamic properties of plasma membranes.

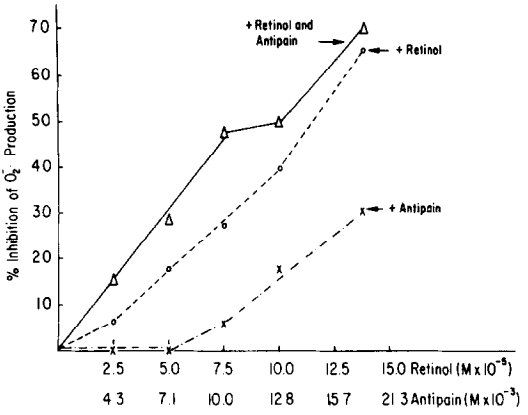

Figure 1. Combined effect of retinol and antipain on O_2^-· production in polymorphonuclear leukocytes stimulated with phorbol myristate acetate. Cells were stimulated with PMA (71ng/ml) in the absence or presence of either retinol, antipain, or both. The percent inhibition of O_2^-· production refers to the reduction in the rate of O_2^-· formation in the presence of inhibitors relative to that of cells stimulated in the absence of inhibitors. Data are average of two experiments.

In previous work, we showed that protease inhibitors effectively inhibit PMN stimulation by PMA and a host of other oxygen burst initiators (1). In order to investigate whether similar membrane interactions are involved in the inhibition by retinoids and protease inhibitors, PMN stimulation was studied in the presence of both types of agents. The results (Figure 1) show that no effect or low-effect concentrations of the protease inhibitor antipain potentiate the inhibitory effect of low concentrations of retinol, while at higher dosages an additive or less than additive inhibitory effect is observed. These data suggest that retinoids and protease inhibitors may inhibit PMN stimulation by different mechanisms. A similar conclusion was recently drawn from mouse skin experiments on the mechanism of tumor inhibition by retinoic acid and the protease inhibitor TLCK (10). Furthermore, combined treatment with retinoic acid and TLCK was shown to result in 100% inhibition of tumor promotion by PMA (10), a finding which correlates

with the combined effect of these types of agents on the stimulation of PMN by PMA.

It was previously shown that the known inhibitors of mouse skin tumor promotion, dexamethasone and protease inhibitors, are capable of inhibiting the stimulation of O_2^-· production by phagocytic cells (11, 1). With the present study, retinoids can be added to the list of compounds. Furthermore, the tumor promoting activity of phorbol esters correlates with their ability to stimulate PMN to produce reactive oxygen species (2). The present data therefore lend additional support to our hypothesis; however, definitive proof for a free radical involvement in tumor promotion requires in vivo experimentation.

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