ORIGINAL PAPER

RJME
Romanian Journal of Morphology & Embryology
http://www.rjme.ro/

Influence of *Staphylococcus aureus* attachment to the herpes simplex virus infected cells

CORALIA BLEOTU[1,2], MARIANA CARMEN CHIFIRIUC[2], VERONICA LAZĂR[2], ROXANA DRĂGUŞEL[1], LILIA MATEI[1], IOANA MĂDĂLINA ALDEA[1], LAURA DENISA DRAGU[1], BOGDAN IONESCU[2], CARMEN CRISTINA DIACONU[1], COSTIN CERNESCU[1], CARMEN AURELIA MOGOANTĂ[3], CRISTIAN RADU POPESCU[4], BOGDAN POPESCU[4], RALUCA GRIGORE[4], ŞERBAN VIFOR GABRIEL BERTEŞTEANU[4]

[1] *Department of Cellular and Molecular Pathology, "Ştefan S. Nicolau" Institute of Virology, Romanian Academy, Bucharest, Romania*

[2] *Department of Botanics–Microbiology, Faculty of Biology, University of Bucharest, Bucharest, Romania; Research Institute of the University of Bucharest (ICUB), Bucharest, Romania*

[3] *Department of ENT, Emergency County Hospital, Craiova, Romania*

[4] *Clinic of ENT, Head and Neck Surgery, "Colţea" Clinical Hospital, Bucharest, Romania*

Abstract

The purpose of this study was to investigate the response of HeLa cells to the interaction with inactivated *Staphylococcus aureus* cells and live challenge with herpes simplex virus (HSV).The results of this study are indicating that the interaction between the HeLa cells and *S. aureus* inactivated whole cells could modulate the host cell apoptosis and cytokine production, and therefore, influence the progression of HSV infection. The pre-treatment of HeLa cells with heat inactivated bacterial whole cells protects them from the occurrence of HSV mediated cytopathic effect, while the post viral infection treatment with bacterial cells prevents the high activation of bax/bcl-2 apoptotic pathway, a process that could change the fate of the infectious process triggered by the virus, and eventually reduce its multiplication rate. The pre-treatment of HeLa monolayer with inactivated bacterial cells 24 hours before the viral infection is increasing the expression level of TNF-α, IL-6 and IL-8 pro-inflammatory cytokines genes, also suggesting that bacterial antigens could contribute to the decrease of viral multiplication rate.

Keywords: *Staphylococcus aureus*, herpes simplex, HeLa cells, inflammatory cytokines, apoptosis.

Introduction

Staphylococcus aureus is a significant human pathogen that causes several diseases in humans, including skin infections, scalded-skin syndrome, toxic shock syndrome, endocarditis, septic arthritis, and osteomyelitis [1]. Although staphylococci have been typically regarded as non-invasive extracellular pathogens that damage host cells after adhering to the extracellular matrix, many studies have shown the ability of *S. aureus* to trigger an endocytic process and to be ingested by non-professional phagocytes, mouse fibroblasts [2], mouse renal cells [3], bovine mammary epithelial cells [4, 5], mouse and human osteoblast cell lines [6, 7], human osteoblasts [8], chicken osteoblasts [9, 10] as well as HeLa cells [11]. Following the internalization, *S. aureus* could modulate the apoptosis of the infected cells. The implication of soluble mediators in the modulation of bacteria–host cell interaction has been intensively studied [5, 12, 13]. On the other hand, viruses could interfere with the cellular events triggered by a bacterial infection, by disrupting the sequence of programmed cellular events, and triggering programmed cell death in the infected cells either intrinsically or by mediators of the host immune response [14]. The herpes viruses are highly disseminated in nature and highly pathogenic for humans causing infections inducing dermatologic, immunologic, or neurologic disorders [15], especially in immunocompromised individuals. Herpes viruses multiply best in metabolically active cells [16]. However, viruses have evolved some mechanisms to block endogenously or exogenously induced apoptosis if this process threatens the capacity of the cell to produce the required number and quality of infectious virus progeny [14]. Therefore, apoptosis during HSV infection represents an important virus-host interaction process, which likely influences viral pathogenesis [17]. The acute and recurrent phases of HSV infection are associated with strong cytokine responses [18]. Although wound infections produced by *S. aureus* are often complicated by other bacteria or virus co-infection, the mechanisms by which a viral infection affecting the epithelial cells can influence *S. aureus* cutaneous opportunistic infections have been poorly studied.

The purpose of this study was to investigate the consequences of the direct contact interaction between the HeLa cells and *S. aureus* inactivated whole cells upon the host cell apoptosis and cytokine production, in the presence of HSV infection.

Materials and Methods

The bacterial strain used in our experiments was one

S. aureus isolate from a wound secretion. In order to obtain heat inactivated bacterial bodies, the bacterial strain was grown in 10 mL of DMEM:F12 (Dulbecco's modified Eagle's medium:nutrient mixture F12) supplemented with 2% fetal bovine serum (FBS) for 18 hours. The obtained culture was centrifuged and the pellet was washed once with phosphate-buffered saline (PBS), resuspended in 10 mL of PBS, heat-inactivated for 20 minutes, centrifuged at 6000×g and resuspended again in 10 mL of DMEM:F12.

Experimental models

In the first experimental model, HeLa cells (ATCC CCL2) monolayers grown in 35 mm Petri dishes were cultured for 24 hours in the presence of heat-inactivated bacterial cells (1:1 in DMEM:F12 supplemented with 2% FBS). After 24 hours, the HeLa cells monolayer was washed with PBS and inoculated with KOS HSV-1 at a multiplicity of infection (MOI) of 0.01 for 30 minutes at 37^0C. Cells were then washed and incubated in DMEM with 2% FBS at 37^0C with 5% CO_2. The experiment was evaluated 24 hours after the viral infection.

In the second experimental model, HeLa cells monolayers were first infected with KOS HSV-1 at a MOI of 0.01, incubated in DMEM with 2% FBS at 37^0C with 5% CO_2 for 24 hours, and then the heat-inactivated bacterial cells were added. The experiment was evaluated 24 hours after the bacterial treatment.

In order to establish a baseline for results interpretation, HeLa cells infected with HSV at a low MOI (0.01) without any bacterial treatments were used as controls.

Immunofluorescence HSV detection

Herpes simplex virus was detected using the Light Diagnostics™ HSV 1/2 Typing DFA Kit (Cat. No. 3291, Millipore, USA) according to the manufacturer's protocol. Cover slips were mounted onto glass slides in 1:1 glycerol: HBSS (Hanks' balanced salt solution) and examined with a Nikon Eclipse E600 Microscope with fluorescence module, using a FITC (Fluorescein Isothiocyanate) filter set. A bright apple-green fluorescence in the cytoplasm and/or cell membrane of the infected epithelial cells indicated the HSV presence, while the absence of fluorescent green and presence of a dull red color due to the Evans blue counterstaining indicated the lack of infection.

Quantitative RT-PCR for apoptosis markers and cytokines assay

Total RNA was extracted with Trizol Reagent (Invitrogen, USA) according to the manufacturer's protocol, and thereafter 2 μg of total RNA, from each sample, was used for reverse transcription with High Capacity cDNA Reverse Transcription Kit with RNAse inhibitor (Applied Biosystems). 50 ng of cDNA was subjected to real time PCR (polymerase chain reaction) for human IL-1, IL-6, IL-8, TNF-α, Bcl2, and Bax, using pre-validated Taqman Gene Expression Assays kits (Applied Biosystems). Human β actin was used as endogenous control. Each experiment was performed three times. The ΔΔCT method was used to compare the relative expression levels.

Results

The effect of heat inactivated bacterial bodies on HSV infected HeLa cells

When HeLa cells were pretreated with heat inactivated bacterial bodies before HSV infection, the cytopathic effect was not observed, although the HSV antigen could be detected by using an immunodetection assay. By contrast, when the bacterial bodies were applied 24 hours post-viral infection, an intense cytopathic effect was observed, similar as magnitude to that observed on HeLa cells infected with HSV for 48 hours. The cytopathic effect figured as small or large syncytia, with alterations in the cell nuclei and cytoplasmic tails resulted from fusion of the cellular membranes harboring viral glycoproteins. The cytopathic effect was confirmed using the HSV antigen immunodetection (Figure 1).

The modulation of the host cell apoptosis by the physical interaction between the HeLa cells, heat inactivated *S. aureus* and HSV

Bax gene expression was significantly increased in the presence of bacterial bodies, but the attachment of bacterial bodies to the cells fails to inhibit the expression of anti-apoptotic genes, accounting for cellular activation.

The qRT-PCR assay clearly showed that the viral infection is triggering a more intensive apoptotic effect (as revealed by the up-regulation of the pro-apoptotic bax gene expression simultaneously with the drastic inhibition of the anti-apoptotic bcl-2 gene), than the bacterial bodies.

The prior contact of HeLa cells with the heat inactivated bacterial bodies followed by virus addition induced only a decrease in bax expression compared to the stimulated control, meaning that these cells are partially protected. If the virus acts first, antiapoptotic gene expression decreases, with an increase of pro-/anti-apoptotic effects ratio, a phenomenon related to the presence of extensive cytopathic effects (Figure 2).

Analysis of some cytokines gene expression in HeLa infected cells

In our experiment, the HSV1 virus increases the expression of tumor necrosis factor alpha (TNF-α), independently to the presence of bacterial bodies. In the presence of concomitant viral infection and bacterial bodies, expression of IL-1 and IL-8 gradually increases from 24 hours to 48 hours accounting for a more pronounced pro-inflammatory effect. IL-6 expression is inhibited by virus, but increased by the bacterial bodies. Post-treatment of HeLa cells with bacterial bodies after the viral infection also increases the IL-6 mRNA level. The addition of bacterial bodies before the viral infection is generally inducing a strong increase in the expression of TNF-α, IL-6 and IL-8 (Figure 3).

Discussion

Epithelial cells are responsible for prevention of invasive infections through the mechanical barrier and also, the initiation of local immune responses. The defense reactions of mammalian cells are triggered or modulated following the interaction between the bacterial components

and the epithelial cells receptors. This may activate various intracellular signaling pathways implicated in the generation of antimicrobial compounds [19] or in the production of pro-inflammatory cytokines [20].

Our aim was to study the effects of the direct contact interaction between the HeLa cells and *S. aureus* inactivated whole cells upon the host cell apoptosis and cytokine production, when a simultaneous viral infection with HSV is occurring.

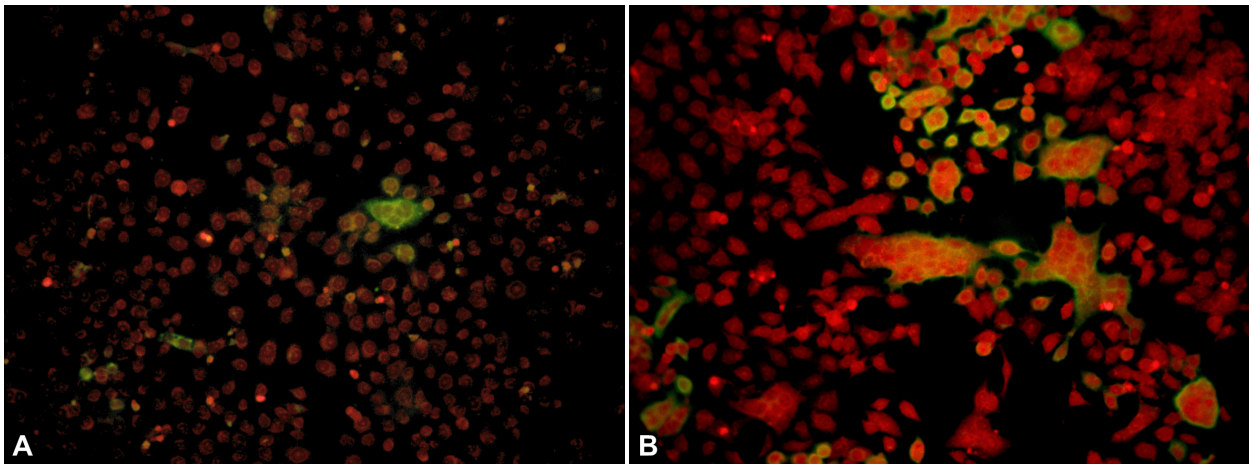

Figure 1 – ***HSV antigen immunodetection: (A) HeLa cells treated 24 hours with heated bacterial bodies followed by HSV infection for another 24 hours; (B) HeLa cells infected with HSV for 24 hours, followed by treatment with heated bacterial bodies for another 24 hours.***

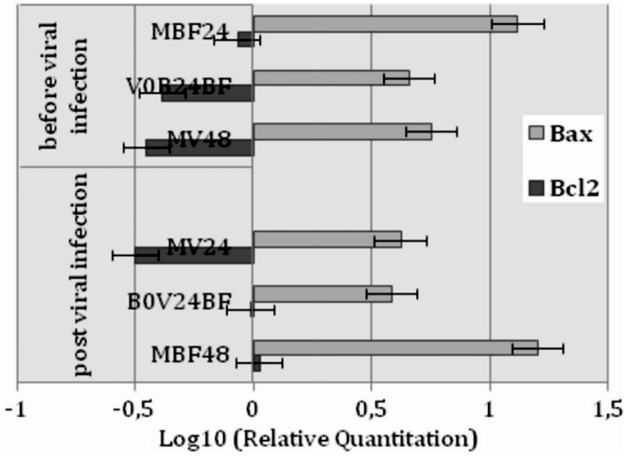

Figure 2 – ***Graphic representation of quantitative expression of bax and bcl-2 genes in HeLa cells. MV24/48: Cell control for viral infection at 24/48 hours; MBF24/48: Control of heated bacterial cells fractions at 24/48 hours; V0B24BF: Viral infection before adding bacterial fraction; B0V24BF: Bacterial infection before HSV infection.***

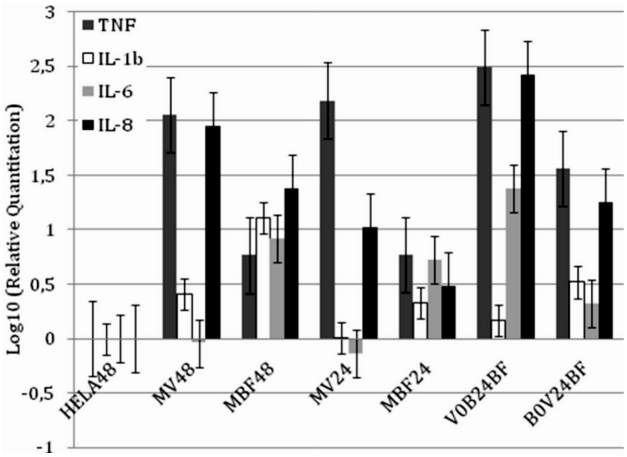

Figure 3 – ***Graphic representation of quantitative expression of IL-1b, IL-6, IL-8 and TNF genes in HeLa cells. HELA cell control; MV24/48: Cell control for viral infection at 24/48 hours; MBF24/48: Control of heated bacterial cells fractions at 24/48 hours; V0B24BF: Viral infection before adding bacterial fraction; B0V24BF: Bacterial infection before HSV infection.***

Both viral and bacterial pathogens have a machinery to modulate the host cells signaling pathways upon infection. One of the major components of microbial pathogenesis is to modulate the apoptosis of the infected host cells at critical time points. The level of apoptosis in HeLa cells infected with HSV is very low, probably due to the manipulation of the host cell for viral multiplication, needing metabolically active cells. However, when bacterial bodies are added at 24 hours after infection, the anti-apoptotic genes expression is decreased, demonstrating that the administration of bacterial cell wall associated antigens following a previous viral infection, could change the fate of the infectious process triggered by the virus, and eventually reduce its multiplication rate.

One of the major pathways, by which the pathogens activate the host cell, is the modulation of the cytokine profile. Different inflammatory mediators are expressed at different times after pathogen exposure. IL-1 and TNF-α, the most important pro-inflammatory cytokines are locally produced by many cell types and are responsible for early responses triggering an inflammatory cascade [21]. The pro-inflammatory cytokine, IL-6, is one of the key mediators of the "acute-phase response" in inflammation [22]. IL-8 is acting both as a chemo-attractant and activator of polymorphonuclear leukocytes and is produced by intestinal and vaginal epithelial cells consecutively to bacterial aggression, demonstrating that epithelial cells act as a link between the bacterial antigens and the specific and nonspecific host immune cells, found in the mucosa-associated lymphoid tissue [23]. The synthesis of cytokines at the site of infection is chronologically coordinated and is decisive for an effective inflammatory

response in order to return to the normal state [24, 25]. The chronic inflammation and/or infection are produced by disturbances in the well-balanced order and extent of the inflammatory response [25].

The efficient clearance of the viral infection is dependent on the occurrence of a pro-inflammatory reaction and of a type 1 immune response, characterized by the activation of immune cells (*i.e.*, macrophages, NK and cytotoxic T-cells). However, the epithelium represents the first line of host defense. After the viral infection, the epithelial cells produce pro-inflammatory cytokines, which attract and activate phagocytes at site of infection. HSV could suppress the pro-inflammatory cytokines expression by decreasing the mRNA stability, and therefore interfering with the antiviral host response [26]. The treatment of HeLa cells with bacterial bodies 24 hours before the viral infection is increasing the expression level of TNF-α, IL-6 and IL-8 pro-inflammatory cytokines genes. The bacterial antigens are also increasing the level of IL-1 in the treated cells, accounting for the occurrence of a more pronounced pro-inflammatory effect, possibly favoring *in vitro* a more rapid and efficient elimination of the viral infection.

Previous studies have shown that the level of IL-6 in eukaryotic cells infected by *S. aureus* is influenced by any change occurred in the adherence phase of this microbial cell to the eukaryotic cell (*i.e.*, the lack of different microbial adhesions) [27, 28]. On the other hand, Strindhall *et al.* (2004) showed that clinical isolates of *S. aureus* express a different capacity to stimulate the cytokine production by the human endothelial cells, which was not directly correlated with the adhesion capacity, indicating the implication of other bacterial components in the eukaryotic cells activation [28].

Taken together, the results of this study could suggest that both viruses and bacterial antigens may interfere with the key processes in host cell by modulating the levels of pro- and anti-inflammatory cytokines and promoting apoptosis.

Conclusions

The results of this study are demonstrating that the direct contact interaction between the HeLa cells and *S. aureus* inactivated whole cells could modulate the host cell apoptosis and cytokine production, and therefore, influence the progress of HSV infection. The pre-treatment with heat inactivated bacterial bodies protects HeLa cells from the occurrence of HSV mediated cytopathic effect, while the post-viral infection treatment with bacterial antigens prevents the high activation of bax/bcl-2 apoptotic pathway, a process that could change the fate of the infectious process triggered by the virus, and eventually reduce its multiplication rate. In our study, the infection of HeLa cells with HSV in the presence of inactivated bacterial bodies is triggering the synthesis of the pro-inflammatory cytokines TNF-α, IL-6 and IL-8, whose levels are generally increasing in time, suggesting that bacterial antigens could contribute to the decrease of viral multiplication rate.

Conflict of interests

The authors declare that they have no conflict of interests.

Acknowledgments

Ioana Mădălina Aldea was financed from the European Social Fund and the Romanian Government under the contract number POSDRU/159/1.5/S/137390.

References

[1] Alexander EH, Hudson MC. Factors influencing the internalization of *Staphylococcus aureus* and impacts on the course of infections in humans. Appl Microbiol Biotechnol, 2001, 56(3–4):361–366.
[2] Usui A, Murai M, Seki K, Sakurada J, Masuda S. Conspicuous ingestion of *Staphylococcus aureus* organisms by murine fibroblasts *in vitro*. Microbiol Immunol, 1992, 36(5):545–550.
[3] Murai M, Usui A, Seki K, Sakurada J, Masuda S. Intracellular localization of *Staphylococcus aureus* within primary cultured mouse kidney cells. Microbiol Immunol, 1992, 36(5):431–443.
[4] Almeida RA, Matthews KR, Cifrian E, Guidry AJ, Oliver SP. *Staphylococcus aureus* invasion of bovine mammary epithelial cells. J Dairy Sci, 1996, 79(6):1021–1026.
[5] Bayles KW, Wesson CA, Liou LE, Fox LK, Bohach GA, Trumble WR. Intracellular *Staphylococcus aureus* escapes the endosome and induces apoptosis in epithelial cells. Infect Immun, 1998, 66(1):336–342.
[6] Ellington JK, Reilly SS, Ramp WK, Smeltzer MS, Kellam JF, Hudson MC. Mechanisms of *Staphylococcus aureus* invasion of cultured osteoblasts. Microb Pathog, 1999, 26(6):317–323.
[7] Jevon M, Guo C, Ma B, Mordan N, Nair SP, Harris M, Henderson B, Bentley G, Meghji S. Mechanisms of internalization of *Staphylococcus aureus* by cultured human osteoblasts. Infect Immun, 1999, 67(5):2677–2681.
[8] Bost KL, Bento JL, Ellington JK, Marriott I, Hudson MC. Induction of colony-stimulating factor expression following *Staphylococcus* or *Salmonella* interaction with mouse or human osteoblasts. Infect Immun, 2000, 68(9):5075–5083.
[9] Hudson MC, Ramp WK, Nicholoson NC, Williams AS, Nousiainen MT. Internalization of *Staphylococcus aureus* by cultured osteoblasts. Microb Pathog, 1995, 19(6):409–419.
[10] Reilly SS, Hudson MC, Kellam JF, Ramp WK. *In vivo* internalization of *Staphylococcus aureus* by embryonic chick osteoblasts. Bone, 2000, 26(1):63–70.
[11] Chifiriuc MC, Lixandru M, Iordache C, Bleotu C, Larion C, Dracea O, Lazar V, Antohe F, Israil AM. Internalization of *Staphylococcus aureus* and *Pseudomonas aeruginosa* bacterial cells by non-phagocytic, epithelial human cells. Rom Biotech Letters, 2008, 13(2):3651–3658.
[12] Gresham HD, Lowrance JH, Caver TE, Wilson BS, Cheung AL, Lindberg FP. Survival of *Staphylococcus aureus* inside neutrophils contributes to infection. J Immunol, 2000, 164(7): 3713–3722.
[13] Proctor RA, Balwit JM, Vesga O. Variant subpopulations of *Staphylococcus aureus* as cause of persistent and recurrent infections. Infect Agents Dis, 1994, 3(6):302–312.
[14] Galvan V, Roizman B. Herpes simplex virus 1 induces and blocks apoptosis at multiple steps during infection and protects cells from exogenous inducers in a cell-type-dependent manner. Proc Natl Acad Sci U S A, 1998, 95(7):3931–3936.
[15] Whitley RJ. Herpes simplex virus. In: Knipe DM, Howley PM (eds). Fields virology. 4th edition. Lippincott Williams & Wilkins, Philadelphia, 2001, 2461–2509.
[16] Schang LM, Rosenberg A, Schaffer PA. Roscovitine, a specific inhibitor of cellular cyclin-dependent kinases, inhibits herpes simplex virus DNA synthesis in the presence of viral early proteins. J Virol, 2000, 74(5):2107–2120.
[17] Nguyen ML, Blaho JA. Apoptosis during herpes simplex virus infection. Adv Virus Res, 2007, 69:67–97.
[18] Hukkanen V, Broberg E, Salmi A, Erälinna JP. Cytokines in experimental herpes simplex virus infection. Int Rev Immunol, 2002, 21(4–5):355–371.
[19] Bals R, Hiemstra PS. Innate immunity in the lung: how epithelial cells fight against respiratory pathogens. Eur Respir J, 2004, 23(2):327–333.
[20] Kato A, Schleimer RP. Beyond inflammation: airway epithelial cells are at the interface of innate and adaptive immunity. Curr Opin Immunol, 2007, 19(6):711–720.
[21] Dinarello CA. Proinflammatory cytokines. Chest, 2000, 118(2): 503–508.

[22] Heinrich PC, Behrmann I, Haan S, Hermanns HM, Müller-Newen G, Schaper F. Principles of interleukin (IL)-6-type cytokine signalling and its regulation. Biochem J, 2003, 374(Pt 1):1–20.
[23] Eckmann L, Kagnoff MF, Fierer J. Epithelial cells secrete the chemokine interleukin-8 in response to bacterial entry. Infect Immun, 1993, 61(11):4569–4574.
[24] Coussens LM, Werb Z. Inflammation and cancer. Nature, 2002, 420(6917):860–867.
[25] Lawrence T, Gilroy DW. Chronic inflammation: a failure of resolution? Int J Exp Pathol, 2007, 88(2):85–94.
[26] Ning R, Zhang X, Guo X, Li Q. Attachment of *Staphylococcus aureus* is required for activation of nuclear factor kappa B in human osteoblasts. Acta Biochim Biophys Sin (Shanghai), 2010, 42(12):883–892.
[27] Söderquist B, Alriksson I, Källman J, Kihlström E. The influence of adhesive and invasive properties of *Staphylococcus aureus* defective in fibronectin-binding proteins on secretion of interleukin-6 by human endothelial cells. APMIS, 2006, 114(2): 112–116.
[28] Strindhall J, Lindgrenz PE, Löfgren S, Kihlström E. Clinical isolates of *Staphylococcus aureus* vary in ability to stimulate cytokine expression in human endothelial cells. Scand J Immunol, 2004, 61(1):57–62.

Corresponding author
Raluca Grigore, Lecturer, MD, PhD, Clinic of ENT, Head and Neck Surgery, "Colţea" Clinical Hospital, 1–3 I. C. Brătianu Avenue, 030171 Bucharest, Romania; Phone +40722–384722, e-mail:raluca.grigore@3f.ro

Received: November 9, 2014

Accepted: May 21, 2015