

Brief Definitive Report

PHORBOL MYRISTATE ACETATE: A MITOGEN SELECTIVE FOR A T-LYMPHOCYTE SUBPOPULATION*

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Phorbol myristate acetate [12-*O*-tetradecanoyl-phorbol-13-acetate (PMA)] is one of several biologically active substances derived from croton oil, which potentiate the action of a carcinogen in inducing epidermoid tumors of mouse skin (cf. reference 1 for review). Interest in the role of immune response in epidermal carcinogenesis prompted us to investigate possible effects of PMA on lymphocytes. We observed that PMA is a potent mitogen for human peripheral blood lymphocytes (2), an observation confirmed by several laboratories (3-6). In this report, we present evidence that PMA approximates phytohemagglutinin (PHA) in its mitogenic potency, yet acts selectively on a T-lymphocyte subpopulation that has high affinity for sheep erythrocytes (SRBC) and is distinct from that responsive to PHA.

Materials and Methods

Human peripheral blood lymphocytes (PBL) were separated by centrifugation on a Ficoll-Hypaque gradient and cultured in tubes as previously described (7). PMA dissolved in dimethylsulphoxide (DMSO) was kindly provided by Dr. A Sivak, New York University, New York. Purified PHA (PHA MR-68-69), concanavalin A (Con A), and pokeweed mitogen (PWM) were used at the optimal mitogenic concentrations. Separation of T lymphocytes was performed by the SRBC rosette technique (8). Ablation of proliferating lymphocytes was performed using the 5'-bromodeoxyuridine (BUdR) and light technique previously described (7). All experimental manipulations were performed in triplicate and repeated at least twice. DMSO, the solvent vehicle for PMA, was inactive at equivalent concentrations in each of the experimental situations.

Results

Fig. 1A presents the dose-response characteristics of PMA stimulation of human PBL proliferation. Maximal stimulation is observed at 100 ng/ml. Variable stimulation occurs at 1,000 ng/ml, and inhibition at greater concentrations. Fig. 1B depicts the time-course of the PMA response. Peak stimulation is observed at 4-5 days of culture, depending on the concentration of PMA. Comparable responses for PHA under these conditions are obtained at 3 μ g/ml, peaking at 3 days (data not shown). In 11 experiments directly comparing PHA (3 μ g/ml) and PMA (100 ng/ml) at 72 h of culture, the incorporation of thymidine

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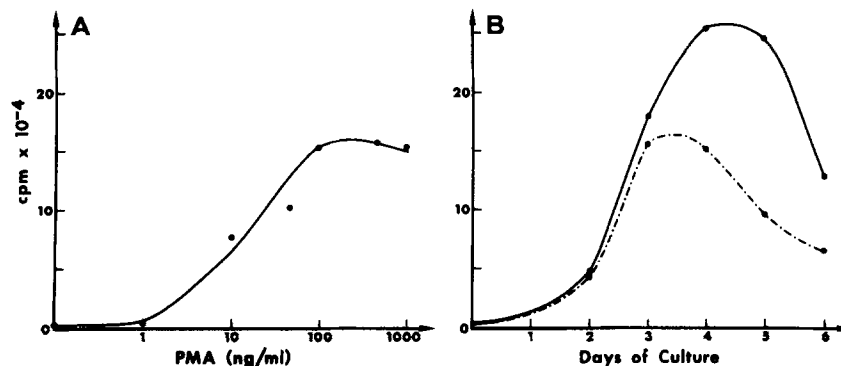


FIG. 1. Incorporation of [³H]thymidine by human PBL after stimulation with PMA. (A) Dose-response curve. (B) Kinetics of the response using 100 ng/ml (○—○) or 1,000 ng/ml (○- -○) of PMA.

was $280 \pm 24 \times 10^3$ cpm/culture and $234 \pm 26 \times 10^3$ cpm/culture, respectively. While the PMA response averages four-fifths of the PHA response, great variability in the relative responses was noted, and in 5 of 11 individuals the stimulation by PMA was greater than the stimulation induced by an optimal dose of PHA. These data suggested that PMA and PHA might be acting on different lymphoid populations. The following experiments were designed to obtain insight into the population of lymphocytes responding to PMA.

To further compare the response of lymphocytes to PHA and PMA, a BUdR and light technique was used to ablate the cells proliferating in response to stimulation by each of the mitogens. With this method, the response to PHA, for example, can be reduced by approximately 75% and, more importantly, no further stimulation can be produced by readdition of PHA or another mitogen acting on the same population of PBL (e.g., Con A) (7). The employment of this technique with PMA is depicted in Fig. 2. When the PHA response is ablated with BUdR and light, the PMA response is unaffected. Similarly, if the PMA response is ablated, the PHA response is left unaffected.

Ficoll-Hypaque-purified lymphocyte preparations were subjected to further fractionation employing the preferential ability of T lymphocytes to form rosettes with SRBC. When T cells are twice sedimented with SRBC then liberated by hypotonic lysis of the erythrocytes, the rosette-forming cell (RFC) population of lymphocytes exceeds 95% pure T lymphocytes, as determined by rosetting or sensitivity to lysis by anti-human T-lymphocyte antiserum. The residual non-rosetted population represents a mixture of B lymphocytes and monocytes, as well as a few T lymphocytes. As shown in Table I, T-cell enrichment segregates the responses to PHA, Con A, and PWM to the RFC fraction, but some residual response occurs in the nonrosetted population. Similarly, the PMA response resides almost exclusively with the rosetted population. In a further experiment with a single rosette sedimentation, which removes only those T lymphocytes with a high affinity for SRBC ($1/3$ to $1/2$ of the total T lymphocytes), an even greater distinction between PMA and the phytomitogens is observed: only 1% of the response to PMA occurred in the non-RFC population in contrast to 60, 45, and 68% for the responses to PHA, Con A, and PWM, respectively. These data

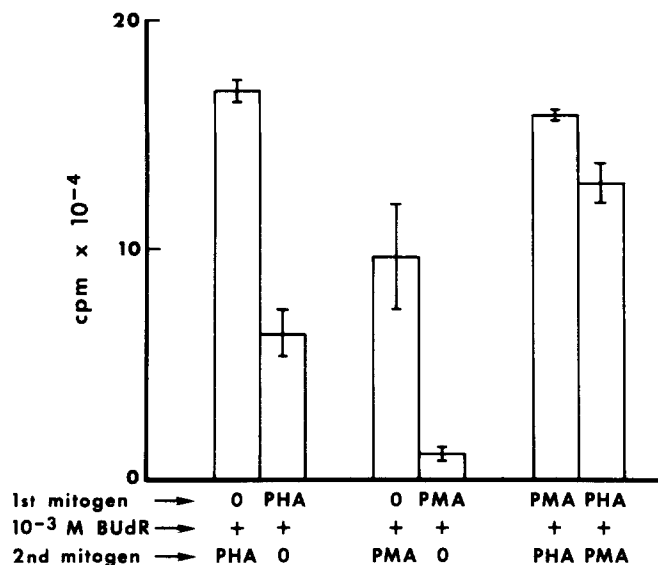


FIG. 2. Inactivation of PHA- or PMA-responsive human PBL by BUdR and light treatment. Experimental protocol: 1st mitogen → indicates 72-h incubation with no mitogen, PMA, or PHA. 10⁻³ M BUdR → indicates treatment with BUdR and light followed by three washes. 2nd mitogen → indicates a second period of 72-h incubation with no mitogen, PMA, or PHA. Cultures were terminated on day 6 and assayed for the incorporation of [³H]thymidine. Data are expressed as cpm ± SE.

TABLE I
Proliferative Responses of Human Lymphocytes Separated by the Rosetting Method*

Lymphocytes	Mitogens				
	None	PMA	PHA	Con A	PWM
Unseparated	205 ± 15	5,914 ± 425	36,225 ± 2,741	14,084 ± 748	9,032 ± 1,320
RFC	131 ± 10	13,931 ± 1,007	23,055 ± 1,069	10,046 ± 846	5,179 ± 944
RFC-depleted lymphocytes	796 ± 84	1,364 ± 55	4,294 ± 554	4,077 ± 223	3,077 ± 184

* Results are lower than those in Fig. 1 and 2 because of the lower specific activity of [³H]thymidine employed.

indicate that the PMA-responsive population may represent a T-cell subset having a high affinity for SRBC.

Discussion

The present experiments indicate that PMA is mitogenic for a subpopulation of thymus-derived lymphocytes having relatively high affinity for SRBC and different from those responsive to PHA. We have previously observed that PMA's mitogenic effect is additive to that of PHA (2), and that PMA induces lymphoblasts that can be distinguished morphologically from those produced by PHA in that they are smaller, more vacuolated, and tend to have a more smooth or ruffled surface appearance (2, 9). We can now interpret these differences between PMA and PHA as resulting principally from their actions to independently activate two different lymphocyte subpopulations in human peripheral blood. This interpretation does not exclude the possibility that PMA has a degree of synergism with other mitogens resulting from their interaction on the

same cell, as is suggested by experiments employing Con A with lymphocytes from different sources and species (4, 5).

According to current evaluations by surface marker criteria, T lymphocytes constitute approximately 75% of the total PBL (8). Of the total PBL, approximately 30% are responsive to PHA and by ablative criteria those responding to PHA are essentially the same as those responding to ConA (10). In independent, unpublished studies of the PMA response of human PBL, we have found that the subpopulation responsive to PMA is also distinct from that responsive to Con A and that the PMA-responsive subset is sensitive to lysis by anti-human T-lymphocyte antiserum and approximates 20% of the original PBL population. The subpopulation responsive to PMA can then be estimated to be one-quarter of the T cells, a subpopulation only slightly smaller than that responsive to PHA and Con A.

The functional significance of the PMA responsive T-cell subpopulation remains to be determined. Obvious candidates include lymphocytes subserving the functions, in addition to proliferation, of cytotoxicity, soluble mediator secretion, and helper or suppressor effects. While no direct information is available, we have observed an extraordinary sensitivity of the PMA-responsive population to inhibition with hydrocortisone.

	Hydrocortisone hemisuccinate $\mu\text{g/ml}$			
	1	10	100	1,000
% inhibition of PHA response	21%	32%	45%	71%
% inhibition of PMA response	66%	84%	91%	100%

The PMA response is sensitive to 2-3 logs lower concentration of hydrocortisone than the PHA response. While it is not clear whether PMA may sensitize the responder to hydrocortisone or is selecting a responder that is intrinsically more sensitive, this finding suggests that the subpopulation is more likely to be a helper T than an allogeneic responder since these two responses are differentially sensitive to steroids (10). The suggestion that the PMA responder is not an allogeneic responder is further supported by our observation that BUdR and light ablation of the PMA-responsive population leaves the mixed lymphocyte culture response unaffected (unpublished observation).

Further insight into the functional responses of T cells subsets is available from recent studies in the mouse. These studies employ antisera to cell surface antigens of the Ly series to identify T-lymphocyte subpopulations, a technique not yet applicable to human PBL. Helper activity is associated with the Ly1 phenotype and cytotoxic and suppressor activity with Ly2,3 (11, 12). PHA and Con A reactivity is associated with both phenotypes (13). These observations are further complemented by the demonstration that in both human peripheral blood and mouse spleen, those lymphocytes responsive to mitogens can be separated from those responsive to allogeneic stimulation (7, 14). Work is needed to resolve the relation of those cells that are responsive to mitogenic, allogeneic, or antigenic stimulation to those that respond with proliferative, cytotoxic, and helper or suppressor activity, and to interrelate these responses to their species of origin, state of differentiation, and location in lymphoid tissues or blood.

The basis of the selectivity of PMA action on a T-cell subpopulation is not known. PMA is mitogenic for 3T3 "fibroblasts" and for epidermal cells and, therefore, may be mitogenic for a large variety of cell types (1, 2, 15). It is known that PMA binds preferentially to plasma membranes and stimulates membrane enzymes like adenosine triphosphatase and 5'-nucleotidase (16) and induces increases in transport and cellular levels of cyclic GMP (2, 15). These actions are similar to those induced by lectin mitogens like PHA and Con A which are thought to bind selectively to plasma membrane receptors. It seems likely then, that the selectivity of PMA action may also reside in specific cell surface receptors.

The high affinity of the PMA-responsive T lymphocyte for SRBC provides some information for understanding the selectivity of PMA action. It is of interest that the population of T cells having high affinity for SRBC forms the basis for the active or early rosette assay (17, 18). It is also this population (approximately 20-25% of the total PBL population) that is reduced in certain autoimmune states and in cancer, and that, when reduced, responds to treatment in vivo and in vitro to stimulation with the thymic hormone preparation thymosin (18). It is tempting to predict that PMA may be a useful diagnostic tool in probing deficits in the size or proliferative function of this subpopulation.

Summary

Phorbol myristate acetate (PMA) is a potent mitogen for human peripheral blood lymphocytes (PBL) comparable to phytohemagglutinin (PHA) in potency. Inactivation of PHA-responsive lymphocytes by 5'-bromodeoxyuridine and light treatment left the PMA response intact and vice versa. Experiments separating lymphocytes by rosetting with sheep erythrocytes (SRBC) demonstrated that the PMA-responsive lymphocytes segregate with those that have a high affinity for SRBC to a greater degree than PHA- or concanavalin A (Con A)-responsive cells. These results indicate that a PMA-responsive population in human peripheral blood resides within the T-lymphocyte population and appears to have a high affinity for SRBC and to be distinct from that responding to PHA and Con A. PMA may be useful clinically to assay the size and function of the high affinity or "active" rosette population.

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