0014-4754/94/010054-06$1.50 + 0.20 

# Retention of *B. burgdorferi* pathogenicity and infectivity after multiple passages in a co-culture system*

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*Received 13 September 1993; accepted 15 October 1993*

*Abstract.* In vitro cultivation of *B. burgdorferi* in BSK medium results in the loss of infectivity and pathogenicity after repeated passages. To prevent this loss, a feeder layer of tibio-tarsal joint tissue derived from newborn LEW/N rats was grown on Cytodex 3 microcarriers in ESG (formerly BSKE), a novel medium developed to support the growth of both the feeder layer and *B. burgdorferi*. A new pathogenic isolate (FNJ) and a high passage, non-pathogenic strain (TNJ) grew well in this co-culture system with high yields of viable organism. FNJ caused no growth inhibition or visible damage to the cells in the feeder layer. FNJ remained arthritogenic for newborn LEW/N rats after 22 passages in the co-culture system, but lost its arthritogenicity after 7 passages when cultured in BSK medium. This borrelia-mammalian tissue co-culture technique presents an experimental system to study the long term interactions of *B. burgdorferi* with the infected host tissues in vitro, as well as facilitate diagnostic tests and vaccine development.
*Key words. B. burgdorferi*; arthritogenicity; co-culture; Lyme; ESG medium.

Tissue culture feeder layers have been used to cultivate many pathogens, including viruses and bacteria. Intracellular pathogens such as Rickettsiae and Ehrlichiae have been grown not only in embryonated eggs but also in tissue cultures derived from susceptible hosts[1,2]. Tissue co-culture may be a promising approach to the challenging problem of in vitro cultivation of *Treponema pallidum*[3,4]. The feeder layer mimics nutritional conditions of the host animals from which they are derived, without the presence of inhibiting components of the host immune system.

In vitro cultivation of *B. burgdorferi*, the etiologic agent of Lyme borreliosis, has been carried out routinely in a modification of Kelly's medium, known as BSK medium[5]. However, loss of infectivity and changes in plasmid content, spirochetal proteins and lipopolysaccharide-like material occur during sequential passages in this medium[6]. Successful cultivation of *B. burgdorferi* in the infectious form is desirable in order to facilitate studies relating to vaccine development, diagnostic tests, immunopathogenic mechanisms of Lyme disease, and spirochete metabolism.

LEW/N rats are susceptible to infection with *B. burgdorferi* and develop arthritis after injection with the organism[7]. The tibio-tarsal joint is one of the target sites in which *B. burgdorferi* resides during this infection. Therefore, tibio-tarsal joint tissue was chosen as the source of cells for the feeder layer in a tissue-*B. burgdorferi* co-cultivation system. A novel culture medium, ESG (formerly BSKE) was developed to facilitate the optimum growth of the host-derived tissue layer and the spirochetes simultaneously[8]. In this study, a co-cultivation system that permits the retention of infectivity and pathogenicity in long-term culture of *B. burgdorferi* in vitro is described.

## *Materials and methods*

*ESG medium.* Ingredients of ESG are listed in table 1. Chemicals were obtained from Sigma (St. Louis, Missouri). pH of the medium was adjusted to 7.4 with 0.1 N HCl. The medium was sterilized by filtration through 0.2 micron pore size nitrocellulose filters and kept in tightly capped containers to prevent pH changes during storage. Dulbecco's modified Eagle's medium (DMEM) was added after pH adjustment and filter sterilization of the other components. 6% FBS (fetal bovine serum; Sigma) was added only during the establishment of the rat joint tissue feeder layer. Shelf life of the medium was one month at 4 °C.

*Strains of* B. burgdorferi. Non-pathogenic, routinely subcultured spirochetes were obtained from the New Jersey Department of Health in Trenton, New Jersey (TNJ). Pathogenic *B. burgdorferi* were isolated from male adult *Ixodes scapularis* ticks sampled in Freehold, New Jersey (FNJ); 10 tick midguts were pooled, minced in BSK and filtered through 0.45 micron pore size filters (Millipore, Bedford, Massachusetts). Cultures were incubated for 4 weeks at 33 °C and inoculated into solid BSK medium for selecting single bacterial colonies[9].

*Identification of borrelia strains. B. burgdorferi* were identified by indirect fluorescence antibody (IFA) technique using anti-OspA and anti-OspB antibodies obtained from Drs J. L. Benach and C. Wheeler, Department of Pathology, State University of New

Table 1. ESG medium for borrelia-rat joint tissue co-cultivation

| Component | g/l |
|---|---|
| Neopeptone | 2.5 |
| Bovine serum albumin Fr. V | 2.5 |
| Yeast extract | 0.5 |
| HEPES | 1.21 |
| Glucose | 1.75 |
| Sodium citrate | 0.0035 |
| Sodium pyruvate | 0.035 |
| N-acetyl glucosamine | 0.2 |
| Sodium bicarbonate | 0.39 |
| Gelatin (2% solution) | 4 ml |
| DMEM (Sigma cat #D-5405) | 500 ml |
| CMRL (Gibco cat #11530-029) | 50 ml |
| d $H_2O$ | 446 ml |
| L-Glutamine | 2 mM |
| FBS | 6% |
| pH | 7.4 |
| Osmolarity | 320 ± 5%mOsm |

York-Health Science Center of Stony Brook. Fluorescein isothiocyanate (FITC)-coupled goat anti-mouse immunoglobulin (Sigma), diluted 1:250 in PBS with 1% BSA (bovine serum albumin) Fraction V (Sigma) was used as the label in all IFA procedures[10].

*Preparation of primary tissue culture feeder layers.* Tibio-tarsal joint tissue from neonatal LEW/N rats (HSD, Indianapolis, Indiana), including synovium, was digested with 0.2% trypsin (type IX) (Sigma) for 30 min at 25 °C with gentle agitation on a rocking platform (Bellco, Vineland, New Jersey). The supernatant was discarded and the tissues were washed twice with Dulbecco's balanced salt solution (DBSS; Sigma). Collagenase (Sigma) (0.2%) in Hanks' balanced salt solution (HBSS; Sigma) was used for further digestions at 25 °C with gentle agitation. Collagenase digestion was repeated 4 times in complete culture medium, ESG with 6% FBS (Sigma, cat. #F 4010), penicillin (100 IU/ml), streptomycin (100 µg/ml), and L-glutamine (2 mM) (Sigma), at 25 °C with gentle agitation. Disaggregated cells were collected from the supernatant by centrifugation at $600 \times g$ for 10 min, and washed with ESG medium supplemented with penicillin (100 IU/ml), streptomycin (100 µg/ml) and L-glutamine (2 mM). T flasks (Nunclon, Nunc, Denmark) were used for all cultivations. Cytodex 3 microcarriers (Pharmacia, Uppsala, Sweden) were used to increase the cell culture surface area by approximately 70-fold. The microcarriers were washed in DBSS, pre-incubated in DBSS at 5 °C for 18 h, autoclaved for 15 min at 150 psi, 115 °C and washed in DBSS before use. The bottoms of empty T flasks were covered with sterilized microcarriers (3 mg/ml) before adding the rat cells. One ml of disaggregated cells ($10^6$ cells/ml) was inoculated into 5 ml of ESG with penicillin (100 IU/ml), streptomycin (100 mg/ml), L-glutamine (2 mM) and 6% FBS (Sigma) per flask and then incubated at 33 °C in sealed containers (Nalgene, Rochester, New York) until the feeder monolayers were formed. Cultures were then fed twice a week with ESG without FBS and antibiotics. Rat joint-derived tissue culture controls were grown in Dulbecco's minimal essential medium (DMEM; Sigma) with 6% FBS and 2 mM L-glutamine.

*Subculture of the primary feeder layers.* Microcarriers covered with feeder layer cells were collected with a pipet from the exponentially growing cultures. T flasks containing 15 mg of sterile Cytodex 3 microcarriers were seeded with 1 ml of this suspension. Five ml of ESG medium with 6% FBS and L-glutamine (2 mM) were added for feeder tissue layer growth.

*Viability test for tissue feeder layers.* The viability of the feeder layer cells was measured by their ability to exclude trypan blue dye, determined both before and after the *B. burgdorferi* co-cultivations. ESG medium was discarded, feeder layers were washed with PBS (pH:7.2), and trypan blue solution (0.4%) (Sigma) was applied. One hundred cells were evaluated per culture.

*Co-culture of borrelia and rat joint tissue feeder layers.* Tissue culture feeder cell layers growing on Cytodex microcarriers in T flasks were subcultured at least once without antibiotics before being inoculated with TNJ or FNJ strains of *B. burgdorferi*. Spirochetes were added ($10^6$/ml) to the tissue cultures and grown in ESG without FBS or antibiotics. The co-cultures were incubated at 33 °C in sealed containers and were fed at 4 d intervals with ESG without FBS or antibiotics.

*Co-inoculation of borrelia and rat joint tissue.* The effects of FNJ and TNJ strains on the rat tissue feeder layer growth were tested. Rat joint tissue cell cultures grown in ESG medium were trypsinized and the detached cells ($10^6$/ml) were washed with fresh ESG. The cell suspension was then mixed with FNJ or TNJ cultures ($1 \times 10^7$ and $2 \times 10^7$ bacteria/ml). The mixtures of *B. burgdorferi* FNJ or TNJ with rat cells were then inoculated into T flasks; 5 ml of ESG medium with 6% FBS and L-glutamine (2 mM) were added. Cultures were incubated at 33 °C and growth of the rat tissue layers in the presence of borrelia was monitored for 10 d.

*Sub-culture of borrelia growing in tissue co-cultures.* FNJ and TNJ strains were grown in the co-culture system for 10 d; 0.1 ml of the culture supernatant with a high number of spirochetes ($10^8$/ml) was collected and confluent feeder tissue layers were then inoculated with these spirochetes. Twenty-two sub-cultures were sequentially carried out in the co-culture system. All co-cultures were fed with ESG medium twice a week.

*Culture of borrelia in BSK.* Modified BSK medium was used as the control medium for all experiments[5]. BSA Fraction V (Sigma, cat #A 4919) was reduced from 50 g to 5 g/l and the gelatin concentration was reduced to 0.2 g/l. *B. burgdorferi* strains were cultured in 5 ml of BSK at 33 °C. The initial concentrations of borrelia were $10^6$/ml, as determined by hemocytometer counts. Subcultivations were done every 10 d.

*Enumeration of borrelia.* The number of borrelia in the suspension was determined by counting in hemocytometer chambers after modified Giemsa staining. Giemsa stain concentrate (Sigma) was diluted (1:10 v/v) in 100 ml of Giemsa buffer and filtered through a Whatman #1 filter. The *B. burgdorferi* culture to be counted was put under the hemocytometer coverslip and the chamber was placed on a hot plate for 2 min until the liquid evaporated. The chamber was cooled and 25 µl Giemsa stain was applied. Spirochetes were counted using a Nikon (Tokyo, Japan) light microscope at 1000 × magnification.

*Bioassays.* One-day-old LEW/N rats (HSD) were injected intra-peritoneally with 0.1 ml of FNJ or TNJ strains ($10^6$/ml) grown in tissue co-culture or in BSK at different passage stages in order to determine the retention of pathogenicity of borrelia. The diameter of the tibio-tarsal joints was measued by using Apprentice Vernier calipers (Ben Meadows Co., Atlanta, Georgia) during the infection to determine the presence and severity of the arthritis.

*Re-isolation of borrelia from rats.* Rats were sacrificed with $CO_2$ 40 d post injection. The liver, kidney, and heart were removed and minced with scalpels in 10 ml BSK. Tissue pieces were ground in a tissue grinder and sieved through a cell dissociation sieve (40 mesh, 0.38 mm opening size; Sigma). The tissue extract was then centrifuged at 9500 × *g* for 15 min and the resulting pellet was inoculated into 5 ml BSK in T flasks. Cultures were incubated at 33 °C in candle jars and monitored for borrelia growth for 5 weeks.

*Statistical methods.* Differences in the diameters of the swollen tibio-tarsal joints of the arthritic rats were analyzed by Student's t-test.

*Results*

*Identification of borrelia.* All passages of FNJ and TNJ strains reacted with FITC labeled anti-OspA and anti OspB-antibodies (data not shown).

*Growth of LEW/N rat joint tissue feeder layers in ESG medium.* Rat joint tissue cultures grown in ESG became confluent in 7–10 d. The cells assumed a fibroblastoid morphology with 90–95% viability.

*Growth of borrelia in tissue co-culture and BSK medium.* ESG medium, in the absence of the rat tissue feeder layers, did not support the growth of either FNJ or TNJ strains of *B. burgdorferi.* ESG medium supplemented with 6% FBS, but without the rat tissue layer, supported only poor growth of the spirochetes compared to the co-cultures with the feeder layer. Conditioned ESG medium was capable of supporting borrelia growth in the absence of FBS.

The initial bacterial counts were $5 \times 10^6$/ml (SD = $0.1 \times 10^6$) for both BSK and tissue co-cultures. The lag period for BSK culture was 4 d; in the rat tissue co-culture system, this was reduced to 3 d ($p < 0.07$). The number of borrelia reached $1 \times 10^8$ (SD = $0.7 \times 10^8$) in BSK cultures on day 6 and $1.0 \times 10^7$ (SD = $0.5 \times 10^7$) for the tissue co-culture grown organisms on day 5.

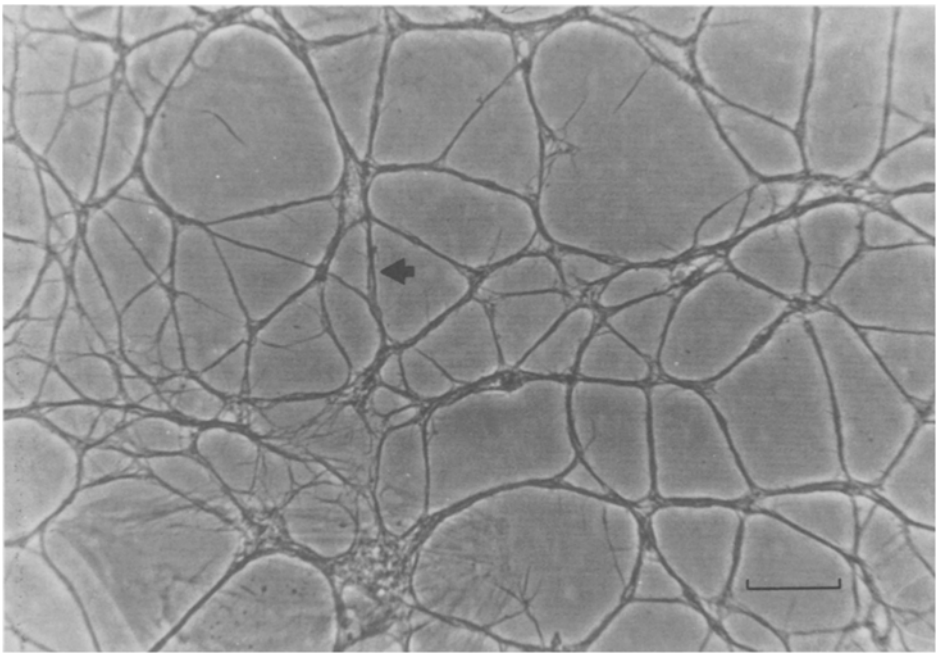
*Aggregation patterns of FNJ and TNJ in rat joint tissue co-culture.* Borrelia attached to the tissue feeder layer by length and by the tips in 15–30 min upon inoculation. The pathogenic FNJ strain grown in the rat tissue co-culture system formed characteristic cord-like aggregates (fig.). Addition of 6% FBS increased cord formation of the FNJ strain in the rat tissue co-culture. These cord-like, intertwined borrelia aggregates were not rigid structures. When examined by phase contrast microscopy at 200 × or higher magnifications, *B. burgdorferi* cells were observed to be in the process of attachment to and detachment from these aggregates. Sterile control rat tissue cultures growing in ESG medium without borrelia did not show any cord-like formations.

The non-pathogenic TNJ strain grew in the co-culture system in a diffuse pattern[8]. TNJ failed to form cords even in co-cultures supplemented with 6% FBS. However, we could not find a correlation between the cord formation of borrelia during growth in tissue co-culture and their arthritogenicity. Non-pathogenic, long term BSK cultured FNJ strain still was able to form cords upon inoculation into the tissue cultures.

*Effects of TNJ and FNJ strains on the rat tissue feeder layers.* FNJ and TNJ strains did not inhibit the attachment or growth of the rat joint cells to the culture flask surface. Tissue feeder layers infected with pathogenic or non-pathogenic borrelia became confluent in 7–10 d with high numbers of spirochetes growing in the co-cultures ($10^7$–$10^8$/ml).

FNJ strain did not cause any visible damage to the cells of the tissue culture feeder layers during 10 d co-culture periods as determined by the dye exclusion assay; 90–95% of the cells in the feeder layer were unstained by trypan blue. Likewise, TNJ strain did not cause cell death; 90% of the tissue culture layer was alive during the 10 d co-culture periods. Feeding co-cultures with fresh ESG medium every 5 d was essential for the viability of the tissue culture monolayers.

The non-pathogenic TNJ strain, in contrast to FNJ strain, caused formation of 2–20 micron diameter vesicles; some of these were found attached to the tissue culture feeder layer, while others floated in the culture supernatant. Some of the released vesicles contained motile spirochetes (1–20 borrelia/vesicle)[8]. The number of vesicles progressively increased with bacterial growth, reaching a final concentration of $2.8 \times 10^6$ vesicles/ml of culture medium. Addition of 6% FBS to the medium inhibited formation of these vesicles. Due to the large number of apparently surface-bound borrelia present, it was not possible to state with certainty if there were any intracellular organisms. There were also large clusters of TNJ spirochetes attached to the tissue feeder layer, although TNJ did not form cord-like aggregates.



*B. burgdorferi* (FNJ) growing in rat joint tissue co-culture. Intertwined borrelia formed cord-like aggregates. Spirochetes were in the process of attachment to and detachment from these cords. Arrow: Inter-twined cord-like borrelia aggregates. Bar: 50 µm.

*Retention of pathogenicity.* In neonatal LEW/N rats, experimental arthritis was manifested by swelling of the tibio-tarsal joints and limping 21 d after injection with the FNJ strain grown in tissue co-cultures. FNJ low passage strain grown in BSK also caused experimental arthritis, while FNJ after the 7th passage in BSK failed to produce experimental arthritis. FNJ strain grown in the tissue co-culture was still pathogenic at the 22nd passage stage (table 2). The diameter of the tibio-tarsal joints of control rats was $6 \pm 0$ mm, whereas the arthritic joints were $7.22 \pm 0.42$ mm in diameter 21 d after injection ($p < 0.04$). TNJ strain did not induce joint inflammation and it did not regain pathogenicity during 11 passages in the co-cultivation system.

*Retention of infectivity upon isolation of borrelia from the rats.* In rats with arthritis induced by FNJ strain grown in the co-culture system for 22 passages, *B. burgdorferi* was re-isolated from rat liver, heart, and kidney tissues 40 d post-injection. Injection of FNJ strain grown in BSK at the 22nd passage stage did not cause experimental arthritis, as noted above, and could not be re-isolated from the rats. The TNJ strain grown in BSK or in the tissue co-cultures failed to cause experimental arthritis and could not be re-isolated from rats.

*Discussion*

*B. burgdorferi* has been isolated and cultured from several tissues of experimentally infected rodents[11,12], and from specimens derived from human patients[13-18] using BSK medium. *B. burgdorferi* interacts with host cells, as demonstrated by in vitro adherence to tick cells[19], human epithelial cells[20,21], human umbilical vein endothelial cells[22,23], Vero cells[24] and cells derived from neonatal rat brain[25]. The adherence of spirochetes to the extracellular matrices produced by endothelial cells in vitro has been described[22]. However, to our knowledge, this is the first report describing long term continuous co-cultivation of *B. burgdorferi* in a mammalian host-derived cell culture.

Hechemy et al. were able to grow *B. burgdorferi* in BSK medium for 3 days in association with an underlying Vero cell feeder layer, which had been previously propagated in Eagle's Minimal Essential Medium (MEM) with 5% fetal calf serum[24]. In previous co-incubation studies, tissue culture monolayers were grown in conventional tissue culture media and after formation of the monolayers, culture media were replaced with BSK to allow borrelia growth. However, this technique has several disadvantages. During the development of the ESG medium, we observed that BSK medium may cause granulation, vacuolation and lysis of mammalian cells[8]. Kurtti et al. reported that tick cells did not grow in BSK medium; the cells assumed a 'rounded up' appearance and detached from the culture surface within 2 to 3 days[19]. The changes in the tissue culture monolayer might be a result of incubation with toxic components of the BSK medium or a result of the high osmolarity or high citric acid content of this medium, and not due to a cytopathogenic effect of *B. burgdorferi*. Therefore, co-culture or co-incubation of borrelia with

Table 2. Bioassay of virulence retention

| Bacterial strain and growth medium | | Subculture number | No. rats injected | No. arthritic rats |
|---|---|---|---|---|
| BSK-grown FNJ strain | | 1 | 14 | 14 |
| | | 4 | 3 | 3 |
| | | 7 | 5 | 0 |
| | | 16 | 4 | 0 |
| | Control[a] | | 5 | 0 |
| ESG-tissue co-culture grown FNJ strain | | 1 | 13 | 13 |
| | | 2 | 5 | 5 |
| | | 3 | 3 | 3 |
| | | 4 | 4 | 4 |
| | | 5 | 2 | 2 |
| | | 11 | 5 | 5 |
| | | 22 | 4 | 4 |
| | Control | | 7 | 0 |
| BSK-grown TNJ strain | | ND[b] | 5 | 0 |
| | Control | | 2 | 0 |
| ESG-tissue co-culture grown TNJ strain | | ND[c] | 7 | 0 |
| | Control | | 5 | 0 |

Neonatal LEW/N rats were injected intraperitoneally with 0.1 ml of *B. burgdorferi* FNJ strain or TNJ strains ($10^6$/ml) grown in BSK medium or joint tissue-borrelia co-culture system at different subculture stages (subculture #). Subcultivations were done every 10 d. Bilateral swelling of tibio-tarsal joints and limping 21 d post injection were the manifestations of experimental Lyme arthritis. Swollen joints were measured with Apprentice Vernier calipers to examine the severity of arthritis. Normal rat tibio-tarsal joints were $6.00 \pm 00$ mm. In the experimental model, the joints were $7.18 \pm 0.38$ mm ($p < 0.04$) for BSK grown FNJ strain during the first 6 subcultures and $7.22 \pm 0.42$ mm ($p < 0.02$) for tissue co-culture system grown FNJ during the 22 passages.

[a]Rats inoculated with sterile culture medium.

[b]Not determined; the precise number of passages for TNJ is unknown.

[c]TNJ, passage number indeterminate, was transferred to the ESG tissue co-culture system and subcultured 11 times prior to use in these experiments.

mammalian tissue in BSK medium cannot accurately define borrelia-tissue interactions. ESG medium supports the healthy growth of the host derived tissue feeder layer and *B. burgdorferi* simultaneously for extended periods of time and represents a suitable culture system to investigate borrelia-host tissue interactions in vitro. *B. burgdorferi*'s loss of pathogenicity and infectivity during long-term culture in BSK medium might be linked to the loss of pathogenicity determinants, e.g. plasmids encoding virulence factors[6]. Loss of infectivity of *B. burgdorferi* in white footed mice[6], mice[26], irradiated hamsters[27] and syrian hamsters[11] upon prolonged cultivation in BSK medium had been described in the previous studies. Loss of virulence coincides with changes in the plasmid content and protein profiles. A subset of low molecular weight proteins are expressed by low passage strains, but they are underexpressed in high passage strains. These low passage associate proteins (Laps) might be associated with virulence of *B. burgdorferi*[28]. We observed differences in the protein profiles of pathogenic and non-pathogenic borrelia grown in BSK and in the tissue co-culture system. A small group of 22–30 kDa range proteins were differentially expressed in BSK grown borrelia cultures compared to tissue co-cultured spirochetes[29]. Artificial introduction of 8 Swiss strains and North American B31 strain of *B. burgdorferi* into tick midgut resulted in modified expression of OspA, OspB and a 22 kDa protein which was either lost or acquired in the re-isolates[30]. Antigenic variation in vertebrate host during the Lyme disease is a suggested but not yet established phenomenon. Growth of spirochetes in association with the host tissues may cause differential expression of *B. burgdorferi* antigens during the infection. Host-derived tissue supported co-culture system mimics the host environment without offensive components of the immune system and this mimicry may promote growth of borrelia in the pathogenic form. The combination of the new co-culture medium, ESG, and the host-derived tissue feeder layer may have provided growth factors or other complex nutrients which supported the growth of pathogenic borrelia during prolonged in vitro cultivation. This suggests that future studies to determine pathogenicity factors may be possible using the borrelia-tissue co-culture system. Of note, the non-pathogenic strain, TNJ, did not regain pathogenicity after prolonged culture in the co-culture system, supporting the contention the virulence factors may be irreversibly lost in long term BSK cultures.

The borrelia-mammalian tissue co-culture system might be useful in the investigation of long term host tissue-*B. burgdorferi* interaction during infection and might facilitate the development of diagnostic tests and the investigation of immunopathogenic mechanisms of this infection. In vitro studies on joint tissue derived chondrocyte development and nervous tissue cytopathogenicity during long term *B. burgdorferi*-host tissue co-cultures are now underway in our laboratory. Experimental in vitro infection of rat joint tissue with *B. burgdorferi* seems to occur without visible damage to the cultured cells. In *Treponema pallidum*-rabbit derived tissue co-incubations, or short term co-cultures, similar observations were reported. As many as $10^7$ treponemes can attach to $10^6$ cultured cells without killing these cells[4].

During our studies, we did not observe any internalized borrelia in rat joint tissue co-cultures. However, tissue co-culture of the non-pathogenic TNJ strain was associated with formation of host derived, monolayer membrane-bound vesicles. These vesicles were not observed in control tissue cultures grown in ESG medium.

We observed a distinctive cord-like aggregation pattern of some borrelia strains grown in rat tissue co-culture system. *B. burgdorferi* may contain specific outer membrane components such as lipoproteins, glycolipids or glycocalyx-like surface layers associated with the loose outer sheet of the microorganisms. Tissue co-culture system may enhance synthesis of these surface layers of some borrelia strains or feeder tissue layer surface components such as collagen may dictate this characteristic cord-like aggregation pattern of spirochetes in tissue co-cultures. Differences in the cell wall components and/or adhesins of different borrelia strains may determine their aggregation patterns on the host tissue monolayers. The vertebrate host tissue-borrelia co-culture technique presents a promising experimental system to study the relationship between *B. burgdorferi* and infected host tissues in vitro. This technique may facilitate the development of diagnostic tests and the investigation of pathogenic mechanisms of this infection.

Acknowledgements. This work was supported in part by funds from the New Jersey Agricultural Experiment Station, and the Lyme Research Foundation. We wish to sincerely thank Dr. K. Maramorosch for his support, guidance, and valuable suggestions during this study. We acknowledge L. Sigal MD for his help and valuable criticism during preparation of this manuscript.

* This article forms New Jersey Agricultural Experiment Station paper no. D8420-19-92.

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