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EXTRACELLULAR POLYMERIC SUBSTANCE OF BIOFILMS IN THE FORMATION OF ANTIMICROBIAL RESISTANCE OF MICROORGANISMS

Abstract. *The analysis of modern literature data on the mechanisms of the formation of antibiotic resistance and the role of extracellular polymeric substance in biofilms, which are the main form of microbial existence. The role of extracellular polymeric substance in limiting of the effect of unfavorable factors as well as the regularity and necessity of its formation for the community of microorganisms were discussed. The position on the permanent character of phenotype dispersion of microorganisms is postulated. This dispersion doesn't provide the formation of more resistant strains only, but plays the prominent role in the permanent formation of various forms, that aren't viable under given conditions but play the role of a depot of building material for extracellular polymeric substance. The mass death of low-resistant forms caused by the action of the antibiotic ensures saturation of the extracellular polymeric substance by dechromatized DNA, that increases the resistance of the microbial socium and contributes to the further formation of multiresistance.*

Key words: infectious diseases, antimicrobial resistance, biofilms, phenotype dispersion, netosis.

Introduction

The rapid spread of antibiotic resistance of microorganisms, that are causative for various diseases, occupies a special place among the numerous medical and social problems of our time. According to the WHO definition, this resistance is due to the development of the sustainability of the microorganisms to the drug which was previously effective for the treatment of the corresponding infection.

This phenomenon has become widespread in the world, and in the last decade the problem has become more acute by polyresistance caused by the resistance of microbiological pathogens to various types of antibiotics (Antimicrobal resistance: global report on surveillance 2014; Antimicrobal Resistance Benchmark 2018).

Aim

The aim of this work is to discuss the reasons for the rapid formation of pathogen resistance to antibiotics. The protective role of the components of the extracellular polymeric substance is substantiated.

Discussions

The emergence and rapid spread of antibiotic-resistant forms of clinically relevant pathogens not only significantly

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complicates the treatment of relevant diseases, but also creates the risk of clinical medicine returning to the pre-antibiotic era (O'Neill, J., 2014). Thus, in 2011, an outbreak of intestinal infection caused by an enterohemorrhagic bowel of E. coli strain O104: H4 or EESC was observed in Europe (Dyachenko, A. G., 2012). Since all the known antibiotics have been proven to be practically ineffective for the treatment this pathogen, it were recommended to abandon the traditional antibiotic therapy.

According to these recommendation, the most patients recover themselves, some require symptomatic treatment, and only in the most common cases two types of antibiotics may chosen by criterion of the minimal release of toxins. In the view of the obvious toothless of such recommendation, the relevance of the study of the mechanisms of the fo-rmation of antibiotic resistance appears obvious.

As to today, more than 6000 antibiotics are known, but only 2-3% of them have been used in clinical practice. Others were either unacceptably toxic or not effective enough. At the same time, no new antibiotic molecule has been created in the last decades, and all the so-called "new" ones are only altered drug forms, different mainly from the ever increasing doses of drugs. Leading pharmaceutical companies are not interested in the production of new antibiotics, since the formation of resistant forms of pathogens occurs very quickly, about a year, that eliminates the proper profit on the money invested in the development and promotion of a new drug (Namazova-Baranova, L., & Baranov, A., 2017).

On these reasons the increase of the resistance of pathogens to antimicrobials leads to millions of deaths annually (Anti-microbal resistance: global report on surveillance, 2014).

What are the cause of the development of antibiotic resistance? First of all, it should be noted that this phenomenon does not represent something qualitatively new, with which various organisms have not dealt with previously. Antibiotics are the means that acquired by microorganisms in the competitive struggle for ecological niches during millions of years of their evolution. No less common is the emergence of resistance to the action of these substances.

That is the eternal competition between the permanent improving the damaging effect of some micro-organisms and the defense mechanisms of others (Dyachenko, A. G., 2012). However, in the case of the antibiotic resistance, the human population becomes the place of these races, and the use of antibiotics occurs in absolutely unnatural, extremely high quantities. There is no consensus regarding the reasons of antibiotic resistance, but there are three leading ones are unanimously recognized:

- the widespread use of antibiotics and structurally similar compounds in veterinary medicine, that leads to their constant supply to the human body with food in low subtherapeutic quantities;
- uncontrolled antibiotic self-medication, which is usually discontinued after the disappearance of the external signs of the disease and, as a consequence, contributes to the formation and spread of resistant strains of the pathogen;
- the formation of polyresistant strains in hospitals, because it is the places where the bacteria undergo the most intense exposure to various antibiotics. Such strains are called "hospital superinfections", which are present in various forms in all clinics without exception (Namazova-Baranova, L., & Baranov, A., 2017; Babady, N., 2016).

This list can be supplemented by the use of overdue drugs, chaotic regimens, unknown strains in hospitals. The formation and distribution of antibiotic resistant strains is facilitated by the neglect of the characteristics of the pathogen of each individual patient. In the latter case, in the absence of laboratory data, the administration of a broad-spectrum antibiotic may temporarily extinguish the external signs of the disease, but will promote the progression of antibiotic resistance and the development of a number of related complications.

Contrary, the timely dete-ction of the nature of the antibiotic resistance of the patient may be the basis for the effective use of so-called reserve antibiotics - rarely used, and therefore very expensive, antibiotics stored as a last resort (Namazova-Baranova, L., & Baranov, A., 2017).

It is accepted that antibiotic resistance arises in one of three ways (Dyachenko, A. G., 2012; Namazova-Baranova, L., & Baranov, A., 2017; Hoffman, S., Outterson, K., Rottigen, G., et al., 2015):

- due to the natural resistance of certain bacteria of this type. Typically, natural infectious agents are a pool of different strains of one pathogenic organism. Under certain conditions, the individual strains may show increased resistance to the action of this antibiotic. Such selective pressure leads to the dominance of the resistant strains;

- due to a genetic mutation that leads to the formation of an antibiotic-resistant form;

- due to the acquisition of resistance by one species of bacteria from others. Various variants of horizontal gene transfer between microorganisms both of the same type and the different ones ensures the dominance of the resistant strains as the result of the rigid selection (Hazen, T., Mettus, R., McElhey, C., et al., 2018; Mahfouz, N., Caucci, S., Achatz, E., et al., 2018).

However, such an important feature of the vast majority of microorganisms as their existence in the form of biofilms remains unaddressed.

According to the conventional definition, a biofilm is an aggregate of microorganisms in which cells are fixed between themselves and / or with an insoluble surface due to the incorporation into the matrix of extracellular polymeric substance (EPS), which is synthesized by the cells themselves (Vert, M., Doi, Y., Hellwich, K-H., Hess, M., et al., 2012). Biofilms exist both in a fixed phase boundary state and in a mobile, unrelated to any substrate form (Flemming, H., Wingender, J., Szewzyk, U., et al., 2016). All multicellular organisms are colonized by a variety of microorganisms, mainly in the form of biofilms (Nikolaev, Yu., Plakunov, V., 2007). It's known that the intensity of reproduction of microorganisms in the composition of biofilms is much inferior to free planktonic forms, but exactly biofilms are the leading form of existence of microorganisms due to the increased resistance to the influence of various negative environmental factors. As the rule, such communities are formed by one or more different types of monocellular micro-organisms (Flemming, H., 2016). EPS includes proteins, lipids, carbohydrates, and nucleic acids, which play an important role in the functioning of the biofilm and ensure the survival of the microorganism community (Fig. 1).

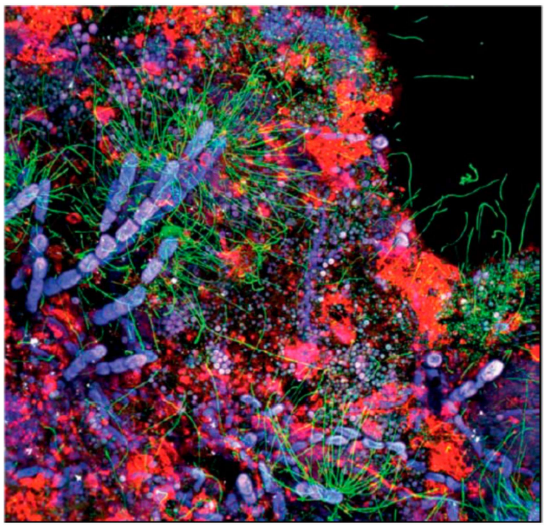

Fig. 1. Fluorescence of the components of biofilms. Nucleic acids are stained with SybrGreen and glow green, lectin inclusions are stained with AAL-Alexa568 and glow red, the autophluorescence of chlorophyll IIA is blue, the autofluorescence of cyano-bacteria is purple and white. The frame size is 246x246 microns (Kamiunke, N., Herzsprung, P., & Neu, T., 2015).

Thus, the proteins, that are secreted by microorganisms, form the fibrils that play the role of a kind of scaffolding of a three-dimensional biofilm grid (Taglialegna, A., Lasa, I., Valle, J., 2016). Carbohydrates provide a high level of hydration of the biofilm, its permeability for the cell-required substances. The insoluble components of the EPS covalently conjugate enzymes which are capable of inactivating various toxic substances in one or another way and playing the role of a kind of external digestive system of microorganisms, till to "cannibalizing" the remains of dead microorganisms (Flemming, H., 2016). At the same time, the composition of the EPS components plays an important role in the ensuring of adhesive bonds both between the individual microorganisms in the biofilm and the biofilm itself with insoluble surfaces. Despite the fact that the concept of biofilms was proposed relatively recently - in the mid 80's of the last century - a great deal of scientific work is devoted to the study of various forms of biofilms as a basic form of life. The stages of biofilm formation and heating, the differentiation of the cell phenotype in its composition, the mechanisms of formation of feeding channels, horizontal gene transfer, the formation of forms different from each other and much more are carefully described (Flemming, H., Wingender, J., Szewzyk, U., et al., 2016; Nikolaev, Yu., Plakunov, V., 2007). However, the reasons for the high resistance of biofilms to a variety of adverse factors remain poorly understood (Flemming, H., 2016).

Regarding the topic of our work, the biofilms' resistance to antibiotics deserves special attention. It has been shown that by this criterion biofilms exceed in two or three orders of magnitude one-celled planktonic forms (Nikolaev, Yu., Plakunov, V., 2007). In the same time, the ability of EPS to absorb and accumulate a lond list of toxins had been shown. As to look from the point of cells' view, this is equals for filtration, which dramatically reduces the possibility of toxic molecules to reach the intracellular space. Particular attention should be paid to the role of extracellular DNA (eDNA). Until recently, extracellular DNA was thought to be one of the remains of dead and destroyed cells. However, its role in adhesive interactions in the early stages of biofilm formation has been demonstrated recently (Tang, L., Schramm, A. & Neu, T., 2013; Vilain, S., Pretorius, J., Theron, J., et al., 2009). Moreover, it is shown that it not only plays a structure-forming function but is also able to efficiently bind toxins and antibiotics (Flemming, H., Wingender, J., Szewzyk, U., et al., 2016; Man, T., Pitts, B., Pellock. B., et al., 2003). As well-known, majority of antibiotics effect their bactericidal action due to

their ability to influence on transcription and translation processes, ie in some way to interact with nucleic acids (Dyachenko, A. G., 2012). Therefore, the presence of dechromatized DNA in the EPS can cause a sharp decrease in the number of antibiotic's molecules capable for penetrating into the cell. Somewhat unexpected conclusions emerge from this assumption. First of all, it is known that the intensity of metabolism at the level of microorganisms on average is much higher than that of macroorganisms. This also applies to EPS, which is undergoing permanent destruction and reproduction. This requires significant amounts of building material coming from destroyed dead cells. That is, the formation and maintenance of biofilm requires the constant formation of large quantities of the cells that are doomed to break down and unviable under the given conditions. The assumption implicits well with the ability of individual toxins to stimulate biofilm matrix formation (Huseby, M., Kruse, A., Digre, J., et al., 2010).

It's generally accepted that in the composition of biofilms there are different forms of the same microorganisms differing in phenotype, but there are no forms different in genotype. One can only guess what exactly leads to a similar divergence of the phenotype of the newly formed cells. The most probable explanation is the asymmetric distribution of the gene material under the conditions of intense horizontal transfer of genes, that is a recognized factor of microbial evolution (Dyachenko, A. G., 2012). In the overwhelming majority, the newly formed mutant forms are non-viable and decay and only a small fraction acquire useful or at least harmless mutations, forming the cellular component of the antibiotic-resistant biofilm. In other words, the impact of any adverse factor stimulates the dispersion of the phenotype, and the formation of non-viable forms becomes the key for the survival of the community of microorganisms as a whole. As noted above, the million-years evolution have brought about the adaptive capacity of microorganisms to ensure their survival under a variety of conditions and under the influence of a variety of intractable factors. Thus, it's noteworthy the message on the formation of antibiotic resistance in certain parts of microorganisms under the influence of heavy metal ions (Seiler, C., Berendonk, T., 2012). It is likely that in this case the mass death of the components of the biofilm's components led to qualitative changes in the composition of the EPS, which led to the development of antibiotic resistance in a certain part of the microbial community. The saturation of extracellular polymeric substance by dechromatized DNA not only increases the resistance of the microorganism society, but also facilitates the subsequent formation of polyresistance. For the same reasons, the transfer of antibiotic-resistant pathogens should be mediated not by single microorganisms, but by more or less formed "floating islands" of biofilms - the traditional form of microbial colonies spread (Flemming, H., 2016; Nikolaev, Yu., Plakunov, V., 2007). This form of transfer not only contributes to the conservation of the resistant species of the microorganism, but also significantly increases their transmissibility (Dyachenko, A. G., 2012). It is also worth noting a certain parallel between the protective action of EPS and the action of a multicomponent three-dimensional net formed by netosis - the third form of the cells' death (Brinkmann, V., Reichard, U., Goosmann, C., et al., 2004; Fuchs, T., Abed, U., Goosmann, C., et al., 2007). In this case, the decay products of dead cells become the components of a three-dimensional network that binds and

neutralizes pathogens, in this time at a much higher, multicellular, level. That is, both at the levels of relatively primitive biofilms and in the incomparably complex system of a multicellular organism, the reutilization of dead cells supports the formation of a protective three-dimensional net from their components.

It is believed that the main mechanisms of antibiotic resistance are mediated by a number of processes, which include the neutralization of the antibiotic by its modification (hydrolysis, phosphorylation, acetylation, glycosylation), active removal of the antibiotic from the cell (efflux), change the properties of the target and the properties of the target (Dyachenko, A. G., 2012). According to the above material, the implementation of all these mechanisms may be associated with the protective effects of biofilms, which reduce significantly the effective concentration of the antibiotic in the immediate environment of thethe microorganism, which, in turn, promotes the selection of antibiotic-resistant strains. However, no less important is the formation of sufficiently large quantities of the forms that are not adapted to survive under the given conditions. They or, rather, the products of their decay, provide the necessary building material for biofilm formation and, as a consequence, the survival of the micro-organism community as a whole.

Conclusions

Summaries of the materials presented in this work allow us to draw the following conclusions:

1. Biofilms are the leading form of existence of most microorganisms that have evolved over the course of evolution and provide significant benefits in terms of resistance to the adverse environmental factors compared to monocellular planktonic forms.

2. Any adverse factors that inhibit the normal life of microorganisms lead not to the selection of more resistant forms only, but also to the enhanced formation of extracellular polymeric substance from the decay products of less resistant cells.

3. Negative factor caused by mass destruction of microorganisms leads to horizontal transfer of genes between microorganisms of both one and different species, which promotes the formation of more resistant forms and less stable ones. The structural components of the latter are transformed into the building material for extracellular polymer substance. The dying of low-resistance microorganisms leads to the saturation of extracellular polymeric substance by de-chromatized DNA molecules, which are able to effectively adsorb not only the molecules of the given antibiotic, but also contribute to the development of polyresistance to other ones.

4. A necessary condition for the implementation of these mechanisms is the permanent divergence of the phenoltype of microorganisms. The most probable it's cause is the asymmetric distribution of the genotype during DNA replication, which has undergone various epigenetic modifications.

5. For these reasons, the formation of antibiotic resistance of pathogens is a manifestation of the evolutionary adaptation mechanism, and the effective counteraction of it is possible only at the account of the characteristics of the resistance of the pathogen in each individual patient and the appointment of a complex of drugs that can overcome it.

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