

Borrelia burgdorferi sensu lato as activators of the complement system in *in vitro* model

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Abstract

Introduction. The following parameters were analyzed: C5a, which is significant in classical and alternative pathways of the complement system activation, and factor H, the major function of which is to regulate the alternative pathway. Factor H, in the case of *Borrelia* infection, is combined by CRASPs proteins of spirochetes, and thus prevents C3b molecules from contact with the pathogen, opsonisation and lysis of bacteria.

Material and Methods. The experimental material in the model for the presented work consisted of whole blood of healthy persons (without the presence of antibodies anti-*Borrelia*) and persons with clinical symptoms of Lyme disease, which was stimulated with three genospecies of spirochetes recognized as pathogenic in Poland and Europe: *B. afzelii*, *B. burgdorferi* s.s. and *B. garinii*.

Results. The increase in the level of C5a in the experimental model after *in vitro* stimulation of whole blood with three genospecies *Borrelia* can be treated as an indicator of an effective activation of the complement's cascade. The increase in level C5a in the plasma relies on the genospecies and the strongest is for *B. garinii*. The decrease in the level of factor H, observed after the incubation of whole blood with three genospecies *Borrelia*, shows that this parameter was included in the spirochetes' mechanisms acting against factors of the innate immunity system of a host, which prevents lysis of bacteria via the alternative pathway.

Conclusions. The results obtained on the basis of the *in vitro* model can be analysed from the aspect of spirochetes' real contact with a host's organism during the bite of infected ticks. Despite blocking of the alternative pathway, *Borrelia* initiate the activation cascade regardless of antibodies via the first contact of a host's organism with spirochetes, or in accordance with antibodies during the infection or subsequent contact with bacteria.

Key words

Complement system, *Borrelia burgdorferi*, Factor H, C5a

INTRODUCTION

Bacteria, on entering the human organism, encounter the defence lines acting as inborn mechanisms, among which the complement system plays the predominant role. The alternative pathway of complement's activation is triggered the earliest because it does not require the presence of specific antibodies. The complement, which is activated by polysaccharide elements of a cell wall of gram-negative bacteria, is able to destroy the pathogen before specific antibodies appear [1, 2, 3]. However, in the case of spirochetes *Borrelia*, only several isolates of *B. garinii* can be effectively eliminated by the complement activated via the alternative pathway, which was experimentally proved [4]. Other pathogenic genospecies of *Borrelia* are able to avoid lysis accompanied by these mechanisms since they developed strategies effectively blocking the key stages of the process [5, 6]. Proteins CFH (factor H) and FHL-1 (factor H-like

protein 1) acting as regulators of the alternative pathway of the complement's activation play the key roles in preventing the killing of components of the complement [1, 2, 3]. All genospecies of *Borrelia* are able to effectively activate the complement by the classical pathway without specific antibodies. This mechanism is predominant during the first bite of a person by a tick infected with spirochetes [3, 7].

The response of the complement system to the presence of *Borrelia* spirochetes is a multifaceted issue, in which the crucial role is played by genospecies causing infection, the presence or absence of specific antibodies of anti-*Borrelia* and, as a result, whether the organism has the first or subsequent contact with the pathogenic factor. The issue concerning repeated contact with spirochetes should be examined from two points of view since it can also be connected with repeated penetration of spirochetes to the organism of a person with Lyme disease, for instance: a subsequent bite by the infected spirochetes, frequently occurring in farmers and foresters, as with the re-infection which cannot be excluded due to the defence strategies of spirochetes (e.g. the forming of cysts in conditions of environmental stress). It is not without significance that the antibiotic therapies previously

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used in the treatment of Lyme disease have an influence on the response of the complement [8].

In the presented study, two parameters were analysed: C5a and factor H, connected with the response of the complement system in the conditions of a contact of whole blood with spirochetes *Borrelia* in an *in vitro* model. Whole blood, which was taken from healthy persons and patients infected with *Borrelia*, was stimulated with genospecies of spirochetes recognised as pathogenic in Poland and Europe – *B. afzelii*, *B. burgdorferi* s.s. and *B. garinii* [9, 10]. Two parameters analyzed: 1) C5a, which is significant in classical and alternative pathways of the complement's activation, and 2) factor H, the major function of which is to regulate the alternative pathway, and in the case of *Borrelia* infection, is bound by CRASPs proteins of spirochetes preventing in this way C3b molecules from the contact with pathogen, opsonisation and lysis of bacteria [1].

MATERIAL AND METHODS

Study group. The study group consisted of 20 patients, men (age 33-55 yrs) and women (age 35-60 yrs) diagnosed with Lyme disease and hospitalized in the Department of Infectious Diseases of the Medical University in Lublin between November 2011 – February 2012, and 20 healthy adult volunteers – 20 women (age 35-55 yrs).

The human experimentation guidelines of the local Ethics Committee were followed in conducting the research.

Equipment and reagents. The polypropylene tubes containing 0.045mg r-hirudin/ml blood (s-Monovette®, SARSTEDT), 24 well suspension culture plate (NUNC), BSK-H Medium Complete (Sigma-Aldrich), Human C5a (BD Biosciences), Human Complement Factor H (Hycult Biotech).

Bacterial reference strains and growth conditions. Reference strains of *B. burgdorferi* sensu lato: *B. burgdorferi* sensu stricto B31 (ATCC 35210), *B. garinii* 20047 (ATCC 51383) and *B. afzelii* VS461 (ATCC 51567) were used in the study. To cultivate spirochetes, 0.1 ml of each strain was inoculated into 5 ml of BSK-H Medium Complete. Strains were incubated in 5% CO₂ atmosphere at 35°C for 7 days, to a cell density of 10⁷/ml [11]. The growth of bacteria was observed in dark field microscopy.

Whole-blood model. Whole blood from volunteers and patients was collected in polypropylene tubes containing r-hirudin. Recombinant hirudin is a highly specific thrombin inhibitor that minimally influences complement activation or cytokine production [12, 13, 14]. Immediately after venipuncture, the sample was split and 1ml blood added to a sterile culture plate. *B. burgdorferi* s.s. B31(ATCC 35210), *B. garinii*, 20 047(ATCC 51383) and *B. afzelii*, VS461(ATCC 51567) – 2 × 10⁷/ml was then added. For the detection of complement activation (C5a, factor H), the samples were rotated for 1h at 37°C. Complement activation was stopped by EDTA (20mM) immediately after incubation. The blood samples were centrifuged for 10 min at 1,400 × g at 4°C. The plasma was collected and stored at -70°C until was analyzed.

The C5a and factor H in each sample was examined before incubation, after incubation without stimulation, and after incubation with stimulation.

Statistical analysis. Student's two-tailed, paired t-test was used for determining the statistical significance of the results; p values of <0.05 were considered statistically significant.

RESULTS

Records were made of the level of antibodies IgM/IgG anti-*B. burgdorferi* in persons from the control group in order to exclude the infection. The results were negative in all cases.

All persons with Lyme disease had positive results in the serological tests and clinical symptoms of the disease.

Level of C5a and factor H in plasma before and after stimulation of whole blood of persons without antibodies anti-*Borrelia* (control group).

The level of the C5a parameter in plasma of persons from the control group was in medium – 16.7ng/ml. The fact of the incubation of whole blood in 37°C caused a small increase in the level of C5a in plasma, compared with the specimens which were not incubated. In order to determine the actual influence of the stimulators on generating the level of C5a of values obtained after stimulation *B. afzelii*, *B. burgdorferi* s.s. and *B. Garinii*, the values obtained for the specimens incubated without the stimulator were decreased. Calculations were made for each specimen in both studied groups. The results of the level of C5a presented in the text and Figures take the above-mentioned calculations into consideration.

The genospecies *Borrelia* inserted into the whole blood of healthy persons (control group) had evident influence on the increase in the level of C5a in plasma, compared with the level of C5a in specimens not stimulated. In each case, the values obtained after stimulation were statistically significantly higher in comparison with the specimens not stimulated (16.7 ng/ml), and were, respectively, for *B. afzelii* 32.2 ng/ml (p<0.00001), *B. burgdorferi* s.s. 38.9 ng/ml (p<0.000001), *B. garinii* 57.4 ng/ml (p<0.000001). The increase in the level of C5a in plasma of the specimens not stimulated correlated with the increase of the level of C5a obtained after incubation of whole blood with *B. afzelii* (p<0.02). A correlation was also present between the increasing level of C5a after stimulation of *B. afzelii* and increasing level of C5a after stimulation of *B. burgdorferi* s.s. (p<0.05). The correlations of the same type were observed for *B. burgdorferi* s.s. and *B. garinii* (p<0.05).

The level of factor H in plasma of persons from the control group was in the median range – 96.8 µg/ml, and the incubation of whole blood at 37°C did not have any effect on the change of its level. *B. afzelii*, *B. burgdorferi* s.s. and *B. garinii* placed in the specimens of whole blood of persons from the control group had an influence on the decrease in the level of factor H in plasma, in comparison with the level of this protein in the specimens not incubated. In each case, the values after stimulation with spirochetes were significantly statistically lower than in the specimens not stimulated (961.8 µg/ml) and amounted, respectively, for *B. afzelii* to 636.8 µg/ml (p<0.0002), for *B. burgdorferi* s.s. – 593.4 µg/ml (p<0.00005), and for *B. garinii* – 617.6 µg/ml (p<0.00005). The decrease in the level of factor H after stimulation of *B. afzelii* correlated with the decreased level of *B. burgdorferi* s.s. (p<0.0005). The correlations of the same type were observed for *B. afzelii*, *B. garinii* (p<0.05), and *B. burgdorferi* s.s. and *B. garinii* (p<0.005).

Level of C5a and factor H in plasma before and after stimulation of whole blood in patients with Lyme disease. The level of the C5a element in plasma in the group of persons with Lyme disease was in the median range – 21.6 ng/ml. Incubation of whole blood of the patients with every genospecies of *B. afzelii*, *B. burgdorferi s.s.* and *B. garinii* resulted in all cases in a statistically significant increase of C5a in the plasma ($p < 0.000001$). The highest increase in the level of C5a was noted for the specimens of whole blood incubated with *B. garinii* – 61.9 ng/ml, and for *B. burgdorferi s.s.* – 51.6 ng/ml, whereas the lowest was for the specimens incubated with *B. afzelii* (48.8 ng/ml). The increase in the level C5a in the specimens stimulated with *B. afzelii* correlated with the increase of the level of C5a obtained after the incubation of whole blood with *B. burgdorferi s.s.* ($p < 0.000005$).

The level of factor H in plasma of patients with Lyme disease was in the median range – 954.9 µg/ml. A significant statistically lowering of its level ($p < 0.005$) in relation to the specimens not incubated was observed after stimulation of blood with three genospecies *Borrelia*. For *B. afzelii* and *B. burgdorferi s.s.* approximate values were obtained, respectively, 810.3 µg/ml and 819.8 µg/ml. The lowest median value of factor H was obtained for *B. Garinii* – 775.7 µg/ml. The level of factor H lowered after stimulation of *B. burgdorferi s.s.* correlated with a lowered level of this regulatory protein after incubation with *B. garinii* ($p < 0.000002$).

Comparison of results obtained for the control group and patients with Lyme disease. The level of C5a in the specimens of whole blood not incubated of the persons with Lyme disease was significantly statistically higher ($p < 0.0005$) in comparison with the control group, respectively, 21.6 ng/ml and 16.7 ng/ml.

The result of placing the spirochetes of three genospecies of *Borrelia* into the blood of persons with Lyme disease was observed in the evident and statistically significant increase in the levels C5a, in comparison with the control group (Fig. 1).

The median values of the level of factor H in the specimens of blood not incubated of the patients with Lyme disease and persons from the control group were approximate and amounted, respectively, to 954.9 µg/ml and 961.8 µg/ml; the differences between them were not statistically significant. Statistically significant differences were obtained by comparing the level of factor H in specimens incubated with individual genospecies of spirochetes. The incubation of blood with *B. afzelii* influenced to a greater degree the decrease in the level of factor H in the control group

(636.8 µg/ml) than in the group of persons with Lyme disease (810.3 µg/ml, $p < 0.002$). A similar tendency was observed for specimens stimulated with the two remaining genospecies. For blood incubated with *B. burgdorferi s.s.* the level of factor H was 593.4 µg/ml in the control group, and 819.8 µg/ml in the group of persons with Lyme disease ($p < 0.0005$). For blood incubated with *B. Garinii*, the level of factor H was 617.6 µg/ml in the control group, and 775.7 µg/ml in the group of persons with Lyme disease ($p < 0.02$) (Fig. 2).

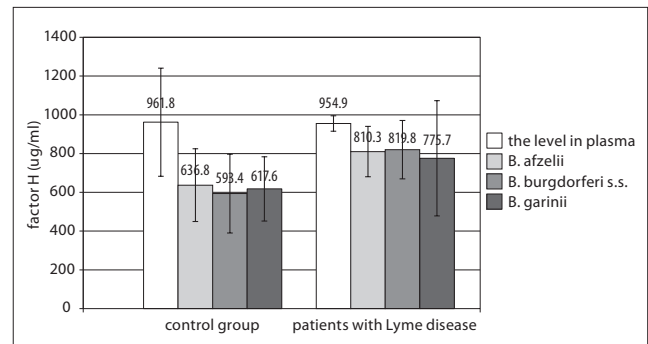


Figure 2. The plasma level of factor H in person with the infection of *B. burgdorferi* and in a control group before and after stimulation with whole blood

DISCUSSION

The activate cascade of the complement can be activated during the contact of a host organism with spirochetes of *Borrelia* via the classical or alternative pathways, and also the lectin pathway [3, 7]. In order to form a membrane attack complex (MAC) it is necessary to dissociate protein C5 through the participation of convertase C5 as an alternative method (C3bBb3b) on two smaller polypeptides – C5a (10kDa) and C5b (180 kDa). The element C5b remains near to the cell membrane and is activated in the cycle of reactions leading to the creation of MAC, whereas a part of C5a is detached and has a strong chemotactic effect on the immune cells. The disintegration of the protein C5a on the components is always caused by convertase C5, regardless of the pathway via which the complement was activated [15].

As observed in the presented study, the incubation of whole blood of healthy persons and persons with Lyme disease with spirochetes *Borrelia* in *in vitro* conditions caused in both groups a statistically significant increase in the level C5a, in comparison with the specimens not incubated. *Borrelia garinii*, which was used in the experiment among three genospecies of *Borrelia*, had the strongest effect on the complement's activation in both studied groups, which resulted in high levels of C5a.

The conclusion can be drawn that in the absence of antibodies against specific proteins of *Borrelia*, which was observed in the control group, the presence of spirochetes in whole blood can have an effect on the activation of the complement system via the alternative or classical pathways, regardless of the antibodies. This conclusion is connected with other studies according to which the spirochetes are able to activate the killing mechanisms via the classical or alternative pathways when there is lack of specific antibodies of anti-*Borrelia*, and both of these pathways are significant in Lyme disease with the participation of complement (complement-mediated bacteriolysis). Krajczy,

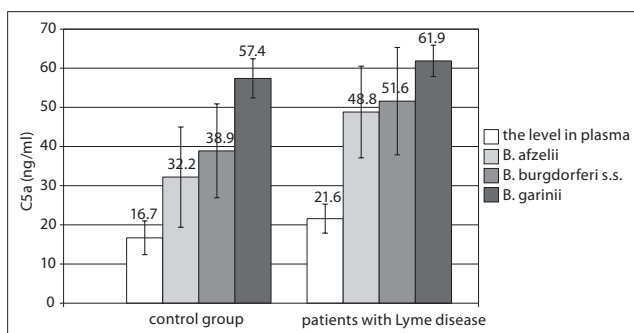


Figure 1. The plasma level of C5a in person with the infection of *B. burgdorferi* and in a control group before and after stimulation with whole blood

in his experiments, demonstrated the domination of the classical over the alternative pathway since larger number of isolates of *Borrelia* underwent lysis with its participation. Simultaneously, the part of isolates of *B. garinii* was effectively destroyed by the complement activated by the alternative pathway [4].

This fatal effect is much higher in the case of the presence of specific antibodies of anti-*Borrelia*. It was shown that the serum of patients with erythema migrans (EM) is less effective in killing *B. burgdorferi* than the serum of patients with late symptoms of the disease, and in whom the levels of specific antibodies against proteins of a spirochete are high. The serum of patients with EM, Lyme arthritis (LA) and acrodermatitis chronica atrophicans (ACA) demonstrated *Borrelia* activity, respectively, 29%, 63% and 86% [4]. According to other data, *Borrelia burgdorferi* s.s. affects the complement's activation where there is lack of antibodies of anti-*Borrelia*, but the evident fatal effect was observed only after the addition of specific antibodies to serum [3].

There were higher median values of C5a noted for specimens of whole blood incubated with spirochetes of three genospecies in the group of persons with clinical symptoms of Lyme disease and serologically confirmed presence of antibodies of anti-*Borrelia* than in the specimens of blood in the control group subjected to the same stimulation. Statistically significant higher values of C5a obtained in the plasma of the group of sick patients for *B. afzelii*, *B. burgdorferi* s.s. and *B. garinii*, in comparison with the control group, proves that the complement was activated by the classical pathway with the participation of specific antibodies. This kind of activation pathway leads to a more effective immunological response against spirochetes of *Borrelia*. The obtained results demonstrating the level of C5a suggest that the level of the intensification of the response of the complement system relies on the genospecies, the strongest of which is for *B. garinii*.

The three pathogenic genospecies mostly identified in Lyme disease: *B. afzelii*, *B. burgdorferi* s.s. and *B. garinii*, to a certain degree, can avoid lysis and resist against the mechanisms of an innate immunity. The complement system's ability to survive in the conditions of being exposed to fatal activity depends on the genospecies, and is higher for *B. afzelii* and *B. burgdorferi* s.s., while most of the isolates of *B. garinii* are effectively destroyed [5, 7]. The effectiveness of the complement concerning the effective destruction of particular genospecies of pathogenic spirochetes of *Borrelia* is varied for humans. In the literature, it is common to classify the isolates of *B. afzelii* as serum-resistant. The majority of isolates of *B. burgdorferi* s.s. are determined as being intermediate serum-sensitive, whereas *B. garinii* as determined as serum-sensitive [3, 4, 7].

One of the most significant mechanisms developed by *Borrelia* in order to protect against the damaging process of the complement's molecules, consists in the acquirement of the regulatory proteins, such as factor H and/or factor H-like protein-1 (FHL-1). Both proteins have similar complement's regulatory functions. They control C3b-inactivation, formed by acting as co-factors for factor I-mediated degradation of C3b, and the decay of the C3bBb convertase.

Borrelia proteins CRASPs serve as ligands for factors H and FHL-1 on *Borrelia* isolates. CRASP-1 (27,5 kDa) preferentially binds FHL-1 and CRASP-2 (20,7 kDa) protein that interacts preferentially with factor H [4]. These proteins contribute

in vitro to the immunity of bacteria against lysis with the participation of the complement [2, 16]. The strong expression of proteins CRASP was demonstrated in all isolates of *Borrelia* resistant to killing with the participation of complement. Some of the isolates of *B. garinii*, which were effectively destroyed by the complement, did not show expression of these peripheral proteins [4].

Factor H, which acts as a regulatory protein of plasma, occurs in serum in the scope of concentrations 110-615 µg/ml [17]. In the experimental model of the presented study, it was assumed that the decrease in the level of factor's H in the blood samples incubated with particular genospecies *Borrelia*, in comparison with samples not incubated, proved that the spirochetes initiate defence strategies which block the activation of the complement via the alternative pathway. The substantial decrease in the level of factor H after incubation of whole blood with three genospecies *Borrelia* observed in the control group, shows that this parameter was included in the defence mechanisms of spirochetes against factors of the innate immunity system of the host. Bacteria, by binding factor H, block the activation of the complement via the alternative pathway. At the same time, it can be deduced that the alternative pathway is not the only significant form in the process of killing spirochetes in people without specific antibodies. This was proved by the presence of the complement's component C5a detected in the plasma of the healthy persons after incubation with spirochetes of *Borrelia*. The presence of C5a can be justified by the process of activation of the complement system without antibodies via the classical pathway.

According to the results obtained, it can be deduced that in the group of people with Lyme disease, in whom specific antibodies of anti-*Borrelia* were present, the response of the classical pathway relying on the antibodies dominated in the response to contact with the spirochetes. The activation of the complement via the alternative pathway was the primary fatal factor. It was proved by the decreased level of factor H, which is bound by spirochetes which, as a result, blocked the alternative pathway. Despite the fact that the alternative pathway was blocked, and lysis of spirochetes with the use of its mechanisms was not possible, the high level detected of complement's component C5a proves that the classical pathway is dominant.

CONCLUSIONS

In the experimental model, during stimulation *in vitro* of whole blood with three genospecies of *Borrelia*, the increased level of C5a can be accepted as an indicator of the effective activation of complement cascade. The increase in the level of C5a in the plasma depends on the genospecies, of which *B. garinii* is the strongest.

The decrease in the level of factor H after incubation of whole blood with three genospecies of *Borrelia* shows that this parameter was included in the defence mechanisms of spirochetes against factors of the innate immunity system of a host, and prevents lysis of bacteria via the alternative pathway.

In spite of the blocking of the alternative pathway, *Borrelia* activates the activation cascade, regardless of antibodies by the first contact of a host organism with spirochetes, or in accordance with antibodies during the infection or another contact with bacteria.

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