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Fibroblasts Protect the Lyme Disease Spirochete, *Borrelia burgdorferi*, from Ceftriaxone In Vitro

Kostis Georgilis, Monica Peacocke, and Mark S. Klempner

Division of Geographic Medicine and Infectious Diseases, Department of Medicine, and Department of Dermatology, New England Medical Center and Tufts University School of Medicine, Boston, Massachusetts

The Lyme disease spirochete, *Borrelia burgdorferi*, can be recovered long after initial infection, even from antibiotic-treated patients, indicating that it resists eradication by host defense mechanisms and antibiotics. Since *B. burgdorferi* first infects skin, the possible protective effect of skin fibroblasts from an antibiotic commonly used to treat Lyme disease, ceftriaxone, was examined. Human foreskin fibroblasts protected *B. burgdorferi* from the lethal action of a 2-day exposure to ceftriaxone at 1 μg/mL, 10–20 × MBC. In the absence of fibroblasts, organisms did not survive. Spirochetes were not protected from ceftriaxone by glutaraldehyde-fixed fibroblasts or fibroblast lysate, suggesting that a living cell was required. The ability of the organism to survive in the presence of fibroblasts was not related to its infectivity. Fibroblasts protected *B. burgdorferi* for at least 14 days of exposure to ceftriaxone. Mouse keratinocytes, HEp-2 cells, and Vero cells but not Caco-2 cells showed the same protective effect. Thus, several eukaryotic cell types provide the Lyme disease spirochete with a protective environment contributing to its long-term survival.

Lyme disease, or Lyme borreliosis, is a complex multisystem disorder caused by the spirochete *Borrelia burgdorferi*, which is transmitted by *Ixodes dammini* and other related ixodid ticks [1, 2]. The typical course of the disease begins with a characteristic skin lesion, called erythema chronicum migrans; this is often followed by involvement of joints, the nervous system, the heart, and the lymphatic system [1, 2]. The pathogenesis of Lyme disease requires that the spirochete survive in the skin at the site of inoculation, possibly multiply locally, and then disseminate hematogenously to

various other sites [1, 3, 4]. The spirochete can persist in the host for prolonged periods of time; viable *B. burgdorferi* can be isolated from tissues of patients many years after the initial infection [5–11]. Many of these patients have high serum concentrations of antibody to *B. burgdorferi* at the time of its isolation [2, 5–12]. Moreover, antibiotic treatment does not preclude isolation of viable spirochetes from some patients. For example, *B. burgdorferi* has been isolated from the skin [8], blood [9], synovial fluid [12], and cerebrospinal fluid (CSF) [8, 13] of patients who had received antibiotic treatment for Lyme disease. These observations suggest that *B. burgdorferi* may be sequestered in a protective niche within the host allowing its long-term survival [1, 3, 4, 13].

The Lyme disease spirochete gains entry into the host through the skin, where it spreads locally and establishes infection [1, 3, 4]. In this study we examined whether fibroblasts, cells that are abundant in the skin, protect *B. burgdorferi* from the action of a clinically useful antibiotic, ceftriaxone [1, 13–15]. We also examined whether the cell-spirochete interaction was related to the organism's infectivity and whether it was specific for fibroblasts.

Materials and Methods

Organisms. All strains and clones of *B. burgdorferi* were cultured at 32°C in modified Barbour-Stoenner-Kelly (BSK) medium formulated as previously described [16]. The cultures were routinely monitored by darkfield microscopy for growth and contamination. The number of spirochetes was determined using a Petroff-Hausser bacteria counting chamber. Viability of the spirochetes was assessed by motility; nonmotile organisms were considered nonviable [16]. For long-term preservation of spirochetes at desired passages, aliquots of the cultures were stored at −70°C in the presence of 15% glycerol. The parent strains used in these experiments were the laboratory-adapted tick isolate G39/40, the tick isolate N40, and the human CSF isolate 297 [16].

Clones of *B. burgdorferi* were obtained by limiting dilution. Inoculations were made from cultures at logarithmic or stationary phase of growth into BSK medium (20 μL in 180 μL) in each of the 8 wells of the first vertical row of 96-well microtiter plates, and 12 serial 10-fold dilutions were made. The plates were placed in a candle jar to provide a microaerophilic environment for optimal growth of *B. burgdorferi* [1, 2] and incubated at 32°C. Wells were checked weekly for the presence of spirochetes by darkfield microscopy. Cultures were considered to contain cloned organisms when ≤3 wells of 8 in the last vertical row that contained spirochetes were positive.

Spirochete infectivity assay. The infectivity of the *B. burgdorferi* strains and clones was assessed by their ability to persist in an experimental rodent as previously described [16]. Spirochetes were injected intraperitoneally into Swiss Webster mice (0.1 mL, 5.0 × 10^7 live organisms/mouse). Three weeks after inoculation the mice were sacrificed, and the urinary bladder and spleen were cultured as previously described [16]. The cultures were examined for the presence of live spirochetes every 2–4 days for 1 month.

MBC. For each strain and clone used in these experiments, the MBC of ceftriaxone (Rocephin; Hoffmann-La Roche, Nutley, NJ) was determined by the broth dilution method [17, 18]. The concentrations of ceftriaxone tested ranged from 0.001 to 10.0 μg/mL. Duplicate wells of 24-well polystyrene plates containing BSK with ceftriaxone and control wells without antibiotic were inoculated with spirochetes at 1.0 × 10^6 viable organisms/mL. The plates were placed in a candle jar and incubated at 32°C. After 48 h, the number of viable spirochetes in each well was determined by darkfield microscopy, and inoculations were made at 1% dilution (vol/vol) in wells containing BSK without antibiotic. The plates were incubated as above and monitored for spirochetal growth over the course of 4 weeks. The MBC was the lowest concentration of ceftriaxone from which viable spirochetes were not detectable in the subculture.

Cell cultures. Human foreskin fibroblasts, mouse keratinocytes, HEp-2 cells (larynx epidermoid carcinoma cells, epithelial-like), Vero cells (green monkey kidney cells, fibroblast-like), and Caco-2 cells (human colon adenocarcinoma cells, epithelial-like) were cultured in multiwell polystyrene tissue culture dishes (35-mm wells in six-well plates) in Dulbecco's modified Eagle medium (GIBCO, Gaithersburg, MD) with 10% heat-inactivated fetal bovine serum (FBS; HyClone Laboratories, Logan, UT) at 37°C in humidified air with 5% CO_2, except for Caco-2

cells, which were cultured in 15% FBS. Confluent cell cultures were split by treatment with trypsin-EDTA (0.05% trypsin, 0.53 mM EDTA · 4Na; GIBCO).

Coculture of spirochetes with eukaryotic cells. Spirochetes at logarithmic phase of growth were centrifuged at 9000 g at 20°C for 20 min and resuspended in RPMI 1640 tissue culture medium (GIBCO) supplemented with 10% heat-inactivated FBS, 1% L-glutamine, and 10 mM HEPES (pH 7.4). The supernatants of confluent cell cultures were decanted, and spirochetes were added to wells containing a monolayer or to wells without cells (2.0–5.0 × 10^7 live organisms/well) and incubated at 37°C with 5% CO_2 in humidified air. Cells and spirochetes were cocultured for 48 h. The cells were then washed twice with warm PBS, pH 7.4, fresh tissue culture medium containing ceftriaxone at 1 μg/mL was added, and the cocultures were further incubated. After 48 h, the cells were washed twice with warm PBS and lysed by adding 0.5 mL of distilled H_2O for 5 min. In preliminary experiments, suspension of spirochetes in distilled H_2O for 5 min did not affect their viability. Adherent cells were then scraped off, and the total content of each well was inoculated into 10 mL of BSK. Cultures were incubated at 32°C and monitored for the presence of viable spirochetes every 2 or 3 days for 4 weeks. Control wells without cells but with spirochetes underwent the same procedure. Experiments were run in duplicate or triplicate.

To exclude the possibility that fibroblast products inactivated ceftriaxone and thus protected the spirochetes, ceftriaxone was dissolved (at 1 μg/mL) in cell-free (filtered through 0.45 μm filters) fibroblast-conditioned medium (supernatant from fibroblast monolayers obtained after 48 h in culture) and added to control wells that had spirochetes without fibroblasts.

In some experiments, fibroblasts were fixed with glutaraldehyde before addition of spirochetes. For glutaraldehyde fixation, wells with or without fibroblast monolayers were incubated with 2% glutaraldehyde (vol/vol) in PBS at 4°C for 2 h. Wells were then washed three times with PBS, and organisms were added as above.

To prepare human foreskin fibroblast lysate, fibroblast monolayers in supplemented RPMI tissue culture medium were scraped off, transferred into tubes, sonicated, and transferred back to wells. Fibroblast lysate was then cocultured with *B. burgdorferi* as above.

To study the survival of spirochetes over periods of antibiotic treatment >48 h, fibroblast monolayers were infected for 48 h and then treated with ceftriaxone for 3–14 days. Each day from day 3 to day 14, duplicate monolayers were washed, lysed, scraped, inoculated into BSK, and incubated at 32°C. For monolayers that were maintained in culture for >3 days, every third day the medium containing ceftriaxone was replaced to provide the cells and spirochetes with fresh medium containing active antibiotic.

Statistics. For statistical evaluation, the χ^2 test with Yates's correction was used.

Results

We first determined the MBC of ceftriaxone for all strains and clones used in these studies. Strains N40 and 297 and clone 297P6E8 had an MBC of 0.05 μg/mL. Each other

strain and clone had an MBC of 0.1 μg/mL. Therefore, we used a concentration of ceftriaxone that was at least 10 times the MBC, 1 μg/mL, for testing the possible protective effect of cells against the antibiotic.

We then examined whether fibroblasts would protect *B. burgdorferi* from the action of ceftriaxone. As shown in table 1, the presence of fibroblasts completely protected the spirochetes, which grew in culture after inoculation of the lysate of fibroblasts infected with strains or clones of *B. burgdorferi* for 2 days and then treated with ceftriaxone at 1 μg/mL for 2 days. In the absence of fibroblasts, the antibiotic was effective in eliminating organisms in 43 of the 46 samples. Cell-free fibroblast-conditioned medium did not protect the spirochetes from the lethal action of ceftriaxone, suggesting that fibroblasts did not protect the organisms by inactivating the antibiotic. To further confirm the absence of a neutralizing effect of fibroblasts on ceftriaxone, fibroblast-conditioned medium that contained ceftriaxone at 1 μg/mL and that had been incubated as above for 48 h was tested for antibiotic concentration in a bioassay using *Escherichia coli* as the test organism. The concentration of ceftriaxone in fibroblast-conditioned medium was equal to that in a sample that contained the antibiotic in tissue culture medium that had also been incubated as above for 48 h; both samples were found to contain ceftriaxone at 1 μg/mL.

To determine whether viable fibroblasts were needed for protection of the spirochetes from the action of ceftriaxone, additional culture wells with or without fibroblasts that had been treated with glutaraldehyde were cocultured with *B. burgdorferi*. As shown in table 1, glutaraldehyde-fixed fibroblasts did not protect the spirochetes from the lethal effect of

Table 1. Protective effect of human fibroblasts against the action of ceftriaxone (1 μg/mL) on *B. burgdorferi*.

Strain or clone	Cocultured with					
	HF	No cells	HF-conditioned medium	Gluta-ralde-hyde-fixed HF	Gluta-ralde-hyde, no cells	HF lysate
Infective						
N40	8/8	1/10	0/4			
297	20/20	1/13				1/6
N40P6D9	8/8	0/3		0/6	0/3	
297P6E8	22/22	1/11	1/4	0/6	0/3	
Noninfective						
G3940A6	3/3	0/3				
297P49C8	6/6	0/3				
297P49E8	5/5	0/3				
Total	72/72*	3/46	1/8	0/12	0/6	1/6

NOTE. Data are no. cultures positive for viable *B. burgdorferi*/total. HF = human fibroblasts.
* *P* < .0005 compared with all others.

Table 2. Time course of the protective effect of human fibroblasts on *B. burgdorferi* against the action of ceftriaxone (1 μg/mL).

Length of antibiotic treatment (days)	Cocultured with	
	HF	No cells
3	5/5	1/3
4	6/6	1/3
5	5/5	0/4
6	6/6	0/4
7	6/6	0/3
8	5/6	0/4
9	5/6	0/4
10	6/6	0/4
11	4/6	0/4
12	4/6	0/4
13	4/6	0/4
14	5/6	0/4

NOTE. Data are no. cultures positive for viable *B. burgdorferi*/total. HF = human fibroblasts.

ceftriaxone. Spirochetes cultured in the absence of cells or in glutaraldehyde-treated wells did not survive. Furthermore, fibroblast lysate (sonicated human foreskin fibroblasts) was not protective for *B. burgdorferi* (table 1). These findings suggest that a living cell was required to promote survival of the organism in the presence of the antibiotic.

We also examined whether there was a relationship between in vivo infectivity of *B. burgdorferi* and the ability of the organism to be protected from ceftriaxone by the presence of fibroblasts. As shown in table 1, both infective (N40, 297, N40P6D9, 297P6E8) and noninfective (G3940A6, 297P49C8, 297P49E8) organisms were protected from the action of the antibiotic in the presence of fibroblasts. Therefore, the protective effect of fibroblasts was not directly related to the infectivity of the organism in the mouse model.

To examine the time course of the protective effect of fibroblasts on *B. burgdorferi*, we extended the treatment with ceftriaxone to 14 days. As shown in table 2, spirochetes (clone 297P6E8) were able to survive in the presence of fibroblasts even after 14 days of ceftriaxone treatment. In the absence of fibroblasts, the antibiotic was lethal for the spirochetes.

Since *B. burgdorferi* do not grow in tissue culture medium, we next defined a mixed culture medium that would permit spirochetal growth and at the same time maintain viable fibroblast monolayers. In a medium consisting of 50% tissue culture medium and 50% BSK (vol/vol), fibroblasts proliferated to 204.7% ± 42.1% and spirochetes to 144.9% ± 41.2% of the initial inoculum in 3 days. Using this medium, we examined the protective effect of fibroblasts on *B. burgdorferi*. In 11 of 12 cultures, spirochetes were recovered from ceftriaxone treated cocultures of spirochetes with fibroblasts. In the absence of fibroblasts, *B. burgdorferi* was isolated in only 1 of 12 cultures (*P* < .0005). Therefore, fibroblasts were

able to protect the Lyme disease spirochete from the action of ceftriaxone in a medium that permits growth of extracellular organisms.

To determine whether the protective effect of fibroblasts for the Lyme disease spirochete was unique, we examined the ability of different eukaryotic cell types to protect *B. burgdorferi* from the action of ceftriaxone. Like human fibroblasts (18 positive of 20 cultures), mouse keratinocytes (8/8), Vero cells (14/18), and HEp-2 cells (6/7) also protected the spirochetes. However, Caco-2 cells did not exert a protective effect, and *B. burgdorferi* did not grow in any of 12 cultures ($P < .0005$ compared with all others).

Discussion

In the present study, fibroblasts and keratinocytes, cells that are abundant in the skin, protected *Borrelia burgdorferi* from the action of an antibiotic commonly used for its eradication, ceftriaxone [1, 13–15]. Even at 10 × MBC of ceftriaxone, as determined by the present study and as previously reported [14, 17, 18], spirochetes cocultured with fibroblasts or keratinocytes survived after 2 days of treatment. Protection of *B. burgdorferi* required viable cells, since no organisms survived in the presence of cell-free fibroblast-conditioned medium, glutaraldehyde-fixed fibroblasts, or fibroblast lysate (table 1).

Unlike our previous findings, in which infective strains of *B. burgdorferi* differed from noninfective ones in their ability to evade phagocytic cells [16], the ability of *B. burgdorferi* to survive in the presence of fibroblasts was not related to the strain's or clone's infectivity (table 1). This implies that the ability to interact with fibroblasts is not strictly linked to the organism's infectivity, at least when infectivity is assessed as persistence of the spirochete in the rodent model that we and others used [16].

One of the regimens most commonly used clinically for treatment of Lyme disease is administration of ceftriaxone for 14 days [1, 14, 15]. Our time course experiments showed that human skin fibroblasts can protect *B. burgdorferi* from ceftriaxone for 14 days (table 2). It will be of interest to examine the maximum duration of this protective effect.

A central question about the pathogenesis of chronic Lyme disease is whether the pathology is related in any way to the presence of viable spirochetes. A strong suggestion that this may be the case comes from studies demonstrating isolation of *B. burgdorferi* from patients many years after the initial infection. On the basis of this observation, several authors have suggested that the organism is sequestered in protective niches [1, 3, 4, 13]. It has been previously demonstrated that *B. burgdorferi* penetrates endothelial cell monolayers and can be observed inside and between these cells; however, the viability of those potentially intra- and intercellular spirochetes was not assessed [19]. In a recent study, *B. burgdorferi* was shown to penetrate cultured human

umbilical vein endothelial cells, but the viability of the intracellularly located spirochetes was not assessed [20]. The organism also has been observed inside phagocytic cells, but electron and fluorescence microscopy studies have provided evidence that it does not survive inside these cells [4].

At least three possibilities could explain our observations. First, the antibiotic could have been inactivated and therefore was not lethal for the spirochetes. This is unlikely, since ceftriaxone was lethal for *B. burgdorferi* in the control wells in the absence of cells (tables 1 and 2). Furthermore, the fibroblasts did not inactivate the antibiotic, since cell-free fibroblast-conditioned medium containing ceftriaxone was borreliacidal (table 1), and a bioassay using another organism showed that ceftriaxone in either tissue culture medium or fibroblast-conditioned medium was bioactive.

A second possibility is that the tissue culture medium in some of these experiments did not permit growth of *B. burgdorferi* and thus might have made spirochetes resistant to the cell wall–active antibiotic ceftriaxone. If this were the case, then organisms in the control wells in the absence of cells should have survived. Furthermore, we confirmed our observation in a medium (50% borrelial growth medium and 50% tissue culture medium) that allows growth of both the spirochetes and the fibroblast monolayers.

A third possibility is that the organism, like other pathogens, penetrates cells and survives in them. An intracellular site of survival would provide protection, since many of the antibiotics are much less concentrated in the cells than in extracellular spaces. Also, it would be consistent with the ability to isolate *B. burgdorferi* from patients with high antibody titers [2, 5–12], since the intracellular site would protect the spirochete from the host's immune system. Possibly fibroblasts and keratinocytes are the initial sites of this intracellular survival. This is especially relevant in that the first contact between the spirochete and the host in Lyme disease occurs in the skin. Furthermore, animal studies have shown that the skin is the optimal site for *B. burgdorferi* inoculation; considerably fewer organisms are needed to induce persistent infection if inoculated intradermally than by other routes [21]. Future studies are required to precisely define the locale of the protective effect of eukaryotic cells for *B. burgdorferi* as well as whether other antibiotics with different modes of action and different ability to penetrate cells, such as macrolides (e.g., erythromycin), would eliminate the Lyme disease spirochete even in the presence of cells.

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