

Calprotectin, an Abundant Cytosolic Protein from Human Polymorphonuclear Leukocytes, Inhibits the Growth of *Borrelia burgdorferi*

Denise Lusitani, Stephen E. Malawista, and Ruth R. Montgomery*

Department of Internal Medicine, Yale University School of Medicine, New Haven, Connecticut 06520

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We previously showed that numerous polymorphonuclear leukocyte (PMN) granule components efficiently kill *Borrelia burgdorferi*, the agent of Lyme disease. In addition, motile, granule-poor cytoplasts (U-Cyt) from human blood PMN can exert anti-*Borrelia* activity against opsonized *B. burgdorferi* independently of oxidative mechanisms. Here we show that lysates of U-Cyt also possess anti-*Borrelia* activity, a portion of which comes from the abundant cytosolic protein calprotectin. The anti-*Borrelia* activity of U-Cyt lysates and recombinant calprotectin was partially or completely reversed by specific antibody to calprotectin and by Zn^{2+} , a cation essential for the growth of *B. burgdorferi* and known to inhibit the antimicrobial activity of calprotectin. Quantitative microscopic and regrowth assays revealed that calprotectin acted in a bacteriostatic fashion against *B. burgdorferi*. We conclude that calprotectin, a potent bacteriostatic agent from a cell primarily recognized for its oxidative and granular antibacterial mechanisms, may play a modulatory role in infection by the Lyme spirochete, particularly at sites of acute inflammation.

The spirochete *Borrelia burgdorferi* is the infectious agent of Lyme disease, and its natural life cycle involves a mammalian and an arthropod host (10). Lyme disease usually begins with a characteristic rash, erythema migrans, and persistent infection leads to a range of inflammatory processes that include arthritis, carditis, and a variety of neurological symptoms (10). Polymorphonuclear leukocytes (PMN), important components of both innate immunity and antibody-dependent clearance, are the first immune cells to respond to *B. burgdorferi* infection in the skin and are heavily represented in the subsequent inflammatory response in joints (10). Experimental studies have indicated that PMN play a critical role in controlling infection by *B. burgdorferi*; beige mice, which have defects in their PMN granules, have significantly worse arthritis (1). Infected mice have tendonitis, synovial tissue hyperproliferation, and a synovial fluid cellular influx predominated by PMN, abnormalities that resemble those observed in human patients (2, 10). The severity of these symptoms has been correlated with the spirochete burden found in joints (28).

The PMN is a potent killer cell with abundant antimicrobial mechanisms that include contributions from a respiratory burst oxidase and from granule constituents. PMN generally kill target organisms in the controlled environment of phagolysosomes following ingestion (16). We have shown that, unlike macrophages, PMN ingest and kill *B. burgdorferi* efficiently only in the presence of specific antibody (14). We previously defined the role of particular granule components and developed evidence for extracellular killing of *B. burgdorferi* by PMN (9, 14). We showed that the PMN granule components elastase, LL-37, bactericidal/permeability-increasing protein, and

human neutrophil peptide-1 have anti-*Borrelia* abilities that are restricted by incubation conditions such as pH and ionic strength (9). In addition, U-cytoplasts (U-Cyt), motile, granule-poor cytoplasts which retain activatable oxidase (12), also reduce the viability of opsonized *B. burgdorferi*, even without the contribution of reactive oxygen intermediates (9). The remarkable anti-*Borrelia* activity retained by granule-poor U-Cyt despite scavenging of reactive oxygen intermediates suggests the presence of an additional, previously unconsidered anti-*Borrelia* mechanism (9).

One PMN antimicrobial agent that is less well studied is the cytosolic protein calprotectin, a heterodimer consisting of one light (11 kDa) and one heavy (14 kDa) chain (5), which has both cellular and extracellular roles. Calprotectin is a particularly abundant protein of the neutrophil, with an estimated cytosolic concentration of 5 to 15 mg/ml, constituting 45% of the cytosolic protein (7, 27). Its effect is exerted primarily by inhibition of growth through competition for zinc (23), although at high concentrations calprotectin may be microbicidal (25). The multifunctional role of calprotectin and its association with a variety of inflammatory diseases have historically created a complex nomenclature; other names given to either the heterodimer or its individual subunits include MRP-8 and MRP-14 (migration-inhibitory factor-related proteins), cystic fibrosis antigen, L1 antigen, calgranulin A and B, and S100A8 and S100A9 (7). Here we show that the cytosolic protein calprotectin is a potent anti-*Borrelia* component of the neutrophil's armamentarium.

MATERIALS AND METHODS

Preparation of *B. burgdorferi* culture. Low-passage, virulent *B. burgdorferi* strain N40 was grown to logarithmic phase in Barbour-Stoenner-Kelley II medium (BSK) with added neomycin and amphotericin at 33°C (9). For experiments, *B. burgdorferi* was pelleted (10 min, 3,000 × g, 25°C), resuspended in BSK, and enumerated with a Petroff-Hausser hemacytometer (Hausser Scientific Partnership, Horsham, Pa.) under dark-field microscopy. *B. burgdorferi* was opso-

* Corresponding author. Mailing address: Department of Internal Medicine, Yale University School of Medicine, 300 Cedar Street, New Haven, CT 06520-8031. Phone: (203) 785-7039. Fax: (203) 785-7053. E-mail: ruth.montgomery@yale.edu.

nized by treatment for 30 min at 37°C with 1% heat-inactivated serum (56°C for 30 min) from a well-characterized Lyme disease patient (recognizing bands at 18, 21, 28, 30, 58, and 60 kDa on the *B. burgdorferi* immunoblot) as described before (9).

Cytoplasm and lysate preparations. Blood donations from healthy volunteers were obtained in accordance with the guidelines of the Human Investigation Committee of Yale University School of Medicine. PMN were isolated from fresh heparinized blood of healthy volunteers that was allowed to sediment in 3% dextran at an angle of 45° for 1 h, followed by hypotonic lysis of contaminating erythrocytes (12). U-Cyt were prepared from purified PMN on a discontinuous Ficoll gradient as described previously (12). Lysates of U-Cyt and PMN were prepared immediately before use by multiple freeze-thaw cycles in liquid N₂ and a 37°C water bath.

[³H]adenine *B. burgdorferi* regrowth assay. Changes in *B. burgdorferi* number were quantified with a regrowth assay which measures the uptake of [³H]adenine by *B. burgdorferi* as we have described previously (9). The [³H]adenine uptake is linear from 10⁴ to 10⁶ *B. burgdorferi* organisms/ml over 48 h. Opsonized spirochetes (5 × 10⁶ opsonized *B. burgdorferi* organisms per ml) were incubated in the presence of intact U-Cyt (5 × 10⁴ to 5 × 10⁶/ml) for 1 h at 37°C in phosphate-buffered saline (pH 7.4) with 5.4 mM glucose and 2% human serum albumin with agitation; this buffer maintains the viability of *B. burgdorferi* throughout the incubation period (9). Triplicate aliquots (50 μl) were plated in 96-well plates in the presence of 200 μl of BSK containing 5 μCi of [³H]adenine, incubated for 48 h at 33°C, and harvested. Incorporation of [³H]adenine by treated *B. burgdorferi* was compared with that of control samples under the same incubation conditions. The data were fit to a first-order exponential decay equation to determine the 50% inhibitory concentration (IC₅₀) obtained with the assay conditions employed. Limits are expressed as standard errors of the mean.

Microscopic assessment of *B. burgdorferi* number. For studies with lysates of U-Cyt and recombinant calprotectin, we employed a direct visual quantification of spirochetes under dark-field microscopy at 1, 24, and 48 h (9). The microscope assay has proven especially useful both for the study of *B. burgdorferi* in the presence of components with limited availability, as results can be obtained from smaller experimental volumes, and for the comparison of immediate versus later effects from the same sample. In this assay, 5 × 10⁶ *B. burgdorferi* organisms/ml are incubated with lysates of U-Cyt or recombinant calprotectin as described below, and the percent cell survival is assessed immediately and after 48 h from 5-μl aliquots of the incubated samples diluted with 10 μl of BSK and enumerated by dark-field microscopy. Spirochete motility and morphology are determined in 10 random fields in a double-blind fashion, and cells are considered killed when complete loss of motility and refractivity is observed (15). The earlier time points were used to distinguish growth inhibition from direct killing, since either effect will result in reduced numbers at 48 h. We have previously shown excellent concordance of the dark-field microscopy assay with the [³H]adenine regrowth assay for determination of *B. burgdorferi* numbers (15).

Lysates of U-Cyt were incubated with *B. burgdorferi* (5 *B. burgdorferi* organisms to 1 U-Cyt equivalent) in 50 μl of assay buffer (50 mM HEPES [pH 7.55], 150 mM NaCl, 5.4 mM glucose, and 1 mM CaCl₂) for 1 h at 37°C prior to threefold dilution with BSK and further incubation at 33°C for 48 h of regrowth. Recombinant human calprotectin (MRP 8/MRP 14) (5) was the generous gift of Craig Vanderkooi and Walter Chazin (Vanderbilt University, Nashville, Tenn.). For studies with multiple concentrations of calprotectin, *B. burgdorferi* was directly incubated in BSK with 20% (vol/vol) assay buffer at 33°C for 1 to 48 h. The IC₅₀ and error were calculated as described above.

For antibody blocking studies, 4 μg of monoclonal anticalprotectin antibody Mac 387 (Dako Corporation, Carpinteria, Calif.) was incubated with 10 μg of U-Cyt lysate protein, as determined by the Bradford assay, or calprotectin (125 μg/ml) in a 45-μl volume for 30 min at 37°C and then for 30 min at 4°C (20) before addition of *B. burgdorferi* (5 × 10⁶ organisms/ml). The final experimental volume (50 μl) was incubated directly in BSK with 20% (vol/vol) assay buffer at 33°C for 48 h. Control incubations of *B. burgdorferi* alone with either Mac 387 or a control monoclonal antibody, mouse anti-CD15 (4 μg), a PMN surface marker (Dako Corporation), showed no effect on spirochete growth. Antibody buffers were exchanged into assay buffer immediately before use. Significance was assessed by analysis of variance and paired *t* tests, one-tailed.

For Zn²⁺ reversal studies, *B. burgdorferi* was incubated directly in the presence or absence of calprotectin (90 and 125 μg/ml) or U-Cyt lysates (5 *B. burgdorferi* organisms to 1 U-Cyt equivalent) and 3 μM ZnCl₂ in 50% BSK with 15 mM HEPES (pH 7.5). BSK medium contains 28 μM Zn²⁺, as determined by inductively coupled plasma-mass spectrometry (Chemical Analysis Laboratory, University of Georgia, Athens). Significance was assessed by paired *t* tests, one-tailed.

Microscopic assessment of *B. burgdorferi* viability was performed to distinguish

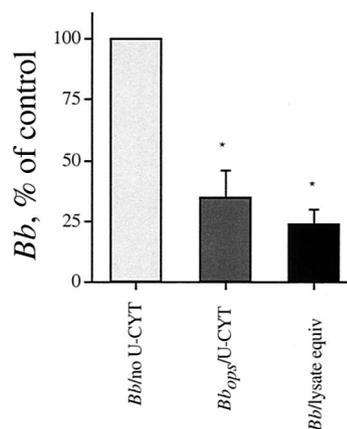


FIG. 1. U-Cyt and lysates of U-Cyt reduce *B. burgdorferi* number. Intact U-Cyt incubated with opsonized *B. burgdorferi* (Bb_{ops}) cells were compared with paired freeze-thawed lysates of U-Cyt incubated with *B. burgdorferi* (Bb; 5 *B. burgdorferi* organisms to 1 U-Cyt, intact or lysate) (*n* = 3). *B. burgdorferi* number was determined by either [³H]adenine uptake or dark-field microscopic assay after 48 h. Experimental significance was assessed by analysis of variance (degrees of freedom = 2, *P* = 0.0012); individual differences were assessed with a paired *t* test. Both intact U-Cyt and paired lysates of U-Cyt reduced *B. burgdorferi* number significantly (*n* = 3). The asterisk indicates that the difference in values for paired U-Cyt or lysates of U-Cyt and *B. burgdorferi* alone is statistically significant (*P* = 0.014 and 0.004, respectively). U-Cyt did not have an appreciable anti-*Borrelia* effect unless the *B. burgdorferi* was opsonized (9). Lysates of U-Cyt given *B. burgdorferi* were at least as effective as intact U-Cyt given opsonized *B. burgdorferi*, but the two groups were not significantly different from each other (*n* = 3, *P* = 0.1).

between growth inhibition and direct killing. *B. burgdorferi* (10⁷ organisms/ml) was incubated for 1 h in the presence of highly inhibitory levels of either calprotectin (225 μg/ml in assay buffer) or LL-37 (200 μg/ml in phosphate-buffered saline–5.4 mM glucose), the active fragment of the PMN granule component cathelicidin, the kind gift of Birgitta Agerberth (Karolinska Institutet, Stockholm, Sweden) (6). After 1 h at 37°C, the *B. burgdorferi* was labeled for viability with a supravital stain (Live/dead kit; Molecular Probes, Inc., Eugene, Oreg.). Cells were examined on a Zeiss Axiovert microscope with a 510 long-pass filter under a 100× oil immersion lens.

Western blot analysis of cellular calprotectin levels. U-Cyt and PMN lysates were separated by sodium dodecyl sulfate–20% polyacrylamide gel electrophoresis (SDS–20% PAGE) and electroblotted to nitrocellulose membranes. The membranes were blocked for 1 h at room temperature in phosphate-buffered saline with 5% bovine serum albumin and then incubated with monoclonal anti-human MAC 387 antibody, specific for both subunits of calprotectin, at 1:500 for 3 h, washed, and incubated with alkaline phosphatase-conjugated anti-mouse immunoglobulin G (Vector Laboratories, Burlingame, Calif.) for 30 min at 1:500. The blots were developed with 5-bromo-4-chloro-3-indolylphosphate–nitroblue tetrazolium (KPL, Gaithersburg, Md.).

RESULTS

U-Cyt and lysates of U-Cyt reduce *B. burgdorferi* number.

We undertook the present study in order to elucidate the anti-*Borrelia* activity of U-Cyt. We showed previously that even after 48 h of incubation, intact U-Cyt significantly reduced the number of *B. burgdorferi* organisms only when the spirochetes had been opsonized with specific antibody (9). A similar reduction in the number of *B. burgdorferi* organisms was now apparent when either intact U-Cyt or their lysates were employed (*n* = 3) (Fig. 1). Lysates of U-Cyt reduced the number of opsonized *B. burgdorferi* organisms over 48 h by 50% when

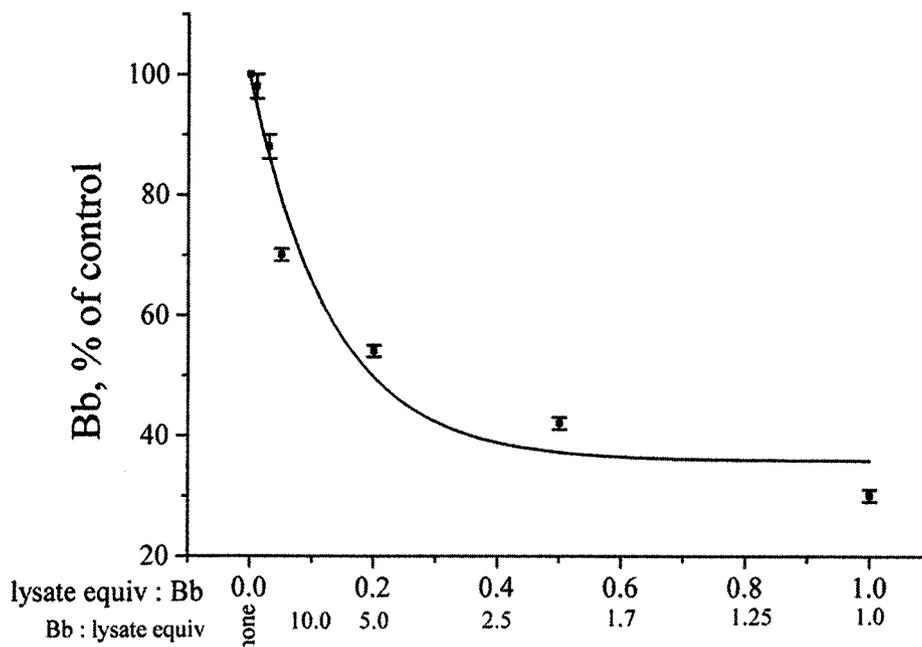


FIG. 2. The inhibitory effect of lysates of U-Cyt on *B. burgdorferi* number is dose dependent. Lysates of U-Cyt were incubated with *B. burgdorferi* (Bb) at 5×10^6 *B. burgdorferi* organisms/ml for 1 h at 37°C and then at 33°C for 48 h for regrowth. The IC₅₀ (50% inhibition) was 8 *B. burgdorferi* organisms to 1 U-Cyt equivalent. The *B. burgdorferi* number was determined microscopically at 48 h. Inhibition is expressed as a percentage of the value for the control, *B. burgdorferi* incubated in buffer alone.

incubated at a ratio of 8 *B. burgdorferi* organisms to 1 U-Cyt equivalent; the maximum inhibition in *B. burgdorferi* number observed was 35% of the control value ($n = 5$) (Fig. 2) under the experimental conditions employed. In granule-poor cytoplasts, these findings suggest the presence of a previously unrecognized cytosolic anti-*Borrelia* activity.

U-Cyt and PMN contain similar amounts of calprotectin. We assessed the levels of calprotectin in serial 10-fold dilutions (10^6 to 10^3) of U-Cyt and PMN by Western blotting. Despite their reduced size, U-Cyt cell lysates and whole PMN contained similar quantities of the heterodimeric calprotectin (Fig. 3). Repeated Western blots of multiple U-Cyt prepara-

tions demonstrated equivalent distributions of the two bands in U-Cyt prepared from PMN from other donors (data not shown).

Anti-*Borrelia* effect of calprotectin. We incubated *B. burgdorferi* cells with calprotectin to determine whether they were sensitive to this protein. We found a dose-dependent reduction in the number of *B. burgdorferi* organisms when the spirochetes were incubated with physiologically relevant concentrations of calprotectin (5 $\mu\text{g}/10^6$ PMN) (Table 1), corresponding to cytosolic concentrations of 5 to 15 mg/ml (4, 7, 27). At 48 h, the IC₅₀ was 140 μg of calprotectin per ml; the maximum inhibition was 72%, i.e., only $28\% \pm 2.2\%$ of the control remained when *B. burgdorferi* cells were incubated with 300 μg of calprotectin per ml (Table 1).

Anticalprotectin antibody reduces the anti-*Borrelia* activity of lysates of U-Cyt and of calprotectin. To confirm that a portion of the anti-*Borrelia* effect of lysates of U-Cyt was a result of the action of calprotectin, we incubated lysates of

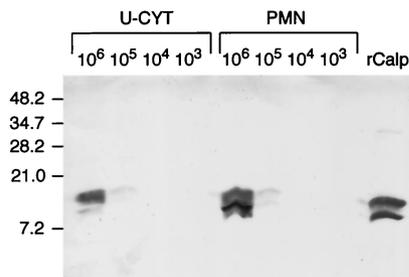


FIG. 3. PMN and U-Cyt contain similar amounts of calprotectin. A total of 10^6 to 10^3 cell equivalents of PMN and U-Cyt were separated on an SDS-20% PAGE gel and electroblotted to a nitrocellulose membrane. Cellular recombinant human calprotectin (rCalp) was visualized with the monoclonal anti-human Mac 387 antibody, specific for both subunits of calprotectin; the control antibody showed no bands (not shown). The bands visualized after 10-fold dilutions of PMN and U-Cyt were similar. Calprotectin (2.5 μg) is shown at the right. Molecular weight standards are indicated on the left.

TABLE 1. Effect of calprotectin on growth of spirochetes at 48 h^a

Calprotectin ($\mu\text{g}/\text{ml}$)	No. of <i>B. burgdorferi</i> organisms (% of control) \pm SEM ($n = 2-4$)
0	100
18	68 ± 0.5
50	65 ± 4.0
150	50 ± 4.2
300	28 ± 2.2

^a Cells were incubated in BSK with 20% assay buffer (50 mM HEPES [pH 7.55], 150 mM NaCl, 5.4 mM glucose, and 1 mM CaCl₂) at 33°C for 48 h. The IC₅₀ of calprotectin was determined to be 140 $\mu\text{g}/\text{ml}$.

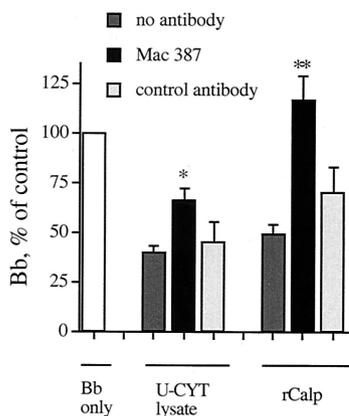


FIG. 4. Anticalprotectin antibody reduces the anti-*Borrelia* activity of lysates of U-Cyt and of calprotectin itself. *B. burgdorferi* (Bb) was incubated with lysates of U-Cyt at a ratio of 5 *B. burgdorferi* organisms per U-Cyt equivalent or with calprotectin (rCalp) at 125 $\mu\text{g/ml}$. The average number of *B. burgdorferi* cells relative to the control after 48 h is shown for lysates of U-Cyt ($n = 7$) and calprotectin ($n = 6$). The anti-*Borrelia* activity of lysates of U-Cyt and calprotectin was inhibited when either was incubated with specific antibody ($n = 6$ to 7). A control PMN monoclonal antibody did not significantly affect *B. burgdorferi* growth ($n = 3$ to 5). These two antibodies incubated with *B. burgdorferi* alone did not significantly affect *B. burgdorferi* growth ($n = 5$, not significant). Experimental significance was assessed by analysis of variance for lysate of U-Cyt (degrees of freedom = 3, $P = 0.0016$) and calprotectin (degrees of freedom = 3, $P = 0.0015$); individual differences were assessed with a paired t test. The asterisk indicates that the differences in values for lysates of U-Cyt with specific antibody and lysates of U-Cyt alone or lysates of U-Cyt with control antibody were significant ($P = 0.001$ and 0.04, respectively). The double asterisk indicates the significance of the corresponding differences for calprotectin ($P = 0.0006$ and 0.03, respectively).

U-Cyt or calprotectin with the blocking monoclonal antibody Mac 387 or a control antibody (anti-CD15). Incubation of *B. burgdorferi* alone with either Mac 387 or control did not significantly affect *B. burgdorferi* growth ($n = 5$, not significant). The reduction in *B. burgdorferi* number resulting from incubation with lysates of U-Cyt was significantly blocked by specific anticalprotectin antibody but not control antibody (Fig. 4). In addition, as expected, Mac 387 completely removed the anti-*Borrelia* effect of calprotectin (Fig. 4); the control antibody did not.

Zn²⁺ reverses the anti-*Borrelia* activity of lysates of U-Cyt and of calprotectin. The antimicrobial effects of calprotectin on *Candida albicans* are reversible by 10 to 30 μM Zn²⁺ (3, 23). We examined the effect of Zn²⁺ on the anti-*Borrelia* activity of lysates of U-Cyt and of calprotectin. The incremental concentration of only 3 μM Zn²⁺ partially reversed the anti-*Borrelia* activity of lysates of U-Cyt and completely reversed the ability of calprotectin to reduce *B. burgdorferi* number (Fig. 5). The BSK medium, used at 50% (vol/vol) in this assay, contributes 14 μM Zn²⁺ (BSK contains 28 μM Zn²⁺, as determined by mass spectrometry; see Materials and Methods). This final concentration (17 μM) is in the range employed for reversal of anti-*Candida* activity (above), suggesting that, as with *Candida* (3, 21, 22), chelation of Zn²⁺ may be responsible for the anti-*Borrelia* effect. Additions of higher concentrations of Zn²⁺ did not increase the reversal of anti-*Borrelia* activity by U-Cyt lysates (data not shown).

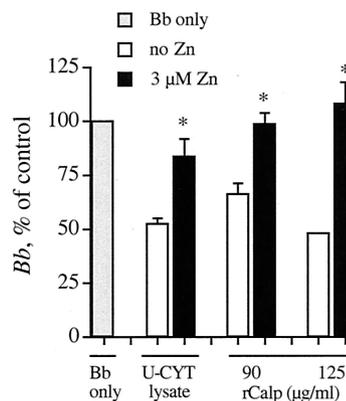


FIG. 5. Zn²⁺ reverses the anti-*Borrelia* activity of lysates of U-Cyt and of calprotectin. *B. burgdorferi* (Bb) was incubated with U-Cyt lysates (5 *B. burgdorferi* organisms to 1 U-Cyt equivalent) and calprotectin (rCalp; 90 or 125 $\mu\text{g/ml}$). Average numbers of *B. burgdorferi* relative to controls after 48 h are shown. The anti-*Borrelia* activity of U-Cyt lysates and calprotectin at either concentration was reversed by 3 μM Zn²⁺. In three experiments, the asterisks indicate statistical significance of the reversals when Zn²⁺ was added ($P = 0.04$, 0.03, and 0.01, respectively).

Calprotectin is bacteriostatic. To clarify the mechanism of the anti-*Borrelia* effect of calprotectin, we examined treated spirochetes microscopically. When enumerated under dark-field microscopy, numbers of *B. burgdorferi* organisms treated with calprotectin remained essentially constant at 1, 24, and 48 h. During the same interval, spirochete controls increased two- to threefold (data not shown). In contrast, spirochetes treated with LL-37, the active fragment of the PMN granule protein cathelicidin (6), employed here for comparison, showed an immediate reduction in the number of viable *B. burgdorferi* organisms by 1 h (Fig. 6), followed by proliferation of the remaining viable *B. burgdorferi* organisms over the subsequent 48 h. With fluorescence microscopy and a supravital stain, we found that after 1 h, *B. burgdorferi* with calprotectin remained viable; *B. burgdorferi* with LL-37 was being killed (Fig. 6). When examined after 48 h of incubation, the numbers of spirochetes treated with LL-37 were 9% of the control numbers (9); those treated with calprotectin were 28% of the control numbers. We conclude that the reduction in the number of *B. burgdorferi* organisms incubated with calprotectin observed at 48 h was the result of bacteriostasis rather than a direct lethal effect, such as that achieved by LL-37.

DISCUSSION

The extensive antimicrobial mechanisms of PMN include contributions from oxygen and nitrogen intermediates and granule constituents that can act both intra- and extracellularly (16). We previously identified the importance of the PMN granule components elastase, bactericidal/permeability-increasing protein, LL-37, and human neutrophil peptide-1 for efficient killing of *B. burgdorferi* and found that killing can also occur extracellularly (9, 14). U-Cyt, anucleate PMN fragments depleted of granules but retaining motility and respiratory burst activity, have proven to be a useful tool for identifying contributions from various portions of the web of cellular an-

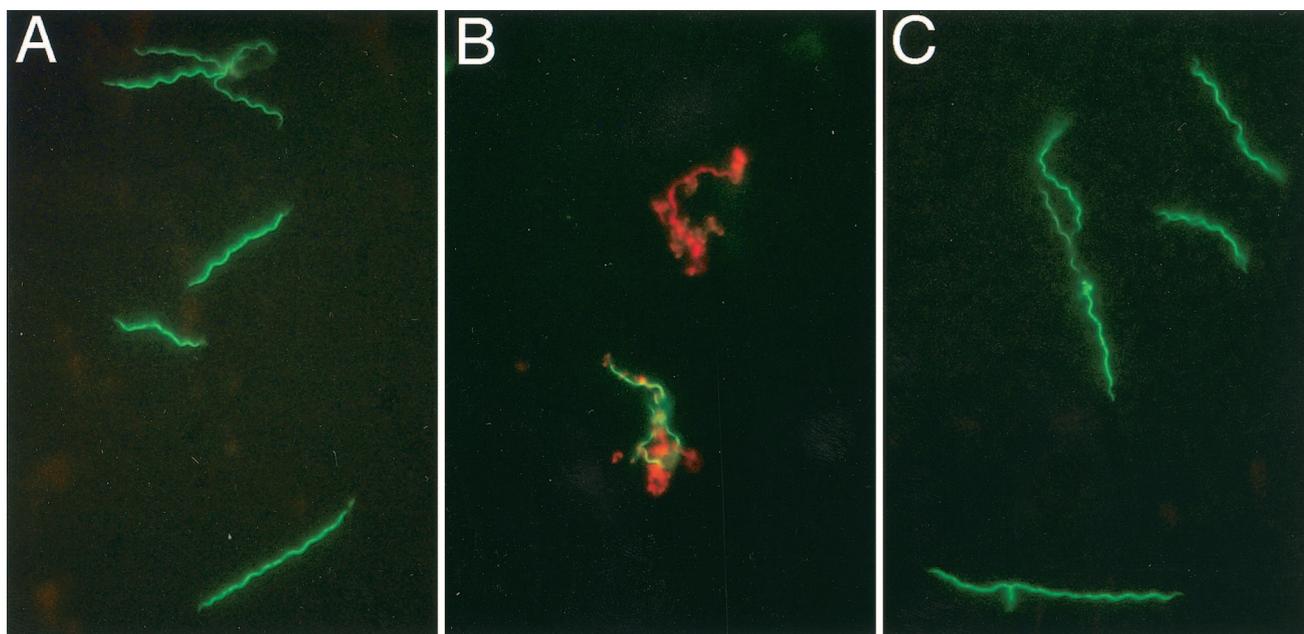


FIG. 6. Calprotectin has no immediate effect on *B. burgdorferi* viability. *B. burgdorferi* was incubated with LL-37, the active fragment of the PMN microbicidal granule component cathelicidin, or with calprotectin for 1 h before being labeled with a vital stain and examined by fluorescence microscopy, with a 100 \times objective and a 510-nm long-pass filter. Live organisms stain green; killed organisms stain red. (A) Buffer control; (B) LL-37 (200 $\mu\text{g/ml}$); (C) calprotectin (300 $\mu\text{g/ml}$). After 1 h in LL-37, *B. burgdorferi* is being killed; *B. burgdorferi* controls and those treated with calprotectin remain viable.

timicrobial mechanisms (9, 11, 24). For example, with cytoplasts we were able to show that nitric oxide, not previously detected in human PMN, contributed to killing of *Staphylococcus aureus* (11) and that targeted oxidant generation by PMN did not kill *Candida albicans* hyphae unless granule constituents were added back (24).

When intact U-Cyt were studied in the presence of inhibitors or scavengers of O_2^- , H_2O_2 , and OH^\cdot , no reduction in their killing of *B. burgdorferi* was found (9). We have now shown that lysates of U-Cyt were also able to reduce the number of *B. burgdorferi* organisms, as well as intact U-Cyt given opsonized *B. burgdorferi*, confirming the lack of contribution of membrane-bound oxidative mechanisms seen previously (Fig. 1) (9). Dose-dependent reduction of *B. burgdorferi* number by lysates of U-Cyt (Fig. 2) established that an additional anti-*Borrelia* mechanism was present, and we now identify it, at least in part, as the cytosolic protein calprotectin. The susceptibility of *B. burgdorferi* to calprotectin was confirmed with recombinant calprotectin (Table 1). Its presence in U-Cyt, in amounts comparable to those found in whole PMN, was confirmed by Western blot analysis (Fig. 3). Finally, the anti-*Borrelia* activity of lysates of both U-Cyt and calprotectin was significantly reversed, in part or completely, by incubation with the calprotectin-specific antibody Mac 387 (Fig. 4).

Competition for iron is a well-documented mechanism of antimicrobial defense; *B. burgdorferi* is unusual in that it requires Zn^{2+} for growth, not Fe^{2+} (17). This is in keeping with our previous demonstration that *B. burgdorferi* is not susceptible to the iron chelator lactoferrin (9). In general, bacteria are reported to require less zinc than fungi (26), so it is not surprising that susceptibility of fungi to calprotectin, which binds zinc, has received the most attention (25). Studies have shown

that the antifungal ability of calprotectin is reversible upon addition of Zn^{2+} (13, 19, 20, 23). Zn binding to calprotectin leads to conformational changes that alter its ability to bind arachidonic acid (8), but it is unclear how this affects its antimicrobial functions. Multiple studies have shown that the mechanism of the antimicrobial action of calprotectin is sequestration or deprivation of Zn (3, 21, 22). Our studies show that *B. burgdorferi* is as susceptible to calprotectin as such fungi as *Candida albicans* and *Cryptococcus neoformans*, for which the MIC is 4 to 128 $\mu\text{g/ml}$ (25), versus a MIC of 18 to 300 $\mu\text{g/ml}$ for *B. burgdorferi*. This susceptibility to calprotectin was reversed upon addition of 3 μM Zn^{2+} (Fig. 5), suggesting that Zn^{2+} deprivation is the probable mechanism of action, as has been shown for *Candida albicans* (3).

In contrast to the previously studied individual granule components, which require specific controlled conditions for optimal activity (9), calprotectin exerted its anti-*Borrelia* activity in the complex growth medium BSK. Thus, unlike granule proteins, many of which are active only in the environment of the phagolysosome or have decreased activity in other physiologically relevant conditions, calprotectin can function as an extracellular antimicrobial factor. Indeed, ambient calprotectin levels correlate with PMN death and lysis (27). In bacterial infections, plasma levels as high as 11 $\mu\text{g/ml}$ are seen; in 41 patients with rheumatoid arthritis, synovial fluid contained a median of 18 $\mu\text{g/ml}$ (range, 2 to 375 $\mu\text{g/ml}$) (plasma controls generally contain ≤ 1 $\mu\text{g/ml}$) (7). A disorder of calprotectin regulation resulting in increased plasma levels has recently been reported; these patients also experience arthritis (18). Our data show that a portion of the anti-*B. burgdorferi* activity of lysates of U-Cyt was reversible by a calprotectin-specific antibody (Fig. 4) and by 3 μM Zn^{2+} (Fig. 5), indicating that

cellular calprotectin contributes to anti-*Borrelia* PMN activity. The absence of early killing of *B. burgdorferi* (Fig. 6) indicates that calprotectin activity against *B. burgdorferi* is bacteriostatic in nature. The possible modulatory role of calprotectin at inflammatory sites in Lyme disease remains to be elucidated.

These studies indicate that physiologic concentrations of the cytosolic protein calprotectin can contribute to the anti-*Borrelia* activity of PMN. Future studies may identify further roles for this abundant cytosolic protein in a cell primarily recognized for its oxidative and granular antibacterial mechanisms, particularly where zinc dependence proves to be important.

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REFERENCES

- Barthold, S. W., and M. de Souza. 1995. Exacerbation of Lyme arthritis in beige mice. *J. Infect. Dis.* **172**:778–784.
- Barthold, S. W., M. de Souza, E. Fikrig, and D. H. Persing. 1992. Lyme borreliosis in the laboratory mouse, p. 223–242. *In* S. E. Schutzer (ed.), *Lyme disease: molecular and immunologic approaches*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- Clohessy, P. A., and B. E. Golden. 1995. Calprotectin-mediated zinc chelation as a biostatic mechanism in host defence. *Scand. J. Immunol.* **42**:551–556.
- Edgeworth, J., M. Gorman, R. Bennett, P. Freemont, and N. Hogg. 1991. Identification of p8, 14 as a highly abundant heterodimeric calcium binding protein complex of myeloid cells. *J. Biol. Chem.* **266**:7706–7713.
- Hunter, M. J., and W. J. Chazin. 1998. High level expression and dimer characterization of the S100 EF-hand proteins, migration inhibitory factor-related proteins 8 and 14. *J. Biol. Chem.* **273**:12427–12435.
- Johansson, J., G. H. Gudmundsson, M. E. Rottenberg, K. D. Berndt, and B. Agerberth. 1998. Conformation-dependent antibacterial activity of the naturally occurring human peptide LL-37. *J. Biol. Chem.* **273**:3718–3724.
- Johne, B., M. K. Fagerhol, T. Lyberg, H. Prydz, P. Brandtzaeg, C. F. Naess-Andresen, and I. Dale. 1997. Functional and clinical aspects of the myelomonocyte protein calprotectin. *J. Clin. Pathol. Mol. Pathol.* **50**:113–123.
- Kerkhoff, C., T. Vogl, W. Nacken, C. Sopalla, and C. Sorg. 1999. Zinc binding reverses the calcium-induced arachidonic acid-binding capacity of the S100A8/A9 protein complex. *FEBS Lett.* **460**:134–138.
- Lusitani, D., S. E. Malawista, and R. R. Montgomery. 2002. *Borrelia burgdorferi* are susceptible to killing by a variety of human polymorphonuclear leukocyte components. *J. Infect. Dis.* **185**:797–804.
- Malawista, S. E. 2001. Lyme disease, p. 2629–2648. *In* W. J. Koopman (ed.), *Arthritis and allied conditions: a textbook of rheumatology*, 14th ed., vol. 2. Lippincott Williams & Wilkins, Philadelphia, Pa.
- Malawista, S. E., R. R. Montgomery, and G. Van Blaricom. 1992. Evidence for reactive nitrogen intermediates in killing of staphylococci by human neutrophil cytoplasm: a new microbicidal pathway for polymorphonuclear leukocytes. *J. Clin. Investig.* **90**:631–636.
- Malawista, S. E., and G. Van Blaricom. 1987. Cytoplasm made from human blood polymorphonuclear leukocytes with or without heat: preservation of both motile function and respiratory burst oxidase activity. *Proc. Natl. Acad. Sci. USA* **84**:454–458.
- Mambula, S. S., E. R. Simons, R. Hastey, M. E. Selsted, and S. M. Levitz. 2000. Human neutrophil-mediated nonoxidative antifungal activity against *Cryptococcus neoformans*. *Infect. Immun.* **68**:6257–6264.
- Montgomery, R. R., D. Lusitani, A. de Boisfleury Chevance, and S. E. Malawista. 2002. Human phagocytic cells in the early innate immune response to *Borrelia burgdorferi*. *J. Infect. Dis.* **185**:1773–1779.
- Pal, U., R. R. Montgomery, D. Lusitani, P. Voet, V. Weynants, S. E. Malawista, Y. Lobet, and E. Fikrig. 2001. Inhibition of *Borrelia burgdorferi*-tick interactions *in vivo* by outer surface protein A antibody. *J. Immunol.* **166**:7398–7403.
- Philips, M. R., and B. N. Cronstein. 2001. Structure and function of neutrophils, p. 358–382. *In* W. J. Koopman (ed.), *Arthritis and allied conditions: a textbook of rheumatology*, 14th ed., vol. 1. Lippincott Williams & Wilkins, Philadelphia, Pa.
- Posey, J. E., and F. C. Gherardini. 2000. Lack of a role for iron in the Lyme disease pathogen. *Science* **288**:1651–1653.
- Sampson, B., M. K. Fagerhol, C. Sunderkotter, B. Golden, P. Richmond, N. Klein, I. Z. Kovar, J. H. Beattie, B. Wolska-Kusnierz, Y. Saito, and J. Roth. 2002. Hyperzincaemia and hypercalprotectinaemia: a new disorder of zinc metabolism. *Lancet* **360**:1742–1745.
- Santhanagopalan, V., B. L. Hahn, B. E. Dunn, J. H. Weissner, and P. G. Sohnle. 1995. Antimicrobial activity of calprotectin isolated from human empyema fluid supernatants. *Clin. Immunol. Immunopathol.* **76**:285–290.
- Sohnle, P. G., C. Collins-Lech, and J. H. Weissner. 1991. Antimicrobial activity of an abundant calcium-binding protein in the cytoplasm of human neutrophils. *J. Infect. Dis.* **163**:187–192.
- Sohnle, P. G., B. L. Hahn, and R. Karmarkar. 2001. Effect of metals on *Candida albicans* growth in the presence of chemical chelators and human abscess fluid. *J. Lab. Clin. Med.* **137**:284–289.
- Sohnle, P. G., B. L. Hahn, and V. Santhanagopalan. 1996. Inhibition of *Candida albicans* growth by calprotectin in the absence of direct contact with the organisms. *J. Infect. Dis.* **174**:1369–1372.
- Sohnle, P. G., M. J. Hunter, B. Hahn, and W. J. Chazin. 2000. Zinc-reversible antimicrobial activity of calprotectin (migration inhibitory factor-related proteins 8 and 14). *J. Infect. Dis.* **182**:1272–1275.
- Stein, D. K., S. E. Malawista, G. Van Blaricom, and R. D. Diamond. 1995. Cytoplasm generate oxidants and kill *Staphylococcus aureus* but require added neutrophil granule constituents for fungicidal activity against *Candida albicans* hyphae. *J. Infect. Dis.* **172**:511–520.
- Steinbakk, M., C. F. Naess-Andresen, E. Lingaas, I. Dale, P. Brandtzaeg, and M. K. Fagerhol. 1990. Antimicrobial actions of calcium binding leukocyte L1 protein calprotectin. *Lancet* **336**:763–765.
- Sugarman, B. 1983. Zinc and infection. *Rev. Infect. Dis.* **5**:137–147.
- Voganatsi, A., A. Panyutich, K. T. Miyasaki, and R. K. Murthy. 2001. Mechanism of extracellular release of human neutrophil calprotectin complex. *J. Leukoc. Biol.* **70**:130–134.
- Yang, L., J. H. Weis, E. Eichwald, C. P. Kolbert, D. H. Persing, and J. J. Weis. 1994. Heritable susceptibility to severe *Borrelia burgdorferi*-induced arthritis is dominant and is associated with persistence of large numbers of spirochetes in tissues. *Infect. Immun.* **62**:492–500.