Selection of a *Borrelia burgdorferi* antigenic variant by cultivation in the presence of increasing amounts of homologous immune serum

Marina Cinco

*Institute of Microbiology, University of Trieste, Trieste, Italy*

Received 11 December 1991
Revision received 16 January 1992
Accepted 21 January 1992

Key words: *Borrelia burgdorferi*; Antigenic variant

1. SUMMARY

This investigation was undertaken to select antigenic variants of a *Borrelia burgdorferi* strain in vitro. The original strain BITS was cultivated in BSK medium supplemented with increasing concentrations of homologous hyperimmune serum raised in rabbits. After a few serial passages starting from a subinhibitory serum dilution of 1:800 in BSK up to 1:200, a variant named BITSv was obtained; it grew abundantly like the control culture in the presence of hyperimmune serum. Analysis of the antigenic pattern of the original and derived variants by Western blotting revealed that BITSv, compared to the original strain BITS, had lost the reactivity with the immune serum at the level of the oligosaccharide moiety. These experiments, designed to mimic the possible action of antibodies that arise during a *Borrelia* infection, suggest that lipopolysaccharides are surface located and that they play a role in the integrity of the outer membrane during the multiplication of *Borrelia burgdorferi*.

2. INTRODUCTION

A series of antigenic variations were described to occur in *Borrelia burgdorferi*, the etiological agent of Lyme borreliosis. Several studies have demonstrated changes in surface proteins [1,2], infectivity [3,4] and plasmid profile [5] during in vitro cultivation. In fact, T.C. Schwan found that early cultivation led to loss of infectivity associated to loss of small 7.6- and 22-kb plasmids. Analysis of the variability within a population of *Borrelia burgdorferi* of the outer membrane protein OspB demonstrated a clonal polymorphism and spontaneous variants of this antigen in the range of 18.5–21 kb [6]. There is also evidence of
antigenic variation in vivo: in fact during experimental infection the spirochete numbers in the blood of animals fluctuate [7], and there are alternating periods during which borreliae are not cultivable from the blood of cotton rats. Antigenic variations in vivo were also suggested to occur in human infection. Some authors [8] demonstrated repeatedly an OspA and pC specific immune response only toward heterologous and not with the autologous Borrelia isolate. Therefore it is tempting to speculate that antibodies arisen during the early infection act as selective agents toward spontaneous antigenic variants and are responsible for the appearance of a Borrelia population which is different from the parental infecting strain. There are no reports which describe the selection of antigenic variations induced by antibodies in vitro, or any indication on the type of antigen involved in the antigenic shift. In the present study we investigated the influence on the antigenic pattern of Borrelia burgdorferi strain BITS by cultivation in vitro in the presence of polyclonal antibodies.

3. MATERIALS AND METHODS

3.1. Strains of Borrelia and in vitro cultivation

Borrelia burgdorferi strain BITS, isolated from Ixodes ricinus [9], was used in this study. The strain has been maintained in the laboratory in liquid nitrogen for several years and, when needed, cultivated in BSK medium [10] at 34°C. Counting of borreliae was carried out by a Thoma counting chamber, under dark-field microscopy (×400).

3.2. Rabbit immune serum:

Polyclonal anti-BITS immune serum was obtained by five i.v. administrations of 1 ml of 10⁹ borreliae washed twice in PBS, in a white rabbit over a 40-day period. The development of antibodies towards BITS was monitored at day 30 by immunoprecipitation, according to Ouchterlony [11]. If no further immunization was needed, the rabbit was bled and the serum inactivated at 37°C for 30 min.

3.3. Cultivation in the presence of homologous antibodies and selection of antigenic variants

Preliminary experiments were done to define the subinhibitory hyperimmune serum concentration towards strain BITS. Sterile rabbit serum was diluted in BSK medium to reach the concentration of 1:1600, 1:400 and 1:200 and 6 ml of each preparation were distributed in tubes in duplicate. 0.1 ml of 10⁵ borreliae in the logarithmic phase were added to each serum supplemented with BSK medium and incubated at 34°C. The cultures were monitored by dark-field microscopy, starting from the 3rd day of incubation. Controls were cultures of BITS subcultivated in BSK without immune serum. The highest dilution of immune serum capable of inhibiting the growth of borreliae, represented by the persistence of a stationary low number of motile organisms observed by dark-field microscopy, was judged subinhibitory (SID). From this initial culture in SID, serial passages were performed in the presence of the same SID, until an actively growing culture, like the control, was obtained. Subsequently the culture was cultivated in the presence of higher serum concentrations and the procedure was repeated serially as described up to the serum dilution of 1:200 in BSK. The antigenic pattern of the borreliae derived from the original strain, was checked before any passage in higher serum concentrations.

3.4. SDS-PAGE and Western blotting

Antigenic analysis of the original and the derived BITS strains was performed by SDS polyacrylamide gel electrophoresis (PAGE) and subsequent Western blot (WB), as described previously [12]. Hyperimmune BITS antiserum was diluted 1:100 for WB. The strains grown in the presence of immune serum used for SDS-PAGE and WB, were grown and maintained in BSK plus immune serum at the SID used for selection. When needed, nitrocelluloses, with blotted material, were treated with Proteinase K (Sigma; 100 μg/ml) as described [12] to destroy protein reactivity.
4. RESULTS

4.1. Selection of antigenic variants of strain BITS

When borreliae were cultivated in BSK medium plus immune serum, the starting subinhibitory dilution was determined as 1:800. Darkfield observations of cultures at this serum dilution revealed a small number of borreliae per field, which appeared to be motile and agglutinated. Only very few borreliae not motile and apparently damaged were seen at 1:400 and 1:200 immune serum dilutions. Subcultures from these mixtures in BSK without serum were sterile. At the dilution of 1:1600 the BITS culture grew rapidly, reaching the cell density observed in the control. The BITS strain surviving in 1:800 diluted serum developed a rapid growth after subculturing in the same medium; subsequently 0.1 ml of this culture was inoculated into BSK plus 1:400 immune serum which resulted to be the next S1D. The results obtained were like those observed with the 1:800 diluted serum and the BITS strain derived was hereafter subcultured in BSK plus 1:200 immune serum. This culture developed rapid growth at the first inoculation like in the control. The Borrelia population obtained by this last passage was named BITSv.

4.2. Antigenic analysis of the BITS variants

Western blot carried out with the polyclonal homologous serum revealed that the BITS cultures selected at 1:800 and 1:400 dilutions of serum had the same antigenic pattern as the original strain and the BITS cultures derived from the control series (data not shown). Western blotting done with the strain BITSv maintained in BSK plus 1:200 immune serum showed (Fig. 1a) a pattern of reactive bands identical to BITS in the protein region of the gel (molecular mass > 13 kDa). In fact, the immunodominant bands at 20, 22, 23, 32, 39, 41, 54, 56 and 68 kDa were visible in both the strains. The only difference observed was in the 8–11 kDa region, which corresponds to the lipooligosaccharides (LOS) recently described in Borrelia [12]. The reactivity towards LOS is evident in the original strain BITS as a typical band of washboard shape and is absent in BITSv. Proteinase K treatment of the blotted sonicated organisms retained the washboard band in the LOS region of original strain BITS (Fig. 1b), thus confirming the polysaccharide nature of this antigen(s). These findings indicate that the antigenic change of the derived strain BITSv occurred in the LOS component.

5. DISCUSSION

This research was designed to investigate the behaviour of a population of Borrelia burgdorferi strain BITS, under the selective pressure of increasing concentrations of homologous antibodies. The data obtained indicate that through a few subcultures the borreliae became progressively less sensitive to the inhibitory action of immune serum, from dilution 1:800 up to 1:200. Microscopic observation of the organisms in the presence of SID suggested that the borreliae were not directly killed by the antibodies, but agglutinated, and were probably not able to divide. At higher serum concentration the cells apparently
lysed and the culture was not viable. This finding indicates that high concentrations of antibodies exert some direct damage on Borrelia, probably at the cell surface, as already suggested [13]. The loss of sensitivity to the inhibitory effect of serum was associated to an antigenic change of the Borrelia population which was evident in the variant BITTs at the level of the LOS fraction. This finding is quite new, because in previous reports antigenic variations were reported to affect only the OspB major antigens [2,6] during serial in vitro cultivation without selecting agents. An apparent increase in molecular mass of proteinase K resistant material was obtained by Schwan to occur in Borrelia after 25 passages [4]. This observation was interpreted as a change in the LPS-like substance which occurred opposite to that seen for other bacteria, in which the LOS undergoes conversion to lower molecular mass LOS. Our data are in agreement to what is commonly reported to occur with the majority of enteric Gram-negative bacteria, in which during infection there is a loss of sugars in the O side chains. This phenomenon corresponds to a decrease in virulence [14]. We can hypothesize that antibodies at increasing concentrations act directly on the surface of borreliae and interfere with substances which are involved in maintaining the outer membrane integrity and consequently cell division. In this view lipooligosaccharides could be a target of antibodies, which would explain the selection of LPS variants. Apparently the LOS component of Borrelia burgdorferi is surface located as in the Gram-negative bacteria. Recently we obtained indirect evidence for the surface location of LOS. Immune serum raised against purified LOS reacted in an immunofluorescence assay with intact borreliae (data not shown). We do not know whether the antigenic changes which we observed in this in vitro model reflect what happens in vivo, during infection. Antibodies against the LOS fraction are actively synthesized during human infection [12]. It seems likely that they play a role in selecting antigenic variants which contribute to the survival and persistence of borreliae in the body.

REFERENCES