Regulation of Fungal Infection by a Combination of Amphotericin B and Peptide 2, a Lactoferrin Peptide That Activates Neutrophils

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To establish a novel strategy for the control of fungal infection, we examined the antifungal and neutrophil-activating activities of antimicrobial peptides. The duration of survival of 50% of mice injected with a lethal dose of Candida albicans (5 x 10⁸ cells) or Aspergillus fumigatus (1 x 10⁸ cells) was prolonged 3 to 5 days by the injection of 10 μg of peptide 2 (a lactoferrin peptide) and 10 μg of α-defensin 1 for five consecutive days and was prolonged 5 to 13 days by the injection of 0.1 μg of granulocyte-monocyte colony-stimulating factor (GM-CSF) and 0.5 μg of amphotericin B. When mice received a combined injection of peptide 2 (10 μg/day) with amphotericin B (0.5 μg/day) for 5 days after the lethal fungal inoculation, their survival was greatly prolonged and some mice continued to live for more than 5 weeks, although the effective doses of peptide 2 for 50 and 100% suppression of Candida or Aspergillus formation were about one-third and one-half those of amphotericin B, respectively. In vitro, peptide 2 as well as GM-CSF increased the Candida and Aspergillus killing activities of neutrophils, but peptides such as α-defensin 1, β-defensin 2, and histatin 5 did not upregulate the killing activity. GM-CSF together with peptide 2 but not other peptides enhanced the production of superoxide (O₂⁻) by neutrophils. The upregulation by peptide 2 was confirmed by the activation of the O₂⁻-generating pathway, i.e., activation of large-molecule guanine binding protein, phosphatidylinositol 3-kinase, protein kinase C, and p47phox as well as p67phox. In conclusion, different from natural antimicrobial peptides, peptide 2 has a potent neutrophil-activating effect which could be advantageous for its clinical use in combination with antifungal drugs.

Phagocytes play an essential role in the host defense against fungal invasion and growth. Functional phagocytes eliminate fungi from infected organs by releasing nitrogen oxide and reactive oxygen species (ROS) (2, 15, 29, 35). One approach to the control of fungal infections is therefore to enhance the generation of ROS in phagocytes. Several kinases are involved in the signal pathways in the generation of ROS, and the phosphorylating signal is eventually transduced to the electron-transporting complex, NADPH oxidase (12, 14, 39). It is therefore indicated that activation of the kinases involved in ROS generation is a useful means of controlling fungal infection (2). However, few agents have been developed for this purpose, and only granulocyte-macrophage colony-stimulating factor (GM-CSF) is in clinical use (27).

During the last decade, biological studies have revealed much about the innate immune system. Along with the signals of bacterial toxins (polysaccharides and lipopolysaccharides) and viral DNA via Toll-like receptors, the biological characteristics of antimicrobial peptides continue to be clarified (19, 28). Each peptide exhibits a wide spectrum of activity against a variety of bacteria and fungi (25, 26). Defensins exhibit superior antimicrobial activities against gram-positive and -negative bacteria as well as fungi (10, 21, 43). α-Defensin 1 and β-defensin 2 are not constitutively expressed but are inducible. They are generated by and released from neutrophils and epithelial cells, respectively, in response to bacterial and fungal infections (13, 23, 32). These defensins therefore appear to play an important role in the protection of the host against microbial invasion. If both defensins have neutrophil-potentiating activities, especially ROS generation-stimulating activities, the defense coordinated with neutrophils and the peptides would be firm and functional. However, no investigation has indicated such neutrophil-activating activities of antimicrobial peptides.

Lactoferrin (Lf) was first found more than 30 years ago in human maternal milk, and its antimicrobial activity was ascertained soon thereafter (1, 34, 41, 42, 45). About 10 years later, the peptides lactoferricins B and H were synthesized, along with the bovine and human Lf amino acid sequences. These peptides possess higher anti-Candida activities than each original Lf (6, 7, 18). With reference to these peptides, our group synthesized a novel peptide (peptide 2) with stronger antifungal activity (2). Peptide 2 is composed of the N-terminal 17 to 26 amino acids (FKCCRWWQWRM) of bovine Lf. We already reported that the injection of peptide 2 and an antifungal drug, amphotericin B, combined prolonged the survival of mice into which Candida cells were injected (37). The in vivo effect of peptide 2 appeared to depend largely on neutrophil activation because the in vitro antifungal activity of peptide 2 was less than 1/10 that of amphotericin B. Previous studies indicate that some antimicrobial peptides enhance neutrophil killing activity by stimulating the signal in the pathway to generate ROS (26). On the basis of this possibility, we examined the ex vivo and in vivo antifungal and anti-Aspergillus activities of peptide 2, α-defensin 1, β-defensin 2, and histatin 5 and the influences of...
these peptides on neutrophils. The present study revealed that peptide 2 upregulates the generation of ROS in mouse neutrophils and protects mice against lethal *Candida* and *Aspergillus* infections in cooperation with amphotericin B.

**MATERIALS AND METHODS**

**Cell preparation and culture.** *Candida albicans* KSC1 was isolated from the oral cavity of a patient with oral candidiasis. The swabbed material was cultivated with Sabouraud dextrose agar (Difco, Detroit, Mich.) at 37°C, and the growth was cultured in yeast extract-peptone-dextrose (YPD) medium for about 20 h at 37°C. The growth in YPD medium was identified as *C. albicans* by using *Candida* check (Iatron Lab. Inc., Tokyo, Japan), and the organism was classified as serotype A according to the criteria of Fukazawa et al. (16). *C. albicans* strain TIMM0134 was used as a standard. *Aspergillus fumigatus* IF033022 was supplied by the Institute for Fermentation (Osaka, Japan). The cells were harvested and fixed by gently scraping the fungal mat into 1% Tween 80 in phosphate-buffered saline (PBS). All blastoconidial cells were filtered through sterile gauze, washed with PBS, and counted with a hemacytometer, and stored at 4°C until they were needed. Cells in the exponential phase of growth were used in all experiments.

**Peptides and antifungal drugs.** Peptide 2 was synthesized by Iwaki Glass Biolab Co. (Chiba, Japan) by a solid-phase method and was purified by high-performance liquid chromatography on a reverse-phase C18 column. The level of purity was >95%, as analyzed from the peak integration with high-performance liquid chromatograms at 214 nm. Both α-defensin 1 and β-defensin 2 were purchased from Peptide Institute Inc. (Osaka, Japan). Amphotericin B and histatin 5 were obtained from Sigma (Steinheim, Germany).

**Determination of ED.** The effective dose (ED) of each agent against *C. albicans* or *A. fumigatus* was determined by a standard microdilution technique. Blastocidinal cells (10^3) were cultured in the presence or absence of each agent for 4 h. They were then transferred onto Sabouraud agar plates and cultured for 24 h at 37°C. The colonies that formed in each plastic dish were then counted. Five plates were used for each sample, and the mean colony counts were calcu-
lated from the colonies on the five plates. ED₅₀ and ED₁₀₀ indicate the concentrations of agent that limited growth to 50 and 0% of the number of CFU of the control (which received no treatment), respectively.

**Treatment of mice.** Specific-pathogen-free inbred CBA/N female mice (age, 8 weeks) were used in the present study. A total of 5 × 10⁶ spores of *C. albicans* were administered intraperitoneally, and 1 × 10⁶ spores of *A. fumigatus* were administered into the lungs of other groups of mice through an intratracheal tube. From the day of challenge, saline, peptide 2 (10 or 20 µg/mouse), α-defensin 1 (10 or 20 µg/mouse), GM-CSF (0.1 or 0.5 µg/mouse), amphotericin B (0.1 or 0.5 µg/mouse), or a combination of these agents was administered intravenously once a day for five consecutive days. The mice were fed solid feed (Clea Japan, Inc.). Ten mice were used in each treatment group.

**Neutrophil preparation.** Neutrophils were separated from heparinized peripheral blood from healthy individuals. After centrifugation at 400 × g for 10 min, the buffy coat layer was collected, diluted in 3 volumes of PBS, and centrifuged on Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, N.J.) gradients by Boyum’s method. The pellets were resuspended in PBS containing 3% (wt/vol) dextran to remove any contaminating erythrocytes. After centrifugation, the neutrophils were resuspended in a hypotonic buffer solution, and the residual erythrocytes were removed. A purity of >95% and cell viability of >98% were confirmed by Giemsa staining and trypan blue exclusion, respectively.

**O₂⁻ generation assay.** The generation of superoxide (O₂⁻) was assayed by the nitroblue tetrazolium (NBT) reduction method. Neutrophils (10⁶ cells/well) were incubated in a 5% CO₂ atmosphere for 1 h at 37°C in Hanks buffered saline solution containing 1 mg of NBT per ml, with or without 10⁻⁷ M phorbol myristate acetate (PMA), 10⁻⁷ M N-formylmethionyl leucyl phenylalanine (FMLP), or 2.5 mg of opsonized zymosan (OZ) per ml. The optical density at 550 nm in each well was examined with a plate reader.

**Killing activities of neutrophils.** *C. albicans* or *A. fumigatus* blastoconidia were labeled with Na₂¹⁵CrO₄ at a concentration of 100 µCi per 10⁸ cells for 1 h at 37°C. The blastoconidia were then washed twice and used as targets. The effector cells were suspended in RPMI 1640 medium supplemented with 2% fetal bovine serum and were then mixed with the ¹⁵Cr-labeled blastoconidia to give an effector cell/target cell ratio of 1:10 in a final volume of 0.2 ml/flat-bottom well. The mixtures were incubated for 4 h at 37°C, and the isotope activity in 0.1 ml of the supernatant from each well was measured with a gamma scintillation counter. The percent cytotoxicity was calculated by the following formula: [(experimental release – spontaneous release)/(maximal release – spontaneous release)] × 100, where spontaneous release is the isotope activity in the target cells incubated without effector cells, maximal release is the isotope activity in the supernatant after treatment of the blastoconidia with 0.1% Triton X-100, and the units for all release values are counts per minute. Values were expressed as the mean ± standard deviation of triplicate assays.

**Western blotting.** Proteins were extracted from the separated neutrophils by lysing them 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 the method of Lowry et al. (24). The protein level in each lysate was adjusted to 40 µg/30 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, anti-PI3-K, and anti-PKC antibodies (Transduction Laboratories, Lexington, Ky.) and anti-Ras GAP antibody (Santa Cruz Biotechnology, Santa Cruz, Calif.).

**PKC activity.** Protein kinase C (PKC) activity was measured with a MESPACU protein kinase assay kit (MBL, Nagoya, Japan), based on the enzyme-linked immunosorbent assay. Briefly, after the fractionation of neutrophils by the method of Balazovich and Boer (4), the cytosol fraction was reacted with

![Figure 2](image.png)
phosphatidylserine peptide-coated microplates in the presence of 1 mM ATP. After 20 min of incubation at 25°C, biotinylated monoclonal antibody 2B9, which bound to the phosphorylated form of the phosphatidylserine peptide, was added, and the mixture was incubated at 25°C for 60 min. After the mixture was washed, peroxidase-conjugated streptavidin was added and the mixture was incubated for 60 min; peroxidase substrate was then added. The PKC activity was determined from the intensity of the color measured photometrically at 492 nm.

**G-protein activation.** Guanine binding protein (G-protein) activation was determined by measuring the stimulation of \(^{35}\)S-GTP binding. After pretreatment with or without 50 ng of pertussis toxin per ml and 100 ng of cholera toxin per ml for 1 h, the neutrophils were washed twice with buffer A, which contained 20 mM HEPES and 150 mM NaCl (pH 7.4). The cells were harvested and homogenized with a kinematica Polytron homogenizer in buffer A. The suspension was centrifuged twice at 20,000 \(\times\) 100 g for 20 min at 4°C. The pellet, which included the membrane fraction, was then preincubated for 15 min with peptide 2 in buffer B, which contained 20 mM HEPES (pH 7.4), 0.1 \(\mu\)M GDP, 50 mM MgCl\(_2\), and 150 mM NaCl; and the reaction was started with 0.2 nM \(^{35}\)S-GTP in a final volume of 200 \(\mu\)l in 96-well plates at room temperature for 60 min. The experiments were terminated by rapid filtration of the mixture through Unifilter-96 GF/B filters by use of a Filtermate harvester. The amount of radioactivity that was retained on the filters was determined with a Top Count microplate scintillation counter. The examination was performed in triplicate, and the values presented in the figures are expressed as the means \(\pm\) standard deviations of triplicate assays.

**Statistical analysis.** All experiments were performed in duplicate, and each value is shown as the mean \(\pm\) 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**

**Antifungal activities of peptides in vitro.** Compared with the activity of amphotericin B, all peptides examined had very...
weak antifungal activities (Fig. 1). The ED_{100} of amphotericin B was 0.62 \mu g/ml against *C. albicans* strain KSC1, obtained from a patient, and those of peptide 2, \(\alpha\)-defensin 1, \(\beta\)-defensin 2, and histatin 5 were 1.22, 1.50, 2.50, and 0.77 \mu g/ml, respectively. The standard strain, TIMM0134, was a little more sensitive to these agents than KSC1, with the ED_{100}s of amphotericin B, peptide 2, \(\alpha\)-defensin 1, \(\beta\)-defensin 2, and histatin 5 against TIMM0134 being 0.50, 1.15, 1.25, 2.25, and 0.70 \mu g/ml, respectively. Amphotericin B and these peptides exhibited similar patterns of inhibition of *A. fumigatus* and *C. albicans* growth, although the ED of each agent was a little higher against *A. fumigatus* than against *C. albicans*; the ED_{100}s of amphotericin B, \(\alpha\)-defensin 1, and peptide 2 were 0.85, 2.25, and 1.65 \mu g/ml, respectively.

**Survival-prolonging effects of peptides for mice infected with *C. albicans* or *A. fumigatus*.** When \(5 \times 10^8\) cells of *C. albicans* were inoculated intraperitoneally, all the mice died within 8 days, with a 50% survival duration (the duration from the time of *Candida* inoculation to the times of death of five mice) of 6 days (Table 1). Serial injection of 10 and 20 \mu g of peptide 2/day for 5 days prolonged the 50% survival rates to 10 and 14 days, respectively. The anticandidal effect of \(\alpha\)-defensin 1 was a little less than that of peptide 2. Compared with the activities of these two peptides, amphotericin B at 0.5 \mu g/day revealed a further strong anticandidal effect, with a 50% survival rate of 19 days, although all the infected mice had died by 25 days after the injection. The mice that underwent treatment with both peptide 2 and amphotericin B or with both \(\alpha\)-defensin 1 and amphotericin B survived for a long time, and four of the mice treated with 20 \mu g of peptide 2 and 0.5 \mu M amphotericin B survived throughout the observation period. The mean length of survival of mice treated with the combination of peptide 2 (20 \mu g/ml) and amphotericin B (0.5 \mu g/ml) was longer than that of mice treated with amphotericin B alone (*P*...
The combination of α-defensin 1 and GM-CSF with peptide 2 had a weak anticandidal effect compared with the effects of the other combinations. The prolongation of survival as a result of treatment with each peptide and combination of peptides with amphotericin B for A. fumigatus-injected mice was similar to that for C. albicans-injected mice. The effect of the combination of peptide 2 and amphotericin B was most prominent in both C. albicans- and A. fumigatus-inoculated mice.

Influences of antimicrobial peptides on Candida and A. fumigatus killing activities of neutrophils and O$_2^-$ generation. Although α-defensin 1, β-defensin 2, and histatin 5 did not upregulate the killing activities of neutrophils against C. albicans and A. fumigatus, peptide 2 did (Fig. 2). The killing activity upregulated by peptide 2 (10 μg/ml)-treated neutrophils was similar to that upregulated by GM-CSF (0.1 μg/ml)-treated neutrophils. Neutrophils pretreated with peptide 2 or GM-CSF and then treated with an O$_2^-$ inducer, PMA, FMLP, or OZ, generated higher levels of O$_2^-$ than control neutrophils (Fig. 3). O$_2^-$ generation was most strongly induced by PMA in peptide 2-pretreated neutrophils. The level of upregulation of O$_2^-$ generated by peptide 2 (10 μg/ml) pretreatment was almost the same as that generated by GM-CSF (0.1 μg/ml) pretreatment.

Influences of peptides on NADPH oxidase and O$_2^-$ signal pathway. The expression of NADPH oxidase components, p47$^{phox}$ and p67$^{phox}$, was increased by peptide 2 as well as by GM-CSF, but the other peptides tested only weakly upregulated the expression of these components (Fig. 4). When neutrophils were pretreated with a tyrosine kinase inhibitor, genistein, or a PKC inhibitor, H-7, the expression of p47$^{phox}$ and p67$^{phox}$ upregulated by peptide 2 and GM-CSF was strongly suppressed, especially by H-7 pretreatment. Phosphatidylinositol 3-kinase (PI3-K) expression was increased by treatment of the neutrophils with peptide 2 or GM-CSF (Fig. 5A). Histatin 5 also upregulated PI3-K expression, but the increase was very low (Fig. 5B). In parallel with PI3-K activation, PKC activity was strongly increased by GM-CSF and peptide 2 treatment and was weakly increased by treatment with histatin 5 and other peptides.
Peptide 2 activated G protein in a dose-dependent manner (Fig. 6). The activation reached a peak after 15 min of treatment with 10 μg of peptide 2/ml. The membrane fraction was obtained from neutrophils pretreated with a G-protein antagonist, pertussis toxin or cholera toxin, and the amount of \([\gamma^{35}S]GTP\) bound was estimated (Fig. 7). G-protein activation by peptide 2 was not influenced by pertussis toxin but was suppressed by cholera toxin.

**DISCUSSION**

Fungal infections are serious problems during the treatment of immunosuppressive viral infections, malignant tumors, and other immunocompromising disorders (17, 30, 31). Novel drugs with potent antifungal activities have been developed (9, 11), but they have insufficiently controlled fungal infections in immunocompromised patients, and a new strategy for control is desired. Recently, antimicrobial peptides have been found, and their biological activities have been studied with the hope that they may be used clinically (10, 22, 25, 26, 33, 36, 40). It was reported, for example, that histatin 5, which is generated from epithelial cells such as salivary gland cells and secreted into saliva, exhibited antifungal activity by binding to the fungal cell membrane and inducing ATP efflux (3, 8, 13, 20, 44). Other investigators have reported that defensins as well as histatins induce histamine release from mast cells (5). These reports suggest that antimicrobial peptides generally possess both fungal growth-inhibitory activities and leukocyte-activating activities. If they do possess both types of activities, combined therapy with antimicrobial peptides and synthesized antifungal drugs can be expected to control fungal infections.

Compared with the antifungal drug amphotericin B, all pep-
tides examined exhibited weaker activities on the inhibition of colony formation. Of the peptides tested, histatin 5 possessed the strongest activity against *C. albicans* and *A. fumigatus*, but its effect was slight lower than that of amphotericin B. The low levels of antifungal activity of the peptides appear to indicate that peptides alone, even at high doses, cannot remove fungi from an infected site if they do not activate phagocytes or if they do not cooperate synergistically with antifungal drugs. In vitro, the *C. albicans* and *A. fumigatus*-killing activities of neutrophils were increased by peptide 2 to levels near those of GM-CSF-treated neutrophils. However, the remaining three peptides, α-defensin 1, β-defensin 2, and histatin 5, did not upregulate the killing activities of neutrophils. Consistent with this result, peptide 2 and GM-CSF primed neutrophils to generate O$_2^-$, although combinations of GM-CSF with other peptides did not. The priming effect of peptide 2 was most strongly observed in PMA-treated neutrophils and was almost completely suppressed by a PKC inhibitor, H-7, while the upregulation of O$_2^-$ production by GM-CSF was nearly the same among PMA-, FMLP-, and OZ-treated neutrophils. This result suggests that peptide 2 activates some molecules on the PKC pathway to O$_2^-$ generation. Dose-dependent increases in the killing activity and O$_2^-$ generation were not observed in the upregulation of neutrophil function by peptide 2; that is, the upregulated activities of the neutrophils were instead decreased by treatment of neutrophils with a high dose (50 μg/ml) of peptide 2. The accurate mechanism is unclear, but the decrease in neutrophil function obtained with 50 μg of peptide 2/ml was consistent with the decreased levels of expression of p47$^{phox}$ and p67$^{phox}$ in neutrophils treated with the same dose of peptide 2. A suppressive signal appears to be induced by the high dose of peptide 2, which was similarly observed with 0.5 μg of GM-CSF/ml.

Although treatment with 20 μg of peptide 2 and α-defensin 1 per mouse revealed the survival prolongation effects in mice, their effects were weaker than that of amphotericin B. The combination of peptide 2 and amphotericin B, however, largely prolonged the survival times of the mice. The effect of the combination was, however, weakly synergistic. The weak synergistic effect appears to be derived from the neutrophil-activating activity of peptide 2.

The generation of O$_2^-$ is under the control of the NADPH oxidase-activating signal (39, 46). For the activation of NADPH oxidase, its cytosol components, p47$^{phox}$, p67$^{phox}$ and GTP-bound Rac, must move to the membrane and form a complex with p22$^{phox}$ and gp91$^{phox}$ (2). Interestingly, the present study revealed that peptide 2 as well as GM-CSF upregulated p47$^{phox}$ and p67$^{phox}$ expression, but the other peptides tested did not. The upregulated expression was almost completely suppressed by H-7 and was only weakly suppressed by a tyrosine kinase inhibitor, genistein. The suppression by H-7 suggested that the activation of PKC was induced by peptide 2 and GM-CSF. As expected, an increase in PKC activity was observed by the treatment of human peripheral blood neutrophils with peptide 2 and GM-CSF. The activation of PKC was supported by the finding that peptide 2 and GM-CSF increased the level of expression of PI3-K, which is the upstream kinase of PKC. Compared with the increase in PKC activity achieved with these two agents, α-defensin 1, β-defensin 2, and histatin 5 increased PKC activity only slightly.

Cell surface receptors and cell membrane-associated kinases play a critical role in the signal pathways associated with cell activation. Large G proteins are involved in the signal pathways in the generation of ROS, and some O$_2^-$ inducers, for example, FMLP, bind to large G proteins; the signal required to generate O$_2^-$ is transduced downward via PI3-K and PKC to NADPH oxidase (2, 14, 39). The present study demonstrated that peptide 2 activated G protein and that the activation was largely suppressed by cholera toxin but not by pertussis toxin, and cholera toxin suppressed p47$^{phox}$ and p67$^{phox}$ activation by peptide 2. From these results, peptide 2 appears to activate the cytosol components of NADPH oxidase through the G protein–PI3-K–PKC pathway and does not appear to use a pathway that involves mitogen-activated kinase. In addition, peptide 2 likely binds to certain receptors on the cell surface which are coupled with G protein, perhaps the Go subunit. However, the result that cholera toxin did not completely suppress G-protein activation by peptide 2 suggests the existence of other pathways in the signaling of peptide 2.

The upregulation of O$_2^-$ generation and the fungicidal activities of peptide 2-treated neutrophils indicate a potent antifungal effect in vivo. In fact, the injection of peptide 2 and amphotericin B combined brought about a far greater prolongation of the survival time in mice inoculated with a lethal dose of *C. albicans* or *A. fumigatus*. A combination of peptide 2 and gentamicin also had a potent antifungal effect in vivo (data not shown). The synergistic effect of peptide 2 in the combination largely appears to depend on the neutrophil-activating activity of peptide 2. However, another mechanism likely appears to be involved in the synergistic cooperation between peptide 2 and amphotericin B. Furthermore, compared with histatin 5, peptide 2 increased the rate of ATP efflux from both *C. albicans* and *A. fumigatus* cells and decreased the intracellular ATP level, although GM-CSF did not affect the ATP level (unpublished data), suggesting that the levels of intracellular antifungal drugs were increased by peptide 2 because of the decreased levels of efflux of antifungal drugs. These effects of peptide 2 on neutrophils and fungal cells therefore appear to indicate a new strategy for the control of fungal infections. For the application of peptide 2 as part of combination therapy, a pharmacological investigation of peptide 2, including the possibility of antibody formation, is required.

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