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Effect of Amorolfine (Ro 14-4767/002) on in vitro Phagocytosis and Intracellular Killing of *Candida albicans* by Human Neutrophils

Die Wirkung von Amorolfin (Ro 14-4767/002) in vitro auf Phagozytose und intrazelluläre Abtötung von *Candida albicans* durch menschlische Neutrophile

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Schlüsselwörter: *Candida albicans* – Neutrophile – Antimyzetika – Amorolfin

Summary: The effect of amorolfine (Ro 14-4767/002) on phagocytosis and intracellular killing of *Candida albicans* blastospores was determined in human neutrophil monolayer assays. At 0.2, 2 and 5 μg/ml amorolfine did not have any significant inhibitory or enhancing effect on phagocytosis whether following simultaneous addition of blastospores and drug to the neutrophils, prior treatment of neutrophils for 2 h before addition of blastospores or prior treatment of blastospores for 2 h. Simultaneous addition of amorolfine resulted in a significant increase in killing at all concentrations. This increase was not significantly enhanced by either preincubation of neutrophils or blastospores for 2 h with the drug.

Zusammenfassung: Es wurde die Wirkung von Amorolfin (Ro 14-4767/002) auf die Phagozytose und die intrazelluläre Abtötung von *Candida albicans*-Blastosporen in Monolayern menschlischer Neutrophiler untersucht. Bei 0,2, 2 und 5 μg/ml Amorolfin wurde keine signifikante Hemm- oder Förderwirkung auf die Phagozytose beobachtet, und zwar weder bei simultaner Zugabe von Wirkstoff und Blastosporen zu den Neutrophilen noch bei 2 h Amorolfin-Vorbehandlung der Neutrophilen oder der Blastosporen. Die Simultanzugabe von Amorolfin bewirkte jedoch einen Anstieg der Abtötungsrate bei allen Konzentrationen. Diese wurde durch 2 h Präinkubation der Neutrophilen oder der Blastosporen mit Amorolfin nicht mehr signifikant gesteigert.

Introduction

The actions of antimicrobial agents on host defence mechanisms are largely ignored in clinical practice, although a wide range of effects (determined mostly in experimental systems), on both cell-mediated and humoral immunity have been recognised (1). The relevance of many of these findings is uncertain since antimicrobial chemotherapy is often short-lived. However, certain infections require prolonged therapy. Immuno-

logical mechanisms play an integral part in the pathogenesis of fungal infections and are also important for recovery. Even a marginal influence on host response may have a significant effect on outcome. However, the literature on the interaction between antifungal drugs and host defence is still limited and often contradictory but the most relevant data concern the effects of these agents on the inflammatory response, in particular the function of neutrophils (2, 11). From an understanding of the major mechanisms used by neutrophils to ingest and kill fungal cells, it is clear that antimicrobial drugs could modify the interaction between phagocytic cells and fungi at each step of the phagocytic process. Although some antifungal agents may have direct effects, on neutrophils for example, it is more likely that those drugs which specifically alter the fungal cell surface can most profoundly influence the fate of a fungal cell within a host effector cell.

Alterations in the cell wall may favourably affect attachment to neutrophil membranes or more likley enhance intracellular killing.

In previous studies using a monolayer assay it has been shown that polyene and imidazole antifungals potentiate the function of neutrophils against *Candida albicans* by pre-exposure of blastospores to the antifungals prior to serum opsonization, phagocytosis and intracellular killing of the yeast (5). We have extended these studies by looking at amorolfine (Ro 14-4767/002), a new compound in a class of phenylpropyl-piperadine and phenylpropyl-morpholine derivatives (4), which has been shown to have high activity against *C. albicans* in experimental superficial and systemic infections (7, 8) and causes ultrastructural changes of the cell wall similar to that seen with imidazole compounds (6).

In the present study, the effect of amorolfine on phagocytosis and intracellular killing of *C. albicans* was investigated following pretreatment of blastospores and neutrophils.

Materials and Methods

Inoculum

A strain of *Candida albicans* was selected for the formation of singlet blastospores and grown as a lawn on 4 % malt agar. Cultures of 2 d growth were emulsified in sterile water and then diluted into Hanks Balanced Salt Solution (HBSS), pH 7.5. Before use, viabilitiy was assessed by means of a methylene blue dye (0.025 % w/v in water) exclusion technique and in all cases was found to be > 95 %.

Antifungal drug solution

Ro 14-4767/002 was prepared as a 5000 µg/ml stock solution in sterile water and stored for up to two weeks at −20 °C. Drug dilutions of amorolfine were prepared in HBSS. Final treatment solutions contained concentrations of 0.2., 2 and 5 µg/ml.

Preparation of neutrophil monolayers

Neutrophil monolayers were prepared by a method similar to that described previously (5, 9), by obtaining drops of blood from a finger tip puncture and placing these on untreated, dust-free coverslips (16 mm diameter). These were incubated at 37 °C in a humidified atmosphere. After 45 min the coverslips were immersed in 0.85 % w/v saline kept at 37 °C and agitated to remove clots and adherent erythrocytes. This procedure was repeated in fresh warm saline. The result was monolayers of neutrophil phagocytes. Viability of the phagocytic monolayer was assessed after the final incubation step by trypan blue dye exclusion (0.16 % w/v) and in all cases was found to be > 97 %. The average area of the monolayers was about 0.138 cm^2 and represented 900−1100 neutrophils.

Autologous serum

Autologous human serum as a source of

Table 1: Effect of Ro14-4767 on phagocytosis and killing of *C. albicans* by human neutrophil PMN cells

Drug Concentration (μg/ml)	Simultaneous addition of drug			Pre-treated phagocytes			Pre-treated blastospores		
	phagocytosis (%)	phagocytic index	killing (%)	phagocytosis (%)	phagocytic index	killing (%)	phagocytosis (%)	phagocytic index	killing (%)
Control	31 ± 5	1.21 ± 0.1	7 ± 5	44 ± 7	1.40 ± 0.1	12 ± 6	34 ± 5	1.18 ± 0.04	10 ± 6
0.2	39 ± 5	1.25 ± 0.1	22 ± 5	45 ± 7	1.50 ± 0.1	26 ± 6	40 ± 5	1.23 ± 0.04	28 ± 6
2	42 ± 5	1.30 ± 0.1	31 ± 5	49 ± 7	1.35 ± 0.1	40 ± 6	40 ± 5	1.11 ± 0.04	36 ± 6
5	40 ± 5	1.26 ± 0.1	40 ± 5	49 ± 8	1.30 ± 0.1	48 ± 7	36 ± 5	1.15 ± 0.04	38 ± 6

Figures are mean ± S.E.M. adjusted for small samples.

Statistical Analysis

Simultaneous addition: significant difference, for increased intracellular killing only, at all concentrations ($p < 0.05$).
significant difference between 0.2 μg/ml and 5 μg/ml ($p < 0.05$),

Pre-treated phagocytes: significant difference, for increased intracellular killing only, 0.2 μg/ml ($p < 0.05$), 2 and 5 μg/ml ($p < 0.02$).

Pre-treated blastospores: significant difference, for increased intracellular killing only, 0.2 μg/ml ($p < 0.05$), 2 μg/ml ($p < 0.02$), 5 μg/ml ($p < 0.01$).

opsonizing complement was stored in the gaseous phase of liquid nitrogen. In all cases serum was warmed to 37 °C before addition to the neutrophil monolayers.

Treatment of neutrophil monolayers with amorolfine prior to blastospore ingestion

Coverslips were placed in the wells of square plastic tissue culture dishes, monolayer side up, covered with 1 ml aliquots of HBSS to which various concentrations of drug had been added, and incubated for 2 h at 37 °C before addition of the blastospore suspensions.

Treatment of blastospore suspensions with amorolfine prior to addition to monolayers

Having previously determined that the strain of *C. albicans* used did not produce germ-tubes within the incubation period, blastospore suspensions (5×10^5 per ml) were incubated in 1 ml volumes of HBSS with or without antifungal at 37 °C for 2 h.

Assessment of blastospore ingestion and subsequent survival in neutrophils

After washing, coverslips with monolayers were placed in the wells of a square plastic tissue-culture dish. For the pre-treated monolayers a concentrated blastospore suspension was added to the HBSS in the wells to give a final blastospore concentration of 1×10^5 cells/ml. This constituted a ratio of ten blastospores to one neutrophil. In the case of simultaneous addition of drug and blastospore suspension, 1 ml aliquots of HBSS with or without drug were warmed to 37 °C and inoculated with 1×10^5 blastospores/ml just prior to addition to the monolayer.

Pre-warmed (37 °C) autologous serum (100 μl) was added to each well before incubation at 37 °C for 20 min. Ingestion was visualised by light microscopy. To determine that blastospores were intracellular and not just adhering to the phagocyte membrane, monolayers were stained with fluorescein-labelled concanavalin A which binds only to extracellular organisms (10).

After the ingestion period the monolayers were transfered into fresh, pre-warmed 1 ml aliquots of HBSS and incubated for a further 60 min to allow time for killing of blastospores without further ingestion.

Following incubation, the viability of ingested blastospores was assessed by a modified methylene blue dye exclusion method (5). The coverslips were rinsed in warm saline to remove any remaining extracellular blastospores. Each monolayer was then flooded with 0.025% (w/v) sodium deoxycholate (SDC) for 15 sec. After this time excess SDC was removed and replaced with 0.02% (w/v) methylene blue in distilled water. After 3 min, excess stain was removed and the coverslips were mounted, monolayer side down, over well slides and examined. Live blastospores remained colourless or faintly green while dead cells took up the dye to stain uniformly blue. The nuclei of the neutrophils stained purple.

Monolayers were examined within 5 min of staining. Ten fields of view were observed at each drug concentration and the numbers of intracellular live and dead blastospores were recorded. The proportion of neutrophils that contained blastospores (% phagocytosis), the mean number of blastospores per infected neutrophil (phagocytic index) and the proportion of live to dead blastospores (% killing) were calculated. At least 3 separate tests were performed for each drug regimen at each concentration to provide mean and standard deviation values.

Statistical Analysis

An analysis of variance was performed on the results with adjustments for the small sample standard error of the mean.

Results

At any of the concentrations used, amorolfine did not have any significant effect on the phagocytosis of *C. albicans* whether following simultaneous addition, prior treatment of the neutrophils for 2h or prior treatment of blastosphores for 2h.

Simultaneous addition of amorolfine resulted in a significant increase in killing at all concentrations ($p < 0.05$). This increase was not significantly enhanced by either preincubation of monoalyers or blastospores for 2h with the drug (Table 1).

Discussion

This investigation has demonstrated that amorolfine does not have an effect on neutrophil phagocytosis. No clear examples of direct phagocytic stimulation by antimicrobial agents have been reported (1). In the present study it is apparent that amorolfine does not affect cell wall biosynthesis in a way that enhances opsonization or attachment to neutrophil membrane receptors. Unlike bacterial virulence factors, it is not known whether *C. albicans* expresses virulence factors similar to the M protein of *Streptococcus pyogenes* which is repressed by subinhibitory concentrations of clindamycin (3). In previous studies it has been shown that blastospores of strains of *C. albicans* differing in virulence are readily ingested by host effector cells in the presence of an opsonin and a range of antifungal compounds (5). It is also apparent from the present study that receptors for complement or other serum opsonins are not damaged or masked by pretreatment of blastospores with amorolfine.

A general trend for increased killing with higher drug concentrations was observed although large replicate variance rendered only the following to be significant; an effect most probably due to weakening of the blastospore wall or repression of the initial stages of germ tube formation. Amorolfine enhanced intracellular killing of *C. albicans* at all drug concentrations with the simultaneous addition experiments. This effect was also observed with pre-treated neutrophils and blastospores as would be expected with

the mimicry of the simultaneous test inherent within both the pretreatment experiments.

In conclusion, the lack of adverse effects on neutrophil function in the presence of amorolfine is encouraging. Furthermore, the enhancement of killing to organisms pretreated with this drug in vitro may indicate a mechanism where the action of amorolfine is potentiated in vivo.

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