

# Inhibition of Phorbol Myristate Acetate and Phytohemagglutinin Stimulation of Human Lymphocytes by Retinol<sup>1</sup>

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## ABSTRACT

Retinol (vitamin A) in concentrations above 5  $\mu\text{g/ml}$  inhibits the stimulation of human lymphocytes by phytohemagglutinin and phorbol myristate acetate. This is in contrast to bovine lymphocytes in which retinol inhibits only phorbol myristate acetate stimulation of lymphocytes. The cause of the inhibition does not appear to be due to a direct toxic action because the viability and ultrastructure of lymphocytes exposed to a concentration of 20  $\mu\text{g/ml}$  for 72 hr were normal. The mechanism of action of the retinol is not known, but it may be mediated by a retinol-binding protein in the cells.

## INTRODUCTION

PMA,<sup>3</sup> a constituent of croton oil, is recognized as a tumor promoter or cocarcinogen in experimental animals (1, 13). It also increases the confluent density and DNA synthesis of a variety of cell types in tissue culture (6, 8), acts as a mitogen for human lymphocytes (9), and is a platelet and lymphocyte agglutinin (20, 26). Retinol and its analogs have a prophylactic effect on experimentally produced benign and malignant tumors in a variety of experimental animals (2-4), restore contact inhibition of cultured L-cells (7), block phenotypic changes induced by sarcoma growth factor in mouse fibroblasts (22), and inhibit the growth of untransformed, transformed, and tumor cells *in vitro* (17). Retinol has also been reported as inhibiting the mitogenic response of bovine lymphocytes to PMA but not to PHA (14, 25). The following study was carried out to assess the effect of retinol on the mitogenic response of human lymphocytes to PHA and PMA.

## MATERIALS AND METHODS

Human lymphocytes from defibrinated and heparinized blood were obtained by separation over a Ficoll-Hypaque (Pharmacia) gradient. They were washed 3 times with phosphate-buffered saline [0.13 M NaCl:3.3 mM  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ :6.7 mM  $\text{Na}_2\text{HPO}_4$  (pH 7.2)] and suspended in GM consisting of RPMI Medium 1640 (Grand Island Biological Co., Grand Island, N. Y.) and 10% fetal calf serum at a concentration of  $6 \times 10^6$  lymphocytes in 3 ml of GM. The mitogenic response of the lymphocytes was assessed by the [<sup>3</sup>H]thymidine (Amersham/Searle, Don Mills, Ontario, Canada) uptake of the cultures after 72 hr (23). A dose-response curve of the PHA (Difco Laboratories, Inc.,

Detroit, Mich.) and the PMA (Sigma Chemical Co., St. Louis, Mo.) showed that PHA (4  $\mu\text{l/ml}$ ) and PMA (50 ng/ml) gave optimal stimulation, and these concentrations were used throughout. DMSO was needed to dissolve the PMA, and with a PMA concentration of 50 ng/ml, the percentage of DMSO in the system was 0.01%. Retinol (Sigma) was dissolved in DMSO and diluted further with GM. Addition of retinol in 0.2 ml GM was made so that final concentrations of 1, 3, 5, 10, and 20  $\mu\text{g/ml}$  were achieved. The final concentration of DMSO with the 20  $\mu\text{g}$  of retinol per ml was 1%. The retinol was added at 0, 24, 48, and 72 hr after the mitogen. GM (0.2 ml) was added at 48 and 72 hr postmitogen as a control on the effect of adding the additional GM at these times. Controls with the appropriate concentration of DMSO (up to 1%) were also carried out to test the effect of DMSO on the mitogenic response of lymphocytes. Viability of the lymphocytes exposed to retinol in doses up to 20  $\mu\text{g/ml}$  for 72 hr was assessed by the trypan blue exclusion test. The morphology of lymphocytes exposed to retinol (10 and 20  $\mu\text{g/ml}$ ) for 72 hr was assessed by fixing the cells in ice-cold 3% glutaraldehyde for 1 hr, postfixing in 2% osmium tetroxide, and examining these sections by electron microscopy to determine if there was any evidence of nuclear or organelle damage.

## RESULTS

The dose-response curve of human lymphocytes to varying degrees of PMA was calculated, and a dose of 50 ng of PMA per ml was chosen as the final concentration to be used. The effect of varying doses of retinol on the mitogenic response of the lymphocytes to PHA and PMA as assessed by the [<sup>3</sup>H]thymidine uptake is shown in Charts 1 and 2. The effect of delaying the addition of retinol by 24, 48, and 72 hr is also shown (Charts 1 and 2). The variation in cpm in the control data (without retinol) for the timed additions of retinol results from interexperimental variation. DMSO at doses up to 1% had no effect on the mitogenic response to either PHA or PMA (data not shown). As assessed by the trypan blue exclusion test, viability of lymphocytes exposed to retinol (20  $\mu\text{g/ml}$ ) for 72 hr in GM was 96 to 100%, and ultrastructure of lymphocytes exposed to retinol (20  $\mu\text{g/ml}$ ) for 72 hr showed no evidence of organelle or cellular damage. Ribosomes, however, were solitary, and much of the chromatin was in the heterochromatin state.

## DISCUSSION

Apart from a slight stimulatory effect of low concentrations at 1 to 3  $\mu\text{g/ml}$ , retinol above a concentration of 5  $\mu\text{g/ml}$  is inhibitory to the stimulated human lymphocytes. This effect is seen with both PHA and PMA and is in contrast to bovine

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<sup>3</sup> The abbreviations used are: PMA, phorbol myristate acetate; PHA, phytohemagglutinin; GM, growth medium; DMSO, dimethyl sulfoxide.

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lymphocytes in which the effect is on PMA stimulation only (14, 25). However, examples of different responses of different cell types to retinol are documented (17). The site of action of the inhibitory effect of the retinol is not known. It is known that PHA does not need to enter the cell to stimulate human lymphocytes (11) and that its ligand action on the cell membrane provides the triggering stimulus (12). PMA also appears to act on the cell membranes (24), and in epidermal cells it at least appears to bind to receptors of the epidermal growth factor (15). It might thus be postulated that retinol interferes with the stimulating effect of PHA or PMA on the cell surface. However, the significant inhibition that occurs even when retinol is added 72 hr later to the already stimulated lymphocytes makes this very unlikely. The finding of a retinol-binding protein in some cells (19) and the correlation between the protein-binding activity of retinol analogs and the effect of these analogs on the growth characteristics of the cells tested (18) offer another hypothesis, namely, that the stimulated lymphocytes contain a retinol-binding protein by which inhibition occurs. It has already been shown that a steroid-binding protein operates in a similar fashion in the hydrocortisone inhibition of stimulated lymphocytes (16, 21). The inhibitory effect cannot be attributed to the DMSO used as a solvent for the retinol because, even at a 1% solution, there was no inhibition of the mitogenic response (data not shown). This is in agreement with other reports that DMSO is not inhibitory to the lymphocyte mitogenic response (5). The effect of retinol cannot be attributed to toxicity, inasmuch as lymphocytes cultured with retinol in the doses used showed no loss of viability as judged by the trypan blue exclusion test, nor was there any ultrastructural evidence of cellular damage. The solitary ribosomes suggest a quiescent cell (10).

It is clear that this inhibitory effect of retinol on the mitogenic response of lymphocytes will not be present *in vivo* at the normal dietary intake level of vitamin A (normal level of 0.2 to

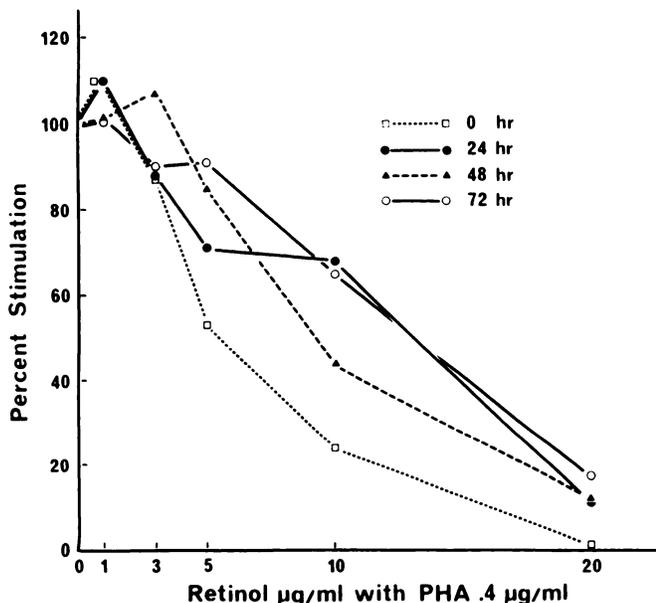


Chart 1. The effect of varying doses of retinol expressed as percentage of stimulation on the response of lymphocytes to PHA. Times refer to the time interval between additions of PHA and retinol. One hundred % represents the following cpm for the curves as indicated: 0 hr, 62,745 ± 1,436 (S.D.); 24 hr, 38,248 ± 1,941; 48 hr, 77,375 ± 920; and 72 hr, 73,225 ± 2,024.

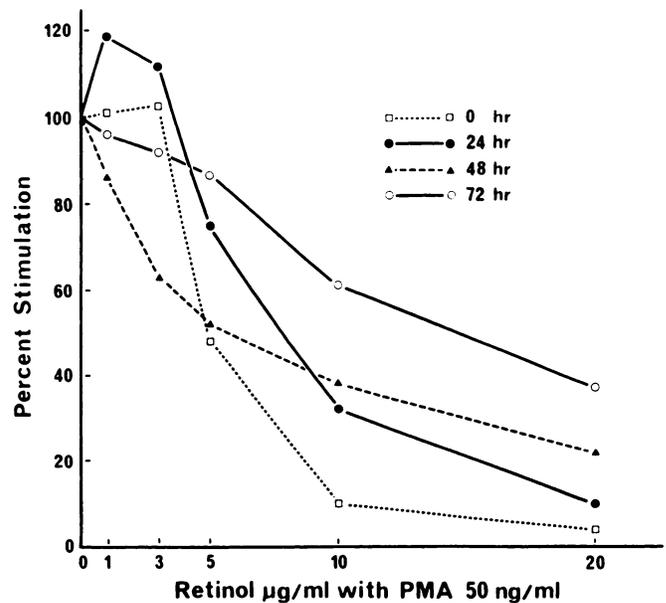


Chart 2. As in Chart 1 but with PMA instead of PHA. One hundred % represents the following cpm for the curves as indicated: 0 hr, 15,242 ± 525 (S.D.); 24 hr, 34,189 ± 1,574; 48 hr, 17,846 ± 937; and 72 hr, 31,922 ± 1,347.

0.4 µg/ml) but, in the present climate of unwarranted expectations of megavitamin therapy, its effect should be considered.

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