

# Interaction of *Borrelia burgdorferi Sensu Lato* with Epstein-Barr Virus in Lymphoblastoid Cells

( lymphoblast / viruses / borrelia / interaction )

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**Abstract.** Since the possibility of interruption of latent EBV infection has been suggested by the induction of the lytic virus cycle with chemical substances, other viruses, and by immunosuppression, we hypothesized that the same effect might happen in *B. burgdorferi sensu lato* infection as happens in Lyme disease patients with positive serology for both agents. We have observed EBV replication in lymphoblastoid cells after superinfection with *B. garinii* and *B. afzelii* strains after 1 and 4 h of their interaction. We found that viral and borrelial antigens persisted in the lymphoblasts for 3 and 4 days. Morphological and functional transformation of both agents facilitate their transfer to daughter cells. Association with lymphoblasts and internalization of *B. garinii* by tube phagocytosis increased replication of viruses more successfully than *B. afzelii* and chemical inductors. Demonstration of such findings must be interpreted cautiously, but may prove a mixed borrelial and viral cause of severe neurological disease.

The Epstein-Barr virus (EBV) as a human lymphotropic herpesvirus may participate in evoking severe neurologic symptoms (Strauss, 1993) in immunodeficient patients. *Borrelia garinii* (*B. garinii*), serotype 4, was also considered to be a neurotropic agent (Marconi et al., 1999).

Unlike viruses, borreliae as a helical form are present in human cerebrospinal fluid (Busch et al., 1996), blood and tissue (Moter et al., 1994) only in low amounts, but under suitable conditions they are able to grow, colonize cell surfaces and form immunocomplexes (Coyle et al., 1995). This allows them to live as intracellular parasites and thus persist in the host tissue (Wang et al., 2000). The association of *Borrelia* with lymphoblastoid cells and possible interaction with EBV that are in

latent phase in these cells was not studied. Serological cross-reaction between anti-viral and anti-borrelial IgM antibodies has been mentioned by Harris (1998). Adherence of spirochetes *Borrelia burgdorferi* (*B. burgdorferi sensu lato*) to monocytes (Wang et al., 2000), neutrophilic leucocytes (Hulínská et al., 1995), macrophages (Linder et al., 2001) and human B lymphocytes (Dorward et al., 1997) could explain this hypothesis of cross-reactivity in cases of superinfection or co-infection with different bacteria and viruses in the host. Knutson and Sugden (1989) described immortalization of B lymphocytes by the EBV. Borrelial adherence, internalization by fibroblasts (Georgilis et al., 1992) and reactivity with decorin (Guo et al., 1995) allow them to become hidden against antibiotics and the defence immunological reactivity of the host. The uptake of *B. burgdorferi sensu lato* occurs predominantly by the coiling process, which can influence the course of the infection (Linder et al., 2001). The lymphoblastoid P3HR-1 line derived from Burkitt lymphoma (Klein, 1989) was used as a model for the study of the interaction of EBV and spirochetes *B. burgdorferi sensu lato*. EBV pathogenic effects are connected with its capacity of inducing latent infection in B lymphocytes, when the lytic function of the virus is suppressed and several non-structural virus proteins with the regulatory function are formed. This ensures long-term persistence of viral DNA in the infected cells (Thorley-Lawson, 1988). Long-term persistence of borrelial antigen or nucleic acids was observed in patients with Lyme arthritis (Hulínská et al., 1999) and in the central nervous system (Luft et al., 1992).

The state of latent EBV infection can be interrupted by factors that induce a lytic virus cycle, for instance a-butyrate (Klein, 1989), phorbol-ester (TPA), antibody against IgG, hydrocortisone. The interruption of EBV latency can also occur upon superinfection of the cell with other viruses. Latent borrelial infection has also been demonstrated (Karch et al., 1994).

Although serological tests using ELISA and IFA are considered to be sensitive both for EBV and *B. burgdorferi sensu lato*, they are not highly specific for both agents. Possible serological cross-reactions

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Received July 18, 2002. Accepted October 24, 2002.

This work was supported by the Ministry of Health Grant Agency (IGA) with grants Nos. NI6880-3 and L31/93:23795.

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Abbreviations: EBV – Epstein-Barr virus, Mab(s) – monoclonal antibodies.

between *Borrelia* and EBV have been presumed. The possible involvement of *B. burgdorferi sensu lato* infection by activation of EBV in Lyme disease patients can be revealed by experimental study of the conditions of their interaction.

## Material and Methods

Spirochetes *B. garinii*, strains 192M and 61E, *Borrelia afzelii* (*B. afzelii*), strains 97 M and Kc90 were isolated from cerebrospinal fluids (CSF) and plasma of patients with clinically and serologically estimated Lyme borreliosis in BSK-H medium (Sigma-Aldrich GmbH, Steinheim, Germany) and kept at  $-80^{\circ}\text{C}$  in aliquots. Borreliac cells were cultured from aliquots in volumes of 10 to 250 ml until they reached the log phase, approximately 3–5 days after inoculation, making up a 1 : 100 dilution. Culture density, approximately  $10^6/\text{ml}$  bacteria, was determined by dark-field microscopy by counting in a Petroff-Hausser chamber. Viability of the spirochetes was assessed by their typical corkscrew motility.

Lymphoblastoid P3HR-1 cells were passaged twice weekly in RPMI 1640 medium (SeVac, Prague, Czech Republic) supplemented with antibiotics (100 i.u./ml penicillin, 100  $\mu\text{g}/\text{ml}$  streptomycin, and 25  $\mu\text{g}/\text{ml}$  gentamycin), sodium bicarbonate, and 10% foetal calf serum. Before infecting, the lytic virus cycle was induced in a part of the culture: the third day after passaging, the cells were spun down at 1500 g for 10 min and seeded into the induction medium containing 1 mM sodium a-butyrate (Merck, BRAND GmbH, Wertheim, Germany) and 20 ng/ml 12-o-tetradecanoyl-phorbol acetate (TPA, Sigma), to obtain a density of  $3 \times 10^5$  cells/ml.

The other part of the culture was seeded similarly into the culture medium containing no inductors. After 24 h the cells were spun down, washed in the culture medium containing no antibiotics, seeded into a new medium in a density  $3 \times 10^5$  cells/ml and infected with borreliac.

Association and internalization of *B. garinii* and *B. afzelii* with P3HR-1 cells was investigated by light and fluorescent microscopy. A freshly prepared bacterial culture in 0.1 ml of BSK-H medium (about  $2 \times 10^5$  cells) was mixed with 0.4 ml of P3HR1 cells (about  $3 \times 10^5$  cells) and added into 4 ml of complete RPMI medium and then incubated for 1 h and 4 h at  $36^{\circ}\text{C}$  in a  $\text{CO}_2$  incubator with regular rocking. Instead of the microbial suspension, 0.1 ml of filtered (Microcon YM-10, YM50, Millipore Carrigtwahille, Co., Cork, Ireland) borreliac-spent medium BSK-H was added to control mock-infected cultures. To remove non-attached bacteria from P3HR-1 cells the cultures were spun down at 3000 g for 2 min. Supernatants containing free borreliac were spun out into other tubes and the numbers of borreliac were counted. Sedimented cells were washed

in sterile PBS and seeded into antibiotics-free RPMI medium at a density of  $3 \times 10^5$  cells/ml and incubated at  $36^{\circ}\text{C}$  in a  $\text{CO}_2$  incubator; on days 3, 4, and 6 the cells were subjected to study. Cells after incubation were briefly centrifuged (at 2300 g) and washed with PBS. The supernatant with non-attached bacteria was serially diluted (10-fold) with BSK-H medium and the number and viability of bacteria were detected by dark-field microscopy. The sedimented cells with bacteria were then fixed on a glass slide for immunofluorescent examination. Slides with cells were fixed for 5 min by ice-cold methanol. Another part of the samples were taken for electron microscopy.

Virus antigens were detected in cells by indirect immunofluorescence using a) human immune serum containing antibody against early and capsid EBV antigen and b) monoclonal antibody against early EBV antigen, which was non-reactive to *Borrelia* (anti-ZEBRA, DAKO, GmbH, Copenhagen, Denmark). Porcine immunoglobulin against human and murine IgG tagged with fluorescein-isothiocyanate (SwAHu-IgG-FITC, SwAM-FITC, SeVac, Prague, Czech Republic) served as the second antibody. The number of cells containing virus antigens was determined in *B. burgdorferi sensu lato*-invaded cells by viewing at least 600 cells on each slide. The same calculation was made in mock-infected and in chemically induced control cell cultures.

Assessment of association and internalization of *Borrelia* was expressed as the ratio of cell-associated bacteria (C) to extracellular supernatant bacteria (S), i.e. the number of free bacteria present in the supernatant after 1 h, 4 h and 3 days of incubation with P3HR-1 cells to the number of cell-associated bacteria. All experiments were performed with multiple observations and were reported as the means  $\pm$  S.D. Results from experiments were analysed by Student's *t*-test with  $P \leq 0.05$  considered to be statistically significant.

For the slide immunofluorescence assay, spirochetes were separated from supernatant by centrifugation at 12 000 g for 10 min and washed in PBS supplemented with 5 mM  $\text{MgCl}_2$ . Borreliac attached or inside cells (as mentioned above) and borreliac from supernatant were applied to slides and fixed by drying overnight at  $4^{\circ}\text{C}$  and then stored at  $-80^{\circ}\text{C}$ . Monoclonal antibodies (Mab) against BmpA (H1141), OspA (336,184) and flagellin (H9724) from CDCP were used. Slides were incubated with Mabs for 1 h at  $37^{\circ}\text{C}$  in moist chambers, washed in two changes of PBS +  $\text{MgCl}_2$ . Incubation with secondary antibodies, SwAM IgG-FITS or SwAM IgG-gold (Jenssen, Redding, CA) was for 1 h at  $37^{\circ}\text{C}$  with two washes in PBS following incubation. Slides were mounted with Slow Fade (Molecular Probes Inc., Eugene, OR).

For electron-microscopic examination, the following material was used: a) P3HR-1 culture cell suspension, b) P3HR-1, mock-infected, c) induced P3HR-1 infected

with *B. garinii*, or other strains for 1 h and for 4 h, sampled on days 3 and 4. One part of each material was processed for the negative staining method (IEM) and one part for preparation of ultrathin sections as published previously (Hulínská et al., 1995, 1999). Drops of the sediment were stained on grids with 1% phosphotungstic acid (PTA) or the material was fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer at 4°C, postfixed in 1% OsO<sub>4</sub>, dehydrated and embedded in Lowicryl K4M for immunocytochemistry as was described elsewhere (Hulínská et al., 1995). Examination was made under a Jeol 200CX electron microscope (Tokyo, Japan).

## Results

Borrelia strains used for experiments were: *B. garinii* strain 192M, low-passaged isolate from CSF, OspA serotype 5 and strain 61E, high-passaged isolate from erythema migrans, OspA serotype 4, which have a sequence similarity with strains SL14 and Pbi; strain Kc90, high-passaged strain which was isolated from the blood, and strain 97M, low-passaged strain isolated from CSF, both with sequence similarity with *B. afzelii* strain BVI. All strains were in the log phase in BSK-H medium.

Visualization of *B. garinii* and *B. afzelii* interaction with P3HR-1 lymphoblastoid cells investigated by light and fluorescence microscopy showed significant differences in the behaviour of both strains. Lymphoblastoid cells incubated with selected pairs of Borrelia strains for 1 and 4 h at 36°C contained a number of associated bacteria. *B. garinii* displayed much higher association with P3HR-1 cells than *B. afzelii* strains. Quantitation of Borrelia association and internalization by lymphoblastoid P3HR-1 cells was expressed as the average C/S ratio. At 4 h this was also higher for the low-passaged *B. garinii* 192M strain ( $0.448 \pm 0.048$ ) as compared with the low-passaged *B. afzelii* strain 97M ( $0.117 \pm 0.041$ ) (shown in Table 1).

Light and fluorescence microscopic examination showed that both *B. garinii* and *B. afzelii* strains influenced the induction of the lytic cycle of EBV in lymphoblastoid P3HR-1 cells. The approximate numbers of viable P3HR-1 cells in different experiments either after chemically induced replication of EBV or after infection or induction of the viral lytic cycle with *B. garinii* were different as illustrated in Fig. 1. In all experiments we started with  $3 \times 10^5$  cells infected 1 and 4 h and sampled on days 3 and 4. The number of viable P3HR-1 cells infected 1 or 4 h with borreliae diminished and after 3- and 4-day incubation they reached only  $2 \times$  or  $1 \times 10^5$  cells. On the other hand, the number of viable mock-infected or chemically induced cells sampled on days 3 and 4 increased (Fig. 1).

The percentage of cells containing EBV antigens was higher in *B. garinii*-infected than in *B. afzelii*-infected or in the control mock-infected cultures. However, it has come to light that just the BSK-H medium alone in which *B. garinii* had been cultured for four hours also had an inducing effect on EBV. Growing Borrelia released microvesicles with outer surface proteins and decreased pH of the medium. In each preparation we inspected about 500–600 cells and about one hundred high-power fields. Viral antigens were seen in 14.0% induced P3HR-1 cells infected for 1 h with *B. garinii*, sampled on 3<sup>rd</sup> day, and in 4.8% cells infected with *B. afzelii* low-passaged strains in IFA observations with polyclonal and monoclonal antibodies (Table 2). Observations of EBV antigens in P3HR-1 cells expressed as the approximate ratio of the number of cells containing the EBV antigen and the number of cells not containing the virus antigen was higher for *B. garinii* ( $0.275 \pm 0.070$ ) than for *B. afzelii* ( $0.103 \pm 0.041$ ) on day 3 of incubation. Depending on the duration of infection with both viral and borrelial antigens on day 4, cell viability decreased and 50–60% of cells were lysed. A longer 6-day period of incubation with Borrelia caused lysis of P3HR-1 cells approximately in 70%. Growing Borreliae decreased the pH of the medium from pH 7.2 to pH 6.9.

Table 1. Time course of Borrelia association in induced lymphoblastoid P3HR-1 cells

Average ratio of cell-associated bacteria (C) to bacteria in supernatant (S)						
Strain	Origin	Phenotype	1 h	4 h	3 days	
Kc90	blood	<i>B. afzelii</i> , type-2 high-passaged	$0.030 \pm 0.002$	$0.040 \pm 0.018$	$0.076 \pm 0.010$	
97M	CSF	<i>B. afzelii</i> low-passaged	$0.161 \pm 0.017$	$0.117 \pm 0.041$	$0.268 \pm 0.072$	
192M	CSF	<i>B. garinii</i> , type-5 low-passaged	$0.310 \pm 0.070$	$0.448 \pm 0.048$	$0.376 \pm 0.134$	
61E	skin	<i>B. garinii</i> , type-4 high-passaged	$0.300 \pm 0.040$	$0.348 \pm 0.072$	$0.301 \pm 0.172$	

Association of *B. afzelii* and *B. garinii* strains was expressed as the ratio  $\pm$  (S.D.) of cell-associated bacteria (C) to bacteria in the supernatant (S) after 1 and 4 h incubation with H3PR-1 cells and sampled on 3<sup>rd</sup> day of interaction. CSF, cerebrospinal fluid.