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RESEARCH ARTICLE

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An in vitro study of the susceptibility of mobile and cystic forms of *Borrelia burgdorferi* to hydroxychloroquine

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Abstract In this work the susceptibility of mobile and cystic forms of *Borrelia burgdorferi* to hydroxychloroquine (HCQ) was studied. The minimal bactericidal concentration (MBC) of HCQ against the mobile spirochetes was >32 μg/ml at 37 °C, and >128 μg/ml at 30 °C. Incubation with HCQ significantly reduced the conversion of mobile spirochetes to cystic forms. When incubated at 37 °C, the MBC for young biologically active cysts (1-day old) was >8 μg/ml, but it was > 32 μg/ml for old cysts (1-week old). Acridine orange staining, dark-field microscopy and transmission electron microscopy revealed that the contents of the cysts were partly degraded when the concentration of HCQ was ≥MBC. At high concentrations of HCQ (256 μg/ml) about 95% of the cysts were ruptured. When the concentration of HCQ was ≥MBC, core structures did not develop inside the cysts, and the amount of RNA in these cysts decreased significantly. Spirochetal structures inside the cysts dissolved in the presence of high concentrations of HCQ. When the concentration of HCQ was ≥MBC, the core structures inside the cysts were eliminated. These observations may be valuable in the treatment of resistant infections caused by *B. burgdorferi*, and suggest that a combination of HCQ and a macrolide antibiotic could eradicate both cystic and mobile forms of *B. burgdorferi*.

Keywords *Borrelia burgdorferi* · Cystic forms · Spirochetes · Spheroplasts · Hydroxychloroquine

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Introduction

Borrelia burgdorferi sensu lato, the causative bacterium of Lyme borreliosis, has been divided into at least ten genospecies, but the multisystem infection is usually caused by the three genospecies of *Borrelia burgdorferi*: *B. garinii, B. afzelii,* and *B. burgdorferi* sensu stricto [51]. A conclusive diagnosis of Lyme borreliosis may be difficult to achieve since the symptoms are complex and may resemble those of many other diseases. Serology, PCR, and cultivation are important tools to make the conclusive diagnosis of Lyme borreliosis, but all these techniques have their shortcomings, and false-positive or false-negative results are frequent [4, 11, 18, 19, 35, 44, 46,47]. *B. burgdorferi* has the ability to adopt cystic forms both in vivo and in vitro, e.g. when exposed to antibiotics commonly used for treating Lyme borreliosis [12, 21, 23, 30, 40,43]. This phenomenon, combined with the ability of the cysts to reconvert to normal mobile spirochetes [1, 5–7], may explain a reactivation of the disease after an illusory cure– and not a "post-Lyme syndrome" as postulated by other researchers. This hypothesis requires susceptibility testing of the cystic forms to antibiotics capable of penetrating the cyst wall, and then eradicating the biologically active structures inside the cyst. *B. burgdorferi* has a very heterogeneous appearance and may be problematic to cure, especially if the infection has lasted for some time [25, 26, 32–34, 37, 39,41]. Since *B. burgdorferi* has the ability to convert to cystic forms both in vivo and in vitro [1, 6, 7, 12, 21, 23, 30, 40,43], this may partly explain the difficulties in the treatment of this infection.

We recently demonstrated that the cystic forms are susceptible to metronidazole [8], but because of the heterogeneity of the bacteria, some strains might be resistant to this agent. Therefore, we propose that antibiotics which are used against protozoa could be of value against cystic forms of *B. burgdorferi*. Malaria infections are treated with hydroxychloroquine (HCQ). The nonprotonated form of chloroquine can permeate the cell

membrane and then be protonated in the acidic environment of the vesicle. More polar molecules are unable to diffuse out of the vesicle [14]. These high concentrations of HCQ inside the cell are able to inhibit RNA and DNA synthesis [14,45]. The purpose of this study was to examine if in vivo concentrations of HCQ may have any effect against both cystic and mobile forms of *B. burgdorferi*.

Materials and methods

The bacterial strain used was *Borrelia burgdorferi ACA-1* (originally isolated in Sweden by Eva Åsbrink, Karolinska Institute, Södersjukhuset, Department of Dermatology, Stockholm, Sweden).

Production of spirochetes

A sample (0.1 ml) of a bacterial culture in exponential growth was transferred to 4 ml BSK-H medium (Sigma B3528) [39]. The concentration of inactivated (56 °C, 30 min) rabbit serum (Sigma R7136) in the BSK-H medium was 6%, and this serum was tested to be free of antibodies against *B. burgdorferi* by the manufacturing company (Sigma, St. Louis, Mo., USA). All culture media were sterile-filtered using a 0.2-μm filter, ensuring both sterility and the absence of mammalian cells from serum. All cultures were incubated in sterile 5-ml closed tubes (Nalgene cryovial; Nalge, Rotherwa, UK) at 30 °C.

Production of cystic forms

After 1 week of cultivation the tube was centrifuged (3,200 *g*, 30 min). The sediment and 0.5 ml of the supernatant were mixed (2×10^8 mobile spirochetes/ml, and no cysts were observed at that moment). This mixture of sediment and supernatant was diluted 1:100 in distilled water at 4 °C. The concentration of bacteria in the distilled water was 2×10^6 bacteria/ml. One part of the culture was incubated for 24 h and the other for 7 days, both at 30 °C. The number of cysts in the water culture was examined by dark-field microscopy (DFM) (400× and 800×) (Zeiss Axiophot; Carl Zeiss, Oberkochen, Germany). The number of spirochetes or cysts was in all cases determined by observation of 10-μl samples by DFM (200–400×).

Susceptibility testing with hydroxychloroquine

Plaquenil (HCQ; 200 mg) (Sanofi Winthrop, UK) was dissolved in distilled water, sterile-filtered using a 0.2-μm filter, and diluted geometrically in 5-ml Nalgene tubes from 512 μg/ml to 1.0 μg/ml in 2 ml of diluted BSK-H medium (dilution 1:100 in distilled water). The control consisted of a tube containing only diluted BSK-H. A 2-ml suspension of cystic forms at the age of 24 h and 7 days was added to each of the HCQ dilutions and to the control tube in a final concentration ranging from 0.5–256 μg/ml.

Susceptibility testing for HCQ was performed for mobile spirochetes in a final dilution of 0.5–256 μg/ml in non-diluted BSK-H medium. The final volume was 4 ml in each tube; 40 μl of 10^7 bacteria in exponential growth was added. Dilutions of HCQ in 0.9% NaCl were additionally made to exclude any inhibiting effect of BSK-H medium on the antibiotic. To examine if HCQ could prevent the conversion of normal mobile spirochetes to cystic forms, susceptibility testing was also carried out in distilled water.

Incubation conditions for susceptibility testing

Susceptibility testing for the cysts was carried out for 1-day-old and 7-day-old cysts. The 1-day-old cysts were incubated at 30 °C for 2 and 4 weeks, and at 37 °C for 2 weeks. The 7-day-old cysts were incubated at 30 °C for 4 weeks. The mobile bacteria were incubated in BSK-H medium at 37 °C for 2 weeks and at 30 °C for 4 weeks; in 0.9% NaCl at 30 °C for 2 weeks, and in distilled water at 30 °C for 2 weeks.

Examination of the hydroxychloroquine-exposed microbes

The tubes with the mobile borreliae in BSK-H medium were examined by DFM (200–800×) 2 or 4 weeks after addition of HCQ to detect the presence of mobile spirochetes. The mobile bacteria in 0.9% NaCl were examined similarly 1 and 2 weeks after addition of HCQ, and the mobile bacteria in distilled water were examined after 3 h and 2 weeks. A 0.1-ml suspension of these non-mobile spirochetes was transferred to fresh BSK-H medium and incubated for 2 months, and subcultured every second week.

The tubes containing cysts were examined by DFM (200× and 800×), and the number of intact cysts was counted. Twenty μl of the culture was transferred to a glass slide, dried, flame-fixed and overlaid with 50 mg/l acridine orange (AO) in phosphate buffer, pH 6.4, for 4 min, and finally rinsed in distilled water. The AO-stained cysts were examined by UV-microscopy (1,000–2,000×). HCQ-exposed cysts at 30 °C and 37 °C were vitally stained by mixing 10 μl AO (100 mg/l in phosphate buffer, pH 7.4) and 10 μl of culture on a glass slide protected with a coverslip. The vitally stained cysts were examined by UV-microscopy (400–2000×).

Reconversion of cystic forms to spirochetal forms

A 0.3 ml sample of each HCQ dilution in distilled water (containing 10^6 cysts/ml) was transferred to 4 ml BSK-H medium (resulting in 8×10^4 cysts/ml) and incubated at 30 °C in tightly capped tubes. The tubes were centrifuged (3,200 *g*, 30 min), and the sediment was mixed with 0.5 ml of the supernatant and examined by DFM. A magnification of 200× was used for the overview, and the formation of spirochetes from the cysts was examined at 800×. Four ml fresh BSK-H medium was added to each tube to examine whether the cysts could convert totally or partly to normal borreliae. This process was repeated every 1–2 weeks and was stopped after 2 months.

To examine whether any normal mobile spirochetes were present in the water culture at the time of the transfer to BSK-H medium, a method previously described [5,6] was used: the water culture (control) was filtered using a 0.45-μm filter (Schleicher and Schuell FP 030/2; Dassel, Germany). A 0.3 ml aliquot of the filtrate was transferred to 4.0 ml BSK-H medium, cultured for 2 months and examined. If normal mobile spirochetes are present, even at extremely low concentrations, they will pass through the filter [22].

Electron microscopy

The following cultures were examined by transmission electron microscopy (TEM): cysts from HCQ-free controls, cysts from cultures with 8, 32, and 64 μg/ml HCQ in distilled water incubated at 37 °C for 14 days. The examination by TEM was carried out according to the following procedure. The cultures were centrifuged (5,000 *g*, 20 min), the medium was removed and replaced with 2% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.3), and the bacteria were fixed for 2 h. The bacteria were post-fixed in 1% osmium tetroxide in 0.2 M cacodylate buffer for 2 h. The pellets were dehydrated, infiltrated and embedded in conventional epoxy resin (LX-112; Ladd, Burlington, Vt., USA) by a method described earlier [9,10]. Ultrathin sections were cut with a diamond knife (Jumdi; Juniper ultra Micro, Stockholm, Sweden) on an ultramicrotome (LKB 2088 Ultrotom V) and mounted on 200-mesh copper grids. The sections were stained with 5% uranyl acetate in 30% ethanol for 20 min and Reynolds lead citrate for 5 min and then examined in a Jeol 1200 EX electron microscope to identify bacterial structures.

Results

Susceptibility testing for cystic forms

Rupturing was observed in 20–95% of the 1-day-old cysts that had been incubated in HCQ dilutions of 8–256 μg/ml at 30 °C for 14 days (most rupturing for the highest concentrations). The same results were obtained when 7-day-old cysts were incubated at 30 °C for 4 weeks. After incubation at 37 °C for 14 days, similar rupturing results occurred, but in the range of 4–256 μg/ml HCQ. In AO-stained fixed smears of cysts, the control cysts (without HCQ incubation) were a bright orange-red color. These cysts contained distinct spirochetal structures. Following incubation for 4 or 2 weeks, 30–40% and 10–20% of the cysts contained core structures, respectively (Fig. 1A, B). At 8 μg HCQ/ml (37 °C) and 16–32 μg HCQ /ml (30 °C), less than 5% of the cysts contained core structures, and the contents of the cysts were dissolved and had a weak green color (Fig. 2A, B). Staining with AO revealed initial disruption of the internal structures and a color change from orange to green even at an HCQ concentration of 4 μg/ml. When spirochetes were incubated in distilled water at an HCQ concentration of 64 μg/ml for 1 week, some spirochetes resembled streptococci when the cysts were stained with AO (Fig. 3). The contents of the cysts seemed to dissolve completely at an HCQ concentration of 32 μg/ml at 37 °C. The blebs showed a pycnotic appearance (Fig. 4).

When cysts that had been incubated at 37 °C for 14 days or at 30 °C for 4 weeks were vitally stained with AO, the cores were bright green when the concentration of HCQ was 4 μg/ml, and both orange and green at an HCQ concentration of 8 μg/ml. All core structures stained red and the membrane was very thin or disrupted at an HCQ concentration ≥16 μg/ml (Fig. 5 A, B, C). When the cysts were incubated at 30 °C for 14 days, they were green at an HCQ concentration of 8 μg/ml, green/orange at 16 μg/ml and orange at 32 μg/ml. When 7-day-old cystic forms were incubated for 4 weeks, they were green at an HCQ concentration of 16 μg/ml, green/orange at 32 μg/ml and orange at 64 μg/ml.

Reemergence of mobile spirochetes from cysts

When transferred to BSK-H medium, 10–20% of the cysts converted to immobile spirochetes for control cysts, or cysts which had been exposed to a low concentration of HCQ. The converted spirochetes were relatively short, but a few of them(1–5%) had a normal appearance, except for being very thin. No further development was observed beyond 1 month of incubation (Fig. 6). When the concentration of HCQ was above the MBC, no beginning spirochetes were seen protruding from the cysts. Only a few blebs (tubuli) were observed, and these looked pycnotic (see Fig. 4).

Susceptibility testing for mobile spirochetes

Susceptibility testing of normal mobile borreliae showed that the MBC to HCQ was >32 μg/ml if the incubating temperature was 37 °C for 14 days, >128 μg/ml if the temperature was 30 °C for 2 weeks, and >32 μg/ml when the temperature was 30 °C for 4 weeks. Only a few cystic forms originated from mobile spirochetes in the BSK-H media at HCQ = MBC. In these cysts, mostly small granula were visible along the degenerated spirochetes (Fig. 7). Susceptibility testing in 0.9% NaCl at 30 °C for 14 days resulted in a MBC of >128 μg/ml. There was an equal number of cystic structures in the HCQ-free control tube as in the tubes containing concentrations of HCQ < MBC.

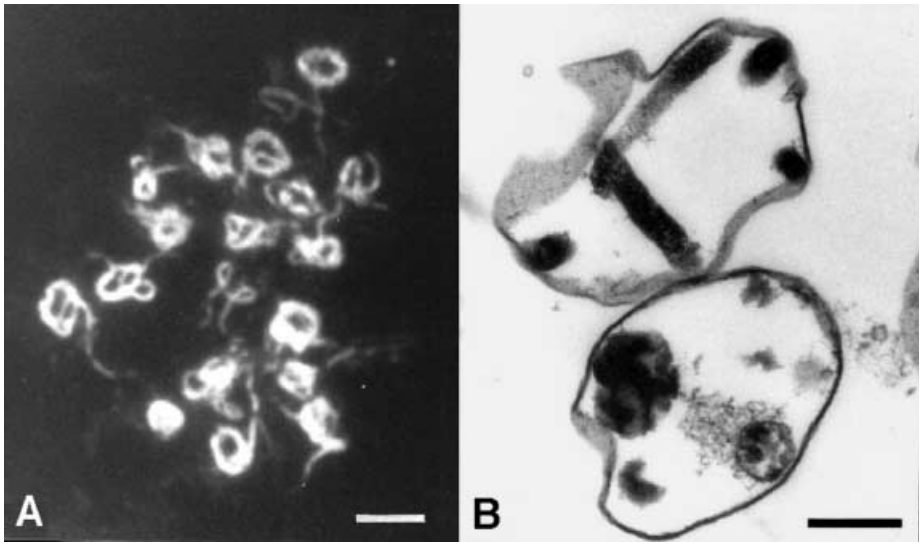

Fig. 1A, B. Cystic forms of *Borrelia burgdorferi* incubated for 14 days in distilled water without hydroxychloroquine (HCQ). **A** At 30 °C, Distinct bacterial structures and cores are observed inside the cysts. The intensely stained bacteria and cores indicate significant amounts of RNA. The cysts were flame-fixed and stained with acridine orange. *Bar* 3 μm. **B** At 37 °C. Distinct spirochetal and core structures are observed inside the cysts. TEM (uranyl acetate and lead citrate). *Bar* 500 nm

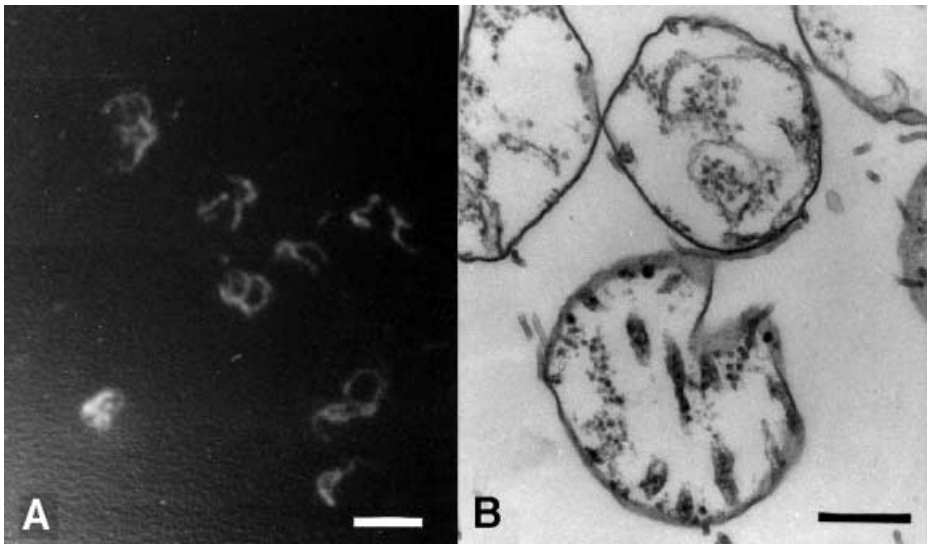

Fig. 2A, B. Cystic forms of *B. burgdorferi* incubated for 14 days. **A** At 30 °C in distilled water with 16 μg HCQ/ml, the cysts are diffuse and no distinct bacteria or cores are observed; only pycnotic bacteria are present. The staining is weak, which illustrates low concentration of RNA. The cysts were flame-fixed and stained with acridine orange. *Bar* 3 μm. **B** At 37 °C and 8 μg HCQ/ml, most of the cysts are intact but the intracystic spirochetal forms are completely dissolved into small granula. Even the core structures show initial disruption. TEM (uranyl acetate and lead citrate). *Bar* 500 nm

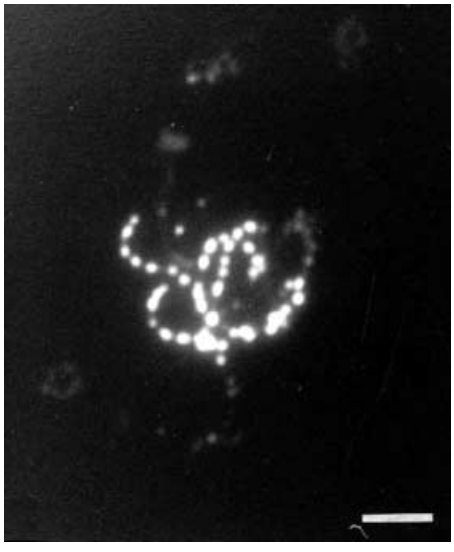

Fig. 3. Spirochetes incubated in distilled water at 37 °C for 1 week with 64 μg HCQ/ml. The intracystic spirochetes appears as small granula, and the intensely stained RNA is only partly present, which makes the spirochetes resemble streptococci. The cysts were flame-fixed and stained with acridine orange. *Bar* 3 μm

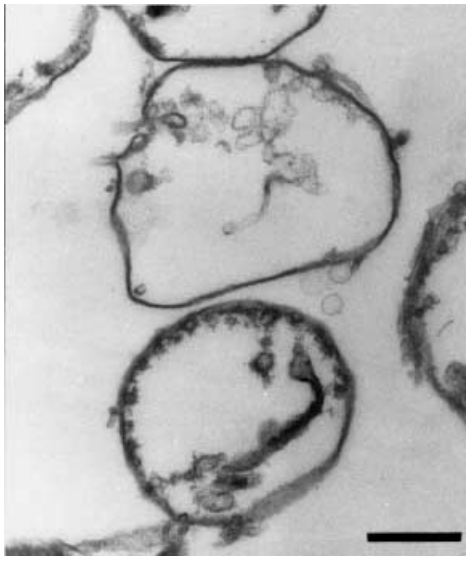

Fig. 4. Cystic forms of *B. burgdorferi* incubated at 37 °C for 14 days in distilled water with 32 μg HCQ/ml. The cysts have less contents than following incubation in 8 μg HCQ/ml. TEM (uranyl acetate and lead citrate). *Bar* 500 μm

The effect of hydroxychloroquine on the conversion of mobile spirochetes to cysts

Susceptibility testing for mobile spirochetes in distilled water resulted in a rate of conversion that was strongly dependent on the HCQ concentration and the incubation time. After incubation at 30 °C for 3 h, the number of converted spirochetes ranged from none at an HCQ concentration of 256 μg/ml, 10% at 128 μg/ml, 30% at 64 μg/ml, 50% at 8–32 μg/ml, and 80% at 2–4 μg/ml. At HCQ concentrations of 0.5 and 1.0 μg/ml, and in the HCQ-free control, more than 95% of spirochetes had converted to normal cysts in 3 h. About 5% of free spirochetes started to show degradation at an HCQ concentration of 4 μg/ml, with increasing rates at higher HCQ concentrations. The content of the cysts showed initial granulation at 32 μg HCQ/ml. After a 2-week incubation, cysts from the 2–256 μg HCQ/ml tubes appeared pycnotic with respect to contents, and looked like small granula. No distinct bacteria or core structures were visible inside the cysts, in contrast to the 1.0 and 0.5 μg/ml concentrations and the HCQ-free control. A few degraded spirochetes could be observed even at a concentration of 2 μg/ml. AO staining (pH 6.4) of cysts exposed to an HCQ concentration of 2 μg/ml were slightly green. The contents of the cysts dissolved if HCQ concentration was ≥2 μg/ml.

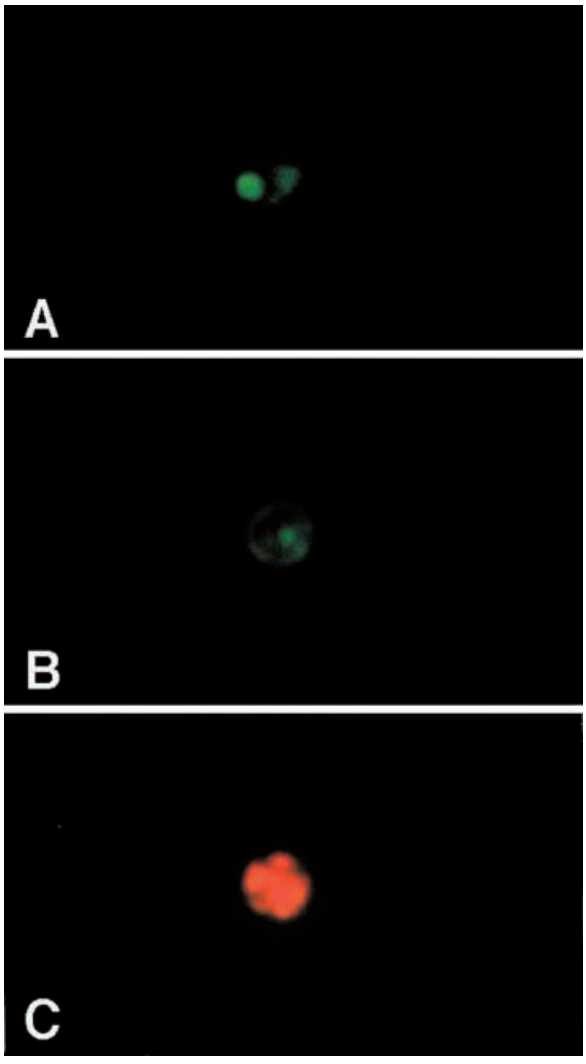

Fig. 5A–C. Susceptibility testing of 1-day-old cystic forms of *B. burgdorferi* to HCQ in distilled water at 37 °C for 14 days. Vital AO staining, pH 7.4. **A** At an HCQ concentration of 4 μg/ml, the cyst contains green cores (living organisms). **B** At an HCQ concentration of 8 μg/ml, the cyst contains both green and orange cores. **C** At an HCQ concentration of 16 μg/ml, the cyst has a diffuse membrane and contains red-orange core/granula (biologically inactive organisms). *Bar* 3 μm

Fig. 6. *B. burgdorferi* cysts were incubated at 30 °C for 14 days in distilled water with 16 μg HCQ/ml and then transferred to BSK-H medium. After incubation for 4 weeks in BSK-H medium, some thin immobile spirochetes protrude from the cysts. Dark-field microscopy. *Bar* 6 μm

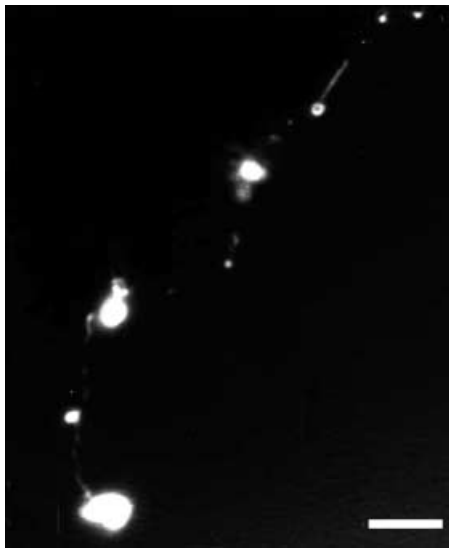

Fig. 7. Susceptibility testing of mobile *B. burgdorferi* in BSK-H medium at 30 °C for 14 days at 256 μg HCQ/ml. Mainly small granula are visible among the pycnotic spirochetes. Dark-field microscopy. *Bar* 6 μm

Discussion

Our results show that HCQ alone may be sufficient in the treatment of intracellular cystic forms of borreliae, since cystic forms are susceptible to this agent at concentrations which are achievable in vivo intracellularly at normal body temperature [45]. However, when the infection is located at the dermis, and when free-living *B. burgdorferi* circulate in the bloodstream, the MBC of HCQ is not achievable. Susceptibility testing for the mobile spirochetes in 0.9% NaCl resulted in the same MBC as obtained for susceptibility testing in BSK-H medium, which means no inhibitory effect of MSB-H medium to HCQ.

The susceptibility testing of *B. burgdorferi* to HCQ in distilled water revealed a significant decrease in the formation of cysts within the first 3 h, but 14 days later no normal spirochetes could be observed. The content of the cysts rapidly degraded, but the rate of degradation increased over time. Therefore, it is possible that HCQ has some rapid inhibitory effect on the development of cystic forms in vitro, e.g. alkaline degradation [6,24], not involving inhibition of RNA and DNA. Further degradation of the spirochetes and the cystic contents may account for the direct inhibition of DNA and RNA. The contents of the blebs is of great pathogenic importance [28], and these structures were pycnotic at HCQ concentrations achievable in vivo.

The orange color of AO-stained fixed cysts (pH 6.4) diminished with increasing concentrations of HCQ. This indicates little or no RNA synthesis, and thereby little or no biological activity. At the highest HCQ concentration, most of the cysts ruptured, and even when the concentration of HCQ was $<$MBC, some of them had

ruptured and were degraded. This means that cysts are affected even at HCQ concentrations lower than the estimated MBC.

However, vital AO staining (pH 7.4) of HCQ-incubated cysts in distilled water revealed the formation of immobile spirochetes. Additionally, the amount of cystic RNA, and also TEM observation argues for the estimated MBC. Living organisms stain bright green, while those that are non-viable stain orange and red. The shifts in AO staining from bright green to orange/red means increasing uptake of AO caused by intercalation of the stain with the phosphate-sugar backbone of DNA as the DNA becomes denatured in non-viable cells [27,31].

An important feature is the temperature-dependent influence of HCQ on mobile bacteria and cysts. At 30 °C, higher amounts of HCQ are required to destroy the cysts than at 37 °C. This is in agreement with the results obtained with other antibiotics [8,42] and may be significant when cysts are located in the dermis. In a previous report of susceptibility testing of normal mobile *B. burgdorferi* to HCQ, the MBCs ranged from 50 to 90 μg/ml for different strains; there was no information, however, about the incubation temperature [13].

The conversion of cysts to mobile spirochetes depends strongly on the composition of the culture medium [1,5–8], and the quality of the rabbit serum is especially important. We have recently shown that old cystic forms are difficult to transform into mobile spirochetes [5,6]. We were not able to obtain mobile spirochetes, but thin immobile spirochetes developed from the core structures of the cysts incubated in HCQ < MBC.

Increased pH in the vesicle may be advantageous for intracellular bacteria, because O^{2-} is absent, but since *B. burgdorferi* produces superoxide dismutase, the bacterium is protected from the disadvantageous acidic environment as well [52]. Since macrolides are also more active in an alkaline environment, a combination therapy with HCQ and a macrolide seems advantageous [15]. *B. burgdorferi* is considered to be a facultative intracellular organism and animal cells may shelter the spirochete [17, 20, 21, 29, 30, 36,51]; therefore, it seems sensible to use an agent like HCQ, which can concentrate inside the cells.

We have established that mobile and cystic forms of *B. burgdorferi* are susceptible to HCQ in vitro in a time-, temperature- and concentration-dependent manner. As far as we know, this is the first susceptibility testing of cystic forms of *B. burgdorferi* to HCQ. We did not use metabolites of HCQ in this study, although these compounds could theoretically be more effective than the parent drug. Since the MBC values for HCQ obtained in this study are achievable intracellularly in vivo [45], and because the effect of the drug is mainly anti-cystic, HCQ may be useful in treating Lyme borreliosis. In addition, HCQ is a safe antibiotic with important anti-inflammatory activity suitable for the treatment of chronic Lyme disease [2, 16, 45,49].

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