which each of these factors contributes to the deposition in the sampling chamber inlet, and ultimately in the inertial impactor, depends upon the actual particle or droplet size of the aerosol. It may not be possible, using this method in a single configuration, to obtain a definitive particle size estimate. Indeed, it may never be possible to achieve this aim. Specifications for the dimensions of inlets to inertial devices are necessary. Beside the need to specify sampling chamber dimensions, the impactor type may also be important due to differences in transit times to impaction on the plates and wall losses for different models (Hickey 1990b). The approach to establishing appropriate inlet dimensions should follow that previously taken for the twin impinger, wherein the data obtained can be shown to have clinical significance for a particular drug.

References

Andersen, A. A. (1958) New sampler for the collection, sizing and enumeration of viable airborne particles. J. Bacteriol. 76: 471–484

Andersen, A. A. (1966) Sampler for respiratory health hazard assessment. Am. Ind. Hyg. Assoc. 27: 160–165

British Pharmacopoeia (1988) Vol II, Appendix XVIIB. HMSO, London, A204–207

Dalby, R. N., Byron, P. R. (1988) Comparison of output particle size distributions from pressurized aerosols formulated as solutions or suspensions. Pharm. Res. 5: 36–39

Diem, K. (1968) In: Scientific Tables. 6th edn, Geigy Pharmaceuticals, Ardsley, UK, p. 281

Division of Bioequivalence of the Food and Drug Administration (1989) Informal communication under 21 CFR 10·90 (b) (9)

Fults, K., Cyr, T. D., Hickey, A. J. (1990) Particle size characterization of inhalation aerosols. Pharm. Res. 7: S–5

Gonda, I. (1981) A semi-empirical model of aerosol deposition in the human respiratory tract for mouth inhalation. J. Pharm. Pharmacol. 33: 692–696

Hallworth, G. W., Westmoreland, D. G. (1987) The twin impinger: a simple device for assessing the delivery of drugs from metered dose pressurized aerosol inhalers. Ibid. 39: 966–972

Hickey, A. J. (1990a) An investigation of size deposition upon individual stages of a cascade impactor. Drug Dev. Ind. Pharm. 16: 1911–1929

Hickey, A. J. (1990b) Factors influencing aerosol deposition in inertial impactors and their effect on particle size characterization. Pharm. Tech. 14: 118–130

Hickey, A. J., Dalby, R. N., Byron, P. R. (1988) Effects of surfactants on aerosol powders in suspension. Implications for airborne particle size. Int. J. Pharm. 42: 267–270

Hickey, A. J., Fults, K., Cyr, T. D. (1989) Aerosol particle size characterization using liquid impinger and two cascade impactors. Pharm. Res. 6: S–48

Hinds, W. C. (1982) In: Aerosol Technology. John Wiley, New York, pp 83–87

Padfield, J. M., Winterborn, I. K., Pover, G. M., Tattersfield, A. (1983) Correlation between inertial impactor performance and clinical performance of a bronchodilator aerosol. J. Pharm. Pharmacol. 35 (Suppl.): 10P

Phillips, E. M., Byron, P. R., Fults, K. A., Hickey, A. J. (1990) Optimized inhalation aerosols II. Inertial testing methods for aerosol particles size analysis. Pharm. Res. 7: 1228–1233

Srinivasan, V. (1991) Drug Standards Division, USP Convention, Inc., Personal Communication

Stahlhofen, W., Gebhart, J., Heyder, J., Scheuch, G. (1983) Deposition pattern of droplets from medical nebulizers in the human respiratory tract. Bull. Eur. Physiopathol. 19: 459–463

US Pharmacopeia (1985) 21st Rev., Suppl. 1, US Pharmacopeial Convention, Rockville, MD: 1743–1744

Vaughan, N. P. (1989) The Andersen Impactor: calibration, wall losses and numerical simulation. J. Aerosol. Sci. 20: 67–90

Zainudin, B. M. Z., Tolfree, S. E. J., Biddiscombe, M., Whitaker, M., Short, M. D., Spiro, S. G. (1989) An alternative to direct labelling of pressurized bronchodilator aerosol. Int. J. Pharm. 51: 67–71

J. Pharm. Pharmacol. 1991, 43: 728–730
Communicated February 16, 1991

The effect of the uptake of particles on the chemotaxis of polymorphonuclear leucocytes in-vitro

R. HYDE, D. A. LEWIS, *P. W. TAYLOR, *Pharmaceutical Sciences Institute, Aston University, Aston Triangle, Birmingham B4 7ET and* **Research Centre, Ciba Geigy Pharmaceuticals, Horsham, Sussex RH12 4AB, UK*

Abstract—The relationship between the phagocytic uptake of latex microspheres (1·1 μm diam.) and the mobility of rat polymorphonuclear leucocytes (PMNLs) has been investigated in-vitro. The movement of PMNLs was found to be independent of the uptake of particles but about half of the PMNLs were not receptive to the chemo-attractant *N*-formylmethionyleucylphenylalanine. However, the uptake of particles was greater when particle/cell ratios were high and the greatest number of particles were carried into a cellulose nitrate filter by chemotaxis at the highest particle/cell ratio of 30:1.

We are investigating the possibility of using polymorphonuclear leucocytes (PMNLs) as carrier vehicles to target drug-loaded microspheres in-vivo. PMNLs leave the blood vessels and accumulate in tissues in various infectious conditions and in non-infectious conditions such as rheumatoid arthritis, myocardial infarction and some malignant tumours (Reba & Chandeysson 1980). Boggs (1974) transfused PMNLs successfully into patients with neutropenia for the treatment of infection. This suggests the possibility of loading PMNLs by phagocytosis in-vitro with drug loaded particles and transfusing the cells into patients, where, by the process of chemotaxis, they may accumulate at sites of disease. The effect of the phagocytosis of particles by PMNLs on their directed mobility (chemotaxis) is unknown. In this communication we have examined this relationship in-vitro.

Correspondence: D. A. Lewis, Pharmaceutical Sciences Institute, Aston University, Aston Triangle, Birmingham B4 7ET, UK.

Materials and methods

Microspheres. Fluoresbrite carboxylated polystyrene latex microspheres (2·5% solids-latex) with a batch diameter of 1·1 μm (s.d. = 0·02) were purchased from Polysciences Inc., Warrington, PA, USA. Particles were dispersed by immersion in a sonic bath for 10 s before dilution and subsequent use. Particle suspensions were diluted with Hank's balanced salt solution (HBSS). Manufacturer's particle size was checked by electron microscopy.

Polymorphonuclear leucocytes. Male Wistar rats, 200–250 g, were purchased from Banting and Kingman (Kingston on Hull, UK). Animals were maintained on rat and mouse breeding diet (Pilsbury's Ltd, Birmingham, UK) and allowed free access to water. Rats were anaesthetized and injected i.p. with 10 mL of sodium caseinate (12% m/v in isotonic saline). The animals were killed after 12 h and the bellies shaved. A small incision was made along the ventral midline of each rat and the exudate removed by lavaging with 4 × 5 mL portions of ice-cold HBSS containing 10 units mL^{-1} heparin. Plastic pasteur pipettes were used to collect the exudate. Subsequent operations were performed at 4°C. The exudate was centrifuged at 100 *g* for 5 min and the supernatant discarded. The cell pellet was dispersed in 9 vol. of water for 30 s, which lysed contaminating erythrocytes. Isotonicity was restored by adding a calculated amount of HBSS. The cells were recovered by centrifugation and washed in HBSS before being finally suspended in 8 mL HBSS (per rat). This suspension was layered on to 3 mL Histopaque-1077 (1·077 g mL^{-1}) (Sigma Chemical Co., Poole, Dorset) in a 15 mL conical plastic tube which was centrifuged at 400 *g* for 30 min. The opaque interface contained mainly mononuclear leucocytes leaving a pellet of PMNLs. The pellet was harvested and washed three times with ice-cold heparinized HBSS before the cells were subjected to total, differential and viability counts. Total counts were made using an improved Neubauer counting chamber. Differential counts were made by centrifuging 10^6 cells on to microscope slides using cytobuckets (Hyde et al, unpublished results). The air-dried slides were stained with Haema-Gurrs stain (BDH Diagnostics, Poole, Dorset, UK) and counted as mononuclear or polymorphonuclear cells under the light microscope. Viability counts were made by trypan blue (1% m/v) exclusion and, where satisfactory (> 95%), the cells were used in various experiments.

Phagocytosis The PMNL cell suspension and a microsphere suspension (with varying particle/cell ratios) were mixed in equal volumes to a total of 5 mL in mini bijoux bottles (Sterilin, Teddington, Middlesex, UK). The bottles were sealed with parafilm and clipped to the wheel of a blood cell mixer (Type BCM, Voss of Malden, Essex, UK) and rotated at 30 rev min^{-1} at 37°C for 1 h. The cell suspension was transferred to a plastic centrifuge tube together with 5 mL of HBSS washings from the bijoux bottle and centrifuged. The cells were washed and centrifuged (100 *g*, 5 min) three times to remove free microspheres. Finally the washed cells were resuspended at 10^7 cells mL^{-1} in HBSS for phagocytic and chemotactic assays. For quantitative data fluorescence microscopy (Giordano & Lichtman 1973) was used for counting on slides prepared by the cytospin/cytobucket technique. This technique was found to be more accurate than counting particles in cell smears or by flow cytometry when large numbers of particles were employed (Hyde et al, unpublished results). Briefly this technique prepares slides where flattened cells outline engulfed particles giving a 'poached egg' appearance allowing the particles to be easily counted.

Chemotaxis. The chemo-attractant used was *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP) (Sigma Chemical Co., Poole, Dorset). A stock solution (10^{-2} M) in dimethylsulphoxide (DMSO) was prepared and working dilutions in HBSS (pH 7·4) containing 1 ng mL^{-1} of bovine serum albumin (BSA) were made.

Chemotaxis was studied in blind well chambers (Buckley Scientific Ltd, Prestwood, Great Missenden, Bucks). The lower chemo-attractant chamber was constructed of polished acrylic and the upper filter retainers of white Delrin. Cellulose nitrate filters (13 mm diam., Sartorius GmbH, Gottingen, Germany) with a 5 μm pore size and an average thickness of 140 μm were used to measure PMNL migration.

Polystyrene latex microsphere suspensions were prepared as described above to final concentrations of 0, 5, 10, 20 and 30 beads per cell in a cell suspension of 10^7 cells mL^{-1} in HBSS (1 mg mL^{-1} BSA, pH 7·4). Subsequent treatment was as described above (phagocytosis). The blind well chambers were prewarmed to 37°C and 200 μL of 10^{-8} M FMLP in buffered HBSS (1 mg mL^{-1} BSA, pH 7·4) pipetted into the lower chamber. A filter was carefully manoeuvred using forceps and a suction line over the fluid in the lower chamber ensuring an even absorbance of fluid and an absence of air bubbles. Filters wetted unevenly (e.g. droplets on the upper surface) or containing entrapped air bubbles were discarded. The upper and lower sides of the filters were defined as in the manufacturers pack. Once in place the filters were fixed in position by screwing down the filter retainers and 200 μL of the cell suspension pipetted into the upper half of the cell. The unit was sealed with a glass coverslip and incubated at 37°C for 60 min. In random migration determinations the FMLP was omitted. After incubation the filters were removed, fixed in methanol for 2 s and stained with Harris's haematoxylin (a nuclear stain (Cates & Quie 1978)). When stained the filters were mounted onto a slide, cell side uppermost, and mounted in DPX mountant (BDH Chemicals, Poole, Dorset). The migration response was measured. The migration response in this communication was measured by the determination of the leucotactic index as described by Maderazo & Woronick (1978). The leucotactic index (LI) was determined by counting the cells at 10 μm intervals from the upper surface to the distal surface, and was calculated from the formula

$$LI = \frac{\Sigma[A]\,[B]}{\Sigma[B]}$$

where B = number of cells per level, A = distance from monolayer of residual cells on the surface.

For counting particles instead of cells the same procedure was used. However, the mountant DPX dissolved the fluoresbrite microspheres and so oil of cedar wood was used in its place. This enabled clarification of the filters without dissolution of the particles and allowed counting of both the cells and fluorescent particles to be made at all levels.

Results and discussion

Determination of the size of the fluoresbrite polystyrene latex particle. The analysis of 200 beads on photoelectron micrographs gave a mean particle diameter of 1·0 ± 0·06 μm which was smaller than the manufacturer's figure of 1·1 ± 0·03 μm. This is a reduction of 25% in bead volume over the manufacturer's stated size.

Optimum FMLP concentration and incubation time. Preliminary experiments showed that the optimum movement of PMNLs occurred when the FMLP concentration was 10^{-8} M. When the blind well chambers were incubated for 90 min, cells appeared on the lower side of the filter showing that they had crossed the filter completely. An incubation time of 60 min was taken as standard for the experiments.

Table 1. Phagocytosis expressed as the mean number of particles/cell ± s.d. after incubations using particle/cell ratios of 5 to 30. Each result is the mean ± s.d. of 6 determinations.

Particle/cell	Particle concn	Cells with particles (%)
5:10	1·22 ± 0·09	27·8 ± 2·4
10:1	3·42 ± 0·33	58·8 ± 4·0
20:1	8·60 ± 0·88	77·1 ± 3·2
30:1	18·20 ± 1·08	87·9 ± 5·07

Phagocytosis and chemotaxis. The results in Table 1 show the mean number of particles/per cell present in PMNLs incubated at various particle/cell ratios. Both the percentage of cells containing particles and the mean number of particles taken up per cell increased at increasing particle/cell ratios. This result was identical to those found in parallel studies (Hyde et al, unpublished results). However, the cells used in this experiment were also examined by the blind well chamber assay for directed migration (chemotaxis) and random migration. These results are given in Table 2 and show that both the random and directed migration of the PMNLs was independent of the particle content since all values were identical to that of normal (empty) cells. The actual numbers of particles carried into the filters is given in Table 3. These show that about half of the particles were left at the surface of the filter in contrast to about 20% of the cells (Fig. 1). This difference suggests heterogeneity in the PMNL population as suggested by others (Gallin 1984; Seligmann et al

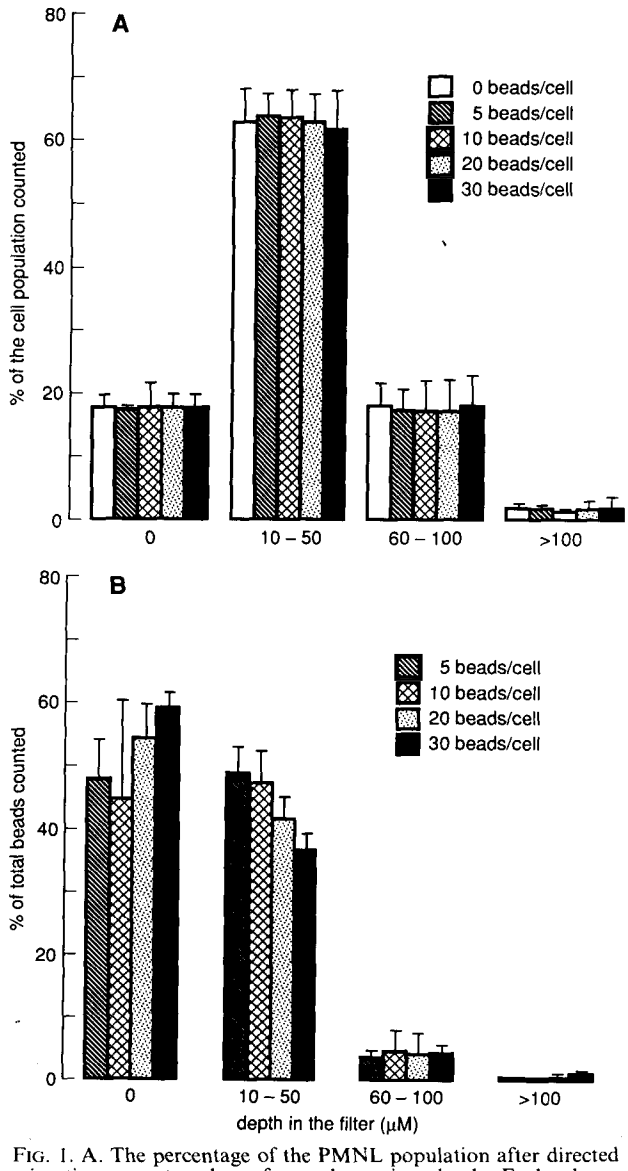

FIG. 1. A. The percentage of the PMNL population after directed migration present on the surface and at various depths. Each column represents the mean ± s.d. of 6 PMNL populations at each bead concentration. B. The percentage of the total number of counted PSL particles carried by PMNLs by directed migration, present on the surface and at various depths. Each column represents the mean ± s.d. of 6 PMNL populations at each bead concentration.

Table 2. The migration of PMNLs with and without particles. The values are the leucotactic index ± s.d. for 6 PMNL populations at each particle/cell ratio used.

Particle/cell	Directed migration	Random migration
0	32·77 ± 2·1	14·76 ± 1·30
5:1	32·20 ± 2·74	14·87 ± 1·12
10:1	32·02 ± 2·75	14·86 ± 1·28
20:1	32·32 ± 2·60	14·39 ± 1·25
30:1	32·36 ± 3·85	14·57 ± 1·06

Table 3. The number of particles/cell carried to various depths in the cellulose nitrate filter. Each value represents the mean ± s.d. of 6 cell populations migrating in response to FMLP (10^{-8} M).

	Incubation concentrations (particle/cell ratio)			
Depth (μm)	5	10	20	30
	No. of particles			
0	102·5 ± 11·4	254·0 ± 28·1	590 ± 34	1370 ± 11
10-50	102·0 ± 6·5	282·0 ± 45·7	469 ± 30	811 ± 53
60–100	8·7 ± 2·7	31·0 ± 9·8	82 ± 43	116 ± 26
>100	0·7 ± 0·2	2·3 ± 0·5	13·6 ± 5·6	25 ± 11

1984). One fraction of the PMNLs consists of active phagocytes but is not responsive to FMLP. A second population of cells is responsive to FMLP but has phagocytosed a smaller load of particles. If the PMNL population had been homogenous then the distribution of both cells and particles would have been similar to Fig. 1. Clearly that was not the case. However, it is of interest that where the greatest numbers of particles were carried into the filter it was from the highest particle/cell incubations.

In conclusion this study has shown that the mobility of PMNLs is independent of the number of particles loaded by phagocytosis. It has also shown that not all cells are responsive to FMLP as a chemo-attractant but that sufficient are so responsive as to warrant further research on PMNLs as potential drug targeting vehicles.

R. Hyde is grateful for a SERC-CASE award.

References

Boggs, D. R. (1974) Transfusion of neutrophils as prevention or treatment of patients with infection or neutropenia. New Engl. J. Med. 290: 1055–1062

Cates, K. L., Quie, P. G. (1978) Modified Boyden chamber method of measuring polymorphonuclear leukocyte chemotaxis. In: Gallin, J. L., Quie, P. G. (eds) Leukocyte Chemotaxis. Raven Press, NY, USA pp 302–328

Gallin, J. L. (1984) Human neutrophil heterogeneity exists, but is it meaningful? Blood 63: 977–983

Giordano, G. F., Lichtman, M. A. (1973) The role of sulphohydryl groups in human neutrophil adhesion movement and particle ingestion. J. Cell. Physiol. 82: 387–396

Maderazo, E. G., Woronick, C. L. (1978) A modified micropore filter assay of human granulocyte leukotaxis. In: Gallin, J. L., Quie, P. G. (eds) Leukocyte Chemotaxis. Raven Press, New York, USA, pp 43–45

Reba, R. C., Chandeysson, P. L. (1980) Imaging infection with Indium ^{111}-labelled leukocytes, platelets and lymphocytes. In: Thaker, M. L., Gottschalk, A. (eds) Proc. State of the Art. Symp. Radiolabelled Cellular Elements-Current Accomplishments, Immediate Potential and Future Problems. Truvernum Pub. New York, pp 305–318

Seligmann, B., Chused, T. M., Gallin, J. I. (1984) Differential binding of chemoattractant peptide to subpopulations of human neutrophils. J. Immunol. 133: 2641–2646