Destruction of spirochete *Borrelia burgdorferi* round-body propagules (RBs) by the antibiotic Tigecycline

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Persistence of tissue spirochetes of *Borrelia burgdorferi* as helices and round bodies (RBs) explains many erythema-Lyme disease symptoms. Spirochete RBs (reproductive propagules also called coccoid bodies, globular bodies, spherical bodies, granules, cysts, L-forms, sphaeroplasts, or vesicles) are induced by environmental conditions unfavorable for growth. Viable, they grow, move and reversibly convert into motile helices. Reversible pleiomorphy was recorded in at least six spirochete genera (>12 species). Penicillin solution is one unfavorable condition that induces RBs. This antibiotic that inhibits bacterial cell wall synthesis cures neither the second ''Great Imitator'' (Lyme borreliosis) nor the first: syphilis. Molecular-microscopic techniques, in principle, can detect in animals (insects, ticks, and mammals, including patients) helices and RBs of live spirochetes. Genome sequences of *B. burgdorferi* and *Treponema pallidum* spirochetes show absence of >75% of genes in comparison with their free-living relatives. Irreversible integration of spirochetes at behavioral, metabolic, gene product and genetic levels into animal tissue has been documented. Irreversible integration of spirochetes may severely impair immunological response such that they persist undetected in tissue. We report in vitro inhibition and destruction of *B. burgdorferi* (helices, RBs = ''cysts'') by the antibiotic Tigecycline (TG; Wyeth), a glycylcycline protein-synthesis inhibitor (of both 30S and 70S ribosome subunits). Studies of the pleiomorphic life history stages in response to TG of both *B. burgdorferi* and *Treponema pallidum* in vivo and in vitro are strongly encouraged.

bacterial resistant stages | doxycycline | medical symbiotics | multiple sclerosis | spirochete cysts

We reexamine evidence and point to mainly Russian studies ignored in English scientific literature that spirochete round bodies (RBs, also called coccoid bodies, globular bodies, spherical bodies, cysts, granules, L-forms, sphaeroplasts, or vesicles) are fully viable. RBs are spherical, membrane-bounded structures that appear in pure cultures as they age in proportion to the disappearance of helical forms. They tend to be immotile or less motile than typical helical-shaped spirochetes although they twitch and may move laterally. Analysis by thin section transmission electron microscopy (tsTEM) has revealed the presence of coiled protoplasmic cylinders and flagella inside RBs that lead investigators to hypothesize that they are pleiomorphic stages of spirochetes (1) or that they are moribund. Anglophone medical discussion of spirochetoses (spirochete-associated infirmities, such as Lyme disease or syphilis) omit mention of ''round bodies'' or state that they have no clinical relevance (2). Yet evidence abounds not only that RBs are viable but also that they may locomote, grow, and reproduce.

Spirochetes threatened by environmental insult form RBs. Unfavorable conditions include changes in solution chemistry: acidity-alkalinity, salts, gas composition; concentrations of antibiotics, sugars, or other organic compounds such as amino acids. Transition from one growth medium to a second of different viscosity or temperature stimulates the formation of RBs. Starvation, threat of desiccation, exposure to oxygen gas, total anoxia and/or sulfide may induce RB formation (3–13). RBs revert to the active helical swimmers when favorable conditions that support growth return (3–5).

That RBs reversibly convertible to healthy motile helices is bolstered by the discovery of a new member of the genus *Spirochaeta*: *S. coccoides* (14) through 16S ribosomal RNA sequences. Related on phylogenies to *Spirochaeta thermophila*, *Spirochaeta bajacaliforniensis*, and *Spirochaeta smaragdinae*, all of which have helical morphology, *S. coccoides* does not form spirals or helices; rather, it grows and reproduces in the RB form (14). Clearly, coccoidal spirochete life history stages are viable.

Penicillin, among many other ''unfavorable growth conditions,'' induces RBs (3, 6, 10–13, 15). That penicillin does not cure either of the two ''Great Imitators'' (Lyme disease and syphilis) is widely accepted in Russian medical literature. In rabbits inoculated for study with the venereal spirochete *Treponema pallidum*, borrelias were seen in testicular-tissue sections (16, 17).

RB formation in the test tube is induced by penicillin, especially in the presence of glycine, a protein amino acid and therefore food. Relevant observations contributed to the PhD dissertation and patent application of Belichenko (18), a student of medical microbiologist Igor Bazikov, Stavropol, Russia. Decrease in penicillin concentration induced reversion of round bodies to active helical spirochetes (18). Russian research (19) and ours (5, 20, 21) on spirochetal life history suggests that the course of Lyme disease, and probably other spirochetoses such as syphilis, is altered by penicillin and other antibiotics.

Spirochetes, motile helical Gram-negative eubacteria, are heterotrophs that at optimal temperatures for growth require abundant moist food. Most ferment sugar in the absence of oxygen. They form a cohesive taxon, a prokaryotic phylum (22) detectable by DNA sequence analysis that corresponds precisely to the 16 Svedberg-unit ribosomal RNA (16S rRNA) component of the small 30S ribosomal subunit. Spirochetes, with their Gram-negative cell walls and internal (i.e., ''periplasmic'') flagella between the inner and outer membranes, are distinctive at the level of thin section-electron microscopy (23). The plasma (inner) membrane is universal in all bacterial (i.e., prokaryotic) and eukaryotic cells, but the outer lipoprotein membrane that surrounds the peptidoglycan layer of the wall characterizes

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Gram-negative bacteria. Spirochete morphology, definitively identified by EM transverse (cross) section, permits assignment of the larger spirochetes (>0.5 μm in diameter) to a lower taxon, "species" or "genus." Negative stain images of high quality may allow assessment of flagella insertions and numbers. Spirochetes display a distinctive flagellar pattern summarized as n:2n:n where the fist "n" is the number of flagella at one end, "2n" (or zero for leptospiras) represents overlap of flagella in the middle, and the second "n" is the number of flagella at the opposite end (24).

Borrelia burgdorferi (4:8:4 or 5:10:5) and other spirochetes in natural habitats [e.g., *Treponema pallidum*:(1:2:1 or 2:4:2) (25)] are detectable by at least three methods: (*i*) morphology, especially active motile behavior in live bacteria, (*ii*) thin section transmission electron micrographs (tsTEM), and (*iii*) negative stain whole mount EMs. Except for quality high magnification observation of spirochetes including their RBs in affected tissue by an experienced microscopist, and to some extent PCR for DNA, no definitive tests for the presence of spirochetes in patients in late stages exist. Cures of Lyme disease or venereal treponeme infection (syphilis) lack this level of verification.

Spirochetoses (e.g., leptospiroses, yaws, syphilis, and Lyme disease) are bacterial diseases correlated with continued presence in the body of potentially identifiable spirochetes. They obey Koch's postulates. The little-known history of Lyme borreliosis begins with the discovery of spiral bacteria in ticks by Dutton before the 1905 publications of Dutton and Todd (26). Spirochetes developed from the large number of "granules" (i.e., RBs) detected in infected ticks when ambient temperatures rose to 25 °C or higher. The spirochete bacteria were later named *Borrelia duttoni*. Hindle (9) injected solutions of "granular forms" (i.e., RBs) in the absence of motile helices (*Borrelia duttoni*) into mice to show that they caused symptoms of infection in the test animals. *Borrelia vincentii* RBs that had remained in their "granular" form for 31 months converted to helical motile spirochetes when transferred to fresh medium under conditions favorable for growth (8). However, when other investigators failed to find propagules, dormant spirochetes or pleiomorphic life history stages the RBs were declared to be dying spirochetes. Although terminologcal confusion (cysts, granules, RBs, vesicles, etc.) exacerbated the problem, the dismissal of an entire scientific literature was unjustified (23).

The presence of the eubacterium spirochete *Borrelia burgdorferi* in human tissue correlates with a syndrome of symptoms in people known to have experienced "erythema migrans," a mobile circular skin blemish related to a blood meal by immature (young means <1 mm) nymphs that are the most active in transmission of the disease. The acarids are *Ixodes scapularis* whereas they are *Ixodes pacificus* in North America and *I. ricinus* in Europe. Arthropods in this genus of ticks (class Arachnida, phylum Chelicerata) (22) lack holometabolous development: eggs hatch into small ticks that molt without metamorphosis into nymphs and later adults. Only ≈50% of those bitten who later test positive for Lyme borreliosis and present other symptoms like "Lyme arthritis" and neuroborreliosis actually develop the tell-tale >50-cent-size, coin-shaped rash at the site of the bite (27). Symptoms vary greatly in severity, frequency, and persistence. When antibodies against tick-borne borreliosis, PCR or cultured borreliosis are detected in patient blood, they are scored "Lyme disease borreliosis positive," but results are frequently negative despite infection (28). An estimated 20,000 cases of Lyme disease are reported annually in the U.S., but the actual number is estimated to be closer to 200,000 cases per year. In central Europe and Scandinavia, the disease is highly endemic. Cases of *B. burgdorferi* in southern Sweden alone rose from 164 (in 1992) to 664 (in 2000) per 100,000 population (29). Over 100 strains of *Borrelia* sp. have been isolated from healthy ticks and grown in rich liquid medium that contains 6% mammalian serum cultured at elevated temperatures (30–36 °C).

At least three immunologically distinguishable strains have been claimed to transmit the tendency toward symptoms: *Borrelia afzelii*, *B. burdorferi sensu stricto*, and *Borrelia garinii*.

Persistence of tissue spirochetes of *B. burgdorferi* has been suggested since Dutton first reported them in the 19th century. The group of isolates in the *B. burdorferii* complex sensu lato includes named "genospecies:" *Borrelia valaisiana*, *Borrelia lucitania*, *Borrelia spielmanii*, and *Borrelia bissettii*, with different organoheterotrophic optimal in vitro growth requirements. Differences in strains relative to symptoms is unclear [Fingerle et al. (30)]. Seabirds in the Arctic regions of Norway carry *Ixodes uriae* ticks from which *B. garinii* spirochetes were isolated (31). In Danish *Ixodes ricinus* three different genospecies were simultaneously present in at least 40% of the ticks (32). *Borrelia* sensu stricto was found in blood plasma from nearly half of the erythema migrans of American patients when large volumes of blood plasma were used as source of spirochete cultivation (33). Lyme borreliosis, with incidence that has increased in northern countries, becomes chronic and variable. The percentage of virulent strains has greatly increased in recent years (34). The site(s) of affected tissue or organ and the intensity, severity, frequency, and duration of symptoms are idiosyncratic. Clinical failures to eradicate all traces of symptoms and correlated immunological indicators of the presence of the spirochetes have been widely reported in mainstream medical literature. Lack of relief with no definitive cure of "Lyme arthritis" or "post-Lyme syndrome" and other borreliosis symptoms has led to a groundswell of health advice, published contradictory claims and other vociferous activity beyond confines of professional medicine. We hypothesize that Lyme borrelioses is an old chronic bacterial symbiosis that tends toward necrotrophy in mammals. In healthy immature ticks, the spirochetes are seasonally transmitted by bite to vertebrates including seabirds, cervids, mice, rabbits, and humans.

Results

Tigecycline (TG) Incubation. When incubated for 3 h (h) in TG concentrations <50 μg/mL, spirochete motility was normal in BSK-H medium to which TG had been added. By use of darkfield microscopy (DFM) at TG concentrations from 50 to 100 μg/mL movement was reduced. When the contents of the incubated tubes were transferred to fresh BSK-H medium, one single motile bacterium was observed after incubating for 8 wk at a concentration of 0.4 μg/mL. Therefore, minimal bacteriocidal concentration (MBC) for 3 h incubation is 0.8 μg/mL.

When motile spirochetes were examined after 24 h incubation in BSK-H medium to which TG was added at 6.25 to 100 μg/mL, the bacterial cells had entirely disintegrated. Those originally exposed to TG concentrations from 3.12 to 0.4 μg/mL had degenerated and were not motile. For concentrations from 0.012 to 0.2 μg/mL, some spirochetes moved normally whereas others hardly moved at all. They failed to survive and reproduce upon transfer to fresh medium after incubation for times up to 2 months. Spirochetes originally exposed to a TG concentration of 0.003 μg/mL grew and reproduced to the same level as the antibiotic-free control. Thus, minimal inhibition concentration (MIC) for 24 h incubation is 0.006 μg/mL, and transfer to fresh BSK-H medium 1/50 showed MBC to be 0.05 μg/mL.

The spirochetes that had been incubated for 7 days (d) with TG/BSK-H were subsequently incubated in fresh BSK-H medium for 8 wk at 34 °C, and the MBC turned out to be 0.012 μg/mL (Table 1). For TG concentration ≤0.006 μg/mL, the spirochetes survived and reproduced in fresh BSK-H. When examined by TEM, a few spirochetes lacking the S-layer of the wall (Fig. 1*A*) and a few RBs with normal ultrastructure were present at the concentration of 0.006 μg/mL (Fig. 1*B*). Spiro-

Table 1. Inhibitory and bacteriocidal effect of TG (TG concentration in μg/ml)

	Spirochete TG culture, incubation time (34°C)		
Concentration	3 h	24 h	7 d
Minimal inhibitory concentration	n/a	0.006	<0.0006
Minimal bacteriocidal concentration	0.8	0.05	0.012

Borrelia afzelii ACA-1 to TG at different incubation times (34 °C).

chetes incubated in growth medium without TG for 7 d showed typical swimming spirochetes with intact cell walls (Fig. 2).

The motile spirochetes incubated for 3 h or 1 d in dH_20 with TG concentrations of 6.25 to 100 μg/mL showed ≈50% reduction in conversion to RBs relative to incubation in distilled water alone. This reduction was 10% for TG concentrations from 0.8 to 3.12 μg/mL. However, when incubated for 2 d, most spirochetes converted to RBs. When the TG concentration was >0.8 μg/mL, the RBs had irregular, pycnotic appearance in DFM.

RBs originally exposed to TG concentrations <0.1 μg/mL for 1 wk, when transferred to BSK-H medium for 2 months (mo), developed into nonmotile spirochetes. RBs that had been incubated in TG concentrations = <0.2 μg/mL for 5 wk, were examined by acridine orange pH 6.4 (Fig. 3). Most RBs disintegrated at least partially and were green in color, from which it is concluded that they are inviable. Very few small, orange cores that had developed from spirochetes were present. For concentrations >0.2 μg/mL only green, disintegrated structure and debris was visible. Healthy cells treated with AO stain with which the RNA orange interacts and the cells are alive. The staining will also show core structures that develop at an age of more than 1 wk in living RB**.** The control incubated for 5 wk lacking TG can be seen in Fig. 3*C*. Many core structures have developed and some are in a state of transverse fission. After 5 d incubation, only intact spirochetes are visible (Fig. 3*B*).

When RBs, originally incubated in TG concentrations <0.1 μg/mL for 5 wk, were stained with BacLight vital stain, a few green cores and spirochetal structures (living organism) were present inside some of the RBs (Fig. 3*A*). When the concentration of TG was 0.1 μg/mL only red RB could be seen (Fig. 3*D*). Therefore, MBC was estimated to 0.1 μg/mL.

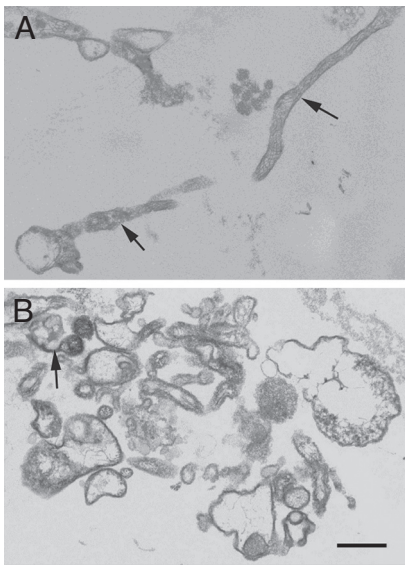

Fig. 1. Spirochetes incubated for 7 d in growth medium with 0.006 μg/mL TG. (*A*) Most spirochetes disintegrated (arrows), but a few spirochetes are recognized that lack the S-layer of the cell wall. (*B*) A few viable RBs (arrow) were visible. (TEM; scale bar: 500 nm.)

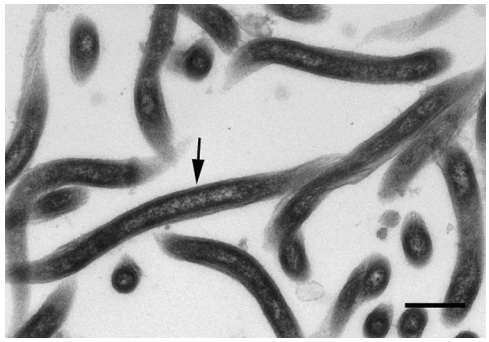

Fig. 2. Spirochetes in growth medium without the antibiotic TG, incubated for 4 d. Shown are typical swimming spirochetes with cell walls intact (arrow). (TEM; scale bar: 500 nm.)

TEM. Spirochetes exposed to TG concentrations >0.006 μg/mL for 7 d had disintegrated; only a few empty membrane bounded RBs were present. Even at the TG concentration of 0.006 μg/mL, only a few bacteria with approximately normal appearance were observed, but even they lacked the normal "sheath" (Fig. 1*A*; the outer membranes, its coat and the adhering peptidoglycan layer; see refs. 23 and 24). The S-layer, the name of the "sheath" around the protoplasmic cylinder of the spirochete, in actuality consists of the periplasmic peptidoglycan inner protein coat of the outer membrane and, if present, its outer coat. The spirochete "sheath" is the distinctive outer layer of the spirochete beyond the plasma membrane (Fig. 2).

At TG concentration of 0.006 μg/mL, a few typical RBs were present (Fig. 1*B*) in stark contrast to the untreated control in which there were no RBs at all (Fig. 2). Rather, only typical helical motile spirochetes were present, indicating conditions optimal for growth. RBs exposed to ≥0.05 μg/mL TG display membrane protrusions, cytoplasmic leakage through membrane rupture, and other abnormalities (Fig. S1). Healthy RBs of normal morphology were present only in untreated controls: those incubated without TG or exposed to very dilute levels (<0.006 μg/mL; Fig. S2). In Fig. 3*E Left*, young RB reveal numerous spirochetes and flagella inside healthy cell walls and a beginning formation of a core structure, and in Fig. 3*E Right* are old RBs with a few core structures, healthy cell wall, and numerous flagella. When replaced into growth media under favorable environmental conditions, helical, motile spirochetes leave the RBs and swim away.

Spirochete RB Formation and Other Pleiomorphy. Life histories of at least six genera of spirochetes (Table 2) permit us to conclude that well-studied healthy spirochetes in nature form viable round bodies (RBs) quickly in response to numerous unfavorable conditions. This morphological transition from periplasmically flagellated, helically motile spirochetes to RBs develops naturally in mixed and pure cultures under conditions that tend to limit growth, and is reversible (23). Many spirochetes under inhibitory changes in oxygen or sulfide concentration fail to form cross walls and elongate into unrecognizable filaments (7). Both the filamentous and round body forms of spirochetes are fully viable. We established in the Berlin conference (Table 2) the identity of various propagules with names that extend from the 1905 granules of Dutton (26) to the 1998 publications by the Brorsons (5). Confusion perpetrated by the plethora of names is resolved here in our recognition of the seme of spirochete RBs. A seme is a trait of evolutionary importance that has been

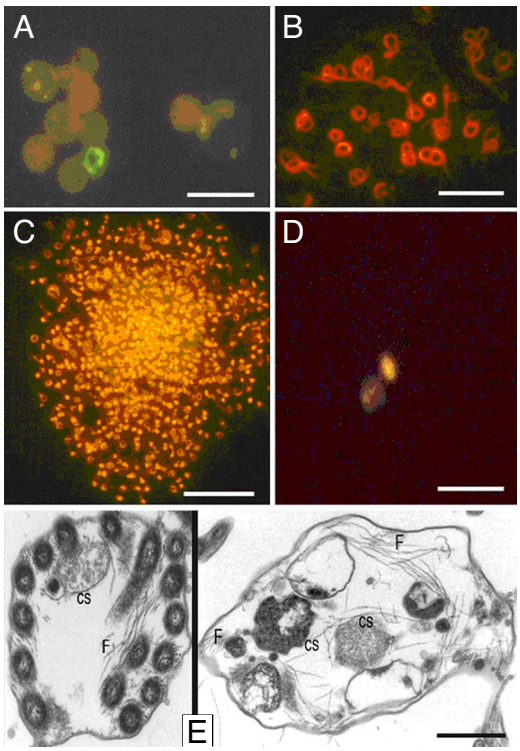

Fig. 3. Spirochete round bodies (RBs). (*A*) BacLight preparations vital stain. RBs formed from healthy spirochetes after 5 wk suspension in dH_2O in 0.05 μg/mL TG. The red stain indicates that the material is dead. Whereas a few green cores and spirochetes in the RBs seen at TG concentrations of 0.05 μg/mL or less indicate some viability after 5 wk, those few RBs with green cores are entirely reversible. (Scale bar: 4 μm.) (*B*) Five-day-old RBs after suspension of healthy spirochetes in dH_2O-acridine orange stain at pH 6.4, flame-fixed. The red-orange stain color indicates the RNA of healthy spirochetes. They do not yet show core structures; the outer membrane is not visible. Core structures develop only in viable, nondegenerating living organisms >1 wk old. (Scale bar: 8 μm.) (*C*) Five-week-old RBs after suspension of healthy spirochetes in dH_2O at pH 6.4 acridine orange flame fixed as in *B*. The red-orange stained RBs show the RNA of these viable propagules that are fully capable of reversion to helical spiral swimmers. Cell division of the core structures inside the RBs leads to proliferation of spirochetes that are not in the motile, helical form. (Scale bar: 10 μm.) (*D*) RBs originally incubated in lethal quantities of TG for 5 wk. Shown is BacLight vital stain. The red stain indicates that the material is dead. Neither spirochetes nor green color (here) was visible in any cultures treated with TG at concentrations >0.05 μg/mL. (Scale bar: 5 μm.) (E) Two RBs in electron micrographic preparation reveal spirochetes and flagella (F) beneath the outer membrane, healthy cell walls and the gradual formation of core structures (CS). When replaced into growth media under favorable environmental conditions, helical, motile spirochetes leave the RBs and swim away. (*Left*) Young RB reveal numerous spirochetes and flagella inside healthy cell walls and a beginning formation of a core structure. (*Right*) Old RBs with several core structures, healthy cell wall, and numerous flagella. (Scale bar: 450 nm.)

naturally selected in any group (taxon) of organisms related by common ancestry. Semes well-known to evolutionists include the following: the Embden–Meyerhof pathway of glycolysis in fermenting bacteria, endospores in bacilli, feathers in birds, flowers in angiosperms, placentas in mammals, and myriad others (see ref. 35). Round bodies, defined both physiologically and morphologically, are characteristic of at least the species listed in Table 2 (all members of the prokaryotic phylum Spirochaetae).

Discussion

Chronic spirochete infections in humans when seen in their ecological-evolutionary context are examples of symbioses that have evolved over geologic time. Symbiotic relationships are characterized by numerous reverse transcriptases and viral-like particles that are posited to be part of the integration process between the symbionts (e.g., human and spirochete) (36, 37).

Our results are consistent with the idea that antibiotic treatment is effective only in the earliest stages of Lyme borreliosis. Antibiotics such as penicillin and its derivative doxycycline induce round body formation and quiescence of symptoms rather than cure. Suspension of round bodies in growth media causes rapid reversion to helical swimmers even in a few days (3–5).

The treatment of spirochetoses, specifically borrelioses, requires knowledge of the natural history, including pleiomorphy, of the borrelia spirochetes in their environmental context. From the profound age of spirochete–animal associations, the permanent, topological and genomic level of integration of spirochetes with tissue suggests a most propitious strategy for amelioration of symptoms in humans. Study and clinical trials of TG, a glycylcycline 30S and 70S ribosomal inhibitor of protein synthesis, untested on spirochetoses (e.g., Lyme disease, syphilis, and perhaps multiple sclerosis) are warranted. TG is related to tetracycline. This broad-spectrum antibiotic is not affected by the kinds of problems of resistance encountered in tetracycline therapies. Resistance of *B. burgdorferi* to tetracycline is probably related to the spirochetes efflux mechanism (38).

Neither ribosomal protection proteins nor efflux by antibiotic-specific pumps has been observed to counteract the efficacy of TG. TG inhibits translation of bacterial proteins by binding both small and large ribosomal subunits of bacterial ribosomes (39–42).

Spirochete Pleiomorphy and Environmental Resistance. Spirochete round bodies (RBs), intraconvertible into helices, are induced by penicillin and many other conditions unfavorable for growth. The seme of formation of viable RBs is widespread in the phylum and no doubt was present in the free-living ancestors of necrotrophic spirochetes. Spore-like structures inside round bodies of *Spirosymplokos deltaeiberi* (1, 23, 46) may be comparable with the cores structures of *Borrelia* described here. In any case, such resistant spirochete forms are fully viable, and in natural habitats (mud and microbial mats) they revert to growing helices when favorable conditions for growth return.

Morphological analyses of Miocene amber 20 million years old revealed an intestinal spirochete. This large *Pillotina* sp. was detected by tsTEM in a *Mastotermes electrodominicus* (family Mastotermitidae, wood-feeding termite) from a Dominican Republic mine. The well-preserved fossil (43) and live observations (44) indicate that spirochetes lived on Earth as healthy symbionts in insects long before the appearance, <0.5 million years ago, of any australopithecine of the genus *Homo*. Clearly spirochete–animal tissue symbioses precede by millions of years the spirochete-induced tissue necrotrophy associated with diseased states, i.e., spirochetoses of Lyme disease or syphilis.

Mainly Russian studies ignored in English literature show that many "unfavorable conditions" including penicillin do not injure round bodies and therefore fail to cure the "Great Imitators," either Lyme borreliosis or syphilis. Both treponeme and *Borrelia* morphotypes persisted as they are simultaneously present in the same EM thin-section in *T. pallidum*-inoculated rabbit testicular tissue (16, 17). In principle, molecular (DNA and RNA hybridization, immunofluorescence, etc.) and microscopic techniques (e.g., tsEM, immunoantibody binding at the light microscopic level combined with electron microscopy, EM autoradiography) can detect live growing spirochetes (helices, RBs) in patients.

Complete genome sequencing reveals enormous deficiencies in the genomes and proteomes of necrotrophic mammalian spirochetes. Only 900 (or 1,100 if plasmid-borne genes are included in the count) chromonemal genes are present in this borrelia. Genomic analysis shows *Borrelia burgdorferi* to be strictly genetically deficient when compared with 4,700 genes in

Table 2. Spirochetes that form RBs

Taxon	Location	Freeliving (F)/ symbiotic (S)	Source
Borrelia duttoni	Africa, Congo	S (ticks)	ref. 26
Borrelia burgdorferi	Norway	S (ticks, humans)	ref. 5
Bradyspira sp.	Denmark	S (pigs)	ref. 36
Clone 16, unnamed spirochete	Spain	F (marine mud)*	ref. 37
Leptospira sp.	Ecuador	F (riparian mud)	Gabriel Trueba†
Mixotricha epibiotic treponemes	Australia	S (termites)	Renate Radek†
Perfilievia russensis, *Spirochaeta perfilievii* ref. 7, †	Crimea, Staraya Russia, Pacific	F (sulfidic mud)* Saline fresh water	ref. 7, †
Spirochaeta coccoides	Caribbean	S (termite)	ref. 14
Spirochaeta bajacaliforniensis	BCN, Mexico	F (marine mud)*	John F. Stolz† Stephen Fracek†
Spirosymplokos deltaeiberi	Spain (Catalanya)	F (marine mud)*	refs. 1, 46
Spirosymplokos mexicanus	BCN, Mexico	F (marine mud)*	ref. 46, Thomas Teal†
Spirosymplokos sippewissettensis	Cape Cod, MA	F (marine mud)*	Thomas Teal† Lois Brynes†
Treponema pallidum	France	S (humans)	ref. 36

Free-living means that the spirochete has been observed to grow unassociated with an animal in nature and/or in culture. Symbiosis, in this table, refers to obligate symbionts of animals that have reduced genomes and are invariably found in nature in association with animal tissue. BCN, Baja California Norte.
*Mud at bottom of laminated microbial mats dominated by cyanobacteria.
†Berlin Natural History Museum Conference on Spirochete Co-evolution in the Proterozoic Eon, May 1–2, 2008, Berlin, Germany.

the cultivable spirochete *Leptospira interrogans* (45). We posit, based on strong inference from scientific literature, that permanent integration into humans of *Borrelia* symbiotic spirochetes at behavioral, metabolic, gene product, and genetic levels may collapse immunologic memory. This hypothesis requires vigorous investigation. Failures to understand chronic diseases of Lyme borreliosis (and syphilis) as symptomatic of ancient coevolved mammalian–bacterial symbioses generate misunderstanding. Little, if any, evidence for cure of late Lyme borreliosis exists in the scientific literature. Independent of serologic test results, investigation to seek *Borrelia burgdorferi*, other borrelias and treponemes in lymph, joints, brain, eyes, etc. where pathology is asserted to "be caused by autoimmune disease" such as multiple sclerosis, is advocated. To avoid perpetuation of underdiagnoses, misdiagnoses, and inappropriate therapies, the borrelia spirochetes, whether as helices or RBs, can and should be directly identified in tissue. Very ill patients are likely to be afflicted simultaneously by more than one kind (strain or species) of these symbiotic spirochetes.

The in vitro inhibition, even destruction, of *B. burgdorferi* (helices, RBs) by the antibiotic TG augurs well for a possibility of a Lyme disease cure. Tygecycline deserves immediate scientific attention. The in vitro effects here of TG on borrelia life history stages help predict successful in vivo results both in laboratory animals and patients. Failure to understand chronic diseases, in this case Lyme borreliosis, as a manifestation of permanent genetically integrated symbionts where disease symptoms are expressions of symbiogenesis to which many aspects of the vertebrate immune system respond, generates tragedy and even death due to ignorance. Misdiagnoses and "therapies" that aggravate conditions of the patient could be avoided if treatment were based on scientific principles. The borrelia–human physical association of Lyme disease, as a spirochete symbiosis, ought to be recognized as a coevolved eubacterial–acarid–mammalian association highly integrated on several levels: the genetic, gene product (RNA, protein, coenzyme), metabolic (metabolites, ion flow, water balance), and behavioral. Both at the cell and organismal level, changes can be documented in the bacterial, acarid, and human partnership. This wider view of Lyme borreliosis, coupled with the efficacy of TG for inhibition and destruction of the pleiomorphic stages, especially the RB propagules in vitro, provides a call to action to test this antibiotic in a clinical setting.

The MBC of doxycycline, one of the recommended antibiotics used for effective early treatment of Lyme borreliosis for this strain of *Borrelia burgdorferi*, was 8 μL/mL for the spirochete morphotype and a concentration thirty-two times higher (256 μL/mL) for the much more resistant RB forms. TG is here far more effective than doxycycline in destruction of the *Borrelia* RB propagules. Therefore, we advocate clinical studies that compare these drugs and begin with comparable dosages. We urge expanded study in vivo and in vitro of Tigecycline's effects on borrelias, leptospiras, perfilievias, *Spirochaeta coccoides*, and especially *Treponema pallidum*.

Materials and Methods

The bacterial strain used in our experiments was *B. afzelii ACA-1*. Production of motile spirochetes and RB forms was performed as described by Brorson and Brorson (20).

Motile Spirochetes: Test of Antibiotic Susceptibility. Tigecycline "Wyeth" 50 mg (Wyeth Pharmaceuticals, New Lane, Havant, Hampshire PO9 2NG, U.K.) was dissolved in sterile 0.9% sodium chloride. TG was tested at 100–0.0006 μg/mL in BSK-H medium (B3528; Sigma, St. Louis, MO). The concentration of inactivated (56 °C, 30 min) rabbit serum (R7136; Sigma) in the BSK-H medium was 6%, and this serum was guaranteed free of antibodies against *B. burgdorferi* by the manufacturing company (Sigma). All culture media were sterile filtered by a 0.2-μm filter, ensuring both sterility and the absence of mammalian cells from serum. Cultures were incubated in sterile 5-mL closed tubes (Nalge cryovial; Nalge, Rotherwa, U.K.) at 34 °C. The spirochetes were suspended in TG antibiotic concentration from 100 μg/mL to 0.0006. An inocula of 40 μL of 10^7/mL bacteria in logarithmic growth was added, to bring the final volume to 4 mL in each tube. The control contained only BSK-H medium. The tubes were incubated for 3 h, 24 h, and 7 d before observation. The spirochete samples were also suspended in antibiotic solution or controls in dH_2O for 1 wk at 34 °C and incubated in tubes with tight caps to minimize gas exchange.

Susceptibility Testing of RBs. Antibiotic was diluted in the range 200–0.0012 μg/mL in 2 mL of diluted BSK-H medium (dilution 1:100 in dH_2O). The control was diluted BSK-H. The RBs were produced by diluting 5×10^7 spirochetes

1:100 in dH_2O. Two-milliliter suspension of RBs of 5×10^5/mL of a 3 h culture was added to each tube to give a final concentration of 100–0.0006 μg/mL. After 1 wk, the 100 μL of growth medium (BSK-H) was added to observe developmental changes in the RBs. The TG-exposed RBs were transferred to fresh BSK-H medium 1/50 and incubated for 2 mo at 34 °C.

Examination of the RBs. The RBs on addition of growth medium were incubated for 4 wk, and samples were taken and acridine orange (AO) stained for examination by UV microscopy to look for conversion of spirochetes inside RBs to core structures. Culture medium samples of 20 μL were transferred to glass slides, dried, flame fixed, overlain with 50 mg/L AO in pH 6.4 phosphate buffer for 4 min and rinsed in distilled water. The AO-stained RBs were examined by UV microscopy. (Magnification: 1,000–1,600×.)

The 4-wk-incubated RBs were also examined with vital stain. A mixture of 10 μL of BacLight with 10 μL of culture fluid was prepared. (Live/dead BacLight bacterial viability kit, L-13152; Molecular Probes Eugene, OR) and placed on glass slides protected by cover slips. After 15-min incubations in darkness, slide preparations were examined by UV microscopy at magnification 800 to 1200×. The RBs were also examined by DFM at magnification 400×.

Microscopic Examination of Motile Helical Spirochetes. The TG tubes that contained motile borrelia in growth medium (and dH_2O controls) were examined for life history-stage change by DFM at magnification 200 and 800× after 3 h, 24 h, and 7 d. The 3-h-, 1-d-, and 7-d-old spirochetes in BSK-H (normal growth medium) were transferred to fresh medium (diluted 1/50) and incubated for 8 wk at 34 °C and examined by DFM (magnification, 400×) to assess effects of TG to confirm or refute the presence of viable bacteria, injured helical spirochetes, or induction of RBs.

TEM Observations. Cultures of spirochetes with and without TG were examined by TEM as described by Brorson and Brorson in ref. 20. Motile spirochetes were incubated for 7 d with TG at dilutions of 0.006, 0.05, or 0.4 μg/mL in normal growth medium and a control that lacked TG. RBs were incubated for 1 wk with TG at dilutions of 0.05, 0.8, and 6.2 μg/mL with a TG-free control.

MBC of the motile spirochetes was determined by transfer to growth medium. The lowest concentration of exposure to antibiotic where no growth occurred was set as the MBC value for the TG-treated motile spirochetes. The MIC value for spirochetes was determined according to the lowest antibiotic concentration that gave reduced multiplication rates when examined in DFM. (Magnification: 400×.)

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