Human Lactoferrin-Derived Peptide’s Antifungal Activities against Disseminated Candida albicans Infection

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Background. Because the human lactoferrin-derived peptide, hLF(1-11), exerts potent in vitro candidacidal activity, we investigated whether it displays antifungal activity against disseminated Candida albicans infections.

Methods. Neutropenic mice were intravenously infected with C. albicans and, 24 h later, were injected with hLF(1-11); 18 h later, the number of viable yeasts in the kidneys was determined microbiologically, the size and number of infectious foci were determined histologically, and serum cytokine levels were determined by immunoassays.

Results. hLF(1-11) was effective (maximum reduction, 1.5 logs) against disseminated C. albicans infections, and its antifungal activity leveled off at a concentration of 0.4 ng of hLF(1–11)/kg of body weight. The antifungal activity of hLF(1-11) was increased in mice injected with interleukin (IL)-10 neutralizing antibodies, which suggests that IL-10 reduces the antifungal activity of hLF(1-11). In agreement with this result was the finding that injection of high doses of hLF(1-11) into infected mice was accompanied by increased levels of IL-10 in serum. Microscopic analysis revealed that infectious foci in kidneys of hLF(1-11)-treated mice contained mainly blastoconidia, whereas filamentous forms were abundant in untreated mice. The peptide inhibited the in vitro morphological transition of C. albicans, in a dose-dependent manner.

Conclusions. hLF(1-11) is effective against disseminated C. albicans infections; and its effects on C. albicans viability and virulence and on host cells may explain this antifungal activity.

The frequency of Candida infections has increased during the past 2 decades, as a consequence of the rising number of immunocompromised hosts. An international program of surveillance of disseminated infections caused by Candida reported that 53% were due to Candida albicans [1], with an overall crude mortality rate of 38% [2]. Moreover, in recipients of bone marrow transplants and in patients undergoing intensive chemotherapy, the crude mortality rate of disseminated Candida infections is >80% [3].

In healthy individuals, C. albicans is a commensal of skin and mucosal surfaces. This commensal status is ensured by an equilibrium between the yeast, the microflora, and the innate and acquired immune systems. The most relevant components of the innate immune system are phagocytes, NK cells, mast cells, and proteins such as complement factors, cytokines, and antimicrobial proteins/peptides (e.g., human lactoferrin [hLF]) [4–8]. hLF, a 77-kDa iron-binding glycoprotein synthesized by mucosal-gland epithelial cells and neutrophils, is present in body secretions such as milk and...
tars and in specific granules of neutrophils. This protein plays a role in the host defense by (1) releasing (during pepsinolysis) lactoferrin (residues 1–47) and various smaller N-terminal peptides with potent antimicrobial activities [5, 7, 8] and (2) modulating immune responses [9, 10]—for example, by inhibiting complement activation [11], affecting the production of reactive oxygen intermediates [12] and cytokines by phagocytes [5, 13, 14] and T lymphocytes [15], and increasing the phagocytosis and intracellular killing of Staphylococcus aureus by bovine phagocytes [16]. Furthermore, hLF triggers the production of antimicrobial peptides by mucosal epithelial cells [17], and it is able to prevent biofilm formation [18]. Therefore, analysis of the mechanisms underlying the antimicrobial activities of (peptides derived from) hLF in experimental animals and humans should consider all biological properties of this peptide.

Elsewhere we have reported that a synthetically prepared, naturally occurring, cationic N-terminal peptide, referred to as “hLF(1-11),” displays fungicidal activity against various Candida species, including fluconazole-resistant C. albicans strains in vitro [19, 20]. The mechanism of action of hLF(1-11) is as follows: via mitochondrial calcium uptake [21], it stimulates an increase in the mitochondrial membrane’s potential and permeability [19], resulting in the synthesis and secretion of adenosine triphosphate and the production of reactive oxygen species [22], thereby leading to C. albicans cell death. In addition, hLF and lactoferrin inhibit filamentous growth of some azole-resistant strains of C. albicans [23]. This is important because the phenotypic transition of C. albicans from the blastoconidial to the filamentous form (together with both expression of adhesion molecules in the cell wall and protease production) is involved in tissue invasion by C. albicans [24–27]. The present study was undertaken to (1) determine whether hLF(1-11) displays antifungal activities in mice with disseminated fluconazole-resistant C. albicans infection and (2) gain more insight into the mechanisms underlying the antifungal activities of this peptide.

**MATERIALS AND METHODS.** The synthetic peptide corresponding to residues 1–11 of hLF (amino acids, GRRRSVQWCA; molecular mass, 1374 daltons), hLF(1–11), was purchased from Peptisyntha; the synthetic peptide comprising alanines at position 2, 3, 6, and 10 of hLF(1–11), hereinafter referred to as a “control peptide” (Peptisyntha), was included as a negative control. The purity of these 2 peptides exceeded 97%, as determined by reverse-phase high-performance liquid chromatography. Stocks of the peptides at a concentration of 1 mg/mL of 0.01% acetic acid (pH 3.7) were stored at −20°C and were dried in a Speed-Vac (Savant Instruments) immediately before being used in in vitro experiments. In addition, synthetic peptides were freshly dissolved at a concentration of 1 mg/mL of saline and were diluted to desired concentrations immediately before being used in in vivo experiments.

**Source of C. albicans strain.** Fluconazole-resistant C. albicans strain Y01-19 was purchased from Pfizer. The yeast was identified by Candiselect (Sanofi Pasteur) and was confirmed by the pattern of sugar utilization (API, ID 32C; bioMerieux). Fluconazole resistance was evaluated, as MIC >256 μg/mL, by the Etest (AB Biodisk). Yeasts were cultured overnight at 37°C and then was subcultured, for 2.5 h at 37°C, on a rotary wheel in Sabouraud broth (Oxoid). Virulent strains of the yeast were obtained after 2 passages in Swiss mice. In brief, ∼1 × 10⁷ cfu of yeasts in 0.1 mL of saline were injected into a tail vein, and the mice were killed 24 h later. The spleen was aseptically removed and homogenized, and serial dilutions of the homogenate were plated onto Sabouraud agar. A single colony-forming unit was transferred into 25 mL of Sabouraud broth and was incubated for 24 h at 37°C, and aliquots of this suspension, containing ∼5 × 10⁶ virulent cfu of yeasts/mL of broth, supplemented with 30% (vol/vol) glycerol, were stored at −80°C.

**Animals.** Specific pathogen–free, female Swiss mice, 8–10 weeks old and weighing 20–25 g (Charles River), were used in the study. The mice were housed in local animal facilities for ≥1 week before the start of the experiments. Food and water were given ad libitum. The animal studies were approved by the Leiden Experimental Animal Committee (approval DEC 02909) and were performed in compliance with Dutch laws related to the conduct of animal experiments.

**Treatment of disseminated fluconazole-resistant C. albicans infections in neutropenic mice.** The mice were rendered neutropenic by intraperitoneal injection, at days −3 and 0 of infection, of 100 mg of cyclophosphamide (Sigma-Aldrich Chemie BV)/kg of body weight. After the second injection of cyclophosphamide, blood-cell counts were randomly performed to confirm neutropenia, and then the mice were anesthetized with a single intraperitoneal injection of 0.1 mL of water containing 11 μg of fluanisone and 330 μg of fentanyl citrate (Hypnorm; Janssen Pharmaceuticals). Next, ∼1 × 10⁶ cfu of fluconazole-resistant C. albicans in 0.1 mL of saline were aseptically injected into a tail vein; 24 h later, the mice were intravenously injected with various doses (range, 0.04 ng–4 mg/kg of body weight) of hLF(1–11). Control mice were injected with saline. The control peptide and fluconazole (Pfizer) were included as negative controls. Fluconazole dissolved in 0.1 mL of saline was subcutaneously injected into mice at a concentration of 5 mg/kg of body weight. Amphotericin B (intraperitoneal injection of 0.5 μg/kg of body weight; Bristol-Meyers Squibb Group) was included as a positive control. After 18 h of treatment, the mice were killed; their kidneys were removed, weighed, and homogenized; and the number of viable yeasts was determined by plating serial dilutions of each sample on
Sabouraud agar. The detection limit was 667 cfu. Results, expressed as the number of colony-forming units per gram of kidney, are from 2–4 independent experiments. Values for each mouse, as well as the median for its group, are reported. In some experiments, one kidney was used for microbiological analysis of the antifungal activities of the peptide whereas the other was processed for histological analysis (see below).

**Measurement of cytokines.** Levels of interleukin (IL)–10 and tumor necrosis factor (TNF)–α in serum were measured by ELISA (Sanquin), according to manufacturer’s instructions. In addition, we took advantage of the Cytometric Bead Assay (Becton-Dickinson), to simultaneously measure multiple cytokines (IL-4, IL-5, IL-6, IL-10, monocyte chemotactic protein [MCP]–1, interferon [IFN]–γ, and IL-12p70) in small samples of serum. This assay was performed according to the manufacturer’s instructions and used FACSCalibur (Becton-Dickinson) for quantitation of the levels in serum. The lower limit of detection of these immunological assays was ~5 ng/mL. Results for each mouse, as well as the median (of 2–4 independent experiments) for its group, are reported.

**Treatment with IL-10 neutralizing monoclonal antibody (mAb), in mice with disseminated fluconazole-resistant C. albicans infection.** The mice were intravenously injected with 250 μg of IL-10 neutralizing mAb (clone JES052A5, rat IgG1; R&D Systems) 2 h before and 4 h after infection. As a control, the isotype-matched mAb anti-keyhole limpet hemocyanin (clone 43414, rat IgG1; R&D Systems) was injected into infected mice. Results for each mouse, as well as the median for its group (4–7 mice/group, in a single experiment), are reported.

**Histology.** Tissue samples taken at autopsy were fixed in 70% alcohol for 24 h and then in 4% formaldehyde and were processed and embedded in paraffin wax. Next, 5-μm–thick sections were stained with Grocott’s modification of Gomori’s methanamine-silver stain [28] and were counterstained with 0.2% light green (Fluka Sigma-Aldrich), for microscopic examination both of the number and size of infectious foci and of the morphology of C. albicans at sites of infection.

**Assay for filamentous C. albicans.** The assay used for determination of the transition of C. albicans from the blastoconidial to the filamentous form has been described elsewhere [29]. In brief, C. albicans cells were harvested in stationary phase by centrifugation at 1500 g for 10 min and then were washed twice in distilled water and were diluted to a concentration of 2 × 10⁸ cfu/mL of Hanks’ balanced salt solution.

![Figure 1](https://academic.oup.com/jid/article-abstract/196/9/1416/2192130/1418.1418.png?w=1024)
Antifungal Activity of hLF(1–11) in Mice

**RESULTS**

**hLF(1–11)’s antifungal activity against disseminated fluconazole-resistant C. albicans infection in neutropenic mice.** The results revealed that hLF(1–11) was effective (P < .05) against disseminated C. albicans infections, with a maximum antifungal effect of an ~1.5-log reduction (figure 1). It is noteworthy that the antifungal activity of this peptide leveled off at concentrations as low as 0.4 ng of peptide/kg of body weight. As expected, the control peptide and fluconazole had no antifungal effect.

In addition, 0.4 μg of hLF(1–11)/kg of body weight and an optimal dose of amphotericin B were equally effective (figure 1). The antifungal activity of hLF(1–11) could already be seen 4 h after treatment; for example, the median level of C. albicans in mice treated with 40 μg of peptide/kg of body weight was 1.9 × 10² (range, 1.7–3.1 × 10³) cfu/g of kidney and that in control mice was (range, ) cfu/g of kidney (n = 3 mice; P < .05).

All mice treated with doses inadequate to reduce antifungal morphological transition of C. albicans in vitro. Correlations between the number of viable C. albicans and the levels of TNF-α and IL-6 in serum were calculated by Spearman’s rank test. In all tests, the level of significance was set at P < .05.

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**Statistical analyses.** The antifungal activities of the various treatments—different doses of hLF(1–11), amphotericin B, fluconazole, control peptide, and saline—in mice with disseminated C. albicans infection, as well as those of different doses of hLF(1–11) in mice either injected or not injected with IL-10 neutralizing antibodies, were compared by Bonferroni’s post-hoc multiple-comparison test. The levels of cytokine in sera of mice treated with the different doses of the hLF(1–11) peptide were also compared by this test. Analysis of variance was used to determine the effect that hLF(1–11) has on the
activity displayed clinical symptoms, such as fever and ruffled fur, and 27% of these mice died on day 2 of infection. None of the mice treated with an effective dose of hLF(1-11) peptide were severely ill during the period of analysis.

**Effect of hLF(1-11) on levels of cytokine in sera of neutropenic mice with disseminated fluconazole-resistant C. albicans infection.** Because we considered the possibility that the leveling off of the antifungal activity of hLF(1-11) peptide can be attributed to the levels of IL-10 in serum, we measured the levels of IL-10 in serum samples collected 18 h after hLF(1-11) was injected into mice with disseminated *C. albicans* infection. The levels of IL-10 in serum samples from mice that were injected with 4 mg of hLF(1-11)/kg of body weight were significantly *(P<.001)* greater than those in serum samples from mice injected with ≤40 μg of peptide/kg of body weight (figure 2). In addition, the levels of TNF-α in serum samples from infected mice treated with 4 mg of hLF(1-11)/kg of body weight did not differ from those in serum samples from untreated infected mice, whereas they were decreased *(P<.03)* in infected mice injected with ≤40 μg of peptide/kg of body weight (figure 3). No detectable levels of IL-10 and TNF-α were seen in uninfected neutropenic mice, and injection of the peptide into them did not induce detectable serum levels of these cytokines (data not shown).

To gain more insight into the cytokine profile of hLF(1-11)-treated mice, we measured the effects of this peptide had on the levels of type 1 (IFN-γ, IL-12p70), type 2 (IL-4, IL-5), and other cytokines (e.g., MCP-1 and IL-6) in the sera of infected mice; we found detectable levels of IFN-γ, IL-12p70, MCP-1, and IL-6 (table 1) but not of IL-4 and IL-5. The levels of IL-12 and MCP-1 in the sera of infected mice were not affected by this peptide, and the levels of IFN-γ in the sera of mice injected with 0.0004–0.4 μg of peptide/kg of body weight were significantly greater than those in untreated mice, whereas the levels of IL-6 were significantly *(P<.05)* decreased by these doses of hLF(1-11). Interestingly, significant correlations were found between the number of viable *C. albicans* and the levels of both TNF-α *(P<.01)* and IL-6 *(P<.01)* that were induced by treatment with 0.0004–0.4 μg of hLF(1-11)/kg of body weight.

**Effect of IL-10 neutralizing mAb on hLF(1-11)’s antifungal activity against disseminated *C. albicans* infection.** To discover whether the level of IL-10 in the mice reduces the antifungal activity of hLF(1-11) peptide, we determined the antifungal activities of 2 different doses—4 μg and 4 mg of hLF(1-11) peptide/kg of body weight—in mice injected with and in

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**Table 1. Effect that human lactoferrin–derived peptide (hLF(1-11)) has on levels of cytokine in sera of mice with disseminated *Candida albicans* infection.**

<table>
<thead>
<tr>
<th>hLF(1-11), μg/kg of body weight</th>
<th>Levels of cytokine in serum, median (range), pg/mL</th>
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<tbody>
<tr>
<td></td>
<td>IL-6</td>
</tr>
<tr>
<td>40</td>
<td>546 (183–2687) (n = 5)</td>
</tr>
<tr>
<td>0.4</td>
<td>207 (71–395) (n = 3)</td>
</tr>
<tr>
<td>0.004</td>
<td>41 (10–118) (n = 5)</td>
</tr>
<tr>
<td>0.0004</td>
<td>99 (63–161) (n = 4)</td>
</tr>
<tr>
<td>0</td>
<td>1665 (154–5000) (n = 5)</td>
</tr>
</tbody>
</table>

**NOTE.** n values are numbers of mice. IFN, interferon; IL, interleukin; MCP, monocyte chemotactic protein.

* Significantly different *(P<.05)* from values for mice injected with 0.1 mL of saline instead of hLF(1-11) peptide.

![Figure 4](https://academic.oup.com/jid/article-abstract/196/9/1416/2192130)
mice not injected with IL-10 neutralizing mAb. Both doses of peptide completely eliminated *C. albicans* from the kidneys of mice injected with IL-10 neutralizing mAb, whereas 4 μg and 4 mg of peptide/kg of body weight resulted, respectively, in 4-log and 3-log reductions in the number of colony-forming units per gram of kidney in mice not injected with this mAb (figure 4). Interestingly, the number of *C. albicans* in mice injected with IL-10 neutralizing mAb was 1.5 log lower (*P* < .01) than that in mice not injected with this antibody. It is noteworthy that the antifungal activity of the hLF(1–11) peptide in infected mice did not differ from that in infected mice injected with the isotype-matched control mAb (data not shown).

**Effect of hLF(1–11) on the size and number of infectious foci and on the morphology of *C. albicans* in kidney tissue sections of mice with disseminated fluconazole-resistant *C. albicans* infection.** Light-microscopic examination of Grocott-stained kidney-tissue sections of mice with disseminated fluconazole-resistant *C. albicans* infection revealed that the size and number of infectious foci decreased with increasing doses of hLF(1–11), up to a dose of 40 μg/kg of body weight; for example, the number and size of infectious foci in mice injected with 40 μg of peptide/kg of body weight (figure 5A and 5B) were considerably lower than those in untreated mice (figure 5C and 5D). Doses >40 μg of peptide/kg of body weight were not more effective (results not shown). In addition, the morphology of *C. albicans* in these tissue sections was studied. The results revealed that *C. albicans* cells, which were mainly filamentous in infectious foci in untreated mice (figure 5D), were growing as blastoconidia in mice effectively treated with hLF(1–11) (figure 5B).

**Effect of hLF(1–11) on the in vitro transition, induced by human plasma, in *C. albicans* morphology.** In order to evaluate whether hLF(1–11) inhibits the transition in *C. albicans* morphology, blastoconidia were incubated with 10% (vol/vol) heat-inactivated human plasma in vitro and then were washed and reincubated in buffer in the presence of increasing concentrations of the peptide. Microscopic analysis revealed that the percentage of *C. albicans* germ-tube formation in vitro decreased (*P* < .05) with increasing concentrations of hLF(1–11) (figure 6).
DISCUSSION

The main conclusion to be drawn from the results of the present study is that the hLF(1–11) peptide is effective against disseminated (fluconazole-resistant) *C. albicans* infections in cyclophosphamide-treated mice. This conclusion is based on the following observations. First, compared with the number of viable yeasts in the kidneys of untreated mice, those in the kidneys of mice treated with a wide range of hLF(1–11) doses was significantly reduced, and the results are in agreement with our earlier reports showing that in vitro hLF(1–11) kills *Candida* species in a dose-dependent manner [19, 20]. Second, microscopic analysis of kidney-tissue sections showed that infectious foci in hLF(1–11)-treated mice contained considerably fewer *C. albicans* than did those in untreated mice, and both the size and number of infectious foci in the kidneys of untreated mice also were greater than those in hLF(1–11)-treated mice. Moreover, it appeared that *C. albicans* was growing mainly as blastoconidia in hLF(1–11)-treated mice, whereas filamentous forms prevailed in untreated mice; consistent with this observation is that the percentage of *C. albicans* germ-tube formation in vitro decreased with increasing concentrations of hLF(1–11). Because, both in animal studies [18] and in cell cultures [30, 31], the filamentous form is associated with the virulence of *C. albicans*, it is conceivable that inhibition of the morphological transition of *C. albicans* effected by this peptide contributes to the amelioration of clinical signs and symptoms of *C. albicans* infection in mice.

It should be noted that the dose of hLF(1–11) peptide necessary to provide antifungal effects (killing and inhibition of the morphological transition of *C. albicans*) in mice was much lower than that in in vitro experiments [19, 20]. A possible explanation for this observation is that the antifungal effects of hLF(1–11) in animals with experimental infections involve synergistic/additive effects between this peptide and host-derived factors, including other antimicrobial molecules, as has been reported for hLF and lysozyme and secretory leukoprotease inhibitor [32], reactive oxygen intermediates [33], and other local factors, such as pH and Ca\(^{2+}\) and Zn\(^{2+}\) concentrations. Another possible explanation is that hLF(1–11) stimulates the antifungal activities of host cells, as has been reported for the effects that lactoferrin has on the antimicrobial activities of macrophages and neutrophils [14, 34, 35] and the production of antimicrobial peptides by mucosal epithelial cells [17].

One of the most remarkable findings of the present study is that the antifungal activity of hLF(1–11) leveled off after a dose as low as 0.4 ng/kg of body weight. This finding is in agreement with our observation that mice treated with low doses of hLF(1–11) did not have significant clinical signs and symptoms of the infection. The most likely explanation for the leveling off of the antifungal effects of hLF(1–11) is that the latter induces multiple processes that contribute differently to its antifungal activity against disseminated *C. albicans* infection in mice and that activation of some of these processes was seen only when high doses of this peptide were used. Consistent with this explanation is our finding that, compared with the levels of IL-10 in the sera of untreated mice, those in the sera of mice treated with high doses—that is, \(\geq 4\) mg/kg of body weight—of hLF(1–11) were elevated whereas those in the sera of mice injected with \(\leq 40\) \(\mu\)g/kg of body weight were not. Because IL-10 suppresses immunity against *Candida* infections [36–39], it could be that the leveling off of its antifungal effects in mice is due partly to the inhibitory effects that IL-10 has on host defense against *C. albicans* infections. Indeed, we found that, compared with the antifungal effects in mice that were injected with either the isotype-matched control mAb or no antibody, those in mice injected with IL-10 neutralizing mAb were increased, indicating that, in this latter group of mice, IL-10 counteracts hLF(1–11)’s antifungal effects in a dose-dependent manner. Further investigation into the effects that hLF(1–11) has on the levels of cytokines in the sera of mice with disseminated *C. albicans* infection have shown that, compared with the levels of TNF-\(\alpha\) and IL-6 in the sera of untreated mice, those in the sera of mice treated with effective doses of hLF(1–11) were significantly decreased whereas those in mice treated...
with the highest tested dose (i.e., 4 mg/kg of body weight) of this peptide were not. These data are in agreement both with the observation that bovine lactoferrin reduces the production of TNF-α and IL-6 by circulating blood cells in mice [13] and with the finding that bovine lactoferrin suppresses the lipopolysaccharide-stimulated production of IL-6 and TNF-α by human monocytes [40]. Our observation that the levels of IFN-γ in the sera of mice treated with 0.4 ng–0.4 μg of hLF(1–11)/kg of body weight were significantly greater than those in the sera of untreated mice may indicate that this peptide acts on T lymphocytes, as has elsewhere been reported for intact human lactoferrin [15]. Obviously, further investigations are necessary to establish whether cytokine production at sites of infection is reflected by the levels of cytokines in serum.

In conclusion, the antifungal activities of hLF(1–11) may involve both its actions on C. albicans viability and virulence and its effects on host cells that are crucial in the defense against disseminated C. albicans infections, as has elsewhere been reported for amphotericin B [41].

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**References**


