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Studies on the interaction of charge-reversed emulsions with the reticuloendothelial system

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Summary

The effect of charge reversal on the uptake of fat emulsions by the reticuloendothelial (RE) system has been assessed by in vitro and in vivo methods. Emulsions stabilized by egg lecithin were charge reversed from negative to positive by added calcium ions that are adsorbed at the emulsion surface. The interaction of positively charged emulsion droplets with rat Kupffer cells was not significantly different to that found with negatively charged emulsion droplets. In all cases the measured uptake was low. Blockade of the reticuloendothelial system by infused fat emulsion was evaluated in vivo using a rabbit model. RE function was quantified by means of a probe in the form of radiolabelled albumin microspheres together with gamma scintigraphy. The kinetics of uptake of the probe into the liver/spleen regions and organ analysis showed that there were no differences between conventional and charge reversed systems. The extent of RE blockade was small in all cases.

Introduction

Total parenteral nutrition systems comprising mixtures of amino acids, carbohydrates, electrolytes and lipids are now well accepted in clinical practice. They provide an increased convenience and can minimize problems in microbial contamination (Washington, 1990). However, it is appreciated that not all such mixtures will demonstrate good physical (and chemical) stability. The component fat emulsions gain their physical stability through mechanical and electrical barriers. The electrical barrier is provided by the ionization of phospholipids in the stabilizing layer of egg lecithin at the oil-water interface. At neutral pH and low ionic strength, a typical fat emulsion carries a negative electric charge with a magnitude between 30 and 50 mV. This is more than sufficient to provide a potential energy barrier to prevent droplet flocculation (Davis, 1983). The addition of electrolytes will cause a reduction in surface charge; the efficiency being related to the valency of the added cations. Divalent cations are much more effective than monovalent cations and because of specific surface interactions, fat emulsion systems containing significant quantities of calcium and magnesium can

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display charge reversal such that the emulsion carries a (small) net positive charge (Davis et al., 1987).

It is likely that the emulsion droplets in some TPN mixtures used clinically are positively charged. While there is no evidence to indicate that such systems are anything less than satisfactory, it has been suggested that a positively charged emulsion droplet could behave differently when introduced into the bloodstream with respect to the uptake of plasma blood components and opsonic factors. In such an event, positively charged droplets might be recognised differently from normal (negatively charged) fat emulsion droplets. Of particular concern is the possible sequestration by elements of the reticuloendothelial system residing in the liver and spleen. As a consequence, we have undertaken experiments in a cell culture system and in an animal model in order to ascertain whether a positively charged emulsion interacts differently with phagocytic cells compared to a negatively charged control emulsion.

The uptake of labelled fat emulsions by isolated cultured rat Kupffer cells has been measured. The extent of reticuloendothelial blockade introduced by injection of fat emulsions (negatively and positively charged) has been evaluated in the rabbit model by gamma camera imaging using administration of a labelled probe (^{99m}Tc-labelled albumin microspheres). Blood clearance data were also obtained and organ levels of the probe at the time of killing were determined. This latter method has been described in detail recently for the evaluation of medium and long chain triglyceride emulsions (Davis et al., 1990).

Materials and Methods

Kupffer cell studies

Radiolabelled emulsions

Emulsions were prepared at 10% phase volume from purified soybean oil labelled with [^{125}I]iodine according to the method described by Lubran and Pearson (1958). Two different phospholipid emulsifiers were used. These comprised egg lecithin with a phosphatidylcholine (PC) content of 80% (emulsion I) and > 90% (emulsion II) (Lucas Meyer, Germany). These emulsifiers were chosen on the basis of their contents of ionizable phospholipids and consequently their different charge reversal characteristics. A concentration of 1.2% lecithin was used in both emulsions.

The emulsions were prepared under aseptic conditions using an ultrasonic probe (Dawe Soniprobe). Previous work with unlabelled oil and the same emulsifiers had demonstrated that the mean particle size as determined by photon correlation spectroscopy would be in the region of 300 nm and contain approx. 1.2×10^{13} droplets per ml. The radioactivities of the prepared emulsions were in the range 0.25–0.50 MBq/ml. Emulsions were charge reversed using 5 mM $CaCl_2$. It was not possible to measure the zeta potentials of the labelled emulsions for reasons based on protection from radiation. Consequently, a parallel set of samples were produced and the zeta potentials of these were measured. Emulsion I had a zeta potential of -40 mV, and was $+4$ mV in the presence of 5 mM $CaCl_2$, while emulsion II had a zeta potential of -13 mV and was $+10$ mV in 5 mM $CaCl_2$.

Isolation and culture of Kupffer cells

Kupffer cells were obtained from perfused rat liver by enzyme digestion, followed by gradient and elutriation centrifugation as described by Knook and Sleyster (1976). The cells were maintained in culture for at least 24 h before use in particle uptake studies. The ability of the cultured cells to take up particles recognised as foreign was evaluated using 1 μm polystyrene latex particles (Polysciences). The uptake for four replicate plates, where 50 cells were counted in each well, gave an overall uptake of 6.7 ± 0.9 particles per cell when presented with a quantity of 10 particles per cell for 1 h.

For the studies on the uptake of labelled emulsions, Kupffer cell plates were prepared at an original cell density of 1.3×10^6. The cells were incubated for 3 days prior to use in 10% heat-inactivated swine serum in minimal essential media. The cells were twice washed with phosphate-

buffered saline. In order to evaluate the effect of possible opsonic factors, the emulsion samples were added to PBS containing 10% Gibco rat serum (not heat inactivated) to give a concentration of 0.4%. This provided a final droplet presentation of 9.6×10^6 droplets per 2 ml, or 74 droplets per Kupffer cell.

The emulsions were incubated in PBS or serum/PBS mixture for 30 min at room temperature. The emulsions were then incubated with the Kupffer cells for 1 h at 36°C in an atmosphere enhanced with CO_2.

The plates were washed three times with PBS and all washings collected for counting as supernatant (S). Cells were then incubated with 1 M NaOH for 3 h and washed with 1 M NaOH and 1% Triton X-100. These washings were collected and counted as uptake (U). Percentage uptake was taken from the ratio of U/S. An average of 85% total activity was accounted for by this method.

Measurement of reticuloendothelial blockade by emulsions

Intralipid (20%) was obtained from the Queen's Medical Centre, Nottingham. A test emulsion (emulsion III) containing 20% soybean oil (Sainsbury PLC) and emulsified using egg lecithin with > 90% PC (1.2%) (Lucas Meyer) was prepared using a Microfluidizer as described by Washington and Davis (1988).

The components were sterilized by autoclaving and the emulsion prepared using an aseptic technique. The emulsions were charge reversed by 5 mM $CaCl_2$. The particle size of the two emulsions was very similar; 300 ± 10 nm as measured by photon correlation spectroscopy.

Testing RE function

Human serum albumin microspheres for assessing the functionality of the RES were obtained as a kit (TCK-9) from C.I.S. (Italy). The wet size of 90% of these particles was stated to be less than 1 μm diameter. The microspheres were labelled with ^{99m}Tc using [^{99}Tc]pertechnetate obtained from a generator (Amersham International). Doses of 0.8 ml of microsphere suspension were given to each rabbit and contained about 4 MBq of radioactivity.

Animal model

Female New Zealand white rabbits (weight 2.5–3.5 kg) were used. These were injected with emulsions via the marginal ear vein. The labelled probe colloid was administered via the contralateral ear. Four rabbits were used in each test group and in a control group.

Infusion of test emulsions and gamma scintigraphy

The groups of four rabbits (that had previously been trained) were infused with the fat emulsions (Intralipid and emulsion III) over a period of 6 h at a rate sufficient to provide a total dose of 3 g fat/kg body weight. Infusion was performed using Braun Infusomat syringe pumps. After termination of the infusion, the rabbits were immediately given a dose of the labelled probe. After the probe particles had been injected the animals were placed on the face of a gamma camera (GE-Maxicamera, 40 cm field of view), fitted with a low-energy collimator. Dynamic images (30 frames of 20 s duration) and static images (0.5, 1, 2 h) were taken. The data were stored on magnetic tape for subsequent analysis. Regions of interest were created for lung/heart, liver/spleen regions and the activity vs time profiles were determined from dynamic views with appropriate corrections for background radiation and radiation decay.

The liver/spleen uptake data (0–160 s) and the lung/heart clearance data (40–220 s) were fitted to monoexponential (first-order) models that were characterized by a rate constant and a corresponding half-life. The maximum quantity of the probe appearing in the liver/spleen region was also determined, as was the content in the lung/spleen and liver/heart regions at the various selected time periods corresponding to the static views.

Circulating levels of activity in the blood were measured by the withdrawal of blood samples (0.5 ml) at suitable time intervals (0, 10, 20, 30 min, 1, 2 h) followed by the counting of gamma activity (LKB, Compugamma gamma counter). Following the killing of an animal at 4 h, the total

weight of the animal was measured and the weights of the individual organs were determined. The gamma activity in these organs was obtained using a 'bucket' counter (Ortec).

A further group of untreated rabbits that had not been infused with fat emulsion acted as a control. The administration of the labelled probe, blood sampling and organ analysis were carried out as before.

Statistical analysis

The data were analysed where appropriate using an unpaired *t*-test. The Bonferroni correction was applied using a 1.6% level of significance.

Results and Discussion

Kupffer cell studies

Table 1 shows the results for the mean uptake of emulsions by cultured Kupffer cells for negatively and positively charged systems after incubation in PBS buffer or in rat serum (not heat activated) in order to assess the influence of possible opsonic factors. In all cases, the extent of uptake was small compared to that found with hydrophobic particles such as polystyrene, ranging from less than 1% for emulsion II systems in the absence of opsonic factors to just less than 6% for emulsion I in the opsonized system. Charge reversal only had a significant effect on uptake for non-opsonized emulsion II. A significant difference was found between the behaviour of the two emulsifiers in all the tests (opsonized/non-opsonized calcium/no calcium studies). The opsonization of the droplets prior to uptake studies had a significant effect for the emulsions that were not charge reversed. The charge reversed systems failed to demonstrate any significant differences whether opsonized or not. Thus, the uptake of labelled fat emulsions by cultured Kupffer cells was very low. The nature of the emulsifier had more influence than did the presence of added calcium in the original emulsion. No effects could be found that suggested that calcium-containing charge reversed emulsions were opsonized in a different way from the corresponding control systems containing no added calcium. It is therefore likely that a charge reversed emulsion, upon dilution in the blood, would revert to its 'original' state. Therefore, charge reversed emulsions would be expected to cause no greater blockade of the reticuloendothelial system than normal systems. Apparently, of

TABLE 1

Uptake of labelled fat emulsion by cultured Kupffer cells (mean ± SD, n = 4)

	Mean % uptake	
	PBS buffer	Rat serum (not heat inactivated)
Emulsion I	3.44 ± 0.17	5.64 ± 0.63
Emulsion I + 5 mM Ca	3.46 ± 0.16	3.99 ± 0.31
Emulsion II	0.85 ± 0.07	1.62 ± 0.13
Emulsion II + 5 mM Ca	1.97 ± 0.31	1.59 ± 0.06

t-tests

	± Ca		Emulsion I/II		PBS/serum
Emulsion I	ns	serum	[a]	emulsion I	ns
Emulsion I + serum	ns	serum + Ca	[b]	emulsion I + Ca	ns
Emulsion II	[a]	PBS	[b]	emulsion II	[a]
Emulsion II + serum	ns	PBS + Ca	[a]	emulsion II + Ca	ns

Statistical significance: [a] $p < 0.016$; [b] $p < 0.001$.

greater importance could be the quality and composition of the emulsifier, a higher PC content being favoured.

Infusion of test emulsions

All rabbits tolerated the infused emulsions without any visible sign of respiratory distress.

The data obtained by dynamic scintigraphy, namely the liver/spleen uptake and the residue in the heart/lung region, both demonstrated that all emulsions caused little or no impairment of reticuloendothelial function as compared to the control (Table 2). The maximum amounts of the probe reaching the liver were unaltered as compared to control values. There was no significant difference in the data for the various emulsions.

A summary of the static scintigraphy data (0.5–2 h) is also given in Table 2. The quantities of the probe found in the liver/spleen region and heart/lung region were very similar for all the emulsions tested and for the untreated control. It will be noted that the probe is cleared less rapidly from the liver/spleen region in the emulsion treated animals than for the control animals. Davis et al. (1990) have suggested that the presence of the emulsion in the liver/spleen affects the metabolism of the probe particles.

The blood level data (not shown) indicated that the probe was rapidly cleared from the circulation and that at the first measurement point at 10 min, the level of circulating probe in the emulsion treated animals was no different from that for the control animals for all the emulsions.

Data from the analysis of the individual organs are listed in Table 3. They show few differences between the control and test emulsion groups, the major difference being the higher level of activity in the carcass for the control group. This reflects the more rapid metabolism of the probe in untreated animals and the presence of technetium label in the kidney, bladder and to a lesser extent, in the thyroid and gastrointestinal tract. There was a suggestion that spleen levels of the probe were higher in the emulsion groups than for the control but only for Intralipid was this difference significant. It is to be expected

TABLE 2

Summary of scintigraphic data

Percentage of probe particles in different regions at designated times

	Liver/spleen region					Lung/heart region			
	Dynamic		Static			Dynamic	Static		
	% max	10 min	30 min	60 min	120 min	10 min	30 min	60 min	120 min
Control									
$\bar{x}$	67.4	65.8	67.4	63.7	59.7	3.8	6.1	7.3	7.9
SE	2.4	2.7	2.2	1.6	2.9	0.3	0.3	1.1	0.8
Intralipid									
$\bar{x}$	70.0	72.0	72.3	69.6	66.4	7.5	7.2	6.0	6.2
SE	1.4	1.8	2.4	2.8	1.0	1.2	1.3	1.1	1.1
Intralipid charge reversed									
$\bar{x}$	72.7	72.7	77.8	75.8	72.8	2.9	4.1	3.5	3.7
SE	1.6	1.6	0.5	0.4	1.2	0.1	0.7	0.4	0.7
Emulsion III									
$\bar{x}$	69.1	71.5	68.6	73.2	67.6	4.1	4.6	4.5	3.5
SE	2.1	1.5	4.1	6.2	1.6	0.6	1.0	1.2	0.8
Emulsion III charge reversed									
$\bar{x}$	68.4	67.9	71.3	65.2	64.2	7.2	5.1	7.4	6.0
SE	3.5	3.3	5.9	3.9	4.7	0.7	1.2	0.8	1.4

TABLE 3
Organ analysis

	Weights				Percentage				Percentage/g tissue			Ratio (counts/g)	
	Rabbit (kg)	Lung (g)	Liver (g)	Spleen (g)	Lung	Liver	Spleen	Carcass	Lung	Liver	Spleen	Liver/ lung	Liver/ spleen
Control													
x	3.8	18.3	105.8	4.3	3.3	54.2	7.2	35.2	0.19	0.53	1.65	3.3	0.41
SE	0.33	1.9	12.0	0.8	0.64	3.0	2.5	2.1	0.03	0.07	0.22	1.0	0.15
Intralipid													
x	4.1	19.6	112.0	2.7	5.3	65.9	9.6	19.2 [a]	0.30	0.61	3.70 [a]	2.5	0.17
SE	0.31	2.8	1.08	0.40	0.95	1.8	1.3	1.7	0.07	0.06	0.27	0.7	0.03
Intralipid charge reversed													
x	4.0	19.8	113.0	4.1	2.7	70.4	10.7	16.1 [a]	0.14	0.63	2.71	6.3	0.25
SE	0.16	0.75	5.4	0.44	0.8	2.5	3.5	0.5	0.05	0.03	0.32	1.9	0.05
Emulsion III													
x	4.4	17.5	96.8	3.3	4.7	66.7	13.3	15.2 [a]	0.26	0.73	4.48	2.9	0.25
SE	0.37	1.4	12.2	0.33	0.8	2.5	3.5	0.5	0.03	0.09	1.6	0.6	0.09
Emulsion III charge reversed													
x	4.3	22.3	109.8	4.2	5.5	65.7	16.4	12.4 [a]	0.25	0.60	4.02	2.8	0.16
SE	0.10	1.5	4.4	0.38	0.9	3.0	2.6	0.75	0.04	0.05	0.65	0.65	0.04

[a] Statistically significant from control: $p < 0.016$.

that more of a probe should be found in the spleen if the reticuloendothelial cells in the liver (Kupffer cells) are increasingly blocked. The liver/spleen ratios also reflected this trend.

The results confirm previous work that has suggested that the infusion of fat emulsions into the rabbit can cause slight temporary impairment of the reticuloendothelial system as determined by the subsequent administration of a radiolabelled colloid probe. An infused emulsion can cause RE blockade by one or both of two mechanisms. Immediately after infusion, some of the particles may be recognised as foreign and are then cleared by the RES (largely the Kupffer cells of the liver). In addition or alternatively, the Kupffer cells of the liver may become overloaded by emulsion 'remnants' that will result from normal metabolism of the emulsion by tissue lipases. The important conclusion is that no differences can be seen between emulsions that are infused as such, or when they have been charge reversed by added calcium. This applies not only to Intralipid but also to an emulsion formulated to carry a positive charge in the presence of added calcium ions.

Clearly, the clinical significance of results obtained in animal studies needs to be interpreted with caution. However, the results suggest that charge reversed emulsions (i.e. those carrying a positive charge) due to the specific adsorption of calcium ions should be handled no differently from conventional emulsion systems that carry a negative charge.

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