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# SHORT COMMUNICATIONS

## Effects of polyene antibiotics on the activation of human polymorphonuclear leukocytes

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Polyene macrolide antibiotics are characterized by a large lactone ring containing conjugated double bonds and usually combined with sugar, aliphatic chains and/or aromatic groups. The drugs bind to membrane sterols, leading to increased permeability and lysis of sensitive cells. Some of them are among the most effective antifungal agents known [1]. The high affinity to fungal membranes together with a relatively low toxicity for patients is the rationale for the clinical use of these compounds in the treatment of systemic mycoses.

It has been recently reported that some of these drugs elicit interesting immunomodulating effects, interfere with the signal cascade and modify many properties of polymorphonuclear leukocytes [2, 3]. Amphotericin B, a polyene antibiotic commonly used in therapy, inhibits phagocytosis and killing of opsonized bacteria [4, 5] and chemotaxis induced by N-formylated oligopeptides [6], opsonized zymosan [5, 6] or *Escherichia coli* filtrate [4]. This antibiotic, as well as its methyl ester, at relatively low concentrations decreases leukocyte adherence to nylon fiber columns, whereas at higher concentrations promotes cell aggregation on the polymer [5, 7]. Nystatin, a less potent polyene antibiotic, prevents leukocyte chemotaxis towards N-formylated oligopeptides [6] but seems to be ineffective in inhibiting chemotaxis triggered by *E. coli* filtrate [4]. As far as the effect on the respiratory burst of leukocytes is concerned, most of the data refer to the inhibition of oxygen radical production in the presence of opsonized zymosan by relatively high amphotericin B concentrations [4, 5].

In this work we describe a dose-dependent modulation of the respiratory burst by amphotericin B. Moreover, we examine whether the alteration of this metabolic event is dissociable from the impairment of other leukocyte functions.

### *Materials and Methods*

Buffer solutions contained 140 mM NaCl, 2.7 mM KCl, 1.5 mM $KH_2PO_4$, 12 mM $Na_2HPO_4$, 0.49 mM $MgCl_2$, 0.3 mM $CaCl_2$ and 5 mM glucose, pH 7.4, unless otherwise specified. In some experiments the concentration of sodium was varied from 24 to 164 mM at constant osmolarity and fixed concentrations of phosphate and chloride by reciprocally adjusting the concentration of NaCl and choline chloride. Phorbol 12-myristate 13-acetate, *N*-formylmethionylleucylphenylalanine, calcium-ionophore A23187 and luminol (5-amino 2,3-dihydro 1,4-phthalazinedione) from the Sigma Chemical Co. and amphotericin B from Squibb & Sons Inc. were solubilized in dimethylsulfoxide. Zymosan (Sigma) was opsonized by dissolving 20 mg of dry powder in 1 mL of freshly-prepared human serum. After 30-min incubation at 37°, the suspension was centrifuged and the pellet was washed once and resuspended in buffer without calcium to give a final concentration of 20 mg/mL. Latex beads (diameter: 0.801 ± 0.036 μm) were purchased from Sigma, washed twice and resuspended in buffer solution. Other reagents were high purity commercial samples from Sigma, Merck A.G. and Fluka A.G.

Human leukocyte preparations containing 90–98% of polymorphonuclear cells and apparently free of contaminating erythrocytes were obtained by a one-step procedure [8] involving centrifugation of heparinized blood, freshly drawn from healthy donors and layered on Mono-Poly Resolving Medium (Flow Laboratories Ltd). Each preparation produced cell populations that were more than 90% viable by trypan blue exclusion [5] up to 6 hr after purification. Dimethylsulfoxide (up to 0.14 M) did not affect dye exclusion. In each group of experiments, comparisons between stimuli were made with cells from the same donor.

Oxygen radical production was studied by measuring light emission [9] in the presence of $10^5$ cells/mL and 1 μM luminol with a LKB luminometer Mod. 1251 thermostated at 30°. The samples were continuously stirred during the assay. Except where otherwise stated, the stimulating agent was added last to the reaction mixture. Since the chemiluminescence response slowly decreased with time, we used only freshly prepared cells. The peak chemiluminescence in the presence of polyenes was expressed as a percentage of the peak chemiluminescence of untreated leukocytes.

Alteration of membrane potential was evaluated by measuring the initial rate [10] as well as the amplitude of fluorescence changes [11] of the probe 3,3'-dipentyloxacarbocyanine iodide (0.1 μM, Molecular Probes Inc.). Oxygen consumption was measured with a Clarke oxygen electrode (Ysi Mod. 53 oxygen monitor) [12]. Potassium efflux was monitored with a Radiometer Type F2312K electrode [13]. Phagocytosis of latex beads was evaluated by measuring the 253-nm absorbance of cell extracts in dioxan [14]. Chemotaxis under agarose gel was assessed by the semi-quantitative method described in Ref. 15.

### *Results and Discussion*

The ability of a variety of stimuli (latex particles, opsonized zymosan, phorbol 12-myristate 13-acetate, *N*-formylmethionylleucylphenylalanine and the ionophore A23187) to trigger oxygen radical production in human polymorphonuclear leukocytes was determined by measuring the chemiluminescence response in the presence of luminol. In most of the cases, light emission with time demonstrated a peak at 2–20 min, followed by a slow decline (Fig. 1). Among all stimuli tested, only the chemotactic peptide *N*-formylmethionylleucylphenylalanine showed a biphasic response (after about 1 min and 10 min, respectively), the second signal being less intense and sharp. The intensity of light signals was roughly hyperbolic function of activator concentration, as reported in Refs 14–16. Peak chemiluminescence was up to three to four times greater in the presence of an excess of particulate stimuli (0.4 mg/mL opsonized zymosan or 2 mg/mL latex) than in the presence of an excess of soluble agents (0.33 μM phorbol ester or 5 μM chemotactic peptide). In the case of A23187, the intensity of cellular response increased by increasing calcium concentration in the suspending medium [17].

The effect elicited by amphotericin B on leukocyte chemiluminescence was dependent on the antibiotic

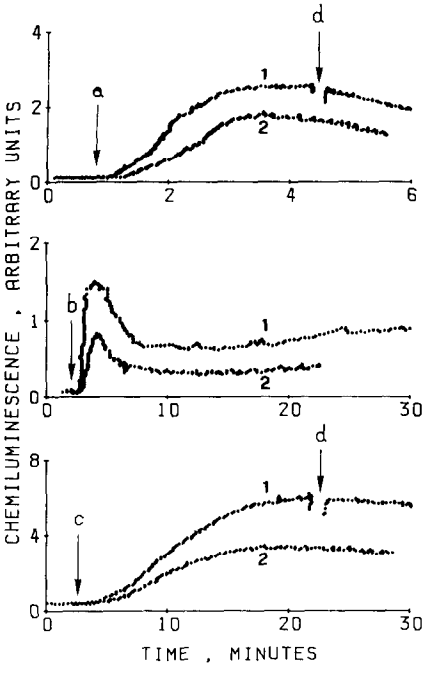


Fig. 1. Time course of the chemiluminescence response of human polymorphonuclear leukocytes. Cells ($10^5$/mL) were preincubated in the absence (1) or in the presence (2) of amphotericin B (top: 0.8 $\mu$M; centre: 5 $\mu$M; bottom: 70 $\mu$M). Arrows indicate the addition of 20 $\mu$M A23187 (a), 5 $\mu$M *N*-formylmethionylleucylphenylalanine (b) and 0.4 mg/mL opsonized zymosan (c) to resting cells and the addition of amphotericin B (d) to activated cells. Other experimental conditions are described in the text.

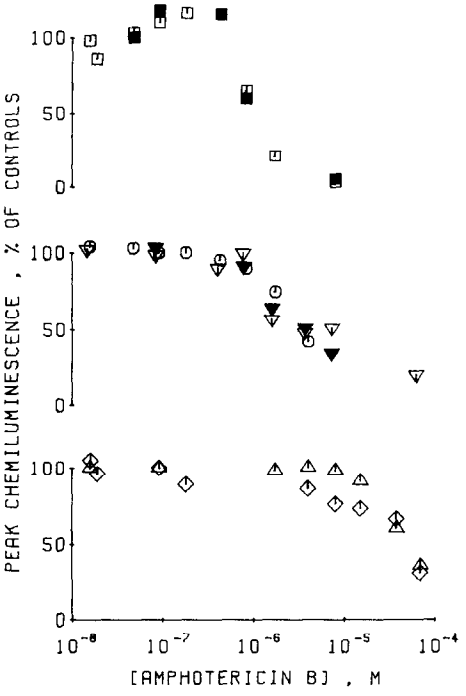


Fig. 2. Effect of amphotericin B on the peak of the chemiluminescence signal of human polymorphonuclear leukocytes. Cells ($10^5$/mL) were activated with 20 $\mu$M A23187 (□, ■), 5 $\mu$M *N*-formylmethionylleucylphenylalanine (▽, ▼), 0.33 $\mu$M phorbol 12-myristate 13-acetate (○), 0.4 mg/mL opsonized zymosan (△) and 2 mg/mL latex (◇). Closed symbols refer to experiments in media containing choline chloride instead of NaCl. Other experimental conditions are described in the text.

concentration and on the nature of the stimulus used to trigger cell response. Low amphotericin B concentrations (0.1–0.5 $\mu$M) slightly enhanced chemiluminescence of ionophore-activated cells, without affecting light emission in the presence of phorbol ester, chemotactic peptide or particulate stimuli (Fig. 2). At higher concentrations, the polyene antibiotic elicited a dose-dependent inhibition of the leukocyte signal, whatever stimulating agent was used. A 50% inhibition of A23187-induced chemiluminescence was observed in the presence of about 1 $\mu$M amphotericin B. Inhibition of the signals triggered by phorbol ester or by chemotactic peptide required about 5 $\mu$M amphotericin B. Signals induced by particulate agents were inhibited by very high polyene concentrations (>10 $\mu$M), as already observed [2, 3]. The effects elicited by amphotericin B did not appear to be affected by the substitution of NaCl with choline chloride in the isotonic buffer.

Kinetics of amphotericin B inhibition were characterized by a decrease in the whole light signal (Fig. 3) rather than by an appreciable variation of the apparent half-saturation constant for the signal-triggering stimulus (A23187, latex, opsonized zymosan). The hypothesis of a non-competitive mechanism for the inhibition appeared to be substantiated by the observation that signal vs amphotericin B concentration plots were not significantly affected by 10-fold lowering of the concentration of the stimulating agent. A similar conclusion, thus excluding a receptor-selective effect, had been reached [4] by studying amphotericin B inhibition of the binding of N-formylated peptides to leukocyte membranes.

Chemiluminescence inhibition could not be exclusively attributed to a scavenger effect of polyene antibiotics on oxygen reactive species produced during cell activation. Firstly, amphotericin B was unable to decrease light emission if added to reaction mixtures containing already-activated leukocytes (Fig. 1). Moreover, during the respiratory burst, oxygen consumption was less intense (in the presence of 20 $\mu$M A23187, 1 $\mu$M amphotericin B reduced oxygen consumption to about half). Finally, amphotericin B (up to 10 $\mu$M) did not appreciably affect luminol-enhanced chemiluminescence of the xanthine–xanthine oxidase peroxide-generating model system [9], whereas more concentrated solutions were inhibitory (data not shown).

As far as the effect on the leukocyte membrane potential is concerned, a 50% decrease in fluorescence of the membrane probe 3,3′-dipentyloxacarbocyanine iodide was obtained by adding 0.2 $\mu$M amphotericin B to unstimulated cells, i.e. a concentration well below that necessary to inhibit oxygen radical production. Signal changes went to completion in about 10 min. In the presence of an excess of antibiotic, the fluorescence of the probe approximated to that elicited in the absence of leukocytes. No fluorescence changes were seen in the absence of cells and/or by adding dimethylsulfoxide (up to 0.14 M). Membrane polarization changes, unlike respiratory-burst inhibition, were influenced by the compostion of the extracellular saline medium. Substitution of extracellular NaCl with choline salt determined a 10-fold decrease in the velocity of the signal that we observed upon amphotericin B addition, without affecting the fluorescence of resting cells. Increasing

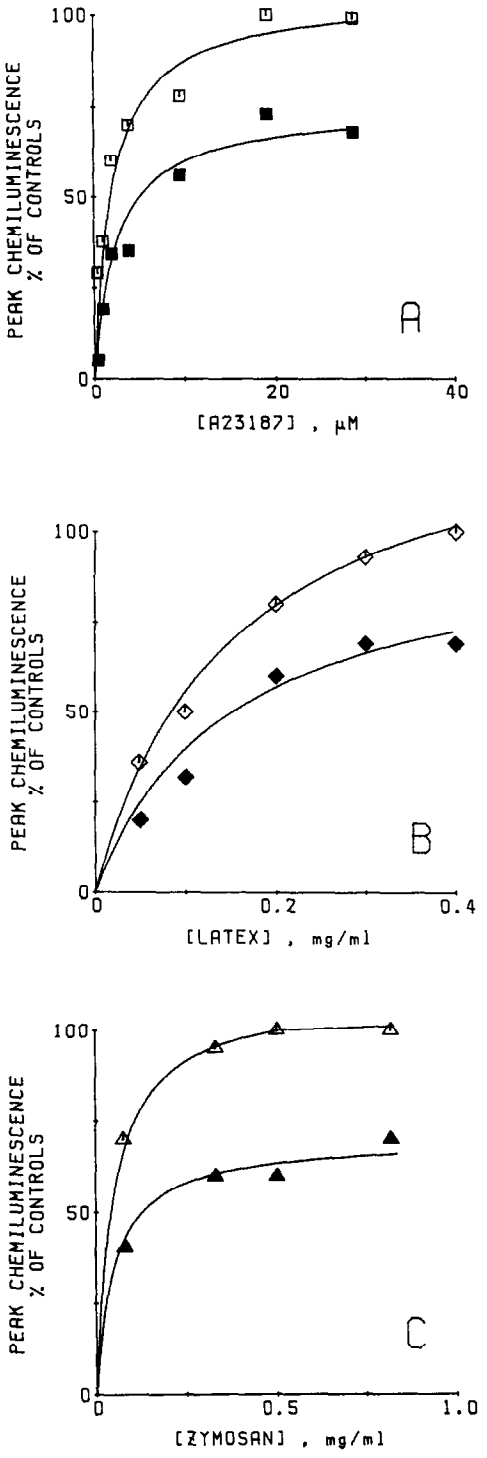


Fig. 3. Peak chemiluminescence of human polymorphonuclear leukocytes as a function of activator concentration. Closed symbols refer to experiments in the presence of amphotericin B (A: 0.8 μM; B: 10 μM; C: 50 μM). Other conditions are described in Fig. 2.

external potassium concentration reduced both the fluorescence intensity of resting cells [18, 19] and the effect of the antibiotic.

To address the possibility that the results obtained in potassium-enriched media were related to a high intrinsic permeability to $K^+$ [18, 19], experiments were performed to evaluate the flux of this cation across leukocyte plasma membranes. In an isotonic medium containing only 10 μM potassium, we observed, using the specific electrode, the loss of 10–20% of intracellular $K^+$ within 1 hr. Intracellular potassium was completely lost by adding 40 μM digitonin to the cell suspension. Amphotericin B (up to 10 μM) did not appreciably modify the kinetics of the $K^+$ efflux, whereas higher antibiotic concentrations accelerated potassium leak.

As a whole, these data seem to indicate that inhibition of respiratory burst is not directly related to the alteration of the cell membrane potential and/or with the formation of pores inducing unspecific leaks for monovalent ions (at least in the case of cells activated by soluble stimuli). This in not surprising, since dissociation between permeability changes and impairment of cellular functions was already observed in mammalian cell cultures [20–22] by studying polyene effects that were attributed to modifications of the structure of some of the plasma membrane components rather than to the ionophoretic properties of the drugs. Respiratory-burst inhibition did not correlate even with the impairment of other cellular functions. It had been reported [4] that amphotericin B inhibits the binding of the chemotactic peptide to leukocyte membranes and chemotaxis at concentrations considerably lower than those required to reduce chemiluminescence of cells triggered by this soluble agent (Fig. 2). In the same way, we found that chemotaxis towards opsonized zymosan was approximately halved by 1 μM amphotericin B, i.e. when light emission induced by the particulate stimulus was not yet influenced by the antibiotic. By contrast, these concentrations of amphotericin B did not affect phagocytosis of latex particles (data not shown) and of opsonized bacteria [2].

In conclusion, our data suggest a stepwise inhibition of the functional properties of human polymorphonuclear leukocytes *in vitro* by amphotericin B. This finding, together with the existence of patients in whom the respiratory burst can be activated by some stimuli but not by others [23, 24], strongly suggests multiple points of entry into the activation process.

*Departments of Biochemical Sciences and Human Biopathology*
*University of Rome La Sapienza*
*and Centre of Molecular Biology*
*National Research Council*
*Rome, Italy*

SOIZIC JULLIEN
ELISABETTA CAPUOZZO
COSTANTINO SALERNO*
CARLO CRIFÒ

* Correspondence: Costantino Salerno, Department of Biochemical Sciences, University of Rome La Sapienza, p. le A. Moro, 5-00100 Rome, Italy.

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# Inhibition of dopamine synthesis in rat striatal minces: evidence of dopamine autoreceptor supersensitivity to *S*(+)- but not *R*(−)-*N*-*n*-propylnorapomorphine after pretreatment with fluphenazine

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Striatal dopamine (DA*) synthesis can be modulated by DA agonists acting at nerve terminal and other autoreceptors to inhibit the rate-limiting tyrosine hydroxylase (EC 1.14.16.2) step in DA biosynthesis [1]. Through an apparent autoreceptor-mediated mechanism, the apomorphine congener *R*(−)-*N*-*n*-propylnorapomorphine (NPA) potently inhibits DA synthesis in rat striatal minces and synaptosomes [2, 3], as well as in an *in vivo* model [4] measuring accumulation of L-dihydroxyphenylalanine (DOPA) in rat striatum [5]. Both *S*(+)- and *R*(−)-NPA are full agonists at postsynaptic $D_2$ receptors in rat pituitary gland, where the *R*(−) enantiomer is about 45-fold more potent [6]. At synthesis-modulating striatal $D_2$ autoreceptors, both isomers also are full agonists but *S*(+)-NPA is nearly equipotent to *R*(−)-NPA [2]. We have proposed [2] that *S*(+)-NPA is a relatively selective full agonist at presynaptic $D_2$ autoreceptors mediating inhibition of DA synthesis, where its efficacy may depend on a relatively high abundance or "reserve" of striatal $D_2$ autoreceptors [7], while *R*(−)-NPA is a nonselective full agonist at both pre- and postsynaptic $D_2$ receptors.

* Abbreviations: DA, dopamine; NPA, *N*-*n*-propylnorapomorphine; DOPA, dihydroxyphenylalanine; and GBL, γ-butyrolactone.