Lactoferrin Peptide Increases the Survival of Candida albicans-Inoculated Mice by Upregulating Neutrophil and Macrophage Functions, Especially in Combination with Amphotericin B and Granulocyte-Macrophage Colony-Stimulating Factor

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Received 20 October 2000/Returned for modification 27 December 2000/Accepted 20 March 2001

To develop a new strategy to control candidiasis, we examined in vivo the anticandidal effects of a synthetic lactoferrin peptide, FKCRWQWRM (peptide 2) and the peptide that mimics it, FKARRWQWRM (peptide 2'). Although all mice that underwent intraperitoneal injection of 5 x 10⁸ Candida cells with or without peptide 2' died within 8 or 7 days, respectively, the survival times of mice treated with 5 to 100 μg of intravenous peptide 2 per day for 5 days after the candidal inoculation were prolonged between 8.4 ± 2.9 and 22.4 ± 3.6 days, depending on the dose of peptide 2. The prolongation of survival by peptide 2 was also observed in mice that were infected with 1.0 x 10⁸ Candida albicans cells (3.2 ± 1.3 days in control mice versus 8.2 ± 2.4 days in the mice injected with 10 μg of peptide 2 per day). In the high-dose inoculation, a combination of peptide 2 (10 μg/day) with amphotericin B (0.1 μg/day) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (0.1 μg/day) brought prolonged survival. With a combination of these agents, 60% of the mice were alive for more than 22 days. Correspondingly, peptide 2 activated phagocytes inducing inducible NO synthase and the expression of p47phox and p67 phox, and peptide 2 increased phagocyte Candida-killing activities up to 1.5-fold of the control levels upregulating the generation of superoxide, lactoferrin, and defensin from neutrophils and macrophages. These findings indicated that the anticasoidal effects of peptide 2 depend not only on the direct Candida cell growth-inhibitory activity, but also on the phagocytes' upregulatory activity, and that combinations of peptide 2 with GM-CSF and antifungal drugs will help in the development of new strategies for control of candidiasis.

Candida albicans is a common commensal organism that occasionally causes opportunistic infections (38). As shown by the increased number of fungal infections in AIDS, the frequency of candidiasis has rapidly increased during the last 2 decades (7, 8, 45, 48). In addition to AIDS, immunosuppression is induced by treatments of solid malignant tumors, lymphoproliferative disorders, and organ transplantation. In immunocompromised patients, Candida cells easily invade the host's organs and multiply, causing lethal damage to the lungs, kidneys, liver, and intestines.

The prevention and treatment of candidial infection have therefore become important for immunocompromised patients. Although the host's defense system against Candida cells has not yet been completely clarified, it has been reported that both humoral and cellular immunities contribute to protection against Candida cells (14, 51). In the former, antibodies to Candida cell antigens enhance phagocytosis of neutrophils and macrophages (30, 36). Salivary proteins, such as secretory immunoglobulin A, secretory components, histatins, lysozyme, lactoferrin, transferrin, lactoperoxidase, mucins, and defensins have also been nominated as the humoral agents that prevent Candida cell adhesion and growth in the oropharyngeal cavity (21, 33, 34, 49, 50, 54, 57, 59, 63), whereas cellular agents, such as neutrophils, macrophages, and T and NK cells, play important roles in the front line against Candida cells, exhibiting phagocytosis and killing (17, 32). For sufficient phagocytosis, opsonization of Candida cells is required (26, 42). However, macrophages can trap nonopsonized blastoconidia by using their mannose receptors (18). To kill the trapped blastoconidia, neutrophils and macrophages generate reactive oxygen intermediates (ROI) and nitric oxide (NO) (16, 19, 58). The generation of ROI and NO is regulated by multiple cytokines (9, 13). Among them, granulocyte-macrophage colony-stimulating factor (GM-CSF), interferons, and prostaglandins strongly induce NO synthase (NOS) (11, 22, 46) and activate other enzymes associated with ROI generation (27, 39, 41). However, the virulence of blastoconidia is correlated with their resistance to phagocytes (24). It has been reported that Candida cells with high levels of hyphal wall protein 1 (HWP1) and C. albicans drug resistance proteins 1 and 2 (CDR1 and -2) were resistant not only to antifungal drugs, but also to phagocytes (15, 43, 52).

Clinically, there are two types of candidiasis: body surface candidiasis, including mucocutaneous candidiasis, and deep (organ) candidiasis. Surface candidal infection is relatively easily cured, but deep candidiasis is highly resistant to antifungal drug therapy (37). To prevent and control candidal infection, extensive efforts to develop excellent antifungal drugs have been undertaken (1). If a drug that possesses high antifungal activity also shows phagocyte-activating activity, a new aspect of treatment of fungal infections will open up. Such agents are likely to be obtained by following the example provided by...
physiologically secreted antimicrobial proteins. Recently, it has been reported that some lactoferrin peptides exhibit an potent anti-Candida cell activity (61). Along with these approaches, we synthesized a short lactoferrin peptide, FKCRRWQRWM, and examined its influences on blastoconidia and phagocytes. We found that the peptide possessed superior activities in both kinds of cells, suggesting its usefulness for the treatment of candidiasis.

MATERIALS AND METHODS

Peptide preparation. The lactoferrin peptide (FKCRRWQRWM; peptide 2) and the peptide it mimics (FKRRWQRM; peptide 2') were synthesized by Iwaki Glass Biolab Co. (Chiba, Japan) by the solid-phase method with Fmoc (9-fluorenylmethoxycarbonyl) as the Nα-amino-protecting group. These peptides were purified by high-performance liquid chromatography on a reverse-phase C18 column. The level of purity was >95%, as analyzed from its peak integration with high-performance liquid chromatograms at 214 nm.

Blastoconidial manipulations. C. albicans TIMM0134 was supplied by the Department of Microbiology, Kochi Medical School, Kochi, Japan, and C. albicans KSC1 was isolated from the oral cavity of a patient with oral candidiasis, which was classified serotype A according to the criteria of Fukazawa et al. (20a). Both strains were grown in Sabouraud’s dextrose agar (Difco, Detroit, Mich.) at 37°C. The Candida cells were cultured at 37°C in yeast extract-peptone-dextrose (YPD) medium for 16 to 20 h, and blastoconidial cells were used in all experiments.

Inoculation of Candida cells and treatment of mice. Specific-pathogen-free inbred CBA/N female mice 8 weeks old were intraperitoneally challenged with 5 × 10⁹ or 1 × 10⁹ blastoconidia. From the day of the challenge, peptide 2 (5 to 100 μg/mouse), peptide 2’ (100 μg/mouse), GM-CSF (Peprotech EC Ltd, London, United Kingdom) (0.1 μg/mouse), amphotericin B (0.1 μg/mouse), combination of these, or saline was injected intravenously for 5 days. Five mice were used in each treatment.

Separation of macrophages and neutrophils. On the 5th day after the start of treatment, 2 ml of 1% thioglycolate was injected intraperitoneally. Neutrophils and macrophages were separated from each peritoneal lavage obtained at 10 and 72 h after the injection of the thioglycolate solutions, respectively. The purity of each population was examined microscopically, and >95% purity was ascertained from the morphology.

Nitrite assay. The separated peritoneal macrophages (10⁷/well) were cultured in 96-well plates (Corning, Corning, N.Y.) by using Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 2 mM L-glutamine, 50 μU of penicillin per ml, and 50 μg of streptomycin per ml. To stimulate the macrophages, lipopolysaccharide (LPS) (from Escherichia coli; Sigma, St Louis, Mo.) was added to DMEM to a concentration of 1 μg/ml. After 24 h of cultivation, the culture supernatants were collected, and the nitrite concentration in each supernatant was assayed by Griess reaction. Briefly, equal volumes of 2% sulfanilamide in 10% phosphoric acid and 0.2% naphthylethylenediamine dihydrochloride were mixed to prepare the Griess reagent. The reagent (100 μl) was added to equal volumes of the supernatant, and the mixture was then incubated for 30 min at room temperature in the dark. The A₅₄₀ of the formed chromophore was measured with a plate reader. The nitrite content was calculated with sodium nitrite as a standard.

O²⁻ generation assay. O²⁻ generation was assayed by the nitroblue tetrazo- lium (NBT) reduction method. In a 5% CO₂ atmosphere, neutrophils (10⁶/well) or peritoneal macrophages (10⁵/well) were incubated for 1 h at 37°C in Hanks buffered saline solution containing 1 mg of NBT per ml, with or without 10⁻⁵ M phorbol myristate acetate (PMA), 10⁻⁷ M N-formyl methionyl leucyl phenylal- anine (FMLP), 2.5 mg of o-phenzoin methanols (OZ) per ml, or heat-treated dead blastoconidia (10⁹ cells/ml, 100°C, 30 min). The optical density at 550 nm in each well was examined with a plate reader.

Phagocytosis of neutrophils and macrophages. C. albicans blastoconidia were labeled with 0.5 μg of fluorescein isothiocyanate (FITC)-concanavalin A (Con A) (Sigma) for 10 min at room temperature and washed three times with phosphate-buffered saline (PBS) (56). The FITC-labeled C. albicans cells were cocultured with effectors (neutrophils or macrophages) at a ratio of 10:1 for 1 h at 37°C. Phagocytosing cells were detected by a FACScan fluorescence-activated cell sorter (Beckton Dickinson, Mountain View, Calif.), and the peak intensity of the fluorescence level (arbitrary units) was determined as the phagocytic index.

Candida killing. C. albicans blastoconidia were labeled with 125I (NaI) for 1 h at 37°C at a concentration of 100 μCi per 10⁹ cells. The blastoconidia were then washed three times and used as the targets. The effectors, neutrophils or macrophages, were mixed with the 125I-labeled blastoconidia to give an effector/target ratio of 1:10 in a final volume of 0.2 ml flat-bottom well. The mixtures were then incubated for 4 h at 37°C, and the isotope activity in 0.1 ml of the supernatant from each well was counted with a gamma counter. The percentage of cytotoxicity was calculated with the following formula: % cytotoxicity = [(experimental release (cpm) − spontaneous release (cpm))/(maximal release (cpm) − spontaneous release (cpm))] × 100, where spontaneous release is the isotope activity in the target cells incubated without effectors, and maximal release is the isotope activity in the supernatant after treatment of the blastoconidia with 0.1% Triton X-100. Values are expressed as the mean ± standard deviation of triplicate assays.

Western blotting. After the mouse treatment indicated above, separated neutrophils and peritoneal macrophages were lysed with TNE lysis buffer (1 M Tris-HCl [pH 7.6], 0.5 M EDTA, 10% Nonidet P-40), and the total protein level in each sample was determined by Lowry’s method. The protein level in each lysate was adjusted to 30 μg/20 μl of sodium dodecyl sulfate (SDS) sample buffer, and the lysate samples were subjected to SDS-polyacrylamide gel electrophoresis and Western blotting, which was performed with anti-p47phox, anti-p67phox, and anti-iNOS antibodies (Transduction Laboratories, Lexington, Ky.)

Lactoferrin release from neutrophils. Lactoferrin levels in the culture supernatants were assayed by sandwich enzyme-linked immunosorbent assay (ELISA). Briefly, microwells were coated overnight with 400-fold-diluted rabbit anti-human lactoferrin serum (Nordic Immunological Laboratories, Tilburg, The Netherlands). After blocking with non-specific reactions with PBS containing 0.5% bovine serum albumin (BSA), the samples and serially diluted standard lactoferrin were poured into the microwells and left overnight at 4°C. After a thorough washing, alkaline phosphatase-conjugated, affinity-purified rabbit anti-human lactoferrin antibody (Jackson Immunoresearch Laboratories, West Grove, Pa.) was added to each well, and the wells were incubated for 90 min at 37°C and washed. A phosphatase substrate (Sigma) was then added, and the developed color was read at 405 nm on an ELISA reader. The findings were calculated from the standard curve.

Measurement of defense concentration. A 10-μl aliquot of the supernatant was assayed by reversed-phase HPLC on a C₁₈ column (4.6 by 250 mm; Nacalai Tesque, Osaka, Japan). HPLC was performed with a 20-min linear gradient from solvent A (0.05% trifluoroacetic acid [TFA], 10% acetonitrile) to solvent B (0.05% TFA and 50% acetonitrile) at a flow rate of 1.0 ml per min. Defense was quantified by comparing the peak heights of the eluted defensin derived from the samples with that of a synthetic human defensin-1 standard (Protein Research Foundation, Osaka, Japan).

Statistical analysis. All experiments were duplicated, and each value is shown as the mean ± standard deviation. The significance of differences between sets of data was determined by Student’s t test. P values of <0.05 were considered significant.

RESULTS

Effects of peptide 2 on survival periods of Candida cell- injected mice. Peptide 2 dose-dependently prolonged the survival periods of Candida-infected mice (Fig. 1A). Although all

FIG. 1. Influence of peptide 2, GM-CSF, and amphotericin B on survival of Candida cell-injected mice. (A) Each mouse was intraperitoneal challenged with 5 × 10⁹ Candida blastoconidia and intravenously treated with saline, 5 ( ), 10 (A), 20 ( ), or 100 ( ) μg of peptide 2 per day or 100 μg of peptide 2’ per day ( ), for 5 days from the day of challenge. (B) After inoculation with 5 × 10⁹ blastoconidia, each mouse was treated with peptide 2 (10 μg/day), GM-CSF (0.1 μg/day), or both together for 5 days. (C) After inoculation with 10⁹ blastoconidia, each mouse was treated with peptide 2 (10 μg/day), peptide 2’ (100 μg/day), GM-CSF (0.1 μg/day), amphotericin B (0.1 μg/day), peptide 2 plus amphotericin B, peptide 2’ plus amphotericin B, peptide 2 plus amphotericin B plus GM-CSF, or peptide 2’ plus amphotericin B plus GM-CSF for 5 days.
control mice died within 8 or 7 days after the inoculation of 5 \times 10^8 blastoconidia with or without 100 \mu g of peptide 2 per mouse per day, mice administered 5, 10, 20, or 100 \mu g of peptide 2 per mouse per day, showed prolonged survival, and their survival times were 8.4 \pm 2.9, 16.2 \pm 3.7, 18.8 \pm 3.4, and 22.4 \pm 3.6 days, respectively. Compared with 10 \mu g of peptide 2 per mouse, a dose of 0.1 \mu g of GM-CSF per mouse per day more strongly suppressed lethality in mice inoculated with blastoconidia (Fig. 1B). Sixty percent of the mice administered GM-CSF survived longer than 3 weeks, while more than half of peptide 2-treated mice died within 10 days after Candida cell inoculation. When mice were treated with both GM-CSF and peptide 2, 40% of the Candida-inoculated mice survived longer than 4 weeks, and 20% survived until the end of the experiment, respectively. Furthermore, the cooperation of peptide 2 with amphotericin B and GM-CSF was observed in mice inoculated with 10^9 blastoconidia (Fig. 1C). Although the survival time of control mice was less than 5 days, the survival time of mice treated with peptide 2 and amphotericin B (0.1 \mu g per mouse per day) was prolonged to 11 days in the mice with the shortest survival time. When Candida (10^9 cells)-inoculated mice were concomitantly treated with the three agents peptide 2, GM-CSF, and amphotericin B, the rate of lethality for the mice was strongly decreased; all mice survived for 18 days, and 6 of 10 mice survived until the end of the study. Peptide 2 did not show such effects in combination with GM-CSF and amphotericin B.

Enhancement of phagocytosis of neutrophils and macrophages by peptide 2. Neutrophils and macrophages phagocytosed FITC-labeled Candida cells more markedly when the donor mice were treated with peptide 2 (Fig. 2). The phagocytic activities of neutrophils and macrophages obtained from control mice were 32.1 \pm 0.9 and 29.8 \pm 0.8, respectively, while those of peptide 2-treated mice were 42.3 \pm 1.8 and 38.6 \pm 1.9, respectively (P < 0.01). The levels of phagocytosis in the peritoneal infiltrates of GM-CSF-treated mice were similar to those in the peptide 2-treated mice, and the phagocytic activities of both kinds of phagocytes, which were obtained from mice treated with both peptide 2 and GM-CSF, were higher than those of phagocytes obtained from peptide 2 or GM-CSF-treated mice (P < 0.05). However, peptide 2 did not upregulate the phagocytic activities.

Influence of peptide 2 on Candida-killing activities of neutrophils and macrophages. Although peptide 2 did not increase the Candida-killing activities of phagocytes, both neutrophils and macrophages obtained from peptide 2-treated mice showed higher Candida-killing activities than those from control mice (Fig. 3). The Candida (TIMM0134)-killing activities of neutrophils and macrophages from peptide-2-injected mice were 31.3% \pm 3.1% and 33.6% \pm 2.6%, respectively, while those in control mice were 21.7% \pm 2.8% and 23.6% \pm 2.5%, respectively. With the combination of peptide 2 and GM-CSF, the Candida-killing activity of macrophages from mice that were treated with both peptide 2 and GM-CSF was significantly higher than that of macrophages from mice treated with peptide 2 or GM-CSF alone.

O$_2^-$ generation and expression of p47$^{phox}$ and p67$^{phox}$ in neutrophils. The generation of O$_2^-$ from neutrophils was enhanced by in vivo treatment of mice with peptide 2 as well as GM-CSF (Fig. 4). O$_2^-$ generation was clearly increased when neutrophils obtained from peptide 2-treated mice were stimulated with PMA, FMLP, OZ, or Candida cells. The upregulatory effect of in vivo treatment was also observed in GM-CSF, and the upregulated O$_2^-$ levels were similar to those obtained with peptide 2. When mice underwent injection of peptide 2 and GM-CSF, further increases in O$_2^-$ generation from neutrophils were observed.

Concomitant with the increase in O$_2^-$ generation by in vivo treatment with peptide 2, the expression of p47$^{phox}$ and p67$^{phox}$ was increased (Fig. 5). P47$^{phox}$ expression was also enhanced by GM-CSF, but upregulation of p67$^{phox}$ expression by the cytokine was not observed. In addition, no additive effect of
peptide 2 and GM-CSF on the expression of the NADPH oxidase components was observed.

**Nitrite generation and iNOS expression in macrophages.** Nitrite generation from macrophages was enhanced by in vivo treatment of mice with peptide 2 and GM-CSF and further enhanced by both agents (Fig. 6). LPS-stimulated and non-stimulated macrophages from control mice generated low levels of nitrite, 7.3 ± 1.2 and 3.7 ± 1.1 μM/10^5 cells, respectively. Macrophages obtained from mice treated with peptide 2, GM-CSF, and peptide 2 and GM-CSF together generated 5.0 ± 1.1, 8.1 ± 1.3, and 13.6 ± 1.8 μM nitrite per 10^5 cells without any stimulation, and they generated 18.5 ± 2.6, 25.4 ± 3.1, and 42.5 ± 3.6 μM nitrite per 10^5 cells with LPS stimulation, respectively. Concomitant with the upregulation of nitrite generation, iNOS expression in macrophages was increased by both agents (Fig. 7).

**Release of lactoferrin and defensin from neutrophils.** Lactoferrin release from neutrophils was upregulated by in vivo treatment of mice with peptide 2, although GM-CSF only weakly increased lactoferrin release (Fig. 8). The levels of released lactoferrin in control and peptide 2-treated mice were 253 ± 44 and 396 ± 61 ng/ml, respectively. The release of defensin was slightly increased by peptide 2 and GM-CSF, but the increases were not significant.
DISCUSSION

Fungal infections, especially candidiasis, have gradually increased during the past few decades, corresponding with the increase in immunosuppression due to viral infections, organ transplantation, and cancer treatments (7, 45, 48). With the increase in candidiasis, highly virulent strains of C. albicans resistant to antifungal agents have appeared, and their lethality has become a serious matter in immunocompromised patients (35). For the control of infections with highly virulent C. albicans strains, potent antifungal drugs are required. However, synthesized antifungal chemicals possess generally severe adverse effects, such as impairment of liver and kidney functions (31, 47). Therefore, new strategies are required for development of physiologically adapted antifungal agents and combined treatments with multiple kinds of agents.

Recently, the antibacterial activities of natural peptides such as defensins, histatins, gramicidins, granulysin, and lactoferrin have been investigated (6, 25, 28, 44). In these investigations, including C. albicans (2, 60, 62). In 1992, Bellamy et al. (5) synthesized a potent antifungal lactoferrin peptide consisting of 25 amino acid residues, lactoferricin B, which lacked iron-binding activity. Following their study, we synthesized a new lactoferrin peptide, named “peptide 2,” which consists of 10 amino acid residues (55). Compared with lactoferricin B, peptide 2 appeared to have a higher antifungal activity in vitro (55). In the present study, we examined the in vivo effects of peptide 2.

Peptide 2 dose-dependently prolonged the survival periods of the Candida-inoculated mice in cooperation with GM-CSF and amphotericin B. Of the mice that were injected with a high dose (10⁹ cells) of C. albicans, more than half (60%) survived when they underwent combined treatment with the three agents, although all mice without any antifungal treatment died within 5 days after injection of C. albicans. As reported previously, peptide 2 inhibits growth of C. albicans by suppressing glucose incorporation and DNA and protein syntheses (55). Differing from peptide 2, amphotericin B binds to the membrane sterols of fungal cells, causing impairment of their barrier function and loss of cell constituents (4). The difference in the antifungal actions of peptide 2 and amphotericin B suggests the cooperation of both agents. In fact, we ascertained previously that peptide 2 inhibited growth of amphotericin B-resistant strains (55). These previous and present study findings recommend a combined use of peptide 2, GM-CSF, and antifungal chemicals such as miconazole, fluconazole, and/or nystatin for control of infections with highly virulent Candida strains.

The in vivo cooperation of peptide 2 with GM-CSF against Candida cells indicates a role of peptide 2 in the activation of phagocytes. As expected, the killing activities of neutrophils and macrophages obtained from the mice treated with peptide 2 were increased to about 1.5-fold of those from untreated mice. Accordingly, the levels of O₂⁻ generated by neutrophils obtained from the peptide 2-injected mice were increased to about twofold those of controls. These upregulations were
confirmed by the enhanced expression of the components of NADPH oxidase, p47phox and p67phox. In the upregulation of O2− generation, Peptide 2 appears to activate many signal pathways, including the protein kinase C pathway, because the enhanced O2− generation was observed in all O2− inducers, PMA, FMLP, and OZ. In addition to the increase in neutrophil O2− generation, iNOS expression and nitrite generation in macrophages were upregulated by peptide 2. These upregulations in neutrophils and macrophages appear to result from both the direct and indirect actions of peptide 2. Previously, we ascertained that peptide 2 primed in vitro neutrophils to generate O2− (55), and peptide 2 induced lactoferrin secretion from neutrophils and GM-CSF release from neutrophils and macrophages (data not shown). Therefore, the upregulation of O2− and nitrite generation by peptide 2 appears to have partially resulted from the priming and partially resulted from indirect induction via the autocrinal and paracrine cytokines. In candidal infections, multiple kinds of cytokines, such as tumor necrosis factor alpha, gamma interferon, interleukin-2, GM-CSF, and interleukin-6, are generated from leukocytes, endothelial cells, and epithelial cells (3, 20). When peptide 2 is administered under such circumstances, the cytokine generation level is increased further, and activation of neutrophils and macrophages as well as NK cells and CD4+ T cells is expected. GM-CSF is one of the cytokines that strongly activates neutrophils and macrophages (10, 40). GM-CSF enhances neutrophil chemotaxis by increasing diacylglycerol generation, enhances phagocytosis by increasing FcR III expression, and enhances killing via increasing O2− generation and cytokine release (12, 23, 29, 53). Therefore, combinations of GM-CSF and macrophages as well as NK cells and CD4+ T cells is expected. The activation of the anti fungal activity and the pharmacodynamics of peptide 2 have not yet been sufficiently explored. To establish a reasonable combined treatment of candidal infections with peptide 2 and other agents, the antifungal mechanism of peptide 2, including its influence on CDR1, CDR2, and nitrite generation, Peptide 2 appears to activate many signal pathways, including the protein kinase C pathway, because CDR1, CDR2, and nitrite generation, Peptide 2 appears to activate many signal pathways, including the protein kinase C pathway, because

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