First publ. in: Journal of Microbiological Methods 65 (2006), 2, pp. 301–310

Quantification of bacterial invasion into adherent cells by flow cytometry

Stefan Pils, Tim Schmitter, Florian Neske, Christof R. Hauck *

Zentrum für Infektionsforschung, Universität Würzburg, Röntgenring 11, 97070 Würzburg, Germany

Received 23 June 2005; received in revised form 10 August 2005; accepted 15 August 2005
Available online 26 September 2005

Abstract

Quantification of invasive, intracellular bacteria is critical in many areas of cellular microbiology and immunology. We describe a novel and fast approach to determine invasion of bacterial pathogens in adherent cell types such as epithelial cells or fibroblasts based on flow cytometry. Using the CEACAM-mediated uptake of Opa-expressing *Neisseria gonorrhoeae* as a well-characterized model of bacterial invasion, we demonstrate that the flow cytometry-based method yields results comparable to a standard antibiotic protection assay. Furthermore, the quantification of intracellular bacteria by the novel approach is not biased by intracellular killing of the microbes and correctly discriminates between cell-associated extracellular and *bona fide* intracellular bacteria. As flow cytometry-based quantification is also applicable to other pathogen-host interactions such as the integrin-mediated internalization of *Staphylococcus aureus*, this approach provides a fast and convenient alternative for the quantification of bacterial uptake and should be particularly useful in elucidating the molecular mechanisms of pathogen-triggered host cell invasion.

Keywords: Bacterial invasion; Intracellular bacteria; Fluorescence labelling; Flow cytometry; *Neisseria gonorrhoeae*; *Staphylococcus aureus*

1. Introduction

Bacterial invasion, the pathogen-induced entry into eukaryotic cells, is a widespread trait associated with disease-causing microbes (Falkow, 1991; Finlay and Cossart, 1997). Invasive bacteria, including *Legionella pneumophila, Listeria monocytogenes, Mycobacterium tuberculosis, Neisseria gonorrhoeae, Salmonella typhimurium, Shigella flexneri* and *Staphylococcus aureus*, exploit host cell components such as the actin cytoskeleton and its regulatory machinery to get access to the inside of a eukaryotic cell. In some instances, bacteria inject proteins, e.g., via type III secretion systems, to directly modulate intracellular regulators of actin dynamics such as the Rho family GTPases (Galan and Zhou, 2000; Tran Van Nhieu et al., 2000). In other cases, the bacteria use an indirect route by engaging cell surface receptors coupled to the cytoskeleton including integrins, cadherins or immunoglobulin-related cell adhesion molecules (Hauck, 2002). For example, *S. flexneri* as well as *S. aureus* are able to connect to cell surface integrins to trigger actin-dependent internalization (Watarai et al., 1996; Sinha et al., 1999; Agerer et al., 2005). *Listeria monocytogenes* targets host E-cadherin by means of the InlA protein to enter epithelial cells (Mengaud et al., 1996; Lecuit et al., 1999). Variants of *N. gonorrhoeae* expressing distinct colony

* Corresponding author. Tel.: +49 931 312137; fax: +49 931 312578.
E-mail address: christof.hauck@mail.uni-wuerzburg.de (C.R. Hauck).

opacity-associated (Opa) proteins bind to receptors of the carcinoembryonic antigen-related cell adhesion molecule (CEACAM) family to direct their uptake by human cells (for review see (Hauck and Meyer, 2003)). Clearly, each pathogen seems to have evolved a specific set of virulence determinants which together orchestrate the sequence of events guiding the uptake by the host cell. Quantitative analysis of bacterial invasion in vitro is key to unravel the molecular basis of the different internalization processes.

In the majority of the cases, bacterial invasion is quantified by antibiotic protection assays that have a convenient read-out with dilution plating and counting of colony forming units released from infected cells after selective antibiotic killing of extracellular bacteria. However, as this sort of assay is based on the recovery of viable bacteria after their internalization by eukaryotic cells, it can severely underestimate the number of intracellular bacteria if intracellular killing by the eukaryotic cell occurs (Booth et al., 2003). A similar underestimation occurs if prolonged treatment with high concentrations of gentamicin leads to significant endocytic uptake of the antibiotic eventually contributing to the killing of intracellular bacteria enclosed in endosomes. Therefore, antibiotic protection assays have to be complemented with microscopic evaluation of infected samples that detect both viable and killed intracellular bacteria. Unfortunately, such approaches involve time consuming staining procedures and often labour-intensive quantification (Heesemann and Laufs, 1985; Drevets and Elliott, 1995).

To allow a convenient and rapid quantification of bacteria residing within attached epithelial cells we have adapted a method originally developed to measure uptake by non-adherent professional phagocytes (Voyich and DeLeo, 2002). This approach is based on direct fluorescein labelling of the bacteria prior to infection followed by flow cytometric detection of cell-associated fluorescence as an estimate of the number of internalized bacteria. In order to adapt this approach to adherent epithelial cells, the infected eukaryotic cells are released from the substrate and taken in suspension at a defined time after addition of the bacteria. Prior to analysis, the fluorescence emitted by extracellular, cell-attached bacteria is quenched by trypan blue. Therefore, the fluorescence signals detected by flow cytometry are derived from intracellular bacteria only. Moreover, flow cytometric analysis not only determines the percentage of infected cells, but also provides the relative amount of internalized bacteria by the strength of the fluorescence signal. Combination of these two read-outs provides a reliable way to estimate the total amount of internalized bacteria, the uptake index, in each sample. Most importantly, we demonstrate that this protocol correctly quantifies intracellular bacteria when compared to the conventional antibiotic protection assay or compared to microscopic techniques. Moreover, flow cytometric evaluation of intracellular bacteria is independent of the viability status of the microorganisms resulting in an unbiased estimate of bacterial uptake. This convenient and fast method allows the quantitative evaluation of intracellular bacteria in adherent cell types and should be a valuable technique in the areas of cellular microbiology and infection biology.

2. Materials and methods

2.1. Cell culture and transfection

The human embryonic kidney cell line 293T (293 cells) was grown in DMEM/10% calf serum (CS) at 37 °C, 5% CO_2. Cells were subcultured every 2–3 days. Transfection with HA-tagged CEACAM3 (CEACAM3-HA), RFP-tagged CEACAM3, or the empty control vector (pCDNA) was accomplished by standard calcium–phosphate-co-precipitation using 4 μg plasmid/10 cm culture dish as previously described (Schmitter et al., 2004). Cells were used 2 days after transfection. CEACAM3-HA expression in the transfected cells was evaluated by flow cytometry after staining the cells with monoclonal anti-CD66 antibody (clone CLB-gran/10; Sanquin, Amsterdam, The Netherlands) followed by Cy2-labelled goat–anti-mouse antibodies (Jackson ImmunoResearch, West Grove, PA). In some cases, cells were treated 15 min prior to infection with 1 μg/ml cytochalasin D (Calbiochem, Schwalbach, Germany) and the inhibitor remained with the cells throughout the infection.

Integrin β1-deficient mouse fibroblasts (GD25 cells) and GD25 cells stably re-expressing human integrin β1A (GD25 hIntβ1) were generously provided by Reinhard Fässler (MPI for Biochemistry, München, Germany) and grown in DMEM supplemented with 10% FCS, non-essential amino acids, and sodium pyruvate on gelatine-coated culture dishes.

2.2. Bacteria

Opa_{CEA}-expressing (Opa_{52}), non-piliated *N. gonorrhoeae* MS11-B2.1 (strain N309) and Opa-negative, non-piliated *N. gonorrhoeae* MS11-B1 (strain N302) were obtained from T.F. Meyer (MPI Infektionsbiologie, Berlin, Germany) and grown at 37 °C, 5% CO_2 on

GC-agar (Gibco BRL, Praisley, UK) supplemented with vitamins and the appropriate antibiotics.

Staphylococcus aureus (strain Cowan) and *S. carnosus* were cultured in Tryptic Soybean Broth (TSB; BD Biosciences, Heidelberg, Germany) at 37 °C and harvested in mid-logarithmic phase.

2.3. Labelling of bacteria

Bacteria (1×10^9/ml) were washed twice with sterile PBS, suspended in 1 μg/ml 5-(6)-carboxyfluorescein-succinimidylester (CFSE; Molecular Probes, Eugene, OR) in PBS, and incubated for 20–30 min under constant shaking at 37 °C. CFSE-labelled bacteria were washed three times with PBS prior to use. For discrimination between extra-and intracellular bacteria by fluorescence microscopy (FBA staining), bacteria were additionally labelled with 0.3 mg/ml sulfo-NHS-LC-Biotin (Perbio Science, Bonn, Germany) in PBS as described before (Agerer et al., 2004).

2.4. Antibiotic protection assay with epithelial cells

Antibiotic protection assays were performed as described (Schmitter et al., 2004). Briefly, transfected 293 cells in DMEM/10% CS were seeded at 5×10^5 cells/well in 24-well plates (coated over-night at 4 °C with bovine fibronectin (5 μg/ml in PBS; Sigma, St Louis, MO) one day before infection. Cells were infected at a multiplicity of infection (MOI) of 20 bacteria/cell. After 1 h of infection, the medium was replaced with DMEM containing 50 μg/ml gentamicin (DMEM-genta). After 30 min, or after the indicated time points in the case of prolonged infections, DMEM-genta was withdrawn and cells were lysed by the addition of 1 ml of 1% saponin in PBS for 15 min. Suitable dilutions were plated on GC agar to determine the number of recovered viable bacteria.

2.5. Flow cytometry of infected cells

For flow cytometric analysis, 1×10^6 293 cells in 6 cm culture dishes were infected at a MOI of 20 CFSE-labelled bacteria/cell. After 1 h (*N. gonorrhoeae*) or 2 h (*S. aureus*) of infection, the infected cells were suspended by 1 min trypsin treatment. For determination of intracellular bacteria after prolonged times of infection, medium was changed to DMEM-genta and the cells were further incubated for the indicated time before trypsin treatment. Suspended cells were washed two times with ice-cold flow buffer (PBS containing 1% CS) and resuspended in 1 ml flow buffer. Samples were kept on ice and dark until analysis. To eliminate signals from extracellular bacteria, trypan blue solution (0.4%; Sigma, Taufkirchen, Germany) was added to a final concentration of 0.2% directly before analysis. Samples were analysed on a FACSCalibur (Becton Dickinson) by gating on the eukaryotic cells based on forward and side scatter and cell-associated fluorescence of 10,000 cells per sample was measured in fluorescence channel 1 (FL1-H) detecting CFSE fluorescence. To obtain an estimate of the amount of internalized bacteria, the percentage of CFSE-positive cells was multiplied by the mean fluorescence intensity of these cells to obtain the uptake index (u.i.).

2.6. Microscopic evaluation of extra- and intracellular bacteria

One day before infection, transfected 293 cells in DMEM/10% CS were seeded on coated glass coverslips at 7.5×10^4 cells/well in 24-well plates. Coverslips had been coated over-night at 4 °C with a combination of bovine fibronectin (4 μg/ml) and poly-L-lysine (10 μg/ml) in PBS. Cells were infected with CFSE-and biotin-labelled *N. gonorrhoeae* for 60 min, fixed with 4% paraformaldehyde in PBS and processed for FBA staining essentially as described previously (Agerer et al., 2004). Briefly, fixed samples were incubated without permeabilization of the infected cells in staining buffer containing streptavidin-AlexaFluor647 (Molecular Probes, Eugene, OR). This results in dual labelling of extracellular bacteria by CFSE and AlexaFluor647, whereas intracellular bacteria are characterized by their CFSE only staining. Samples were viewed with a LSM 510 confocal laser scanning microscope (Zeiss, Oberkochen, Germany) using a 40×, 1.3 NA Plan Neofluar oil-immersion objective. Fluorescence signals of labelled specimens were serially recorded with appropriate excitation and emission filters for CFSE, RFP, and AlexaFluor647, respectively, to avoid bleed-through. Images were digitally processed with Photoshop 6.0 (Adobe Systems, Mountain View, CA).

3. Results

3.1. Flow cytometry to detect Neisseria gonorrhoeae within epithelial cells

To analyse the potential of this flow cytometry approach to quantify intracellular bacteria, we took advantage of the well characterized uptake of Opa_{CEA}-expressing gonococci by specific members of the human CEACAM family (see (Hauck and Meyer,

2003)). Previous investigations have established an essential role of the Opa_{CEA}-CEACAM interaction for uptake into epithelial cells in vitro ((Virji et al., 1996; Bos et al., 1997; Chen et al., 1997; Gray-Owen et al., 1997; Schmitter et al., 2004). In this experimental model, invasion only takes place if the bacteria possess Opa_{CEA} proteins and the infected cells express particular CEACAM family members (i.e., CEACAM1, CEACAM3, CEA or CEACAM6). Accordingly, in a conventional antibiotic protection assay, viable bacteria were recovered from CEACAM3-transfected human 293 cells infected with Opa_{CEA}-expressing *N. gonorrhoeae* (Fig. 1A). However, when the same CEACAM3-expressing cells were infected with an isogenic, non-opaque variant, no bacterial internalization was detected and the number of recovered viable bacteria was less than 5% of the sample infected with Opa_{CEA}-expressing gonococci (Fig. 1A). As expected, neither non-opaque nor Opa_{CEA}-expressing *N. gonorrhoeae* were able to invade into CEACAM-negative 293 cells transfected with the empty control vector (Fig. 1A). The presence of CEACAM3 on the surface of transfected cells and the absence of detectable CEACAM expression by the control cells was confirmed by flow cytometry with CEACAM-specific monoclonal antibodies (Fig. 1B).

Using the same set of pCDNA or CEACAM3 transfected samples, we infected the cells with CFSE-labelled *N. gonorrhoeae* strains. 1 h after infection, cells were quickly trypsinized and taken up in ice-cold flow buffer (PBS with 1% CS). After three washes with flow buffer, infected cells were analysed by flow cytometry after quenching of extracellular bacteria-derived fluorescence by trypan blue. Around 20% of the cell population containing CEACAM3-transfected 293 cells was observed to have a strong fluorescence signal indicating intracellular bacteria (Fig. 1C). As the transfection efficiency was around 40% (Fig. 1B), these results suggest that about 50% of the CEACAM3-expressing cells contained intracellular bacteria after 1h. In contrast, CEACAM3-expressing cells infected with CFSE-labelled non-opaque bacteria or control transfected cells (pCDNA) infected with CFSE-labelled Opa_{CEA}-expressing gonococci were found to emit only a very weak fluorescence signal, suggesting that the method identified intracellular bacteria (Fig. 1C). Furthermore, when we calculated the relative amount of Opa_{CEA} bacteria taken up by CEACAM3-expressing cells versus control transfected cells, only a small percentage of intracellular bacteria in control transfected cells (ranging from 1% – 5% in four independent experiments) was detected by flow cytometry (Fig. 1D). These results are in agreement with the values obtained by the antibiotic protection assay (Fig. 1A and 1D), where values for intracellular bacteria in control transfected cells ranged from 1% – 3% in parallel experiments. Together, these data demonstrate that the novel approach gives an accurate estimate of bacterial uptake similar to the widely used gentamicin protection assay.

3.2. Pharmacological blockage of internalization can be evaluated by flow cytometry

As the novel assay relies on the use of trypan blue to quench fluorescence emitted from extracellular, cell-associated bacteria, it is instrumental to demonstrate that bacteria closely associated with the eukaryotic cell surface can be correctly discriminated from intracellular bacteria. Therefore, we infected CEACAM3-expressing cells with Opa_{CEA}-positive *Neisseria gonorrhoeae* and either allowed uptake or specifically interfered with actin polymerization and bacterial internalization by cytochalasin D. In this situation, the pathogens still attach to cell surface accessible CEACAM3, but uptake into the cell is blocked. In the absence of cytochalasin D, intracellular bacteria were again observed, when the cells were infected with Opa_{CEA}-positive, but not in the case of non-opaque gonococci (Fig. 2A). Moreover, upon cytochalasin D treatment detection of intracellular Opa_{CEA}-positive bacteria was reduced by more than 90% (Fig. 2A) demonstrating that the flow cytometric analysis correctly identifies intracellular bacteria and is not prone to detect extracellular, cell-bound bacteria as false positives. These results also imply that inhibitors of the uptake process could be identified by such a flow cytometry approach.

To corroborate the results, 293 cells were transfected with CEACAM3-RFP and infected for 1 h with CFSE- and biotin-labelled bacteria in the presence or absence of cytochalasin D. Fixed samples were processed for microscopic evaluation of extra-and intracellular bacteria according to the FBA staining (Agerer et al., 2004). Inspection of the infected samples confirmed the results obtained by flow cytometry. In particular, Opa_{CEA}-expressing gonococci were taken up by CEACAM3-expressing cells as indicated by those bacteria not being labelled with AlexaFluor647 (arrowhead; Fig. 2B). Importantly, cytochalasin D treatment still allowed attachment of the Opa_{CEA}-expressing bacteria to CEACAM3-transfected cells (small arrows; Fig. 2B). However, in agreement with the flow cytometric analysis, no intracellular bacteria were detectable upon microscopic evaluation (Fig. 2B). These results verify the inhibitory effect of the cytochalasin D

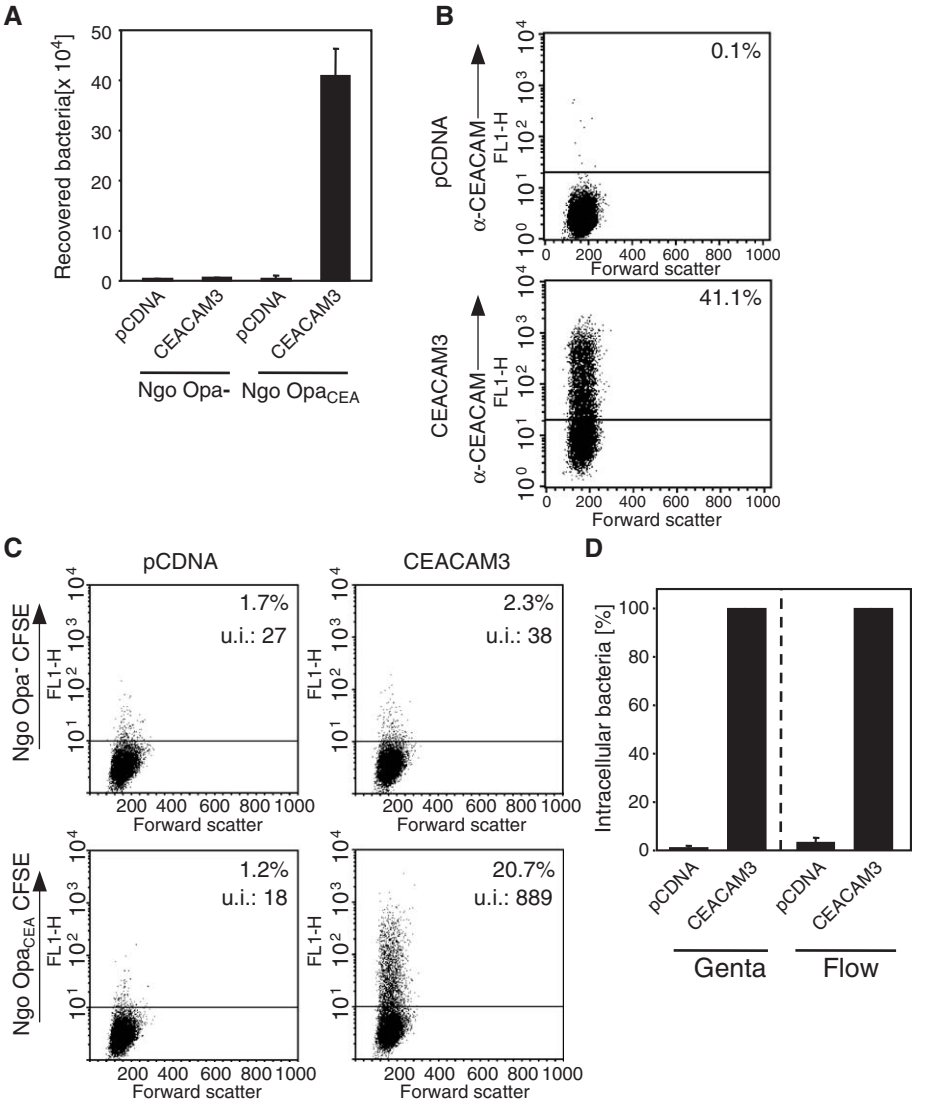

Fig. 1. Comparison between conventional antibiotic protection assay and flow cytometric determination of intracellular bacteria. (A) 293 cells transfected with control vector (pCDNA) or expressing CEACAM3-HA were infected with non-opaque *N. gonorrhoeae* (Ngo Opa-) or Opa_{CEA}-expressing gonococci (Ngo Opa_{CEA}) for 1 h. After 30 min gentamicin treatment to eliminate extracellular bacteria, intracellular bacteria were released by addition of saponin and numbers of viable bacteria were determined upon plating on GC agar. The graph shows means ± SD of a representative experiment done in triplicate. (B) Transfected cells as in (A) were stained with monoclonal anti-CD66 antibody and Cy-2-coupled goat–anti-mouse antibodies to determine expression of CEACAM3. (C) The same cell population as in (A) was infected for 1 h with CFSE-labelled gonococci and taken in suspension. After addition of trypan blue (final concentration of 0.2%), samples were analysed by flow cytometry. Shown are the original dot plots of a representative experiment depicting the percentage of cells containing intracellular bacteria as well as the respective uptake index (u.i.). Similar results were obtained in three independent repetitions. (D) Comparison of the relative uptake of Opa_{CEA}-expressing *N. gonorrhoeae* by the indicated cells as measured by antibiotic protection assay (Genta) or flow cytometry-based uptake index (Flow). Uptake of Opa_{CEA}-expressing *N. gonorrhoeae* by CEACAM3-expressing cells was set to 100%. Bars represent the mean values ± SD of the intracellular bacteria as observed in four independent experiments. In each experiment, the antibiotic protection assay and the flow cytometry-based approach were conducted in parallel with cells taken from the same transfected cell populations.

treatment and further support the conclusion that the addition of trypan blue effectively quenches the fluorescence of extracellular, cell-associated bacteria allowing selective detection of intracellular bacteria by flow cytometry.

3.3. Bacterial killing following CEACAM3-mediated uptake of Opa_{CEA}-expressing N. gonorrhoeae

Antibiotic protection assays rely on the recovery of viable intracellular bacteria and, therefore, can underes-

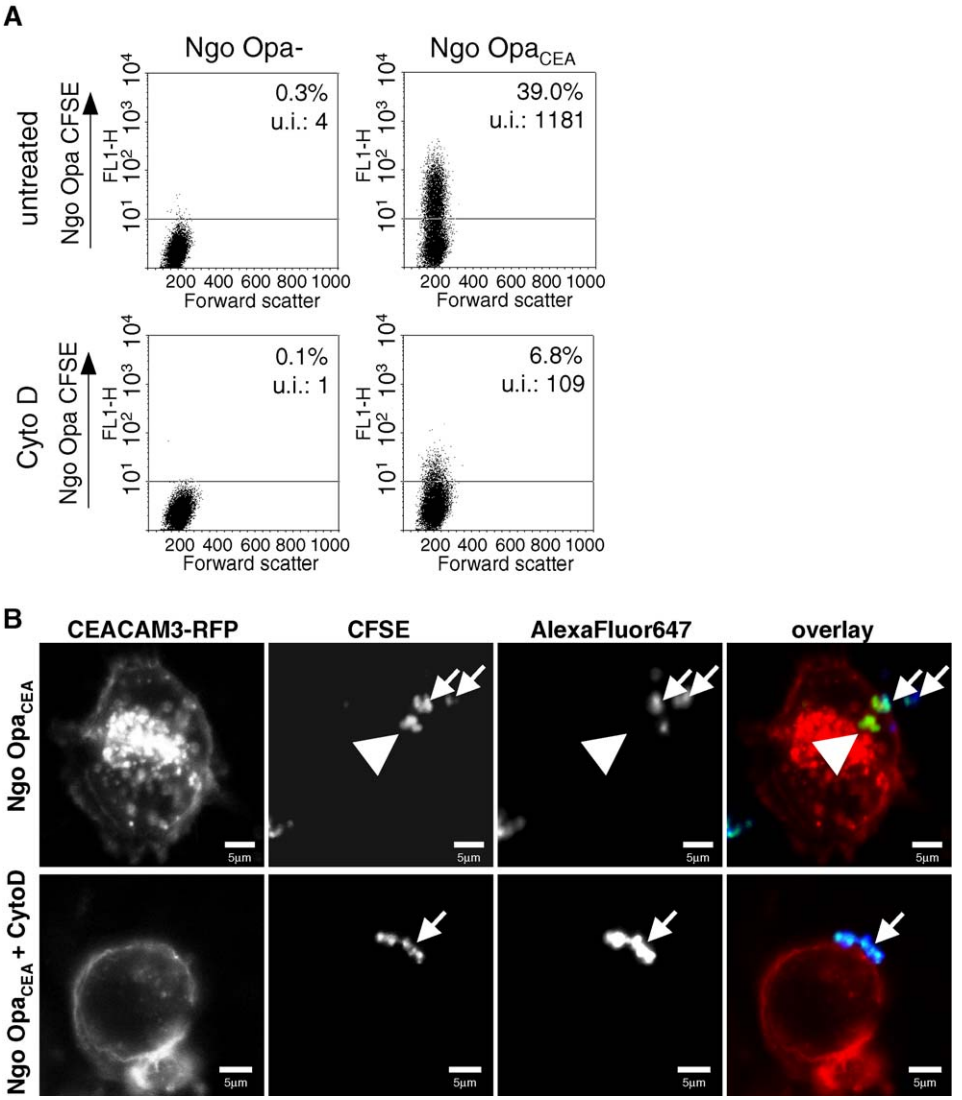

Fig. 2. Flow cytometry in the presence of trypan blue discriminates cell-associated, extracellular bacteria from intracellular bacteria. (A) 293 cells transfected with CEACAM3-HA were infected with non-opaque *N. gonorrhoeae* (Ngo Opa-) or Opa$_{CEA}$-expressing gonococci (Ngo Opa$_{CEA}$) for 1h in the presence or absence of 1 µg/ml cytochalasin D as indicated. Cells were taken in suspension and, after addition of trypan blue (final concentration of 0.2%), analysed by flow cytometry. Shown are the original dot plots of a representative experiment depicting the percentage of cells containing intracellular bacteria as well as the respective uptake index (u.i.). Similar results were obtained in two independent repetitions. (B) CEACAM3-RFP expressing cells were treated or not with cytochalasin D (1 µg/ml) and infected with CFSE/biotin labelled Opa$_{CEA}$-expressing *N. gonorrhoeae* for 1 h. Infected samples were fixed and extracellular bacteria stained with AlexaFluor647-coupled streptavidin. Accordingly, extracellular bacteria are positive for both CFSE and AlexaFluor647 (small arrows), whereas intracellular bacteria can only be detected in the CFSE channel (arrowhead). Bars represent 5 µm.

timate the amount of intracellular bacteria, if the internalized microorganisms are killed. This is particularly evident with receptors that are specialized in phagocytosis and efficiently deliver their cargo to lysosomal compartments (Booth et al., 2003). CEACAM3 represents a phagocytic receptor that not only efficiently internalizes bound particles, but also initiates the elimination of the internalized bacteria (McCaw et al., 2004; Schmitter et al., 2004). Importantly, determination of bacterial uptake by the flow cytometric approach should, theoretically, not be influenced by intracellular killing, as within a reasonable time the CFSE-label should stay intact. Therefore, we changed the experimental layout of our antibiotic protection assay to observe intracellular killing. CEACAM3-transfected 293 cells were infected with CFSE-labelled Opa$_{CEA}$-expressing gonococci for

60 min and at this point, the medium was replaced as before by DMEM containing 50 μg/ml gentamicin. Instead of analysing the whole sample after 30 min gentamicin treatment, an aliquot of the sample was taken out after 30 min, 60 min, or 120 min. Setting the viable bacteria recovered after 30 min as 100%, the amount of intracellular viable gonococci isolated at 60 min or 120 min was significantly lower (~70% or 27%, respectively)(Fig. 3A). These results demonstrated that bacteria taken up via CEACAM3 are rapidly killed inside human epithelial cells and similar observations have been reported by others (McCaw et al., 2004; Schmitter et al., 2004). Importantly, when the flow cytometric analysis was performed on samples infected and gentamicin treated identical to the ones used in the antibiotic protection assay, the fluorescence signal obtained 30, 60 and 120 min after gentamicin addition did not decrease (Fig. 3B). These results demonstrate that the flow cytometry evaluation of bacterial uptake is independent of intracellular viability of the bacteria. In addition, these results suggest that the time course of bacterial killing could be monitored by a combination of flow cytometry and antibiotic protection assays.

3.4. Evaluation of integrin-dependent invasion of S. aureus by flow cytometry

To further demonstrate that the application of the novel technique is not limited to the example of CEACAM-mediated cellular invasion of Opa_{CEA}-expressing *Neisseria gonorrhoeae*, we applied the flow cytometry approach to the integrin-mediated uptake of *Staphylococcus aureus* by human epithelial cells. Strains of *S. aureus* can express two fibronectin-binding proteins (FnBP-A and FnBP-B) that recruit the host extracellular matrix protein fibronectin (Fn) to the bacterial surface. Fn then serves as a molecular link connecting the bacteria with the principal host cell fibronectin receptor, the integrin $\alpha_5\beta_1$ (Fowler et al., 2000). Previously, we and others have observed that FnBP-initiated uptake via integrin $\alpha_5\beta_1$ is the major route of *S. aureus* internalization in vitro (Sinha et al., 1999; Sinha et al., 2000; Agerer et al., 2003). Therefore, *S. aureus* and the non-Fn-binding, non-pathogenic *S. carnosus* were labelled with CFSE and used to infect fibroblasts isolated from integrin β1-deficient mouse embryos (GD25 cells) as well as GD25 cells stably expressing human integrin β1 (Fig. 4A). Clearly, barely any uptake into integrin β1-deficient cells was observed for either of the two bacterial species (Fig. 4B). However, upon integrin β1 expression by the fibroblasts *S. aureus* was found in a large proportion of the cells, whereas no intracellular *S. carnosus* were detected (Fig. 4B). In these assays, more than 20% of the infected integrin β1 expressing cells harboured intracellular *S. aureus*, whereas only about 1% of the integrin β1-deficient fibroblasts contained intracellular pathogens (Fig. 4C). Together these results demonstrate that the flow cytometry-based detection of intracellular bacteria is a widely applicable approach to quantify bacterial invasion into adherent cell types.

4. Discussion

Quantitative analysis of bacterial invasion into non-professional phagocytes is an important pre-requisite to

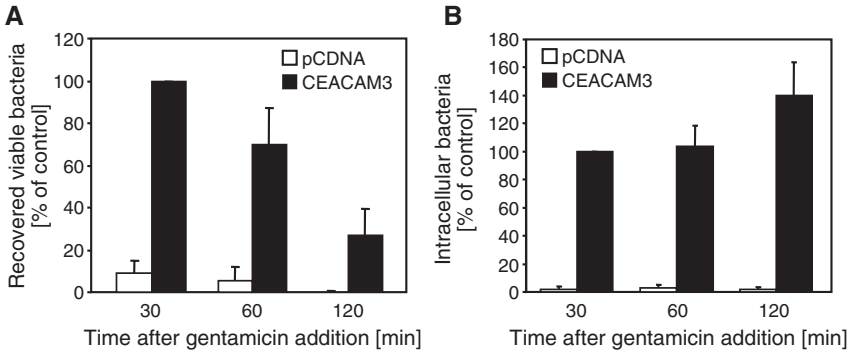

Fig. 3. Flow cytometric detection is not biased by intracellular killing of bacteria. (A) 293 cells transfected with control vector (pCDNA) or expressing CEACAM3-HA were infected with Opa_{CEA}-positive *N. gonorrhoeae* (Ngo Opa_{CEA}) for 1 h. After 30, 60, or 120 min gentamicin treatment, intracellular bacteria were released and the number of viable intracellular bacteria was determined by plating on GC agar. Bars represent the mean values ± SD of four independent experiments done in triplicate. (B) 293 cells transfected as in (A) were infected with CFSE-labelled Ngo Opa_{CEA} for 1 h. After incubation in gentamicin containing medium for the indicated time, cells were taken in suspension and analysed by flow cytometry for intracellular bacteria. The uptake index of the samples was calculated and expressed as relative value compared to CEACAM3-transfected samples incubated for 30 min with gentamicin. The bars represent the mean values ± SD of four independent experiments.

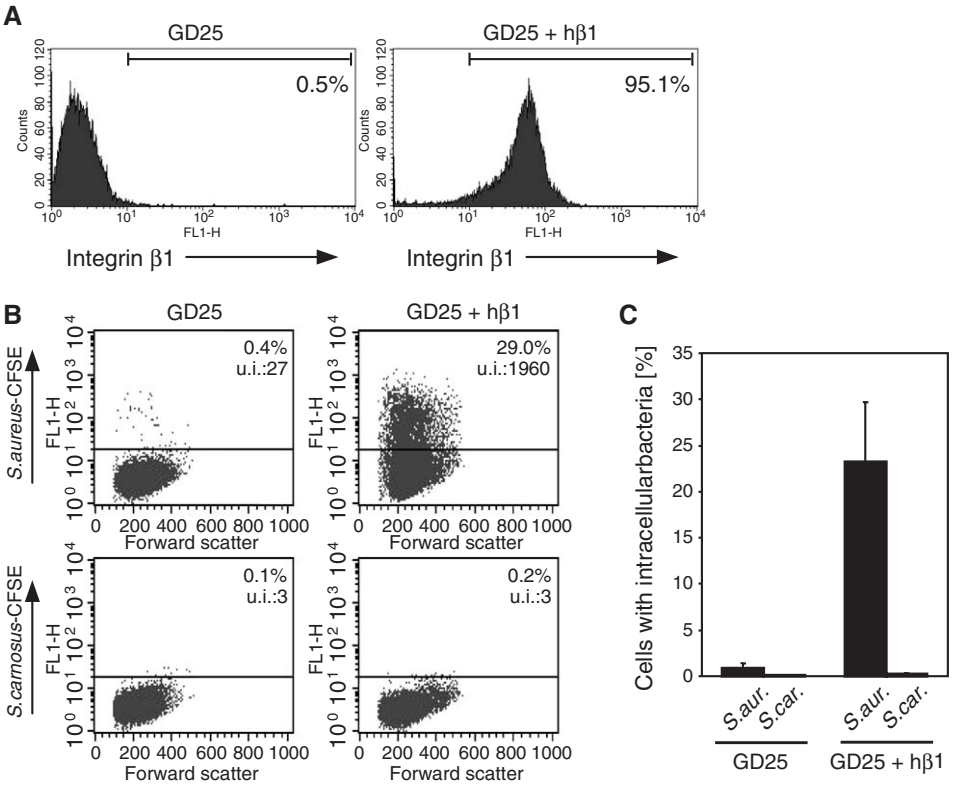

Fig. 4. Quantification of *S. aureus* invasion in integrin-deficient and reconstituted fibroblasts by flow cytometry. (A) GD25 cells and GD25 cells re-expressing human integrin β_{1A} (GD25+hβ1) were stained with a monoclonal antibody against human integrin β_1. and a Cy-2-conjugated goat–anti-mouse antibody before flow cytometry. Numbers indicate the percentage of integrin β_1 positive cells. (B) GD25 and GD 25+hβ_1 were infected for 2 h with CFSE-labelled *S. aureus* or *S. carnosus*, respectively. The infected cells were taken in suspension and, after addition of trypan blue (final concentration of 0.2%), analysed by flow cytometry. Shown are the original dot plots of a representative experiment depicting the percentage of cells containing intracellular bacteria as well as the respective uptake index (u.i.). (C) GD25 and GD 25+hβ_1 were infected and analysed as in (B). The bars represent the mean values ± SD of the percentage of cells containing intracellular bacteria as observed in four independent experiments.

unravel the molecular details of pathogen-host cell interaction. Both, the widely used antibiotic protection assay as well as microscopic evaluation of intracellular bacteria have considerable disadvantages regarding the correct enumeration of the total number of intracellular bacteria or with respect to the labour-intensive visual quantification of the samples, respectively. We present here an adapted flow cytometry-based method that allows the rapid quantitative analysis of intracellular bacteria in different adherent cell types. Comparison of this approach with other assays and the specific inhibition of the uptake process by pharmacological agents demonstrate that this methodology correctly discriminates between *bona fide* intracellular and cell-associated, extracellular bacteria. Furthermore, the cytometric evaluation allows the detection of intracellular microbes for a prolonged time after internalization and is not compromised by intracellular killing of the bacteria that occurs rapidly upon CEACAM3-mediated internalization of Opa_{CEA}-expressing *N. gonorrhoeae*. As the novel protocol delivers quantitative results faster than other assays, it will be a valuable addition to the current experimental approaches in infection biology.

Whereas the conventional antibiotic protection assay allows the direct enumeration of each viable intracellular bacterium by plating and colony counting, the flow cytometer-based method detects intracellular bacteria only in the context of the infected host cell. This is due to the fact that data acquisition by the flow cytometer is focussed on the eukaryotic cells, which are selectively recognized based upon their forward and side light scatter properties. Therefore, the fluorescence signals are measured in a population of cells that remain either non-fluorescent (no intracellular bacteria) or have varying strength of the fluorescence signal depending on the amount of internalized labelled bacteria. Accordingly, the flow cytometer does not provide an exact count of intracellular bacteria. Nevertheless, if the per-

centage of fluorescent cells is combined with the mean fluorescence intensity of the infected cell population, this uptake index (u.i.) gives an estimate of the total amount of internalized bacteria. The uptake index appears to be a reliable indicator for the intracellular bacteria as it produces similar differences between non-invasive (e.g., non-opaque gonococci) versus invasive strains (e.g., Opa_{CEA}-expressing gonococci), when compared to the conventional antibiotic protection assay or microscopic means of evaluation.

An important criterion to be fulfilled by a proper invasion assay is its ability to differentiate between intracellular bacteria and extracellular bacteria in close association with the eukaryotic cell surface. Several alternative approaches have been described that exploit the speed and the quantitative nature of flow cytometric detection to measure bacterial interaction with host cells. For example, GFP-expressing *Yersinia pseudotuberculosis* as well as *Pseudomonas aeruginosa* have been used to infect human and murine cells and the GFP-derived fluorescence of the infected cells has been used as a measure of bacterial-eukaryotic cell association (Valdivia et al., 1996; Feldman et al., 1998). In other cases, bacteria such as *Salmonella typhimurium* or *Listeria monocytogenes* have been directly labelled with PKH-2 (a lipophilic dye that unspecifically stains membranes), rhodamine, or fluorescein derivates to measure interaction of these pathogens with mouse macrophage cell lines (Raybourne and Bunning, 1994; Drevets and Elliott, 1995). Though these investigations have shown that GFP expression or direct fluorescent labelling of the microorganisms did not interfere with viability and cell association, all these approaches do not discriminate between cell-bound, extracellular and true intracellular bacteria. Therefore, the effect of inhibitors that target bacterial uptake, but that do not interfere with bacterial adherence, cannot be appreciated with these methods. Such a scenario for example is observed in cells that are treated with inhibitors of actin cytoskeleton dynamics (e.g., cytochalasin D) or inhibitors of signaling enzymes. These compounds generally still allow pathogen attachment to cellular receptors, but block the receptor-triggered internalization process, resulting in extracellular bacteria intimately associated with the host cell. Indeed, fluorescence microscopy confirms that, in contrast to untreated control cells, cytochalasin D-treated cells do not harbour intracellular bacteria, but still have numerous bacteria attached to the surface. These results clearly demonstrate the effectiveness of trypan blue as a quenching agent for extracellular CFSE-labelled bacteria and point to the usefulness of the flow cytometry-based approach in analysing inhibitors of the uptake process.

An important additional feature of the novel methodology pertains to the fact that the fluorescence signal derived from the CFSE-labelled intracellular bacteria does not decrease over time. In fact, the CFSE-signal slightly increases over the course of 1 to 2 h even after addition of gentamicin suggesting that some cell-bound bacteria are further internalized after the addition of the antibiotic (see Fig. 3B). In contrast, gentamicin assay-based viable counts of gonococci internalized via CEACAM3 decline rapidly, consistent with previous reports (McCaw et al., 2004). These results further stress the limitations of antibiotic protection assays, where the read-out (recovered viable bacteria) is influenced by both the efficiency of uptake as well as rate of intracellular killing or multiplication of the bacteria. As these types of assays are sometimes conducted over periods of several hours, the influence of intracellular events on bacterial viability should not be underestimated. Accordingly, the flow cytometry approach provides a convenient and rapid alternative to directly and specifically quantify bacterial uptake by different adherent cell types. Therefore, this method will be a valuable addition to the experimental toolbox available to researchers investigating the molecular mechanisms guiding the uptake of microorganisms by eucaryotic cells.

Acknowledgments

We thank T.F. Meyer (MPI für Infektionsbiologie, Berlin) for the Neisseria strains used in this study, R. Fässler (MPI für Biochemie, München) for GD25 cell lines, and D. Deininger for expert technical assistance. This study was supported by funds from the DFG (Ha2568/3-2) to C.R.H.

References

Agerer, F., Michel, A., Ohlsen, K., Hauck, C.R., 2003. Integrin-mediated invasion of Staphylococcus aureus into human cells requires Src family protein tyrosine kinases. J. Biol. Chem. 278, 42524–42531.

Agerer, F., Waeckerle, S., Hauck, C.R., 2004. Microscopic quantification of bacterial invasion by a novel antibody-independent staining method. J. Microbiol. Methods 59, 23–32.

Agerer, F., Lux, S., Michel, A., Rohde, M., Ohlsen, K., Hauck, C.R., 2005. Cellular invasion by *Staphylococcus aureus* reveals a functional link between focal adhesion kinase and cortactin in integrin-mediated internalisation. J. Cell Sci. 118, 2189–2200.

Booth, J.W., Telio, D., Liao, E.H., McCaw, S.E., Matsuo, T., Grinstein, S., Gray-Owen, S.D., 2003. Phosphatidylinositol 3-kinases in carcinoembryonic antigen-related cellular adhesion molecule-

mediated internalization of *Neisseria gonorrhoeae*. J. Biol. Chem. 278, 14037–14045.

Bos, M.P., Grunert, F., Belland, R.J., 1997. Differential recognition of members of the carcinoembryonic antigen family by Opa variants of *Neisseria gonorrhoeae*. Infect. Immun. 65, 2353–2361.

Chen, T., Grunert, F., Medina-Marino, A., Gotschlich, E.C., 1997. Several carcinoembryonic antigens (CD66) serve as receptors for gonococcal opacity proteins. J. Exp. Med. 185, 1557–1564.

Drevets, D.A., Elliott, A.M., 1995. Fluorescence labeling of bacteria for studies of intracellular pathogenesis. J. Immunol. Methods 187, 69–79.

Falkow, S., 1991. Bacterial entry into eukaryotic cells. Cell 65, 1099–1102.

Feldman, M., Bryan, R., Rajan, S., Scheffler, L., Brunnert, S., Tang, H., Prince, A., 1998. Role of flagella in pathogenesis of *Pseudomonas aeruginosa* pulmonary infection. Infect. Immun. 66, 43–51.

Finlay, B.B., Cossart, P., 1997. Exploitation of mammalian host cell functions by bacterial pathogens. Science 276, 718–725.

Fowler, T., Wann, E.R., Joh, D., Johansson, S., Foster, T.J., Hook, M., 2000. Cellular invasion by *Staphylococcus aureus* involves a fibronectin bridge between the bacterial fibronectin-binding MSCRAMMs and host cell beta1 integrins. Eur. J. Cell Biol. 79, 672–679.

Galan, J.E., Zhou, D., 2000. Striking a balance: modulation of the actin cytoskeleton by Salmonella. Proc. Natl. Acad. Sci. U. S. A. 97, 8754–8761.

Gray-Owen, S.D., Lorenzen, D.R., Haude, A., Meyer, T.F., Dehio, C., 1997. Differential Opa specificities for CD66 receptors influence tissue interactions and cellular response to *Neisseria gonorrhoeae*. Mol. Microbiol. 26, 971–980.

Hauck, C.R., 2002. Cell adhesion receptors — signaling capacity and exploitation by bacterial pathogens. Med. Microbiol. Immunol. 191, 55–62.

Hauck, C.R., Meyer, T.F., 2003. 'Small' talk: Opa proteins as mediators of Neisseria-host-cell communication. Curr. Opin. Microbiol. 6, 43–49.

Heesemann, J., Laufs, R., 1985. Double immunofluorescence microscopic technique for accurate differentiation of extracellularly and intracellularly located bacteria in cell culture. J. Clin. Microbiol. 22, 168–175.

Lecuit, M., Dramsi, S., Gottardi, C., Fedor-Chaiken, M., Gumbiner, B., Cossart, P., 1999. A single amino acid in E-cadherin responsible for host specificity towards the human pathogen Listeria monocytogenes. EMBO J. 18, 3956–3963.

McCaw, S.E., Liao, E.H., Gray-Owen, S.D., 2004. Engulfment of *Neisseria gonorrhoeae*: revealing distinct processes of bacterial entry by individual carcinoembryonic antigen-related cellular adhesion molecule family receptors. Infect. Immun. 72, 2742–2752.

Mengaud, J., Ohayon, H., Gounon, P., Mege, R.M., Cossart, P., 1996. E-cadherin is the receptor for internalin, a surface protein required for entry of *L. monocytogenes* into epithelial cells. Cell 84, 923–932.

Raybourne, R.B., Bunning, V.K., 1994. Bacterium-host cell interactions at the cellular level: fluorescent labeling of bacteria and analysis of short-term bacterium-phagocyte interaction by flow cytometry. Infect. Immun. 62, 665–672.

Schmitter, T., Agerer, F., Peterson, L., Muenzner, P., Hauck, C.R., 2004. Granulocyte CEACAM3 is a phagocytic receptor of the innate immune system that mediates recognition and elimination of human-specific pathogens. J. Exp. Med. 199, 35–46.

Sinha, B., Francois, P.P., Nusse, O., Foti, M., Hartford, O.M., Vaudaux, P., Foster, T.J., Lew, D.P., Herrmann, M., Krause, K.H., 1999. Fibronectin-binding protein acts as *Staphylococcus aureus* invasin via fibronectin bridging to integrin alpha5beta1. Cell. Microbiol. 1, 101–117.

Sinha, B., Francois, P., Que, Y.A., Hussain, M., Heilmann, C., Moreillon, P., Lew, D., Krause, K.H., Peters, G., Herrmann, M., 2000. Heterologously expressed Staphylococcus aureus fibronectin-binding proteins are sufficient for invasion of host cells. Infect. Immun. 68, 6871–6878.

TranVan Nhieu, G., Bourdet-Sicard, R., Dumenil, G., Blocker, A., Sansonetti, P.J., 2000. Bacterial signals and cell responses during Shigella entry into epithelial cells. Cell. Microbiol. 2, 187–193.

Valdivia, R.H., Hromockyj, A.E., Monack, D., Ramakrishnan, L., Falkow, S., 1996. Applications for green fluorescent protein (GFP) in the study of host-pathogen interactions. Gene 173, 47–52.

Virji, M., Makepeace, K., Ferguson, D.J.P., Watt, S.M., 1996. Carcinoembryonic antigens (CD66) on epithelial cells and neutrophils are receptors for Opa proteins of pathogenic Neisseriae. Mol. Microbiol. 22, 941–950.

Voyich, J.M., DeLeo, F.R., 2002. Host-pathogen interactions: leukocyte phagocytosis and associated sequelae. Methods Cell Sci. 24, 79–90.

Watarai, M., Funato, S., Sasakawa, C., 1996. Interaction of Ipa proteins of *Shigella flexneri* with alpha5beta1 integrin promotes entry of the bacteria into mammalian cells. J. Exp. Med. 183, 991–999.