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Research Papers

In vitro uptake of polystyrene latex particles and parenteral fat emulsions by human granulocytes

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Summary

Suspensions of human granulocytes were used to study the in vitro phagocytosis of polystyrene latex particles (1030 nm) as i.v. model drug carriers and of commercial fat emulsions for parenteral nutrition (Lipofundin, Intralipid). Chemiluminescence (CL) was employed to follow the time-dependent uptake of the particles. The total uptake was quantified by the area under the curve of the CL intensity/time profiles and comparitively by a modified fluorimetric assay. The data proved that a linear relationship existed between the AUC of the CL assay and the total mass of internalized polymer. The uptake of fat emulsions could not be followed directly by CL because the CL intensity signal was too weak. Their uptake could be quantified via the impairment of the phagocytic function of granulocytes after pre-incubation with emulsions. The polystyrene particles were used as test colloid. The phagocytic function was reduced up to 50% by 10% fat emulsions.

Introduction

Cell cultures are frequently used to study the affinity of particulate drug carriers to cell populations (Johnson et al., 1986; Illum et al., 1987). Detection of internalized particles is mainly performed by microscopy (Illum et al., 1987), chemical assays such as fluorimetry (Muller and Schuber, 1986) or the use of fluorescently (Dunn et al., 1983) or radioactively labelled particles (Roerdink et al., 1983). The major disadvantage of these methods is that they are invasive. In contrast, chemiluminescence (CL) is a non-invasive method allowing the continous measurement of time-dependent phagocytosis (Allen and Loose, 1976; Blair et al., 1988; Thomas et al., 1988). CL can be performed on microtitre plates and is therefore ideal for the processing of large sample numbers. Quantification of uptake is possible by measuring the maximum intensity (I_{max}) of the CL signal or by calculating the area under the curve (AUC) of the intensity/time profile. The method was successfully employed to study the uptake of differently sized polymeric model drug carriers (polystyrene particles), optimum analytical parameters could be established (Rudt and Müller, 1992). However, the question remains to

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which extent I_{max} and AUC correlate with the total mass of internalized polymer. Therefore, the uptake of polystyrene particles determined by CL was compared with the internalized polymer mass quantified by a modified fluorimetric assay.

Furthermore, emulsions are also of interest as potential drug carriers for parenteral administration (e.g., egg lecithin stabilized systems). They possess relatively hydrophilic surfaces (Carstensen et al., 1991), resulting in a relatively low uptake by phagocytic cells (Davis et al., 1987) which might be close to the detection limit of the CL. In addition, the amount of reactive oxygen species formed might be different during the ingestion of liquid (oil) droplets and the internalisation of solid particles. A lower amount of oxygen species would result in a weak CL intensity signal. Therefore, the applicability of the method to non-polymeric particulates needed to be investigated and possibly the analytical parameters required to be modified.

Materials and Methods

Materials

Polystyrene particles (diameter 1030 nm) were purchased from Polysciences (St. Goar, Germany). Fat emulsions were obtained from various suppliers as follows: Endolipide 10% and 20% (Laboratoires Bruneau, France), Intralipid 10% (Kabi. Vitrum, Germany), Lipofundin MCT 10% and 20% (B. Braun Melsungen AG, Germany) and Lipovenös 10% (Fresenius AG, Germany).

Poloxamers 181, 184, 188, 235 and 407 were a gift from ICI (Middlesbrough, Cleveland, U.K.). The materials for cell culture, Lowry protein determination and CL assay (Luminol) were obtained from Sigma Chemie (Deisenhofen, Germany). Acetonitrile was purchased from Merck (Darmstadt, Germany).

Methods

Human granulocytes were obtained from blood by density centrifugation (Ferrante and Thong, 1980), dispersed in phosphate-buffered saline (PBS) and distributed in wells of microtitre plates (250 000 cells in 50 μl PBS).

Latex particles were surface modified by adsorption of poloxamer polymers (coating) as described previously (Wallis and Müller, 1990). Equal volumes of polystyrene latex particles (2.5% w/w) and poloxamer solution (0.05% w/w) were mixed and incubated overnight. The coated particles were diluted with PBS to yield a final particle concentration of 0.25% (w/w).

For the CL assay, 100 μl luminol solution (5×10^{-4} M in PBS) was added to the cells and incubated for 30 min in an Amersham research luminometer (Amersham-Buchler, Braunschweig, Germany). The CL intensity was recorded to determine the background (typical background intensity: 0.1–0.5 arbitrary units). Then 100 μl of the polystyrene latex particles dispersed in PBS (0.25% polymer w/w) were added to each well. This corresponded to 250 μg polymer per well. The CL intensity was recorded for 180 min (one CL measurement every 3 min in each well, measurement time 1 s).

All CL experiments were performed in triplicate, and the data are the means with a relative standard deviation of less than 1%. The particle uptake was quantified by calculating the area under the curve (AUC) of the intensity/time profiles. The maximum intensity (I_{max}) was found to be less consistent than the AUC when comparing results obtained with granulocytes from different donors. Therefore, the AUC instead of I_{max} was used to establish the correlation between CL and fluorimetric assay.

To determine the internalized mass of polystyrene particles by fluorimetry, 50 μl cells were incubated with 100 μl particles (uncoated and coated with the different poloxamers) in 1.5 ml Eppendorf vials for 180 min at 37°C. Parallel experiments were conducted at 4°C to allow compensation for particles adhering to the cell membranes. After incubation the cells were centrifuged ($250 \times g$, 3 min) and the supernatant discarded which contained non-internalized particles. The cells were resuspended and washed three times with PBS ($250 \times g$, 3 min). The final cell pellet was lysed with 1 N NaOH overnight. The polystyrene particles in the lysate were centrifuged ($15\,000 \times g$, 10 min), washed three times with water and finally redispersed in 300 μl wa-

ter. 100 μl of the redispersed particles were added to 2 ml of acetonitrile for fluorescence determination, yielding a total sample volume of 2.1 ml (excitation at 255 nm, emission at 320 nm; LS-5B Luminescence Spectrometer (Perkin Elmer, Germany) (Rudt, 1992).

The distribution of cells to the wells was quantified by determining the protein mass per well (mg/well) using the method of Lowry et al. (1951). The protein determination compensated for possible loss of cells during the washing procedure. The mass of internalized polystyrene is given per unit protein in the well (μg polystyrene/mg protein).

Particle size determinations of fat emulsions were performed by photon correlation spectroscopy (PCS) using a Malvern RR 102 spectrometer (Malvern Instruments, Malvern, U.K.) in connection with an ALV 1000 channel correlator (ALV Laservertriebsgesellschaft, Langen, Germany).

For the CL assay of fat emulsions, 100 μl luminol solution was added to 50 μl cells and incubated for 30 min in the luminometer. Then 100 μl of fat emulsion diluted with PBS (final concentration: 1% fat) were added to each well. This corresponded to 400 μg fat per well. The CL intensity was recorded for 180 min as described for polystyrene particles but no distinct uptake could be determined.

Consequently, the CL assay was modified for the fat emulsions, 50 μl of cells were distributed in each well, 100 μl of diluted fat emulsion were added and incubation carried out for 90 min. The fat emulsion was removed from the cells and the cells washed twice with PBS. Then 50 μl PBS, 100 μl of luminol solution and 100 μl of uncoated polystyrene latex dispersion (0.25% w/w) were added and the CL intensity determined for 180 min.

Results and Discussion

In vitro uptake determined by chemiluminescence

In order to determine the correlation between CL intensity and internalized polymer mass, different extents of particle uptake by cells are required. This can be achieved by surface modification of the polystyrene particles. Surface modification was performed by adsorption of poloxamer polymers. Reducing the surface hydrophobicity of the particles by poloxamer adsorption (coating) layers was found to result in reduced phagocytic uptake as determined by microscopy (Illum et al., 1987). A similar behaviour was observed in the CL assay. Coating of the particles with poloxamer 181 and 184 reduced the I_{max} and AUC to a limited extent, whilst distinct reductions were observed with poloxamer 188 and 235 coated particles, and uptake was almost prevented after coating the particles with poloxamer 407 (Fig. 1 and Table 1).

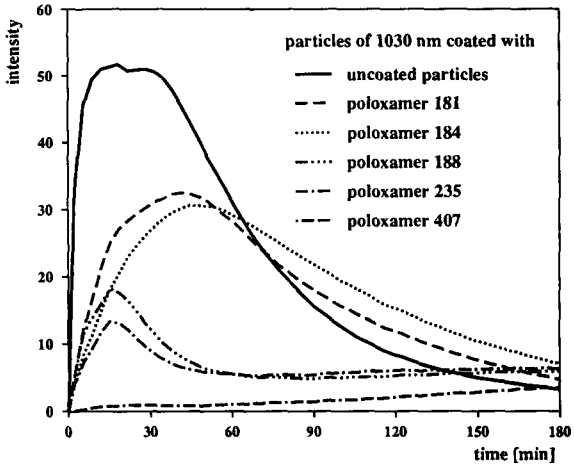

Fig. 1. CL intensity/time profiles of uncoated polystyrene particles and particles coated with poloxamer 181, 184, 188, 235 and 407 (from uppermost to lowest curve, respectively).

In vitro uptake determined by fluorimetry

Fluorimetric analysis requires polymers such as polystyrene which can undergo excitation in organic solvents. The use of 1,2-dimethoxyethane as solvent, described previously by Muller and Schuber (1986), has the disadvantage of being expensive and very sensitive towards the formation of fluorescent products during storage. The solvent must be freshly distilled before the measurement in order to minimize the background fluorescence. Dimethoxyethane was therefore replaced by acetonitrile which is less expensive and does not require redistallion prior to use. Excita-

TABLE 1

AUC and I_{max} (arbitrary units) of polystyrene particles of 1030 nm (uncoated and coated with poloxamer polymers) determined in the CL assay and corresponding mass of internalized polystyrene polymer (μg) per mg protein including standard deviation (μg / well) determined by fluorimetry

Particles	AUC	I_{max}	μg polymer per mg protein	SD
Uncoated	4135	51.7	3.13	0.43
Coated with poloxamer 181	3330	32.5	2.82	0.56
Coated with poloxamer 184	3605	30.6	3.56	0.82
Coated with poloxamer 188	1590	18.1	1.94	0.23
Coated with poloxamer 235	1505	13.3	2.22	0.48
Coated with poloxamer 407	500	4.2	0.78	0.17

tion and emission wavelengths were chosen to provide the maximum differences between the solvent and solvent/polystyrene spectra. A linear relationship was obtained between fluorescence intensity and polymer mass from 0.5 to 10 μg/sample (volume 2.1 ml) (Fig. 2), the range required for analysis. Linearity was also observed for concentrations up to 50 μg/sample.

At 37°C, maximum binding (adherence and internalization) of 8.93 μg polystyrene per mg protein was observed for uncoated latex particles, while reduced uptake was found for the surface-modified particles. The data obtained at 4°C (particle adherence) indicate that a large number of particles were bound by the cell membranes. Subtracting the masses at 37 and 4°C yielded the mass of internalized polymer as being 3.13 μg per mg protein for uncoated particles (Table 1). This corresponded to approx. 40 particles per granulocyte. This is similar to the uptake of 1.1 μm latex particles reported in splenocyte suspensions (approx. 30 per cell (Muller and Schuber, 1986)). For the coated particles lower masses of polymer were internalized (Table 1).

Comparison between CL and fluorimetric assay

Plotting the internalized polymer mass related to 1 mg protein in the wells vs the AUC determined in the CL assay resulted in a linear relationship (Fig. 3). A linear fit yielded the equation $y = 0.839 + 0.0064x$ with a correlation coefficient of 0.9322. Calculating the polymer mass per unit protein proved necessary because of the observed cell loss during centrifugation.

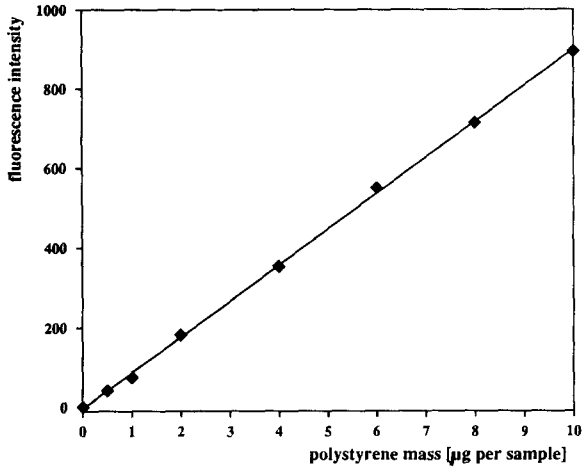

Fig. 2. Calibration curve of the fluorimetric assay. A linear relationship exists between fluorescence intensity and polystyrene mass up to 10 μg/2.1 ml sample volume (excitation, 255 nm; emission, 320 nm; polystyrene was solubilized in a mixture of 2.0 ml acetonitrile and 0.1 ml water). Standard deviation ($n = 3$) was less than 1%.

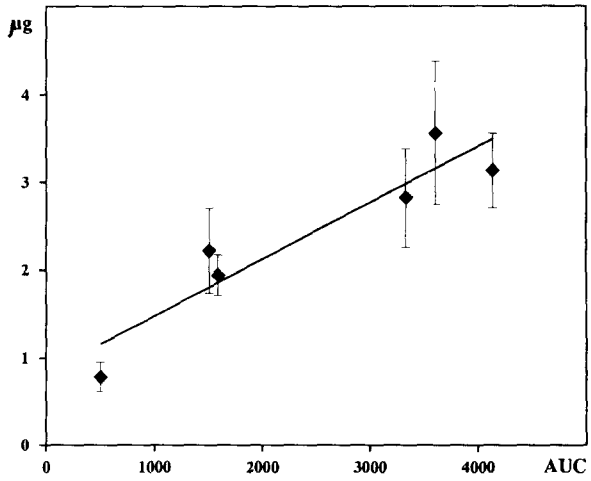

Fig. 3. Uptake of uncoated polystyrene particles and particles coated with various poloxamers. Plot of internalized polymer mass per mg protein in the well (determined by Lowry assay) vs the AUC calculated from the CL intensity time profiles.

Comparison of CL and fluorimetric results proved that a linear relationship exists between the AUC and the internalized polymer mass for the optimized analytical parameters used in this study (particle size, 1030 nm; polymer mass, 250 μg/well; number of cells, 250000 per well; luminol concentration, 5×10^{-4} M in the assay). Changing the analytical parameters was found to affect drastically the I_{max} and AUC (Rudt and Müller, 1992). Consequently, the above relationship is inapplicable and must be established for each cell culture system. In addition, the nature of the internalized material (polymeric particles, liquid oil droplet, liposome) can also affect the CL signal.

Uptake of fat emulsions determined by chemiluminescence

Internalisation of oil droplets by the granulocytes produced an extremely weak CL signal intensity in the range of the background noise (0.1–0.5) or slightly above (up to approx. 1 arbitrary unit). This demonstrated the effect of the nature of the internalized material on the CL assay. The weak CL signal was attributed to a low amount of oxygen species formed during the ingestion of oil droplets. The reduced uptake of hydrophilic emulsion droplets will of course contribute to the observed low CL intensity. The CL assay was therefore not regarded as suitable for a direct measurement of emulsion internalization.

However, the correlation between AUC of polystyrene particles and internalized polymer mass/volume allowed us to establish an indirect assay of emulsion phagocytosis. Internalization of emulsion droplets by the granulocytes will impair their ability to phagocytose polystyrene particles. Therefore, the granulocytes were pre-incubated with the emulsions and then polystyrene particles added as a test colloid to determine the impairment of phagocytic function.

The AUC of the test colloid should decrease with increasing impairment, i.e., increasing phagocytosis of emulsion droplets will occur. Pre-incubation of granulocytes with commercial 10% fat emulsions reduced the AUC to about 50–70% of the control (= cells not pre-incubated) (Table 2 and Fig. 4). The comparable impairment observed for emulsions from different suppliers can be explained by the similar composition of the emulsions and their identical low surface hydrophobicity (Carstensen et al., 1991).

TABLE 2

AUC and I_{max} obtained with polystyrene particles after pre-incubation of the granulocytes with commercial fat emulsions (per cent of control without fat emulsion = 100%)

Fat emulsion	AUC (%)	I_{max} (%)	Diameter of droplets (nm)
Lipofundin MCT 10%	49.0	76.2	274
Endolipide 10%	51.9	50.7	284
Intralipid 10%	72.5	58.7	292
Intravenös 10%	51.6	51.3	236
Lipofundin MCT 20%	87.9	83.5	322
Endolipide 20%	99.0	104.9	402
Control (no fat emulsion)	100.0	100.0	–

The diameters of emulsions droplets were determined by photon correlation spectroscopy.

The impairment of phagocytic function was distinctly less pronounced on pre-incubation of the granulocytes with 20% fat emulsions (Table

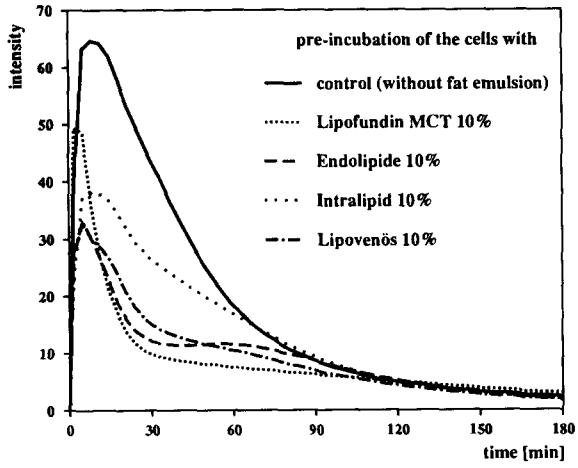

Fig. 4. Uptake of polystyrene particles by granulocytes after pre-incubation of the cells with 10% commercial fat emulsions (upper curve: control, i.e., cells not pre-incubated with emulsion). The reduction in AUC reflects the impairment of phagocytic function due to phagocytosis of emulsion droplets.

2). This is in contrast to literature data reporting increased in vitro uptake with increasing particle size (Karino et al., 1987; Rudt and Müller, 1992). The droplet sizes in the 20% emulsions were 322 and 402 nm as compared to droplets in the range of 236–292 nm in the 10% emulsions. At least similar impairment could be expected because the surface properties of the 10 and 20% fat emulsions affecting the phagocytic uptake were identical (Carstensen et al., 1991). The lower uptake of 20% emulsions can be explained on considering the droplet numbers in the well. Previously, the uptake was found to be distinctly reduced for lower particle numbers (Rudt and Müller, 1992). In those studies, identical masses of oil were added. This corresponds to an 8-fold decrease in the number of 400 nm droplets as compared to 200 nm droplets. At present, further studies are therefore being conducted to establish the effect of analytical parameters on emulsion uptake as performed previously for the CL assay with polymeric particles (Rudt and Müller, 1992).

Conclusions

The AUC of the CL assay is linearly related with the mass of internalized polymeric particles. Chemiluminescence can therefore be used to compare the affinity of surface-modified particles to cell populations with phagocytic activity. The intensity of the CL signal also depends on the nature of the internalized material. Particulates resulting in excessively weak signals are accessible by using polystyrene particles as 'test colloid'.

The established relations are only valid for the applied analytical parameters and must be determined for each cell culture system. This makes the CL assay tedious during the validation procedure, however, CL has the distinct advantage of being a rapid method for the non-invasive screening of large sample numbers.

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