The Antimicrobial Agent Melittin Exhibits Powerful In Vitro Inhibitory Effects on the Lyme Disease Spirochete

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Borrelia burgdorferi **has demonstrated a capacity to resist the in vitro effects of powerful eukaryotic and prokaryotic metabolic inhibitors. However, treatment of laboratory cultures on Barbour-Stoenner-Kelly medium with melittin, a 26–amino acid peptide contained in honeybee venom, showed immediate and profound inhibitory effects when they were monitored by dark-field microscopy, field emission scanning electron microscopy, and optical density measurements. Furthermore, at melittin concentrations as low as 100 μg/mL, virtually all spirochete motility ceased within seconds of inhibitor addition. Ultrastructural examination of these spirochetes by scanning electron microscopy revealed obvious alterations in the surface envelope of the spirochetes. The extraordinary sensitivity of *B. burgdorferi* to mellitin may provide both a research reagent useful in the study of selective permeability in microorganisms and important clues to the development of effective new drugs against lyme disease.**

Many microorganisms appear resistant to the effects of inhibitory agents, at least in part because of the molecular composition of their outer membranes and the selective permeability that these structures provide [1, 2]. Outer envelopes may form an effective barrier to certain macromolecules while at the same time allowing others (such as nutrients) to enter. As might be expected, a great deal of interest has been focused on the structure and function of these microbial membranes and upon a small group of agents that appear to have the ability to alter membrane permeability. It has been the hope that these agents might provide important clues about the molecular nature of selective permeability in microorganisms and about its role in microbial pathogenesis. It has also been the hope that, once past this permeability barrier, antimicrobial agents previously thought to be ineffective might prove to be therapeutically useful.

It has been recognized for some time that a variety of naturally occurring, low-molecular-weight substances bind avidly with the lipopolysaccharide moiety of certain bacteria and, in the process, dramatically disrupt outer membrane integrity [3, 4]. This allows access through the outer membrane to their final target, the cytoplasmic membrane, where the primary antimicrobial activity is thought to take place [1]. Melittin, the principle toxic component of honeybee (*Apis mellifera*) venom, is a 26-amino acid, cationic oligopeptide with strong channel-forming activities previously demonstrated in eukaryotic cells [5], in multilamellar phospholipid vesicles [6], and in *Escherichia coli* [7, 8]. As we report here, the effect of this molecule upon *Borrelia burgdorferi* cells growing in culture, as monitored by dark-field and scanning EM and by spectrophotometry, is both immediate and profound.

Methods

Purified (85% by HPLC) melittin (no. M 2272) with <5 units of phospholipase activity per mg was obtained from Sigma Chemical (St. Louis) and used without further treatment or purification. Phospholipase A_2 (no. P 9279), used in control experiments, was also purchased in lyophilized form from Sigma Chemical.

All spirochetes were cultured in modified Barbour-Stoenner-Kelly (BSK-II) medium and monitored over time by optical density measurements of culture aliquots. Melittin was added to the BSK-II-medium cultures at the times and concentrations indicated. Culture aliquots were removed at various time points, placed under glass coverslips, and examined immediately by dark-field microscopy.

Fifty to 100 spirochetes over several fields were monitored at each time point to determine motility values. Cells were also removed from culture, fixed within minutes with glutaraldehyde, collected on Pronectin F (no. PF1001; Protein Polymer Technologies, San Diego) pretreated cyclopore membranes (Falcon no. 3096-A; Daigger Scientific, Manassas, VA), dehydrated with a graded series of ethanol, critical-point dried, and sputter-coated (as previously described [9]) for examination in an S-4500 field emission scanning electron microscope (Hitachi, Tokyo).

Results

In contrast to a number of known metabolic inhibitors similarly tested, melittin appeared to produce a profound inhibitory effect on cultures of *B. burgdorferi* that was detectable shortly

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after the addition of the peptide to the culture (figure 1). While untreated cultures continued their growth over time, the optical density of melittin-treated cultures appeared to decrease dramatically soon after addition of the insect-derived oligopeptide.

Untreated *B. burgdorferi,* when observed with the scanning electron microscope, appears as smooth, undulating cells, well dispersed across the field of view and relatively free of detectable extracellular material except for occasional blebs [9, 10]. In contrast, however, ultrastructural examination of melittin-treated spirochetes revealed a greatly diminished number of spirochetes with distorted shapes, profoundly altered surface envelopes, and much extracellular debris, including an enormous increase in vesicular bleb formation (figure 2).

The surface envelope appeared to have been disordered, stripped from the surface of virtually all of the spirochetes, and either lay strewn about the field of view or appeared wound loosely around the cells. Cells were highly aggregated, with an adherent matrix often holding a number of spirochetes together. Close examination of membrane material at high magnification occasionally revealed numerous holes in their surfaces.

Free endoflagella and endoflagellar fragments were commonly visible about the field of view as well. Increased blebbing of surface components could be documented, with numerous blebs visible both free in the background and still attached to cells. In addition to their dramatically increased numbers, their size and shape appeared to vary more widely than those of untreated controls. Furthermore, individual spirochetes appeared to have lost some structural integrity, as the characteristic undulating form was absent and cells appeared tightly kinked and twisted.

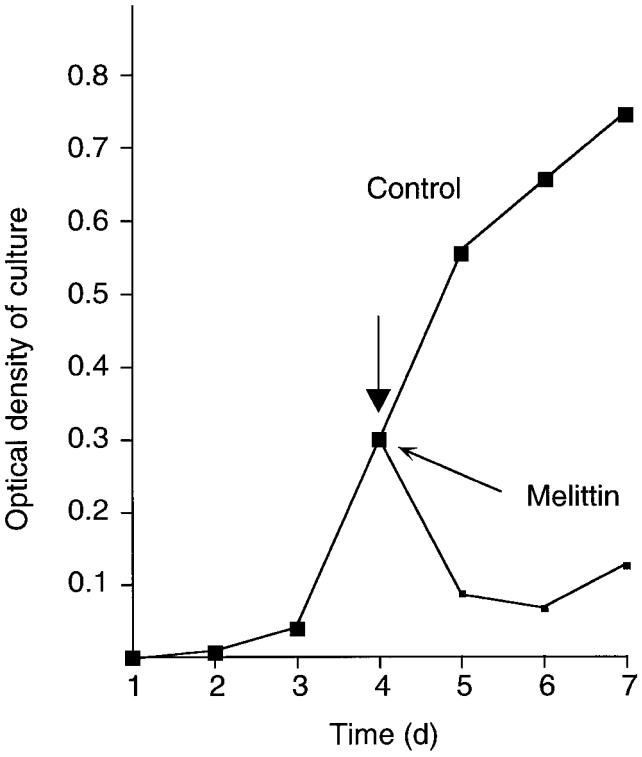

Figure 1. The effect of melittin treatment on the growth of *Borrelia burgdorferi* in BSK-II medium. Melittin was added to a 100-mL culture, producing a 400-μg/mL final concentration, at a time indicated by the arrows. Five-mL aliquots were removed at various time points from the untreated control culture (■) and the melittin-treated culture (■), and the optical density at 600 mμ was determined.

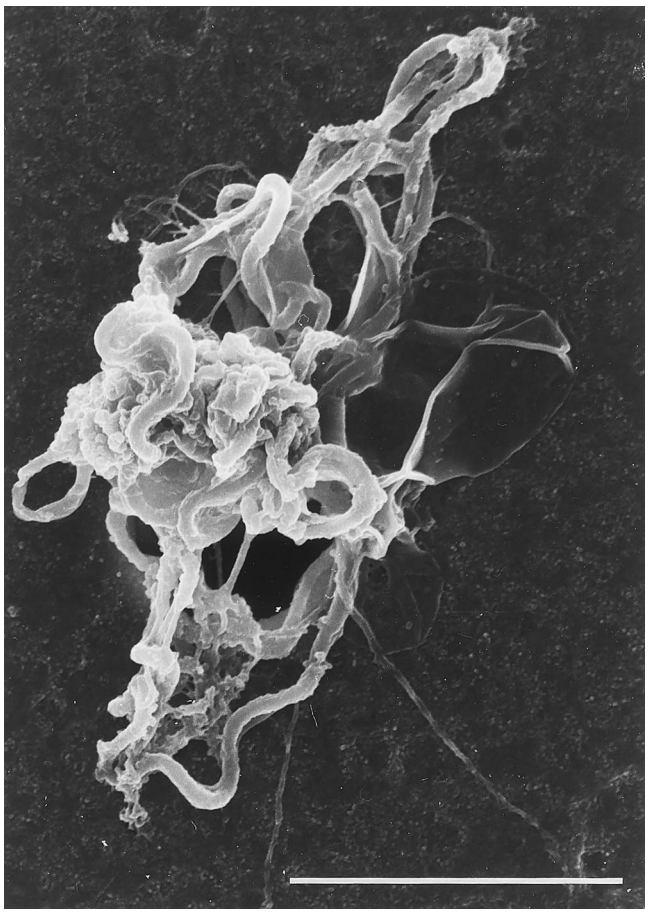

Figure 2. Scanning electron micrograph of *B. burgdorferi* 24 hours after treatment with melittin (400 μg/mL) in BSK-II culture medium. Spirochetes were mounted for microscopy as described in the Methods section (bar = 3.75 μm).

In view of the apparent disruption of the surface envelope with the release of flagellar material, we wondered if motility might not also have been affected. Melittin was added to growing cultures and the motility was monitored directly by dark-field microscopy at various time points and at various concentrations. As shown in figure 3, both motility and optical density dropped precipitously immediately following the addition of melittin. These data were confirmed by a dose-response curve showing the effect of various concentrations of melittin on the motility of *B. burgdorferi.* Those data are presented in figure 4 and show that 100-μg/mL concentrations of melittin are sufficient to abolish motility in virtually all spirochetes within minutes of treatment.

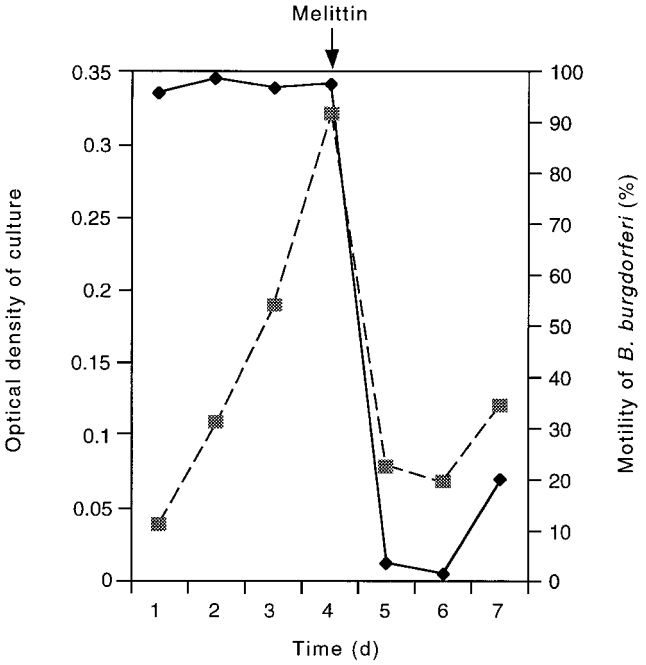

Figure 3. The effect of melittin (400 μg/mL) on the growth and motility of *B. burgdorferi* in BSK-II-medium culture. The culture was monitored for both optical density at 600 mμ (*dashed line*) and spirochete motility ($n = 50$; *solid line*) by dark-field microscopy at each of the time points presented. Melittin was added to the culture at the time indicated by the arrow.

Subsequent experiments (data not shown) allowing melittin to seep under the coverslip of a motile culture being viewed under the dark-field microscope revealed that virtually all detectable spirochete motility ceased within seconds of melittin addition as the peptide diffused across the field of view.

Commercially available melittin may be contaminated with small amounts (<5 units/mg) of phospholipase A. Therefore, control experiments (data not shown) using concentrations of purified phospholipase A that might be expected to contaminate our experiments were conducted. Phospholipase-treated cells were indistinguishable from control cells in both motility and ultrastructure, a finding suggesting that the effects described were correctly assigned to melittin.

Discussion and Conclusion

A family of small, cationic polypeptides produced by insects, amphibians, and mammals has recently been shown to exhibit powerful cytolytic activity against both eukaryotic and prokaryotic cells [1]. Several mechanisms have been proposed to account for this biological effect [1, 2, 3, 6]. Although not totally resolved, one common activity thought to be at work in this cytotoxic effect involves insertion of the alpha helical form of the peptide into the surface membranes of the cells, causing pores or channels that subsequently cause inappropriate permeability characteristics, followed by membrane disintegration and osmotic cytolysis [11–15].

Regardless of the exact mode of action, it is clear from data presented here that the structural integrity of the surface envelope of *B. burgdorferi* is also profoundly affected by treatment in vitro with the insect oligopeptide melittin. Increased blebbing of surface membranes and disintegration of outer envelope integrity are effects seen on spirochetal surface components. However, purification of membrane blebs with subsequent enzyme and organic solvent extraction showed intact DNA molecules packaged in blebs, as previously reported with regard to blebs from untreated spirochetes [9, 10].

In addition, probably because of the unique location of the motility apparatus in *B. burgdorferi*—namely, between the inner and outer membranes of the cell—profound effects on spirochete motility are also detectable very soon after treatment. This effect has not been reported in other systems that have been studied. At the very least these data suggest that melittin is a useful and readily available reagent for controlling spirochete motility and vesicular blebbing in laboratory experiments.

Although antimicrobial agents may act to exert their effects in various ways, all must be internalized effectively before they can produce any effect on the pathogen. Experiments are under way in our laboratory to further characterize the effects of pore-forming substances like melittin on the microbial cell outer membrane, with the aim of designing therapeutically useful permeabilizers that might serve to widen the spectrum of clinically useful antimicrobial agents.

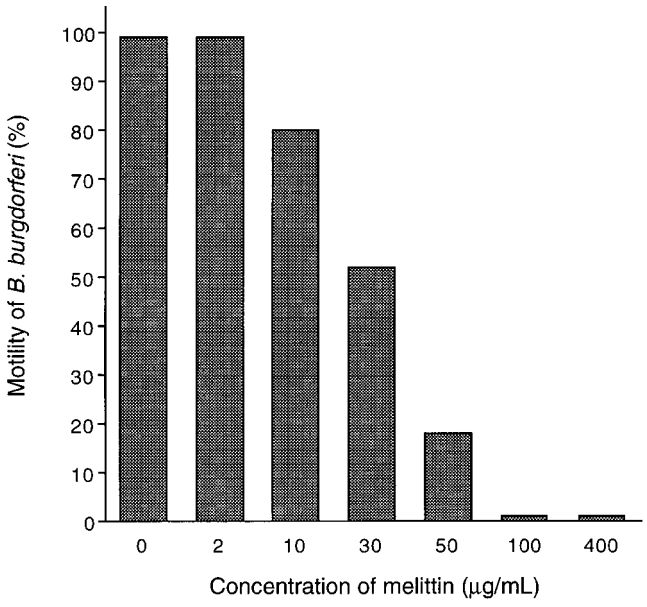

Figure 4. The effect of various concentrations of melittin on the motility of *B. burgdorferi* within 1 hour following treatment in BSK-II medium. The motility of at least 50–100 spirochetes was assessed by dark-field microscopy at each melittin concentration tested.

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