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In vivo evaluation of a reverse water-in-fluorocarbon emulsion stabilized with a semifluorinated amphiphile as a drug delivery system through the pulmonary route

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Abstract

The potential of a reverse water-in-fluorocarbon (w-in-FC) emulsion stabilized with a semifluorinated amphiphile, namely $C_8F_{17}(CH_2)_{11}OP(O)[N(CH_2CH_2)_2O]_2$ (F8H11DMP) for drug delivery through intrapulmonary administration was investigated in the mouse. This study involved assessment of the effect of single or repeated intranasal instillations of a plain emulsion on lung tissue integrity, and evaluation of blood glucose levels in mice treated with an insulin-loaded emulsion. When instilled intranasally to mice, the plain emulsion did not alter lung tissue integrity, as demonstrated by histological staining, and did not induce any airway inflammatory reaction. Treated mice exhibited decreased body weight within the 3–4 days that followed the first emulsion administration, but this decrease was reversible within few days. Mice instilled intranasally with the insulin-loaded emulsion displayed decreased blood glucose levels within the 20 min that followed the administration, thus demonstrating the potential of the reverse w-in-FC emulsion stabilized with F8H11DMP to systemically deliver drugs, including peptides, upon lung administration.

Keywords: Drug delivery; Lung; Reverse water-in-fluorocarbon emulsion; Fluorinated surfactant; Insulin

1. Introduction

Advances in engineering procedures aimed at controlling the dispersibility of drugs, the particle size

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of vectors, and the consistency of the delivery have made pulmonary delivery a feasible approach for many therapeutics. As clinical experience and data accumulate in support of the efficacy and safety of inhaled drugs, pharmaceutical developers are recognizing that pulmonary delivery offers unique advantages. This delivery route has a substantial impact on four areas of pharmaceutical development: proteins and peptides, fast-acting drugs, localized lung infections and other pulmonary disease treatments, and vaccines (Courrier et al., 2002).

Inhaled versions of proteins and peptides, presently which are chiefly administered via injection to avoid breaking down in the gastrointestinal tract, have shown promise. Pulmonary insulin, in particular, is in late-stage human clinical testing, its most advanced version being Exubera®, which is under development by Pfizer, Aventis and Nektar. Clinical data for Exubera® indicate that the drug can provide better glycaemic control than combinations of oral diabetic therapies. Fast onset is a key consideration for many drugs, particularly for treating pain, nausea, anxiety and hypertension.

Infections remain a leading cause of death worldwide, pneumonia and other lung infections account for a large proportion of those deaths. Such diseases respond well when treated locally with pulmonary-administered antibiotics. A drug delivered via the pulmonary route acts directly at the infected site, offering the potential for faster, more efficient therapy with reduced risk of drug resistance and fewer side effects. Similar treatment benefits may be found with pulmonary drugs destined to local treatment of lung diseases such as severe asthma, emphysema or cancer (Gonda, 2000). In addition, inhaled vaccines are of great interest to pharmaceutical developers because many currently approved vaccines address respiratory diseases. Almost all of these vaccines are currently injected; one intranasal vaccine against flu is nearing commercialization. Pulmonary vaccines offer major benefits, including greater efficacy through the stimulation of mucosal and humoral immunity, which could help stopping infections at their port of entry.

We have recently investigated the potential of reverse water-in-fluorocarbon (w-in-FC) emulsions as drug delivery system through the pulmonary route. Fluorocarbons have a wide potential of medical applications (Krafft, 2001; Riess, 2001; Schutt et al., 2003) that is related to their specific physical and chemical characteristics (Riess, 2002). Progress in FC emulsion research has recently been reviewed (Krafft et al., 2003). The stability and in vitro toxicity of a range of w-in-FC emulsions emulsified with a range of fluorinated amphiphiles having a dimorpholinophosphate polar head group (FnHmDMP, with n = 4, 6, 8, 10 and m = 2, 5, 11) were investigated (Courrier et al., 2003, 2004). These studies led us to select $C_8F_{17}(CH_2)_{11}OP(O)[N(CH_2CH_2)_2O]_2$ (F8H11DMP) among the various amphiphiles tested. Indeed, F8H11DMP, when incubated for 48 h at a concentration of 1.85×10^{-2} M (corresponding to 1.5%, w/v) in perfluorooctyl bromide (PFOB) exhibited little (12%) toxicity towards human lung epithelial cells in culture. Likewise emulsions stabilized with 1.5s F8H11DMP appeared to be non-cytotoxic (Courrier et al., 2003). The present study was designed to evaluate the in vivo safety of the reverse w-in-FC emulsion stabilized with F8H11DMP, and the capacity of this emulsion to deliver drugs upon intrapulmonary administration. This study was conducted in mice instilled intranasally once or once daily for four consecutive days with a w-in-PFOB emulsion stabilized with 1.5% F8H11DMP. Each emulsion administration represented a total dosage of 15 mg per kg body weight of F8H11DMP, which is far below the LD_{50} of the compound (4 g per kg body weight) administered through the intraperitoneal route (Sadtler et al., 1998). Safety was assessed by daily observation of the animals and determination of their body weight as a general health index, histological examination of lung sections to assess tissue integrity, and determination of total and differential cell counts in bronchoalveolar lavage (BAL) fluids to underline a possible inflammatory reaction. The capacity of the emulsion to deliver drugs was in mice instilled intranasally with a stabilized emulsion loaded with insulin and by measuring serum glucose levels.

2. Materials and methods

2.1. Animals

Nine-to-ten weeks old male BALB/c mice (25–30 g) were purchased from Charles River Laboratories (Saint-Germain-sur-l'Abresle, France). The animals

were maintained under controlled environmental conditions with a 12 h/12 h light/dark cycle according to EU guidelines for use of laboratory animals. Food (UAR-Alimentation, Villemoisson, France) and tap water were available ad libitum.

2.2. Synthesis and characterization of the semifluorinated amphiphile

The (perfluorooctyl)undecyl dimorpholinophosphate amphiphile F8H11DMP was synthesized from perfluorooctyl iodide as previously reported (Sadtler et al., 1998). It was thoroughly purified by successive recrystallizations from hexane. Its purity was checked by ^{1}H, ^{31}P and ^{13}C NMR (Brucker AC 200), and by elemental analysis. Perfluorooctyl bromide (PFOB) was a gift from Alliance Pharmaceutical Corp. (San Diego, CA, USA). All the other reagents used in this study were of analytical grade.

2.3. Preparation of the reverse water-in-fluorocarbon emulsions

Plain and insulin-loaded w-in-PFOB emulsions stabilized with F8H11DMP were prepared as follows: F8H11DMP (1.5 g) was solubilized in 100 ml of PFOB under gentle agitation. Five ml of saline (NaCl 0.9%, w/v) or of a commercial aqueous solution of insulin at 100 U/ml (Umuline humaine rapide, Lilly France S.A., Saint-Cloud, France) were added dropwise to the PFOB phase under agitation using an Ultra Turax mixer (model T25, Ika-Labortechnik, Stanfen, Germany) at 8000 rpm. The mixture was then homogenized at 24000 rpm for 10 min. The resulting coarse emulsion was further homogenized under high pressure (1000 bar, 10 cycles) using a Microfluidizer (model 110, Microfluidics Corp., Newton, MA, USA). The mean diameter of the water droplets was assessed by quasi-elastic light scattering (Zetasizer 3000 HS Malvern Instruments, UK). The emulsion containing insulin was not heat-sterilized in order to avoid degradation of the peptide, and was used within 3 days after its preparation (Patel et al., 1991).

2.4. Administration of the emulsions

Plain or insulin-loaded w-in-FC emulsions were administered to mice one time, or once daily for four consecutive days by intranasal instillation. Each instillation (25 μl) represented a total dosage of 15 mg per kg body weight of F8H11DMP and 5 U per kg body weight of insulin in the case of mice instilled with the insulin-loaded emulsion. Control animals received intranasal instillations of either saline (NaCl 0.9%, w/v), B. Braun, Boulogne, France) or of PFOB that constituted the continuous phase of the reverse emulsions. Intranasal instillations were performed under anaesthesia (50 mg per kg body weight ketamine (Imalgene® 1000, Merial, France) and 3.33 mg per kg body weight xylazine (Rompun® 2%, Bayer, France given i.p.).

2.5. Clinical observation of the mice

The mice were carefully observed each day during 2 weeks for signs of suffering, state of their fur, general behaviour and body weight.

2.6. Determination of total and differential cell counts in bronchoalveolar lavage

Determination of total and differential cell counts in BAL fluids of treated mice was used to assess a possible inflammatory reaction in response to the single or repeated administrations of the emulsion. Bronchoalveolar lavage were achieved 18–24 h after the last emulsion delivery was administered. The mice were anaesthetized by i.p. injection of ketamine (150 mg per kg body weight) and xylazine (10 mg per kg body weight). The trachea was canulated and the lungs were washed using 10 instillations of 0.5 ml of ice-cold saline supplemented with 2.6 mM of EDTA (saline-EDTA). The fluids recovered from the instillations were centrifuged (4100 rpm for 5 min at 4 °C) to pellet cells and the erythrocytes were lysed by hypotonic choc. The cells were then resuspended in 500 μl of ice-cold saline-EDTA and total cell counts were determined using a hemocytometer (Neubauer's chamber). Differential counts were assessed on cytological preparations. Slides were prepared by cytocentrifugation at 700 rpm for 10 min (Cytospin 3, Shandon Ltd., Runcorn, Chershire, UK) of 200 μl of diluted BAL fluids (250,000 cells/ml in ice-cold saline-EDTA) and the slides were stained with Diff-Quick (Dade Behring, Narburg, Germany). Determinations were performed by counting at least 400 cells for each preparation. The cells were identified

as macrophages, neutrophils, eosinophils and lymphocytes.

2.7. Histological studies

Histological studies were achieved 18–24 h or two weeks after the last emulsion delivery was administered. The mice were anaesthetized as previously described. The lungs were dissected out and rinsed free of blood by perfusing ice-cold phosphate-buffered saline (PBS) through the left ventricle. The lungs were then inflated with 4% paraformaldehyde (PFA) in PBS and immersed in a fixative for 24 h at 4 °C. The fixed lungs were rinsed in PBS, dehydrated and embedded in paraffin using standard procedures. Five-micrometer tissue sections were prepared, stained with haematoxylin-eosin and observed under light microscopy.

2.8. Determination of serum glucose levels in mice treated with the insulin-loaded emulsion

The time-course of the effect of intranasal instillation of the insulin-loaded emulsion on serum glucose levels was assessed in individual animals taking into account the small blood volume of the mouse. At the chosen time (10, 20 min or 40 min) after administration of the emulsion, the mice were anaesthetized by i.p. injection of ketamine (150 mg per kg body weight) and xylazine (10 mg per kg body weight). Blood was drawn from the abdominal vein and incubated for 1 h at room temperature. Sera were then collected by centrifugation (4100 rpm for 15 min at 4 °C) and stored at 4 °C until analysis. Glucose levels in sera were determined using a Synchron CX5® Delta analyzer (Beckman Coulter, Brea, CA, USA). Control mice included mice instilled with the plain emulsion or with free insulin (5 U per kg body weight).

2.9. Statistical analysis of the data

Results are presented as means ± S.E.M. Statistical differences between controls and treated groups were analyzed from raw data by analysis of variance, followed by unpaired two-tailed Student's *t*-test. Data were considered as significantly different when $P < 0.05$.

3. Results and discussion

3.1. Evaluation of safety of the emulsion

3.1.1. Clinical observation and assessment of body weight

Mice instilled intranasally once, or once daily on four consecutive days, with either PFOB (the continuous phase of the emulsion) or with the emulsion did not exhibit any particular evidence of suffering, as compared to mice treated with saline.

A slight decrease in body weight was observed for mice treated once or four times with saline or PFOB (Fig. 1a and b). This decrease reached significance 2 days after administration in animals treated once with PFOB (3% decrease, $P < 0.05$). However, the decrease in body weight was not significant for mice instilled repeatedly with PFOB. Moreover, in all groups, whether treated with saline or PFOB, once or four times, body weight returned progressively to normal, and then increased as compared to the initial body weight. Intranasal administration of the reverse emulsion led to similar variations in body weight than those observed for controls (Fig. 1c). However, the decreases in body weight were much more pronounced. The mice that received a single administration of emulsion lost 1.9 ± 0.3 g (6.8% decrease, $P < 0.05$) during the day that followed administration, with no further significant decrease thereafter and restoration of a normal weight within 3 days. Mice that received repeated instillations lost 4.5 ± 0.9 g ($P < 0.01$) during the first 2 days and recovered their initial weight after 9 days, namely 5 days after the last administration.

3.1.2. Lung tissue integrity

As shown in Fig. 2, histological studies carried out 18–24 h after a single or four repeated instillations of the reverse emulsion did not show any particular sign of lung tissue injury. Indeed, normal appearance of both epithelium (Fig. 2, left panels) and alveoli (Fig. 2, right panels) was observed in animals treated with the emulsion (Fig. 2c and d), as compared to saline- (Fig. 2a) or PFOB- (Fig. 2b) exposed animals. Epithelial cells displayed normal morphology with intact cilia. The number of macrophages present in alveolar spaces was unchanged as compared to controls. No inflammatory cell infiltrate was observed in airway lumen or tissue.

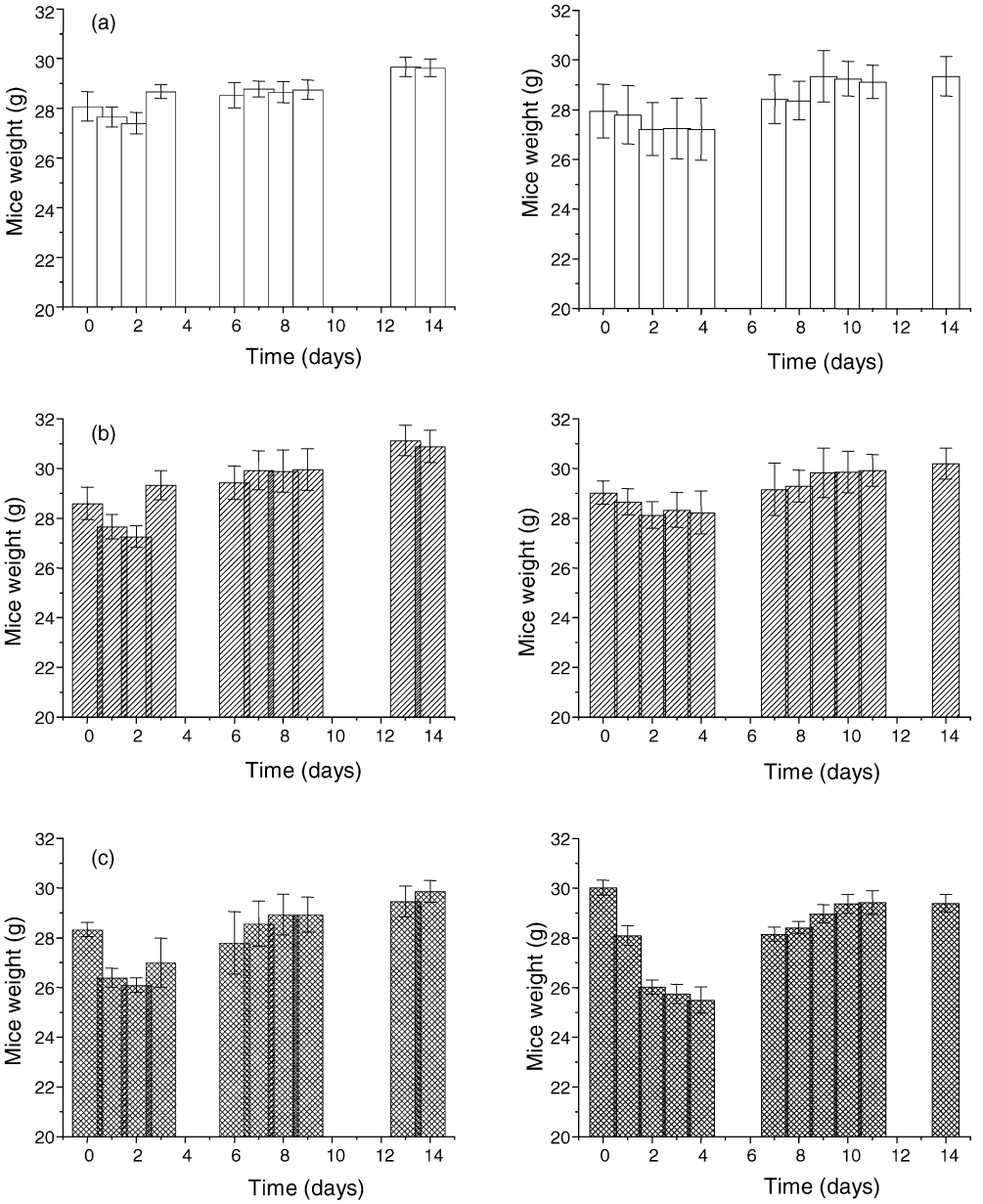

Fig. 1. Body weight of mice instilled intranasally once (left panel) or once daily for four consecutive days (right panel) with: (a) saline; (b) neat PFOB or; (c) a reverse w-in-PFOB emulsion stabilized with F8H11DMP. Body weight measurements started the day of the first administration and lasted for 14 days.

3.1.3. Assessment of airway inflammation

Fig. 3 shows total cell numbers found in BAL fluids from mice exposed to four intranasal instillations of PFOB or to one or four intranasal instillations of the reverse water-in-fluorocarbon emulsion, as measured 18–24 h after the last instillation. No significant change in total cell concentrations was observed, whatever the treatment, as compared to numbers found in

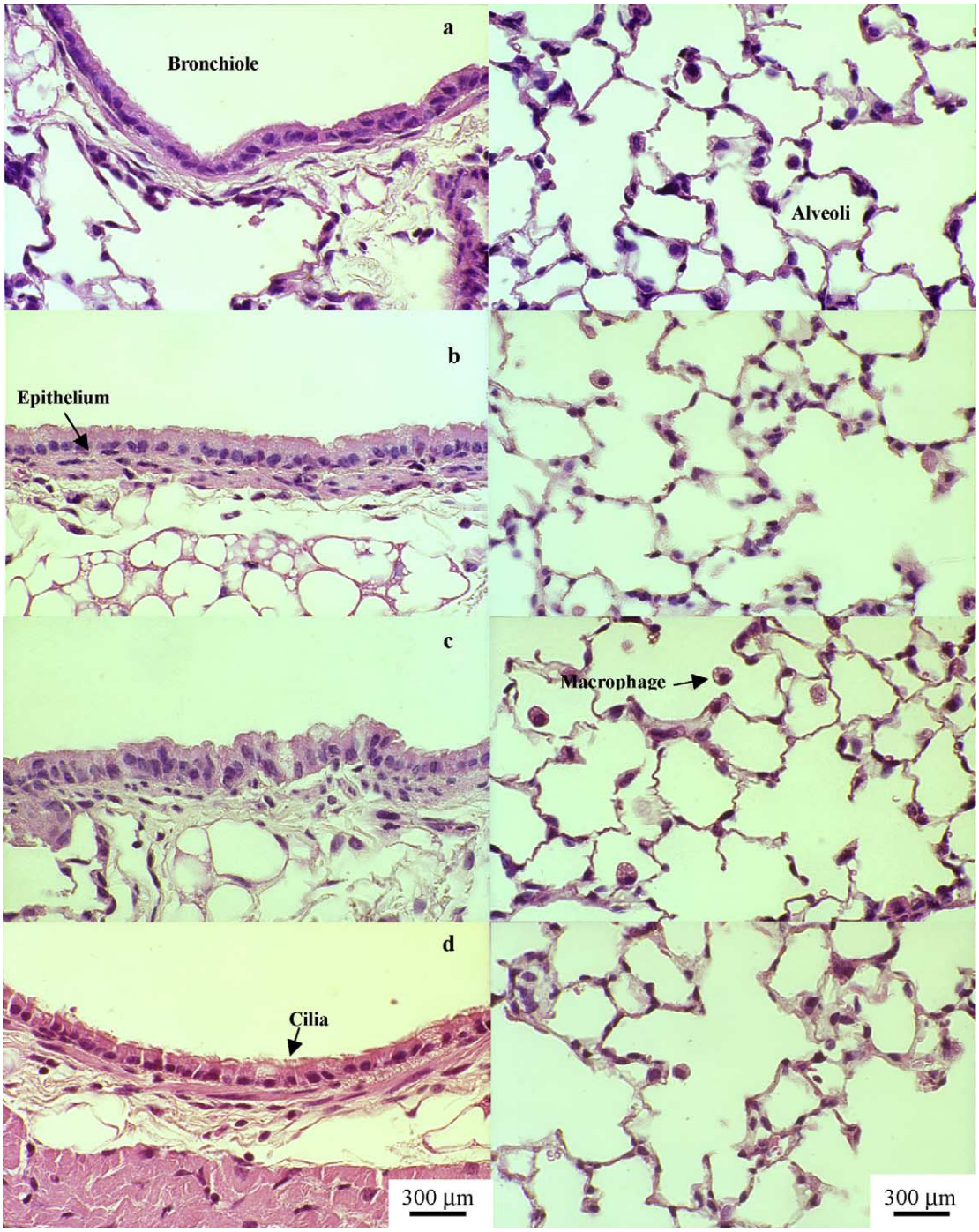

Fig. 2. Light micrographs of lung tissue from mice instilled intranasally once or once daily for four consecutive days with saline, PFOB or the reverse w-in-PFOB emulsion stabilized with F8H11DMP. Left panels: epithelium. Right panels: parenchyma. (a) Mice exposed once to saline; (b) Mice exposed once to PFOB; (c) Mice exposed once or; (d) four times to the emulsion. Initial magnification ×40.

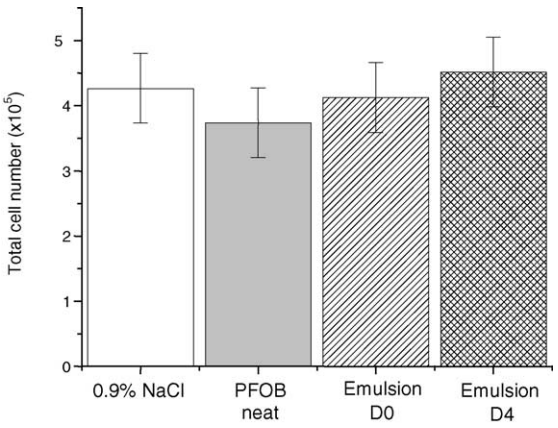

Fig. 3. Total cell numbers in bronchoalveolar lavage fluids from mice exposed to four intranasal instillations of saline or PFOB, or to one (D_0) or four (D_4) intranasal instillations of the reverse w-in-PFOB emulsion stabilized with F8H11DMP. Bronchoalveolar lavage fluids were collected 18–24 h after the last instillation. Data are means ± S.E.M. of n = 2–4 animals.

saline-treated mice, suggesting the absence of inflammatory reaction in response to administration of the emulsion. In agreement with this observation, cytological preparations from BAL fluids revealed that BAL cells were composed essentially of macrophages, whatever the treatment.

Taken together, these results provide encouraging data on the in vivo safety of our reverse w-in-FC emulsion stabilized with F8H11DMP. Indeed, locally, single or repeated administrations of the emulsion did not result in any sign of tissue injury or pro-inflammatory activity that could result in loss of lung integrity and function. This observation is in agreement with our previous results obtained on human lung epithelial cells in culture (Courrier et al., 2003). Clinical observation did not show evidence of any major sign of toxicity, but assessment of body weight revealed some weight loss. Although more pronounced for mice treated with the reverse emulsion, weight losses were also observed for control mice (exposed to either saline or PFOB), and were reversible. Therefore, these effects most probably result from the stress caused to the animals by anaesthesia and the intranasal administration procedure, rather than from a toxicological effect of the emulsion.

3.2. Evaluation of delivery of insulin from the emulsion

3.2.1. Physical stability of the insulin-containing emulsion

The stability of the insulin-containing reverse w-in-PFOB emulsion was determined by monitoring the variation of the mean diameter of the water droplets as a function of time (Fig. 4). The presence of insulin influenced the initial mean diameter of the water droplets and its variation upon time. When measured

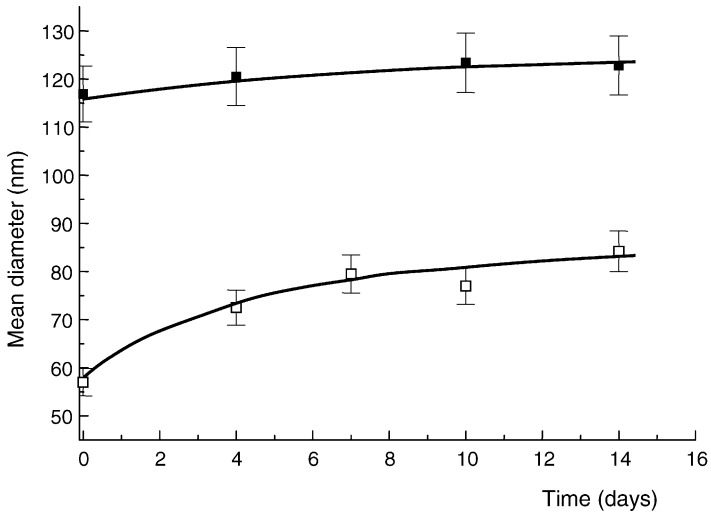

Fig. 4. Mean diameter of water droplets of a plain (□) or an insulin-loaded (■) reverse water-in-PFOB emulsion as a function of time at room temperature (n = 3).

immediately after preparation, this mean diameter was higher for the insulin-loaded emulsion than in the unloaded control emulsion (115 ± 8 nm versus 60 ± 5 nm). Upon ageing at room temperature, the insulin-containing emulsion was at least as stable as the control emulsion. The reverse emulsion that contained insulin had a milky appearance and did not cream.

3.2.2. Serum glucose levels in mice injected with the insulin-loaded emulsion

The time-courses of the changes in serum glucose levels induced by intranasal administration of the insulin-loaded emulsion, as compared to the changes induced by intranasal administration of the plain emulsion or of a solution of free insulin as controls, are shown on Fig. 5. Administration of the plain emulsion resulted in a slight decrease in blood glucose levels, which reached 23% at 10 min and tended to reverse upon time. In contrast, administration of the insulin-loaded emulsion resulted in a more pronounced and more sustained reduction in blood glucose levels. This reduction was progressive and reached 47% after 20 min ($P < 0.01$). At 40 min, glucose levels were lowered by 70%. Administration of an insulin solution also resulted in decreased blood glucose levels. This decrease was similar in amplitude to the one triggered by the insulin-loaded emulsion (70% decrease at 40 min). However, the decrease was faster with the insulin solution, since it was already substantial 10 min after administration.

These results allow us to conclude that insulin could be incorporated into the internal phase of the reverse emulsion without destabilizing this emulsion. The reverse emulsion was capable of delivering insulin systemically after intrapulmonary administration, as evidenced by the observed decrease in serum glucose levels. Although the hypoglycaemic effect due to the insulin incorporated into the internal phase of the emulsion was somewhat delayed as compared to the effect triggered by the free insulin solution, it occurred with the same kinetic as the effect induced by insulin encapsulated in liposomes. Indeed, Liu et al., 1993 demonstrated that insulin encapsulated into dipalmitoylphosphatidylcholine/cholesterol DPPC/CHOL (7:2) liposomes lowered blood glucose levels to about 70% of their initial levels after 40 min and led to maximal hypoglycaemic effect after 60 min. On the other hand, the hypoglycaemic effect induced by the insulin-loaded reverse emulsion

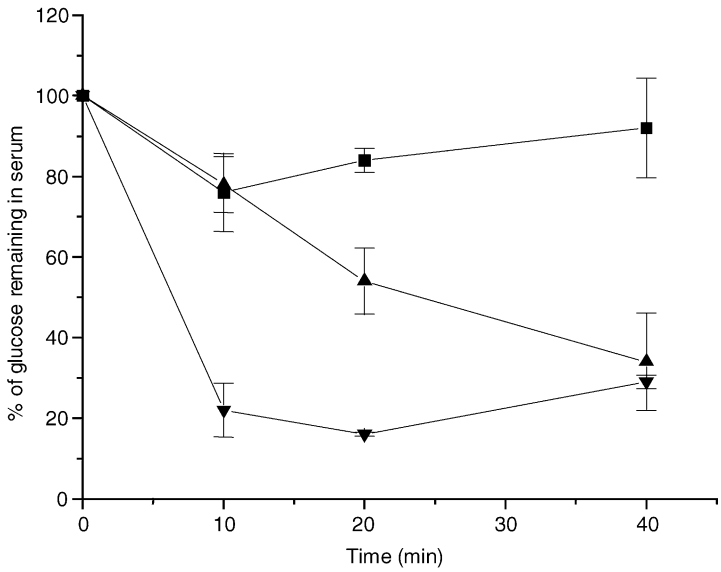

Fig. 5. Time-course of changes in blood glucose levels in mice instilled intranasally with a plain (■) or an insulin-loaded reverse w-in-PFOB (▲) emulsion or with a solution of free insulin (▼). Final dosage of insulin was of 5 U per kg body weight in both cases. Data were expressed as percent of serum levels found in the blood of untreated mice. They are means ± S.E.M. of $n = 4$ animals.

occurred more quickly than that evoked by insulin encapsulated in polymeric particles. Indeed, administration of porcine zinc insulin encapsulated into poly(butylcyanoacrylate) nanoparticles lowered blood glucose levels by less than 20% of their initial value after 1 h and led to maximal hypoglycaemic effect after 4 h (Zhang et al., 2001).

Whereas, several studies describe the release of insulin administered by the intrapulmonary route, the mechanisms of release and absorption of insulin encapsulated into carriers like emulsions are not known. A recent study (Mitra et al., 2000) reported that absorption of insulin encapsulated into oil-in-water (O/W) emulsions after intranasal administration is more effective than that of insulin encapsulated into water-in-oil (W/O) emulsions. The slower release of insulin was attributed to the oily phase that constituted an additional barrier during the nasal absorption process of the peptide by spreading on the epithelium of the nasal mucous membrane. In our study, the fluorocarbon that constitutes the external phase of the emulsions has very different physicochemical properties (Riess, 2002). Indeed, PFOB has a high fluidity and is able to flow easily into the deep respiratory part after an intranasal administration. The reverse emulsion, due to the high spreading coefficient of PFOB, likely forms a thin film on the mucous membrane containing the aqueous droplets. Perfluorooctyl bromide tends also, at a much slower rate, to evaporate due to its high vapor pressure. As a consequence, the water droplets of the internal phase readily come in contact with the pulmonary surfactant. As observed with a monolayer of DPPC, used as a model of pulmonary surfactant (Hirana et al., 2003), the fluorinated surfactant used to stabilize the emulsion is miscible with the DPPC monolayer, which may facilitate the release of the drug from the water droplets, and its absorption. The mechanism of pulmonary absorption of drugs described by Li et al. (1993) supports our hypothesis. These authors concluded that a surfactant was able to decrease the surface tension between the aqueous insulin-containing solution and the pulmonary surfactant, reduce the viscosity of the mucous layer and enhance the pulmonary absorption of insulin. In addition, Mitra et al. (2001), Todo et al. (2001), Suarez et al. (2001), and Kumar and Misra (2003) showed, in the same way, that the bioavailability of insulin, after pulmonary administration, was enhanced by phospholipids, Span 85 and other surfactants.

4. Conclusions and prospects

The present work shows that a reverse water-in-fluorocarbon emulsion stabilized with a fluorinated amphiphile having a dimorpholinophosphate polar head group, F8H11DMP, may be a valuable vehicle for administering systemic drugs through the pulmonary route. Indeed, both encouraging toxicological data and evidence of systemic delivery of insulin are here reported in mice intranasally injected with this emulsion. Further studies on this vehicle will be devoted to gathering further safety data, to studying the extent of the release of encapsulated peptides by the emulsion, and to assessing whether the reverse emulsion can be delivered into the lung as an aerosol, taking into account that this reverse emulsion is very stable in hydrofluoroalkane (HFA 227) (Butz et al., 2002).

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