

Synthetic Peptides That Exert Antimicrobial Activities in Whole Blood and Blood-Derived Matrices

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Peptides that exert antimicrobial activity in artificial media may lack activity within blood or other complex biological matrices. To facilitate the evaluation of antimicrobial peptides for possible therapeutic utility, an *in vivo* assay was developed to assess the extent and durability of peptide antimicrobial activities in complex fluid biomatrices of whole blood, plasma, and serum compared with those in conventional media. Novel antimicrobial peptides (RP-1 and RP-11) were designed based in part on platelet microbicidal proteins. RP-1, RP-11, or gentamicin was introduced into biomatrices either coincident with, or 2 h prior to, inoculation with an *Escherichia coli* target organism. Antimicrobial activities of peptides were assessed by quantitative culture 2 h after bacterial inoculation and compared to those of peptide-free and gentamicin controls. In whole blood and homologous plasma or serum, introduction of RP-1 or RP-11 coincident with *E. coli* was associated with a significant reduction in CFU per milliliter versus the respective peptide-free controls. Moreover, substantial antimicrobial activity remained when RP-1 or RP-11 was placed into whole blood or plasma 2 h prior to *E. coli* inoculation. These results suggest that the peptides were not rapidly inactivated within these biomatrices. Peptide antimicrobial activities were negatively affected by preincubation in serum or in heat-inactivated serum, compared with those of the respective controls. Peptides RP-1 and RP-11 were consistently effective at lower concentrations in biomatrices than in artificial media, indicating favorable antimicrobial interactions with components of blood or blood fractions. Collectively, these findings support the concept that synthetic peptides can be designed to exert potent antimicrobial activities in relevant and complex biological matrices.

Antimicrobial peptides have been isolated and characterized from virtually every organism surveyed to date, ranging from prokaryotes to humans. Among the most thoroughly studied of these molecules are those from mammalian leukocyte granules and the epithelial mucosa (6, 25). We hypothesize that, for such peptides, localization to these sites likely contributes to optimal antimicrobial functions and minimization of host toxicity by limiting their distribution to these restricted environments. Antimicrobial peptides from these or other sources often exhibit strong antimicrobial activities when tested in artificial media or austere buffer systems *in vitro*. However, in complex fluid matrices such as whole blood, plasma, and serum, they tend to have relatively poor activity or target selectivity and thus are typically rapidly inactivated or are cytotoxic and/or erythrolitic.

The potential interactions between a given antimicrobial peptide and blood components are multifactorial. For example, whole blood and other fluid biomatrices may contain binding or blocking proteins that may inactivate such a peptide. Similarly, blood may contain peptidases which degrade the peptide over time. Alternatively, blood and blood fractions may contain components that potentiate antimicrobial peptide actions against target pathogens or that are enhanced by antimicrobial peptides, resulting in amplified pathogen killing. Un-

derstanding such factors is of direct relevance to the potential therapeutic applications of antimicrobial peptides.

Recent studies have demonstrated that human and other mammalian platelets contain a group of potent antimicrobial peptides, termed platelet microbicidal proteins (PMPs) and thrombin-induced PMPs (tPMPs) (5, 22–24). In comparison to leukocytes and the epithelial mucosa, mammalian platelets respond to microorganisms or soluble mediators of inflammation by liberating the majority of their PMPs and tPMPs directly into the bloodstream. Antimicrobial polypeptides from platelets have some structural and functional similarities with peptides from leukocyte and epithelial sources: PMPs and tPMPs are relatively small (e.g., <15 kDa), and some have a net cationic charge associated with antimicrobial activity and appear to interact with microbial cytoplasmic membranes, followed by intracellular targeting to achieve their ultimate mechanism of action (19, 22, 23). Despite their potent and broad-spectrum antimicrobial activities, these peptides have relatively limited cytotoxicity for relevant mammalian cells (e.g., vascular endothelial cells), as assessed by preliminary studies *in vitro* (M. R. Yeaman, A. S. Ibrahim, J. A. Ritchie, S. G. Filler, A. S. Bayer, J. E. Edwards, Jr., and M. A. Ghannoum, IDSA Annu. Meet., abstr. 491, 1995). Thus, PMPs and tPMPs likely possess structure-activity relationships optimally suited for antimicrobial functions without concomitant host cytotoxicity in complex biomatrices such as whole blood, plasma, and serum (8, 23, 24).

Based in part on these concepts, the present investigation tested novel synthetic peptides intended to exert optimal antimicrobial functions in relevant settings, including blood and blood fractions. An *ex vivo* screening assay was developed to

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define potential antimicrobial peptide-biomatrix interactions by using a conventional antibiotic, gentamicin, as a comparator. The results demonstrate that the test peptides exert potent and remarkably durable antimicrobial activity in whole blood and homologous plasma when coincubated with serum compared to conventional artificial media.

MATERIALS AND METHODS

Antimicrobial agents. RP-1 is an 18-amino-acid synthetic peptide designed to incorporate structure-activity attributes characteristic of PMPs and tPMPs (23). In this respect, RP-1 (N-ALYK⁵FKK¹⁰LKSLK¹⁵RLG-C; mass, 2,162.8 kDa) has some structural similarities with the antimicrobial peptide C18G, previously described by Darveau et al. (1). However, RP-1 is structurally distinct, as it contains a signature dipeptide motif, believed to influence antimicrobial activity. RP-11 is a novel 13-amino-acid synthetic peptide (N-ALYKR⁵LFKK¹⁰KKF-C; mass, 1,683.1 kDa) also designed to reflect structure-function attributes of PMPs and tPMPs. RP-1 and related peptides have been previously demonstrated to exert antimicrobial activities in a chemically defined medium by radial diffusion assay in vitro (M. R. Yeaman, M. D. Joshi, K. D. Gank, D. Kahler, and W. H. Welch, Abstr. 38th Intersci. Conf. Antimicrob. Agents Chemother., abstr. F-170, 1999).

For the present study, peptides were made with a Symphony multiplex synthesizer (Rainin, Woburn, Mass.) located in the Biopolymer Synthesis Core Facility at Harbor-UCLA Research and Education Institute (Torrance, Calif.). Standard *N*^α-9-fluorenylmethoxy carbonyl chemistry with appropriate R-protecting groups was used in coupling on polyethylene Wang resin (American Peptide Company, Sunnyvale, Calif.), and 1-hydroxybenzotriazole activation was used to optimize coupling efficiency. The resulting peptides were completed, cleaved, and deprotected by routine methods. The cleaved peptide was then precipitated, washed, and lyophilized. Following this process, the crude peptide was dissolved in 0.1% acetic acid (pH 5.5). Samples were then subjected to analytical and preparative reversed-phase high-pressure liquid chromatography (RP-HPLC) and matrix-assisted laser desorption/ionization–time of flight mass spectroscopy (UCLA Protein Microsequencing Facility) to confirm homogeneous purification and verify correct mass, respectively. Efficiency of solid-phase synthesis of RP-1 and RP-11 was typically $\geq 75\%$, and RP-HPLC allowed rapid baseline purification of each of the peptides, which were confirmed to have masses indicative of correct synthesis. Purified antimicrobial peptides or gentamicin sulfate (Fujiisawa USA Inc., Deerfield, Ill.) was suspended and diluted in sterile phosphate-buffered saline (PBS; pH 5.5 or 7.2 for the respective assay conditions) prior to use.

Microorganism. The well-characterized *Escherichia coli* strain ML-35 was used as the target organism in these studies (7). In pilot experiments (data not shown), this strain was confirmed to be susceptible to gentamicin: the gentamicin MIC and minimum bactericidal concentration (MBC) for the strain in standard broth microdilution assays in accordance with National Committee for Clinical Laboratory Standards guidelines were each $\leq 1 \mu\text{g/ml}$ (12). In addition, this strain was resistant to serum to a degree sufficient to permit survival to be assessed in the absence of antimicrobials, thus allowing for its use in the development of the biomatrix assay (see below). For assays described below, the organism was cultured to mid-logarithmic phase in brain-heart infusion broth (Difco Laboratories, Detroit, Mich.) at 37°C with agitation, harvested by centrifugation, washed, and resuspended in PBS (Irvine Scientific, Santa Ana, Calif.; pH 7.2) and the inoculum was determined by spectrophotometry and validated by quantitative culture (see below).

Antimicrobial assay in biomatrices. The antimicrobial activities of RP-1, RP-11, and gentamicin in human whole blood and homologous plasma and serum fractions were assessed. For a given experiment, 30 ml of fresh whole blood was obtained from a healthy volunteer in compliance with institutional policies and practice for informed consent. Citrate anticoagulant was used to anticoagulate two-thirds (20 ml) of the whole-blood sample (final concentrations, 16.8 μM sodium citrate and 4.2 μM citric acid) (19, 22). Six milliliters of the citrated whole blood was set aside, and the other 14 ml was subjected to low-speed centrifugation ($210 \times g$), yielding a platelet-rich plasma supernatant. These preparations comprised the whole-blood and plasma biomatrices, respectively. The remaining 10 ml of nonanticoagulated whole blood was allowed to spontaneously clot in Vacutainer tubes (red top; Becton Dickinson, Franklin Lakes, N.J.; 1 h) and centrifuged ($300 \times g$) to yield homologous serum (“homologous” refers to a blood fraction, in this case serum, derived from the same donor whose whole blood was also directly studied). In selected experiments, antimicrobial peptides and gentamicin were compared for activities in pooled normal human serum (PNHS; Gemini Scientific, Woodland, Calif.) and heat-inactivated PNHS (56°C

for 30 min; ΔPNHS). Routinely, samples at dilutions of between 10^{-2} and 10^{-5} were quantitatively cultured in parallel from each condition at the 0 and 2 h time points. Because sonication of whole blood or plasma risked liberating leukocyte and platelet antimicrobial peptides, samples were incubated with continuous agitation and vortexed immediately prior to quantitative culture. These methods achieved reproducible results, with typical standard deviations of $\leq 10\%$ within and across individual experiments, despite day-to-day variations in blood donors. Additionally, these approaches provided a concurrent comparison of antimicrobial effects and corroboration of CFU and established a reliable limit of sensitivity of 1 \log_{10} CFU/ml in test biomatrices and media. Thus, apparent counts of less than \log_{10} 1.0 CFU/ml were conservatively interpreted as being \log_{10} 1.0 CFU/ml when calculating log survival. This approach likely underestimated the true antimicrobial activity of study peptides. For purposes of comparison in the present study, concentrations of RP-1, RP-11, or gentamicin yielding 50 or 90% reductions in CFU per milliliter were considered 50 or 90% inhibitory concentrations (IC₅₀ or IC₉₀, respectively) and concentrations yielding no detectable surviving *E. coli* were considered MBC.

For assays, whole blood, plasma, homologous serum, PNHS, and ΔPNHS were distributed in 85- μl aliquots into 96-well microtiter plates (Corning Glass Works, Corning, N.Y.). Two strategies were employed to compare the extent and durability of antimicrobial activity of RP-1, RP-11, and gentamicin in these biological matrices. In one series of experiments, peptide or gentamicin (in 5- μl volumes) was introduced into each matrix simultaneously with pathogen inoculation (10 μl), yielding a final inoculum of 10^5 CFU/ml and a final range in RP-1, RP-11, and gentamicin concentrations of 1 to 50 $\mu\text{g/ml}$ in a 100- μl total volume (of which 85% represented the fluid matrix of interest). In parallel experiments performed concurrently, RP-1, RP-11, or gentamicin was introduced into each matrix prior to pathogen inoculation, incubated with constant agitation for 2 h at 37°C, and then inoculated with the organism. In either strategy, pathogen inoculation and exposure to antimicrobial agent initiated an additional incubation period of 2 h at 37°C with agitation. At the end of the 2-h exposure period, mixtures were vortexed and diluted in PBS to ensure dispersion and quantitatively cultured in triplicate onto blood agar as outlined above. Colonies representative of surviving organisms were enumerated and calculated in units of CFU per milliliter after a 24-h incubation of blood agar plates at 37°C.

Antimicrobial assay in conventional media. For comparison, the antimicrobial activities of RP-1, RP-11, and gentamicin were tested in a conventional-medium system employing cation-supplemented Mueller-Hinton II broth (MHB; Becton Dickinson, Sparks, Mo.) as appropriate in accordance with National Committee for Clinical Laboratory Standards guidelines (11). Assays performed with conventional media were conducted in parallel with biomatrix assays. Because whole blood and blood fractions, as well as sites of inflammation or serum generation, may undergo a shift in pH, the efficacies of the study antimicrobial agents were compared in MHB adjusted to pH 5.5 or 7.2. As described for the biomatrix assay, the peptide was introduced into the media either 2 h prior to, or simultaneously with, the pathogen inoculum; the introduction of the peptide was followed by a 2-h exposure period. At the end of the 2-h exposure period, reaction mixtures were processed for quantitative culture as described above. As in the biomatrix assays, the limit of detection in conventional media assays was considered 1 \log_{10} CFU/ml.

Data analysis. Experiments were performed a minimum of two independent times on different days and with different blood donor sources. Two-way analysis of variance was used to compare differences in antimicrobial agent activities in distinct test conditions (K.Yoshioka, KyPlot, version 2.0 beta, public domain, <http://www.quelest.co.jp>, 2000). The Bonferroni correction for multiple comparisons was used where appropriate. *P* values ≤ 0.05 were considered significant.

RESULTS

The antimicrobial activities of RP-1, RP-11, and gentamicin in whole blood and homologous plasma and serum fractions, as well as in conventional media at pH 5.5 and 7.2, were simultaneously compared. In selected experiments, antimicrobial agent efficacies in PNHS or ΔPNHS were compared. The pHs of whole blood, homologous plasma, and homologous serum were 7.3, 7.7, and 6.6, respectively, prior to preincubation and 7.2, 7.8, and 6.6, respectively, following 2 h of incubation.

Antimicrobial activities of RP-1, RP-11, and gentamicin coincubated with the organism. (i) Efficacy in whole blood. In whole blood, an inoculum of \log_{10} 5 CFU/ml yielded a mean

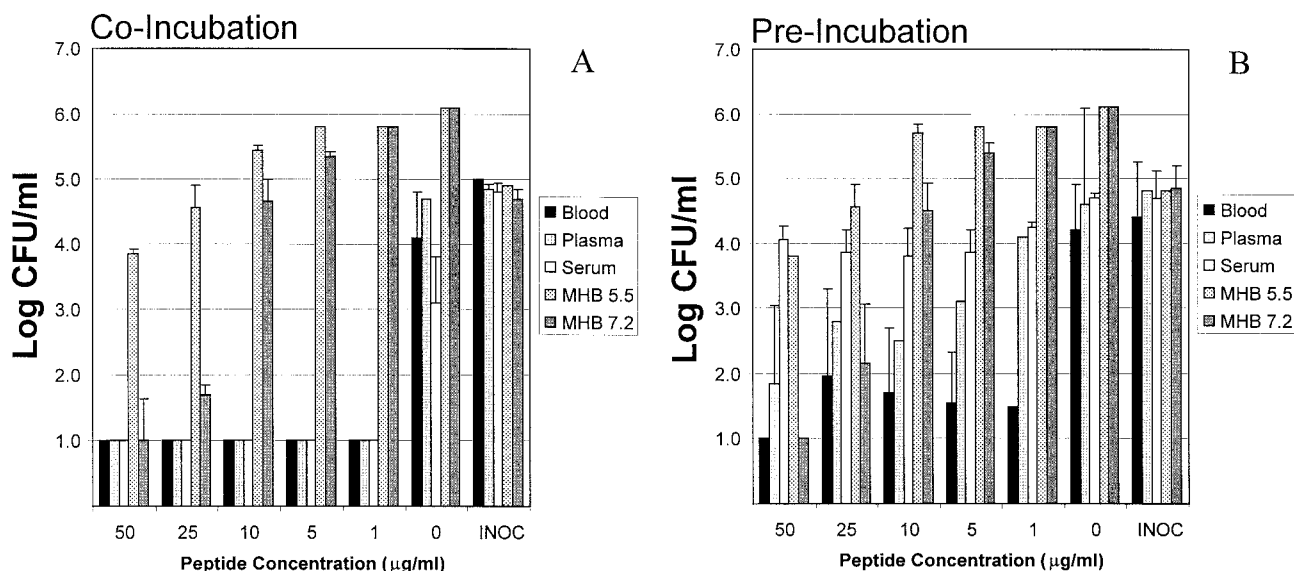


FIG. 1. Comparative efficacies of RP-1 versus *E. coli* ML-35 in biomatrices or in MHB at pH 5.5 and 7.2. (A) Coincubation of the peptide and organism simultaneously added to the test biomatrix or medium; (B) preincubation of the peptide in the biomatrix or medium for 2 h at 37°C prior to introduction of the organism. The *E. coli* inoculum (INOC) was 10^5 CFU/ml, and the threshold of sensitivity was considered $1 \log_{10}$ CFU/ml.

end point in *E. coli* viability of \log_{10} 4.1 CFU/ml (Fig. 1A) after a 2-h incubation at 37°C. This reduction of $-\Delta\log_{10}$ 0.9 CFU/ml was attributed to the inherent antimicrobial activity of whole blood. The comparative antimicrobial effects of peptides and gentamicin were normalized to the same biomatrix in the absence of an antimicrobial agent. By comparison, in whole blood containing RP-1 at concentrations ranging from 50 to 1 μg/ml, no viable *E. coli* cells were detectable after a 2-h incubation. The presence of as little as 1 μg of RP-1 per ml was associated with a mean reduction of $\geq\log_{10}$ 3.1 CFU/ml in whole blood upon coincubation with organisms for 2 h at 37°C. The MBC of RP-1 in whole blood (MBC_{blood}) was ≤ 1 μg/ml (Table 1). In comparison, viable *E. coli* cells were not detectable in whole blood containing RP-11 at concentrations ranging from 5 to 50 μg/ml but were present (\log_{10} 1 CFU/ml) in whole blood containing RP-11 at 1 μg/ml (Fig. 2C). Thus, the MBC_{blood} of RP-11 was 5 μg/ml (Table 1). Similar to what was found with RP-1, *E. coli* was undetectable in whole blood containing gentamicin at concentrations ranging from 1 to 50 μg/ml. Therefore, gentamicin exhibited an MBC_{blood} of ≤ 1 μg/ml versus *E. coli* (Table 1). Vehicle alone caused no significant reduction in *E. coli* viability in whole blood compared with controls.

(ii) Efficacy in homologous plasma. In homologous plasma, a \log_{10} 5 CFU/ml inoculum yielded a mean end point *E. coli* viability of \log_{10} 4.7 CFU/ml after a 2-h incubation at 37°C (Fig. 1A). This change of $-\Delta\log_{10}$ 0.3 CFU/ml was not significantly different from control, suggesting that plasma had minimal inherent anti-*E. coli* activity in this assay. However, no viable *E. coli* cells were detectable in plasma containing RP-1 at concentrations ranging from 50 to 1 μg/ml when the peptide and organism were introduced simultaneously and incubated for 2 h at 37°C (Fig. 1A). Therefore, RP-1 exhibited an assay MBC_{plasma} of ≤ 1 μg/ml (Table 1), corresponding to a mean reduction of $\geq\log_{10}$ 3.7 CFU/ml versus *E. coli* in this biomatrix.

By comparison, RP-11 exerted considerably less anti-*E. coli* activity in plasma than did RP-1. For example, RP-11 displayed dose-dependent efficacy in plasma, with surviving \log_{10} CFU/ml of 1.4, 1.6, 1.9, 2.9, and 3.1 in the presence of RP-11 at 50, 25, 10, 5, and 1 μg/ml, respectively. However, since viable *E. coli* cells were detectable in plasma containing RP-11 at concentrations as high as 50 μg/ml, the apparent RP-11 MBC_{plasma} was interpreted to be ≥ 50 μg/ml (Table 1). The efficacy of gentamicin in plasma against *E. coli* was significantly lower than that of RP-1 ($P < 0.05$ for concentrations of 10 μg/ml or less; Table 1). For example, exposure to gentamicin at 50 or 25 μg/ml in plasma yielded no detectable *E. coli*, while 10 μg/ml caused a mean reduction of $-\Delta\log_{10}$ 2.1 CFU/ml compared with controls ($P < 0.05$ versus the respective controls). However, 1 or 5 μg of gentamicin/ml in plasma failed to cause a significant reduction in *E. coli* viability compared to controls. From these data, gentamicin MBC_{plasma} was observed to be 25 μg/ml (Table 1). Vehicle alone caused no significant reduction in *E. coli* viability in plasma compared with controls.

(iii) Efficacy in homologous serum. In fresh homologous human serum, an inoculum of \log_{10} 5 CFU/ml yielded a mean *E. coli* survival of \log_{10} 3.1 CFU/ml (Fig. 1A) after a 2-h incubation at 37°C. This reduction of $-\Delta\log_{10}$ 1.9 CFU/ml was attributed to the intrinsic antimicrobial activity of serum. As with whole blood, the comparative antimicrobial effects of peptides and gentamicin in serum were normalized to the serum biomatrix lacking an antimicrobial agent. Coincubation in serum containing RP-1 at concentrations ranging from 50 to 1 μg/ml yielded no viable *E. coli* cells detectable after a 2-h incubation. As little as 1 μg of RP-1 per ml of serum introduced simultaneously with *E. coli* was associated with a reduction of $\geq\log_{10}$ 2.1 CFU/ml, translating to an RP-1 MBC_{serum} of ≤ 1 μg/ml (Table 1). Likewise, RP-11 at 50 μg/ml of serum yielded no detectable *E. coli* survival; however, RP-11 concentrations of 25 μg/ml or less failed to render *E. coli* undetectable

TABLE 1. Comparative efficacies of antimicrobial peptides RP-1 and RP-11 and gentamicin in fluid biomatrices and artificial media under specified assay conditions^a

Biomatrix or medium and peptide or drug	IC or MBC ($\mu\text{g/ml}$)					
	Coincubation			2-h preincubation		
	IC ₅₀	IC ₉₀	MBC	IC ₅₀	IC ₉₀	MBC
Biomatrices						
Whole blood						
RP-1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	50
RP-11	≤ 1	≤ 1	5	≤ 1	50	>50
Gentamicin	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1
Plasma						
RP-1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 5	>50
RP-11	≤ 1	≤ 1	>50	≤ 1	>25	>50
Gentamicin	10	10	25	10	10	25
Homologous serum						
RP-1	≤ 1	≤ 1	≤ 1	≤ 1	>50	>50
RP-11	≤ 5	≤ 5	50	>50	>50	>50
Gentamicin	≤ 1	≤ 1	≤ 1	≤ 1	5	5
PNHS						
RP-1	≤ 1	≤ 5	>50	≤ 1	≤ 5	>50
RP-11	25	>50	>50	>50	>50	>50
Gentamicin	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1
Δ PNHS						
RP-1	50	>50	>50	>50	>50	>50
RP-11	50	>50	>50	>50	>50	>50
Gentamicin	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1
Conventional media						
MHB at pH 5.5						
RP-1	5	≤ 25	>50	10	≤ 25	>50
RP-11	>50	>50	>50	>50	>50	>50
Gentamicin	5	5	>50	5	5	>50
MHB at pH 7.2						
RP-1	≤ 5	≤ 10	50	≤ 5	≤ 10	50
RP-11	>50	>50	>50	>50	>50	>50
Gentamicin	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1

^a See Materials and Methods for specific conditions of assay.

in serum. Therefore, the RP-11 MBC_{serum} under these assay conditions was interpreted as 50 $\mu\text{g/ml}$ (Table 1). No surviving *E. coli* cells were detectable in serum containing gentamicin across the 50- to 1- $\mu\text{g/ml}$ range tested. These results indicate an apparent gentamicin MBC_{serum} of ≤ 1 $\mu\text{g/ml}$ versus *E. coli* as defined in this assay (Table 1). As with the whole-blood and plasma assays above, vehicle alone caused no significant reduction in *E. coli* viability in serum compared with controls.

(iv) Efficacy in MHB at pH 5.5. In MHB at pH 5.5, an *E. coli* inoculum of log₁₀ 5 CFU/ml produced a mean cell density of log₁₀ 6.1 CFU/ml after a 2-h incubation at 37°C (Fig. 1A). The comparative antimicrobial effects of peptides and gentamicin were normalized to this increase in log₁₀ CFU/ml of 1.1 in this medium. Under this condition, RP-1 exerted a significant and dose-dependent anti-*E. coli* effect at concentrations of 50 and 25 $\mu\text{g/ml}$ ($P < 0.05$ versus controls) but no significant difference from controls at 10, 5, or 1 $\mu\text{g/ml}$ (Fig. 1A). These findings correspond to RP-1 MBC_{MHB5.5}, IC_{90, MHB5.5}, and IC_{50, MHB5.5} values of >50, 50, and 25 $\mu\text{g/ml}$, respectively (Table 1).

RP-11 exhibited no anti-*E. coli* activity in MHB at pH 5.5 at the concentrations tested, yielding MBC, IC₉₀, and IC₅₀ values ≥ 50 $\mu\text{g/ml}$ under these conditions (Table 1). In contrast, gentamicin concentrations as low as 5 $\mu\text{g/ml}$ were associated with no detectable *E. coli* recoverable from this medium, while 1 $\mu\text{g/ml}$ achieved no reduction compared with controls. These data correspond to gentamicin MBC_{MHB5.5}, IC_{90, MHB5.5}, and IC_{50, MHB5.5} values of 5 $\mu\text{g/ml}$ each (Table 1).

(v) Efficacy in MHB at pH 7.2. In conventional medium at pH 7.2, an *E. coli* inoculum of log₁₀ 5 CFU/ml produced a mean cell density of log₁₀ 6.1 CFU/ml after a 2-h incubation at 37°C (Fig. 1A). As above, the comparative antimicrobial effects of peptides and gentamicin were normalized to an increase in log₁₀ CFU/ml of 1.1 in this medium in their absence. In these conditions, RP-1 at 50 $\mu\text{g/ml}$ yielded no detectable surviving *E. coli*, mean survivals of log₁₀ 1.7 and log₁₀ 4.7 CFU/ml at 25 and 10 $\mu\text{g/ml}$ ($P < 0.05$ versus controls), respectively, and no reduction compared with controls at either 5 or 1 $\mu\text{g/ml}$ (Fig. 1A). These findings yield RP-1 MBC_{MHB7.2}, IC_{90, MHB7.2}, and IC_{50, MHB7.2} values of 50, 10, and 5 $\mu\text{g/ml}$, respectively (Table 1). It is notable that RP-1 exerted greater antimicrobial activity in MHB at pH 7.2 than at pH 5.5 in preincubation studies ($P < 0.05$; Table 1). By comparison, RP-11 exhibited no anti-*E. coli* activity in MHB at pH 7.2, yielding MBC_{MHB7.2}, and IC_{90, MHB7.2}, and IC_{50, MHB7.2} concentrations of ≥ 50 $\mu\text{g/ml}$ (Table 1). In contrast, a gentamicin concentration as low as 1 $\mu\text{g/ml}$ yielded no detectable *E. coli* after the 2-h incubation period, corresponding to MBC_{MHB7.2}, IC_{90, MHB7.2}, and IC_{50, MHB7.2} values all ≤ 1 $\mu\text{g/ml}$ by definition in this assay system (Table 1).

Antimicrobial activities of RP-1, RP-11, and gentamicin following 2-h preincubation. **(i) Efficacy following preincubation in whole blood.** Consistent with the results above, an inoculum of log₁₀ 5 CFU/ml in whole blood preincubated for 2 h at 37°C yielded a mean end point in *E. coli* viability of log₁₀ 4.2 CFU/ml (Fig. 1B). Thus, preincubation had no significant effect on the intrinsic anti-*E. coli* activity of whole blood. The antimicrobial effects of RP-1, RP-11, and gentamicin were normalized to those of the respective controls in this matrix. After preincubation in whole blood, the apparent potency of RP-1 was modestly, but not significantly, reduced compared with that found during coincubation ($P > 0.05$; Fig. 1B, 2A and B, and 3A). For example, in the preincubation assay, only RP-1 at a concentration of 50 $\mu\text{g/ml}$ yielded undetectable *E. coli*; lower concentrations achieved strong and equivalent antimicrobial activities but not complete killing of the organism (Fig. 1B and Table 1). The MBC_{blood}, IC_{90, blood}, and IC_{50, blood} values reflect the comparative reduction in antimicrobial activity of RP-1 due to preincubation versus coincubation (Tables 1 and 2; Fig. 2 and 3). Of note, preincubation of RP-11 in whole blood resulted in significantly less antimicrobial activity than coincubation of the peptide and organism ($P < 0.05$; Fig. 2C and D and 3B). Reductions in log₁₀ CFU/ml ranged from 1.0 at 50 $\mu\text{g/ml}$ to 0.6 at 1 $\mu\text{g/ml}$, yielding increased MBC_{blood} and IC_{blood} values for RP-11 when it was preincubated in whole blood compared with those when it was coincubated (Fig. 2D and 3B; Table 1). In contrast, across the test range from 50 to 1 $\mu\text{g/ml}$, gentamicin reduced the amount of viable *E. coli* to levels below the threshold of detection even after a 2-h preincubation in whole blood (Fig. 2C and D and 3B; Table 1). As before, vehicle alone caused no significant reduction in

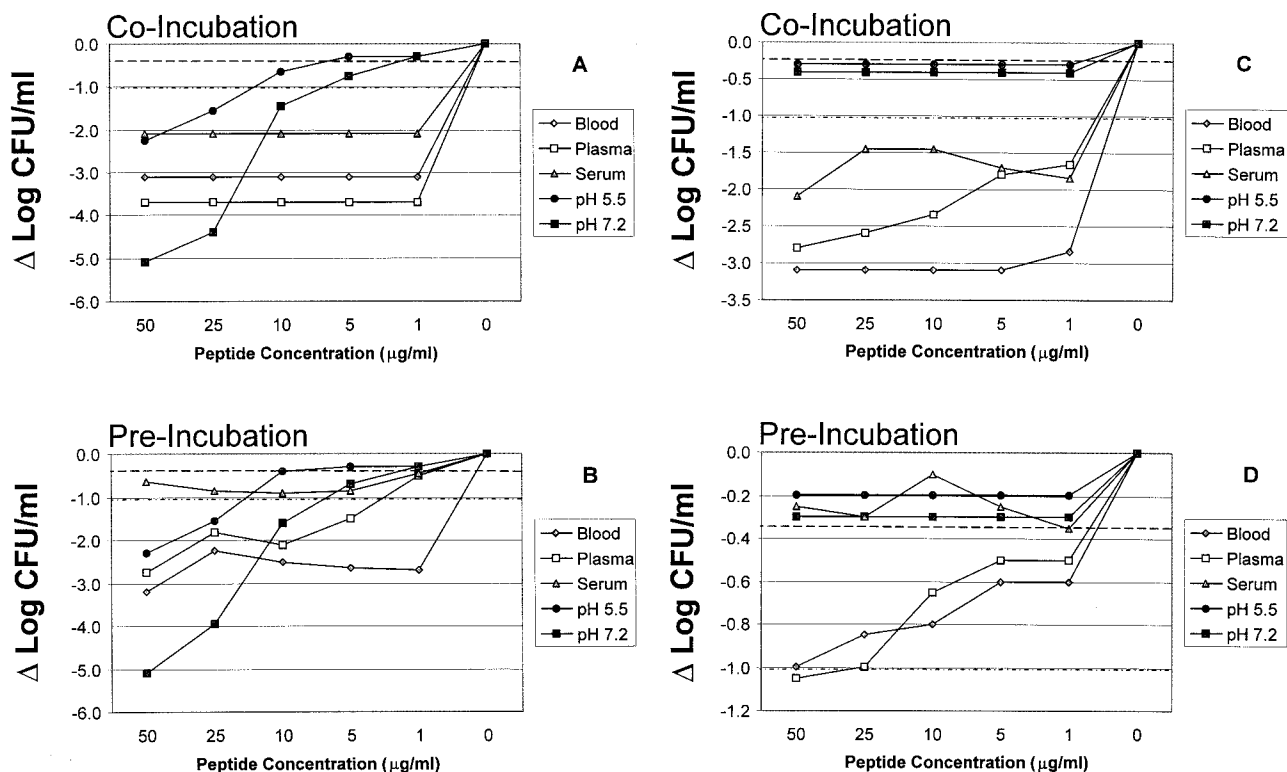


FIG. 2. (A and B) Comparative impact of RP-1 on survival of *E. coli* ML-35 when coincubated with the organism (A) or preincubated for 2 h at 37°C prior to introduction of the organism into test biomatrices or MHB (pH 5.5 or 7.2) (B). (C and D) Comparative impact of RP-11 on survival of *E. coli* ML-35 when RP-11 was coincubated with the organism (C) or preincubated for 2 h at 37°C prior to introduction of the organism into biomatrices or MHB (pH 5.5 or 7.2) (D). The *E. coli* inoculum was 10^5 CFU/ml, and the threshold of sensitivity was considered 1 \log_{10} CFU/ml. Data are normalized to the respective controls. Each point represents the mean of two independent experiments with $\leq 10\%$ standard error. Error bars are intentionally omitted for clarity. Note the change in scale of the ordinate for the different panels. Dashed and dot-dashed lines indicate logs of survival corresponding to the respective IC_{50} and IC_{90} levels, allowing estimation of peptide concentrations translating to these inhibitory concentrations (see also Table 2).

E. coli viability in preincubated whole blood compared with controls.

(ii) Efficacy following preincubation in homologous plasma.

Similar to results from coincubation studies, inoculation of \log_{10} 5 CFU of *E. coli*/ml into plasma preincubated for 2 h at 37°C yielded a mean end point viability of \log_{10} 4.6 CFU/ml (Fig. 1B) following a 2-h incubation postinoculation. This change of $-\Delta \log_{10}$ 0.4 CFU/ml compared with control was not significant and suggested that the limited anti-*E. coli* activities of coincubated and preincubated plasma were equivalent. RP-1 and RP-11 maintained anti-*E. coli* activity after a 2-h incubation in homologous plasma. As observed in whole blood, higher peptide concentrations were required to achieve levels of killing in plasma following preincubation equivalent to those following coincubation (Fig. 2 and 3). For example, RP-1 under this condition exerted considerable antimicrobial activity but did not completely sterilize *E. coli* even at a concentration of 50 $\mu\text{g/ml}$ (Fig. 1B, 2B, and 3A). These effects translated to corresponding increases in preincubation RP-1 $\text{MBC}_{\text{plasma}}$ and $\text{IC}_{\text{plasma}}$ values compared to coincubation results (Tables 1 and 2). In contrast, RP-11 exerted significantly reduced anti-*E. coli* bioactivity in plasma following preincubation compared to that when it was introduced simultaneously with the organism ($P < 0.05$ versus coincubation; Fig. 2D and 3B; Tables 1 and 2).

Consistent with its activity in plasma coincubation experiments, gentamicin at concentrations of 50 or 25 $\mu\text{g/ml}$ preincubated with plasma yielded undetectable *E. coli*, while 10 $\mu\text{g/ml}$ caused a mean reduction of \log_{10} 2.0 CFU/ml compared with the control, but 5 or 1 $\mu\text{g/ml}$ failed to reduce *E. coli* viability. Therefore, the preincubation $\text{MBC}_{\text{plasma}}$ and $\text{IC}_{\text{plasma}}$ values for gentamicin were not different from those seen with coincubation ($P > 0.05$; Table 1). Vehicle alone caused no significant reduction in *E. coli* viability in preincubated plasma versus controls.

(iii) Efficacy following preincubation in homologous serum.

An *E. coli* inoculum of \log_{10} 5 CFU/ml yielded a mean survival of \log_{10} 4.7 CFU/ml (Fig. 1B) in serum preincubated for 2 h at 37°C. Thus, preincubated serum did not cause a significant reduction in CFU per milliliter compared with the inhibitory effect of coincubated serum ($P > 0.05$; see above). However, preincubation of RP-1 in homologous serum resulted in a dramatic loss of antimicrobial activity compared with coincubation in this biomatrix ($P < 0.05$; Fig. 2A and B; Tables 1 and 2). Similarly, RP-11 failed to achieve significant reductions in *E. coli* CFU following preincubation at any test concentration in serum compared with control ($P > 0.05$ versus control; Fig. 2C and D; Table 1), in contrast to its antimicrobial activity in coincubation experiments ($P < 0.05$ versus coincubation).

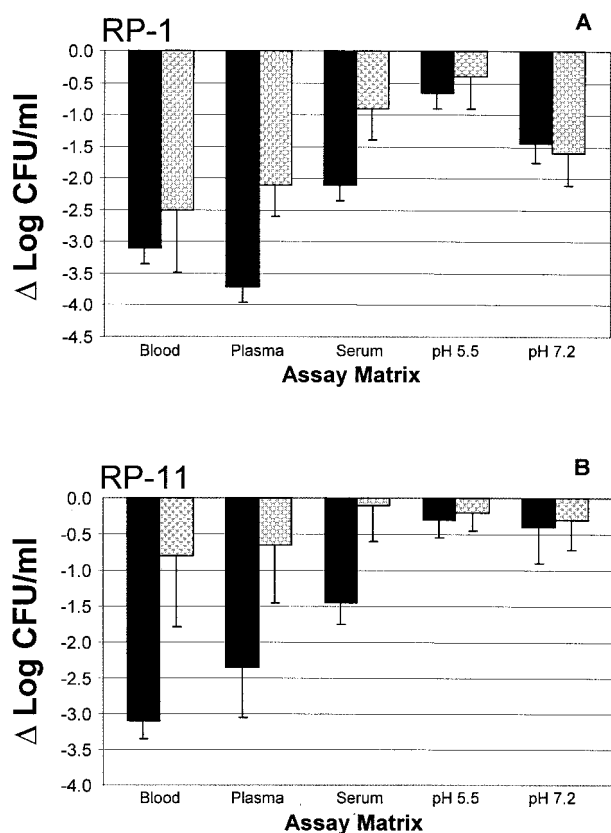


FIG. 3. Comparative influences of coincubation (solid bars) versus preincubation (cross-hatched bars) on the antimicrobial efficacies of RP-1 (A) and RP-11 (B) in biomatrices or MHB (pH 5.5 or 7.2). These data illustrate the effects of these antimicrobial peptides at a concentration of 10 $\mu\text{g/ml}$. Data are normalized to the respective controls.

Gentamicin at concentrations ranging from 50 to 5 $\mu\text{g/ml}$ retained its efficacy against *E. coli* following preincubation in serum but failed to significantly reduce organism viability when tested at 1 $\mu\text{g/ml}$. These data yielded gentamicin $\text{MBC}_{\text{serum}}$ and $\text{IC}_{90, \text{serum}}$ values that were generally 1 dilution greater than those observed in serum with coincubation (Table 1). As before, vehicle alone produced no significant reduction in *E. coli* viability in preincubated serum compared with controls.

(iv) **Efficacy in preincubated MHB at pH 5.5 or 7.2.** Anti-*E. coli* activities of RP-1, RP-11, and gentamicin following preincubation in artificial medium at pH 5.5 or 7.2 were not significantly different from those observed in the same media with coincubation (Fig. 1 to 3 and Table 1). As in the coincubation studies (above), the IC_{90} and MBC for RP-1 in MHB were modestly lower at pH 7.2 than at pH 5.5 ($P < 0.05$ at concentrations of 50 and 25 $\mu\text{g/ml}$; Fig. 1; Table 1)

Influence of serum status on antimicrobial efficacy. The results observed above suggested that components present in blood or blood fractions influenced the antimicrobial activities of RP-1 and RP-11 but had comparably less impact on the anti-*E. coli* activity of gentamicin. Moreover, the observation that peptide antimicrobial activities were most negatively affected by preincubation in serum suggested that they may be influenced by complement, peptidases, or other heat-labile factors in this biomatrix. Therefore, the efficacies of RP-1,

TABLE 2. IC_{50} and IC_{90} of RP-1 and RP-11 versus *E. coli* ML-35 in test biomatrices and MHB estimated from co- and preincubation assay results^a

Biomatrix or medium	Condition ^b	IC_{50} ($\mu\text{g/ml}$)	IC_{90} ($\mu\text{g/ml}$)
RP-1 ^c	Whole blood	Co	≤ 1
	Pre	≤ 1	≤ 1
Homologous plasma	Co	≤ 1	≤ 1
	Pre	≤ 1	3
Homologous serum	Co	≤ 1	≤ 1
	Pre	≤ 1	> 50
MHB at pH 5.5	Co	6	15
	Pre	10	18
MHB at pH 7.2	Co	2	6
	Pre	3	7
RP-11 ^d	Whole blood	Co	≤ 1
	Pre	≤ 1	50
Homologous plasma	Co	≤ 1	≤ 1
	Pre	≤ 1	30
Homologous serum	Co	≤ 1	≤ 1
	Pre	≥ 50	≥ 50
MHB at 5.5	Co	≥ 50	≥ 50
	Pre	≥ 50	≥ 50
MHB at 7.2	Co	≥ 50	≥ 50
	Pre	≥ 50	≥ 50

^a See text for detailed assay protocols.

^b Co, coincubation; Pre, preincubation.

^c Values for RP-1 were approximated by interpolation of data from Fig. 2A and B.

^d Values for RP-11 were approximated by interpolation of data from Fig. 2C and D.

RP-11, and gentamicin in PNHS and ΔPNHS with and without preincubation for 2 h were assessed as described above (Fig. 4). PNHS consistently achieved a somewhat greater killing of *E. coli* than ΔPNHS (Fig. 4); this was attributed to the respective intrinsic antimicrobial characteristics of these matrices. Similar to homologous serum (above), RP-1 at concentrations of 50 to 5 $\mu\text{g/ml}$ exerted significant anti-*E. coli* activity when introduced into PNHS simultaneously with the organism (Fig. 4A). However, this effect of RP-1 was absent on coincubation in ΔPNHS or in PNHS or ΔPNHS following a 2-h preincubation prior to organism inoculation (Fig. 4B; Table 1). In PNHS, RP-11 exerted modest antimicrobial activity only at concentrations of 50 and 25 $\mu\text{g/ml}$ (Fig. 4C). Corresponding RP-11 $\text{IC}_{90, \text{plasma}}$ and $\text{MBC}_{\text{plasma}}$ values for PNHS differed somewhat from those for fresh homologous serum (Table 1). In contrast, no significant antimicrobial activities against *E. coli* were observed when RP-11 was preincubated in PNHS or ΔPNHS for 2 h prior to introduction of the organism (Fig. 4D; Table 1). Consistent with results observed in fresh homologous serum, the anti-*E. coli* activities of gentamicin in PNHS and ΔPNHS

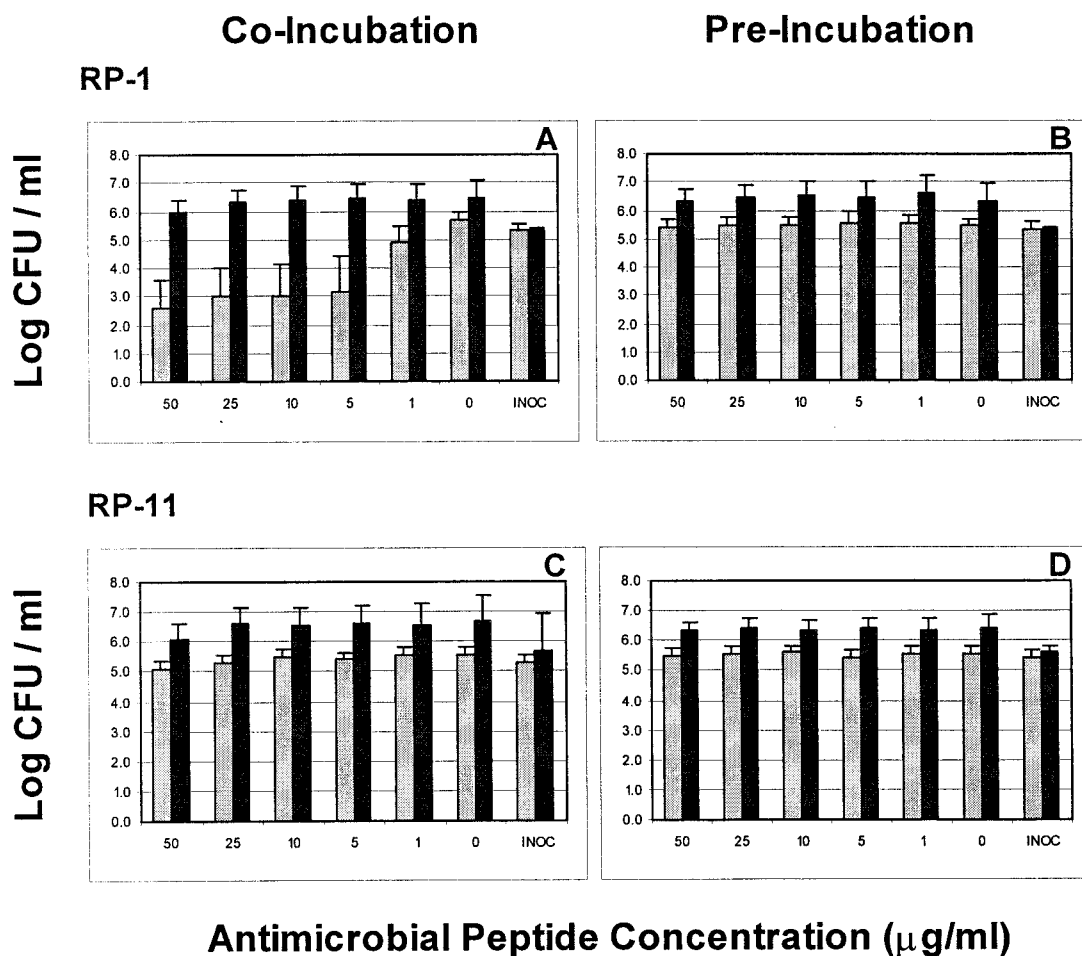


FIG. 4. Comparative efficacies of RP-1 (A and B) and RP-11 (C and D) versus *E. coli* ML-35 in coincubation (A and C) and preincubation (B and D) conditions in PNHS (light bars) and Δ PNHS (dark bars). As in other experiments, the *E. coli* inoculum (INOC) was 10^5 CFU/ml and the threshold of sensitivity was considered $1 \log_{10}$ CFU/ml.

prior to and following preincubation were equivalent (Table 1).

DISCUSSION

An ex vivo assay was developed to provide a relevant challenge to the extent and duration of antimicrobial peptide efficacy in complex biomatrices including whole blood, plasma, and serum. Plasma differs from whole blood in that the former is essentially devoid of leukocytes and erythrocytes. Moreover, serum is differentiated from either whole blood or plasma by being a cell-free biomatrix generated following the coagulation of whole blood and plasma components, with associated activation of proteases and other factors. The activities of two antimicrobial peptides were compared with that of gentamicin in parallel in each of these matrices and in conventional artificial media at pH 5.5 or 7.2. The assay design included simultaneous introduction of the antimicrobial agent and organism into biomatrices or media or a 2-h preincubation of the antimicrobial agents in test matrices or media prior to inoculation of organisms. In conventional antimicrobial assays, gram-negative organisms may exhibit a "regrowth" phenomenon follow-

ing exposure to cationic agents such as aminoglycoside antibiotics (20), obscuring the differentiation of bacteriostatic from bactericidal effects. Awareness of this potential effect resulted in a biomatrix assay that integrated a quantitative culture over a 24-h period prior to colony enumeration to verify bactericidal effect. Thus, the present assay was developed to screen for the extent and durability of antimicrobial activity of peptides in complex biological matrices. In addition, the activities of the antimicrobial peptides in PNHS and Δ PNHS were examined to assess the influence of heat-labile components on their efficacy. The assay system described differs from other methods that focus on whole blood (18) and that do not include the preincubation step integral to the assay developed for the present investigation.

Several important observations emerged from these investigations. RP-1 exerted anti-*E. coli* activity in conventional media; this activity was modestly enhanced at pH 7.2 compared to that at pH 5.5 and was retained following a 2-h preincubation in media. Importantly, RP-1 exhibited potent antimicrobial activities in whole blood and homologous plasma or serum. In some conditions tested, RP-1 exerted anti-*E. coli* effects that were equivalent to, or exceeded, those of gentamicin. Addi-

tionally, the antimicrobial activities of RP-1 in the biological matrices were generally greater than those in conventional media. In contrast to RP-1 and peptides structurally related to RP-11, which demonstrate robust antimicrobial activities in nutrient broth and radial diffusion assays *in vitro* (Yeaman et al., 38th ICAAC), RP-11 exerted essentially no anti-*E. coli* activity in MHB at either pH 5.5 or 7.2. Nonetheless, as intended based on its derivation from PMP and tPMP structural themes (i.e., antimicrobial peptides active in the cardiovascular milieu), RP-11 exhibited striking antimicrobial activity in whole blood, plasma, and serum, and this activity endured in whole blood and plasma following a 2-h preincubation prior to organism inoculation. These observations suggest that RP-11 is inhibited in the relatively complex MHB medium, has activity that is amplified by components in biomatrices, or potentiates these components to exert antimicrobial activity. The reduced antimicrobial effect of gentamicin at pH 5.5 versus pH 7.2 is consistent with previous studies regarding pH optima for aminoglycoside antibiotics (2, 10, 15, 20).

Modification of the biomatrix, through either changes to its constituents (e.g., whole blood versus serum) or preincubation with a peptide, may affect antimicrobial peptide activity in a variety of ways. As noted above, the biomatrix may directly bind the peptide, leading to inactivation. The necessary binding components may be present in some of the matrices but not others; for example, binding to erythrocytes would occur only in whole blood. Alternatively, biomatrices may contain peptidases or other reactants that degrade the peptide in a time-dependent fashion. Such degradation would contribute to loss of activity in the 2-h-preincubation assay compared to that in the assay involving immediate coincubation of the antimicrobial peptide and target organism. The activation of plasma components during the generation of serum may also yield peptidases not present in native whole blood or plasma and may contribute to any loss of activity observed during serum preincubation compared with the activity in plasma. Alternatively, biomatrices may contribute components that potentiate peptide antimicrobial activity. Thus, both RP-1 and RP-11 appear to be more potent in blood and blood fractions than in artificial media. However, RP-11 was considerably more sensitive than RP-1 to inhibition or inactivation due to preincubation in biomatrices. For example, activation of blood to generate serum or preincubation in serum may eliminate positive cooperative factors or effects that enhance the net bactericidal activities of antimicrobial peptides, resulting in an apparent decrease in their efficacy. Given the distinct structures of RP-1 and RP-11, these results may provide insights into structure-activity relationships governing the fate of antimicrobial peptides such as RP-1 and RP-11 in complex biological settings such as blood or blood fractions.

Screening for activity in biological matrices is an important step in identifying antimicrobial peptides with potential as systemic therapeutic agents. Based on experiences with RP-1 and RP-11, care in the selection of an appropriate matrix for, and in interpreting the results of, antimicrobial assays evaluating such agents must be exercised. In this regard, fresh whole blood or blood fractions may represent particularly relevant matrices for evaluation of potential systemic therapeutic antimicrobial peptides. For example, RP-1 and RP-11 antimicrobial activities in such matrices appeared to be potentiated

compared with those in conventional test media. As anticipated, blood itself exerted a modest degree of intrinsic antimicrobial activity against *E. coli*. Thus, one or more blood components may interact with the peptides or this target organism to enhance killing. Such potentiation has been observed with other conventional antibiotics (3, 9, 14) and tPMPs *in vitro* (4). Likewise, studies by Yan and Hancock (21) implicated synergistic interactions of antimicrobial peptides and lysozyme *in vitro*. Varra et al. suggested that an outer membrane-disorganizing peptide sensitized *E. coli* to the bactericidal activity of serum *in vitro*, hypothetically by deshielding the organism to the effects of complement fixation (16). Subsequently, this same group demonstrated that the polymyxin B nonapeptide (PMBN) acted synergistically with fresh human serum to effect bactericidal activity against gram-negative bacteria *in vitro* (17). It has also been suggested that complement contributes to the potentiation of other antimicrobial peptides (1). Importantly, the present findings indicate that RP-1 does not require complement to demonstrate antimicrobial activity, as evidenced in the defined-medium experiments. In contrast, neither RP-1 nor RP-11 demonstrated antimicrobial activity in heat-inactivated serum (Δ PNHS). Consistent with these results, Oh et al. (12) found that short peptides consisting of L-amino acids lost antimicrobial activity in heat-inactivated serum. Additionally, low-density lipoprotein has been implicated as an inhibitor of small- α -helical-peptide activity in serum (13). It may also be hypothesized that heat inactivates potentiating factors in serum, but the loss of RP-1 antimicrobial activity in this matrix implies influences beyond this effect. As RP-1 is a potent antimicrobial agent in conventional media, its lack of efficacy in Δ PNHS suggests that a blocking or inhibitory factor(s) is generated during heat inactivation of serum. Ostensibly, this effect could result from one or more simple binding reactants or specific inhibitors of the antimicrobial action(s) of RP-1 and RP-11.

The above considerations emphasize the validity and importance of assays which evaluate the antimicrobial activities of peptides under rigorous conditions potentially relevant to their development to function as therapeutic agents *in vivo*. The assay described was designed and developed to provide a relevant assessment of the degree and duration of peptide antimicrobial activities in complex biological matrices. However, the differences between this assay and conventional assays should be understood. For example, even in preincubation assays, the antimicrobial agent and target organism are subsequently incubated for 2 h prior to quantitative culture. Substantial inactivation of the antimicrobial agent may occur over this period, leading to false-negative results (e.g., apparent lack of antimicrobial activity of a peptide possessing intrinsic activity). The potential for this effect can be minimized if shortened exposure of the organism (e.g., shortened incubation periods) are found to be sufficient to detect efficacy. Additionally, the biological matrices themselves (e.g., whole blood and serum) may exhibit differential antimicrobial effects, making quantitative expression of the antimicrobial activity of the agent of interest more difficult. For example, antimicrobial activities of the synthetic peptides in fresh homologous human serum were greater than those in frozen PNHS. The present study utilized killing in the presence of the antimicrobial agent, compared to killing by the matrix itself, to best represent the additional

effects of the agent of interest. Differential potencies in distinct biomatrices or conventional media with differing pHs may reflect influences of pH on intrinsic structure-activity relationships of antimicrobial peptides such as RP-1 and RP-11. These influences may be significant in specific contexts in which peptides may exert optimal antimicrobial activities *in vivo*.

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