INFECTION AND IMMUNITY, Apr. 1991, p. 1359–1367
0019-9567/91/041359-09$02.00/0

Vol. 59, No. 4

Antimicrobial Activity of Rabbit Leukocyte Defensins against *Treponema pallidum* subsp. *pallidum*

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Received 17 August 1990/Accepted 9 January 1991

Defensins, which are peptides with broad antimicrobial activity, are major constituents of rabbit neutrophils and certain macrophages. We tested six rabbit defensins, NP-1, NP-2, NP-3a, NP-3b, NP-4, and NP-5, for activity against *Treponema pallidum* subsp. *pallidum*. Mixtures of *T. pallidum* and defensin in 10% normal rabbit serum (NRS) or heat-inactivated NRS (HI-NRS) were incubated anaerobically for various time periods ranging between 0 and 16 h and then examined by dark-field microscopy for treponemal motility or inoculated intradermally into rabbits to assess treponemal virulence. Immobilization of *T. pallidum* by NP-1 (400 μg/ml) occurred after 4 and 8 h of coincubation in mixtures containing NRS and HI-NRS, respectively. Similarly, neutralization of *T. pallidum* by NP-1 occurred more rapidly and was complete when incubations were performed in NRS as compared with that in HI-NRS. Endpoint titration confirmed the augmentation of NP-1 antitreponemal activity by heat-labile serum factors; NP-1 showed neutralizing activity at 4 μg/ml (about 1 μM) in NRS and at 40 μg/ml in HI-NRS. When NP-1 was tested in serum that was deficient in C6, the *T. pallidum* neutralizing activity of NP-1 was reduced to levels slightly greater than that observed in HI-NRS. NP-1 that had been reduced and alkylated was inactive against *T. pallidum*. When NP-2, NP-3a, NP-3b, NP-4, and NP-5 were tested at 400 μg/ml, all exerted potent treponemicidal activity, manifested by abrogation or delayed development of cutaneous lesions relative to that of controls. These data suggest that defensins may equip certain macrophages and neutrophils to participate in host defense against *T. pallidum*, that the direct activity of defensins against *T. pallidum* is enhanced by heat-labile serum factors (presumably complement), and that conformational factors influence the biological activity of the defensin molecule.

Professional phagocytes have two principal mechanisms of defense against invading microbes. The first relies on reactive oxygen intermediates generated in association with particle ingestion (3, 45); these include superoxide, H_2O_2, and the hydroxyl radical (43–45). Polymorphonuclear leukocytes and circulating monocytes also contain an enzyme, myeloperoxidase, that can amplify the killing activity of H_2O_2 by catalyzing formation of hypohalous acids (25, 30).

Phagocytes also possess oxygen-independent microbicidal mechanisms that equip them to destroy certain infectious agents. These mechanisms arise from diverse antimicrobial molecules that include acid hydrolases, neutral proteases, lysozyme, lactoferrin, and basic polypeptides such as bactericidal permeability-increasing protein (50 to 58 kDa), cathepsin G (25 to 28 kDa), and defensins (3.3 to 3.9 kDa) (15, 29, 30, 44, 57).

Defensins, which are arginine- and cystine-rich microbicidal peptides, are abundant in the cytoplasmic granules of rabbit alveolar macrophages and polymorphonuclear leukocytes (41, 55). Alveolar macrophages possess two defensins that were originally designated as MCP-1 and MCP-2 (41), whereas neutrophils contain six defensins that were designated NP-1, NP-2, NP-3a, NP-3b, NP-4, and NP-5 (51). Amino acid sequence analysis subsequently demonstrated that MCP-1 and MCP-2 were identical to NP-1 and NP-2, respectively (51, 52). The defensins exert in vitro microbicidal activity against many gram-positive and -negative bacteria (31, 40, 41, 55), fungi (32, 47, 55), and some metazoan parasites (unpublished observations). In addition, they directly inactivate certain enveloped viruses (13, 28) and exert cytotoxic or antitumor activity (33, 34, 56).

Although *Treponema pallidum* subsp. *pallidum*, the etiologic agent of syphilis, has been shown to activate complement by both the classical and alternative pathways, it is relatively resistant to the lytic effects of the complement cascade (17). Both immobilization (38) and neutralization (7) of the organism by antibody and complement require 16 h of incubation, an unusually long period when compared with the kinetics of antibody-mediated complement lysis of other bacteria (19). Since defensins also have membrane active properties (24, 27), we examined these peptides for bactericidal activity against *T. pallidum* and looked for synergistic activities between defensins and complement.

(This work was done by L.A.B. in partial satisfaction of the requirements for the Ph.D. degree in Microbiology and Immunology at the University of California, Los Angeles. It was presented in part at the 88th Annual Meeting of the American Society for Microbiology, Miami Beach, Fla., 8 to 13 May 1988.)

MATERIALS AND METHODS

Peptides. Rabbit defensins were purified to homogeneity from alveolar macrophages or peritoneal exudate granulocytes as previously described (41, 51, 54). The purity of all defensin preparations was verified by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and acid-urea-PAGE and reversed-phase high-pressure liquid chromatography, defensins were quantitated by amino acid anal-

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ysis as described elsewhere (53). Before use in bioassays, defensins in the desired quantities were rendered sterile by vacuum centrifugation in the presence of acetonitrile. Inasmuch as macrophage-derived MCP-1 and MCP-2 are identical to neutrophil-derived NP-1 and NP-2, respectively (52, 55), we will refer to the macrophage- and granulocyte-derived defensins as NP-1 and NP-2 in this report.

Reduction and alkylation of NP-1. Lyophilized NP-1 was dissolved in reducing buffer containing 6.0 M guanidine hydrochloride, 0.1 M Tris-hydrochloride (pH 8.5), and 0.002 M EDTA. The solution was heated to 55°C for 30 min and then cooled to room temperature. Dithiothreitol was added to a concentration of 1.0 mg/ml, and the mixture was incubated for 4 h at 55°C under N_2. The solution was brought to room temperature, and either iodoacetic acid or iodoacetamide was added in a fourfold molar excess relative to dithiothreitol. After 20 min of incubation in the dark, carboxymethylated or carboxyamidomethylated NP-1 was purified by reversed-phase high-pressure liquid chromatography. The purity of the carboxyamidomethylated NP-1 and carboxymethylated NP-1 was assessed, and their concentrations were determined as described above for nonderivatized defensins.

Gel electrophoresis. SDS-polyacrylamide gradient slab gels of 10 to 30% with 0.45% cross-linking were used with the discontinuous buffer system of Laemmli (26). In preparation for electrophoresis, samples were diluted 1:1 in final sample buffer (62.5 mM Tris-hydrochloride [pH 8.8], 4% SDS, 50 mM dithiothreitol, 20% glycerol, 0.001% bromophenol blue) and boiled for 10 min. Electrophoresis was performed at 125 mV until the dye front entered the separating gel, at which time the voltage was increased to 300 mV until the dye front reached the base of the gel. Proteins were identified by using a Formalin-Coomassie blue stain as previously described (11). Prestained high- and low-molecular-weight markers (Bethesda Research Laboratories) were used to estimate the molecular masses of identified proteins.

Rabbits. Adult male New Zealand White rabbits with nonreactive Venereal Disease Research Laboratory (59) and *T. pallidum* immobilization (16, 59) tests were used for the virulence neutralization assays. Breeding stock of an autosomal recessive complement factor 6-deficient strain of rabbit was generously provided by William T. Watson of the National Institutes of Health Small Animal Section (46). All rabbits were housed individually, maintained at 18 to 20°C, and given antibiotic-free food and water.

Source of treponemes. The Nichols strain of *T. pallidum* subsp. *pallidum*, herein referred to as *T. pallidum*, was maintained by intratesticular passage in normal rabbits without the use of cortisone acetate as described by Miller et al. (36).

Sera. Male or female rabbits that were nonreactive in the Venereal Disease Research Laboratory test were used as a source of normal rabbit serum (NRS). Sera from both normal and C6-deficient (C6D) rabbits were used within 48 h of blood collection and processing. Sera were used in the antimicrobial assays unheated or after heat inactivation (56°C, 30 min). Serum complement activity was determined by a microtiter plate hemolysis assay with 1% opsonized sheep erythrocytes as the indicator (60). Hemolytic complement titers for the NRS pools ranged between 1/8 and 1/32, whereas pools of heat-inactivated NRS (HI-NRS) and C6D (HI-C6D) sera had no demonstrable titer; undiluted C6D serum showed trace hemolysis.

Antitreponemal assays. Defensins were tested for antitreponemal activity in the presence of various concentrations of NRS and HI-NRS in an in vitro immobilization assay. The treponemes were harvested in either NRS or HI-NRS, centrifuged at low speed to remove tissue debris, and adjusted with the same medium to a concentration of 5×10^7 treponemes per ml. One part of the appropriate treponemal suspension was combined with nine parts of phosphate-buffered saline (PBS), NRS, HI-NRS, or serum-PBS mixtures with or without defensin (400 μg/ml) such that the final serum concentrations ranged between 10 and 100%. Mixtures were incubated anaerobically at 34°C for 0, 4, 8, and 16 h. Aliquots of each mixture were then scored for percent motility by counting 25 random organisms with a dark-field microscope. Native and derivatized defensin preparations were also tested for treponemicidal activity by a modification of the in vitro-in vivo neutralization test of Bishop and Miller (7). Treponemes were extracted in HI-NRS from 9- to 11-day infected rabbit testes adjusted to a concentration of approximately 5.0×10^7 organisms per ml, and then serially diluted with the appropriate sera to a concentration of 1.0×10^5 treponemes per ml. One volume of treponemal suspension was combined with 9 volumes of defensin solution such that the final defensin concentrations ranged between 0.4 and 400 μg/ml. Lyophilized defensin preparations were suspended in either PBS or a serum-PBS solution such that the final serum concentration was 10 or 20%. Unless otherwise stated, test mixtures were incubated anaerobically for 4 h at 34°C. After incubation, 0.1 ml of each test suspension was inoculated intradermally into the clipped backs of five serologically nonreactive rabbits. Control suspensions were prepared as described above without the inclusion of defensins. All animals were observed daily for a minimum of 30 days for the appearance and development of lesions. Aspirates of representative lesions were examined by dark-field microscopy to confirm their treponemal etiology.

Statistical analysis. The mean incubation periods for intradermal lesions were compared by the two-tailed *t* test for the comparison of paired means (12). When lesions failed to develop at a fraction of the sites inoculated, each negative site was assigned a value equal to the greatest lesion incubation period in that data set for calculation of the *t* statistic; a *P* value of ≤ 0.05 was considered statistically significant.

RESULTS

Identification of NP-1 activity against *T. pallidum*. Although the antibacterial activity of defensins is generally best expressed in serum-free, low-ionic-strength buffers (21), *T. pallidum* requires serum for in vitro maintenance of virulence and dies rapidly under serum-free conditions. Therefore, in a preliminary experiment we determined whether relatively low (10%) serum concentrations would support the motility and virulence of *T. pallidum* and examined NP-1 for antitreponemal activity. Suspensions of treponemes (5×10^7/ml) were incubated with or without NP-1 (400 μg/ml) in PBS solutions containing 0, 10, 50, or 100% NRS or HI-NRS under anaerobic conditions. After 0, 4, 8, and 16 h of incubation, each suspension was examined by dark-field microscopy. Treponemal motility exceeded 90% throughout the 16-h incubation in suspensions containing 50 and 100% serum, with and without NP-1; no differences were observed between the NRS or HI-NRS preparations (data not shown). In contrast, after 16 h in 10% NRS and 10% HI-NRS devoid of NP-1, treponemal motility was reduced to 46 and 76%, respectively; the motility of *T. pallidum* at 0, 4, and 8 h in both, however, exceeded 70% (Fig. 1).

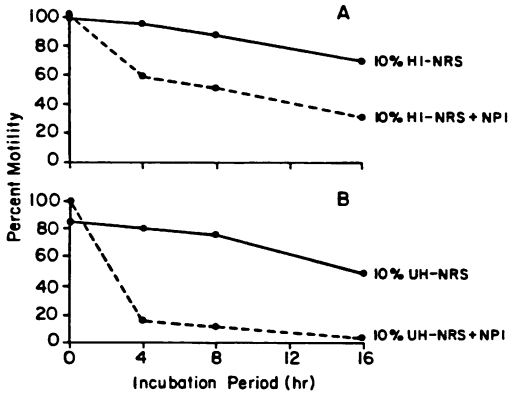

FIG. 1. Effect of 10% heated (A) and unheated (B) normal rabbit serum on *T. pallidum* motility in the presence and absence of NP-1. These results are from a representative experiment based on single tube motility determinations.

Motility after 4 and 8 h in the 10% NRS–NP-1 and 10% HI-NRS–NP-1 suspensions was reduced to 16 and 60%, respectively (Fig. 1). To test for virulence, we injected 0.1 ml of each of the above suspensions at their 16-h time points into the clipped backs of two rabbits. Absence of lesion development and delays in the incubation periods correlated well with the observed loss of motility. No lesions developed at the two sites inoculated with *T. pallidum* suspensions containing NP-1 in 10% NRS, and only one of two sites receiving organisms incubated with NP-1 in 10% HI-NRS developed a lesion, which appeared 5 days after that in the appropriate control (data not shown).

Kinetics of *T. pallidum* neutralization by NP-1. In the above experiment, animals were infected with 5×10^6 treponemes per inoculation site. To increase the sensitivity of the infectivity assay, subsequent experiments characterizing defensin-mediated neutralization of *T. pallidum* were carried out with an infectivity assay in which the defensins were incubated with 10^4 treponemes per ml and then 10^3 organisms were inoculated intradermally. With this inoculum, characteristic lesions usually develop after 11 to 14 days. Further, each 4-day delay in the lesion incubation period reflects 1 log unit of killing (8).

The kinetics of NP-1-mediated antitreponemal activity were examined by injecting *T. pallidum* suspensions containing 400 μg of NP-1 in either 10% HI-NRS or NRS after 0, 4, and 16 h of anaerobic incubation. The neutralization of *T. pallidum* by NP-1 was rapid and complete when the suspending medium was 10% NRS (Table 1). Lesions failed to develop at each of the six sites inoculated after 0, 4, and 16 h of incubation; in contrast, each of the six control sites developed lesions within the expected time frame (mean, 12.5 days). By comparison, NP-1-mediated neutralizing activity in 10% HI-NRS suspensions was slower and considerably less dramatic. Appreciable antitreponemal activity was not observed until after 4 h of in vitro incubation; lesions developed at each of the six inoculation sites in an average of 19.3 days compared with a mean of 12.2 days for the 10% HI-NRS control ($P < 0.001$, Table 1). Slightly greater activity was observed at 16 h; lesions developed at five of six sites with a mean incubation period of 20.0 days compared with a mean of 14.3 days for the six control sites ($P < 0.001$). After 16 h of incubation in the absence of NP-1, lesion development at sites receiving *T. pallidum* in 10% NRS was delayed significantly ($P < 0.001$) compared with that in the 10% HI-NRS control. Inasmuch as this difference was not observed at the 4-h time point, an in vitro incubation period of 4 h was adopted for subsequent neutralization assays.

Dose titration of NP-1 neutralizing activity. To determine the minimal neutralizing concentration of NP-1 and further examine the augmenting effect of heat-labile serum factor(s), NP-1 was tested as described above at peptide concentrations of 4, 40, 200, and 400 μg/ml. NP-1-mediated antitreponemal activity was demonstrable at 4 μg/ml in the presence of 10% NRS and at 40 μg/ml in 10% HI-NRS as indicated by significant delays in lesion incubation periods (Table 2). Abrogation of lesion development in 10% HI-NRS was observed only at the highest peptide concentration (400 μg/ml), whereas 40% (two of five) of the lesions failed to develop at sites receiving 40-μg/ml NP-1 in NRS (Table 2). These endpoint titration data confirmed the differential treponemicidal activity of NP-1 in 10% HI-NRS and 10% NRS.

The ability of NP-1 to neutralize *T. pallidum* was critically dependent on serum concentration. Table 3 compares the results obtained in 10% NRS and 10% HI-NRS with those obtained in 20% NRS and 20% HI-NRS. Note that lesions developed at all 12 sites inoculated with suspensions con-

TABLE 1. Kinetics of *T. pallidum* neutralization by NP-1[a,b]

In vitro incubation time (h)	NP-1 (μg/ml)	10% HI-NRS		10% NRS	
		Lesions/sites inoculated[c]	Days to lesion appearance,[d] mean ± SD (range)	Lesions/sites inoculated[c]	Days to lesion appearance,[d] mean ± SD (range)
0	400	6/6	14.8 ± 3.3 (12–21)	0/6[e]	
4	400	6/6	19.3[f] ± 2.1 (17–21)	0/6[e]	
16	400	5/6	20.0[f] ± 3.7 (16–24)	0/6[e]	
0	0	6/6	12.0 ± 1.3 (11–14)	6/6	12.5 ± 0.8 (12–14)
4	0	6/6	12.2 ± 1.2 (11–14)	6/6	13.0 ± 0.6 (12–14)
16	0	6/6	14.3 ± 1.4 (13–16)	4/6	17.5 ± 2.7 (15–21)

[a] *T. pallidum* suspensions were prepared by extraction of a 9- to 11-day orchitis with 100% HI-NRS or NRS and diluted to 10^5 treponemes per ml with the appropriate serum.

[b] Incubation mixtures were prepared by combining one part of *T. pallidum* suspension with nine parts of PBS–NP-1 solution (10^4 treponemes per ml) and incubated for 4 h at 34°C under an atmosphere of 95% N_2–5% CO_2 before animal inoculation.

[c] Each of six rabbits was inoculated intradermally with 0.1 ml of each test mixture containing 10^3 *T. pallidum* organisms.

[d] Lesion appearance was defined as the presence of erythema and induration.

[e] Lesions failed to develop through day 42, when all control sites had healed.

[f] $P \leq 0.05$, *t* test for comparison of paired means.

TABLE 2. Dose titration of *T. pallidum* neutralization by NP-1[a]

NP-1 (μg/ml)	10% HI-NRS		10% NRS	
	Lesions/sites inoculated[b]	Days to lesion appearance, mean ± SD (range)	Lesions/sites inoculated[b]	Days to lesion appearance, mean ± SD (range)
400	0/5[c]		0/5[c]	
200	5/5	19.8 ± 1.79 (17–21)	2/5	27.0[d] ± 1.40 (26–28)
40	5/5	17.0 ± 1.58 (15–19)	3/5	18.0[d] ± 2.65 (15–20)
4	5/5	15.6 ± 0.55 (15–16)	5/5	16.8[d] ± 0.84 (16–18)
0	5/5	14.4 ± 1.14 (13–16)	5/5	14.8 ± 1.30 (13–16)

[a] See footnotes *a*, *b*, and *d* of Table 1.
[b] Each of five rabbits was inoculated intradermally with 0.1 ml of each test mixture containing 10^3 *T. pallidum* organisms.
[c] Lesions failed to develop through day 33, when all control sites had ulcerated.
[d] $P \leq 0.05$, relative to defensin-free control; *t* test for comparison of paired means.

taining NP-1 in 20% NRS or 20% HI-NRS and that the lesion incubation periods were similar. The basis of the apparent serum-mediated inhibition of NP-1 activity was suggested when we observed that a flocculent precipitate formed in suspensions containing NP-1 and was most prominent in 20% serum.

To ascertain the nature of this precipitate, we combined NP-1 with 20% NRS or a *T. pallidum* suspension in 20% NRS as with the neutralization assay, recovered and washed the precipitate, and analyzed it by SDS-PAGE (Fig. 2). The key findings in this gel are the presence of NP-1 in the initial reaction mixture (lane 3) and the washed precipitate (lane 5) and its absence in the supernatant after centrifugation (lane 4). Since equivalent amounts of supernatant and initial reaction mixture were loaded, virtually all of the NP-1 had precipitated out of solution. In addition to NP-1, the precipitate contained approximately 20 serum polypeptide bands whose estimated molecular masses ranged between 32 and 248 kDa (lane 5). Although the most prominent polypeptide comigrated with serum albumin at 68 kDa (lanes 1 and 7), additional polypeptides with bands at 248, 137, and 120 kDa and a doublet at 53.5 kDa were also precipitated preferentially. Similar results were obtained with the *T. pallidum* suspensions (lanes 8 through 11). The faint NP-1 band seen in the supernatant (lane 9) may reflect peptide bound to *T. pallidum* rather than serum proteins.

Role of complement. The contribution of complement to the enhancement of defensin-mediated antitreponemal activity by heat-labile serum factor(s) was examined by comparing NP-1 neutralizing activity in unheated and heat-inactivated 10% NRS and 10% C6D. NP-1 was tested at 200 μg/ml, a concentration expected to inhibit lesion development at 50% of the inoculated sites, when in the presence of 10% NRS. The NRS and C6D used were matched for total protein and albumin as determined by biuret assay and quantitative densitometry of electrophoresed agarose gels of each serum (Paragon system; Beckman).

The results of three such experiments are presented in Table 4. Although the level of NP-1-mediated neutralizing activity varied from experiment to experiment, the greatest level of NP-1 activity was present in suspensions containing 10% NRS, followed by the 10% C6D suspensions and then by the 10% HI-NRS suspensions. Antitreponemal activity by NP-1 in the presence of 10% HI-C6D was demonstrable only in experiment 2, in which there were unexpectedly high levels of NP-1 activity in both the unheated NRS- and C6D-containing suspensions. Although NP-1-mediated neutralizing activity was observed in the absence of C6, it was less potent than that observed in serum with an intact complement cascade. This suggests that, although full activation of the complement pathway in vitro is not required for expression of enhanced NP-1-mediated antitreponemal activity, it contributes substantially to the augmentation phenomenon.

Contribution of native structure and charge. To determine the relative contribution of cationicity and native conformation to its antitreponemal activity, native NP-1 was reduced with dithiothreitol and alkylated with either iodoacetic acid or iodoacetamide. Although both treatments unfold NP-1 by cleaving its disulfide bonds and preventing their reformation, carboxymethylation of NP-1 with iodoacetic acid reduced its net positive charge by 1 for every sulfhydryl alkylated (total of six), whereas the net charge of the native molecule was maintained in the carboxyamidomethyl derivative formed with iodoacetamide. Minimal treponemicidal activity was detected with preparations of either carboxymethylated or carboxyamidomethylated NP-1 (Table 5), indicating that cationicity alone was insufficient to account for the antitreponemal activity of NP-1. The role of positive charge was further explored by comparing the treponemicidal activities

TABLE 3. Serum inhibition of *T. pallidum* neutralization by NP-1[a,b]

Serum concn (%)	HI-NRS		NRS	
	Lesions/sites inoculated	Days to lesion appearance, mean ± SD (range)	Lesions/sites inoculated	Days to lesion appearance, mean ± SD (range)
10	6/6	19.3[c] ± 2.1 (17–21)	0/6[d]	
20	6/6	12.2 ± 0.8 (11–13)	6/6	14.8 ± 3.1 (13–20)

[a] See footnotes *a*, *c*, and *d* of Table 1.
[b] Incubation mixtures were prepared by combining one part of *T. pallidum* suspension with nine parts of a solution containing NP-1 in either PBS or 11% NRS–PBS (10^4 treponemes per ml, 400 μg/ml) and incubated for 4 h at 34°C under an atmosphere of 95% N_2–5% CO_2 before animal inoculation.
[c] $P \leq 0.05$, *t* test for comparison of paired means.
[d] Lesions failed to develop through day 42, when all control sites had healed.

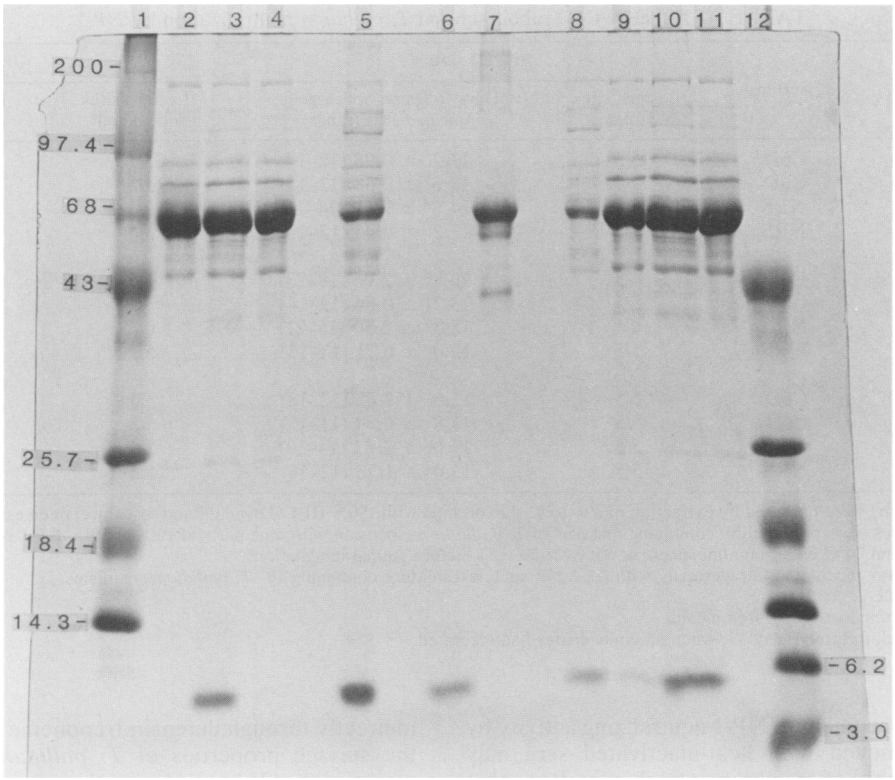

FIG. 2. Coomassie blue-stained 10 to 30% polyacrylamide gradient SDS-PAGE demonstrating precipitation of serum proteins by NP-1 (400 μg/ml) from 20% NRS (lanes 2 through 5) or a treponemal suspension containing 10^4 *T. pallidum* in 20% NRS (lanes 8 through 11): 2.0 μl of 20% NRS (lane 2) or treponemal suspension (lane 11); 2.0 μl of mixture containing NP-1 and 20% NRS (lane 3) or treponemal suspension (lane 10); 2.0 μl of supernatant after centrifugation of NP-1 mixtures containing 20% NRS (lane 4) or treponemal suspension (lane 9); 20% of precipitate formed by interaction of NP-1 with 20% unheated NRS (lane 5) or treponemal suspension (lane 8). Size standards include high (lane 1)- and low (lane 12)-molecular-mass markers, 1 μg of purified NP-1 (lane 6), and 8 μg of threefold-crystallized rabbit serum albumin (lane 7).

of all six rabbit polymorphonuclear leukocyte defensins, whose net charges range from +9 for NP-1 to +4 for NP-5 (Table 6). Each of the six defensins was tested at a concentration of 100 or 400 μg/ml in the presence of 10% NRS. Although all of the NP molecules had demonstrable neutralizing activity when tested at 400 μg/ml, NP-3a and NP-3b (net charge, +8) did not completely inhibit lesion development (Table 6). Moreover, at 100 μg/ml the activity exhibited by NP-4 (net charge, +5) was approximately 1.5 log units greater than that obtained with the more cationic peptides, NP-1 and NP-2, as indicated by the mean lesion incubation periods; NP-3a, NP-3b, and NP-5 had no detectable activity at this concentration. The above findings confirm that the *T. pallidum*-neutralizing activity of the defensins is not solely dependent on their net cationicity or charge density.

DISCUSSION

The macrophage has been implicated as the central effector cell in the destruction and clearance of *T. pallidum* from human and rabbit syphilitic lesions (36, 48, 49); however, the mechanisms by which *T. pallidum* may be destroyed by these phagocytes has not been explored. In this study we examined the rabbit defensins for antitreponemal activity. Our initial studies focused on NP-1, a defensin originally isolated from rabbit lung macrophages and shown to be the most active of the rabbit defensins in other microbicidal assays (41, 51).

The ability of NP-1 to immobilize *T. pallidum* in vitro was the first indication of its antitreponemal potential. This activity was remarkable in two respects. First, immobilization was observed within 4 h of coincubation, which is in marked contrast to the 16 h required for *T. pallidum* immobilization by specific antibody and complement (38). Second, the extent of NP-1-mediated immobilization was markedly increased by the presence of heat-labile serum components. When NP-1 was tested in the more sensitive infectivity assay, it exerted potent rapid virulence-neutralizing activity that was both time and dose dependent. As in the immobilization assay, maximal activity required heat-labile serum component(s) that augmented the level of NP-1-mediated treponemicidal activity and accelerated its kinetics (Table 1). The augmentation of NP-1 antitreponemal activity by heat-labile serum factor(s) was confirmed by endpoint titration (Table 2). NP-1 exhibited definitive antitreponemal activity (ca. 0.5 log unit) at concentrations of 4 μg/ml in the presence of 10% NRS and 40 μg/ml in 10% HI-NRS.

The conditions used for serum inactivation (56°C for 30 min) characteristically remove antibody-dependent and antibody-independent hemolytic activity of complement (14), suggesting that complement activation is responsible for augmented defensin antitreponemal activity. The experiments with C6D sera further support this contention (Table 4). Although *T. pallidum* neutralization by NP-1 in 10% C6D was greater than that achieved in either 10% HI-C6D or 10% HI-NRS, maximal activity was observed in the presence of

TABLE 4. Effect of C6D rabbit sera on *T. pallidum* neutralization by NP-1[a]

Expt no.	NP-1 (μg/ml)	Serum	10% HI serum		10% serum	
			Lesions/sites inoculated[b]	Days to lesion appearance,[c] mean ± SD (range)	Lesions/sites inoculated[b]	Days to lesion appearance,[c] mean ± SD (range)
1	200	C6D	5/5	13.2 ± 0.98 (12–14)	5/5	15.6[d] ± 0.90 (15–17)
	0	C6D	5/5	12.6 ± 0.55 (12–14)	5/5	12.6 ± 0.89 (12–14)
	200	NRS	5/5	14.6[d] ± 0.89 (14–16)	2/5	21.5[d] ± 0.71 (21–22)
	0	NRS	5/5	12.2 ± 0.45 (12–13)	5/5	12.6 ± 0.55 (12–13)
2	200	C6D	4/5	20.8[d] ± 2.06 (18–23)	1/5	23.0[d]
	0	C6D	5/5	13.2 ± 0.84 (12–14)	5/5	13.0 ± 1.00 (12–14)
	200	NRS	4/5	18.3[d] ± 2.50 (15–21)	0/5[e]	
	0	NRS	5/5	12.0 ± 0.71 (11–13)	5/5	12.4 ± 0.55 (12–13)
3	200	C6D	5/5	13.6 ± 2.88 (12–18)	5/5	16.2[d] ± 2.59 (14–19)
	0	C6D	5/5	11.8 ± 0.84 (11–13)	5/5	11.8 ± 0.84 (11–13)
	200	NRS	5/5	17.0[d] ± 2.83 (14–20)	3/5	19.0[d] ± 3.61 (15–22)
	0	NRS	5/5	13.0 ± 1.73 (12–16)	5/5	13.0 ± 1.73 (12–16)

[a] *T. pallidum* suspensions were prepared by extraction of a 9- to 11-day orchitis with 100% HI-C6D and diluted to 10^5 treponemes per ml with the appropriate serum. Incubation mixtures were prepared by combining one part of *T. pallidum* suspension with nine parts of PBS or PBS–NP-1 solution (10^4 treponemes per ml) and incubated for 4 h at 34°C under an atmosphere of 95% N_2–5% CO_2 before animal inoculation.
[b] Each of five rabbits was inoculated intradermally with 0.1 ml of each test mixture containing 10^3 *T. pallidum* organisms.
[c] See footnote *d* of Table 1.
[d] $P \leq 0.05$, *t* test for comparison of paired means.
[e] Lesions failed to develop through day 33, when all control sites had ulcerated.

10% NRS. The enhancement of NP-1 neutralizing activity by C6D over that achieved with heat-inactivated sera may reflect in vivo assembly of membrane attack complexes that use functional C6 provided by the inoculated animal. Other explanations for the limited enhancement of NP-1 antitreponemal activity by C6D, such as in vitro generation of complement opsonins, cannot be excluded.

The mechanisms by which heat-labile serum component(s), presumably complement, enhance defensin-mediated treponemicidal activity are unclear. Binding of defensins to *T. pallidum* may activate complement directly. In this regard, modulation of complement activity by synthetic and naturally occurring polycations was reported by Baker et al. (5, 6), who demonstrated that cationic polypeptides obtained from acid extracts of rabbit leukocyte lysosomal granules potentiate C567-initiated lysis of sheep erythrocytes (6). Although the macrophage and neutrophil defensins used in this study were isolated and purified in a manner similar to that of Baker et al., their exact relationship to the polypeptide(s) involved in potentiation of sheep erythrocyte lysis is not known. The complement cascade may be activated indirectly through defensin-treponeme interactions that alter the surface properties of *T. pallidum*. Baseman and coworkers (2, 42) have shown that serum proteins, including albumin, fibronectin, immunoglobulin, and C3, bind to *T. pallidum*. Defensin binding may alter the association of host material (18) with the surface of the organism, exposing moieties that potentiate complement activation (17). Since it has been shown that immunoglobulin is associated with freshly extracted *T. pallidum* (1, 23, 35, 38), defensin binding may promote complement activation by aggregating prebound, host-derived specific immunoglobulin (9). This hypothesis is particularly attractive in light of the recent report by Blanco et al. that the rate-limiting step in antibody-mediated complement-dependent killing of *T. pallidum* is the time required for aggregation of antibody-bound rare outer membrane proteins and consequent complement activation (9). Our demonstration that NP-1 precipitates as many as 20 serum proteins (Fig. 2) provides a potential mechanism for outer surface pertubation or immunoglobulin aggregation. The above interpretations presuppose that the augmentation of NP-1-mediated treponemicidal activity in unheated serum

TABLE 5. Effect of reduction and alkylation of NP-1 on *T. pallidum* neutralization[a]

NP-1 prepn[b]	10% HI-NRS		10% NRS	
	Lesions/sites inoculated[c]	Days to lesion appearance,[d] mean ± SD (range)	Lesions/sites inoculated[c]	Days to lesion appearance,[d] mean ± SD (range)
NP-1	0/5[e]		0/5[e]	
CAM NP-1	5/5	12.6 ± 0.89 (12–14)	5/5	13.8[f] ± 1.92 (11–16)
CM NP-1	5/5	13.0 ± 1.01 (12–14)	5/5	14.6[f] ± 1.52 (13–16)
Control (no NP-1)	5/5	11.8 ± 1.64 (10–14)	5/5	11.8 ± 0.84 (11–13)

[a] *T. pallidum* suspensions were prepared by extraction of a 9- to 11-day orchitis with 100% HI-NRS or NRS and diluted to 10^5 treponemes per ml with the appropriate serum. Incubation mixtures were prepared by combining one part of *T. pallidum* suspension with nine parts of PBS–NP-1 solution (10^4 treponemes per ml) and incubated for 4 h at 34°C under an atmosphere of 95% N_2–5% CO_2 before animal inoculation.
[b] Carboxyamidomethylated (CAM) and carboxymethylated (CM) NP-1 preparations were tested at a concentration of 400 μg/ml.
[c] Each of five rabbits was inoculated intradermally with 0.1 ml of each test mixture containing 10^3 *T. pallidum* organisms.
[d] See footnote *d* of Table 1.
[e] Lesions failed to develop through day 33, when all control sites had ulcerated.
[f] $P \leq 0.05$, *t* test for comparison of paired means.

TABLE 6. Comparison of *T. pallidum* neutralizing activity of rabbit defensins[a]

Defensin	Net charge	100-μg/ml defensin		400-μg/ml defensin	
		Lesions/sites inoculated[b]	Days to lesion appearance,[c] mean ± SD (range)	Lesions/sites inoculated[b]	Days to lesion appearance,[c] mean ± SD (range)
NP1	+9	5/5	16.8[d] ± 2.95 (12–19)	0/5[e]	
NP2	+8	5/5	15.8[d] ± 2.77 (12–19)	0/5[e]	
NP3a	+8	5/5	13.8 ± 1.79 (12–16)	2/5	24.5 ± 0.71 (24–25)
NP3b	+8	5/5	14.2[d] ± 1.92 (12–17)	1/5	15.0
NP4	+5	4/5	22.0[d] ± 1.16 (21–23)	0/5[e]	
NP5	+4	5/5	14.0[d] ± 2.00 (12–16)	0/5[e]	
Control (no NP)		5/5	12.4 ± 0.89 (12–14)		

[a] *T. pallidum* suspensions were prepared by extraction of a 9- to 11-day orchitis with 100% NRS and diluted to 10^5 treponemes per ml with the same serum. Incubation mixtures were prepared by combining 1 part of *T. pallidum* suspension with nine parts of PBS-defensin solution (10^4 treponemes per ml) and incubated for 4 h at 34°C under an atmosphere of 95% N_2–5% CO_2 before animal inoculation.

[b] Each of five rabbits was inoculated intradermally with 0.1 ml of each test mixture containing 10^3 *T. pallidum* organisms.

[c] See footnote *d* of Table 1.

[d] $P \leq 0.05$, *t* test for comparison of paired means.

[e] Lesions failed to develop through day 45, when all control sites had healed.

results from an additive or synergistic effect between lethal actions of defensin and complement lysis. The recent studies indicating that the lethal event in human defensin bactericidal activity against *Escherichia coli* is inner membrane permeabilization (27) raise the possibility of an alternate interpretation. In the presence of unheated serum, sublethal activation of complement by *T. pallidum* via either the alternative or the classical pathway (17) results in outer membrane breeches that facilitate access of defensin to the bacterial inner membrane, the site of lethality. More refined analysis is required to delineate between these possibilities.

Our observation that carboxymethylated and carboxyamidomethylated NP-1 retain minimal antitreponemal activity is consistent with previous reports on antifungal (16) and antiviral (13, 28) activities of both rabbit and human defensins and further illustrates the importance of three-dimensional conformation to biological action. Structural studies with crystallographic techniques (58) and two-dimensional nuclear magnetic resonance (4, 39) on representative human and rabbit defensins in solution have revealed an amphiphilic three-dimensional structure consisting of spatially separated hydrophobic and charged regions. Reduction and alkylation of NP-1 alter this amphiphilic structure and likely alter its membrane active properties. It is interesting that carboxymethylated NP-1, in contrast to native NP-1, does not induce voltage-dependent ion channels in black lipid membranes (24).

Among the rabbit defensins, the more basic peptides NP-1 and NP-2 in general possessed the greatest bactericidal activity against multiple strains of gram-positive and -negative organisms (41, 55). The least cationic of the rabbit defensins, NP-4 and NP-5, had the narrowest spectrum of antibacterial activity and were usually of lesser potency (55). In contrast, we found that at limiting peptide concentrations NP-4 (net charge, +5) was the most effective rabbit defensin against *T. pallidum*, followed closely by both NP-1 (+9) and NP-2 (+8). Neither NP-5 (+4) nor NP-3A (+8) possessed activity, suggesting that structural features other than net charge were critical determinants of defensin action against *T. pallidum*.

Our observation that serum concentrations in excess of 20% inhibited defensin antitreponemal activity (Table 3) was consistent with previous findings that both herpes simplex virus type 1 inactivation by human neutrophil defensins and tumor cell cytolysis by rabbit and human defensins were impaired by the addition of either albumin or serum (13, 34). In light of this inhibition, the antitreponemal effects of defensins are likely limited to the intracellular environment of the leukocyte phagolysosome, which may contain concentrations of peptide in excess of 10 mg/ml (13) or, after secretion, to the adjacent interstitial spaces (20), where fluid protein and albumin concentrations approximate our in vitro conditions. The reported total protein and albumin contents of interstitial fluid are approximately 20 and 1% of those of blood plasma, respectively (10, 22), which is at the threshold of inhibitory concentrations of NP-1 treponemicidal activity in serum. Since defensins have activity against mammalian cells and since polymorphonuclear leukocytes turn over rapidly, it is reasonable that their extracellular activity is tightly regulated to minimize tissue destruction.

Although much remains to be learned about the molecular effectors of antitreponemal activity in macrophages and granulocytes, the experiments presented here suggest that defensins or defensinlike leukocyte components may contribute to the ability of phagocytes to contain or terminate infections by *T. pallidum*.

ACKNOWLEDGMENTS

We thank Sylvia Harwig for expert technical assistance in the purification and quantitation of native and derivatized defensins, David R. Blanco and Eldon M. Walker for helpful discussions and critical reading of the manuscript, and Laurie Han for typing the manuscript.

This work was supported by Public Health Service grants AI-22931 (M.E.S.), AI-22839 (R.I.L.), and AI-12601 (J.N.M.) from the National Institutes of Health, by Office of Naval Research grant N0001490-1310 (M.E.S.), and by World Health Organization agreement V3-181-26 (J.N.M.).

REFERENCES

1. **Alder, J. D., L. Friess, M. Tengowski, and R. F. Schell.** 1990. Phagocytosis of opsonized *Treponema pallidum* subsp. *pallidum* proceeds slowly. Infect. Immun. **58**:1167–1173.
2. **Alderete, J. F., and J. B. Baseman.** 1979. Surface-associated host proteins on virulent *Treponema pallidum*. Infect. Immun. **26**:1048–1056.
3. **Babior, B. M.** 1978. Oxygen-dependent microbial killing by phagocytes. N. Engl. J. Med. **298**:659–668.
4. **Bach, A. C., II, M. E. Selsted, and A. Pardi.** 1987. Two-dimensional NMR studies of the antimicrobial peptide NP-5. Biochemistry **26**:4389–4397.

5. **Baker, P. J., T. F. Lint, B. C. Mcleod, C. L. Behrends, and H. Gewurz.** 1975. Studies on the inhibition of C56-induced lysis (reactive lysis). VI. Modulation of C56 induced lysis polyanions and polycations. J. Immunol. **114**:554–558.
6. **Baker, P. J., T. F. Lint, J. Siegel, M. W. Kies, and H. Gewurz.** 1976. Potentiation of C567-initiated lysis by leukocyte cationic proteins, myelin basic proteins, and lysine-rich histones. Immunology **30**:467–473.
7. **Bishop, N. H., and J. N. Miller.** 1976. Humoral immunity in experimental syphilis. II. The relationship of neutralizing factors in immune serum to acquired resistance. J. Immunol. **117**:197–207.
8. **Blanco, D. R., J. N. Miller, and P. A. Hanff.** 1984. Humoral immunity in experimental syphilis: the demonstration of IgG as a treponemicidal factor in immune rabbit serum. J. Immunol. **133**:2693–2697.
9. **Blanco, D. R., E. M. Walker, D. A. Haake, J. N. Miller, and M. A. Lovett.** 1990. Complement activation limits the rate of *in vitro* treponemicidal activity and correlates with antibody-mediated aggregation of *Treponema pallidum* rare outer membrane protein. J. Immunol. **144**:1914–1921.
10. **Blomback, B., and L. A. Hanson.** 1979. Plasma proteins. John Wiley & Sons, Inc., New York.
11. **Burk, R. R., M. Eschenbruch, P. Leuthard, and G. Steck.** 1983. Sensitive detection of proteins and peptides in polyacrylamide gels after formaldehyde fixation. Methods Enzymol. **91**:247–253.
12. **Colton, T.** 1974. Statistics in medicine. Little, Brown and Co., Boston, Mass.
13. **Daher, K. A., M. E. Selsted, and R. I. Lehrer.** 1986. Direct inactivation of viruses by human granulocyte defensins. J. Virol. **60**:1068–1074.
14. **Donaldson, D. M., and J. G. Tew.** 1966. Differentiation of muramidase and β-lysin. Proc. Soc. Exp. Biol. Med. **122**:46–49.
15. **Elsbach, P., and J. Weiss.** 1983. A reevaluation of the roles of the O_2-dependent and O_2-independent microbicidal systems of phagocytes. Rev. Infect. Dis. **5**:843–853.
16. **Fehniger, T. E., A. M. Walfield, T. M. Cunningham, J. D. Radolf, J. N. Miller, and M. A. Lovett.** 1984. Purification and characterization of a cloned protease-resistant *Treponema pallidum*-specific antigen. Infect. Immun. **46**:598–607.
17. **Fitzgerald, T. J.** 1987. Activation of the classical and alternative pathways of compliment by *Treponema pallidum* subsp. *pallidum* and *Treponema vincentii*. Infect. Immun. **55**:2066–2073.
18. **Fitzgerald, T. J., and R. C. Johnson.** 1979. Surface mucopolysaccharide of *Treponema pallidum*. Infect. Immun. **24**:244–251.
19. **Frank, M. M.** 1985. Current concepts. A Scope Publication, The Upjohn Co., Kalamazoo, Mich.
20. **Ganz, T.** 1987. Extracellular release of antimicrobial defensins by human polymorphonuclear leukocytes. Infect. Immun. **55**: 568–571.
21. **Ganz, T., M. E. Selsted, D. Szklarek, S. S. L. Harwig, K. Daher, D. F. Bainton, and R. I. Lehrer.** 1985. Defensins: natural peptide antibiotics of human neutrophilis. J. Clin. Invest. **76**:1427–1435.
22. **Guyton, A. C.** 1976. Textbook of medical physiology. The W. B. Saunders Co., Philadelphia.
23. **Hardy, P. H., and E. E. Nell.** 1955. Specific agglutination of *Treponema pallidum* by sera from rabbits and human beings with treponemal infection. J. Exp. Med. **101**:367–382.
24. **Kagan, B. L., M. E. Selsted, T. Ganz, and R. I. Lehrer.** 1990. Antimicrobial defensin peptides form voltage-dependent ion permeable channels in planar lipid bilayer membranes. Proc. Natl. Acad. Sci. USA **87**:210–214.
25. **Klebanoff, S. J.** 1980. Myeloperoxidase-mediated cytotoxic systems, p. 279–308. *In* A. J. Sbarra and R. Strauss (ed.), The reticuloendothelial system—a comprehensive treatise: biochemistry and metabolism. Plenum Publishing Corp., New York.
26. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
27. **Lehrer, R. I., A. Barton, K. A. Daher, S. S. L. Harwig, T. Ganz, and M. E. Selsted.** 1989. Interaction of human defensins with *Escherichia coli*. Mechanism of bactericidal activity. J. Clin. Invest. **84**:553–561.
28. **Lehrer, R. I., K. Daher, T. Ganz, and M. E. Selsted.** 1985. Direct inactivation of viruses by MCP-1 and MCP-2, natural antibiotics from rabbit leukocytes. J. Virol. **54**:467–471.
29. **Lehrer, R. I., T. Ganz, and M. E. Selsted.** 1988. Oxygen-independent bactericidal systems. Mechanisms and disorders. Hematol./Oncol. Clin. N. Am. **2**:159–169.
30. **Lehrer, R. I., T. Ganz, M. E. Selsted, B. M. Babior, and J. T. Curnutte.** 1988. Neutrophils and host defense. Ann. Intern. Med. **109**:127–141.
31. **Lehrer, R. I., M. E. Selsted, D. Szklarek, and J. Fleischman.** 1983. Antibacterial activity of microbicidal cationic proteins 1 and 2, natural peptide antibiotics of rabbit lung macrophages. Infect. Immun. **42**:10–14.
32. **Levitz, S. M., M. E. Selsted, T. Ganz, R. I. Lehrer, and R. D. Diamond.** 1986. In vitro killing of spores and hyphae of *Aspergillus fumigatus* and *Rhizopus oryzae* of rabbit neutrophil cationic peptides and bronchoalveolar macrophages. J. Infect. Dis. **154**:483–489.
33. **Lichtenstein, A., T. Ganz, T. M. Nguyen, M. E. Selsted, and R. I. Lehrer.** 1988. Mechanism of target cell cytolysis by peptide defensins: target cell metabolic activities, possibly involving endocytosis, are crucial for expression of cytotoxicity. J. Immunol. **140**:2686–2694.
34. **Lichtenstein, A., T. Ganz, M. E. Selsted, and R. I. Lehrer.** 1986. In vitro tumor cell cytolysis mediated by peptide defensins of human and rabbit granulocytes. 1986. Blood **68**:1407–1410.
35. **Logan, L.** 1974. Rabbit immunoglobulin and antiglobulin factors associated with *Treponema pallidum* grown in rabbits. Br. J. Vener. Dis. **56**:421–427.
36. **Lukehart, S. A., S. A. Baker-Zander, R. M. C. Lloyd, and S. Sell.** 1980. Characterization of lymphocyte responsiveness in early experimental syphilis. II. Nature and cellular infiltration and *Treponema pallidum* distribution in testicular lesions. J. Immunol. **124**:461–467.
37. **Miller, J. N., S. J. Whang, and F. P. Fazzan.** 1963. Studies on immunity in experimental syphilis. I. Immunologic response of rabbits immunized with Reiter protein antigen and challenged with virulent *Treponema pallidum*. Br. J. Vener. Dis. **39**:195–198.
38. **Nelson, R. A., and J. A. Diesendruck.** 1951. Studies on treponemal immobilizing antibodies in syphilis. I. Techniques of measurement and factors influencing immobilization. J. Immunol. **66**:667–685.
39. **Pardi, A., D. R. Hare, M. E. Selsted, R. D. Morrison, D. A. Bassolino, and A. C. Bach II.** 1988. Solution structures of the rabbit neutrophil defensin NP-5. J. Mol. Biol. **201**:625–636.
40. **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.
41. **Patterson-Delafield, J., D. Szklarek, R. J. Martinez, and R. I. Lehrer.** 1981. Microbicidal cationic proteins of rabbit alveolar macrophages: amino acid composition and functional attributes. Infect. Immun. **31**:723–731.
42. **Peterson, M., J. B. Baseman, and J. F. Alderete.** 1983. *Treponema pallidum* receptor binding proteins interact with fibronectin. J. Exp. Med. **157**:1958–1970.
43. **Roos, D., and A. J. M. Balm.** 1980. The oxidative metabolism of monocytes, p. 189–229. *In* A. J. Sbarra and R. Strauss (ed.), The reticuloendothelial system—a comprehensive treatise: biochemistry and metabolism. Plenum Publishing Corp., New York.
44. **Root, R. K., and M. S. Cohen.** 1981. The microbicidal mechanisms of human neutrophils and eosinophils. Rev. Infect. Dis. **3**:565–598.
45. **Rossi, F., P. Pierluigi, and D. Romeo.** 1980. Metabolic changes accompanying phagocytosis, p. 153–188. *In* A. J. Sbarra and R. Strauss (ed.), The reticuloendothelial system—a comprehensive treatise: biochemistry and metabolism. Plenum Publishing Corp., New York.
46. **Rother, K., U. Rother, J. J. Mueller-Eberhard, and U. R.**

Nilsson. 1966. Deficiency of the sixth component of complement in rabbits with an inherited complement defects. Exp. Med. **124**:773–785.

47. **Segal, G. P., R. I. Lehrer, and M. E. Selsted.** 1985. Effects of rabbit granulocyte cationic peptides on the growth of *C. immitis*: a preliminary report, p. 104–111. *In* H. E. Einstein and A. Catanzaro (ed.), Coccidiomycosis. National Foundation for Infectious Diseases, Washington, D.C.
48. **Sell, S., S. Baker-Zander, and H. C. Powell.** 1982. Experimental syphilitic orchitis in rabbits: ultrastructural appearance of *Treponema pallidum* during phagocytosis and dissolution by macrophages in vivo. Lab. Invest. **46**:355–364.
49. **Sell, S., D. Gamboa, S. A. Baker-Zander, S. A. Lukehart, and J. N. Miller.** 1980. Host response to *Treponema pallidum* in intradermally-infected rabbits: evidence for persistence at local and distant sites. J. Invest. Dermatol. **75**:470–474.
50. **Selsted, M. E., and H. W. Becker III.** 1986. Eosin Y: a reversible stain for detecting electrophoretically resolved protein. Anal. Biochem. **155**:270–274.
51. **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.
52. **Selsted, M. E., D. M. Brown, R. J. DeLange, and R. I. Lehrer.** 1983. Primary structures of MCP-1 and MCP-2, natural peptide and antibiotics of rabbit lung macrophages. J. Biol. Chem. **258**:15585–15589.
53. **Selsted, M. E., S. S. L. Harwig, T. Ganz, J. W. Schilling, and R. I. Lehrer.** 1985. Primary structure of three human neutrophil defensins. J. Clin. Invest. **76**:1436–1439.
54. **Selsted, M. E., D. Szklarek, T. Ganz, and R. I. Lehrer.** 1985. Activity of rabbit leukocyte peptides against *Candida albicans*. Infect. Immun. **49**:202–206.
55. **Selsted, M. E., D. Szklarek, and R. I. Lehrer.** 1984. Purification and antimicrobial activity of antimicrobial peptides of rabbit granulocytes. Infect. Immun. **45**:150–154.
56. **Sheu, M. J. T., W. W. Baldwin, and K. W. Brunson.** 1985. Cytotoxicity of rabbit macrophage peptides MCP-1 and MCP-2 for mouse tumor cells. 1985. Antimicrob. Agents Chemother. **28**:626–629.
57. **Spitznagel, J. K., and W. M. Shafer.** 1985. Neutrophil killing of bacteria by oxygen-independent mechanisms: a historical perspective. Rev. Infect. Dis. **7**:398–403.
58. **Stanfield, R. L., E. M. Westbrook, and M. E. Selsted.** 1988. Characterization of two crystal forms of human defensin netrophil cationic peptide 1, a naturally occurring antimicrobial peptide of leukocytes. J. Biol. Chem. **263**:5933–5935.
59. **US Department of Health, Education and Welfare.** 1964 and 1969. Manual of serologic tests for syphilis. Centers for Disease Control, Atlanta, Ga.
60. **Williams, C. A., and M. W. Chase.** 1967. Complement, p. 134–165. *In* C. A. Williams and M. W. Chase (ed.), Methods in immunology and immunochemistry, vol. 4. Academic Press, Inc., New York.