In Vivo ESR Studies on Pharmacokinetics and Metabolism of Parenteral Lipid Emulsion in Living Mice

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Received November 27, 1995; accepted February 6, 1996

Purpose. We applied non-invasive and real-time method with *in vivo* ESR spectroscopy to determining pharmacokinetics and metabolism of lipid emulsion as a drug carrier in living mice.

Methods. A spin-labeled triglyceride (SL-TG) was newly synthesized and lipid emulsion containing SL-TG was prepared. *In vivo* ESR spectra in mice were observed after intravenous administration of the lipid emulsion.

Results. *In vivo* ESR spectra consisted of three components, coinciding with the *in vitro* spectra of SL-TG particles, free and immobilized fatty acids. The amount of the components depended on both the observing domain and the period after administration. In the chest, all three components were observed, while SL-TG particle was lacking in the abdomen. The half-life of the lipid particles in the chest was 2 hr.

Conclusions. Non-invasive and real-time analysis of drug carriers in living animal is successfully accomplished using an *in vivo* ESR method.

KEY WORDS: *in vivo* ESR; spin-label; lipid emulsion; pharmacokinetics.

INTRODUCTION

Lipid emulsions are dispersions of lipid particles in aqueous solution, and are commonly used as nutrients. Recently, lipid emulsions have come to be used as drug carriers in drug delivery systems (1). For example, Prostaglandin E_1 in injectable lipid emulsion form has been well established, and is commercially available as "Palux®". This lipid emulsion retains a significant amount of Prostaglandin E_1 in lipid particles (2), and exhibits remarkably high efficacy in clinical treatment (3). However, neither the lifetime nor the metabolic fate of lipid particles in the body has been clearly determined.

The radioautography method is widely used to determine the disposition of lipid emulsions (4, 5). A disadvantage of this method is that an animal must be sacrificed at each point of measurement, and that real-time data concerning tissue distribution cannot be obtained from a whole living animal. The recent development of the L-band electron spin resonance (ESR) apparatus has enabled *in vivo* measurement of radicals in whole animals. We have used non-invasive methods to measure the *in vivo* ESR spectrum with the nitroxide radical as a spin-probe in whole mice after the intravenous (6, 7), intraperitoneal (8), intramuscular (6), or intratracheal (9) injection, and found that the signal decay of the nitroxide radical varied in the presence of certain physiological or pathological phenomena. The spectral pattern of the nitroxide radical is very characteristic and sensitive to the dynamic state of the probe, and pattern analyses make possible estimation of molecular state. These findings suggested the use of *in vivo* ESR measurement to determine the pharmacokinetics and metabolism of lipid emulsion in the living body.

In the present study, we investigated, using non-invasive methods, the distribution, pharmacokinetics, and metabolism of lipid particles, which were prepared as the same procedure as the commercially available drug carrier, in living mice with *in vivo* ESR spectroscopy. For this purpose, we synthesized spin-labeled triglyceride (SL-TG). The behavior of SL-TG, which constituted the lipid particles, was analyzed qualitatively and quantitatively with this method.

MATERIALS AND METHODS

Materials

Egg yolk phosphatidylcholine and concentrated glycerin JP XII were purchased from Asahi Kasei Co., Ltd. (Tokyo, Japan) and Kozakai Pharmaceutical Co., Ltd. (Tokyo, Japan), respectively. Bovine serum albumin (BSA) and 1,2-dioleoyldiglycerol were from Sigma Chemical Company, (St. Louis, USA). Oleic acid and lipoprotein lipase (LPL) from *Pseudomonas* spiritus were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Ketamine hydrochloride used for anesthesia was from Sankyo Co., Ltd. (Tokyo, Japan). All other chemicals were of reagent grade.

Synthesis of Spin-Labeled Triglyceride

12-(N-oxyl-4,4′-dimethyloxazoline)-stearic acid (12SLS) was prepared using the method of Waggnor et al. (10). The spin-labeled triglyceride (1,2-dioleoyl-3-12SLS-glycerol) was synthesized by the condensation of 1,2-dioleoyl-glycerol with 12SLS in the presence of N,N′-carbonyldiimidazole, and purified by silica-gel column chromatography. The SL-TG prepared was an orange oil, and gave a single spot on thin-layer-chromatography. ESR spectrum of SL-TG in hexane exhibited triplet sharp lines (hyperfine splitting, hfs = 1.56mT).

Preparation of Lipid Emulsion

Lipid emulsion, which contains 36 mg of egg yolk lecithin, 4.8 mg of oleic acid, 200 mg of SL-TG, and 44.2 mg of concentrated glycerol in 2 ml of the water for injection, was prepared using the same procedure as for a commercially available lipid emulsion, as previously described (11). The pH of the emulsion was adjusted to 5.4 with sodium hydroxide aqueous solution. The particle size of the lipid emulsion obtained was determined to be about 200 nm using a light scattering spectro-

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0724-8741/96/0500-0729$09.50/0

meter (Nicomp Model 370 Submicron Particle Sizer, Pacific Scientific Instrument Division, Germany).

In Vitro ESR Measurement by X-band ESR Spectrometry

10 μl of lipid emulsions (equivalent to 1 μmole SL-TG) and LPL (0.1 unit) with or without the 12 mg of BSA were added to 290 μl of phosphate buffered solution (0.1 M, pH 7.4) and incubated at 37°C for 0, 5 or 30 min. After incubation, the samples were transferred to disposable capillary tubes (Drummond Scientific Co., U.S.A.) and X-band ESR spectra were obtained at room temperature with a JES-RE-1X (JEOL, Tokyo, Japan) with the following parameters: microwave frequency, 9.4 GHz, microwave power, 5 mW; modulation width, 0.125 mT; external magnetic field, 336.3 ± 5.0 mT; sweep time, 2 min; and time constant, 0.03 sec.

In Vitro ESR Measurement by L-band ESR Spectrometry

100 μl of lipid emulsion was added to 900 μl of phosphate buffered solution (pH 7.4) with either or both of LPL (1 unit) and/or 40 mg of BSA and incubated at 37°C for 30 min. After incubation, the samples were transferred to a glass tube and L-band ESR spectra were obtained at room temperature with a JES-RE-1L (JEOL) with the following parameters: microwave frequency, 1.1 GHz, microwave power, 3 mW; modulation width, 0.1 mT; external magnetic field, 40.0 ± 5.0 mT; sweep time, 8 min; and time constant, 1 sec.

In Vivo ESR Measurement

A male mouse (ddY strain) weighing about 20 g was anesthetized with ketamine hydrochloride, and 3 μl of the lipid emulsion/g body weight of mice (equivalent to 0.3 μmole SL-TG/g body weight) were intravenously injected. *In vivo* ESR spectra were obtained in each cross-section (5 mM length) of the mouse at room temperature with a JES-RE-1L with the following parameters: microwave frequency, 1.1 GHz, microwave power, 3 mW; modulation width, 0.1 mT; external magnetic field, about 40.0 ± 5.0 mT; sweep time, 8 min; and time constant, 1 sec.

RESULTS

In Vitro ESR Measurement

Lipid emulsion was prepared using SL-TG as an oil phase. Fig. 1 initial shows typical ESR spectrum of SL-TG in lipid emulsion, which exhibits a singlet broad line (ΔH_{pp} = 1.6mT). The line width of SL-TG in the lipid particles was close to that of spin-labeled phosphatidylcholine (1.525 mT) (12). This finding suggests that SL-TG itself forms the lipid particles and undergoes strong spin exchange interaction. Figure 1 shows x-band ESR spectra of SL-TG in the lipid emulsion after treatment with LPL and/or BSA at appropriate intervals of time. Treatment of the emulsion with LPL induced a large change in the ESR spectra, resulting in the appearance of triplet sharp lines (Fig. 1A). The ESR parameter of the triplet sharp lines (g = 2.0054,

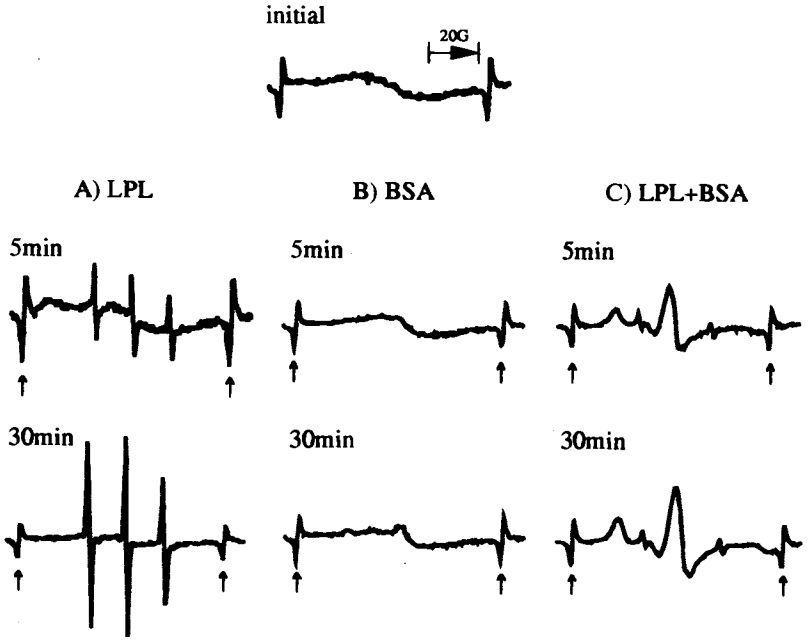

Fig. 1. *In vitro* ESR spectra at X-band (9.4 GHz) of lipid emulsions containing SL-TG before (initial) and after treatment with LPL (A), BSA (B), and the both of LPL/BSA (C). 300 μl of lipid emulsion (0.33% w/w) containing 3.3 μM of SL-TG were incubated at 37°C for 5 or 30 min with 0.1 unit of LPL, with 4% of BSA, and with both LPL and BSA. ESR spectra were obtained at room temperature with an X-band ESR spectrometer with the following conditions: power, 5 mW; modulation width, 0.125 mT; field, 336.3 ± 5.0 mT; sweep time, 2 min; and time constant, 0.03 sec. Arrows indicate the resonance lines of Mn^{2+} used for internal standard.

hfs = 1.59 mT) agreed with those for 12SLS in aqueous solution, suggesting that the lines were due to 12SLS, the hydrolyzed product of SL-TG, in aqueous phase. Further incubation of lipid emulsion with LPL decreased the singlet broad signal and increased the triplet sharp lines over time. Fig. 1B shows the spectral change of SL-TG spectra in the lipid emulsion after addition of BSA. 30 min after incubation, an immobilized ESR signal was superimposed on the broad singlet line due to SL-TG in the lipid particles. The immobilized signal coincided with that of 12SLS bound to BSA, indicating that disintegration of SL-TG in the lipid particles must have occurred. The degree of disintegration, however, appeared to be very small, since further incubation only slightly increased the immobilized signal. The singlet broad line was not obtained in the sample treated with both BSA and LPL, but the anisotropic signal was predominant (Fig. 1C). These anisotropic signals agreed with that of 12SLS-BSA (data not shown). The intensity of the anisotropic signal in Fig. 1C was much higher than that in the Fig. 1B, although SL-TG-BSA complex gave also the same ESR signal to the 12SLS-BSA complex. These findings indicate that SL-TG in the lipid emulsion must be immediately hydrolyzed and bound to BSA.

Fig. 2 shows typical ESR spectra at L-band of SL-TG in the lipid emulsion itself and those after treatment with LPL or BSA. The spectra were fundamentally similar to those at X-band, although the SL-TG/LPL/BSA spectrum at L-band differed slightly from that at X-band, probably due to resonance frequency dependence of the g-value. We used these ESR spectra at L-band for the analysis of the following *in vivo* ESR measurement.

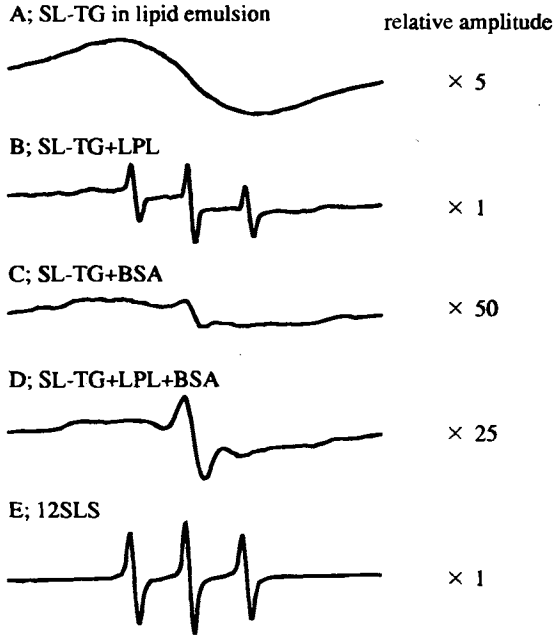

Fig. 2. *In vitro* ESR spectra at L-band (1.2 GHz) of SL-TG in lipid emulsions (A), of 12 SLS aqueous solution (E), and those after treatment with LPL (B), BSA (C), and the both (D). Lipid emulsion (10% w/w) containing 0.1 M of SL-TG was prepared as described in Materials and Methods. 100 μl of the lipid emulsions were incubated for 30 min at 37°C with 900 μl of phosphate buffered solution containing 1 unit of LPL, 40 mg of BSA, or the both. ESR spectra were obtained at room temperature with an L-band ESR spectrometer with the following conditions: power, 3 mW; modulation width, 0.1 mT; field, 40.0 ± 5.0 mT; sweep time, 8 min; and time constant, 1 sec.

In Vivo Measurement of Lipid Emulsion in Mice

In vivo ESR measurements were carried out in living mice after intravenous administration of lipid emulsion using an L-band ESR spectrometer. We used a resonator of 5 mm length × 35 mmØ, and therefore the spectra obtained comprised the total signal within the cross-sections (5mm length) of mouse. Fig. 3 shows spectral changes in the abdomen and in the chest. In the abdomen, a small peak was observed 20 min after administration, which became larger with the appearance of the two small satellite peaks 60 min after administration. The resonance fields of the two satellites coincided with that of the anisotropic signal shown in Fig. 2D, which were due to the 12SLS-BSA complex. This finding indicated that SL-TG in the lipid emulsion has been hydrolyzed to 12SLS and that the 12SLS bound to serum proteins immediately. 150 min after administration, triplet sharp lines were superimposed on the anisotropic signal, and the hyperfine splitting constant (hfs) of the triplet sharp lines coincided with that of free 12SLS (Fig. 2B). A part of the anisotropic signal should remain in the spectra of the abdomen, since the ratio of two peak-heights, h(0)/h(+1), of the spectrum was larger than that in Fig. 2E. It is noteworthy that signal due to the lipid particles was not observed in the abdominal domain.

In the chest, only a singlet broad line, 2.5 mT of the ΔH_{pp}, was observed 8 min after administration (Fig. 3B). Since the spectral pattern coincided with that of Fig. 2A, the spectrum must have been due to the lipid particles. The spectral change in the chest was similar to that in the abdomen, except that a broad singlet line was superimposed on two signals, anisotropic

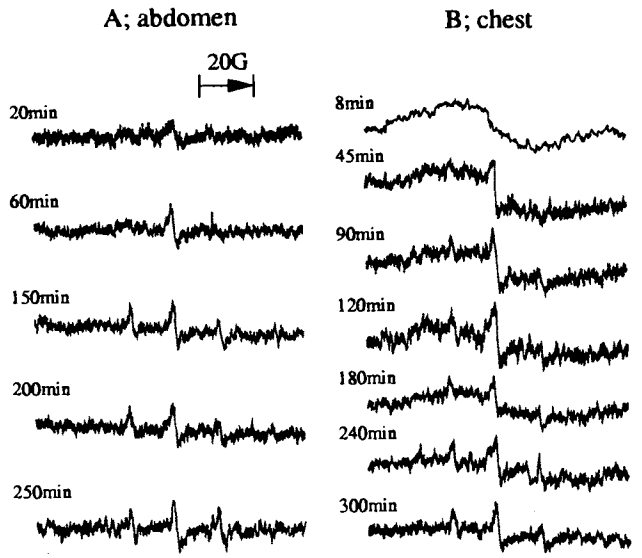

Fig. 3. *In vivo* ESR spectra of SL-TG in lipid emulsion in a living mouse after intravenous administration. 3 μl of the lipid emulsion (10% w/w, 0.1 M of SL-TG)/g body weight were injected to mouse (ddY, male, 20g) and then the ESR spectra were, by turns in the abdomen (A) and in the chest (B), observed with an L-band ESR spectrometer at the time indicated in the left side of each spectrum with following conditions: power, 3 mW; modulation width, 0.1 mT; field, about 40.0 ± 5.0 mT; sweep time, 8 min; and time constant, 1 sec.

and triplet sharp ones, which were observed in the spectra of the abdomen. This finding suggested that the fate of SL-TG in the lipid particles in chest and abdomen differed. In the chest, three states were present: SL-TG in lipid particles, free fatty acid (FFA, hydrolyzed product) and fatty acid bound to protein (FA-protein), while FFA and FA-protein were present in the abdomen. The spectral patterns depended strongly on the period after administration, indicating that the amounts of these states in living mice were time-dependent.

Quantitative Analysis of *in Vivo* ESR Spectra

In order to analyze the *in vivo* ESR spectra shown in Fig. 3, computer simulation was carried out using the *in vitro* ESR spectra at L-band shown in Fig. 2. Fig. 4 shows some examples of the simulated ESR spectra for mixtures of the lipid particles, FFA and FA-protein. The spectra in Figs. 4A, B, C, and D were corresponding to those shown in Fig. 3A-60 min, in Fig. 3A-150 min, in Fig. 3B-240 min, and in Fig. 3B-180 min, respectively. These simulated spectra fairly agreed with the observed ones, suggesting that this simulation was appropriate to analyze the components of superimposed spectra. With this method, superimposed components in each observed spectra were separated using the simulated spectra by the least squares method, and the amounts of lipid particles, FFA and FA-protein were calculated as the absorption areas of the resonance line.

Fig. 5 shows the means ± SD of percentages of the remaining lipid particles in the chest from 4 individual experiments, in which the value obtained at 8 min after administration was taken to be 100%. The half-life of the lipid particles was roughly 2 hours in the chest in living mice.

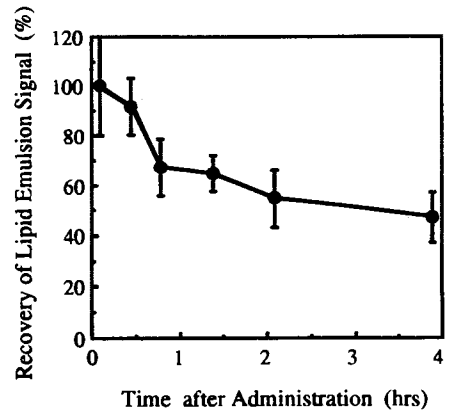

Fig. 5. Decay curves for lipid particles in chest after intravenous administration of lipid emulsion. 3 μl of the lipid emulsion (10% w/w, 0.1 M of SL-TG)/g body weight were injected to mice and then the ESR spectra were measured. The absorption areas of lipid emulsion signals were obtained from the corresponding simulated spectra, some of which are shown in Fig. 4, as described in the Results. Plots and error-bars show means ± SD of percentages of that at 8 min after administration for 4 mice.

Fig. 6 shows the means ± SD of the percentages of FFA or FA-protein in the chest and in the abdomen to the amounts of lipid particles, for which the value of the lipid particles at 8 min was taken to be 100%. The amounts of FA-protein in

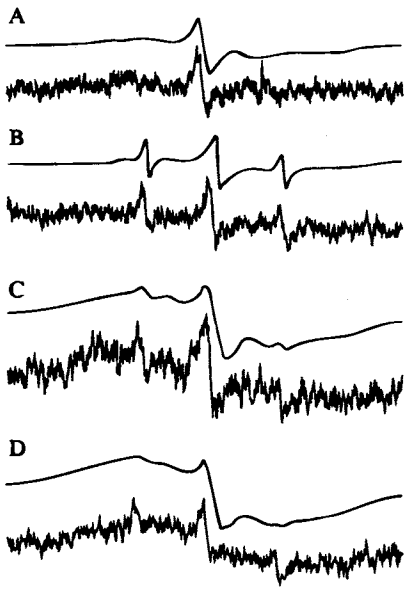

Fig. 4. Simulation of *in vivo* ESR spectra of SL-TG in lipid emulsion intravenously injected into mice. The spectra shown with solid line at the upper part of A, B, C, and D were the calculated one corresponding to those of Fig. 3A-60 min, Fig. 3A-150 min, Fig. 3B-240 min, and Fig. 3B-180 min, respectively. Calculations were carried out using the observed *in vitro* signals of the lipid particles, FFA and FA-protein shown in Fig. 2. The observed signals were once nummerized, averaged with the values of 4 points, and then the averaged values of 3 components were added for simulation of each spectrum. Composition of peak areas of the lipid particles, FFA and FA-protein were (A) 100:0:0.5, (B) 100:0.5:0.5, (C) 30:0.5:0, and (D) 100:0.5:0, respectively.

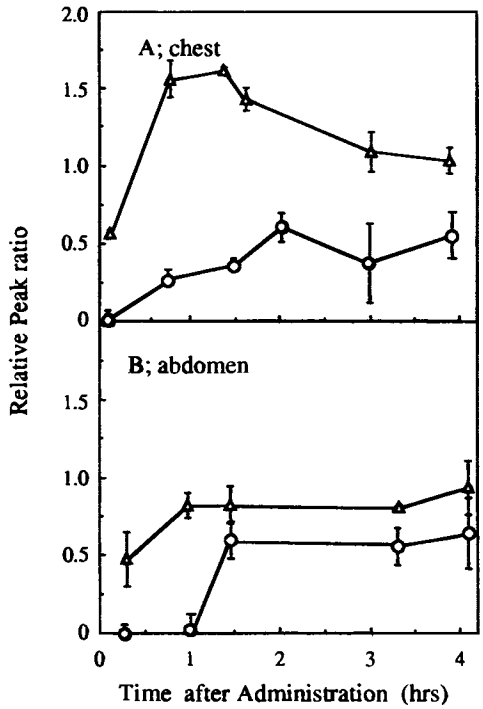

Fig. 6. Curves for appearance of FFA (○) and FA-protein (△) in the chest (A) and the abdomen (B) after intravenous administration of lipid emulsion. The absorption areas of FFA and FA-protein signals were obtained from the corresponding simulated spectra as described in the legend of Fig. 5. Plots and error-bars show means ± SD of percentages of the absorption area to that of lipid particles at 8 min after injection shown in Fig. 5.

the chest initially increased, reached a maximum at 90 min, and then decreased (Fig. 6A). On the other hand, the amount of FFA increased gradually, reached plateau at 120 min, and then remained constant until 300 min after administration. The amounts of FFA and FA-protein in the abdomen also increased gradually and then reached plateau at 60–90 min (Fig. 6B). The amounts of FFA and FA-protein at plateau were almost the same to those in the chest at 4hr.

DISCUSSION

In this study, the pharmacokinetics of lipid particles and their decomposition in living mice were analyzed by *in vivo* ESR spectrometry. Lipid particles themselves were clearly distinguished in the ESR spectrum from their decomposed products, a free fatty acid (FFA) and fatty acid bound to macromolecule such as serum protein (FA-protein). The predominant ESR signal due to lipid particles was observed in the chest immediately after administration. The clearance of the lipid particles followed first-order kinetics, and their half-life was 2 hours in the chest. However, any ESR spectrum due to lipid particles was not observed in the abdomen. In addition to the signal of lipid particles, two signal components due to FFA and FA-protein were superimposed on the spectra for the chest. The finding in *in vitro* and *in vivo* experiments suggested that triglycerides in the lipid emulsion was initially hydrolyzed to fatty acid by LPL in the blood. The time course of the appearance of signals for FFA and FA-protein in the abdomen suggested that fatty acid was initially bound to serum protein, and that FFA appeared after saturation of serum proteins with fatty acid. It should be mentioned, however, that total spin observed in this study gradually decreased over time. As we noted in a previous study (8), the nitroxide radical itself is converted to the corresponding hydroxylamine, which is ESR silent. In the present study, the conversion of nitroxide radical to the hydroxylamine in SL-TG, FFA, and FA-protein may have contributed to the signal decay observed.

The distributions of the lipid particles and FA-protein differed significantly between in the chest and in the abdomen. This phenomena are unexpected and there should be many factors to determine the distribution in tissue. The reduction of nitroxide radical might be one of the factors, because the reducing activity for nitroxide is very high in liver. In addition, the interaction of lipid particle/FA-protein with the reticuloendothelial system of liver should also contribute to the distribution in the chest and abdomen.

Two studies on the distribution of lipid emulsion have been reported. Yoshioka et al. (5) determined the distribution of lipid emulsions in rats. Lipid emulsion containing ^{14}C-labeled soybean oil decayed with first-order kinetics in the blood after intravenous administration with a half-life of 58 min. This value roughly agreed with that determined in the present study. Illum et al. (4) prepared lipid emulsion with egg lecithin and soybean oil labeled with ^{123}I; this lipid emulsion was eliminated with first-order kinetics with a half-life of 5 min, although more than 20% of it remained in the blood 6 hours after intravenous administration. The findings by Illum et al. (4) thus appeared to be inconsistent. In both reports, lipid particles themselves distributed in the abdomen; 2–3% of the total radio-activity injected were recovered from liver (5), and 10% of that from liver and spleen (4). It should be mentioned that any direct evidence has not been provided in both reports that lipid emulsion itself remains intact in living body. The present study should, however, not neglect the possibility that the small amount of lipid particles may contaminate in the spectrum because of its large line-width.

The present method using *in vivo* ESR technique is quite unique and completely different from tracer method using radioisotope label. The intensity of ESR signal reflects the amount of lipid particles, FFA and FA-protein in both blood and tissue, while tracer method demonstrates the relative amount of radioisotope in between tissue. This difference in the principle of two method must contribute the discrepancy results observed by the two method.

In conclusion, we studied, for the first time within our knowledge, the pharmacokinetics of lipid emulsion in non-invasive fashion by *in vivo* ESR spectroscopy. We demonstrated the usefulness of *in vivo* ESR spectrometry for determination of the pharmacokinetics of lipid emulsions. The method used is entirely non-invasive and real-time analysis in individual living animals. Oxidative damage of lipoproteins or blood cells in living body will become possible to be analyzed with this method.

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