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Fate of *Mycobacterium tuberculosis* in peroxidase-loaded resting murine macrophages

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ABSTRACT

Background: Myeloperoxidase (MPO), in the presence of hydrogen peroxide and a halide represent an efficient microbicidal mechanism of phagocytic cells. MPO is abundant in neutrophils which also respond to infection by producing large amounts of reactive oxygen species (ROS). MPO, ROS and halide constitute a very toxic antimicrobial system (called the Klebanoff system or KS). Resting mature macrophages do not contain granular MPO and thus are unable to kill pathogenic mycobacteria and some other microorganisms by this system. *Experimental:* Under the hypothesis that transforming macrophages into peroxidase-positive (PO^+) cells, these cells would be able to kill *Mycobacterium tuberculosis*, in this study, mature macrophages were loaded with exogenous peroxidase and were tested for their capacity to kill the *Mycobacterium* in the presence or in the absence of hydrogen peroxide. *Results:* It was found that PO-loaded macrophages eagerly ingest *M. tuberculosis*, but do not show a significant mycobactericidal activity on this microorganism despite that it is highly susceptible to the Klebanoff system *in vitro*. Failure of PO-loaded macrophages to kill *M. tuberculosis* may obey either to an inappropriate location of the exogenous PO in these cells or more likely, to the presence of efficient detoxifying mechanisms in the bacteria. On the contrary, MPO-loaded or unloaded macrophages efficiently killed *Listeria monocytogenes*. *Conclusion:* The lack of granular MPO in mature macrophages, and the predilection of mycobacteria to infect these cells are two situations that favor the development of tuberculosis and related diseases, such as leprosy and Buruli ulcer.

Introduction

Since the identification, by Seymour J. Klebanoff, of the myeloperoxidase–hydrogen peroxide–halide (MPO–H_2O_2–h) system as a microbicidal weapon of utmost importance for phagocytic cells [1], this system has been the subject of intense research. Nowadays, this system continues to be researched, not only as a microbicidal weapon, but also as an important pathogenetic mechanism, because MPO-derived oxidation has been implicated in degenerative processes and cancer development, among other pathologies [2–6].

No doubt exists that MPO-derived oxidation is a key mechanism for the destruction of most invasive microorganisms and that failure of this mechanism very often leads to disease. The clearest example of its importance is chronic granulomatous disease (CGD). This is an illness in which both neutrophils and monocytes fail to produce hydrogen peroxide (the substrate of MPO) because of an alteration in the NADPH–

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oxidase (NOX2) system, resulting in patients who remain persistently infected [7,8]. Hydrogen peroxide itself is toxic to most microorganisms, but its toxicity increases greatly in the presence of MPO [9,10]. Although CGD neutrophils are able to kill some microorganisms, they cause little damage to catalase-positive germs because this enzyme destroys the minute amounts of H_2O_2 produced via NOX2-independent mechanisms or by the microorganisms themselves [1,10].

In the case of tuberculosis, it has been largely debated whether mature macrophages can kill *Mycobacterium tuberculosis*. The consensus is that unless these cells become activated by lymphocyte-derived cytokines, namely interferon gamma (IFNγ) or tumor necrosis factor alpha (TNFα), they possess a limited capacity to kill this microorganism [11]. Four main reasons for this have been proposed: (a) virulent mycobacteria, including *Mycobacterium leprae*, *M. tuberculosis*, *Mycobacterium avium*, and *Mycobacterium lepraemurium*, enter the macrophages without significantly triggering the production of reactive oxygen and nitrogen species (ROS and RNS) [12,13]; (b) mature macrophages lack MPO and therefore do not complement the microbicidal Klebanoff system (KS) [14]; (c) these bacteria possess a cell wall with a complex structure that is difficult for most toxic agents to penetrate [15]; and (d) pathogenic mycobacteria are endowed with efficient detoxifying mechanisms (see Discussion below). In the absence of ROS or MPO, the microbicidal KS is incomplete and is therefore barely functional. Nevertheless, *in vitro*, *M. tuberculosis* is killed by the KS [16] and also by nitric oxide (NO) and NO-derivatives [17]. *In vivo*, macrophages are activated through type 1 cytokines, namely IFNγ and TNFβ, to produce NO, which is a potent microbicidal agent [11]. It is unclear, however, whether the KS operates in mature macrophages, as these cells lack MPO [16,18,19]. In the granulomas of leprosy, mycobacteria appear within MPO-negative macrophages located at the center of the granulomas, surrounded by a multilayer of bacilli-free MPO-positive cells, which are likely immature monocytes or neutrophils (Fig. 1) [20–22]. The theory that arose from these findings was that if macrophages possessed MPO, they would be able to kill the mycobacteria; using this hypothesis, the anti-*M. tuberculosis* microbicidal capacity of macrophages that are supplied with exogenous peroxidase (horse radish peroxidase, HRPO), in the presence or the absence of hydrogen peroxide, was investigated in this study. HRPO was used because it has been observed in this study and others that this enzyme efficiently substitutes for MPO in the KS *in vitro* [13].

Materials and methods

The strategy followed in this work included the assessment of: (a) the *in vitro* susceptibility of *M. tuberculosis* (MTB) to the KS; (b) the ability of MTB to trigger the respiratory burst in intact macrophages (measured as the production of H_2O_2 and chemo-luminescence emission); and (c) the anti-MTB activity of intact macrophages and of macrophages loaded with HRPO in the presence of non-toxic amounts of hydrogen peroxide. Some of these assays were performed in comparison to the behavior of *Mycobacterium bovis* BCG (BCG) or *Listeria monocytogenes* (LIS).

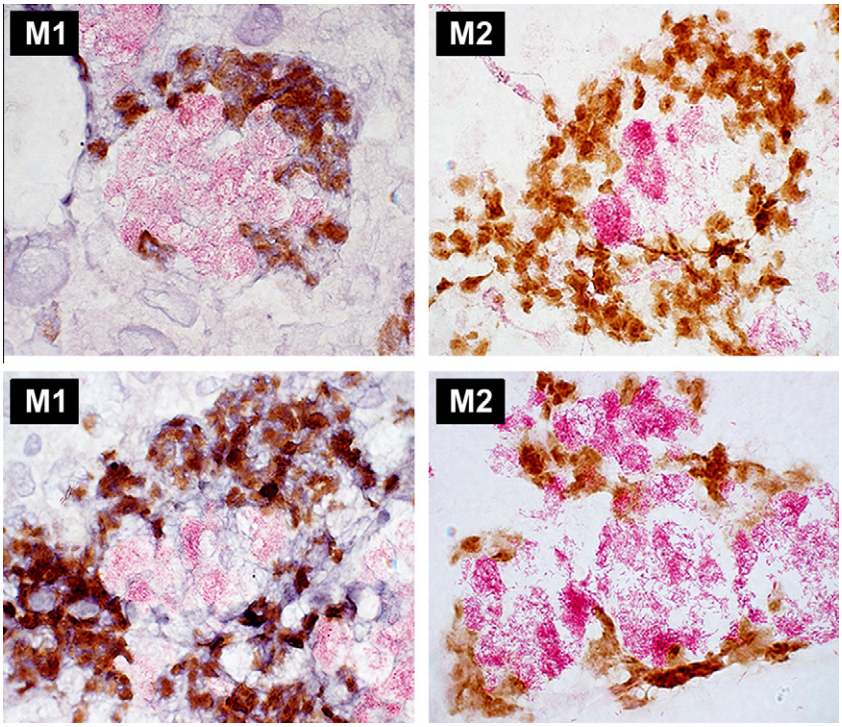

Fig. 1 – Mature granulomas in the liver of a mouse infected with *M. lepraemurium*. Notice the presence of bacilli in the MPO-negative macrophages at the center of the lesions, and the presence of MPO activity in the bacilli-free peripheral cells. Ziehl–Neelsen and MPO stains. 10 × 12.5X.

Chemicals

Unless otherwise specified, the chemicals were purchased from Sigma–Aldrich Chemical Co., St. Louis, MO, USA.

Mice

Healthy, albino NIH adult (24–26 g) female mice were used in this study. They were manipulated following the Mexican Official Norm (NOM-062-ZOO-1999) for the Correct and Humane Handling of Experimental Animals.

Bacilli

The H37Rv strain of *M. tuberculosis* (MTB) (ATCC 25618) was used as the test microorganism. When not in use, this microorganism was kept under refrigeration, growing on Lowenstein–Jensen medium. When in use, a loop-full portion of the grown bacteria was deposited onto the surface of 50 ml of 7H9 Middlebrook medium supplemented with 10% OADC (Becton Dickinson Co., Sparks, MD, USA) and was incubated at 37 °C. At confluence, the superficial bacterial mass was collected, suspended in sodium glutamate, stirred with the aid of a magnetic bar for 4 h at room temperature, washed by centrifugation with PBSG (0.1% glucose in 0.01 M phosphate–0.15 M sodium chloride, pH 7.4), and the pellet was suspended in RPMI medium supplemented with 10% FCS. The bacterial concentration was determined and adjusted in reference to a nephelometer calibration curve previously prepared in the laboratory.

Control microorganisms included *M. bovis* (BCG) and *L. monocytogenes* (LIS) (ATCC 43249, Manassas, VA, USA). BCG was expanded from a BCG vaccine and was prepared in a similar manner as MTB. LIS was cultured in BHI medium at 37 °C for 6 h, harvested by centrifugation (6000 rpm/5 min/4 °C), washed, and suspended in BHI medium to the desired concentration.

Macrophages

Non-elicited macrophages were collected from the peritoneal cavity of adult female NIH mice. In the chemiluminescence experiments, because of the number of cells needed, the macrophages were induced by the intraperitoneal injection of 1.0 ml of sterile light mineral oil per mouse, 5 days before cell collection. It was observed that mineral oil itself does not stimulate the oxidative response of macrophages.

Collection of macrophages

Mice were sacrificed by CO_2 inhalation, heart-exsanguinated, and prepared for peritoneal cell collection. The collection of cells was a simple procedure and consisted of pin-fixing the dead mice upward on a dissection board. The abdominal skin was lifted, and 5 ml of Alsever's solution (20.5 g of glucose, 8.0 g of sodium citrate, 0.55 g of citric acid, 4.2 g of sodium chloride, and water to 1.0 L) was injected into the peritoneum through the xiphisternum. After a gentle massage, a region in the middle part of the abdomen was pinched with a "mosquito" forceps, and a small incision was made on the abdomen. Through this incision, a bent-tip Pasteur pipette was introduced to withdraw the injected fluid. The procedure was repeated, and the washings from five mice were pooled and centrifuged at 1500*g* for 5 min at 4 °C. The cell pellet was suspended in 3 ml of 0.2% NaCl for 1 min to lyse erythrocytes, and then, 3 ml of 1.6% NaCl was added to restore isotonicity. Finally, Alsever's solution up to 12 ml was added, and the cell suspension was centrifuged as above. The final cell pellet was suspended in 1 ml of RPMI 1640 with 10% of FCS (Fetal Calf Serum), and the cells were counted in a hemocytometer and adjusted to 50×10^6 cells per ml.

Viability of *M. tuberculosis* and mouse macrophages through reduction of Alamar Blue

The Alamar Blue reduction test was used to monitor the viability of both bacteria and macrophages that had been subjected to different treatments. Alamar Blue (DAL 1100 Biosource, Invitrogen, CA, USA) is a resazurin that detects all of the oxide-reduction reactions related to cellular metabolism that occurs within a cell [23]. Routinely, the viability of *M. tuberculosis* was monitored with the bacteria suspended in Middlebrook 7H9 broth (Beckton Dickinson [BD], Sparks, MD, USA) supplemented with 10% OADC (BD), while the viability of macrophages was monitored with the cells suspended in RPMI-FCS medium. The metabolic activity, which can refer to cell number or viability, is reflected in the amount of emitted fluorescence (544 nm/590 nm) that is read by a fluorometer (Fluoroskan Ascent FL, Thermo Fisher Scientific Inc., Vantaa, Finland). When the number of bacteria had to be counted as colony forming units (CFU), this was done by the micro drop technique on Middlebrook 7H10 agar (BD) plates.

Cultivation of macrophages

For the cultivation of macrophages, acid-washed, sterile cover slips were placed into 3-cm Petri dishes, and 2 ml of the macrophage suspensions (with 4×10^6 mononuclear cells) were deposited per dish. The Petri dishes were incubated at 37 °C/5% CO_2 for 5 days to allow the monocytes to differentiate into macrophages. At this time, non-adherent cells and cell debris were removed by gently washing with RPMI-FCS, and the viability of the adherent cells was assessed by staining with Trypan blue solution for 10 min. Only macrophage mono layers showing over 90% viability were used in this study.

Infection of macrophages

Infection of the macrophages was performed on macrophage mono layers that were prepared as described above. The culture medium from each dish was removed, and fresh medium with bacilli (MOI 10:1) was added. After 0.5, 1, 2, 4 or 6 h of incubation at 37 °C/5% CO_2, the cell cultures were gently washed with sterile PBSG, then 1.0 ml of fresh medium with 40 μg of amikacin was added per dish to inactivate the extracellular bacteria, and the cultures were incubated for 40 min. After rinsing the cell cultures with PBSG, the cell-containing slides were recovered, fixed in 4% paraformaldehyde for 10 min, rinsed with water, stained for acid fast bacilli (Ziehl–Neelsen stain) and counterstained with Harris' Hematoxylin.

Chemiluminescent response of macrophages

To obtain an enriched population of macrophages, mice were injected in the intraperitoneal space with 1.0 ml of light mineral oil 5 days before macrophage harvesting. Mineral oil does not biochemically stimulate macrophages in a noticeable manner. The macrophages were collected according to the procedure mentioned earlier, and the final suspension was adjusted to 5.0×10^6 cells per ml in RPMI-FCS.

Chemiluminescence (CL) was measured in a Beckmann scintillation counter (LS 6000SE, Brea, CA, USA) in plastic vials containing 5×10^6 cells in 1.0 ml RPMI-FCS, 20 μl of luminol (1 mg/ml of dimethyl sulfoxide, DMSO), and the corresponding stimulus (1.0 μg of PMA, *M. tuberculosis* or BCG at the selected MOI, or PBS as a negative control). Immediately after adding the stimulus, CL was registered for 20 min, and the results were plotted using Sigma Plot.

In vitro susceptibility of M. tuberculosis to the Klebanoff system

To assess the susceptibility of *M. tuberculosis* to the Klebanoff system, aliquots of 1.195×10^9 bacilli were incubated in the presence of 0.5 μg of HRPO (100 IU) in PBSG and 0.25 or 0.88 mM H_2O_2, at 37 °C/5% CO_2 for different time periods (0, 1, 2 and 4 h). The aliquots were washed with PBSG (8000*g*/1–3 min) to eliminate the unreacted components, and the bacilli were suspended and diluted to contain 67×10^6 bacilli in 200 μl of RPMI-FCS. Triplicate 200-μl samples and 20 μl of stock Alamar Blue (BioSource, Camarillo, CA, USA) were deposited in 96-well plates, and the plates were incubated at 37 °C/5% CO_2. Emission of fluorescence was recorded at 0, 1, 24 and 48 h in a fluorometer at 544/590 nm. The 0.25 mM concentration of H_2O_2 was reported to be highly microbicidal for *M. tuberculosis* in the presence of eosinophil peroxidase (EPO) by Borelli et al. [24].

In vitro susceptibility of M. tuberculosis to supra physiologic concentrations of hydrogen peroxide and nitric oxide

To assess the toxicity of higher concentrations of hydrogen peroxide and nitric oxide on *M. tuberculosis*, the protocol described by Firmani and Riley [25] was used. In this study, 2×10^7 bacteria suspended in 0.1 ml of PBSG were mixed with 0.9 ml of Middlebrook 7H9 culture medium (pH 5.4) containing different amounts of sodium nitrite (0, 2, 4, 6, and 8 mM) or 0.9 ml of Middlebrook 7H9 (pH 7.0) containing differing amounts of hydrogen peroxide (0, 2, 4, 6, and 8 mM). Control bacteria were suspended in plain PBSG. Each bacterial suspension was then incubated for 16 h at 37 °C/5% CO_2, and the incubated suspensions were diluted from 1:10 to 1:10000 in Middlebrook 7H9, pH 7.0. Then, 10 μl volumes of each suspension were plated on Middlebrook 7H10 (semisolid) medium supplemented with 10% OADC, and the plates were incubated at 37 °C for 12 days; then, the number of colonies was counted, and the value was transformed to colony forming units (CFU) per ml. This method for counting CFUs was used in all of the experiments in which the number of viable bacteria had to be assessed.

Effects of HRPO on the viability of mouse macrophages

Cultures of the mouse peritoneal macrophages (5×10^5 cells in 0.1 ml of RPMI-FCS per well) were incubated in the presence of 2–40 μg of HRPO for 1 h at 37 °C/5% CO_2. The medium was then discarded and replaced with fresh medium. The cultures were further incubated at 37 °C in the presence of 20 μl per well of fluorescent Alamar Blue (stock) for 1, 2, 3, 4, 5, 6, 24, and 48 h. Cell viability was assessed from the emitted fluorescence (relative fluorescence units) using a fluorometer.

Effects of hydrogen peroxide on the viability of mouse macrophages

Cultures of 5×10^5 cells in 0.1 ml of RPMI-FCS were inoculated in triplicate in 96-well plates and were incubated in the presence of twofold increasing amounts of hydrogen peroxide (from 0.6 to 150 μg per well) plus 20 μl of Alamar Blue, for 0–8 h at 37 °C/5% CO_2. The emitted fluorescence was determined using a fluorometer.

Ex vivo susceptibility of M. tuberculosis to the Klebanoff system

Macrophage cultures (2×10^6 cells per ml, per well) in RPMI-FCS were treated with: (a) 20×10^6 MTB (MOI 10:1); (b) MTB plus the Klebanoff system (3.33 IU of HRPO and 0.1 mM H_2O_2; (c) HRPO-coated MTB; and (d) MTB-HRPO and 0.1 mM H_2O_2, or MTB-HRPO plus PMA (1.0 μg per well). After 24 h of incubation at 37 °C/5% CO_2, the cultures were treated with Amikacin (20 μg per well) for 2 h to kill the extracellular bacilli. The cultures were gently washed to eliminate any non-phagocytosed bacteria; then, the cells were lysed (with 0.5 ml of 0.25% Triton X-100 per well) for 1 min to release the ingested bacteria, and the lysates were recovered and centrifuged at 10,000*g* for 1 min. The sediments were suspended in 1 ml of PBSG and serially diluted from 1:10 to 1:1000; then, 10 μl of each dilution was added to Middlebrook 7H10 medium to determine the number of the colonies (CFU) after 12 days.

Ex vivo susceptibility of L. monocytogenes to the Klebanoff system

Macrophage cultures (2×10^6 cells per ml/well), intact, or preloaded with HRPO (3.33 IU per well), were infected with *L. monocytogenes* (ATCC 43249) (MOI 10:1) for 1 h at 37 °C. The non-ingested bacteria were washed away with warm PBSG, and the cultures were incubated for 24 h (37 °C, 5% CO_2) in medium containing 10 μg per ml of gentamicin. After removing the medium, the macrophage mono layers were lysed with 0.25% Triton X-100 for 1 min, and the lysates were collected and centrifuged (10,000*g*/3 min); the resulting sediments were suspended in 1.0 ml of PBSG, diluted from 1:10 to 1:1000, and 10 μl aliquots of each dilution were plated on BHI agar medium. After 16 h of incubation (37 °C), the number of CFUs was determined and used to calculate the number of CFUs per ml. Analogous dilutions and aliquots of *L. monocytogenes* were used as a control system.

Statistical analysis

As comparisons were direct between control and individual experimental groups and the samples were small ($n = 9–12$), the two-sample *t*-test was used to analyze the results comparatively.

Results

Infection of macrophages with M. tuberculosis

The infection of macrophages with MTB occurred rapidly and increased in a time-dependent manner. By 1 h of infection,

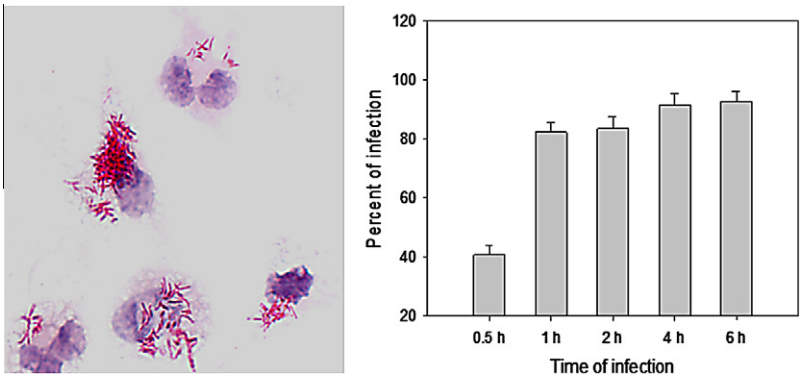

Fig. 2 – Infection of macrophages by MTB at different incubation times (MOI of 10:1). Left panel: a representative image of cells containing bacilli at 1 h of infection (Ziehl–Neelsen and Harris' Hematoxylin stains, 100 × 12.5X). Right panel: percentage of cells that ingested bacilli at the indicated infection times (percent).

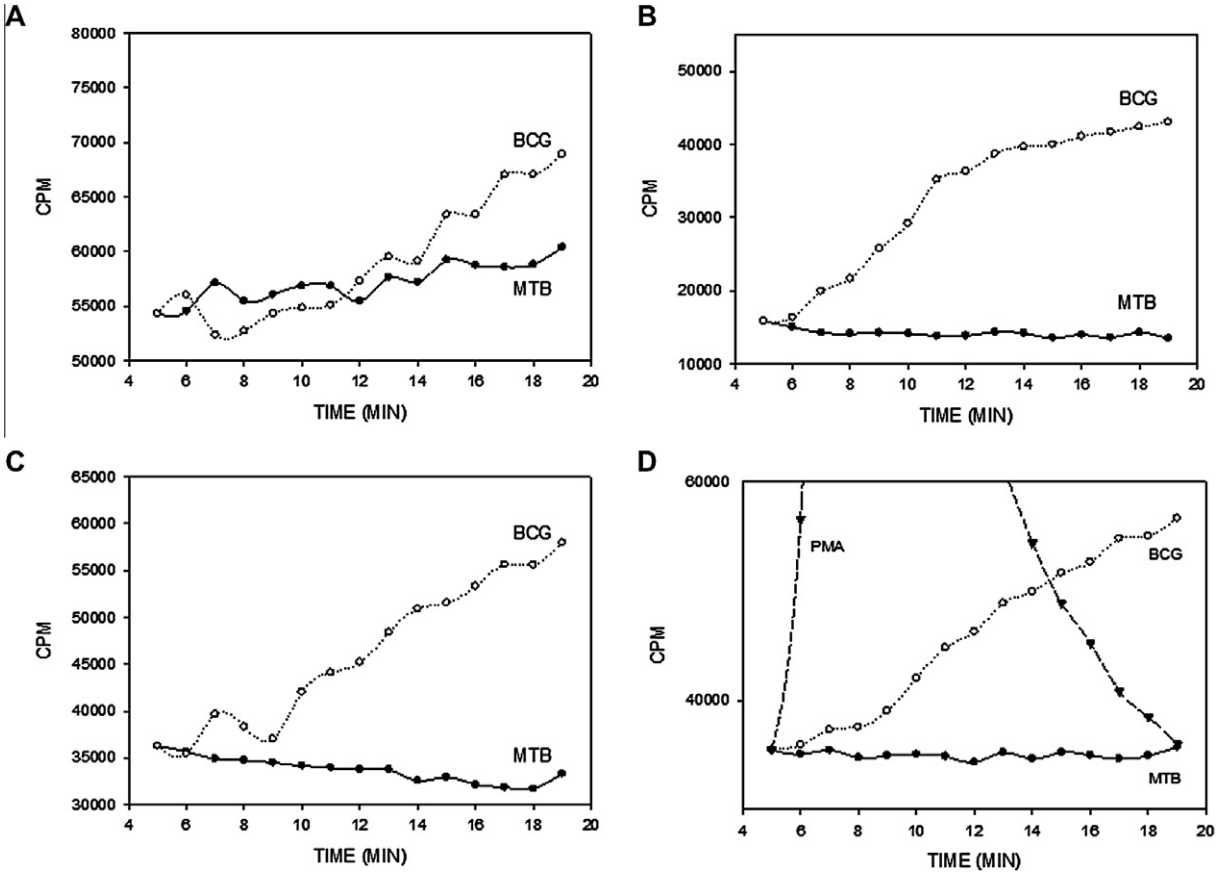

Fig. 3 – Chemiluminescence emitted by mouse macrophages infected with BCG or MTB at a MOI of 10:1. A–C, three independent experiments; D, average CL value from experiments A–C, and average CL response of macrophages stimulated with PMA. At this MOI, BCG, but not MTB, is able to induce the CL response at a significant level.

over 80% of the macrophages became infected, and the degree of infection increased to 90% after 4 or more hours. The number of bacilli per cell was highly variable and ranged from one to uncountable (Fig. 2). From this result, the standard infection time of macrophages for all of the experiments was fixed at 1 h.

Chemiluminescent response of macrophages infected with mycobacteria

When used at a MOI of 10:1, *M. tuberculosis* H37Rv did not induce a detectable CL response of macrophages; BCG, however, stimulated a significant CL response, which did not surpass the CL response induced by PMA (Fig. 3). Nevertheless, when tested at a MOI of 50:1, both mycobacteria induced a significant CL response, which was slightly higher with BCG than with MTB (Fig. 4).

In vitro susceptibility of M. tuberculosis to the Klebanoff system

Under the conditions described in the "Materials and methods" section, *M. tuberculosis* was not susceptible to the toxic effects of the Klebanoff system in the presence of 0.25 mM

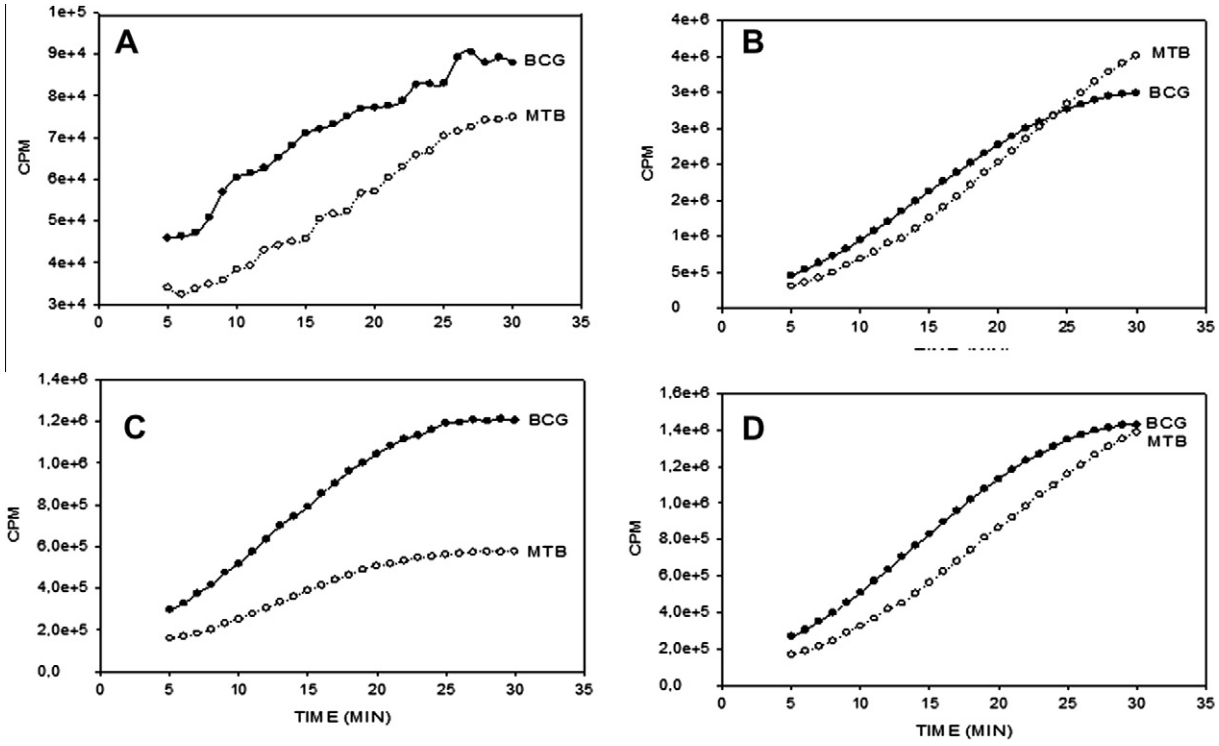

Fig. 4 – Chemiluminescence (CL) emitted by mouse macrophages stimulated with BCG or MTB at a MOI of 50:1. A–C, three separate experiments; D, average CL value from experiments A–C. CL was always, though variably, higher when macrophages were stimulated with BCG.

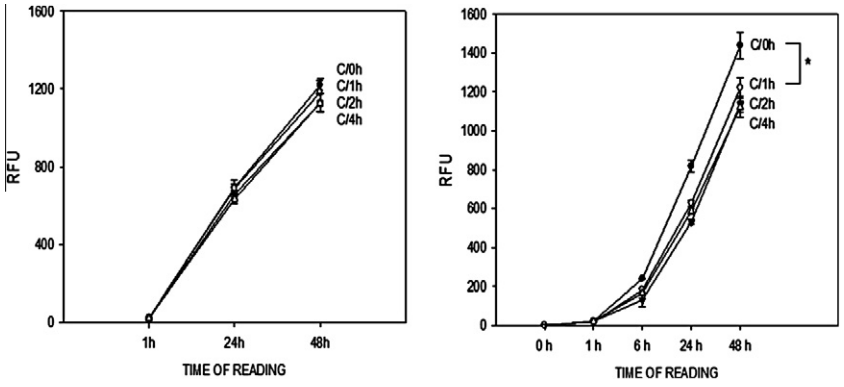

Fig. 5 – *In vitro* susceptibility of MTB to the Klebanoff system (KS). Fluorescence emitted (relative fluorescence units, RFU) by MTB kept in contact with the KS for 0 h (C/0 h), 1 h (C/1 h), 2 h (C/2 h) or 4 h (C/4 h). Viability assessed by the Alamar blue test with readings at the indicated time. Panel on the left: 0.25 mM H_2O_2; panel on the right: 0.88 mM H_2O_2. *P (C/0 h vs. C/1 h) < 0.001.

hydrogen peroxide, but it was susceptible to the KS when hydrogen peroxide was added at 0.88 mM concentration. This finding was deduced (a) from the fluorescence emitted by the bacterium cultivated in the presence of the KS for 0–4 h (with readings at 0, 1, 6, 24, and 48 h in the Alamar Blue assay) (Fig. 5), and (b) from the number of colony forming units (CFU) on Middlebrook 7H10 medium (Fig. 6).

Effects of supraphysiologic concentrations of hydrogen peroxide and nitric oxide on the viability of M. tuberculosis

At concentrations ranging from 1 to 10 mM, both hydrogen peroxide and sodium nitrite (the source of nitric oxide) had strong microbicidal effects on *M. tuberculosis* H37Rv, and this effect was even stronger with sodium nitrite. A total microbicidal effect was noticed with 2–4 mM sodium nitrite, as well as with 6–8 mM hydrogen peroxide (Fig. 7).

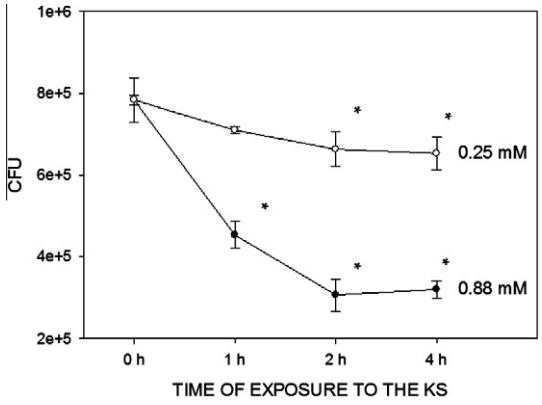

Fig. 6 – Effect of the Klebanoff system (KS) on the viability of *Mycobacterium tuberculosis* (MTB) assessed by CFU. MTB was incubated for the indicated periods in the presence of the KS with 0.25 or 0.88 mM H_2O_2 and then plated onto Middlebrook 7H10 for CFU counting. A colony count was performed after 12 days of incubation at 37 °C. Points are the mean values ± 1 SD from three individual experiments. Asterisks mean statistically significant differences ($P < 0.001$) in relation to control (0 h) bacilli.

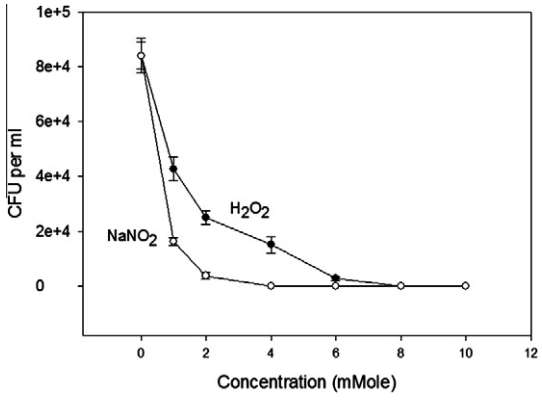

Fig. 7 – The toxic effects of hydrogen peroxide and sodium nitrite on *M. tuberculosis* H37Rv at concentrations from 1 to 10 mM. See details in the text.

Susceptibility of macrophages to hydrogen peroxide

Mouse macrophages resisted the effects of hydrogen peroxide up to 1.2 μg per 10^5 cells. Doubling the amount of H_2O_2 increased the death of the cells to nearly 80% (Fig. 8). Therefore, 1.0 μg of this agent was used in the experiments in which macrophages were supplemented with H_2O_2.

Peroxidase loading of macrophages and infection

Macrophages incorporated most of the exogenous HRPO within the first hour of incubation, and kept the enzyme active for at least 12 days, at which time most of the cells continued to appear to be morphologically healthy. Concentrations of HRPO

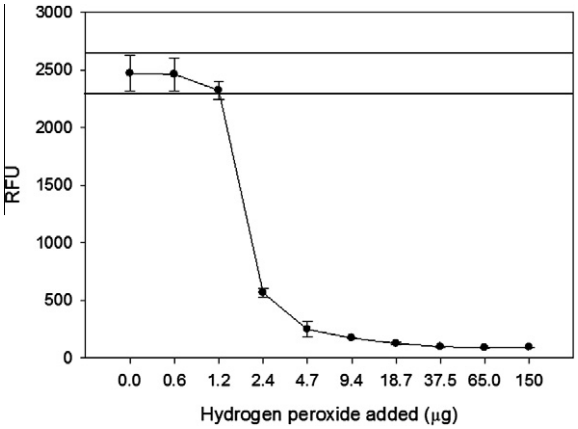

Fig. 8 – The effect of hydrogen peroxide on the viability of mouse peritoneal macrophages. Cell viability was assessed by the Alamar blue test, and the results are reported in relative fluorescence units (RFU). The upper horizontal lines indicate the mean RFU ± 1 SD values for the cells cultured in the absence of hydrogen peroxide. The fluorescence readings were made at 24 h of treatment with hydrogen peroxide.

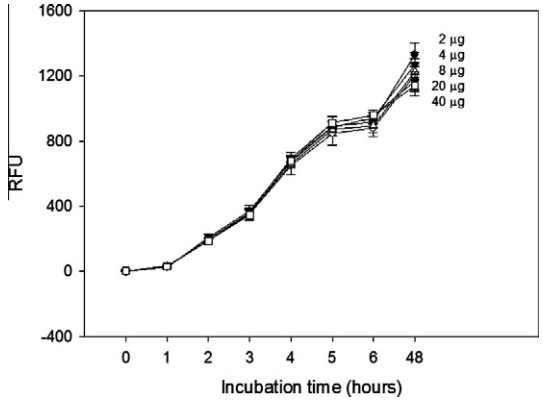

Fig. 9 – None of the concentrations of HRPO tested (0–40 μg) had a harmful effect on the viability of macrophages up to 24 h of incubation. Cell viability was assessed from the emitted fluorescence when the cells reduced the Alamar blue. RFU = relative fluorescence units. Average values of three independent experiments.

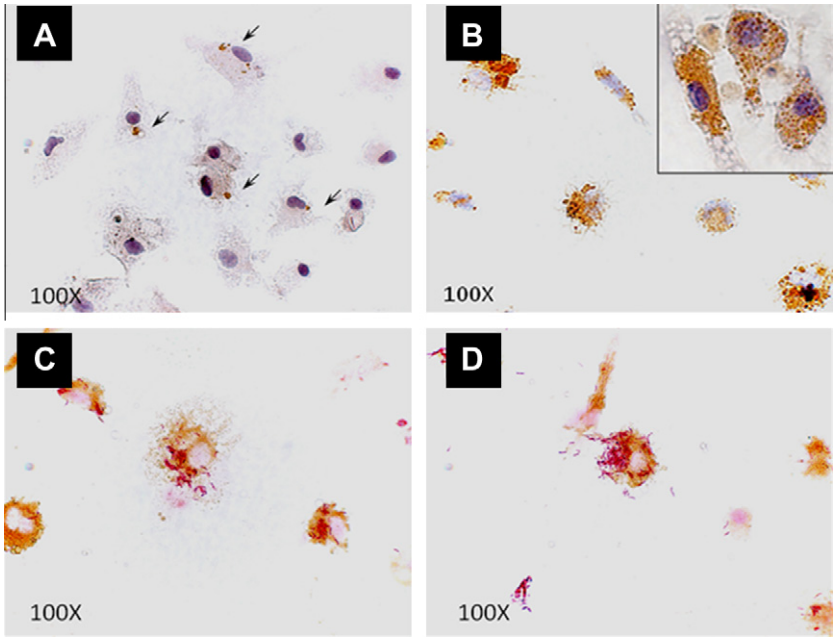

Fig. 10 – (A) Vestigial amount of MPO in resting mouse peritoneal macrophages (arrows); (B) Macrophages loaded with HRPO (a detail in the insert); (C and D) Macrophages loaded with HRPO and infected with *M. tuberculosis*. Stain with Ziehl–Neelsen (red) and hydrogen peroxide and o-dianisidine (brown). Magnification: 100 × 12.5X.

from 2 to 40 μg per 5×10^5 cells did not have any deleterious effect on the cells up to 48 h (Fig. 9), as determined by the Alamar Blue reduction test. Under the microscope (Fig. 10), a few resting macrophages showed only remnants of the granular enzyme (arrows in panel A); the incorporated HRPO presented a granular pattern (panel B) and did not interfere with the ingestion of *M. tuberculosis* (panels C and D).

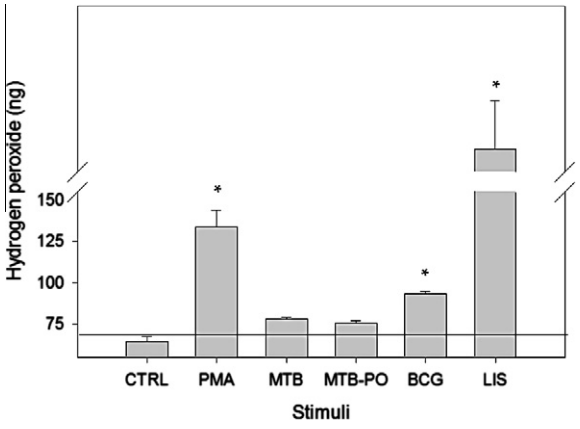

Fig. 11 – Production of hydrogen peroxide (nanograms per 10^6 cells) by control peritoneal macrophages (CTRL), and macrophages stimulated with PMA, intact MTB, peroxidase-coated MTB (MTB-PO), BCG or *Listeria* (LIS) (bacteria at a MOI 10:1). The horizontal line indicates the average hydrogen peroxide production (+1 SD) by the non-stimulated control cells. Bars are the mean ± 1 SD values from three independent experiments. Asterisks indicate those stimuli that gave statistically significant differences ($P = 0.01 < 0.001$) with the control cells (CTRL).

Production of H_2O_2 by macrophages infected with mycobacteria and L. monocytogenes

The mouse peritoneal macrophages produced small, but detectable amounts of hydrogen peroxide under resting

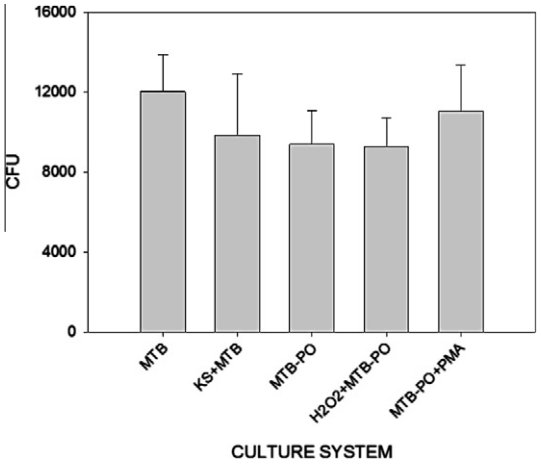

Fig. 12 – Effects of HRPO, H_2O_2, HRPO + H_2O_2, and PMA on the viability of MTB ingested by macrophages (2×10^6 cells per well). MTB: bacteria alone (MOI 10:1); KS (Klebanoff system): 3.33 IU HRPO + 0.1 mM hydrogen peroxidase; MTB-PO: peroxidase coated bacilli; H2O2 + MTB-PO: 0.1 mM hydrogen peroxide + peroxidase-coated MTB; MTB-PO + PMA: peroxidase-coated bacilli + 1.0 μg of PMA. Infection and treatments were maintained for 24 h. The results (colony forming units, CFU per ml) are the mean values from three different experiments ± 1 SE. There were no statistical differences between the groups ($P > 0.05$).

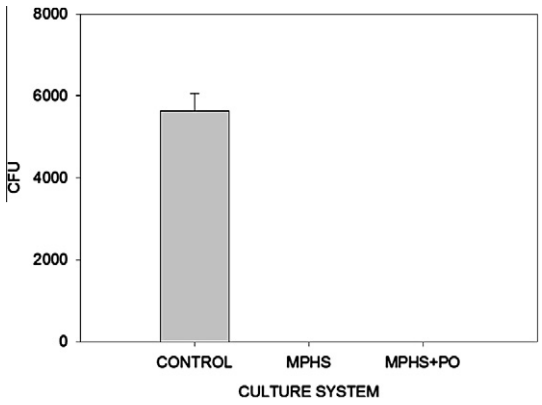

Fig. 13 – Bactericidal activity of resting macrophages (MPHS) and peroxidase-loaded macrophages (MPHS + PO) on *Listeria monocytogenes*.

conditions; stimulation of macrophages with PMA and BCG notably increased the production of this oxygen derivative. Stimulation of the macrophages with intact or peroxidase-coated *M. tuberculosis* (MTB) at a MOI of 10:1 induced a weak production of hydrogen peroxide, which was minimally higher than the levels found in the control, non-stimulated, macrophages (Fig. 11). *L. monocytogenes* (LIS) was a strong hydrogen-peroxide inducer in mouse macrophages.

Ex vivo effect of ROIs on MTB ingested by macrophages

The *ex-vivo* effects of HRPO, H_2O_2, HRPO + H_2O_2, and PMA on MTB ingested by macrophages is shown in Fig. 12. Some deleterious effects were noticed when HRPO and H_2O_2, alone or combined, and PMA, were added to the cultures of macrophages infected with MTB; however, the differences were not statistically significant ($P > 0.05$).

Bactericidal activity of macrophages on L. monocytogenes

Intact or HRPO-loaded macrophages efficiently killed *L. monocytogenes* within 1 h of its ingestion (Fig. 13).

Discussion

Since Klebanoff [1] described the bactericidal effect of the myeloperoxidase–hydrogen peroxide–halide system, myeloperoxidase (MPO) has been recognized as a prime microbicidal component of neutrophils, which have abundant granular MPO. It is not clear, however, whether this mechanism also operates in mature macrophages, which have only a vestigial amount of MPO. MPO is present in promonocytes in the bone marrow of humans, mice and guinea pigs; it is less abundant in the blood monocytes of the same species and is absent in the granules of mature macrophages. A role for MPO has been suggested in the pathogenesis of leprosy, which is another chronic granulomatous disease. Mature granulomas of leprosy (and also of murine leprosy) have been described as a core of highly bacilliferous, MPO-negative, macrophages surrounded by a multilayer of bacilli-free cells strongly positive for MPO [22]. The nature of these MPO-positive cells has not been studied in detail, but they could be newly recruited monocytes or mature neutrophils.

The present results indicate that *M. tuberculosis* H37Rv is not an efficient inducer of the oxidative response of macrophages that accompany the phagocytosis of other microorganisms. MTB did not induce the emission of luminescence or the production of significant amounts of hydrogen peroxide. This finding suggests that the bacteria enter the macrophages through a pathway that does not involve the participation of ROS-generating systems as deduced from the null production of hydrogen peroxide and the weak emission of chemiluminescence. Chemiluminescence is believed to reflect the general activity of diaphorases within a cell; diaphorases are flavin-bound enzymes that catalyze the reduction of various substrates, which act as hydrogen acceptors from the reduced NADH and NADPH; diaphorases may be unrelated to members of the NADPH oxidase family (NOXs 1-5, DUOX1 and DUOX2), all of which transport an electron to oxygen and other acceptors across cellular membranes to produce a superoxide anion [26]. Because of its high instability, superoxide decomposes itself, releasing energy that can be measured in the form of luminescence [27]. *M. tuberculosis* is susceptible to the *in vitro* effect of the Klebanoff system (peroxidase–hydrogen peroxide–halide), but it is resistant to the microbicidal mechanisms of resting macrophages and to the microbicidal mechanisms of macrophages supplemented with exogenous peroxidase and hydrogen peroxide. The resistance of *M. tuberculosis* in macrophages supplemented with the Klebanoff system may be due to the low toxic microenvironment reached within these cells, compared with the high toxic environment in the *in vitro* situation, in which the concentration of these reagents is certainly higher. Alternatively, and more probably, *M. tuberculosis* may resist the toxic intracellular environment because of the presence of potent detoxifying systems in the viable bacteria. Some of these systems include the following: the gene *noxR1*, which confers resistance to both reactive oxygen- and reactive nitrogen-intermediaries, although the precise molecular mechanism of resistance is not well understood [28]; the product of the controversial alkyl hydroperoxide reductase (*ahpC*) gene, which was originally regarded as a key participant in the protection of *M. tuberculosis* against oxidative stress and subsequently found to be suppressed in the bacteria growing in the infected cells [29]; the catalase-peroxidase *KatG* system that detoxifies reactive oxygen species generated by the macrophage upon infection [30]; the FTsH stress protein from the *ftsH* gene that enables *M. tuberculosis* to confront ROS; the superoxide dismutase C (*SodC*), which is an enzyme that transforms superoxide anion into hydrogen peroxide, which is further decomposed to water and oxygen by catalase (*Cat*) [31]; the *mel2* locus that also confers resistance of *M. tuberculosis* to the toxic effects of ROS [32]; and some other protecting mechanisms. The thick and complex cell wall of *M. tuberculosis*, composed of lipoarabinomannan, mycolic acids and phenolic glycolipids, which function as efficient scavengers of ROS and RNIs, also contribute to the survival of the intracellular mycobacteria.

Conclusion

In conclusion, incorporating exogenous peroxidase into peritoneal macrophages does not enable these cells to efficiently kill *M. tuberculosis*. Three reasons might account for this finding: (1) horseradish peroxidase does not functionally substitute for natural myeloperoxidase (however, there are reports on the efficient effect of HRPO in experiments similar to the one described in the present report) [13,33]; (2) horseradish peroxidase incorporated by pinocytosis does not locate, in an adequate manner, within the cell, and peroxidase pinosomes do not fuse with *M. tuberculosis* phagosomes, so that peroxidase and bacteria never come in contact (however, there are reports in which added peroxidase substitutes for the lack of peroxidase in macrophages infected with other microorganisms [34]; (3) peroxidase pinosomes fuse efficiently with mycobacterial phagosomes, but no anti-mycobacterial effects are observed because of: (a) the poor oxidative response induced by *M. tuberculosis* in macrophages (hydrogen peroxide being required for the optimal bactericidal effect of peroxidase); and (b) the multiple protective detoxifying mechanisms of *M. tuberculosis*. As *in vitro M. tuberculosis* is harmed by the Klebanoff system using horseradish peroxidase in a dose- and time-dependent manner, this study indicates that the third reason is the most probable explanation for the inefficacy of the KS *in vivo*.

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