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Lung surfactant phospholipids inhibit the uptake of respirable microspheres by the alveolar macrophage NR8383

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Abstract

Fluorescent poly(lactic-co-glycolic acid) microspheres of a respirable size were fabricated for use in a fluorescent activated cell sorting assay utilizing the continuous alveolar macrophage NR8383. This is a suitable model of alveolar phagocytosis, which permitted an investigation of the influence of phospholipid structure on the inhibition of phagocytosis of microspheres. Phospholipid inhibition was found to be independent of phosphatidylcholine alkyl chain length. Head group effects were investigated by studies employing phosphatidyl-choline,-serine, and -ethanolamine, and inhibition was shown to be independent of head group. Closer modelling of the lung environment by co-culturing NR8383 on A549 alveolar epithelium showed type II secretions to also down-regulate phagocytosis. In addition, pre-incubation with microspheres coated with dipalmitoylphosphatidyl-choline reduced the uptake of a second microsphere (fluorescein isothiocyanate-labelled latex).

Introduction

The lung has been investigated as a potential systemic portal for biotechnology products that are degraded by the digestive enzymes of the gastrointestinal tract if administered orally. The mucociliary escalator does not extend to the alveoli, which are instead patrolled by alveolar macrophages. Inhaled drug delivery systems such as bioerodible polymer microspheres deposited in the alveoli will remain there until they dissolve, disintegrate or are phagocytosed by mobile alveolar macrophages. For maximum drug release and bioavailability, the drug delivery system must either avoid uptake by the alveolar macrophage or be readily soluble in the alveolar fluid (Patton & Platz 1992).

Alveolar macrophages occur on all lung epithelial surfaces, although they are more prevalent in the distal regions (Bezdicek & Crystal 1997). The number of macrophages in the normal human lung is variable, but they compose over 95% of the mobile cell population. Macrophages account for 2–5% of total alveolar cells, numbering between 50 and 100 per alveolus (Crapo et al 1982; Bezdicek & Crystal 1997).

Throughout these studies, NR8383 alveolar macrophages were used. This continuous cell line, derived from bronchio-alveolar lavage of a normal adult male Sprague–Dawley rat, provides more reproducible data than primary cultures of limited lifespan. Helmke et al (1987, 1989) have demonstrated that NR8383 and freshly isolated alveolar macrophages both possess the Fc receptor, produce interleukin (IL)-1, respond to phagocytic challenge by ingestion, and demonstrate a characteristic respiratory burst.

Drug delivery systems depositing in the alveolar compartment will come into intimate contact with lung surfactant. It is well established that lipids compose over 90% of pulmonary surfactant, the remainder being made up of the surfactant proteins SP-A, -B, -C and -D. Phospholipids form 80–90% of the lipids, while the remainder is composed largely of neutral lipids such as cholesterol. Phosphatidylcholine lipids account for 70–80% of surfactant phospholipid, ~60% of which is dipalmitoyl phosphatidylcholine (DPPC). Phosphatidylglycerols (PG) constitute up to 10% of the remaining phospholipids, with phosphatidylinositol (PI), phosphatidylethanolamine (PE) and phosphatidylserine (PS) making up the rest (Batenburg 1992).

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Both liposomes and microspheres have been extensively studied (and recently reviewed by Ahsan et al 2002) as carriers for drug delivery to macrophages. Various carrier properties such as hydrophobicity, surface charge, composition, concentration and inclusion of surface ligands can influence specificity and efficacy.

In previous studies (Jones et al 2002), fluorescent poly(lactic-co-glycolic acid) (PLGA) microspheres of a respirable size were fabricated for use in a fluorescent activated cell sorting (FACS) assay to investigate phagocytosis. The continuous cell line NR 8383 was verified as a good model of alveolar phagocytosis by comparison with primary cultures. This work revealed that the inclusion of DPPC to the cell media or as microsphere coatings significantly reduced phagocytosis. This phospholipid, when included in PLGA microsphere production, has also been shown to inhibit phagocytosis (Evora et al 1998). DPPC is the major component of lung surfactant and in this study we investigated this phenomenon further by using other airway surfactant lipids, A549 alveolar epithelium and incubations with secondary non-DPPC-coated microspheres.

Materials and Methods

Materials

Analytical grade dichloromethane (DCM) was purchased from Fisher Scientific, Loughborough, UK. Water was prepared using a Millipore Milli-Ro system. PLGA 75:25, 90–126 kDa, poly(vinyl alcohol) (PVA) 13–23 kDa, 87–89% hydrolysed, rhodamine B (RB) and latex microspheres (2.73 μm) were obtained from Sigma Chemicals, Poole, UK.

Methylcellulose was purchased from ICI Pharmaceuticals Limited, Alderley Park, UK.

Dimyristoylphosphatidylcholine (DMPC), DPPC and distearoylphosphatidylcholine (DSPC) were purchased from Avanti Polar Lipids, Alabaster, USA. L-α-Lysopalmitoylphosphatidylcholine (LPC), dipalmitoylphosphatidylserine (DPPS), dipalmitoylphosphatidylethanolamine (DPPE) and L-arginine methyl ester hydrochloride (L-NAME) were purchased from Sigma Chemicals.

NR8383 cells were from the American Tissue Culture Collection, A549 cells were from the European Collection of Animal Cell Cultures, and the TR146 cells were a kind gift from the Imperial Cancer Research Laboratories, London, UK. Cell media, fetal bovine serum (FBS), penicillin/streptomycin (P/S) and trypsin–ethylenediamine tetraacetic acid (EDTA) solution (0.5 g trypsin/0.2 g EDTA mL^{-1}), Dulbecco's minimum essential media (DMEM) deficient in L-methionine, L-glutamine, sodium phosphate, L-cysteine, glucose, L-leucine, L-arginine and inositol were all purchased from Gibco Life Technologies, Paisley, UK.

L-Methionine, L-glutamine, sodium phosphate, L-cysteine, glucose, L-leucine and inositol were added at appropriate concentrations (glucose was supplemented to 4.5 g L^{-1}) to produce L-arginine-free DMEM.

Microsphere fabrication and coating

PLGA microspheres were prepared by a method previously described (Jones et al 2002). Briefly, 20 mg of PLGA was dissolved in 1 mL DCM containing 0.2 mg of RB and dispersed in 4 mL of an aqueous phase of 4% PVA and 0.25% methylcellulose. The resultant oil-in-water emulsion was homogenized for 10 min with an Ultraturax homogenizer at 13 500 rev min^{-1}. Subsequent evaporation of the DCM was carried out with mechanical stirring for 6 h at room temperature.

Microspheres were collected by centrifugation and washed by dispersion in water ($\times 3$), with subsequent centrifugation. Microspheres were freeze-dried to remove remaining water and the yield determined ($>90\%$). Laser diffraction analysis (Malvern 2600; Malvern Instruments, Malvern, UK) indicated a narrow size distribution (D[v, 0.9] 2.91 ± 0.18 μm; D[v, 0.1] 2.42 ± 0.04 μm; D[v, 0.5] 2.71 ± 0.11 μm).

To coat the microspheres in phospholipid, 5 mg of microspheres were incubated in aqueous 0.1% dispersions of phospholipid for 12 h at 37°C on a plate shaker. Microspheres were washed three times by centrifugation in water to remove excess or unattached lipid. The presence of lipid was investigated by Fourier transform infrared spectroscopy (FTIR) (Perkin Elmer 1625 FTIR). Samples were suspended in a ground matrix of barium chloride and spectra of coated microspheres compared against substrate and uncoated microsphere controls.

NR8383 cell culture

NR8383 cells were cultured in F12K (Kaighn's modification) nutrient media supplemented with 0.5% P/S and 15% FBS under standard culture conditions (37°C, 5% CO_2/95% air, humidified). Two phenotypes are expressed, an adherent macrophage population used for experimental purposes, and a floating, regenerating phenotype from which sub-cultures are taken. Floating cells were passaged by centrifugation (95 *g* for 8 min) and the cell pellet reconstituted in fresh media. Cells were seeded at 0.4–0.5 million mL^{-1} to achieve a floating concentration of approximately 0.25 million mL^{-1}. For experimental purposes, adherent macrophages were covered by fresh media and removed by light scraping or agitation, before re-suspension at 100 000 cells mL^{-1} in the wells of 24-well plates.

Culture of epithelial cells and isolation of pulmonary surfactant

Pulmonary surfactant was harvested from A549 cell layers grown to confluency in 75-cm^2 T-flasks. Medium was removed and the cell layer washed three times with 10 mL phosphate-buffered saline (PBS). The media and washings were collected in centrifuge tubes and spun at 95 *g* for 10 min to pellet any removed A549 cells. The supernatant

was then transferred to an ultracentrifuge tube and spun for 1 h at 100 000 *g* to pellet surfactant secretions. The supernatant was removed and the pellet harvested from each T-flask re-suspended in 15 mL of fresh F12K media.

Both A549 and TR146 cells were passaged by trypsinization and experimental cultures seeded at a density of 10 000 per cm^2 in the wells of 24-well plates.

FACS assay

Cell phagocytosis was assayed by a FACS assay coupled with acid washing (Jones et al 2002), where it was validated by isolating the fluorescent intensity of internalized microspheres. FACS allows fluorescent measurements to be made on single cells at high speed. Assays utilizing this instrumentation are based on the cellular uptake of fluorescent particles. Cells can be discriminated from particulates or other cell types by the unique way in which they scatter a laser beam. Side scatter (90°) is attributable to granularity and forward scatter to size; thus, both fluorescent intensity of each cell and the number of fluorescent cells per sample can be determined. Cells aliquoted into the wells of 24-well plates were exposed to PLGA microspheres (5, 10 or 100 (excess) per cell) for 2 h. Cells were then removed and acid washed by centrifugation (2 min at 550 *g*) in a buffer of 28 mM sodium acetate, 117 mM sodium chloride adjusted to pH 5 with 2 M HCl, to remove microspheres bound externally to the cell membrane. Inhibitors (845 ng mL^{-1} sodium azide and 15 ng mL^{-1} cytochalasin D) were included with some cultures for the experimental duration and a preincubation stage of 1 h, serving as negative controls.

Cells and particulates were analysed using a FACScan flow cytometer (Becton Dickinson, Oxford, UK) and interpreted via WinMDI software. Results were collected for 5000 cells (n = 3) and expressed as the mean of three median fluorescent intensity (MFI) values for each of three experiments.

Influence of DPPC-coated microspheres on the phagocytosis of a second particulate

NR8383 macrophages were incubated with DPPC-coated and uncoated PLGA microspheres at ratios of 2.5, 5 and 100 (excess) microspheres per cell for 2 h. Microspheres were removed by a gentle washing in PBS. Cells were then bathed in fresh media containing fluorescein isothiocyanate (FITC)-labelled latex microspheres and incubated for a further 2 h. Cells were transferred to microcentrifuge tubes, acid washed and analysed on channel one (FITC detector) of the FACScan flow cytometer.

Non-professional epithelial phagocytosis

The phagocytic ability of the epithelial cell lines TR146 and A549 was investigated. A549 is a continuous cell line derived from a human pulmonary adenocarcinoma that has morphological and biochemical features of the pulmonary alveolar type II epithelial cell (Smith 1977). TR146 is a cell line established from human biopsy specimens of a squamous cell carcinoma of the buccal mucosa, which in culture differentiates to form a stratified epithelium (Jacobsen et al 1995). An excess of PLGA microspheres was added to epithelial cells that had reached confluency. Culture media were removed and the cells washed in PBS before trypsinization. After trypsinization, 1 mL of fresh medium was added to each well and the cells collected in microcentrifuge tubes. Cells were centrifuged, the supernatant removed and the resultant cell pellet acid washed. Cells were reconstituted in fresh media and analysed by FACS.

Epithelial and macrophage co-cultures

A549 and TR146 cells were seeded in the wells of 24-well plates. The cell medium was replaced after approximately 3 days (the time taken for the epithelial cells to reach confluency) with 1 mL of F12K containing 100 000 NR8383 macrophages. For some cultures, F12K media containing A549 secretions were used. A549 cell layers were carefully washed in PBS to remove any surfactant, with care taken to preserve the integrity of the epithelial layer. Macrophages and epithelial cells were co-incubated for 12 h under normal culture conditions before microspheres were added at ratios of 5 and excess, and the system then incubated for a further 2 h.

PBS washings of A549 removed surfactant before the 12-h co-incubation stage, limiting surfactant concentration to that secreted during this and the experimental duration. In addition, to some cultures, previously isolated surfactant was added. In further controls, the incubation period was removed, limiting the time for surfactant secretion.

Influence of NO on phagocytosis

NR8383 cells were reconstituted in modified DMEM (L-arginine 0, 84 and 840 mg mL^{-1}) and 1 mL^{-1} of the cell suspension (100 000 cells mL^{-1}) aliquoted into the wells of 24-well plates. Cells were incubated for 12 h in these modified media, which were subsequently replaced with fresh modified DMEM. To some wells, L-NAME (1 or 5 mM), and/or DPPC (0.1 mg mL^{-1}) were included for a preincubation stage of 1 h. PLGA microspheres were added to excess and the system incubated for a further 2 h. Cells were removed from the plates, acid washed and the extent of phagocytosis assayed by FACS analysis.

Statistics

A single factor analysis of variance was performed with significance quoted at the level of $P < 0.05$ for data in Table 1 (between DPPS, DPPE, DPPC, DMPC and DSPC coatings and comparing LPC with each of these lipids;

Table 1 The effect of various phospholipid coatings of PLGA microspheres on phagocytosis by NR8383 macrophages.

Phospholipid	% Population phagocytosing microspheres	Number of microspheres phagocytosed per cell
DMPC	28.75±2.54	48.46±9.26
DPPC	24.53±0.39	54.95±3.40
DSPC	31.85±12.87	42.28±12.96
DPPS	32.72±3.76	42.39±1.18
DPPE	42.58±8.59	50.55±10.08
LPC	101.2±6.07	90.98±12.84

Microspheres were added to excess and incubated for 2 h. Results are expressed as a percentage of the control (uncoated) microspheres. Data are mean±s.d., n = 3 for each of three experiments.

Table 2 (each ratio of PLGA microsphere per cell to latex microsphere per cell); and Table 3 (for both ratios of excess and 5 microspheres per cell between TR146, A549 and $A549_{t=0}$ and surfactant, TR146+surfactant and A549 +surfactant. In Table 4, some important comparisons were between (i) ×10 arginine and no arginine, 1 mM L-NAME and DPPC (NO cannot be produced in the latter) and (ii) between no arginine, 1 mM L-NAME and DPPC, and ×10 arginine and DPPC, to investigate whether DPPC inhibition of phagocytosis is augmented by NO production.

Results

FTIR demonstrated the presence of DPPC, DMPC, DSPC, DPPE and DPPS in microsphere samples incubated in aqueous suspensions of the aforementioned phospholipids (data not shown). The spectral evidence for DPPC coating of the PLGA microspheres is provided in Jones et al (2002). However, for microspheres incubated in LPC, the phospholipid could not be identified by FTIR, suggesting the absence of LPC or very low surface coverage (i.e. below detection limits).

Table 2 NR8383 phagocytosis of FITC-labelled latex microspheres after initial exposure to DPPC-coated and uncoated PLGA microspheres.

PLGA microspheres per cell/latex microspheres per cell	% Macrophages phagocytosing microspheres		Number of latex microspheres per cell	
	Uncoated PLGA microspheres	Coated PLGA microspheres	Uncoated PLGA microspheres	Coated PLGA microspheres
2.5/2.5	23.04±2.64	5.52±1.51	0.41±0.002	0.29±0.001
2.5/5	28.33±0.23	6.10±2.08	0.43±0.000	0.30±0.002
2.5/excess	70.55±1.02	28.10±3.07	1.11±0.001	0.44±0.002
5/2.5	10.22±0.35	4.31±0.62	0.38±0.001	0.28±0.000
5/5	19.55±0.85	3.55±2.63	0.39±0.002	0.39±0.008
5/excess	67.74±0.15	15.17±0.42	0.94±0.000	0.35±0.000
0/2.5	26.01±3.39	–	0.44±0.004	–
0/5	33.43±1.76	–	0.51±0.001	–
0/excess	75.18±1.88	–	1.37±0.001	–

Table 3 Phagocytosis of PLGA microspheres by NR8383 macrophages co-incubated with A549 epithelia, TR146 epithelia or A549 isolated surfactant.

	% of NR8383 cells phagocytosing PLGA microspheres		Number of PLGA microspheres per NR8383 cell	
	Microspheres added to excess	Microspheres added at ratio of 5 per cell	Microspheres added to excess	Microspheres added at ratio of 5 per cell
TR146	94.93±3.22	92.98±11.26	87.14±9.97	87.41±14.81
A549	86.67±8.79	101.53±13.57	73.63±1.53	109.63±7.41
$A549_{t=0}$	75.18±8.70	84.27±8.22	73.60±1.92	93.66±7.20
Surfactant	35.51±8.54	33.97±2.46	43.41±7.72	72.59±11.11
TR146+surfactant	53.10±3.37	51.22±5.25	55.63±4.82	75.56±7.41
A549+surfactant	44.19±11.51	55.50±2.91	48.55±0.97	80.15±8.15

Microspheres were incubated with cells for 2 h. Data are expressed as a percentage of phagocytosis under control conditions (NR8383 in the absence of surfactant and epithelial cells). Data are mean±s.d., n = 3 for each of three experiments. TR146 = NR8383 incubated on TR146 epithelium; A549 = NR8383 incubated on A549 epithelium; $A549_{t=0}$ = cell layer washed with PBS immediately before the study; surfactant = A549 isolated surfactant included in the media.

Table 4 The phagocytosis of PLGA microspheres by NR8383 in L-arginine-modified media, and/or the presence of surfactant (DPPC) and L-NAME.

Media modification	% Macrophages phagocytosing microspheres	Mean number of microspheres per cell
No arginine	73.72±4.88	4.43±0.35
Normal arginine	73.72±0.43	4.00±0.39
×10 Arginine	74.54±1.03	3.85±0.25
No arginine, 1 mM L-NAME	75.11±0.86	4.44±0.26
Normal arginine, 1 mM L-NAME	72.34±2.84	3.79±0.60
×10 Arginine, 1 mM L-NAME	76.12±0.87	4.10±0.30
No arginine, 1 mM L-NAME+DPPC	32.18±1.91	3.12±0.39
Normal arginine, 1 mM L-NAME+DPPC	32.82±2.80	3.41±0.11
×10 Arginine, 1 mM L-NAME+DPPC	35.45±2.15	3.59±0.36
No arginine, 5 mM L-NAME+DPPC	40.94±10.94	3.63±0.02
Normal arginine, 5 mM L-NAME+DPPC	46.87±9.41	3.83±0.18
×10 Arginine, 5 mM L-NAME+DPPC	35.68±0.09	3.94±0.35
No arginine+DPPC	29.84±1.96	3.01±0.66
Normal arginine+DPPC	32.00±2.54	3.26±0.21
×10 Arginine+DPPC	31.61±3.18	3.33±0.21

Data are mean±s.d., n = 3 for each of three experiments.

Effect of phosphatidylcholine structure on phagocytosis

NR8383 cells phagocytosed a mean of 3.24±0.65 uncoated microspheres per cell and 70.1±1.81% of the cell population were found to contain microspheres. The inhibition of phagocytosis by microspheres coated in phosphatidylcholines of three different chain lengths (DMPC, C14; DPPC, C16; and DSPC, C18) is shown in Table 1. There was no significant difference in the degree of inhibition ($P > 0.05$). The number of cells containing microspheres reduced to approximately 30% and the mean number of microspheres per cell approximately halved. Microsphere coatings of DPPE and DPPS similarly reduced phagocytosis (Table 1); LPC, in contrast, failed to decrease microsphere phagocytosis ($P < 0.01$).

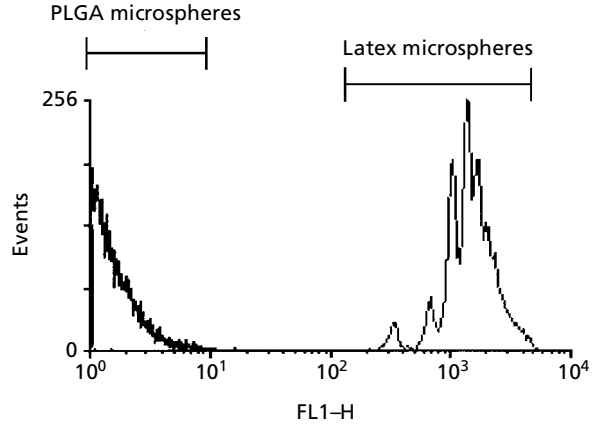

Figure 1 Representative histograms of latex and PLGA microsphere fluorescence detected using channel one of the flow cytometer.

Uptake of a second fluoroscein isothiocynate (FITC)-labelled microsphere

Figure 1 shows two representative histograms of latex microsphere (FITC-labelled) and PLGA microsphere (RB-labelled) fluorescence. Fluorescence data were collected on channel one of the flow cytometer. The median fluorescence of latex microspheres is approximately 1000-times that of PLGA microspheres when detected using this channel.

The uptake of latex microspheres subsequent to a preincubation with DPPC-coated or uncoated PLGA microspheres is shown in Table 2. Increasing the ratio of latex microspheres from 2.5 or 5 microspheres per cell to excess increases the number of microspheres phagocytosed ($P < 0.01$). Further, except at the ratio of 5:5, initial exposure to DPPC-coated microspheres reduced the uptake of the second particulate ($P < 0.01$).

The final three lines in the table represent cultures that were not preincubated with PLGA microspheres. For excess additions of latex microspheres, uptake is significantly greater by cultures not previously exposed to PLGA microspheres ($P < 0.05$), preincubation with PLGA microspheres significantly reducing the mean number of latex microspheres per cell.

Effects of epithelial co-culture and A549 isolated surfactant on NR8383 phagocytosis

Neither TR146 nor A549 epithelia phagocytosed PLGA microspheres. When microspheres were added in excess, MFIs did not exceed those of autofluorescence subsequent to acid washing.

From Table 3, where NR8383 have been cultured on TR146 and A549 epithelial cells, phagocytosis approximates that of control (NR8383 cultured on plastic wells) populations. No significant differences were found between groups incubated on epithelium in the absence of surfactant and their controls.

The inclusion of surfactant caused a decrease in phagocytosis compared with populations from which surfactant had been excluded when microspheres were added to excess ($P < 0.01$). However, when microspheres were added at a ratio of 5 per cell, a significant difference ($P < 0.01$) was found only in respect of the percentage of the macrophage population participating in particle uptake.

NO inducing experiments

The percentage of the population containing microspheres and the number of microspheres phagocytosed per cell correlate closely, following the same trend, and thus any discussion here can be extended to either parameter (Table 4). Adjusting the concentration of L-arginine did not affect the phagocytic capacity of NR8383. The inclusion of L-NAME at 1 mM did not alter phagocytosis. Where DPPC was included, the percentage of the macrophage population containing microspheres was reduced irrespective of other manipulations of the media such as the exclusion of L-arginine and inclusion of L-NAME. Comparing experiments in which elevated levels of arginine ($\times 10$) were added to no arginine, 1 mM L-NAME and DPPC, there was a significant inhibition of phagocytosis in the latter, with a significant reduction in the number of phagocytosing cells ($P < 0.01$) and the number of microspheres per cell ($P < 0.02$). There was no significant difference ($P > 0.5$) in DPPC inhibition of phagocytosis when NO levels were raised by the addition of $\times 10$ arginine.

Discussion

DMPC, DPPC and DSPC possess dialkyl chains of 14, 16 and 18 carbon atoms respectively, with an identical phosphatidyl head group. Each reduced phagocytosis to the same extent (Table 1). Inhibition is therefore not dependent on phospholipid acyl chain length. The phospholipid molecules are likely to be adsorbed to the microsphere surface through hydrophobic interaction between their alkyl chains and the PLGA surface. The polar head groups would therefore protrude from the microsphere surface to make contact with the macrophages. In this arrangement, the length of the alkyl chain is unlikely to effect phagocytosis.

DPPS and DPPE, like DPPC, have two palmitoyl chains, but differ in their head group charge. DPPS has a net negative charge, whereas DPPE and DPPC head groups carry no net charge at pH 7.4. All however reduce phagocytosis (Table 1), suggesting that head group charge does not effect phagocytosis. Alkyl chain length and the substitution of serine, choline and ethanolamine do not influence phagocytic inhibition, suggesting that the active moiety is the phosphate group. In contrast, LPC with a single palmitoyl chain was not detectable by FTIR as an adsorbed film and predictably had no effect on phagocytosis.

In FACS analysis, RB fluoresces largely outside the wavelengths detected by channel one, which is usually reserved for FITC fluorescence. As a consequence the MFI of FITC microspheres was approximately 1000-times that of RB encapsulated microspheres using this detector (Figure 1). Hence, 99.9% of acquired cellular fluorescence can be attributed to the uptake of FITC, negating the fluorescent contribution of phagocytosed PLGA microspheres. Rhodamine fluorescence is detected by channel two of the flow cytometer. The uptake of PLGA microspheres, however, could not be isolated since FITC fluorescence can also be detected by channel two. Cellular MFI quantified by this channel would be a summation of phagocytosed rhodamine and FITC labelled microspheres. Hence, only data from channel one, the uptake of latex microspheres, was analysed.

Macrophages initially incubated with DPPC/PLGA microspheres phagocytosed fewer latex microspheres in comparison with control cultures preincubated with PLGA microspheres (Table 2). Preincubation with DPPC/PLGA microspheres reduced latex microsphere phagocytosis from a mean (all PLGA microsphere per cell to latex per cell ratios) of 0.61 to 0.34 microspheres per cell. There was a corresponding decrease in the percentage of phagocytosing macrophages from 36.6 to 10.5%. It is doubtful whether internalization is necessary for DPPC to induce a reduction in phagocytosis, since relatively small numbers of microspheres are internalized by a reduced percentage of the macrophage population. If internalization were necessary for further inhibition, then the number of phagocytic cells would remain similar to controls, with the number of microspheres per cell dampened. It is more likely that interaction between DPPC and the membrane is sufficient to trigger inhibition, although it is possible that those cells containing microspheres could release anti-inflammatory mediators into the media. Since DPPC-coated microspheres were removed by washing and the phagocytosis of a secondary fluorophore depressed for the 2-h experimental period, it can be concluded that inhibition has a longevity of at least 2 h.

As the ratio of latex microspheres to cells is increased, so is their uptake, presumably as the chances of contact between cell and particulate are raised (Table 2). When added to excess, uptake of latex microspheres is significantly greater by cultures not previously incubated with PLGA microspheres. Significance is limited to cultures incubated with an excess of latex microspheres, since only here does latex phagocytosis reach its maximum. Prior uptake of PLGA microspheres reduces that maximum. This suggests that cells have a finite capacity and is supported by time-dependent studies, which show the number of microspheres per cell to reach a maximum by 45 min, phagocytosis continuing through the recruitment of more cells (Jones et al 2002).

Neither A549 nor TR146 phagocytosed PLGA micro-

spheres, suggesting that such cells do not act as Rabinovitch's non-professional phagocytes (Rabinovitch 1995). This is an important find in itself, since epithelial cells heavily outnumber macrophages. However, phagocytosis is seemingly confined to the smaller population.

FACS allows cell types to be distinguished on the basis of size and granularity; hence NR8383 macrophages can be harvested from co-cultures without fear of contaminated results. A549 cells are capable of producing pulmonary surfactant in-vitro (Smith 1977). Such secretions were isolated and proved to reduce phagocytic function when microspheres were added at both 5 and excess microspheres per cell (Table 3). The exposure of NR8383 to epithelial cell layers (TR146 and A549) in the absence of surfactant did not reduce phagocytosis.

Miles et al (1998) reported the incubation of alveolar macrophages with DPPC to increase NO production in a time- and concentration-dependent manner, reaching a maximum of 150 μg mL^{-1} after 2 h. This increase could be blocked by co-incubation with L-NAME at 1 mM. Increasing concentrations of L-arginine, the precursor to NO, increased NO production. The authors proposed that increases in NO provoked by DPPC could be responsible for maintaining an anti-inflammatory state in-vivo. Studies recreated here used these conditions and assayed the physiological response of phagocytosis rather than NO production directly. Without a quantitative measure of NO, we can only presume that the system recreated here had a similar effect to that described by Miles et al (1998) and that additions of DPPC up-regulated NO production.

L-NAME is a methylated ester of L-arginine and a competitive inhibitor of L-arginine for the binding site in nitric oxide synthase, which synthesizes NO from its arginine precursor. Presuming the system behaved accordingly, if DPPC decreases phagocytosis through a mechanism augmented by an up-regulation of NO, then phagocytic inhibition should be inhibited by the addition of L-NAME. In addition, elevated levels of arginine should have an effect similar to DPPC, since both elevate NO production (Miles et al 1998). Further, if DPPC exerts its effects by a mechanism dependent on enhanced NO production, then it should be ineffective in L-arginine-free media. None of these hypotheses were found to be true; DPPC inhibited phagocytosis in the absence of arginine, elevated concentrations of arginine did not reduce phagocytosis, and L-NAME did not inhibit the decrease in phagocytosis caused by DPPC (Table 4). This suggests that DPPC inhibition of phagocytosis and indeed the phagocytic process is independent of NO.

NO, however, does not only serve as an anti-inflammatory cytokine, it is also a potent oxidizing agent released by macrophages and other leukocytes as an integral part of host defence. Soluble in lipids and water, it passes through cell membranes to induce cytotoxicity in tumour cells and parasites. DPPC-induced NO production may preserve the sterility of the lung. NO is utilized in this way in the stomach where, preserved by acidity, it acts as an antimicrobial agent. Indeed, the lung is also slightly acidic, with a pH of 6.4.

DPPC and the surfactant secretions of pulmonary epithelial cells down-regulate phagocytic function by a mechanism that can perhaps be exploited in-vivo to produce particulates capable of evading phagocytosis. Its application in-vivo to a pulmonary environment remains to be investigated. It is also clear that alveolar surfactants down-regulate phagocytosis. This is of great consequence to pulmonary drug delivery since particulates deposited in the alveoli may remain for longer than was first calculated from extrapolations of systemic phagocytosis. The pulmonary milieu and indeed the heterogeneity of the alveolar macrophage (Jones et al 2002) affect phagocytosis in a way that must be characterized if we are to estimate bioavailability after the pulmonary deposition of aerosolized drugs.

Conclusion

Phagocytic inhibition, previously demonstrated for DPPC, has been shown to be independent of head group charge and chain length. Inhibition occurs if the DPPC is adsorbed onto the microsphere before incubation or is dispersed in the media. The ingestion of DPPC-coated microspheres inhibits the uptake of a second microsphere with an established longevity of at least 2 h. DPPC and the surfactant secretions of pulmonary epithelial cells down-regulate phagocytic function by a mechanism that may be exploited in-vivo to produce particles capable of avoiding phagocytosis.

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