tophores darken with age[8,9], the black ones developing from brown and the red ones from orange, etc., though we also know, on physiological grounds, that the different members of the coloured series are separately innervated and make distinct contributions to patterning at any one moment[10,11].

1 This work was prepared while A.P. was on study leave in Germany supported by the Deutscher Akademischer Austauschdienst.

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Studies on the activity of phorbol myrystate acetate on the human polymorphonuclear leukocytes

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Summary. Phorbol myrystate acetate (PMA) activates nitroblue tetrazolium reduction in human polymorphs. The activation is inhibited by dibutyryl cyclic AMP, theophylline and phenylbutazone, but is not influenced by hydrocortisone in vitro, nor is it inhibited by leukocytes from patients treated with prednisone. Peptide analogues of Tuftsin also had no effect on this stimulatory activity. We conclude that the action of PMA on the nitroblue tetrazolium reduction is mediated through cyclic nucleotides.

Recently it was shown that phorbol myrystate acetate (PMA), the active substance of croton oil, when added to polymorphonuclear (PMN) leukocytes stimulates events occurring during phagocytosis of particles, i.e. the increase of oxygen consumption, hexose monophosphate shunt (MMP) activity and reduction of nitrous blue tetrazolium (NBT)[1,2]. The mechanism of action of PMA has not yet been explained, but there are some very important observations[1] prompting further studies.

We have previously reported that dibutyryl cyclic AMP and related substances decrease the NBT reduction by PMN leukocytes, while cGMP and related substances increase this reaction[3]. We have also demonstrated that when PMN leukocytes are preincubated with tuftsin (phagocytosis-stimulating peptide) analogs, the ability of the cells to reduce NBT is inhibited[4]. These findings and the importance of the model of PMA-stimulated intracellular changes led us to investigate the influence of cAMP, theophylline, cGMP, tuftsin and its analogs, hydrocortisone and phenylbutazone on the stimulatory effect of PMA in the system of quantitative NBT reduction test[5].

Material and methods. Samples of 20 ml venous blood from healthy young medical students were collected in 30 ml disposable plastic tubes containing 50 units heparin (thromboliquin) per 1 ml. 5 ml of 6% dextran solution (Fluka AG) were added to each tube and the mixture was allowed to settle for 1 h at room temperature.

The next steps were essentially as described by Baehner and Mathan[5] with the following changes: To each test tube containing 2.5×10^6 leukocytes in 0.1 ml, we added the following substances (instead of latex particles):

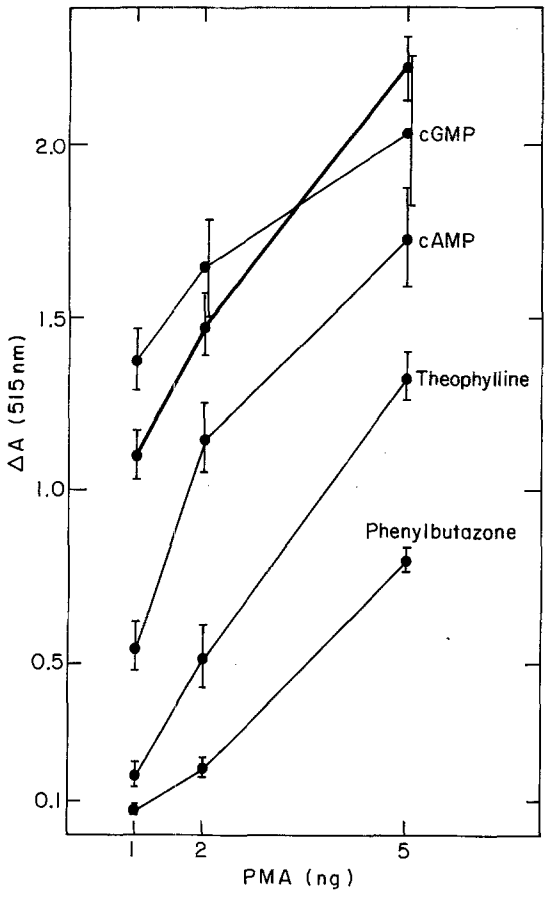
1. 20 µg endotoxin (B_4 lipopolysacharride, Difco, Detroit);
2. 1, 2, 5 ng phorbol myrystate acetate (12-O-tetradecanoyl phorbol-13-acetate, Cons. Midland Corp. Brewster N.Y.;
3. 1 µg or 10 µg of tuftsin (phagocytosis stimulating peptide L-Thr-L-Lys-L-Prol-Arg);
4. tuftsin analogs: Des^4-threonyl-tuftsin 0.1 µg - alanyl^4 tuftsin 0.1 µg (synthesized by us);
5. methylene blue $0.22 \times 10 \times 10^{-3}$ M;
6. hydrocortisone 100 ng, 200 ng, 500 ng;
7. theophylline (Sigma) 10^{-5} M;
8. carbacholamine 10^{-5} M (Sigma);
9. cAMP dibutyryl adenosine 3′5′cyclic monophosphoric acid 5×10^{-6} M (Sigma), cGMP Guanosine 3′5′cyclic monophosphoric acid (Sigma) 3×10^{-3} M;
10. phenylbutazone (Sigma) 5×10^{-3} M.

In addition, we studied the effect of PMA and endotoxin on PMN leukocytes obtained from 4 children treated by prednisone 2.5 mg/kg for 10 days at least (rheumatic fever (2), dermatomyositis (1) and nephrotic syndrome (1). After addition of 0.2 ml of 0.1% NBT (sigma), the mixtures were incubated for 30 min at 37 °C. The reaction was stopped by addition of 10 ml 0.5 HCl and centrifuged. The reduced NBT was extracted from the cells once in 3 ml pyridine (BDH) under a boiling water bath. Then the colour intensity was measured spectrophotometrically (Zeiss PM20DL) at 515 nm.

In the 2nd experiment we preincubated the leukocytes (handled as in the 1st experiment) separately with the following substances: Des-Thr tuftsin, Ala Tu, phenylbutazone, theophylline, dibutynyl cAMP, cGMP, carbachol, cAMP′-theophylline, carbachol′-cGMP, in the same concentrations as in the 1st experiment; then to each test-tube we added endotoxin or phorbol myrystate acetate and NBT suspension and continued as previously described.

The results of each test were expressed as ΔA per 2.5×10^6 leukocytes per 30 min, calculated as the difference in optical density between each reaction tube ('stimulated') and a blank.

Results. Figure 1 summarizes the principal data obtained from our experiments. PMA has a marked stimulatory



The effect of phorbol myrystate acetate (PMA) on NBT reduction by polymorphonuclear leukocytes. The influence on PMA action is related to a constant concentration of phenylbutazone, theophylline, cAMP and cGMP in all the 3 concentrations of PMA (The dark line expresses PMA alone). ΔA is a difference between the optic density of 'stimulated' cells and blank 'resting' (mean±SEM).

effect on the NBT reduction by human PMN leukocytes. This effect is stronger than that of endotoxin and similar to that of methylene blue[4] and is dose-dependent up to 5 ng. At higher amounts (7.5 ng, 10 ng, 20 ng) a plateau is reached (ΔA 2.31'-0.06, 2.27'-0.09, 2.24'-0.07 respectively). When the leukocytes are incubated with theophylline, cAMP (or with both) before exposure to PMA and then PMA added, the stimulatory effect is reduced. Similar results, even more prominent, are obtained when phenylbutazone is added to the reaction. The inhibitory effect of these substances is shown in all three concentrations of PMA.

On the other hand, cGMP, carbachol (or both) have a mild additive action with the lower concentration of PMA but not a significant effect at higher concentrations. Tuftsin analogs are without any effect in all 3 concentrations of PMA (ΔA: 1.09, 1.45, 2.30). Hydrocortisone, while demonstrating a clear inhibitory effect on the stimulatory action of endotoxin[6], has no effect on the action of PMA (ΔA similar as for PMA alone). This is demonstrated when hydrocortisone is added in vitro or when the donor of leukocytes is treated with steroids in therapeutic doses.

Discussion. It was previously shown that phorbol myrystate acetate in µg quantities stimulates the oxidative metabolism of normal human polymorphonuclear leukocytes[1,2]. It increases the glucose utilization via the HMP shunt[1] oxygen consumption[2] and enhances the NBT reduction[2]. PMA fails to act on leukocytes from patients with chronic granulomatous disease[7] and its action is expressed differently in carriers of this disease[8].

We have shown that PMA is a potent stimulator of NBT reduction even in ng amounts in the quantitative system. Its stimulatory effect is stronger than that of endotoxin and tuftsin, and of the same degree as methylene blue[3,4]. While methylene blue acts on both living and dead cells, PMA acts on living cells only[1].

While the effect of endotoxin and tuftsin can be blocked by tuftsin analogs[4], the effect of PMA, like that of methylene blue cannot be blocked by these peptides. These findings indicate 2 different mechanisms for the PMN leukocytes activation.

Discussing the mode of action of PMA, De Chatelet et al.[1] raised the possibility that it stimulated the NASPH oxidase or NADH oxidase. It seems that the fact that preincubations of leukocytes with various concentrations of hydrocortisone, a known inhibitor of NADH oxidase activity[9], has no effect on the PMA action indicates that the action of PMA is not via the NADH oxidase.

It was previously shown that PMA can raise the intracellular cGMP concentrations in a variety of cell types[10,11]. We have previously demonstrated that cGMP or carbocholamine (which increases its intracellular concentration) enhances the reducing ability of NBT by the human PMN leukocytes, while cAMP or substances which increase its intracellular concentration decrease this ability.

Our findings that cAMP and/or theophylline reduce markedly the influence of PMA on the cell, while cGMP has a mild additive action, suggested that like in other systems, PMA acts in our system also through the cyclic nucleotides. It is possible that PMA reduces the concentration of cAMP in the cell and elevates that of cGMP. Phenylbutazone, which decreases G6PD and HMP shunt activity and is able to produce bactericidal defects in PMN leukocytes[12] inhibits the action of PMA. The finding that most of the pharmacological agents, which have been found to inhibit the influence of PMA, inhibit also the events during phagocytosis of a particular matter, can be a further confirmation that PMA action may contribute to the understanding of the biochemical events during phagocytosis.

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