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Lymphoid tissue responses to a novel perfluorochemical emulsion in rats*

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The effects of a novel perfluorochemical emulsion on rat lymphoid tissues and antibody production against sheep red blood cells (SRBC) have been studied. The responses were compared with those following injection of identical doses of the proprietary emulsion, Fluosol-DA 20% (F-DA). Liver weight was increased up to 15% at 8 days following intravenous (i.v.) or intraperitoneal (i.p.) injection of the novel emulsion but was unaffected by F-DA injection. Spleen weight also increased by a maximum of 20% in response to i.p. injection of the novel emulsion but this was less than increases of up to 44% which occurred in F-DA-injected rats. Thymus weight decreased ($P < 0.05$) following i.p. injection of the novel emulsion whereas mesenteric lymph node (MLN) weight remained unchanged. However, MLN weight was increased in response to i.v. injected F-DA, while thymus weight showed a small increase following i.p. F-DA injection. Mean plasma antibody titres to SRBC were significantly ($P < 0.01$) increased at 7 days after immunization in rats pretreated with i.p. injections of either the novel emulsion or F-DA; titres in animals pretreated with i.v. injections of either emulsion were similar to control.

Emulsified perfluorochemicals (PFCs) have properties which make them attractive as physiological oxygen-transport fluids. Such properties include: the ability to dissolve substantial volumes of oxygen and other respiratory gases; and small particle sizes (<0·25 μm) which enable them to pass readily through capillary beds. In addition, due to the strength of the carbon–fluorine bond (ca 116 kcal mol^{-1} kJ mol^{-1}), PFC molecules are generally regarded as being both chemically and biologically inert (Riess & Le Blanc 1982). Some of the physiological effects of emulsified PFCs have been studied in several species, and also in experiments using cells in culture (Lowe & Bollands 1985; Lowe 1986, 1987).

A commercial emulsion, Fluosol-DA 20% (F-DA; Green Cross, Japan), which contains perfluorodecalin (FDC) and perfluorotripropylamine (FTPA) emulsified with the poloxamer surfactant, Pluronic F-68, has been tested in human trials in several countries (Mitsuno et al 1982; Tremper et al 1982; Waxman et al 1984; Stefaniszyn et al 1985; Gould et al 1986). This emulsion is also being evaluated for possible therapeutic uses related to tumour oxygenation (Lustig & McIntosh 1986) and perfusion of ischaemic tissues (Forman et al 1985). However, one inherent problem with F-DA is its tendency for destabilization on storage, resulting in increased particle size (Riess & Le Blanc 1982). This effect is minimized by storage of the stem emulsion component of F-DA in a frozen state, as recommended by the manufacturers (Naito & Yokoyama 1978).

The major cause of droplet growth in emulsions is coalescence, but this can normally be retarded using emulsifying agents which form electrostatic and mechanical barriers at the oil-water interface. However, a more subtle means of instability can occur by a process of molecular diffusion known as Ostwald ripening and this can occur even if particles have excellent barriers to coalescence (Davis et al 1981).

We have recently reported the development and preliminary physicochemical assessment of novel compositions of emulsified PFCs for possible biological uses related to oxygen transport (Davis et al 1986; Sharma et al 1986). The new emulsions were based on FDC and contained small quantities of polycyclic, perfluorinated, higher boiling point oil (HBPO) additives to stabilize against instability caused by Ostwald ripening. Since there have been no biocompatibility studies using these preparations, the present experiments were undertaken to investigate the effects of one such emulsion on lymphoid tissues and antibody production against an antigenic 'challenge' in the form of sheep red blood cells (SRBC) in rats. A particular objective was to compare the responses with those following injection of identical doses of F-DA. A preliminary report of some of these results has already been published (Bollands et al 1986).

Materials and methods

Care of animals and experimental procedures. Female Wistar rats (140–160 g, n = 37) were used. They 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 food

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concentrate diet (Rat and Mouse Breeding Diet, Haygates, Birmingham). Before experimentation, they were allocated randomly into one of six experimental groups as follows: Group I (n = 14): saline controls, i.v. injection. Group II (n = 5): saline controls, i.p. injection. Group III (n = 4): FDC emulsion with C-16 oil additive, i.v. injection. Group IV (n = 4): FDC emulsion with C-16 oil additive, i.p. injection. Group V (n = 5): F-DA, i.v. injection. Group VI (n = 5): F-DA, i.p. injection.

All animals were initially anaesthetized with ether and i.p. injections were then performed in the inguinal region while i.v. injections were made into a tail vein. A 20% (w/v) FDC (Flutec PP5: I.S.C. Chemicals Ltd, Avonmouth) emulsion containing 1% of a C-16 HBPO additive, perfluoroperhydrofluoranthrene (I.S.C. Chemicals Ltd., Avonmouth), was prepared by sonication (Dawe Automatic 7532A Soniprobe: Dawe Instruments Ltd, London) for 30 min with 4% Pluronic F-68 in an aqueous phase consisting of 0·9% (w/v) NaCl (Table 1). The emulsion was prepared within 24 h of injection and the aqueous phase was passed through a Millipore filter (0·22 μm) before use. F-DA (Table 1) was freshly prepared by mixing stem emulsion and annex solutions immediately before injection; emulsions were prewarmed to ca 37 °C before administration. Control animals were injected with sterile saline solution (0·9% w/v NaCl). The dose of emulsion or saline injected was 10 mL kg^{-1} weight throughout.

Table 1. Composition of Fluosol-DA 20% and the novel emulsion.

	Fluosol-DA	Novel emulsion
Perfluorodecalin	14·0	20·0
Perfluorotripropylamine	6·0	—
Perfluoroperhydrofluoroanthrene	—	1·0
Pluronic F-68	2·7	4·0
Yolk phospholipids	0·4	—
Glycerol	0·8	—
NaCl	0·600	0·900
KCl	0·034	—
$MgCl_2$	0·020	—
$CaCl_2$	0·028	—
$NaHCO_3$	0·210	—
Glucose	0·180	—
Hydroxyethyl starch	3·0	—

All values are w/v (%).

At 24 h following injection with either emulsion or saline, animals were again lightly anaesthetized with ether and a small blood sample (approx. 0·2 mL) was collected into heparinized capillary tubes (Hawksley, Lancing) from the retro-orbital plexus. Animals were then given a single i.p. injection of ca 5×10^8 double-washed SRBC suspended in 1·0 mL Hank's balanced saline solution.

At seven days after immunization, all animals were anaesthetized with ether and exsanguinated by cardiac puncture. The weights of liver, spleen, thymus and mesenteric lymph nodes (MLN) were then measured following careful dissection of individual tissues.

Treatment of blood samples and analytical procedures. Blood samples were placed into Eppendorf tubes (Sarstedt, Leicester) and stored on ice. They were then centrifuged at 2500 rev min^{-1} for 10 min at 4 °C and plasma was subsequently removed and stored at −20 °C until required for analysis. The specific antibody titre to SRBC was measured using a conventional haemagglutination technique with a 0·5% dilution of cells (Pritchard & Eady 1980).

Particle size analysis. Particle size and distribution for both freshly prepared novel emulsion or F-DA were determined by photon correlation spectroscopy (Malvern Instruments, Malvern).

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

Results

Particle size and distribution. Mean (±s.d.) particle size for the novel emulsion and F-DA were 238 ± 4 nm (n = 14) and 266 ± 1 nm (n = 10) respectively; corresponding mean polydispersity values were 0·072 and 0·062.

Changes in tissue weights. Mean liver weight was increased by 12–15% ($P < 0·001$) following i.p. or i.v. injection of FDC emulsion (Table 2). In contrast, liver weight was unchanged at 8 days after injection of F-DA, irrespective of route of administration (Table 2). Spleen weight was also increased by a maximum of 20% ($P < 0·01$) in animals injected i.p. with the novel FDC emulsion but this was less than increases of up to 44% ($P < 0·001$) which occurred in F-DA-injected rats (Table 2). Thymus weight decreased following i.p. injection of the novel emulsion but was similar to the mean control value (0·26 ± 0·01% body weight) in all other animals, except for a small increase following i.p. F-DA injection. MLN weight following administration of FDC emulsion was not significantly different from control (0·08 ± 0·01% body weight) but was increased by 38% ($P < 0·01$) in response to i.v. injection of F-DA (Table 2).

Changes in antibody titres. Mean plasma antibody titres to SRBC measured 7 days after immunization in rats

Table 2. Lymphoid tissue weights in rats after treatment with either Fluosol-DA 20% or FDC emulsion containing C-16 oil.

Treatment	n	Tissue weights (% body weight)			
		Liver	Spleen	Thymus	MLN†
Controls	19	4·62 ± 0·05	0·25 ± 0·01	0·27 ± 0·01	0·08 ± 0·01
Fluosol-DA					
i.v.	5	4·65 ± 0·12	0·36 ± 0·03***	0·27 ± 0·02	0·11 ± 0·01**
i.p.	5	4·53 ± 0·04	0·35 ± 0·03***	0·29 ± 0·01	0·09 ± 0·01
FDC emulsion					
i.v.	4	5·32 ± 0·10***	0·27 ± 0·01	0·24 ± 0·02	0·06 ± 0·01
i.p.	4	5·18 ± 0·07***	0·30 ± 0·01**	0·22 ± 0·01*	0·07 ± 0·01

Values are given as mean ± s.e.m. $*P < 0·05$; $**P < 0·01$; $***P < 0·001$.
† Mesenteric lymph node.

injected i.v. with either FDC emulsion or F-DA were similar to the corresponding mean control value (Fig. 1). However, day 7 $\log_2$ titres were significantly ($P < 0·01$) increased in rats injected i.p. with either emulsified FDC (8·9 ± 0·3) or F-DA (7·8 ± 0·4).

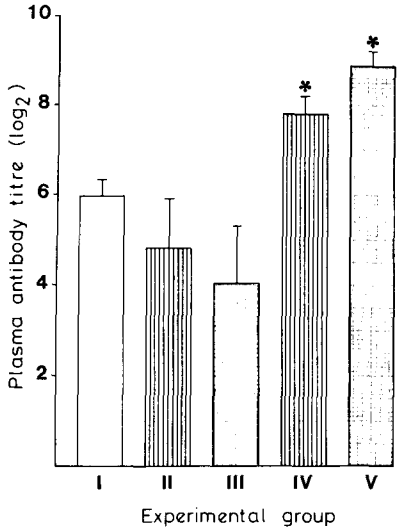

FIG. 1. The mean plasma haemagglutination response (expressed as $\log_2$ titre) of rats at seven days after intraperitoneal injection of SRBC. Experimental pretreatments were as follows: Group I, saline-injected controls (n = 19); Group II, F-DA i.v. (n = 5); Group III, FDC emulsion containing C-16 oil i.v. (n = 4); Group IV, F-DA i.p. (n = 5); Goup V, FDC emulsion containing C-16 oil i.p. (n = 4). Vertical bars represent s.e.m. $*P < 0·01$ compared with mean control value.

Discussion

The results of the present experiments show that an FDC emulsion containing a C-16 HBPO additive can be used in-vivo in rats with no obvious adverse effects. The results show that a single i.v. or i.p. injection of the emulsion is followed by changes in the weights of lymphoid tissue components. Moreover, injection of the emulsion into the peritoneal cavity potentiated the humoral immune response to i.p. injected SRBC.

The increases in tissue weights which followed injection of the novel FDC emulsion were in general agreement with previous findings that PFC particles can accumulate in reticuloendothelial and other tissues (Okamoto et al 1975; Lutz & Metzenauer 1980; Kitazawa & Ohnishi 1982; Lowe & Bollands 1985; Bollands & Lowe 1986, 1987). The variation in lymphoid tissue responses to the new emulsion and F-DA probably reflects the differences in their compositions, since the latter contains 6% FTPA (Naito & Yokoyama 1978; Table 1). The estimated whole body half-life of FTPA in rats is approximately 60 days compared with a corresponding value of about 7 days for FDC (Naito & Yokoyama 1978). Thus, it is reasonable to conclude that FTPA will make a significant contribution to differences in lymphoid tissue responses to the new emulsion and F-DA. However, the effects of the C-16 HBPO component of the novel emulsion cannot be discounted although in-vivo half-life data for this compound are not yet available.

Variations in liver and spleen responses to different emulsified PFCs have been reported previously: for example, Caiazza et al (1984) noted that no significant alterations in tissue weights occurred in rats following i.v. injection of emulsified perfluorotributylamine (FC-43; Green Cross, Japan) in doses comparable with those administered in the present experiments.

While it is recognized that particle size and distribution are of fundamental importance in determining the biological fate of PFC emulsions, the similarity of these variables for both the novel emulsion and F-DA suggests that they were probably less important determinants of the different tissue weight changes observed.

In addition to the effects of different PFC preparations, the responses of individual lymphoid tissues also vary according to route and dose of emulsion administered (Bollands & Lowe 1986, 1987). It is likely that the lack of significant hepatomegaly after injection with either emulsified FDC or F-DA reflected the use of relatively low doses of these emulsions. For example, in previous studies, injection of greater doses of either

F-DA or FC-43 in rodents produced proportionately greater increases in liver weights (Lutz & Metzenauer 1980; Lutz et al 1982; Mason et al 1985).

Species variability is also an important determinant of lymphoid tissue responses to PFC emulsions: for example, there are marked differences in the extent to which identical doses (per unit body weight) of F-DA can produce increases in liver or spleen weights in rats and mice (Bollands & Lowe 1986, 1987). This has inevitably introduced complications in identifying the effects of different PFC emulsions and their components upon normal physiological variables, especially in relation to immune system function.

The increases in plasma antibody titres to SRBC following i.p. injection of the novel emulsion or F-DA were in agreement with previously published observations of immunopotentiating effects produced by emulsified PFCs (Bollands & Lowe 1986, 1987). One explanation for these findings is that the PFCs or other emulsion constituents act as an adjuvant when injected into the peritoneal cavity of either rats or NIH mice 24 h before SRBC (Bollands & Lowe 1986, 1987). However, inconsistencies have been noted in the humoral responses in mice injected with F-DA since Shah et al (1984) reported that pretreatment of Balb/C mice with F-DA or FC-43 i.v. led to a decrease in the in-vivo production of antibodies of SRBC. While this difference may reflect strain variations in response, other factors, especially timing and route of presentation of PFCs relative to the immune challenge, must also be considered. Further work is needed to determine the mechanism(s) by which emulsified PFCs and their components can alter immune system function and identify the active principle(s) involved.

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