Inhalable Microparticles Containing Drug Combinations to Target Alveolar Macrophages for Treatment of Pulmonary Tuberculosis

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Purpose: Drug therapy of tuberculosis (TB) requires long-term oral administration of multiple drugs for curing as well as preventing and/or combating multi-drug resistance. Persistent, high blood levels of antitubercular drugs resulting from prolonged oral administration of anti-TB drugs may be neither necessary nor sufficient to kill mycobacteria residing in macrophages (Mϕ). Inhalable biodegradable microparticles containing two of the first-line anti-TB drugs, isoniazid (H), and rifampicin (R), were prepared and tested for (i) phagocytosis by mouse Mϕ, (ii) administration as a dry powder inhalation to rats, and (iii) targeting alveolar Mϕ with high drug doses when administered to rats.

Methods: *poly*(D-L lactic acid) microparticles were prepared by emulsion methods and their drug content and size distribution determined. These were tested for uptake by murine Mϕ in culture and resultant intracellular drug concentrations determined by high performance thin-layer chromatography (HPTLC). Rats were administered an inhalation of microparticles using an inhalation chamber developed in the lab. The extent of microparticle delivery *in vivo* was examined by flow-cytometry. Drug concentrations in the blood and in alveolar Mϕ were estimated by high-performance liquid chromatography after oral, vascular, intratracheal, and inhalation administration.

Results: Inhalable microparticles could be prepared and were taken up by cultured Mϕ. Large numbers of particles could be delivered to the bronchiopulmonary system through a 2-min exposure to fluidized particles. The intracellular drug concentrations resulting from vascular delivery of soluble drugs were found to be lower than those resulting from particle inhalation.

Conclusions: Inhalable microparticles containing multiple anti-TB drugs offer promises of dose and dosing-frequency reduction, toxicity alleviation, and targeting Mϕ-resident persistent mycobacteria.

KEY WORDS: tuberculosis; isoniazid; rifampicin; alveolar macrophages; dry powder inhalation; biodegradable microspheres.

INTRODUCTION

Directly observed therapy (short course) or DOTS, recommended for patients with pulmonary TB (1), is reported to cure approximately 80% of Indian (2) and 95% of Chinese (3) patients completing the full course. These observations suggest that 5 to 20% of Asian patients are not cured by DOTS. One reason for treatment failure could be that drug concentrations achieved in the cytosol of target cells through oral administration are not sufficient to kill bacteria residing within Mϕ.

Several investigators have proposed the administration of antitubercular drugs in the form of vesicular systems as inhalations (4) or injectable preparations (5), as also microparticulate systems for injection (6,7). The objectives of these investigators include reduction of drug dose, dose frequency and toxicity, improvement in patient compliance and, most importantly, targeting Mϕ that harbor the TB bacteria. Recently, O'Hara and Hickey suggested the administration of biodegradable microspheres through the bronchio-pulmonary route for better therapy of TB (8). We have also been working in this area with the long-term goal of preparing inhalable particles for TB therapy.

Dry powder inhalations formulations have been developed for asthma (9) and for deep-lung delivery of various agents (10,11). It has been observed that particles reaching the lungs are phagocytosed rapidly by alveolar Mϕ (12). Although phagocytosis and sequestration of inhaled powders may be a problem for drug delivery to other cells comprising lung tissue, it is an advantage for chemotherapy of TB. Phagocytosed microparticles potentially can deliver larger amounts of drug to the cytosol than oral doses. Moreover, microparticles have the potential for lowering dose frequency and magnitude, which is especially advantageous for maintaining drug concentrations and improving patient compliance. It may therefore be advantageous to incorporate inhalable microparticles containing multiple drugs in a dry powder inhalation for chemotherapy of TB.

Single-drug therapy of TB is disfavored for fear of drug resistance (1). We report here our initial experiments on developing inhalable microparticles containing two of the first-line anti-TB drugs, H and R. The ratio of H:R in these particles was set at 1:3 from an appraisal of maximal plasma concentration (C_{max}) values obtained in humans when these drugs are administered at the lowest reported efficacious doses compatible with DOTS. The C_{max} values of R reach 9.2 μg/ml, and those for H attain 1.7 to 3 μg/ml (in fast and slow acylators of H respectively) (13). We used these observations to design our microparticles, reasoning that since drug concentrations attained within Mϕ as a consequence of exposure to blood levels in the above ratio (roughly, 1:3) are efficacious, it would be acceptable to formulate particles that deliver the two drugs to Mϕ in a similar proportion when phagocytosed.

Processing H and R together during microparticle preparation led to the formation of an adduct–rifamycin isonicotinyl hydrazone (RIH)—that showed up consistently in HPTLC and high performance liquid chromatography (HPLC) analyses. RIH is an off-patent anti-tubercular agent (14,15) and was demonstrated recently to form within an hour at gastric pH when H and R are present together in solution (16). The formulations we report contain significant but unquantified amounts of RIH in addition to H and R.

MATERIALS AND METHODS

Materials

Isoniazid, I.P. and Rifampicin, I.P. were gifts from Ranbaxy, New Delhi, and Themis, Bombay, respectively. These

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were stored at 4°C and recrystallized before use. *Poly*(D,L-Lactic Acid) (PLA) of intrinsic viscosity 1.11 cps (Birmingham Polymers, Inc., Birmingham, USA) was generously shared with us by Dr. A.K. Panda, National Institute of Immunology, New Delhi. All other chemicals and solvents were of HPLC or Analytical Grade. Dichlorofluorescein, cell culture media and supplements were obtained from Sigma Chemical Co. (St. Louis, MO).

Formulation

Microparticles containing one part of drug to three parts of PLA were prepared by a combination of solvent extraction and evaporation. Dichlorofluorescein (F) was incorporated in addition to H and R in some of the batches for use as a probe in microscopy and flow cytometry studies. We used a mixture of methanol and dichloromethane (DCM) to dissolve R and H (and F where applicable) in a single, non-aqueous phase. This was added to chilled silicone oil under sonication or homogenization. It was expected that DCM would be extracted into the oil. Methanol was evaporated by stirring at ambient temperature (~20 to 30°C) overnight. Particles were recovered by centrifugation at 2,500 g, washed three times by centrifuging with hexane, and dried under reduced pressure for 4 h.

Characterization

Particle size and size distribution were determined using a laser-based analyzer (Mastersizer 2000, Malvern Instruments, UK). Particles were dry-mixed with a roughly equal amount of sodium dodecyl sulfate, suspended in distilled water and an aliquot observed under an optical microscope to confirm absence of aggregates. The suspensions were examined using the size analyzer at laser obscuration factors between 7.5 and 15. A few batches were evaluated by optical microscopy, counting 200 particles (17).

Drug content was analyzed by HPTLC (Camag, Switzerland; ATS3 sample applicator, Scanner 3 densitometer and Cats4 software) on silica gel plates (Merck) using 9:1 chloroform:methanol as the mobile phase. Particles were dissolved in tetrahydrofuran before bandwise application, and peak areas compared with those of standards run on the same plate.

Phagocytosis *In Vitro*

The murine Mϕ line J774 A.1 was maintained in DMEM supplemented with 10% fetal bovine serum, and antibiotics, at 5% CO_2 and 37°C. Cells were plated at a density of 10^6/well in 24-well culture plates and allowed to adhere overnight. Duplicate wells were exposed to R and H, at concentrations either equivalent to C_{max} values (13) or at 10 and 100 times the C_{max} values. The drugs were added either as solutions in DMSO-saline or as microparticles suspended in a dispersion medium of identical composition. Blank microparticles were added to control wells. After 0, 5, 10, 15, and 30 min of exposure, the drug solution or suspension was removed, and the wells washed three times with saline. The cells were fixed with methanol and observed under an inverted phase-contrast/fluorescent microscope (Nikon). Subsequently, cells were lysed with 0.5% sodium dodecyl sulfate and the drug concentrations estimated in cell lysates by HPTLC. It is important to note that the lysis protocol did not lyse microparticles, which were separated along with cell debris during filtration prior to spotting on the HPTLC plate.

Administration to Rats

A "nose-only" inhalation exposure apparatus was designed to administer dry powder aerosols to rats (Fig. 1) (18). The delivery chamber consisted of a 50-ml plastic centrifuge tube with a hole of ~0.5 cm diameter at a distance of about 2.5 cm from the rim. The powder for inhalation was weighed in the cap. A length of tubing (i.d. ~ 2mm) was inserted into the tube from the apex of the taper (through another orifice) to a clearance of about 2.5–5mm from the inner surface of the cap. The tubing was connected to the exhaust port of a vacuum pump (Millipore), power supply to which was regulated by a dimmer-type rheostat such that it operated at a minimal speed to just fluidize the powder bed.

Sixteen adult (10 weeks old), male Wistar rats bred and housed ethically in the institution's Laboratory Animal Sciences Division under guidelines embodied in NIH publication #85–23 were obtained following approval for the study from the institutional ethics committee. Animals were randomly assigned to 4 groups. and administered urethane anesthesia one by one. Rats assigned to Group 1 were administered 0.75 mg H and 1.5 mg R by gavage. Each animal in Group 2 received 50 μl of a solution containing 100 μg R and 30 μg H dissolved in DMSO-saline by intra-cardiac injection. Group 3 was administered microparticles equivalent to 10% of the oral doses by intratracheal instillation (19). For intra-cardiac and intra-tracheal administration, the thoracic cavity was opened and the trachea, lungs and heart exposed when the animal was under deep anesthesia. The last group (Group 4) was exposed for 2 min to microparticles fluidized in the exposure chamber described above.

Sampling

Because cardiac puncture was required for exsanguination before bronchoalveolar lavage (BAL), the blood sample was obtained during this process itself. Blood was allowed to coagulate at room temperature, the clot resected, and serum

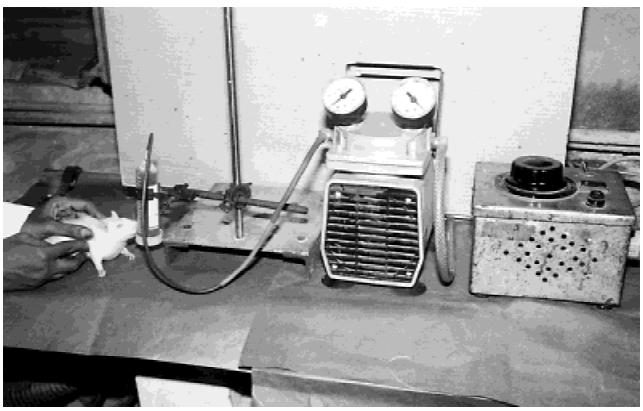

Fig. 1. "Nose-only" inhalation apparatus used for administering inhalable microparticles to rats. A vacuum pump operating at low voltage through a control rheostat fluidizes a powder bed in a plastic centrifuge tube. Restraining an anesthetized rat with its muzzle in an orifice in the centrifuge tube enables fluidized microparticles to be inhaled.

separated by centrifugation. Serum was stored at –20°C till analyzed.

BAL was performed as described by Kouish (20) with minor modifications. BAL fluid was immediately centrifuged at 2,500 *g* at 4°C and the cell pellet and supernatant stored as described for serum samples.

Blood as well as BAL from animals from Groups 2 through 4 was obtained after no more than 5 min after dosing. Two hours were allowed for C_{max} values to be attained in orally dosed animals. Samples were analyzed by ion-pair, reverse phase HPLC (Perkin Elmer) as described earlier (21) with modifications.

Flow Cytometry

A Becton-Dickinson FACSCalibur™ flow cytometer and CellQuest software was used to record 50,000 fluorescent events in samples of BAL fluid from rats administered blank or fluorescent microparticles by inhalation or intra-tracheal instillation.

RESULTS AND DISCUSSION

Table I shows the characteristics of the formulation used in the study. The size range of 0.5–3 micron, which is the most relevant for inhalation delivery to rats (19), is reported in the last column.

The drug content assay (Table II) was complicated by the presence of the RIH adduct, but chromatographic conditions were adequate to resolve the analytes of interest. The analyses reported, however, do not satisfy material balances, since various amounts of adduct were present in the samples.

Cultured Mϕ ingested fluorescent microparticles within 15 min of exposure, even in adherent cultures in ambient atmosphere and temperature (~30°C). This result indicated that phagocytosis is likely to be much more efficient in suspension cultures in 5% CO_2 / 37°C or *in vivo*. (22) Having established that the particles prepared were amenable to phagocytosis by Mϕ, we determined the concentrations of R and H established within these cells after different times of exposure by HPTLC (Fig. 2). Intracellular drug levels observed on exposure to the reported C_{max}were below quantitation limits. At 100 × C_{max}, there was little difference between intracellular drug levels resulting from exposure to soluble or microparticle-incorporated drugs, indicating saturating conditions (data not shown).

The results of the above experiments were taken to signify that microparticle-incorporated R and H could establish higher drug concentrations in Mϕ as compared to equivalent amounts of drugs in solution; within a concentration 'window' imposed by the relative rates of: (i) phagocytosis, (ii) drug release from microparticles, and (iii) intracellular diffusion of soluble drugs. Although it may be conceded that some extra drug would have leached out from intact microparticles into the analyte solutions as a consequence of the cell lysis protocol, our argument that a larger amount of drug (in the same time period) was delivered to the cells using microparticles still holds good.

We then tested whether phagocytosis could be observed when microparticles were administered to intact animals. Rats were exposed to a fluidized bed of fluorescent or blank particles for a period of two minutes using the "nose-only" exposure chamber and protocol described (Fig. 1). One rat, administered fluorescent, drug-containing microparticles as an intratracheal instillation, was used as a positive control. The BAL fluid recovered from these animals and a sample of microspheres alone were examined under a fluorescent microscope and by flow cytometry. It was possible to discern fluorescent microparticles internalized by or associated intimately with BAL cells using microscopy. In flow cytometry experiments, fluorescent microparticles showed high fluorescence intensity (Fig. 3, a), which dropped in association with lung lavage components; but larger cell numbers exhibited high-intensity fluorescence in the case of lavage recovered from rats exposed to fluorescent particles by intra-tracheal instillation (b) or inhalation for 5 min. (c) than background fluorescence in the case of rats exposed to blank microparticles (d). As expected, more free-floating microparticles could be observed in the case of intra tracheal instillation than when rats were made to inhale fluidised microparticles.

Analysis of serum and BAL cell samples yielded results shown in Fig. 4. It is apparent that drug concentrations developed in the serum do not correlate with those developed within cells. For example, vascular administration of H yielded serum concentrations in the vicinity of 2.5 μg/ml, (Fig. 4a) but the amount permeating into alveolar Mϕ was quite low (Fig. 4b). This was in contrast to the observation when microspheres were instilled into the trachea, leading to high concentrations in cells despite low serum concentrations. Cytosolic concentrations of H and R after intra-tracheal instillation and inhalation were consistently higher than those observed with the other two routes of administration. This observation reconfirms an axiom of drug targeting—that phagocytosis of particulate drugs should result in the delivery of larger amounts to the cytosol than would be possible by diffusive uptake of drugs dissolved in body fluids. The fact that serum concentrations of both H and R were significantly lower when microparticles were instilled into the trachea or inhaled further highlights that effective targeting strategies render the familiar metric of blood concentration irrelevant. The apparent lack of correlation between serum and cytosolic

Table I. Characteristics of Inhalable Microspheres

	Amounts taken (mg)			Incorporation (%)		Drug content (w/w)		Yield	Particle size characteristics			
Batch	H[a]	R	PLA	H	R	H	R	(%)	Microscopy	$d_{0.5}$	Uniformity	% 0.5–3 μ
Initial	4.9	15	61.2	30.6	20.7	0.004	0.0097	32	<4–12 μ	ND	ND	ND
Midway	13.5	33.4	120.4	24	18.2	0.021	0.04	94.7	<4–12 μ	6.817 μ	1.541	27.7
Final	15.1	42.4	165	49.55	45.2	0.043	0.110	78.2	<4–12 μ	6.214 μ	1.288	38.0

H = isoniazid, R = rifampicin, PLA = *poly* (D, L-lactic acid), ND = not determined.

Table II. Characteristics of Drug Assay Protocols

Amount taken (μg)	Amount found (μg) mean ± standard deviation	Percent C.V.[a]	Percent D.F.A.[b]
Rifampicin in formulation			
0.3	0.31 ± 0.05	14.59	3.32
0.75	0.72 ± 0.04	4.92	–3.33
1.5	1.57 ± 0.03	1.90	4.82
2.25	2.25 ± 0.06	2.74	–0.19
Isoniazid in formulation			
0.1	0.10 ± 0.02	20.96	1.66
0.25	0.23 ± 0.02	8.079	–5.67
0.5	0.51 ± 0.01	0.31	2.55
0.75	0.73 ± 0.02	3.31	–2.81
Rifampicin in cell lysate			
0.2	0.20 ± 0.006	2.93	2.13
0.3	0.29 ± 0.009	3.37	–2.35
0.4	0.40 ± 0.003	0.96	0.69
Isoniazid in cell lysate			
0.2	0.21 ± 0.006	2.78	5.90
0.3	0.28 ± 0.007	2.65	–6.02
0.4	0.41 ± 0.002	0.39	1.57

[a] Coefficient of variation.
[b] Deviation from actual.

concentrations points to the different mechanisms of drug uptake by BAL cells-diffusion in the case of soluble drugs and phagocytosis when particles were administered.

Results shown in Fig. 4 also indicate that the dose of microspheres inhaled on exposure to fluidized microspheres was lower than 1/10th of the oral dose, since intra tracheal instillation of the latter dose led to higher concentrations in both serum and cell lysate. Alternatively, it is possible that both groups received very similar doses of microparticles, but bioavailability was much higher in case of instillation. This is a likely outcome, since some of the particles entering the nostrils would be trapped in the nasopharynx, while microparticles instilled into the trachea would not need to negotiate this barrier (19).

A third conclusion we would like to draw is that microspheres delivered by inhalation or intra tracheal instillation release significant amounts of drug almost instantaneously, so that appreciable amounts can be recovered from serum within the short sampling time. This is in agreement with results of *in vitro* drug release studies conducted with other batches of microspheres (data not shown), as well as those of other investigators. Typically, microparticles prepared by us release 25–30% of their content into buffered saline within 15 minutes when placed in an incubator-shaker at 37°C in stoppered, glass vials. The remaining amount is released slowly over 4 weeks.

This report demonstrates phagocytic uptake of inhalable microspheres *in vitro* and *in vivo*. It has also been shown here that phagocytosis results in higher intracellular drug concentrations, confirming drug targeting to the site of infection. Targeting drugs to alveolar Mϕ appears to have additional merits apart from the obvious. Alveolar Mϕ migrate to secondary lymphoid organs after taking up particulate material in the lungs. Thus, mycobacteria disseminate not only through the bloodstream (hematogenous dissemination), but also to sites where Mϕ traffic. Loading resident alveolar Mϕ with

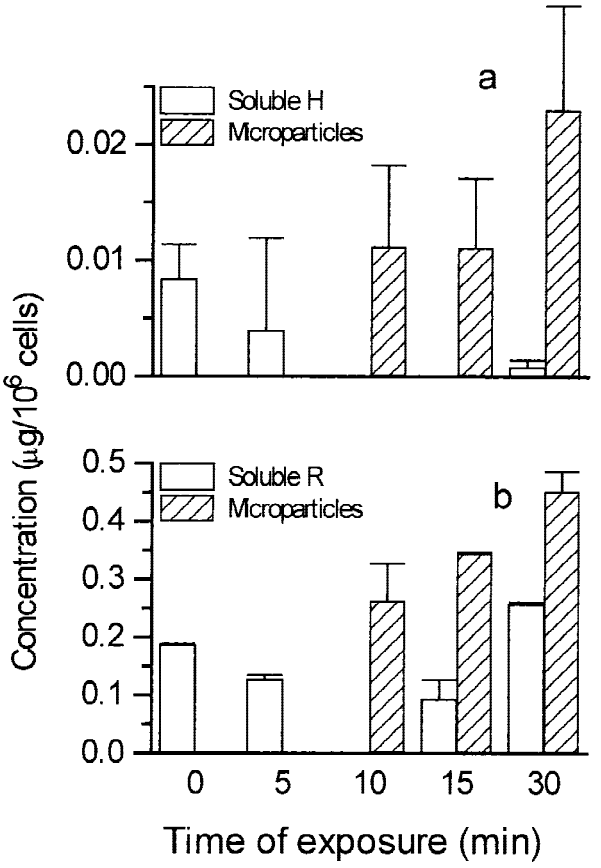

Fig. 2. Concentrations of H (a) and R (b) resulting from exposure of cultured J774 cells to equivalent amounts of drugs, either dissolved in culture medium (open bars) or incorporated in microparticles (hatched bars). Error bars represent standard deviations between replicates.

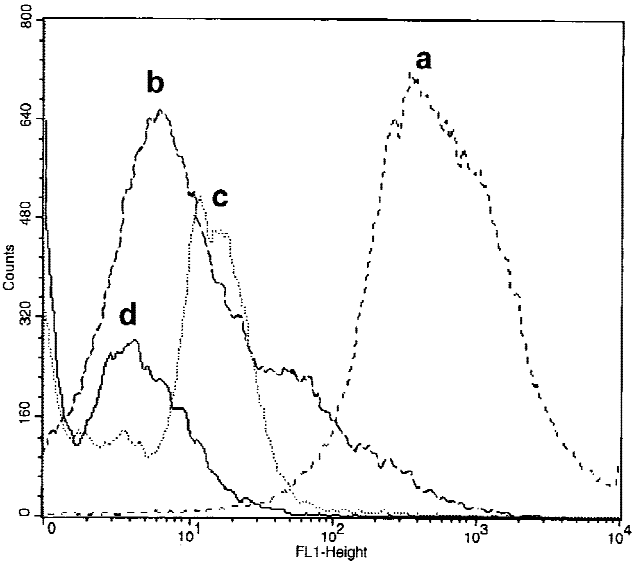

Fig. 3. Flow cytometry histograms of fluorescent microparticles alone (a), and cells lavaged from rats administered such particles by intratracheal instillation (b), or inhalation (c). Autofluorescence of the sample is depicted by the histogram obtained with lavage from rats administered dummy (non-fluorescent) microparticles.

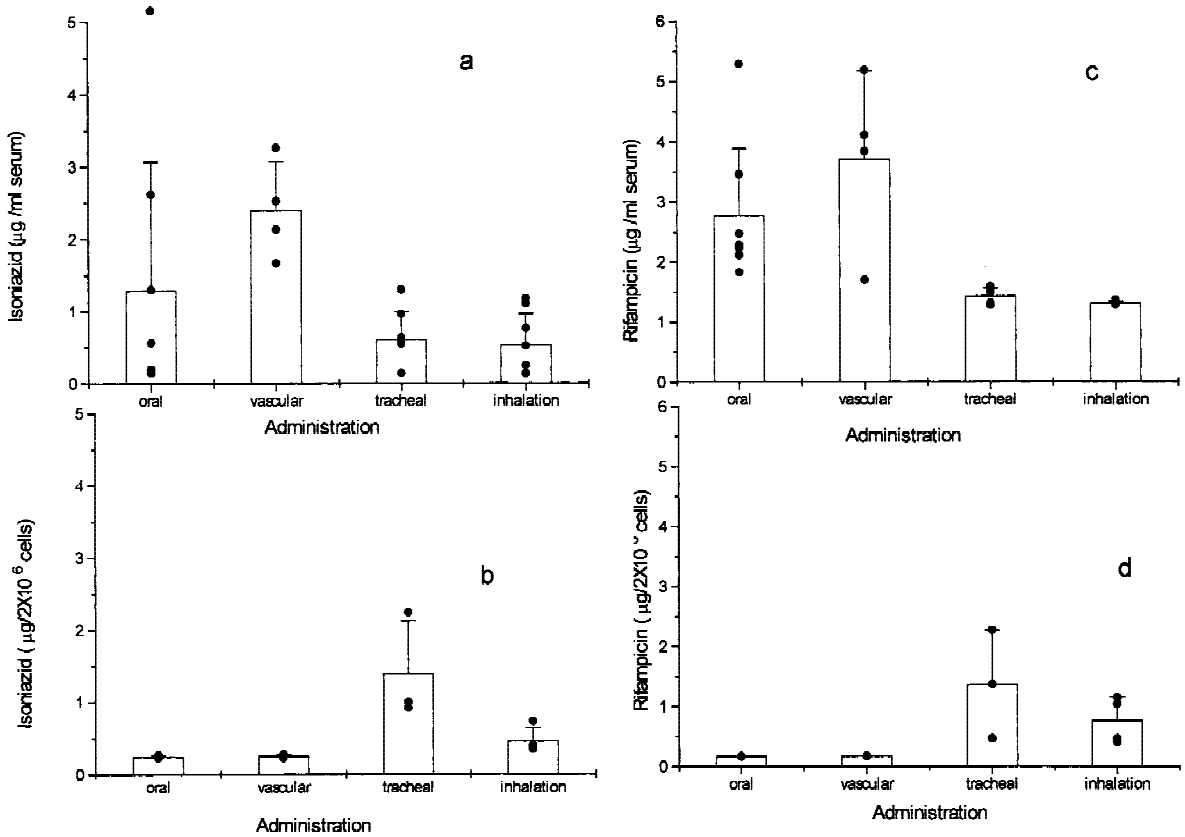

Fig. 4. Concentrations of H in serum (a) or alveolar Mϕ (b) recovered from rats dosed via several routes as shown on the X axis. Panels (c) and (d) show R concentrations in the same samples. Bars represent arithmetic means, error bars show standard deviations between samples and filled circles represent individual data points.

drug-containing particles might conceivably lead to transport of drugs to those very sites where migrating Mϕ go-mimicking the course of spread of mycobacteria.

O'Hara and Hickey (8) have earlier proposed a formulation for the purpose that we outline. This report addresses two aspects that were not given prominence by these authors. First, we show that our formulation is amenable to rapid phagocytosis by macrophages, *in vitro* and *in vivo*. This is important, since the expected surface hydropathy of polyester microspheres does not encourage assumptions that phagocytosis would be facile. Second, in what we believe is a first report of data on comparison of drug concentrations in serum and target cell cytosol, we show that high serum concentrations do not lead to high intracellular concentrations, and *mutatis mutandis,* high intracellular drug concentrations achieved by the administration of microparticles do not lead to high serum concentrations. Thus, we provide numbers to confirm an axiomatic principle of drug targeting.

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