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Identification of a *Staphylococcus aureus* strain with increased intracellular growth and reduced intracellular susceptibility to gentamicin.

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Abstract

Objectives: The persistence and recurrence of some *S. aureus* infections like endocarditis has been ascribed to its intracellular character. Our aim was to examine the intracellular development of *S. aureus* strains differing by their hemolysin (hl) production as well as their intracellular response to antibiotics, using both phagocytic (THP-1) and endothelial cells (HUVEC).

Methods: Intracellular growth was measured by the delta log CFU/mg prot. at 24h post infection. Controls were maintained with gentamicin (1 x MIC) to prevent the development of extracellular bacteria. For confocal microscopy, bacteria were stained with fluorescein isothiocyanate, and lysosomes with LysoTracker® Red.

Results :

S. aureus	Characteristics	Intracellular growth in THP-1	Gentamicin MIC[a] (µg/ml)	Gentamicin concentration (in x MIC) allowing to reach a static effect: Intracell.	Extracell.
8325-4	Laboratory strain cured of all prophages.	0.2 ± 0.1	0.2	2.3 x MIC	1.5 x MIC
DU5942	Mutant derived from 8325-4. Disrupted for gamma-hemolysin (hlg)	1.9 ± 0.1 *	0.3	5.0 x MIC	1.3 x MIC
DU5942-M1	Mutant derived from 8325-4. Disrupted for gamma-hemolysin and complemented with pCU1-*hlg+*	1.8 ± 0.1 *	0.3	5.0 x MIC	1.3 x MIC
DU5938	Mutant derived from 8325-4. Disrupted for alpha- (hla), beta- (hlb), and gamma- (hlg) hemolysins	0.3 ± 0.1	0.2	/	/

n=3; * p < 0.001 (ANOVA)
[a] : microdilution in Mueller-Hinton broth pH 7.4

S. aureus DU5942 showed a more important intracellular growth than the other strains (also observed in HUVEC). This is not due to hlg disruption, since it was also observed for the complemented strain DU5942-M1 but not for the multidisruptant strain DU5938. Dose-effects studies with gentamicin showed a similar response for all tested strains extracellularly. In contrast, DU5942 and its M1 mutant were less sensitive to gentamicin than 8325-4 intracellularly, exposure to higher extracellular concentrations being required to prevent intracellular growth. No difference was seen between strains with other antibiotics (oxacillin, vancomycin or telavancin).

Confocal microscopy showed that, after 24h of infection in THP-1 macrophages, all strains are confined in lysosomes. In endothelial cells, most bacteria colocalised with lysosomes but a small number appeared free in the cytoplasm.

Conclusion: We identified a strain with high capacity of intracellular growth in both professional phagocytes and endothelial cells. This effect seems to be related to a lower intracellular susceptibility of the strain to gentamicin but not to its hl status.

Background and aims

S. aureus is an important human pathogen responsible for severe infections such as osteomyelitis, endocarditis or skin and soft tissues infections. These infections are often persistent and recurrent, which has been ascribed to the ability of *S. aureus* to penetrate, survive and proliferate in various cell types including professional phagocytes [2,3,4,7]. *S. aureus* also produces a wide range of virulence factors among which, haemolysins alpha, beta and gamma are known to display a lytic action on cellular membranes [5].

Our aims were i) to examine the intracellular development of several *S. aureus* strains differing by their hemolysin production to assess the effect of these virulence factors using both phagocytic (THP-1) and endothelial cells (HUVEC) and ii) to study the intracellular response of these strains to antibiotics.

Results

Intracellular infection by *S. aureus* (24h)

After 24h of infection, *S. aureus* DU5942 (hlg-) shows a much more pronounced intracellular growth than its parental strain 8325-4. This important growth is not due to hlg disruption since the complemented mutant DU5942-M1 presents the same important intracellular growth but not the multidisruptant strain DU5938. In addition, all strains show similar growth in phagocytic cells (THP-1) **[Fig. 1]** and non phagocytic cells (HUVEC) **[Fig. 2]**.
In both models, cells are incubated with gentamicin (1 x MIC) to prevent development of extracellular bacteria. We therefore examined dose-effect relationships for gentamicin against extracellular and intracellular bacteria.
When exposed to increasing doses of gentamicin in Muller-Hinton broth, *S. aureus* 8325-4, DU5942 and DU5942-M1 present identical responses **[Fig. 3]**. In contrast DU5942 and its complemented mutant appear less sensitive to gentamicin during intracellular infections **[Fig. 4 and 5]**. The difference of intracellular growth observed with 1 x MIC is represented by orange arrow. Exposure to higher extracellular concentrations of gentamicin is required to reach a static effect (black arrow).

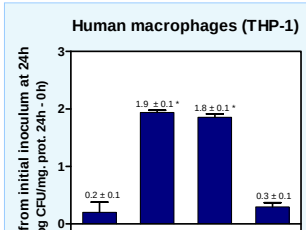

Fig. 1: n=3; * p < 0.001 (ANOVA)

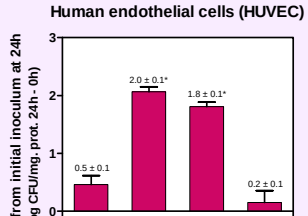

Fig. 2: n=3; * p < 0.001 (ANOVA)

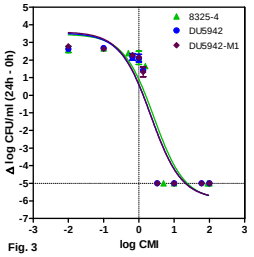

Fig. 3

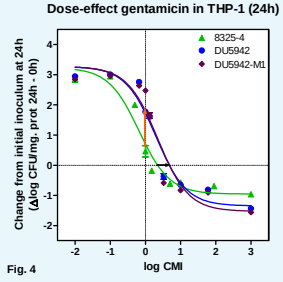

Fig. 4

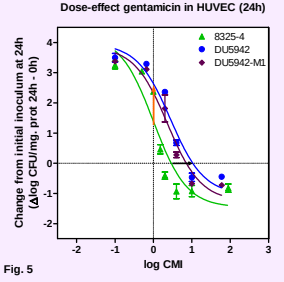

Fig. 5

Confocal microscopy showed that, in endothelial cells, most bacteria colocalised with acidic compartment but a small number appeared free in the cytoplasm **[Fig.8]**. No differences were observed between all the strains tested. In macrophages, *S. aureus* appears exclusively confined in these acidic vacuoles **[Fig.7]**. Electron microscopy demonstrated that *S. aureus* were able to multiply in these vacuoles **[Fig.6]**.

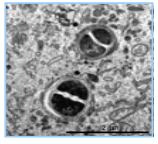

Fig. 6

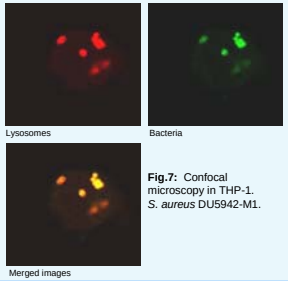

Fig.7: Confocal microscopy in THP-1. *S. aureus* DU5942-M1.

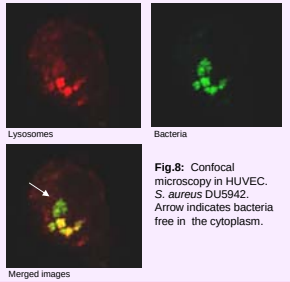

Fig.8: Confocal microscopy in HUVEC. *S. aureus* DU5942. Arrow indicates bacteria free in the cytoplasm.

Methods

Bacteria: The study was performed with the reference strain 8325-4 [6] and mutant strains with specific gene disruption(s): *S. aureus* DU5942 disrupted for gamma-hemolysin [5], DU5942-M1 disrupted for gamma-hemolysin and complemented with pCU1-*hlg+* [this study] and DU5938 disrupted for alpha-, beta-, and gamma-hemolysins [5].

Intracellular infections: THP-1 or HUVEC cells were incubated with opsonised *S. aureus* for 1h to allow phagocytosis. The extracellular bacteria were eliminated with 45 min incubation with gentamicin (100 x MIC). To assess intracellular growth (controls), cells were incubated for 24h in presence of gentamicin (1 x MIC) to prevent development of extracellular bacteria [1]. For dose-effect studies, cells were incubated with increasing concentrations of antibiotic (0.01 to 1000x MIC). The intracellular growth was measured by the number of CFU/mg of cell proteins at 24h post infection.

Microscopy: For confocal microscopy, the bacteria were stained with FITC (fluorescein isothiocyanate) and the lysosomes with LysoTracker® Red DND 99 (Invitrogen). For electron microscopy, Infected macrophages were washed with PBS, fixed with glutaraldehyde and osmium tetraoxyde, and stained "en bloc" with uranyl acetate.

Discussion

These intracellular infection models demonstrate that *S. aureus* is able to survive and multiply in phagocytic cells as well as in endothelial cells. In macrophages, *S. aureus* seems exclusively confined in phagolysosomes whereas in endothelial cells, *S. aureus* appears in phagolysosomes and in the cytoplasm.

Hemolysins disruption doesn't seem to affect the development of intracellular infection, since no difference has been observed between the parental strain 8325-4 and the multidisruptant strain DU5938. Furthermore, tow additional strains disrupted for alpha-hemolysin (DU1090) or beta-hemolysin (DU5719) alone presented the same intracellular growth than the parental strain 8325-4. (data not shown)

We identified a strain with high capacity of intracellular growth in both professional phagocytes and endothelial cells. This effect seems to be related to a lower intracellular susceptibility of the strain to gentamicin but not to its hemolysins status. In addition, this decreased susceptibility to gentamicin seems to be expressed only in intracellular environment. No difference was seen between strains with other antibiotics (oxacillin, vancomycin or telavancin).

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