ФАГОЦИТОЗ И ОКИСЛИТЕЛЬНАЯ АКТИВНОСТЬ НЕЙТРОФИЛОВ ПРИ ВЗАИМОДЕЙСТВИИ С БИОПЛЕНКАМИ УРОПАТОГЕННЫХ *ESCHERICHIA COLI*

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Резюме. Развитие рецидивирующих инфекций мочевыводящих путей (ИМП) связано, в первую очередь, со способностью *Escherichia coli* образовывать биопленки. Взаимодействие нейтрофилов, факторов врожденного иммунитета с микроорганизмами в биопленках затруднено по сравнению с планктонными формами из-за отсутствия прямого контакта, а также из-за антифагоцитарного действия внеклеточного матрикса биопленок. Цель данного исследования – оценка фагоцитарной и окислительной активности нейтрофилов при взаимодействии с биопленками уропатогенных *E. coli* (UPEC) DL82 и R44. Нейтрофилы периферической крови здоровых мужчин выделяли с помощью двухградиентного фиколла-урографина, инкубировали в течение 1 ч с клетками бактерий из биопленок или их супернатантами, после чего оценивали функциональную активность лейкоцитов. Фагоцитарную активность нейтрофилов определяли по степени гашения биолюминесценции светящегося штамма *E. coli* K12 TG1 lux$^+$ (pXen) при их поглощении нейтрофилами. Продукцию внеклеточных активных форм кислорода (АФК) анализировали по интенсивности люминолзависимой хемилюминесценции в спонтанном и стимулированном клетками *E. coli* K12 вариантах. Достоверность различий определяли с помощью критерия Стьюдента при $p < 0,05$. Установлено, что взаимодействие нейтрофилов с клетками или супернатантами биопленки UPEC не влияло на фагоцитарную активность. Супернатанты *E. coli* DL82 снижали спонтанную продукцию АФК нейтрофилами по сравнению с контролем и клетками биопленок. Супернатанты *E. coli* R44 с низким вирулентным потенциалом не влияли на продукцию АФК нейтрофилами, в то время как клетки биопленки ее стимулировали. При оценке стимулированной продукции АФК воздействие супернатантов штамма R44 не вызывало снижения способности нейтрофилов к активации в ответ на внешний раздражитель (клетки *E. coli* K12). Предварительный контакт нейтрофилов с бактериями *E. coli* R44 приводил к высокому и длительному уровню продукции АФК по сравнению с контролем. Взаимодействие нейтрофилов с клетками DL82 приводило к более высокому уровню АФК по сравнению с супернатантами, однако наблюдалось по-

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следующее быстрое истощение окислительного потенциала нейтрофилов. Таким образом, клетки и супернатанты биопленок UPEC могут определять активацию нейтрофилов.

Ключевые слова: нейтрофилы, активные формы кислорода, фагоцитоз, биопленки, Escherichia coli, UPEC

PHAGOCYTOSIS AND OXIDATIVE ACTIVITY OF NEUTROPHILS AFTER INTERACTION WITH UROPATHOGENIC *ESCHERICHIA COLI* BIOFILMS

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Abstract. Recurrent urinary tract infections (UTIs) are associated primarily with the ability of *Escherichia coli* to form biofilms. The interaction of neutrophils, factors of innate immunity, with microorganisms in biofilms is difficult compared to planktonic forms due to the lack of direct contact, as well as due to the antiphagocytic action of the extracellular matrix of biofilms. The purpose of this study was evaluation of neutrophils phagocytic and oxidative activity during interaction with biofilms of uropathogenic *E. coli* (UPEC) DL82 and R44. Peripheral blood neutrophils from healthy men were isolated using ficoll-urographin double gradient, incubated for 1 h with bacterial cells from biofilms or their supernatants, then leukocytes functional activity was evaluated. Phagocytic activity of neutrophils was determined by the degree of bioluminescence inhibition of bioluminescent strain *E. coli* K12 TG1 lux^+ (pXen) upon their absorption by neutrophils. Production of extracellular reactive oxygen species (ROS) was analyzed by the intensity of luminol-dependent chemiluminescence in spontaneous and stimulated by *E. coli* K12 variants. Significance of differences was determined using Student's t-test at $p < 0.05$. It was found that neutrophils interaction with UPEC biofilm cells or supernatants did not affect the phagocytic activity. *E. coli* DL82 supernatants reduce neutrophils spontaneous ROS production compared to control and biofilm cells. *E. coli* R44 supernatants with a low virulence potential did not affect ROS production, while biofilm cells stimulated it. When assessing stimulated ROS production, exposure to R44 strain supernatants did not cause a decrease in neutrophils activation in response to an external stimulus (*E. coli* K12 cells). Preliminary contact of neutrophils with *E. coli* R44 bacteria resulted in a high and prolonged level of ROS production compared to the control. Neutrophils interaction with DL82 cells resulted in a higher level of ROS compared to supernatants, however a subsequent rapid depletion of neutrophils oxidative potential was observed. Thus, cells and supernatants of UPEC biofilms can determine the activation of neutrophils.

Keywords: neutrophils, reactive oxygen species, phagocytosis, biofilms, Escherichia coli, UPEC

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Introduction

During urinary tract infections (UTI), *Escherichia coli* must overcome several lines of defense [3], among which neutrophils are the most effective [6]. Counteraction to antimicrobial mechanisms of host innate immunity may depend on the virulent potential of "biofilm" bacteria [8] and be achieved by enhanced synthesis of structural and functional proteins of *E. coli* cell wall, allowing the integrity of the bacterial cell to be maintained.

Neutrophils play a key role in the fight against pathogenic microorganisms in biofilm during the infection by performing phagocytosis, releasing toxic enzymes and reactive oxygen species (ROS) from

granules, and extracellular traps (NETs) [7]. However, the interaction of neutrophils with microorganisms in biofilms is difficult compared to planktonic forms due to the lack of direct contact between neutrophils and bacteria in biofilm [2] as well as the antiphagocytic effect of extracellular matrix of biofilms [9].

The current research aims are to estimate phagocytosis and oxidative activity of neutrophils after interaction with biofilms of uropathogenic *E. coli* (UPEC) DL82 and R44.

Materials and methods

Neutrophils from peripheral blood of healthy men (n = 3-6) were isolated using double gradient Fikoll–Urografin (1.077 and 1.112 g/mL) centrifugation at 400 × g. Cell purity and viability of neutrophils were 97% (Trypan Blue assay).

Escherichia coli DL82 was a reference strain from Ex culture collection of the University of Ljubljana, Slovenia. *E. coli* R44 was isolated from patients with UTI in Perm, Russian Federation. Bacteria (10^6/mL) were grown in 96-well plates in LB (Sigma-Aldrich, USA) for 24 h. Cell-free supernatants (CFS) were collected from the wells and sterilized by filtration (pore diameter 0.22 μm). Bacteria were released from biofilms by ultrasound (Elma Ultrasonic 30S; 5 times for 1 min) and resuspended in RPMI-1640. In the "neutrophil-bacteria" system, neutrophils (250 μL in RPMI; 10^6 cells/mL) were cultured with UPEC biofilm cells (100 μL of suspension) for 1 h. Then the supernatant was removed, and the pellet was resuspended in 400 μL of colorless Hanks' Balanced Salt Solution (HBSS). In the "neutrophil – CFS" system, neutrophils (250 μL in RPMI; 10^6 cells/mL) were cultured with UPEC CFS (250 μL) for 1 h, and then supernatants were removed and the pellet was resuspended in 400 μL of HBSS.

Analysis of the phagocytic activity of neutrophils was performed as described previously [4]. Lyophilized bioluminescent *E. coli* K12 TG1 lux$^+$ (pXen) [1] were rehydrated in cold 0.89% NaCl for 30 min at 4°C and then for 30 min at 20°C. A reaction mixture consisted of 20 μL of *E. coli* K12 TG1 lux$^+$ (10^8 CFU/mL), 20 μL of serum (50% solution in 0.89% NaCl), and neutrophils (160 μL, 10^6 cells/mL) in 96-well white plates. The control was 20 μL *E. coli* K12 TG1 lux$^+$ (10^8 cells/mL), 180 μL of HBSS or 20 μL *E. coli* K12 TG1 lux$^+$ (10^8 cells/mL), 20 μL 50% serum, 160 μL HBSS. Bioluminescence was measured at 37 °C for 40 min on a microplate reader (Synergy H1, BioTek, USA). Phagocytic activity was calculated as the degree of bioluminescence inhibition: (Ik – Io)/Ik × 100%, where Ik, Io are the bioluminescence of *E. coli* K12 TG1 lux$^+$ without/with neutrophils, respectively.

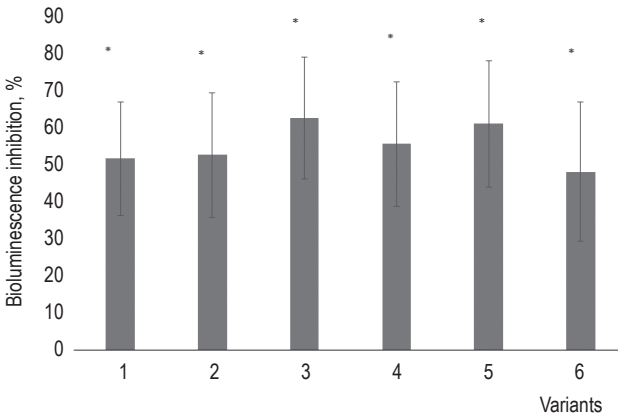

Figure 1. Phagocytosis of neutrophils exposed to CFS (2, 3) and biofilm bacteria (5, 6) of UPEC DL82 (2, 5) and R44 (3, 6)
Note. *, significant difference with the control without neutrophils. 1, LB; 4, 0.89% NaCl.

The production of extracellular reactive oxygen species (ROS) was analyzed as described previously [4]. To 20 μL of neutrophils (10^6 cells/mL) was added 220 μL of the reaction mixture to assess either spontaneous level of ROS production (180 μL of 1 mM luminol sodium salt (Sigma, USA), 40 μL of HBSS) or stimulated ROS production (180 μL of 1 mM luminol sodium salt, 20 μL *E. coli* K12 (10^8 CFU/mL), 20 μL serum pool (50% solution in 0.89% NaCl)) in a 96-well plate. Luminescence was assessed for 60 min at 37 °C using a microplate reader (Synergy H1, BioTek, USA).

The results were processed statistically using Microsoft Office XP Excel. The data are presented as M±SD. The significance of differences was determined with a Student's t-test at $p < 0.05$.

Results and discussion

The strains of *E. coli* DL82 and *E. coli* R44 were characterized by the same biofilm biomass (OD570: 0.90-1.14), but *E. coli* DL82 had a wide range of virulence factors (*fimH, papC, papGII, sfaDE, hlyA, usp, fyuA, iucD, iroCN, iroN*) as opposed to R44 [5].

Figure 1 suggests that inhibition of *E. coli* TG1 lux$^+$ luminescence was observed by 51-62% in all variants with neutrophils. Thus, pretreatment of neutrophils with CFS of *E. coli* DL82 and *E. coli* R44 or their interaction with UPEC biofilm cells did not affect neutrophil phagocytosis.

We observed that CFS of *E. coli* DL82 strain reduced spontaneous ROS production of neutrophils compared to LB and biofilm cells (Figure 2A, B). The CFS of *E. coli* R44 with a low virulence potential had no effect on ROS production of neutrophils, while the biofilm cells stimulated it (Figure 2A, B). This, apparently, may indicate about more effective

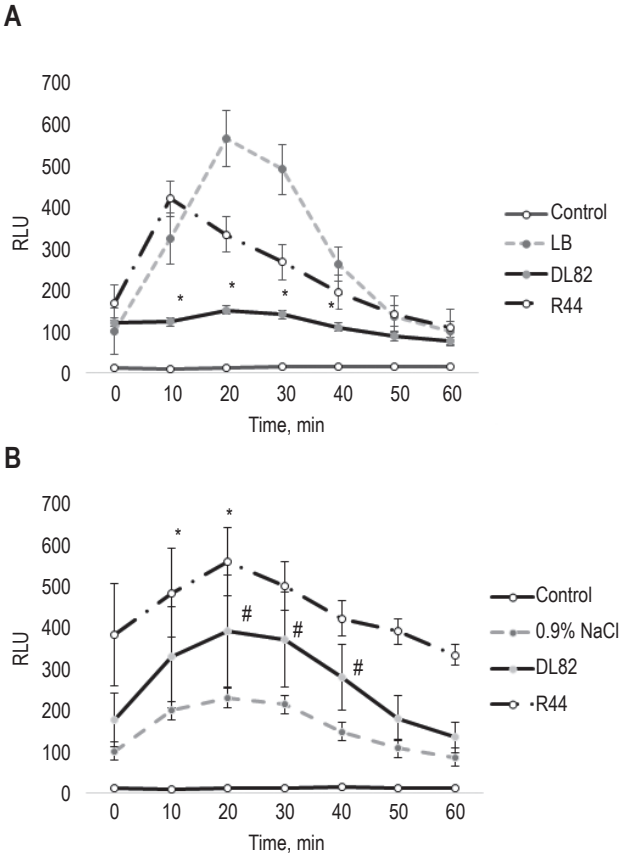

Figure 2. Extracellular spontaneous ROS production of neutrophils exposed to CFS (A) and biofilm cells (B) of *E. coli* DL82 and *E. coli* R44 strains

Note. *, significant differences compared to the control; #, significant differences between biofilm cells and CFS effect. RLU, relative light units.

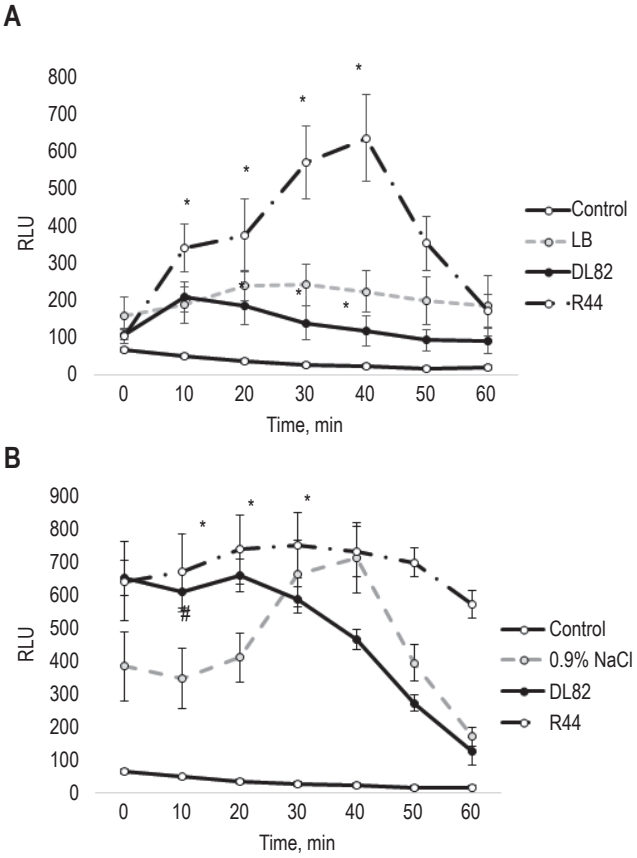

Figure 3. Extracellular stimulated ROS production of neutrophils exposed to CFS (A) and biofilm cells (B) of *E. coli* DL82 and *E. coli* R44 strains

Note. *, significant differences compared to the control ; #, significant differences between biofilm cells and CFS effect.

elimination of UPEC pathogens with a low virulence potential from the inflammation sites.

In the system of stimulated ROS release, exposure to CFS of the R44 strain did not cause a decrease in the ability of neutrophils to be activated in response to an external stimulus (*E. coli* K12 cells) (Figure 3A). The low activation of neutrophils by CFS of DL82, comparable to control, is probably associated with the damaging effect of "biofilm" supernatants on neutrophils [5].

Preliminary contact of neutrophils with bacteria of *E. coli* R44 resulted in a high and prolonged level of ROS production compared to the control (Figure 3B). The interaction of neutrophils with DL82 cells led to a higher level of ROS compared to CFS, but the subsequent rapid depletion of the oxidative potential of neutrophils was observed.

Conclusion

Thus, the virulence potential of UPEC strains can determine neutrophil activation upon direct contact with both bacteria cells and their "biofilm" supernatants. It is possible that the severity of UTI may be determined by the functional activity of neutrophils, namely, the features of their interaction with bacterial biofilm structures.

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