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Human Neutrophil Calprotectin Reduces the Susceptibility of *Borrelia burgdorferi* to Penicillin

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***Borrelia burgdorferi*, the spirochetal agent of Lyme disease, is susceptible to killing by a variety of polymorphonuclear leukocyte (PMN) components. Some are most effective against metabolically active *B. burgdorferi*. The abundant PMN cytoplasmic protein calprotectin, elevated 10- to 100-fold in inflammation, inhibits the growth of spirochetes through chelation of the essential cation, Zn. Since the action of some therapeutic antibiotics depends on bacterial division, we investigated the antibiotic sensitivities of spirochetes in calprotectin. In physiologic calprotectin, *B. burgdorferi* is not eliminated by therapeutic doses of penicillin G; in contrast, doxycycline is effective. Calprotectin may modify the clearance of spirochetes at sites of inflammation.**

Lyme disease is caused by the spirochete *Borrelia burgdorferi* and is characterized by the hallmark rash, erythema migrans, and subsequent inflammatory processes that include arthritis, carditis, and neurological symptoms (14). An enigma is the persistence of spirochetes despite an intact host defense. We have previously shown appropriate site-specific activation of the innate immune response in Lyme borreliosis in vivo and thus have no evidence for impaired immune responses as an explanation for spirochete persistence (18). Spirochetes in vitro are eliminated by numerous effective clearance mechanisms including phagocytic and extracellular killing by macrophages, neutrophils (polymorphonuclear leukocytes [PMN]), and granule components of PMN (4, 12, 16, 17). However, the adaptive arm of the immune system is key for resolution of disease, as can be seen from the prolonged and severe infection in SCID mice which lack that response (1, 30) and impaired efficiency of clearance of *B. burgdorferi* by PMN when specific antibody is not present (12, 16). Possible explanations for *B. burgdorferi* evasion of host killing mechanisms include the well-characterized changes in spirochete protein expression in the host-adapted state and residence in immunoprivileged sites (3, 21, 23, 26).

Recently, we found that the abundant PMN cytoplasmic protein calprotectin is a potent bacteriostatic compound for spirochetes (13). Calprotectin (L1, MRP8/14, S100A8/A9) is a noncovalently associated heterodimeric protein that comprises 45% of the cytosolic protein of the PMN and inhibits the growth of *Candida* or bacteria by chelation of zinc (6, 9, 11, 15, 19, 22, 31, 32). Using quantitative microscopic and regrowth assays, we demonstrated that the anti-*B. burgdorferi* activity of PMN lysates and recombinant calprotectin is reversed by specific antibody to calprotectin and by Zn^{2+}, a cation essential for the growth of *B. burgdorferi* (13, 25).

Calprotectin can be elevated 10- to 100-fold in disease states and can be used as a measure of inflammation in several pathological conditions including rheumatoid arthritis and inflammatory bowel disease (9). In our studies, we noted that when *B. burgdorferi* was exposed to a physiological concentration of calprotectin, spirochetes entered a static, nondividing state but were not directly killed. A natural consequence of growth inhibition is that such organisms are no longer sensitive to antibiotics that target dividing organisms. We have investigated whether the nondividing state of spirochetes in calprotectin, such as may occur in an inflamed joint, renders them less susceptible to killing by therapeutic doses of certain antibiotics.

Cultivation of bacteria and assessment of *B. burgdorferi* number. Low-passage, virulent *B. burgdorferi*, strain N40, was grown to logarithmic phase (10^6 to 10^8/ml) in Barbour-Stoenner-Kelley II (BSK) media with added antibiotics (2 mg/ml phosphomycin, 5 mg/ml rifampin, 250 μg/ml amphotericin B; Sigma, St. Louis, MO) at 33°C (12). *B. burgdorferi* spirochetes were pelleted (10 min, 3,000 × *g*, 25°C) and enumerated in BSK using a Petroff-Hausser hemocytometer (Hausser Scientific Partnership, Horsham, PA) under dark-field microscopy. For the growth inhibition assay, 5×10^6 *B. burgdorferi* spirochetes/ml were incubated in 0.05 ml BSK containing 20% (vol/vol) assay buffer (50 mM HEPES, 150 mM NaCl, 5.4 mM glucose, 1 mM $CaCl_2$, pH 7.55). Duplicate samples of untreated spirochetes were used in each experiment. For treated samples, assay buffer contained, as needed, the following: calprotectin (150 to 600 μg/ml), penicillin (0.01, 0.05, and 0.1 μg/ml), or doxycycline (0.1, 0.5, and 1.0 μg/ml; made on the day of the experiment) in the presence or absence of added $ZnCl_2$ (3, 10, or 30 μM). *B. burgdorferi* viability was assessed after 48 h of incubation as described previously (12, 13) either by vital staining using the Live/dead kit (*Bac*Light; Molecular Probes/Invitrogen, Carlsbad, CA) under fluorescence microscopy with a 510 long-pass filter or by direct visual quantitation of spirochete motility and morphology using dark-field microscopy. For vital staining, a 20-μl aliquot was stained with 1 μl of *Bac*light dye and incubated for 15 min in the dark before assessment of viability. For dark-field examination, a 5-μl aliquot of the *B. burgdorferi* sample was diluted with 10 μl fresh BSK and 4.5 μl was placed under an 18- by 18-mm coverslip for enumeration of living *B. burgdorferi.* Spirochetes in 10 to 15 random fields were enumerated in a double-blind fashion, and

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B. burgdorferi was considered killed when stained red by a fluorescent vital stain (×100 objective) or when complete loss of motility and refractivity was observed in dark-field examination (×43 objective). Determination of viability was equivalent from the two assays, and we have previously shown excellent concordance of the microscopic assay with the [^{3}H]adenine regrowth assay for detection of *B. burgdorferi* viability (24). The percentage of viable spirochetes was calculated as (no. of living *B. burgdorferi* spirochetes treated/no. of living *B. burgdorferi* spirochetes untreated) × 100. Significance was assessed by the paired *t* test, two-tailed. Lysates of PMN were prepared by freeze-thaw of cells isolated from healthy volunteers in accordance with guidelines of the Human Investigation Committee of Yale University School of Medicine (12).

Purification and detection of calprotectin. Clones for the light and heavy chains of calprotectin were generously provided by Walter Chazin, Vanderbilt University, Nashville, TN. Recombinant calprotectin (rCalp) proteins were produced according to the standard procedures of the Chazin laboratory (8). Recombinant monomer proteins were purified by high-performance liquid chromatography, and calprotectin heterodimer (rCalp) was further purified by fast-performance liquid chromatography. Purity of calprotectin was confirmed by Western blotting. Calprotectin levels in stored arthritis patient joint fluids were determined by enzyme-linked immunosorbent assay using rCalp as a standard. Samples from stored patient joint fluids were used without patient identifiers in accordance with the regulations of Yale University's Human Investigations Committee. Plates were coated with rCalp standard or joint fluid samples in 1 M $NaHCO_3$ buffer, pH 9.6, for 2 h at room temperature, washed three times in phosphate-buffered saline (PBS)–0.1% Tween 20, blocked with PBS–3% bovine serum albumin at room temperature for 1 h, washed three times before detection of protein by biotinylated anti-calprotectin antibody at 0.5 μg/ml (Bachem Bioscience, Inc., King of Prussia, PA), and visualized using the Vectastain Elite colorimetric assay (Vector Laboratories, Burlingame, CA). Detection is linear from 10 ng to 20 μg of calprotectin. Data shown are means ± standard errors of means for three determinations per patient sample.

Calprotectin inhibition of *B. burgdorferi* growth is dose dependent and long lasting. We have shown previously by quantitative culture and by vital staining that calprotectin inhibits the growth of *B. burgdorferi* in a zinc-reversible manner but does not lead directly to spirochete death (13). To assess what level of inhibition is likely to occur in vivo, we incubated spirochetes in BSK growth medium with doses of calprotectin for up to 96 h. A dramatic reduction in growth of spirochete numbers was observed (Fig. 1A) at concentrations of calprotectin reported previously at sites of inflammation (9). When spirochetes were incubated with calprotectin at 300 μg/ml for 7 days and then restored to fresh BSK medium, growth remained inhibited for several days but recovered thereafter (Fig. 1B). After 14 days in calprotectin (300 μg/ml), spirochetes remained viable and showed a similar slow recovery when restored to fresh BSK (data not shown).

Calprotectin protects spirochetes from killing by penicillin G. Antimicrobial therapy that depends on cell wall synthesis targets dividing bacteria, and thus, we hypothesized that spirochetes incubated in calprotectin would be resistant to killing by

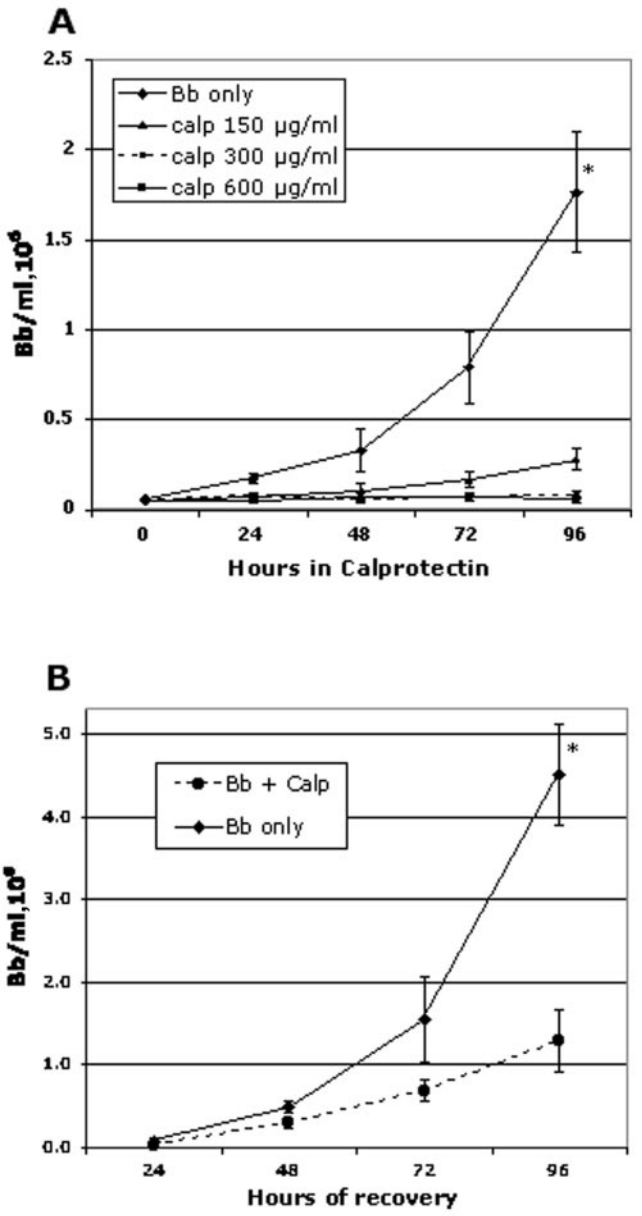

FIG. 1. Calprotectin inhibition of spirochete growth is profound and long lasting. Spirochetes (5×10^6) were incubated in 0.05 ml BSK containing 20% (vol/vol) assay buffer and (A) doses of calprotectin (150 μg/ml, 300 μg/ml, 600 μg/ml) for 96 h or (B) calprotectin (300 μg/ml) for 7 days followed by recovery in fresh BSK. *B. burgdorferi* spirochetes were enumerated daily during regrowth periods. A 5-μl aliquot was diluted with 10 μl fresh BSK for enumeration of living *Borrelia burgdorferi* in 10 to 15 random fields under dark-field microscopy, and the average percent viability was determined as described previously (13). The statistical significance of difference in growth of spirochetes without treatment (Bb only) versus those treated with calprotectin (calp) (all concentrations) is a *P* value of 0.0001; $n = 3$. The statistical significance of difference in recovery growth of spirochetes after treatment for 7 days with calprotectin at 300 μg/ml at 96 h is a *P* value of 0.005; earlier time points do not reach significance. Data shown are averages for four separate experiments.

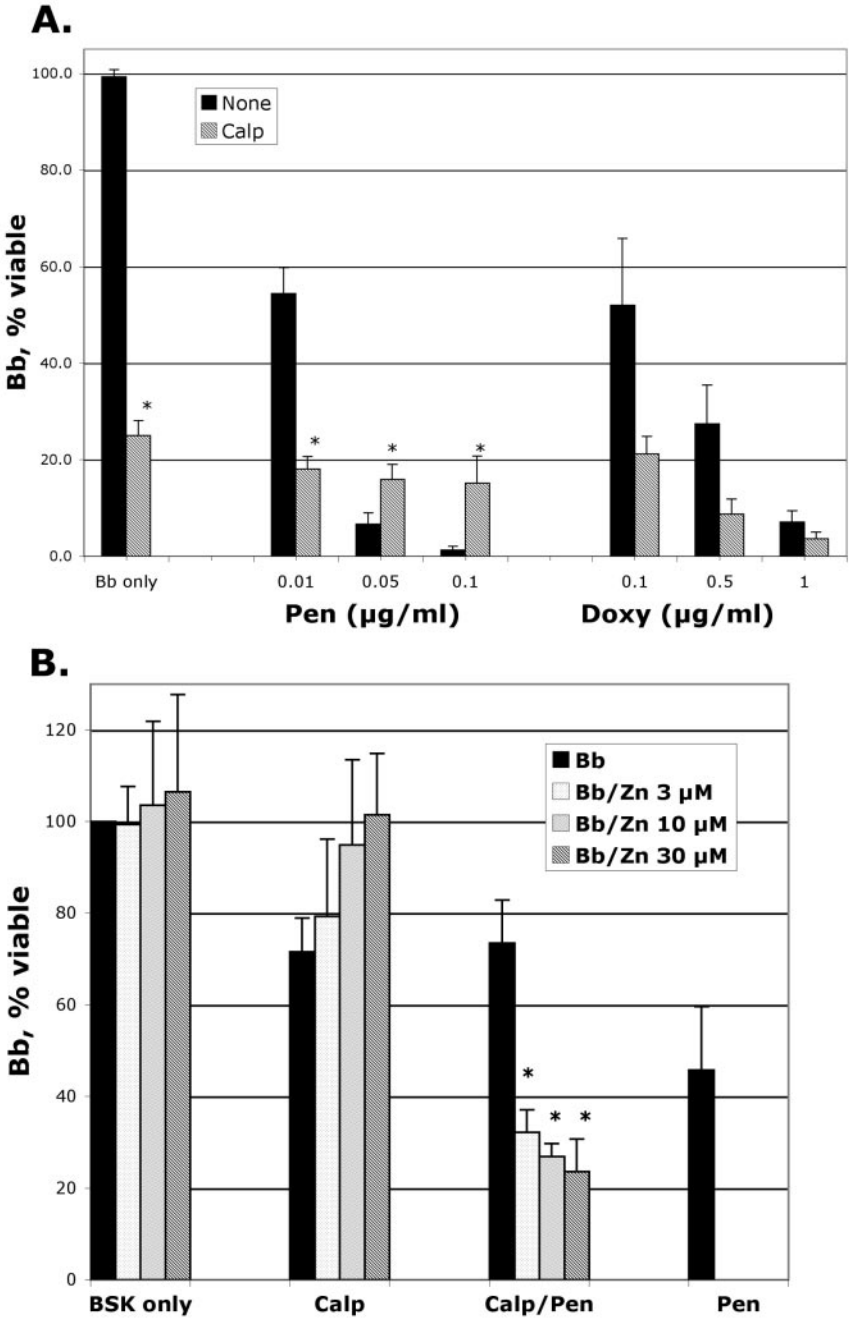

FIG. 2. Calprotectin alters spirochete susceptibility to antibiotic killing. Spirochetes (5×10^6) were incubated for 48 h at 33°C in 0.05 ml BSK with 20% (vol/vol) assay buffer containing (A) penicillin (Pen) (0.01, 0.05, or 0.1 μg/ml), doxycycline (Doxy) (0.1, 0.5, or 1.0 μg/ml), and rCalp (300 μg/ml) in six to eight separate experiments or (B) penicillin (0.05 μg/ml), rCalp (300 μg/ml), and $ZnCl_2$ (3, 10, or 30 μM) in three separate experiments. Percent viable spirochetes was determined by microscopic examination (dark-field or vital stain) at 48 h. Untreated spirochetes increased from 5×10^5 to (2 to 5) $\times 10^6$ during the 48 h of incubation. The statistical significance between spirochete numbers in untreated versus rCalp incubations is a P value of 0.001; results with antibiotic alone or antibiotic with calprotectin for Pen G are as follows: 0.01 μg/ml, $P = 0.001$; 0.05 μg/ml, $P = 0.02$; 0.1 μg/ml, $P = 0.04$. For doxycycline: 0.1 μg/ml, not significant; 0.5 μg/ml, $P = 0.04$; 1 μg/ml, $P = 0.04$. The statistical significance between numbers of spirochetes incubated without Zn versus Zn (3 μM) is $P = 0.04$; for Zn (10 μM), $P = 0.02$; and for Zn (30 μM), $P = 0.02$.

select antibiotics. When incubated in calprotectin at 300 μg/ml for 48 h, the number of viable spirochetes was 25% of that for the untreated controls (Fig. 2A) ($P = 0.001$, paired t test, $n = 8$). This is due to inhibition of growth of treated *B. burgdorferi* in comparison to the dividing controls and reproduces our earlier findings (13). The percentage of spirochetes alone was reduced by half when *B. burgdorferi* was incubated with a sublethal concentration of penicillin (0.01 μg/ml), and spirochetes

TABLE 1. Levels of calprotectin in stored patient joint fluids

Patient diagnosis	No. of samples	Amt of Calp (μg/ml)[b]
Pseudogout	3	377.82 ± 121.4
Lyme arthritis	32	362.1 ± 69.2
Inflammatory arthritis	5	379.6 ± 33.0
Other[a] and unknown	26	382.8 ± 103.6
Total	63	

[a] Includes juvenile rheumatoid arthritis, seronegative Lyme, leukemia, sickle cell disease, and Still's disease.

[b] Patient samples were assayed by specific enzyme-linked immunosorbent assay for calprotectin (Calp) using rCalp as a standard. Data shown are means ± standard errors of the means for three determinations per patient sample.

were efficiently eliminated by penicillin at 0.05 and 0.1 μg/ml. However, in the presence of calprotectin, spirochetes were maintained at 15 to 18% of the level of controls and were not cleared even in the presence of the higher doses of penicillin (Fig. 2A). In contrast to penicillin, doxycycline is a bacteriostatic antibiotic that inhibits protein synthesis. Viable spirochetes were reduced by incubation in doxycycline at 0.1, 0.5, and 1.0 μg/ml and were similarly eliminated even in the presence of calprotectin (Fig. 2A).

The mechanism of action of penicillin targets dividing bacteria; *B. burgdorferi* growth is inhibited by calprotectin. However, the addition of excess Zn^{2+} overcomes the inhibition induced by calprotectin and restores *B. burgdorferi* growth (13). In the presence of calprotectin and added Zn^{2+}, *B. burgdorferi* growth resumed, and consequently, killing by penicillin was restored (Fig. 2B).

Calprotectin effects on spirochetes are rapid. To investigate the mechanism of calprotectin's effects on spirochete growth inhibition, we examined whether a longer preincubation with calprotectin would enhance *B. burgdorferi* resistance to antibiotic killing. Although a 24-h preincubation reduced the numbers of all *B. burgdorferi* spirochetes, effects of additional preincubations with calprotectin were not statistically different from results for samples treated only during the antibiotic incubations (data not shown; $n = 4$). This suggests a rapid effect of calprotectin on spirochetes, consistent with chelation of essential Zn^{2+} ions. The addition of calprotectin (300 μg/ml) to PMN lysate during the assay period slightly reduced the efficiency of PMN lysate to kill *B. burgdorferi*, most likely due to reducing the contribution of PMN components that require active *B. burgdorferi*, e.g., defensins (data not shown).

Calprotectin levels in inflammatory joint fluids. We have detected levels of calprotectin in stored joint fluids of patients with a range of arthritic complaints including pseudogout, Still's disease, and Lyme disease. The fluids from 32 patients with Lyme arthritis showed an average of 362 ± 69.2 μg/ml, an elevated level that reflects the inflammation of the aspirated joints and which is significantly elevated over reported levels from joint fluids of osteoarthritis patients (range, 0.2 to 2 μg/ml [9]). We detected similar levels of calprotectin in our stored joint fluids from patients with pseudogout, unspecified inflammatory arthritis, and other conditions including juvenile rheumatoid arthritis, leukemia, sickle cell disease, and Still's disease (Table 1). These physiological levels were sufficient to confer protection against penicillin G in vitro.

Calprotectin is a member of the highly conserved S100 family of proteins and has numerous names and varied functions (6, 9). It is bacteriostatic and a powerful regulator of PMN mobilization from the bone marrow, and it accounts for much of the chemotactic activity in abscess fluids and in experimental gout (active at 10^{-12} to 10^{-9} M) (5, 10, 28, 31, 33). Peptides from the calprotectin monomer S100A9 activate the integrin Mac-1 (CD11b/CD18) and promote adhesion in tissues (6, 7, 20, 29, 34). Calprotectin also functions as the major intracellular transporter of fatty acids in PMN (27) and activator of the NADPH oxidase (2). The potent functions of this abundant protein, elevated in numerous inflammatory reactions, contribute to leukocyte recruitment and activation.

We have assessed the role of PMN calprotectin in *B. burgdorferi* persistence and have shown that calprotectin's inhibition of growth of spirochetes translates to the promotion of resistance to killing by certain antibiotics. Penicillin G, which relies on cell division for its action, does not eliminate spirochetes when they are incubated in the presence of calprotectin. In contrast, doxycycline eradicates spirochetes regardless of the presence of calprotectin. In our examination of the mechanism of this effect, we noted no additional protection to spirochetes from longer preexposure to calprotectin. In the case of penicillin, this suggests that the mechanism of action is consistent with chelation of Zn^{2+}, essential for growth of *B. burgdorferi*. Indeed, addition of Zn^{2+} to the incubation restores killing by penicillin even in the presence of calprotectin. Elimination by doxycycline, a bacteriostatic antibiotic, suggests that spirochetes for which growth is inhibited by calprotectin require protein synthesis for maintenance in the resting state. Our experiments suggest that the action of calprotectin to inhibit growth of *B. burgdorferi* may contribute to resistance to certain antibiotics in vivo.

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