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Research Article

Screening for Potential Adjuvants Administered by the Pulmonary Route for Tuberculosis Vaccines

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Abstract. Tuberculosis (TB) infects one third of the world's population, and new infections occur at a rate of 1/s. Better vaccines are needed than the live mycobacterium Bacille Calmette-Guérin (BCG). Alveolar macrophages (AMΦs) play a central role in pulmonary manifestations of TB. Targeting immunomodulators to AMΦs, the first line of defense against *Mycobacterium tuberculosis* (Mtb), may initiate a potent cell-mediated immune response. Muramyl dipeptide (MDP) and trehalose dibehenate (TDB) have elicited strong immune response when delivered to the lungs as aerosols. AMΦs show toxicity in response to some immunomodulators. The objective of this work was to screen the immunomodulators MDP and/or TDB encapsulated in microparticles (MPs) and to evaluate certain indicators of toxicity in human AMΦ-like cells. Poly(lactide-*co*-glycolide) (PLGA) MPs containing MDP and/or TDB were prepared by spray-drying. The morphology, particle size distribution, and immunomodulator encapsulation efficiency of MPs were examined. THP-1 cells were exposed to these MPs for 24 h and characteristics of cell morphology, tumor necrosis factor-alpha (TNF-α) release, lactate dehydrogenase (LDH), *N*-acetyl-β-D-glucosaminidase (NAG) and alkaline phosphatase (ALP) activity in AMΦ culture supernatants were measured. MTT assay was used to assess the viability of cells. Spray-drying produced low-density MPs having volume median diameters between 4 and 6 μm as measured by laser diffraction and projected area diameter between 3 and 5 μm calculated by microscopy. More TNF-α was produced by THP-1 cells exposed to MPs composed of PLGA-MDP or PLGA alone than PLGA-TDB. LDH release following exposure to MPs of PLGA-MDP and PLGA alone was greater than controls. NAG release was higher following exposure to MPs of PLGA alone or PLGA-MDP 0.1% than PLGA-TDB (0.1% and 1.0%). Cells remained viable after exposure to MPs as per MTT assay. PLGA-MDP MPs demonstrated statistically elevated indicators of biochemical responses in cell culture compared to PLGA-TDB MPs, but the extent of their potential to elicit adverse effects *in vivo* must be studied independently.

KEY WORDS: adjuvants; cytotoxicity; immunostimulant; microparticles; pulmonary.

INTRODUCTION

Tuberculosis (TB) caused mortality and morbidity which included 9.2 million new cases and 1.7 million deaths in 2006, mostly in developing countries (1). These statistics reveal that Bacille Calmette-Guérin (BCG), the only TB vaccine used in humans for almost a century, had limited success against this epidemic in the developing world. In fact, BCG fails to protect against the most common form of the disease, pulmonary TB in adults (2). *Mycobacterium tuberculosis* (Mtb), the causative agent for TB, normally enters the host through the respiratory tract after inhalation of droplets from an infected individual. They are taken up by antigen-presenting cells (APCs), especially alveolar macrophages (AMΦs), which provide the first line of immune response for the infected individual. This may lead to a rapid inflammatory response characterized by the release of various antimicrobial effector molecules, including cytokines and chemokines, and with time, to the formation of granulomas in the lungs (3). This inflammatory response is accompanied by a cell-mediated immune response consisting of $CD4^+$ and $CD8^+$ effector T cells (4). A Th1 immune response, rather than a Th2 response, is critical in containing the infection in granulomas and thus preventing progression to active disease (5).

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ABBREVIATIONS: ALP, alkaline phosphatase; AMΦ, alveolar macrophage; APC, antigen-presenting cell; BCG, Bacille Calmette-Guérin; LDH, lactate dehydrogenase; Mtb, *Mycobacterium tuberculosis*; MDP, muramyl dipeptide; MP, microparticle; NAG, *N*-acetyl-β-D-glucosaminidase; PLGA, poly(lactide-*co*-glycolide); TB, tuberculosis; TDB, trehalose dibehenate; TNF-α, tumor necrosis factor-alpha.

Certain cell wall components of Mtb are known to elicit an immune response, one of them being mycolic acid (6); its derivatives are known to stimulate the host inflammatory response, leading to the protective granuloma formation (7) and the T cell response in animal models (8). The best-characterized mycolate, trehalose dimycolate (TDM) or cord factor, is used as adjuvant and selectively induce a Th1 response (9). Any adjuvant system is guided by its safety, stability, and immunogenicity.

Muramyl dipeptide (MDP) and trehalose dibehenate (TDB), immunogenic components and synthetic analogs of the cell wall of Mtb, are capable of eliciting a strong adjuvant activity. TDB is a synthetic analog of TDM (10) and MDP has the minimal chemical structure needed for adjuvant activity similar to complete Freund's adjuvant (11). MDP has previously been administered with mineral oil; the oil prolongs its residence time in the body as a depot and enhances the cell-mediated immune response (12). But mineral oil leads to greater toxicity as it is not metabolized (13).

Immunization by the pulmonary route against TB has gained considerable interest in recent years (14), as it eliminates the requirement for needles and the associated discomfort, while stimulating a cell-mediated immune response. The pulmonary route being the route of infection in humans, the lungs should be the gateway organ to confer protection. This noninvasive route reduces the need for medical assistance and is suitable for mass vaccination.

We have previously shown that guinea pigs exposed to aerosols of MDP microparticles (MPs) lead to morphological changes in AMΦ like pseudopodia formation and spreading (15). Nagao *et al.* had targeted MDP to peritoneal macrophages (MΦs) in guinea pigs and showed that it induced activation (16). AMΦ are the first line of defense to inhaled foreign antigens. These antigens may cause release of cytoplasmic enzymes which are characteristic of damaged or lysed cells including AMΦs.

Lactate dehydrogenase (LDH), a cytoplasmic enzyme released from damaged or lysed cells, can be used as a marker of cytotoxicity. *N*-acetyl-β-D-glucosaminidase (NAG), a lysosomal enzyme, is an indicator of increased phagocytic activity in response to inhaled particles (17). Alkaline phosphatase (ALP) is again a lysosomal enzyme indicative of tissue damage. An increase in these enzymes after exposure of AMΦs to MDP and TDB MPs can be used to distinguish between deleterious effects and physiologic responses. The role of TNF-α in TB is paradoxical. Although it has been shown to have a protective role, there is also evidence that it plays a part in tissue damage that characterizes human disease. Potential cytotoxicity is demonstrated directly in the viability assay (MTT) performed using cells exposed to MDP and TDB MPs.

MATERIALS AND METHODS

Preparation of MPs Containing Immunostimulatory Adjuvants

Poly(lactide-*co*-glycolide) (PLGA, lactic/glycolic ratio 75:25, MW 84.7 kDa, intrinsic viscosity 0.68 dL/g in $CHCl_3$) was purchased from Durect (Lactel® Absorbable Polymers, Pelham, AL, USA). Adjuvants MDP and TDB were purchased from Sigma (St. Louis, MO, USA). Dichloromethane (DCM) was purchased from Burdick & Jackson (high-performance liquid chromatography [HPLC] grade, Muskegon, MI, USA). MPs were prepared by spray-drying a primary emulsion. PLGA was dissolved in DCM (0.35%, *w*/*v*) and MDP and TDB dissolved either alone or together in 20 mM, pH 7.0 phosphate buffer. A water-in-oil emulsion (phosphate buffer/DCM=1:20, *v*/*v*) was formed by emulsifying the mixture using a probe sonicator.

The emulsion was spray-dried through a 0.7-mm diameter nozzle of a Buchi mini spray-dryer (B-191 Buchi Labortechnik AG, Flawil, Switzerland). The following conditions were used: inlet temperature 65°C, outlet temperature 40°C, aspirator setting 50%, feed flow rate 7.3 mL/min, and compressed nitrogen flow rate 600 L/h.

Characterization of Particle Morphology and Size

MPs were examined by a scanning electron microscope (JSM 6300V SEM, JEOL USA, Peabody, NY, USA). The surface morphology and shape were examined by spreading particles on a conductive double-sided tape attached to aluminum stubs and sputter-coating with gold–palladium alloy using Polaron 5200 (Structure Probe Supplies, West Chester, PA, USA).

Particle size distribution and median volume diameter were measured using a laser diffraction size analyzer (Series 2600C Malvern Instruments, Malvern, UK). Briefly, MPs were dry-mixed with sodium lauryl sulfate (SDS, Sigma) and suspended in distilled water. The suspensions were vortex-mixed for 1 min and sonicated for 30 s (Fisher Scientific FS21H) to break the aggregates. It was added dropwise in a stirred sample cell containing distilled water until the obscuration between 15% and 30% was achieved. The samples were directed through the monochromatic laser (He–Ne) light beam. The analyses were carried out in triplicate for each batch and type of MPs.

Density Determination

MP densities were evaluated by tap density and by the relationship between mass median aerodynamic diameter (MMAD) and median volume diameter (Dv_{50}, 50% by volume of particles below this value). Briefly, known quantities ($v \times \rho$) of MPs were added to a 10-mL graduated cylinder. Tap densities of MPs were determined by measuring the volumes occupied after 100 uniform taps on a flat surface. The MMAD and Dv_{50} acquired from the Anderson cascade impactor and laser particle size analyzer, respectively, were used to calculate the apparent density using the equation:

$$\mathrm{MMAD} = \mathrm{Dv}_{50}\sqrt{\rho}.$$

Adjuvant Encapsulation Efficiency

About 20 mg of MPs were dissolved in 1 mL DCM and extracted with 0.5 mL ammonium phosphate buffer (25 mM, pH 7). The amount of MDP was analyzed by Hewlett Packard 1100 series HPLC (Agilent Technologies, Santa Clara, CA, USA) using the Waters Spherisorb S5 ODS2

(4.0×250 mm) analytic column. The mobile phase consisted of ammonium phosphate buffer and methanol (97:3). A UV-visible detector was used at a maximum wavelength of 200 nm. The encapsulation efficiency was calculated as a ratio of observed to theoretical MDP content.

The encapsulation efficiency of TDB in the formulations could not be measured because of the absence of an HPLC method for its detection.

Cell Line and MP Exposure

Human monocytic THP-1 cells were obtained from American Type Culture Collection and cultured in complete RPMI 1640 media (10% fetal bovine serum, 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, 1.0 mM sodium pyruvate, and 0.05 mM 2-mercaptoethanol) at 37°C in a 5% CO_2. THP-1 cells were plated in 24-well plates at a density of 10^6 cells per well and treated with 30 ng/mL of phorbol myristate acetate (PMA). One day after stimulation with PMA, adherent cells were maintained in the same media without PMA for an additional 3 days and used as a source of MΦs. The MPs were kept under UV light overnight prior to exposure to cells for sterility. They were suspended in complete RPMI 1640 media to have a concentration of 3×10^6 particles per well. Varying particle suspensions were added to constant cell numbers depending upon the experiments and incubated for 24 h. Control groups consisted of untreated cells and cells receiving MDP and TDB solutions.

Macrophage Activation and Biochemical Assays

Cell morphology after MP exposure was observed by phase contrast microscopy (Olympus IMT2, Lake Success, NY, USA). TNF-α concentrations in culture supernatants were determined using a commercial ELISA kit (BioSource Europe S. A., Nivelles, Belgium). The enzymes ALP, LDH, and NAG are widely used markers for tissue damage, cell lysis, and phagocytic activity, respectively. Cell death by necrosis is typically measured by assays for plasma membrane permeability such as LDH assay. Assays for these markers were performed on cell-free supernatant with modifications for use on the Cobas Fara II Autoanalyzer (Hoffman-LaRoche Branchburg, NJ, USA). Activities of ALP and LDH were assayed using commercially available kits from Sigma (St. Louis, MO, USA) and NAG levels were determined by a kit from Boehringer Mannheim (Indianapolis, IN, USA). Each formulation was tested at least in triplicate.

MTT Cell Viability Assay

The viability of the cells were evaluated by the reduction of yellow MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) to purple formazan. THP-1 cells (1.8×10^5 cells per well) were plated in 96-well plates and stimulated with PMA as described above. This assay provides a sensitive measurement of the normal metabolic state of cells, particularly that of mitochondria, and hence, is an indication of early cellular changes. Differentiated THP-1 cells were exposed to MPs at various concentrations for 24 h. MPs were uniformly suspended in media (with antibiotics) before replacing the original media in the 96-well plate with the MP suspension. Final MP numbers achieved were one, five, ten, and 30 per three THP-1 cells (100 μL per well) for all the exposure groups. Controls consisted of cells incubated in the absence of any formulations. After removing the media, cells in each well were incubated with MTT in phenol red-free RPMI 1640 media for 3.5 h according to the manufacturer's protocol. The assay was stopped by adding MTT solubilization solution (10% Triton X-100, 0.1 N HCl in anhydrous isopropanol). The plates were stored overnight at 37°C to completely dissolve formazan crystals. The formazan was quantified using Bio-Rad Model 3550 microplate reader (Bio-Rad Laboratories, Hercules, CA, USA) to measure the absorbance at a wavelength of 570 nm and background absorbance at 690 nm. Results of cell viability were calculated as the percentage of formazan formed by cells coincubated with MPs with respect to the formazan formed after incubation of cells in a particle-free medium.

Statistical Evaluation

The data were analyzed by one-way analysis of variance for multiple comparisons (Dunnett's t tests) with $P<0.05$. SAS software version 8.2 (SAS institute, Cary, NC, USA) was used for statistical analysis.

RESULTS

Characterization of MPs

MPs analyzed for particle size by laser diffraction produced unimodal or bimodal distributions indicating some agglomeration with median volume diameters between 4 and 6 μm. This confirms the size range expected for particles to be engulfed by dedicated phagocytic APCs including AMΦs (Fig. 1; Table I).

The MP morphology in SEM micrographs (Fig. 2) was influenced by the adjuvant encapsulated. The spray-drying procedure produced PLGA and PLGA-MDP MPs that were wrinkled and had collapsed perforated hollow sphere appearance.

The particle densities obtained by tap density method and the cascade impactor were similar with densities of 0.1–0.3 mg/mL; PLGA-TDB MPs were more dense than PLGA-MDP MPs. Encapsulation efficiency between 86% and 100% was achieved for MDP in MPs as estimated by HPLC (Table I).

Morphology

When exposed to MPs, THP-1 cells demonstrated signs of activation after 24 h incubation based on morphological changes as shown in Fig. 3. THP-1 cells exposed to PLGA particles, 0.1% MDP and TDB MPs, and 1% MDP-TDB MPs (Fig. 3b–e, respectively) had extensive cellular extensions and elongated morphology, suggesting that theses cells were activated and phagocytosed the particles. Extensive cellular extensions were not observed in control cells (Fig. 3a).

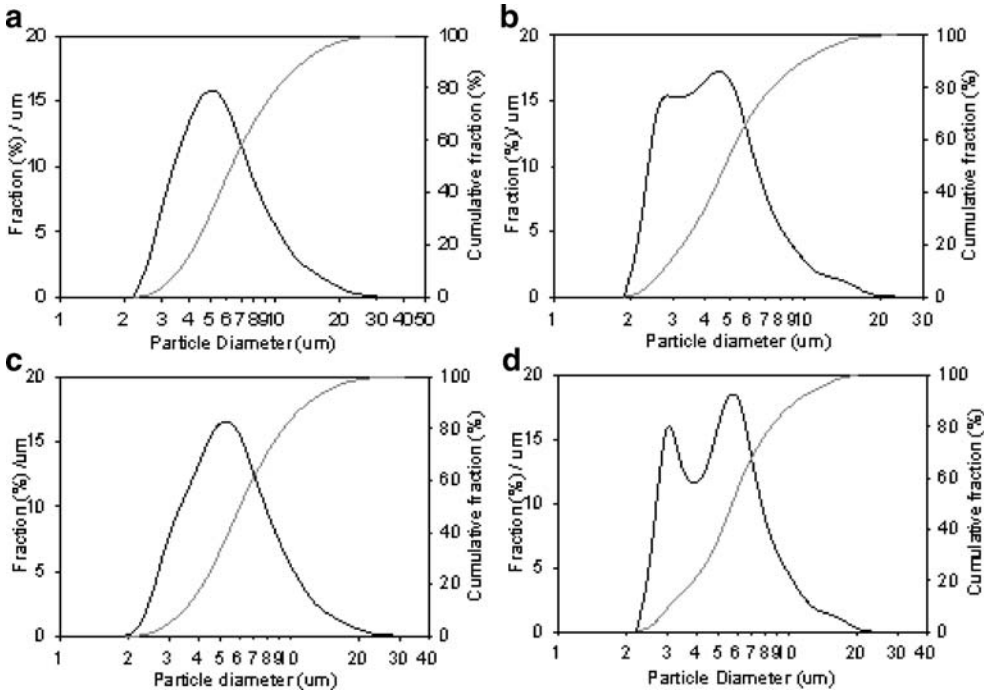

Fig. 1. Volume particle size distribution of PLGA (**a**), PLGA-MDP 1% (**b**), PLGA-TDB 1% (**c**), and PLGA-TDB-MDP 1% (**d**) determined by laser diffraction

Macrophage Activation upon MP Uptake

TNF-α activity in the culture supernatant of cells exposed for 24 h to MPs was determined. TNF-α secretion increased by treating THP-1 cells with either PLGA MPs alone (192.70±24.05 pg/mL) or different concentrations of PLGA-MDP MPs. PLGA-MDP 0.1% (244.81±12.63 pg/mL), PLGA-MDP 0.5%, and PLGA-MDP 1% MPs had higher TNF-α release than PLGA-TDB MPs (Fig. 4).

Substantial LDH was released following exposure to PLGA alone (87.15 IU/L), PLGA-MDP 0.1% (93.79 IU/L), and PLGA-MDP 0.5% MPs (90.51 IU/L) than TDB MPs and control (Fig. 5) ($P<0.05$).

NAG release was statistically higher following PLGA alone (22.73 IU/L) and PLGA-MDP 0.1% and 0.5% MPs exposure (23.24 and 21.98 IU/L, respectively) compared to control cells (Fig. 6) ($P<0.05$).

The ALP levels from the cells exposed to PLGA-MDP and PLGA-TDB MPs showed no significant difference compared to the control values.

Cytotoxicity Studies

MTT assays were conducted on THP-1 cells after exposure to the MPs. Figure 7 compares the effect that MPs have on the metabolic state of the cells over a 24-h exposure period, showing the cellular viability as a function of both formulation type and dosage level. The general cellular viability of AMΦs was estimated with respect to the addition of either blank MPs or mycobacterial adjuvants entrapped in MPs. PLGA and PLGA-TDB 0.1% MPs at less than ten particles per three cells produced more formazan than control resting cells, which may be indicative of an activation of the metabolic state of the cells. All particles showed cell

Table I. Summary of the Physicochemical Characteristics of the MPs

Particles	Concentration of adjuvant (%)	Encapsulation efficiency (%)	Tap density (mg/mL)	SEM		Volume diameter distribution	
				Projected area median diameter (μm)	GSD	Dv_{50} (μm)	Span
PLGA	–	N.A.	0.133	3.66	1.36	6.29	1.60
PLGA-MDP	0.1	94.5	0.129	3.25	1.37	6.41	1.61
PLGA-MDP	0.5	86.3	0.162	3.16	1.39	5.70	1.41
PLGA-MDP	1	102.7	0.169	3.86	1.41	5.57	1.48
PLGA-TDB	0.1	N.D.	0.343	2.73	1.31	3.94	1.14
PLGA-TDB	1	N.D.	0.220	3.35	1.33	6.07	1.41
PLGA-MDP-TDB	1	N.D.	0.161	4.35	1.40	5.72	1.36

N.A. not applicable, *N.D.* not determined, *GSD* geometric standard deviation

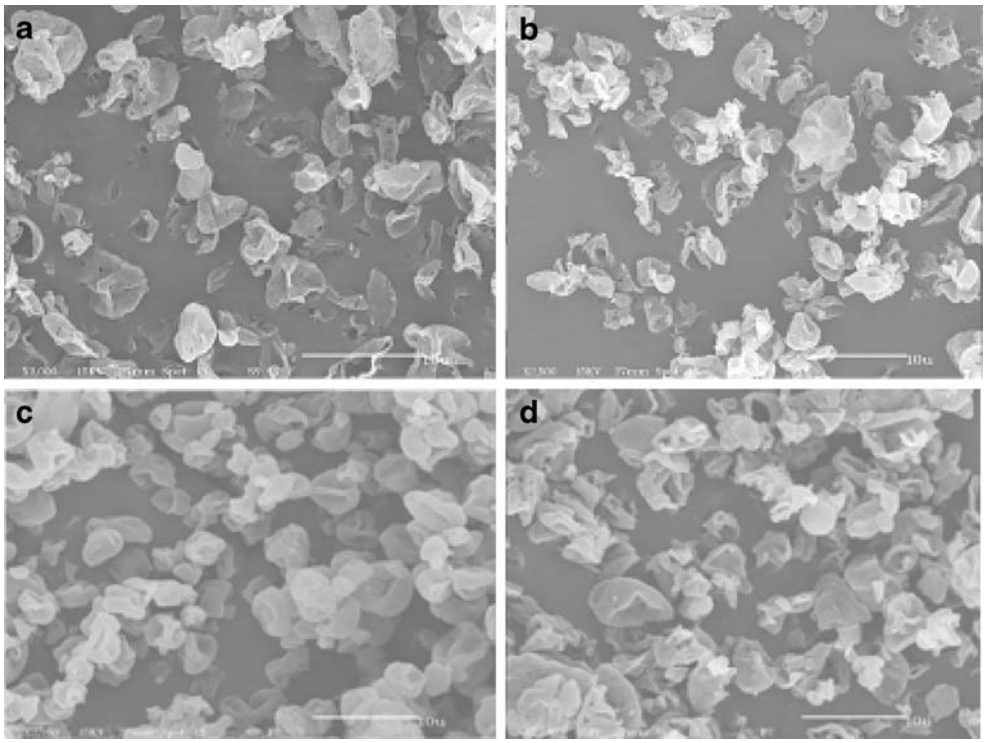

Fig. 2. SEM micrographs of PLGA particles (**a**), PLGA-MDP 0.1% particles (**b**), PLGA-TDB 1% particles (**c**), and PLGA-TDB-MDP 1% particles (**d**). Scale bar represents 10 μm

viabilities above 84% except PLGA-TDB 1% MPs exposed to one particle per three cells (cell viability 72%). Thus, no formulation showed detectable decrease in cell viability in all dosages used.

DISCUSSION

The objective of this study was to screen potential adjuvants entrapped in MPs for their effect on phagocytic

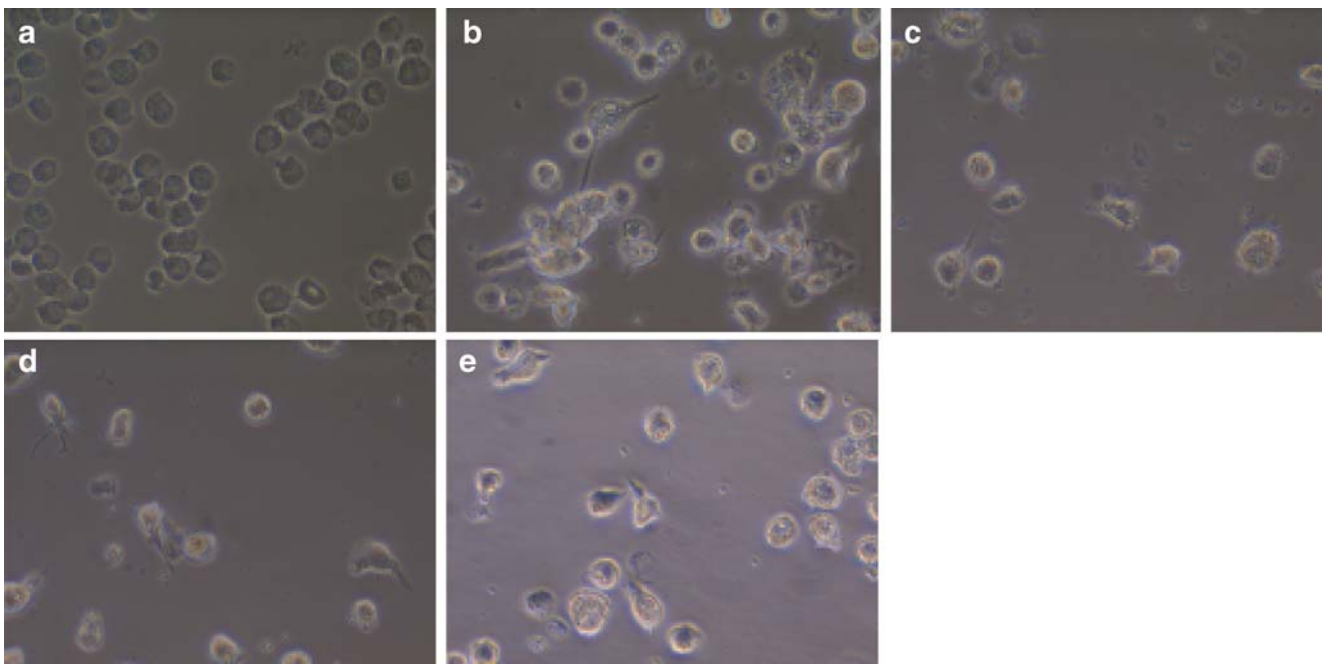

Fig. 3. Activation of THP-1 cells after 24 h incubation with the microparticles: control or no treatment (**a**); PLGA particles (**b**); PLGA-MDP 0.1% particles (**c**); PLGA-TDB 0.1% particles (**d**); PLGA-TDB-MDP 1% particles (**e**). Photographs taken at ×40 magnification

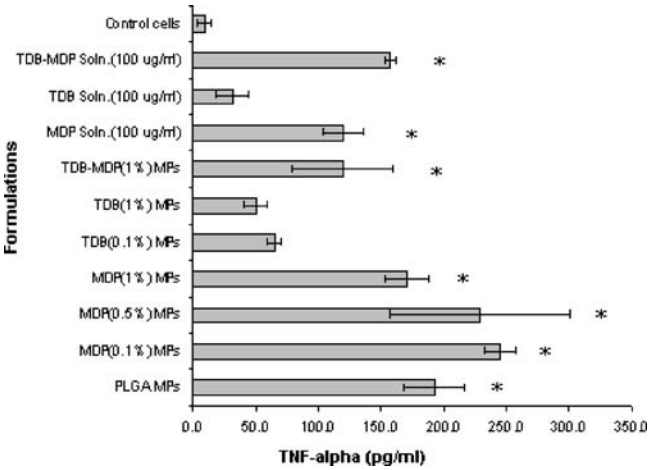

Fig. 4. Release of TNF-α in the culture supernatant. Data expressed as mean values±SD of the means from studies conducted in triplicate. *$P<0.05$ of TNF-α compared with negative controls (Dunnett's t test was used)

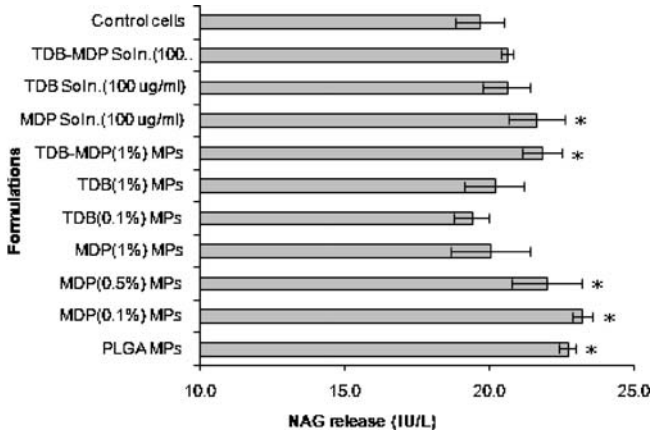

Fig. 6. Release of NAG from THP-1 cells after exposure to microparticles for 24 h. Data expressed as mean values±SD of the means from studies conducted in triplicate. *$P<0.05$ of NAG compared with negative controls (Dunnett's t test was used)

APCs following administration by the pulmonary route. Therefore, the cytotoxicity of MDP and TDB immunomodulators entrapped in biodegradable PLGA polymer in THP-1 cells was assessed. Most of the antigens showing protection in animal models have been effective when administered with a Th1-inducing adjuvant (18).

PLGA particles containing MDP and TDB immunomodulators may act as adjuvants when targeted to AMΦs in the lungs, causing cell activation. These activated MΦs may trigger multiple microbicidal mechanisms, including phagolysosomal fusion, respiratory burst, and the production of proinflammatory cytokines, which restrict the growth of ingested organisms and the recruitment of additional immune cells (19,20). This phenomenon may also lead to the initiation of a Th1-mediated immune response (21). Targeting immunomodulators to AMΦs could also contribute to the reduction in toxicity observed in other pulmonary cells. Cellular and biochemical changes in THP-1 cells could serve as markers for pulmonary injury induced by these particles.

The electron micrograph images of these MPs indicated that they were perforated collapsed hollow spheres consistent with their low densities. The production of such particles is known to be possible from the adoption of specific processing parameters for spray-drying (22). The volume median diameters, obtained by laser diffraction, of these MPs were larger than the projected area diameters obtained from SEM, consistent with the presence of agglomerates in the formulations.

It is known that materials incorporated in PLGA MPs show extended dissolution profile. MDP and TDB incorporated in MPs may, thus, show extended release inside MΦs and exhibit sustained immune response. Once phagocytosed,

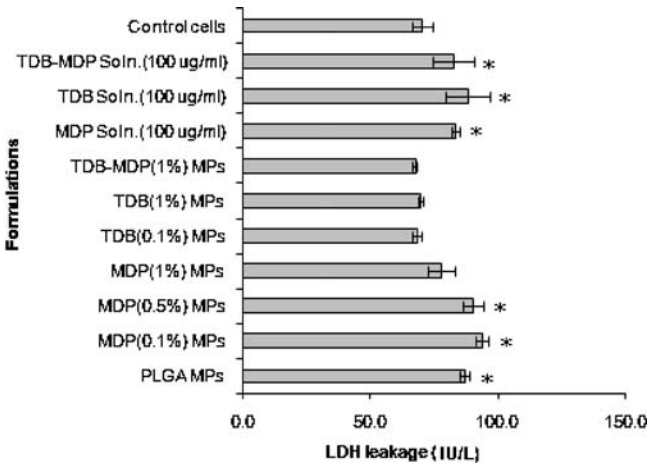

Fig. 5. Measure of LDH activity (mean±SD) on the culture medium of cells after 24 h exposure to different formulations. Data expressed as mean values±SD of the means from studies conducted in triplicate. *$P<0.05$ of LDH compared with negative controls (Dunnett's t test was used)

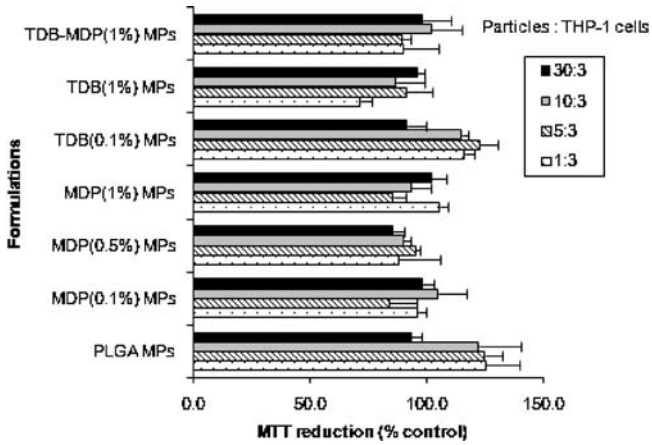

Fig. 7. Cell viability of THP-1 cells exposed to one (*white fill*), five (*hatched fill*), ten (*gray fill*), and 30 (*black fill*) MPs per three cells for 24 h with different microparticle formulations of MDP and TDB using MTT assay. The data expressed as mean±SD of triplicate experiments per condition and are expressed as a percentage of the control value

the majority of particles are trafficked within the cell for periods that may be sufficient for clearance by MΦ migration from the lungs (23,24). Thus, it is anticipated that particle clearance is linked to the rate of degradation of the particles in the intracellular compartments and to the exit of AMΦs from the lungs leading to sustained release of the molecules within AMΦs.

These MPs are not expected to induce any cytotoxicity due to the biodegradable nature of the polymer. MDP has been reported to be a potent immunoadjuvant that enhances antigen processing by stimulating immune-component cells, such as monocytes and MΦs (25). Therefore, it is anticipated that MDP and TDB MPs will serve as effective adjuvants.

Human monocytic THP-1 cells can be differentiated into mature MΦ-like cells and develop AMΦ-like functions in response to PMA. It has been shown that differentiated THP-1 cells closely model the behavior of primary human AMΦs in various aspects (26). Differentiated THP-1 cells adhere to dish surfaces (27) and resemble primary MΦs in morphology. Mechanistic studies using human AMΦs have been challenging because of the difficulty of obtaining these cells in large numbers and, in addition, their appearance in a homogeneous population of cells. Cytokine production is highly variable among human donors, and apoptosis does not proceed in a synchronous manner, making experiments difficult to interpret. We, therefore, selected the THP-1 cell line as a representative model for studying functional, biochemical, and morphological changes in MΦs.

TNF-α appears to have numerous protective functions in TB, but there are instances where it causes tissue damage similar to human disease (28). However, it should be noted that, in the broader context of immunization, TNF-α is known to have positive implications. It is required for the formation and maintenance of granulomas, in animal models of TB (29,30), and for the control of latent TB, in humans (31). TNF-α supports apoptosis of MΦs infected with Mtb, thereby contributing to the clearance of the pathogen (32). TNF-α promotes the maturation of dendritic cells, thereby inducing the transport of mycobacterial antigens to the lymph nodes and the priming of T cell subsets that traffic to the site of infection to complement effector mechanisms of innate immunity. TNF-α has also been reported to increase MΦ phagocytosis and activation, leading to the induction of a Th1 immune response. An increase in TNF-α release was observed in cells treated with MDP and PLGA MPs in comparison to TDB MPs as depicted in Fig. 4. TDB MPs (0.1% and 1.0%) and solution (100 μg/mL) both had significantly lower TNF-α release. Additional *in vivo* studies are required to establish the beneficial or deleterious effects of TNF-α release in response to the presence of MDP MPs.

In our previous studies, we had shown that the inclusion of TDB (0.1% *w*/*w*) in PLGA-Ag85B MPs enhanced their adjuvant effect when administered to infected AMΦ cells as indicated by the release of the cytokine IL-2 (33). TDB in combination with the cationic surfactant dimethyl dioctadecyl ammonium bromide promoted an immune response similar to potential TB vaccines against Mtb infection in a murine model (34). This combination of TDB and cationic surfactant has also been effective in the more sensitive guinea pig model of TB infection (35).

THP-1 cells exposed to MDP and TDB MPs exhibited altered morphology relative to controls. Enzyme levels of NAG and LDH were elevated in the case of cells exposed to MDP MPs compared to TDB MPs, indicating a nonspecific potentially cytotoxic response.

The *in vitro* cytotoxicity of these immunomodulators formulated in biodegradable MPs were quantified by the release of cytosolic enzyme LDH into cell culture medium as the marker for membrane integrity and the MTT assay representing the metabolic and mitochondrial activity of treated cells. LDH is a stable cytoplasmic enzyme stored in viable cells and any increase in its leakage indicates that the integrity of cell membranes is compromised, possibly due to oxidative injury (36). The TDB MPs (0.1% and 1.0%) and TDB-MDP MPs (1.0%) both had significantly lower LDH release than MDP-only MPs. The leakage of LDH to the outside media shows that MDP MPs leads to higher loss of membrane integrity compared to TDB MPs. For the MTT assay, all cells showed viabilities above 84% except 1.0% TDB MPs (exposed to one particle per three cells) which had a lower viability of 72%. There were no significant differences between MDP and TDB particles when assayed by MTT.

These results showed that the LDH assay was more sensitive than the MTT assay. Lower concentrations of MDP in MPs caused greater changes in LDH release compared with their effects on mitochondrial dehydrogenase activity by MTT assay. This may suggest that mitochondria may not be a primary target for MDP released from MPs. MDP MPs may interact with the plasma membrane causing cell lysis but may not be significantly taken into cells thus not reaching intracellular targets such as mitochondria and impairing their functions. MDP may cause a progressive accumulation in the phospholipid bilayer resulting in disruption of the membrane architecture and lysis. Thus, the LDH test provides valuable additional information to that generated by the MTT test. The LDH test is widely used in modern toxicology; hence, there is great value in using the combined approach of MTT and LDH.

The increase in NAG release relative to LDH can be used to estimate how much of this increase in NAG is due to lysed cells (increase in both NAG and LDH enzymes) and how much is due to stimulated phagocytic cells (increase in NAG only). PLGA MPs, PLGA-MDP MPs (0.1% and 0.5%), as well as MDP solution showed higher levels of both NAG and LDH release, suggesting MDP causes more cell lysis than TDB MPs.

Exposure time may also significantly influence apparent cytotoxicity of these MPs. In the present study, differentiated THP-1 cells were exposed to a variety of MP concentrations containing MDP and TDB for 24 h. Though we did not evaluate different exposure times, it may be suggested that the maximal release of these immunomodulators was essentially complete in 24 h and that the most toxic effects occurred during the first 24 h. We had previously demonstrated that approximately 85% of MDP was released from MPs in a linear fashion with respect to time over the first 24 h reflecting the release of surface associated adjuvant (33) and phagocytosis by AMΦs occurred within 6 h after particle exposure (37). Since the supernatant sampling was performed at 24 h, it is reasonable to assume that 75–80% of immunomodulators were presented to the AMΦs. However,

the initial rapid release is followed by extended release of the remaining adjuvant. PLGA formulated MPs are known to release measurable amounts of compounds for periods beyond 24 h, although the rate of release decreases with time due to the significantly depleted reservoir of the active ingredient (38).

CONCLUSION

TB being an insidious worldwide disease of immense significance, new approaches to delivery and effectiveness of vaccines to treat this disease is desirable. Two potential adjuvants, MDP and TDB, were encapsulated in polymeric MPs. These particles were prepared in sizes suitable for aerosol delivery to the lungs and appeared to be perforated collapsed hollow spheres. Further work is required to study the relevance of the statistical increases in biochemical markers of adverse effects evident from the *in vitro* studies to the function of AMΦs *in vivo* and the impact on the surrounding tissues using different animal species following the administration of immunomodulators.

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