Effect of Chloroquine on Phagolysosomal Fusion in Cultured Guinea Pig Alveolar Macrophages: Implications in Drug Delivery

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ABSTRACT The aim of this study was to evaluate the effects of chloroquine on phagolysosomal fusion (PLF) in cultured guinea pig alveolar macrophages (AMs). This technique may be of significance for antitubercular drugs, because the survival of *Mycobacterium tuberculosis* is linked to evasion of PLF. Guinea pig AMs were obtained from anesthetized animals after exsanguination. The AMs were cultured at a density of 1×10^6 cell/mL in 24-well plates after attachment to 13-mm coverslips. Culture conditions were at 37°C, with 95% air/5% CO₂ in Roswell Park Memorial Institute (RPMI) 1640 medium with 10% heat-inactivated fetal bovine serum. Rhodamine-dextran (70 kd) was incubated with the cells at 0.25 mg/mL for 24 hours to label the lysosomes. Chloroquine treatment where indicated was performed at 10-20 μ g/mL for 1 hour. Fluorescent BioParticles were then added, and PLF was monitored by formation of an orange-yellow fluorescence on fusion of green fluorescent BioParticles with rhodamine-labeled lysosomes. PLF endpoints were measured by scoring for the percentage of orange-yellow cells in the field of view. Image analysis to measure the intensity of the orange-yellow color was performed by obtaining *a*, *b* values for 5 x 5 pixel areas using the PhotoAdobe program 4.0.1.

The results indicated that the rate of PLF was enhanced by chloroquine. Thus, chloroquine may be used to potentiate the effects of rifampicin. This may be confirmed by studies involving similar dual fluorophore labeling techniques of fluorescein-labeled formulation in macrophages infected with *M. tuberculosis*. Preliminary studies with the rhodamine-labeled formulation confirmed cellular uptake and persistence for up to 7 days in culture.

INTRODUCTION

Worldwide, tuberculosis (TB) remains the single most important infectious disease in adults. Each year, 8 million people develop new cases of TB and 3 million die (1,2). Pulmonary deposition of the causative organism *Mycobacterium tuberculosis* (MTB) leads to 1 of 4 possible outcomes: 1) immediate clearance of the organism, leaving no trace of infection; 2) asymptomatic chronic infection; 3) active disease soon after infection; or 4) active disease many years after the primary infection. The fate of inhaled bacilli at the site of infection depends on the capacity of the organism to proliferate after the alveolar macrophages (AM) ingest them and the AM's capacity to arrest their growth (3,4). Hence, the interaction of the pathogen with the AM is a key factor in the outcome.

The intracellular growth of MTB has been attributed to various suggested pathways. Evasion of phagolysosomal fusion (PLF) and thereby evasion of the lysosomal hydrolases is one of the putative mechanisms (5,6). Various mechanisms by which the bacillus is able to accomplish this objective have been suggested. Studies by Goren et al (7) demonstrated that purified sulfatides from MTB strain H37Rv displayed potent inhibition of phagolysosome formation. This inhibition was attributed to the accumulation of the sulfatides in secondary lysosomes, rendering them incompetent to fuse with phagosomes. Studies by another group (8) demonstrated inhibition by filtrates from tubercle bacilli cultures, attributed to the large amounts of ammonia present as PLF activity was restored on replacement with normal culture media. *Mycobacterium microti* was found to inhibit PLF by inhibition of lysosomal movements, possibly by modifying microtubule controls (9).

The evasion of PLF may also explain the added difficulty in obtaining proximity of drug to pathogen that stays intracellularly sequestered in a protective

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environment. As reported earlier (10), modest doses of chloroquine were used to enhance PLF. Potentially, chloroquine may be used to modulate PLF to enhance the proximity between antitubercular drugs and the intracellular pathogen. The effect of chloroquine at the dose employed was studied by using a dual fluorophore PLF assay as reported by Duzgunes et al (11). The aim of the study was to adopt the above technique for cultured guinea pig alveolar macrophages (AMs) and examine the role of chloroquine on the PLF in guinea pig AM. Preliminary studies examined the intracellular fate of rhodamine-labeled rifampicin poly(lactic-co-glycolic acid) (RR-PLGA) microspheres.

MATERIALS AND METHODS

Materials

Rifampicin was obtained from Sigma Chemicals, St Louis, MO; 75:25 poly(lactic-co-glycolic acid) (PLGA), 80 000 MW, was obtained from Birmingham Polymers, Birmingham, AL; and glycerol and formaldehyde were obtained from Fisher Scientific, Pittsburgh, PA. Polyvinyl alcohol, 78 000 MW, was obtained from Polysciences Inc, Warrington, PA; isopropyl alcohol and methylene chloride were from Mallinckrodt, St Louis, MO; and rhodamine 6G chloride, rhodamine-dextran 70 000 MW, and fluorescein-labeled BioParticles were from Molecular Probes, Portland, OR. Hank's Balanced Salt Solution, Ca^{2+} - and Mg^{2+} -free, RPMI 1640 medium, fetal bovine serum, and penicillin-streptomycin solution were obtained from Gibco BRL, Grand Island, NY, and chloroquine was from Fluka, Milwaukee, WI.

Equipment

The following equipment was used in the study: 22-gauge stainless steel 10-inch-long luer hub and deflecting tip needle (Aldrich Co., Milwaukee, WI), 10 cc Micromat® syringes (Popper and Sons, New Hyde Park, NY), lab dispersator (Premier Mill Corp, Reading, PA), centrifuge (IEC-22M), Napco vacuum oven #5831 (Precision Scientific, Chicago, IL), sonicator (Fisher Scientific FS21H), 13-mm round glass coverslips (Ernest Fullam Inc, Latham, NY), 24-well culture plates (Costar, Cambridge, MA), electron microscope (JEOL 6300 system electron microscope, Peabody, NY), fluorimeter (Perkin-Elmer

luminescence spectrometer LS50B, Norwalk, CT), microscope (Nikon B-2A, Nikon Inc, Garden City, NY), Sigma Scan Software, and Adobe Photoshop 4.0.1.

Methods

Study of cellular uptake and intracellular fate of rhodamine-labeled formulation.

Microsphere manufacture

Rifampicin-rhodamine-loaded poly(lactic-co-glycolic acid) (RR-PLGA) microspheres were manufactured by the emulsification solvent evaporation technique using the solubility of rifampicin in organic solvents such as methylene chloride (12). The polymer and the drug-dye mixture were separately dissolved in methylene chloride in equal volumes. Sonication was used for 20 seconds to aid dissolution of the polymer. Subsequently the polymer solution was added to the drug-dye solution. The continuous phase was a 70% vol/vol glycerol and a 0.05% wt/vol polyvinyl alcohol (PVA) aqueous solution. The PVA was added to the requisite amount of distilled water and warmed to 65°C. The solution was mixed with glycerol to achieve the desired concentration. The microspheres were prepared by the addition of 4 mL of the polymer-drug-dye solution in methylene chloride to 100 mL of the continuous phase, chilled between 4°C and 10°C, and administered with a syringe attached to a 10-inch-long needle. The mixture was agitated with a dispersator at 5500 rpm. After 15 minutes, the ice bath was replaced with a water bath warmed to 45°C for 5 minutes to enhance the evaporation of organic solvent with continued dispersion at 5500 to 6000 rpm. The contents were then transferred to a 5% isopropyl alcohol solution and stirred vigorously for 30 minutes with a magnetic stirrer (Corning PC 351, Corning, NY). The microsphere suspension was subjected to repeated centrifugation at 12 000 rpm to remove residual isopropyl alcohol. The centrifuge vials were then placed overnight in a vacuum oven under a vacuum of 27 in Hg without heating, and the microspheres obtained were collected on drying. They were then placed over desiccant at -20°C.

Terminal sterilization cannot be employed because PLGA has been known to deteriorate as a result of irradiation (13). Hence, the entire manufacture operation described above was carried out in a clean environment to prevent the appearance of contamination under culture conditions. Briefly, all glassware and stainless steel components were autoclaved before use. Equipment was swabbed with 70% ethanol, and the manufacture was performed under UV illumination (Blak-Ray long-wave UV lamp, Upland, CA). Sterile water was used for injection, and the drug-polymer solution was filter sterilized using a Millex-FG 0.22 μ m filter (Millipore, CA). No contact with the ice bath occurred during manufacture. The product was dried under a laminar flow hood. The product obtained by this modified technique did not exhibit altered characteristics compared with that prepared without clean technique.

Microsphere characterization

The microspheres were dispersed in distilled water, applied to a stub, and dried overnight in a vacuum oven. Microspheres were sputter coated with gold-palladium before examination using a scanning electron microscope. Photomicrographs of the particle images were obtained (Polaroid, Cambridge, MA).

Particle size analysis

The photomicrographs were used to perform particle size analysis (Sigma Scan software, Jandel, Sunnyvale, CA). The dimensions of 500 microparticles were measured, and the mass median diameter and the geometric standard deviation were calculated, assuming a log-normal distribution (14).

Rhodamine-release profile

The rhodamine loading was determined by dissolving 10 mg RR-PLGA microspheres in 1 mL chloroform followed by precipitation of the polymer with 9 mL methanol as described by Denkbass et al (15). The supernatant was analyzed for rhodamine by fluorimetric analysis employing excitation wavelength at 529 nm and emission wavelength of 551 nm. The release of rhodamine was determined over 24 hours by analyzing the supernatant after suspending 15 mg of RR-PLGA microspheres in 5 mL of phosphate buffer at 37 °C, with a pH of 7.4.

Uptake and degradation studies with RR-PLGA microspheres

Alveolar macrophages were harvested from Dunkin-Hartley guinea pigs by bronchoalveolar lavage with Hank's Balanced Salt Solution (HBSS), Ca²⁺- and Mg²⁺-free buffer after anesthetization and exsanguination of the animal. The cells obtained were washed by alternate centrifugation and resuspension at 500g. The viability and cell count were determined by employing a 1:1 mixture of cell suspension and trypan blue solution. The cells were then plated onto 13-mm glass coverslips at a density of 1×10^5 cells/mL in 24-well plates. The cell culture medium was RPMI 1640 with 10% vol/vol heat-inactivated fetal bovine serum, penicillin G-100 U/mL, and streptomycin-100 μ g/mL. After 2 hours the non-adherent cells were gently washed and, where indicated, chloroquine (10-20 μ g/mL) was added to the wells for 1 hour and then replaced with fresh media. RR-PLGA microspheres were added to the wells at differing (3:1,10:1) sphere:cell ratios for 6 hours and then replaced with media. The excess spheres were washed away and the cells resuspended in RPMI medium. At discrete timepoints over 7 days, the coverslips were removed and the cells fixed by addition of 2% HCHO and visualized by fluorescence microscopy employing a rhodamine filter.

Measurement of PLF and study of the effect of chloroquine

Uptake studies with fluorescein-labeled BioParticles

Similar studies with BioParticles were conducted to evaluate PLF in cultured guinea pig AMs as reported earlier (11). The lysosomes were labeled with rhodamine-dextran (0.25 mg/mL) for 24 hours. The excess dye was washed and the cells were then incubated at a sphere:cell ratio of 3:1 with fluorescein-labeled BioParticles, thereby labeling the endosomes green. Phagolysosomal fusion events were measured by scoring individual cells over 10 randomly chosen fields of view. The cells were scored as fusion/non-fusion events based on the appearance of an orange-yellow color obtained by the merging of the green fluorescence with the red fluorescence of rhodamine.

PLF intensity was studied by measuring the intensity of the orange-yellow color. The intensity was obtained by measuring arbitrary *a*, *b* values for 5 x 5 pixel areas

from cells with the Adobe Photoshop 4.0.1 software program at 60X magnification. As reference points and to validate the software, images were collected from control cells with no fluorophores, cells loaded with rhodamine-dextran only, and fluorescein-labeled BioParticles. These measurements were performed to determine whether the intensity of the color changed as a result of probable fusion of more lysosomal compartments with the endosome possibly leading to subtle differences in the hue obtained.

RESULTS AND DISCUSSION

Study of rhodamine-labeled formulation

Microsphere manufacture and characteristics

The solvent evaporation technique produced smoothly rounded microspheres (Figure 1), indicating uniform evaporation and solvent extraction from the droplet. The median diameter was found to be 0.5 μm with a geometric standard deviation of 1.8 (Figure 2). Generally, these microspheres were smaller in diameter than rifampicin-loaded microspheres, probably because of the additive effect of the planar configurations of rhodamine and rifampicin molecules and their possible surface adsorption. The size profile determined demonstrated the microspheres to be in the size range for optimal phagocytosis by the alveolar macrophages.

The formulation manufactured under a clean environment was found to be suitable as it did not contribute any confounding contamination when incubated with the guinea pig AMs. The limiting condition remained the limited lifetime of primary culture of up to 7 days. Rhodamine was the fluorophore of choice to examine the behavior of the formulation because rhodamine is uniformly intense over a broad pH range (pH 4-9).

Rhodamine-release profile

The rhodamine loading was found to be $0.42\% \pm 0.04\%$ versus a theoretical loading of 1%. The release pattern reflected a slow rhodamine release of only 2.5% over a day (Figure 3). This reflected release restricted by polymer degradation because of its high molecular weight of 80 000. The slow release of rhodamine indicated suitability of the use of rhodamine as a marker for long-term studies of the formulation under culture conditions.

Particle size analysis of rhodamine-rifampicin microspheres

Figure 1. Log-probability plot of the size analysis of rhodamine-labeled rifampicin poly(lactic-co-glycolic acid) microspheres (Median diameter 0.473 μm [GSD 2.09]).

Figure 2. Representative photomicrograph of rhodamine-labeled rifampicin poly(lactic-co-glycolic acid) microspheres.

Figure 3. Rhodamine release profile from rhodamine-labeled rifampicin poly(lactic-co-glycolic acid) microspheres (n=5).

Uptake and degradation studies with RR-PLGA microspheres

The studies indicated a persistence of intact microspheres within cultured guinea pig AMs up to 7 days in culture (Panel 1). This time frame was limited mainly due to detachment of cells from the coverslip surface owing to the typical characteristics of a primary culture. The persistence of intact microspheres within cells may be attributed to the composition of the polymer (75:25 PLGA) and its high molecular weight. Studies by Tabata and Ikada (16) demonstrated that the lifetime of PLGA microspheres within mouse peritoneal macrophages was found to be dependent on the monomer composition and polymeric weight. The authors reported persistence of polylactic acid microspheres (MW 13 000) up to 7 days in culture. Varying degrees of diffuse red fluorescence were observed in the cytoplasm, indicating degradation of the microsphere over time, a pattern witnessed by Tabata and Ikada in similar studies (16).

Chloroquine has been reported to be a phagolysosomal fusion enhancer when employed at modest doses, while at higher doses it has been reported to be capable of shutting down intracellular trafficking due to lysosomal rupture (17). Chloroquine treatment (10 μ g/mL) did not sharply alter the time point for the appearance of a diffuse fluorescence, which may partly be a shortcoming of the technique, as it relied on the individual perception for subtle differences in the extent of diffuseness of the intensely bright rhodamine. However, chloroquine did exert a protective effect on the cells at a sphere:cell ratio of 10:1 compared with the control group as fewer cells detached over time. This may be due to rapid turnover of excess polymeric by-products.

Measurement of PLF and Study of the Effect of Chloroquine

Uptake with BioParticles

The further investigation of doses of chloroquine employed in our system on PLF was carried out using a dual fluorophore system. This system enhances the visualization of PLF events by the formation of an orange-yellow color on fusion of the green fluorescent endosomal compartment with the red fluorescent lysosomal compartment.

Panel 1. Photomicrographs of alveolar macrophages containing rhodamine-labeled rifampicin poly(lactic-co-glycolic acid) (RR-PLGA) microspheres at a) 24 hours, b) 4 days, and c) 6 days. Cells stayed in culture for 7 days and began detaching. Patterns of punctate to diffuse fluorescence was observed over time, indicating the slow degradation of RR-PLGA microspheres.

Panel 2. Photomicrographs of alveolar macrophages with BioParticles and rhodamine-dextran. a) cells depicting pinpoint red lysosomes b) few cells with freshly phagocytosed BioParticles c) cells displaying orange-yellow fluorescence indicating phagolysosomal fusion

Panel 2 depicts various stages in the phagolysosomal process, with the formation of orange-yellow fluorescence resulting from the fusion events that are occurring. The rate of fusion was measured by scoring fields of view for cells exhibiting orange-yellow fluorescence over time at 40X magnification. The phagocytic index was also compared to ensure that chloroquine did not alter the phagocytic uptake on incubation with the cells. Figure 4 depicts the kinetics of PLF in the different groups. The PLF kinetics were

enhanced in the treatment groups, which were enhanced compared with the control group. This trend was, however, equalized over a period of 4 hours. D'Arcy Hart and Young reported acceleration of PLF at a 1 hour time point after treatment with 20 $\mu\text{g/ml}$ for 45 minutes (10). The mechanism of action of chloroquine is in part a result of its basic nature and its sequestration within lysosomes where it exerts its effect (11). Such subtle manipulation may be employed to improve the vicinity of surface-associated drug in microsphere formulations to intracellular pathogens.

An image analysis of cells at 60X magnification with scoring of 5 x 5 pixel areas for the intensity of color showed a different stagger pattern of the *a*, *b* values for cells treated with rhodamine-dextran alone or those incubated with fluorescein-labeled BioParticles alone (Figure 5). The *Lab* color analysis system distinguishes between colors and hues based on an arbitrary numeric scale. The *Lab* color consists of a luminance, or brightness component, and 2 chromatic component - the *a* component, which ranges from green to red, and the *b* component, which ranges from blue to yellow. The *Lab* mode has been employed to edit the luminance and color values in the images independently.

The *a* component (green-red) axis and the *b*-components (blue to yellow) axis can range in scale from -120 to +120 (18). The *Lab* scale is clearly suitable to measure a transition from green to red as the *a* component measures the green to red transition. The stagger pattern of the cells depicting an orange-yellow color yielded a pattern with points that fell between the red only or green only zones (Figure 6). Similar data presentation and interpretation have been reported by Bruce et al (19). The authors employed a Hoechst 33342 staining of lung fibroblasts and compared the scatter values between the red and blue fluorescence obtained by flow cytometry to study apoptosis in postnatal lung fibroblasts.

The process did not reveal any differences in the intensity of color, especially visually to the experimenter. However, a set of *a*, *b* values intermediate to that between the green and red values were obtained as evidenced by Figures 5 and 6. This may result from the cells reaching a similar endpoint in the fusion process (e.g., a similar number of

Figure 4. Kinetics of phagolysosomal fusion over time in control and treatment groups ($n=5$). For each timepoint, cells over 10 fields of view at 40X were scored as fusion/non-fusion events depending on visualization of the orange-yellow color formed on fusion between endosomes and lysosomes. The percentage of phagolysosomal fusion was then obtained by dividing the number of cells by the total number of cells in the field of view.

Figure 5. Scatter patterns for 5 x 5 pixel areas of *a*, *b* values of rhodamine-dextran-treated cells alone (red fluorescence) or fluorescein-labeled BioParticles exposed cells alone (green fluorescence). In the *Lab* scale system, green to red values range from -120 to +120. The cluster of scatter points on the left-hand side of the graph corresponds to *a*, *b* values for 5 x 5 pixel areas (at 60X) treated with only fluorescein-labeled BioParticles, while the cluster of points on the right-hand side corresponds to cells treated with only rhodamine-labeled dextran (70 kd).

Figure 6. Scatter patterns for 5 x 5 pixel areas of *a*, *b* values of rhodamine-dextran-treated and fluorescein-labeled BioParticles exposed cells. The profile of the *a*, *b* values for the 5 x 5 pixel areas of cells visually displaying an orange-yellow color falls in between the values for the corresponding *a*, *b* values for 5 x 5 pixel areas for fluorescein-labeled BioParticles only and rhodamine-dextran only-treated cells displayed in Figure 5.

lysosomes may fuse with an endosome leading to the formation of the same "hue"). It is equally likely that the modest dose of chloroquine served only to affect the kinetics and not the extent of fusion. The studies reflected that it is possible to monitor the intracellular behavior of formulations that treat intracellular pathogens or exert an action intracellularly. Chloroquine was found to modestly enhance the rate of PLF over 4 hours, and such strategies might safely manipulate naturally occurring physiological mechanisms that aid health and combat disease.

Similar studies in survival of the intracellular TB in infected macrophages on treatment with rifampicin microspheres and chloroquine in tandem remains to be assessed to further understand the subtle intricacies of physiological functioning of the AM resulting in health or disease.

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