INFECTION AND IMMUNITY, July 1985, p. 202–206
0019-9567/85/070202-05$02.00/0

Vol. 49, No. 1

Activity of Rabbit Leukocyte Peptides Against *Candida albicans*†

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

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

Received 14 December 1984/Accepted 2 April 1985

Six related cysteine-rich, low-molecular-weight peptides were purified from rabbit peritoneal granulocytes and tested in vitro for fungicidal activity against *Candida albicans*. Two peptides (NP-1 and NP-2) were highly effective, one (NP-3a) was moderately active, and three (NP-3b > NP-4 >> NP-5) had substantially less potency. There was a general, but imperfect, correlation between the candidacidal potency of each peptide and its net cationic charge. Candidacidal activity by NP-1 was concentration and time dependent and occurred rapidly under optimal low-ionic-strength conditions. It was inhibited by increasing either the ionic strength or Ca^{2+} concentration of the incubation mixtures, but was relatively unaffected by Mg^{2+}. Candidacidal activity was independent of H^+ concentrations between pH 5 and 8, but decreased below pH 5. Candidacidal activity was temperature sensitive and was virtually abolished when NP-1 was incubated with *C. albicans* at 0°C. Cysteine-rich antimicrobial peptides such as NP-1 and NP-2 may equip leukocytes to deal with infections caused by *C. albicans* and other fungi that are susceptible to their microbicidal effects.

Rabbit granulocytes (11a, 13–15) and alveolar macrophages (11, 12) contain cysteine-rich, low-molecular-weight peptides whose antibacterial (9, 13, 15), antiviral (R. I. Lehrer, T. Ganz, M. Sherman, and M. E. Selsted, *in* E. Pick, ed., *Lymphokines*, in press), and antifungal (8, 10) properties may contribute to host defense mechanisms against infection. We recently isolated six related peptides, NP-1, NP-2, NP-3a, NP-3b, NP-4, and NP-5 from rabbit granulocytes and have described their antibacterial activity (13) and amino acid sequences (11a, 12). In this report we describe the activity of these peptides against *Candida albicans*, and we identify factors that modulate the candidacidal activity of NP-1, the most potent of these peptides.

MATERIALS AND METHODS

Peptide preparation and nomenclature. The peptides used in this study were purifed from granulocyte-rich sterile peritoneal exudates raised in male or female adult New Zealand White rabbits as recently described in detail (11a, 13). Rabbit granulocytes contain six distinct, homologous peptides that we have designated NP-1, NP-2, NP-3a, NP-3b, NP-4, and NP-5. By sequence analysis and crystallographic data, NP-1 and NP-2 are identical in structure to MCP-1 and MCP-2, respectively, earlier purified from rabbit alveolar macrophages (11a, 12).

C. albicans. *C. albicans* strain 820 has been used extensively in our laboratory to probe the candidacidal activity of rabbit and human leukocytes (5). It was cultured on Sabouraud 2% dextrose agar petri dishes at 37°C for 24 h and then kept at 4°C for up to 2 weeks. Organisms picked from a single colony were inoculated into 10 ml of Sabouraud 2% dextrose broth (Difco Laboratories, Detroit, Mich.) and grown for 18 h at 37°C. From this intermediate culture, 1 ml was inoculated into 50 ml of Sabouraud broth and incubated at 37°C for 4, 18, or 66 h. The test organisms were washed two times by centrifugation in the buffer to be used in the experiment, counted in a hemocytometer, and adjusted to the desired concentration in buffer.

Fungicidal testing. Unless otherwise noted, *C. albicans* at a final concentration of 10^6 CFU/ml was exposed to selected concentrations of the purified leukocyte peptides in 100 μl (final volume) of standard low-ionic-strength (1.36 mS) 10 mM sodium phosphate buffer (pH 7.4) as previously described (9). Incubation time and temperature were varied as described below. In some experiments, the buffer was modified by the addition of 0.025 to 0.14 M NaCl to alter its ionic strength or by varying its pH while maintaining a constant ionic strength (1.45 mS), as determined with a Sybron/Barnstead PM-70 CB conductivity bridge. In experiments to ascertain the effects of Ca^{2+} and Mg^{2+} on candidacidal activity, the buffers were modified to contain 10 mM Tris (Sigma Chemical Co., St. Louis, Mo.) instead of phosphate to insure solubility of the added Ca^{2+}. The mean number of *C. albicans* CFU per milliliter in all control incubation mixtures varied by <15% from the initially added inoculum. Experiments were typically confirmed, in whole or in part, on 3 to 10 separate occasions. The figures show representative experimental results.

RESULTS

Candidacidal activity. The activities of all six rabbit leukocyte peptides against *C. albicans* in the early logarithmic phase (4 h) are compared in Fig. 1, which demonstrates the $\log_{10}$ reduction of CFU per milliliter after 20 min. Note that NP-1 and NP-2 were essentially equipotent and that 10 μg (approximately 2.6 μM) of either peptide per ml reduced the colony count by >3 $\log_{10}$. NP-3a, although less potent than NP-1 or NP-2, also had substantial activity in that 10 μg/ml reduced the colony count by 1.8 $\log_{10}$ in 20 min under these conditions. NP-3b and NP-4 were considerably less effective, and NP-5 appeared virtually devoid of activity.

The susceptibility of *C. albicans* to these peptides was influenced by the growth phase of the yeast and by the duration of exposure to the peptide (Fig. 2). When *C. albicans* organisms grown in Sabouraud broth for 4, 18, or 66 h before the assay (early log phase, early stationary phase, and stationary phase, respectively) were compared, their relative susceptibility toward each granulocyte peptide was 4

* Corresponding author.

† Publication no. 96 of the Collaborative California Universities—Mycology Research Unit.

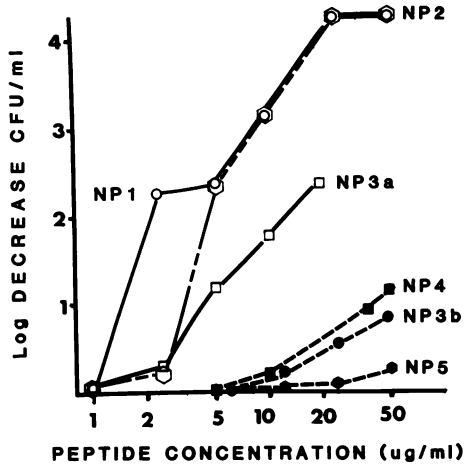

FIG. 1. Candidacidal activity of granulocyte peptides. *C. albicans* (4 h), 10^6 CFU/ml, was incubated for 20 min with the indicated peptide in 10 mM phosphate buffer (pH 7.4) at 37°C. The abscissa shows $\log_{10}$ reduction in CFU per milliliter relative to the control, as determined by quantitative plate counts.

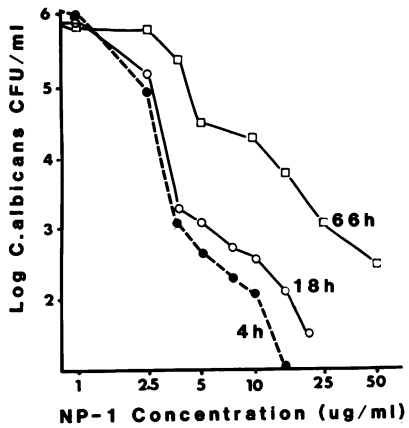

FIG. 3. Effect of culture age on susceptibility. *C. albicans*, 10^6 CFU/ml, was incubated for 20 min at 37°C in 10 mM phosphate buffer (pH 7.4), and surviving CFU were enumerated by colony counting. The culture ages (4, 18, and 66 h) are indicated on the figure.

h > 18 h >> 66 h. Figure 2b shows that 50 μg of NP-3b or NP-4 per ml caused appreciable (2.0 or 1.35 $\log_{10}$, respectively) reductions in CFU of *C. albicans* per milliliter after 2 h of incubation, whereas NP-5 still showed relatively weak activity.

The effect of culture age on susceptibility of *C. albicans* to NP-1 in a 20-min incubation is shown in Fig. 3. To secure the first $\log_{10}$ fall in CFU per milliliter, the addition of approximately 2.3 μg of NP-1 per ml was required for the 4-h culture, and 4.6 μg/ml was required for the 66-h culture. Thereafter, the curves diverged. Elimination of 2 $\log_{10}$ required 3.0 μg of NP-1 per ml for 4-h cultures and 12 μg/ml for 66-h cultures, whereas elimination of 3 $\log_{10}$ required 4.0 and 30 μg/ml, respectively, at 66 h. These results suggest the presence of heterogeneity with more resistant subpopulations in the older culture. The 18-h *C. albicans* culture was only slightly less susceptible than the 4-h culture.

The kinetics of candidacidal activity, as manifested by exposure of an early log phage (4-h) culture to 10 μg of NP-1 per ml in 10 mM phosphate buffer (pH 7.4) is demonstrated in Fig. 4. Note that only 90 s of exposure to peptide sufficed to bring about a 2 $\log_{10}$ (99%) reduction in CFU.

Modulating factors. The effect of pH on the candidacidal activity of NP-1 is shown in Fig. 5. In contrast to our earlier findings with bacteria, candidacidal activity was essentially independent of H^+ concentrations between pH 5 and 8. Diminished candidacidal activity was noted at pH 3 to 4, and peptide-mediated candidacidal activity was abolished at pH 2. *C. albicans* controls were remarkably unaffected by 20 min of exposure to the various buffers used in these experiments, and NP-1 was completely stable at pH 2 to 4.

The effect of ionic strength on the candidacidal activity of NP-1 is shown in Fig. 6 and 7. Candidacidal activity was optimal under the low-ionic-strength conditions selected for our routine assays and was progressively inhibited when the incubation mixture was supplemented with increasing concentrations of NaCl (Fig. 6a). Comparative dose-response curves for *C. albicans* (18 h) exposed to NP-1 in standard buffer with or without 25 mM NaCl (Fig. 6b) showed a rightward displacement of approximately 0.6 $\log_{10}$ in the salt-supplemented system. Thus, approximately fourfold higher peptide concentrations were required to give equiva-

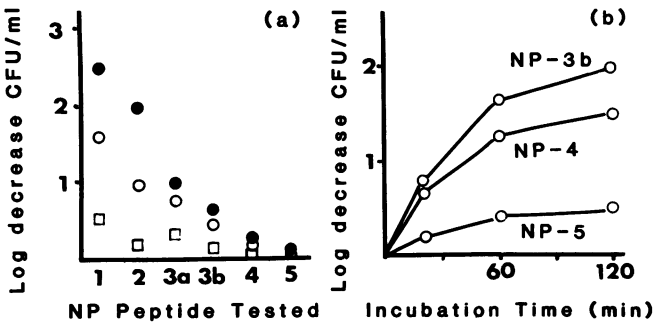

FIG. 2. Effect of culture age and incubation time on candidacidal activity. (a) *C. albicans*, 10^6 CFU/ml, was incubated for 20 min at 37°C in 10 mM phosphate buffer (pH 7.4) with 10 μg of NP-1 or NP-2 per ml, 25 μg of NP-3a per ml, or 50 μg of NP-3b, NP-4, or NP-5 per ml. The symbols represent the age of the culture, as follows: ●, 4 h; ○, 18 h; □, 66 h. (b) *C. albicans* (4 h), 10^6 CFU/ml, was exposed to 50 μg of NP-3b, NP-4, or NP-5 per ml at 37°C in 10 mM phosphate buffer (pH 7.4).

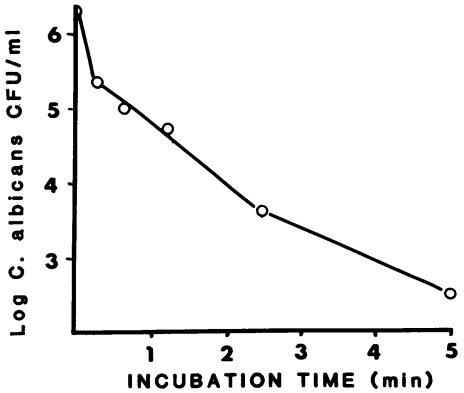

FIG. 4. Kinetics of candidacidal activity. *C. albicans* (4 h), 10^6 CFU/ml, was incubated with 10 μg of NP-1 per ml in 10 mM phosphate buffer at 37°C. Samples were serially diluted with phosphate-buffered saline, and surviving CFU were enumerated by quantitative plate counts.

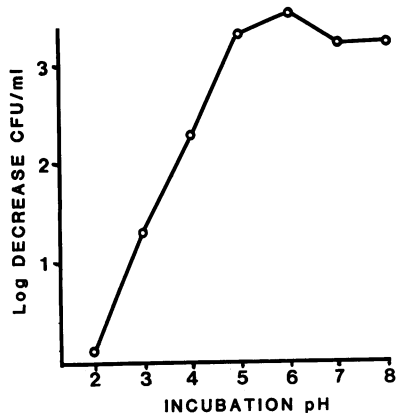

FIG. 5. Effect of pH on candidacidal activity. *C. albicans* blastoconidia (18 h), 5×10^6 CFU/ml, were incubated for 30 min at 37°C in constant-ionic-strength buffers (1.4 mS) with or without 5 μg of NP-1 per ml. The abscissa shows $\log_{10}$ reductions in CFU per milliliter relative to peptide-free controls. The control *C. albicans* was minimally affected by exposure to any peptide-free buffers at pH 2 to 8.

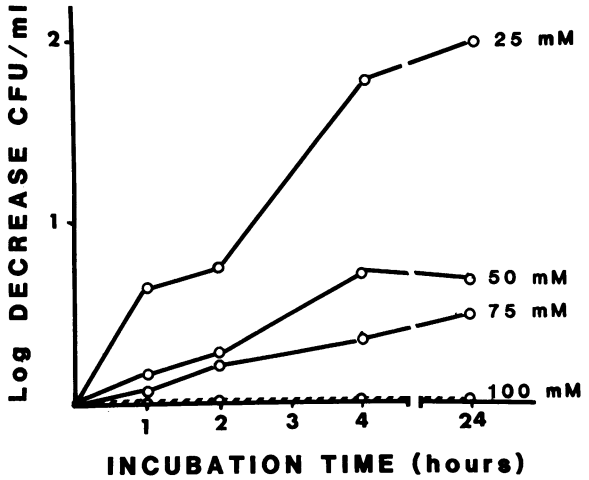

FIG. 7. Effect of ionic strength on candidacidal activity. *C. albicans*, 10^6 CFU/ml, was incubated with 10 μg of NP-1 per ml at 37°C in 10 mM phosphate buffer (pH 7.4) supplemented with 25 to 100 mM NaCl as shown. *C. albicans* incubated with 10 μg of NP-1 per ml in unsupplemented phosphate buffer underwent a 5.1 $\log_{10}$ reduction in CFU per milliliter after 1 h.

lent candidacidal activity after 20 min in the presence of 25 mM NaCl, relative to standard conditions. Figure 7 shows the effects of various degrees of NaCl supplementation on the candidacidal activity of 10 μg of NP-1 per ml. Although candidacidal activity increased gradually with time, up to 4 h of incubation in the presence of 25 to 75 mM NaCl, virtually no additional killing occurred when the incubations were prolonged to 24 h in these solutions. The addition of 100 mM NaCl sufficed to completely inhibit candidacidal activity by 10 μg of NP-1 per ml. When we exposed *C. albicans* (4 h) to 100 μg of NP-1 per ml in 10 mM phosphate buffer (pH 7.4) that had been supplemented with 100 mM NaCl, the reduction in CFU per milliliter relative to the control was 82% after 4 h and 87.5% after 24 h. Thus, the inhibitory effects of increasing NaCl were relative, and they could be partially overcome by increasing the concentration of NP-1 used in the assays.

In addition to the inhibitory effect of ionic strength per se, the candidacidal activity of NP-1 was profoundly affected by the addition of calcium ions, but not magnesium ions, to the incubation mixtures (Fig. 8a). To preclude precipitation of Ca^{2+} by our standard phosphate-buffered incubation mixture, these studies were performed in a Tris-buffered medium. Figure 8b demonstrates that NP-1 was even more active in the Tris-buffered system than it was in our customary phosphate-containing, low-ionic-strength buffer.

The ability of NP-1 to kill *C. albicans* depended markedly on incubation temperature. The candidacidal activity of NP-1 was virtually absent when incubations were conducted at 0°C and was greater at 37°C than at room temperature (Fig. 9).

We previously reported that MCP-1 and MCP-2 (identical

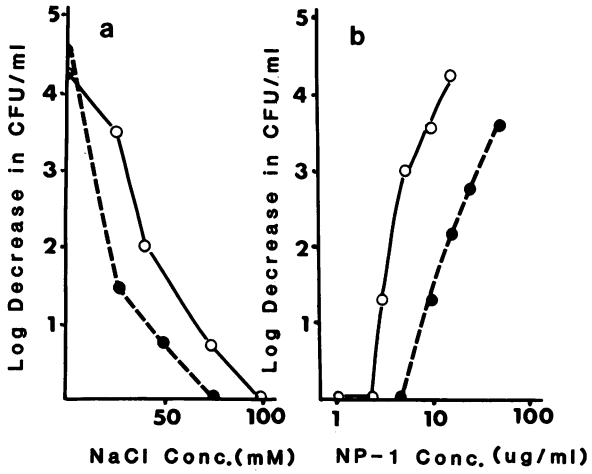

FIG. 6. Effect of ionic strength on candidacidal activity. (a) *C. albicans* 4 h [○], 18 h [●]), 10^6 CFU/ml, was incubated for 20 min at 37°C with 10 μg of NP-1 per ml in 10 mM phosphate buffer (pH 7.4) supplemented with NaCl as shown. (b) *C. albicans* (18 h) was incubated for 20 min at 37°C with 1 to 50 μg of NP-1 per ml in unsupplemented 10 mM phosphate buffer, pH 7.4 (○), or in 10 mM phosphate buffer containing 25 mM NaCl (●).

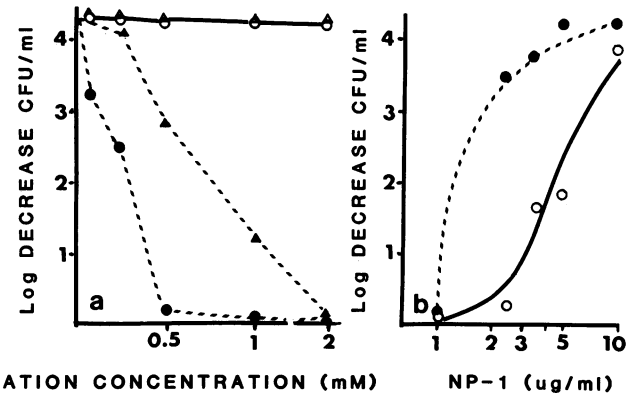

FIG. 8. Effect of calcium and magnesium on candidacidal activity. (a) *C. albicans* (18 h), 10^6 CFU/ml, was incubated for 20 min at 37°C with 5 μg (○, ●) or 10 μg (△, ▲) of NP-1 per ml in 10 mM Tris buffer (pH 7.4) supplemented with calcium (●, ▲) or magnesium (○, △). (b) *C. albicans* (18 h) was incubated for 20 min at 37°C with 1 to 10 μg of NP-1 per ml in 10 mM phosphate (○) or 10 mM Tris (●) buffer, pH 7.4.

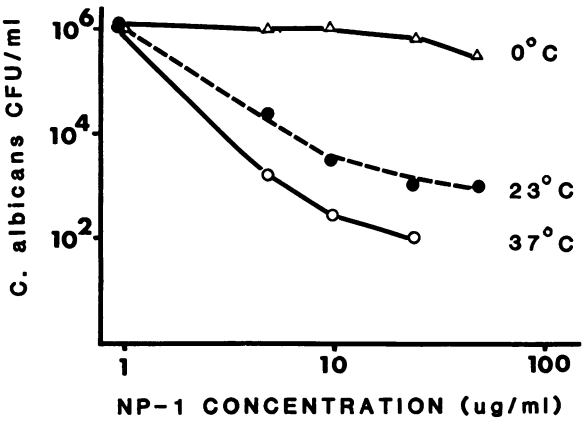

FIG. 9. Effect of temperature on candidacidal activity. *C. albicans* (4 h), 10^6 CFU/ml, was incubated for 20 min with NP-1 in 10 mM phosphate buffer (pH 7.4) at 0, 23, or 37°C.

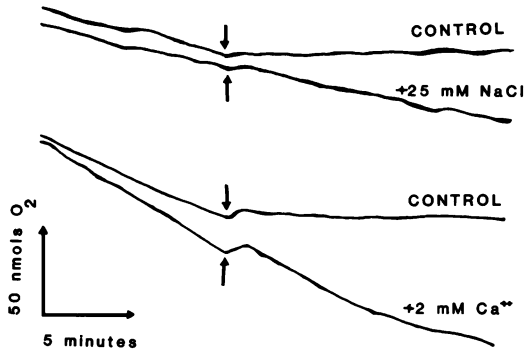

FIG. 10. Ionic strength and calcium modulate respiratory inhibition by NP-1. These polarigraphic traces of oxygen consumption by *C. albicans*, 5×10^6 blastoconidia per ml, were made at 37°C with a Gilson KM-1 oxygraph with dual thermostatted Clark-YSI electrodes. After basal O_2 consumption was recorded for approximately 10 min, 5 μg of NP-1 per ml was added (vertical arrows). The top pair of tracings was done with 18-h blastoconidia in 10 mM phosphate buffer with or 25 mM NaCl (pH 7.4). The bottom pair was shows 4-h blastoconidia incubated in 10 mM Tris buffer with or 2 mM calcium chloride (pH 7.4). Time and O_2 scales are shown in the bottom left corner.

to NP-1 and NP-2) rapidly abolished O_2 consumption by *C. albicans* exposed to microbicidal concentrations of these peptides (11). Figure 10 demonstrates the protective effects of NaCl or Ca^{2+} addition on peptide-mediated respiratory suppression.

DISCUSSION

Granulocytes are major contributors to host defenses against the opportunistic fungal pathogen *C. albicans* (1a). Two distinct types of microbicidal mechanisms allow granulocytes to kill *C. albicans* blastospores (blastoconidia). One of these depends on the production of H_2O_2 by phagocytic cells and its potentiation by myeloperoxidase and halide ions (3). The other, which operates independently of the oxidative metabolism of the granulocyte (4), may reflect the activity of intrinsically microbicidal proteins (1) and peptides (8) in granulocytes.

In previous studies we used *C. albicans* blastoconidia, typically cultured for 3 to 5 days in Sabouraud broth before testing, to probe the candidacidal mechanisms of human neutrophils (5). Our present studies suggest that organisms prepared in this manner may be relatively resistant to O_2-independent microbicidal mechanisms and may explain why human granulocytes deficient either in myeloperoxidase content (6) or the ability to generate O_2^- and H_2O_2 (7) have a markedly impaired ability to kill such blastoconidia.

In this study we have demonstrated that *C. albicans* blastoconidia are susceptible to several peptides purified from rabbit granulocytes. The order of the anticandidal activity of the peptides was NP-1 = NP-2 > NP-3a > NP-3b > NP-4 >> NP-5. Candidacidal activity was optimal in low-ionic-strength buffers and was both time and peptide concentration dependent. In each of these respects, our present studies with *C. albicans* generally parallel those earlier reported with various bacteria (13).

NP-1 and NP-2 are highly cationic peptides, due to their high content of arginine residues. Although various other cationic peptides have been reported to exert antibacterial (3) and antifungal (2) effects in vitro, cationicity alone cannot explain the marked candidacidal activity of NP-1 and NP-2. This is made apparent by comparing the relative candidacidal activity of the six rabbit granulocyte peptides (Fig. 1) to their net molecular charge (Table 1). NP-2, NP-3a, and NP-3b have identical net charges (+8), yet they varied substantially in relative potency. Although net positive charge cannot be the sole determinant of candidacidal activity, the least candidacidal peptides, NP-4 and NP-5, were also the least cationic peptides in this series.

Whereas the bactericidal effects of NP-1 and other peptides were minimal at pH <6.0 (9, 13), the candidacidal activity of NP-1 was relatively independent of H^+ concentration from pH 5 to 8. NP-1-mediated candidacidal activity was inhibited by Ca^{2+}, low temperature, and increased NaCl concentrations. Although these observations provide intriguing hints about the potential mechanism of peptide-mediated antifungal activity, additional studies in this realm are required.

Our studies indicate that the small, cysteine-rich antimicrobial peptides described in this report may contribute to the ability of rabbit granulocytes to kill *C. albicans* and other fungi. The concentrations of NP-1 and NP-2 in rabbit granulocytes are remarkably high. Together, these two peptides account for approximately 4% of the total protein-peptide content of these cells (Selsted et al., unpublished data). Even if homogeneously distributed, their intracellular concentration in granulocytes would exceed 10 mg/ml.

We have recently discovered that cysteine-rich antimicro-

TABLE 1. Net charge of rabbit granulocyte peptides at pH 7.4[a]

Peptide	Total amino acid residues	No. of charged residues per molecule					Net charge
		Arg	Lys	Glu	Asp	His	
NP-1	33	10	0	1	0	1	+9
NP-2	33	9	0	1	0	1	+8
NP-3a	34	9	0	1	0	0	+8
NP-3b	34	8	2	1	1	0	+8
NP-4	33	6	0	1	0	1	+5
NP-5	32	4	0	1	0	1	+3

[a] The number of positively charged (arginine and lysine) and negatively charged (glutamic and aspartic acid) amino acid residues per molecule of peptide is tabulated. Net charge was calculated as (arginine + lysine) − (glutamic acid + aspartic acid). Histidine residues, which have minimal net charge at pH 7.4, are also shown, but are not included in the calculation of overall net charge. This table is adapted from the description of amino acid compositions and sequences of these peptides reported elsewhere (12, 14).

bial peptides, homologous by amino acid sequence analysis to the family of rabbit granulocyte peptides examined in this report, exist in human neutrophils (T. Ganz, M. E. Selsted, D. Szklarek, and R. I. Lehrer, Fed. Proc., in press; M. E. Selsted, T. Ganz, J. W. Schilling, and R. I. Lehrer, Fed. Proc., in press). The human peptides, collectively referred to as "defensins," are also extremely active against *C. albicans* in vitro. We suggest, therefore, that such peptides may represent an important component of the antifungal defense mechanisms in mammalian phagocytes.

ACKNOWLEDGMENTS

This study was supported, in part, by Public Health Service grants AI 16252 and 18754 from the National Institutes of Health. T.G. is a Parker B. Francis fellow of the Puritan-Bennett Foundation.

LITERATURE CITED

1. **Drazin, R. E., and R. I. Lehrer.** 1977. Fungicidal properties of a chymotrypsin-like cationic protein from human neutrophils: adsorption to *Candida parapsilosis*. Infect. Immun. **17**:382–388.

1a. **Fleischman, J., and R. I. Lehrer.** 1985. Phagocytic mechanisms in host response, p. 123–149. *In* D. H. Howard (ed.), Fungi pathogenic for humans and animals. Part B. Pathogenicity and detection: II. Marcel Dekker Inc., New York.

2. **Gadebusch, H. H.** 1972. Natural host resistance to infection with *Cryptococcus neoformans*. IV. The effect of some cationic proteins on the experimental disease. J. Infect. Dis. **116**:551–565.

3. **Klebanoff, S. J., and R. A. Clark.** 1978. The neutrophil: function and clinical disorders. North Holland Publishing Co., Amsterdam.

4. **Lehrer, R. I.** 1972. Functional aspects of a second mechanism candidacidal activity in human neutrophils. J. Clin. Invest. **51**:2566–2572.

5. **Lehrer, R. I.** 1981. Ingestion and destruction of *Candida albicans*, p. 693–708. *In* D. A. Adams, H. Koren, and P. Edelson (ed.), Methods for studying mononuclear phagocytes. Academic Press, Inc., New York.

6. **Lehrer, R. I., and M. J. Cline.** 1969. Leukocyte myeloperoxidase deficiency and disseminated candidiasis: the role of myeloperoxidase in resistance to *Candida* infection. J. Clin. Invest. **48**:1478–1488.

7. **Lehrer, R. I., and M. J. Cline.** 1969. Interaction of *Candida albicans* with human leukocytes and serum. J. Bacteriol. **98**:996–1004.

8. **Lehrer, R. I., K. M. Ladra, and R. B. Hake.** 1975. Nonoxidative fungicidal mechanisms of mammalian granulocytes: demonstration of components with candidacidal activity in human, rabbit, and guinea pig leukocytes. Infect. Immun. **11**:1226–1234.

9. **Lehrer, R. I., M. E. Selsted, D. Szklarek, and J. Fleischmann.** 1983. Antibacterial activity of MCP-1 and MCP-2, natural peptide antibiotics of rabbit lung macrophages. Infect. Immun. **42**:10–14.

10. **Patterson-Delafield, J., R. J. Martinez, and R. I. Lehrer.** 1980. Microbicidal cationic proteins in rabbit alveolar macrophages: a potential host-defense mechanism. Infect. Immun. **30**:180–192.

11. **Patterson-Delafield, J., D. Szklarek, R. J. Martinez, and R. I. Lehrer.** 1980. Microbicidal cationic proteins of rabbit alveolar macrophages: amino acid composition and functional attributes. Infect. Immun. **31**:723–731.

11a. **Selsted, M. E., D. M. Brown, R. J. DeLange, S. S. L. Harwig, and R. I. Lehrer.** 1985. Primary structures of six antimicrobial peptides of rabbit peritoneal neutrophils. J. Biol. Chem. **260**:4579–4584.

12. **Selsted, M. E., D. M. Brown, R. J. DeLange, and R. I. Lehrer.** 1983. Primary structures of MCP-1 and MCP-2, natural peptide antibiotics of rabbit lung macrophages. J. Biol. Chem. **258**:14485–14489.

13. **Selsted, M. E., D. Szklarek, and R. I. Lehrer.** 1984. Purification and antibacterial activity of antimicrobial peptides of rabbit granulocytes. Infect. Immun. **45**:150–154.

14. **Zeya, H. I., and J. K. Spitznagel.** 1966. Cationic proteins of polymorphonuclear leukocyte lysosomes. II. Composition, properties, and mechanism of antibacterial action. J. Bacteriol. **91**:755–762.

15. **Zeya, H. I., and J. K. Spitznagel.** 1968. Arginine-rich proteins of polymorphonuclear leukocyte lysosomes. Antimicrobial specificity and biochemical heterogeneity. J. Exp. Med. **127**:927–941.