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Terbinafine induces the PMNL priming effect and enhances *in vitro* PMNL fungicidal activity against *Candida albicans* blastospores

Terbinafin-bedingte Induktion eines Primingeffekts und Steigerung der Fungizidie von polymorphkernigen Granulozyten gegenüber *Candida albicans*-Blastosporen *in vitro*

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Schlüsselwörter. *Candida albicans*, Antimyzetika, Terbinafin, Primingeffekt, Rasterelektronenmikroskopie, REM.

Summary. Terbinafine, a new antifungal drug of the allylamine category, has been shown not to interfere with some polymorphonuclear leucocyte (PMNL) functions such as chemotaxis and chemiluminescence. The aim of the present study was to elucidate the effects of terbinafine on PMNL respiratory burst activation and killing of *Candida albicans* blastospores by PMNLs at the biochemical and ultrastructural level by means of scanning electron microscopy (SEM). Terbinafine is shown to enhance the respiratory burst and superoxide anion release in human PMNLs stimulated by phorbol esters or chemotactic peptides, and to have a priming effect on PMNL functions. Moreover, in our experiments we found that terbinafine does not interfere with PMNL killing of *C. albicans* blastospores but, in fact, at the concentration found in tissues after oral administration, slightly increases it. As PMNLs play a key role in the early stages of fungal infections we suggest that *in vivo* terbinafine induces priming of PMNLs, and that this effect is related to enhanced candidacidal activity independent of direct drug damage to fungal particles.

Zusammenfassung. Terbinafin, ein Antimyzetikum der Allylamin-Gruppe, hat keinen Einfluß auf Chemotaxis und Chemolumuneszenz polymorphkerniger Granulozyten (PMNL). Ziel der vorliegenden Studie war, die Terbinafin-Wirkungen auf die Respiratory-Burst-Aktivität von PMNL und deren Abtötungsfähigkeit gegenüber *Candida albicans*-Blastosporen biochemisch und ultrastrukturell mittels Rasterelektronenmikroskopie zu untersuchen. Terbinafin steigert die Respiratory-Burst-Aktivität und die Freisetzung von Peroxid-Anionen in PMNL, die mit Phorbolestern oder chemotaktischen Peptiden zur Erzielung eines Primingeffektes stimuliert waren. Hierbei wurde kein Einfluß auf die Abtötungsfähigkeit der PMNL für *C. albicans*-Blastosporen beobachtet; Terbinafin in der Größenordnung von Gewebskonzentrationen erhöht jedoch diese Abtötungsfähigkeit geringgradig. Da PMNL im Frühstadium von Pilzinfektionen eine Schlüsselrolle spielen, wird gefolgert, daß Terbinafin auch in vivo einen solchen Primingeffekt bewirkt, der, zusätzlich und unabhängig von der Direktschädigung der Pilzzelle, zu erhöhter candidazider PMNL-Aktivität führt.

Introduction

Allylamines inhibit ergosterol biosynthesis by blocking squalene epoxidase, a key enzyme in the transformation of squalene to squalene epoxide [1]. Terbinafine is a new allylaminic antimycotic drug that is fungicidal to dermatophytes and fungistatic against *Candida albicans* [2–4]. In addition,

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terbinafine has an *in vitro* effect on human neutrophil polymorphonuclear leucocyte (PMNL) functions [5]. PMNLs are one of the major effectors in the early stages of fungal infections, generating highly reactive oxygen metabolites through respiratory burst activation [6]. PMNLs can be 'primed' for respiratory burst activation by exposure to priming agents. Following such priming PMNLs show an enhanced response to stimuli, responding to below-threshold doses of stimulating agents [7–9]. To assess the influence of terbinafine on PMNL functions [10], besides its antimycotic activity, this study aimed to investigate the *in vitro* effect of terbinafine on (a) the respiratory burst, (b) killing of *Candida albicans* blastospores by PMNLs and (c) scanning electron microscopy (SEM) features that may be considered to be terbinafine-induced effects on PMNLs.

Materials and methods

The terbinafine solution and buffer used were tested for the presence of bacterial lipopolysaccharide (LPS) using the *Limulus* amebocyte lysate test (Whittaker Bioproducts, Walkersville, MD, USA) and were found to be free of contamination. All separation steps were performed at 4 °C in order to avoid PMNL preactivation. PMNLs were isolated from fresh citrate acid glucose (ACD) anticoagulated blood drawn from healthy human donors by dextran sedimentation, Histopaque 1077 (Sigma, St Louis, MO, USA) density-gradient centrifugation and subsequent hypotonic lysis of remaining erytrocytes. Cells were washed, resuspended in HBSS (Hanks' balanced salt solution) and placed in 2 ml polystyrene cuvettes at a final concentration of 1–4 10^6 cells $2\,ml^{-1}$ in the presence of 0.08 mM ferricytochrome *c*. After addition of variable amounts of stimulants and terbinafine, generation of superoxide anion by PMNLs was evaluated by spectrophotometric determination (Beckman DU-50 spectrophotometer) of cytochrome *c* reduction at 450 nm. As previously described [11], stimulating substances tested were phorbol myristate acetate (PMA) and formyl methionyl leucyl phenylalanine (fMLP) (both from Sigma) using dimethylsulphoxide (DMSO) as solvent at a final concentration never exceeding 0.1%, which did not affect superoxide release in our system. To measure killing, 5×10^6 *C. albicans* blastospores were suspended with 2.5×10^6 PMNLs in 1 ml of Dulbecco's phosphate-buffered saline (PBS) supplemented with 5.5 mM glucose, 1.3 mM calcium, 0.8 mM magnesium and 10% autologus serum in the presence of terbinafine at a final concentration of 1, 10 or 100 mM, or without terbinafine. After 1 h incubation, 0.3 ml of 2.5% deoxycholic acid in normal saline was added to lyse leucocytes. Specimens were centrifuged at 1000 *g* for 10 min. Supernatants were discarded and the remaining fungi were washed twice with 10 ml of distilled water and finally suspended in yeast nitrogen base broth containing MTT and incubated for 3 h at 30 °C. Then MTT converted to MTT formazan by viable *Candida* blastospores was extracted and *Candida* killing was calculated according to the following equation:

$$\text{Killing (\%)} = 1 - \frac{A_{570}\text{ fungi incubated with PMNLs}}{A_{570}\text{ fungi not incubated with PMNLs}} \times 100$$

as described by Levitz & Diamond [12].

Ultrastructural study

For SEM observation *C. albicans* was cultivated on Sabouraud periodic acid–Schiff (PAS) agar medium at 30 °C for 1 week. The colonies obtained were collected using 4-mm punch biopsies from the culture edge. Colonies obtained were subsequently exposed to terbinafine at a final concentration of 1×10^{-5} M in DMSO for 30 min, 1 h, 3 h, 8 h or 24 h with: (a) a suspension of human PMNLs; (b) terbinafine; (c) PMNLs and terbinafine. Specimens were fixed in Karnowsky solution for 2 h at room temperature, dehydrated in ethanol (50%–70%–90%–100%, 10 min each passage), dried in a Baltzer CPD 030 device, metallized with gold palladium and examined under a Philips 505 scanning electron microscope. To study the effects of terbinafine on PMNLs we evaluated four PMNL morphofunctional parameters: (a) shape modification of cellular bodies (see Fig. 3); (b) protrusion of pseudopodia (see Fig. 4); (c) the presence of PMNL granules outside the cell (see Fig. 5); (d) phagocytosis against yeasts (see Fig. 6). Each parameter considered was scored subjectively at 30 min, 1 h, 3 h, 8 h and 24 h by three independent observers, according to the following scheme:

0 = no detectable effect
1 = small effect
2 = moderate effect
3 = intense effect.

Results

Preincubation of a PMNL suspension with terbinafine (1, 10 or 100 μM) for 10–60 min did not by itself trigger the release of superoxide anion by

PMNLs. After preincubation with terbinafine for 10 min, superoxide anion production induced by different stimuli (i.e. 100 mM fMLP and 10 nM PMA) was increased compared with that in cells not treated with terbinafine (Figs 1 and 2). Killing of *C. albicans* blastospores by PMNLs was slightly

Table 1. Effects of varying concentrations of terbinafine on PMNL candidacidal activity

	Killing (%) ± SD
PMNLs	30 ± 8
PMNLs + terbenafine 1 μM	32 ± 7
PMNLs + terberafine 10 μM	35 ± 10*
PMNLs + terberafine 100 μM	37 ± 10*

* $P < 0.01$ *vs.* PMNLs alone.

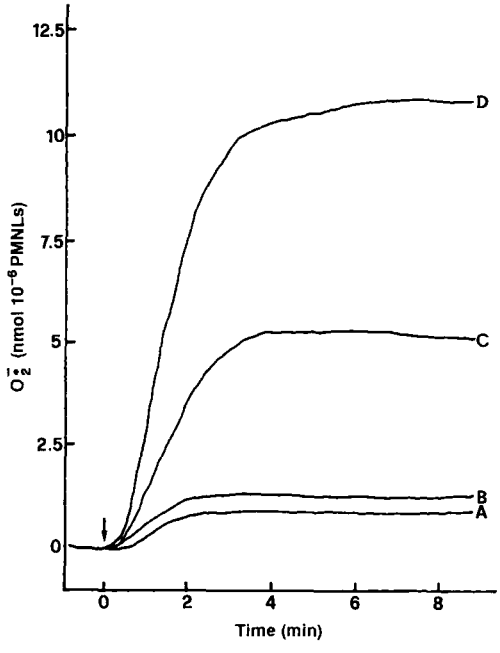


Figure 1. Effect of fMLP on superoxide anion release by human PMNLs expressed as cytochrome *c* reduction with or without preincubation with terbinafine. A = fMLP 1 μM; B = fMLP 1 μM + terbinafine 1 μM; C = fMLP 1 μM + terbinafine 10 μM; D = fMLP 1 μM + terbinafine 100 μM.

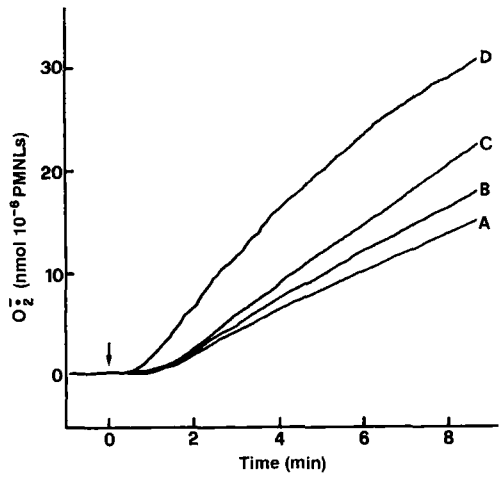


Figure 2. Effect of varying concentrations of terbinafine on the oxidant production by neutrophils elicited by PMA. A = PMA 10 nM; B = PMA 10 nM + terbinafine 1 μM; C = PMA 10 nM + terbinafine 10 μM; D = PMA 10 nM + terbinafine 100 μM.

increased by pretreating PMNLs with 1 mM terbinafine. Terbinafine at 10 and 100 mM significantly increased killing of *C. albicans* blastospores by PMNLs (Table 1). Ultrastructural observations are summarized in Table 2 and demonstrated in representative examples in Figs. 3–6.

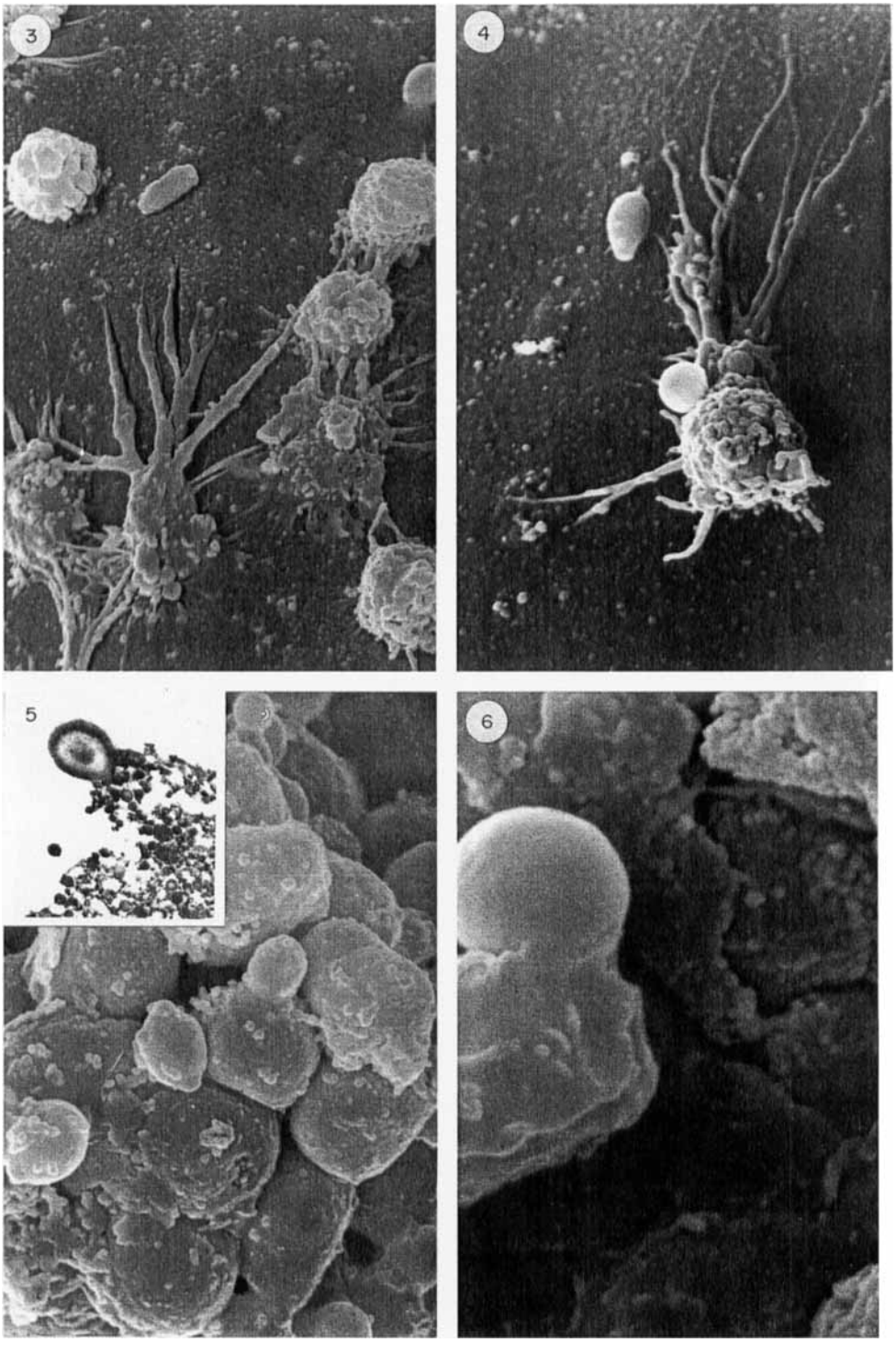
Discussion

The treatment of fungal infections by modern chemotherapeutic agents achieves the aim of controlling fungal proliferation but may affect the immune response of patients [13–16]. PMNLs are specialized effectors of the immune response during the early stages of fungal infections. The eradication of the fungus from the infection site is strictly dependent on conserved PMNL functions (generation of oxidant molecules, phagocytosis, etc.). For this reason, adverse effects of drugs on PMNL functions are undesirable. Recently, some reports have drawn attention to the fact that terbinafine does not affect PMNL chemotaxis and

Table 2. PMNL activation: mean score

PMNL parameters	PMNL + fungi					Terbinafine + PMNLs + fungi				
	30 min	1 h	3 h	8 h	24 h	30 min	1 h	3 h	8 h	24 h
A	0	1, 3	1	0	0	3	3	2, 3	1, 3	0
B	0	1	1, 3	0	0	3	2, 6	2	1	0
C	0	0	1	1	0, 3	1	1	2	1	0, 3
D	0	0	0	1	0	1	1	2, 3	2	0

A, shape modification of entire cellular bodies; B, protrusion of pseudopodia; C, presence of PMNL granules outside the cell; D, phagocytosis against yeasts.
0, no detectable effect; 1, small effect; 2, moderate effect; 3, intense effect.



Figures 3–6. Scanning electron micrographs of PMNLs under terbinafine-induced activation. **Figure 3.** Some PMNLs with ruffled surfaces and cellular shape modifications. Cell-to-cell contacts are visible (× 3150). **Figure 4.** High-power view of an activated PMNL. Polarization of cell shape with emission of long pseudopodia towards two *C. albicans* blastospores (× 4125). **Figure 5.** Presence of granules outside the cells as a result of PMNL membrane damage following phagocytosis of *C. albicans* blastospores and release of enzymatic granules (× 3000). Inset shows transmission electron microscopy counterpart. **Figure 6.** Phagocytosis of one *C. albicans* blastospore by a PMNL (× 7500).

chemiluminescence [5]. In this work we have considered the activity of terbinafine—an antimycotic drug of the allylamine group—on PMNL respiratory burst activation and fungicidal activity against *C. albicans* blastospores. Terbinafine enhances superoxide anion release in PMNLs exposed to classical stimuli such as PMA and fMLP, having a priming effect on PMNL function. Priming, although not necessary for activation of neutrophil NADPH oxidase, allows cells to

increase their response to subsequent stimuli and thus to increase the effectiveness of PMNL defence functions against yeast infections.

In our hands terbinafine not only failed to depress *C. albicans* blastospore killing by PMNLs but, at the concentration found in tissues, and in contrast to other antimycotic drugs, slightly increased it.

Ultrastructural observation of PMNLs allowed us to recognize a relation with the quantitative data biochemically assessed.

All PMNL activation parameters considered showed an early appearance and a greater intensity after incubation of PMNLs with terbinafine before exposing them to *C. albicans* blastospores. These *in vitro* observations suggest that *in vivo* terbinafine could act in two ways: by a direct antimycotic effect on ergosterol biosynthesis by the fungal cells and by an indirect effect via host PMNL priming.

This fact may explain why the activity of terbinafine against *Candida* cells observed in clinical studies is somewhat better than predicted by *in vitro* reported findings [3]. The exact mechanisms by which terbinafine induces priming of PMNLs need further experimental studies.

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