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Generation of Free Radicals in Lipid Emulsion Used in Parenteral Nutrition

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ABSTRACT. Lipid emulsions used in parenteral nutrition are prone to peroxidation that may be an important feature of oxygen-associated tissue damage. Incubation of lipid emulsion [Intralipid (IL)] with H_2O_2 and $FeCl_2$ increased lipid peroxidation, measurable as increased production of pentane, from 0.39 ± 0.33 to 0.99 ± 0.18 μM ($p < 0.0001$). Malondialdehyde was increased from 0.010 ± 0.005 mM to 0.380 ± 0.025 mM ($p < 0.001$). Superoxide dismutase and catalase (each 100 U/mL) or vitamin C (10 mM) inhibited pentane and malondialdehyde production ($p < 0.0001$). Incubation of human erythrocytes in the presence of $FeCl_2$ caused 11.0 ± 3.2% hemolysis (control 0.95 ± 0.14%). Addition of 0.44% IL increased hemolysis to 66.5 ± 3.4%, whereas further addition of vitamin E or C significantly inhibited hemolysis to 16.4 ± 8.1 and 38.9 ± 7.1%, respectively ($p < 0.0001$). IL was administered i.v. to eight preterm infants. It increased 3- to 28-fold ($p < 0.001$) the amount of pentane in expired breath. Partly, this increase was due to pentane dissolved in IL as a result of lipid peroxidation during storage. After discontinuing IL infusion, the elimination of pentane was nonexponential, consisting of a rapid and a slow component. According to our results, IL undergoes peroxidation causing free-radical-dependent damage to human cells. We propose that the adverse effects of parenteral IL are partially caused by free oxygen radicals generated by lipid peroxidation. (*Pediatr Res* 29: 56–59, 1991)

Abbreviations

CAT, catalase
IL, Intralipid
MDA, malondialdehyde
SOD, superoxide dismutase

Premature infants frequently require parenteral nutrition during the first weeks of life. Despite unquestionable nutritional advantages, the infusion of lipids has been associated with decreased oxygenation and accumulation of lipids in pulmonary vessels (1–6). In addition, the use of i.v. lipids in small, premature infants has been related to increased chronic morbidity such as bronchopulmonary dysplasia and retinopathy of prematurity (7).

Several lines of evidence suggest that partially reduced, highly reactive oxygen species, free oxygen radicals, are important in oxidant injury (8). The significance of free-radical-mediated damage for the outcome of small premature infants has previously been proposed (9). The vulnerability of premature infants may be caused by their inability to resist free oxygen radicals, or by incidents capable of liberating excess free oxygen radicals, such as hyperoxia, respiratory burst by activated phagocytic cells, and episodes of hypoxia-reperfusion (9–12). Lipid peroxidation, free-radical-induced autocatalytic breakdown of polyunsaturated fatty acids, involves a cascade of reactions further liberating reactive intermediates, lipid peroxides (13, 14). Therefore, the infusion of a lipid emulsion rich in polyunsaturated fatty acids may cause lipid peroxidation and, consecutively, tissue injury.

This investigation was undertaken to study whether lipid emulsion used in parenteral nutrition undergoes lipid peroxidation *in vitro*, whether this peroxidation can generate free radicals that damage human cells, and whether generation of free radicals can be demonstrated *in vivo* during infusion of lipid emulsion to premature infants.

MATERIALS AND METHODS

Patients. Eight premature infants were studied at the Children's Hospital, University of Helsinki. The mean gestational age was 30.0 ± 2.5 wk (range 27.6–35.6 wk) and birth weight 1150 ± 375 g (range 715–1850 g). Seven of them initially required intubation and mechanical ventilation due to respiratory distress syndrome. One infant, also requiring mechanical ventilation, had early neonatal septicemia due to *Haemophilus influenzae*, but leukocyte count and C-reactive protein had become normal before the study. The other patients did not have infections during the first 10 postnatal days. More than 2 d before the study, two patients received indomethacin for treatment of patent ductus arteriosus. None of the patients had malformations or surgery. The study protocol was approved by the Ethics Committee of the Children's Hospital, University of Helsinki, and informed consent was obtained from the parents.

Parenteral nutrition with glucose, amino acids, and lipid was started on clinical indications at the age of 5.0 ± 1.2 d (range 4–7 d). Vitamins (A, 350 IU; D, 60 IU; E, 0.7 IU; C, 15 mg; and K, 0.02 mg daily) were given parenterally together with the lipid. IL (KabiVitrum, Stockholm, Sweden), a soybean oil mixture, was used in parenteral nutrition. The patients received 10% IL at constant rates of 1.3 to 2.5 mg/kg/min; mean 1.6 ± 0.5 mg/kg/min. Measurements of expired pentane were performed on every patient one or two times before the onset of the first lipid infusion. They were repeated at the end, and again at 2 and 5 h after cessation of the infusion. To five of the six patients, this protocol was repeated on the next day. In three patients, the measurements were performed 0, 10, 20, 50, and 160 min after lipid infusion at a rate of 1.4 ± 0.2 mg/kg/min. On two patients, the procedure was repeated on the following day.

During collection of expired breath, the patients were ventilated manually with a hydrocarbon-free mixture of air and

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oxygen via a nonrebreathing valve for 5 min, after which expired breath was collected for 2 min for analysis.

One of the patients remained on ventilator until death at the age of 48 d due to uremia, bronchopulmonary dysplasia, and septicemia. Another patient required mechanical ventilation up to the age of 3 wk. The others did not require ventilation beyond 10 d of age. Two developed grade I intraventricular hemorrhage, but none had bronchopulmonary dysplasia at the age of 4 wk.

Generation of pentane and MDA from IL in vitro. Twenty μL of 10% IL were incubated in 0.5 mL of 10 mM Tris-Cl, pH 7.4, in airtight ampoules (5 mL). The head space was replaced with hydrocarbon-free air. Lipid peroxidation was propagated by 10 mM H_2O_2 and 200 μM $FeCl_2$ for 2 h at 37°C. SOD and CAT (SOD from bovine erythrocytes and CAT from bovine liver, Sigma Chemical Co., St. Louis, MO), 100 U/mL each, or 10 mM vitamin C were added where indicated.

The amount of pentane present in commercial preparations of lipid emulsion was measured in a 20-μL aliquot of 10% IL. The samples were transferred to the ampoules filled with hydrocarbon-free air containing 0.5 mL of Tris-Cl, pH 7.4, and the internal standard. Pentane and ethane in the lipid preparation were trapped during 2 min and quantitated as described below. MDA was measured as described (15).

In vitro hemolysis. Fresh leukocyte-free human erythrocytes were incubated in 4.5 mL of 10 mM Tris-Cl, pH 7.4, in isotonic saline (final hematocrit 0.25), at 37°C under continuous shaking (100/min). Where indicated, a final concentration of 4.4 or 0.44% (vol/vol) IL with or without 200 μM Fe^{2+} was present. For determination of hemolysis, aliquots of 0.5 mL were taken at 0, 4, 8, 12, 16, and 22 h. The sample was diluted in 4.5 mL of isotonic saline and centrifuged at 600 × *g* for 10 min. To sediment the lipids, 600 μL of the supernatant was vigorously shaken with 400 μL Lipoclean (Behringwerke AG, Marburg, West Germany) and centrifuged for 5 min at 1000 × *g*. The lipid-free supernatant was separated and used for measurement of Hb (16). At the end of the experiment (22 h), a 0.5-mL aliquot was taken for the determination of total hemolysis by adding 4.5 mL distilled water. After centrifugation, the supernatant was used for determination of Hb as above.

The effect of free radical scavengers on hemolysis was studied by adding 1 mM vitamin C or 0.25 mM vitamin E (α-tocopherol, Sigma Chemical Co.) to a mixture containing erythrocytes and 0.44% IL. After incubation for 22 h, the hemolysis was quantitated as above.

Analysis of pentane and ethane. For the quantitation of pentane and ethane, 2-pentene was used as the internal standard. In *in vitro* experiments, 25 pmol of 2-pentene was added to the reaction vial before collection of the gas phase. To the samples of expired breath, 50 pmol of 2-pentene were added, after which 300 mL of the specimen were taken for analysis. Ethane and pentane were measured by gas-chromatography with capillary column as previously described (17).

Statistical analysis. The data are presented as mean ± SD. Statistical significance was analyzed using the two-tailed *t* test.

RESULTS

Commercial 10% IL contained 2.10 ± 0.72 μM ($n = 5$, range 1.2 to 3.0 μM) pentane, suggesting lipid peroxidation during storage. Extensive purging of IL with helium for 10 min completely removed the dissolved pentane. After incubation of helium-washed 10% IL at 37°C for 2 h in air, the concentration of pentane was 0.39 ± 0.33 μM. Addition of 10 mM H_2O_2 and 200 μM $FeCl_2$ increased this to 0.99 ± 0.19 μM. SOD and CAT inhibited the induction by 89.1%, and vitamin C inhibited induction by 69.7%. The amount of ethane in the 20-μL aliquots was under the detection limit (1 pmol). In the experiment with 10% IL alone, 0.010 ± 0.001 μM MDA was detected as compared with 0.380 ± 0.025 μM in the presence of H_2O_2 and ferrous ion. Addition of SOD + CAT or vitamin C to the incubation mixture decreased MDA by 96 and 95%, respectively (Table 1).

In erythrocytes incubated for 22 h with ferrous iron, hemolysis was 11.0 ± 3.2% (Fig. 1). Addition of IL (4.4% final concentration) with or without ferrous iron caused significant hemolysis beginning at 12 h. After 16 h, there was 65.2 ± 13.3% hemolysis in the presence and 23.0 ± 5.2% in the absence of iron, and at 22 h hemolysis was complete in both (Fig. 1). Hemolysis in the presence 0.44% IL and ferrous iron for 22 h was 66.5 ± 3.4%. Addition of vitamin E or C reduced this to 16.4 ± 8.1% ($p < 0.0001$) and 38.9 ± 7.1% ($p < 0.0001$), respectively (Fig. 2).

In the patients, the basal amount of pentane in expired breath was 12.2 ± 5.5 pmol/kg/min. At the end of the IL infusion, this

Table 1. *Production of pentane and MDA in IL after induced lipid peroxidation**

	Pentane (μM)	MDA (mM)
Control	0.39 ± 0.33	0.010 ± 0.001
H_2O_2	0.99 ± 0.19†	0.380 ± 0.025†
H_2O_2 + SOD + CAT	0.46 ± 0.18‡	0.023 ± 0.007‡
H_2O_2 + vitamin C	0.57 ± 0.15‡	0.029 ± 0.003‡

* Twenty μL of IL were incubated with 200 μM $FeCl_2$ and 10 mM H_2O_2. In the control experiment, H_2O_2 was omitted. SOD and CAT were added at 100 U/mL each. The final concentration of vitamin C was 10 mM. Results are presented as means ± SD of six parallel experiments.

† Different from control at $p < 0.0001$.

‡ Different from H_2O_2 at $p < 0.0001$.

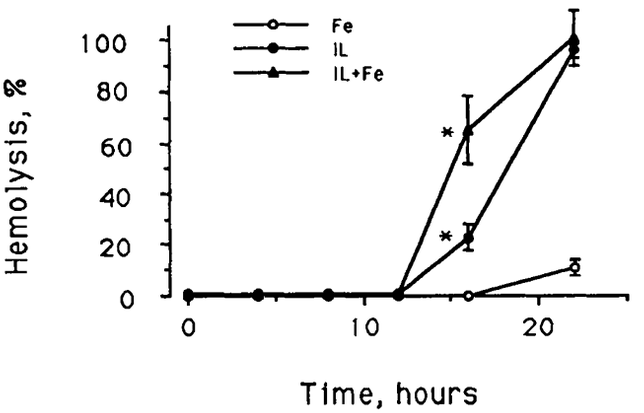

Fig. 1. The effect of IL on human erythrocytes *in vitro*. Erythrocytes were incubated in the presence of 200 μM $FeCl_2$ (*Fe*), or in 4.4% IL without (*IL*) or with 200 μM $FeCl_2$ (*IL+Fe*). In the control experiment without Fe or IL, hemolysis was less than 1%. Results are presented as means ± SD of six parallel experiments. *Different at $p < 0.001$.

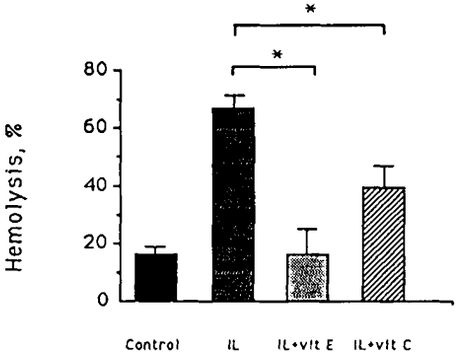

Fig. 2. The effect of IL and antioxidants on human erythrocytes. Erythrocytes were incubated in 0.44% IL or buffer alone, both containing 200 μM $FeCl_2$. The final concentration of vitamin C was 1 mM, and that of vitamin E 250 μM. Results are presented as means ± SD of six parallel experiments. *Different at $p < 0.0001$.

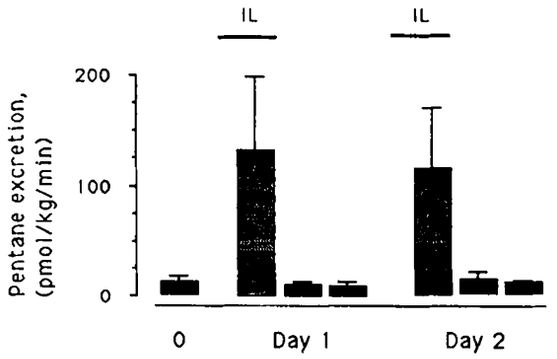

Fig. 3. Expired pentane in premature infants receiving IL. Ten percent IL was infused at a rate of 1.3 to 2.5 mg/kg/min for 6 h on 2 subsequent days to six infants; gestational age 31.1 ± 3.3 wk; postnatal age 5.0 ± 1.2 d at the beginning of IL. Expired pentane in breath was measured before IL, at the end of the infusion, and 2 and 5 h after cessation of the infusion. Results are presented as means ± SD of six measurements.

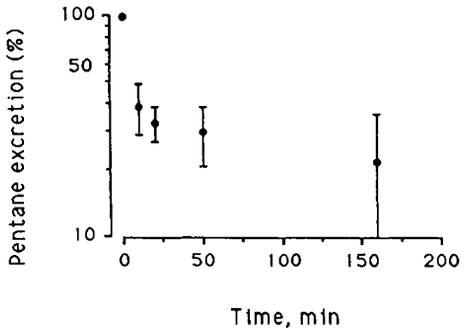

Fig. 4. Elimination of pentane after the lipid infusion. Ten percent IL was infused to three premature infants (gestational age 28.9 ± 1.2 wk, birth weight 853 ± 136 g) at a rate of 1.4 ± 0.2 mg/kg/min. Expired pentane immediately before cessation of the infusion (range 76.3–127 pmol/kg/min) was used as a reference and assigned the value of 100%. The values are presented as mean ± SD on a logarithmic scale.

increased to 117 ± 61.5 pmol/kg/min ($p < 0.001$, $n = 13$). Two h after cessation of lipid infusion, pentane had declined to 9.5 ± 2.8 pmol/kg/min ($n = 13$), and 3 h later it remained low at 12.4 ± 5.9 pmol/kg/min ($n = 7$) (Fig. 3). At the end of the IL infusion, ethane varied widely and was exhaled at a rate of 21.6 ± 25.3 pmol/kg/min compared with the value 5.3 ± 7.6 pmol/kg/min measured before the infusion (NS).

In three patients, the decline of pentane in breath was followed at closer intervals after discontinuing the lipid infusion. Initially, a rapid phase of elimination with a $t_{1/2}$ of 12 min or less was evident. Thereafter, pentane in breath decreased more gradually toward the basal level (Fig. 4).

DISCUSSION

In our study, we have demonstrated that a lipid emulsion used in parenteral nutrition undergoes peroxidation that causes free-radical-mediated damage to human cells *in vitro*. The generation of free radicals in the lipid emulsion was documented by several findings. Incubation of IL with hydrogen peroxide and ferrous iron increased the generation of pentane. This alkane is produced in free-radical-induced peroxidation of linoleic acid, the main constituent of IL (4, 13, 17). The production of MDA also increased significantly. The generation of both these products of lipid peroxidation was inhibited by antioxidants. Furthermore, the experimental system demonstrated lipid emulsion-induced hemolysis in which erythrocytes were protected by the naturally occurring radical scavengers, vitamin E and vitamin C.

Recently, the significance of lipid peroxidation has been under discussion (18). Although, to a degree, peroxidation is part of normal metabolism, profound lipid peroxidation is associated with definite tissue injury *in vivo* (18, 19). As shown here and in other studies, human cell membranes are susceptible to damage mediated by free radicals (20, 21). Lipid emulsions for parenteral nutrition are also prone to lipid peroxidation upon storage, as indicated by the presence of pentane in IL. The amount of vitamin E in IL (0.02 IU/mL = 36 μM) is not sufficient to inhibit the peroxidation.

The infusion of IL to premature babies, at rates used in clinical practice, caused a nearly 10-fold increase in expired pentane. This is in accordance with the results of Wispe *et al.* (22). A fraction of the increase is derived from preformed pentane infused to the patient in the lipid emulsion. However, the amount of pentane measured in expired breath during the 2-min collection exceeded the amount of pentane given to the patient in the infusate by 3- to 4-fold. Therefore, it is evident that pentane is generated in the process of lipid peroxidation *in vivo*. The maximum amount of pentane expired during infusion of IL was close to that observed by Wispe *et al.* (22). Smaller amounts of pentane have been found in adult patients receiving parenteral lipid emulsion. However, those results were obtained after discontinuing the infusion (23). We could not show any clear correlation between ethane production and IL infusion. In general, the basal ethane and pentane excretion rates were low in our patients, reflecting the stable clinical condition of the patients (9).

The elimination rate of pentane in breath after discontinuation of IL was nonexponential. The initial rapid phase may mainly be due to dissolved pentane formed during storage of IL. However, further elimination of pentane produced *in vivo* was more gradual (Fig. 4). In our study, in contrast to a previous report (22), pentane returned to the basal level by 5 h after cessation of IL. Wispe *et al.* (22) examined pentane and ethane production after continuous infusion of safflower oil emulsion to one term infant not receiving supplementary oxygen. Initially, pentane decreased with an apparent $t_{1/2}$ of 2 h, followed by a more gradual decline (43–59% in 24 h). The difference in the elimination rate of pentane after the lipid emulsion may be explained either by the differences in the quality and quantity of lipid emulsion or by differences in patient characteristics.

In this investigation, only one of the eight preterm infants died and none of the others developed severe sequelae (bronchopulmonary dysplasia, retinopathy of prematurity, cerebral palsy). However, in a study of sick premature infants, the use of lipid emulsions was associated with increased incidence of complications such as bronchopulmonary dysplasia and retinopathy of prematurity (7). We have previously shown that, in very-low-birth-weight newborns who later died or developed chronic disease, lipid peroxidation was significantly higher during the first days of life than it was in patients of comparable gestational age with a favorable outcome (9). As shown here, lipid emulsions are prone to peroxidation and generation of free radicals. Therefore, it is possible that in susceptible patients the lipid peroxidation products generated by lipid emulsions exceed the capacity of the organism to inactivate free radicals (10). The excess of free radicals may in turn be a factor that contributes to the complications encountered in premature infants receiving IL. It remains to be studied whether the generation of free radicals and complications in patients receiving parenteral nutrition can be decreased by antioxidant therapy.

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