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Cooperative Anti-*Candida* Effects of Lactoferrin or Its Peptides in Combination with Azole Antifungal Agents

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Abstract: **The effects of lactoferrin (LF), an antimicrobial protein secreted in body fluids, and its peptides in combination with azole antifungal agents were investigated by the micro-broth-dilution method in a study of *Candida albicans*. In the case of LF, its pepsin hydrolysate (LFhyd) or the LF-derived antimicrobial peptide Lactoferricin® B (LF-B), the concentrations required to inhibit the growth of *Candida* decreased in the presence of relatively low concentrations of clotrimazole (CTZ). The minimum inhibitory concentration (MIC) of all azole antifungal agents tested was reduced by 1/4-1/16 in the presence of a sub-MIC level of each of these LF-related substances. Polyene and fluoropyrimidine antifungal agents did not show such a combined effect with these LF-related substances. The anti-*Candida* activity of LF or LF-B in combination with CTZ was shown to be synergistic by checkerboard analysis. These results indicate that LF-related substances function cooperatively with azole antifungal agents against *C. albicans*.**

Key words: **Lactoferrin, Lactoferricin®, Azole antifungal agents, *Candida albicans***

Azole antifungal agents are widely used in the chemotherapy of mycoses where they act in a fungistatic fashion, however, there is recently a trend of caution due to the rising incidence of failures in the treatment of mycoses in the case of severely immunosuppressed patients on azole therapy. Lately, the isolation of azole-resistant *Candida* species has been reported (11, 15) and azole antifungal chemotherapy may become insufficient in the near future. This clinical situation points to the need for the development of new therapeutic agents which augment the antifungal activity of azoles.

Lactoferrin (LF) is an iron-binding glycoprotein found in many exocrine secretions of mammals and in the secondary granules of neutrophils. LF is known to display antimicrobial activity against fungi such as *Candida* as well as bacteria (5, 12), and is considered to play an important role in the host defense against infections on mucosal surfaces. It has been recently reported that Lactoferricin®, an antimicrobial peptide derived from the N-terminal region of the LF molecule (2), exhibits potent disruptive effects on cell membrane and has fungicidal activity against *Candida albicans* (16).

It seems likely that some antifungal agents may act cooperatively with host defense factors to inhibit fungal growth in the body, and we have attempted to ascertain whether such natural factors contribute to the therapeutic efficacy of antifungal agents. Here, we have studied the inhibitory effects of LF and its peptides in combination with several antifungal agents by testing their cooperative activity against *C. albicans in vitro*.

Materials and Methods

LF-related substances. Bovine LF was produced by Morinaga Milk Industry Co. (Tokyo). Pepsin hydrolyzate of bovine LF (LFhyd) and the antimicrobial peptide "Lactoferricin® B" (LF-B), which is derived from bovine LF, were produced by methods previously reported (2). The fraction of LFhyd without LF-B (LF (−)) was obtained by repeated removal of LF-B from LFhyd by

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Abbreviations: AMPH, amphotericin B; *C. albicans*, *Candida albicans*; CTZ, clotrimazole; DMSO, dimethylsulfoxide; 5-FC, flucytosine; FIC index, fractional inhibitory concentration index; FLCZ, fluconazole; IC80, 80% growth inhibitory concentration; ITCZ, itraconazole; KCZ, ketoconazole; LF, lactoferrin; LF-B, an antimicrobial peptide derived from bovine lactoferrin, Lactoferricin® B; LFhyd, pepsin hydrolyzate of bovine lactoferrin; LF (−), a fraction of LFhyd without LF-B; MIC, minimum inhibitory concentration; NYS, nystatin; SDB, Sabouraud dextrose broth.

the above method (2). All LF-related substances were dissolved in sterile distilled water.

Antifungal agents. Amphotericin B (AMPH) and clotrimazole (CTZ) were purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Nystatin (NYS) and flucytosine (5-FC) were purchased from Nacalai Tesque Co. (Kyoto, Japan). Ketoconazole (KCZ), itraconazole (ITCZ) and fluconazole (FLCZ) were prepared from Nizoral® Cream (Kyowa Hakko Co., Tokyo), Itrizole® Capsules 50 (Kyowa Hakko) and Diflucan® Capsules (Pfizer Pharmaceutical Japan, Tokyo), respectively. 5-FC, ITCZ and FLCZ were dissolved in sterile distilled water, dimethylsulfoxide (DMSO) containing 0.1 N HCl and phosphate buffered saline, respectively. Other antifungal agents were dissolved in DMSO.

C. albicans *strain.* *C. albicans* TIMM1768, a clinically isolated serotype A strain obtained from the Research Center for Medical Mycology, Teikyo University (Tokyo), was used.

*Assay of anti-*Candida *activity.* The anti-*Candida* activities of antifungal agents and LF-related substances were assayed by the micro-broth-dilution method (14). Yeast-form cells of *C. albicans* were collected from stock cultures on Sabouraud dextrose agar (1% Bactopeptone [Difco Laboratories Co., Detroit, Mich., U.S.A.], 2% glucose, 1.5% agar) slants, washed in saline and suspended in Sabouraud dextrose broth (SDB; 1% Bactopeptone, 2% glucose, pH 6.5) at 10^5 cells/ml. 20 μl of *Candida* suspension, 10 μl of stock solution of LF-related substances, 2 μl of stock solution of the antifungal agent and 168 μl of SDB were mixed and incubated at 37 C for 17 hr. We used SDB as a medium since *C. albicans* TIMM1768 grew almost completely in yeast form in this medium but partially in mycelial form in other medium such as RPMI1640. The addition of LF-related substances at tested concentrations caused slight decreases in the pH (Δ0.1−0.2) of the SDB medium. The medium containing solvents (1%) used for the dissolution of antifungal agents were confirmed not to affect the growth of *Candida*. The absorbance at 630 nm of triplicate samples was measured spectrophotometrically at the start and end of incubation, and the difference in absorbance was taken to indicate the extent of *Candida*. The percent growth inhibition of *C. albicans* was calculated as follows: [1 − absorbance (*C. albicans* with drug) / absorbance (*C. albicans* alone)] × 100 (%). The 80% growth inhibitory concentration (IC80) was taken to represent the minimum inhibitory concentration (MIC).

Checkerboard analysis. For the assessment of antimicrobial combinations, the checkerboard method was employed and results were evaluated according to standard criteria (4). Concentrations of each of the drug combinations showing IC80 against *C. albicans* as determined in triplicate tests were plotted on arithmetic scales. The fractional inhibitory concentration index (FIC index) was calculated as follows: (lowest inhibitory concentration of drug A in combination/MIC of drug A alone + lowest inhibitory concentration of drug B in combination/MIC of drug B alone). FIC index values of ≤ 0.5, 1.0 and >4.0 represent synergism, additivity and antagonism, respectively.

Results

*Anti-*Candida *Activities of LF-Related Substances Combined with CTZ*

The growth inhibitory activities of LF, LFhyd, LF-B and LF (−) combined with CTZ against *C. albicans* TIMM1768 were tested (Fig. 1a–d). CTZ by itself caused marginal inhibition of *Candida* at 12.5 ng/ml and 80% growth inhibition at 50 ng/ml. LF alone at 200 μg/ml completely inhibited the growth of *Candida*. In the presence of CTZ at 3.1 or 12.5 ng/ml, LF caused complete growth inhibition at 100 and 50 μg/ml, respectively (Fig. 1a). LFhyd at 400 μg/ml and LF-B at 12.5 μg/ml caused 80% growth inhibition, and the IC80 of both agents decreased to 1/2 or 1/4 when combined with 3.1–12.5 ng/ml of CTZ (Fig. 1, b and c). In contrast, LF (−) enhanced the growth of *Candida* dose-dependently, and this tendency was observed even in the presence of 3.1–50 ng/ml of CTZ (Fig. 1d). These results indicate that a combination of LF, LFhyd, or LF-B with CTZ inhibits the growth of *Candida* more effectively than any one of these agents alone, and cooperative effects were evident from the decrease in MIC value (IC80).

Combinations of Antifungal Agents and LF

The effects of LF, LFhyd and LF-B on the anti-*Candida* activities of various antifungal agents were investigated. In the presence of 100 μg/ml of LF, which scarcely inhibits the growth of *Candida* by itself, the anti-*Candida* activity of two polyenes, one fluoropyrimidine, two imidazoles containing CTZ, and two triazoles were tested (Table 1). The MIC values (IC80) of AMPH, NYS, polyene antifungal agents and 5-FC, a fluoropyrimidine antifungal agent, did not change following the addition of LF. The MIC of KCZ, an azole antifungal agent, decreased by 1/16 in the presence of LF. The MICs of the other three azoles were also reduced by 1/4 in the presence of LF.

Combinations of Antifungal Agents and LFhyd

In the presence of LFhyd at concentrations of 50 or 200 μg/ml, which just weakly inhibit the growth of *Candida*, the MIC values (IC80) of various antifungal

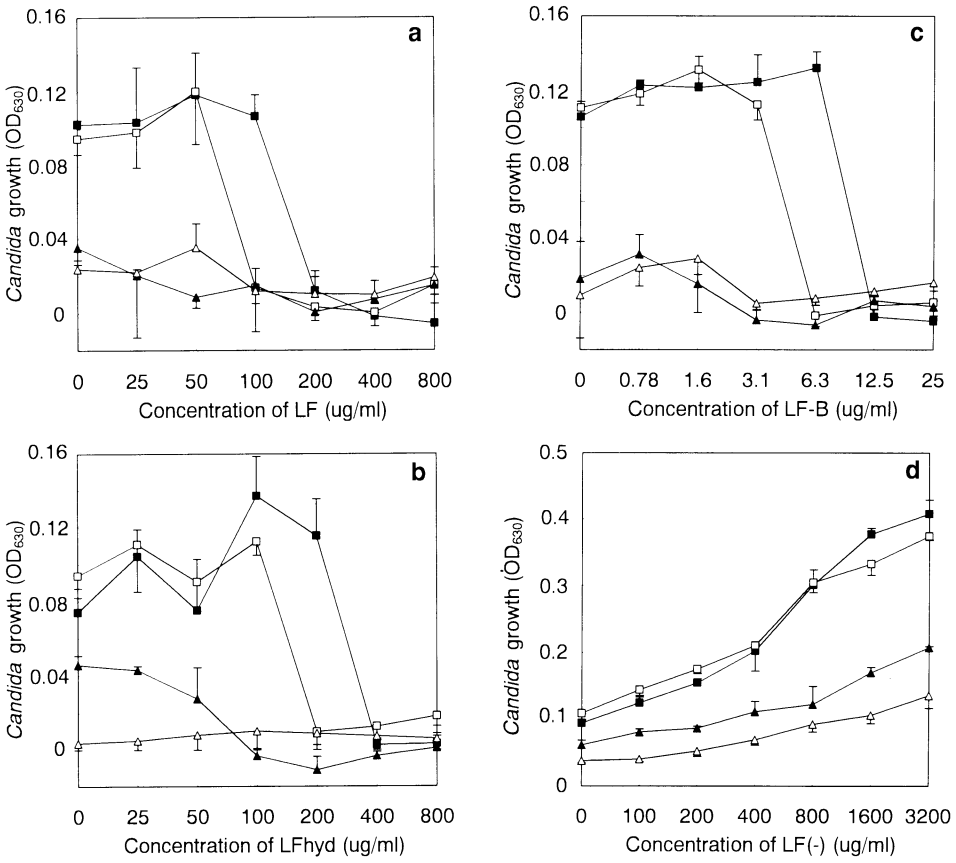

Fig. 1. Growth inhibitory effects of LF (Panel a), LFhyd (Panel b), LF-B (Panel c) and LF (−) (Panel d) with and without CTZ. The growth of *C. albicans* TIMM1768 was estimated by measuring OD_{630}. Data are means of three samples ± SD. CTZ concentrations were 0 (■), 3.1 (□), 12.5 (▲), and 50 (△) ng/ml.

Table 1. Effect of LF on the anti-*Candida* activity of various antifungal agents

Antifungal agent	MIC (ng/ml)	
	Alone	+LF
AMPH	62.5	62.5
NYS	1,000	1,000
5-FC	4,000	4,000
CTZ	50	12.5 (1/4)
KCZ	50	3.1 (1/16)
FLCZ	4,000	1,000 (1/4)
ITCZ	50	12.5 (1/4)

The MIC value was defined as the IC80 of each antifungal agent. LF was added at a sub-MIC level (100 μg/ml). The ratio of the MIC value in the presence of LF to that in the absence of LF is indicated in parentheses.

Table 2. Effect of LFhyd on the anti-*Candida* activity of various antifungal agents

Antifungal agent	MIC (ng/ml)		
	Alone	+LFhyd 50	+LFhyd 200
AMPH	62.5	62.5	62.5
NYS	1,000	1,000	1,000
5-FC	4,000	4,000	1,000 (1/4)
CTZ	50	50	3.1 (1/16)
KCZ	50	50	3.1 (1/16)
FLCZ	16,000	4,000 (1/4)	1,000 (1/16)
ITCZ	50	50	3.1 (1/16)

The MIC value was defined as the IC80 of each antifungal agent. LFhyd was added at a sub-MIC level (50 or 200 μg/ml). The ratio of the MIC value in the presence of LFhyd to that in the absence of LFhyd is indicated in parentheses.

agents were examined (Table 2). The MICs of AMPH and NYS did not change following the addition of LFhyd. Combined with 200 μg/ml of LFhyd, MICs of 5-FC and four azole antifungal agents were decreased by 1/4 and 1/16 as compared with the MIC of each antifungal agent alone. Furthermore, the MIC of FLCZ decreased by 1/4 in the presence of only 50 μg/ml of LFhyd.

Combinations of Antifungal Agents and LF-B

In the presence of LF-B at a concentration of 3.1 μg/ml, which scarcely affected the growth of *Candida*,

Table 3. Effect of LF-B on the anti-*Candida* activity of various antifungal agents

Antifungal agent	MIC (ng/ml)	
	Alone	+LF-B
AMPH	62.5	62.5
NYS	1,000	1,000
5-FC	4,000	4,000
CTZ	50	12.5 (1/4)
KCZ	50	12.5 (1/4)
FLCZ	16,000	4,000 (1/4)
ITCZ	50	12.5 (1/4)

The MIC value was defined as the IC80 of each antifungal agent. LF-B was added at a sub-MIC level (3.1 μg/ml). The ratio of the MIC value in the presence of LF-B to that in the absence of LF-B is indicated in parentheses.

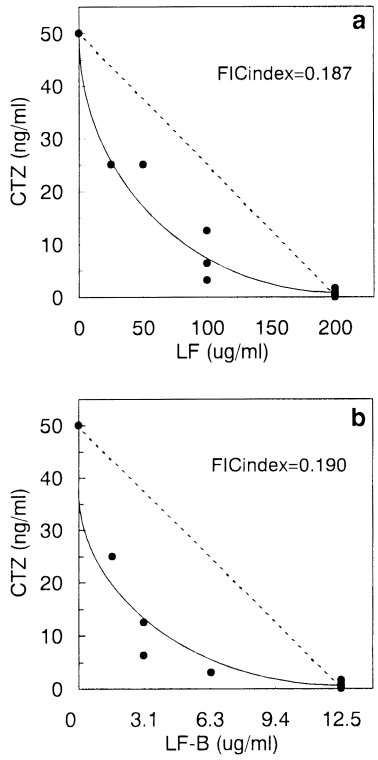

Fig. 2. Anti-*Candida* activity of the combinations of LF (Panel a) and LF-B (Panel b) with CTZ were analyzed by the checkerboard method. For each combination, the IC80 of CTZ and LF-related substance against *C. albicans* TIMM1768 was determined in triplicate tests and plotted in closed circles on arithmetic scales. A putative additive effect is represented by the dashed line. The FIC index calculated for combinations of the two drugs is indicated on each panel.

the anti-*Candida* activity of various antifungal agents was tested (Table 3). No change in the MICs of AMPH, NYS or 5-FC was observed following the addition of LF-B. On the other hand, in the presence of LF-B, the MICs of four azole antifungal agents were decreased by 1/4 as compared with the MIC of each azole alone.

Analysis of Combined Effects by the Checkerboard Method

The combined effects of LF-related substances and CTZ were characterized by the checkerboard method. The anti-*Candida* activity of combinations of LF and CTZ at various concentrations was examined, and points representing each concentration giving 80% growth inhibition of *Candida* were plotted. The plotted curve suggests the existence of a "synergistic" combination effect as shown in Fig. 2a. The FIC index of this combination was 0.187, and this value indicates synergy ($\leq$0.5). In the case of varied concentrations of LF-B and CTZ as well, the plotted points create a synergistic curve (Fig. 2b). The FIC index of this combination was 0.190, indicating synergy.

Discussion

LF and LF-B, at relatively high doses, are known to inhibit the growth of *C. albicans* (3, 12, 16). Here, we found that LF-related substances at sub-MIC levels function cooperatively with azole antifungal agents against *C. albicans*. The MIC of each azole antifungal agent tested decreased by about 1/4 in the presence of LF-related substances. The effects of LF or LF-B combined with CTZ on the inhibition of *Candida* were characterized as synergistic by checkerboard analysis. Although LF, LFhyd and LF-B showed no cooperative effect with non-azole types of antifungal agents such as AMPH or 5-FC, all of the azoles tested showed significant combined effects. The azoles tested differ from each other in physical features (e.g., FLCZ is hydrophilic and ITCZ is hydrophobic) but all azole antifungal agents are known to inhibit fungal growth by interference with cytoplasmic membrane synthesis through the inhibition of ergosterol synthesis. Therefore, we speculate that such interference with membrane synthesis may have a role in the cooperative inhibitory effects observed here.

The active component of each of the LF-related substances contributing to the cooperative inhibitory effect with azoles against *C. albicans* is assumed to be lactoferricin (LF-B), since LF, LFhyd and LF-B each contain the LF-B domain or the LF-B peptide. LF (−), which was removed from LF-B, did not inhibit but rather enhanced the growth of *Candida* even in the presence of CTZ. The mechanism of action of the LF-related substances against *C. albicans* has not been fully elucidated. Advanced glycosylation end products which bind to the lactoferricin domain are reported to block the antibacterial activity of LF against *Micrococcus luteus* (6). This finding tends to support our assumption that the anti-*Candida* activity of LF depends on the lactoferricin region. We have shown that LF-B directly binds to

Candida cells (3) and is highly effective to disrupt the cell membrane of *Candida* (16). Therefore, the combination of LF-related substances and azoles may cooperatively inhibit the growth of *Candida* by causing the dysfunction of fungal membranes.

Since our experiments were performed using SDB medium known to be relatively acidic, it is possible that the cooperative effects of LF-related substances and azoles may be influenced by the biochemical characteristics of SDB. However, we believe that the combined anti-*Candida* activities were not dependent on the pH change of the medium because non-effective LF (−) caused a slight decrease in the pH (Δ0.1–0.2) of the medium, similar to that of other effective LF-related substances.

LF and some β-lactam antibiotics are reported to have cooperative antimicrobial effects against *Klebsiella pneumoniae* and *Salmonellae* (8, 10). More recently, azoles and lysozyme were shown to have synergistic anti-*Candida* activity (13). These findings also suggest that agents having different modes of action, each causing damage to microbial cells, can exhibit synergistic antimicrobial effects.

The levels of LF in exocrine secretions have been reported as: 0.5–1.0 mg/ml in cervical mucus (7), 0.4–1.2 mg/ml in tears (1) and $\geq$2 mg/ml in milk (9). These levels of LF are higher than 100 μg/ml, which is the concentration required to exhibit a synergistic effect in combination with azoles as demonstrated in our assays. Therefore, it is conceivable that LF in exocrine secretions, such as vaginal mucus, may contribute to the therapeutic efficacy of azole antifungal agents against *Candida* infection. Moreover, we hope that combination therapies using azole antifungal agents and LF-related substances, especially the low molecular weight peptide LF-B, prove to be therapeutically effective in clinical trials. In our future studies, we intend to investigate the combined effectiveness of LF-B and azoles against pathogenic fungi other than *C. albicans,* not only *in vitro* but also *in vivo* in animal models.

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