

RESEARCH ARTICLE

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Susceptibility of motile and cystic forms of *Borrelia burgdorferi* to ranitidine bismuth citrateReceived: 30 May 2001 / Accepted: 30 August 2001 / Published online: 1 December 2001
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Abstract Gastrointestinal symptoms accompanying Lyme disease have not been considered in the treatment of Lyme patients yet. Here we examine the effect of ranitidine bismuth citrate (RBC) on motile and cystic forms of *Borrelia burgdorferi* in vitro, to determine whether it could cure this bacterial infection in the gastrointestinal tract. When motile forms of *B. burgdorferi* were exposed to RBC for 1 week at 37 °C, the minimal bactericidal concentration (MBC) was >64 mg/ml. At 30 °C, the MBC was >256 mg/ml. When the incubation lasted for 2 weeks at 37 °C, the MBC dropped to >2 mg/ml. Bismuth aggregates were present on the surface of *B. burgdorferi* when $RBC \geq MBC$, as shown by transmission electron microscopy (TEM). Cystic forms of *B. burgdorferi*, exposed to RBC for 2 weeks at 37 °C, were examined by cultivation in BSK-H medium (Sigma B3528). They were stained with acridine orange (pH 6.4, pH 7.4) and studied by TEM. The MBC for RBC for young cystic forms (1 day old) and old cysts (8 months old) was estimated to be >0.125 mg/ml and >2 mg/ml, respectively. Bismuth aggregates were attached to the cysts and, in some, the pin-shaped aggregates penetrated the cyst wall. The bismuth aggregates also bound strongly to blebs and granules of *B. burgdorferi* when $RBC \geq MBC$. When *B. burgdorferi* is responsible for gastrointestinal symptoms, bismuth compounds may be candidates for eradication of the bacterium from the gastrointestinal tract.

Keywords *Borrelia burgdorferi* · Cystic forms · Spirochetes · Spheroplasts · Bismuth compounds

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Introduction

Lyme disease, caused by *Borrelia burgdorferi* and difficult to cure, may develop into a chronic infection if not treated early. All antibiotics commonly used have shortcomings. Eventual relapse may be highly dependent on the choice of antibiotics and the stage of the disease [22, 23, 32, 33, 34, 35, 37, 38]. One reason why the infection can relapse may be that commonly used antibiotics stimulate the conversion of *B. burgdorferi* from mobile to cystic forms [17, 18, 27, 39, 40]. The cysts convert back to motile spirochetes under favorable circumstances; and this may be one reason for reactivation [1, 5, 6, 7]. The bacteria spread early in the progression of infection [25], such that all organs and tissues may be infected. Until recently, *B. burgdorferi* was thought to be manifest in the skin, muscles, skeleton, cardiovascular system, eyes, central and periphery nervous system, genitourinary tract, lymphatic system, liver, kidneys, bronchi and lungs [16, 41]. Recently, PCR was used on biopsies from the gastrointestinal (GI) tract to detect *B. burgdorferi* infection in the gut of patients with erythema migrans and gastrointestinal pain [15]. New insights into the life history of the bacterium show that the GI tract must be considered a sanctuary for this bacterium, implying insufficient treatment of *Borrelia* infections [15].

For the past 300 years, bismuth salts have been used successfully in gastroenterology for the treatment of stomatitis, flatulence, diarrhoea, abdominal pain, constipation, dyspepsia and peptic ulcer disease [19]. Certain enteropathogenic bacteria and viruses are also susceptible to bismuth salts, including *Clostridium difficile* and *Helicobacter pylori* [12, 14, 28]. Bismuth subsalicylate, which also has antisecretory properties, prevents fluid accumulation caused by enterotoxins and is widely used for travellers' diarrhoea [4, 13]. Before the introduction of penicillin, bismuth was reported to cure neurosyphilis [43]. The mechanism of action of bismuth is not fully understood, but it has been proposed to

inhibit the following functions: ATPase function, protein synthesis, membrane function and cell wall synthesis [19]. In *H. pylori*, another bacterium that transforms to coccoid (cystoid) forms and reverts to normal motile forms [2], treatment with two or more antibiotics has been established in combination with bismuth compounds. Bismuth compounds act synergistically with antibiotics. Infections with antibiotic-resistant strains of *H. pylori* have been cured by a combination of bismuth and antibiotics [20, 30].

High concentrations of bismuth are tolerated in the GI tract [19, 20, 21] without severe adverse effects [14]. Therefore, our hypothesis is that bismuth compounds may inhibit motile and cystic forms of *B. burgdorferi*, which portends well for the eradication of *B. burgdorferi* from GI tract-infected patients.

Materials and methods

The bacterial strain used in our experiments was *B. burgdorferi* ACA-1 (originally isolated in Sweden by Eva Åsbrink, Department of Dermatology, Karolinska Institute, Södersjukhuset, Stockholm, Sweden) [3].

Production of spirochetes

A bacterial culture in exponential growth (0.1 ml) was transferred to 4 ml BSK-H medium (Sigma B3528) [36]. 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.). Passage of all culture media through a sterile 0.2-µm filter ensued both sterility and the absence of mammalian cells from the serum. All cultures were incubated in sterile 5-ml closed tubes (Nalgene cryovial; Nalge, Rotherwick, UK) at 30 °C.

Production of cystic forms

After 1 week of cultivation, the tube was centrifuged at 3,200 g for 30 min. The sediment and 0.5 ml of the supernatant were mixed (10^8 motile spirochetes/ml, no cysts observed). 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 10^6 bacteria/ml. Samples of the culture were incubated for 24 h or for 8 months, both at 30 °C. The number of spirochetes and/or cysts was in all cases determined by microscopy in 10 µl of medium by dark field microscopy (DFM), (200–800 ×, Zeiss Axiophot; Carl Zeiss, Oberkochen, Germany).

Susceptibility testing with ranitidine bismuth citrate to cystic forms

Bismuth salts are sparingly soluble, so ranitidine bismuth citrate (RBC) was chosen because it is completely soluble in water [19]. RBC (400 mg; Glaxo Welcome, Oslo, Norway) was dissolved in distilled water, sterile-filtered using a 0.2-µm filter and diluted geometrically in 5-ml Nalgene tubes from 1,024 µg/ml to 0.06 µg/ml in 2 ml of diluted BSK-H medium (diluted 1:100 in distilled water). A 2-ml suspension of cystic forms (24 h, or 8 months) was added to each of the RBC dilutions (and to a control tube containing only diluted BSK-H) in a final concentration between 0.03 µg/ml and 512 µg/ml – a total of 15 tubes. Susceptibility

testing for RBC was performed for motile spirochetes in a final dilution from 0.03 µg/ml to 256 µg/ml in non-diluted BSK-H medium. The final volume was 4 ml in each tube, and 40 µl of 10^7 bacteria/ml in exponential growth was added.

Incubation conditions for susceptibility testing

The cysts were tested at the ages of 1 day and 8 months. Both categories of cysts were incubated for 2 weeks at 37 °C. The motile bacteria were incubated for 2 weeks at 30 °C or 37 °C in BSK-H medium.

Examination of the ranitidine bismuth citrate-exposed microbes

The tubes with motile borrelia in BSK-H medium (RBC concentration: 0.03–512 mg/ml; 0 mg/ml in the control) were examined by DFM (200–800×) 1 week and 2 weeks after the addition of RBC to detect the presence of any motile spirochetes. Of these non-motile spirochetes, a 0.1-ml suspension was transferred to fresh BSK-H medium, incubated for 2 months and subcultured every second week.

Examination of the ranitidine bismuth citrate-exposed cysts

A total of 20 µl of the culture was transferred to a glass slide, dried, flame-fixed and overlaid with acridine orange (AO; 50 mg/l) in phosphate buffer (pH 6.4) for 4 min and rinsed in distilled water. The AO-stained cysts were examined by UV microscopy (1,000–2,000×). Vital AO-staining was performed on RBC-exposed cysts at 37 °C by mixing 10 µl of AO (100 mg/l) in phosphate buffer (pH 7.4) with 10 µl of culture on a glass slide protected with a coverslip. The vitally stained cysts were examined by UV microscopy (400–2,000×).

Reconversion of cystic forms to spirochetal forms

Each RBC dilution in distilled water (0.3 ml containing 10^6 cysts/ml) was transferred to 4 ml of BSK-H medium (resulting in 7.5×10^4 cysts/ml) and incubated at 30 °C in tightly capped tubes. The tubes were centrifuged at 3,200 g for 30 min. The pellet was mixed with 0.5 ml of the supernatant and examined by DFM at 200 × magnification. The formation of spirochetes from cysts was examined at 800×. Fresh BSK-H medium, 4 ml, was added to each tube to determine the extent of cyst conversion to normal borrelias. This process was repeated every second week for 2 months.

Examination by transmission electron microscopy

The following cultures were examined by transmission electron microscopy (TEM): (a) 8-month-old cysts which had been incubated in distilled water with 0 µg RBC/ml (control), 1, 4, or 256 µg RBC/ml for 2 weeks at 37 °C, (b) motile bacteria which had been incubated in BSK-H medium with 0 µg RBC/ml (control), 4 µg RBC/ml, or 64 µg/ml RBC for 2 weeks at 37 °C. The cultures were centrifuged at 5,000 g for 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 postfixated 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.) 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 Ultratome 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. The sections were examined in a Jeol 1200 EX TEM.

Results

Susceptibility testing for cystic forms

No rupturing was observed (DFM) for any of the cysts when incubated with RBC (0.03–512 $\mu\text{g}/\text{ml}$) for 14 days at 37 °C. When fixed smears of cysts were AO-stained at pH 6.4, the control cysts (incubated without RBC) showed a bright orange-red color and contained distinct core structures (Fig. 1a). When old cysts were exposed to 4 μg RBC/ml (37 °C), fewer than 10% of the cysts revealed an orange color (Fig. 1b). The majority of the

cysts revealed partly dissolved contents and their color was a weak green. Incubated in 8 μg RBC/ml (37 °C), the content of the cysts dissolved without distinct core structures and appeared a weak green color. The 1-day-old control cyst contained distinct spirochetal structures (Fig. 2a). When 1-day-old cysts were exposed to concentrations of RBC higher than 0.125 $\mu\text{g}/\text{ml}$ (37 °C), no distinct spirochetal structures could be seen and only green, swollen cysts with dissolved content were observed (Fig. 2b).

When old cysts that had been incubated for 14 days at 37 °C in RBC environment were exposed to vital AO-staining (pH 7.4), the cores of the cysts revealed the

Fig. 1a, b Old cysts of *Borrelia burgdorferi*. **a** Old cysts (8 months) in distilled water incubated at 37 °C for 14 days without ranitidine bismuth citrate (RBC). Mainly core structures and a few short rod structures are observed inside the cysts. The intensely stained bacteria and cores indicate the presence of significant amounts of RNA. Flame-fixed and stained with acridine orange. **b** Old cysts incubated at 37 °C for 14 days in distilled water with 4 μg RBC/ml weakly stained green, which illustrates a low concentration of RNA. Flame-fixed and stained with acridine orange. *Bar* 6 μm

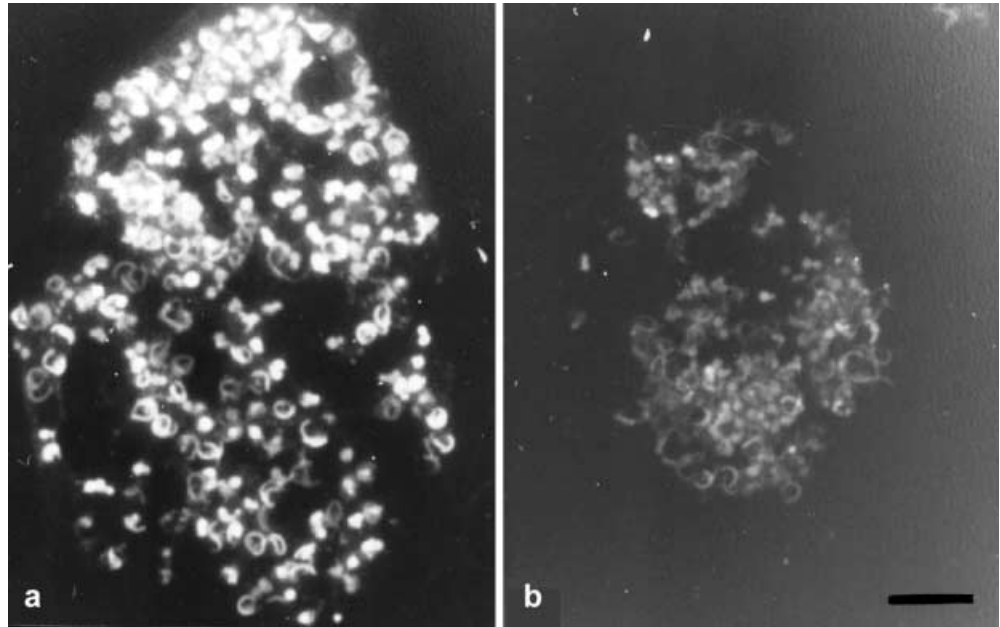
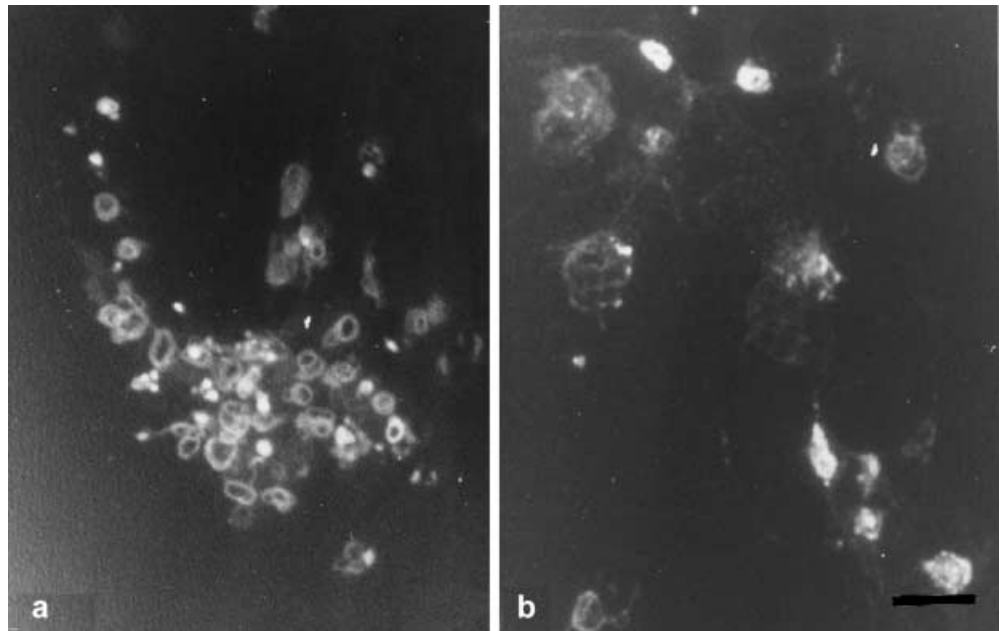


Fig. 2a, b Young cysts. **a** Young cysts (1 day) incubated in distilled water without RBC. Distinct orange spirochetal forms are observed inside the cysts. **b** Same as **a**, but with a RBC concentration of 4 $\mu\text{g}/\text{ml}$. The swollen cysts are partly dissolved and no distinct spirochetal structures are present. The color is weak green. Flame-fixed and acridine orange (AO)-stained, pH 6.4. *Bar* 6 μm



following colors, depending on the concentration of RBC: bright green for $\text{RBC} < 2 \mu\text{g/ml}$ (Fig. 3a), 50% orange and 50% green cores for $\text{RBC} = 2 \mu\text{g/ml}$ (Fig. 3b) and 100% yellow/orange/red core structures for $\text{RBC} > 2 \mu\text{g/ml}$ (Fig. 3c). For the young cysts, the corresponding limits for color shift by vital AO-staining were: green for $\text{RBC} < 0.06 \mu\text{g/ml}$, 90:10 orange:green for $\text{RBC} = 0.125 \mu\text{g/ml}$ and yellow/orange/red for $\text{RBC} \geq 0.25 \mu\text{g/ml}$.

Bismuth aggregates attached to the cell envelope of the old cysts (Fig. 4). The aggregates seemed to penetrate some of the cell walls when the RBC concentration was higher than $2 \mu\text{g/ml}$ (Fig. 4a). Surprisingly, most of the blebs were covered with bismuth aggregates. When $\text{RBC} = 256 \mu\text{g/ml}$, the 8-month-old cysts were attached to large clusters of needle-like aggregates which penetrated the cyst wall (Fig. 4b). No aggregates were seen attached to cysts or blebs when $\text{RBC} = 1 \mu\text{g/ml}$ (Fig. 4c). Only a few intact blebs were visible when the concentration of RBC was higher than $2 \mu\text{g/ml}$.

Re-emergence of motile spirochetes from the cysts

When transferred to BSK-H medium, about 50% of the cysts which had been exposed to RBC concentrations from $0 \mu\text{g/ml}$ (control) to $2 \mu\text{g/ml}$ converted to immotile spirochetes. Most of the converted spirochetes were relatively short and they were attached to cysts (Fig. 5). Spirochetes that detached from the cyst (about 5%) were normal in appearance, except they were so thin ($0.1 \mu\text{m}$) that they were almost impossible to photograph. No further development of these spirochetes was observed beyond incubation for 1 month. When the concentration of RBC was above $2 \mu\text{g/ml}$, no spirochetes began to protrude from cysts.

Susceptibility testing for motile spirochetes

Susceptibility testing of normal motile borrelias showed that MBC vs RBC was $> 256 \mu\text{g/ml}$ when incubation took place at 30°C for 14 days, it was $> 64 \mu\text{g/ml}$ when the incubation occurred at 37°C for 7 days, and it was $> 2 \mu\text{g/ml}$ when the temperature was 37°C for 14 days. When the motile spirochetes were incubated at 37°C for 14 days, the production of cysts decreased as the concentration of RBC reached the MBC.

Transmission electron microscopy

No bismuth aggregates were observed by TEM on the bacteria or the cyst when the concentration of RBC was lower than MBC (37°C for 14 days) (Fig. 6a). At a RBC concentration of $4 \mu\text{g/ml}$, only a few bismuth aggregates were visible on the spirochetal envelope (Fig. 6b), but at RBC concentrations $> 64 \mu\text{g/ml}$, many aggregates were attached to the spirochetes, cyst and blebs (Fig. 6c).

Discussion

Our investigation revealed high susceptibility of motile and cystic forms of *B. burgdorferi* to RBC. The MBC achieved in our study is far below the concentration

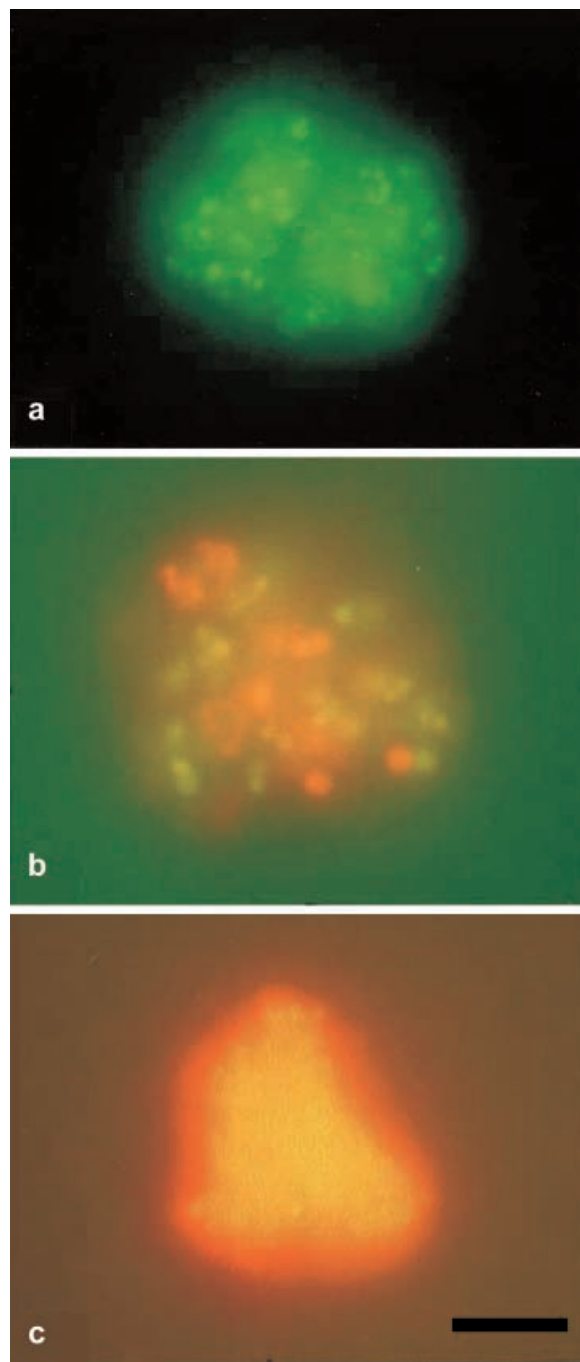
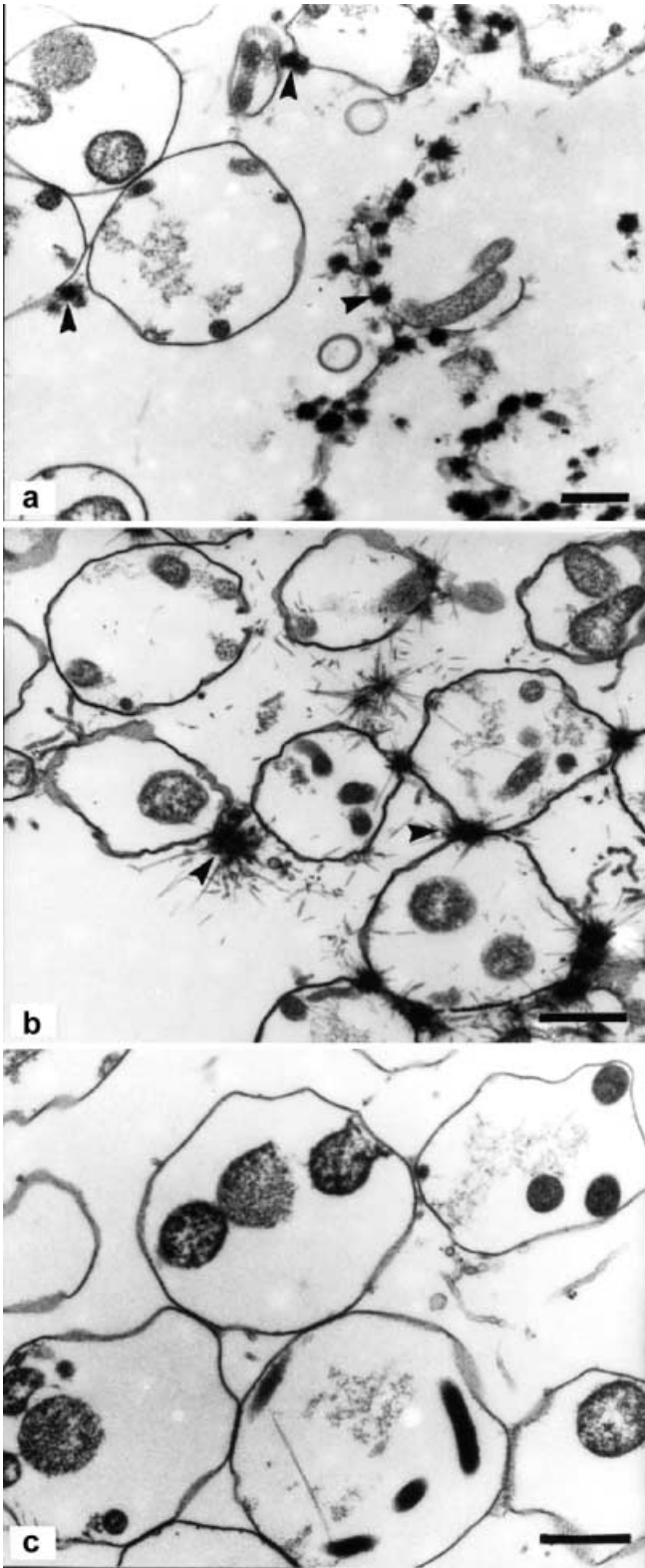


Fig. 3a-c Susceptibility testing of old cystic forms of *B. burgdorferi*, using RBC in distilled water at 37°C for 14 days. **a** $\text{RBC} = 1 \mu\text{g/ml}$: the cyst contains green cores (living organisms). **b** $\text{RBC} = 2 \mu\text{g/ml}$: the cyst contains both green and orange cores. **c** $\text{RBC} = 256 \mu\text{g/ml}$: The cyst contains only red core/granules (biologically inactive organisms) and no distinct cyst/core can be seen. Vital AO-staining, pH 7.4. Bar $5 \mu\text{m}$



achievable in the GI tract [19, 20, 21]. Our present study shows that RBC also attacks the granules and blebs. Bleb production from cysts has been demonstrated earlier by us [6, 7]. Our observation that blebs and granules were targets for bismuth and that few blebs

Fig. 4a–c Old cystic forms. **a** Old cystic forms incubated at 37 °C for 14 days in distilled water with 4 µg RBC/ml. Some aggregates of bismuth (*arrows*) are attached to the cysts. **b** The same conditions, but the RBC concentration is 256 µg/ml. Note the increased aggregation of bismuth (*arrows*) to cysts and blebs, compared with 4 µg/ml. The bismuth aggregates seem to penetrate the envelope of the cyst. No normal blebs are seen. **c** Not exposed to RBC: distinct core structures and a few short spirochetal structures are observed inside the cysts. Some blebs are also present. No clusters of aggregates are observed attached to the cyst. Transmission electron microscopy (TEM), staining: uranyl acetate and lead citrate. Bar 750 nm

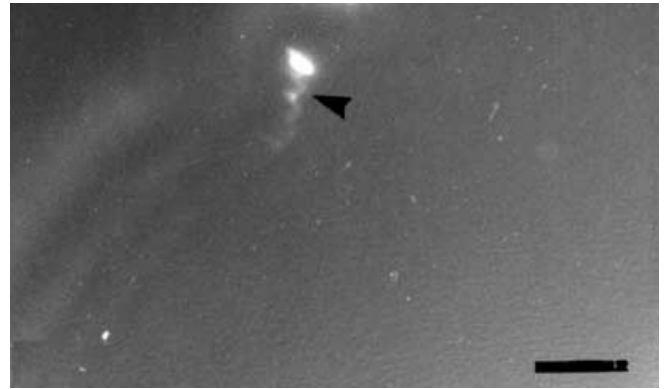


Fig. 5 Old cysts were incubated at 37 °C for 14 days in distilled water with 1 µg RBC/ml and then transferred to BSK-H medium (Sigma B3528). After incubation for 1 week in this medium, some thin, immobile spirochetes (*arrow*) protrude from the cysts. Dark field microscopy. Bar 8 µm

were observed when RBC ≥ MBC may be of relevance in order to decrease the amount of cytokines and CD8 + T-lymphocytes, thereby reducing the ability of the bacterium and cysts to induce disease [26, 44]. Earlier, we demonstrated the *in vitro* susceptibility of young cystic forms of *B. burgdorferi* to metronidazole (MZ) in concentrations which are achievable *in vivo*. However, the motile forms of this spirochete were resistant to MZ and the blebs were not observably affected [8].

The adherence of bismuth aggregates to motile and cystic forms of *B. burgdorferi* and penetration into the bacterium are very similar to what happens when *H. pylori* is exposed to this polycation. The latter recognizes the glycocalyx, resulting in cell damage and death [42]. We demonstrated that new spirochetes emerge from cystic core structures [6]. New spirochetes emerged from the cysts when vital AO-staining (pH 7.4) revealed green core structures inside the cysts (RBC < MBC), whereas yellow/orange/red AO-coloration was observed when conversion to the spirochete form was inhibited (RBC ≥ MBC). A bright green color indicated living organisms, whereas yellow/orange/red indicated dead organisms. The chain of shifts in AO-coloration (bright green/yellow/orange/red) means (from left to right) increasing uptake of AO caused by intercalating with the phosphate–sugar backbone of DNA, as the DNA becomes denatured in non-viable cells [24, 31].

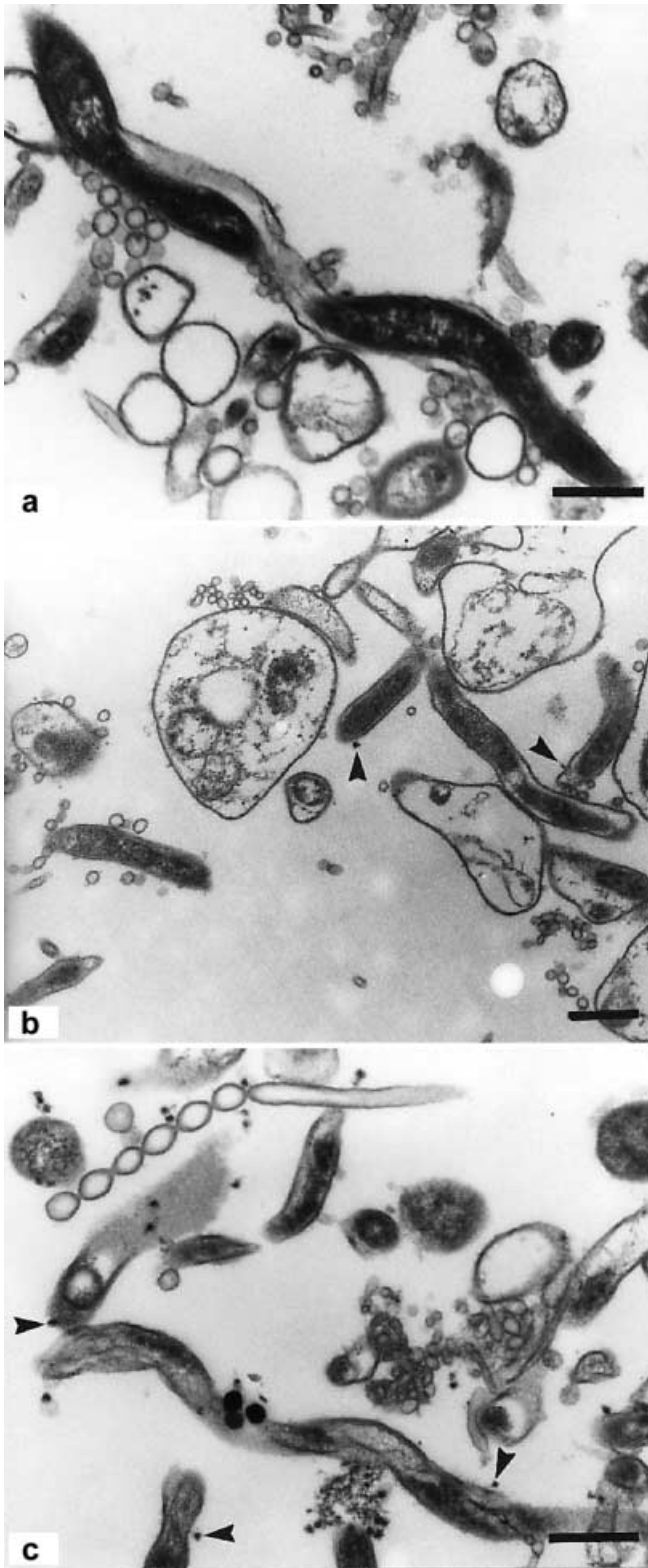


Fig. 6a–c Susceptibility testing of mobile *B. burgdorferi*. **a** Susceptibility testing in BSK-H medium at 37 °C for 14 days without RBC. Normal, motile spirochetes, cores and blebs are present. **b** Same as in **a**, but the RBC concentration is 4 µg/ml. Only a few spirochetes have aggregates of bismuth (arrows) attached to their cell envelopes. **c** At a RBC concentration of 64 µg/ml, bismuth aggregates (arrows) are attached to *B. burgdorferi*. TEM. Bar 500 nm

When $RBC \geq MBC$, AO-stained fixed smears at pH 6.4 revealed cysts with a green color and no distinct core structures could be seen, which implied no or little intact RNA. When $RBC < MBC$, the cysts were red, which implied large amounts of intact RNA [29]. The lack of distinct orange core structures corresponded to concentrations of RBC where spirochetes were no longer formed from cysts.

Our study revealed an enhanced action of bismuth when the temperature and length of incubation increased. When motile spirochetes were incubated at the relatively high temperature of 37 °C, the production of cysts was induced and was independent of the concentration of RBC. However, when the concentration of RBC reached the MBC, the number of cysts decreased. After 14 days incubation at 37 °C with 2 mg RBC/ml, about 100 spirochetes/ml of degraded, slightly motile forms were observed. When subcultured in fresh medium, these bacteria did not regenerate. They died within 14 days. Considering the sparse amount of bismuth crystals attached to the spirochetes at this concentration, the motile spirochetes are apparently more susceptible to RBC than the cysts.

Bismuth is cytoprotective and increases the dermal growth factor [11, 12, 13]. Therefore, bismuth is theoretically advantageous when *B. burgdorferi* causes bleeding in the GI tract [31]. Bismuth salts may also have beneficial properties regarding action on *C. difficile* [28], which can be troublesome for prolonged Lyme treatment.

This is the first susceptibility testing of motile and cystic forms of *B. burgdorferi* to bismuth salts. Further, this is the first indication of viable cystic forms older than 2 months [5] and the first indication that old cysts are more resistant to adverse environment than young cysts. The facts that *B. burgdorferi* can infect the GI tract and that both motile and even old cystic forms of *B. burgdorferi* are susceptible to RBC in vitro, suggest the agent may be of value in vivo when *B. burgdorferi* infects the GI tract.

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