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STUDIES OF SKIN-WINDOW EXUDATE HUMAN NEUTROPHILS: Increased Resistance to Pentoxifylline of the Respiratory Burst in Primed Cells

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Abstract—Human neutrophils were isolated both from peripheral blood (PB) and from aseptic inflammatory exudates obtained by the Senn's skin window technique (SW). The respiratory burst (O_2^- production) induced by in response to *n*-formyl-methionyl-leucyl-phenylalanine (fMLP) and by serum-treated zymosan (STZ) was investigated using a microplate assay. SW neutrophils were primed to enhanced fMLP-dependent O_2^- production in response to fMLP but not to STZ. Pentoxifylline, a cAMP-elevating drug, dose-dependently inhibited the respiratory burst in any experimental condition, but the dose-effect curves were markedly different according the stimulant used and the source of the cells. With fMLP as stimulant, a significant inhibition of the O_2^- production by PB neutrophils was obtained using doses of 10 μg/ml, while SW neutrophils were inhibited only by doses equal or higher than 100 μg/ml. With STZ as stimulant, the inhibition of the respiratory burst of PB neutrophils and of SW neutrophils was obtained only with doses higher than 400 μg/ml and 1 mg/ml respectively. Pentoxifylline dose-dependently (10 μg/ml to 1 mg/ml) increased the intracellular adenosine 3′–5′-cyclic monophosphate (cAMP) to the same extent in SW and in PB neutrophils. These data indicate that the priming of neutrophil oxidative metabolism by in vivo inflammation is associated with an increase in the resistance to the regulating effect of cAMP on the fMLP-dependent activation pathway of NADPH oxidase. The fact that therapeutic doses of pentoxifylline do not inhibit the respiratory burst of primed neutrophils may have relevance in the interpretation of the clinical effects of this drug.

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INTRODUCTION

Previous investigations on animal and human models have demonstrated that neutrophils harvested from inflammatory exudates show enhanced responses to various membrane stimulants with respect to peripheral blood neutrophils (1–5). One frequently observed metabolic modification related to this phenomenon, called "priming", is the enhanced production of oxygen free radicals. The study of the mechanism(s) of priming and the search of pharmacological agents that could modulate this phenomenon are the object of active investigations because priming may have pathophysiological importance both in strengthening resistance against invading microorganisms and in predisposing the host to increased tissue damage during the inflammatory process (6–10).

Uncertainty still persists on the molecular nature of the priming phenomenon and various hypotheses have been proposed (10, 11). A reasonable target for amplification of the metabolic responses in primed cells would be the signal transduction pathways connecting extracellular receptors with the terminal free-radical generating NADPH oxidase. Among the various events of signal transduction mechanisms, the possibility that the priming phenomenon was linked to regulation of cAMP intracellular levels has been explored in a number of investigations. In fact, it has been reported that priming of neutrophils by lipopolysaccharide (LPS) and tumor necrosis factor-α (TNF) is inhibited by increase in cAMP levels (12, 13) and that TNF exerts its priming effect on human neutrophils by decreasing the intracellular cAMP (14). Moreover, we and others have shown that a number of cAMP-elevating agents such as adenosine (15, 16), PGE2 (17), forskolin (18), pentoxifylline (19, 20) and the membrane-permeable analogue of cAMP, dibutyrryl cAMP (21) suppress several neutrophil responses including the respiratory burst.

On the other hand, according to other lines of investigation, the priming effects of GM-CSF on the respiratory burst, was not inhibited by the cAMP-elevating agent pentoxifylline (20), suggesting that primed neutrophils may "escape" the regulation by cAMP elevation. Moreover, in a previous study (22) we did not find any significant difference either in the basal cAMP content of primed versus normal neutrophils or in the kinetics of the cAMP response to fMLP, indicating that priming can not be directly ascribed to increased cAMP levels.

We therefore made the hypothesis that the primed state could be associated not to a change of cAMP levels, but to a decrease of sensitivity to the regulating effect of cAMP, possibly due to adaptation mechanisms. To test this hypothesis we took advantage of a human ex-vivo model system of priming consisting in the accumulation of neutrophils in a sterile skin-window exudate. The oxidative metabolic responses (O_2^- production) of peripheral blood (PB) neutrophils were

compared with those of neutrophils isolated from a skin experimental exudate (SW) for their sensitivity to pentoxifylline, an agent that is known to inhibit phosphodiesterase and to increase intracellular cAMP. Since we (5) and others (23) have previously shown that the priming state achieved in the skin-window inflammatory cells is markedly stimulus-specific, depending on the stimulatory agents employed, we used two stimulants, the soluble chemotactic agent fMLP and the opsonized particles STZ, that activate the oxidative metabolism through different transduction pathways. We reasoned that, if the sensitivity to cAMP is changed in primed cells and if priming is factor-specific, the effect of pentoxifylline should be different in SW and PB cells and according to the stimulant used. The results here reported confirmed this hypothesis.

METHODS

Reagents. The chemotactic peptide fMLP and Zymosan A were purchased from Sigma Chemical Company, St. Louis, Missouri; cytochrome c from Boehringer, Mannheim, Germany; purified serum bovine albumin and human albumin from Behring Institut, Marburg, Germany; Percoll was from Pharmacia, Uppsala. Sterile 96-well microtiter plates with flat-bottomed wells (Linbro type) were from Flow Laboratories. The microplates were pre-coated with fetal bovine serum (Flow Laboratories) in order to abolish nonspecific cell activation and to provide physiological adherence surface, as described (16). Skin chambers for in vivo chemotaxis were from FAR Italia, Verona, Italy. Hank's balanced salt solution (without calcium and magnesium) (HBSS) and Dulbecco's phosphate buffered saline (PBS) were from Gibco Ltd., Paisley, Scotland; the composition of HBSS was 0.4 g/l KCl, 0.06 g/l KH_2PO_4, 8 g/l NaCl, 0.09 g/l Na_2HPO_4, 1 g/l D-glucose (pH 7.4); the composition of PBS was 0.2 g/l KCl, 0.2 g/l KH_2PO_4, 0.047 g/l $MgCl_2$, 8 g/l NaCl, 1.15 g/l Na_2HPO_4 (pH 7.4). Other materials and reagents were of the highest purity available.

Sterile apyrogenic solutions and disposable plasticware were used in all experiments, which were carried out, whenever possible, under a laminar flow hood.

Cell Preparation. Neutrophils were obtained from blood and from SW exudates of healthy human volunteers. Blood neutrophils were prepared from EDTA-anticoagulated blood by centrifugation over Percoll gradients (24). The final cell preparation was suspended in HBSS supplemented with 0.2% human serum albumin, 0.5 mM $CaCl_2$ and 1 mM $MgSO_4$ (medium H-GACM). Exudate neutrophils were isolated according to the skin-window technique proposed by Senn (25) with modifications (5). Briefly, an abrasion of 1 cm^2 was obtained with a rotating sterile abrasive cylinder on the volar surface of the nondominant forearm. A bell-shaped, sterile and disposable plastic skin chamber was put on the skin abrasion and fixed with a fenestrated sticking plaster. One ml of autologous serum was then injected into the chamber through a hole and twenty-four hours later the exudate was collected by aspiration. The exudate cells (>95% neutrophils, >99% viable) were then centrifuged at 1200 rpm, washed twice with phosphate buffered saline (PBS) and finally suspended in H-A and kept at room temperature until use. Before use, the cell suspensions were supplemented with $CaCl_2$ and $MgSO_4$ as described above for blood cells.

Superoxide Anion Production. Superoxide anion was measured by the superoxide-dismutase-dependent reduction of ferricytochrome c with a microplate assay (5, 16). The wells of fetal bovine serum-coated microplates were supplemented with 25 μl of 0.6 mM cytochrome c and with either 25 μl of 5×10^{-7} M fMLP (final concentration) diluted in H-GACM, or 0.1 mg/ml STZ (final

concentration), or 25 μl of H-GACM (unstimulated cells). The plate was then brought to 37°C, and 50 μl of the neutrophil suspension (2×10^5 cells), pre-warmed at 37°C, were added to each well. At the end of incubation (10 minutes and 40 minutes with fMLP and STZ respectively), the reduction of cytochrome c was measured with a microplate reader (Reader 400, SLT Labs Instruments) at 550 nm using 540 nm as reference wavelength (16).

cAMP Concentration. Neutrophils (10^7/ml) were suspended in 0.5 ml of H-GACM and incubated for 10 minutes at 37°C in the absence and in the presence of the indicated doses of pentoxifylline. Then, the neutrophil suspension was added to 1 ml of cold (−20°C) absolute ethanol containing 0.8 mM IBMX. The samples were held at −20°C for 1 h, then denatured protein was removed by centrifuging at 10,000 × g for 3 min and the supernatants were dried under vacuum. The lyophilates were suspended in 200 μl of 0.05 M acetate buffer, pH 5.8, containing 0.02% serum albumine and 0.005% thimerosal. cAMP content of duplicate samples was measured using cAMP enzyme-immunoassay kit from Amersham Int. (Amersham, U.K.), based upon the competition between unlabelled cAMP and fixed quantity of peroxidase-labelled cAMP on a specific antibody according to Volker et al. (26).

RESULTS

Peripheral blood and SW neutrophils from the same subject were treated with increasing doses of pentoxifylline. Table 1 shows that the drug caused a dose-dependent increase in their cAMP content, the extent of the modification being comparable in the two cell populations. Also the cytosolic cAMP concentration in the absence of drug was similar in PB and SW cells.

The O_2^- production by PB and SW following the challenge with fMLP and STZ was assayed in the presence of increasing concentrations of pentoxifylline. Figure 1 reports the dose-dependence curves in these four different experimental conditions, obtained in a series of 10 separate experiments. Absolute values of O_2^- production (1A) show that SW stimulated with fMLP had an activity

Table 1. Effects of Pentoxifylline on the Intracellular cAMP Content of Human Neutrophils from Blood (PB) and from Skin Window Exudate (SW)

	cAMP (fmol/5 × 10^6 neutrophils)[a]	
Pentoxifylline (μg/ml)	PB neutrophils	SW neutrophils
0	410 ± 112	430 ± 44
10	670 ± 265	662 ± 163
100	1041 ± 339	966 ± 137
400	1267 ± 404	1250 ± 294
1000	1591 ± 469	1575 ± 270

[a]Values are mean ± SD of three separate experiments.

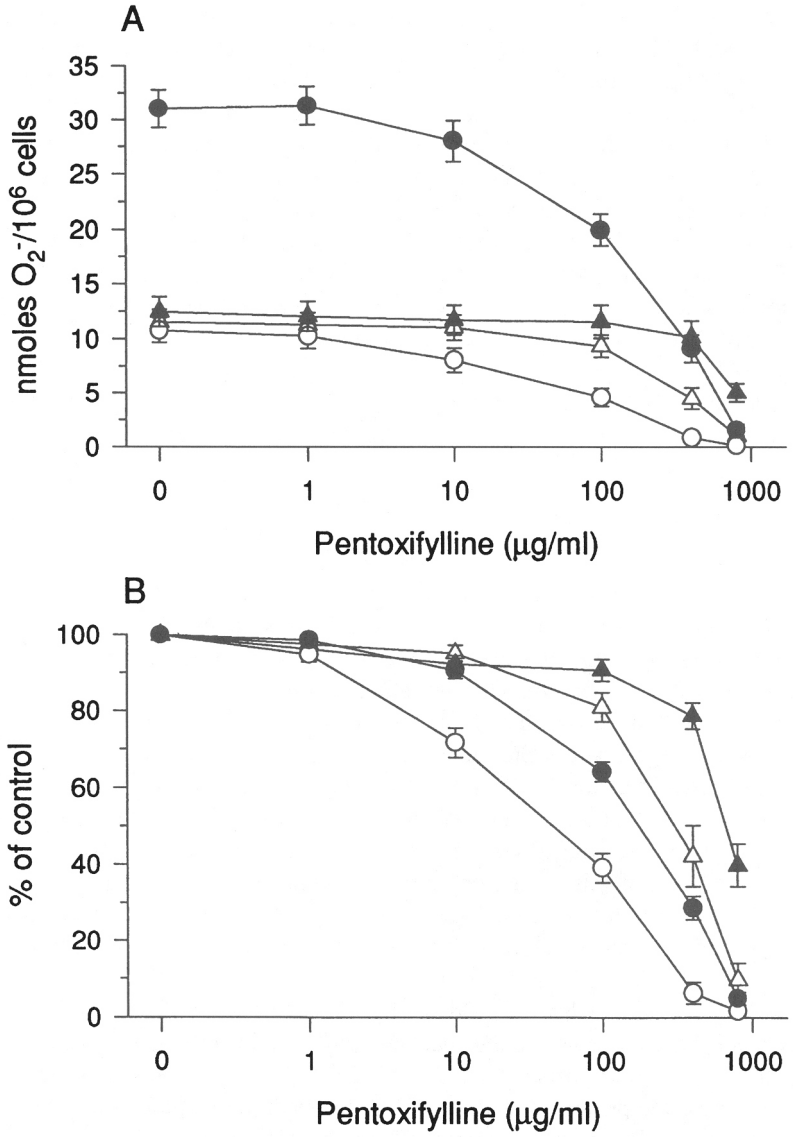

Fig. 1. Effect of pentoxifylline on the O_2^- production by human neutrophils. Neutrophils from peripheral blood (open symbols) and from skin window exudates (closed symbols) from the same subjects were activated with fMLP (circles) or with STZ (triangles) in the presence of increasing doses of pentoxifylline. The O_2^- production was assayed as described in the methods section. A, absolute values, B, % of control. Values are mean ± *SD* of 10 separate experiments.

about three times higher than that of PB cells, while the activity of SW and PB cells with STZ as stimulant was almost identical, as previously noted (5). Increasing doses of pentoxifylline inhibited the respiratory burst, but the effect of the drug appeared to be quantitatively different in the four different experimental conditions. The dose-effect relationship of pentoxifylline is better appreciated as percent of control (untreated) cells (Figure 1B). fMLP-activated PB cells were significantly inhibited by as low as 10 μg/ml pentoxifylline and in these conditions the E.D.$_{50}$ resulted of 41 μg/ml; the dose dependence curve of fMLP-activated SW cells was shifted to the right by about one order of magnitudo: significant inhibition required at least 100 μg/ml pentoxifylline and the E.D.$_{50}$ was 202 μg/ml; STZ-activated PB cells were more resistant to the effect of pentoxifylline (E.D.$_{50}$ = 320 μg/ml) than fMLP-activated PB and SW cells; STZ-activated pentoxifylline were the most resistant, being inhibited by pentoxifylline only at doses higher than 800 μg/ml (E.D.$_{50}$ = 610 μg/ml).

DISCUSSION

Neutrophils exist in one of four functional states: quiescent, primed, active, and deactivated (or unresponsive). In the primed state, there is no increase in oxidase activity, yet subsequent stimulation provokes a response that is larger than in non-primed, activated cells. Such enhanced responsiveness to stimulants occurs as an integral part of the early inflammatory reaction to infections and has been implicated in a number of infectious and inflammatory diseases (8–10, 27). Thus, the cellular and molecular mechanisms of priming and the possible strategies for its pharmacological control are the object of active investigation (10), but many aspects of this intriguing phenomenon have still to be clarified. Some authors have found that priming is associated with an increase in the number of receptors for various agents including the chemotactic peptide fMLP (4, 5, 28), in cytosolic free Ca^{2+} (29–31) and in other steps of the activation cascade such as phospholipid metabolism (32–34) and protein kinase C (35, 36) while other experiments have contradicted the necessary participation of one or more of these biochemical events in primed cells (22, 35, 37–41). These differences suggest a variety of possible priming states, according to the experimental models utilized or, in vivo, according to the various types of infection or inflammation (42–44).

In this paper, a specific aspect of the cellular and molecular mechanisms involved in the regulation of priming has been investigated, by testing the effect of a classical cAMP-elevating drug, pentoxifylline, on a well established ex vivo model of inflammation, the skin-window technique, by which consistent and reproducible lots of primed neutrophils can be obtained from healthy human subjects. Pentoxifylline is a potent inhibitor of phagocyte phosphodiesterase activity

and elevates cAMP levels in the micromolar range. For their ability to inhibit free radical generation and TNF-α synthesis, pentoxifylline and its derivatives have been recently proposed as effective antiinflammatory agents in experimental models (45–50) and human inflammatory diseases (51–53). However, the actual mechanisms by which pentoxifylline exerts its therapeutic effects are not fully clarified, since the drug has many potential targets (54) and the interpretation of the effects on phagocyte function are confused in many cases by the use in in vitro experiments at much higher concentrations than are obtained clinically (20).

Here we have shown that normal resting neutrophils and unstimulated SW neutrophils have the same basal cAMP content and pentoxifylline treatment increases the cAMP content to the same extent in the two cell populations. This indicates that the uptake of the drug is similar to the two cell populations and that the rate of synthesis of this messenger in not affected by priming, a conclusion that is in agreement with previous observations (22).

Although pentoxifylline caused similar increase on the cAMP intracellular concentration, the regulating effect of the drug on the oxidative metabolism was markedly affected by priming and by the type of stimulant used. The response to fMLP is primed and is inhibited by pentoxifylline, while the response to STZ is not primed by inflammation and is much more resistant to the drug. The range of doses which raise cAMP correspond approximately to the doses that inhibit fMLP-dependent O_2^- production by PB cells, indicating that in these conditions the inhibitory effect of the drug is actually due to its cAMP-elevating effect. This finding confirms previous indications that a cAMP-sensitive regulation step is involved in the transduction mechanism of fMLP and strengthen the hypothesis that this step may be the site of the regulation of priming itself. In other words, our data indicate that in the signal transduction cascade from fMLP receptor to the oxidase there exist a negative cAMP-dependent feed-back, potentially capable of controlling the neutrophil response to fMLP and the priming state and susceptible to amplification by phosphodiesterase-inhibiting compounds. The efficiency of this regulation appears to be relatively insufficient in SW, in vivo primed, neutrophils. A decrease of the inhibitory feedback mechanism controlled by cAMP has been suggested also by others for the in vitro priming effect of cytokines on neutrophil functions (55).

The cAMP pathway was paradigmatic for the concept of second messengers and signal transduction, but in white cells this pathway can function as a "gating" pathway (56), i.e. a pathway that regulate signal flow through other pathways. The mechanism of the gating effects of cAMP presumably involves the regulation of phospholipase C and of Raf-1 by cAMP-dependent protein kinase (11, 57, 58), but also a direct effect on the activity or the assembly of NADPH oxidase is conceivable because components of this enzyme complex are substrates of protein kinase-A (59, 60). The fact that cAMP-elevating agents do not affect the

O_2^- production in response to STZ (this work) and to PMA (11, 17, 22) indicate that they act by interfering with the assembly of the NADPH oxidase complex at a point proximal to the fMLP receptor rather than by directly inhibiting the activity of the already working enzyme.

Taking into account that during the chemotactic extravasation and following the accumulation into the inflammatory focus the SW neutrophils are exposed to repeated and multiple stimulatory signals that trigger the cAMP metabolism, we suggest that the priming state is associated with some adaptation mechanism at the level of protein kinase A and/or of its substrates. However, our data do not exclude the possibility that other mechanisms are involved in priming itself. Whether priming of the metabolic response to fMet-Leu-Phe might be accounted for by the changes in sensitivity to cAMP or whether it requires other changes of specific transduction pathways (membrane receptors, phospholipid metabolism, protein phosphorylation, G-proteins, etc.) that have not been considered here, is a matter of investigation.

The stimulus-dependence of the effect of pentoxifylline and the increased resistance of SW primed cells to this drug is a challenge for therapeutic strategies. Neutrophils present into inflammatory exudates are functionally modified with respect to "virgin" cells, which may be isolated from peripheral blood, and also their sensitivity to drugs appears to be modified. Clinically achievable levels of pentoxifylline and of its active metabolites are in the range of 1 to 10 μM (0.3–3 μg/ml) (54, 61) although considerably higher doses have been used in septic patients (52) and experimental studies (62). Therefore, assuming that the doses acting on neutrophils in vitro and in vivo are similar, our data demonstrate that a (partial) inhibition of the respiratory burst triggered by specific stimuli may occur only on blood cells. On the other hand, the effect of pentoxifylline on the cells involved in inflammatory sites, i.e. on primed cells, and on the cells activated by cAMP-independent stimuli, e.g. serum-opsonized fungi or bacteria, should be expected to be almost null. This fact may be interpreted as a positive aspect in view that the retention of activity in primed cells would not compromise the phagocytosis and oxidative killing of microbial invaders, thus preserving the biological defenses against infections, but may be an important handicap for the anti-inflammatory activity of the drug. If exudate cells "escape" the inhibition by clinically achievable doses of pentoxifylline, the therapeutic strategies for ameliorating neutrophil-mediated tissue injury in patients with inflammation syndromes and the interpretation of pharmacokinetic data should be reconsidered.

In this respect, our data are in significant agreement with those showing that pentoxifylline at doses of 5–10 μmole/l inhibited the responses (O_2^- and chemotactic migration) to fMLP but not the same responses of in vitro GM-CSF-primed neutrophils (20) and with those showing that in a rabbit model, pentoxifylline did not prevent the neutrophil sequestration and emigration in the lung induced by infusion of complement fragments (63) and it is well known

that activated complement is a powerful neutrophil priming agent (64). These data suggest that, in vivo, pentoxifylline does not protect against lung injury by inhibiting neutrophil sequestration or emigration and may act to alter the generation of mediators that affect neutrophil behavior, rather than acting directly on neutrophils.

On the other hand it has been recently reported that high doses of pentoxifylline administered intravenously in septic patients (5 mg/kg over a period of 180 minutes) actually reduced the fMLP-stimulated neutrophil oxidative metabolism (52), indicating that intensive course of this drug may be beneficial also in a case where the neutrophils are presumably highly primed. It is also worth of note that in a porcine model of gram-negative sepsis a high dose of pentoxifylline (20 mg/kg bolus followed by a 6 mg/kg per hour infusion) protected from hemodynamic derangement and pulmonary dysfunction (62). However, in the latter work it has been shown that if administration is delayed until overt septic shock occurs, the beneficial effects of the treatment are lost, indicating that pentoxifylline has a "therapeutic window" when given during the course of the disease.

In conclusion, the evidence provided in this paper and the clinical studies of the effect of pentoxifylline strongly suggest that the activation state of neutrophils is a critical factor determining its therapeutic effectiveness. The changes of cellular sensitivity to pentoxifylline due to priming should be taken into account for the interpretation of its pharmacodynamic properties.

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