

Effects of Amphotericin B and Fluconazole on the Extracellular and Intracellular Growth of *Candida albicans*

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The effects of amphotericin B and fluconazole on the extracellular and intracellular growth of *Candida albicans* were studied. With respect to the extracellular growth of *C. albicans*, antifungal activity was measured in terms of MICs and minimal fungicidal concentrations as well as by determination of the concentration that effectively killed (>99.9%) *C. albicans* in the absence or presence (amphotericin B only) of serum. Amphotericin B was highly active in terms of killing, even at an increased inoculum size. In the presence of serum, amphotericin B activity was substantially reduced. For fluconazole, activity was restricted to inhibition of fungal growth, even after the inoculum size was reduced. With respect to the intracellular growth of *C. albicans*, antifungal activity was measured by using monolayers of murine peritoneal macrophages infected with *C. albicans* and was measured in terms of inhibition of germ tube formation as well as effective killing (>99%) of *C. albicans*. Amphotericin B was highly active against *C. albicans*. At an increased ratio of infection, amphotericin B activity was slightly reduced. Fluconazole had no antifungal activity. Neither a reduction in the ratio of infection nor exposure of *C. albicans* to fluconazole prior to macrophage ingestion resulted in activity against intracellular *C. albicans* by fluconazole. Previous exposure of *C. albicans* to amphotericin B resulted in increased intracellular activity of amphotericin B. The intracellular antifungal activity of the combination of fluconazole with amphotericin B was less than that of amphotericin B alone. Amphotericin B showed fungicidal activity against *C. albicans* growing both extracellularly and intracellularly, whereas fluconazole inhibited growth only of extracellular *C. albicans*. A slight antagonistic effect between fluconazole and amphotericin B was found with respect to intracellular as well as extracellular *C. albicans*.

Deep-seated candidal infections are an important cause of morbidity and mortality in immunocompromised patients (15, 21, 22). The current drug of choice for most systemic mycoses is still amphotericin B (AmB), but its use is restricted by a variety of toxic side effects (6, 13, 15, 21, 22). Consequently, there is a need for effective and less toxic drugs for the treatment of patients with these infections. One of these antifungal agents is the triazole fluconazole (Flu) (4, 10, 14, 33, 34, 41). In vivo experimental studies suggested that Flu is active against disseminated candidiasis (8, 9, 29, 31, 32, 35, 37). Recently, the antifungal efficacy of Flu was demonstrated in persistently granulocytopenic rabbits when it was used for prevention or early treatment (39, 40). Little is still known, however, on the role of Flu in the treatment of systemic candidiasis in immunocompromised patients. Since the host defense mechanisms in these patients are severely impaired, the intrinsic antifungal activity of the antifungal agent is of great importance for effective treatment. Unfortunately, standardized in vitro methods for assessment of the intrinsic activity of antifungal agents are not available. In addition, most of the in vitro methods that are used are criticized because of the poor correlation with in vivo antifungal activity (5, 6, 11, 12, 18, 27, 28, 36).

To gain more insight into the intrinsic antifungal activity of AmB and Flu, the activities of both agents against extracellular *Candida albicans* in relation to inoculum size were investigated in this study. Known antagonizing factors for the assessment of in vitro antifungal activity were excluded

by choosing the recommended optimal test conditions for each agent.

The in vivo efficacy of an antifungal agent is determined not only by its intrinsic antifungal activity but also by factors that influence the bioavailability of the agent, such as protein binding and intracellular penetrating capacity. Therefore, the effect of serum on the antifungal activity against extracellular *C. albicans* was investigated. Next, the antifungal activities of AmB and Flu against intracellular *C. albicans* were studied. Activity was determined in relation to the ratio of infection. In addition, the effect of exposure of *C. albicans* to one of the antifungal agents prior to macrophage ingestion as well as the effect of a combination of both antifungal agents on intracellular *C. albicans* were investigated.

MATERIALS AND METHODS

Candida strains. *C. albicans* ATCC 44858 was used in the experimental studies. *C. albicans* ATCC 28516 and *C. kefyr* (formerly *C. pseudotropicalis*, Carshalton strain; Pfizer Code Y0601; kindly provided by Pfizer Central Research, Sandwich, England) were used as reference strains in the MIC and minimal fungicidal concentration (MFC) determinations with AmB and Flu, respectively. The yeasts were maintained at -80°C in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) containing 10% (vol/vol) glycerol.

Antifungal agents. AmB (Fungizone for intravenous infusion; Bristol Myers-Squibb, Woerden, The Netherlands) was reconstituted with 10 ml of distilled water to give a standard solution of 5 mg/ml. Further dilutions were made in distilled water. Flu was kindly provided by Pfizer Nederland B.V. as a standard solution of 2 mg/ml in saline (Flu for

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intravenous infusion; Pfizer France). Further dilutions were made in phosphate-buffered saline (PBS).

Antifungal activity of AmB versus that of Flu against extracellular *C. albicans*. (i) MIC and MFC. Stationary-growth-phase cultures were obtained after incubation of the yeasts in Sabouraud maltose broth (Difco Laboratories) for 24 h at 37°C. Yeasts were washed in PBS, counted in a hemacytometer, and adjusted to the desired inoculum in assay medium. For experiments with AmB, the assay medium was Antibiotic Medium no. 3 (Penassay broth; Difco Laboratories), whereas for Flu the assay medium was high-resolution medium (kindly provided by Pfizer Central Research). At twofold increasing inocula ranging from 1.3×10^4 to 1×10^5 CFU/ml, the MICs and MFCs of AmB and Flu at twofold increasing concentrations ranging from 0.025 to 102 µg/ml were determined. In order to ensure accurate determination of the 99.9% endpoint, the MFC was determined after a total volume of 1.0 ml was subcultured onto Sabouraud dextrose agar (Oxoid, Basingstoke, England). Before plating, the concentrations of AmB and Flu in these specimens were reduced to an inactive level by washing the samples three times with PBS.

(ii) Short-term growth. Logarithmic-growth-phase cultures were prepared by reincubation of a stationary-phase inoculum in assay medium for 5 h under continuous agitation at 37°C. The desired inoculum was obtained after dilution of this *C. albicans* suspension in assay medium at 37°C. The extracellular activity of the drug in terms of effective killing (>99.9%) of *C. albicans* during 6 h of incubation was determined. At eightfold increasing inocula, ranging from 1.3×10^4 to 8.0×10^5 CFU/ml for AmB and from 2.0×10^2 to 1.3×10^4 CFU/ml for Flu, the extracellular activity of AmB versus that of Flu was assessed at various concentrations ranging from 0.05 to 12.8 µg/ml for AmB and 0.4 to 102 µg/ml for Flu. The controls contained only the solvent of the antifungal agent in the appropriate dilution. During incubation, under continuous rotation at 8 rpm at 37°C and protected from light, the numbers of viable organisms were determined at 2-h intervals by making plate counts of 10-fold serial dilutions of the washed specimen on Sabouraud dextrose agar. Additionally, at the concentrations that effected >99.9% killing of *C. albicans*, the effect of serum on the activity of the antifungal agent was measured. At an inoculum of 1.3×10^4 CFU/ml, *C. albicans* was exposed during 6 h of incubation to antifungal agent in assay medium supplemented with various amounts (5, 50, and 90% [vol/vol]) of pooled human serum (PHS; from a pool of 350 serum samples from healthy volunteer donors). Because the presence of serum induces the formation of germ tubes by *C. albicans*, the antifungal activity was expressed not only in terms of effective killing (>99.9%) of *C. albicans* but also in terms of inhibition of germ tube formation, which was determined microscopically in a hemacytometer.

Antifungal activity of AmB versus that of Flu against intracellular *C. albicans*. (i) Activity in relation to ratio of infection. Macrophages were obtained from peritoneal cavities of 10- to 13-week-old specific-pathogen-free BALB/c mice (Iffa Credo, L'Arbresle, France). The macrophages were washed twice in Dulbecco modified Eagle medium (Flow Laboratories, Irvine, Scotland) supplemented with 1% glutamine. Monolayers of peritoneal macrophages were cultured at 37°C on chamber slides (Lab-Tek, Bayer, The Netherlands) under a humidified atmosphere of 7.5% CO₂ in air in culture medium containing Dulbecco modified Eagle medium supplemented with 1% glutamine and 15% fetal bovine serum (FBS; Hy-Clone, Logan, Utah). After the first

2 h of incubation, fresh culture medium was added to the monolayer, and the macrophages were incubated for 24 h. After this incubation period, various inocula of opsonized *C. albicans* were added to the monolayers, resulting in increasing *C. albicans*-to-macrophage ratios ranging from 1:40 to 5:8. *C. albicans* was opsonized by incubation of a washed stationary-growth-phase suspension with 10% normal mouse serum (BALB/c) under continuous rotation at 8 rpm for 15 min at 37°C, and then the yeasts were washed twice in PBS. After a 30-min uptake period, the noningested yeasts were removed by washing the monolayer three times with Dulbecco modified Eagle medium supplemented with 1% glutamine and 5% FBS at 37°C (time zero). The macrophages were reincubated for 24 h in the presence of twofold increasing concentrations of the antifungal agent in culture medium supplemented with 5% FBS; the concentrations ranged from 0.05 to 1.6 µg/ml for AmB and 0.4 to 102 µg/ml for Flu. Control monolayers were incubated with the solvent of the antifungal agent in the appropriate dilution. Parameters for intracellular antifungal activity were inhibition of germ tube formation as well as killing of intracellular *C. albicans* (>99% of the number of viable intracellular yeasts at time zero). Inhibition of germ tube formation was determined microscopically. At 6 and 24 h of incubation, after the monolayer was washed three times with PBS, the monolayers were fixed in methanol and stained with May-Grünwald-Giemsa, after removal of the plastic chamber from the slide. At the concentrations that inhibited germ tube formation, intracellular killing was determined by washing the monolayers three times with ice-cold (0°C) PBS; this was followed by disruption of the macrophages by quickly freezing and thawing them in the presence of distilled water containing 0.1% bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.). Suspensions were mixed vigorously. Freezing and thawing did not affect the viability of *C. albicans*. The number of viable intracellular yeasts was determined by making plate counts of 10-fold serial dilutions of the specimen.

The toxicities of AmB up to 1.6 µg/ml and Flu up to 102 µg/ml to the macrophages were assessed by determining the effects of the agents on the phagocytic capacity and the membrane integrity of the macrophages. The effects of the antifungal agents on the phagocytic capacity of the macrophages were determined after 24 h of exposure of uninfected macrophage monolayers to the antifungal agents. Control monolayers were incubated with the solvent of the antifungal agent in the appropriate dilution. After the monolayer was washed three times, opsonized *Escherichia coli* 128K⁻ was added to the monolayers. After a 30-min uptake period, the uningested bacteria were removed by washing the monolayer and the number of viable intracellular bacteria was determined. The effects of the antifungal agents on the membrane integrity of the macrophages was determined by ⁵¹Cr release. Uninfected macrophage monolayers were labeled during 1 h with Na₂⁵¹CrO₄ (Amersham International plc., Buckinghamshire, England). After labeling, the monolayer was washed to remove non-cell-associated ⁵¹Cr; this was followed by 6 h of exposure to the antifungal agents. Control monolayers were incubated with the solvent of the antifungal agent in the appropriate dilution. By this method, exposure to antifungal agent for 24 h could not be tested because of the high spontaneous ⁵¹Cr release, because of passive labeling, from the control monolayers after 24 h. After incubation, ⁵¹Cr release was determined by measuring the radioactivity in the supernatants as well as in the

disrupted monolayers in a gamma counter (Minaxy 5530; Packard Instrument Co. Inc., Downers Grove, Ill.).

(ii) **Effect of previous exposure of *C. albicans* to antifungal agent.** Monolayers of peritoneal macrophages were cultured as described above. After 24 h of incubation, opsonized *C. albicans* was added to the monolayers, resulting in a *C. albicans*-to-macrophage ratio of 1:8, together with twofold increasing concentrations of the antifungal agent in culture medium supplemented with 5% FBS; antifungal agent concentrations ranged from 0.05 to 1.6 $\mu\text{g/ml}$ for AmB and 0.4 to 102 $\mu\text{g/ml}$ for Flu. After a 30-min uptake period, the monolayer was washed to remove uningested yeasts and antifungal agent and was reincubated for 24 h in the presence of antifungal agent. Control monolayers were reincubated with the solvent of the antifungal agent in the appropriate dilution. Intracellular antifungal activity was determined at 6 and 24 h of incubation, as described above.

Antifungal activity of a combination of AmB and Flu against intracellular *C. albicans*. Monolayers of peritoneal macrophages were cultured as described above. After 24 h of incubation, opsonized *C. albicans* was added to the monolayers, resulting in a *C. albicans*-to-macrophage ratio of 1:8. After a 30-min uptake period, the uningested yeasts were removed by washing the monolayer. The macrophages were reincubated for 24 h in the presence of combinations of the antifungal agents in culture medium supplemented with 5% FBS; antifungal agent concentrations ranged from 0.1 to 1.6 $\mu\text{g/ml}$ for AmB and 0.4 to 102 $\mu\text{g/ml}$ for Flu. Control monolayers were reincubated with the solvent of the agent in the appropriate dilution. Intracellular antifungal activity was determined at 6 and 24 h of incubation, as described above. The effect of combinations of AmB and Flu on the extracellular growth of *C. albicans* during 24 h of incubation was tested, after the addition of fourfold increasing Flu concentrations ranging from 0.4 to 102 $\mu\text{g/ml}$ to AmB at 0.2 $\mu\text{g/ml}$, by the method described above.

RESULTS

Antifungal activity of AmB versus that of Flu against extracellular *C. albicans* in relation to inoculum size. (i) **MIC and MFC.** At a *C. albicans* inoculum of 1.3×10^4 CFU/ml, the MIC and MFC of AmB were 0.1 and 3.2 $\mu\text{g/ml}$, respectively, and did not change at an inoculum size increased to 1.0×10^5 . The MIC of Flu was 0.8 $\mu\text{g/ml}$ and could be assessed only by using the lowest inoculum (1.3×10^4 CFU/ml). Flu did not show fungicidal activity when it was tested up to 102 $\mu\text{g/ml}$.

(ii) **Short-term growth.** The survival of *C. albicans* at various concentrations of AmB or Flu during 6 h of incubation at 37°C is shown in Fig. 1. Because the MIC of Flu could be assessed only at 1.3×10^4 CFU/ml, the antifungal effect on the short-term growth of *C. albicans* was initially determined at this inoculum size. With AmB, killing of *C. albicans* (>99.9%) was obtained at 0.2 $\mu\text{g/ml}$. Killing was not affected by an increase in the inoculum by 8- or 64-fold, respectively (data not shown). Flu tested up to a concentration of 102 $\mu\text{g/ml}$ did not result in the killing of *C. albicans* but only inhibited fungal growth. A reduction in the inoculum size by 8- or 64-fold, respectively, did not result in the killing of *C. albicans* (data not shown).

The effect of serum on the antifungal activity of AmB against extracellular *C. albicans* is presented in Table 1. The minimal AmB concentrations required for the inhibition of germ tube formation as well as for effective killing (>99.9%) of *C. albicans* in the presence of serum are given. Flu was

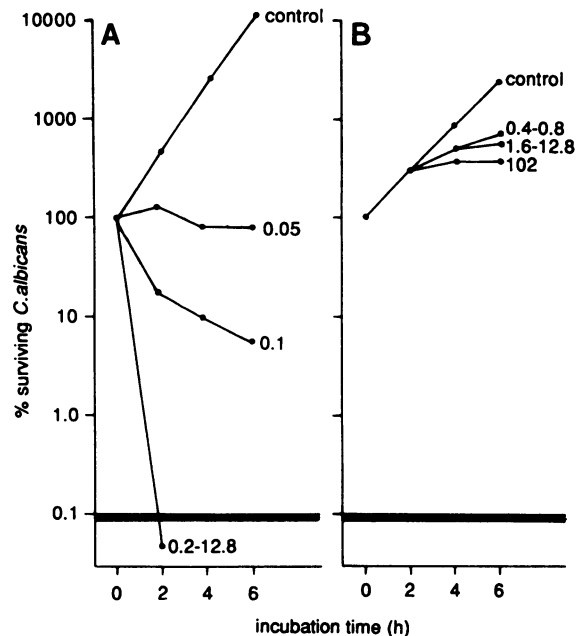


FIG. 1. Antifungal activity of AmB (A) and Flu (B) at the indicated concentrations (in micrograms per milliliter) against extracellular *C. albicans* during short-term growth at an inoculum size of 1.3×10^4 CFU/ml in the logarithmic phase of growth. The control contained only the solvent of the antifungal agent at the appropriate dilution. Each symbol represents the mean of three experiments.

not tested, since it is known that protein binding is low for Flu. In the presence of 5% PHS, inhibition of germ tube formation as well as effective killing were observed at an AmB concentration of 0.2 $\mu\text{g/ml}$. An increase in the amount of serum did result in an increase in the concentration of AmB required to obtain the effects. Eventually, compared with the effect of 5% PHS, the presence of 90% PHS resulted in a 4-fold increase in the concentration required to inhibit germ tube formation, whereas a 16-fold increase in concentration was needed to obtain effective killing.

Antifungal activity of AmB versus that of Flu against intracellular *C. albicans*. (i) **Activity in relation to ratio of infection.** The minimal concentrations of AmB and Flu required for the inhibition of germ tube formation as well as for effective killing (>99.9%) of *C. albicans* at 6 and 24 h of incubation at various ratios of infection are given in Table 2. In the absence of antifungal agent, *C. albicans* grows intracellularly by the formation of germ tubes, and within 24 h

TABLE 1. Effect of serum on the antifungal activity of AmB against extracellular *C. albicans* during 6 h of incubation^a

Medium	Minimal AmB concn ($\mu\text{g/ml}$) required for:	
	Inhibition of germ tube formation	Effective killing (>99.9%)
Serum-free		0.2
PHS		
5%	0.2	0.2
50%	0.4	1.6
90%	0.8	3.2

^a *C. albicans* was used at an inoculum size of 1.3×10^4 CFU/ml. Percentages of PHS are by volume.

TABLE 2. Antifungal activity of AmB versus that of Flu after 6 and 24 h of incubation against intracellular *C. albicans* in relation to ratio of infection^a

Antifungal agent and <i>C. albicans</i> /macrophage ratio	Minimal concn (µg/ml) required for:			
	Inhibition of germ tube formation		Effective killing (>99%)	
	6 h	24 h	6 h	24 h
AmB				
1:8	0.2	0.2	0.4	0.4
5:8	0.4	0.4	0.8	0.8
Flu				
1:8	>102	>102	ND ^b	ND
1:40	>102	>102	ND	ND

^a Antifungal agent was present in the incubation medium after *C. albicans* ingestion.

^b ND, not done.

mycelium is formed. At a *C. albicans*-to-macrophage ratio of 1:8, inhibition of germ tube formation and killing were observed within the first 6 h of incubation at AmB concentrations of 0.2 and 0.4 µg/ml, respectively. After prolonged incubation (24 h), this activity was not changed. At a fivefold increased ratio of infection, twofold increased AmB concentrations were required to obtain both effects. Flu tested up to 102 µg/ml was not effective intracellularly during 6 h of incubation. After prolonged incubation (24 h), germ tubes and hyphae were still observed when Flu was tested up to 102 µg/ml. However, the formation of mycelium was inhibited. A fivefold reduction in the ratio of infection also did not result in intracellular activity of Flu. These data were derived from three separate experiments, in which each concentration was tested in triplicate.

Cellular toxicities of the antifungal agents measured in terms of inhibition of the phagocytic capacity of the macrophages or a decrease in membrane integrity were not observed with AmB or Flu when they were tested at concentrations up to 1.6 and 102 µg/ml, respectively (data not shown).

(ii) **Effect of previous exposure of *C. albicans* to antifungal agent.** The minimal concentrations of AmB and Flu required for the inhibition of germ tube formation as well as for effective killing (>99%) of *C. albicans* at a *C. albicans*-to-macrophage-ratio of 1:8 after 6 and 24 h of incubation are given in Table 3. Exposure of *C. albicans* for 30 min to one of the antifungal agents at the concentrations used during macrophage ingestion did not reduce the number of viable intracellular yeasts after 30 min of ingestion. With AmB, inhibition of germ tube formation and killing were observed within 6 h of incubation at concentrations of 0.1 and 0.2 µg/ml, respectively. Flu tested up to 102 µg/ml was not effective intracellularly. These data were derived from three separate experiments, in which each concentration was tested in triplicate.

Antifungal activity of a combination of AmB and Flu against intracellular *C. albicans*. The antifungal activity of combinations of AmB and Flu against intracellular *C. albicans* at a *C. albicans*-to-macrophage ratio of 1:8 after 24 h of incubation in terms of inhibition of germ tube formation as well as effective killing (>99%) are shown in Tables 4 and 5, respectively. In the absence of Flu, inhibition of germ tube formation and effective killing were observed at AmB concentrations of 0.2 and 0.4 µg/ml, respectively. The addition

TABLE 3. Antifungal activity of AmB versus that of Flu after 6 and 24 h of incubation against intracellular *C. albicans*: effect of previous exposure to antifungal agent

Antifungal agent and <i>C. albicans</i> /macrophage ratio	Minimal concn (µg/ml) required for:			
	Inhibition of germ tube formation		Effective killing (>99%)	
	6 h	24 h	6 h	24 h
AmB				
1:8 ^a	0.2	0.2	0.4	0.4
1:8 ^b	0.1	0.1	0.2	0.2
Flu				
1:8 ^a	>102	>102	ND ^c	ND
1:8 ^b	>102	>102	ND	ND

^a Antifungal agent was present in the incubation medium after *C. albicans* ingestion.

^b Antifungal agent was present in the incubation medium during and after *C. albicans* ingestion.

^c ND, not done.

of Flu up to 102 µg/ml to AmB did not result in a reduction of the AmB concentrations required for inhibition of germ tube formation and effective killing. In contrast, at combinations of 0.2 µg of AmB per ml plus 1.6 µg of Flu per ml and 0.4 µg of AmB per ml plus 1.6 µg of Flu per ml, inhibition of germ tube formation and effective killing, respectively, were no longer observed. In the presence of Flu at concentrations from 1.6 µg/ml, an increase in the AmB concentration up to 0.8 µg/ml was required to obtain both effects. After 6 h of incubation, this antagonistic effect of Flu was not observed (data not shown). These data were derived from three separate experiments, in which each concentration was tested in triplicate. Regarding the effect of combinations of AmB with Flu on the extracellular growth of *C. albicans*, we did observe a slight antagonistic effect of Flu during 24 h of incubation after the addition of fourfold increasing Flu concentrations, ranging from 0.4 to 102 µg/ml, to AmB at 0.2 µg/ml (data not shown).

DISCUSSION

The observations of the activity of AmB against extracellular *C. albicans* after an incubation period of 24 h showed that, in the absence of serum, AmB is highly active in terms of growth inhibition and killing of *C. albicans*. This antifungal activity was maintained when the inoculum was increased. The presence of serum greatly influenced the activity of AmB against extracellular *C. albicans*. This is in

TABLE 4. Antifungal activity of combinations of AmB and Flu at various concentrations against intracellular *C. albicans* after 24 h of incubation: inhibition of germ tube formation^a

AmB concn (µg/ml)	Antifungal activity with Flu concn (µg/ml) of:									
	102	51.2	25.6	12.8	6.4	3.2	1.6	0.8	0.4	0
1.6	+		+		+		+	+	+	+
0.8	+		+		+		+	+	+	+
0.4	-		-		-		-	+	+	+
0.2	-		-		-		-	+	+	+
0.1	-		-		-		-	-	-	-
0	-	-	-	-	-	-	-	-	-	-

^a The *C. albicans*-to-macrophage ratio was 1:8. +, inhibition of germ tube formation; -, no inhibition of germ tube formation.

TABLE 5. Antifungal activity of combinations of AmB and Flu at various concentrations against intracellular *C. albicans* after 24 h of incubation: effective killing (>99%)^a

AmB concn ($\mu\text{g/ml}$)	Antifungal activity with Flu concn ($\mu\text{g/ml}$) of:									
	102	51.2	25.6	12.8	6.4	3.2	1.6	0.8	0.4	0
1.6	+	+	+	+	+	+	+	+	+	+
0.8	+	+	+	+	+	+	+	+	+	+
0.4	-	-	-	-	-	-	-	+	+	+
0.2	-	-	-	-	-	-	-	-	-	-
0.1	-	-	-	-	-	-	-	-	-	-
0	-	-	-	-	-	-	-	-	-	-

^a The *C. albicans*-to-macrophage ratio was 1:8. +, effective killing; -, no effective killing.

agreement with observations of others (23), who found a twofold increase in the MIC and MFC of AmB when *C. albicans* was exposed to AmB in the presence of 25% PHS. This reduction of antifungal activity in the presence of serum may be explained by the high protein binding of AmB, resulting in reduced bioavailability.

Flu was not active in terms of killing of extracellular *C. albicans*. Only an inhibitory effect by Flu was observed at a low inoculum. This is in agreement with the observations of other investigators (16, 28, 32, 37, 38). Since protein binding is reported to be low for Flu (1, 14, 17), the effect of serum on its antifungal activity was not tested.

The antifungal effect against intracellular *C. albicans* was studied by using monolayers of murine peritoneal macrophages infected with *C. albicans*. The experimental design varied with respect to different ratios of infection, exposure of *C. albicans* to one of the antifungal agents prior to macrophage ingestion, and exposure to the antifungal agents alone or in combination. It is known that resident peritoneal macrophages are unable to kill *C. albicans* during incubation *in vitro* and are even unable to prevent intracellular germ tube formation (2, 3, 7, 24, 38). As a consequence, the antifungal effect on intracellular *C. albicans* could be investigated without interference from the direct fungistatic or fungicidal effects of the macrophages themselves. The intracellular efficacies of the antifungal agents were determined in terms of inhibition of germ tube formation as well as effective killing of *C. albicans* intracellularly. With AmB, inhibition of the development of germ tubes was found at concentrations lower than those required for killing of intracellular *C. albicans*, which has also been reported by others (25, 26, 38). After an increase in the ratio of infection, slightly increased AmB concentrations were required to obtain both effects. The cellular toxicity of AmB in terms of a decrease in membrane integrity (⁵¹Cr release) or a reduction in the phagocytic capacity of the macrophages (*E. coli* phagocytosis) was not observed when macrophages were exposed to AmB for 6 and 24 h, respectively. Other investigators (20) reported, however, that AmB is highly suppressive to macrophage differentiation, as measured by transglutaminase induction, and to effector functions, as measured by superoxide anion release after 16 h of incubation. The immunostimulatory effects of AmB after *in vitro* exposure of peritoneal macrophages have also been reported. Increased phagocytosis of polystyrene beads in macrophages after *in vitro* exposure to AmB was observed (19). In our study, however, an increase in the phagocytosis of *E. coli* was not found. The discrepancies in these observations cannot readily be explained.

Exposure of *C. albicans* to AmB prior to macrophage ingestion slightly influenced the antifungal activity of AmB in terms of a decrease in the AmB concentrations required for inhibition of germ tube formation as well as for the effective killing of intracellular *C. albicans*.

The antifungal activity of AmB was observed within 6 h. Prolonged incubation (24 h) did not change this effect, provided that AmB was present in the incubation medium. It seemed that the continuous presence of AmB extracellularly was necessary to maintain intracellular antifungal activity.

Flu was not effective intracellularly when macrophages were exposed to Flu for 6 h, not even at a reduced *C. albicans*-to-macrophage ratio, nor after exposure of *C. albicans* to Flu prior to macrophage ingestion. Although the formation of mycelium was inhibited after prolonged incubation (24 h), germ tubes and hyphae were still observed with Flu, and therefore, Flu was not effective intracellularly. The results of the activity of Flu against intracellular *C. albicans* are in agreement with the results of activity against extracellular *C. albicans*, in which no complete growth inhibition was observed. It has been reported by others (42) that Flu, despite its observed good intracellular penetrating capacity, did not have a significant effect on the killing of intracellular *C. albicans* or on the formation of germ tubes within 4 h of incubation of *C. albicans*-infected macrophage monolayers derived from human blood monocytes, which is in agreement with the results of our study. Those investigators (42) also mentioned the prevention of the formation of mycelium by Flu after 24 h of incubation and reported a significant growth inhibition of *C. albicans*, as measured by CFU counts of viable intracellular *C. albicans*. CFU counts of intracellular *C. albicans* can only be determined accurately when germ tube formation is completely inhibited. In the presence of germ tubes, the CFU count may be an overestimation of viable *C. albicans* because of fragmentation of the germ tubes during disruption of the monolayer, giving rise to an increase in CFU. On the other hand, the CFU count can be an underestimation of viable *C. albicans*, since viable *C. albicans* may be washed away after destruction of the macrophages by germ tubes. Other investigators (38) described that Flu had only a minimal effect on the number of viable intracellular *C. albicans* in murine macrophage monolayers, but that it significantly inhibited germ tube formation within 4 h of incubation. The number of yeasts that produced germ tubes as well as the length of the germ tubes appeared to be significantly reduced, as measured microscopically. The intracellular activities of antifungal agents can only be defined as complete inhibition of germ tube formation, at the least.

To investigate whether the AmB concentrations required for intracellular activity could be reduced further, combinations of AmB and Flu were tested. However, the potentiation of intracellular activity was not observed after coinubation of AmB with Flu. In contrast, antagonism between Flu and AmB was found with respect to intracellular *C. albicans*. This antagonism was observed only after 24 h of incubation but was not observed after 6 h of incubation. With respect to extracellular *C. albicans*, a slight antagonistic effect between Flu and AmB measured during 24 h of incubation was observed, as was also recently described by others (30). They speculated that competition for binding sites on *C. albicans* could be responsible for this antagonism. Intracellular activity could also be reduced by competition during intracellular penetration. However, these explanations for the observed antagonism are speculative, and further study is needed for elucidation.

In summary, it was observed that although the activity of AmB against extracellular *C. albicans* was high, the antifungal activity was influenced by factors relevant to its efficacy in the treatment of infection. The antifungal activity of AmB was substantially reduced by the presence of serum; however, it was only slightly hindered by the intracellular localization of the yeasts. At an increased ratio of infection of intracellular *C. albicans*, the antifungal activity of AmB was slightly reduced. For Flu, antifungal activity against extracellular *C. albicans* was restricted to inhibition of fungal growth, despite the use of optimal test conditions, such as the recommended assay medium and the use of low *C. albicans* inocula, whereas Flu was not effective against intracellular *C. albicans*. Therefore, it is questionable whether Flu effectively contributes to the eradication of *C. albicans* when host defense mechanisms are severely impaired. A slight antagonistic effect between Flu and AmB was found with respect to intracellular as well as extracellular *C. albicans*.

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