ABSTRACT INTRODUCTION:

*Staphylococcus aureus* is the major causative pathogen in osteomyelitis, responsible for approximately 50% of all cases. In some cases, *S. aureus* can internalize into non-phagocytic mammalian cells to allow persistent infection. Others have measured internalized bacteria after removal of non-internalized bacteria by a 2 h treatment in gentamicin. Based on our knowledge of adherent bacteria and our experimental observations, this gentamicin treatment is inadequate as it fails to eradicate external bacteria. We have therefore developed a quantitative immunofluorescent assay to differentiate between internalized bacteria and adherent, external bacteria. We anticipate that this assay will supply an important tool for investigating how *S. aureus* and other pathogens are able to cause persistent orthopaedic infection.

METHODS:

Mouse embryonic fibroblasts (line FN +/- H4) were cultured in DMEM + 10% PBS (complete media). All experiments were performed at cell densities of 5,000 cells/cm², and after cells had reached 90% confluency. For imaging analysis, *S. aureus* strain AH1710, a strain expressing GFP on a chloramphenicol resistance plasmid was used. For the lyse and plate analysis reported for literature internalization studies, *S. aureus* ATCC 25923 were also used; these same bacteria were used for the quantitative immunofluorescent (immunoquant) method. All bacteria were grown overnight in trypticase soy broth (TSB), 37°C, with agitation; AH1710 media included 10μg/ml chloramphenicol. This overnight culture was subcultured for 2-4 h, rinsed in phosphate buffered saline (PBS), and diluted to a concentration of 10⁷ colony forming units (CFU)/ml in complete media. Prior to co-incubation, fibroblasts were rinsed with PBS, fresh media was added, and 10⁶ CFU of bacteria added to the fibroblast culture. Bacteria and cells were co-incubated for 2 hours at 37°C, 5% CO₂. For studies with gentamicin, at 2 h, cells were rinsed with PBS and incubated with complete media or complete media plus 100 μg/ml of gentamicin. In all experiments, bacterial inoculant was verified by serial dilution and plating on aerobic-count Petrifilms (3M, 6406). For experiments using the immunofluorescence method, a standard curve of bacterial concentrations was prepared on a separate 96-well plate. The plate was incubated concurrently with the cellular experiments, and then treated as described below for quantImmunofluo. *Lyse and plate method:* fibroblasts were lysed in PBS + 0.3% Tween-20 with sonication, 10 min. Recovered bacteria in the lysates were serially diluted in PBS, and plated on Petrifilms, and incubated overnight at 37°C. QuantImmunofluo and microscopic visualization: cells were rinsed 4 times in PBS and then fixed in 4% paraformaldehyde for 10 minutes. To determine total number of bacteria (external + internal), fibroblasts were permeabilized, 30 minutes in 0.3% Triton-X 100, and rinsed with PBS 3X. To determine number of external bacteria, cultures were not permeabilized. After this step, bacterial and cellular alkaline phosphatase activity was inactivated by incubation at 72°C, 2 h. Plates were blocked with 0.3% nonfat dry milk in PBS for 30 minutes, followed by incubation with rabbit anti-*Staphylococcus aureus* polyclonal IgG (1:100, Abcam, ab20920), 2 h, at room temperature. Plates were rinsed in PBS 4X, incubated in blocking buffer, 30 min, room temperature, and incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG (H+L) (1:200, Invitrogen, G-21079) After 4 PBS rinses, alkaline phosphatase activity was analyzed using a PNPP substrate kit (Thermo Scientific Pierce, 37620). A₉ₐ was measured and values normalized to controls that had no primary antibody. Numbers of bacteria were calculated by comparison to the standard curve. To determine number of internalized bacteria, external bacterial count (not permeabilized) was subtracted from the total bacterial count (permeabilized). Statistics were performed using the Student’s t-test, with significance at p < 0.01. Experiment were performed each performed three times. Image analysis was performed on cultures stained as above, except primary antibody: 1:500, and secondary antibody: Alexa Fluor 568 goat anti-rabbit IgG (H+L) (1:500 Invitrogen, A-11011). Slides were mounted using Vectorshield + DAPI (Vector Laboratories, H-1200). Digital images were recorded with a 20x lens. Composites were prepared using ImagePro 6.2.

RESULTS SECTION:

We first showed that external, adherent bacteria survived despite a 2 h gentamicin treatment (Figure 1). When numbers of internalized vs. external GFP-expressing bacteria were compared on the basis of staining, our images suggested that external bacteria outnumbered bacteria harbored within the cells (Figure 2). By direct comparison, standard lyse and plate method vs. the ImmunoQuant method showed an underestimate of internalized bacteria with the former method (p < 0.001 for ATCC 25923, p < 0.01 for AH 1710 strains) (Figure 3).

DISCUSSION:

Osteomyelitis and chronic infections are more recalcitrant to treatment when bacteria become adherent and internalize. Adherent bacteria are known to be relatively antibiotic resistant and their resistance to treatment with gentamicin is in keeping with this. We thus questioned if the standard lyse and plate methods are accurate. The ImmunoQuant method that we describe differentiates between total and externalized bacteria, allowing accurate measurement of internalization. This becomes of special importance when exploring mechanisms and developing methods to block establishment of orthopaedic infection.

SIGNIFICANCE:

To develop therapies to block osteoblastic internalization of bacteria and establishment of chronic infection, accurate assessments of these processes are required. We have described a new method that allows differentiation between adherent, external bacteria and cellular internalized bacteria that will allow a more rapid development of means to combat this important clinical problem.

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