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Fluosol 43 particle localization pattern in target organs of rats

An electron microscopy study

Salvatore Caiazza [1], **Carla Fanizza** [1], **and Marco Ferrari** [2]

[1] Department of Ultrastructures and [2] Department of Physiopathology, Istituto Superiore di Sanità, Viale Regina Elena, 299, I-00161 Roma, Italy

Summary. Perfluorocarbons (PFCs), chemically inert fluorinated organic compounds with high binding capacity for O_2, are under discussion as substitutes for erythrocytes. Given the paucity of E.M. observations after their use, the aim of this study was to provide more extensive ultrastructural data about PFC particle size in the emulsion and about the localization pattern in organs of rats after infusion of Fluosol 43. Attention has been focused on the particle relationships with subcellular structures and on the subsequent histopathological implications.

Key words: Fluorocarbons, electron microscopy, subcellular pathology.

Perfluorocarbons (PFCs) are chemically inert fluorinated organic compounds with high binding capacity for O_2 and CO_2 (Dixon and Holland 1975; for a review see Le Blanc and Riess 1982). The availability of stable PFC emulsions, such as Fluosol 43 (FC-43) and Fluosol DA, has made possible their use as breathing liquids (Clark and Gollan 1966) and as substitutes for red blood cells in laboratory animals (Geyer 1973). Recently PFCs emulsions have been tested as blood supplements in patients who required emergency surgical treatment or who refused blood transfusions, as reported in promising clinical studies (Mitsuno et al. 1982; Oda et al. 1982; Tremper et al. 1982).

However, much has been written in the recent past about the large number of possible therapeutic applications of PFCs (For a review see Naito and Yokoyama 1978 and 1981), while tissue response and histotoxicity of such substances are only now receiving the required attention. Most studies have been performed by light microscopy, very few papers reporting ultrastructural observations.

Offprint requests to: S. Caiazza at the above address

In additions to various evidence suggesting that PFCs are not biologically inert materials (Lutz and Metzenauer 1980; Vercellotti et al. 1982), recent reports have described relevant histological changes in PFC treated animals (Kitazawa and Ohnishi 1982; Pfannkuch and Schnoy 1983) and human autopsy cases (Ohnishi and Kitazawa 1980; Mitsuno et al. 1983).

The aim of the present study is to provide more extensive ultrastructural data about the assessment of PFC particle size in the emulsions and about the localization pattern in target organs of rats after infusion of FC-43. Attention has been focused on the particle relationships with subcellular structures and on the subsequent histopathological implications.

Materials and methods

Accordingly to an established procedure (Naito and Yokoyama 1978 and 1981), FC-43 emulsion was prepared by adding to 25 g of perfluorotributylamine (3M Company) and 3.2 g of non-ionic surfactant polyoxypropilene-polyoxyethylene copolymer (Pluronic F-68, BASF Corporation) the amount of Krebs-Ringer hypertonic bicarbonate solution required to obtain 100 g of emulsion with physiological osmolarity, oncotic pressure and buffer capacity.

FC-43 emulsion particles were negatively stained with 2% sodium phosphotungstate (NaPT) and observed with a Zeiss EM 10C electron microscope. Electron micrographs were processed for particle dimension control with an IBAS image automatic analysis system (Kontron), accordingly to a previously reported method (Caiazza et al. 1983).

For the infusion experiment a set of 10 normal healthy young male rats (Charles River strain) weighing 165–210 g was kept in a restraining apparatus, two rats being utilized as control. Each of the other 8 rats was slowly injected via the tail vein with 1–2 ml (4.7–10 ml/kg b.w.) of FC-43 emulsion, without blood depletion. Thereafter animals were kept on normal feed and breathing pure O_2. After 24 h, all of them were sacrified by decapitation and their organs (liver, spleen, lung, heart and kidney) were quickly removed. Tissue samples from each organ were immediately fixed for 50 min in 2.5% glutaraldehyde in Millonig buffer pH 7.4 and postfixed for 60 min in 1% OsO_4 in the same buffer. Embedding was in Epon 812 and sectioning was carried out by means of a Porter-Blum MT2 ultramicrotome. Ultrathin sections were stained accordingly to Karnovsky procedure and observed with a Zeiss EM 10C electron microscope.

Results

A typical E.M. pattern of FC-43 emulsion is shown in Fig. 1. Particles are scarcely electron dense, almost circular in shape, well divided from each other and with an average diameter of 0.08 μm. This value is considered suitable for safe particle infusion in animals (Fujita et al. 1973).

No anomaly in rat behaviour or signs of distress were recorded in rats during the infusion or before the sacrifice. Autopsy examination did not show any significant alteration in organ size and weight. Light microscopy of semi-thin sections did not reveal pathological changes such as acute inflammation or necrosis in any organ.

The initial survey at ultrastructural level was performed to verify the distribution of FC-43 particles in the different organs. We could not demonstrate PFC particles in heart and kidney, therefore we focused E.M. observations on storage (liver, spleen) and elimination (lung) organs.

Generally, intracellular FC-43 particles are not so regular in shape and size as in the original emulsion, frequently appearing gathered and double-

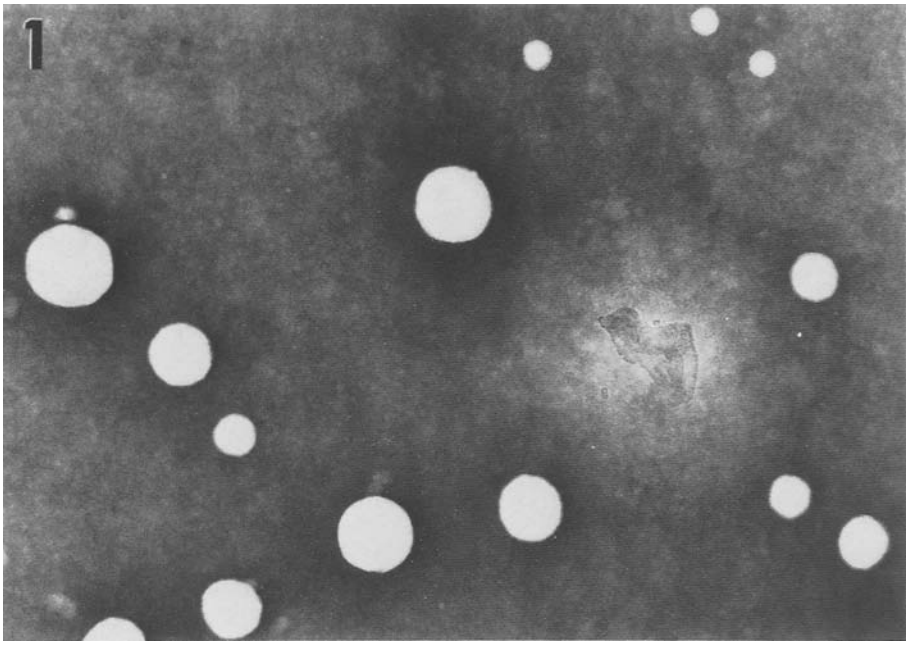

Fig. 1. FC-43 particles in emulsion negatively stained with NaPT. A 0.08 μm average diameter was calculated for over 1,000 particles. × 70,000

membrane bounded (Fig. 2). In contrast, closely aggregated particles seem to be surrounded by a single membrane which may present more than one peculiar indentation by the membranes of contiguous particles (Fig. 3). In addition, a large number of single-membrane bounded particles may be contained in double-membrane limited vacuoles (Fig. 4) and in lysosome-like structures whose matrix is almost completely obliterated (Fig. 5).

In the liver, FC-43 particles at different levels of coalescence are generally found in Kupffer cells (Fig. 6) as well as in hepatocytes where the massive presence of vacuoles in the perinuclear cytoplasm is often observed (Fig. 7).

In the spleen, FC-43 particles at various coalescence levels are found in RES cells, in large vacuolar complexes and in lysosome-like structures as shown in Fig. 8. Paranuclear arrangements of coalescing particles with the above mentioned indentations are frequently observed (Fig. 9).

In the lungs, FC-43 particle aggregates of varying extension are found in alveolar cells (Fig. 10) as well in interstitial cells (Fig. 11).

Discussion

For over 15 years PFC emulsions have been proposed as potential artificial blood substitutes.

However, papers which have been published to date are concerned with animal experimentation and testing in humans (For a review see Le Blanc

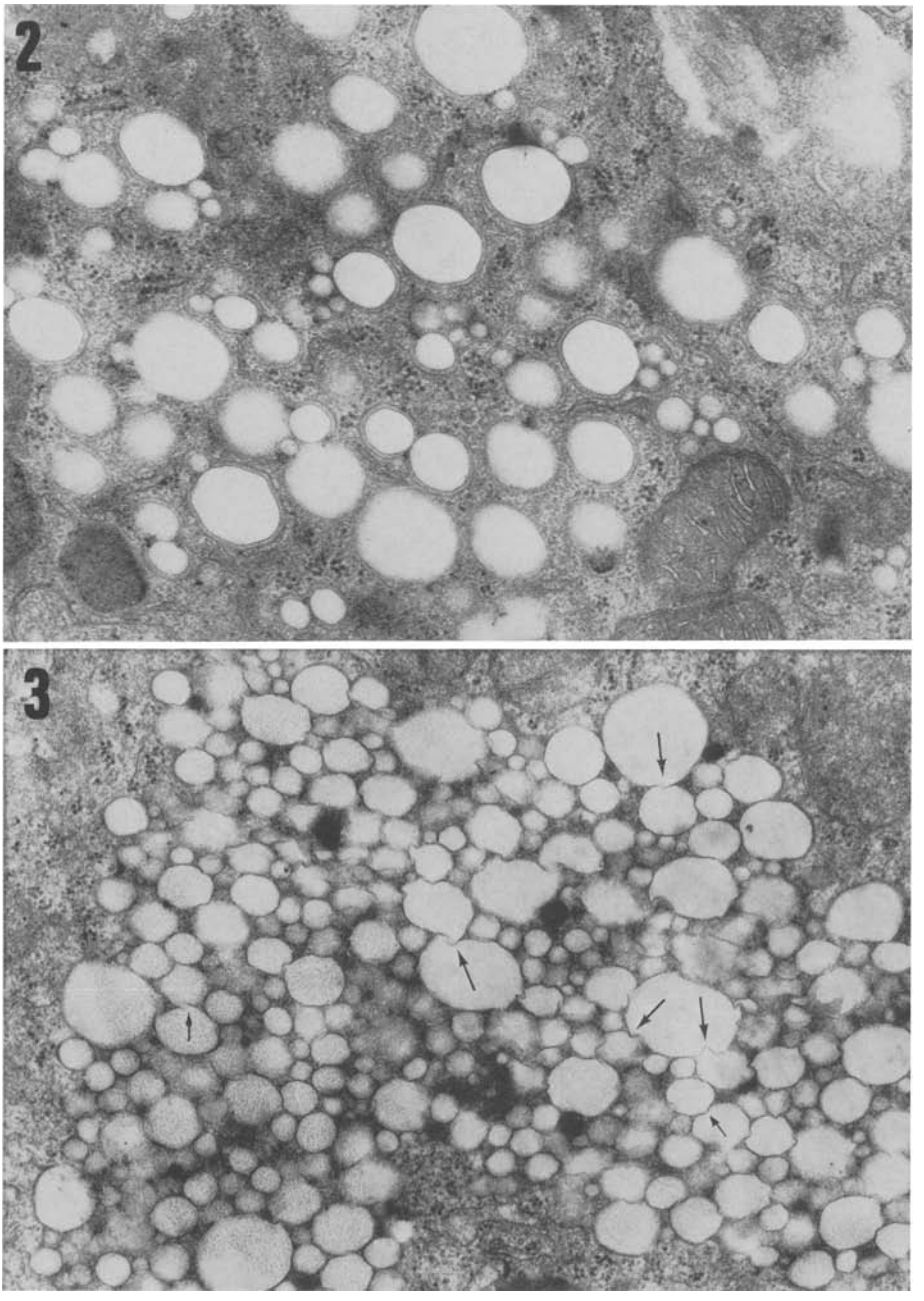

Fig. 2. Rat spleen, 24 h after infusion of FC-43. Double membrane bounded FC-43 particles gathered in RES cell cytoplasm. × 28,000

Fig. 3. Rat spleen, 24 h after infusion of FC-43. Single membrane bounded FC-43 particles massed in RES cell cytoplasm with indentations among contiguous particles (*arrows*). × 28,000

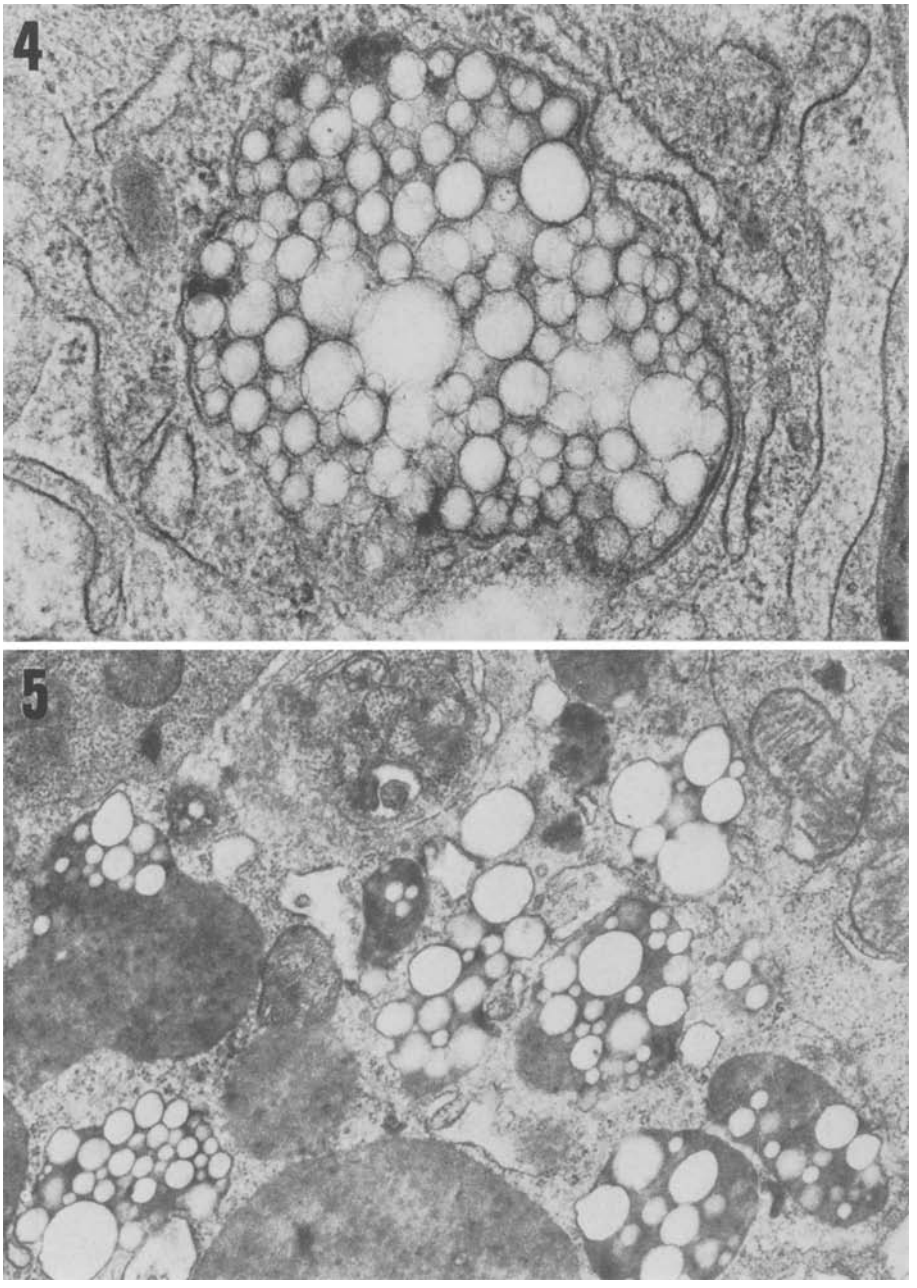

Fig. 4. Rat liver, 24 h after infusion of FC-43. Single membrane bounded FC-43 particles in double membrane limited vacuole of hepatocyte. × 60,000

Fig. 5. Rat spleen, 24 h after infusion of FC-43. Lysosome-like structures containing FC-43 particles in RES cell cytoplasm. × 26,000

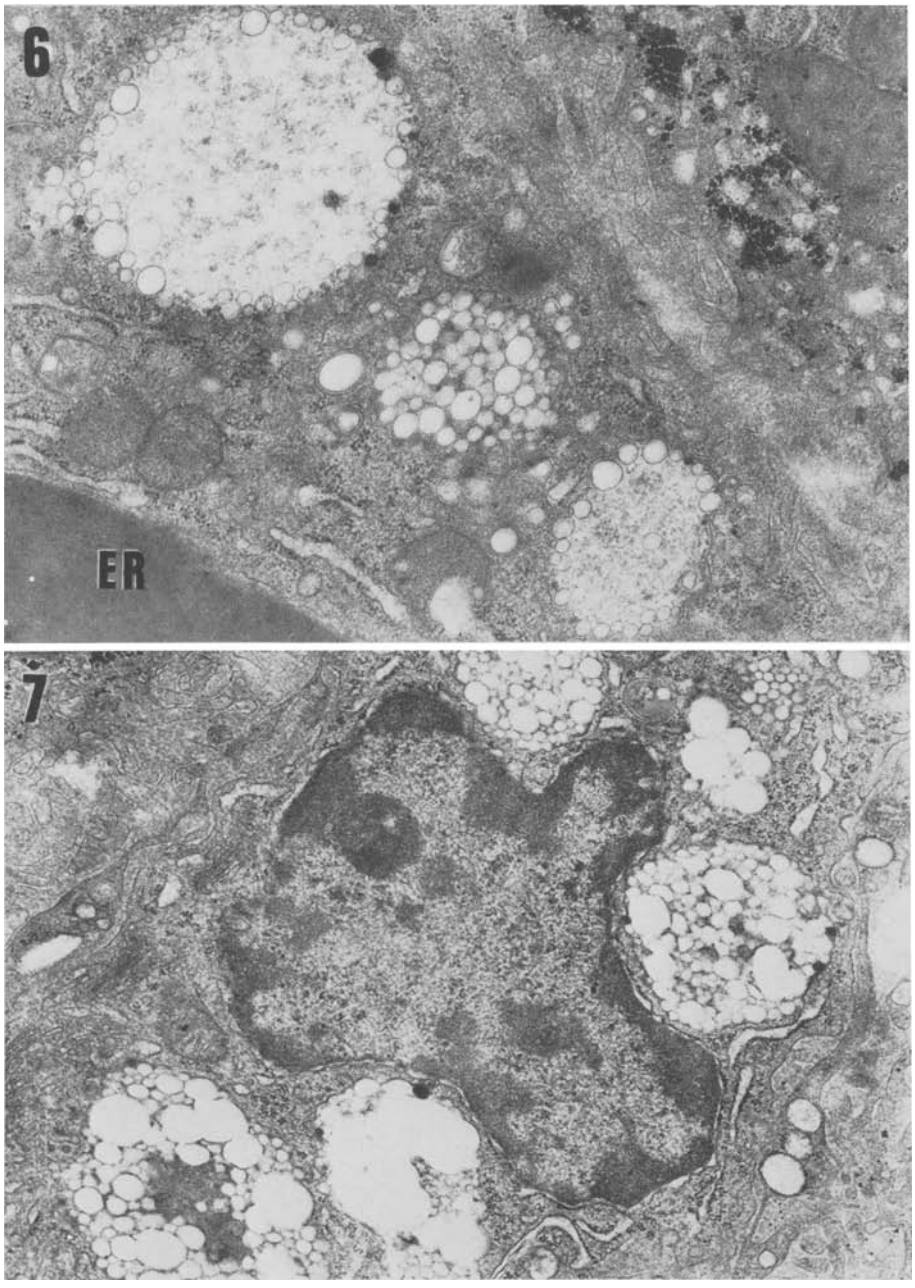

Fig. 6. Rat liver, 24 h after infusion of FC-43. Kupffer cell contains particles at different levels of coalescence in vacuoles. ER = erythrocyte. × 28,000

Fig. 7. Rat liver, 24 h after infusion of FC-43. Hepatocyte contains a typical pattern of perinuclear vacuoles with particles at different levels of coalescence. × 18,000

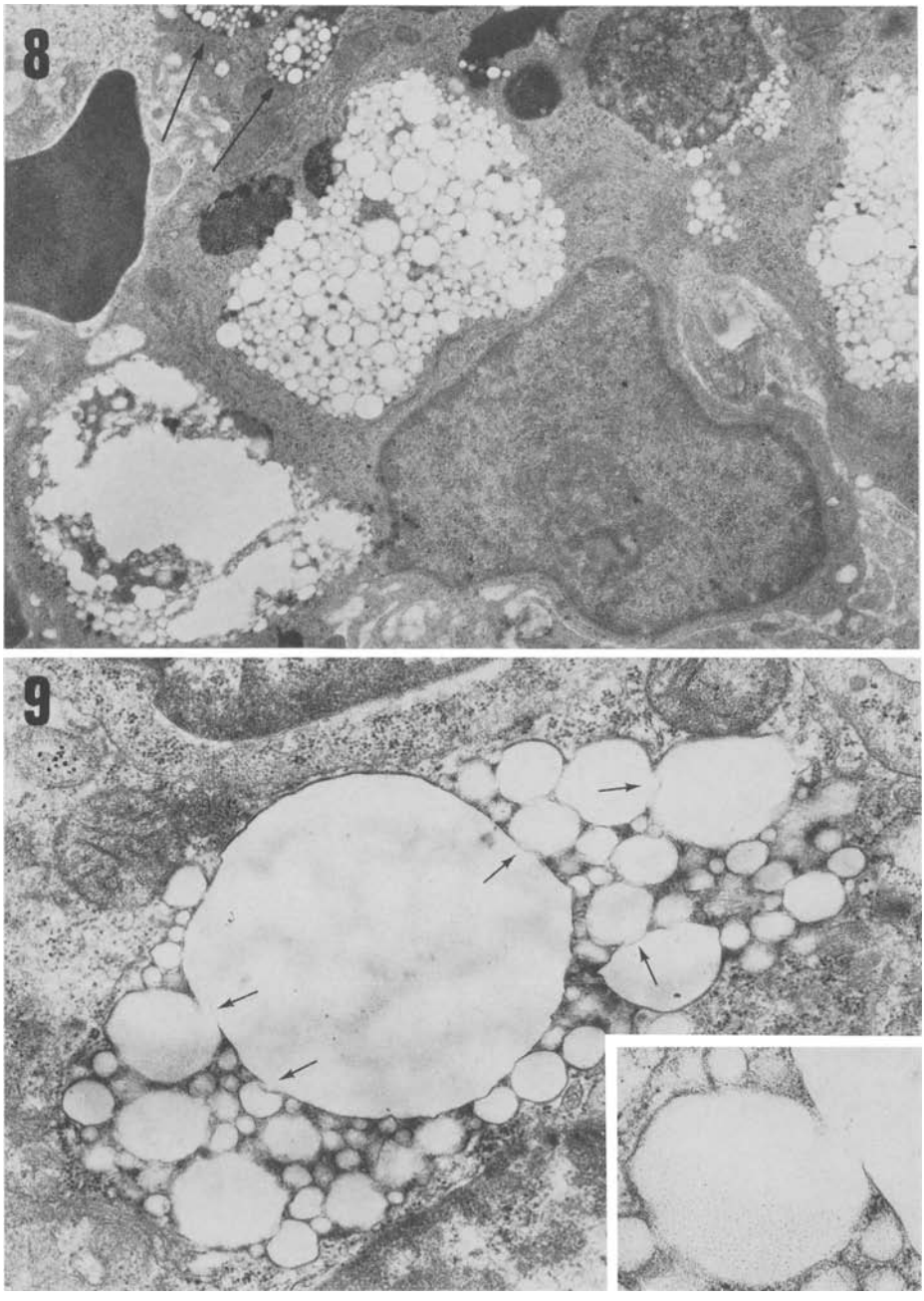

Fig. 8. Rat spleen, 24 h after infusion of FC-43. RES cell with large vacuoles and lysosome-like structures (*arrows*) containing particles at different levels of coalescence. × 10,000

Fig. 9. Rat spleen, 24 h after infusion of FC-43. Paranuclear vacuole, in parenchymal cell, containing particles at advanced level of coalescence with indentations (*arrows*). × 22,000. *Inset:* Detail of indentation. × 53,000

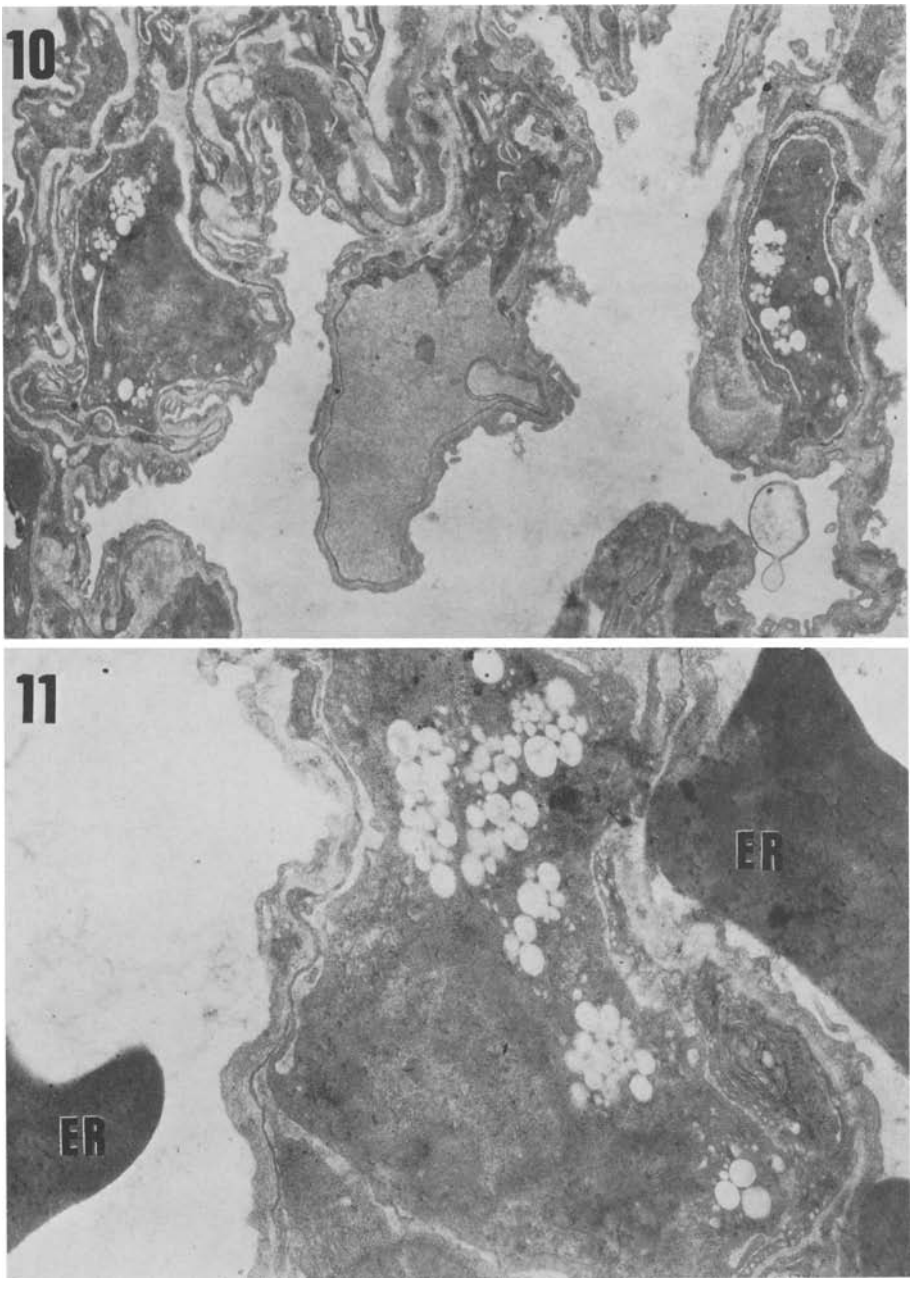

Fig. 10. Rat lung, 24 h after infusion of FC-43. Alveolar cells with vacuoles containing particles at different levels of coalescence. × 6,300

Fig. 11. Rat lung, 24 h after infusion of FC-43. Interstitial cell with vacuoles containing particles at different levels of coalescence. *ER* = erythrocyte. × 12,600

and Riess 1982), the clinical safety of PFC being not yet demonstrated. In spite of some encouraging Japanese results (Mitsuno et al. 1982), a well monitored multipatient study described short-term reactions in two out of seven treated patients. The activation of plasma complement cascade was suggested as underlying mechanism (Tremper et al. 1982) and shown through in vitro studies by other authors (Vercellotti et al. 1982). Furthermore, a specially appointed FDA commitee recently questioned Fluosal efficacy, affirming that the PFCs are not yet ready for marketing (Marwick 1983).

Apart from these and other data, such as the antigenic properties of PFC (Hammarstrom et al. 1983), the alteration of neutrophile and macrophage function (Bucala et al. 1983; Virmani et al. 1983) and reticuloendothelial blockade (Lutz 1983; Castro et al. 1983), lack of biological reactivity of such substances cannot be claimed as a necessary consequence of their chemical inertia. No evidence of PFCs being metabolized has been ever found, the intravascular persistance mainly depending on the injected dose, the emulsion particle size and the surfactant nature. In studies concerning retention and elimination of PFCs the available data have been obtained by chemical methods, i.e. the measure of PFC amount in the air expired by transfused laboratory animals (where the amount expired is related to vapour tension in the lung) and the quantitative analysis of PFC content in storage organs. Finally, long-term histopathological monitoring studies are up to now woefully scarce.

In this framework, electron microscopy allowed us to observe the typical pattern of FC-43 particles in the emulsion (Fig. 1) and the particle arrangement 24 h after rat infusion. Single and variously aggregated PFC particles surrounded by a double membrane (Fig. 2), in complexes without limiting membrane (Fig. 3), in typical vacuoles limited by a double membrane (Fig. 4) as well as in lysosomes (Fig. 5) were described in the cytoplasm of different cells and organs.

The observed variety of particle arrangement could be related to their intracellular dynamics, as seem to be indicated by the massive coalescence figures previously described as foamy cells by light microscopy and considered to be the histopathological indicator of PFC storage (Kitazawa and Ohnishi 1982). We observed significant coalescence phenomena within one day, FC-43 emulsion half-persistance time in the blood stream being about 52 h (Yokoyama et al. 1975). These coalescence phenomena were particularly evident in storage organs such as liver and spleen. Kupffer cells were the place of early intensive coalescence which seemed to proceed in a sequence from the inside to the outside of the vacuoles (Fig. 6). Early particle coalescence was also demonstrated in hepatocytes and, when affecting perinuclear cytoplasm, it may represent a stress for the whole cell (Fig. 7). In this connection, the severe pathological changes described by Rosenblum (1976) in parenchymal and reticuloendothelial cells of the liver could also be the consequence of early nuclear compression.

Miller (1978) suggested intracytoplasmic re-emulsification as a three step process probably leading to particle elimination. In our experience, we can

neither confirm nor reject this model, even if the possible mechanism suggested is very attractive.

The early coalescence phenomena occurring in spleen parenchymal and reticuloendothelial cells, (Figs. 8 and 9) were probably due to the same mechanism observed in liver cells. In this context, the indentations between two or more contiguous particles (Figs. 3 and 9) could play a dynamic role in determining those gaps in the continuity of the particle membranes which allow coalescence.

Given the paucity of data on particle localization in lungs (Schnoy and Pfannkuch 1980; Nanney et al. 1983; Pfannkuch and Schnoy 1983), it seems interesting to point out our results showing early coalescence figures morphologically analogous to those described in liver and spleen in the lung (Fig. 10, 11). It is therefore reasonable to affirm that the same ultrastructural particle arrangement occurred almost contemporaneously in storage organs as well as in the lung.

We underline that, with our experimental FC-43 dosage, the subcellular damage might be considered close to that expected from the dosage commonly proposed for humans (6–25 ml/kg b.w.) (Le Blanc and Riess 1982).

The results of our E.M. observations show the possibility of a kind of damage affecting cell nuclei of both liver and spleen in which particles accumulate. We can merely consider such a mechanical stress as one of the possible hazardous events whose consequences must be evaluated in the framework of the PFC effectiveness and safety assessment.

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