Respirable PLGA Microspheres Containing Rifampicin for the Treatment of Tuberculosis: Screening in an Infectious Disease Model

Sandra Suarez,[1] Patrick O'Hara,[1] Masha Kazantseva,[1] Christian E. Newcomer,[2] Roy Hopfer,[3] David N. McMurray,[4] and Anthony J. Hickey[1,5]

Received March 12, 2001; accepted May 14, 2001

Purpose. Targeted delivery of rifampicin loaded microspheres to the alveolar macrophage, the host cell for *Mycobacterium tuberculosis* (MTB), may be an effective targeted approach to pulmonary tuberculosis therapy. A guinea pig infection model has been adopted as a post-treatment screening method for antimicrobial effect. Insufflation and nebulization methods of drug delivery were evaluated.

Methods. Rifampicin alone (RIF, 1.03–1.72 mg/kg), within poly(lactide-co-glycolide) microspheres (R-PLGA, equivalent to 1.03–1.72 mg/kg) or polymer microparticles alone (PLGA) were administered by insufflation or nebulization, 24 h before bacterial aerosol exposure. Animals were infected with an aerosol containing a small number (2×10^5 cfu/mL) of virulent H37Rv strain of MTB. Lung and spleen tissue samples were collected 28 days after infection for quantitative bacteriology and histopathological analysis.

Results. There was a dose-effect relationship between insufflated R-PLGA and burden of bacteria in the lungs. In addition, guinea pigs treated with R-PLGA had a significantly smaller number of viable bacteria ($P < 0.05$), reduced inflammation and lung damage than lactose or saline control, PLGA or RIF treated animals.

Conclusions. These studies indicate the potential of R-PLGA, delivered by insufflation or nebulization directly to the lungs, to affect the early development of pulmonary TB.

KEY WORDS: pulmonary tuberculosis; rifampicin-loaded poly(lactide-co-glycolide) microspheres; low dose aerosol infection.

INTRODUCTION

Pulmonary tuberculosis (TB) is the most common form of tuberculosis, involving alveolar macrophages containing large numbers of the bacilli (1). The current treatment of pulmonary TB involves prolonged oral administration of high systemic doses of combined antibiotics, which is associated with unwanted side effects and poor compliance (2–5). The resident alveolar macrophage is the first defense against lung infection. Targeting drug to the alveolar macrophage may improve efficacy and potentially reduce systemic toxicity. Several studies (6–8) have shown that the activity of anti-TB drugs encapsulated in liposomes, administered parenterally at below therapeutic concentration of free drug, was considerably increased. In addition, encapsulated drugs had marginal side effects compared to the free drug. Also, a high localized concentration of the drug can be given in cases where the organisms are resistant to the therapeutic oral dose (9,10). Microsphere technology has been used to develop formulations of rifampicin (RIF) for targeted delivery to host macrophages (11). Treatment of MTB-infected monocyte cell lines with RIF-loaded microspheres resulted in a significant decrease in the number of viable bacteria at 7 days following initial infection (12). Thus, a microparticle drug delivery system targeting to the alveolar macrophages might contribute to improved chemotherapy of TB.

In the present study, the RIF, alone or encapsulated in microspheres were delivered by insufflation or nebulization to a post treatment low level respiratory challenge animal model (13). This model was used to screen the ability of the drug to reduce the accumulation of virulent bacilli and concomitant histopathological effects during the first two weeks of infection. The postulated mechanism of action is macrophage uptake of drug loaded particles increasing the local dose at the residence site of MTB.

MATERIALS AND METHODS

Chemicals and Reagents

Rifampicin loaded poly(lactide-co-glycolide) (75:25, MWt 85,200 D) microspheres (R-PLGA) and RIF were prepared by solvent evaporation method and characterized (14). The count median diameters (CMD) of the R-PLGA, PLGA microparticles and RIF were (CMD [μm]/GSD): 1.2/2.0, 1.8/1.7, and 2.8/1.5 respectively. Microparticles were smooth and spherical. The dissolution release rate up to 24 h for 4.3% w/w loaded R-PLGA microspheres was 20–40% and 5–25% at pH 7.4 and pH 5.2, respectively. Terminal rate constant for both pHs was 1.4–3.6%/$h^{1/2}$. RIF was micronized using the bench top air-jet mill (Trost GEM-T, Glenn Mills, Clifton, NJ). RIF dissolved completely within 6 h at pH 7.4 and 70% dissolved in 24 h at pH 5.2. MTB strain H37Rv (lot # 27294) was purchased from American Type Culture Collection (ATCC, Rockville, MD). Bactec™ 12B Mycobacteria culture vials (Becton Dickinson & Co., Sparks, Maryland) containing Middlebrook 7H12 broth were used for radiometric assay. Lactose (Mallinckrodt, Paris, KY) in the 45–125 μm size range was used as a carrier to aid particle dispersion in air. Sodium pentobarbital (Sigma, St. Louis, MO) was used for euthanasia.

Animals

All animal procedures were approved by the UNC-CH Institutional Animal Care and Use Committee. Specific-pathogen free male Dunkin-Hartley guinea pigs (150–200 g) (Hilltop, Scottsdale, PA) were housed individually in a biosafety level 3 (BL-3) containment area with a 12 h light/dark cycle. Animals were allowed free access to water and food

[1] School of Pharmacy, University of North Carolina, Chapel Hill, North Carolina 27599.
[2] Department of Pathology and Laboratory Animal Medicine, School of Medicine, University of North Carolina, Chapel Hill, North Carolina 27599.
[3] Laboratory of Special Microbiology, University of North Carolina Hospitals, Chapel Hill, North Carolina 27599.
[4] Department of Medical Microbiology and Immunology, College of Medicine, Texas A & M University, System Health Science Center, College Station, Texas 77843.
[5] To whom correspondence should be addressed. (e-mail: ahickey@unc.edu)

0724-8741/01/0900-1315$19.50/0

(Prolab guinea pig 5P18, PMI feeds, Inc., St. Louis, MO), but were fasted overnight before each experiment.

In-Vitro Performance of R-PLGA microspheres

Bactec™ Radiometric Susceptibility Assay

MTB suspensions were prepared according to (15) and adjusted to McFarland #1 standard. Aliquots of 0.1 mL were added to the vials containing Middlebrook 7H12 broth. Aliquots of 0.1 mL of the R-PLGA suspensions (2, 20, and 100 Mg/mL of RIF) and RIF in solution (1, 2 or 4 Mg/mL) were aseptically added to the vials. Controls consisted of PLGA suspension samples (equivalent to 100 Mg/mL R-PLGA) and untreated MTB suspension samples. All samples were prepared in triplicate. The susceptibility of the MTB was assessed by a radiometric assay (Bactec™ Model 460 Instrument, Johnston Labs., Coxieville, MD), in which the amount of $^{14}CO_2$ detected, reflected the bacterial growth rate by consumption of ^{14}C-palmitic acid, present as a substrate in the vial. β-radiation emitted at 0.025 μCi from $^{14}CO_2$ corresponds to 100 growth index units. The data was represented graphically as growth index versus time of incubation, in days.

Chemotherapy and Experimental Infection

Dose Selection

The macrophage concentration of rifampicin calculated from oral doses of 600mg is twice that of serum (16), which is 7–9 μg/mL (2–3 h after administration) (17). Assuming sphericity of macrophages, cell diameter of 10 μm, and 10^6–10^7 cells/lung the total dose required for efficacy is ~5–50 ng (10 μg × # Cells [10^6–10^7] × volume of cell [500 nL]). This is much less than the total dose delivered to the lungs [1.03–1.72 mg/kg RIF] in the following studies. However, the release rate of drug will dictate the amount available at any point in time making larger doses a necessity to achieve the effect of an oral dose in the macrophage. From a practical standpoint the doses were limited by the maximum drug load (4.3% w/w R-PLGA) in a lactose blend (90:10 rifampicin:lactose) that could be delivered in a single bolus of powder (10 mg) from the insufflator. The dose delivered from the insufflator was >95% of the nominal dose. Nebulizer delivery was assumed to be no more than 10% of the emitted dose (18).

R-PLGA Microspheres Administered by Insufflation

Guinea pigs were anesthetized (50 mg/kg ketamine: 5 mg/kg xylazine: 2 mg/kg acepromazine by intraperitoneal injection), endotracheally intubated and the powder (10 mg) was insufflated using 3 mL of air (Insufflator, PennCentury, Philadelphia, PA). Four treatment groups of animals were employed. The treatment groups (n = 3–7) were: micronized RIF/lactose (1.03–1.72 mg/kg RIF), R-PLGA/lactose (1.03–1.72 mg/kg RIF), PLGA/lactose and the control group (lactose). Animals were allowed to recover for 24 h in their cages while being monitored and were infected with MTB as a group.

R-PLGA Microspheres Administered by Nebulization

Drug or control materials were delivered by nebulization (Acorn II nebulizer, Marquest Medical Products, Inc. Englewood, CO) under controlled conditions (15 min delivery period, 5 mL of a 40mg suspension, inlet pressure 40 psig). Suspensions of each formulation were prepared in normal saline containing 0.05% of Tween 80: 1) R-PLGA (1.0–1.3 mg/kg RIF); 2) RIF (1.0–1.3 mg/kg); 3) PLGA or 4) Normal saline containing 0.05% of Tween 80 (control group). Twenty four hours later animals were infected with MTB.

Experimental Infection

Animals were infected with an inoculum of MTB strain H37Rv and were placed randomly in the aerosol exposure chamber (19). Suspensions of bacteria (5 mL, 2×10^5 cfu/mL) were nebulized (modified MRE-3 Collison, BGI Inc., Waltham, MA) into the chamber.

Assessment of the Number of Viable Bacteria

Four to five weeks after infection the lungs were inspected to determine the number of superficial lesions. The caudal right lung lobe and approximately three quarters of spleen tissue were homogenized (2–3 min, 1300 rpm, 4.5 mL sterile saline). The caudal left lung lobe and residual spleen tissue were placed in 10 % neutral buffered formalin for histopathologic evaluation. Aliquots of 0.1 mL of diluted homogenates were inoculated into duplicate M7H10 agar plates (Hardy Diagnostics, Santa Maria, CA). The plates were incubated (37°C, 21–28 days). Visible colonies were counted and data was expressed as logarithm to base 10 (log) to meet the assumption of parametric statistics.

Histopathology

Formalin-fixed lung and spleen tissues were embedded in paraffin wax and sectioned at 5 μm. The sections were mounted on glass slides and stained with hematoxylin-eosin.

Statistical Analysis

The data was analyzed by Scheffe's multiple comparison statistical test (SAS/STAT User's guide, version 6, 4th edition, volume 2, 1989. The SAS Institute Inc., Cary, NC).

RESULTS

In-Vitro Efficacy

Figure 1 shows the results of the BACTEC™ radiometric assay for the RIF, R-PLGA, PLGA and the untreated control. RIF and R-PLGA exhibited action in limiting the growth of MTB in this culture system. RIF inhibited MTB growth at both concentrations employed (1 and 4 μg/mL). R-PLGA limited MTB growth by approximately 50% at the lowest concentration (2 μg/mL) and by 100% at both of the higher concentrations (20 and 100 μg/mL). R-PLGA was not as effective as RIF as the total drug load was only available on dissolution. Indeed, the release rate of drug from R-PLGA was not sufficient to inhibit growth at the low dose of 2 μg/mL. PLGA treatments did not have any effect on the growth of MTB.

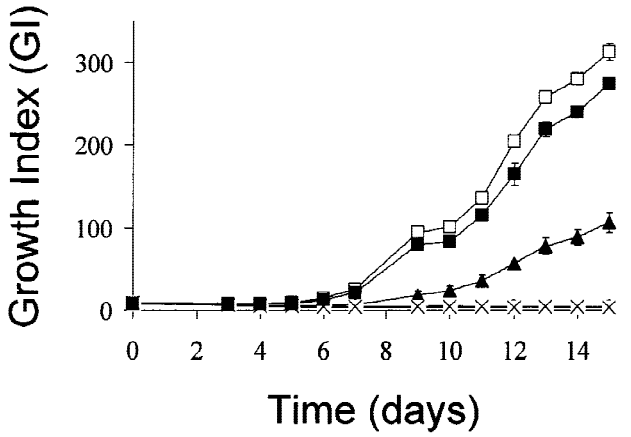

Fig. 1. *In-vitro* growth of MTB in the presence of: 2 (▲), 20 (△) and 100 (x) Mg/mL RIF in R-PLGA; 1 (○) and 4 (●) Mg/mL RIF and (□) PLGA (equivalent to PLGA in 100 Mg/mL of R-PLGA). Untreated control is represented by (■) symbol. Points represent mean * SD for n = 3.

Bacteriology

R-PLGA Microspheres Administered by Insufflation

Figure 2 shows a comparative dose-response relationship following lung insufflation of escalating doses of R-PLGA. The number of viable bacteria presented was normalized by the lactose control values (3.81 ± 0.2 log cfu/mL). There was a significant reduction ($P < 0.05$) in number of viable bacteria following insufflation of the highest dose (1.72 mg/mL) of R-PLGA microspheres (log cfu/mL = 2.99 ± 0.23) compared with the other doses employed (1.03 and 1.46 mg/kg). In contrast, no trend occurred for spleen tissues, which suggests the inefficiency of the insufflation method in distributing the drug uniformly in the lungs. Bacteria grow in untreated areas from which dissemination into systemic circulation can then occur.

R-PLGA Microspheres Administered by Nebulization

Antitubercular action of nebulized R-PLGA, RIF and PLGA, based on the number of viable bacteria cultured in lung and spleen is shown in Fig. 3. There was a significant difference in the viable microorganisms counted following treatment with nebulized R-PLGA microspheres (log cfu/mL = 3.8 ± 0.4) compared to either the RIF (log cfu/mL = 4.8 ± 0.08) or saline control group (log cfu/mL = 4.9 ± 0.06). No trend was observed for the spleen samples.

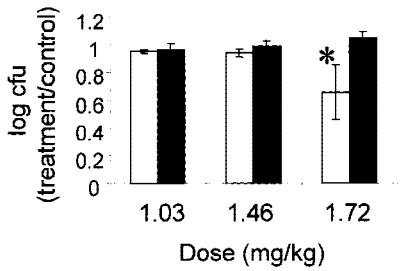

Fig. 2. Comparative dose-response relationship following lung insufflation of RIF (□) and R-PLGA microspheres (■) based on lung bacteriology, 4–5 weeks post-infection. Bars represent mean ± SD for n = 3–7. *$P < 0.05$ (level of significance for R-PLGA microspheres).

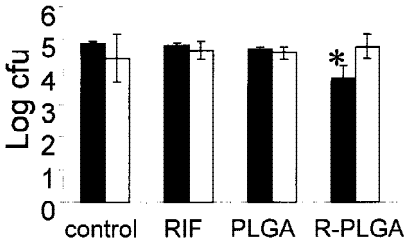

Fig. 3. Number of viable bacteria (cfu/mL) in lung (■) and spleen (□) tissues (4–5 weeks post-infection) following nebulization of R-PLGA microspheres (1.03–1.72 mg/kg), RIF (1.03–1.72 mg/kg) and PLGA. Animals, including control group, were exposed to MTB 24 h after drug administration. Bars represent mean ± SD for n = 3-5. *$P < 0.05$ (level of significance for R-PLGA microspheres).

Histopathological Studies

The guinea pig lung tissues from all treatment groups employed had characteristic histopathological lesions 4–5 weeks post-infection, which consisted of multiple large sheets of epithelioid macrophages with abundant foamy cytoplasm (Fig. 4A–C). These lesions were present in the peripheral parenchyma and peribronchial/perivascular areas. In many samples, mature tubercular granulomas with caseation and lymphoid aggregates in the peribronchial/perivascular zones were evident. In addition, peribronchial and perivascular edema was observed in some control tissues. Lung histopathology findings from animals insufflated with R-PLGA were less severe; foci of epithelioid macrophages were smaller and less had progressed to caseous necrosis (Fig. 4D). No histopathological differences were observed for spleen tissue samples between groups, the majority of them had several caseous tubercular granulomas.

DISCUSSION

RIF was used as a candidate antitubercular drug for incorporation in microspheres as its solubility was suitable for the manufacturing procedure, and from the perceived benefit that would accrue from extending the usefulness of this first line drug. Poly (DL-lactide-co-glycolide) was used for the production of all batches of microspheres. The use of slow release preparations such as microspheres presents several possible advantages over conventional preparations: 1. higher patient compliance; 2. drug targeting to alveolar macrophages by inhalation of the microsphere preparation; and 3. reduction of the systemic side effects by significantly decreasing the total dose and frequency of drug administration. In addition, microspheres may be prepared in sizes appropriate for pulmonary delivery and macrophage uptake (1–5 μm). These attributes make microspheres a promising alternative for the treatment of mycobacterial infections, especially pulmonary tuberculosis.

R-PLGA, at 2 μg/mL, appeared to inhibit the growth of MTB by approximately 50% at 5–7 days. Figure 1 shows that the use of 20 and 100 μg/mL of RIF in R-PLGA microspheres completely inhibited the TB growth, indicating the importance of an appropriate dose and release rate for the inhibition of the bacterial growth.

In the present studies, a guinea pig model for tuberculosis, which includes a low dose aerosol challenge (19–21), was adopted. In addition, to circumvent the toxicity resulting from

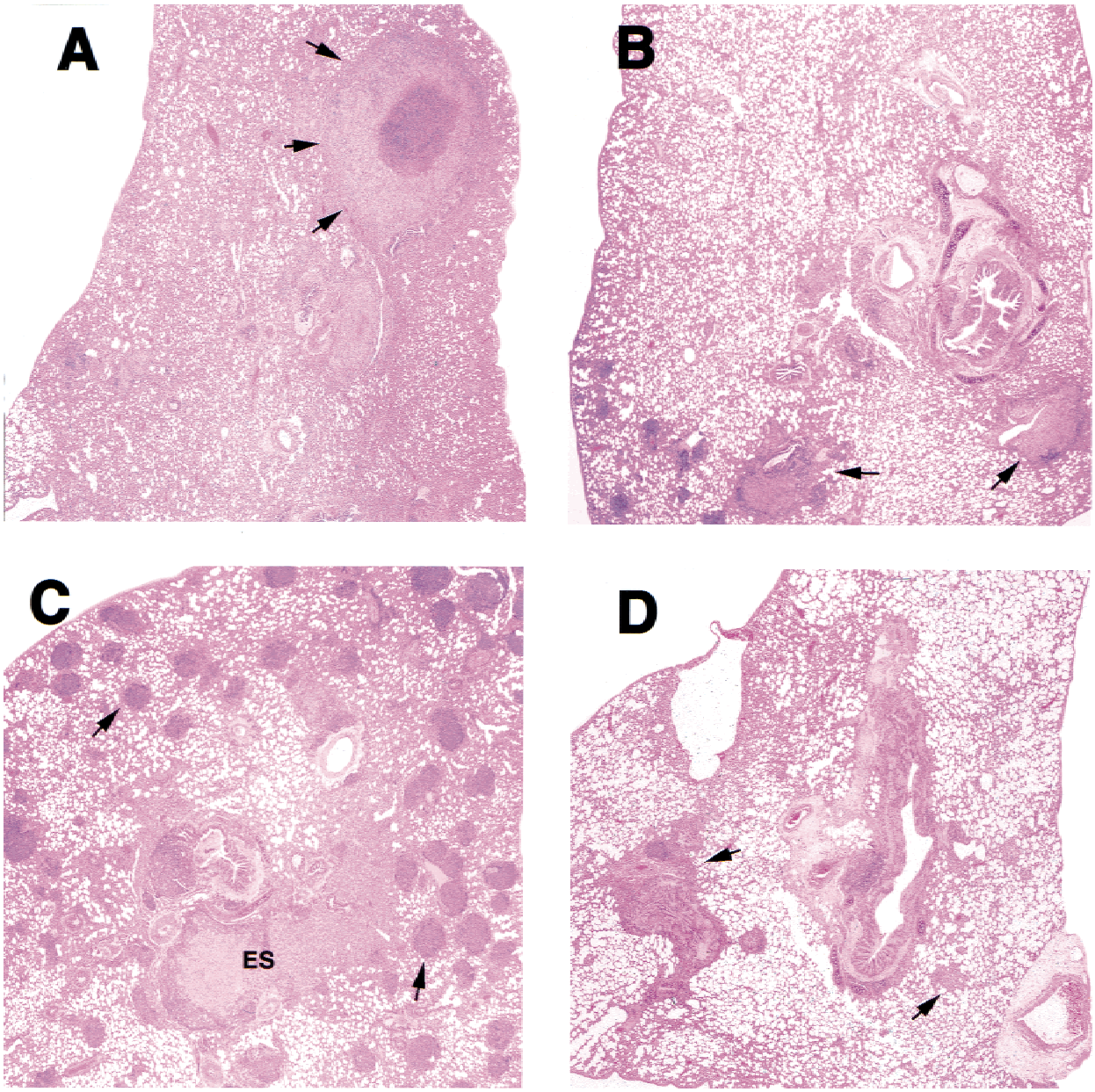

Fig. 4. Lung histopathology in guinea pigs treated by insufflation and infected for 4–5 weeks with virulent MTB. **(A)** Lactose control animal. A large granuloma is adjacent to an arteriole and two bronchioles. **(B)** PLGA treated animal. Sheets of epithelioid macrophages encircle and invade a pulmonary vein and bronchiole (arrows). **(C)** RIF treated animal. There is an extensive sheet of epithelioid macrophages (ES) and lymphoid follicle development (arrows). **(D)** R-PLGA (1.72 mg/kg) insufflated animal. The granulomas (arrows) were typically smaller and without caseation in this group. Samples were stained with hematoxylin-eosin. Mag. 25x.

high oral doses, R-PLGA microspheres were administered directly to the site of infection, the lungs. Under these conditions it is possible to mimic the pathogenesis of TB as it occurs in humans infected with virulent organisms (21): bacilli develop at the site of implantation in the lung and some bacilli escape via the lymphatics to the regional node with subsequent leaking to the bloodstream and eventual colonization of the spleen and other organs. The guinea pig was chosen as a model for tuberculosis due to its similarity to humans in terms of the pathophysiology of the disease.

A dose-effect relationship for R-PLGA microspheres and the number of bacteria cultured in the lungs was observed, while no trend was found for RIF. The ratio of log cfu/mL cultured in the lungs following administration of the 1.72 mg/kg of R-PLGA to that of control animals (log cfu R-PLGA/log cfu control $= 0.66 \pm 0.12$) was significantly different than that obtained at lower doses and after the administration of RIF (log cfu RIF/log cfu control $= 1.05 \pm 0.03$) ($P < 0.05$) (Fig. 2). These findings suggest that the effective treatment of pulmonary tuberculosis using sustained release preparations may consist of formulations with high loading efficiency (high doses of encapsulated drug) and preparations exhibiting a large initial release. This release profile may be considered in terms of an initial dose to achieve the minimum

inhibitory concentration (MIC) locally followed by a sustained release to maintain the MIC. However, the local dose required to achieve the desired MIC at the level of the subcellular organelle in which the MTB resides is not known. Future studies are needed to challenge this hypothesis.

The administration of R-PLGA microspheres by both nebulization and insufflation methods resulted in a significant reduction of lung bacterial burden compared to that obtained for the RIF or control groups. Assuming that 15 to 20% of the nebulized drug reaches the lungs (22), the dose of R-PLGA administered using this method was estimated as 1–1.3 mg/kg. Nebulization was more efficient in reducing the number of viable microorganisms in the lungs at equivalent doses of R-PLGA than was insufflation, the ratios of [log cfu treated] /[log cfu control] were 0.95 ± 0.01 and 0.79 ± 0.1 ($P < 0.05$), for the insufflation and nebulization method, respectively. Although there was a significant difference in the number of viable bacteria in the lung as a function of the dose of RIF in R-PLGA microspheres, no clear differences were observed in the degree of damage or inflammation between these treatments. A higher dose of encapsulated drug and a more efficient mode of aerosol delivery might reduce deterioration in lung pathology if delivered early in the course of primary infection. Further studies are required to evaluate the effect of therapeutic aerosols delivered at defined intervals following infection and possibly in conjunction with oral or parenteral doses. The performance of such studies would not be a trivial undertaking but the outcome would define a relevant therapeutic dosing regime. The present studies are the basis for the future optimization of this drug delivery system to achieve maximum therapeutic effect.

In summary, R-PLGA microspheres manufactured in the respiratory range (1–5 μm), showed a dose-effect relationship between the amount of insufflated R-PLGA microspheres and the number of viable microorganisms in the lungs. A single dose (1.7 mg/kg) of R-PLGA microspheres significantly reduced the lung bacterial burden (10 fold) when compared to that for the control or RIF treated guinea pigs infected with MTB. In addition, R-PLGA treated animals exhibited reduced inflammation and lung damage compared to untreated control or RIF-alone treated animals. The potential use of R-PLGA microspheres for aerosol treatment of primary pulmonary tuberculosis is supported by these preliminary studies.

ACKNOWLEDGMENTS

This work was supported by the National Heart, Lung and Blood Institute (NHI Grant # HL 55789). The technical assistance of Ms. Susan Phalen of Texas A&M University was much appreciated.

REFERENCES

1. P. Deol and G. K. Khuller. Lung specific stealth liposomes: stability, biodistribution and toxicity of liposomal antitubercular drugs in mice. *Biochim. Biophys. Acta* **1334**:161–172 (1997).
2. J. Schreiber, G. Zissel, U. Greinert, M. Schlaak, and J. Muller-Quernheim. Lymphocyte transformation test for the evaluation of adverse effects of antituberculous drugs. *Eur. J. Medical Res.* **4**:67–71 (1999).
3. S. K. Teo. Assessment of a combined preparation of isoniazid, rifampicin and pyrazinamide in the initial phase of chemotherapy in three 6-month regimens for smear-positive pulmonary tuberculosis: a five-year follow-up report. *Inter. J. Tuber. Lung Dis.* **3**:126–132 (1999).
4. D. J. Girgling. The hepatic toxicity of antituberculosis regimens containing isoniazid, rifampicin and pyrazinamide. *Tubercle*. **59**: 13–32 (1978).
5. M. Zierski and E. Bek. Side effects of drug regimens used in short course chemotherapy for pulmonary tuberculosis: a controlled study. *Tubercle*. **61**:41–49 (1980).
6. L. C. Orozco, F. O. Quintana, R. M. Beltran, I. Moreno, and G. Rodriguez. The use of rifampicin and isoniazid entrapped in liposomes for the treatment of murine tuberculosis. *Tubercle*. **67**: 91–97 (1986).
7. A. Agarwal, H. Kandpal, H. P. Gupta, and N. B. Singh. Tuftsin-bearing liposomes as rifampicin vehicles in treatment of tuberculosis in mice. *Antimic. Agents Chem*. **38**:588–593 (1994).
8. P. Deol, G. K. Khuller, and K. Joshi. Therapeutic efficacies of izoniasid and rifampicin encapsulated in lung-specific stealth liposomes agaist Mycobacterium tuberculosis infection induced in mice. *Antimic. Agents Chem*. **41**:1211–1214 (1997).
9. T. Allen and J. Everest. Effect of liposome size and drug release properties on pharmacokinetics of encapsulated drug in rats. *J. Pharm. Exp. Ther.* **226**:539–546 (1983).
10. T. S. Moulding. Should isoniazid be used in treatment of tuberculosis despite acquired isoniazid resistance. *Am. Rev. Resp. Dis*. **123**:262–269 (1981).
11. D. C. Quenelle, J. K. Stasss, G. Z. Winchester, E. L. Barrow, and W. W. Barrow. Efficacy of microencapsulated rifampicin in mycobacterium tuberculosis-infected mice. *Antimic. Agents Chem*. **43**:1144–1151 (1999).
12. E. L. Barrow. Use of microsphere technology for targeted delivery of rifampin to mycobacterium tuberculosis-infected macrophages. *Antimic. Agents Chem*. **42**:2682–2689 (1998).
13. D. W. Smith and G. R. Harding. Animal model : experimental airborne tuberculosis in the guinea pig. *Am. J. Path*. **89**:273–276 (1972).
14. P. O'Hara and A. J. Hickey. Respirable PLGA microspheres containing rifampicin for the treatment of tuberculosis: manufacture and characterization. *Pharm. Res*. **17**:955–961 (2000).
15. A. A. Grover, H. K. Kim, E. H. Wiegeshaus, and D. W. Smith. Host-parasite relationships in experimental airborne tuberculosis II. Reproducible infection by means of an inoculum preserved at -70 degrees C. *J. Bacteriol*. **94**:832–835 (1967).
16. H. Thadepalli. Lower respiratory tract. In A.M. Ristuccia, B.A. Cunha (eds.), Antimicrobial therapy, Raven Press, New York, 1984, pp. 439–451.
17. A. Kucers, N. M. Bennet, and R. J. Kemp. The use of antibiotics. J.B. Lippincot Company, London, GB, 1988.
18. T. B. Martonen and Y. Yang. A mathematical model for aerosol deposition in the respiratory tract of the guinea pig. *Inhalation Toxicology* **6**:1–19 (1994).
19. E. H. Wiegeshaus, D. N. McMurray, A. A. Grover, G. E. Harding, and D. W. Smith. Host-parasite relationships in experimental airborne tuberculosis. III. Relevance of microbial enumeration to acquired resistance in guinea pigs. *Amer. Rev. Resp. Dis*. **102**:422–428 (1970).
20. V. Balasubramanian, E. H. Wiegeshaus, and D. W. Smith. Mycobacterial infection in guinea pigs. *Immunobiology* **191**:395–401 (1994).
21. D. N. McMurray. Guinea pig model of tuberculosis. In B.R. Bloom (ed.), Tuberculosis: pathogenesis, protection, and control. American Society of Microbiology, Washington, DC, 1994, pp. 135–147.
22. S. J. Smith and J. A. Bernstein. Therapeutic uses of lung aerosols. In A.J. Hickey (ed.), Inhalation aerosols. Physical and biological basis for therapy. Marcel Dekker, Inc., New York, 1996, pp. 233–269.