

# Synergistic Interactions between Mammalian Antimicrobial Defense Peptides

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**A single animal can express several cationic antimicrobial peptides with different sequences and structures. We demonstrate that mammalian peptides from different structural classes frequently show synergy with each other and selectively show synergy with human lysozyme.**

Cationic antimicrobial peptides are nature's antibiotics (5). They are being increasingly recognized as a component of the innate immune systems of all species of life, and more than 500 natural peptides are known. A single animal, e.g., a cow, can produce as many as three dozen antimicrobial peptides (3; <http://www.univ.trieste.it/~tossi>). These can include representatives of all four known structural classes, which comprise  $\beta$ -sheet peptides stabilized by two to three disulfide bridges, amphipathic  $\alpha$ -helical peptides, loop peptides, and extended peptides, in addition to peptides generated by proteolysis of larger proteins in the host.

Cationic antimicrobial peptides as a class have the ability to kill both gram-negative and gram-positive bacteria, fungi, eukaryotic parasites, and even enveloped viruses. Indeed, a single peptide can have most or all of these activities (3, 5). In addition, it has been demonstrated that such peptides can trigger the transcription of numerous genes in eukaryotic cells (3), and they appear to have multiple effects on the inflammatory response (2, 4). Given this extraordinary diversity of activities, it is worth asking why so many peptides can be observed in a single host. Possible explanations include (i) the fact that individual peptides are preferentially expressed under specific circumstances and/or in specific sites in the body, (ii) the fact that different peptides cover gaps in the activity spectrum of the other peptides expressed at a given location in the body, and (iii) the fact that different peptides act in synergy with one another to reduce the concentrations required to effectively kill microorganisms. Each of these explanations is credible. Inducibility or constitutivity, as well as tissue tropism, has been demonstrated for different peptides in many host organisms (reviewed in reference 3). Similarly, it is well established that insect immunity peptides tend to be preferentially antifungal or antibacterial in nature (9). The third concept, that of synergy among individual peptides, was first observed with frog peptides, including members of the dermaseptin family (10) and the  $\alpha$ -helical peptides magainin and PGLa (8, 13), and between  $\beta$ -defensins and the cationic protein BPI (7). Since then, there have been few reports demonstrating synergy between individual peptides, and generally speaking these have

not addressed synergy by using the well-established methods developed by clinical microbiologists, namely, checkerboard titration.

In this paper, we examine the synergy of peptides representing each of the structural classes found in mammals against four of the more serious pathogenic bacteria in our society. In addition, we perform a limited study to examine if such synergy can also be observed between these peptides and the moderately cationic innate defense protein lysozyme.

Four of the peptides were synthesized at the University of British Columbia's Nucleic Acid and Protein Sequencing Facility using *tert*-butoxycarbonyl chemistry and were purified by reversed-phase high-pressure liquid chromatography. These were the  $\beta$ -hairpin pig neutrophil peptide protegrin-1, the  $\alpha$ -helical human peptide LL-37, the loop-structured bovine neutrophil peptide bactenecin, and the extended-structure bovine neutrophil peptide indolicidin. The disulfide bonds of protegrin and bactenecin were formed by oxidation and confirmed by matrix-assisted laser desorption ionization mass spectrometry as previously described (10). The human  $\beta$ -defensin peptide HNP-1 was a kind gift from Bob Lehrer and Tom Ganz at the University of California at Los Angeles. Human lysozyme was purchased from Calbiochem (La Jolla, Calif.). The bacterial strains used included *Pseudomonas aeruginosa* PAO1 strain H103, *Escherichia coli* strain HB101, a methicillin-resistant *Staphylococcus aureus* (MRSA) strain, SAP0017, and *Enterococcus faecalis* ATCC 29212 (14). They were cultured on Mueller-Hinton broth, solidified when necessary with Bacto Agar (Sigma Chemical Co., St. Louis, Mo). MIC assays and checkerboard titrations were performed using the modified microtiter dilution assay (1, 11, 14).

The peptides examined showed only modest activities (e.g., relative to the best synthetic variants [12]) against the four tested bacteria, with MICs ranging from 3.1 to 64  $\mu$ g/ml (Table 1). HNP-1 and human lysozyme were not active against the gram-negative bacterium *P. aeruginosa* but readily killed the gram-positive bacterium *E. faecalis*. It should be noted, however, that this represents a very stringent test of antibacterial activity, in that Mueller-Hinton medium has a high ionic strength that can inhibit the action of such peptides. Indeed, in other assays employing dilute buffers, much more impressive activities can be observed (6). However we employed Mueller-Hinton medium because it is considered to give MICs that are

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TABLE 1. MICs of mammalian peptides

Species	MIC ( $\mu\text{g/ml}$ )					
	Bovine indolicidin	Pig protegrin 1	Bovine bactenecin	Human LL-37	Human HNP-1	Human lysozyme
<i>P. aeruginosa</i>	64	8	32	64	>50	>50
<i>E. coli</i>	16	4	32	4	ND <sup>a</sup>	ND
MRSA	8	8	16	64	ND	ND
<i>E. faecalis</i>	32	8	16	64	3.1	6.3

<sup>a</sup> ND, not done.

clinically meaningful (i.e., equivalent to activity in the human host) for cationic antibiotics such as the aminoglycosides.

Synergy was measured by checkerboard titrations (1), in which one peptide is diluted along the rows of a microtiter tray and the other is diluted along the columns. In this method, one is looking for a reduction in the MIC of each compound in the presence of the other. The result is expressed as the fractional inhibitory concentration (FIC) index, which is assessed as follows:  $\text{FIC} = [\text{A}]/\text{MIC}_A + [\text{B}]/\text{MIC}_B$ , where  $\text{MIC}_A$  and  $\text{MIC}_B$  are the MICs of peptides A and B alone and [A] and [B] are the MICs of A and B when in combination. An FIC index of 0.5 is taken to indicate good synergy (representing the equivalent of a fourfold decrease in the MIC of each compound in combination). An FIC index of 1.0 represents additive activity (a twofold decrease in the MIC of each compound in combination), and an index of >4 indicates antagonism. Table 2 describes the results obtained with these peptides. In general, we observed synergy (FIC < 0.5) for several combinations of peptides in *P. aeruginosa* (three of six combinations), *E. coli* (four of six combinations), and *E. faecalis* (two of six combinations) but not in any instance with MRSA. The best peptide in combination was protegrin, which showed synergy with most peptides against most bacteria. We also examined the synergies of these peptides with the human neutrophil defensin HNP-1, although the limited availability of this peptide (which has three disulfide bonds and is very difficult to synthesize) reduced the scope of these experiments to two strains of bacteria and a single concentration of HNP-1. No synergy was observed with 25  $\mu\text{g}$  of HNP-1/ml and any peptide against *P. aeruginosa*. However, since we could not measure an MIC for this bacterium, we did not know what multiple of the MIC was represented by 25  $\mu\text{g/ml}$  and thus whether there was any possibility of seeing synergy. With *E. faecalis* we again failed to observe synergy, even though an MIC could be measured. Consistent with this, Levy et al. failed to observe synergy between de-

fensins and a peptide, P15a (7). Nevertheless, our results overall were consistent with the conclusion that peptide-peptide synergy does occur and is peptide specific.

We also examined the synergy of peptides with human lysozyme against *P. aeruginosa* and *E. faecalis* (again, our experiments were somewhat constrained by the high cost of human lysozyme). Human lysozyme by itself is poorly antimicrobial against wild-type *P. aeruginosa* strains under the conditions tested here, causing approximately 90% killing in 60 min (15) but not demonstrating a definite MIC. A single concentration of lysozyme, 25  $\mu\text{g/ml}$ , consistently reduced the MICs of protegrin and bactenecin by twofold but had no effect on the MICs of indolicidin, LL-37 (Table 3), or HNP-1 (data not shown). It is possible that better synergy would have been observed if higher lysozyme concentrations were available, since we have observed synergy of many peptides with hen egg white lysozyme, which is relatively inexpensive and thus far more available (R. Hancock, unpublished data). In contrast, lysozyme showed higher natural activity and good synergy with two peptides against *E. faecalis* (Table 3), with protegrin leading the way. In checkerboard titrations, MICs of protegrin and lysozyme were lowered from 16 and 6.3  $\mu\text{g/ml}$  alone to 4 and 0.39  $\mu\text{g/ml}$ , respectively, in combination.

Of the peptides studied in detail, only indolicidin and bactenecin from cattle actually coexist in nature. A major reason that we chose the studied peptides was that information on the types of peptides in any given species remains quite fragmentary, with defensins as the major structural type identified in mammals (3, 5). Therefore, in this study we chose peptides primarily to represent the individual structural classes, which differ maximally from one another. It is thus an assumption here that peptides of given structural classes, regardless of their mammalian origin, will act in similar ways. Consistent with this, we have observed synergy similar to that observed

TABLE 2. Synergy among peptides expressed as the FIC

Species	Lowest FIC index ([A]/[B]) <sup>a</sup>					
	Indolicidin + protegrin 1	Indolicidin + bactenecin	Indolicidin + LL-37	LL-37 + protegrin 1	LL-37 + bactenecin	Bactenecin + protegrin 1
<i>P. aeruginosa</i>	0.25 (8/1)	0.75 (16/16)	>1.0 (32/64)	0.31 (4/1)	1.0 (32/16)	0.50 (8/2)
<i>E. coli</i>	0.25 (2/1)	0.5 (4/8)	0.75 (4/1)	0.31 (0.5/0.5)	0.56 (4/1)	0.50 (8/1)
MRSA	0.75 (2/4)	1.0 (4/8)	>1.0 (32/64)	1.0 (64/2)	0.75 (16/16)	1.0 (8/4)
<i>E. faecalis</i>	0.75 (8/4)	0.75 (8/8)	0.56 (8/4)	0.32 (4/1)	0.50 (16/4)	0.56 (8/0.5)

<sup>a</sup> FIC index =  $[\text{A}]/\text{MIC}_A + [\text{B}]/\text{MIC}_B$ , where  $\text{MIC}_A$  and  $\text{MIC}_B$  are the MICs of peptides A and B alone and [A] and [B] are the MICs of peptides A and B in combination. The MICs for the peptides alone are as given in Table 1. The numbers in parentheses are the MICs in combination, with the first number corresponding to the first antibiotic named in the column heading.

TABLE 3. Synergy of peptides with human lysozyme against *E. faecalis*

Bacterium	Peptide	MIC ( $\mu\text{g/ml}$ )				FIC index
		Peptide alone	Lysozyme alone	Peptide in combination	Lysozyme in combination	
<i>E. faecalis</i>	Indolicidin	128	6.3	8	3.1	0.56
	Protegrin	16	6.3	4	0.39	0.31
	LL-37	128	6.3	32	3.1	0.75
	Bactenecin	32	6.3	8	1.6	0.5
<i>P. aeruginosa</i>	Indolicidin	128	>50	128	25 <sup>a</sup>	>1.0
	Protegrin	32	>50	16	25	0.75
	LL-37	128	>50	128	25	>1.0
	Bactenecin	128	>50	64	25	0.75

<sup>a</sup> Due to the high cost of lysozyme, a single concentration of lysozyme (25  $\mu\text{g/ml}$ ) was used for these experiments.

with LL-37 with an insect-derived  $\alpha$ -helical peptide (R. Hancock, unpublished observations).

Together, these data are consistent with the proposal that antimicrobial peptides demonstrate synergy with each other and with lysozyme in a peptide-specific manner. It is assumed that this reflects the cooperative interactions of the peptides with the outer membranes of gram-negative bacteria and/or cooperative interaction with lipid bilayers in general (8, 15). We conclude, therefore, that given the substantial diversity of peptides in any given location in the host, synergistic interactions are an important determinant of the overall effectiveness of the peptides.

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