

Use of a colorimetric assay to measure differences in cytotoxicity of *Mycobacterium tuberculosis* strains

Jorge Castro-Garza,¹ Hugo B. Barrios-García,^{1,2} Delia Elva Cruz-Vega,¹ Salvador Said-Fernández,¹ Pilar Carranza-Rosales,¹ Carmen A. Molina-Torres³ and Lucio Vera-Cabrera³

Correspondence

Jorge Castro-Garza
jorge.castro@biomedicas.net

¹División de Biología Celular y Molecular, Centro de Investigación Biomédica del Noreste, Instituto Mexicano del Seguro Social, Monterrey, NL, Mexico

²Facultad de Ciencias Biológicas, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, NL, Mexico

³Servicio de Dermatología, Hospital Universitario 'José E. González', Monterrey, NL, Mexico

Several techniques have been used to quantify the cytotoxicity produced by *Mycobacterium tuberculosis* bacilli on cell monolayers; however, they are semi-quantitative or time consuming. Herein, a method based on crystal violet (CV) uptake by THP-1 cell monolayers is described. This colorimetric method quantifies the cytotoxic effect as a function of the number of remaining cells after the infection with *M. tuberculosis*. Since this micro-organism is not stained by the dye, it does not produce a background that affects absorbance readings. As determined by CV assay (CVA), *M. tuberculosis* strain H37Rv destroyed 10.5 % of THP-1 cell monolayers at 24 h and 50.52 % at 72 h, while *M. tuberculosis* strains lacking the complete phospholipase C locus produced a reduced cytotoxic effect. The damage estimated by microscopy corresponded to the effect quantified by CVA. The results show that the use of CVA is a rapid, sensitive and reliable quantitative assay to measure the cytotoxicity of different *M. tuberculosis* strains.

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INTRODUCTION

Mycobacterium tuberculosis is a bacterial pathogen that produces a detrimental effect on mammalian cell cultures. This effect can be due to the induction of cell apoptosis or necrosis leading to cell death. The phenotype is reported as a cytotoxic or cytopathic effect (Castro-Garza *et al.*, 2002; Danelishvili *et al.*, 2003; Dobos *et al.*, 2000; McDonough & Kress, 1995) and it can be roughly quantified by analysing the altered cell morphology (cell rounding and loss of monolayer integrity) (Daniel *et al.*, 2004; Fischer *et al.*, 1996), setting up a scale to estimate the percentage of rounded or detached cells (Read *et al.*, 1974) or counting the amount of degraded cells by electron microscopy (McDonough & Kress, 1995). A more precise quantitative assay, such as measuring lactate dehydrogenase (LDH) release by using a colorimetric kit, has also been reported (Danelishvili *et al.*, 2003; Dobos *et al.*, 2000). However, most of the above experimental procedures are only semi-quantitative or are time-consuming.

There are other methods to quantify cytotoxicity; however, they are not practical for use with *M. tuberculosis* infection systems: exclusion or inclusion of vital dyes requires direct handling of samples, the release of radiolabelled substances increases the biosafety level, and the reduction of coloured compounds such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1) and other tetrazolium salts by bacteria (Franzblau *et al.*, 1998; Gomez-Flores *et al.*, 1995) as well as macrophages (Ferrari *et al.*, 1990) in *in vitro* infection models would produce a high background and the results obtained would not be accurate.

Crystal violet (CV) is a triphenylmethane dye also known as gentian violet. The most commonly used application for this dye is as the primary stain in the Gram-staining procedure. Gillies *et al.* (1986) used CV to quantify the cell number in monolayer cultures as a function of the absorbance of the dye taken up by the cells. This method has been used with modifications for a wide number of applications, most of them to determine cytotoxicity or cell death produced by chemicals, drugs, or toxins from pathogens (Harhaji *et al.*, 2004; Shaik *et al.*, 2004;

Abbreviations: CV, crystal violet; CVA, crystal violet assay; PLC, phospholipase C; WST-1, 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate.

Rothman, 1986), and to determine cell viability (Thomas *et al.*, 2004) or cell proliferation (Zivadinovic *et al.*, 2005) under different conditions.

A unique characteristic of *M. tuberculosis* bacilli is their outer lipid bilayer, which is the thickest biological membrane known. Because of its hydrophobic nature, the bacterial wall is not accessible to hydrophilic substances such as CV, and *M. tuberculosis* cannot be stained with the regular Gram-staining reagents, resulting in a remarkable Gram-neutral (neither positive nor negative) or Gram-ghost appearance of mycobacteria (Trifiro *et al.*, 1990). Therefore, the CV assay (CVA) becomes a very attractive method to quantify mycobacterial cytotoxicity, because CV will not react or be absorbed by the mycobacteria and any change in absorbance reading is due solely to the difference in the number of mammalian cells in a culture.

METHODS

Mycobacterial strains, culture and growth conditions. *M. tuberculosis* clinical isolates DR-689, DR-448 and DR-1289 lacking the *plcA-plcB-plcC* phospholipase C (PLC) locus were used for the present study. The extension of the deletions and the description of the changes in the locus are described in a previous paper (Vera-Cabrera *et al.*, 2007). *M. tuberculosis* H37Rv was included as a positive control for cytotoxicity. Stock cultures of mycobacterial strains were prepared in Middlebrook 7H9 broth supplemented with oleic acid/albumin/dextrose/catalase (OADC), grown to mid-logarithmic phase, and stored at -70°C until needed. Cell density for each stock culture was determined in triplicate as c.f.u. using Middlebrook 7H10 agar.

Cell cultures. Human monocyte cell line THP-1 was maintained in RPMI 1640 medium (Gibco-BRL) supplemented with 10 % fetal bovine serum (FBS; Gibco-BRL) and 1 mM sodium pyruvate (Sigma). In order to transform the cells into macrophages, the cells were subcultured four times without sodium pyruvate and then seeded into 24-well microplates (Costar Corning) at a concentration of 5×10^5 cells per well in complete RPMI 1640 supplemented with phorbol-12-miristate-13-acetate (PMA; Calbiochem Biosciences) at a concentration of 6.25 ng ml^{-1} . Cell cultures were washed twice with RPMI 1640 every 48 h for no longer than 4 days. Before infecting the cell cultures, the number of viable cells per well was determined using the WST-1 (Roche Molecular Biochemicals) reduction assay (Ishiyama *et al.*, 1993), plotting the A_{545} obtained in a standard curve of cell density. WST-1 is a tetrazolium salt that is cleaved by viable cells into a coloured product that can be measured spectrophotometrically. A_{545} values have a linear relationship with the number of viable cells. The average cell density of three wells per plate determined by WST-1 was used as the number of cells per well for each plate.

CVA. Control and infected cell cultures were fixed at the time points indicated for each experiment in 10 % buffered formalin for 24 h at 4°C . In all cases, the volume added to each well of the different solutions was 500 μl . Fixative solution was discarded and a 0.1 % aqueous CV solution was added to each well. The samples were incubated at room temperature for 30 min with gentle shaking. The plates were washed by submersion in flowing tap water for 15 min. Microplates were allowed to air dry and 0.2 % Triton X-100 in water was added to each well and incubated for 30 min at room temperature with gentle shaking to dissolve the dye. Then, 100 μl from each well was transferred into a fresh 96-well microplate and the A_{600} read in a microplate reader (EIA Microwell Reader II, Sigma).

Mycobacterial cytotoxicity assay. Before the beginning of the experiment, bacteria were thawed at 37°C , mixed vigorously using a vortex, and diluted in tissue-culture medium to obtain the desired density to infect the cells with an m.o.i. of 1 : 10 (bacilli : cells). Bacterial c.f.u. from infected cultures was determined at the beginning and end of each experiment in 7H10 medium supplemented with OADC.

Macrophage (THP-1 cell line) monolayers were infected in triplicate with the *M. tuberculosis* strains tested. Infected cultures were incubated at 37°C and 5 % CO_2 for up to 72 h. Every 24 h, the culture media from three wells from each strain and controls was discarded and the cells fixed in 10 % buffered formalin for at least 24 h at 4°C . A different microplate was used for each time point, to avoid the effect of formaldehyde vapour on the rest of the cell culture. The cytotoxic phenotype was observed qualitatively as disruption of the confluent monolayers over time using a phase-contrast light inverted microscope Axiovert 25 (Carl Zeiss).

The quantitative analysis of cytotoxicity on the stained cultures infected with *M. tuberculosis* was based on the percentage of dead cells, employing the following equation:

$$\text{Percentage dead cells} = ((\text{control } A_{600} - \text{sample } A_{600}) / \text{control } A_{600}) \times 100$$

All experiments were repeated three times in triplicate.

RESULTS AND DISCUSSION

A_{600} readings were proportional to the number of cells per well. Regression and correlation analysis of data (1.5×10^4 – 5×10^5 cells per well) showed a slope value of 0.2019 with an r^2 value of 0.9465 (Fig. 1). This result confirms the utility of CVA to determine viability or cell culture density, as reported previously (Gillies *et al.*, 1986).

In order to test a cytotoxic agent, we used Triton X-100, which is an anionic detergent that dissolves the cell membrane leading to cell death, and the effect depends on the concentration used. Triton X-100 produces a clear

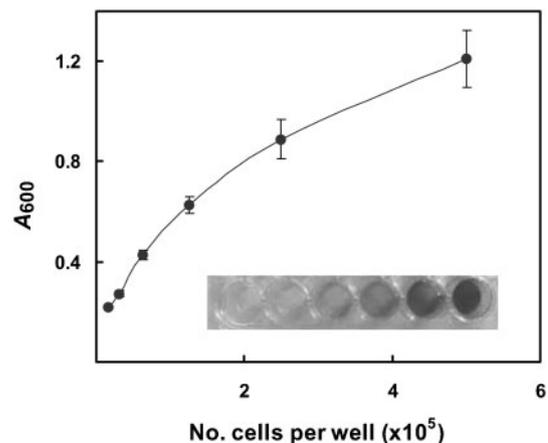


Fig. 1. CVA in the THP-1 cell line. Bars show mean \pm SD of three independent experiments done in triplicate. A row of an experimental microtitre plate is shown in which the wells correspond to the numbers of cells.

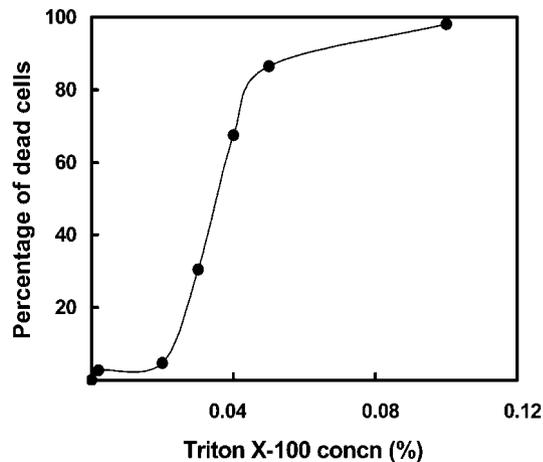


Fig. 2. Cytotoxic effect of Triton X-100 on the THP-1 cell line.

and increasing cytotoxic effect starting at a concentration of 0.05 % and reaching a maximum at 0.1 %, as measured with the CVA (Fig. 2). Regression and correlation analysis of data (three experiments each in triplicate) between 0.02 and 0.1 % Triton X-100 gave a slope value of 2821.5 and an r^2 value of 0.9865.

To test the ability of the method to quantify the cytotoxicity produced by *M. tuberculosis*, it was necessary that no background was caused by staining of the bacteria. As mentioned above, *M. tuberculosis* is not stained by CV and we found this feature very useful for this model. THP-1 cell monolayers were infected with a variable number of mycobacteria with an m.o.i. up to 10 : 1 (bacteria : cell), incubated for 6 h to allow the internalization of bacteria into the cells, and then immediately subjected to the CVA before any effect was produced by *M. tuberculosis* on the monolayers. Absorbance readings for all the different bacterial densities were very similar and no significant differences were found by an ANOVA one-way analysis ($P < 0.05$). This result supports the use of the CVA to quantify mycobacterial cytotoxicity, since no background was produced by the mycobacteria. Accordingly, any change in absorbance readings in infected monolayers would be produced exclusively by *M. tuberculosis* cytotoxicity.

In order to compare the cytotoxicity of different *M. tuberculosis* strains using the CVA, THP-1 cell monolayers were infected with *M. tuberculosis* H37Rv strain and *M. tuberculosis* clinical isolates DR-689, DR-448 and DR-1289

(all of them lacking the PLC locus). *M. tuberculosis* H37Rv produced a cytotoxic effect on THP-1 cell monolayers that was observed microscopically 24 h post-infection. After 72 h, it could be visually estimated that around 50 % of the cell monolayer was destroyed (Fig. 3). On the other hand, *M. tuberculosis* DR-689 and DR-1289 did not disrupt the cell monolayer 72 h post-infection. Strain DR-448 produced cytotoxicity at 72 h as determined by CVA. The CVA values obtained from these experiments are shown in Table 1. All the strains infected the cell cultures, as shown by the c.f.u. values for each strain determined at the end of the experiment, which were: DR-689, 3.5×10^4 ; DR-448, 2.3×10^4 ; DR-1289, 2.2×10^4 ; and H37Rv, 3.7×10^4 . The strain DR-689, with a yield of 3.5×10^4 c.f.u., produced no effect on cell cultures, while H37Rv, with a similar yield, had the highest cytotoxic effect. This result shows that the cytotoxicity is not only due to the intracellular growth but also the strain producing the activity. We have previously reported similar results with *M. tuberculosis* strains Erdman and CDC-1551, and *Mycobacterium bovis* BCG (Castro-Garza *et al.*, 1997). Although the clinical isolates used in this work lacked the PLC locus, they still had residual PLC activity (data not shown). There is a fourth phospholipase gene (*plcD*) in a different region of the genome. In H37Rv, the *plcD* gene is truncated and interrupted by a copy of the IS6110 insertion sequence. Raynaud *et al.* (2002) have demonstrated the importance of phospholipases in virulence by inactivating either each individual ORF or all of them concurrently and testing for virulence to mice; however, phospholipases are not the only mycobacterial product able to produce cytotoxicity.

Recently, Takii *et al.* (2005) have reported the use of CV to test the pyrazinamide susceptibility of *M. tuberculosis* in a fibroblast-based assay, in which the host-cell viability is reflected by the state of the bacilli inside the host cells. In this study, we demonstrated that the CVA can quantify the cytotoxicity produced by *M. tuberculosis* in a THP-1 cell monolayer. The ability of the CVA to measure this effect with other mycobacterial species remains to be tested, although a biofilm produced by *Mycobacterium avium* can be stained with CV (Carter *et al.*, 2003). Mycobacteria other than *M. tuberculosis*, such as *Mycobacterium ulcerans* and *Mycobacterium marinum*, are natural pathogens of poikilothermic organisms (Trucksis *et al.*, 2005), but produce only localized skin infections in humans (Ranger *et al.*, 2006), while *Mycobacterium abscessus* is commonly associated with contaminated traumatic skin wounds and

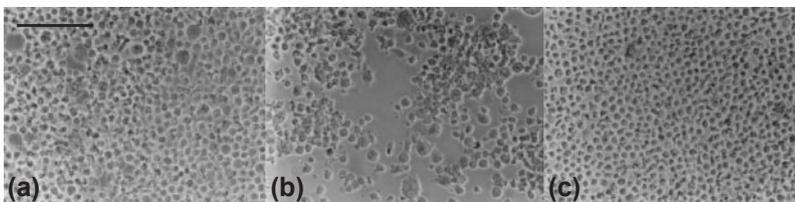


Fig. 3. Cytotoxicity of *M. tuberculosis* towards THP-1 cells. Cell monolayers 72 h post-infection are shown ($\times 250$). (a) Uninfected cells, (b) *M. tuberculosis* H37Rv infected cells, (c) *M. tuberculosis* DR-689 infected cells. Bar, 200 μm .

Table 1. Cytotoxicity of *M. tuberculosis* strains

Values show the percentage of dead cells, and represent the mean \pm SD of three independent experiments done in triplicate.

Incubation time	<i>M. tuberculosis</i> strains			
	DR-689	DR-448	DR-1289	H37Rv
24 h	0	0	0	10.50 \pm 1.05
72 h	0	20.51 \pm 1.53	0	50.52 \pm 6.32

with post-surgical soft-tissue infections (Petrini, 2006). These mycobacterial species exhibit cytotoxicity towards cell cultures or have a necrotic effect in their pathologies (Torrado *et al.*, 2007; Ranger *et al.*, 2006). It would be very interesting to apply the CVA to measure and compare their activities, either between species or among mutant strains of a single species, such as the *M. ulcerans* mutant lacking the mycolactone toxin, which has only been analysed by altered cell morphology (Daniel *et al.*, 2004).

The results of this work support the use of the CVA as a rapid, sensitive and reliable quantitative assay to measure the cytotoxicity of *M. tuberculosis* and to compare differences in activity between strains that could potentially be related to virulence.

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