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Analysis of nanomechanical properties of *Borrelia burgdorferi spirochetes* under the influence of lytic factors in an *in vitro* model using atomic force microscopy

Analiza własności nanomechanicznych krętków *Borrelia burgdorferi* pod wpływem działania czynników litycznych w modelu *in vitro* z zastosowaniem mikroskopu sił atomowych (AFM)

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A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
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Summary

Background: Atomic force microscopy (AFM) is an experimental technique which recently has been used in biology, microbiology, and medicine to investigate the topography of surfaces and in the evaluation of mechanical properties of cells. The aim of this study was to evaluate the influence of the complement system and specific anti-*Borrelia* antibodies in *in vitro* conditions on the modification of nanomechanical features of *B. burgdorferi* B31 cells.

Material and methods: In order to assess the influence of the complement system and anti-*Borrelia* antibodies on *B. burgdorferi* s.s. B31 spirochetes, the bacteria were incubated together with plasma of identified status. The samples were applied on the surface of mica disks. Young's modulus and adhesive forces were analyzed with a NanoScope V, MultiMode 8 AFM microscope (Bruker) by the PeakForce QNM technique in air using NanoScope Analysis 1.40 software (Bruker).

Results/Conclusion: The average value of flexibility of spirochetes' surface expressed by Young's modulus was 10185.32 MPa, whereas the adhesion force was 3.68 nN. AFM is a modern tool with a broad spectrum of observational and measurement abilities. Young's modulus and the adhesion force can be treated as parameters in the evaluation of intensity and changes which take place in pathogenic microorganisms under the influence of various lytic factors. The visualization of the changes in association with nanomechanical features provides a realistic portrayal of the lytic abilities of the elements of the innate and adaptive human immune system.

Keywords: ***Borrelia burgdorferi*** **• AFM • Young's modulus • adhesion force**

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Introduction

Atomic force microscopy (AFM) is an experimental technique which recently has been used in biology, microbiology, and medicine to investigate the topography of surfaces and in the evaluation of mechanical properties of cells. The forces acting between the AFM probe and the examined surface are measured with the accuracy of piconewtons (pN), and as a result it is possible to obtain precise information about the shape of biological structures and size of studied objects as well as nanomechanical properties of studied surfaces [19]. Nanomechanical properties constitute the most important features of living cells and tissues. The measure of springiness and stiffness of biological surfaces is done through the determination of Young's modulus, i.e. the ratio in which the studied structure can be stretched or tightened, and is provided in N/m^2 [20]. The other parameter which can be used in the study of cells is the adhesion force (nN), which describes the mechanical properties of studied surfaces resulting from the intermolecular forces which act on the surface of their junction. In the case of bacterial cells, the changes of adhesion force can accurately define their ability to colonize tissues or environmental elements and create a biofilm [4,14]. The evaluation of changes of mechanical properties of living Eukaryote and Prokaryote cells provides information about transformations which occur in their structures under the influence of environmental, immunological or chemical factors. The cells of healthy tissues are characterized by different values of Young's modulus or the adhesion force in comparison with cells of pathologically changed tissues. The changes also occur in the cells of bacteria exposed to the activity of lytic factors [21,22]. The contact of a microorganism with potentially lytic factors such as complement's components or specific antibodies can result in damage of a bacterial cell. The outer structures are destroyed as a result of the action of a damaging factor. Hence, the turgor of a cell is violated and in consequence the flexibility of layers also changes. Therefore, all changes concerning mechanical features of living cells inform about modifications which occur in the structure of a cell [10,20]. The modifications of the robustness of a cell wall and associated disturbance of turgor have an influence on weakening of the infectious potential of a microorganism. The scale of its damage cannot be reliably estimated using only the method of visual assessment of the surface of a pathogen cell exposed to the action of damaging factors. AFM as an experimental technique, used in testing the mechanical features of cells, enables one to estimate the level of destruction of bacteria's surface using measurable parameters, such as Young's modulus and the adhesion force [21,22]. Broadening the scope of research concerning the analysis of mechanical parameters of cells of pathogenic microorganisms not only has theoretical value but also allows the development of new, more precise diagnostic and therapeutic methods, and evaluation of the effectiveness of antibacterial agents' forces.

The aim of the present study was to evaluate the influence of the complement system and specific anti-*Borrelia* antibodies in *in vitro* conditions on the modification of nanomechanical features of *B. burgdorferi* B31 cells.

Materials and methods

Bacterial strains and culture conditions

A reference strain of *B. burgdorferi* s.s. B31 (ATCC 35210) was used in the study. To cultivate spirochetes, 0.1 ml of strain was inoculated in 5 ml of BSK-H Complete medium. The strain was incubated in a 5% CO_2 atmosphere at 35°C for 7 days, to a cell density of 10^7/ml [9].

Acquisition of plasma of identified status

In order to assess the influence of the complement system and anti-*Borrelia* antibodies on *B. burgdorferi* s.s. B31 spirochetes, the bacteria were incubated together with plasma of identified status. A blood sample taken from a person who did not reveal the presence of IgM/IgG anti-*Borrelia* was the source of plasma with the active complement system. A blood sample taken from a person with clinical symptoms of Lyme disease and IgM/IgG anti-*Borrelia* was the source of plasma with the active complement system and specific antibodies. Whole blood (2.5 ml) was collected in polypropylene tubes containing r-hirudin (SARSTEDT). Recombinant hirudin is a highly specific thrombin inhibitor that minimally influences complement activation [3,8,13]. The complement system activity was blocked as a result of taking a blood sample (2.5 ml) from a person with clinical symptoms of Lyme disease and IgM/IgG anti-*Borrelia* using syrin-

ges coated with EDTA (SARSTEDT). The blood samples were centrifuged for 10 min at 1000 x g. The plasma was separated. The bacteria were suspended in specific status plasma (200 µl), and the samples were incubated for 3 h at 37°C [18].

Microscopic imaging of bacterial cells

B. burgdorferi s.s. B31 in BSK-H medium and in specific status plasma was centrifuged for 10 min at 2500 rpm. The supernatant was removed from the precipitate of bacteria. Bacteria samples were prepared according to the procedure given by Zdybicka-Barabas [21]. The samples were applied on the surface of mica disks and allowed to dry at 28°C before imaging. Young's modulus and adhesive forces were analyzed with a NanoScope V, MultiMode 8 AFM microscope (Bruker) by the PeakForce QNM technique in air using NanoScope Analysis 1.40 software (Bruker).

Results

Analysis of nanomechanical properties of *B. burgdorferi* s.s. B31 spirochetes expressed by Young's modulus and adhesion force

The image record of bacteria in DMT Modulus format (Fig. 1) enables one to perform a series of measurements of Young's modulus on the precisely determined operative fields of the analyzed spirochete's surface. The image record of bacteria in the Adhesion format (Fig. 2) enables one to take measurements of the adhesion factor, i.e. intermolecular forces on the surface of a junction between the AFM probe tip and a bacterial cell. The average value of flexibility of spirochetes' surface expressed by Young's modulus is 10 185.32 MPa (min. 5812.00 MPa – max. 15 181.0 MPa; SD 2590.34), whereas the adhesion force is 3.68 nN (min. 3.05 nN – max. 4.37 nN, SD 0.45). Young's modulus and the adhesion force were designated for *Borrelia* spirochetes which were not subjected to the action of destructive factors. They were compared to results achieved for spirochetes incubated with plasma of identified status.

Young's modulus determined for *Borrelia* spirochetes incubated with plasma of different status

The value of Young's modulus measured for incubated bacteria with plasma containing active complement was significantly lower (4511.70 MPa, p≤0.0000001) in comparison with the control (10 185.33 MPa). Young's modulus determined for spirochetes incubated with plasma containing active complement and specific anti-*Borrelia* antibodies was significantly lower (3075.82 MPa, p≤0.0000001) in comparison with a control and significantly lower (p≤0.0000001) in comparison with the value measured for spirochetes exposed to plasma with active complement. Spirochetes incubated with plasma containing specific anti-*Borrelia* antibodies and blocked complement obtained lower values of Young's modulus (3752.72 MPa, p≤0.0000001) in comparison with control cells. The obtained values were also significantly lower (p≤0.000008) in comparison with Young's modulus measured for spirochetes incubated with plasma with active complement (4511.70 MPa) and significantly higher (p≤0.0000001) in comparison with the value measured for bacteria incubated with plasma with active complement and specific anti-*Borrelia* antibodies (3075.82 MPa) (Fig. 3).

The adhesive factor for spirochetes *Borrelia* incubated with plasma of different status

The value of the adhesion force for *Borrelia* cells incubated with plasma containing active complement was significantly lower (3.11 nN; p≤0.000004) in comparison with the value achieved for control cells (3.68 nN). The measures taken for spirochetes incubated with plasma containing active complement and specific anti-*Borrelia* antibodies revealed a significant decrease of the adhe-

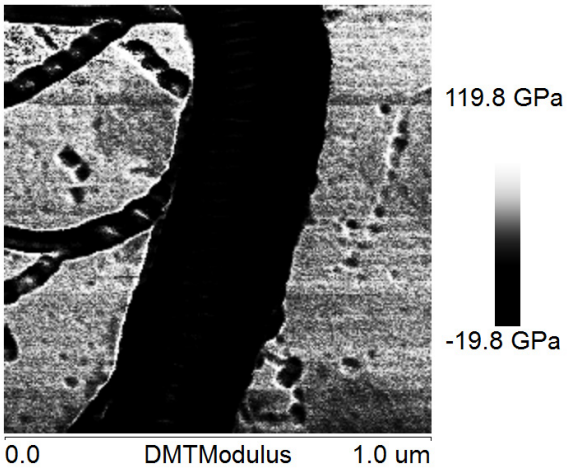

Fig. 1. The image record of bacteria *B. burgdorferi s.s.* B31 in the DMTM Modulus format (NanoScope Analysis 1.40; Bruker)

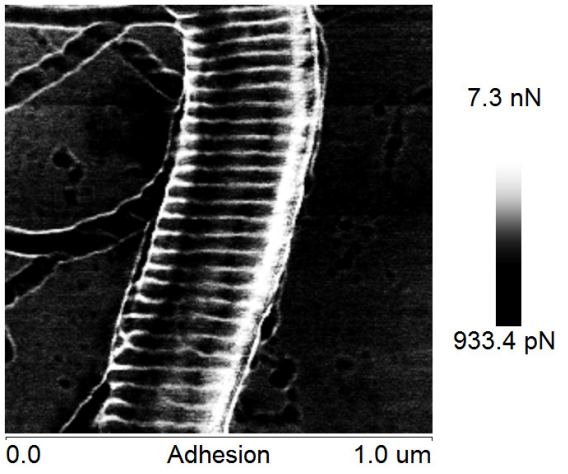

Fig. 2. The image record of bacteria *B. burgdorferi* s.s. B31 in the Adhesion format (NanoScope Analysis 1.40; Bruker)

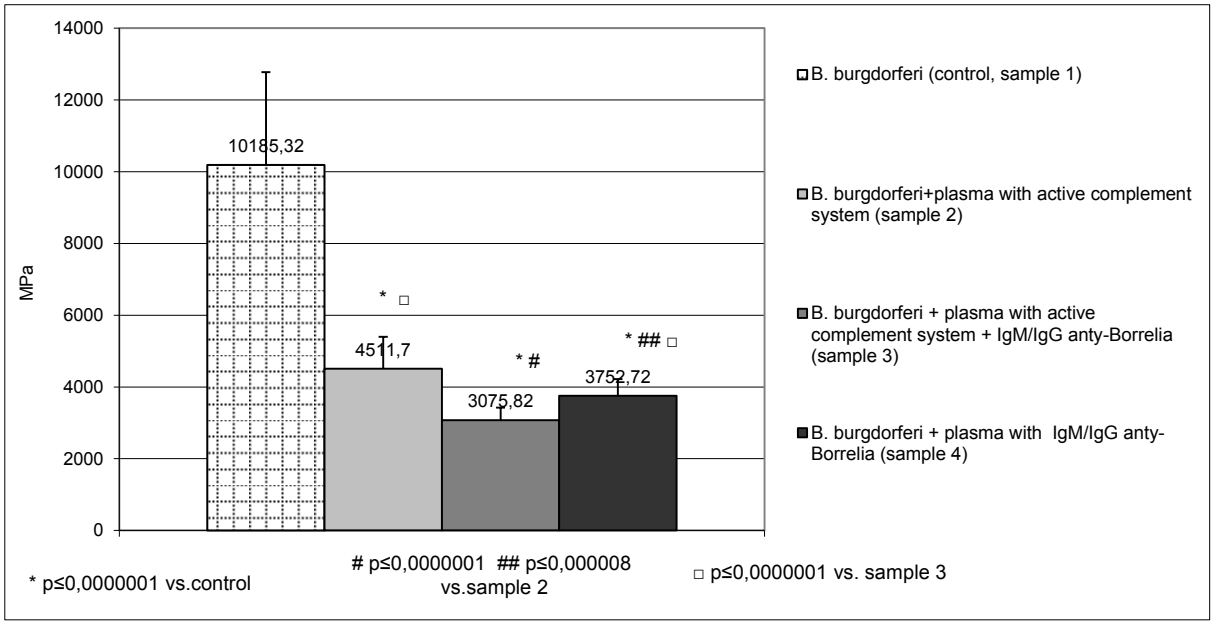

Fig. 3. Young's modulus determined for spirochetes *Borrelia* incubated with plasma of different status

sion force (2.07 nN; p≤0.0000001) in comparison with the control. The obtained adhesion force value was also significantly lower (p≤0.0000001) in comparison with the value measured for spirochetes which were exposed to plasma with active complement (3.11 nN). The adhesion force was significantly lower (2.96 nN) (p≤0.0000001) for *B. burgdorferi* spirochetes incubated with plasma containing specific anti-*Borrelia* antibodies and blocked complement, in comparison with the control. At the same time, the obtained adhesion force value was significantly higher (p≤0.000002) in comparison with the measured value for spirochetes incubated with plasma containing active complement and specific anti-*Borrelia* antibodies (2.07 nN), and significantly lower (p≤0.0005) in comparison with the value measured for bacteria incubated with plasma and active complement (3.11 nN) (Fig. 4).

Discussion

AFM is one of the most modern tools with a very broad spectrum of observational and measurement capabilities, and its significance in the study of microorganisms is immense [7,12]. The cell wall of gram-negative bacteria is one of the most complex structures in prokaryotic cells. The foundation consists of a thin layer of peptidoglycan and a double external layer with a a rich protein-lipid fraction. If the external layer of a cell is damaged as a result of the activity of lytic factors, e.g. the complement system, the pressure present inside the cell radically decreases, which has an effect on the flexibility parameters. In conclusion, the shape of a cell is a resultant of all factors: turgor pressure, thickness of the peptidoglycan and the external membrane [20]. The mutual reaction between a pathogen and tissues of an infected organism is described as a decisive stage of colonization. During this process, the mechanical parameters of external structures of a bacterial cell are very important [1,2,4,14].

Specific antibodies enable one to eliminate spirochetes when acting as described and favor their destruction via the complement system, whereas antibodies attached to the surface of a bacterial cell cannot lead to its lysis. Non-specific factors such as complement and immunologically competent cells with the participation of immunoglobulin can lead to the effective lysis of a pathogen. The presence of specific antibodies or non-specific mechanisms solely is insufficient in order to effectively eliminate an infection from the organism [6,15,16]. In the case of cooperation of specific and non-specific mechanisms, the killing effect is immensely greater. It was confirmed that the specimen of patients with erythema migrans (EM) is less effective in killing *B. burgdorferi* than the specimen of patients with later symptoms of the disease, who had high levels of specific antibodies against spirochete proteins. The specimen of patients with EM, Lyme arthritis and acrodermatitis chronica atropicans confirmed the activity of the killing effect as respectively 29.63 and 86% [5]. According to other data, *B. burgdorferi* has an influence on the activation of a complement without anti-*Borrelia* antibodies, although the killing effect was distinctly visible only after the addition of specific antibodies to the specimen [6]. AFM was used to observe how the surface of *B. burgdorferi* and its mechanical parameters change as a result of a reaction of complement and specific anti-*Borrelia* antibodies. The changes of flexibility of bacterial cells and their prospective abilities to adhere were determined by measuring Young's modulus and the adhesion force. The values of Young's modulus and the adhesion force were decreased due to *B. burgdorferi* spirochetes being subjected to the effect of plasma with active com-

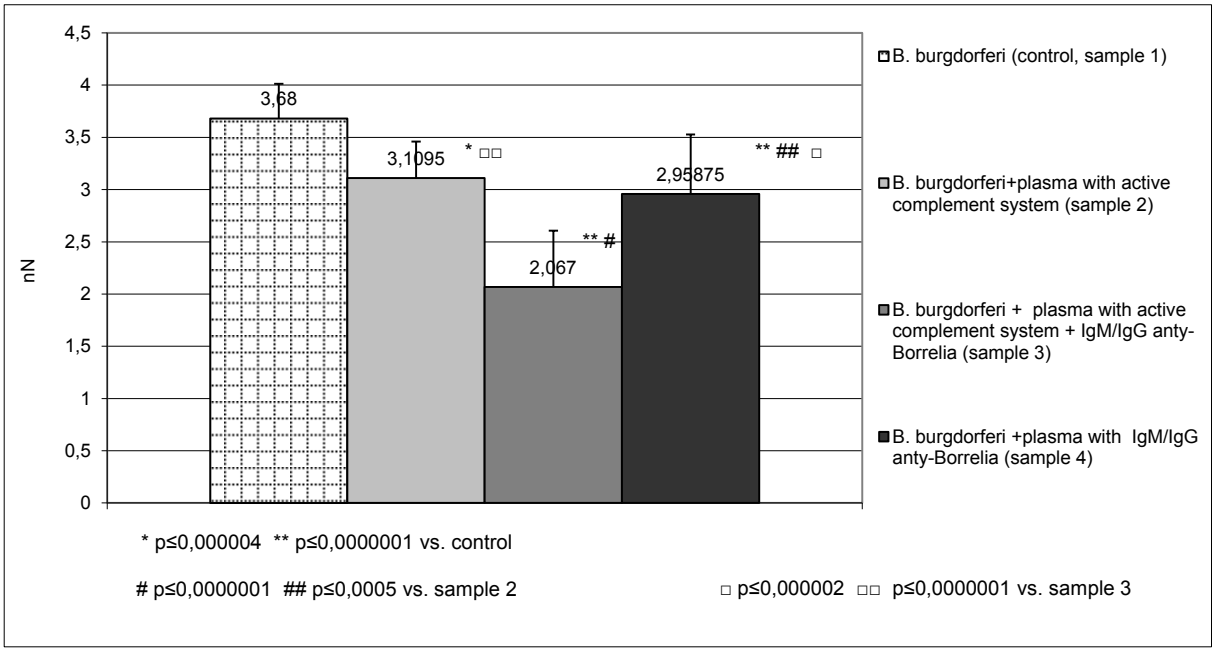

Fig. 4. The adhesive factor for spirochetes *Borrelia* incubated with plasma of different status

plement in comparison with bacteria which were not subjected to the complement's activity. This indicates what changes took place on the surface of bacteria as a result of an action of this potentially lytic factor. The spirochetes subjected only to the action of specific anti-*Borrelia* antibodies also showed a decrease in the flexibility of outer cell layers. However, the ultimate changes of Young's modulus and the adhesion force were confirmed for spirochetes which were subjected to the complement action and specific anti-*Borrelia* antibodies. In the case of infection with *Borrelia*, the coexistence of a humoral response and the complement's response seems to create the greatest possibility of spirochetes' elimination via the destructive influence on the cell wall. The low values of Young's modulus which were obtained in the conducted tests for spirochetes subjected to plasma with active complement and anti-*Borrelia* antibodies demonstrate the strong destructive influence of those factors on a bacterial wall and confirm their effectiveness in lysis. The analysis of mechanical features of bacteria in AFM is performed to other microorganisms. The influence of destructive factors on biophysical features of *E. coli* JM83 revealed in changes of Young's modulus and the adhesion force were analyzed by Zdybicka [22]. However, Powell et al. in *in vitro* tests proved the decrease of Young's modulus for *P. aeruginosa* and *A. baumannii* biofilms under the influence of OligoG in comparison with control biofilms [11]. The knowledge of mechanisms responsible for the ability of bacteria to adhere to surfaces is crucial in relation not only to pathogenic bacteria but to probiotic ones as well. Tripathi et al. using AFM analyzed the issues connected with adherence of *L. rhamnosus GG* [17]. The notion of adhesion is also crucial in terms of biology of pathogenic microorganisms including *B. burgdorferi* as regards their participation in pathogenicity of diseases. The destruction of outer structures as a result of the activity of complement and specific antibodies can lead to a decrease of adhesive abilities of bacteria. Spirochetes subjected to the reaction of a complement and antibodies demonstrate the lowest adhesion force. Thus, it is possible that these two factors can have the greatest influence on a decrease of abilities of *B. burgdorferi* to adhere to colonized surfaces.

Conclusions

Young's modulus and the adhesion force can be treated as parameters in the evaluation of intensity and changes which take place in pathogenic microorganisms under the influence of various lytic factors. The visualization of the changes in association with mechanical features provides a realistic portrayal of the lytic abilities of the elements of the innate and adaptive human immune system.

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