Borrelia burgdorferi Are Susceptible to Killing by a Variety of Human Polymorphonuclear Leukocyte Components

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The killing of *Borrelia burgdorferi* by intact human polymorphonuclear leukocytes (PMNL) and by individual PMNL components was compared. Intact PMNL killed *B. burgdorferi* 6.5-fold more efficiently and 5-fold more completely when spirochetes were opsonized with specific antibodies. U-cytoplasts, which have activatable oxidase, killed opsonized *B. burgdorferi* with an efficiency similar to that of intact PMNL in killing unopsonized *B. burgdorferi.* Although *B. burgdorferi* were susceptible to H_2O_2 and nitric oxide, PMNL lysates killed *B. burgdorferi* nearly as well as intact PMNL killed opsonized *B. burgdorferi,* suggesting a critical role for granule contents. *B. burgdorferi* were killed by the PMNL antimicrobial components elastase, LL-37, bactericidal/permeability-increasing protein, and human neutrophil peptide–1. *B. burgdorferi* had limited susceptibility to killing by lysozyme and were not killed by azurocidin, proteinase 3, or lactoferrin. The efficient killing of *B. burgdorferi* by a variety of PMNL mechanisms highlights the paradoxical persistence of spirochetes in vivo.

Lyme disease, a tick-transmitted multisystem disease, is caused by the spirochete *Borrelia burgdorferi.* It is characterized by a transient rash, erythema migrans, and a range of subsequent inflammatory processes involving most notably the joints, nervous system, and heart [1]. Polymorphonuclear leukocytes (PMNL) arrive at infected sites and are heavily represented, for example, in the inflammatory response in joints [1]. Signs of arthritis in mice are found as early as 7–10 days after infection [2]. The symptoms of Lyme disease are related to the capability of spirochetes to disseminate and persist in a variety of host tissues. Such persistence in vivo requires that *B. burgdorferi* avoid host defense mechanisms.

Experimental studies show that PMNL play a critical role in controlling infection by *B. burgdorferi* and that defects in PMNL cause worse arthritis [3]. PMNL in vitro phagocytose *B. burgdorferi* both in the presence and in the absence of specific antibody [4]. *B. burgdorferi* stimulate the oxidative burst mechanism of PMNL [4–6], yet they are susceptible to killing by PMNL from patients with chronic granulomatous disease who lack this mechanism [4]. In some experimental systems, the contribution of complement factors to PMNL interactions with *B. burgdorferi* is critical [7]; in others, complement factors contribute little or nothing [4, 8, 9], perhaps because complement sensitivity may vary by spirochete strain [10]. Elastase, a serine proteinase, has a role in the killing of *B. burgdorferi* in vitro [11]. Also, in vivo studies of beige mice, which have defects in PMNL neutrophil elastase and cathepsin G activities [12], detected higher *B. burgdorferi* loads in their joints than in those of their wild-type counterparts [3].

PMNL contain many other antimicrobial proteins, including hCAP18/LL-37, bactericidal/permeability-increasing protein (BPI), defensins (i.e., human neutrophil peptide–1 [HNP-1]), lysozyme, azurocidin, proteinase 3, and lactoferrin, which contribute significantly to the killing of numerous organisms [13, 14]. To clarify the role of the various PMNL killing mechanisms in interactions with *B. burgdorferi,* we examined PMNL killing of spirochetes, using intact cells, PMNL lysates, phagocytic motile anucleate granule–poor cytoplasts [15], and individual granule components, individually and in concert. Features from both oxidative and nonoxidative killing mechanisms were included.

Materials and Methods

Reagents. Reagents, including human neutrophil lactoferrin and lysozyme, were purchased from Sigma, unless otherwise specified. Human neutrophil elastase was purchased from Calbiochem. Human granule components were the generous gifts of colleagues: neutrophil elastase, azurocidin, and proteinase 3 [16–18], from J. E. Gabay (Columbia University, New York); LL-37, from B. Agerberth (Karolinska Institute, Stockholm) [19]; HNP-1, from T. Ganz (UCLA School of Medicine, Los Angeles) [20]; human cathelicidin (hCAP18), from O. E. Sørensen (Rigshospitalet, Copenhagen) [21]; and recombinant BPI ($rBPI_{21}$), derived from the N-terminal domain of human BPI, from Xoma [22].

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Polymorphonuclear leukocytes were obtained from healthy volunteers in accordance with guidelines of the Yale University School of Medicine Human Investigation Committee.

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B. burgdorferi *culture.* Low-passage, virulent strain N40 *B. burgdorferi* were propagated in Barbour-Stoenner-Kelley II (BSKII) medium at 33°C, as described elsewhere [23]. *B. burgdorferi,* grown to logarithmic phase, were pelleted (10 min, 3000 *g,* 25°C), resuspended in BSKII medium, and counted under darkfield microscopy, using a Petroff-Hausser hemocytometer (Hausser Scientific Partnership). BSKII medium was washed off, and *B. burgdorferi* were resuspended at the desired concentration in PBS (5 m*M* $NaPO_4$ and 150 m*M* NaCl [pH 7.4], with variations as indicated). *B. burgdorferi* were opsonized for 30 min at 37°C with 1%–10% heat-inactivated human serum (56°C, 30 min) from a well-characterized Lyme disease patient (recognizing bands at 18, 21, 28, 30, 58, and 60 kDa on *B. burgdorferi* IMMUNOBLOT).

PMNL and cytoplast preparations. PMNL were isolated from fresh, heparinized blood of healthy volunteers that was allowed to sediment in 3% dextran at a 45° angle for 1 h before hypotonic lysis of contaminating erythrocytes [15]. This process yields about 98% PMNL, as determined by microscopic examination of sampled cells. Lysates of intact PMNL equivalents were prepared immediately before use by several brief (~5 s) periods of sonication on ice, followed by centrifugation (5000 *g* at 4°C) to remove cell nuclei. U-cytoplasts (U-CYTs) were prepared from purified PMNL on a discontinuous ficoll gradient, as described elsewhere [15].

[^{3}H]adenine B. burgdorferi *regrowth assay and microscopic killing assay. B. burgdorferi* viability was quantified by a modified regrowth assay [24] that measures the uptake of [^{3}H]adenine by *B. burgdorferi* [25]. We previously showed that [^{3}H]adenine uptake is linear, from 10^4 to 10^6 *B. burgdorferi*/mL, over 48 h [24]. This method correlates well with a microscopic assay [24] and is clearly superior for examination of opsonized spirochetes, which tend to clump. We usually use the [^{3}H]adenine assay, because it measures regrowth and allows for *B. burgdorferi* that may be damaged but not killed to recover and be measured as viable. The microscopic assay has proved to be useful for study of *B. burgdorferi* in the presence of components with limited availability and for the immediate assessment of any effects on *B. burgdorferi* viability.

For the [^{3}H]adenine assay, 5×10^6 *B. burgdorferi*/mL were incubated in the presence of a killing agent, with agitation, for 1–3 h at 37°C. We plated triplicate aliquots (50 μL) in 96-well plates in the presence of 200 μL of BSKII containing 5 μCi of [^{3}H] adenine and incubated the plates for 48 h at 33°C. We compared results with those obtained by using control samples that contained *B. burgdorferi* in only the buffer condition appropriate for the particular incubation. In some instances, spirochete viability was also determined by direct visual assessment of *B. burgdorferi* under darkfield microscopy. We assessed the percentage of cell survival immediately and 48 h after diluting 5-μL aliquots of the incubated samples with 10 μL of BSKII. We determined spirochete motility and morphology in 10 random fields and assessed samples in a double-blind fashion [24]. Cells were considered to be killed when complete loss of motility and refractivity was observed.

The antimicrobial components of PMNL are specialized. They work against target cells in particular environments, and environmental variations affect their ability to act [13]. Thus, for optimal killing, assay length and buffer conditions were as follows: H_2O_2 and nitric oxide (NO) killing was for 1 h in PBS. For studies of whole cells and cell lysates, including PMNL, U-CYTs, and PMNL lysates, *B. burgdorferi* were incubated with the killing agent for 1 h in PBS containing physiologic glucose (5.4 m*M*) and 2% human serum albumin (HSA; PBS/glu/HSA). Elastase, BPI, HNP-1, and lysozyme were each incubated with *B. burgdorferi* in 50 m*M* Na-phosphate buffer (pH 7.4) for 2 h, with variations as follows: Elastase was incubated for 2 h in buffer and then was diluted 2× with BSKII before an additional 20 h of incubation prior to plating with [^{3}H]adenine. HNP-1 was incubated with or without 5.4 m*M* glucose at either pH 6 or pH 7.4 or with 150 m*M* NaCl at pH 7.4. Lysozyme incubations were done with or without 150 m*M* NaCl. Azurocidin and proteinase 3 incubations were tested in 5 m*M* phosphate buffer at pH 7.4 or in PBS. LL-37 and lactoferrin incubations were done in 10 m*M* phosphate with 5.4 m*M* glucose, at pH 7.4, or in PBS at pH 7.4, for 1 or 3 h, as indicated.

Results

The study included both intact neutrophils and neutrophil lysates (free granule contents). For the investigation of oxidative mechanisms, we used U-CYTs, motile, granule-poor cytoplasts developed in our laboratory, which have activatable oxidase [15]. U-CYTs retain killing capacity for particular bacteria [15, 26]. We also studied the effect of the inorganic disinfectants H_2O_2 and NO. For the investigation of nonoxidative mechanisms, we used various purified neutrophilic proteins. By fitting the data to a first-order exponential decay equation, we determined the point of 50% viability (V_{50}). We also determined the minimum viability, or the point of least viability obtained with the assay conditions used (V_{min}). All errors are expressed as SEM. All results were obtained with the [^{3}H]adenine assay, unless otherwise stated.

Cellular Effects on *B. burgdorferi* Viability

Intact PMNL. We compared PMNL killing of *B. burgdorferi* at a single ratio (3 *B. burgdorferi* [Bb]:1 PMNL) after various pretreatments of spirochetes. Although *B. burgdorferi* viability was reduced in the absence of added protein (PBS alone), no significant differences were noted when we compared PMNL killing of *B. burgdorferi* incubated in PBS/glu/HSA or after opsonization in 10% normal human serum or heat-inactivated normal human serum (percentages of viability ± SD after 60 min were 55 ±11, 57 ± 14, or 55 ± 4, respectively; $n = 4$). We chose the simplest buffer system for further study. With intact PMNL, the V_{50} for nonopsonized *B. burgdorferi* was 6 Bb:1 PMNL, and the V_{min} was 45% ($n = 3$; figure 1*A*). Killing was both more efficient and more complete when the *B. burgdorferi* spirochetes were opsonized (Bb_{ops}; V_{50}, 40 Bb_{ops}:1 PMNL; V_{min}, 9% ($n = 3$; figure 1*A*). Thus, specific antibody dramatically enhanced the efficiency of killing (V_{50} of 6 vs. V_{50} of 40—a factor of 6.5).

PMNL lysates. Sonicated PMNL lysates killed unopsonized *B. burgdorferi* with efficiency approaching that of intact PMNL given opsonized *B. burgdorferi* and 4 times more efficiently than intact PMNL given unopsonized *B. burgdorferi:* V_{50}, 22 Bb:1 PMNL lysate equivalent; V_{min}, 13% ($n = 3$; figure 1*A*). Lysate killing represents the synergistic effects of all the internal contents of PMNL. Its equivalence to intact PMNL given opsonized spirochetes suggests a critical role for PMNL granule contents in the killing mechanism of *B. burgdorferi.*

Intact U-CYTs. To further clarify the role of nongranule mechanisms in the killing of *B. burgdorferi,* we determined the viability of virulent *B. burgdorferi,* opsonized and unopsonized, after exposure to U-CYTs. U-CYTs are granule poor but maintain oxidative killing mechanisms [15, 26]. U-CYTs killed spirochetes opsonized with specific patient antibodies: V_{50}, 5 Bb_{ops}:1 U-CYT; V_{min}, 29% ($n = 3$; figure 1*B*). In contrast, no significant killing of unopsonized *B. burgdorferi* by U-CYTs was seen under the same conditions (figure 1*B*). These results suggest that nongranule factors have a role in the PMNL killing of *B. burgdorferi* or that a few remaining granules in U-CYTs are sufficient to kill.

We evaluated the role of NO production in U-CYT killing of *B. burgdorferi* by using the reversible NO synthase inhibitor, N^{ω}-monomethyl-L-arginine (NMMA), an analogue of L-arginine. We previously used U-CYTs to show that PMNL use the NO pathway to kill microbes [26]. Inhibition of NO had no effect on the killing of *B. burgdorferi* by U-CYTs. For killing at a ratio of 5 Bb_{ops}:1 U-CYT, 41% ± 2% were viable ($n = 4$). The presence of 1 m*M* NMMA had no significant effect on the killing seen. This suggests that in vitro killing of *B. burgdorferi* by U-CYTs does not depend on NO production. We also tested the effects of preincubation of U-CYTs for 30 min with the O_2^- scavenger superoxide dismutase (SOD; 40 μg/mL); with the H_2O_2 scavengers catalase (200 μg/mL) and peroxidase (200 μg/mL); and with the $OH^{\bullet}$ scavengers mannitol (100 m*M*) and dimethyl sulfoxide (50 and 200 m*M*). As expected, the addition of SOD had no significant effect ($n = 3$) on the viability of opsonized *B. burgdorferi* exposed to U-CYTs, since *B. burgdorferi* have the O_2^- degrading enzyme SOD [27]. No significant effect on *B. burgdorferi* viability ($n = 3$) was seen, compared with that of controls, for each of the scavengers individually or for all of them incubated in concert. Together, the results suggest that U-CYT killing of *B. burgdorferi* does not depend on the oxidative intermediates O_2^-, H_2O_2, or $OH^{\bullet}$, at least not by themselves.

Nevertheless, both oxidants can kill *B. burgdorferi* when present in sufficient concentrations. Reagent H_2O_2 was effective in killing the spirochete: V_{50}, 0.49 m*M* H_2O_2; V_{min}, 15% ($n = 5$; figure 2*A*). *B. burgdorferi* are susceptible to killing by H_2O_2 in a range comparable to that reported for other organisms (e.g., *Cryptococcus neoformans,* 0.8 m*M* H_2O_2; *Trypanosoma cruzi,* 0.6 m*M* H_2O_2) [28, 29].

We evaluated the role of NO by using reagent NO spontaneously generated by the NO-producing compound *s*-nitroso-*N*-acetyl-penicillamine (SNAP). A stock solution in PBS (pH 7.4) was prepared immediately before each experimental use. When

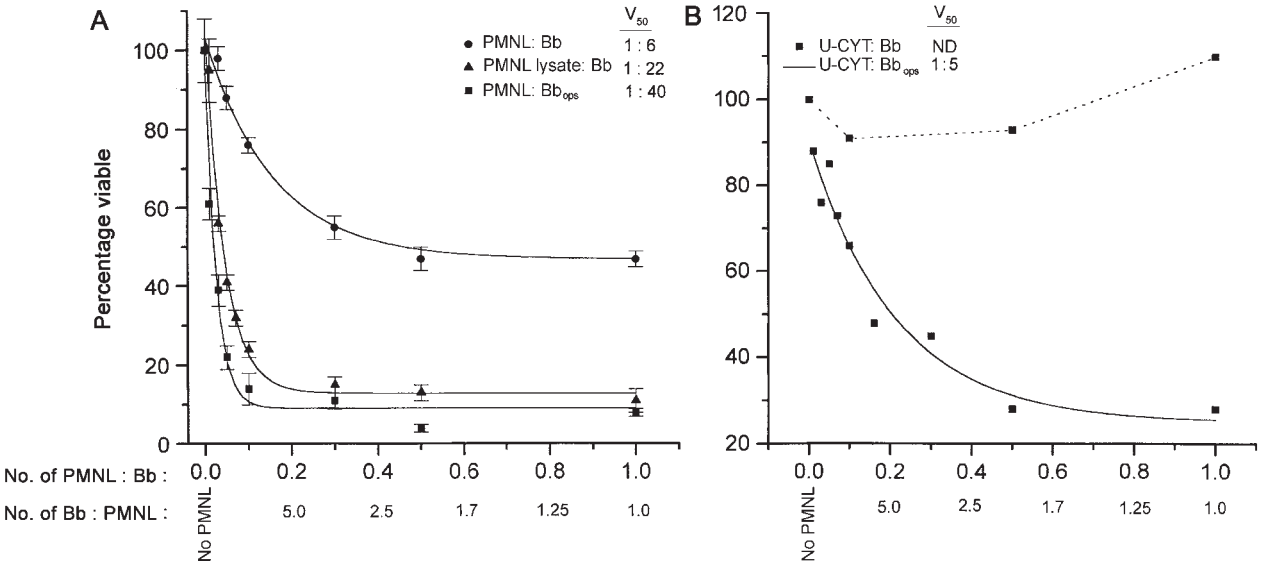

Figure 1. Cellular effect on the viability of opsonized and unopsonized *Borrelia burgdorferi* (Bb_{ops} and Bb). In total, 10^6 Bb in PBS/glucose/human serum albumin were incubated with 10^4–10^6 polymorphonuclear leukocytes (PMNL) or U-cytoplasts (U-CYTs) for 1 h at 37°C ($n = 3$). *A,* PMNL and lysate effect. V_{50} (50% viability) was 40 Bb_{ops} per PMNL, 22 Bb per PMNL lysate equivalent, and 6 Bb per PMNL. *B,* U-CYT effect. V_{50}, 5 Bb_{ops} per U-CYT. There was no significant killing of Bb by U-CYTs. ND, not determined. Results of a representative experiment are shown.

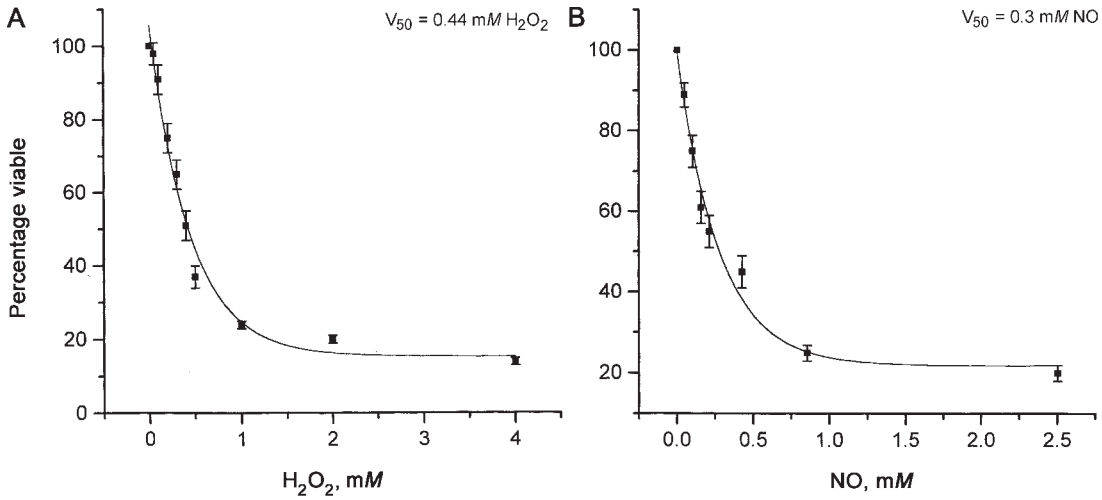

Figure 2. Reagent oxidative effects on *Borrelia burgdorferi* viability (percentage viable). 5×10^6 *B. burgdorferi*/mL were incubated in the presence of H_2O_2 *(A;* 0.05–4.0 m*M)* or NO *(B;* 0.05–1.0 m*M)* in PBS for 1 h at 37°C. V_{50} (50% viability), 0.44 m*M* H_2O_2 and 0.3 m*M* NO.

we examined its ability to kill *B. burgdorferi,* we found effective killing: V_{50}, 1.4 m*M* SNAP, which is equivalent to 0.3 m*M* NO; V_{min}, 12% ($n = 5$; figure 2*B*). In comparison, the parasite *Leishmania major* was almost completely killed by a similar concentration of SNAP [30].

Effect of Human PMNL Granule Components

The high susceptibility of *B. burgdorferi* to PMNL lysate points to a large contribution of killing from the granule components. To elucidate their relative contributions to *B. burgdorferi* killing, we examined individual components.

Elastase, azurocidin, and proteinase 3. Elastase is an abundant serine proteinase located in PMNL primary granules at a concentration of 1.5 μg/10^6 PMNL [13]. *B. burgdorferi* were susceptible to elastase: V_{50}, 6 μg/mL; V_{min}, 4% ($n = 4$; table 1). To achieve more complete killing of *B. burgdorferi,* it was necessary to use 30 μg/mL elastase, at which point 5×10^6 *B. burgdorferi*/mL were ~5% viable. This is comparable to the findings of an earlier study, in which 5×10^5 *B. burgdorferi*/mL (i.e., 10-fold less) were >99% killed by elastase concentrations of 2 μg/mL [11]. Elastase killed *B. burgdorferi* equally as well at 7 μg/mL (32% ± 2% viable; $n = 4$) as when proteolytically inactivated either by pretreatment with phenylmethylsulfonyl fluoride (PMSF; 29% ± 3% viable) or by heating to 100°C for 5 min (27% ± 3% viable), as reported elsewhere [11]. Both azurocidin, which lacks protease activity, and proteinase 3 are part of the serine proteinase family of proteins located within the azurophil granules of PMNL [16, 17]. Neither protein killed *B. burgdorferi* at concentrations up to 100 μg/mL, as observed microscopically at neutral pH (pH 7.4) both in PBS and in 5 m*M* phosphate buffer. Because azurocidin was reported to synergize with neutrophil elastase [31], both azurocidin and proteinase 3 were tested independently at 100 μg/mL (pH 7.4) in its presence (1 μg/mL elastase). No synergy was seen for either protein with elastase.

BPI. BPI is an antimicrobial protein present in the azurophil granules of PMNL at a concentration of 1.5 μg/10^6 PMNL [13, 14]. BPI consists of a 25-kDa highly cationic antimicrobial N-terminal domain and a 30-kDa nonantimicrobial C-terminal domain. The mechanism of action of BPI involves lipopolysaccharide (LPS) binding; thus BPI is expected to have activity against gram-negative bacteria [32]. Because *B. burgdorferi* do not have LPS but express a variety of lipoproteins, it is possible that BPI is also able to bind these lipoproteins. In this study, $rBPI_{21}$ (derived from human BPI) was used, and *B. burgdorferi* were sensitive to killing by this protein: V_{50}, 41 μg/mL $rBPI_{21}$; V_{min}, 14% ($n = 4$) in 50 m*M* phosphate/5.4 m*M* glucose (pH 7.4; table 1).

HNP-1. HNP-1 is the most common human defensin and is present in azurophil granules at 3–5 μg/10^6 PMNL [21]. HNP-1 killed the spirochetes efficiently, achieving a V_{50} of 131 μg/mL HNP-1 and a V_{min} of 5% ($n = 4$) in 50 m*M* phosphate/5.4 m*M* glucose (pH 7.4; table 1). *B. burgdorferi* were killed by HNP-1 at a concentration similar to that for other organisms: *Escherichia coli, Staphylococcus aureus,* and *Pseudomonas aeruginosa* were all susceptible to HNP-1 in the 50 μg/mL range when in the presence of specific nutrients [33]. As expected, HNP-1 most efficiently killed *B. burgdorferi* in the presence of glucose [21], presumably because the spirochetes were metabolically active (table 2). The susceptibility of *B. burgdorferi* to HNP-1 was eliminated at high ionic strength and was reduced at lower pH (table 2), also as expected [21]. To investigate the possibility of synergistic effects, HNP-1 and $rBPI_{21}$ were incubated together with *B. burgdorferi.* No synergy was seen; the 2 components killed *B. burgdorferi* in an additive manner (table 3).

Table 1. Effect of polymorphonuclear leukocyte (PMNL) granule components on *Borrelia burgdorferi* viability.

Granule component, μg/mL	% Viable ± SEM	V_{50}, μg/mL
PMNL elastase		
1	82 ± 2	6
3	65 ± 2	
7	41 ± 2	
10	11 ± 2	
30	5 ± 1	
$rBPI_{21}$		
16	87 ± 1	41
24	55 ± 3	
32	48 ± 4	
64	38 ± 3	
96	34 ± 2	
128	11 ± 2	
HNP-1		
50	95 ± 3	131
70	59 ± 4	
100	47 ± 2	
200	21 ± 3	
400	14 ± 2	
LL-37[a]		
2	97 ± 2	9
4	69 ± 2	
8	46 ± 2	
12	26 ± 2	
16	15 ± 2	
20	6 ± 1 [98 ± 1]	[68]
40	2 ± 1 [66 ± 2]	
80	[43 ± 1]	
120	[21 ± 2]	
160	[10 ± 1]	
200	[9 ± 1]	
Control	100	100

NOTE. Percentage of viable spirochetes after incubation with the indicated component, expressed relative to buffer controls, as described in Materials and Methods; $n = 4$. HNP-1, human neutrophil peptide–1; $rBPI_{21}$, recombinant bactericidal/permeability-increasing protein; V_{50}, 50% viability.

[a] LL-37 effect on *B. burgdorferi* viablility is given at low ionic strength (10 m*M* phosphate; nonbracketed data) and/or high ionic strength (5 m*M* phosphate and 150 m*M* NaCl; bracketed data).

hCAP18. hCAP18 is a protein expressed as a 19-kDa precursor in the secondary granules of PMNL [34]. The active 4.6-kDa cationic peptide, LL-37, is proteolytically released from its proform by proteinase 3 [35]. No killing was seen with intact hCAP18 (100 μg/mL). LL-37 killed *B. burgdorferi* (observed microscopically) in both low- and high-concentration salt conditions: V_{50}, 9 μg/mL LL-37; V_{min}, 1% ($n = 4$) in 10 m*M* phosphate/5.4 m*M* glucose (pH 7.4). At higher ionic strength, the amounts of LL-37 needed to effect killing were increased: V_{50}, 68 μg/mL LL-37; V_{min}, 10% ($n = 4$) in PBS (pH 7.4; table 1). LL-37 (20 μg/mL) and HNP-1 (70 μg/mL) were incubated together in PBS (pH 7.4) to investigate any synergistic effects in the killing of *B. burgdorferi.* PMNL release cathelicidins and defensins extracellularly and were reported to synergize for the killing of *E. coli* and *S. aureus* [36]. The individual sublethal amounts used did not cause killing of *B. burgdorferi* when the 2 were incubated in concert. Thus, no synergy was found.

Lysozyme. Lysozyme is a 14.4-kDa cationic protein with enzymatic activity, which, unlike that of the other constituents studied, is present in both the primary and secondary granules (1.5–3 $\mu g/10^6$ PMNL) [13, 14]. *B. burgdorferi* were partially killed by a high concentration of lysozyme (300 μg/mL) when tested in an NaCl-free buffer (50 m*M* phosphate [pH 7.4]): 68% ± 2% viability ($n = 4$). No killing was seen at the lower concentrations tested, 50 and 100 μg/mL, in this salt-free buffer. No killing was seen at 300 μg/mL when 150 m*M* NaCl was added to the buffer.

Lactoferrin. Lactoferrin belongs to the transferrin family of iron-binding proteins and is found in the specific granules (3–8 $\mu g/10^6$ PMNL) [13, 14]. *B. burgdorferi* were not killed by lactoferrin, as observed microscopically in 10 m*M* phosphate/5.4 m*M* glucose (pH 7.4) or PBS (pH 7.4) at concentrations $\leqslant$250 μg/mL and with incubation for up to 3 h. Enhancing conditions by the use of detergents [37] was not possible, as *B. burgdorferi* did not survive well under these conditions. To investigate the possibility of synergistic effects [38], lactoferrin and lysozyme were incubated together with *B. burgdorferi* in either 10 m*M* phosphate/5.4 m*M* glucose (pH 7.4) or PBS (pH 7.4). No synergy (i.e., no killing of *B. burgdorferi*) was seen for any combination of the components at 10, 50, or 100 μg/mL.

Discussion

This study of mechanisms used by PMNL for killing *B. burgdorferi* revealed that the neutrophil is well equipped to kill spirochetes. *B. burgdorferi* were susceptible to a variety of the components within the PMNL arsenal, including nonoxidative components from granules and reactive oxygen and nitrogen intermediates.

The killing capacity of intact PMNL for *B. burgdorferi* increased dramatically in the presence of specific antibody: The V_{50} increased from 6 to 40 *B. burgdorferi* killed per 1 PMNL. PMNL killing within a given period was also more complete in the presence of specific antibody: The V_{min} decreased from

Table 2. Human neutrophil peptide–1 (HNP-1) effect at 100 μg/mL.

Buffer condition	% Viable ± SEM
pH 7.4	81 ± 2
5.4 m*M* glucose	52 ± 2
150 m*M* NaCl	104 ± 2
pH 6.0	97 ± 2
5.4 m*M* glucose	86 ± 2

NOTE. Spirochetes were incubated with 100 μg/mL HNP-1 in 50 m*M* phosphate buffer, at pH indicated, with NaCl or glucose; $n = 3$.

Table 3. Additive killing effects of human neutrophil peptide–1 (HNP-1) and recombinant bactericidal/permeability-increasing protein ($rBPI_{21}$).

Granule component (μg/mL)	% Viable ± SEM
HNP-1 (70)	58 ± 2
$rBPI_{21}$ (24)	46 ± 1
HNP-1 (70) + $rBPI_{21}$ (24)	13 ± 3

NOTE. Spirochetes were incubated in 50 m*M* phosphate buffer, pH 7.4, with 5.4 m*M* glucose; $n = 4$.

45% to 9%. Opsonization by specific antibody presumably enhances killing by allowing binding of spirochetes to Fc receptors [9] that fix the rapidly moving spirochete and activate the process of invagination and fusion of phagosome with lysosome. We showed earlier that unopsonized spirochetes do not interact directly with the phagocyte Fc receptor [39]. A significant portion of PMNL killing of spirochetes may take place extracellularly, as seen microscopically (authors' unpublished data), which is not surprising, considering the size of the spirochete.

Opsonization with specific antibody caused agglutination of *B. burgdorferi* into clumps much larger than an individual PMNL, yet these clumps were still killed by the PMNL. PMNL lysate killed *B. burgdorferi* (V_{50}, 22 Bb:1 PMNL) at a 13% efficiency, approaching the killing capability of whole cells with opsonized *B. burgdorferi* (V_{50}, 40 Bb_{ops}:1 PMNL), at an efficiency of 9%. This supports the observation of extracellular killing. Moreover, concentrations of extracellular granule components are likely to be high, because of targeting to adherent portions of the spirochete.

Although *B. burgdorferi* stimulate the respiratory burst of PMNL [4–6, 40], NO and other reactive oxygen intermediates are not required for the successful killing of the spirochete [4, 40, 41]. Indeed, PMNL from patients with chronic granulomatous disease, who have defective generation of reactive oxygen intermediates, kill *B. burgdorferi* equally as well as control PMNL [4]. Despite the report that inhibition of NO and O_2 species over 24 h in vitro led to a partial reduction of macrophage killing of *B. burgdorferi* [42], in another report in vivo, the absence of NO production did not worsen *B. burgdorferi* infection [40, 41].

We found that U-CYTs can kill spirochetes, but only when opsonized; inhibitors or scavengers of the oxidants did not affect this capability. *B. burgdorferi* are susceptible to reagent H_2O_2 and NO. Thus, although oxidants may not be necessary for successful PMNL killing of *B. burgdorferi,* these organisms are susceptible to them. Also, since oxidative killing requires intact cells, it would not be expected to contribute to killing by PMNL lysates. The lysates kill nearly as well as the intact cell, suggesting that an intact cell would not require oxidative mechanisms to kill *B. burgdorferi.* It is difficult to estimate to what capacity various components are able to compensate for what is lacking in disease states (e.g., in patients with chronic granulomatous disease); yet, taken together, these observations suggest that multiple mechanisms of PMNL killing may be in play in vivo.

To investigate the role of granules in spirochete killing, we used purified granule components. We found 5 granule constituents to be capable of killing *B. burgdorferi* in their purified active states. The experiments were designed so that conditions were optimized for each constituent. Thus, a direct quantitative comparison between them may not be appropriate.

B. burgdorferi were susceptible to killing by elastase both in its proteolytically active state and after inactivation by pretreatment with PMSF or heat [11]. The importance of elastase to PMNL killing of *B. burgdorferi* may be increased in vivo, since elastase is required to activate other granule components that contribute to PMNL killing. *B. burgdorferi* were not susceptible to azurocidin or proteinase 3 under the experimental conditions used. Although the antimicrobial activity of azurocidin is enhanced under slightly acidic conditions or lower ionic strength [16], because *B. burgdorferi* do not survive well under these conditions, such testing was not feasible.

B. burgdorferi were also sensitive to $rBPI_{21}$ and to the human neutrophil defensin HNP-1. The effectiveness of killing for HNP-1 was influenced by the metabolic state of the target cell, as expected [20]: *B. burgdorferi* were most efficiently killed when they were metabolically active (i.e., in the presence of glucose). Lowering the pH or increasing the ionic strength caused a loss of HNP-1 activity against the spirochete. The 2 proteins did not act synergistically. When they were incubated in combination with *B. burgdorferi,* their contribution to killing was additive (table 3).

Although LL-37 was able to kill *B. burgdorferi* (table 1), its proform, hCAP-18, as expected, was not [43]. Of interest, a study of the bovine cathelicidins Bac5 and Bac7 found that, whereas the spirochetes *Leptospira interrogans* and *L. biflexa* were susceptible, *B. burgdorferi* were not ($\leqslant$200 μg/mL) [44]. Thus, although the proregion of the cathelicidin family is highly conserved, the susceptibility of *B. burgdorferi* to LL-37, but not to Bac5 or Bac7, may highlight the heterogeneous nature of the antimicrobial portion of this family of proteins [43]. LL-37 killed *B. burgdorferi* in buffers of both low (10 m*M* phosphate/5.4 m*M* glucose [pH 7.4]) and high (PBS [pH 7.4]) ionic strength, suggesting that it may contribute to killing both extracellularly and after internalization into a phagolysosome. LL-37 and HNP-1 did not synergize to kill *B. burgdorferi* in physiologic ionic strength at the concentrations studied.

B. burgdorferi were apparently susceptible to lysozyme, albeit only at the very high concentration of 300 μg/mL. Although the lysozyme preparation we used was 99% pure, at the level at which killing was found (300 μg lysozyme/mL), contaminants could reach concentrations of 3 μg/mL, or levels at which killing was found for other highly pure neutrophilic components (i.e., elastase or LL-37; table 1).

An earlier study showed that elastase was the only granule protein that could kill *B. burgdorferi* independently (i.e., without other granule factors also present) [11]. We have shown that multiple highly pure granule components kill *B. burgdorferi.*

Thus, when assay conditions are optimized for individual granule components, their ability to kill *B. burgdorferi* is clear. PMNL in vivo can provide a variety of conditions in which their components would have the opportunity to act, either alone or in concert, including the possibility of high concentrations being reached when PMNL granules emit their antimicrobial components directly onto a bound target cell or into a phagosome.

In summary, we found that *B. burgdorferi* in contact with PMNL are susceptible to killing by a variety of PMNL mechanisms. Yet, in vivo, spirochetes often survive, disseminate, and continue to cause disease. The reasons for their survival are of intense current interest and study. Early on, they may persist because of the anti-inflammatory properties of saliva [45] and lack of antibody. Later, they appear to stay out of harm's way by finding privileged sites (or situations) from which they may periodically emerge to provoke clinical illness [46].

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