Inhalable microparticles containing isoniazid and rifabutin target macrophages and ‘stimulate the phagocyte’ to achieve high efficacy†

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Macrophage responses to infection with Mycobacterium tuberculosis (MTB) and treatment with soluble isoniazid (INH) plus rifabutin (RFB) versus microparticles containing equivalent amounts of drugs were compared. It was investigated whether macrophages driven to alternative activation upon infection with MTB could be rescued to display the classical activation phenotype. It was established that microparticles sustain high levels of drugs in cytosol of macrophages for longer period as compared to soluble drugs. Microparticles co-localized with intracellular bacteria, and induced a variety of innate bactericidal responses, including induction of free radicals, alteration of mitochondrial membrane potential and apoptosis. The data strongly suggest that additional benefit may be derived from the nature of the drug delivery system, which fulfils Koch’s dictum ‘stimulate the phagocyte’ for curing tuberculosis.

Keywords: Inhalable microparticles, Isoniazid, Macrophage, Mycobacterium tuberculosis, Phagocyte, Rifabutin, TB

Innate responses of the host macrophage infected with Mycobacterium tuberculosis (MTB) have not been traditionally considered as a possible component of chemotherapeutic strategy against the infection. In recent years, it has been proposed that alveolar macrophage-resident MTB may be targeted by inhaled drug delivery systems, and encouraging results reported1-12.

The present report is intended to highlight two aspects of inhaled therapies in TB. First, the delivery of a large amount of anti-TB drugs to macrophage where MTB may be residing; and second, the possibility that phagocytosis of particulate material may activate classical bactericidal macrophage responses that the cell type has evolved against generic particulate matter inhaled in physiological breathing.

Materials and Methods

Anti-TB drugs, isoniazid (INH) and rifabutin (RFB), and inhalable microparticles containing these drugs1 were provided by Lupin Research Park. MTB was grown in Sauton’s medium to log phase, harvested and dispersed in RPMI-1640 by sonication for 20 sec and OD at 600 nm was used as a measure of CFU, employing a standard curve prepared in advance. THP-1 human monocytic cells were induced to differentiate to the macrophage phenotype by treatment with phorbol ester and infected at a MOI of 10 for 3 h. Extracellular bacteria were washed off with 3 portions of RPMI and the infected cells treated with drugs and/or microparticles2,4. Uninfected cells were used to establish the time-course of intracellular drug concentrations following administration of drugs in solution or in microparticles.

HPLC on a C-18 column was used to estimate drug concentrations in cell lysates prepared at 24, 48, 72 and 96 h after treatment of 2×10⁶ cells with 3 µg/mL each of INH and RFB in solution in the culture medium, or in microparticles1,5.

The following experimental groups were formed to evaluate cellular and biochemical phenomena associated with microparticle phagocytosis: Normal (uninfected) cells (N), uninfected cells treated with drug-containing microparticles (NMP), infected cells receiving no treatment (I), infected cells treated with 3 µg/mL each of INH and RFB (ISD), or with an equal amount of the two drugs in inhalable microparticles (IMP), or an equivalent amount of drug-free (blank) microparticles (IBMP).
Reactive oxygen species (ROS) induced in each group were estimated by flow cytometry. The indicator 2’, 7’-dichlorodihydrofluorescein diacetate (DCHF-DA) was used for the estimation of ROS, particularly peroxides. DCHF-DA solution was prepared by diluting a 20 mM DMSO stock solution kept in dark to 25 μM DCHF-DA with RPMI. For equal loading of all cells with the dye, cells were pelleted by centrifugation (400×g for 10 min at 20°C), resuspended in RPMI and incubated with 25 μM DCHF-DA solution at 37°C for 1 h. DCHF-DA-loaded cells were used in the experimental groups described above. To estimate intracellular peroxide production, fluorescence emissions at 520 nm were recorded after 4 h. Flow cytometric analysis was carried out with a Becton-Dickinson FACS Calibur equipped with an argon laser using an excitation wavelength of 488 nm.

Mitochondrial membrane potential was estimated by flow cytometry following loading cells with the fluorescent dye Rhodamine 123. A 96-well flat-bottom culture plate was seeded with 0.2×10^6 cells/well and the cells differentiated, infected and treated as described for 14, 24 and 48 h. The lipophilic, cationic dye Rhodamine 123 (Sigma), was then added to wells (1 μL of a 0.5 mg/mL solution) for 15 min at 37°C in complete medium. Cells were washed twice with PBS, re-suspended in 300 μL of PBS and stained with PI. Samples were analyzed on the flow cytometer with the CellQuest software. The intensity of staining was used as a measure of Δψ_m.

DNA fragmentation was studied as a measure of apoptosis of infected cells subjected to different modes of treatment. DNA was extracted according to the procedure of Miller et al. Briefly, DNA was extracted from 1- 3×10^7 cells by adding 3 mL of nuclei lysis buffer (10 mM, Tris-CI; 400 mM, NaCl; and 2 mM, Na2EDTA at pH 8.2), 0.2 mL of 10% SDS and 0.5 mL of proteinase K solution (1 mg, proteinase K; 2 mM, Na2EDTA; and 1%, SDS) and incubated at 37 °C for overnight. NaCl (6 M) was added and the supernatant collected by spinning at 1300 × g for 15 min. Two volumes of ethanol (95%) were added to the supernatant and gently mixed. DNA precipitate was dissolved in 200 μL of Tris-EDTA and quantitated spectrophotometrically. Electrophoresis was performed with equal amounts of DNA on agarose gel (1.4%).

Results

Concentrations of RFB observed from 0-96 h after administration of microparticles or drugs in solution to uninfected THP-1-derived macrophages are depicted in Fig 1. Similar intracellular concentrations were observed immediately after adding the drug, either in soluble or microparticulate form, as well as when sampled 24 h later. Significant differences were discernible from 48 h onwards.

Intracellular reactive oxygen species were observed in flow cytometry using DCHF-DA (Fig. 2). Even uninfected cells apparently comprised two distinct sub-populations, differing in their intracellular ROS. This observation was probably due to phorbol treatment of THP-1 cells in order to induce differentiation. The ratio of ROS_to ROS_cells was 5.7 in uninfected cells (N), however, infection (I) brought this ratio down to 3.5. Treatment of infected macrophages with soluble drugs (ISD) resulted in marginal recovery of ratio to 4.0. Microparticles significantly enhanced ROS to yield a ratio of 7.9.

It was investigated whether mitochondrial integrity would correlate with microparticle treatment. The lipophilic cationic dye rhodamine 123 was used as an indicator of Δψ_m in flow cytometry experiments. Figure 3 shows data from one of three experiments, where forward light scatter (cell size) is plotted versus rhodamine fluorescence intensity on the abscissa (Δψ_m) observed after 12 h of treatment. After 6 or 18 h of treatment, no differences were discernible between the groups.

Reduction in Δψ_m was observed at 12 h following uptake of either drug-containing microparticles or

![Fig. 1](image-url)
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The drop in Δψm after treatment with drug-free microparticles was maximal, with drugs containing microparticles and dissolved drugs showing progressively smaller effects, though the difference between drug-containing and drug-free microparticles was not statistically significant.

Disruption of mitochondrial membrane integrity is likely to result in apoptosis of the infected cell. Flow cytometric analysis of DNA damage, such as that induced in late apoptosis, showed minor differences between the proportions of apoptotic cells in various treatment groups (data not shown). However, DNA fragmentation was clearly visible when DNA isolated from various groups after 48 h of treatment was resolved on a 1.4% agarose gel. As shown in Figure 4, a ladder pattern was observed in case of infected cells. Densitometric comparison of band intensities within a window on the gel indicated that drug-free microparticles induced the highest fragmentation (IBMP), followed by drug-containing microparticles (IMP) and dissolved drugs (ISD) respectively. Normal controls (N and NMP) did not yield a ladder pattern.

Discussion

Virtually all forms of TB begin with pulmonary infection, when droplet nuclei are inhaled into the lungs and phagocytosed by alveolar macrophages. These cells usually serve as the first line of defense against invading microorganisms, but several

M. tuberculosis H37Ra. The drop in Δψm after treatment with drug-free microparticles was maximal, with drugs containing microparticles and dissolved drugs showing progressively smaller effects, though the difference between drug-containing and drug-free microparticles was not statistically significant.

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intracellular parasites including MTB have evolved to survive and replicate within maturation-arrested phagosomes that do not fuse with lysosomes. The ability of the macrophage to mount an appropriate response has long been known to affect the course of TB, as enunciated in Koch’s dictum, ‘Stimulate the Phagocytes’. Effective host response to infection, including responses to the survival strategies of the pathogen, is mediated by several mechanisms. Early induction of innate responses such as ROS and reactive nitrogen intermediates are now recognized as examples of innate mechanisms. When the host defense fails to counter the infection, disease ensues and chemotherapy is required. Prolonged multi-drug regimens, however, have several well-known drawbacks. The present data illustrates how host innate responses may be recruited to synergise with targeted chemotherapy against macrophage-resident MTB.

Maintenance of high intracellular drug concentrations over long periods following a single exposure to particles (Fig. 1) promises direct benefits related to killing intracellular MTB. Targeting the alveolar macrophage upon inhalation of these particles has been reported several times elsewhere. It is possible that administration of inhalable microparticles to patients harbouring MTB in lung macrophages would show similar results.

The consequences of targeting alveolar macrophages by inhaled particles, however, were not limited to sustaining drug concentrations in cytosol. As demonstrated in Figure 2, microparticles sustained the production of ROS, but untreated infection depleted intracellular ROS. While the ability to scavenge ROS produced by the host macrophage is an important aspect of pathogenicity, the sustained induction of ROS in an infected macrophage could prove inimical to bacterial survival.

Apart from induction of ROS, it has been demonstrated elsewhere that phagocytosed microparticles induce Th1 cytokines, predominantly, TNF, in murine macrophages. TNF and ROS are both pro-apoptotic, whereas pathogenic MTB induces necrotic or caspase-independent death of the infected macrophage. There is reason to believe that M. tuberculosis-induced cell death correlates not only with virulence, but also with the microbial burden in the macrophage. At a low MOI, virulent strains inhibit host macrophage apoptosis while attenuated strains such as H37Ra or Bacille Calmette-Guerin induce it. Molecular mechanisms of such host-pathogen interaction continue to be worked out, and yield interesting. The pathogen is known, for instance to neutralize TNF signalling by enhancing host IL-10; which leads to shedding of TNF receptor proteins from the infected cell surface. Induction of host Bcl-2 (Ref. 25) and congener anti-apoptotic proteins such as Mcl-1 (Ref. 27) is another documented strategem. At the transcript level, anti-apoptotic bcl-w is upregulated by virulent bacteria, whereas attenuated bacteria show upregulation of proapoptotic genes designated as “upregulated during

![Fig. 4](image_url) DNA fragmentation induced by infection and treatment. [M- marker (1 kb ladder); I- DNA from infected cells; ISD- from infected cells treated with soluble drugs; IMP- treated with microparticles; IDMP- treated with dummy microparticles; and N- uninfected cells]. Densitometry results are shown to the right of the gel image, with an asterisk to denote significant (P<0.05) differences between the ratios of genomic to fragmented DNA.
macrophage has finally succumbed to infection. In response, therefore, and rather indicates that the host death under these circumstances is not a defense progress rapidly from apoptosis to necrosis. Cell strain. Further, cells carrying a large bacillary load apoptosis, regardless of the virulence of the infecting bacteria, whereas it is upregulated when the infectious strain is avirulent. This distinction, too, hinges on TNF signalling, such that treatment of cells infected with avirulent bacteria with anti-TNF-α antibodies results in downregulation of mitochondrial superoxide dismutase-2 (Ref. 23).

At higher MOI and early in infection, however, cell death induced by both strains H37Rv and H37Ra shows features associated with necrosis. Lee et al.21 have demonstrated that a MOI ≥ 25 does not inhibit apoptosis, regardless of the virulence of the infecting strain. Further, cells carrying a large bacillary load progress rapidly from apoptosis to necrosis. Cell death under these circumstances is not a defense response, therefore, and rather indicates that the host macrophage has finally succumbed to infection. In contrast, inhibition of host macrophages apoptosis by stabilization of mitochondrial membrane potential promotes innate macrophage bactericidal activity against M. tuberculosis.15

Figure 3 demonstrates that microparticles, regardless of drug content, reduce mitochondrial membrane potential. Thus, microparticle treatment may be expected to induce apoptosis as a defense response. Figure 4 bears out this expectation, demonstrating the greatest amount of DNA fragmentation in cells treated with blank microparticles. Although, bactericidal effects of microparticle-induced apoptosis in the cell culture model has not been observed, it is possible that apoptotic bodies containing MTB may contribute to the generation of a bactericidal acquired immune response, whereas the entry of naked or opsonized MTB into bystander macrophages is a step in pathogenesis. Therefore, it can be conclude that the proposed drug delivery system has a role in stimulating the phagocyte to mount innate defense responses, and possesses potential in contributing to the chemotherapy of TB.

The indicators of macrophage activation outlined in the present report have prompted us to undertake a genome-wide transcription analysis of gene expression induced by MTB H37Rv in the THP-1-derived macrophage. These studies have been carried out on a microarray platform using Affymetrix chips. The data are currently under analysis and shall shortly be available openly for mining at the website of CSIR’s OSDD programme (http://sysborgtb.osdd.net/bin/view/OpenLabNotebook/WebHome).

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