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Modification of neutrophil functions by naftifine

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Summary

Naftifine (NF), a topical antimycotic agent, is highly active *in vitro* and *in vivo* against a wide range of pathogenic fungi. NF inhibits human polymorphonuclear leucocyte (PMN) chemotaxis. Following stimulation with zymosan-activated serum, 85–97% of the PMNs exhibited detectable membrane ruffling and polarity. In contrast, NF-treated PMNs did not exhibit such chemotactic factor-induced shape changes. We also analysed the effect of NF on PMN superoxide anion (O_2^-) and chemiluminescence (CL) production, as a measure of respiratory burst activity. Stimulation of PMNs pre-incubated with NF (37°C for 30 min at 1–150 μg/ml) by FMLP, PMA and zymosan resulted in a dose-dependent inhibition in PMN CL. Doses of NF which depressed chemotaxis, inhibited CL and diminished O_2^- production in a statistically significant manner ($P<0{\cdot}05$–$0{\cdot}001$). In conclusion, NF alters membrane-related responses in PMNs, and this alteration may be associated with a change in PMN morphology. Binding of NF to PMN membrane sterol, with a subsequent alteration in membrane configuration, is the most likely cause of the inhibition of PMN function. The data collectively document biochemical and morphological differences between control and NF-treated PMNs as determined by stimulus-specific CL and O_2^- generation and membrane shape change. Such differences may account, in part, for its efficacy in inflammatory fungal skin diseases.

Several generations of topical antifungals have evolved over the past decade. Concomitant with this evolution was the recognition of the role of human polymorphonuclear leucocytes (PMNs) in acute inflammation associated with fungal infections.

A new generation of broad-spectrum synthetic antifungal agents, the allylamines, has recently been developed.[1] Naftifine ((E)-N-Methyl-N-(1-naphthylmethyl)-3-phenyl-2-propen-1-amine-hydrochloride), an allylamine, has exhibited therapeutic activity *in vitro* against a variety of organisms including dermatophytes and yeasts. NF appears to interfere with sterol biosynthesis by selectively inhibiting squalene 2,3 epoxidase, which results in decreased amounts of the principal fungal membrane sterols, especially ergosterol.[1,2]

The purpose of the present study was to investigate, *in vitro*, the possible anti-inflammatory effects of NF, and present preliminary data regarding the drug's effect on PMN chemotaxis and respiratory burst activity.

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Methods

Cell preparation

Heparinized peripheral venous blood was obtained from healthy donors, and allowed to sediment at room temperature over an equal volume of a methocel–isopaque density gradient, by a modification of the Boyüm method.[3] The sedimented cells were exposed to 0·87% NH_4Cl for 35 s at room temperature, in order to lyse the remaining erythrocytes. PMNs were washed in Hank's balanced salt solution (HBSS), without Ca^{2+} or Mg^{2+}, centrifuged at 500 ***g*** for 10 min, and then resuspended in HBSS with ions, supplemented with 0·4% bovine serum albumin (BSA; Sigma) (incubating medium). The concentration was adjusted to $2–5 \times 10^6$ PMN/ml in the incubating medium. Collected PMNs were greater than 95% pure, and 98% viable as assessed by the trypan blue dye exclusion assay.

Drug preparation

NF (Naftin®) was provided by Herbert Laboratories

(Santa Ana, CA, U.S.A.), and was prepared in stock solutions (12 mg/ml) in DMSO, and stored at −70°C until used. Tested cells were suspended in various concentrations of the drug (1–150 μg/ml). PMNs were pre-incubated for 30 min at 37°C in the incubating medium containing the drug or DMSO, before being tested for viability or function.

PMN activation

Membrane stimulants used included zymosan-activated serum (ZAS; 1 mg/ml [Sigma], serum opsonized zymosan (SOZ; 50 mg/ml), *f*-Met-Leu-Phe (FMLP; 10^{-6}–10^{-8} M) [Sigma], phorbol meristate ester (PMA; 1–1000 ng/ml) [Sigma] and latex particles (40 μl, 0·795 μm) [Seradyn]. Pooled human serum was used to activate and opsonize zymosan.[4]

1. *PMN chemotaxis.* PMN chemotaxis was evaluated by a modified Boyden chamber assay using blind well chambers with 5 μm pore size filters (Millipore).[5] A 200-μl cell suspension ($2·5 \times 10^6$ PMN/ml) in the incubating medium was layered on the top of the filter, and 100 μl of chemotactic factors was placed in the lower compartment. Following incubation at 37°C for 90 min, under a humidified atmosphere with 5% CO_2, the filters were fixed with propanol and stained with haematoxylin and eosin. The PMN chemotactic response was determined by the number of cells which migrated through the entire thickness of the filter. Migration distance was determined at ×400 magnification by the leading front of cells. The results were expressed as the mean number of cells/high-power field (PMN/HPF).

2. *Luminol-enhanced chemiluminescence (CL) assay.* The PMN–CL assay[4] was a modification of the method used by DeChatelet.[6] The assay was performed in polypropylene scintillation vials using a Beckman LS 100 beta liquid scintillation counter set on an out-of-coincidence mode. The counter was set on repeat cycle, and sequential 0·2 min counts (c.p.m.) were taken for each vial over a 24-min period.

3. *Measurement of superoxide anion production.* Production of superoxide anions (O_2^-) was measured as the superoxide dismutase-inhibitable reduction of ferricytochrome c.[7] The optical density of the supernatants at 550 nm was determined in a LKB spectrophotometer, and the O_2^- released was calculated using an extinction coefficient of $21·0 \times 10^3$ M^{-1} cm^{-1} for cytochrome c.[8] Superoxide release was expressed as nmoles $O_2^-/10^6$ PMN/15 min.

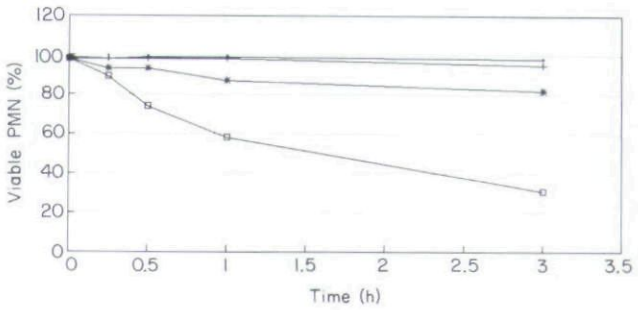

Figure 1. Effect of BSA on PMN viability. PMNs were incubated with either a low (50 μg/ml with BSA, ●; without BSA, +) or high (150 μg/ml with BSA, *; without BSA, □) concentration of NF at 37°C for 180 min. Cell viability tests were performed with the trypan blue exclusion assay at various time intervals.

Results

Viability data

PMNs ($1–4 \times 10^6$/ml) were incubated with either a low (50 μg/ml) or high (150 μg/ml) concentration of NF at 37°C for 180 min. Cell viability tests were performed with the trypan blue exclusion assay at various time intervals. As shown in Figure 1, selective cytotoxicity of PMNs was associated with the high concentraion of NF used in this study. The addition of BSA to the incubating medium increased the viability of PMNs by a factor of 2–3·5.

Chemotaxis and chemokinesis

We initially attempted to determine whether NF (at concentrations of 5–50 μg/ml), when incubated with PMNs, could inhibit PMN chemotaxis. Two tests were utilized. Both attempted to examine NF-treated PMNs for morphology and polarization changes at specific time periods, as well as depth of cell migration or the number of cells which had migrated (PMN/HPF).

In the first test we utilized a stained Millipore filter in a chemotactic chamber containing PMNs pretreated with NF at 5–50 μg/ml. In the second test, we examined a fresh PMN cell preparation in which a control PMN preparation was compared with PMNs incubated with 20 μg/ml of NF. The results with the stained Millipore demonstrated that NF inhibited PMN locomotion, and caused PMN membrane retraction and nuclear consolidation, thus having induced a 'lymphocyte-like' morphology of PMNs. Light microscopy (Fig. 2a and b) showed that PMNs treated with NF remained on the surface of the filter as non-polar cells. Phase-contrast microscopy (Fig. 2c and d) showed that the PMNs assumed a round shape with smooth surfaces, retracted

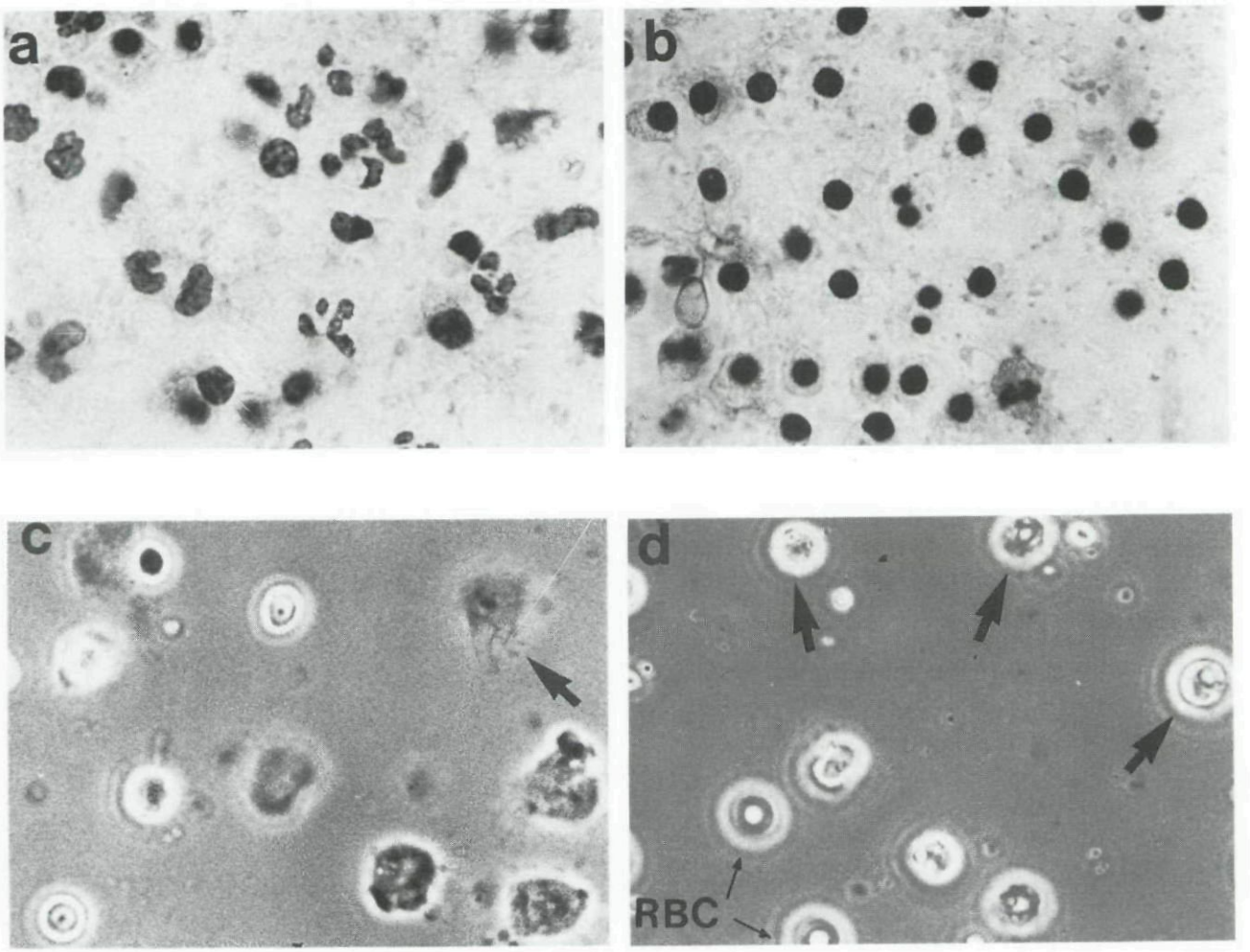

Figure 2. Effect of NF on PMN morphology—microscopic comparison. Microscopic detection of PMN activation induced by ZAS. (a) Control PMNs stimulated with ZAS showing the characteristic morphology of PMNs, with a multi-lobed nucleus (haematoxylin and eosin ×400). (b) NF-treated PMNs stimulated with ZAS displaying morphological changes, including compact nuclei and cytoplasm (haematoxylin and eosin ×400). (c) Control PMNs were polarized and adhered to the slide by developing pseudopodia-like protrusions (arrow) (phase contrast microscopy ×700). (d) NF-treated PMNs exhibited a 'lymphocyte-like' morphology (arrows) as noted by the presence of round cells with decreased cytoplasmic/nuclear ratio (phase-contrast microscopy ×700). RBC, red blood cells.

their plasma membrane, and exhibited a compact nucleus.

Figure 3 shows the chemotactic results obtained with PMNs incubated in various concentrations of NF compared with the controls obtained with cells in the incubating medium. Maximal inhibition occurred at 45 μg/ml, whereas no effect was detected at concentrations less than 5 μg/ml. The data suggest that NF inhibition of PMN-induced chemotaxis by ZAS and FMLP occurs in a dose-dependent manner. In the same concentration range, NF appeared to have a similar effect on chemokinesis (data not shown).

Chemiluminescence

PMNs were incubated with increasing concentrations of NF to determine the effect on CL production. The dose–response curve of NF was measured at concentrations of 5 and 30 μg/ml. As shown in Figure 4, interaction of FMLP (10^{-7} M) with PMNs produced a rapid burst of CL. A distinct CL response, with the characteristic two-peak pattern, was observed, which rapidly reached peak intensities of approximately 300,000 c.p.m. When PMNs activated with FMLP were added to NF (30 μg/ml), a significant decrease was noted in the peak CL

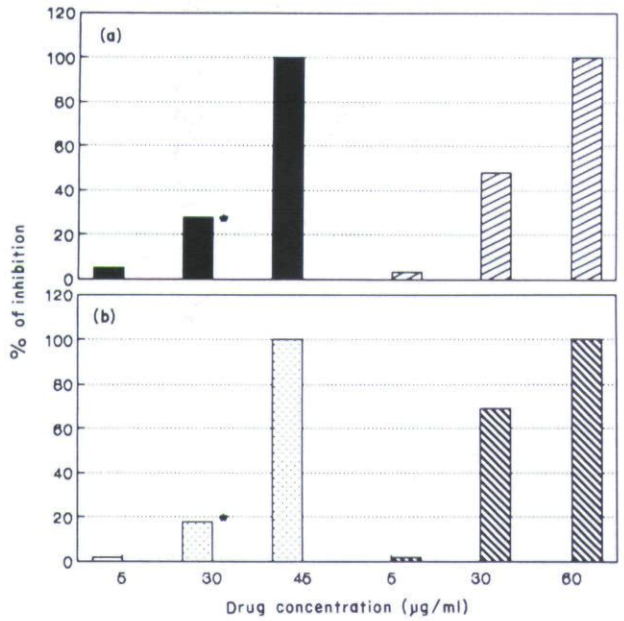

Figure 3. Comparison of chemotactic activity of PMNs induced by ZAS and FMLP. The inhibitory effect of NF on PMN chemotactic activity was compared as the percentage of inhibition in number of migrating PMNs/high-power field, as shown in (a) and in migrating distance as shown in (b), of five experiments *($P<0{\cdot}05$). ■ ZAS (PMNs/HPF); ▨ FMLP (PMNs/HPF); ⊡ ZAS (mμ); ▧ FMLP (mμ).

production, as well as the proportion of the area under the CL curve, compared with the control. Low concentrations of NF (5 μg/ml) did not have any significant effect with respect to the peak CL and CL profile. Similar results were obtained for SOZ, PMA and latex-induced

Table 1. Effect of NF on the oxidative burst in PMNs

Stimulant	Peak CL (log)		O_2^- released (nmol)	
Latex	5·82±0·56	5·03±0·87*	—	—
Opsonized Zy	5·54±0·31	3·12±0·28**	16·5±2·6	9·8±1·2**
PMA	5·11±0·38	4·03±0·27**	37·5±2·7	12·4±1·8**
FMLP	5·36±0·45	3·23±0·36**	17·8±1.9	5·5±0.3**

Measurements of CL peak maximum and O_2^- production are expressed as peak response in log and nmol of cytochrome c reduced/15 min/$2{\cdot}6\times10^6$ PMNs, respectively. Values represent the mean and standard error of three experiments performed in duplicate (±SEM). At a dose (30 μg/ml) similar to that necessary for depression of chemotactic activity, NF was demonstrated to be a statistically significant (*$P<0{\cdot}05$ to **$P<0{\cdot}001$) inhibitor of respiratory burst activity as measured by both CL and O_2^- producton.

PMNs (Table 1). As shown, pre-incubation of PMNs for 30 min with NF (30 μg/ml) effectively reduced stimulant-induced generation of peak CL by 0·5–2·5 log.

Superoxide anion

To evaluate the potential anti-inflammatory effect of NF, PMNs were incubated with 30 μg/ml of NF. The effect of NF on O_2^- generation by FMLP- and PMA-stimulated PMNs, as determined by the cytochrome c assay, is summarized in Table 1. When FMLP (10^{-6}–10^{-8} M) was used, O_2^- production of NF-treated PMNs was significantly reduced. NF interfered with the O_2^- production

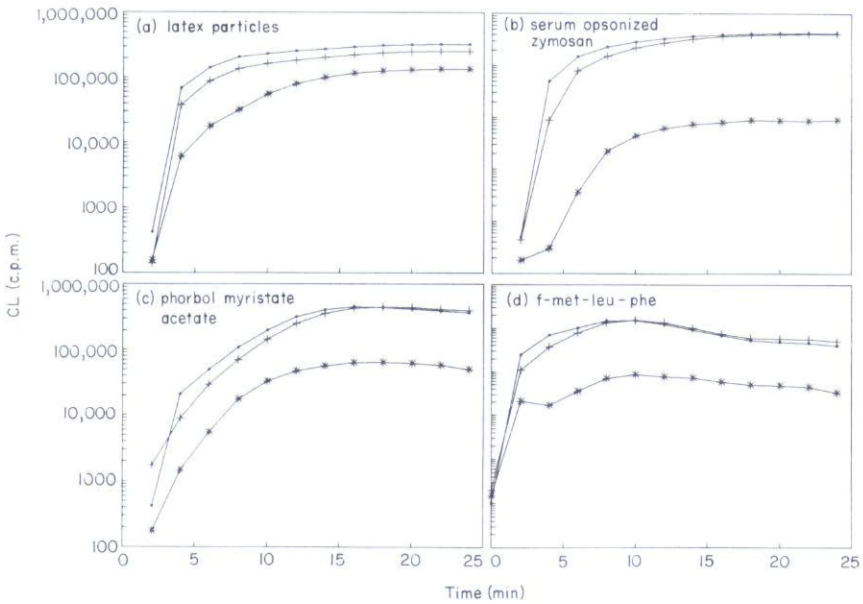

Figure 4. Effect of NF on stimuli-induced PMN CL. The graphs display representative temporal curves for the stimulation of CL by a series of soluble or particulate stimulants. —●—, control; —+—, NF-5 μg/ml; —*—, NF-30 μg/ml.

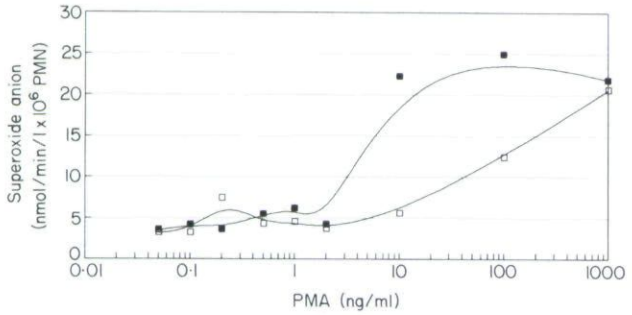

Figure 5. Inhibition of O_2^- production by NF-treated PMNs. O_2^- values from the control and NF-treated PMNs were plotted against various concentrations of PMA. The graph shows dose–response curves from the stimulation of O_2^- production induced by various PMA concentrations. —■—, control PMNs; —□—, NF-treated PMNs.

induced by PMA but only at a concentration range of 10–100 ng/ml (Fig. 5).

Discussion

Phagocytic stimuli or high concentrations of chemoattractants in the skin stimulate PMNs, causing PMN chemotaxis, increased oxygen consumption, and the production of large amounts of microbial oxidants. This process, called the PMN respiratory burst, results in the reduction of molecular oxygen to O_2^-.[9] The O_2^- is rapidly converted to H_2O_2 and hydroxyl radicals, which provide most of the microbicidal oxidative activity within the phagosome and extracellular environment.

Effective methods of fungal treatment, therefore, not only require the use of appropriate antifungals, but must also result in a reducton in excess PMN accumulation and/or activity within the host tissue, in order to reduce host inflammatory tissue damage, and promote healing.

NF is a member of the allylamines, a new class of synthetic broad-spectrum antifungal agents. NF selectively inhibits squalene epoxidase. As a result of this inhibition, the principal fungal membrane sterol, ergosterol, can no longer by synthesized. A similar effect on the synthesis of PMN cell membranes is a possibility.

We have shown that the topical antimycotic NF has several important effects on the normal functions of PMNs, i.e. the inhibition of local PMN chemotaxis, and the respiratory burst of PMNs.

Our results suggest that NF, through an unknown mechanism, inhibits PMN pseudopodia extension and hence locomotion, and does so in a dose-dependent manner. Furthermore, NF inhibits CL activity and O_2^- generation. CL is the result of activation of the PMN respiratory burst, which is associated with the production and release of potentially toxic oxygen metabolites including O_2^-, H_2O_2, hydroxyl radical, and singlet oxygen. The light emission is dependent on the interaction of O_2^- (and possibly other oxidizing agents) with the particles used as stimuli.[10,11] We found that NF at a concentration of 5 μg/ml, when compared with controls, did not exhibit a significant effect on the CL profile or peak. At a concentration of 30 μg/ml, however, a statistically significant ($P<0{\cdot}001$–$0{\cdot}05$) reduction in the CL profile, as measured by CL peak and percentage of the area under the CL control curve (data not shown), was observed. The inhibition was over the narrow range of 20–45 μg/ml; concentrations equivalent to those MIC found for *C. albicans*, but considerably higher than the MIC used for the treatment of dermatophytes, aspergillus and sporothrix infections.[1,12]

The available data from our experiments suggest that NF may have an effect on stimulant-induced PMN responses by altering: (i) membrane viscosity,[13] (ii) recognition of membrane receptors,[14,15] and/or (iii) ligand-mediated transduction events.[16] These mechanisms may provide a subcellular basis for the decreased oxidative metabolism which may block the generation of CL and other reactive oxygen species. We are also investigating the possibility that the alteration of several PMN functions by NF is associated with inhibition of microtubule assembly, similar to that seen with vitamin K.[17]

The demonstrated effects of NF on PMNs, in many respects, are similar to the actions of certain cationic local anaesthetics (e.g. tertiary amines, such as tetracaine) and vitamin K which have been shown to cause smoothing of the cell surface, and reduction in both lysosomal enzyme release and O_2^- production.[18,19] Also, the inhibition of PMN respiratory burst has been previously reported with NSAIDs such as aspirin, piroxicam and flunoxaporafen, and as with NF, their mechanism of action has not been elucidated.[15,20,21] Currently, we are attempting to discern the effects NF may have on PMN membrane function and intracellular reactions, in an attempt to provide a working mechanism for some of the complex interactions between NF and PMNs.

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