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Effects of Podophyllum peltatum compounds in various preparations and dilutions on human neutrophil functions in vitro

S. CHIRUMBOLO,* A. CONFORTI,** S. LUSSIGNOLI,* H. METELMANN,***
P. BELLAVITE*

Abstract

Human blood neutrophil granulocytes (neutrophils) treated with *Podophyllum peltatum* L.-derived compounds exhibited an enhanced oxidative response to subsequent challenge with bacterial formyl peptides. This priming effect was concerned with superoxide anion (O_2^-) release (respiratory burst). The phenomenon was observed with a potentized preparation containing, among other things, *podophyllum* extract (*Podophyllum compositum*), with *Podophyllum* 4x (final concentration of active principle about 0.025 µg/ml), whereas enhancement of O_2^- release was not caused by homoeopathic *Podophyllum* 12x or other components of the complex homoeopathic preparation. Purified podophyllotoxin had the same effect at doses of 0.1–10 µg/ml, whereas doses higher than 100 µg/ml of podophyllotoxin inhibited the respiratory burst, so that pure toxin showed a typical bi-phasic dose–response curve. Similar effects were obtained with purified colchicine (1–1000 µg/ml), a microtubule-disrupting agent. No priming by a *Podophyllum*-derived compound was observed on neutrophils stimulated with 50 ng/ml phorbol ester. Further, both potentized *podophyllum*-derived compounds and pure podophyllotoxin-inhibited cellular adhesion to the serum-coated surface of culture microplates. These results show that low potencies of a drug extract have specific stimulating effects on the activation of neutrophil metabolism. The same stimulating effects are also caused by low doses of the active principle of the drug, which is an inhibitor when used at high doses.

KEYWORDS: Leukocyte function; Superoxide anion; Adhesion; *Podophyllum peltatum* L.; Podophyllotoxin; Colchicine; Hormesis.

Introduction

Podophyllum belongs to a series of purgative drugs derived from the Berberidaceae (*Podophyllum peltatum* L.). Podophyllin, the raw extract containing the active principle podophyllotoxin, is drawn from the rhizome. *Podophyllum* has proved an irritant on patch testing[1] and a mixture of natural and semisynthetic (modified) lignan from *Podophyllum emodi* (proreside) has been used for many years in the treatment of rheumatoid arthritis, but its use was hampered by gastrointestinal side-effects.[2] In addition *podophyllum* and podophyllotoxin have been used in dermatological conditions such as condylomata acuminata.[3, 4] Podophyllotoxin and its chemical derivatives like epipodophyllotoxins (teniposide) affect cell division. As they interact with microtubules or with associated protein or modify tubulin GTP binding and also affect DNA, they have been widely investigated as anti-cancer drugs.[5, 6] The action of *podophyllum* derivatives on leukocytes has been studied principally as regards the effect of its main component, the podophyllotoxin and its derivatives. Its action is to inhibit lymphocyte proliferation,[7] probably because it affects microtubule dynamics[8] and DNA topoisomerases.[9] Podophyllotoxin molecular action could be ascribed to its chemical resemblance to colchicine and vinca alkaloids. These compounds have a significant effect on microtubule function.

*Institute of Clinical Chemistry and Microscopy, University of Verona; **Institute of Pharmacology, University of Verona; ***Biologische Heilmittel Heel GmbH, Baden Baden.

Podophyllum peltatum L. extracts (Bajiaolian) have been widely used in Chinese herbal medicine[10] and in homoeopathy for many symptoms related to dysentery, gastroenteritis, proctitis, fevers and bronchitis.[11, 12] Clarke[11] noted the irritant and inflammatory effects of *podophyllum*, with external application to the skin producing an intertrigo-like rawness; the dust of the powdered root getting into the eyes causes intense inflammation, ulceration and leukoma. Fevers of many kinds are treated with *podophyllum*.

Special interest in the drug arises from the fact that it is currently used in both conventional medicine (mainly for its anti-mitotic properties) and in various potencies in homoeopathy (on a number of indications, including regulation of inflammatory processes). The present study was designed to investigate the effect of potentized preparations of *podophyllum* and its main active principle on a sensitive cellular model in our laboratory, exploring the metabolic and adhesion functions of human neutrophils exposed to drugs and stimulants.[13, 14] At present there is no evidence that *Podophyllum peltatum* L. derivatives act on phagocytes (neutrophils, monocytes, macrophages), important cell types involved in the first line of immunological surveillance (natural immunity) and in inflammatory reactions.

The functional parameters explored in this study are superoxide (O_2^-) release and adhesion to serum-coated plastic surfaces. Superoxide is the fundamental product of oxidative metabolism in all phagocytes when their membrane interacts with specific particulate (e.g. opsonized bacteria and yeast) or soluble (e.g. formyl peptides, phorbol esters, lectins) agents. Adhesion is a recently re-evaluated function of leukocytes that allows them to migrate outside the blood vessels, spread onto various surfaces and adhere to connective tissue structures. Measurement of these functional responses to various agonists in the absence and presence of drug compounds evaluates the impact drugs may have at cell level on a broad range of targets like membrane receptors, metabolic enzymes, anchoring proteins, signal transduction pathways and cytoskeleton structures.

To assess the effect of *podophyllum* compounds on these functions under various conditions of cell responsiveness, we also carried out a series of assays on neutrophils pre-treated with tumour necrosis factor (TNF). This cytokine induces a pre-activated state (priming) *in vitro*, similar to the modifications occurring in diseases like endotoxaemia, severe shock, terminal cancer, etc.

We studied drug preparations made in physiological saline and thus fully compatible with cell viability. All the drug preparations were compared with matched control assays where the cells were incubated with the same physiological saline. As a first empirical approach, we looked for possible effects of commercially available complex homoeopathic (compound) preparations based on *Podophyllum* extracts. Then the effects of single components present in the same extract were investigated to identify possible active components and the dilutions at which the effect was present. Finally, various doses of pure podophyllotoxin were tested on the described leukocyte functions.

As some pharmacological actions of podophyllotoxin on cell division are also exhibited by other alkaloids,[15] we used the same model system to test the effects of colchicine, an alkaloid of *Colchicum autumnale,* that is known to bind to microtubular proteins and cause the disappearance of fibrillar microtubules in granulocytes and other motile cells.[15]

Materials and methods

Reagents

Podophyllotoxin, colchicine, formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP, a synthetic bacterial tripeptide), phorbol-13 myristate-12 acetate (PMA, a phorbol ester), the cytokine tumour necrosis factor-alpha (TNF-α) were purchased from Sigma, St Louis, Mo, USA. Fe^{3+} cytochrome c was purchased from Boehringer, Mannheim, Germany, foetal bovine serum (FBS) and 96 flat bottom well microtitre plates from ICN Flow, Costa Mesa, Ca, USA, Percoll from Pharmacia, Uppsala, Sweden. Hank's balanced salt solution (HBSS) without calcium and magnesium was purchased from Gibco Ltd, Paisley, Scotland, human serum albumin (HSA) from Behring Institute, Marburg, Germany. Other materials and reagents were of the highest purity available. To avoid contamination which is a possible cause of artefacts or priming of neutrophils, sterile solutions and disposable plastic ware were used throughout, with experiments carried

out, whenever possible, under a laminar flow hood. Reagents were prepared and/or diluted using pyrogen-free water or 0.9% sodium chloride solutions.

Drugs
Preparations were made by Biologische Heilmittel Heel, GmbH, Baden Baden, Germany, according to the *German Homoeopathic Pharmacopoeia*[16] (GHP 1, method 3a), from the fresh rhizome of *Podophyllum peltatum* L. Starting from the crude extract (mother tincture), a series of 1:10 dilutions (indicated by x) in sterile physiological saline (0.9% NaCl), followed by vigorous succussion, were prepared and provided in 2.2 ml glass vials. The plant material content of the mother tincture was 1/3, and had a density of 0.89 g/ml. The podophyllotoxin content was determined by HPLC analysis, using dichloromethane/methanol 98 + 2 as a solvent. It was 329 ± 6 µg/ml (n = 3) in the mother tincture and 0.1 µg/ml in *Podophyllum* 4x. Considering the dilution of the drug to produce the assay mixture (see below), the concentration of podophyllotoxin in the leukocyte suspensions using *Podophyllum compositum* and *Podophyllum* 4x was approximately 0.025 µg/ml.

Podophyllum compositum was prepared by adding 22 µl of the following decimal dilutions in a final volume of 2.2 ml of physiological saline: *Podophyllum* 2x, *Podophyllum* 10x, *Ignatia* 3x, *Acidum muriaticum* 4x, *Acidum muriaticum* 10x, *Mercurius sublimatus corrosivus* 8x. According to these dilution procedures, the final doses in the *Podophyllum compositum* preparation corresponded to those contained in the following dilutions: *Podophyllum* 4x, *Podophyllum* 12x, *Ignatia* 5x, *Acidum muriaticum* 6x, *Acidum muriaticum* 12x, *Mercurius sublimatus corrosivus* 10x. These doses were used to test the effect of individual components. Heel also provided the physiological saline for the dilution of drugs; the same physiological saline was used in parallel assays as control.

Solutions of pure podophyllotoxin and colchicine obtained from Sigma were made in our laboratory by making serial 1:10 dilutions in sterile physiological saline (0.5 ml of the more concentrated solution plus 4.5 ml diluent), followed by vigorous succussion (6 seconds at 1,500 strokes/min using a Dyna HV1 dynamizator). All drug preparations were stored at +4 °C and were brought to room temperature at least one hour before the experiment. Pre-incubation and assays were done at 37 °C.

Preparation of neutrophils
Human neutrophils were prepared by centrifugation over 62/73% discontinuous Percoll gradients, using ethylene diamine tetra-acetate anticoagulated blood from healthy donors or otherwise from buffy coats rich in leukocytes prepared by centrifugation of citrate anticoagulated whole blood.[13,17]

Cells were finally suspended at a concentration of 2.67 x 10^6 cells/ml in HBSS supplemented with 0.2% human serum albumin (buffer H-GA). Cell recovery was > 95% neutrophils and > 99% viable cells as judged by the trypan blue exclusion test. 10 minutes before starting the assay, cell suspensions were supplemented with 0.5 mmol/l $CaCl_2$ and 1 mmol/l $MgCl_2$ (buffer H-ACGM) and kept at 37 °C. Whenever indicated, cells were pretreated with 1 ng/ml TNF-α for 15 minutes at 37 °C.

Assay of superoxide release and adhesion
Tests were performed in microtitre plates previously coated with inactivated bovine serum and washed.[13] The assays were done in a final volume of 150 µl, in triplicate for each experimental condition as follows. Human neutrophils (75 µl/microwell) were incubated at 37 °C for 15 minutes (dose–response assays) or for various times (time evolution assay) with 25 µl/microwell of the test compound or with physiological saline (test compound solvent), as control. Soon after the pre-incubation period, 50 µl of an H-ACGM solution containing the stimulant and 450 µmol/l of Fe^{3+}cytochrome c were added to each microwell. Where indicated, stimulation was done with 5 x 10^{-7} M (final concentration) of fMLP or 50 ng/ml (final concentration) of PMA. Incubation at 37 °C was maintained for the indicated time (10–60 minutes). During this time superoxide anion (O_2^-) release was measured by superoxide dismutase-inhibitable reduction of ferricytochrome c. Plates were transferred rapidly into a microplate reader (Reader 400, SLT Labs Instruments) and the reduction of cytochrome c was measured at 550 nm using 540 nm as reference wavelength and 0.037

optical density units as the quenching coefficient for 1 nmol of reduced cytochrome c.

For adhesion evaluation the microplates were transferred to an automatic washer (Easy Washer 2, SLT Labs Instruments) and subjected to 2 washing cycles with PBS Dulbecco at room temperature. The incubation time for the adhesion assay was 40 minutes, unless indicated otherwise. Cells adhering to the microwell bottom were quantified by measuring membrane enzyme acid phosphatase, using 4-nitrophenylphosphoric acid as substrate, with the percentage of adhesion calculated by referring to a standard curve obtained with known numbers of neutrophils from the same subject.

Results

Table 1 summarizes the effect of *Podophyllum compositum* on human neutrophil O_2^- release. *In vitro* pre-treatment of human neutrophils at 37 °C for 15 minutes with *Podophyllum compositum* alone did not affect O_2^- release of resting (i.e. non-stimulated) cells, either in untreated cells (Table 1a) and TNF-α treated cells (primed neutrophils) (Table 1b). Otherwise, when human granulocyte neutrophils were stimulated by the chemotactic peptide fMLP we observed that pre-treatment with *Podophyllum compositum* increased O_2^- release by stimulated neutrophils relative to the control (cells pre-treated with physiological saline) by about 70% (8.47 versus 4.99 nmol O_2^-). The increase was comparable to that caused by TNF-α pre-treatment in the absence of *Podophyllum compositum* (8.61 versus 4.99 nmol O_2^-, see Table 1). *Podophyllum compositum* caused a small, but significant further increase of superoxide release also in TNF-α pre-treated cells (10.27 versus 8.61 nmol O_2^-, see Table 1b).

Table 1 also shows that following stimulation with phorbol esters (PMA), the increase due to *Podophyllum compositum* was minimal and not significant in normal cells (Table 1a) as well as in TNF-α treated cells (Table 1b).

In a subsequent series of experiments, we tested the single components of the *Podophyllum compositum* preparation. Each component was added to the assay mixture in exactly the same dose as used in *Podophyllum compositum*. As may be seen from Table 2, no preparation was observed to have a direct effect on resting cells, whereas a priming effect on fMLP-stimulated cells was observed on pre-treating the cells with *Podophyllum* 4x and *Podophyllum compositum*. Other components caused minor, non-significant stimulation of fMLP-activated neutrophils, suggesting that most if not all of the effect of *Podophyllum compositum* in this assay system was due to *Podophyllum* 4x.

The extent of the priming phenomenon in

a) Normal cells

	Superoxide (nmol/10 min/10^6 cells)			
	No drug (control)	+ drug	% effect	paired t-test
Resting (n = 5)	0.20 ± 0.35	0.18 ± 0.22	– 10.0	n.s.
fMLP (n = 5)	4.99 ± 1.26	8.47 ± 1.77	+ 69.7	p < 0.01
PMA (n = 3)	23.00 ± 3.27	27.35 ± 7.97	+ 18.9	n.s.

b) TNF-treated cells

	Superoxide (nmol/10 min/10^6 cells)			
	No drug (control)	+ drug	% effect	paired t-test
Resting (n = 5)	1.28 ± 0.95	1.67 ± 1.5	+ 30	n.s.
fMLP (n = 5)	8.61 ± 1.83	10.27 ± 1.43	+ 19.2	p < 0.05
PMA (n = 3)	20.97 ± 1.48	24.14 ± 3.53	+ 15.1	n.s.

Values are mean ± SD of the indicated number of experiments. fMLP = formyl-L-methionyl-L-leucyl-L-phenylalanine (a synthetic bacterial tripeptide); PMA = phorbol-13-myristate-12-acetate (a phorbol ester).

TABLE 1. Effect of *Podophyllum compositum* on O_2^- release by human neutrophils.

	Superoxide (nmol/10 min/10^6 cells)			
	No drug (control)	+ drug	% effect	paired t-test
Resting cells				
Podophyllum comp	0.44 ± 0.07	0.31 ± 0.05	-	n.s.
Podophyllum 4x	0.07 ± 0.03	0.15 ± 0.04	-	n.s.
Podophyllum 12x	0.09 ± 0.04	0.09 ± 0.03	-	n.s.
Mercurius subl 10x	0.37 ± 0.09	0.33 ± 0.10	-	n.s.
Ignatia 5x	0.10 ± 0.06	0.18 ± 0.06	-	n.s.
Ac muriaticum 6x	0.06 ± 0.04	0.02 ± 0.01	-	n.s.
+ fMLP				
Podophyllum comp	3.58 ± 0.47	5.86 ± 0.75	+ 63.7	$p < 0.001$
Podophyllum 4x	3.54 ± 0.64	5.22 ± 0.73	+ 47.5	$p < 0.001$
Podophyllum 12x	3.30 ± 0.57	3.53 ± 0.55	+ 7.0	n.s.
Mercurius subl 10x	3.71 ± 0.47	3.43 ± 0.38	+ 7.6	n.s.
Ignatia 5x	3.21 ± 0.52	3.83 ± 0.63	+ 16.4	n.s.
Ac muriaticum 6x	3.14 ± 0.52	3.45 ± 0.39	+ 9.9	n.s.

Values are mean ± SEM of 9 separate experiments.

TABLE 2. Effect of *Podophyllum compositum* and its single components on O_2^- release by human neutrophils.

dependence on various pre-incubation times with *Podophyllum compositum* was evaluated (Figure 1). In the absence of the drug, the cell response to fMLP (solid circles) showed a slow decline on increasing the pre-incubation time. The increase in O_2^- response to fMLP caused by *Podophyllum compositum* (solid squares) was very rapid, appearing in the first 5 minutes of incubation. The primed state lasted for at least 45 minutes of pre-incubation time.

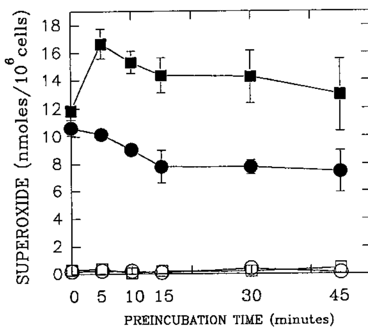

FIGURE 1. Effect of *Podophyllum compositum* on O_2^- release by human neutrophils as a function of pre-incubation time with the drug. Neutrophils were incubated for various time intervals with *Podophyllum compositum* (squares) or physiological saline as a control (circles) and stimulated with 5 x 10^{-7} M fMLP (solid symbols) or treated with saline (outline symbols) (see Methods). Values are mean ± SD of triplicates of a typical experiment out of 3 performed.

We then examined the effect of *Podophyllum peltatum* L. pure podophyllotoxin under the same test conditions. The toxin, like *Podophyllum compositum*, pre-activated (primed) human neutrophils to subsequent challenge with fMLP in the range of 0.1–10 µg/ml (240 nmol/l–24.1 µmol/l) (Figure 2a). The maximum priming dose corresponded to 1 µg/ml podophyllotoxin. High doses of the toxin (> 100 µg/ml) markedly inhibited O_2^- release. Human neutrophil priming by podophyllotoxin was not observed using PMA as stimulant (Figure 2b), thus confirming the data obtained with homoeopathic preparations.

Effects on adhesion

The action of *Podophyllum compositum* on cell adhesion to serum-coated microplates was also investigated. *In vitro* pre-treatment of human neutrophils at 37 °C for 15 minutes with *Podophyllum compositum* decreased the adhesiveness of human neutrophils on serum-

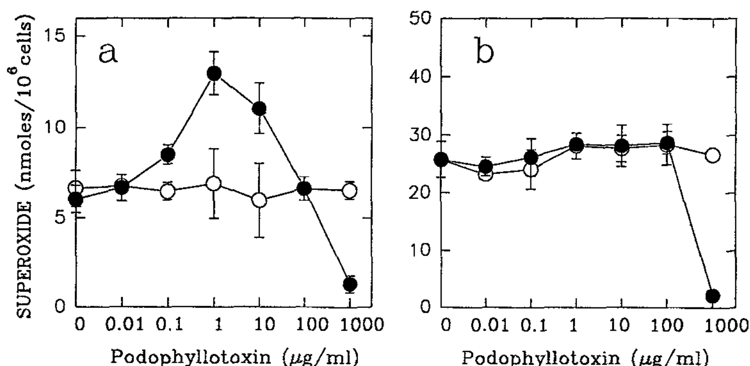

FIGURE 2. Effects of pure podophyllotoxin on O_2^- release by human neutrophils. Neutrophils were incubated for 15 min with the indicated doses of podophyllotoxin and stimulated with 5 x 10^{-7} M fMLP (a) or 50 ng/ml PMA (b). O_2^- released was evaluated after 10 min as described under Methods. Solid circles: + drug; outline circles: matched replicates of physiological saline (control). Values are mean ± SD of triplicates of a typical experiments out of 3 performed.

coated microplates by about 30%, both in the absence (resting) and in the presence of fMLP (Table 3a). This partial inhibition of cell adhesion was observed with fMLP but not PMA challenge, thus confirming the stimulus specificity of the effects. Inhibition of adhesion was observed with *Podophyllum* 4x but not with other components of *Podophyllum compositum* (Table 4) and was also confirmed with podophyllotoxin. The dose-dependence of the inhibitory effect of podophyllotoxin (Figure 3) showed that doses of 1–10 µg/ml, causing peak increase in oxidative metabolism (see Figure 2a), gave only slight inhibition of cell adhesion, whereas complete inhibition was obtained using high doses (100–1000 µg/ml).

Effects of colchicine

Figure 4 shows the dose-dependent curves for the effects of colchicine on the functions of fMLP-stimulated neutrophils. Low doses

a) Normal cells

	Adhesion (%)			
	No drug (control)	+ drug	% effect	paired t-test
Resting (n = 5)	4.96 ± 4.50	3.80 ± 2.70	– 22.4	n.s.
fMLP (n = 5)	29.28 ± 12.22	16.91 ± 6.54	– 42.2	p < 0.05
PMA (n = 3)	45.45 ± 4.46	41.65 ± 3.54	– 8.3	n.s.

b) TNF-treated cells

	Adhesion (%)			
	No drug (control)	+ drug	% effect	paired t-test
Resting (n = 5)	22.20 ± 8.43	17.11 ± 6.15	– 22.9	p < 0.05
fMLP (n = 5)	38.09 ± 10.03	31.61 ± 9.04	– 17.0	p < 0.01
PMA (n = 3)	34.29 ± 2.21	34.99 ± 2.37	+ 2.0	n.s.

Values are mean ± SD of the indicated number of experiments.

TABLE 3. Effect of *Podophyllum compositum* on adhesion of human neutrophils to serum-coated surfaces.

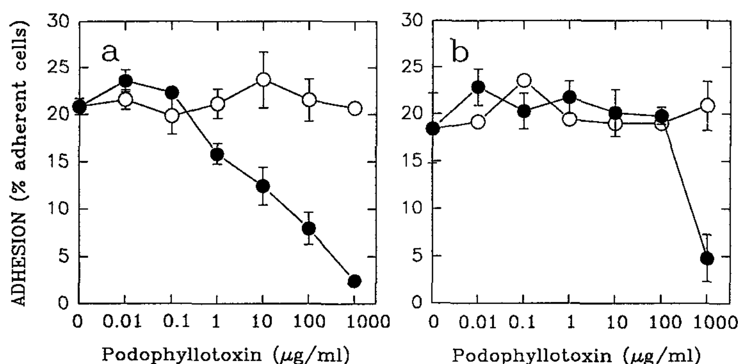

FIGURE 3. Effects of pure podophyllotoxin on adhesion of human neutrophils. Neutrophils were incubated for 15 min with the indicated doses of podophyllotoxin and stimulated with 5 x 10^{-7} M fMLP (a) or 50 ng/ml PMA (b). Solid circles = drug; outline circles = matched replicates of physiological saline (control). Values are mean ± SD of triplicates of a typical experiment out of 3 performed.

of this compound (1–10 μg/ml) enhanced fMLP-stimulated O_2^- release, whereas high doses (1000 μg/ml) were inhibitory (Figure 4a). Colchicine caused dose-dependent inhibition of cell adhesion (Figure 4b).

Discussion

We have described new regulatory effects of *Podophyllum peltatum* L. derivatives in an *in vitro* model system investigating various functional responses of neutrophils to agonist challenge. These compounds, used mostly for their cellular toxic and inhibitory properties in conventional medicine, have shown stimulatory effects on neutrophil oxidative metabolism where tested in low doses. *Podophyllum*

	Adhesion (%)			
	No drug (control)	+ drug	% effect	paired t-test
Resting cells				
Podophyllum comp	10.80 ± 2.54	6.92 ± 1.59	− 35.9	p < 0.001
Podophyllum 4x	11.08 ± 2.57	8.24 ± 1.99	− 25.6	p < 0.01
Podophyllum 12x	11.34 ± 2.81	11.57 ± 2.82	+ 2.0	n.s.
Mercurius subl 10x	11.13 ± 2.46	10.62 ± 2.24	− 4.6	n.s.
Ignatia 5x	10.37 ± 2.13	12.95 ± 2.15	− 24.9	n.s.
Ac muriaticum 6x	11.14 ± 2.49	12.05 ± 2.01	+ 8.1	n.s.
+ fMLP				
Podophyllum comp	40.17 ± 4.83	26.94 ± 3.66	− 32.9	p < 0.001
Podophyllum 4x	40.15 ± 3.96	26.56 ± 3.14	− 33.8	p < 0.001
Podophyllum 12x	43.70 ± 3.80	41.46 ± 4.05	− 5.1	n.s.
Mercurius subl 10x	40.78 ± 3.07	40.83 ± 3.47	+ 0.1	n.s.
Ignatia 5x	38.21 ± 2.91	40.28± 2.96	+ 5.4	n.s.
Ac muriaticum 6x	43.82 ± 3.19	39.40 ± 2.76	− 10.1	n.s.

Values are mean ± SEM of 9 separate experiments.

TABLE 4. Effect of *Podophyllum compositum* and its single components on adhesion of human neutrophils.

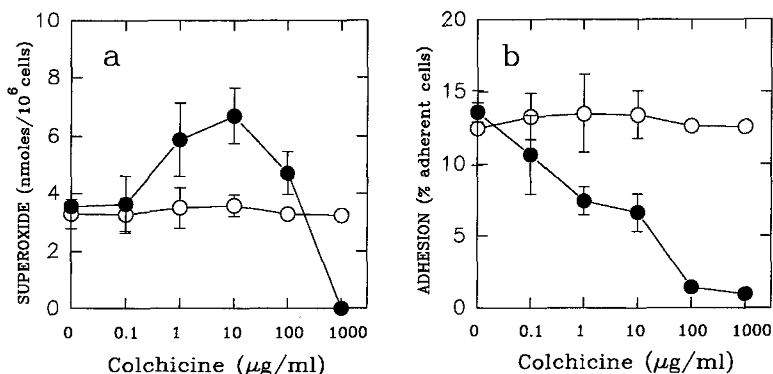

FIGURE 4. Effects of various doses of colchicine on O_2^- release by (4a) and adhesion of human neutrophils. Neutrophils were incubated for 15 min with the indicated doses of colchicine and stimulated with 5×10^{-7} M fMLP. See under Methods. Solid circles = drug; outline circles = matched replicates of physiological saline (control). Values are mean ± SD of triplicate determinations.

compounds did not exhibit direct agonist properties, i.e. they did not behave like bacterial peptides or phorbol esters. Instead, they enhanced the response to N-formylated peptides, an effect of the same extent of the cell priming caused by the cytokine TNF-α. Priming phenomenon induced by *Podophyllum compositum* was found to be accounted for by the action of *Podophyllum* 4x which is included in the *compositum* drug complex and could also be observed using low, sub-toxic doses of pure podophyllotoxin. No effect was obtained with *Podophyllum* 12x and with doses of pure podophyllotoxin below 0.1 μg/ml, indicating that this cellular model is not sensitive to very low doses of these compounds.

The potentiation of the fMLP-dependent O_2^- release by *Podophyllum compositum* and *Podophyllum* 4x occurred at nominal doses of podophyllotoxin which were much lower (0.025 μg/ml) than the doses of pure podophyllotoxin that caused the same effect (about 1 μg/ml). The difference between the 2 different preparations containing podophyllotoxin could have several reasons, including differences in raw materials, loss of activity during extraction and purification of the toxin, or the presence in crude extract and in active potentized preparations (*Podophyllum compositum* and *Podophyllum* 4x) of other compounds that synergize with podophyllotoxin. Further studies are needed to address these questions.

Our findings show that the same toxin causes enhancement of oxidative metabolism at low doses and inhibition at high doses. It is noteworthy that potentized dilutions of *Podophyllum* show only the priming effect typical of low doses of pure toxin on oxidative metabolism. This type of dual effect at different doses of agonists, antagonists or toxins has been described in various experimental systems and is often called 'hormesis'. Hormetic effects may have several explanations at the level of receptors, signal transduction mechanisms, enzyme regulation, gene expression, according to the test compound and the system involved.[18–23] The phenomenon could be regarded as an application of the homoeopathic 'similia principle' in biological systems exposed to specific experimental conditions.[24–26]

The mechanism of improved superoxide release is still unknown but it may be at least in part related to fMLP receptor up-regulation[27] and receptor mobilization and recycling on the cellular membrane, which is linked to cytoskeleton modifications and changes in cellular shape (shape change).[28, 29] Evidence has been reported that cytoskeletal modification can affect neutrophil membrane fluidity and membrane transport.[30] Given the close association of actin and microtubule with plasma membrane and trans-membrane location of free radical-generating NADPH oxidase cytoskeletal modifications may influence neutrophil membrane responses, such as superoxide release, affecting NADPH-oxidase complex assembly-disassembly.[31, 32] This hypothesis is in agreement with the fact that the effects of

podophyllotoxin are shared also by colchicine, a well-known agent that affects cellular microtubule dynamics in a variety of cells including granulocytes.[15]

We have shown that *podophyllum* compounds and colchicine inhibited neutrophil adhesion, and in our assay system this distinguished their effect from the priming effects of TNF-α, which did not inhibit but slightly stimulated leukocyte adhesion. It is likely that, as a microtubule disrupting agent, *podophyllum* derivatives could inhibit neutrophil adhesion on serum-coated microplates because cell flattening on the serum protein-coated microwell can imply microtubule polymerization. However, the effect on cell adhesiveness was only partial when potentized preparations or low doses of podophyllotoxin were used, and it was stimulant (fMLP)-specific, indicating that it was not due to cell toxicity but to fine regulation of this function. Adhesion is a very complex phenomenon involving a series of receptors and intracellular transduction controls (gating systems) that make this function particularly subject to fine regulation.[13, 23, 33, 34] In fact, in order to perform their functions and in particular chemotactic migration into tissue exudates, neutrophils have to adhere and detach alternatively from their anchoring surfaces, according to the different doses of exogenous (bacterial) or endogenous (inflammatory) factors.

Whether priming of oxidative metabolism and inhibition of adhesion are expressions of the same or different biochemical mechanisms caused by *podophyllum* and podophyllotoxin (microtubule/fMLP receptor interaction, membrane fluidity increase or, even, to intracellular messengers variations) is an intriguing problem. It has been previously shown that adhesion to specific surfaces may represent a negative signal affecting the metabolic responses[35] and this is confirmed at least in part by our time-evolution data (Figure 1). It is therefore possible to suggest that in our experimental conditions a certain degree of microtubule disassembly caused by low doses of podophyllotoxin or colchicine stimulates the neutrophil metabolic response by down-regulating the negative signal provided by adhesion. Certainly, further insights about the molecular mechanism of priming and the possible action of microtubule disrupting compounds on the intracellular signal transduction may complete the picture and give promising new clues to these plant compounds.

Apart from intrinsic biological interest the phenomenon may have pharmacological implications. Delivering a high dose of podophyllotoxin in a local tissue, one would expect the compound to have an inhibitory effect at the point of injection or application. This is, for instance, the anti-mitotic or cytotoxic effect obtained with topical application of 2–5 mg/ml podophyllotoxin against condylomata acuminata.[3, 4] On the other hand, considering the tissues at a certain distance from the application point which are exposed to low doses of the toxin, a stimulatory effect on oxidative metabolism of phagocytic cells, and therefore an increase in their resistance, could be envisaged as a possible mechanism of action of the drug.

Showing an *in vitro* effect of a potentized drug demonstrates that it has a precise cellular target, in this case at the level of a fundamental cell involved in the inflammatory process. However, as we have already pointed out in a previous paper,[14] a similar finding is far from representing the explanation of the action of classical homoeopathic medicines that are prescribed—often at potencies much higher than those shown to be effective *in vitro*—according to similarity of symptoms. The identification of a cellular mechanism does not rule out the possibility that other different cellular or systemic levels of organization are modulated by the same drug when administered *in vivo*. In this context, our data have to be regarded as a small piece of a complex picture.

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Address for correspondence
Professor P. Bellavite
Istituto di Chimica e Microscopia Clinica
Università di Verona
Ospedale Policlinico-Laboratorio
37134 Verona
Italy