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NOTES

Synergistic Activity of Rabbit Granulocyte Peptides against *Candida albicans*†

ROBERT I. LEHRER,[1,2]* DOROTHY SZKLAREK,[1] TOMAS GANZ,[1] AND MICHAEL E. SELSTED[1,3]

Departments of Medicine[1] and Pathology,[3] University of California, Los Angeles, Center for the Health Sciences, Los Angeles, California 90024, and Veterans Administration Medical Center, West Los Angeles, California 90073[2]

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Rabbit granulocytes contain six antimicrobial peptides that are structurally homologous to the human neutrophil "defensins." NP-5, a rabbit defensin, lacks significant activity against *Candida albicans*. Nevertheless, its addition to submicromolar concentrations of rabbit NP-1, NP-2, or NP-3a potentiates their candidacidal effect. Thus, granulocyte defensins can act synergistically against potential pathogens.

Rabbit granulocytes contain six cysteine-rich antimicrobial peptides (2, 5, 6). Three of these peptides, NP-1, NP-2, and NP-3a, are highly effective against yeast-phase *Candida albicans* (5). Although NP-5, a congener of these active peptides (4), is abundant in rabbit granulocytes (6), it manifests little direct candidacidal activity (5, 6). The contrast between its relative abundance and its lack of direct antimicrobial activity prompted us to examine the effects of adding NP-5 to *C. albicans* that were simultaneously exposed to low concentrations of NP-1, NP-2, or NP-3a.

The rabbit granulocyte peptides used in this study were purified to homogeneity from sterile peritoneal exudates (4, 6). Briefly, this involved extraction with acetic acid followed by polyacrylamide gel electrophoresis and reversed-phase high-pressure liquid chromatography. *C. albicans* 820 was cultured for 4 or 18 h as previously described (5). Candidacidal activity was tested by incubating 10^5 CFU at 37°C in 100 μl (final volume) of 10 mM sodium phosphate buffer (pH 7.4; ionic strength, 1.36 mS) containing purified granulocyte peptides.

Figure 1 compares the susceptibility of logarithmic-phase (4 h) *C. albicans* blastoconidia to NP-1 and NP-5 after exposure to these peptides for 20 min at 37°C. In nine such experiments wherein *C. albicans* was exposed to 25 μg of NP-5 per ml, the $\log_{10}$ mean ± standard error of the mean CFU per milliliter after exposure (6.00 ± 0.03) was only slightly lower than the control value (6.07 ± 0.02). We therefore used 25 μg of NP-5 per ml in most of the studies.

Addition of NP-5 to fixed concentrations (1 or 2.5 μg/ml) of NP-1 progressively increased overall candidacidal efficacy after 20 min (Fig. 2). To determine whether NP-5 enhanced the rate or extent of candidacidal activity mediated by NP-1, early-stationary-phase (18 h) blastoconidia were exposed to 2.5 μg of NP-1 per ml ± 25 μg of NP-5 per ml for up to 4 h (Fig. 3). We concluded that the synergistic candidacidal effect of NP-5 reflected increases in both the initial rate and the extent of candidacidal activity.

We confirmed the ability of NP-5 to potentiate candidacidal activity by NP-1 (Table 1) under different experimental conditions from those shown in Fig. 2. NP-2 and NP-3a were also potentiated by NP-5, but NP-3b and NP-4 were not (Table 2).

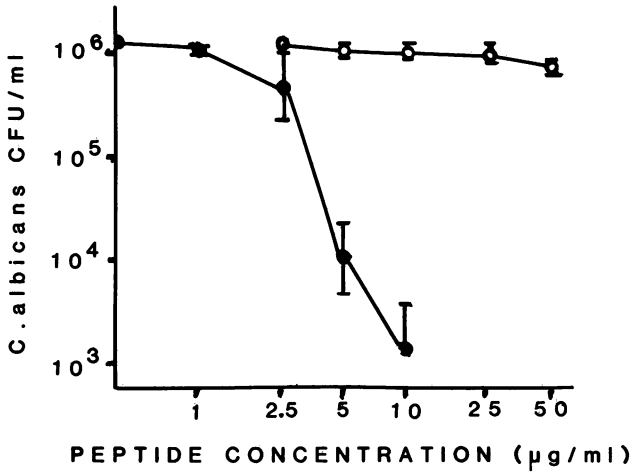

FIG. 1. Susceptibility to NP-1 and NP-5. *C. albicans* (4 h) was incubated for 20 min with NP-1 (●) or NP-5 (○). Data show surviving CFU per milliliter ± the standard error. NP-1, $n = 4$; NP-5, $n = 9$.

The remarkable abundance of six cysteine-rich, relatively small (33 to 34 amino acid residues per molecule) peptides in

TABLE 1. Synergistic effect of NP-5 on *C. albicans*[a]

NP-5 (μg/ml)	$\log_{10}$ reductions (CFU/ml)[b] at following NP-1 concn (μg/ml):					
	0		0.5		1.0	
	Expt 1	Expt 2	Expt 1	Expt 2	Expt 1	Expt 2
0	0.00	0.00	0.06	0.07	0.16	0.17
5	0.07	0.03	0.59	0.76	0.97	0.96
10	0.10	0.03	0.92	1.06	1.26	1.21
25	0.37	0.17	1.61	1.50	1.89	1.81
50	0.78	0.27	1.78	1.88	2.51	2.44

[a] *C. albicans* (18 h) was incubated for 2 h at 37°C.
[b] The data represent values from two separate experiments, each performed in duplicate.

* Corresponding author.
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TABLE 2. Synergistic candidacidal activity of NP-5[a]

Peptide (μg/ml)	Log_{10} decreases (CFU/ml), relative to control, with the following peptides:									
	NP-1		NP-2		NP-3a		NP-3b		NP-4	
	0	+	0	+	0	+	0	+	0	+
1	0.08	(0.99)[b]	0.04	(0.89)	0.05	(0.99)	0.07	0.05	0.01	0.01
2.5	0.58	(1.77)	2.30	2.22	0.38	(1.37)	0.24	0.18	0.03	0.01
5	2.48	2.71	2.41	2.69	1.70	1.81	0.34	0.28	0.08	0.19
10	3.39	3.16	3.13	3.23	2.05	2.21	0.52	0.50	0.20	0.39
25	4.32	4.28	4.31	4.01	2.37	2.98	0.90	0.87	0.33	0.53

[a] A 10^6-CFU/ml amount of *C. albicans* (4 h) was incubated for 20 min at 37°C with 1 to 25 μg of each of the indicated peptides per ml alone (0) or in combination with 25 μg of NP-5 per ml (+). Data are means from two separate experiments for each peptide.
[b] Parentheses indicate synergistic combinations.

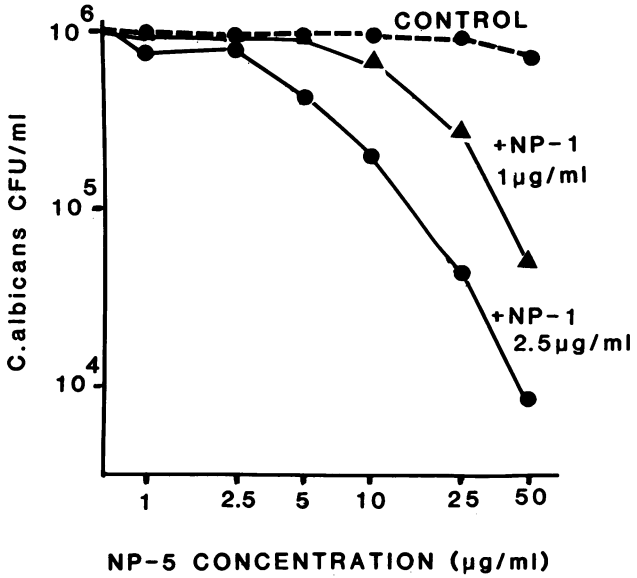

FIG. 2. Synergy between NP-5 and NP-1. *C. albicans* (4 h) was incubated for 20 min at 37°C with NP-5 alone (control) or with NP-5 that had been supplemented with NP-1 at either 1 μg/ml (▲) or 2.5 μg/ml (●). Data show surviving organisms, which were enumerated by colony counting. Each data point is a mean value from two replicate experiments.

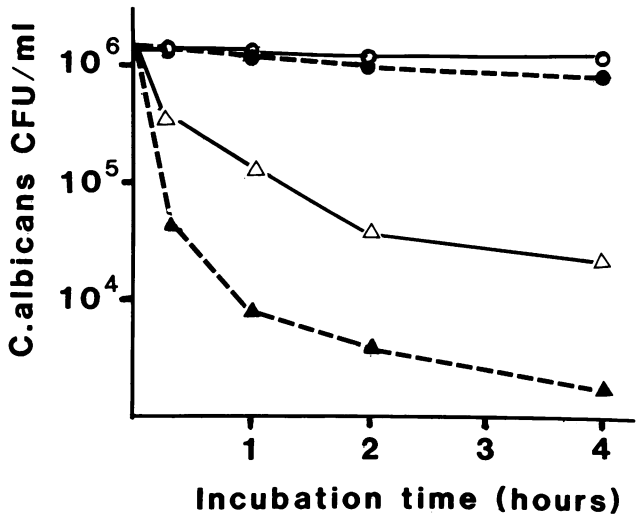

FIG. 3. Kinetics of candidacidal activity. *C. albicans* (18 h) was incubated at 37°C with 2.5 μg of NP-1 per ml (△), 25 μg of NP-5 per ml (●), 2.5 μg of NP-1 plus 25 μg of NP-5 per ml (▲), or no added peptide (○). Each data point is a mean value from two replicate experiments. Data show surviving organisms.

the cytoplasmic granules of rabbit polymorphs suggests that the peptides play a substantial role in the antimicrobial activity of these phagocytes (6, 7). Homologous peptides ("defensins") also exist in azurophil granules of human neutrophils (1).

NP-1 and NP-2 display the greatest antibacterial (6), antifungal (5), and antiviral (2) activities of the rabbit defensins. They are also the only defensins expressed in rabbit alveolar macrophages (3) and are more abundant than their less potent congeners, NP-3a, NP-3b and NP-4, in rabbit neutrophils (Selsted et al., unpublished data). Thus, the relative abundance of these five rabbit defensins is roughly proportional to their in vitro efficacy. However, NP-5, a minimally efficacious direct microbicide, is paradoxically quite abundant in rabbit neutrophils.

Although NP-5 might have been more actively antimicrobial if tested with different microorganisms or under different in vitro conditions, we observed no increase in its candidacidal activity when our incubation media were supplemented (data not shown) by various nutrients, including glucose, succinate, pyruvate, dilute nutrient broths, etc. It is possible that NP-5 contributes primarily to aspects of phagocyte function unrelated to direct microbicidal activity.

The low-molecular-weight antimicrobial peptides of rabbit polymorphs are contained within a distinct class of dense cytoplasmic granules (8). It is likely, therefore, that postphagocytic degranulation exposes intravacuolar microorganisms to mixtures of the rabbit defensins, rather than to individual molecular species. Synergistic antimicrobial effects, such as those demonstrated to occur in vitro between NP-5 and NP-1, -2, and -3a, could therefore contribute to the resultant microbicidal activity of the phagocyte and partially account for the surprising relative abundance of NP-5.

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