Available online at www.sciencedirect.com
SCIENCE DIRECT®
Peptides 24 (2003) 1741–1746
PEPTIDES
www.elsevier.com/locate/peptides

Susceptibility of *Treponema pallidum* to host-derived antimicrobial peptides

David L. Cox[a,*], Yongcheng Sun[a], Hsi Liu[a], Robert I. Lehrer[b], William M. Shafer[c,d]

[a] *Sexually Transmitted Infections Branch, Division of AIDS, STD and TB Laboratory Research, Center for HIV and STD Prevention, Centers for Disease Control and Prevention, Atlanta, GA 30333, USA*
[b] *Department of Medicine, UCLA School of Medicine, Los Angeles, CA 90095, USA*
[c] *Microbial Pathogenesis Laboratory, Research Service, Department of Veterans Affairs Medical Center, Decatur, GA 30033, USA*
[d] *Department of Microbiology and Immunology, Emory University School of Medicine, Atlanta, GA 30322, USA*

Received 1 April 2003; accepted 13 July 2003

Abstract

LL-37 displays potent broad-spectrum activity against a number of pathogenic bacteria and is the only cathelicidin thus far identified in humans. In this study, we examined the capacity of human LL-37 and the similar CAP-18-derived peptide from rabbits to exert antimicrobial activity against the causative agent of syphilis, *Treponema pallidum*. We found that both peptides, as well as a truncated version of human LL-37 that contains its bactericidal domain, could exert rapid, but salt-sensitive antimicrobial activity against *T. pallidum*. Infectivity of *T. pallidum* in a rabbit model could effectively be blocked with the synthetic truncated LL-37-derived peptide WS22-N-amide.

Keywords: Antibacterial peptide; Outer membrane; WS22; Tissue culture; Spirochete

1. Introduction

Recently, extensive efforts have been made to identify and characterize antibacterial peptides which contribute to host defenses and to ascertain their effectiveness against a variety of pathogens [1,10,18]. A number of antibacterial peptides have been identified from both vertebrate and invertebrate sources. The activities of these peptides against many Gram-negative and -positive organisms have been determined, but only three studies have tested the effects of antimicrobial peptides on spirochetes [2–4,16]. The first study demonstrated that the spirochete *Treponema pallidum* could be immobilized by high concentrations of the rabbit β-defensin, NP-1, when exposed for long periods of time (4–16 h) [2,3]. The second demonstrated *Treponema denticola*, an oral spirochete, was resistant to the bactericidal activities of human β-defensins HD-1 and HD-2 [4]. In the most recent report, the activities of five cathelicidins (including LL-37) against *Leptospira interrogans*, *Borrelia burgdorferi*, and *T. pallidum* were determined. Leptospires appeared to be more sensitive to cathelicidin action than did the other two spirochetes [16].

T. pallidum, the etiologic agent of syphilis, is an atypical bacterium that cannot be cultivated with typical laboratory media. However, we and others have successfully cultivated *T. pallidum* in the presence of rabbit skin (Sf1Ep) cells. In this model, cultures of *T. pallidum* can be grown for 17–21 days with >80% viability [5,11]. This spirochete has an outer membrane similar to that of Gram-negative organisms but lacks lipopolysaccharide. Furthermore, the cell envelope structure is not typical of Gram-negative bacteria and the cell wall structure is more related to Gram-positive bacteria. However, the *T. pallidum* lacks teichioic acid, a potential binding site for cationic antimicrobial peptides. Between the cell wall and the outer membrane is a periplasmic space that contains endoflagella for motility. Most importantly, the surface of this spirochete is almost devoid of intramembranous proteins [15,20], and is very lipid rich. This unusual cell architecture presents a unique set of parameters that the host immune system likely encounters during infection.

T. pallidum has a limited range of hosts. Humans are the only natural host, although rabbits can be used for propagation of the spirochete in the testis and in the skin. In fact, the "gold standard" laboratory test to determine treponemal viability is the rabbit infectivity test (RIT), in which serial dilutions of a suspension are injected intra-dermally into the backs of shaved rabbits [13]. Furthermore, in the

* Corresponding author. Tel.: +1-404-639-3446; fax: +1-404-639-3976.
E-mail address: dlc6@cdc.gov (D.L. Cox).

0196-9781/$ – see front matter © 2003 Published by Elsevier Inc.
doi:10.1016/j.peptides.2003.07.026

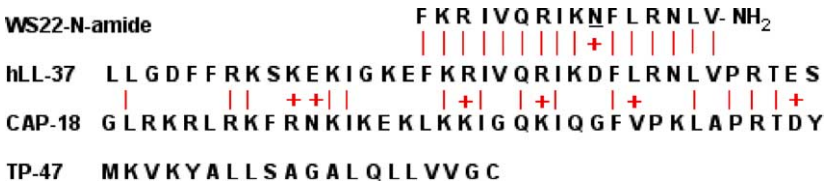

Fig. 1. Amino acid sequence of human LL-37, rabbit CAP-18, WS22-N-amide, and Tp-47. The bars between the sequences highlight residues that are identical; a "+" indicates a conservative substitution. The substitution of asparagine in WS22-N-amide is highlighted (underlined).

natural course of the disease, the primary route of infection of the syphilis spirochete is via dermal tissues, both keratinized and non-keratinized. Antimicrobial peptides, such as human LL-37 and rabbit CAP-18, have been demonstrated to be synthesized by epithelial and testicular tissues during infection [1–3]. With respect to LL-37, recent evidence implicated it as a major contributor to the innate host defense response against the Group A *Streptococcus* [14]. However, the role that LL-37 and other antibacterial peptides may play in the innate host defense during the initial and subsequent stages of syphilis and in its transmission are largely unknown. Therefore, we undertook this study to begin to answer some of these questions. We examined the sensitivity of *T. pallidum* to human and rabbit LL-37 as well as to the truncated peptide derivative, WS22-N-amide (Fig. 1), which represents the antimicrobial core of LL-37. The antimicrobial activity of these cathelicidins was further demonstrated by examining the viability of the treated suspensions of *T. pallidum* using the RIT.

2. Materials and methods

2.1. Spirochetes

T. pallidum subsp. *pallidum* (Nichols strain) was propagated in rabbit testes. Briefly, 10^8 treponemes were injected into each testis of 4 kg male New Zealand white rabbits. At the peak of orchitis (approximately 10 days later), the animals were sacrificed, and the testes excised. The testes were minced and placed in a 50 ml screw-cap flask with 10 ml of *T. pallidum* culture medium (TpCM) [5]. The flask was briefly gassed with 95% N_2/5% CO_2 and then placed on a reciprocal shaker for 20 min. The medium was then removed from the flask and placed in a 15 ml conical centrifuge tube. The gross tissue debris was removed by centrifugation at $500 \times g$ for 15 min. For the antimicrobial assays, spirochetes were diluted in TpCM containing 3 mg/dl dithiothreitol (DTT) and 2% (v/v) fetal bovine serum (FBS).

2.2. Antimicrobial peptides

The four peptides, human LL-37, rabbit CAP-18, WS22-N-amide, and Tp-47 (Fig. 1) were synthesized by solid phase chemistry and purified using reverse-phase high pressure liquid chromatography. Briefly, solid phase Fmoc methodology for synthesis of peptides was performed on 0.02 mM of Wang Resin reacted sequentially in an Advanced Chemtech 396 Peptide Synthesizer with the appropriately Fmoc amino acids using double coupling cycles (0.5 h each) with HOBT, DIC, and two cycles of deprotection (5 and 30 min) with 25% piperdine in DMF. The resulting peptido-resin was deprotected and then cleaved from the resin using TFA (90%), phenol (2%) triisopropylsilan (2%), thioanisol (2%), dithioethane (2%), and water (2%) at room temperature for 2.5 h. They were then precipitated in cold ether, centrifuged and washed four times with cold ether. The residues were dried in vacuo and the structures were confirmed by matrix-associated laser desorption time-of-flight mass spectroscopy (MALDITOF-MS). Tp-47 is a truncated peptide consisting of the first 20 amino acids of the 47 kDa lipoprotein (gene sequence Tp0574) of *T. pallidum*.

2.3. Bactericidal assay

To assess the activity of these three peptides, the appropriate amounts of peptide were added directly to the modified TpCM containing the spirochetes and 2% fetal bovine serum (FBS). Control samples containing no or an irrelevant peptide were simultaneously established. In low salt experiments, the TpCM was diluted 1:3 by adding two volumes of distilled water before adding 10^7 treponemes per ml. One-half milliliter portions of the cell suspensions were placed in 1.5 ml microfuge tubes and the appropriate amount of peptide was added. The tubes were gassed with 95% N_2/5% CO_2, and incubated at 35 °C for 2 h unless otherwise noted. The EC_{50} (Effective Concentration-50%) is the amount of antimicrobial peptide required to kill 50% of the spirochetes during the 2 h incubation.

2.4. Viability measurement

To ascertain the bactericidal effects of the peptides, three microliters of the Live/Dead BacLight™ (Molecular Probes, Eugene, OR) was added to each suspension and mixed by gentle mixing. Five to ten minutes later, 10 μl of each suspension were placed on a slide and observed by both dark-field and fluorescence microscopy (Nikon, Melville, NY). Viability was monitored by counting both the number of motile organisms, and the number of organisms exhibiting green (live) and red (dead) fluorescence. For each condition analyzed by microscopy, two or three

samples were scored; in each sample approximately 100 organisms were observed.

2.5. Flow cytometry

In addition to microscopy, samples were analyzed by flow cytometry using a Bruker ACS100 equipped with a mercury arc lamp. The wavelength of light used to excite the samples was 488 nm and the fluorescence signals were measured at 520 nm. The instrument was set to trigger on fluorescent particles rather than forward light scatter because of the small cell volume of the spirochetes. The flow rate was maintained at 500 events per second. Samples were spiked with a small quantity of 0.7 μM green beads (Duke Scientific, Palo Alto, CA). The signal from these beads permitted monitoring the alignment of the instrument and ensured quality control of the collected data. The fluorescence of approximately 25,000 spirochetes was recorded per determination and determinations were repeated three or four times per sample.

2.6. Rabbit infectivity tests (RIT)

The ability of treated organisms to establish infections was determined by the RIT [7]. Briefly, suspensions containing 10^7 *T. pallidum* per ml were incubated at 35 °C with either cathelicidins, a sham protein, or alone for 2 h. Organisms were diluted to 10^3, 10^2, 10^1 and 1 organism per ml. Mature (≥4 kg) New Zealand white rabbits were anesthetized with 50 mg of ketamine and 1 mg of acepromazine per kg body weight and 0.1 ml of the above dilutions was injected intra-dermally into their shaved backs. Ten sites were injected in each animal. The animals were housed in a room equipped with a refrigeration unit that maintained the air temperature between 62 and 65 °F. The animals were also re-shaved at least once a week and observed on a daily basis for 30 days for the production of a chancre at each site treated. A site was considered positive only if it produced an ulcerative chancre that contained motile spirochetes.

3. Results

3.1. Selection of anti-treponemal assay

Conventional methods for determining the bactericidal activity of peptides against typical bacteria are not applicable to studies with *T. pallidum* because this pathogen is not readily cultivated in vitro; it has very long generation time (>35 h); and methods for plating the organism on semi-solid media have not been developed. Therefore, we explored alternative methods.

Since these organisms possess endoflagella, motility is typically used to ascertain treponemal viability [12]. However, we also wanted to develop a method that would reflect the ability of the cathelicidins to compromise cell membrane integrity. Therefore, staining the spirochetes with a vital dye was another option chosen. When we compared the data from motility and fluorescence analysis of peptide-treated organisms, the values for the percentage of killed spirochetes were virtually identical (data not shown).

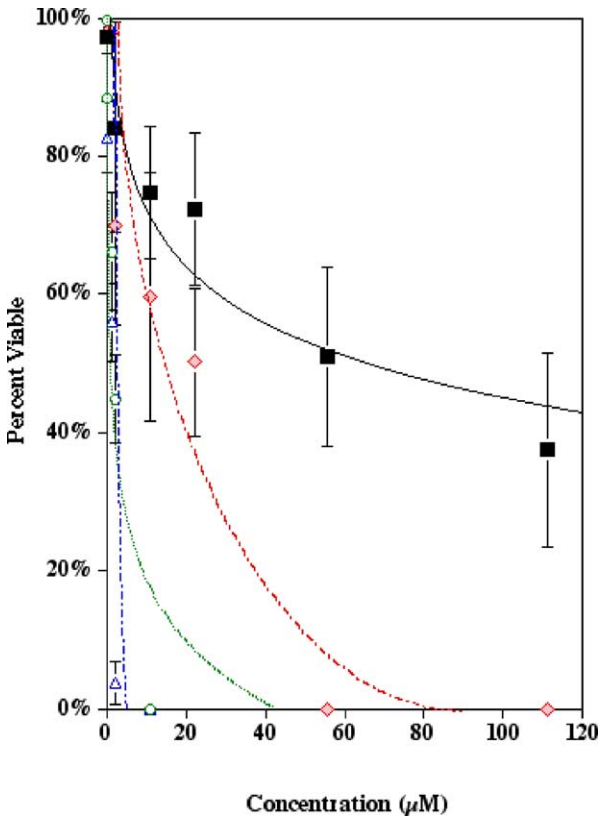

Fig. 2. Effects of human LL-37 and WS22-N-amide on *T. pallidum* viability. Suspensions containing 10^7 treponemes per ml were incubated at 35 °C for 2 h with various concentrations of LL-37 or WS22-N-amide in either TpCM (■, ○) or in TpCM diluted 1:3 with distilled water (◆, △ low salt). The samples were then scored for motility (viability) and fluorescence. Experiments were repeated three times.

3.2. Effects of cathelicidins on the viability of T. pallidum

In the first series of experiments, we varied the concentration of human LL-37, rabbit CAP-18, and WS22-N-amide during a 2 h incubation. The results measuring activity of human LL-37 against *T. pallidum* can be seen in Fig. 2. The EC_{50} of LL-37 was determined to be slightly above 66 μM (300 μg/ml) for human LL-37 in physiological salt concentrations and 11 μM (50 μg/ml) for the peptide under low salt conditions (Table 1). During these incubations, the viability of the control (no peptide) bacteria remained at or above 99%. We did not attempt to test concentrations of any peptide above 500 μg/ml, and therefore 100% of the spirochetes could not be killed with LL-37 in TpCM. However, after 2 h, there were no viable organisms in suspensions treated with 55 μM of the LL-37 under low salt conditions. This result was due to either enhanced activity of the peptide

Table 1
Effectiveness of antimicrobial peptides against *Treponema pallidum*

Cathelicidin	EC_{50}[a] normal (μM)	EC_{50} low salt (μM)
Human LL-37	66.4	11.1
WS22-N-amide	4.37	1.4
Rabbit CAP-18	89.3	ND

[a] The EC_{50} was calculated by plotting viability vs. concentration of each peptide and determining the point at which 50% of the organism were killed.

under these conditions, osmotic stress on the organisms, or a combination of both. The activity of the truncated peptide WS22-N-amide is also reported in Fig. 2. The EC_{50} for WS22-N-amide was approximately 4.4 μM in TpCM and 1.5 μM under low salt conditions. In both assays, we did observe 100% killing. The EC_{50} for CAP-18 was determined to be nearly 90 μM (Table 1). In this case as well, 100% killing was not achieved during the 2 h incubation period.

The activity of these peptides was both dose- and time-dependent. To examine the kinetics of these killing reactions, we selected several concentrations and performed time-course assays. Fig. 3 shows the results of four of these assays using two different concentrations each of LL-37 (22 and 44 μM) and WS22-N-amide (2.4 and 4.8 μM). The kinetics of the action of whole LL-37 were much slower than that for WS22-N-amide. Nevertheless, most of the activity of both occurred in the first 30 min. Similar results were seen at other concentrations (data not shown).

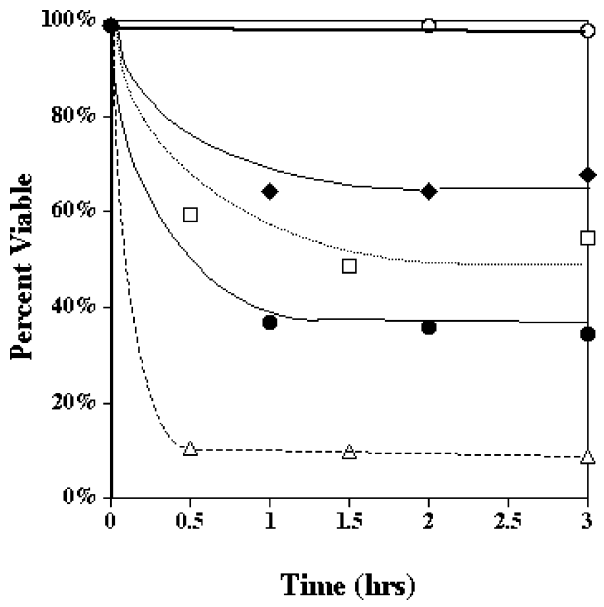

Fig. 3. Kinetics of human LL-37 and the WS22-N-amide peptide on *T. pallidum* viability. Suspensions containing 10^7 treponemes per ml were incubated at 35 °C for 2 h in TpCM with either 22.1 or 44.2 μM LL-37 (◆, □); with 2.4 or 4.8 μM WS22-N-amide peptide (●, △); or without peptide, control (○). At various times, samples were removed and then scored for fluorescence using flow cytometry.

Table 2
Rabbit infectivity[a] tests with *T. pallidum*

Organisms/site	hLL-37	WS22-N	Tp-47	None
100	6/6	0/6	6/6	3/3
10	6/7	0/7	4/6	4/4
1	4/8	0/8	2/6	1/3
0.1	0/6	0/6	0/4	0/2

[a] The ratios above represent the number of positive lesions per number of sites injected.

3.3. Rabbit infectivity tests

To determine and further demonstrate that the peptides could impart anti-treponemal activities, we conducted bactericidal assays and then diluted some of the suspension for use in the RIT to test for both the viability of treated organisms and the infectivity of those surviving. It is important to note that the sensitivity of this assay is between 1 and 10 organisms per injection site and a single organism can theoretically produce a chancre [7]. However, the probability that a site injected with this concentration of organisms will produce a lesion is determined by Poisson distribution.

The experiments were repeated three times and the cumulative data is reported in Table 2. At 66 μM, which is near the EC_{50} of LL-37, exactly half of the infected sites produced lesions when the samples were diluted to one organism per site. At a concentration of 10 organisms per site, 6 of 7 produced lesions. In contrast, when 9.7 μM WS22-N-amide was used in the assay, no lesions developed even at 100 organisms per site. This finding correlates well with the data presented in Fig. 2 showing no survival at this peptide concentration. When an irrelevant peptide, a 20-mer of the 47 kDa antigen, was used in the assay, about half of the injection sites produced lesions at 1 organism per site and 67% at 10 organisms per site. When the antimicrobial peptide was absent from the assay, 33% of the injections produced a lesion at 1 organism per site and 100% at 10 and 100 organisms per site, as would be expected. Thus, these RITs demonstrated that the activity of these peptides was bactericidal and could reduce the infectivity of *T. pallidum* exposed to them.

4. Discussion

LL-37 and other antimicrobial peptides or proteins are likely to be important in the human innate host defense during infection. Their constant presence in phagocytic cells or their synthesis during infection or inflammation permits the host to rapidly respond to invading pathogens prior to the development of acquired immunity systems. LL-37 is a member of the cathelicidin family of antimicrobial peptides and is the sole cathelicidin thus far recognized in humans. Recent studies strongly show the importance of LL-37 in host defense against pathogens infecting the skin [4,6] and gut mucosal surface [10]. In addition, we have previously

shown that LL-37 has significant bactericidal activity in vitro against *Neisseria gonorrhoeae* [17].

Because LL-37 is likely to be present at the genital mucosal surface [19], we asked whether it might be active against sexually transmitted pathogens other than gonococci. During our study, Sambri et al. [16] reported the anti-spirochetal activity (including *T. pallidum*) of a number of cathelicidins. While significant differences exist in the antimicrobial assay system employed by our two groups, both results indicate that human LL-37 has antimicrobial action against. *T. pallidum*. However, it must be stressed that the assay system employed by Sambri et al. may not accurately measure the absolute bactericidal potential of LL-37. Thus, it is relevant to note that their use of PBS as the incubation media, even with the addition of serum (2%, v/v), may have created architectural stress on the spirochetal surface, rendering it more sensitive to LL-37 and thus reducing the viability. In addition, because *T. pallidum* needs a somewhat reduced environment to remain viable [8,21], our use of DTT (3 mg/dl) in the TpCM more likely mimicked the in vivo environment. Under these conditions, *T. pallidum* can replicate in TpCM and survive for 14–17 days [6].

We found that *T. pallidum* is much more sensitive to LL-37 under low salt conditions than in complete TpCM. However, with respect to *T. pallidum*, it is not clear whether this was due to the increased activity of the peptide or to an increased vulnerability of the spirochete to low osmotic pressure. This result was most likely due to a combination of both. We must also note that the only natural biological niche of *T. pallidum* is human tissue, and the spirochete is totally adapted to this limited environment, where it is bathed in interstitial fluids and serum at physiological concentrations of salt. Thus, it would not be surprising that at salt and serum concentrations lower than this, the viability of *T. pallidum* might be severely compromised.

Our studies suggest that the full length LL-37 peptide is less active against *T. pallidum* than its truncated synthetic variant, WS22-N-amide. This decreased sensitivity to LL-37 activity may be explained by the architectural structure of the spirochete's cell envelope. The outer membranes (OMs) of spirochetes are unique and different from those of typical Gram-negative bacteria. First, lipopolysaccharides are absent from species of *Treponema* and *Borrelia*. Second, typical Gram-negative OMs have numerous porins which appear to be lacking in spirochetal OMs. In *T. pallidum*, it is uncertain whether their OMs are totally devoid of porins or whether porins are present in very small numbers. In either case, the OMs of these spirochetes certainly possess far less porins than typical Gram-negative bacteria. Third, typical spirochetal OMs are very lipid rich, which tends to make the surface less charged and more hydrophobic than typical Gram-negative bacteria. Finally, the amount of surface-exposed proteins which also carry a charge is greatly diminished when compared with other bacteria. Freeze-fracture electron microscopy of its outer membrane reveals that the organism has only a few hundred intramembranous particles (IMPs) present per cell [15], compared to several thousand for *Escherichia coli*. *T. pallidum* is an extreme example of this characteristic.

Cathelicidins are positively charged peptides and part of their action depends upon binding to targets on bacterial surfaces. Typical Gram-negative bacteria tend to be negatively charged and thus the initial process of cathelicidin binding involves ionic bonding to these charged targets. Two of these targets are LPS and lipoteichoic acids, neither of which are found in the cell architecture of *T. pallidum*. Other targets may include negatively charged surface proteins. Again, those targets are either nonexistent or very scarce on the surface of *T. pallidum*. Thus, the unique architecture of the OM of *T. pallidum* confers its resistance to cathelicidin activity because of its low surface charge and more hydrophobic nature.

T. pallidum was shown to be much more sensitive to the truncated peptide, WS22-N-amide. The EC_{50} was over 30 times less than that with the whole peptide. The explanation for this increased sensitivity lies in two differences between the truncated peptide and whole LL-37. First, WS22-N-amide consists of the 17 residues of the activity core of LL-37. Second, asparagine was substituted for the aspartic acid residue in this core sequence, thus removing a negatively charged amino acid residue and replacing it with a positively charged residue. This substitution gives WS22-N-amide an even higher charge to residue ratio than native LL-37. In fact, this would make most of the entire peptide either hydrophobic or positively charged (Fig. 1). Hong et al. [9] have shown that the antibacterial activity of short peptides is increased as the net charge of the peptide increases. Furthermore, the large number of uncharged residues that could be attracted to the lipid rich OM of *T. pallidum* because of hydrophobic interaction may explain why this derivative is much more potent than the native peptide.

To prove that the nonmotile organisms observed in the bactericidal assays were killed, infectivity assays using the RIT were performed. This test is considered the "gold standard" to determine viability of *T. pallidum*. The rabbit is the most practical animal because (1) a local lesion is produced, (2) the tissues remain infective for the life of the animal, and (3) the sensitivity is between 1 and 10 viable spirochetes [13]. Furthermore, the RIT is much less complicated than tests using hamsters where internal organs need to be excised and examined. The animals do not need to be sacrificed to determine if organisms are in the lesions, and therefore the incubation periods for sites containing very small numbers of viable organisms can be extended as long as needed. The treponemes were pre-treated with specific peptides before injection in order to tightly control the peptide concentration and incubation conditions, and to ensure reproducibility of the assays.

The data in Table 1 shows that the truncated peptide WS22-N-amide was very effective in killing the treponemes at a concentration of 10 μM. None of the injected sites produced lesions, even with 100 spirochetes per site injected.

In contrast, 66 μM of the whole peptide produced ≤50% killing according to the RIT. It should be noted that the difference between the LL-37-treated and untreated organisms would have been more pronounced if concentrations above the EC_{50} had been used. The narrow difference is may also be due in part to the small numbers of animals tested.

In conclusion, this study demonstrated that viable *T. pallidum* was resistant to the bactericidal action of human LL-37 and rabbit CAP-18, but was much more sensitive to a truncated peptide of LL-37, WS22-N-amide. We suggest that this resistance is most likely due to the unique cell architecture of the spirochete that renders it less susceptible to the bactericidal mechanism of LL-37 than other bacteria. This resistance could be overcome by the removal of the N- and C-terminal amino acids of full length LL-37. Hence, the bactericidal domain of LL-37 might be less available to the targets on the surface due to the presence of these extensions.

Acknowledgments

We wish to express our gratitude to Dr. Ron Ballard for his review of the manuscript and his insightful discussions, and to Dr. Jan Pohl and Dr. Danny Jue for preparing the peptides. This project was supported in part by NIH grant AI 37945. W.M.S. is the recipient of a Senior Research Career Scientist Award from the VA Medical Research Service.

Use of trade names is for identification only and does not constitute an endorsement by the U.S. Department of Health and Human Services, the Public Health Service, or the Centers for Disease Control and prevention.

References

[1] Agerberth B, Gunne H, Odeberg J, Kogner P, Boman HG, Gudmundsson GH. FALL-39, a putative human peptide antibiotic. Proc Natl Acad Sci USA 1995;92:195–9.

[2] Borenstein LA, Ganz T, Sell S, Lehrer RI, Miller JN. Contribution of rabbit leukocyte defensins to the host response in experimental syphilis. Infect Immun 1991;59:1368–77.

[3] Borenstein LA, Selsted ME, Lehrer RI, Miller JN. Antimicrobial activity of rabbit leukocyte defensins against *Treponema pallidum* subsp. *pallidum*. Infect Immun 1991;59:1359–67.

[4] Brissette CA, Lukehart SA. *Treponema denticola* is resistant to human beta-defensins. Infect Immun 2002;70:3982–4.

[5] Cox DL, Riley B, Chang P, Sayahtaheri S, Tassell S, Hevelone J. Effects of molecular oxygen, oxidation-reduction potential, and antixoidants upon in vitro replication of *Treponema pallidum* subsp. *pallidum*. Appl Environ Microbiol 1990;56:3063–72.

[6] Dorschner RA, Pestonjamasp VK, Tamakuwala S, Ohtake T, Rudisill J, Nizet V, et al. Cutaneous injury induces the release of cathelicidin anti-microbial peptides active against group A *Streptococcus*. J Invest Dermatol 2001;117:91–7.

[7] Graves SR, Johnson RC. Effect of pretreatment with *Mycobacterium bovis* (strain BCG) and immune syphilitic serum on rabbit resistance to *Treponema pallidum*. Infect Immun 1975;12:1029–36.

[8] Graves SR, Sandock PL, Jenkin HM, Johnson RC. Retention of motility and virulence of *Treponema pallidum* (Nichols strain) in vitro. Infect Immun 1975;12:1116–20.

[9] Hong SY, Park TG, Lee KH. The effect of charge increase on the specificity and activity of a short antimicrobial peptide. Peptides 2001;22:1669–74.

[10] Islam D, Bandholtz L, Nilsson J, Wigzell H, Christensson B, Agerberth B, et al. Downregulation of bactericidal peptides in enteric infections: a novel immune escape mechanism with bacterial DNA as a potential regulator. Nature 2001;7:180–5.

[11] Konishi H, Yoshii Z, Cox DL. Electron microscopy of *Treponema pallidum* (Nichols) cultivated in tissue cultures of Sf1Ep cells. Infect Immun 1984;53:32–7.

[12] Larsen SA, Hunter EF, Kraus SJ. A manual of tests for syphilis. American Public Health Association Washington, DC; 1990.

[13] Larsen SA, Steiner BM, Rudolph AH. Laboratory diagnosis and interpretation of tests for syphilis. Clin Microbiol Rev 1995;8:1–21.

[14] Nizet V, Ohtake T, Lauth X, Trowbridge J, Rudisill J, Dorschner RA, et al. Innate antimicrobial peptide protects the skin from invasive bacterial infection. Nature 2001;414:454–7.

[15] Radolf JD, Norgard MV, Schultz WW. Outer membrane ultrastructure explains the limited antigenicity of virulent *T. pallidum*. Proc Nat Acad Sci USA 1989;86:2051–5.

[16] Sambri V, Marangoni A, Giacani L, Gennaro R, Murgia R, Cevenini R, et al. Comparative in vitro activity of five cathelicidin-derived synthetic peptides against *Leptospira borrelia* and *Treponema pallidum*. J Antimicrob Chemother 2002;50:895–902.

[17] Shafer WM, Qu X, Waring AJ, Lehrer RI. Modulation of *Neisseria gonorrhoeae* susceptibility to vertebrate antibacterial peptides due to a member of the resistance/nodulation/division efflux pump family. Proc Natl Acad Sci USA 1998;95:1829–33.

[18] Turner J, Cho Y, Dinh NN, Waring AJ, Lehrer RI. Activities of LL-37, a cathelin-associated antimicrobial peptide of human neutrophils. Antimicrob Agents Chemother 1998;42:2206–14.

[19] Valore EV, Park CH, Quayle AJ, Wiles KR, McCray Jr PB, Ganz T. Human beta-defensin-1: an antimicrobial peptide of urogenital tissues. J Clin Invest 1998;101:1633–42.

[20] Walker EM, Zampighi GA, Blanco DR, Miller JN, Lovett MA. Demonstration of rare protein in the outer membrane of *Treponema pallidum* subsp. *pallidum* by freeze-fracture analysis. J Bacteriol 1989;171:5005–11.

[21] Weber MM. Factors influencing the in vitro survival of *Treponema pallidum*. Am J Hyg 1960;71:401–17.