**Mycobacterium “habana” TMC 5135 as a Vaccine Candidate against Tuberculosis: In vitro Studies**

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**Abstract**

**Background:** Tuberculosis (TB) is one of the biggest problems of global health, at present. Bacillus–Calmette–Guérin is the only vaccine available against this disease. It protects only against the severe forms of TB in the childhood, which is a challenge in the search of new vaccine candidates. Taking into account the protective history of Mycobacterium “habana” against experimental TB, we proposed to provide the elements that support the use of M. “habana” TMC 5135 as a vaccine candidate against TB by infection studies in murine macrophages cell cultures.

**Methods:** The production of microbicidal compounds dependent on oxygen metabolism as nitric oxide and hydrogen peroxide by murine peritoneal macrophages was detected. The invasive and toxigenic capacity of M. “habana” to infect this cell type was also evaluated through the quantification of intracellular alive bacillus and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide assay, respectively.

**Results:** The results suggest that M. “habana” TMC 5135 is able to persist into peritoneal macrophages, to resist the effectors mechanisms of respiratory burst, and to keep the viability of the target cell. The demonstration of these effector mechanisms and the survival capacity of M. “habana” in this niche are relevant aspects of this research assuring the continuity of this candidate to next phases of preclinical development.

**Conclusion:** The present investigation contributes to the characterization of the infection by this mycobacteria in its main target cells of innate immunity and it suggest future investigations to evaluate the activation of effector mechanisms of the innate immunity against this candidate.

**Keywords:** Macrophages, Mycobacterium “habana”, vaccine

**Introduction**

The Bacillus–Calmette–Guérin (BCG) vaccine is the only regimen available for tuberculosis (TB) prevention. The vaccination with BCG presents a low cost and a long history of safety as well as a protective efficacy of >80% for several forms of disease in childhood. However, studies show that although its protection is transitory, in a range of 10–20 years, its efficacy is variable (0%–80%) in the different geographical regions and does not protect against lung disease.[1]

It is precisely for these disadvantages that the search of new alternatives of vaccination against TB has increased in recent years. *In vivo* experimental studies have proved the protection after *Mycobacterium “habana”* vaccination in murine models challenged with *Mycobacterium tuberculosis* H37Rv, reporting percentage of survival (20%) higher than BCG vaccination under the same experimental conditions. However, we should take into account that for the development and approbation of all vaccines, candidate is necessary to know its behavior during *in vitro* infection of its main cell targets.[2]

The present work evaluates the phagocytosis process of M. “habana” by murine macrophages (Mφ) cell cultures, aspects of innate immune response of this cell type, as well as invasive and toxigenic capacity of these mycobacteria.

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Methods

This was an experimental descriptive study carried out at The National Reference Laboratory of Tuberculosis, PAHO/WHO Collaborating Center, Center for Research, Diagnosis and Reference (CIDR), Tropical Medicine Institute “Pedro Kouri” (IPK), Havana, Cuba.

Bacteria

*M. habana* TMC 5135 strain deposited in Trudeau Mycobacteria International Collection (Trudeau Institute, Saranac Lake, NY 12983, EUA) was inoculated in 250 mL of Middlebrook 7H9 medium (DifcoLabs, Detroit, EUA) supplemented with oleic acid-albumin-dextrose-catalase (OADC, Becton Dickinson, EUA). The culture was incubated at 37°C for 10 days in an orbital agitator (IKA Labortechnik) at 300 rpm. The macrophages were infected attendand to two infection multiplicity (MOI), one bacteria for macrophage (MOI 1:1) and five bacteria for macrophage (MOI 5:1).[3]

Cell culture

The mice were sacrificed by cervical dislocation[4] and were retired the peritoneal skin to exhibit the peritoneum. They were injected 5 mL of RPMI medium (Sigma) supplemented with bovine fetal serum (BFS) cold and sterile in the peritoneal cavity and were given a slight massage. The liquid deposited was extracted and was passed to sterile cold tubes to avoid the cell adherence. Later, it was diluted 1/10 of the cell suspension with trypan blue (Sigma) and the cells were counted in Neubauer camera (Western Germany). The cell concentration was adjusted to 2 × 10⁵ cell/well.[5] The plates were incubated overnight at 37°C in CO₂ atmosphere to guarantee the cell adherence and the monocape formation.

Detection of nitric oxide and hydrogen peroxide

The bacterial lipopolysaccharide (LPS) was used as positive activation control to a reason of 10 µg/mL. The same medium of cell culture was used as negative activation control. The bacterial inoculum was applied and the plates were incubated at 37°C in CO₂ 5% for 2 h. At this time, the bacterial stimulus was retired and was made successive washed with RPMI medium supplemented with BFS 10%. The plates were incubated again in the same conditions for 72 h.

For the nitric oxide (NO) detection, 50 µL of equal combination of reactive of Griess and N-1 naftilan diamine hydrochloride (0.1%) was added and the plate was incubated for 10 min at the environmental temperature. The reading was executed in a spectrophotometer (λ = 560 nm).[3] In parallel a patron curve was elaborated since a solution of sodium nitrite 625 µM in order to extrapolate the results.

For the hydrogen peroxide (H₂O₂) detection, one time that the supernatant was eliminated; 100 µL of a buffer compound solution was added and was incubated at the environmental temperature in darkness during 1 h. At this time, 10 µL of NaOH 1M was applied. In parallel, a patron curve since H₂O₂ 2000 µM was elaborated. The reading was executed in a spectrophotometer at λ = 630 nm.[5]

Quantification of intracellular alive bacillus

The bacterial inoculum was applied for 2 h, and then, the supernatant was removed to eliminate the nonphagocyte bacilli and was made washed thrice with RPMI medium. The first assay was executed at 2-h postinoculation (considered as zero time). The supernatant was removed and 100 µL of sterile distilled water was added in each well to break the cells and to guarantee the bacillus liberation. The lysed was vigorously homogenized and incubated during 1 h at 37°C in CO₂ 5%. Later of this time, serial dilutions in PBS to the content of each well were done and were inoculated in plates with Middlebrook 7H10 medium supplemented with OADC. These were incubated at 37°C for 14 days. The assay was repeated at 24, 48, and 72 h postinoculation.[6]

Cell cytotoxicity assay

Murine Mφ without infecting was used as negative control. The nonphagocyte bacillus was eliminated through two washed with RPMI medium, and the infected cells were incubated for 72 h in the same conditions.[7] At this time, were added 10 µL of 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) solution (thiazohyl blue tetrazolium bromide, Sigma) 5 mg/mL suspended in PBS previously filtered. After 3 h of incubation at 37°C in CO₂ 5%, it was given a dry beat to the plates to eliminate the supernatant and was added 150 µL of (dimethyl sulfoxide, Sigma). The plates were incubated in the same conditions during 30 min. The reading was executed in a spectrophotometer (λ = 630 nm as reference).[5] Later, the viability percentage (VP) was calculated for each MOI.[3]

\[ \text{VP} = \frac{\text{OD average of problem well}}{\text{OD average of control well}} \]

Where OD refers to optic density.

Statistics analysis

The results of this research are showed in tables and graphics using The Excel Microsoft Program (2013). The statistics analysis of these results was done through NCSS program (version 10.0.9). This experiment account with several replies and in all them was calculated; the average and standard deviation were analyzed through analysis of variance of one factor. In all cases were considered significant differences for values of \( P < 0.05 \).

Ethics considerations

In the work with animals of laboratory, the 3 Rs principle (reduction, replace, and refinement) was respected.[9] The experimental design was in concordance with the international regulations for the manage and care of experimentation animals[10] and good practices of laboratory for preclinical studies.[11]

Results

Nitric oxide and hydrogen peroxide detection

Table 1 shows the values of NO concentration associated at positive control and the MOI.

The concentrations of NO calculated were in patron curve range, giving accuracy to the results. In this assay, significant
differences were not obtained among the MOI and the activation positive control.

The values of H$_2$O$_2$ concentration corresponding to the MOI and positive control are showed in the Table 2. The H$_2$O$_2$ concentrations detected were not statistically different.

**Quantification of intracellular alive bacillus**

The kinetic of bacillary multiplication inside infected Mφ is showed in Figure 1. In the case of MOI 1:1, a decreasing kinetic throughout all experiment was observed. At 2 h, the cell concentration was of $2.5 \times 10^3$ cell/mL. This result revealed that of the initial concentration applied ($2 \times 10^5$ cell/well) around the half was phagocyted by Mφ. At 72 h, a decrease of bacillary survive of around 80% was showed. For MOI 5:1 at 24 h post-inoculation, there was a considerable increase of UFC number regarding 2 h of assay with a maximum pick of concentration, while that the following times of assay showed a gradual decrease of bacillary concentration. At 72 h, the concentration of bacillus inside Mφ fell in around 75%.

**Cell cytotoxicity assay**

Figure 2 shows the survival percentages of infected cells with different concentrations of *M. “habana”* regarding control without infect. The results did not show significant differences ($P > 0.05$) among MOI 1:1, MOI 5:1, and control.

**DISCUSSION**

Prophylactic vaccines are one of the most useful and cost-effective tools in reducing morbidity and mortality associated with infectious diseases.[12] The experts in anti-TB vaccines of the WHO defend the use of attenuated alive vaccines against this disease.[13] These immunogens constitute potent stimulators of a protective immune response taking as advantage the spectrum of antigenic expression as well as the ability to stimulate a combination of different subpopulations of T-lymphocytes.[14,15]

Phagocytic cells as Mφ are the first line of defense against the invasion of bacterial pathogens. Their activation includes the generation of Reactive oxygen intermediates and Reactive nitrogen intermediates as result of respiratory burst. This is a fast process as response to phagocytosis of microorganisms accomplished by inducible enzymatic systems as phagocytic oxidase and syntase of inducible NO, respectively.[16]

The concentration values of NO and H$_2$O$_2$ obtained were not significantly different in comparison with positive activation control (LPS). These experiments confirm that this mycobacteria induce in murine peritoneal macrophages the production of these compounds in the same concentration of LPS; therefore, these mycobacteria are a powerful stimulator of effector mechanisms of innate immune response in this cell type.

The ability of microorganisms for cause disease is known as virulence and for some of them; this depends on their capacity to survive inside host cells. The experiment of intracellular survival allowed to evaluate the persistence of *M. “habana”*
TMC 5135 in murine peritoneal Mφ, phenomenon highly related with mechanisms of evasion of immune response.

In this experiment for the MOI 5:1, a duplication of bacillary concentration was observed at 24 h in comparison with 2h of assay. This result can be explained because a higher bacillary concentration slowed the activation of effector mechanisms of Mφ and allowed the bacillary multiplication. This is corroborated if we take into account that the mycobacteria multiplication time is between 12 and 24 h. In our study with none MOI, the whole sterilization at 72 h persisting these mycobacteria inside Mφ was obtained. This result guarantees a sustained antigenic stimulation that could be related with evasion mechanisms of immune response. This study was in correspondence with Welin and Lerm, who proved that the Mφ infection to low MOI allows the phagosome effective maturation accompanied of a decrease of intracellular growth but not of bacterial elimination.

The cell cytotoxicity is the alteration of basic cell functions that generate a damage that can be detected. The cytotoxicity assay-employed MTT is based in its metabolic reduction executed by mitochondrial enzyme succinate-dehydrogenase in a blue-colored compound (formazan). The cell capacity to reduce the MTT is an indicator of mitochondria integrity and its functional activity is interpreted as cell viability.

Our results suggest that the viability of Mφ infected with M. “habana” TMC 5135 was not different among the two MOI and the control without infect. This proves that M. “habana” generates low cytotoxicity in murine peritoneal Mφ.

**Conclusion**

In the long way that imply to demonstrate the vaccine potential of M. “habana,” this research contributes with the infection characterization in its main cell target of innate immunity. The demonstration effector mechanisms dependent of oxygen activation and the survival capacity of M. “habana” in this niche are relevant aspects of this research guaranteeing the continuity of this candidate to next phases of preclinical development.

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**Conflicts of interest**

There are no conflicts of interest.

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