J. Pharm. Pharmacol. 1993, 45: 182–185
Received May 11, 1992

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Uptake of Concentrated Perfluorocarbon Emulsions into Rat Lymphoid Tissues

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Abstract—The effects of injecting (10–30 mL kg^{-1}) either perfluorodecalin (FDC) emulsions of increasing phase fraction (20–60% w/v) or the commercial formulation, Fluosol, on lymphoid tissues have been studied for up to 7 days in male rats. Tissue weights increased by up to 123% ($P < 0.05$) in proportion with quantity of perfluorochemical (PFC) injected, with spleen responses consistently greater than those of the liver. PFC droplets recovered from these tissues at 72 h after injection of 30% (w/v) FDC emulsion (10 mL kg^{-1}) had mean diameters in the 1–10 μm range, with those from the spleen being larger than those from the liver. Recovered droplet diameters were considerably greater than freshly-prepared emulsion mean particle sizes (0·21–0·25 μm). These results suggest that coalescence of emulsion droplets following accumulation in lymphoid tissues is a pre-requisite for the eventual excretion of PFC vapour through the lungs.

Perfluorochemical (PFC) emulsions are attracting increasing interest for biomedical uses as, for example, vehicles for respiratory gas transport and contrast agents for diagnostic tissue imaging (Lowe 1988a, 1991, 1992). PFCs are considered as chemically inert owing to the high strength of the carbon-fluorine bond (ca. 480 kJ mol^{-1}). PFC emulsion droplets can accumulate in lymphoid tissues and such uptake depends upon the composition and dose of emulsion injected, route of injection, tissue, and species (Lowe 1988b). The droplets are detectable in the liver and spleen as clusters, often termed 'foamy vesicles', owing to their electron microscopical appearance (Caiazza et al 1984; Nanney et al 1984). PFCs are eventually released into the blood where they are believed to be carried bound to lipoproteins before being excreted by expiration through the lungs (Tsuda et al 1988). The relevance of this transient tissue uptake to the overall PFC excretion process is, however, poorly understood.

Much of the previously published work in this area has been concerned with biological studies using emulsions containing approximately 20% (w/v) of the PFC component (Lowe 1988b). The most widely tested emulsion of this type is the proprietary formulation, Fluosol (Green Cross, Japan—available in the UK through Alpha Therapeutic, Thetford), which has recently been approved both in the UK and USA for myocardial oxygenation during angioplasty (Lowe 1991, 1992). The use of formulations containing an increased PFC phase fraction would, in principle, improve the performance of these materials.

The present study has therefore investigated the effects of injection of perfluorodecalin (FDC) emulsions, of increasing phase fraction, on liver and spleen weights in rats. Additionally, we have extracted PFC droplets from these tissues and measured the changes in their size. Some of these results have already been published in a preliminary form (Washington et al 1990; Bentley et al 1991).

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Materials and Methods

Preparation of emulsions

The FDC emulsion consisted of 20–60% (w/v) perfluorodecalin (Flutec PP5; Rhône-Poulenc, ISC Division, UK), 2% (w/v) soya oil (Sainsbury, UK) and 4% (w/v) Pluronic F-68 (ICI/Atochem, UK) in an aqueous phase containing 0·9% (w/v) NaCl. Emulsions were prepared using a Microfluidizer (Microfluidics, Newton, MA, USA) as described previously (Johnson et al 1990a, b). The mean droplet diameters of these emulsions, as measured by photon correlation spectroscopy (Malvern Instruments K7025, Malvern Instruments, UK), are shown in Table 1. Fluosol (Green Cross, Japan), was freshly reconstituted according to the manufacturer's instructions (Naito & Yokoyama 1978) and contains 14·0% (w/v) FDC and 6·0% (w/v) perfluorotripropylamine (Table 2). The mean droplet diameter of Fluosol was 0·23 μm (Table 1).

Care of animals and experimental procedures

Mature male Wistar rats, 150–300 g, were maintained in the laboratory animal house under controlled conditions (13 h light, 11 h dark; temperature 24 ± 1°C) and had free access to a standard diet (Rat and Mouse Breeding Diet, Haygates, UK). Before any experimental treatment, animals were allocated randomly into 1 of 7 experimental groups as follows: group I (n = 7) controls; group II (n = 9) FDC emulsion (20% w/v); group III (n = 3) FDC emulsion (30% w/v); group IV (n = 9) FDC emulsion (40% w/v); group V (n = 9) FDC emulsion (60% w/v); group VI (n = 12) Fluosol (low dose); group VII (n = 12) Fluosol (high dose).

Animals were lightly anaesthetized with ether and then injected intraperitoneally with 10 mL kg^{-1} of either FDC emulsion or Fluosol (low dose); animals in group VII received 30 mL kg^{-1} of Fluosol (high-dose). This latter dose was chosen as a comparison with group V animals injected with an equivalent quantity of PFC. Control animals in group I received 10 mL kg^{-1} of sterile saline (0·9% w/v NaCl; Viaflex, Travenol, UK). At 24 h, 72 h or 7 days after injection, animals were killed by cervical dislocation and their livers and spleens removed and weighed. Tissues from

Table 1. Mean droplet diameters for FDC emulsions measured by photon correlation spectroscopy immediately after preparation.

Emulsion composition	Mean droplet diameter (μm)*
20% FDC	0·21
30% FDC	0·23
40% FDC	0·25
60% FDC	0·25
Fluosol	0·23

* All values ± 5%.

Table 2. Composition of Fluosol (Green Cross, Japan).

Perfluorodecalin	14·0
Perfluorotripropylamine	6·0
Pluronic F-68	2·72
Yolk phospholipids	0·40
Potassium oleate	0·032
Glycerol	0·8
NaCl	0·6
$NaHCO_3$	0·21
Glucose	0·18
$MgCl_2$	0·043
$CaCl_2$	0·036
KCl	0·034

All values are w/v (%).

group III and group VI animals were used immediately for extraction of PFC droplets.

Extraction and sizing of PFC droplets

Approximately 0·1 g spleen or 0·5 g liver was gently macerated in a tissue grinder with 5·0 mL of 4% (w/v) Pluronic F-68 solution. The resulting homogenate was centrifuged at 3000 rev min^{-1} for 30 min. The supernatant was discarded and the cell pellet carefully rinsed with 1·0 mL of 4% (w/v) Pluronic F-68 to give a 'clean' PFC pellet. This was resuspended in 1·0 mL of 4% (w/v) Pluronic F-68 solution. The presence of PFC droplets and absence of cellular debris was confirmed by light microscopy (Nikon Ultraphot; 400 x). Droplet diameters of these suspensions were measured using a Malvern Mastersizer with 45 mm lens (0·1–80 μm size range). Sizes measured by light diffraction were in approximate agreement with those estimated by microscopy.

Statistical analyses

Statistical analyses were performed according to the methods of Snedecor & Cochran (1980). Means and either standard deviations (s.d.) or standard errors (s.e.m.) have been used throughout and are indicated accordingly. Statistical significance between mean values was assessed using a conventional Student's *t*-test; a probability of $P<0·05$ was considered significant.

Results

Emulsion droplet sizes

The mean droplet diameters of all FDC emulsions were in the range 0·21–0·25 μm. This compared well with a mean droplet diameter of 0·23 μm for the commercial Fluosol emulsion (Table 1). Emulsions containing increasing amounts of FDC showed gradually increasing mean droplet diameters, as would be expected. Emulsions stored at room temperature (ca. 25°C) were stable for over 3 months (data not shown).

Table 3. Mean (±s.e.m.) spleen and liver weights (% control) at 24 h, 72 h or 7 days after injection of either Fluosol (10, 30 mL kg^{-1}) or FDC emulsion containing 20, 40 or 60% (w/v) FDC.

Time after injection	Treatment	n	Spleen	Liver
24 h	Fluosol (10 mL kg^{-1})	4	132±16	107±7
	Fluosol (30 mL kg^{-1})	4	144±5*	112±1*
	FDC emulsion (%)			
	20	3	140±3*	110±3
	40	3	157±17*	100±1
	60	3	167±13*	104±3
72 h	Fluosol (10 mL kg^{-1})	4	106±16	105±4
	Fluosol (30 mL kg^{-1})	4	206±4*	113±2*
	FDC emulsion (%)			
	20	3	145±13*	117±3*
	40	3	181±3*	139±2*
	60	3	184±23*	138±7*
7 days	Fluosol (10 mL kg^{-1})	4	104±13	114±8
	Fluosol (30 mL kg^{-1})	4	215±7*	142±4*
	FDC emulsion (%)			
	20	3	146±15*	121±4*
	40	3	223±33*	136±1*
	60	3	223±6*	129±17*

* $P<0·05$ compared with mean control (100%) value.

Tissue weights

Table 3 shows liver and spleen weights, as a percentage of controls (group I), 24 h, 48 h and 7 days after injection of emulsions. Significant weight increases ($P<0·05$) of spleen, and, to a lesser extent liver, occurred in response to injection of FDC formulations and high dose Fluosol, but not following low dose Fluosol injection. The weight increase of the spleen occurred more rapidly than that of the liver but this depended on the composition of emulsion injected. In the case of animals injected with the FDC emulsions and the high dose of Fluosol, both spleen and liver weights were still greater than control after 7 days. In contrast, tissue weights in rats receiving the low dose of Fluosol were similar to control within 72 h.

Recovery of droplets from tissues

The mean diameters of droplets recovered from liver or spleen at 3 days after injection (groups III and VI) were within the range 1–10 μm, with no droplets detectable below 1 μm (Fig. 1). To confirm that the droplet recovery procedure did not cause artifacts due to coalescence, a control experiment was performed in which the emulsions were mixed with liver or spleen homogenates, extracted and sized; the mean diameter of recovered droplets from these controls was less than 0·25 μm. Droplets recovered from the liver were significantly smaller ($P<0·05$) than those recovered from the spleen for either treatment group; the mean (±s.d.) diameter of droplets recovered from the liver in group III animals (30% FDC emulsion) was 3·4±0·1 μm, whereas for the spleen it was 6·9±1·1 μm. Corresponding values for Fluosol-injected (group VI) animals were 5·0±0·4 and 6·3±0·7 μm,

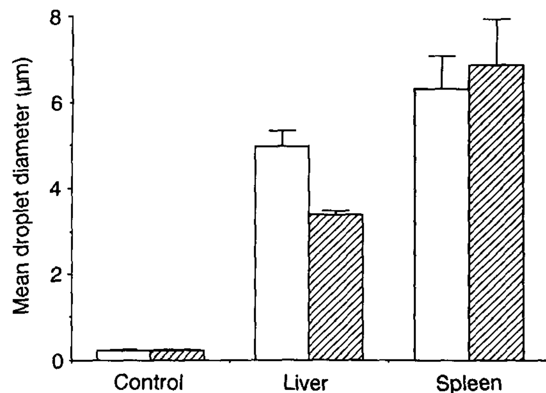

FIG. 1. Mean diameter of PFC droplets extracted from rat liver or spleen at 72 h after injection of low dose Fluosol (group VI, □) or 30% (w/v) FDC emulsion (group III, ■). Control mean values are droplet diameters of emulsions exposed to the extraction procedure (see text for details). Vertical bars represent s.d.

respectively. The mean diameter of droplets recovered from the livers of group III animals was significantly smaller ($P<0{\cdot}05$) than for group VI animals (Fig. 1). In contrast, there was no significant difference in the diameters of droplets recovered from the spleen between these two treatment groups. Liver homogenates from FDC emulsion-injected rats appeared to contain greater amounts of fat compared with those from Fluosol-injected animals. This was attributed to the soya oil component of the FDC emulsion.

Discussion

It is only recently that concentrated PFC emulsions, prepared for in-vivo application, have been successfully produced. Many such emulsions contain FDC as the sole or major PFC component (Magdassi & Siman-Tov 1990; Mukherji & Sloviter 1991), primarily because of its good biocompatibility and excretion characteristics (Lowe 1991, 1992). However, the composition of other emulsions, including those based on perfluorooctylbromide (PFOB) or bis-perfluorohexylethene (F-66E), is unclear, especially regarding the choice of surfactant (Mattrey et al 1989; Thomas et al 1989). The emulsions studied in the present paper are similar to those previously described by our group (Johnson et al 1990a, b), containing Pluronic F-68 as emulsifier and soya oil as stabilizer. These formulations display good stability characteristics, similar to the 20% (w/v) emulsions which we have studied extensively (Bollands et al 1987; Sharma et al 1987). All of these emulsions can be readily sterilized by autoclaving (Johnson et al 1990b), avoiding the need for filter sterilization which is impractical with these systems.

The observed increases in liver and spleen weights were in broad agreement with previous studies in female rats injected intravascularly or intraperitoneally with FDC-based emulsions, including Fluosol, containing approximately 20% by weight of PFCs (Bollands & Lowe 1986, 1988; Bollands et al 1987; Lowe 1988b; Lowe & Bollands 1988; Lowe et al 1989). Furthermore, in earlier related studies in which male rats were injected intravascularly with a total of 5×10 mL kg^{-1} Fluosol over a 15 day period, PFC accumulation was similarly greatest in the spleen (West et al 1986). The increase in spleen weight was relatively greater and more rapid than that of the liver, irrespective of emulsion injected, as seen previously in female rats injected with 10 mL kg^{-1} Fluosol (Bollands & Lowe 1986, 1988). This is consistent with the higher density and greater accessibility of phagocytic cells per unit mass of spleen compared with the liver (Wisse & Knook 1977).

The present results further show that the magnitude of spleen and, to a lesser extent, liver weight increases depends upon the total quantity of PFC injected, although the changes following injection of the 60% (w/v) emulsion were similar to those induced by the 40% (w/v) emulsion. This implies that some degree of tissue saturation by FDC had occurred.

One finding from the present work was that the overall increase in spleen weight over the 7 day period in male rats injected with the higher dose of Fluosol (group VII) or 60% (w/v) FDC (group V) were similar despite the differences in emulsion composition. However, animals receiving the low dose of Fluosol (group VI) showed no significant increase in spleen weight, and a lower increase in liver weight, than animals injected with an identical load of FDC in the form of a 20% (w/v) emulsion (group II). This suggests that while emulsion composition appears to be an important determinant of PFC uptake and clearance from lymphoid tissues, any tissue selectivity conferred by the formulation can be overcome by injection of a sufficiently high dose.

The excretion of PFCs appears to be closely related to their emulsion droplet size. Those of injected FDC emulsions or Fluosol were sufficiently small to circulate freely in the bloodstream. However, the size of droplets recovered from the spleen and liver was up to 30 times larger, and they would thus be expected to be trapped in the lung capillaries when released into the circulation, either by diffusion or following release from senescent tissue phagocytes. This may optimize the excretion of PFCs via the vapour phase by expiration. This suggests that coalescence during tissue uptake is an important part of the overall PFC excretion mechanism. The present results additionally show that, in the rat, such PFC coalescence occurs to a greater extent in the spleen than in the liver.

These findings provide qualitative support for previous observations of PFC coalescence in liver and spleen, as revealed by electron microscopical studies following intravascular injection of comparable doses of emulsified perfluorotributylamine (FC-43) in rats (Caiazza et al 1984) or rabbits (Nanney et al 1984). However, the tissue droplet sizes reported in these earlier experiments were much smaller than those in the present study. This may be due to the different PFCs administered since FC-43 can be much more readily emulsified than FDC and its emulsions are more stable (Naito & Yokoyama 1978). There were also differences in the time after PFC injection at which tissue samples were collected. Caiazza et al (1984) removed tissue samples from rats at 24 h after injection of FC-43, whereas Nanney et al (1984) collected rabbit tissues at 7, 14 and 21 days after FC-43 injection.

It is difficult to compare the present findings with those following injection of concentrated FDC (Mukherji &

Sloviter 1991), PFOB (Mattrey et al 1989) or F-66E (Thomas et al 1989) emulsions since their effects on lymphoid tissues were not reported. However, because of the increasing interest in the in-vivo applications of such emulsions (Lowe 1991, 1992) and the evidence that uptake of PFCs into lymphoid tissues can alter immunological competence (Lowe 1988b), such studies are an essential pre-requisite to further clinical acceptance of these materials. It is also important to distinguish between the effects of the PFCs and other emulsion components since the Pluronic F-68 surfactant is known to induce hepatomegaly in rats (Goodman et al 1984; Bentley et al 1989).

Acknowledgements
We are grateful to Rhône-Poulenc Chemicals Ltd (ISC Division), UK, and the Green Cross Corporation, Japan, for their generous gifts of perfluorodecalin and Fluosol, respectively. P. K. Bentley was the recipient of an SERC/CASE Studentship, sponsored by Rhône-Poulenc Chemicals. We thank Professor D. Wakelin for helpful comments.

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