The toxicity of naturally occurring or designed antimicrobial peptides is a major barrier for converting them into drugs. To synthesize antimicrobial peptides with reduced toxicity, several amphipathic peptides were designed based on the leucine zipper sequence. The first one was a leucine zipper peptide (LZP); in others, leucine residues at the a- and/or d-position were substituted with single or double alanine residues. The results showed that LZP and its analogs exhibited appreciable and similar antibacterial activity against the tested Gram-positive and Gram-negative bacteria. However, the substitution of alanine progressively lowered the toxicity of LZP against human red blood cells (hRBCs). The substitution of leucine with alanine impaired the binding and localization of LZP to hRBCs, but had little effect on the peptide-induced damage of *Escherichia coli* cells. Although LZP and its analogs exhibited similar permeability, secondary structures, and localization in negatively charged membranes, significant differences were observed among these peptides in zwitterionic membranes. The results suggest a novel approach for designing antibacterial peptides with modulation of toxicity against hRBCs by employing the leucine zipper sequence. Also, to the best of our knowledge, the results demonstrate that this sequence could be utilized to design novel cell-selective molecules for the first time.

There is a demand for the design and identification of novel antibiotics because of the emergence of bacterial strains resistant to conventional drugs. A large number of antimicrobial peptides, which exhibit a broad-spectrum activity against microorganisms, including Gram-positive and Gram-negative bacteria, fungi, protozoa, viruses, and even tumors, have been identified from a wide variety of animals, including humans (1–4). Recent studies indicate that these antimicrobial peptides are important and effective components of innate immunity of the hosts (1, 2, 5).

Because the antimicrobial peptides cause damage to microbial cell membranes and thereby destroy their cellular integrity within a short time, it is believed that bacterial resistance will not be developed easily against these peptides (4). Therefore, antimicrobial peptides have drawn the attention of many researchers as the lead molecules for the development of new antimicrobial drugs.

Structure-function studies of naturally occurring antimicrobial peptides have demonstrated the requirements of the amphipathic properties and the presence of positive charges in their microbicidal activities. Several amphipathic cationic antimicrobial peptides with approximately 10–20 amino acid residues (mostly leucine, alanine, valine, lysine, and arginine) have been designed and characterized (6–12). Although most of these peptides are random coils in aqueous environments, they adopt helical structures in hydrophobic environments.

Novel antimicrobial peptides that adopt amphipathic β-sheet structures have also been designed (13, 14). Antimicrobial β-peptides containing β-amino acids have been characterized recently (15, 16).

Despite extensive studies, the molecular basis of cell selectivity of an antimicrobial peptide is not clearly understood, and the design of antimicrobial peptides with reduced toxicity is a challenging issue. Recent studies suggest that self-association of an antimicrobial peptide in an aqueous environment and its localization and secondary structure in membranes could influence its target cell specificity (17–19). For the frog skin peptides dermaseptin-3 and -4 (18) and the cathelicidin-derived human antimicrobial peptide LL-37 and its N-terminally truncated analog FF-33 (20), the peptide with the higher aggregation property in an aqueous environment also showed higher toxicity than the other. Recently, we identified a leucine zipper motif in melittin, which seemed to play a crucial role in maintaining the toxicity of melittin, but not its antibacterial activity (19). The substitution of heptad leucine with alanine disturbed the assembly in aqueous environments, zwitterionic membrane interaction, and the toxic activity of melittin against human red
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blood cells (hRBCs) without affecting its interaction with negatively charged membranes and its antibacterial activity. The results indicated that assembly or peptide-peptide interaction in melittin is a key factor in maintaining its interaction with zwitterionic membranes, and therefore, the substitution of heptad leucine with alanine affected its toxic activity. The results further suggested that this alteration in the assembly of melittin associated with the same amino acid substitution had a little effect on its amphipathic properties and probably therefore did not affect its interaction with negatively charged membranes and thus its antibacterial activity. We hypothesized that it is possible to modulate the toxic activity of novel model antimicrobial peptides designed based on the leucine zipper sequence by altering their assembly without affecting their antibacterial activities.

To develop model antimicrobial peptides with simultaneous modulation of their toxicity by altering their assembly, we characterized several novel amphipathic antibacterial peptides designed based on the leucine zipper sequence. To understand the molecular basis of the antibacterial and toxic activities of the designed peptides, their assembly in an aqueous environment and their detailed interaction with zwitterionic and negatively charged lipid vesicles were studied. Furthermore, to find a correlation between their phospholipid membrane interaction and biological activities, their localization was studied in live hRBCs, and also the peptides’ effect on bacteria was examined under an electron microscope.

EXPERIMENTAL PROCEDURES

Materials—Rink amide-4-methylbenzhydrylamine resin (loading capacity, 0.63 mmol/g) and all of the N-α-Fmoc- and side chain-protected amino acids were purchased from Novabiochem. Coupling reagents for peptide synthesis (1-hydroxybenzotriazole, N,N′-disopropylcarbodiimide, 1,1,3,3-tetramethyluronium tetrafluoroborate, and trifluoroacetic acid, and cholesterol (Chol) were purchased from Sigma. Dichloromethane, N,N′-dimethylformamide, and piperidine were standard grade and procured from reputed local companies. Acetonitrile (HPLC grade) was obtained from Merck. Egg phosphatidylcholine (PC), egg phosphatidylglycerol (PG), and dimyristoylphosphatidylethanolamine (PE) were procured from Northern Lipids Inc. (Vancouver, Canada). 3,3′-Dipropylthiacarbocyanine iodide and 4-fluoro-7-nitrobenz-2-0xa-1,3-diazole (NBD) were purchased from Molecular Probes (Eugene, OR). The rest of the reagents were analytical grade and procured locally; buffers were prepared in Milli-Q water.

Peptide Synthesis, Fluorescent Labeling, and Purification—Stepwise solid-phase syntheses of all of the peptides were carried out manually on rink amide-4-methylbenzhydrylamine resin (0.15 mmol) utilizing standard Fmoc chemistry as reported previously (19, 21). Labeling at the N termini of peptides with a fluorescent probe, cleavage of the labeled and unlabeled peptides from the resin, and their precipitation and purification by reverse-phase HPLC were achieved by standard procedures (19, 21, 22). The purified peptides were ~95% homogeneous as shown by HPLC (supplemental Fig. 1). The experimental molecular masses of the peptides (detected by MALDI-TOF analysis) corresponded very closely to the desired values (see Table 1 and supplemental Fig. 2). The concentrations of the peptides were determined by measuring their absorbances at 280 nm in 6 × guanidine hydrochloride using tryptophan extinction coefficients of 5690 M⁻¹ cm⁻¹.

Preparation of Small Unilamellar Vesicles—Small unilamellar vesicles were prepared by a standard procedure (19, 21, 22) with the required amount of PC/Chol (8:1, w/w), PC/PG (1:1, w/w), or PE/PG (7:3 w/w) using a bath-type sonicator (Laboratory Supplies Co., Inc., Hicksville, NY). The lipid concentration was determined by phosphorus estimation (23).

Circular Dichroism Experiments—CD spectra of the peptides were recorded in phosphate-buffered saline (PBS) and in the presence of phospholipid vesicles utilizing a Jasco J-810 spectropolarimeter. The samples were scanned at room temperature (~30 °C) in a capped quartz cuvette (0.20-cm path length) at a wavelength range of 250–195 nm. The fractional helicities were calculated using mean residue ellipticity values at 222 nm by the following equation as reported previously (24, 25): Fh = ([θ]222 − [θ]0222)/([θ]100222 − [θ]0222), where [θ]222 is the experimentally observed mean residue ellipticity at 222 nm, [θ]100222 and [θ]0222 (which correspond to 100 and 0% helical content, respectively) were considered to have mean residue ellipticity values of ~32,000 and ~2000, respectively, at 222 nm.

Determination of Membrane Permeability of the Peptides—The ability of the peptides to destabilize the PE/PG vesicles was determined by assay of the peptide-induced dissipation of the diffusion potential (26–28). In brief, lipid vesicles prepared in K⁺ buffer (50 mM K₂SO₄ and 25 mM HEPES, pH 6.8) were mixed with isotonic (K⁺-free) Na⁺ buffer, followed by the addition of the potential sensitive dye 3,3′-dipropylthiacarbocyanine iodide. After the addition of valinomycin to the vesicle suspension when the fluorescence of the dye exhibited a steady level, the respective peptide was added. The peptide-induced dissipation of the diffusion potential as detected by an increase in fluorescence (at 670 nm with excitation at 620 nm) was measured in terms of the percentage of fluorescence recovery (Fₜ), defined by Fₜ = (Iₜ − I₀)/(Iₑ – I₀) × 100%, where I₀ is the fluorescence after the addition of a peptide at time t, Iₑ is the fluorescence after the addition of valinomycin, and I₀ is the total fluorescence observed before the addition of valinomycin.

The membrane permeability of the peptides in PC/Chol and PC/PG vesicles was determined by the peptide-induced release of calcein from calcein-entrapped lipid vesicles (21, 29). Calcein-entrapped lipid vesicles were prepared with a self-quenching concentration (60 mM) of the dye in 10 mM HEPES at pH 7.4. A thin film of lipid (either PC/PG or PC/Chol) was resuspended in calcein solution, vortexed for 1–2 min, and then sonicated in a bath-type sonicator. The non-encapsulated calcein was removed from the suspension of lipid vesicles by gel filtration.
using a Sephadex G-50 column. Usually, lipid vesicles are diluted to ~10-fold after passing through a Sephadex G-50 column. The eluted calcine-entrapped vesicles were diluted further to the desired final lipid concentration for the experiment. Peptide-induced release of calcine from the lipid vesicles was monitored by the increase in fluorescence due to the dilution of the dye from its self-quenched concentration. Fluorescence was monitored at room temperature with excitation and emission wavelengths fixed at 490 and 520 nm, respectively. Calcine release as measured by the fluorescence recovery is defined by the same equation as used to determine the dissipation of the diffusion potential. However, in this case, $I_p$, the total fluorescence, was determined after the addition of Triton X-100 (0.1% final concentration) to the dye-entrapped vesicle suspension.

**Enzymatic Cleavage Experiments**—Enzymatic cleavage experiments were performed to detect the location of the peptides in their membrane-bound states by employing their NBD-labeled versions (19, 21, 28). In brief, lipid vesicles were first added to their membrane-bound states by employing their NBD-labeled counterparts (NBD-LZP, NBD-LZP(L8A) (a single alanine-substituted analog (SASA)), or NBD-LZP(L4A/L11A) (a double alanine-substituted analog (DASA))) for 15 min at 37 °C. Cells were washed, and confocal microscopic images of the live cells were taken with an argon ion laser set for NBD excitation at 457 nm. The setting of the photomultiplier was constant during the entire experiment.

**RESULTS**

**Design of Novel Peptides Based on the Leucine Zipper Sequence**—The leucine zipper is a special class of a heptad repeat in which the a- and d-positions are occupied mostly by leucine and isoleucine. Mere substitution of amino acids at the a- and d-positions might affect the toxicity of the leucine zipper-based antimicrobial peptide. Substitution of one or two amino acids at the a- and/or d-position might affect the toxicity of the leucine zipper-based antimicrobial peptides. Therefore, a series of 21-residue amphipathic peptides were designed based on the leucine zipper sequence with minor changes at the a- and/or d-position (Table 1). The first one was designed based on a classical leucine zipper motif sequence (LZP), comprising leucine, alanine, and

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**TABLE 1**

| Peptide          | Amino acid sequences (X = H or NBD) | Calculated mass | Observed mass | RP-HPLC retention time |
|------------------|-------------------------------------|-----------------|--------------|------------------------|
| LZP              | X-NH-LKAKALKALWLKAKALKAKALKAKA-NH2 | 2348.069        | 2348.865     | 20.0                   |
| LZP(1A)          | X-NH-LKAKALKALWLKAKALKAKALKAKA-NH2 | 2348.069        | 2348.993     | 19.9                   |
| LZP(1A/8A)       | X-NH-LKAKALKALWLKAKALKAKALKAKA-NH2 | 2348.069        | 2348.865     | 20.0                   |
| LZP(1A/8A/11A)   | X-NH-LKAKALKALWLKAKALKAKALKAKA-NH2 | 2348.069        | 2348.993     | 19.9                   |
| LZP(1A/8A/11A)   | X-NH-LKAKALKALWLKAKALKAKALKAKA-NH2 | 2348.069        | 2348.865     | 20.0                   |
| LZP(1A/8A/11A)   | X-NH-LKAKALKALWLKAKALKAKALKAKA-NH2 | 2348.069        | 2348.993     | 19.9                   |

**Hemolytic Activity Assay**—The hemolytic activity of these designed peptides was assayed against fresh hRBCs that were collected in the presence of an anticoagulant from a healthy volunteer by a standard procedure (8, 19). Peptides (dissolved in water) were added to the suspension of hRBCs (6% v/v) final concentration) in PBS to a final volume of 200 μl and incubated at 37 °C for 35 min. The samples were then centrifuged for 10 min at 2000 rpm, and the release of hemoglobin was monitored by measuring the absorbance at 540 nm. hRBCs in PBS ($A_{\text{blank}}$) and in Triton X-100 (0.2% v/v) final concentration; $A_{\text{Triton}}$) were used as negative and positive controls, respectively. The percentage of hemolysis was calculated according to the following equation (8, 19): % hemolysis = $\left(\frac{A_{\text{sample}} - A_{\text{blank}}}{A_{\text{Triton}} - A_{\text{blank}}}\right) \times 100$. 

**Assay of the Antibacterial Activity of the Peptides**—The antibacterial activity of the peptides was assayed in LB medium under aerobic conditions (8, 19, 30). Different concentrations of each of the peptides (dissolved in water) were added in duplicate to 100 μl (final volume) of medium containing the inocula of the test organism (~10⁶ colony-forming units) in the mid-logarithmic phase of growth and incubated for 18–20 h. The peptides’ antibacterial activity, expressed as their minimum inhibitory concentrations (MICs; the peptide concentration that resulted in 100% inhibition of microbial growth), was assessed by measuring the absorbance at 492 nm. The microorganisms used were Gram-positive bacteria (*Bacillus subtilis* ATCC 6633 and *Staphylococcus aureus* ATCC 9144) and Gram-negative bacterium (*Escherichia coli* ATCC 10536 and DH5α).
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![Peptide Structures](image)

**TABLE 2**

| Peptide/antibiotic | S. aureus ATCC 9144 | B. subtilis ATCC 6633 | E. coli ATCC 10536 | E. coli DH5α |
|--------------------|----------------------|-----------------------|-------------------|-------------|
| LZP                | 5.8 ± 0.6            | 5.8 ± 0.6             | 6.0 ± 0.6         | 7.2 ± 0.7   |
| LZP(L8A)           | 5.6 ± 0.6            | 6.2 ± 0.6             | 6.2 ± 0.6         | 7.4 ± 0.8   |
| LZP(L8A/L11A)      | 6.2 ± 0.6            | 6.2 ± 0.6             | 6.2 ± 0.6         | 6.8 ± 0.7   |
| LZP(L8A/L11A)      | 6.2 ± 0.6            | 6.2 ± 0.6             | 6.2 ± 0.6         | 6.8 ± 0.7   |
| LZP(L8A/L11A)      | 6.6 ± 0.8            | 7.8 ± 0.8             | 6.2 ± 0.6         | 7.4 ± 0.8   |
| Tetracycline       | 1.0 ± 0.2            | 1.0 ± 0.2             | 1.1 ± 0.2         | 1.2 ± 0.2   |

lysin residues (Fig. 1). To induce antibacterial activity, the amino acids were arranged so that the peptide was amphipathic with broad hydrophobic and hydrophilic surfaces. Tryptophan was included to study the intrinsic fluorescence of the peptides. In the first three analogs, a single leucine from the a- or d-position of LZP was substituted with alanine (SASA). In the other three analogs, two leucine residues in the a-position and the a- or d-position were replaced with two alanine residues (DASA). The number and position of the positive charges were kept the same in all of the peptides. Alanine was chosen for its helix propensity and appreciable hydrophobicity so that the amphipathic characteristics of these peptides would be maintained.

The Designed Novel Peptides Exhibit Appreciable Antibacterial Activity—The designed novel peptides were tested for growth-inhibiting activity in liquid cultures against two Gram-positive and one Gram-negative bacteria of two different strains. Tetracycline was used as a positive control. LZP exhibited appreciable antibacterial activity (Table 2) against the tested bacteria with a MIC (~6 μM) comparable with those of the other designed and naturally occurring antimicrobial peptides (7). Interestingly, the antibacterial activities of SASAs and DASAs were close to that of LZP. The results indicated that the antibacterial activity of LZP was almost intact after the substitution of leucine with alanine at its a- and/or d-position.

To understand the mode of action of the peptides on the bacteria, electron microscopic experiments were performed. A total change in the morphology and lysis of E. coli cells was observed in the presence of LZP, SASAs, and DASAs at their MICs after 2 h of treatment under a transmission electron microscope (Fig. 2). The electron micrographs of the bacteria after treatment with LZP, LZP(L8A) (a SASA), and LZP(L8A/L11A) (a DASA) are shown (Fig. 2).

An initial change in morphology and damage to the bacterial cell wall were evident upon treatment with LZP(L8A/L11A) at 75% of its MIC.

Modulation of the Hemolytic Activity of the Peptides by Substitution of Leucine at the α- and/or δ-position with Alanine—The hemolytic activity of all of the designed novel peptides against hRBCs was examined to measure their toxic activity. LZP exhibited the maximum hemolytic activity against hRBCs. Hemolytic activity was significantly reduced in SASAs and was further decreased in DASAs, rendering them negligibly active up to a concentration of 30 μM (Fig. 3A). Thus, the results indicated that modulation of the toxic activity of the designed peptides against hRBCs was achieved by the substitution of leucine at the a- and/or d-position with alanine.

To investigate the basis of reduction of the toxic activity of LZP analogs, the localization of the peptides to hRBCs was studied by confocal microscopic experiments with NBD-labeled LZP, LZP(L8A) (a SASA), and LZP(L8A/L11A) (a DASA). The green fluorescence of NBD-LZP was observed particularly densely in the hRBC membrane and also significantly in the cytosol (Fig. 3B). However, NBD-LZP(L8A) distributed poorly in a diffused manner and mostly in the membranes of the hRBCs, whereas NBD-LZP(L8A/L11A) showed negligible binding. Clearly, the results indicated that substitution of leucine at the a- and/or d-position progressively impaired the binding and localization of LZP to hRBCs.

Contrasting Differences in the Membrane Permeability of the Designed Peptides in Zwitterionic and Negatively Charged Membranes—To understand the possible mode of action and the basis of contrasting hemolytic but very similar antibacterial activities of LZP and its analogs, their membrane permeability was
checked in zwitterionic and negatively charged lipid vesicles. The membrane permeability (expressed as a percentage of fluorescence recovery) of a SASA was appreciably lower than that of LZP in zwitterionic PC/Chol vesicles and further decreased for a DASA (Fig. 4A). In contrast, SASAs and DASAs exhibited very similar membrane permeability to LZP in the negatively charged PE/PG (Fig. 4C) and PC/PG (data not shown) lipid vesicles. Fig. 4 (B and D) shows the profiles of membrane permeation induced by LZP and a representative SASA and DASA at two different concentrations in the PC/Chol and PE/PG lipid vesicles, respectively. The results indicated that, although the substitution of leucine with alanine appreciably impaired the permeability of LZP in zwitterionic membranes, it had almost a negligible effect on negatively charged membranes.

Differences in the Secondary Structures of LZP and Its Analogs in Zwitterionic Membranes, but Not in Negatively Charged Membranes—CD experiments were performed to determine the secondary structures of LZP and its analogs in an aqueous

FIGURE 2. Electron microscopic visualization of the effect of peptides on E. coli DH5α. Shown are E. coli cells without any treatment (A); after treatment with LZP(L8A/L11A) at 75% of its MIC (5.5 μM; B); and after treatment with LZP (C), LZP(L8A) (D), and LZP(L8A/L11A) (E) at their MICs.

FIGURE 3. Decrease in the hemolytic activity of LZP against hRBCs after substitution of leucine at the α- and/or β-position with alanine. A, dose-dependent hemolytic activity of LZP (line a), LZP(L11A) (line b), LZP(L8A) (line c), LZP(L4A) (line d), LZP(L8A/L11A) (line e), LZP(L4A/L8A) (line f), and LZP(L4A/L11A) (line g). B, detection of localization of NBD-labeled LZP, LZP(L8A), and LZP(L8A/L11A) to hRBCs by confocal microscopy. Panels 1 and 2 show the fluorescence and overlay of fluorescence and transmission images of hRBCs, respectively, either with 4.0 μM free NBD probe or with 4.0 μM NBD-labeled peptide: NBD probe (row a), NBD-LZP (row b), NBD-LZP(L8A) (row c), and NBD-LZP(L8A/L11A) (row d).
environment (PBS, pH 7.4) and in the presence of zwitterionic (PC/Chol) and negatively charged (PC/PG) phospholipid vesicles. (PE/PG vesicles were avoided because of excessive scattering.) Only LZP (but not its analogs) adopted significant helical structures (35%) in PBS (Fig. 5A). In the presence of PC/Chol and PC/PG lipid vesicles, LZP maintained the helical structure (35.6%) that it adopted in PBS. There was no significant induction of secondary structures in SASAs and DASAs in the presence of PC/Chol vesicles, although the former showed a slightly higher helical content than the latter (Fig. 5B). However, contrasting results were obtained when the CD spectra of these peptides were recorded in the presence of negatively charged lipid vesicles. Unlike in the presence of PC/Chol and in aqueous buffer, SASAs and DASAs adopted appreciable helical structures (~35%) in the presence of PC/PG lipid vesicles (Fig. 5C). Because the alanine-substituted analogs of LZP exhibited insignificant structures in aqueous buffer and zwitterionic membranes and because there was very little difference among the three SASAs and DASAs, the CD spectra of one of the representatives are shown (Fig. 5, A and B). The results thus suggested that substitution of leucine with alanine affected the secondary structure of LZP in the aqueous environment and in the presence of zwitterionic lipid vesicles, but not in the presence of negatively charged lipid vesicles.

**Substitution of Leucine with Alanine Affects the Localization of LZP Only in Zwitterionic Membranes, but Not in Negatively Charged Membranes**—The localization of these designed peptides to the phospholipid membrane was analyzed by recording their tryptophan fluorescence in the presence of PC/Chol and PE/PG vesicles. In the presence of increasing amounts of PC/Chol vesicles, the emission maximum of LZP in aqueous buffer shifted appreciably (~8 nm) toward the shorter wavelength (Fig. 6A), suggesting the localization of the tryptophan residue in a hydrophobic environment probably due to the insertion of the peptide in the lipid bilayer. However, the emission maxima of SASAs and a DASA shifted only slightly (~2 and 1 nm, respectively) toward the shorter wavelength, indicating their possible localization to the surface of the membrane and poor penetration into the zwitterionic lipid bilayer (Fig. 6A). Interestingly, the fluorescence studies of the peptides with PE/PG vesicles yielded a different result. The emission maxima of LZP, SASAs, and DASAs shifted (~15 nm) in a similar way toward a shorter wavelength (Fig. 6B). The results suggested that, unlike in zwitterionic membranes, the alanine-substituted LZP analogs inserted into the negatively charged lipid vesicles as did LZP. For simi-
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Additional evidence regarding the binding and localization of these peptides to the phospholipid membrane was collected from the proteolytic cleavage experiments with their NBD-labeled analogs in their membrane-bound states (19, 21, 28). The addition of protease K (at time point 3) to NBD-LZP bound to the PC/Chol vesicles resulted in almost no decrease in NBD fluorescence (Fig. 6E), indicating that the N terminus of the peptide protected itself from digestion by the proteolytic enzyme probably because of its insertion into the lipid bilayer. NBD-LZP(L8A) (a SASA) was cleaved partly, suggesting that substitution of leucine at the a- and/or d-position of LZP with alanine impaired its localization and, to some extent, binding to zwitterionic membranes. Interestingly, the proteolytic cleavage of NBD-labeled LZP, LZP(L8A), and LZP(L8A/L11A) was insignificant (Fig. 6F) when these peptides were bound to the PE/PG or PC/PG (data not shown) phospholipid membrane, indicating the possible insertion of the N termini of all three kinds of peptides into the negatively charged lipid bilayer.

**DISCUSSION**

We have used an amphipathic leucine zipper sequence to design novel antibacterial peptides with simultaneous modulation of their toxicity against hRBCs. LZP exhibited appreciable antibacterial activity and moderate toxicity against hRBCs. Although the substitution of leucine with alanine at the a- and/or d-position had a little effect on the antibacterial activity and/or d-position had a little effect on the antibacterial activity and moderate toxicity against hRBCs. However, the substitution of leucine at the a- and/or d-position with alanine impaired the self-association of LZP as evidenced by the significantly lower induction of helical structures in SASAs and DASAs (Fig. 7, A and B). Any peptide concentration, the molar ellipticity values (the measure of helical content) of SASAs and DASAs were significantly lower than that of LZP, with SASAs eluting at slightly lower volumes. However, the elution volumes of these alanine-substituted analogs indicated that their molecular masses were close to their dimeric molecular masses in aqueous environments.
buffer, which provided the probable explanations of the contrasting activities of LZP analogs against the bacterial cells and hRBCs.

Because the outer membranes of eukaryotic cells are composed mostly of zwitterionic lipids, whereas the major lipids of bacterial membranes are negatively charged, lipid vesicles composed of PC/Chol and PE/PG or PC/PG lipids were employed to understand the possible interaction of LZP and its analogs with eukaryotic and prokaryotic membranes, respectively. Several studies show a nice correlation between the toxic and antibacterial activities of these peptides with the results obtained from their interaction with model membranes of different lipid compositions (8, 19, 34). Although the alanine-substituted analogs had very similar permeability to LZP in the negatively charged lipid vesicles, their permeability to the zwitterionic lipid vesicles was reduced significantly (Fig. 4).

It is increasingly clear that certain amino acids assist in maintaining the secondary structure of a peptide in zwitterionic membranes. Although LZP maintained its α-helical structure in aqueous buffer and zwitterionic membranes, the alanine-substituted analogs exhibited hardly any structure in both environments. In contrast, unordered SASAs and DASAs in PBS adopted a significant helical structure in the presence of negatively charged lipid vesicles (Fig. 5).

Tryptophan fluorescence studies indicated that only LZP penetrated the zwitterionic lipid bilayer, whereas its alanine-substituted analogs were located onto its surface (Fig. 6, A and C). Proteolytic cleavage studies of NBD-labeled peptides also indicated the insertion of the N terminus of LZP inside the zwitterionic membrane. Moreover, these suggested that a DASA was more exposed on the surface of the zwitterionic phospholipid membrane compared with a SASA. Confocal microscopic studies (Fig. 3B), which revealed significant differences among NBD-labeled LZP, LZP(L8A), and LZP(L8A/L11A) in terms of their binding and localization to hRBCs, supported the tryptophan fluorescence and proteolytic cleavage studies of the peptides in the presence of zwitterionic lipid vesicles.

However, both the tryptophan fluorescence and proteolytic cleavage experiments with NBD-labeled peptides suggested that LZP and its analogs were located inside the bilayer of negatively charged lipid vesicles (Fig. 6, B and F). The results support other evidence in the literature indicating a different sequence

FIGURE 7. Detection of self-association and oligomerization states of LZP and its analogs by CD and gel filtration experiments with the peptides in PBS containing 1.5 m NaCl. A, CD spectra (in millidegrees (mdeg)) of 119.2 (line a) and 138 (line b) μM LZP, 106.0 (line c) and 122.7 (line d) μM LZP(L8A), and 123.6 (line e) and 143.1 (line f) μM LZP(L8A/L11A). ———, LZP; -----, LZP(L8A); ———, LZP(L8A/L11A). B, plots of mean residue ellipticity values at 222 nm ([θ]) versus peptide concentrations (micromolar). ■, LZP; □, LZP(L4A); ○, LZP(L8A); ○, LZP(L11A); △, LZP(L4A/L8A); A, LZP(L8A/L11A); plus-centered triangle, LZP(L4A/L11A). C, plot of the molecular masses of myoglobin (point a), cytochrome c (point b), aprotinin (point c), and the insulin B-chain (point d) with respect to their elution volumes after passing through a Sephadex G-50 column (diameter of 1.4 cm, length of 38 cm) with PBS containing 1.5 m NaCl in an AKTA FPLC system (Amersham Biosciences). The elution volumes of LZP, LZP(L4A), LZP(L11A), LZP(L4A/L11A), and LZP(L8A/L11A) are indicated (arrows 1–5, respectively), and the profiles are shown in the inset: LZP(L8A) and LZP(L4A/L8A) eluted at 36.0 and ~38 ml, respectively (data not shown). The flow rate was 1.0 ml/min, and the absorbance was recorded at 280 nm for each peptide at 210 μM and at a 0.2-ml volume.
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microscopic studies and probably therefore exhibited much reduced and almost zero hemolytic activity, respectively, against hRBCs.

CD and gel filtration studies clearly indicated that the self-assembly and extent of oligomerization of LZP were disturbed as a result of the substitution of its leucine residues with alanine at the a- and d-positions (Fig. 7). The tryptophan emission maximum of LZP at a shorter wavelength in PBS compared with those of SASAs and DASAs also indicated that LZP was more self-associated than its analogs (supplemental Fig. 3).

Altogether, the results indicated that substitution of leucine at the a- and/or d-position of LZP with alanine progressively impaired its permeability, secondary structure, and localization in zwitterionic membranes, which could contribute to reduction of the hemolytic activity of alanine-substituted LZP analogs against hRBCs. The higher toxicity of a SASA compared with a DASA was probably due to its slightly higher helical content, better penetration into the zwitterionic lipid vesicles, and higher self-association property in aqueous environments compared with the DASA. On the other hand, the substitution of leucine had negligible effects on the same properties when the peptides bound to negatively charged lipid vesicles, which could result in the similar antibacterial activity of LZP and its analogs.

How the alteration in assembly affects the biological activity and membrane interaction of an antimicrobial peptide is not clearly known. Melittin is a naturally occurring antimicrobial peptide that, in addition to the leucine zipper motif (which is located in the amino acid region 6–20), also contains a small hydrophobic stretch from amino acid 1 to 5 and a hydrophilic region from residues 21 to 26, consisting mainly of positive charges. To further investigate the role of a leucine zipper motif and peptide assembly in an antimicrobial peptide, as observed in melittin (19), it was of interest to examine the effect of substitution of leucine at the a- and d-positions with alanine on the biological activity and membrane interaction of a model peptide with no sequence homology to melittin and containing identical repeated heptads throughout the sequence. However, the experimental data clearly indicated an interesting similarity between the alanine-substituted melittin analogs (19) and the designed LZP analogs in terms of their impaired assembly in aqueous environments, reduced interaction with zwitterionic membranes, and decreased toxic activity against hRBCs, from which the role of a leucine zipper motif or peptide assembly in maintaining the zwitterionic membrane interaction and toxic activity of an antimicrobial peptide can be stated more convincingly.

In our previous work (19), the effect of substitution of leucine with alanine at only the heptadic a-position was examined. Although it was known that amino acids at the d-position also play a crucial role in the assembly of a protein containing this motif, it was not tested how the same amino acid substitution at the d-position affects the membrane interaction and toxic activity of an antimicrobial peptide containing a leucine zipper sequence. The present study clearly indicates that the leucine at the d-position of LZP has an equally important role in maintaining the interaction with zwitterionic membranes and toxic activity against hRBCs.

It has to be mentioned that, although modulation of toxic activity against hRBCs for both melittin and LZP without affecting their antibacterial properties was achieved in a similar way and that the latter has three heptads, whereas the former peptide contains only two, LZP was a less biologically active molecule than melittin in terms of both activities. These data suggest that, although the leucine zipper motif plays a crucial role in maintaining the toxic activities of both peptides, their overall properties are determined by the total amino acid sequence and composition. Therefore, to confer a certain level of antibacterial or toxic activity to a peptide is still a complex issue and requires extensive work.

In conclusion, this study has provided a framework consisting of the leucine zipper sequence for designing antibacterial peptides with modulation of toxicity against hRBCs. Thus, not only does the leucine zipper motif play an important role in the toxic activity of melittin (19), but this motif (as suggested by the results described here) can be further utilized for the rational design of novel antimicrobial peptides with modulation of their membrane/cell selectivity by altering their structural parameters in a simple and well-defined way, which may be useful in designing therapeutically relevant molecules.

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