

Phorbol Myristate Acetate - Fate and Usability in a Human Interleukin-1 Assay

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Phorbol myristate acetate (PMA) has been widely used to produce interleukin-1 (IL-1) from monocytes (Mø) of various species, including man. Supernatants from cultures of human Mø, stimulated with PMA, 2×10^{-6} M, contained IL-1-like activity, as judged by their high co-mitogenic effect on mitogen-stimulated human T-cells. However, a solution of PMA in culture medium was equally as active as the supernatants. By way of ^3H -PMA it was demonstrated that the IL-1-like effect of PMA-induced supernatants was non-dialyzable, was coupled to proteins with a molecular weight $\geq 70,000$ Dalton and could be dependent mainly upon the PMA content. Thus, previous reports dealing with basic immunological as well as immunopharmacological aspects of IL-1, must be interpreted with caution if the conclusions have been based on results obtained with crude or high molecular weight forms of PMA-induced IL-1. It could be that PMA can be used as an IL-1 inducer. However, it must first be demonstrated that PMA does not interfere with the detection of IL-1 or, if so, that PMA has been removed sufficiently from the test material to avoid interference. Secondly, the specificity of PMA with respect to IL-1 inducing effect must be demonstrated under endotoxin-free culture conditions.

Key words: interleukin-1; lymphocyte transformation; monocytes; phorbols.

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CLINICAL ASPECTS

Interleukin-1 (IL-1) is the designation for one or several factors of protein character, produced by cells of the monocyte/macrophage line. Among the multiple biological activities of IL-1 are induction of fever, release and activation of neutrophils from the bone marrow, production of collagen from fibroblasts, release of acute phase proteins from the liver, and activation of various cells in the immune system. During the past 10 to 15 years numerous basic immunological *in vitro* studies have been carried out to elucidate the role of IL-1 in the immunologic response and, hereby, its possible pathogenetic role in allergic and immunologic diseases. Further, results have been recently published concerning the effect of immune suppressive agents on the production and activity of IL-1. Phorbol myristate acetate (PMA), a croton-oil derived tumor promoter, has been widely used for IL-1 production in the above mentioned experiments. However, PMA has a biological effect on some of the cells in the immune system, and in the present paper it is demonstrated that PMA-interference in the detection of IL-1 is almost impossible to avoid. Thus, the clinician who wants to be kept currently informed of the pathogenetic and therapeutic aspects in immunologic diseases, must have in mind that the use of PMA for IL-1 induction might cause incorrect interpretations of the obtained results.

Interleukin-1 (IL-1), perhaps the most fundamental factor in the immunological response, is a soluble, species-unrestricted-factor produced by mononuclear phagocytes (2). Among the various activities of IL-1 is activation and proliferation of T-lymphocytes. Phorbol myristate acetate (PMA), a croton oil-derived tumor promoter, has been one of many activators used *in vitro* to produce IL-1 from monocytes (M ϕ) of various origins. Thus, PMA-induced IL-1 has been widely used in basic immunological and immunopharmacological experiments, either as crude supernatants from M ϕ -cultures or, after partial purification, as high molecular (70–80 kilodalton (KD)) or low molecular weight (12–16 KD) IL-1 (1, 6, 9, 10, 15). However, in none of these studies was it possible to exclude that the apparently increased IL-1 activity of PMA-induced M ϕ supernatants was partly due to carry over of PMA to the test assay.

Experiments of our own demonstrated that the co-mitogenic effect of PMA-induced supernatants on mitogen-stimulated proliferation of human T-cells, indicating IL-1 content, was probably due to PMA. Thus, a solution of PMA in culture medium was as effective as the PMA-induced supernatant with respect to co-mitogenic effect. These results were in agreement with the results of other investigators (4, 5, 18). As a consequence of our results and in order to correctly interpret previously reported results with PMA-induced IL-1, the following questions were raised: does PMA appear in high or in low molecular weight fractions after sieve chromatography of supernatants from PMA-stimulated M ϕ cultures, and does it interfere with the test assay and thereby with the detection of IL-1? If so, is it possible to eliminate PMA from the supernatants?

MATERIAL AND METHODS

Culture medium

RPMI-1640 with 25 mM Hepes (Gibco, Paisley, Scotland) was supplemented with 0.8 μ mol/ml L-glutamin (Gibco), 500 IU/ml penicillin and 500 μ g/ml streptomycin (Gibco).

Preparation of cell suspensions

The preparation of peripheral blood mononuclear cells (MNC), monocytes (M ϕ) and monocyte-depleted mononuclear cells (MDC) has been described in detail previously (3). In brief: MNC from a Lymphoprep[®]-gradient (Nygaard, Oslo, Norway) were suspended in culture medium with 15 % pooled normal human serum (NHS) and plated for 90 min at 37°C on plastic petri dishes. The non-adherent cells were decanted from the dishes, incubated for 30 min at 37°C on nylon-wool columns and finally eluted with 37°C culture medium with 15 % NHS. These MDC typically contained 77 % $\pm$ 11 E-rosetting positive cells, 8 % $\pm$ 6 surface membrane Ig positive cells and 0.2 % $\pm$ 0.2 esterase positive cells (3). The petri dish adherent cells constituted the M ϕ . These cells were cultured in medium with 1 % NHS and stimulated on the dishes, or they were detached from the dishes with 10⁻² M EDTA in phosphate buffered saline (PBS), pH 7.4, at 4°C and cultured in polystyrene tubes (Nunc, Roskilde, Denmark) in appropriate concentrations. These cells typically contained 78 % $\pm$ 9 esterase positive cells, 15 % $\pm$ 13 E-rosetting positive cells and 8 % $\pm$ 4 surface membrane Ig positive cells.

Production of M ϕ supernatants

M ϕ , 0.3 $\times$ 10⁶ cells/ml, if not otherwise stated, were stimulated either with 2 $\times$ 10⁻⁶ M PMA or with NaCl 0.9 % and cultured for 24 h as previously described (12). In some experiments cultures were performed either with heat-inactivated M ϕ , 56°C for 30 min (3), or without cells. After centrifugation (600 *g* for 10 min) supernatants were decanted and tested as crude supernatants or dialyzed at 4°C against 25 mM NH₄HCO₃, pH 7.2 (Dispensary, Rigshospitalet, Copenhagen, Denmark), volume $\times$ 100, with 2 changes every 24 h. Pore size of the dialysis membrane was 15 Å (Union Carbide Corporation, Chicago, Ill., USA).

Sephadex gel filtration chromatography

Dialyzed supernatants were lyophilized, reconstituted in the NH₄HCO₃-buffer to 1/7 of the

original volume. Samples of 4 ml were applied to a 1.5×90 cm Sephadex G-100 column (Pharmacia, Uppsala, Sweden), and the samples were eluted with the same buffer at $4 \pm C$ at a rate of approx. 8 ml/h. Fractions of 4 ml were lyophilized, reconstituted in Hank's balanced salt solution (HBSS) (Dispensary, Rigshospitalet) to an appropriate concentration and tested for IL-1 activity, for absorbance at 280 nm, and for content of ^3H -PMA. The column was calibrated with Blue Dextran, Bovine serum albumin and Cytochrome C (Sigma, St. Louis, Mo, USA).

Interleukin-1 assay

The content of IL-1 in supernatants was determined by the co-mitogenic effect on a phytohemagglutinin (PHA) (PHA-P, Difco, Detroit, Mich., USA) stimulated, $3 \mu\text{g}/\text{ml}$, blast transformation of MDC, 1×10^5 cells/ $500 \mu\text{l}$. After 72 h of culture the incorporation of ^{14}C -thymidine (New England Nuclear, Dreieich, W. Germany) was measured by a liquid scintillation counter as previously described (3). The results are given in counts per minute (CPM) as medians of triplicate determinations, or as ΔCPM (CPM of MDC-cultures with supernatant - CPM of MDC-cultures with medium). For comparison, MNC, 1×10^5 cells/ $500 \mu\text{l}$, were stimulated with PHA $3 \mu\text{g}/\text{ml}$ and cultured for 72 h.

Quantitation of the PMA content

^3H -PMA (New England Nuclear) was dissolved in toluene/ethanol. The solvent was evaporated

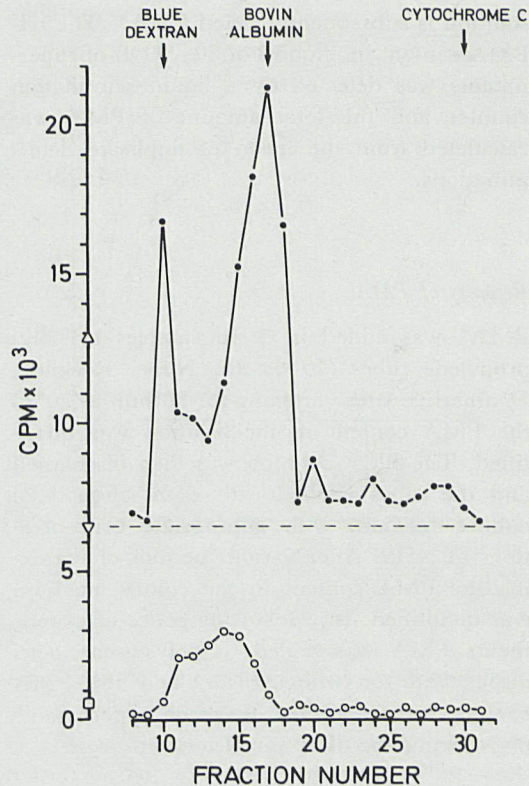


Fig. 1. The mitogenic (○—○) and co-mitogenic (●—●) effect on PHA-stimulated MDC of fractions from Sephadex G-100 chromatography of PMA-induced Mø supernatant compared to PHA-stimulated proliferation of MNC (△) and MDC (▽) and NaCl-stimulated MDC (□). One representative experiment, ($n = 3$).

under streaming air, and the ^3H -PMA residue was redissolved in culture medium with 1 % NHS and 2×10^{-6} M unlabeled PMA resulting in a final solution of 0.05 Ci/mmol. This PMA

Table 1

Effect on PHA-stimulated MDC proliferation of supernatants from cultures of intact or heat-inactivated Mø, stimulated with PMA. Supernatants were tested in 2 % (v/v), results given in CPM

Mø con- centration	$3 \times 10^5/\text{ml}$		$3 \times 10^4/\text{ml}$		$3 \times 10^3/\text{ml}$		$3 \times 10^2/\text{ml}$		Cell-free cultures	MNC-pro- liferation
	Mø	Mø ⁵⁶	Mø	Mø ⁵⁶	Mø	Mø ⁵⁶	Mø	Mø ⁵⁶		
Exp. 1	13794	13131	—	11591	10908	9816	11992	10695	13504	4025
Exp. 2	17251	17381	17571	15113	16765	16171	19270	13215	18342	5135
Exp. 3	13273	13841	13415	13003	—	13504	13003	13641	8197	7109
Exp. 4	10346	—	10010	—	9845	—	8599	—	4263	15709

Abbreviations: PHA: phytohemagglutinin; MDC: monocyte-depleted mononuclear cells; Mø: monocytes; Mø⁵⁶: Heat-inactivated monocytes; PMA: phorbol myristate acetate; CPM: counts per minute; MNC: mononuclear cells.

solution is subsequently called PMA*. The ^3H -PMA content in aliquots of 50–200 μl of supernatants was detected by a liquid scintillation counter and the total amount of PMA was calculated from the mean of duplicate determinations.

Recovery of PMA

PMA* was added in 1 ml samples to polypropylene tubes (10 $\times$ 15, Nunc, Roskilde, Denmark). After vortexing for 30 min at 20°C the PMA content in the solution was quantified. The PMA solution was then discharged and the tubes washed with 1 ml aliquots of culture medium with appropriate concentrations of NHS. After various periods of vortexing the PMA content in the culture medium was quantified. In a following series of experiments PMA was added to polystyrene petri dishes with or without M ϕ , and the PMA content was quantified in crude supernatants and during the IL-1 purification process.

Table 2

Recovery of PMA* in percent of initial PMA*, 2×10^{-6} M in culture medium with 1% NHS, 0.05 Ci/mmol. Recovery was calculated after successive steps in purification of supernatants from M ϕ cultures (+M ϕ) or cell-free "cultures" (-M ϕ)

	Percent of initial PMA	
	+ M ϕ	-M ϕ
Incubation on petri-dishes		
1 h ¹	71 $\pm$ 7	82 $\pm$ 5
22 h ¹	65 $\pm$ 4	76 $\pm$ 5
Before dialysis ²	68 $\pm$ 3	74 $\pm$ 4
After dialysis ²	59 $\pm$ 2	69 $\pm$ 4
After lyophilization and resuspension ²	53 $\pm$ 2	58 $\pm$ 5
After chromatography		
total in fract. 9–23 ³	11 $\pm$ 2	10 $\pm$ 2
total in fract. 24–40 ³	< 0.85	< 0.85

Results from one experiment.

Mean $\pm$ SD ¹of single samples from 6 dishes, ²of triplicate determinations from one sack and ³of duplicate determinations on single fractions.

Abbreviations: PMA: phorbol myristate acetate; NHS: pooled normal human serum; M ϕ : monocytes.

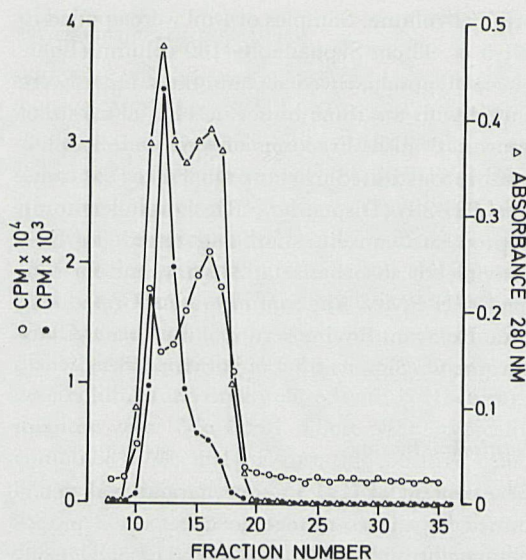


Fig. 2. Co-mitogenic effect on PHA-stimulated MDC (○), absorbance at 280 nm (Δ) and ^3H -PMA content (●) in fractions from Sephadex G-100 chromatography of "supernatant" from cell-free "culture" ($n = 1$).

RESULTS

Four supernatants from PMA-stimulated M ϕ cultures were tested on different MDC-suspensions. The supernatants had a minor mitogenic effect (data not shown), and in three of four experiments they raised the PHA-stimulated MDC-proliferation to a level far above the proliferation of MNC (Table 1). The effect was obtained regardless of the M ϕ concentration and heat inactivation of M ϕ .

By Sephadex G-100 chromatography of three PMA-induced M ϕ supernatants a mitogenic effect was eluted in fractions with molecular weights > 70 KD (bovine serum albumin) (Fig. 1). In fractions with proteins with a molecular weight equal to bovine serum albumin the mitogenic effect almost disappeared, whereas a synergistic co-mitogenic effect to PHA-stimulated MDC-proliferation showed up. In two of the three supernatants low molecular weight fractions had a minor co-mitogenic effect.

After Sephadex G-100 chromatography of supernatants from M ϕ cultures stimulated with PMA*, 10–11% of the PMA originally admitted to the cultures could still be recovered, primarily from the high molecular weight frac-

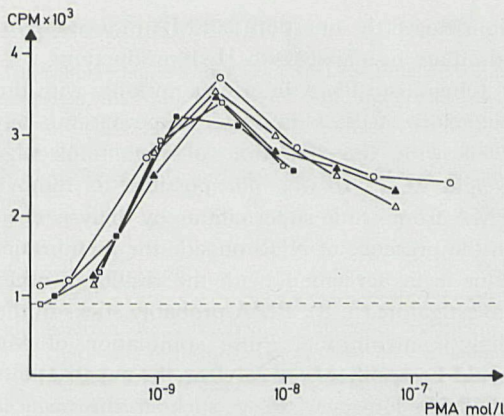


Fig. 3. Co-mitogenic effect on PHA-stimulated MDC of serial dilutions of 2×10^{-6} M PMA in culture medium with 1% NHS (O) and of fractions from Sephadex G-100 chromatography of supernatant from PMA-induced Mø culture (open symbols) or cell-free "culture" (closed symbols). Fractions with maximum PMA content (Δ , $\blacktriangle$) and fractions with maximum co-mitogenic effect ($\square$, $\blacksquare$). PHA-stimulated MDC (---), ($n = 1$).

tions (Table 2). Almost 80% of the PMA admitted to the column stuck to the Sephadex beads, etc. The column was cleared of PMA by eluting with medium + 20% NHS between the runs (data not shown).

By Sephadex G-100 chromatography of "supernatants" from cell-free "cultures", stimulated with PMA*, the synergistic effect was demonstrated in fractions with a molecular weight equal to bovine serum albumin (Fig. 2). This synergistic effect did not coincide with the PMA elution peak. Identical elution profiles were obtained with supernatants from PMA-stimulated Mø cultures (data not shown). From these two chromatographies, fractions with maximum content of PMA, as well as fractions with maximum synergistic effect, were retested in serial dilutions together with dilutions of PMA* (Fig. 3). The experiment demonstrated that the synergistic effect was correlated to and seemed to be dependent solely on the PMA content in the solutions. PMA at or below 1×10^{-9} M, had no mitogenic effect, and PMA concentrations at or below 1×10^{-10} M were without co-mitogenic effect.

When PMA* was added to polypropylene tubes, $20\% \pm 1.5$ of the PMA stuck to the tubes ($n = 8$). Of these 20% almost 30% was

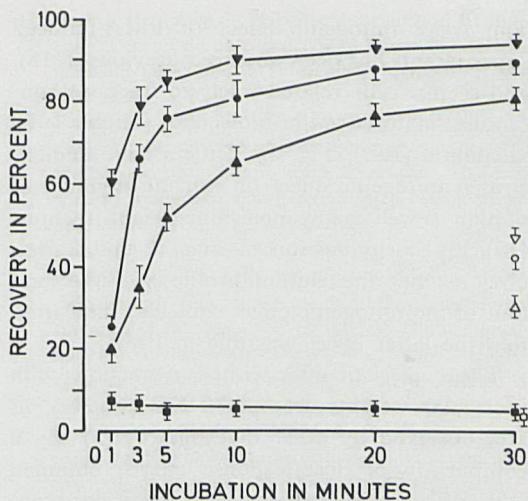


Fig. 4. Recovery of PMA stuck to the polypropylene tubes after washing, without (open symbols) or with (closed symbols) continuously vortexing, with culture medium without ($\square$, $\blacksquare$) or with 1% (Δ , $\blacktriangle$), 4% ($\circ$, $\bullet$) and 16% (∇ , $\blacktriangledown$) NHS. Mean $\pm$ SEM, ($n = 2$).

recovered by incubation with fresh medium + 1% NHS for 30 min without vortexing (Fig. 4). By vortexing for 30 min with medium + 16% NHS, 95% was recovered. Thus, still 1% of the originally admitted PMA stuck to the tube.

Dialysis of PMA* against medium for up to 48 h, at 5°C or 20°C , with or without 1% NHS resulted in almost 20% loss of PMA from the samples no matter the procedure. However, PMA could not be recovered in the medium outside the membrane (data not shown).

DISCUSSION

The results presented in Table 1 indicated that the synergistic effect of supernatants from PMA-stimulated Mø was caused by PMA. This highly effective co-mitogenic effect of PMA might hide the effect of IL-1, which was possibly released from the Mø. The presumption that the co-mitogenic effect was mainly due to PMA is in agreement with results of other investigators, who have demonstrated a synergistical co-mitogenic effect of PMA on guinea pig T-cells (14), murine thymocytes (5), human MDC (4) and human thymocytes (18).

The weak mitogenic effect of PMA-induced supernatants has been observed previously (16), and seems to be related solely to fractions containing material with molecular weight > 70 kilodalton (KD) (Fig. 1). Others have demonstrated mitogenic effect on murine thymocytes of high as well as low molecular weight fractions (15). By a comparison of Figs. 1 and 2 it is obvious that the elution profile of PMA and that of the mitogenic effect coincide, indicating that the latter effect was due to PMA.

The synergistic effect eluted in fractions with molecular weights around 70 KD (Fig. 1), as also observed by other investigators (5). By a comparison of dose-response curves obtained with PMA, with fractions with maximum content of PMA and with fractions with maximum synergistic effect, it is demonstrated that the synergistic effect in these PMA-containing solutions is well correlated to the PMA content (Fig. 3). A similar dose-response curve has been demonstrated with guinea pig T-cells (14) and with murine thymocytes (7, 15).

In two of the three chromatographed supernatants a co-mitogenic effect was observed in fractions with molecular weights around 12–16 KD. None of these fractions had a mitogenic effect (Fig. 1), nor detectable amounts of PMA (Fig. 2). The magnitude of this co-mitogenic effect is comparable to the effect of supernatants from apparently unstimulated Mø, and to highly purified IL-1 (3), as well as to the effect of supernatants from lipopolysaccharide-stimulated Mø cultures (12). Thus, this less pronounced co-mitogenic effect is probably due to IL-1. As culture medium often contains endotoxins, i.e. lipopolysaccharide from Gram-negative bacteria, in concentrations active in biological assays (11, 12, 13), it cannot be excluded that the variable presence of a co-mitogenic effect in low molecular weight fractions might be due to a variable degree of endotoxin contamination of the Mø cultures and not to the presence of PMA. A further indication that PMA probably do not induce Mø to IL-1 production, is the observation that neither high nor low molecular weight fractions of PMA-induced supernatants contained endogen pyrogen (17), which is supposed to be identical to IL-1. These observa-

tions raised the question: is PMA in fact able to stimulate human Mø to IL-1 production?

Obviously, PMA interferes not only with the detection of IL-1 in crude supernatants but even with the detection of high molecular weight IL-1. It was not possible to remove PMA from crude supernatants by dialysis even in the presence of NHS outside the membrane. This is in agreement with the results of other investigators (5, 8). PMA probably sticks to the dialysis membrane. Pulse stimulation of Mø could be another way to clear the supernatants of PMA. However, PMA sticks to the surfaces of polystyrene (Table 2) and polypropylene (Fig. 4). Thus, we have calculated that even after vortexing twice with medium + 16 % NHS for 30 min, the surface of a polypropylene tube still contains PMA in sufficient amounts to result in a concentration of 8×10^{-10} M in medium + 1 % NHS within a few hours. This concentration is sufficiently high to cause a synergistically co-mitogenic effect on PHA-stimulated MDC-proliferation as demonstrated in Fig. 3 and by other investigators (7). But, even if one could clear the tubes sufficiently of PMA, it has been shown that PMA is released from the intracellular compartment during the Mø culture period, causing subsequent contamination of the supernatants (17).

In conclusion: PMA supernatants act mitogenically on human MDC and synergistically co-mitogenically on PHA-stimulated MDC proliferation. This interference with IL-1 detection seems to be due to PMA coupled to serum components with molecular weights ≥ 70 KD. Thus, previous reports concerning basic immunological as well as immunopharmacological aspects, using crude or high molecular weight preparations of PMA-induced IL-1 supernatants, must be interpreted with caution. It could be that PMA is usable as an IL-1 inducer. However, firstly, it seems extremely difficult to remove PMA sufficiently from the Mø supernatants and thereby to avoid PMA-interference in a human IL-1 assay based on PHA-stimulated MDC-proliferation. Secondly, the specificity of PMA with respect to IL-1-inducing effect must be demonstrated under endotoxin-free culture conditions.

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