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INFLUENCE OF PATHOGENIC FACTORS OF CANDIDA ALBICANS AND STAPHYLOCOCUS AUREUS ON THE PHAGOCYTIC ACTIVITY OF NEUTROPHILS

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SUMMARY. The association of *Candida albicans* and *Staphylococcus aureus* microorganisms causes various clinical forms of purulent-inflammatory diseases. They are often isolated in cases of infections associated with the formation of biofilms. These pathogens are the causative agents of nosocomial infections that cause severe illness and mortality even with appropriate treatment.

The aim – to study the ability of microorganisms to form biofilms in clinical and reference strains of *C. albicans* and *S. aureus*, to determine the enzymatic activity of phospholipase and protease of *C. albicans* strains. Determine the phagocytic activity of neutrophils against clinical and reference strains of *C. albicans* and *S. aureus* in vitro.

Material and Methods. Neutrophil phagocytic activity was identified by experiments in vitro using standard methods. The reference strains of *C. albicans* and *S. aureus* were used as a control group. The ability of microorganisms to form biofilms was determined using of plastic plates for immuno-enzyme analysis.

Results. When studying the ability of microorganisms to form biofilms, the indicators for clinical strains of the association were – (1.0987±0.007) units OD for reference strains – (0.0776±0.004) units OD. It has been established that clinical strains of *C. albicans* had a high activity of the aggressive enzymes as phospholipase and protease. There decrease of all indicators of phagocytic activity of neutrophils relative to the association of *C. albicans* and *S. aureus* was found. The phagocytic index for clinical strains was (3.03±0.07), for the reference strains – (3.36±0.27).

Conclusion. *C. albicans* and *S. aureus* in the association can enhance their virulent properties, and presence of pathogenicity factors, such as aggression enzymes and biofilm formation, help to suppress phagocytic reactions and the immune response generally.

KEY WORDS: mixed infection; phospholipases; proteases; microbial biofilms; phagocytosis.

Introduction. Pyoinflammatory infections caused by association of microorganizms *Candida albicans* and *Staphylococcus aureus* are important issue studied by researchers from different countries. According to the literature, in 27 % this consortium is the cause of nosocomial infections and in 11 % causes catheter-associated infections [1]. Australian researchers had found out that *Candida-Staphylococcal* infection in 20 % of cases causes women the acute parturient mastitisin women [2].

Based on the work of scientists, these microorganisms in association enhance their virulence properties in many folds [3].

This is mainly explained, by the ability of microorganisms to form biofilms, while their resistance to the antibiotic increases up to 1000 times, which aggravates the course of the disease and treatment [4]. To understand the development of the inflammatory process caused by the association of bacteria, it is important to study the immune response of a macroorganism when these pathogens invade [5].

One of the mechanisms of immune responses is phagocytosis. This is a fundamental process of cells for destruction and absorptionof foreign particles. In multicellular organisms, phagocytosis is a universal phenomenon that all cells are able to perform (including epithelial, endothelial, fibroblasts, etc.). Some specialized cells (such as neutrophils and macrophages) perform this very effectively and are therefore professional phagocytes [6].

Phagocytosis involves a series of steps from recognizing the target particle, absorption it by the phagosome (phagocytic vacuole), maturing this phagosome into the phagolysosome to the final destruction of the absorbed particle in the agressive antimicrobial phagolysosome medium. Thus, phagocytosis is an effective process that eliminates the invasion of pathogenic microorganisms and helps maintain homeostasis. However, some pathogens have also developed various strategies to prevent the normal process of phagocytosis. These pathogens have a clear advantage in infection development [7].

Phagocytic activity against *C. albicans* fungi is sometimes complicated due to the size of these cells and the presence of hyphae elements. Phagocytic cells lack myeloperoxidase required to kill yeast-like cells. Sometimes the phagosome is not formed, and phagocyte pseudopodia overlap each other [8]. Beside this microbial cells of *S. aureus* synthesize substances that inhibit phagocytic activity [9, 10]. When the association between *C. albicans* and *S. aureus* occurs the is virulent activity of these pathogens increases. It contributes to a strengthening of phagocytosis inhibition [11, 12].

The researchers identified 27 proteins that become active in the association *C. alibicans* and *S. aureus*. This special role is played by L-lactic dehydrogenase (LDH1) of *S. aureus* which provides stability during oxidative stress. This enzyme takes part in

generating of L-lactic acid during fermentation; it activity enhances activity upon binding *S. aureus* and hyphae elements of *C. albicans*. In experimental studies virulence factor *S. aureus* transcriptional repressor protein (CodY) have been found. It blocks the formation of biofilms and synthesis of *S. aureus* toxin. Increased protein expression (Cod Y) and decreased of L-lactate dehydrogenase (Ldh1) activity can indicate that *S. aureus* can suppress virulence. Thus immune system does not recognize it. When the association *S. aureus* and *C. alibicans* creats, the virulence factor protein (CodY) is deactivated and the amount of L-lactate dehydrogenase (Ldh1) increases, which contributes to the aggressiveness of *S. aureus* [13].

Pathogenicity factors of *C. albicans* such as phospholipases and acidic proteases play an important role in suppressing the immune response. They are able to mask receptors for complement components and opsonins, which reduce the effectiveness of phagocytic reactions. With superficial skin lesions, fungi penetrate and are absorbed by macrophages, which do not completely destroy them. Due to the presence of the protease enzyme, *Candida* limits the production of secretory immunoglobulin A. Thus, candida remains viable for a long time due to incomplete phagocytosis. *Candida* also interacts with CD4 and CD8 lymphocytes, while forming cellular immunity and local granuloma. It should be noted that these microorganisms have antilysocyme activity. So *Candida* is able to populate various ecological niches and stay in the human body for a long time [14, 15].

The main strategy for preserving the life of microorganisms is the ability to form biofilms. The causative agents of chronic infections can form biofilms. They have an increased resistance to antibiotics, are able to withstand the action of antibodies, phagocytes and other environmental factors that are potentially dangerous for them. The study of the ability to form biofilms by various microorganisms is an urgent issue in modern medicine [16].

According to the literature, the association of bacteria *C. alibicans* and *S. aureus* in the biofilm increases their pathogenic properties several times. These virulent properties can have a direct effect on suppressing the immune response.

Despite the researches being conducted it still remains unknown, which reactions occur during the immune response in the presence of bacterial and fungal pathogens simultaneously. Therefore, research in this area remains relevant today.

The aim of the study – to study the ability of microorganisms to form biofilms in clinical and reference strains of *C. albicans* and *S. aureus*, to determine the enzymatic activity of phospholipase and protease of *C. albicans* strains. Determine the phagocytic activity of neutrophils against clinical and reference strains of *C. albicans* and *S. aureus* in vitro.

Material and Methods. This article is a part of the dissertation "Combined action of antimicrobial agents against the consortium of bacteria *C. albicans* and *S. aureus*". The studies were conducted on the basis of the laboratory of the Department of Microbiology Kharkiv National Medical University. To study the phagocytic activity of neutrophils, clinical and reference strains were used. We have the approval of the Bioethics Committee for research.

An experimental study was performed on 16 clinical strains of *S. aureus* taken from patients with various pyoinflammatory infections and 16 strains of *C. albicans* taken from patients with pneumonia. The following reference strains of the microorganisms: *Candida albicans* CCM 885, *Staphylococcus aureus* ATCC 25923 = NCDC 25923 = F-49 were used as a control group.

Neutrophil phagocytic activity was identified by experiments in vitro using standard methods. 0.1 ml of 2 % sodium citrate sterile solution and 0.2 ml of fresh blood group O were put into tubes. 0.25 ml of *S. aureus* and *C. albicans* microbial suspension concentrated 2 billion microbial cells in 1 ml was added to the mixture, mixed and placed in an incubator at 37°C for 30 min. The mixture was then centrifuged at 1500 r/min for 5 min, then we carefully selected thin layer of white blood cells, applied it to a glass slide, dried, fixed it with methanol for 5 min, and stained it with azur-eosin solution for 30 minutes. The smears were observed in terms of immersion microscopy (ok.7×ob.90), and there were 100 (sometimes 50) white blood cells counted. The absorbing effect of neutrophils was characterized by three parameters: the phagocytosis percentage – the ratio of neutrophils that captured microorganisms to the total number of counted neutrophils; and the phagocytic index – the number microorganisms captured by one neutrophil and phagocytic number – the percentage and index of neutrophils digestion for each strain.

The phospholipase activity of *C. albicans* was studied using the titrometric method. The indicators were evaluated in mmol/l × hour. To study protease activity, the biuret method was used; the indicators were evaluated in mg/min. × ml.

The ability of microorganisms to form biofilms was determined using of plastic plates for immunoenzyme analysis. The obtained biofilms were washed and stained. The results were taken into account by optical density on a biochemical analyzer.

Results and Discussion. The first step of the experiment was research of pathogenicity enzymes of clinical and reference strains of *C. albicans* (table 1).

As a result of the study, it was found that the phospholipase activity indicators for clinical strains

Table 1. Indicators of the enzymatic activity of clinical and reference strains of *C .albicans*

Enzymes of *C.albicans*	Indicators of the enzymatic activity of clinical strains of *C.albicans*	Indicators of the enzymatic activity of reference strains of *C. albicans*
phospholipase (mmol/l×hour.)	27.3±1.9	20.1±2.2*
protease (mg/min.×ml)	0.37±0.04	0.24±0.05*

Note: * – significant difference p <0.05; the results of a study of 3 repetitions are presented.

were (27.3±1.9) mmol/l × hour, for reference strains – (20.1±2.2) mmol/l × hour. When studying the protease activity in clinical isolates of *C. albicans*, significantly higher values (p≤0.0001) were obtained, which amounted to (0.37±0.04) mg/min. × ml, while in reference strains they amounted to (0.24±0.05) mg/min. × ml.

The ability to form biofilms by the association of bacteria *C. albicans + S. aureus* also were performed. The ability to form biofilms of clinical strains was compared with ability of reference strains (table 2).

Table 2. Determination of the level of film formation by *C. albicans + S. aureus* strains by optical density indices

No. group	The number of strains *C. albicans + S. aureus* (n)	Average optical the density of the test specimens (OD) λ=545 нм (M±m)	Number of colony forming units × 10^9 per ml of the culture medium of the test samples (M±m)	The average optical density of the control samples (nutrient medium) (OD) λ=545 нм (M±m)
1	Clinical strains *S. aureus*	1.0865±0.008	3.9±0.1	0.354±0.003**
2	Reference strains *S. aureus*	0.0550±0.007*	2.5±0.3	0.276±0.006
3	Clinical strains *C. albicans*	1.0690±0.007	3.7±0.1	0.256±0.005**
4	Reference strains *C. albicans*	0.0650±0.006*	2.2±0.2	0.348±0.004
5	Clinical strains *C. albicans + S. aureus*	1.0987±0.007	4.5±0.1	0.0277±0.00**
6	Reference strains *C. albicans + S. aureus*	0.0776±0.004*	3.5±0.2	0.0284±0.007

Note: * – significant difference p <0,05, * – difference between groups, ** – difference with control; results of studies of 3 repetitions are presented.

When determining the ability to form biofilms of *S. aureus + C. albicans* it was found that microorganisms are in associations have a higher ability to form biofilms than single strains. The average optical density of biofilms (OD) formed by the association of clinical isolates was higher (p<0.05) and amounted to (1.0987±0.007) units. The OD of clinical strains compared with reference strains, the value of which was – (0.0776±0.004) units of OD. So, the ability of clinical isolates in the association of *C. albicans* and *S. aureus* to form biofilms is significantly higher (p<0.05) than in reference strains.

The next stage of the study was the determination of the phagocytic activity of neutrophils in vitro (table 3).

Based on these studies, it was found that phagocytic activity of immunocompetent cells decreased in the clinical strains compared to the reference strains. Phagocytic activity indicators of *S. aureus* clinical strains showed: absorption index – (60.1±3.3); diges-

Table 3. Average values of neutrophil phagocytic activity against association of strains *S. aureus* and *C. albicans*

Strain groups researched *C. albicans +S. aureus*	Absorption index	Digestion index	Phagocytic index
Clinical strains *S. aureus*	60.1±3.33*	0.65±0.04*	3.2±0.05*
Reference strains *S. aureus*	78.3±5.21	0.85±0.05	4.66±0.37
Clinical strains *C. albicans*	57.8±2.34*	0.59±0.03	3.74±0,17*
Reference strains *C. albicans*	69.3±4.32	0.77±0.04	4.14±0.21
Clinical strains *C. albicans + S. aureus*	54.23±4.06*	0.69±0.05*	3.03±0.07*
Reference strains *C. albicans + S .aureus*	65.0±6,39	0.75±0,03	3.36±0.27

Note: the differences are significant between clinical and reference strains: * – (p<0.05); the results of studies of 3 experiments.

tion index – (0.65±0.04); phagocytic index – (3.2±0.05). For reference strains of *S. aureus* these figures were: absorption index – (78.3±5.21); digestion index – (0.85±0.05); phagocytic index – (4.66±0.37).

In the study of phagocytic activity against *C. albicans* strains an increasing tendency was observed for all parameters in the reference strains: the absorption index – (69.3±4.32); digestion index – (0.77± 0.04); phagocytic index – (4.14±0.21). Phagocytic activity indices were reduced in *C. albicans* clinical strains: absorption index – (57.8±2.34); digestion index – (0.59±0.03); phagocytic index – (3.74±0.17).

The research results may proof that *C. albicans* hyphae elements promote oxygen-dependent biocidal effect of phagocytes and are more active indicating the neutrophils' synthesis of molecules destabilizing homeostasis. This means that the mycelial form of clinical strains of *C. albicans* compared with reference strains has a greater virulence.

There was found an increase of aggressive properties on the phagocytes in the association of *C. albicans* + *S aureus*. Also there were found increased aggressive properties on phagocytic indices in *C. albicans* + *S. aureus* association: the absorption index amounted to (54.23±4.06) for clinical strains, and (65.0±6.39) for reference strains; the digestion index was – (0.69±0.05) for clinical strains, and (0.75± 0.03) for reference strains; the phagocytic index for clinical strains was (3.03±0.07), for reference strains – (3.36±0.27)

Thus, researchers have found a significant difference between the phagocytosis indices in clinical and reference strains ($p<0.05$). The association of clinical strains of *C. albicans* + *S. aureus* showed the most aggressive properties to phagocytes.

As a result of research, it was found that the phagocytic activity of neutrophils in clinical strains is 34 % lower compared to reference strains. This fact can be explained that patients have strains are more adapted to different parts of the immune system, antibiotics and disinfectants. Virulence of candida and staphylococcus associated with adhesion to sensitive cell receptors, colonization, synthesis of aggressive enzymes and protection, various toxins, the ability to counteract the protective factors of the macroorganism was detected during inhibition of phagocytosis. The aggression factors of microorganisms should also include the waste products of pathogens that contribute to their survival, reproduction, distribution in tissues, and the ability to influence the functions of a macroorganism, which leads to resistance by phagocytosis.

Unlike clinical strains, reference strains are less aggressive due to the loss of their virulence. In their existence, they do not meet the action of the human immune system, with antibiotics, disinfectants, therefore they lose their pathogenicity enzymes.

The lowest values of phagocytic activity parameters were observed in strains isolated in the association of *C. albicans* + *S. aureus*. The phagocytic index of these strains was (3.03±0.07), which can be explained by blocking the corresponding phagocytic receptors or by a decrease in their number under the influence of microorganism aggressive enzymes, which leads to inhibition of immune response mechanisms. There is evidence that microorganisms that are in association have not only antibiotic resistance, but also resistance to phagocytic activity. An increase in protein expression (CodY) and a decrease in the activity of L-lactate dehydrogenase (LDH1) indicates that *Staphylococcus aureus* can suppress its virulence so that the immune system cannot recognize it. When an association of bacteria *S. aureus* with *C. albicans* is created, the virulence factor (CodY) is inactivated, and the amount of L-lactate dehydrogenase (LDH1) increases, which enhances the aggressiveness of *Staphylococcus aureus*.

To deeply understand the mechanisms of the immune response in mixed infections, the pathogenicity enzymes of *C. albicans* strains and their ability to form biofilms were studied.

In consequence, it has been demonstrated that among the studied strains, enzymatic activity was more pronounced in clinical strains of *C. albicans* than in reference strains. It can be explained by the degree of aggressiveness of the microorganism. A significant increase in the activity of the phospholipase enzyme in clinical strains ($p\leq0.0001$) was also reported. This phenomenon contributes to the hydrolytic cleavage of fatty acids in phospholipids and the destruction of immunoglobulins, thereby increasing the resistance of *C. albicans* to the oxygen-dependent bactericidal mechanism of action of phagocytes.

During the experiment, it was found that association of microorganisms *C. albicans* + *S. aureus* enhance their pathogenic properties and increase the ability to produce biofilms. As follows, the strains together are more aggressive and able to suppress the immune response and reduce the phagocytic activity of neutrophils.

Despite the fact that knowledge of mixed infection has expanded significantly lately, there are problems that need to be solved in the future. The effect of multimicrobial infections on the host's immune response is still unclear and requires further study.

A perspective area of research is the study of the ability to form biofilms by the association of bacteria *S. aureus* and *C. albicans*, and the mechanism of the development of the immune response in catheter-associated infections.

Conclusions. Summarizing, we can conclude that the biofilms that are formed as a result of the

association of microorganisms *C. albicans* + *S. aureus* had higher optical densities than biofilms formed by individual microorganisms. Therefore, we can assume in connection with this. The pathogenic properties of these microorganisms are enhanced, as well as increased resistance to chemotherapeutic drugs. Our data are consistent with the results of scientific studies obtained by other microbiologists.

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ВПЛИВ ФАКТОРІВ ПАТОГЕННОСТІ CANDIDA ALBICANS І STAPHYLOCOCUS AUREUS НА ФАГОЦИТАРНУ АКТИВНІСТЬ НЕЙТРОФІЛІВ

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РЕЗЮМЕ. Асоціація мікроорганізмів *Candida albicans* та *Staphylococcus aureus* викликає різні клінічні форми гнійно-запальних захворювань. Вони часто виділяються при інфекціях, пов'язаних із утворенням біоплівок. Ці патогени є збудниками внутрішньолікарняних інфекцій, що викликають тяжкі захворювання та смертність навіть при відповідному лікуванні.

Мета роботи – вивчити здатність до утворення біоплівок у клінічних та референтних штамів *C. albicans* і *S. aureus*, визначити ферментативну активність фосфоліпази і протеази штамів *C. albicans*. Визначити фагоцитарну активність нейтрофілів щодо клінічних і референтних штамів *C. albicans* і *S. aureus* in vitro.

Матеріал і методи. Фагоцитарну активність нейтрофілів визначали в дослідах in vitro за стандартними методами. Референтні штами *C. albicans* і *S. aureus* використовували в якості контрольної групи. Здатність мікроорганізмів утворювати біоплівки визначали за допомогою пластикових планшет для імуноферментного аналізу.

Результати. При вивченні здатності мікроорганізмів до формування біоплівок показники для клінічних штамів склали – (1,0987±0,007) од. ОП, для референтних штамів – (0,0776±0,004) од. ОП. Установлено, що клінічні штами *C. albicans* мали підвищену активність ферментів агресії, таких як фосфоліпази та протеази. Також було виявлено зниження всіх показників фагоцитарної активності нейтрофілів щодо асоціації *C. albicans* і *S. aureus*. Фагоцитарний індекс для клінічних штамів склав (3,03±0,07), для референтних – (3,36±0,27).

Висновки. *C. albicans* і *S. aureus* в асоціаціях можуть посилювати свої вірулентні властивості, а наявність факторів патогенності, таких як ферменти агресії та утворення біоплівок, сприяє пригніченню фагоцитарних реакцій та імунної відповіді в цілому.

КЛЮЧОВІ СЛОВА: змішана інфекція; фосфоліпази; протеази; мікробні біоплівки; фагоцитоз.

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