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Antimicrobial Activities of Amphiphilic Peptides Covalently Bonded to a Water-Insoluble Resin†

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A series of polymer-bound antimicrobial peptides was prepared, and the peptides were tested for their antimicrobial activities. The immobilized peptides were prepared by a strategy that used solid-phase peptide synthesis that linked the carboxy-terminal amino acid with an ethylenediamine-modified polyamide resin (PepsynK). The acid-stable, permanent amide bond between the support and the nascent peptide renders the peptide resistant to cleavage from the support during the final acid-catalyzed deprotection step in the synthesis. Select immobilized peptides containing amino acid sequences that ranged from the naturally occurring magainin to simpler synthetic sequences with idealized secondary structures were excellent antimicrobial agents against several organisms. The immobilized peptides typically reduced the number of viable cells by ≥5 log units. We show that the reduction in cell numbers cannot be explained by the action of a soluble component. We observed no leached or hydrolyzed peptide from the resin, nor did we observe any antimicrobial activity in soluble extracts from the immobilized peptide. The immobilized peptides were washed and reused for repeated microbial contact and killing. These results suggest that the surface actions by magainins and structurally related antimicrobial peptides are sufficient for their lethal activities.

Microbial contamination and possible infection of the host are major concerns in the area of therapeutic medical devices (13, 15). Ongoing studies are directed toward understanding the mechanism of microbial adsorption and proliferation on material surfaces (13, 15, 40) and devising new approaches that can be used to avoid this microbial contamination (3, 8, 9, 34). Sequestering the antimicrobial agent close to the device surface has been shown to be an effective approach to reducing the potential for catheter-related infections in patients (18). We believe that the immobilization of the antimicrobial agent by covalent bonding has the advantage of long-term stability and lower toxicity than that by a leach- or a release-based system (10, 36). We chose to explore whether a variety of peptide sequences ranging from naturally occurring magainin to synthetic idealized amphiphilic peptides, as described by DeGrado (7) and Blondelle and Houghton (1), and several closely related analogs would be bactericidal when they are bound to an insoluble support. Antimicrobial peptides such as the magainins, cecropins, and defensins are attractive candidates for such a support-bound antimicrobial agent because of their broad-spectrum activities, their relatively low cytotoxicities, and a body of basic evidence that suggests that the membrane surface is their lethal site of action (2, 12, 16, 22, 23, 35, 37–39, 41).

In addition to seeking an improved approach to lower microbial proliferation on polymer surfaces, we were also interested in exploring an alternative experimental design for evaluating the mechanisms of action of these particular antimicrobial peptides. The stable chemical immobilization of these peptides to a water-insoluble support is perhaps a simpler experimental approach to testing the hypothesis that these antimicrobial agents are surface active. Immobilization restricts the range of penetration of the antimicrobial agent to loci at the surface of the microbial wall. Investigators of enzyme mechanisms have exploited the placement of the catalyst on an insoluble support as a means of studying the roles of subunit and protein-protein interactions in the catalytic activity (25, 31, 36). In a study of the bactericidal activity of bovine serum albumin (BSA)-gentamicin conjugates, Kadurugamuwa et al. (17) demonstrated the power of this general approach in ascribing the contribution of surface action to the lethal effects of gentamicin. In our study we used a water-insoluble, nondegradable polymer support.

The synthetic route described herein is modeled after the approach of solid-phase peptide synthesis (27), except that the elongating peptide chain was anchored to the polymer support by a noncleavable linkage. This convenient route to a polymer-bound peptide avoids the pitfalls of the other approaches since the peptide is bound via a single linkage and in a way that controls its orientation.

Our results demonstrate the antimicrobial activities of an immobilized magainin 2 and several idealized amphiphilic peptides. We observed no activity by an immobilized peptide whose amino acid sequence was the exact reverse of that of magainin 2 or by several nonamphiphilic, helical peptides. These results are consistent with findings from other studies that support a mechanism by the magainins and related peptides that involves a specific membrane-peptide interaction and that results in the disruption of the outer membrane (5). The activity appears to be distinguishable from the charge-based mode of action of other water-insoluble cationic polymers (10, 29). The proven bactericidal activities of these insoluble immobilized antimicrobial peptides have demonstrated to us the feasibility of preparing antimicrobial articles (e.g., catheters, wound dressings, and food preservation systems [28]) comprising these peptide agents bound to the surfaces of the article.

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MATERIALS AND METHODS

Chemicals. The 9-fluorenylmethoxycarbonyl or Fmoc-N-protected amino acids and the activating reagents hydroxybenzotriazole (HOBt), benzotriazole-1-yl-oxy-tris (dimethylamino)phosphonium-hexafluorophosphate (BOP or Castro's

reagent), or benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyl-uronium-tetrafluoroborate (TBTU) were obtained from the Milligen Division of Millipore (Bedford, Mass.) or Bachem Biosciences, Ltd. (Philadelphia, Pa.), and were used without further purification. Diisopropylcarbodiimide, piperidine, diisopropylethylamine (DIEA), *N*-methylmorpholine, dimethylaminopyridine, trifluoroacetic acid (sequencing grade), and picric acid were purchased from Aldrich (Milwaukee, Wis.). All solvents (dimethylacetamide [DMAc], dimethylformamide [DMF], dichloromethane [DCM], methanol [MeOH]) were glass distilled and were used fresh without further purification. The methylester-activated polyamide resin Pepsyn K, which was purchased from Milligen, was used for the preparation of immobilized peptides. Soluble peptides were prepared either by automated peptide synthesis (EXCELL; Milligen) with the PAC resin (methylbenzhydrylamine polystyrene support with a hydroxymethylphenoxyacetic acid linker) or by manual methods with either PAC resin or Sasrin (D-1295, Bachem), a super acid-sensitive resin.

Bacterial strains and growth conditions. The microorganisms used in the tests of antimicrobial activity were *Escherichia coli* MC4100 (ATCC 35695; a wild-type, bacteriophage lambda-negative strain, *E. coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923 and ATCC 6538, *Klebsiella pneumoniae* ATCC 4352, *Bacillus subtilis* ATCC 6051, *Candida albicans* ATCC 10231, *Aspergillus niger* ATCC 6275, and *Pseudomonas aeruginosa* ATCC 27853. All cells were grown by transferring an inoculum from the frozen stock culture into tryptic soy broth. The cells were grown to the stationary phase and were then subcultured and grown to the mid-log phase in an incubator shaker (New Brunswick Scientific, Edison, N.J.) at 37°C. *E. coli* TV1058 (a bioluminescent strain with a plasmid containing the *Vibrio fischeri* luciferase [*lux*] genes and a kanamycin resistance marker), provided courtesy of Tina VanDyk, Dupont Central Research, was grown in Luria-Bertani (LB) broth supplemented with kanamycin (50 μg ml^{-1}). Cell growth was monitored by optical density measurements (Cary 219 UV/visible spectrophotometer or Klett-Sommerson photoelectric colorimeter).

Synthesis of immobilized peptides. All syntheses except those involving the preparation of those polymers containing the magainin sequence were performed manually. Pepsyn K, a methylester-activated polyamide resin, was first reacted with neat ethylenediamine (EDA) for 18 to 24 h and was then washed three times each with DMF, MeOH, DCM, and MeOH. The resin had a functional group substitution level of 0.20 to 0.25 meq of amine g^{-1} of polymer; the average diameter of an individual resin particle was 300 μm. Picric acid analyses (33) performed on the resin after derivatization confirmed that a nearly quantitative reaction with EDA had occurred. The first Fmoc-protected amino acid (carboxyl terminus) was coupled to the support via standard in situ-activated ester coupling chemistry with either BOP or TBTU in the presence of DIEA. Following washes, the resin was deprotected with piperidine and washed, and then the cycle was repeated until the specific peptide sequence was completed. A final piperidine deprotection was performed to remove the Fmoc group from the amino terminus prior to full deprotection of the peptide with a cleavage cocktail containing thioanisole (5%), anisole (2%), ethane dithiol (3%), and trifluoroacetic acid (90%) to remove the side chain-protecting Boc groups. The resin with bound peptide was washed in sequence with ether, DMF, DCM, 5 to 10% DIEA in methylene chloride, DCM, MeOH, and DCM and was then vacuum dried. Automated peptide synthesis was performed by using the Milligen EXCELL peptide synthesizer. For automated synthesis, prepackaged cartridges of the Fmoc-amino acid reagents were used; however, the polymer support was a Fmoc-amino acid-derivatized PepsynK-EDA (~0.3 to 0.4 g; preparation described above) that was inserted into an empty EXCELL reaction column. We confirmed the sequence and amino acid composition of the bound peptide by two approaches. In the first approach, we applied the standard amino acid composition and sequence methods directly on the insoluble resin-bound peptide; following acid hydrolysis, an appropriately diluted sample of the soluble hydrolysate was transferred to the analyzer. In the second approach, we synthesized a blend of noncleavable and cleavable peptide resins by mixing the noncleavable PepsynK-EDA–Fmoc-amino acid$_1$ with approximately 10 to 20% (by weight) of a traditional ester-coupled amino acid support (e.g., PepsynKA-Fmoc-amino acid$_1$). At the completion of the synthesis, the sequence and amino acid composition of the bound peptide could be confirmed by analyses on the isolated soluble peptide. Amino acid compositions were determined of a Beckman 6300 amino acid analyzer following 6 N HCl hydrolysis of the peptides; amino acid sequences were determined on a Beckman 3000 protein sequencer. The substitution level of peptide on the resin was typically 0.05 ± 0.01 meq of peptide g^{-1} of solid.

Synthesis of soluble peptides. Soluble peptides were prepared by the same method described above for the preparation of immobilized peptides except that Milligen PepsynKA and the PAC supports were used for manual and automated syntheses, respectively. After deprotection, the filtrate was treated with cold, anhydrous diethyl ether to precipitate the peptides. The peptide was then solubilized in 10% acetic acid and was lyophilized to yield a crude crystalline solid. The crude product was further purified by high-performance liquid chromatography (HPLC) on a preparative reversed-phase column (Vydac C-4, 2.5 by 25 cm) by using a Waters Delta Prep HPLC system. The peptides were eluted with a linear gradient composed of buffer A (0.1% trifluoroacetic acid in water) and buffer B (0.1% trifluoroacetic acid in CH_3CN-H_2O [90/10]). The gradient was run from 0 to 100% buffer B in 100 min at a flow rate of 1.0 ml/min at ambient temperature. The peptides were detected at 215 nm. The compounds were collected from the column, lyophilized, and characterized by amino acid analyses.

Antimicrobial tests. All manipulations with the microbes involved standard sterile techniques used for microbiology. A starter culture was grown in a nutrient-rich medium (LB or tryptic soy broth) overnight, and on the following morning a subculture from this dense culture was grown in the same medium until the mid- to late-log phase as determined by optical density measurements (~0.1 to 0.6 A at 600 nm; 50 to 300 on Klett-Sommerson colorimeter). Typically, the cell densities were ca. 5×10^8 cells ml^{-1}. This subculture was diluted to a cell density of between 10^6 and 10^7 cells ml^{-1} to prepare an inoculum for the reactions. The reaction vessel (culture tube or microtiter plate) containing the test sample was inoculated with cells in growth medium so that the cell concentration was 10^5 cells ml^{-1}. The reaction volumes were 1.0 and 0.160 ml for tests performed in culture tubes and microtiter plates, respectively. The cells were maintained in an incubator at 37°C with shaking (~250 rpm). The MBC was determined by performing a dose-response experiment with the test sample. Soluble peptide was tested by preparing a set of twofold serial dilutions and transferring an aliquot to the test cell suspension so that the soluble peptide concentrations ranged from 3.5 to 125 μg ml^{-1}. Routinely, we evaluated the insoluble polymer-peptide conjugates at solid concentrations ranging from 4.0 to 64 mg ml^{-1} (the quantity of chemically titratable peptide ranged from 330 to 5,400 μg ml^{-1}). Agar plate enumeration was used to determine the number of viable cells. To enumerate cells on agar plates, an aliquot (100 μl) was removed from the cell suspension and 10-fold serial dilutions were made. The diluted cell suspensions (100 μl) were transferred and uniformly spread onto agar plates. For those samples in which there was clear suppression of growth (by visual inspection), the supernatant directly from the reaction was also plated onto an agar plate. The colonies on the agar plates were counted after incubation at 37°C for 24 h. The lower limit of cell detection was ca. 10 CFU ml^{-1} for the agar plate viability test method. The MBC was defined as that concentration at which there were no surviving cells. Since the immobilized peptides were insoluble, carryover effects were eliminated in these antimicrobial tests; thus, the MBC could be defined with a greater accuracy than it could for typical soluble drugs.

Bioassay for presence of soluble antimicrobial agent in supernatants of cell suspensions in contact with immobilized peptide. Supernatants were generated by placing either soluble or immobilized peptide at a concentration twice that of the MBC in 2.0 ml of LB broth with kanamycin (50 μg ml^{-1}) and *E. coli* TV1058 (10^5 cells ml^{-1}) into sterile culture tubes: soluble KG(LK$_2$L$_2$KL)$_2$, 62 μg ml^{-1}; immobilized peptide, E16KGL, 50 mg ml^{-1}. Two control tubes were prepared: a growth control, which contained cells with no bactericidal agent, and an antimicrobial activity control, which contained KG(LK$_2$L$_2$KL)$_2$ peptide (62 μg ml^{-1}) in medium only with no cells. The tubes were shaken in an air incubator at either 27°C (maximum temperature permissible when measurement of the light emission from cells is desired) or 37°C for 3 h to allow the growth control to reach the mid-log phase. The supernatants from each sample were removed from the original culture tube. The supernatants were divided and transferred into five wells of a microtiter plate in aliquots of 150 μl. Cells (10 μl) from a newly grown mid-log-phase culture were added to each well so that the final cell concentrations were: 1×10^5, 5×10^5, 1×10^6, 5×10^6, and 1×10^7 cells ml^{-1}. The microtiter plate was covered, placed in an incubator, and allowed to shake overnight. The CFU values were determined by agar plate enumeration as described above and were averaged for the entire range of cell concentrations tested in this antimicrobial assay.

RESULTS

Preparation of the immobilized peptides. We applied standard solid-phase chemistry using Fmoc-amino acids (11). HPLC analyses of the crude isolates from cleavable resins showed typical profiles, with a dominant peak in a mixture of other smaller peaks (data not shown). Amino acid analyses of the immobilized peptide yielded the expected results: the dominant sequence was the desired product. Typical purity was ca. 85% because the insoluble, immobilized peptide also contained deletion sequences that resulted from incomplete reaction coupling. This purity was comparable to that of the crude isolates from soluble peptides generated from cleavable solid-phase peptide resins. The major disadvantage of this stepwise method of attaching peptide monomers onto the polymer support was that there was no convenient way to separate and purify the attached peptides. Thus, the antimicrobial activities measured by the peptide-polymer conjugates reflect the combined activities of the desired peptide and the shorter deletion peptides that are created during the course of synthesis.

Antimicrobial activities of the immobilized peptides. We prepared and tested the bactericidal activities of a collection of

TABLE 1. Sequences and antimicrobial activities of various immobilized peptides

Entry no.	Immobilized peptide[a]	Amino acid sequence of the immobilized peptide (C→N terminus)[b]	Bactericidal activity[c]
1	EDA		−
2	HMD		−
3	E08KKK	K K K K K K K K	−
4	E16LLL	K K K K K K K K L L L L L L L L	−
5	E16KLK	L K L K L K L K L K L K L K L K	−
6	E14KLK	L K L K L K L K L K L K L K	−
7	E07LKK	L K L L K K L	+[d]
8	E14LKK	L K L L K K L L K L L K K L	+
9	H14LKK	L K L L K K L L K L L K K L	+
10	E16KGL	L K L L K K L L K L L K K L G K	+
11	H16KGL	L K L L K K L L K L L K K L G K	+
12	E17KGG	L K L L K K L L K L L K K L G G K	+
13	E18KGG	L K L L K K L L K L L K K L G G G K	+
14	E16LKL	K G L K K L L K L L K K L L K L	+
15	E10KKL	L K K L L K K L K K	+
16	E12LLK	L K K L L K K L K K L L	+
17	E14KKL	L K K L L K K L K K L L K K	+
18	E23GIG magainin 2	S N M I E G V F A K G F K K A S H L F K G I G	+
19	E17HSA magainin 2 deletion	S N M I E G V F A K G F K K A S H	+
20	E23SNM reversed magainin 2	G I G K F L H S A K K F G K A F V G E I M N S	−

[a] All sequences that have names that begin with E are attached to the resin with EDA as the linker. Samples with labels that begin with H are attached via the hexamethylenediamine linker.

[b] The amino sequences are written in the direction that the peptide is attached to the resin, i.e., beginning with the carboxyl terminus at the left; the amino terminus is at the right.

[c] Bactericidal activity was determined by incubating various quantities of the immobilized peptide or control sample with any of the three *E. coli* strains or *S. aureus* in growth medium as described in Materials and Methods. Bactericidal activity is positive when reductions of $\geq 10^5$ cells ml^{-1} are achieved in the presence of 12.5 to 37.5 mg of the test material ml^{-1} of the cell suspension. Samples with a bactericidal label indicated by a minus sign did not inhibit growth; final cell counts were comparable to those of inoculated controls.

[d] This sample was a modest suppressor; cell populations were reduced by 90 to 99.9% at high concentrations ($\geq$50 mg ml^{-1}) of the polymer-peptide conjugate in the reaction.

immobilized peptides (Table 1) against *E. coli* and *S. aureus*. Four of the immobilized peptides (Table 1, entries 3 to 6) had peptide sequences that are not predicted to form amphiphilic helices (1); these samples were nonbactericidal and served as controls for the study. Only those immobilized peptides with sequences that are known to have significant bactericidal activity (1, 6, 41) eliminated all ($\geq 10^5$ cfu ml^{-1}) cells in growing cultures of gram-positive and gram-negative bacteria (Table 1, entries 7 to 19). In addition, we reversed the order of amino acid sequences for two peptides: a synthetic analog, KG $(LK_2L_2KL)_2$ = KG$(LKP)_2$, and magainin 2. Antimicrobial activity was not lost when we reversed the amino acid sequence of the simple sequence of KG$(LKP)_2$, because the compositions in either direction were nearly identical (compare entries 10 and 14 in Table 1). In contrast, however, the reversal of the magainin 2 amino acid sequence essentially destroyed the bactericidal activity (compare entries 18 and 20 in Table 1).

The quantity of total solid required to kill all of the cells was typically between 12 and 40 mg ml^{-1}. We applied picric acid, amino acid sequence, and amino acid composition analyses to

TABLE 2. Comparison of immobilized peptide (E14LKK) and soluble peptide ($[LK_2L_2KL]_2$) MBCs for a variety of microorganisms

Organism	MBC (μg ml^{-1})	
	Immobilized peptide[a]	Soluble peptide
E. coli ATCC 25922	1,496	31
K. pneumoniae ATCC 4352	1,496	31
P. aeruginosa ATCC 27853	>4,300[b]	125
S. aureus ATCC 6538 and ATCC 25923	1,496	31
B. subtilis ATCC 6051	1,995	31
A. niger ATCC 6275	>4,300[b]	>500[b]
C. albicans ATCC 10231	3,200[c]	500

[a] MBCs for immobilized peptides report only the total amount of peptide bound on the solid support, and the values are based on picric acid and amino acid analyses. These calculated values are not measurements of the quantity of peptide available for contact and killing of the microorganism.

[b] No reductions in cell counts were observed.

[c] Total inhibition of the viable cell population was not observed. This value reflects a 90 percent reduction in the original cell count.

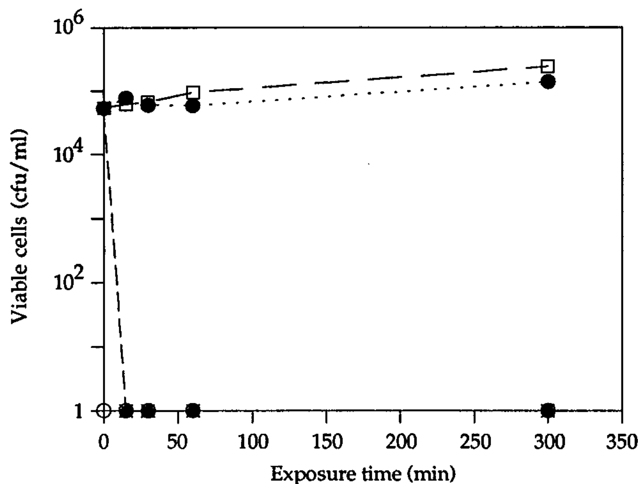

FIG. 1. Concentration of viable cells (*E. coli* MC4100) versus exposure time against uninoculated control (○), inoculated control (□), EDA-modified PepsynK resin (50 mg ml^{-1}) (●), soluble peptide 14LKK (62 μg ml^{-1}) (◆), and immobilized peptide E14LKK (50 mg ml^{-1}) (×).

TABLE 3. Bioassay for presence of soluble antimicrobial agent in supernatants of cell suspensions in contact with soluble and immobilized peptide

Sample[a]	CFU ml^{-1}[b]
Supernatant, soluble peptide $KG(LK_2L_2KL)_2$	0.00 ± 0.00
Supernatant, immobilized peptide, E16KGL	$(8.53 \pm 0.96) \times 10^8$
Inoculated control	$(8.22 \pm 1.79) \times 10^8$
Soluble peptide, $KG(LK_2L_2KL)_2$	0.00 ± 0.00

[a] Samples were generated as described in the Materials and Methods section.
[b] The values for CFU milliliter^{-1} are the averages of the values from three experiments.

estimate the peptide substitution levels on the immobilized supports. On the basis of the average peptide substitution levels (ca. 0.05 ± 0.01 meq g^{-1} of solid) for these polymer-peptide conjugates, these quantities of total solid translate to a range of ca. 1,000 to 3,400 μg of bound peptide ml^{-1}.

Antimicrobial activity of immobilized E14LKK against several organisms. We selected one of the immobilized peptides, E14LKK, to be studied in greater detail, and we examined its activity against a broader spectrum of organisms (Table 2). E14LKK reduced the total cell populations of *E. coli*, *S. aureus*, *B. subtilis*, and *K. pneumoniae*. It displayed a concentration-dependent reduction against *C. albicans*, with the proportion of the reduction ranging from 90% at the lowest quantity to 99.9% at the highest quantity of immobilized peptide in the cell medium. However, the immobilized peptide was ineffective against *P. aeruginosa* and *A. niger* even at the highest practical quantity of solid.

Kinetics of antimicrobial activity of immobilized E14LKK. Figure 1 displays the number of surviving cells after the cells (*E. coli* MC4100) were exposed to the antimicrobial agents for times of up to 300 min. No viable cells remained in the cell suspension after 15 min of exposure to either the immobilized peptide or the soluble peptide. In contrast, the polymer support (EDA-treated PepsynK) with no immobilized peptide did not alter the population in the medium, nor did it inhibit their subsequent growth.

Nature of the antimicrobial agent. We applied bioassays and used semipermeable membranes to determine whether the antimicrobial agent is a soluble peptide (leached or hydrolyzed) from the bactericidal polymer-peptide conjugates. Our test sequence for both experiments was the soluble peptide $KG(LKP)_2$ and the same peptide conjugated to the resin (E16KGL). In the first experiment, we used a bioassay method to look for the presence of a soluble antimicrobial agent in the supernatant of bacterial cultures treated with the immobilized peptide preparation (Table 3). The supernatants were removed from contact with the immobilized agent, transferred to sterile tubes, and reinoculated with more *E. coli* cells (final cell concentration range, 10^5 to 10^7 cells ml^{-1}). We observed cell growth in each sample containing supernatant that was in contact with the bactericidal immobilized peptide E16KGL. This result contrasts sharply with the persistence of bactericidal activity in the supernatants of cells treated with soluble peptide. When the supernatants from soluble peptide-treated cell samples were treated with additional *E. coli* cells, these added cells were also killed. Thus, soluble peptide, if it is present, should be stable, persist in the supernatant, and retain its bactericidal capacity in subsequent cell exposures. We have no evidence that the soluble peptide was captured by negatively charged elements of bacterial origin that would render the peptide inaccessible for either analytical detection or bactericidal action.

In the second experiment, the immobilized peptide was placed inside two semipermeable membrane bags; one membrane bag contained a small volume of medium (Fig. 2a) and the other contained *E. coli* cells suspended in medium (Fig. 2b). The bags were suspended in a suspension of *E. coli* cells sufficient to cover the depth of the bag. The pores of the semipermeable membrane were large enough (molecular mass cutoffs of 10 and 50 kDa were tested in separate experiments) to allow for the bidirectional flow of either soluble peptide or any small proteases released from the microbe. For comparison, the following control samples were tested: immobilized peptide in direct contact with the cell suspension, an inoculated control with no bactericidal agent, and medium only (uninoculated control). Cells lying outside of the dialysis membrane were not killed, nor was their growth inhibited by the membrane-encapsulated immobilized peptide samples. Bactericidal activity was only observed in the immobilized peptide preparation that came into direct contact with the cell suspensions (Table 4). We also subjected these immobilized preparations to boiling water for periods of time ranging from 10 to 1,000 min and observed no extraction of soluble peptide or any agent with antimicrobial activity or the loss of antimicrobial activity in the polymer system.

DISCUSSION

In the approach we describe herein, we exploited solid-phase peptide synthesis in order to rapidly prepare a collection of polymer-peptide conjugates to demonstrate the efficacies of polymer-bound magainin and related antimicrobial peptides.

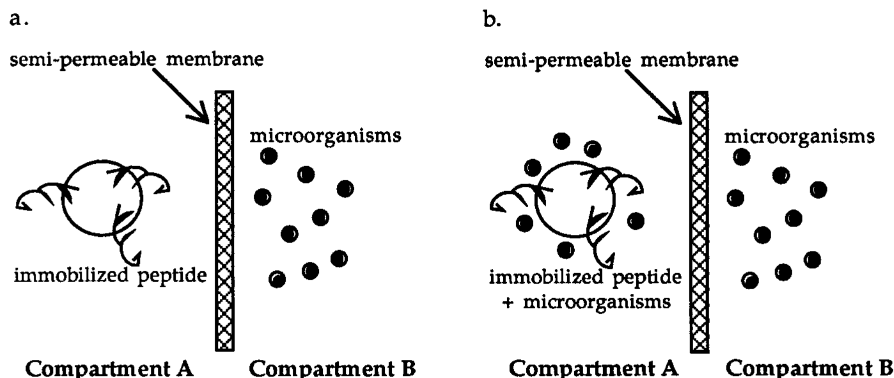

FIG. 2. Illustration of test designs for presence of a leachable antimicrobial agent (a) or a microorganism-induced hydrolysis of antimicrobial peptide agent (b).

TABLE 4. Effect of compartmentalization on bactericidal activity by immobilized peptides[a]

Sample	System component		Observed response
	Outside bag	Inside bag	
A	Medium		
B	Medium, cells		Growth
C	Medium, cells, immobilized peptide (no bag)		No growth
D	Medium, cells	Immobilized peptide	Growth
E	Medium, cells	Immobilized peptide + cells	Growth

[a] The experiment was performed with semipermeable dialysis bags with molecular mass cutoffs of 10 and 50 kDa. In the absence of a direct polymer interaction with cells, the immobilized peptide does not express bactericidal activity.

This approach has been used by others to prepare polymer-peptide conjugates for biological study (4, 19, 32).

Antimicrobial immobilized peptides. We surveyed the antimicrobial activities of a collection of diverse immobilized peptides in order to ascribe the antimicrobial activities to the peptide components of the peptide-polymer conjugates. The activity profile that we observed among the immobilized peptide compositions paralleled that among the soluble peptides. This is evidence that the peptide and not the resin is responsible for the activities of the polymer-peptide conjugates. The sequences of the synthetic and natural antimicrobial peptide compounds either have been predicted or have been experimentally demonstrated to form amphiphilic helices in hydrophobic solvents (1, 25). No antimicrobial activity is displayed by polymer-peptide conjugates whose predicted secondary structures are not classical amphiphilic helices, even though their leucine and lysine contents may be similar (Table 1, E08KKK, E16LLL, E16KLK, and E14KLK) to those of the antimicrobial compound.

Consistent and efficient antimicrobial expression occurs when a compound has a sequence length equal to or greater than 10 amino acids. We saw no significant differences between peptides conjugated to the support with a two-carbon or a six-carbon chain linker. Immobilized magainins also showed an activity dependence consistent with previous work showing that carboxy-terminal deletions are tolerated (6, 37) (Table 1, E17HSA); however, the reverse sequence is nonactive against bacteria (24) (Table 1, E23SNM). Although secondary structure is a major contributor to bactericidal activity, these and other data suggest that sequence-specific interactions of magainins with microbes may also contribute to the activity profile.

Nature of the antimicrobial agent. If we apply the criteria outlined by Venter (36), we present three general pieces of data that demonstrate that the active immobilized peptides meet the requirements for an agent that is active as an insoluble complex. First, the results of our experiments that probed for bactericidal activity in the supernatants of immobilized peptide samples convincingly demonstrate that a soluble agent capable of inhibiting cell growth was absent from these media (Table 3). Second, we consistently observed no antimicrobial activity under conditions in which the immobilized peptide was separated from the cells (Fig. 2a). Any antimicrobial agent released inside of the semipermeable membrane should have diffused to kill those cells lying outside of the bag. It is possible that a mechanism of peptide hydrolysis might require intimate contact with a microbe-associated protease; therefore, we examined the activity of a compartment that contained immobilized peptide and *E. coli* cells on an *E. coli* cell population that was located outside of the compartment (Fig. 2b). In that experiment, if a microbe-bound protease could cleave peptide from the immobilized peptide, then the active peptide fragment could diffuse through the semipermeable bag and act on the cells that lie in the suspension surrounding the bag. We consistently observed no antimicrobial activity in that experiment (Table 4, sample E) or under any other condition in which the immobilized peptide was physically segregated from the cell population under evaluation. Finally, we could recover and wash the immobilized peptides, challenge them repeatedly with fresh cell cultures, and observe antimicrobial activity (data not shown).

Our measured value for the quantity of peptide bound to the minimal bactericidal quantity of the immobilized preparation, E14LKK, was significantly higher than the MBC for its soluble peptide counterpart, $(LKP)_2$: ~1,500 versus 31 $\mu g\ ml^{-1}$. By design, for its optimal use in continuous-flow solid-phase peptide synthesis, the highly permeable, porous Pepsyn K support would have most of the covalently bound peptide chains in the interior, not the surface, of the porous matrix in order to achieve the highest densities of the desired peptide product by this synthetic method. The porosity range of 0.1 to 0.2 μm (11) is too small to allow for the entry and penetration by the *E. coli* bacteria that were used in the primary screening tests. Only those peptide chains immobilized on the surfaces of the resins were readily accessible for bactericidal action. Although we have some concern that the high concentrations of immobilized peptide required for total killing might reflect the slow release of a small percentage of the immobilized peptide by some hydrolysis mechanism, it is highly unlikely that a protease would selectively cleave these peptides with repeated patterns at a single cleavage site to yield an active molecule. Data from preliminary enzyme studies indicate that trypsin cleaves soluble and immobilized peptides (E14LKK, E16KGL) into several inactive fragments. When comparable levels of soluble and immobilized peptide are present, the immobilized systems are also more resistant to proteolytic attack: trypsin catalyzes the cleavage of the pendant peptide chain from the polymer at a rate approximately two to three decades slower than the soluble peptide.

Mechanism of action by magainins. An anticipated outcome of the present investigation was the opportunity to explore the question of whether the lethal activities of magainin and related peptides could result from mere surface activity on the targeted microorganism. We have shown that these insoluble, immobilized peptides are effective in arresting microorganism growth and proliferation. Although another immobilized peptide system comprising silicon rubber-immobilized magainin has been demonstrated to be effective in reducing the population of *Staphylococcus epidermidis* cultures by factors of 10 to 100, the previous study cannot provide significant mechanistic insight into the issue of lethal surface activity because the magainin is bound to the polymer via a long polyethylene oxide tether. By contrast, the immobilized antimicrobial peptide system that we describe herein consists of a short two- or six-carbon-atom chain linking the peptide to the support. The restricted penetration depth of the peptide provides a more convincing demonstration that outer membrane interaction by magainin is sufficient for lethal activity. Kadurugamuwa and others (17) arrived at a similar conclusion for gentamicin by applying a membrane-impenetrable BSA-gentamicin complex in an investigation of the bactericidal activity of the bound complex with *P. aeruginosa*. A recent study (21) of the effect of immobilizing the antimicrobial peptide nisin showed an effect that was different from that which we found. In that study, the

investigators concluded that when nisin is immobilized on either an organic or an inorganic matrix, it is inactive unless the peptide is desorbed or released from the polymer. Although this finding may accurately reflect the mode of action of this narrow-spectrum antimicrobial peptide, we believe that the results of the study by Lante et al. (21) are not conclusive. The investigators did not take into account the fact that the loss of antimicrobial activity by their glutaraldehyde, polyethyleneimine-treated supports might have been due to chemical modification of key amino acids necessary for the antimicrobial action of nisin and not simply due to the immobility of nisin. A comparison of the effect of the derivatization conditions on the activity of soluble peptide is crucial to discerning this key issue. This is where our employment of the controlled solid-phase peptide synthesis approach in the present study has a significant advantage: we avoided the creation of inactive molecules that may result from the reaction of the functionalized polymer with side chain residues on the peptide that are critical for activity.

There are still insufficient data available to determine how similar or dissimilar the modes of action of these particular immobilized peptides are to the actions of numerous nonpeptide, soluble (26, 29), and insoluble (10, 40) polycationic agents that have been used to control bacterial growth. These nonpeptide agents have been widely described in the literature as potent disruptants of the bacterial membrane. Sawyer and others (30) have studied the interactions of naturally occurring cationic proteins with resistant *P. aeruginosa*. In their report they concluded that the peptides behave like other polycations and probably interact with the bacteria's outer membrane since they display susceptibility to competition with divalent cations.

There are several important aspects that should be pursued further to address the questions of the modes of action and the natures of the antimicrobial agents in these polymer-peptide conjugates. Since adhesion to the immobilized peptide by the microorganism is a necessary initial step in this scheme, it is important to determine whether cell depletion is due solely to adhesion or whether the cells lose viability by the action of the peptide. First, we will follow up observations obtained from preliminary experiments with the luminescent bacterial strain *E. coli* TV1058 (20) in the antimicrobial assays (14). The absence of light emission from the reaction mixtures of *E. coli* TV1058 and immobilized peptide parallels the loss of viable CFU in the cell suspension and suggests that a bactericidal step accompanies binding. Second, we will probe the influence of peptide aggregates (and possibly assess the contribution of ion channel formation) by studying the low immobilization densities of the peptides. Third, we will prepare radiolabelled immobilized peptides for experiments that will enable us to better address the question of whether the polymer-bound peptide is the sole participant in the antimicrobial activity. Finally, we are examining other polymer-peptide systems to overcome the deficiency of low surface accessibility that is inherent in this porous model system.

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