Annuaire de l'Université de Sofia "St. Kliment Ohridski"
Faculte de Biologie
2017, volume 102, livre 4, pp. 170-175
Youth Scientific Conference "Kliment's Days", Sofia 2016

INTRACELLULAR SURVIVAL OF *PSEUDOMONAS AERUGINOSA* PAO1 IN A549 CELLS

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Keywords: *Pseudomonas aeruginosa*, human lung carcinoma A549 cells, GFP

Abstract: In their interaction with host epithelia, different strains of *Pseudomonas aeruginosa* manifest different phenotypes: they may remain adherent to the surface of the eukaryotic cells (adherent phenotype), or penetrate and survive intracellularly (invasive phenotype). The aim of the study is to characterize the interaction of strain *P. aeruginosa* PAO1 with human lung carcinoma A549 cells. Initially, we performed a co-cultivation of the cells with $1x10^4$ bacteria for 2 hours followed by incubations for 1 or 24 hours of the infected cells in the absence of bacteria. We determined the number of viable intracellular bacteria by plating on agar. The result indicated that the bacterium is capable for intracellular replication. Using a three-parental labelling system (including *E. coli* MT 102 pBAH 8 as donor, and *E. coli* HB 101 pR K600 as helper) the strain was labelled with green fluorescent protein. Fluorescent microscopy of the GFP-carrying PAO1-GFP strain after co-cultivation with A549 cells confirmed the intracellular survival of the bacteria, as well as progressive disruption of the actin cytoskeleton.

INTRODUCTION

It is already well-recognized that, in order to avoid innate defense mechanisms of the host, many bacterial species and strains have developed the ability to invade and persist in non-phagocytic eukaryotic cells (Sana et al., 2015). Cell culture-based infection models are essential in examining in detail the interaction between

the pathogens and host epithelial and endothelial surfaces (Bucior et al., 2014). The outcome of the relationship may depend on the growth and development of both the bacterial culture and the cultured eukaryotes (Plotkowski et al., 1999; Ha and Jin, 2005).

In their interaction with host epithelia, different strains of *Pseudomonas aeruginosa* manifest different phenotypes: they may remain adherent to the surface of the eukaryotic cells (adherent phenotype), or penetrate and survive intracellularly (invasive phenotype). Most extensive data on invasion of *P. aeruginosa* strains were obtained on the infection model of corneal cells (Cowell et al., 1995; Fleiszig et al., 1995). The host-bacterial interaction was shown to affect the actin cytoskeleton of the host cells (Cowell et al., 1995).

The strain PAO1 used in the present study is invasive in several tested cultured cell lines including A549 cells (Angus et al., 2008; Ahmed et al., 2014). The experimental protocols applied include co-cultivation of the two types of cells for different time intervals, up to 8 hours (Fleiszig et al., 1996; Robertson et al., 2007; Angus et al., 2008). The present study focuses on time-related phenomena, and the following questions are addressed: (i) what is the situation at time intervals longer than the above-cited, and (ii) do bacteria replicate intracellularly in A549 cells.

MATERIALS AND METHODS

Intracellular survival/replication assay

Intracellular survival/replication assay was performed as it was described previously by Angus et. al., 2008 with some modifications. An overnight culture of *P. aeruginosa* PAO1 was prepared in TSB. The bacteria were centrifuged at 10 000 g for 5 min., washed and diluted to $1x10^9$ bacterial cells/ml using McFarland standard. For co-cultivation experiments, they were further diluted to $1x10^4$ bacterial cells/ml in antibiotic-free DMEM medium supplemented with 10% FBS. Confluent monolayers of A549 cells were cultivated in 25 ml flasks in DMEM supplemented with 10% FBS and antibiotics/antimycotics. A549 cells were washed in 3 changes of PBS to remove antibiotics, and then the DMEM-suspended bacteria were added. Co-cultivation was performed for 2 h at 37°C, 5% CO_2. Then the bacterial suspension was withdrawn, the flasks were washed, antibiotic-supplemented DMEM (+10% FBS) was added and the cells were again incubated at 37°C, 5% CO_2. After 1 or 24 h post co-cultivation, flasks were washed, and treated for 15 min with 0.2% Tween 20. The released bacteria were diluted serially and plated on (TSA – Tryptic Soya Agar) for enumeration of CFU.

Labeling of strain PAO1 with green fluorescent protein (GFP) by tri-parental mating

GFP labeling of PAO1 was performed by the tri-parental protocol described by Huber et al. (2002). *E. coli* HB101 pRK600 was used as a helper strain, and *E. coli* MT102 pBAH8 – as a donor of the GFP tag. The selection of the GFP-labeled PAO1 was done on cetrimide agar supplemented with 10 μg/ml of gentamycin. The resulting strain *P. aeruginosa* PAO1-GFP was kept frozen in 20% glycerol at -80°C until use.

Fluorescence microscopy

Fluorescence assay for actin cytoskeleton was performed as it was described previously (Angus et. al., 2008) with some modifications. Confluent A549 cells were cultivated on cover glasses placed in 24-well plate and co-cultivated for different intervals with PAO1-GFP. Preparation of the bacterial suspension, the co-cultivation and post co-cultivation procedures were performed under conditions similar to the described above. At intervals, the bacteria were withdrawn, the wells were fixed for 30 min with 10% buffered neutral formalin, and washed with PBS. Cells were permeabilised for 15 min with 0.2% Tween 20. Actin filaments were colored with TRITC-phalloidin (0.3 µg/ml, incubation 30 min), followed by 5 min incubation in 10 µg/ml DAPI for staining of nuclei. The samples were rinsed in distilled water and mounted with glycerin-gelatin. The observations were made on Nikon TiU microscope, and images were registered and merged using NIS Elements software.

RESULTS AND DISCUSSION

The intracellular survival/replication assay showed that the 2 h co-cultivation followed by 1 h post co-cultivation incubation resulted in the detection of only insignificant number of intracellular viable bacteria. This is in contrast with the significant amounts of viable bacteria on hour 24 incubation after co-cultivation (Fig. 1). This result implies significant replication of the bacteria in the intracellular niche. The result is in agreement with data of Bucior et al. (2014) who observed c.a. fourfold increase of intracellular *P. aeruginosa* PAO1 on the 8-h time point. However, here we show that the increase is significantly more advanced, within the range of $1x10^3$-10^4 cfu/ml on 24 h with more bacteria than on 1 h were the amount of bacteria were under the $1x10^1$ cfu/ml. This significant increase indicates intracellular replication of the bacteria.

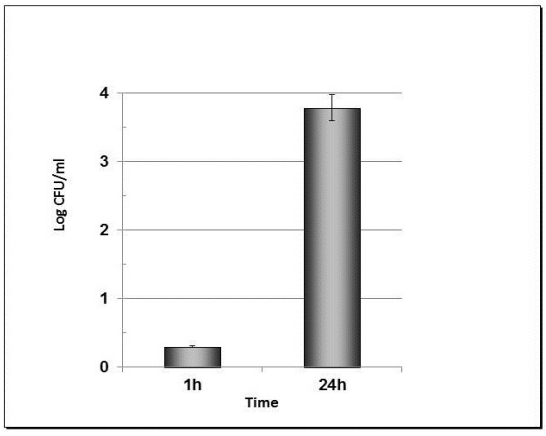

Fig. 1. Estimation of the cfu/ml of *P. aeruginosa* PAO1 released from the infected A549 cells within a 24 h interval.

It is known that *P. aeruginosa* interacts with host cells via its Type III secretion system that may explore one or more exotoxins. Among them, PAO1 possesses Exo-S, Exo-T and Exo-Y that interfere with the actin cytoskeleton (Cowell et al., 1995). In order to discriminate between the effects of the bacteria during co-cultivation, here we examined two comparatively remote time points, 1 and 24 h post co-cultivation. Notably, we used $1x10^4$ cfu/ml bacteria which is several orders of magnitude lower than the applied in previous studies. At this amount of the bacteria, initially (1 h post co-cultivation) the cytoskeleton of A549 cells remained intact, with only fine actin patches surrounding the engulfed bacteria (Fig. 2 A-C).

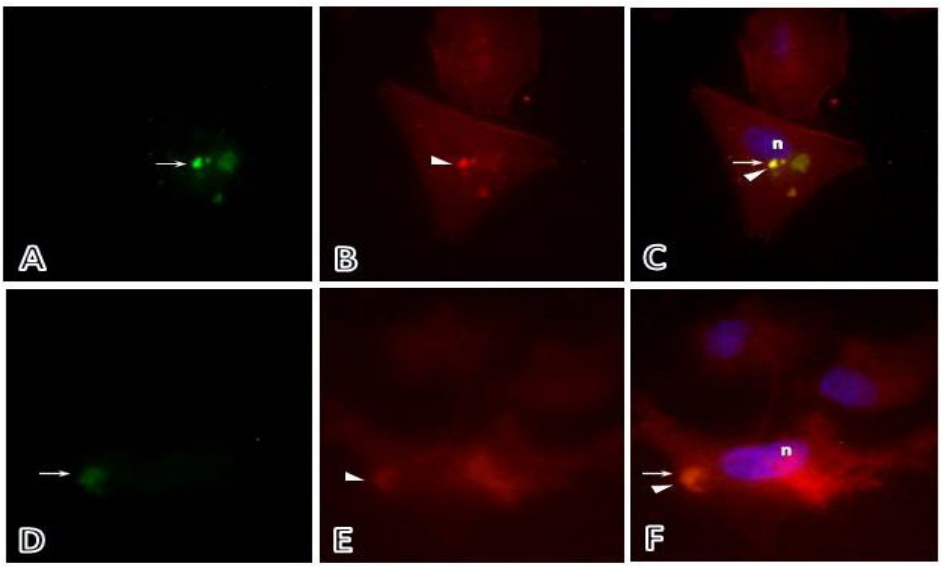

Fig. 2. Fluorescence microscopy of the interaction of PAO1-GFP with A549 cells. (A-C) 1 h post co-cultivation; (D-F) 24 h post co-cultivation. A, D (green signal only) – arrows point to groups of GFP-labeled bacteria; B, C (red signal only) – arrowheads point to patches of actin; C, F – merged images show co-localization of bacteria and condensed actin patches. N, nuclei (colored with DAPI).

The situation on 24 h differed significantly. In parallel with the increase of intracellular bacteria (see Fig. 1), disintegration of the actin cytoskeleton was registered (Fig. 2 D-F). This could indicate increased amounts of the exotoxins, as a contribution of the significantly increased bacterial burden as a result of the intracellular replication.

CONCLUSIONS

The results characterize *P. aeruginosa* PAO1 as an invasive strain in A549 cells. The present study performed in dynamics until hour 24 provides information indicating that this strain not only survives in the host cells, but is also replicated intracellularly. The progress of the infection is accompanied by progressive disruption of the host-cell actin cytoskeleton.

Acknowledgements: This study was supported by "Program for career development of young scientists". BAS, Contract № DFNP – 55/27.04.2016

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