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Conversion of *Borrelia garinii* cystic forms to motile spirochetes in vivo

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Cystic forms (also called spheroplasts or starvation forms) and their ability to reconvert into normal motile spirochetes have already been demonstrated in the *Borrelia burgdorferi* sensu lato complex. The aim of this study was to determine whether motile *B. garinii* could develop from cystic forms, not only in vitro but also in vivo, in cyst-inoculated mice. The cysts prepared in distilled water were able to reconvert into normal motile spirochetes at any time during in vitro experiments, lasting one month, even after freeze-thawing of the cysts. Motile spirochetes were successfully isolated from 2 out of 15 mice inoculated intraperitoneally with cystic forms, showing the infectivity of the cysts. The demonstrated capacity of the cysts to reconvert into motile spirochetes in vivo and their surprising resistance to adverse environmental conditions should lead to further studies on the role and function of these forms in Lyme disease.

Key words: Lyme borreliosis; *Borrelia*; cystic forms; infectivity.

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The first accurate report focusing on cystic forms of *Borrelia burgdorferi* spirochetes was published in 1997 (1). Similar and probably equivalent borrelial structures (so-called blebs, *gemmae*, spheroplasts, L-forms) were described even before this by different researchers (2–5). Outer surface blebs and *gemmae* containing granular material were observed in *borreliae* as long as 50 years ago (6). These structures were associated with spirochetal cells undergoing degenerative changes in a hostile environment (ambient changes, antibiotic treatment, host immune defence activity – antibodies and complement, aging of in vitro borrelial culture etc.), until surprising findings regarding cysts were encountered by Brorson & Brorson in 1997 (1). They showed that normal motile *borreliae* could develop from *B. burgdorferi* cysts and that the cysts were metabolically active, at least under in vitro conditions (1). The same authors observed that motile spirochetes also matured from cysts formed in distilled water, even after being kept in such a medium for over a month (7). These experiments clearly demonstrated that cystic forms were not necessarily only a sign of spirochete degeneration. Cystic forms might just as well represent a low metabolic activity state or phase of *B. burgdorferi* bacterial cells that allows the spirochete to survive in a hostile environment until conditions are favourable for the *borreliae* to grow and replicate again.

The aim of our study was to examine whether cystic forms were able to develop into motile vegetative spirochetes also in vivo, and to verify the infectivity of the cysts in a mouse model.

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Low-passage *B. garinii* was chosen for our experiments as one of the species from the *B. burgdorferi* complex reported to infect humans.

MATERIAL AND METHODS

B. garinii *spirochetes and cysts*

Preparation of *B. garinii* cysts: *B. garinii* cysts were prepared in sterile distilled water as previously described (7). Briefly, *B. garinii* M3S (isolated from the heart of an experimentally tick-infested mouse) in its third passage was grown in 100 ml BSK-II medium (Sigma). The culture was centrifuged (3200 *g*/30 min at room temperature) and the sediment was resuspended in 500 µl of the supernatant and then transferred to 50 ml sterile double distilled water at 4°C. Spirochetes should convert into cystic forms in less than a minute due to the hypotonic environment (7). The cysts were then kept in distilled water at room temperature in the dark. The formation of the cysts was controlled and the cysts, as well as motile spirochetes, were counted by darkfield microscopy at 250 and 400× magnification using a Petroff-Hauser chamber.

In vitro viability testing of the cysts – examination of the capacity of cysts of different age to reconvert into normal, motile spirochetes

As already described by Brorson & Brorson (7), 100 µl of cyst suspension was transferred to 5 ml BSK-II medium 2, 7 and 28 days after preparation in order to determine the time needed for the cysts of different age to reconvert into normal spirochetes. The cultures were incubated at 33°C and examined by darkfield microscopy at least once a week for the presence of motile spirochetes. The cultures were centrifuged at 3200 *g*/30 min every 2 weeks and the sediment was resuspended in a tube with 5 ml fresh BSK II medium. This procedure lasted 3 months starting from the day of BSK-II inoculation. At the same time that the BSK-II was seeded with the cystic forms of specific age, control BSK-II tubes were inoculated with 100 µl of 0.45 µm filtrate of a corresponding cyst preparation. These controls were performed in order to confirm or exclude the possibility that motile spirochetes which might be present in the subsequent BSK-II culture grew from the remaining non-encysted *borreliae*. If such spirochetes were present in the original preparation of the cysts, they would pass through the 0.45 µm filter, as exhaustively demonstrated by quantitative studies (8). If viable, positive

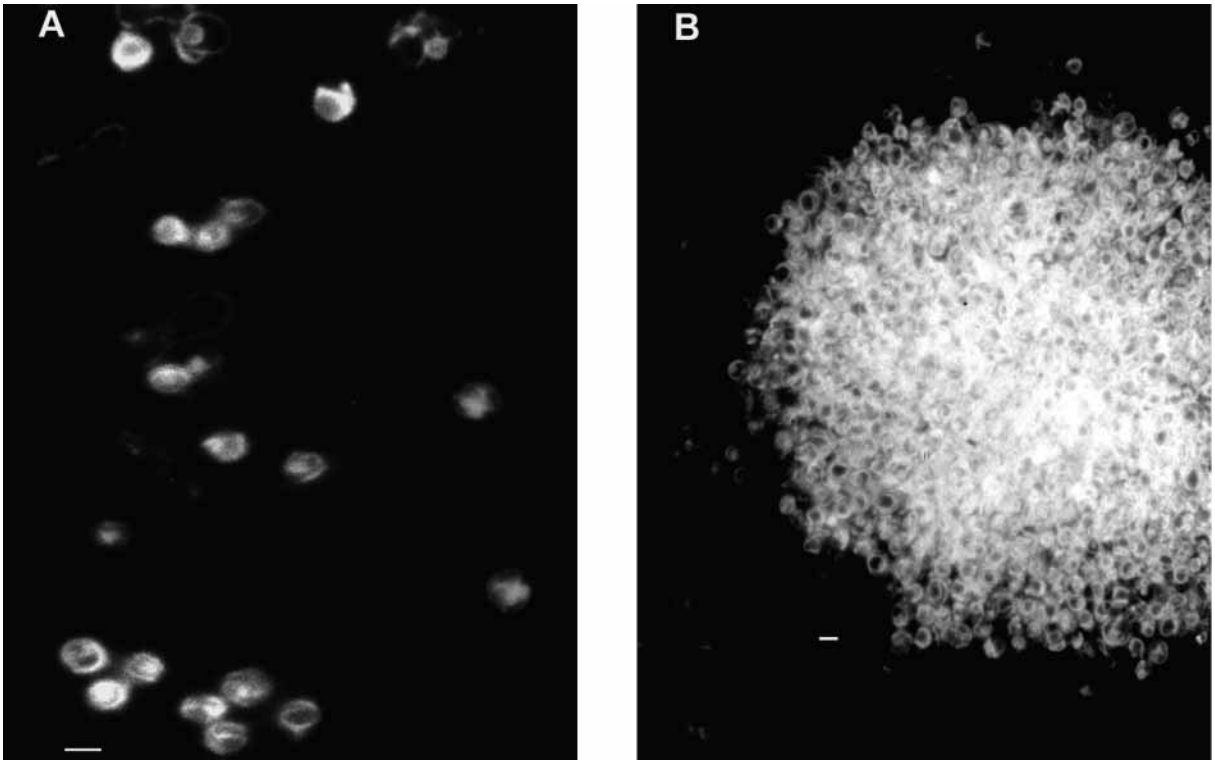

Fig. 1. (A): *B. garinii* cystic forms in distilled water, 24 h after their formation: the cysts are singular and globularly shaped. (B): *B. garinii* cystic forms in distilled water, 7 days after formation: a typical aggregate of the cysts. Spirochetal structures are visible inside the cysts in both A and B. Darkfield microscopy, original magnification 400 ×; bar=1 µm.

TABLE 1. *The results of in vitro viability testing of* B. garinii *cysts of different age by BSK II culture, with data on the time needed to reconvert into normal motile spirochetes*

Age of *B. garinii* M3S cysts in distilled water	2 hours	2 days	3 days	7 days	10 days	14 days	21 days	28 days
Reconversion time (days)	1	9	15	20	14	26	29	33

B. garinii culture would then be scored in the control tube. If the non-encysted spirochetes were absent from the cyst preparation or were dead, the control should remain sterile.

Infectivity testing of B. garinii *cystic forms*

Experimental inoculation of mice with B. garinii *cystic forms, blood sampling procedures and isolation of spirochetes.* Fifteen female BALB/c mice (aged 6–10 weeks) divided into three separately caged groups of five animals were inoculated intraperitoneally with the *B. garinii* M3/S cystic forms (2.5×10^6 cysts in 500 µl distilled water per mouse) of different age. The first group of mice (group A) was injected with 2-day-old cysts, the second (group B) with 7-day-old cysts, and the third (group C) with 28-day-old cysts. Five mice (group D) were infected with the original *B. garinii* M3S (1×10^6 spirochetes in 100 µl BSK-II medium per mouse) as the positive control group. Five mice (group E) were inoculated with 0.5 ml sterile BSK-II medium, and served as negative controls. Mice from group F were injected with 0.5 ml sonicated *B. garinii* cysts held in distilled water for 2 days (400 ng/ml) to evaluate mouse humoral response to dead cysts. Blood samples were taken once a week for 2 months starting from the day of inoculation. Sera were stored at −20°C until the day of analysis. Two months after the initial inoculation the animals were sacrified and the dissected organs (heart, urinary bladder and kidney) were placed in separate tubes containing 5 ml of fresh BSK-II medium. These BSK-II mouse organ cultures were processed and examined as described above for the cyst viability experiments.

ELISA for the detection of B. garinii*-specific antibodies in sera from mice experimentally inoculated with* B. garinii *cystic forms.* The development of the humoral immune response was monitored by indirect ELISA as previously described (9). Sonicated *B. garinii* M3S was used as antigen. Sera from group D mice served as the positive and those from uninfected group E mice as the negative controls. Sera yielding optical densities higher than the calculated average + 3 SD of sera derived from uninfected controls were considered positive.

RESULTS

B. garinii *cysts*

According to darkfield microscopy, more than 95% motile *B. garinii* transformed into cysts 2 h after the initial contact with distilled water. However, the non-encysted spirochetes never completely disappeared from the cyst water suspension. The non-encysted spirochetes had a regular elongated appearance and were clearly non-motile. The cysts had a globular shape and were mostly single at the beginning of the experiment (Fig. 1A). However, aggregates of cysts were also present, which, as time progressed, grew in size due to the increasing number of aggregated cysts (Fig. 1B). One week after their formation, the cysts were mostly aggregated and single cysts were rare.

Viability testing of B. garinii *cystic forms*

As reported by others (1), *B. garinii* cysts were able to reconvert into normal mobile spirochetes even after being kept in distilled water for 4 weeks (Table 1). Younger cysts generally

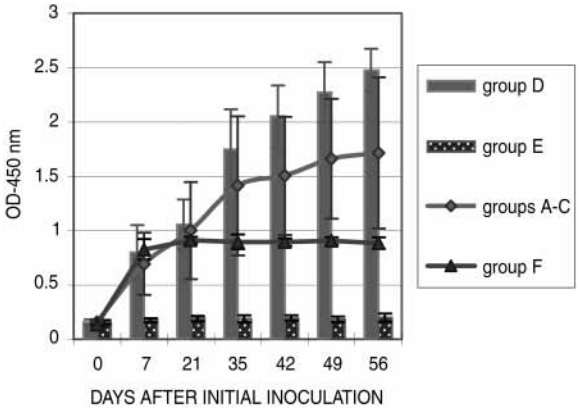

Fig. 2. Humoral response of mice inoculated experimentally with *B. garinii* cystic forms of different age, motile *B. garinii* or BSK II medium, as determined by indirect ELISA. Animals were inoculated with *B. garinii* cysts kept in distilled water for 2 days (group A), 7 days (group B) and 28 days (group C). Mice from the positive control group (D) received motile *B. garinii*, mice from the negative control group (E) received plain BSK II medium, and mice from group F received sonicated *B. garinii* cysts (2 days in distilled water). The data are presented as average OD of ELISA results from mice belonging to groups A–C, D, E and F.

needed less time to reconvert than the older ones. All the control tubes inoculated with 0.45 μm filtrates of corresponding cyst suspensions remained sterile after 3 months' culture. Our impression was that the cysts reconverted to normal spirochetes simply by the cyst membrane opening and subsequent release of the preformed spirochete, with brusque convulsive movements in order to get rid of residual fragments of the former cyst. Normal motility was then gradually regained. Progressive growth of initially small and thin spirochetes, described by Brorson & Brorson (1, 7), was not observed.

Infectivity testing of B. garinii *cystic forms*

None of the mice inoculated with *B. garinii* M3/S cystic forms, *B. garinii* M3/S spirochetes and BSK-II medium developed clinical symptoms of the disease during the 2-month experiment. The humoral responses of mice from each group are shown in Fig. 2. Generally, rising serum titres were observed in ELISA in virtually all the animals with the exception of the negative control group of mice, independently of the age of the cysts being inoculated. Constant specific antibody titres were registered in mice from group F (inoculated with sonicated cysts) after an initial rise during the first week. Motile *B. garinii* was isolated from the hearts of 2 of 15 cyst-inoculated mice (13.3%). Culture-positive mice were inoculated with *B. garinii* cystic forms kept in distilled water for 7 and 2 days, respectively. The spirochetes were first noticed in the third and fifth blind passage tube 40 and 70 days after the beginning of BSK-II organ culture, respectively. The other 13 heart, 14 bladder and 13 kidney seeded tubes from the remaining 13 animals inoculated with cysts from distilled water were all judged negative 3 months after the beginning of each culture. Three out of forty-five cultures (6.6%) were discarded because of contamination. *B. garinii* was reisolated from all the examined organs of each animal from the positive control group (group D). The negative control mice (group E) and mice inoculated with sonicated cysts (group F) showed negative results in isolation attempts. The results of cyst inoculation experiments in mice, and subsequent attempts at *B. garinii* reisolation, are given in Table 2.

DISCUSSION

The reports on *B. burgdorferi* cystic forms (2, 5), their presence in tissues and body fluids of borreliosis patients (3–5), demonstration of their survival for extended periods of time in distilled water and their in vitro transformation to normal mobile spirochetes (1, 7, 10) raise inevitable questions regarding the function of these borrelial structures in Lyme disease. Our in vivo infectivity testing of *Borrelia garinii* cystic forms described in the present study might provide some useful additional information about this extremely interesting argument.

The results we obtained during in vitro viability testing of *B. garinii* cystic forms essentially confirm previously reported data on cyst reversal to mobile spirochetes (1, 7, 10). As shown in Table 1, the cysts we prepared maintained the capability of in vitro transformation in normal *B. garinii* even after being kept in distilled water for 4 weeks, indicating the surprising survival of *Borreliae* under adverse living conditions. In agreement with Brorson & Brorson (7), the older the cyst water suspension, the more time was needed for the cysts to reconvert. The non-converted spirochetes, as estimated from their elongated form and absolute non-motility, ap-

TABLE 2. *The results of in vivo infectivity testing of* B. garinii *cystic forms* via *cyst inoculation experiments on mice and subsequent* B. garinii *reisolation attempts*

Group	Mice Inoculum	Heart	BSK-II organ culture		Total
			Bladder	Kidney	
A	Cysts aged 2 days	1/5	0/5	0/5	1/15 (6.6%)
B	Cysts aged 7 days	1/5	0/5	0/5[a]	1/15 (6.6%)
C	Cysts aged 21 days	0/5	0/5[a]	0/5[a]	0/15 (0%)
	Total	2/15 (13.3%)	0/15	0/15	
D	Motile *B. garinii*	5/5	5/5	5/5	15/15 (100%)
E	BSK-II	0/5	0/5	0/5	0/15 (0%)

[a] contamination of one of the cultures.

parently did not survive in distilled water. This was also confirmed by negative culture results when the cyst preparations were 0.45 μm filtered and then seeded in BSK-II medium.

Cyst inoculation experiments on mice were designed to assess the infectivity of *B. garinii* cystic forms in vivo. Rising specific antibody titres as a sign of vigorous humoral immune response were recorded in virtually all mice injected intraperitoneally with water suspension of cystic forms of different age throughout the 2 months of the experiment (Fig. 2), thus indicating probable active infection. Antibody titres in these mice were not as high as in mice inoculated with vegetative motile spirochetes; this might reflect the fact that not all the cysts converted to vegetative forms, resulting in the comparatively lower number of infecting spirochetes. However, specific antibody titres were substantially higher and rising in mice that received intact *B. garinii* cysts from distilled water than in mice inoculated with sonicated cysts, where the amount of specific antibody was relatively low and constant after the initial rise. This might illustrate the fact that the spirochetes reconverted from the cysts and replicated, inducing a much more evident immune response than in the case of sonicated dead cysts.

B. garinii was successfully reisolated from the hearts of 2 out of 15 (13.3%) cyst-inoculated BALB/c mice. Difficulties regarding reisolation of reconverted spirochetes from cyst-inoculated mice might be comparable to those in *B. burgdorferi* isolation from patients treated with antibiotics (4). The BSK II medium might not be the most suitable medium to recover such spirochetes (11). On the other hand, extended culture preceding successful *B. burgdorferi* isolation is not rare in Lyme disease, and the importance of sufficiently long BSK cultivation with several blind passages has been underlined once again.

Interesting conclusions can be drawn from the results of cyst inoculation experiments on mice: *B. garinii* cystic forms maintain their capability to reconvert into normal spirochetes not only in vitro but also in vivo and can therefore be considered infective, at least in BALB/c mice.

Our results demonstrate that *Borrelia garinii* cystic forms should be taken into consideration when trying to explain the enigmas of Lyme disease. Resistant cysts and their capacity to reconvert into motile spirochetes both in vitro and in vivo indicate that *B. burgdorferi* is not such a fragile and delicate microorganism as might be expected from its difficult isolation. If we accept the thesis that the spirochetes convert to cysts by taking off the outer surface envelope with virtually all the principal antigens and enter the torpor state, then their chances of survival in adverse conditions are not negligible. Dormant *borreliae* evacuated in cysts might be resistant to antibiotics (due to low metabolic activity), and stable under non-optimal environmental conditions. Borrelial cystic forms could therefore be responsible for the frequent failures of antibiotic therapy and for the commonly reported relapses of Lyme disease. These borrelial forms might also be involved in the survival of *borreliae* in unfed ticks.

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