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The influence of emulsifying agents on the phagocytosis of lipid emulsions by macrophages

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Summary

The phagocytosis of lipid emulsion droplets prepared from soybean oil and using various emulsifying agents has been studied in vitro using mouse peritoneal macrophages. The process of phagocytosis was first-order and dependent upon the particle size and the nature of the surface of the droplet. Systems containing anionic lipids and a non-ionic surfactant (Poloxamer) were taken up more slowly than those stabilized by simple phosphatide mixtures.

Introduction

Lipid emulsions prepared using vegetable oils and lecithin stabilizers have been used clinically for parenteral nutrition for several years (Davis, 1983). More recently, similar systems have been used for the administration of poorly water-soluble drugs (Davis, 1982) as well as for drug targeting (Mizushima et al., 1982) and computed tomography (Vermess et al., 1980).

When colloidal particles of a similar size to fat emulsions are administered intravenously they are normally taken up very rapidly by the cells of the reticuloendothelial system; in particular the fixed macrophages in the liver (the Kupffer cells) (Illum et al., 1982). However, the process is dependent on the size and nature of the particle and it has been suggested that the hydrophilic or hydrophobic properties of the surface could be important (Van Oss, 1978; Roerdink et al., 1983; Illum and Davis, 1984). The literature on the phagocytosis of intravenously administered fat

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emulsions is contradictory (Scholler, 1973; Van Haelst and Sengers, 1976; Jarstrand et al., 1978) and some reports show clearly that infused emulsions can be partly cleared by the Kupffer cells of the liver (Lemperle and Reichelt, 1973). Indeed emulsion particles coated by gelatin and other natural materials have been used to test the functionality of the reticuloendothelial system (DiLuzio and Riggi, 1964; Tonaki et al., 1976). Davis and Hansrani (1982) studied the uptake of radiolabelled soybean emulsions by various organ sites in the rabbit, including the liver, in order to investigate the physicochemical determinants of the phagocytic process. Emulsions stabilized using a non-ionic surfactant (Poloxamer) were handled in a different manner to those stabilized with phosphatide mixtures. The results indicated that surface charge might be an important factor and that a proper choice of the phosphatide emulsifier combination could be used to minimize the uptake of fat emulsions by the reticuloendothelial system.

The mouse peritoneal macrophage system has been a popular method for measuring phagocytic uptake of colloidal particles in vitro (Roerdink et al., 1983). The present report describes studies on the uptake of emulsion droplets by the test macrophage system. The emulsions have been stabilized by pure phosphatide mixtures as well as a commercially available egg lecithin and a non-ionic surfactant (Poloxamer).

Materials and Methods

Fractionated soybean oil (Archer Daniels Midland Co., U.S.A.) was used as the oil phase for all emulsions. It had the following specification, peroxide value $\nearrow$ 0.5 meq./kg, saponification value 188–196, iodine value 130–150, free fatty acid $\nearrow$ 0.05% and unsaponifiable matter $\nearrow$ 0.5%. The phosphatide emulsifier combinations investigated were made from purified egg yolk phosphatidylcholine (PC), phosphatidylethanolamine (PE), lysophosphatidylcholine (LPC), phosphatidylserine (PS), phosphatidylinositol (PI) (British Drug Houses) and phosphatidic acid (Sigma). The purity of these samples was assessed by TLC on silica glass plates (Tyrrell, 1976). A commercial egg lecithin was used to make laboratory equivalents to commercial fat emulsions. A typical commercial egg lecithin is composed of phosphatidylcholine (PC) (73%), lysophosphatidylcholine (LPC) (6%), phosphatidylethanolamine (PE) (15%), lysophosphatidylethanolamine (LPE) (2.1%) with possible traces of phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidic acid (PA), cholesterol (CH) and cholesterol esters (Davis, 1983). The lecithin (Lucas-Meyer—lot 42001) had the following composition (%) (PC 76.7, PE 7.6, LPC 2.1). Cholesterol (CH) and cholesterol oleate (CHO) were obtained from Sigma. The non-ionic surfactant Poloxamer 338 (Pluronic F108) was used as received from Wyandotte Corporation. This material is a polyoxyethylene (Eo) polyoxypropylene (Po) block copolymer. The average molecular weights for the two blocks were Eo = 11264 and Po = 3132.

Preparation and characterization of the emulsions

Phospholipid stabilized emulsions of soybean oil (10% w/v) in water were

prepared with 1.2% w/v PC/PE (4:1) using an ultrasonic (probe) homogeniser (Dawe Instruments). The added minor phosphatide components were at a concentration of 0.1% w/v. An equivalent emulsion was also prepared using 1% w/v Poloxamer 338. The homogenization conditions (sonication time) were adjusted to give emulsions of different mean diameters in the range 1–3 μm as measured using the Coulter Counter (Model TAI) fitted with a 50 μm orifice tube.

The surface charge on the droplets over the pH range was determined using micro-electrophoresis (Bangham et al., 1958) at 25 ± 0.1°C.

Mouse peritoneal macrophages

Female Laca mice weighing 20–25 g were used. The animals were killed by dislocation of the neck and 4.0 ml of tissue culture medium M199 (containing swine serum and sodium bicarbonate) (Flow Laboratories) (Hansrani, 1980) and a smaller volume of sterile air were injected into the peritoneal cavity. The abdominal wall was then subjected to gentle massage to distribute the media throughout the cavity. The resultant suspension containing macrophage was then withdrawn and collected in a sterile container held at 0°C. The exudates from several animals were routinely collected in this way and pooled. A cell count was conducted using a haemocytometer and the viability of the phagocytes was tested by exclusion of trypan blue. The viability of the system was better than 90%. Systems containing red blood cells were not used.

The phagocytosis of emulsion particles by the macrophages was measured by a turbidometric method (Stossel et al., 1972; Davies et al., 1975) that determines the number of particles which had not undergone 'uptake', that is those not ingested by or adhering to the macrophages.

A standard incubation system of $2–4 \times 10^6$ cells/ml was incubated in siliconized Erlenmeyer flasks at 37°C in a shaking waterbath at 100 strokes per minute. A known volume of freshly prepared emulsions containing about $1–5 \times 10^8$ particles/ml (determined by Coulter Counter), prewarmed to 37°C was then added. Samples were removed at selected time intervals, diluted with cold EDTA/saline in order to inhibit phagocytosis and centrifuged to remove the cells and then the turbidity of the supernatant measured at 560 nm using a spectrophotometer (Kontron).

Control systems of cells but no emulsion, and emulsion particles and cells inhibited by added cold EDTA/saline solution were used. The experiments and sampling were conducted in duplicate; the differences between duplicate samples from the same flasks were always less than 4%.

Results

The effect of particle size

The emulsion particles were taken up by the macrophage system at a rate that depended on the size of the particle and the surface characteristics. No uptake was observed if the incubation medium was deprived of serum.

Fig. 1 shows the effect of particle size on the phagocytosis of droplets stabilized by the commercial egg lecithin sample and Poloxamer 338 expressed in terms of change in turbidity with time. It is clear that both size and surface character are of relevance to the process of uptake; the smaller the particle the slower the rate for a given emulsifier, and that for a given size the particles stabilized with Poloxamer were cleared more slowly than those stabilized with lecithin.

The effect of emulsifier

Emulsion systems were prepared with a mean droplet size in the range 1–2 μm in order to minimize the effects of particle size differences on phagocytosis. The results (Fig. 2) show that the nature of the emulsifier can be of importance. Emulsions stabilized by simple mixtures of PC and PE, with added CHO, CH and PA were cleared at a similar rate, whereas those with added PI and PS were cleared more slowly and those with added LPC much more quickly. The system stabilized by Poloxamer 338 was cleared at the slowest rate. The data in Fig. 2 are summarized in Table 1 in terms of the first-order rate constant for phagocytosis (obtained from the data for the first 10 min of incubation) as well as the arbitrary quantity; the percentage uptake in 600 s. The zeta potentials calculated from the measured electrophoretic mobility at pH 7.0 ranged from −12 mV for the Poloxamer system to −45 for the PC/PE/LPC system.

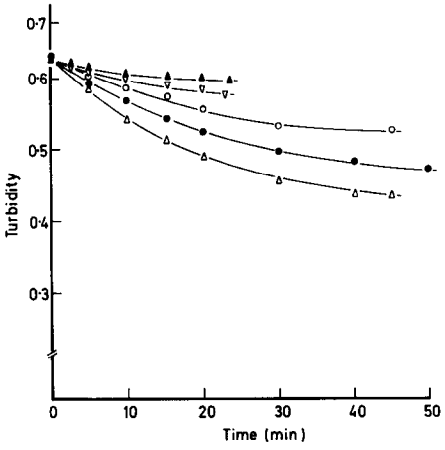

Fig. 1. The effect of particle size on the phagocytosis of fat emulsions by mouse peritoneal macrophages.

Emulsifier		Mean particle (size (μm)
Poloxamer 338	▲	1.3
	▽	2.1
Commercial egg lecithin	○	1.8
	●	2.2
	△	3.0

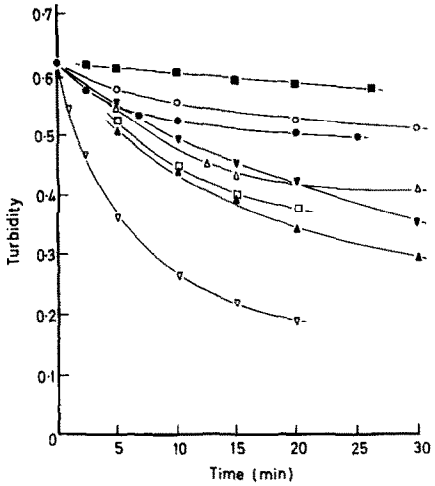

Fig. 2. The effect of the nature of the emulsifier on the phagocytosis of fat emulsions by mouse peritoneal macrophages. Emulsifier: PC/PE (4:1), △. PC with added lipids (0.1%); ○, PS; ●, PI; □, PA; ▽, LPC; ▼, CH; ▲, CHO; ■, Poloxamer 338.

TABLE 1

PHAGOCYTOSIS OF FAT EMULSIONS BY MOUSE PERITONEAL MACROPHAGE

System	1st-order rate constant (min^{-1})	Percentage uptake in 600 s	Zeta potential (mV) at pH 7.0 in buffer
PC/PE (4:1)	6.5×10^{-3}	23	−29
Lecithin (42001)	4.5×10^{-3}	14	−34
PC/PE/PS (0.1%)	2.6×10^{-3}	11	−38
PC/PE/PI (0.1%)	3.3×10^{-3}	16	−44
PC/PE/PA (0.1%)	8.3×10^{-3}	27	−41
PC/PE/CH (0.1%)	5.6×10^{-3}	26	−37
PC/PE/CHO (0.1%)	9.1×10^{-3}	20	−42
PC/PE/LPC (0.1%)	2.0×10^{-2}	59	−45
Poloxamer 338 (1%)	1.0×10^{-3}	7	−12

Discussion

Phagocytosis and opsonization

The mechanism of phagocytosis has been well reviewed by Munthe-Kaas and Kaplan (1980). The process can be divided into two phases. The first phase is the attachment of the particles to the surface of the macrophage followed by a process of ingestion or engulfment. The requirements for the two phases can be different, for instance under certain conditions a particle can adhere to the macrophage surface but may not be ingested (Rabinovitch, 1967a and b).

In the present study it was not possible to determine the contributions from the two phases since the emulsion particles were too small to allow microscopic

estimation of numbers within the individual macrophages, and washing and recovery experiments to remove adhering particles can only be undertaken if appropriately labelled particles are available. The emulsion particles were not removed by macrophage when a simple incubation medium was employed, but measurable rates of phagocytosis were observed when a medium containing swine serum was used. The serum of animals contains various components called opsonins that will promote phagocytosis, these include specific materials such as antibodies and complement as well as glycoproteins (Munthe-Kaas and Kaplan, 1980). The nature and extent of uptake of a particular opsonin is determined by the physicochemical properties of the particle surface (Van Oss et al., 1975) and our previous studies have shown that the modification of the surface of a fat emulsion droplet upon incubation with blood plasma is rapid and that the adsorbed components are not removed by washing with water or saline (Davis and Galloway, 1981).

The effect of particle size

The results show that the particle size of the emulsion system can affect the rate of uptake by mouse peritoneal macrophages. The smaller the particles the slower the apparent rate of clearance and the effect can be related to the number of particles used in the phagocytosis experiments; the smaller the mean particle diameter the greater the number of particles that will be created from a given volume of oil. Such results indicate the importance of conducting phagocytosis experiments under conditions of controlled particle size notwithstanding the difficulty of producing emulsions of identical size and distribution using emulsifiers that have different surface activities. The present results are in agreement with those of Schneider (1979) who reported on the uptake of egg and synthetic lecithin vesicles by stimulated mouse peritoneal macrophage in the presence of calf serum. The macrophages phagocytized large vesicles more rapidly than small vesicles. Similarly, in vivo studies on the clearance of colloidal particles from the circulation and their uptake by liver macrophages have demonstrated particle size effects (Yokoyama et al., 1975; Scott et al., 1967; Juliano and Stamp, 1975).

The effect of surface characteristics

The results demonstrate that rate of uptake of emulsion droplets by mouse peritoneal macrophage is greatly influenced by the characteristics of the stabilizing surface layer. However, there are no clear trends with known physicochemical characteristics such as surface charge. Van Oss (1978) has stated that zeta potential does not appear to be directly linked to the facility with which cells become phagocytized, and that the connection between phagocytosis and zeta potential (if any) is far from simple. In contrast Stossel et al. (1972) have concluded that particles with a large (sic) surface charge (negative or positive) are cleared rapidly by macrophage in vitro while systems with a low surface charge were cleared slowly.

The PC/PE system can be used as the baseline when examining the results for the phosphatide stabilized systems. The addition of CH, CHO and PA had little effect on the rate of phagocytosis; added PI and PS reduced the rate, while LPC caused a marked increase in phagocytosis. Emulsion droplets stabilized by phospholipids

carry a negative charge at neutral pH. The magnitude of this charge at fixed ionic strength is determined by the nature of the phosphatide or mixed phosphatide system (Bangham, 1968; Davis, 1983). PC and LPC are unionized over a wide range of pH while PE, PA, PS and PI will confer a negative charge to emulsion droplets at pH values above 3. Thus, the mixed system PC/PE (4:1) will carry a negative charge at pH 7.4 and this charge will be increased by the addition of small quantities of PA, PI and PS. Cholesterol and its ester cholesterol oleate would be expected to have little effect on surface charge. The marked increase in surface charge produced by the addition of LPC is somewhat surprising since the LPC molecule is uncharged over a wide range of pH values. The increase in charge could be due to the presence of a small amount of surface active impurity in LPC (undetected by the TLC technique) or some effect of LPC on the PC/PE system that changes the relative configurations of the two species at the interface, thereby affecting surface charge (Kurakata, 1981; Saunders, 1975).

Studies on the effect of incorporated PI and PS on the uptake of liposomes by mouse peritoneal macrophages have been reported recently by Roerdink et al. (1983) where the opsonization process was by a specific antibody plus complement. They concluded that the process was strongly dependent on the charge of the liposomal lipids, and that opsonization and negative surface charge could be opposing forces that worked against each other. A similar explanation can be put forward for the results of the present work. Added CH and CHO have little effect on the surface charge and thus the coating process by blood components is little affected. PI and PS increased the negative charge on the particles and thereby reduced the opsonization process by a repulsion effect which in turn leads to a reduced rate of uptake. PA would also be expected to have a similar effect to PI and PS but this was not the case and this result may be related to the presence of divalent ions in the system. Calcium ions are known to have specific effects on the properties of systems containing PA (Rand, 1981) and complexation between PA and Ca^{2+} will result in the removal of the acid from the interface (Hauser and Philips, 1979; Rand, 1981).

The enhanced effect on phagocytosis of added LPC is more difficult to explain but may be associated with the membrane-active properties of the materials. A similar enhancing effect of LPC on phagocytosis has been reported by Davis and Hansrani (1981) for the uptake of similar emulsions by a phagocytic soil amoeba (Acanthamoeba Castellani (Neff)).

The emulsion system with the lowest surface charge, namely that stabilized by the non-ionic surfactant Poloxamer 338, gave the lowest rate of uptake. This result is in good agreement with the studies of Stossel et al. (1972) who showed that emulsions coated with Poloxamers 338 and 188 were not taken up at all by macrophages in vitro and that the uptake of those coated by Tween 20 was low. The results also support the hypothesis of Van Oss (1978) who has related phagocytosis to surface hydrophobicity. According to his suggestions colloidal particles presenting hydrophilic surfaces with a low contact angle will be almost ignored by phagocytic systems. In this connection, Illum and Davis (1984) have administered polystyrene latex coated with Poloxamer 338 to rabbits and have found a markedly reduced uptake by the liver as compared to a control with uncoated particles. The mecha-

nism was believed to be related to changes in surface hydrophobicity as well as to steric stabilization and thus inhibition of particle adhesion.

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