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Antimicrobial Activity of Two Bactenecins against Spirochetes

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Bac5 and Bac7 are antimicrobial peptides of bovine neutrophils that act on enteric gram-negative bacteria. We report here that these two peptides immobilize and kill *Leptospira interrogans* and *Leptospira biflexa* with MBCs of 6 to 25 μg/ml. Conversely, although both peptides bind to *Borrelia burgdorferi*, the organism is resistant to their action.

Bactenecins (specifically, Bac5 and Bac7) are cationic, proline-and-arginine-rich polypeptides, which were extracted from the large granules of bovine neutrophils (9, 23). In vitro, bactenecins exert a potent bactericidal activity toward several gram-negative organisms such as *Escherichia coli*, *Salmonella typhimurium*, *Enterobacter cloacae*, and *Klebsiella pneumoniae* (9). Bac7 also arrests the growth of *Pseudomonas aeruginosa* and neutralizes human herpes simplex virus (9, 25). The bactericidal activity of Bac5 and Bac7 is very likely causally related to the permeabilization of both the outer and inner membranes of susceptible bacteria (16).

In this study, we have analyzed the potential bactericidal effects of bactenecins on spirochetes. Members of the species *Leptospira interrogans* and *Borrelia burgdorferi* are known to be common parasites of humans and other animals and to frequently produce deleterious effects in the host. In particular, *Leptospira* strains belonging to serovar hardjo and biovars hardjoprajitno and hardjobovis are the most important pathogen worldwide of cattle, which is believed to be the maintenance host (4, 7). Furthermore, although little is known about the pathogenetic interaction between *B. burgdorferi* and cattle, *B. burgdorferi* infections and serological evidence of this organism in cows are documented in Europe and the United States (3, 10).

Bac7 and Bac5 were purified to homogeneity from extracts of granules of bovine neutrophils, using ion-exchange chromatography and reverse-phase high-performance liquid chromatography (RP-HPLC) as previously described (9). Purity of peptides was checked by both analytical RP-HPLC and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The purified peptides were stored in 0.05% trifluoracetic acid and their concentrations were determined by the method of Waddell (20).

The following strains of the genera *Leptospira* and *Borrelia*, all belonging to the reference collection of the Institute of Microbiology of the University of Trieste, were used: *L. interrogans* Hardjoprajitno and 70 (a local isolate) (both belonging to serovar hardjo) and Wijnberg (serovar copenhageni), *L. biflexa* RPE, and *B. burgdorferi* B31 (ATCC 35210) and BITS (a local tick isolate) (6). *Leptospira* and *Borrelia* cells were grown at 30°C for 5 to 7 days in EMJH medium (11) and BSK medium (1), respectively.

The MICs of bactenecins were assayed by the microdilution susceptibility test in 96-well microdilution plates. Serial 1:1 dilutions of each peptide in 100 μl of EMJH or BSK medium (starting from a 400-μg/ml stock solution in 0.002% trifluoracetic acid) were pipetted into the wells of microtiter plates to which 10-μl samples of bacteria (10^8/ml) in the same medium were then added. The MIC was defined as the lowest concentration of peptide preventing bacterial growth after a 7-day incubation at 30°C, as evaluated by dark-field microscopy (magnification of ×400). For each bactenecin, the bacteriostatic assays on the spirochetes were performed at least three times.

Bactericidal assays were carried out at the end of the MIC determinations. Twenty microliters of culture was transferred from each well showing no visible bacterial growth into 2 ml of fresh medium, and the incubation was continued for 7 days at 30°C. The MBC was defined as the lowest concentration of peptide causing no bacterial growth after 1 week, as determined by dark-field microscopy analysis. To test the time dependence of *Leptospira* killing by Bac5, an in vitro immobilization assay was performed as follows: one part of leptospiral suspension (10^6/ml) was combined with one part of EMJH medium in the absence or presence of 10 μg of bactenecin per ml. Mixtures were then incubated at 37°C for 10, 30, and 90 min, and aliquots of each mixture were scored for percent motility by counting the motile and nonmotile organisms in 25 randomly chosen fields with a dark-field microscope (18).

To evaluate the binding of bactenecins to bacteria (13, 14), 2 μg of either Bac5 or Bac7 was added to *Leptospira* or *Borrelia* cells (10^8/ml) in 250 μl of fresh medium. After a 15-min incubation at 37°C with gentle stirring, bacteria were recovered from the medium containing unbound bactenecins by centrifugation at 10,000 × *g* for 5 min in an Eppendorf microcentrifuge. The cell pellet was washed twice with phosphate-buffered saline and resuspended in 200 mM $MgCl_2$–10 mM sodium acetate-acetic acid, pH 5. After incubation for 15 min at 37°C with shaking, the bacteria were sedimented as described above and the proteins in the supernatant were precipitated with 6% trichloroacetic acid at 0°C. The precipitate was dissolved in sample buffer, boiled for 3 min under reducing conditions, and analyzed by SDS-PAGE (14% acrylamide) (12). Western blotting and immunodetection of Bac5 were performed as previously described (23).

As shown in Table 1, all the *Leptospira* strains tested are susceptible to the two bactenecins, with MICs ranging from 1.6 to 25 μg/ml. Of the two antibiotic peptides, Bac5 appears

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TABLE 1. Antimicrobial activities of Bac7 and Bac5[a]

Organism and strain	Bac7		Bac5	
	MIC	MBC	MIC	MBC
L. interrogans Hardjoprajitno	6.2	12.5	1.6–3.1	12.5
L. interrogans 70	6.2	12.5	1.6–3.1	6.2
L. interrogans Wijnberg	12.5	25.0	6.2–12.5	12.5
L. biflexa RPE	25.0	25.0	12.5	25.0
B. burgdorferi B31	>200		>200	
B. burgdorferi BITS	>200		>200	

[a] MIC assays were carried out with 10^7 bacteria per ml in EMJH (*Leptospira* strains) or BSK (*Borrelia* strains) medium. For MBC determination, 20 μl of culture medium of a well showing no visible bacterial growth was transferred to 2 ml of fresh medium and the incubation was continued for 7 days. Both MIC and MBC are measured in micrograms per milliliter.

to be the most potent. Strains Hardjoprajitno and 70, belonging to the serovar hardjo, are the most susceptible. Unlike *Leptospira* strains, strains B31 and BITS of *B. burgdorferi* are resistant to both bactenecins, at least at the concentrations employed (MIC of >200 μg/ml).

Bac5 and Bac7 also kill *Leptospira* strains, with MBCs ranging from 12.5 μg/ml for strains Hardjoprajitno and 70 to 25 μg/ml for strains Wijnberg and RPE (Table 1). The rate of killing was then evaluated by observing the loss of motility of *L. interrogans* Hardjoprajitno in contact with 10 μg of Bac5 per ml. After 90-min incubation at 37°C, the peptide is capable of immobilizing more than 70% of bacterial cells (Table 2). Since a reduction in motility of spirochetes is a direct measure of their viability, this result indicates a significant inhibition of the vital functions of *L. interrogans* by Bac5.

In an attempt to understand the difference in resistance of the two genera of spirochetes to bactenecins, we examined the binding of Bac5 or Bac7 to one *Leptospira* strain (Hardjoprajitno) and one *Borrelia* strain (BITS). Although Western blots do not permit quantitative comparisons of protein bands, the analyses carried out with such a technique indicate that Bac5 (not shown) and Bac7 (Fig. 1) bind to both spirochetes. It is thus likely that the different susceptibilities of the two genera of spirochetes to the antimicrobial activity of bactenecins actually reflects their deep difference in metabolism and outer envelope organization. Leptospires are aerobic, while borrelias are microaerophilic. Furthermore, while *L. interrogans* serovar hardjo contains a lipopolysaccharide (LPS) with a high lipid content (19), *B. burgdorferi* is devoid of classic enterobacterium-type LPS (17). We suggest that the absence of antimicrobial effects by Bac5 and Bac7 on *B. burgdorferi* is to be ascribed to the absence of LPS. In fact, the interaction of neutrophil antimicrobial peptides with the inner core of LPS is in general considered essential for the initiation of bactericidal activities (8, 14, 15, 21).

TABLE 2. Immobilization of *L. interrogans* Hardjoprajitno by Bac5[a]

Incubation time (min)	Mobility (%)	
	Without Bac5	With Bac5
0	96	97
10		81
30		65
90	90	28

[a] *L. interrogans* Hardjoprajitno was incubated at 37°C in EMJH medium in the absence and presence of 10 μg of Bac5 per ml. Motile and nonmotile cells were counted with a dark-field microscope.

FIG. 1. Immunodetection (Western blot) of Bac7 bound to and then desorbed with 200 mM $MgCl_2$ from spirochetes. Lane a, control, trichloroacetic acid-precipitated Bac7; lane b, Bac7 desorbed from *L. interrogans* Hardjoprajitno; lane c, Bac7 desorbed from *B. burgdorferi* BITS.

Previous experiments have suggested that after binding to the bacterial surface, bactenecins reach the inner membranes of gram-negative bacteria, where they cause a fall in respiration-linked proton motive force (16). The immobilization effect observed on the most susceptible *Leptospira* strain (Hardjoprajitno) is consistent with a derangement of the proton motive force across the bacterial membrane with an attendant impairment of flagellar motility.

It is interesting that the antibacterial activity of Bac5 and Bac7, which thus far has been found only in bovine neutrophils (23), is exerted toward strains of serovar hardjo, which is selectively adapted to parasitize cattle (7).

Leptospires are phagocytized and killed by various mammalian phagocytes when opsonized (4, 5). However, the rate of the killing process and the intraphagolysosomal events responsible for their inactivation are still unknown. Our results indicate that Bac5 and Bac7, which are rapidly discharged into the phagocytic vacuoles (24), might effectively contribute to the reduction in *Leptospira* viability.

These conclusions are corroborated by recent observations of microbicidal effects on *L. interrogans* serovar lai (22) by other neutrophil antimicrobial peptides, the defensins, which are also active on *Treponema pallidum* (2).

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