ELSEVIER

International Journal of Pharmaceutics 170 (1998) 41–49

international journal of pharmaceutics

Alveolar macrophage cell line is not activated by exposure to polymeric microspheres

Ka-yun Ng [a,*], Kathleen A. Stringer [b], Zoe Cohen [b], Robert Serravo [a], Bin Tian [a], Jeffrey D. Meyer [a], Richard Falk [c], Theodore Randolph [c], Mark C. Manning [a], David C. Thompson [a]

[a] *Department of Pharmaceutical Sciences, School of Pharmacy, Campus Box C-238, University of Colorado Health Sciences Center, 4200 East Ninth Avenue, Denver, CO 80262, USA*

[b] *Department of Pharmacy Practice, School of Pharmacy, University of Colorado Health Sciences Center, Denver, CO 80262, USA*

[c] *Department of Chemical Engineering, University of Colorado at Boulder, Boulder, CO 80309, USA*

Received 20 November 1997; received in revised form 10 February 1998; accepted 4 March 1998

Abstract

An in vitro cell culture model based on a rat alveolar macrophage (AM) cell line, NR8383, was used to determine if poly(L-lactide) (PLA) microspheres prepared by the precipitation with a compressed antisolvent (PCA) method can be taken up by AMs and activate AMs. To examine cellular uptake of microspheres, microspheres were labeled with rhodamine 6G. Using fluorescence microscopy, the uptake of microspheres by NR8383 cells was followed as a function of time, microsphere concentration, and susceptibility to lysosomotropic agents. To determine if microspheres can activate NR8383 cells, the oxidative burst and production of TNF-α by NR8383 cells following microsphere treatment was measured. Uptake of microspheres by NR8383 cells was dependent on microsphere concentration and appeared to occur via endocytosis, as uptake was significantly inhibited by the putative lysosomotropic agents, ammonium chloride and chloroquine. Furthermore, the microspheres do not appear to activate NR8383 cells, since microsphere exposure results in negligible oxidative burst and TNF-α production in NR8383. Microspheres prepared by the PCA method hold great potential in targeting drugs to AMs and, therefore, may be of utility for the treatment of diseases in which AMs play an important role, such as tuberculosis (TB).

Keywords: Alveolar macrophage; NR8383; Endocytosis; Poly(L-lactide) microsphere; Precipitation with compressed anti-solvent; Oxidative burst and TNF-α

* Corresponding author. Tel.: +1 303 3156997; fax: +1 303 3154630; e-mail: Lawrence.Ng@UCHSC.edu

0378-5173/98/$19.00
PII S0378-5173(98)00138-0

1. Introduction

Tuberculosis (TB) is a communicable infectious disease caused by *Mycobacterium tuberculosis*. It most commonly affects the lungs, although any body site can be involved (CDC, 1989). Despite the availability of therapies for controling the vast majority of existing cases, the incidence of TB is increasing (ATS/AAP/CDC/IDSA, 1992). This is because many patients with TB (such as AIDS patients, elderly, drug abusers, and the urban poor) usually have significant social problems, and compliance with a drug therapy that lasts for 6–12 months is frequently poor (CDC, 1989; ATS/CDC, 1990; ATS/AAP/CDC/IDSA, 1992; Raviglione et al., 1995). Current therapy requires large oral doses of antibiotics which often leads to significant side effects (Kucers and Bennett, 1987; McEvoy, 1993). This serves to further reduce compliance, contributing to an increased rate in drug resistance.

TB in a susceptible host is caused by inhalation of tubercle bacilli in fresh airborne droplets (< 10 μm in size) emitted by the coughing pulmonary TB patient (CDC, 1989). Upon deposition in the alveolus, the tubercule bacilli are phagocytosed and destroyed by alveolar macrophages (AMs) (Dannenberg, 1991). However, in some instances, virulent bacilli continue to grow within the AMs, eventually releasing additional bacilli (Dannenberg, 1991; McDonough et al., 1993; McEvoy, 1993). Thus, the failure of TB patients to comply with drug therapy, the manifestation of significant side effects, and the need to target the drug therapy to the AMs, emphasizes the inadequacy of current oral therapies for TB.

Pulmonary delivery of biodegradable microspheres containing antitubercular drugs is a particularly attractive alternative to massive oral dosing since the lungs are the organs most commonly affected (CDC, 1989). The ability to produce microspheres capable of releasing drugs in a sustained fashion should lead to reduced doses and less frequent administration. Lower doses will, in turn, lead to fewer side effects, create cost savings and potentially enhance patient compliance. In addition, pulmonary delivery of sustained release formulations provides an opportunity for direct targeting of drug to the AM, where the mycobacterium is highly protected (Dannenberg, 1991; McDonough et al., 1993).

Recently, utilizing a technology developed in our laboratory, we have successfully incorporated antitubercular drugs into poly(L-lactide) (PLA) microspheres of an appropriate size for delivery into the alveoli (unpublished results). This approach uses either precipitation with a compressed anti-solvent (PCA) or a combination of PCA and hydrophobic ion pairing (HIP) to incorporate lipophilic or non-lipophilic antitubercular agents into the PLA microspheres. Detailed description of these procedures have been reported previously (Matsuura et al., 1993; Powers et al., 1993; Randolph et al., 1994). For microspheres prepared by this method to be useful in the treatment of TB, it is necessary to evaluate if PLA microspheres prepared by the PCA method can interact favorably with AMs. In this study, interaction of PLA microspheres prepared with the

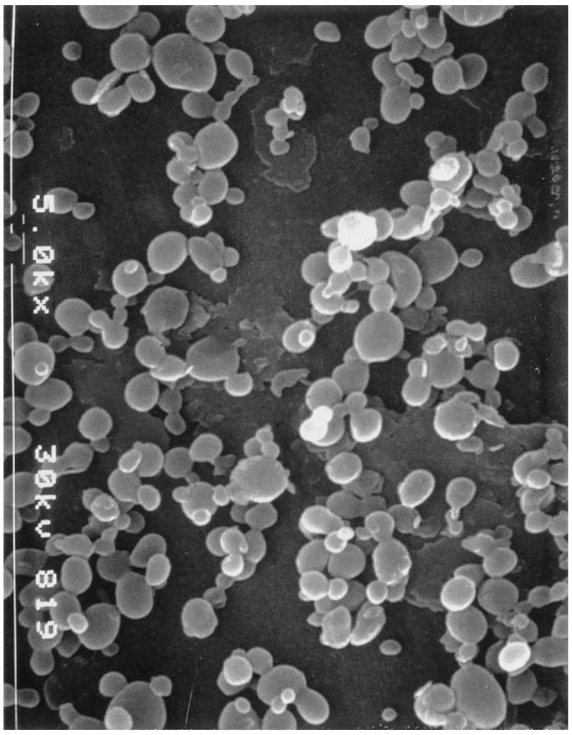

Fig. 1. Scanning electron micrograph of rhodamine 6G-labeled PLA microspheres (magnification $\times$ 5000).

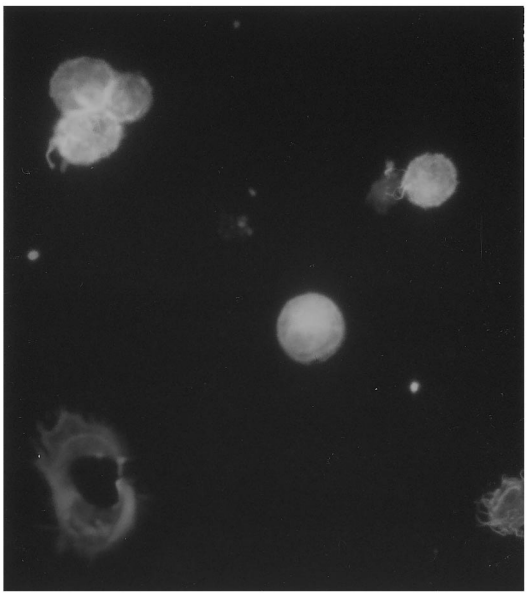

Fig. 2. Fluorescence micrograph of NR8383 cells showing cell-associated rhodamine 6G-labeled microspheres (magnification $\times 400$). Microspheres fluoresce yellow whereas cells are labeled green.

PCA method with a rat AM cell line, NR8383, was investigated. Since AMs contribute to early pulmonary defenses against a variety of external stimuli (Hocking and Glode, 1979) and are known to respond to these stimuli by undergoing increased oxidative metabolism (Fantone and Ward, 1982) and production of inflammatory cytokines such as interleukin-1 (IL-1) and tumor-necrosis factor-alpha (TNF-α) (Driscoll and Maurer, 1991), the tendency of these microspheres to activate NR8383 cells was also investigated.

2. Materials and methods

2.1. Materials

Rhodamine 6G; aerosol-OT (AOT); penicillin/streptomycin; ammonium chloride (NH_4Cl); chloroquine; horse radish peroxidase; luminol (Sigma, St. Louis, MO); PLA (ICN Biomedical, Costa Mesa, CA); spectrophotometric grade methylene chloride (Aldrich, Milwaukee, WI) were of analytical grade. Carbon dioxide, medical grade, was obtained from US Welding. HAMS F12 cell culture medium and heat-inactivated fetal calf serum (FCS) were obtained from Gibco (Grand Island, NY).

2.2. Preparation of rhodamine 6G-labeled microspheres

Rhodamine 6G-labeled PLA microspheres were prepared by ion-pairing the rhodamine 6G with AOT, dissolving the resulting ion-paired complex in a PLA/methylene chloride solution, and spraying the mixture through an ultrasonicated (120 kHz) nozzle into supercritical CO_2 at 85 bar and 35°C. Fresh CO_2 was co-flowed into the spraying chamber at a volumetric flow rate 20 times that for the PLA/methylene chloride solution. Microspheres were washed by flowing approximately three spray chamber volumes of pure CO_2 through the system after spraying was terminated. The details of the process have been previously described (Falk et al., 1997). Microspheres were later collected for scanning electron microscopic

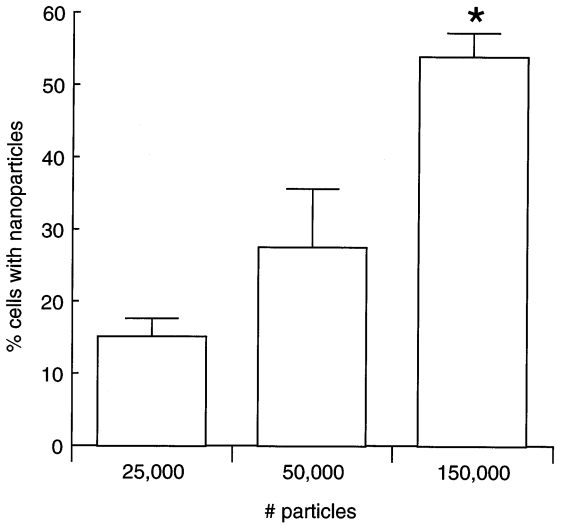

Fig. 3. Effect of amount of microspheres on cell association. To determine the proportion of cells found associated with fluorescently-labeled microspheres, cells were fixed and photographed for scoring. Each column is the mean $\pm$ S.D. of six visual fields (each field contains about 200 cells). * $P < 0.05$, Scheffe's F-test, compared with the percentage cells incubated with 25000 or 50000 particles.

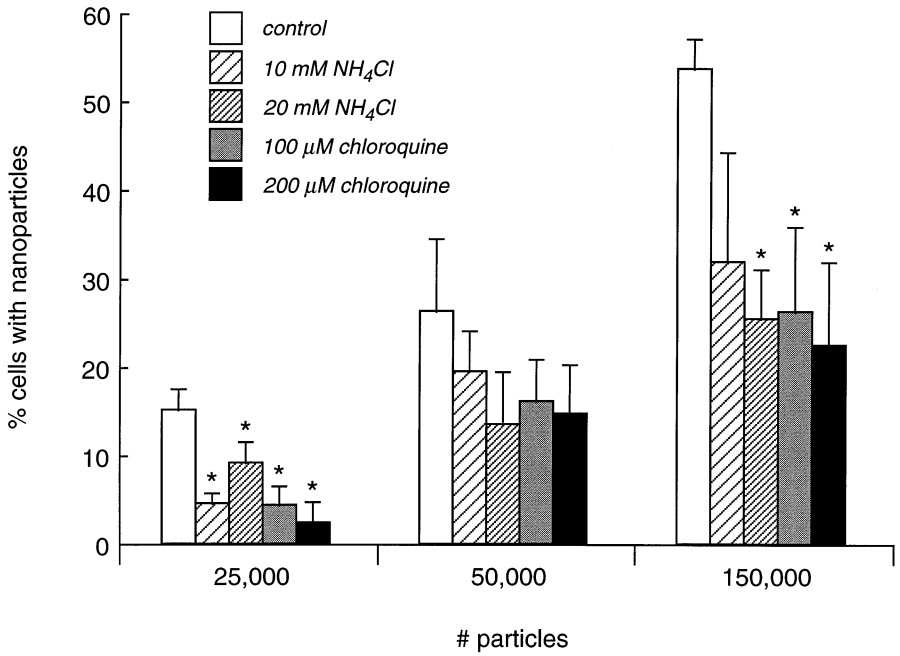

Fig. 4. Effects of lysosomotropic agents, chloroquine and ammonium chloride (NH_4Cl), on cell association of microspheres. To determine the proportion of cells found associated with fluorescently-labeled microspheres, cells were fixed and photographed for scoring. Each column is the mean ± S.D. of six visual fields (each field contains about 200 cells). * $P < 0.05$, Scheffe's F-test, compared with respective controls.

(SEM) analysis on an aluminum SEM stub, and gold sputter coated for 5 min using an Anatech Hummer II sputter coating apparatus. Scanning electron micrograph was taken at × 5000 with a Cambridge Stereoscan S250 MKIII scanning electron microscope using a previously described procedure (Falk et al., 1997).

2.3. Cell culture of NR8383

NR8383 (ATCC CRL-2192, Rockville, MD), a rat AM cell line, was cultured in a flask (75 cm^2, Costar, Boston, MA) in Hams F12 medium supplemented with 15% FCS, penicillin (50 U/ml) and streptomycin (50 μg/ml) (culture medium) under standard culture conditions of 37°C, 5% CO_2 and 95% humidity. These cells exhibit many characteristics of AMs, such as response to appropriate microbial, particulate or soluble stimuli with phagocytosis and production of reactive oxygen species (ROS) and inflammatory cytokines such as IL-1 and TNF-α (Helmke et al., 1987).

2.4. Examination of uptake of microspheres by NR8383

To determine if PLA microspheres prepared by the PCA method are taken up by AMs, the time-course of uptake of different concentrations of rhodamine 6G-labeled microspheres in the presence or absence of lysosomotropic agents was examined in vitro using NR8383. Briefly, 500 μl aliquots containing 10^5 cells in culture medium were added to tissue cell culture inserts (12 mm Transwell, 0.4 μm pore size, Costar) that had previously been coated with type I rat tail collagen (Becton Dickinson, Bradford, MA). The inserts were then placed in the wells of a 12-well cluster plate. To each well, 1.5 ml of the culture medium were added and the cells were allowed to grow overnight in a cell incubator (37°C, 5% CO_2) prior to the uptake studies.

To initiate uptake studies in the absence of lysosomotropic agents, cells were rinsed twice with serum-free culture medium. After rinsing, 1.5

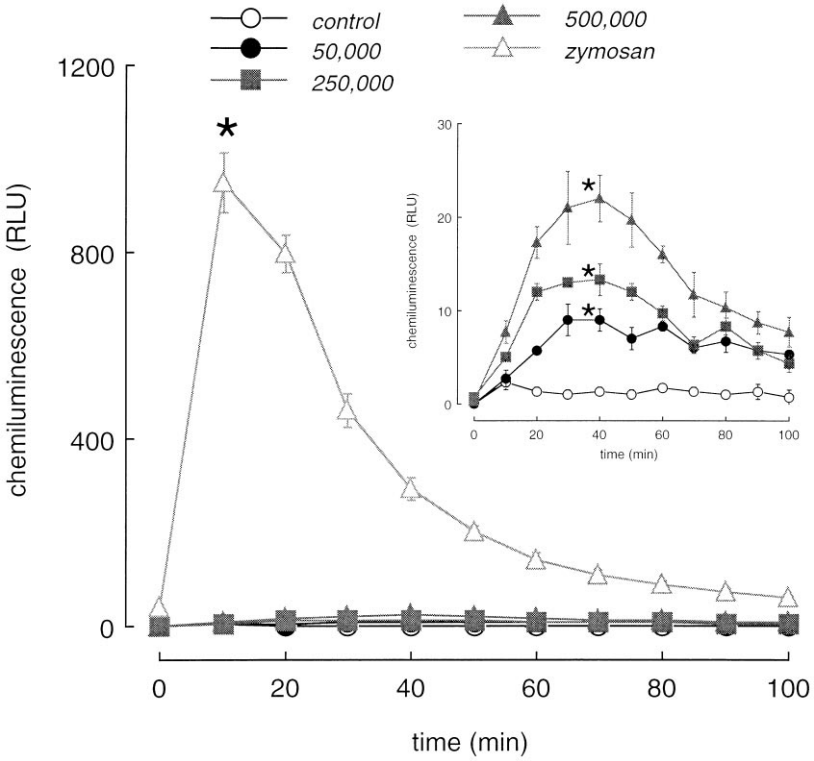

Fig. 5. Time course of oxidant production by NR8383 cells induced by microspheres and zymosan. Inset figure shows oxidant production induced by the different amounts of microspheres using a larger scale. Oxidant production was measured as increased chemiluminescence of luminol (in relative light units, RLU). Data represent the mean ± S.D. from three experiments. $*P < 0.05$, two-way ANOVA, compared with oxidant production in untreated (control) cells.

ml of serum-free culture medium were then added to each well. Subsequently, 500 μl of different concentrations of rhodamine 6G-labeled microspheres (average size ≈ 1 μm) suspended in serum-free culture medium (50000, 100000 or 300000 microspheres/ml) were added to the cell culture inserts and allowed to settle onto the cells. Microsphere concentrations were determined by counting on a hemocytometer. After a 1-h incubation, the medium was aspirated and the cells rinsed twice with Hanks Balanced Salt Solution (HBSS, pH 7.4) buffer. The cells were then fixed, stained with fluorescein-labeled phalloidin (Molecular Probes, OR) to label the actin filaments (Phillips and Tsan, 1988) and examined under a Nikon Microphot-FX fluorescence microscope (excitation λ: 490 nm; emission λ: 520 nm) for evidence of uptake of microspheres. At these specific wavelengths, rhodamine 6G-labeled microspheres appear as yellow particles whereas the NR8383 cells appear green due to the staining of actin molecules by fluorescein-conjugated phalloidin.

In uptake studies conducted in the presence of lysosomotropic agents, cells were first rinsed twice with serum-free culture medium. Subsequently, the cells were preincubated with serum-free culture medium containing either NH_4Cl (10 or 20 mM) or chloroquine (100 or 200 μM). After 1 h of incubation, the medium in the inserts was aspirated and replaced with 500 μl fresh serum-free culture medium containing the appropriate lysosomotropic agents and microspheres suspended at different concentrations (50000, 100000 or 300000 microspheres/ml). After an additional 1 h incubation, the medium was aspirated and the

cells rinsed twice with HBSS buffer. The cells were then fixed and examined under a fluorescence microscope for evidence of uptake of microspheres (as described above).

2.5. Measurement of oxidant production by NR8383 cells

Oxidant production as a function of microsphere concentration was measured using chemiluminescence using a previously described procedure (Archer et al., 1989). Prior to the start of the experiments, cells were harvested from the flask and subjected to centrifugation (1000 rpm) for 10 min. The cells were resuspended in serum-free culture medium to a final concentration of 2×10^6 cells/ml. Cell suspension (100 μl) was placed in each well of an opaque, 96-well plate (Dynex Technologies, Chantilly, VA). The cells were allowed to adhere to the plate (approximately 1 h) and, prior to the addition of the non-fluorescently labeled microspheres, 200 μl of buffered luminol solution (10^{-4} M) containing horseradish peroxidase (0.5 mg/ml) were added to each well. Oxidant production by AMs was determined by luminol chemiluminescence using a luminometer (Lumistar, BMG, Durham, NC) at 37°C. After measurement of baseline chemiluminescent light emission (baseline, time 0), 100 μl of serum-free culture medium containing different concentrations of microspheres (500000, 2.5 million or 5 million microspheres/ml) or opsonized zymosan (60 μg/ml) were added to the wells. Chemiluminescent light emission was measured every 10 min thereafter for 2 h. Experiments were carried out in triplicate. The assay has a detection limit of approximately 100 nM hydrogen peroxide.

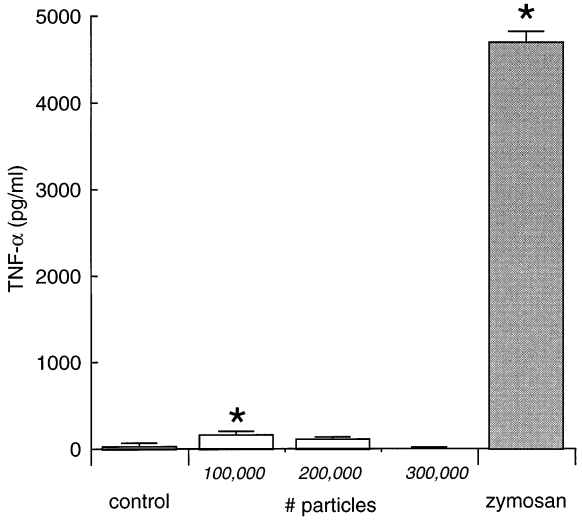

Fig. 6. Microsphere-induced production of TNF-α by NR8383 cells. Zymosan is included as a positive control. TNF-α production was measured using an ELISA kit by Genzyme Corp. Data represent the mean $\pm$ S.D. ($n = 8$). *$P < 0.05$, Scheffe's F-test, compared with TNF-α produced by untreated (control) cells.

2.6. Measurement of TNF-α production by NR8383 cells

Measurement of TNF-α production as a function of microsphere concentration was conducted using an enzyme-linked immunosorbent assay (ELISA) method for TNF-α. Briefly, 200 μl aliquots containing 2×10^5 cells in culture medium were added to the wells of a 96-well cluster plate and the cells were allowed to grow overnight in a cell incubator (37°C, 5% CO_2). On the second day, the medium in each well was aspirated and replaced with 200 μl of fresh culture medium containing either zymosan (60 μg/ml) or various concentrations of microspheres (500000, 1 million or 1.5 million particles/ml) for overnight incubation in a cell incubator (37°C, 5% CO_2). TNF-α production by NR8383 cells was then assayed 24 h later by evaluating media TNF-α levels using an ELISA assay kit for TNF-α (Genzyme Cat # 80-3905-01).

2.7. Statistical analysis

Comparisons were made between groups by analysis of variance (ANOVA) and post hoc Scheffe's F-test. In time-course experiments, two-way ANOVA was conducted to delineate differences between groups. Statistical significance was arbitrarily defined as $P < 0.05$.

3. Results

3.1. Preparation of PLA microspheres

In general, the rhodamine 6G-labeled microspheres prepared by the PCA method exhibit a narrow size distribution, centered between 0.5 and 1.0 μm. Further, the shape of the microspheres tends to be spherical or slight ellipsoidal, as shown in the accompanying scanning electron micrograph (Fig. 1). For this study, a low loading factor of rhodamine 6G (1% by weight) was used, although higher loads are possible.

3.2. Examination of uptake of microspheres by NR8383 cells

One means of studying particle–cell interaction is to fluorescently label the particles and observe their interaction with the targeted cells by fluorescence microscopy. The advantages of using fluorescently-labeled particles rest in the ability to visualize such interactions and to quantify the extent of such interaction. As indicated in Fig. 2, extensive interaction between rhodamine 6G-labeled microspheres and NR8383 cells (as reflected by the yellowing of the otherwise green-stained cells) was observed. Since it is possible that cellular uptake of free rhodamine, which might have leached out from the labeled particles, could have contributed to the same observation, control experiments investigating the interaction of free rhodamine with NR8383 cells were conducted. Our results indicated no evidence of cellular uptake of rhodamine (data not shown), thus, excluding free rhodamine as a source of cellular fluorescence. The particle–cell interaction appeared to depend on the incubating concentrations of the microspheres as evidenced by the increase seen in the proportion of NR8383 cells found associated as the concentration of the microspheres was increased (Fig. 3).

While these data imply that the microsphere–cell interaction is a concentration-dependent event, it was unclear if the majority of the cell-associated microspheres were merely adhering to the external surface of the cells or were actually taken into the cells. Since most cellular uptake processes occurs via endocytosis, it was reasoned that cell-associated microspheres should be reduced in the presence of inhibitors of endocytosis. To verify this contention, microsphere–cell interaction studies were carried out in the presence of different concentrations of NH_4Cl or chloroquine, lysosomotropic agents which inhibit endocytosis (Sandvig et al., 1979). As indicated in Fig. 4, incubating the cells with lysosomotropic agents substantially reduced the extent of interaction of the microspheres with the NR8383 cells, with most extensive reductions ($>50\%$) seen when higher concentrations of lysosomotropic agents were used. In addition, chloroquine appeared to exert more inhibiting effects than NH_4Cl (100–200-fold) on the number of microspheres associated with the cells (Fig. 4).

3.3. Measurement of oxidant production by NR8383 cells using chemiluminescence

Exposure of NR8383 cells to 50000, 250000, 500000 microspheres induced small, but significant, concentration-dependent production of ROS by AMs (Fig. 5 inset). However, the production was insignificant when compared with that produced by opsonized zymosan, an agent known to be phagocytosed and to activate macrophages (Synderman et al., 1969) (Fig. 5).

3.4. Measurement of TNF-α production by NR8383 cells using ELISA

Exposure of AMs to 100000, 200000, 300000 microspheres induced low level production of TNF-α by AMs (Fig. 6). By contrast, significant generation of TNF-α was produced by opsonized zymosan.

4. Discussion

AMs are phagocytic cells that play an important role in early pulmonary defenses against inhaled substances (Hocking and Glode, 1979) and are known to undergo increased oxidative metabolism (Fantone and Ward, 1982) and release of inflammatory cytokines (IL-1 or TNF-α)

(Driscoll and Maurer, 1991) following their activation by phagocytosis of particulates. Accordingly, a concern with microsphere delivery to the peripheral airways relates to the potential of the microsphere to encounter AMs, undergo phagocytosis and activate AMs and, thereby, initiate an inflammatory response in the lung. Under in vitro conditions, this would manifest as induction of an oxidative burst and release of cytokines such as IL-1 and TNF-α by the AMs. To examine this possibility, the interaction between microspheres and a line of cells (i.e. NR8383 cells) with features similar to AMs was investigated. The results indicate that microspheres generated by the PCA method appear to be taken up by AMs. This process occurred in a manner which was dependent on the concentration of microspheres to which the cells were exposed. Further, the microspheres seem to be taken up by endocytosis as evidenced by the ability of the lysosomotropic agents, NH_4Cl and chloroquine, to inhibit association of the microspheres with the cells. Hence, it would appear that microspheres can 'target' the AM. Most importantly, the interaction between the microspheres and the NR8383 cells is not associated with significant activation of the cells. Indeed, the magnitude of the oxidative burst as well as release of TNF-α induced by the highest concentration of microspheres was negligible when compared with that induced by opsonized zymosan, a known phlogistic agent that activates macrophages during phagocytosis (Synderman et al., 1969). These results lend credence to the notion that these microspheres can target AMs without eliciting a pulmonary inflammatory response promoted by AMs. However, it is currently unclear what mechanisms are responsible for the endocytosis of these microspheres by NR8383 cells. Our results would indirectly rule out the possible influence of microsphere interaction with serum constituents or opsonins on macrophage uptake since microsphere induction of oxidative burst and production of TNF-α in NR8383 cells are both negligible despite the fact that one experiment was carried out in the absence of serum (oxidative burst) and the other was not (TNF-α). These results support the notion that pulmonary surfactant (which contains opsonins) has only a small effect on the endocytosis of microspheres by macrophages (Stringer and Koszik, 1996).

In conclusion, targeting drugs to AMs has the distinct advantage of delivering high concentrations of drug to a cell which plays a central role in the progression of TB. The present results indicate that microspheres prepared by the PCA method are a feasible means of delivering drug to AMs in that endocytosis of the microspheres occurs in the absence of significant activation of the macrophage. Accordingly, the likelihood of induction of pulmonary inflammation by inhalation of PCA-processed microspheres seems remote.

Acknowledgements

Presented in part at the American Association of Pharmaceutical Scientists 1996 Annual Meeting and Exposition. The authors want to thank Lisa Liem for her editorial assistance. This work was supported in part by the Colorado RNA Center and Colorado Advanced Technology Institute.

References

American Thoracic Society, American Academy of Pediatrics, Centers for Disease Control, Infectious Disease Society of America, 1992. Joint statement: control of tuberculosis in the United States. Am. Rev. Respir. Dis. 146, 1623–1633.

American Thoracic Society, Centers for Disease Control, 1990. Joint statement: diagnostic standards and classification of tuberculosis. Am. Rev. Respir. Dis. 142, 725–735.

Archer, S.L., Nelson, D.P., Weir, E.K., 1989. Detection of activated oxygen species in vitro and in rat lungs by chemiluminescence. J. Appl. Physiol. 67, 1912–1921.

Centers for Disease Control, 1989. A strategic plan for the elimination of tuberculosis in the United States. Morb. Mortal. Wkly. Rep. 38 (S-3), 1–25.

Dannenberg, A.M., 1991. Delayed-type hypersensitivity and cell-mediated immunity in the pathogenesis of tuberculosis. Immunol. Today 12, 228–233.

Driscoll, K.E., Maurer, J.K., 1991. Cytokine and growth factor release by alveolar macrophages: potential biomarkers of pulmonary toxicity. Toxicol. Pathol. 19, 398–405.

Falk, R., Randolph, T.W., Meyer, J.D., Kelly, R. M., Manning, M.C., 1997. Controlled release of ionic compounds from poly (L-lactide) microspheres produced by precipitation with a compressed antisolvent. J. Control. Release 44, 77–85.

Fantone, J.C., Ward, P.A., 1982. Role of oxygen-derived free radicals and metabolites in leukocyte-dependent inflammatory reactions. Am. J. Pathol. 107, 397–418.

Helmke, R.J., Boyd, R.L., German, V.F., Mangos, J.A., 1987. From growth factor dependence to growth factor responsiveness: the genesis of an alveolar macrophage cell line. In Vitro Cell. Dev. Biol. 23, 567–574.

Hocking, W.G., Glode, D.W., 1979. The pulmonary-alveolar macrophage. New Engl. J. Med. 301, 580–587.

Kucers, A., Bennett, N.M., 1987. The Use of Antibiotics, 4th. J.B. Lippincott, Philadelphia.

Matsuura, J., Powers, M.E., Manning, M.C., Shefter, E., 1993. Structure and stability of insulin dissolved in 1-octanol. J. Am. Chem. Soc. 115, 1261–1264.

McDonough, K., Kress, Y., Bloom, B., 1993. Pathogenesis of tuberculosis: interaction of mycobacterium tuberculosis with macrophages. Infect. Immun. 61, 2763–2773.

McEvoy, G.K., 1993. AHFS Drug Information 93. American Society of Hospital Pharmacists, Bethesda, MD.

Phillips, P.G., Tsan, M.F., 1988. Direct staining and visualization of endothelial monolayer culture on synthetic polycarbonate filters. J. Histochem. Cytochem. 36, 551–554.

Powers, M.E., Matsuura, J., Manning, M.C., Shefter, E., 1993. Enhanced solubility of proteins and peptides in nonpolar solvents through hydrophobic ion pairing. Biopolymers 33, 927–932.

Randolph, T.W., Manning, M.C., Shefter, E., Falk, R.F. Method for producing ultrafine pharmaceutical powders using a compressed gas antisolvent, filed as a continuation-in-part of PCT Int. Pat. Appl. WO 94 08577, June 7, 1994.

Raviglione, M.C., Snider, D.E., Kochi, A., 1995. Global epidemiology of tuberculosis: Morbidity and mortality of a worldwide epidemic. J. Am. Med. Assoc. 273, 220–226.

Sandvig, K., Olsnes, S., Pihl, A., 1979. Inhibitory effect of ammonium chloride and chloroquine on the entry of the toxic lectin modeccin into Hela cells. Biochem. Biophys. Res. Commun. 90, 648–655.

Stringer, B., Koszik, L., 1996. Alveolar macrophage uptake of the environmental particulate titanium dioxide: role of surfactant components. Am. J. Resp. Cell Mol. Biol. 14, 155–160.

Synderman, R., Shin, H.S., Phillips, J.K., Gewurz, H., Mergenhagen, S.E., 1969. A neutrophil chemotactic factor derived from C'5 upon interaction of guinea pig serum with endotoxin. J. Immunol. 103, 413–422.