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Quantitative flow cytometric monitoring of invasion of epithelial cells by *Mycobacterium tuberculosis*

Julie A. Chapeton-Montes [1], David F. Plaza [1], Carlos A. Barrero [1], Manuel A. Patarroyo [1, 2]

[1] Molecular Biology Department, Fundacion Instituto de Inmunologia de Colombia, Bogota, Colombia, [2] Universidad Nacional de Colombia, Bogota, Colombia

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1. ABSTRACT

Current methods for evaluating mycobacterial invasion of target cells pose technical difficulties including a long turn-around time. Thus, new methodologies must be developed that allow rapid and reliable monitoring of host cell invasion. Here, the invasion of A549 cell line by SYBR safe-labeled *Mycobacterium tuberculosis* (*M. tuberculosis*) H37Rv was assessed by flow cytometry and was expressed as the percentage of cells infected by *M. tuberculosis*. Over a third of A549 cells were invaded by *M. tuberculosis*, two hours after infection. The specificity of the invasion was confirmed in assays using red blood cells as target cells and *Escherichia coli* as the non-invasive bacterial control. These findings show that invasion of pulmonary epithelial cells by *M. tuberculosis* can rapidly and quantitatively be assessed with a great sensitivity by flow cytometric detection of SYBR safe-labeled *M. tuberculosis.*

2. INTRODUCTION

Tuberculosis is one of the greatest global health problems since one third of the population of the world is infected and 8 to 10 million new cases develop each year. In over 90% of cases, the disease does not progress, however, over 2 million people die each year from fulminant form of the disease (1-2). Appearance of multi-drug resistant strains, infection of patients with AIDS and the variable efficacy of the existing BCG vaccine (0-80%) have prompted search for new therapies and preventive measures, such as improving or developing a new vaccine which can effectively control this disease (3-4).

Infection begins when bacilli are inhaled and localize in the alveoli of the distal airways. At these sites, bacilli invade epithelial cells and alveolar macrophages. In many cases, the infection remains latent but in others

particularly those with AIDS or other forms of immune deficiency , the disease progresses to an active form (5).

The ability of *M. tuberculosis* H37Rv to invade type II A549 alveolar epithelial cells and macrophages (6) has been used for identifying different host cell receptors involved in the invasion process such as the mannose receptor (7, 8), surfactant protein A (9, 10), complement receptors (11), scavenger receptors, Toll like receptors (TLR) TLR 2 and TLR4 (12) and Fc receptors (13). These are used by the bacteria for binding to and entering the cell through phagocytic or endocytic vesicles in an actin filament- and microtubule-dependant process (5, 14).

Fluorescein isothiocyanate (FITC) and antibodies coupled to different fluorochromes have been used in labeling *M. tuberculosis* to identify freely suspended, bound or intracellular bacteria (15, 16); however, it has been shown that these substances alter bacterial receptor availability for their interaction with eukaryotic cells. On the other hand, enteropathogenic intracellular bacteria stained with acridine orange and counter-stained or quenched with crystal violet for eliminating extra-cellular microorganisms' fluorescence have been observed after infecting HeLa cell monolayers or in phagocytosis assays using macrophages (17). However, such staining affects bacterial viability and therefore prevents them from being counted as colony-forming units (CFU) in data analysis (18). SYBR green, a fluorochrome used for staining nucleic acids and binding, especially to double-stranded DNA, has been used for its great sensitivity in quantifying different microorganisms stained for FC (19). No negative effects have been reported to date regarding SYBR green labeled microorganism's viability.

Mycobacterial invasion of host cells has also been evaluated by the microbiological method consisting of quantifying the CFU of intracellular bacilli obtained from infected-cell lysate (20). Techniques such as microscopy have also been implemented, allowing the percentage of invaded cells to be measured with greater accuracy (14). Microscopy and FC are the only methods which can detect this parameter. Although microscopy is cumbersome, its greatest advantage is that it provides direct visual evaluation and is thus useful for confirming the validity of results obtained using other methods (20).

Intracellular bacterial interaction with host cells has been studied using techniques such as fluorescence microscopy (FM) and FC, based on fluorescent labeling of different cell structures and components. FC has been used for measuring the ability of intracellular bacteria (i.e. *Listeria monocytogenes* and *Salmonella typhimurium*) to invade the J774A.1 murine macrophage cell line (18). This technique has also been used in studying the biology of *M. tuberculosis* to identify cell surface antigens (21), *M. tuberculosis*-secreted proteins, interaction with the immune system (22) and to determine susceptibility to drugs (23, 24).

The time needed for obtaining results from *M. tuberculosis* invasion assays using traditional methodology continues to be long, ranging from 20 to 30 days (25). Alternatively, FC is a rapid, reproducible technique for measuring the phagocytosis of cells in suspension, providing results in a very short time (26) and offering a diagnostic tool for slow-growing microorganisms such as mycobacteria (27). An FC assay for evaluating SYBR Safe stained *M. tuberculosis* H37Rv invasion to alveolar epithelial cells (A549) was standardized in this work.

Standardizing these methodologies has led us to develop a rapid, simple and reliable tool available for evaluating the biological relevance of a large number of peptides or proteins directly implied in target cell invasion.

3. MATERIALS AND METHODS

3.1. Bacterial cultures

The *M. tuberculosis* H37Rv (ATCC 27294) strain was grown for 20 days in Middlebrook agar 7H10 (Difco Lab, Detroit, MI, USA) and then suspended in 7H9 Middlebrook broth (Difco Lab, Detroit, MI, USA) for 5 to 15 days. Bacteria were spun down, washed, and suspended in phosphate-buffered saline (PBS) prior to performing the assays. Due to mycobacteria's tendency to clump, the suspension was briefly sonicated (30 W for 5 s) in a Branson sonifier (VWR Scientific, Boston, MA); suspension turbidity was adjusted to a $3x10^8$ CFU/ ml according to McFarland's pattern and verified by FM once the bacteria had been labeled. *E. coli* M15 was grown in 2XYT medium.

3.2. Cell line

A549 (ATCC No. CCL-185), an adherent epithelial cell line derived from a pulmonary carcinoma was used in this study. The cell line was obtained from ATCC (Rockville, MD), maintained in culture using RPMI 1640 (Gibco, NY) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Logon, UT) at 37°C and 5% CO_2 (at 10^5 cell ml^{-1} density) in 275 ml culture dishes. Adherent A549 cells were dislodged with 0.01% EDTA-PBS. The cell line was collected in 50 ml tubes and centrifuged at 2500 rpm for 5 min, washed with PBS and counted in a Neubauer chamber.

3.3. Bacterial staining

M. tuberculosis and *E. coli* were labeled with SYBR Safe (Applied Biosystems) at 20X final concentration at 37°C for 20 min in the dark. They were washed twice and centrifuged at 12,000 rpm for 20 min and excess dye was removed. Bacteria were then suspended in RPMI 1640 and labeling was further verified by FM and FC.

3.4. Cell invasion assays

The bacterial suspension was added to 1 x 10^6 A549 cells in complete RPMI 1640 medium without antibiotics to a 1:10 multiplicity of infection (MOI) for a final 200 µl volume. They were incubated at 37°C in 5% CO_2 for variable lengths of time with constant shaking and then placed in medium supplemented with 20 µg/ml amikacin (ICN Biomedicals, Costa Mesa, CA, USA), for 30 min to kill off extra-cellular bacteria while intracellular

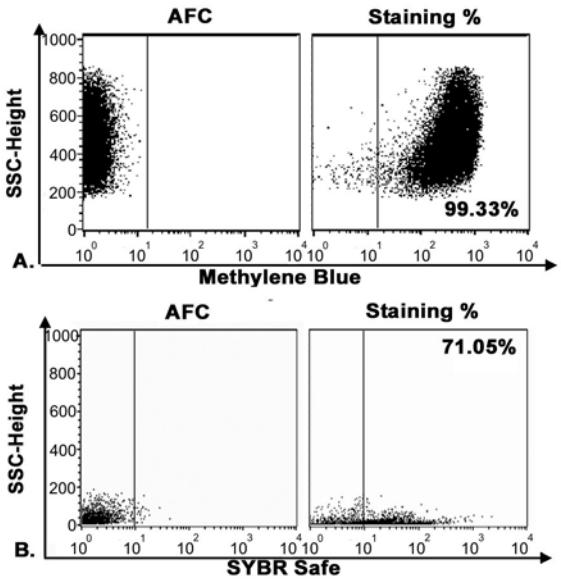

Figure 1. A. Labeling A549 cell line with MB. Dot plot at the left pane shows the AFC level for A549 cells. Dot plot at the right pane shows the A549-labeling percentage with MB. The cells were treated with 3% MB in 30% ethanol five minutes before FC. **B.** Labeling *M. tuberculosis* H37Rv with SYBR Safe. Dot plot at the left pane shows the AFC level for mycobacteria. Dot plot at the right pane shows the percentage of SYBR Safe-labeled mycobacteria. A 200µl aliquot of bacterial suspension was incubated in the presence of SYBR Safe at 20X final concentration for 20 min at 37ºC and treated with 40µg/ml amikacin and 2% paraformaldehyde for later handling at a biosafety level lower than 3 and FC reading. N =4; the dot plot shown represents a significant example of all the replicates that were carried out.

bacteria remained viable. Two RPMI 1640 washes were then carried out followed by centrifuging at 2,500 rpm for 5 min. The cells were fixed in 1% paraformaldehyde (Sigma) in RPMI 1640 at 4°C for 1 hour, then washed and suspended in 50µl RPMI 1640. The cells were counterstained with freshly-prepared 3% Methylene Blue (MB) in 30% Ethanol five minutes before FC. Red blood cells in the presence of *M. tuberculosis* as well as A549 cells in the presence of *E. coli* were used as negative invasion controls, incubating them for two hours. Cells used for determining autofluorescence (AFC) were treated under the same experimental conditions as those incubated with bacteria.

As a control, invasion was carried out in the presence of either microtubule- or actin-polymerization inhibitors such as 30 µM colchicine and 12 µM cytochalasin B (Sigma), respectively. Inhibitors were added to A549 cells in suspension 1 h prior to adding bacteria for inhibiting mammalian cell internalization (14).

3.5. Flow cytometry quantification

Samples were analyzed on a FACScan (Becton Dickinson, Mountain View, CA, USA), equipped with 488 nm argon lasers. The cytometer was routinely calibrated with Calibration beads (Becton Dickinson). Cellquest software (Becton Dickinson) was used for FC capture and analysis. PBS, which was filter-sterilized with a 0.2 µm filter, was used as particle-free sheath fluid. Uninfected epithelial cells were distinguished from infected epithelial cells based on light FL1 characteristics. Samples were run at 1500 events/s; 40000 events were collected.

3.6. Quantifying invasion by fluorescence microscopy

The invasion assay was evaluated by FM to verify the percentage of invasion, counting infected and non-infected cells having one or more bacilli.

3.7. Statistical analyses

Results are shown as means ± SD. Differences between various treatments were analyzed by unpaired Student's t test with p values <0.05 or 0.01 considered as significant.

4. RESULTS

4.1. Fluorescent labeling of A549 cells

Different reagents were tested for staining the A549 cell line used, obtaining 20.8% maximum staining percentage with CD83 for A549 cells (data not shown). Total labeling of the cell population used (>99.33%) was obtained by using 3% MB in 30% ethanol (Figure 1A).

4.2. Fluorescent labeling of *M. tuberculosis* H37Rv

FM was used for monitoring different fluorescent dyes binding to DNA. The bacteria were successfully labeled when using $3x10^{-4}$ % Acridine Orange for 10 minutes, obtaining 56.7% labeled bacteria. However, its toxicity for the microorganism made it impossible to recover viable bacteria upon completing the assay. SYBR Safe revealed high fluorescence intensity without altering bacterial viability as determined by plating on 7H10 medium. The efficacy of such staining in *M. tuberculosis* was shown by FC at different incubation times and different dye concentrations, obtaining 71.13% labeled bacteria after 20 min incubation at 37ºC had elapsed (Figure 1B). Such conditions were thus chosen for carrying out the invasion assays.

4.3. Kinetics of *M. tuberculosis* binding to A549 cells

After bacterial fluorescence labeling had been conducted, *M. tuberculosis* H37Rv invasion to type II A549 epithelial cells was evaluated at different times to determine invasion behavior. The sample was treated with 20 µg/ml amikacin and 1% paraformaldehyde to reduce the risk of contamination by mycobacteria to less than bio-safety level 3. Invasion percentages increased with time, showing 23.94 ± 2.79%, 39.09 ± 0.37%, 56.57 ± 2.01% and 61.31 ± 3.41% at 0, 2, 4 and 6 hours, respectively (Figure 2). Invasion percentages after 2 hours of infection as determined by FM were 36.90 +/- 3.02%, confirming the results observed by FC. In our study, the inter-experiment values of M. tuberculosis invasion to A549 cells range between 33% and 39% (data not shown) with intra-experiment triplicates displaying little variation as shown above.

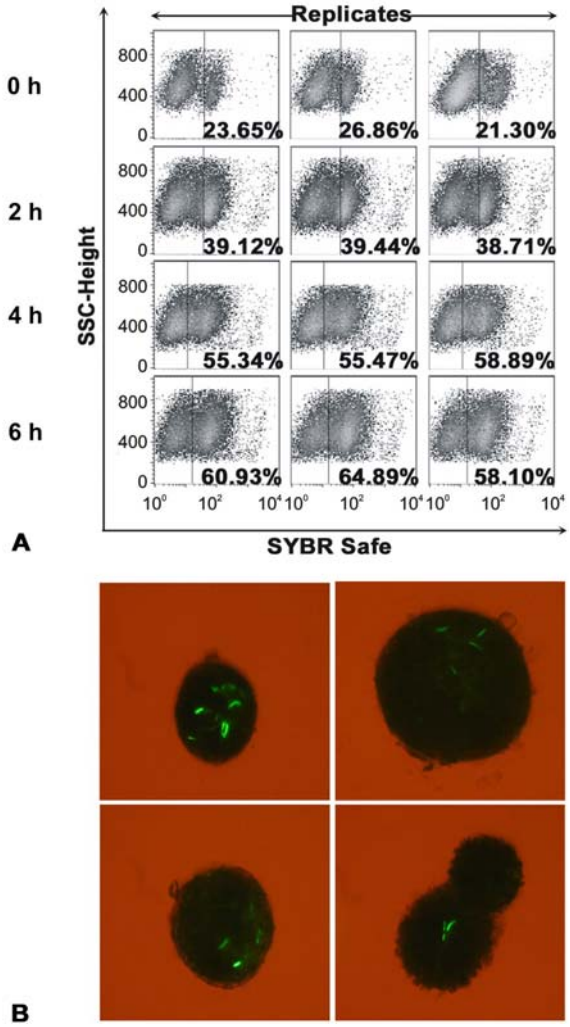

Figure 2. A. Kinetics of *M. tuberculosis* H37Rv invasion to type II A549 epithelial cells. Density plots correspond to triplicate assays for each incubation time point. A time dependant increase of *M. tuberculosis* invasion to A549 cells can be observed. The cells were incubated at 1:10 MOI for 0, 2, 4 or 6 hours with constant shaking in the presence of *M. tuberculosis* previously labeled with SYBR Safe. The sample was then treated with 20µg/ml amikacin for 30 min, washed twice to remove extracellular bacteria and fixed with 1% paraformaldehyde in RPMI 1640. The samples were counterstained with 3% Methylene Blue in 30% ethanol five minutes before FC. N =3. **B.** Fluorescence microcopy (100X) showing A549 cells invaded with *M. tuberculosis* after 2 hour incubation.

4.4. Low *Escherichia coli* binding to A549 cells

A549 cells were incubated for two hours with *E. coli* M15 in the same conditions used for infection with *M. tuberculosis* to prove that non-invasive bacteria presented low association to these cells. Relatively little *E. coli* invasion to the cell line was found once the incubation time had elapsed (Figure 3B) when comparing the results with those obtained for *M. tuberculosis* (Figure 2).

4.5. Low *M. tuberculosis* H37Rv binding to human erythrocytes

Mycobacteria were incubated with human erythrocytes to determine whether *M. tuberculosis* H37Rv binding to pulmonary cells was specific. 2.66 ± 0.58% cell–mycobacteria association was observed (Figure 3C).

4.6. Invasion assay in the presence of inhibitors

M. tuberculosis infection to either colchicine- or cytochalasin B-preincubated A549 cells was tested at concentrations of each inhibitor that could not affect target cell viability. *M. tuberculosis* invasion was diminished by 36.45% when 30 µM colchicine was used ($p < 0.05$) and by 14.32% when 12µM cytochalasin B was used ($p < 0.05$), with respect to the 2 hours' incubation without inhibitors (Figure 3A).

5. DISCUSSION

The release of the complete *M. tuberculosis* genome sequence (28) and studies in proteomics (29) have led to great advances in understanding the biology of the bacillus, the survival mechanisms used within phagocytic cells, as well as the identification of many vaccine candidate antigens (30). Even though several proteins have been described, the molecules implicated in adhesion to and invasion of host cells have not been completely characterized. Many hypothetical membrane proteins having as of yet unknown function could be involved in this process, confirming the necessity to develop methodologies which can evaluate putative membrane protein activity (31).

Studies determining mycobacterial surface antigens (21) have used a hydrophobic fluorochrome (HEDAF) inserted into the mycobacterial cell envelope while labeling the bacteria. A fluorochrome which would not bind to the cell envelope was needed for developing this study in order to avoid affecting *M. tuberculosis* invasion to A549 cells. SYBR Safe is a molecule which binds to double-strand DNA, and a greater percentage of labeled bacteria was achieved with it compared to the other fluorochromes evaluated in the standardization of this technique (acridine orange, FITC, rhodamine and hydroethydine). It was found that labeling with acridine orange gave a lower percentage of stained bacteria. We have compared SYBR Safe and acridine orange stained mycobacteria and found that their passage through the mycobacterial cell membrane was hampered when the former was used, most likely due to its larger molecular weight (509.27 g/mol and 179.2 g/mol respectively), thereby reducing cross-reactivity with uninfected cells.

Bacterial labeling has facilitated the observation of bacterial invasion processes, allowing the distinction to be made between invaded and non-invaded target cells. A potential problem for any bacterial invasion assay lies in the self-replication of the viable microorganism during the assay. The resulting bacteria will thus be less labeled (20). There is no such difficulty with *M. tuberculosis* since its replication rate is around 24 hours (31) and the assay

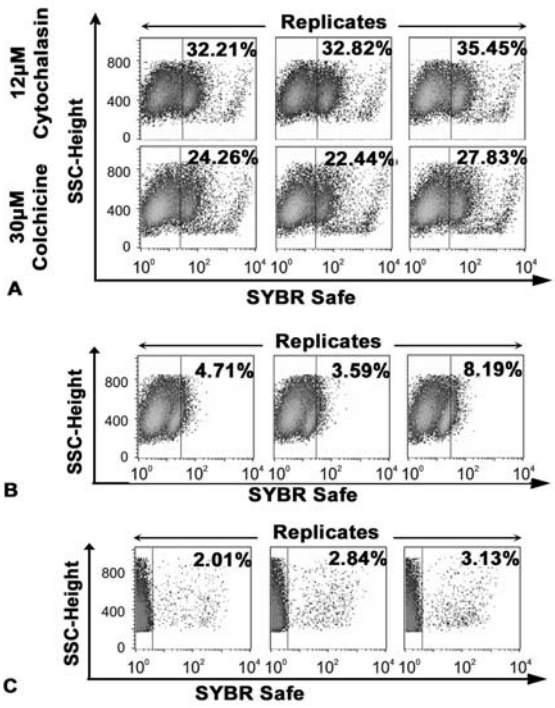

Figure 3. A. Invasion assay in presence of inhibitors. As observed, 12µM cytochalasin B decreases invasion to 33.49 +/- 1.72% while 30 µM colchicine decreases invasion to 24.84 +/- 2.74 %. **B.** Low level *E. coli* invasion to A549 cells, showing invasion percentages of 5.50 +/- 2.40%. **C.** Low level *M. tuberculosis* H37Rv invasion to human erythrocytes, showing an invasion percentage of 2.66 +/- 0.58 %. The cells were incubated at 1:10 MOI for two hours with constant shaking with *M. tuberculosis* or *E. coli* previously labeled with SYBR Safe and processed similarly as in the previous assays with antibiotics, fixing solution and MB. N = 3 data are Mean +/- SD; * $p < 0.05$ and ** $p < 0.01$.

presented here can be carried out in no more than 5 hours when 2 hours incubation is tested.

Target cell labeling was conducted using surface antigen-specific antibodies, showing that a limited amount of the cell population was expressing the markers tested and therefore, labeling percentages obtained were not high enough for our study. When testing a less specific label such as MB, we were able to identify 99% of the cell population that was emitting a signal in FL3 channel on the FC.

The invasion kinetics study (Figure 2) showed an increasing proportion of invaded cells as a function of time. This study sought a direct approach to the percentage of cells invaded with *M. tuberculosis* H37Rv. 39.09% of A549 cells invaded with the bacteria was found after 2 hours incubation. Bermudez *et al*. (14) have shown 41% to 90% *M. tuberculosis*-infected cells after two hours of incubation compared to 0% to 1% *E. coli* HB101-infected cells after the same period of time. The results obtained using our technique are similar to those mentioned above.

Cytochalasin induces actin disruption and has been associated with lowered bacterial internalization by epithelial cells. It has also been observed that cytochalasin diminishes *S. typhimurium* and *Proteus mirabilis* internalization in non-enterocytic cell lines (HeLa and Hep-2) (32). Our results show an important *M. tuberculosis* invasion inhibition to A549 cell lines when either cytochalasin B or colchicine are used as inhibitors. Similar results have been previously reported by Bermudez *et al*. (14).

The results obtained in our assays lead us to believe that the percentages obtained correspond to infection values rather than adhesion values. We base this conclusion on several observations. First, since amikacin treatment has been reported to damage bacterial membrane (33), this may lead to the release of host cell-adhered bacteria and further removal of this bacterial population in the washing processes. The removal of most extracellular bacteria after antibiotic incubation and washing was confirmed by FM. Second, the fluorescence emitted by the few extracellular bacteria that remained attached after washing was quenched by the MB. Third, the results obtained when using invasion inhibitors further demonstrate that the observed data agree with an invasion process, since no reduction in the percentages would be expected if bacteria were only adhering to the target cells.

The controls used show that the results obtained with *M. tuberculosis* were reliable since a low percentage of *E. coli*-infected A549 cells were observed and few red blood cells were found to be associated with *M. tuberculosis*. These results also suggest the mycobacterium's high invasion specificity to target cells (Figures 3B and 3C).

We thus conclude that the technique described here using FC constitutes a powerful tool for evaluating *M. tuberculosis* invasion to pulmonary cells, providing several advantages over other methods due to its rapid detection and sensitivity. This assay is also able to give quantitative and reproducible information. Future studies aimed at measuring the biological activity of different peptides and proteins possibly involved in this microorganism's binding and invasion could benefit from using this approach.

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Abbreviations: FC: flow cytometry; FM: fluorescence microscopy; MB methylene blue; AFC: autofluorescence; FITC: fluorescein isothiocyanate; CFU: colony-forming units

Key Words: *Mycobacterium tuberculosis*, Invasion, Flow Cytometry, Epithelial Cells

Send correspondence to: Dr Manuel A. Patarroyo, Molecular Biology Department, Fundacion Instituto de Inmunologia de Colombia, Bogota, Colombia, Tel: 571-4815219, Fax: 571-4815269, E-mail: mapatarr@fidic.org.co

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