Appearance and Characterization of Lipoprotein X during Continuous Intralipid Infusions in the Neonate

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Research Article

The development of hyperphospholipidemia and hypercholesterolemia was studied in infants that required total parenteral nutrition and given a continuous infusion of Intralipid, (1-4 g/kg body wt per 24 h. Detailed studies were carried out on infusion periods lasting 1-10 d. After 24 h there was a marked increase in plasma free cholesterol (68%) and phospholipid (77%) concentrations. Based on the amount of cholesterol in Intralipid, and the rate of infusion, it was estimated that at least 50% of the plasma cholesterol increment during 64-h infusions was derived from endogenous sources. By contrast, the hyperphospholipidemia could be attributed to the Intralipid as the rise in plasma was calculated to be equivalent to only 16% of the exogenous phospholipid infused. Approximately 10% of the phospholipid in Intralipid was in a triglyceride-free mesophase form with a free cholesterol:phospholipid molar ratio of 0.063. There were no systematic changes in plasma concentrations of cholesterol ester or triglyceride during Intralipid infusions. The increase in free cholesterol and phospholipid was localized in the low density lipoproteins (d = 1.006-1.063 g/ml). The presence of lipoprotein X (Lp-X) in the low density lipoprotein fraction was demonstrated by electrophoresis in agar and by isolation and chemical characterization with hydroxylapatite chromatography. Isoelectric focusing of urea-soluble protein of Lp-X revealed that albumin and apolipoproteins CII and CIII were major components, whereas apolipoprotein […]

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ABSTRACT The development of hyperphospholipidemia and hypercholesterolemia was studied in infants that required total parenteral nutrition and given a continuous infusion of Intralipid, (1–4 g/kg body wt per 24 h. Detailed studies were carried out on infusion periods lasting 1–10 d. After 24 h there was a marked increase in plasma free cholesterol (68%) and phospholipid (77%) concentrations. Based on the amount of cholesterol in Intralipid, and the rate of infusion, it was estimated that at least 50% of the plasma cholesterol increment during 64-h infusions was derived from endogenous sources. By contrast, the hyperphospholipidemia could be attributed to the Intralipid as the rise in plasma was calculated to be equivalent to only 16% of the exogenous phospholipid infused. Approximately 10% of the phospholipid in Intralipid was in a triglyceride-free mesophase form with a free cholesterol:phospholipid molar ratio of 0.063. There were no systematic changes in plasma concentrations of cholesterol ester or triglyceride during Intralipid infusions. The increase in free cholesterol and phospholipid was localized in the low density lipoproteins ($d = 1.006$–1.063 g/ml). The presence of lipoprotein X (Lp-X) in the low density lipoprotein fraction was demonstrated by electrophoresis in agar and by isolation and chemical characterization with hydroxylapatite chromatography. Isoelectric focusing of urea-soluble protein of Lp-X revealed that albumin and apolipoproteins CII and CIII were major components, whereas apolipoprotein E and AI were minor constituents. The abnormal lipoprotein was apparent by 16 h

during 64 h of infusion. After 6 d of continuous infusions the free cholesterol in Lp-X was 30 ± 10 mg/dl (mean$\pm$SD), which represents a total Lp-X mass of 90 mg/dl. After cessation of the infusion, Lp-X, as monitored by electrophoresis in agar, disappeared within 72–96 h. Thus, during infusion of Intralipid in infants at rates commonly employed, the capacity of the clearance mechanisms for phospholipid are exceeded, which causes the accumulation of phospholipid and free cholesterol in the form of Lp-X particles. It is suggested that mesophase phospholipids in Intralipid may play a significant role in this process.

INTRODUCTION

Although fat emulsions are needed to supply additional calories and to avoid essential fatty acid deficiency during intravenous nutrition in the neonate (1), their use has been associated with various hyperlipidemias. Intralipid, the most commonly used parenteral fat solution, is an emulsion of soybean triglyceride that contains 1.2% egg lecithin as a stabilizing agent. Hypertriglyceridemia, defined as plasma triglyceride concentration exceeding 100 mg/dl, has been noted in small-for-gestational-age infants (2, 3), and in the premature, < 32 wk (3, 4); and is manifested by a delay in clearing bolus injections or infusions (4 h) of Intralipid. This apparent intolerance to parenteral lipid has been attributed to reduced hydrolysis of Intralipid triglyceride as well as to a diminished uptake and utilization of the liberated FFA in peripheral tissue. In recent reports a rise in plasma free cholesterol and phospholipid concentrations has been observed in both the neonate (5, 6) and adults (7–9) after infusions of Intralipid. In view of these observations a detailed study was undertaken to investigate the alterations in plasma lipids and lipoproteins during continuous administration of Intralipid to neonates dependent

upon total parenteral nutrition (TPN).[1] On the basis of this study it is suggested that the hypercholesterolemia is a consequence of phospholipid overload, which causes the accumulation of a lipoprotein with chemical composition and electrophoretic properties of lipoprotein X.

METHODS

Patients. The infants studied required TPN for a variety of congenital and acquired gastrointestinal anomalies. All were appropriate for gestational age that ranged from 26- to 40-wk gestation at birth, and were from 4-d- to 3-wk-old when Intralipid (Cutter Medical, Div. Cutter Laboratories, Berkeley, Calif.) was started. Intralipid[2] (containing per 100 ml: 10 g soybean triglyceride, 1.2 g egg lecithin, and 2.5 g glycerol) was administered via a scalp vein needle using a Sage syringe pump (model 255, Sage Instruments Div., Orion Research Inc., Cambridge, Mass.) to maintain a constant rate of infusion. The infants received glucose and casein hydrolysate for at least 24 h before and during the period of Intralipid infusion. None of the infants was acidotic, septic, or had bilirubin concentrations exceeding 5 mg/100 ml. Infants with minor elevations in direct reacting bilirubin (<2 mg/100 ml) were included because these infants did not differ from those in whom there was no such increase.

Based on Intralipid tolerance tests, individual infusion rates were calculated[3] for the majority of infants studied as follows: a bolus injection of 200 mg of Intralipid triglyceride/kg body wt was given; the fractional elimination constant, K_2, and the Intralipid volume of distribution were determined, and these values were applied to the formula of the plateau principle (10, 11) to calculate an infusion rate to achieve a steady plasma Intralipid concentration of 100 mg/dl or less. This target concentration was chosen to avoid hyperlipidemia (12).[3] All the infants in this study received, by continuous infusion, 1–4 g Intralipid triglyceride/kg body wt per d.

Lipoprotein analyses. Heel prick blood samples (200 μl) were taken into capillary tubes that contained EDTA or heparin for studies involving agarose electrophoresis or lipid analyses, respectively. For lipoprotein isolation 1.3–2.0 ml of blood was collected into EDTA by venous puncture. Plasma lipoprotein fractions were isolated (13) in a Beckman rotor (40.3, Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) fitted with a Teflon (E. I. DuPont, de Nemours & Co., Inc., Wilmington, Del.) insert that would accept 2.0-ml

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[1] *Abbreviations used in this paper*: HDL, high density lipoprotein(s); LCAT, lecithin-cholesterol acyltransferase; LDL, low density lipoprotein(s); Lp-X, lipoprotein X; TMU, tetramethylurea; TPN, total parenteral nutrition; VLDL, very low density lipoprotein(s).

[2] Detailed analyses of Intralipid (three preparations) revealed the presence of free cholesterol (4 mg/g Intralipid triglyceride) as well as plant sterols (2 mg/g Intralipid triglyceride). Two Intralipid preparations were subjected to ultracentrifugation at saline density (1.6 × 10⁶ g min). Analysis of the supernatant and infranatant fractions obtained after tube slicing revealed that 8–12% of the total phospholipid and free cholesterol was associated with the infranatant fraction as a triglyceride-free micelle. The molar ratio of free cholesterol:phospholipid in the triglyceride-rich supernatant and the infranatant mesophase was 0.063.

[3] Griffin, E. A., M. H. Bryan, and A. Angel. Intralipid tolerance tests and continuous infusion in neonates. Manuscript submitted for publication.

cellulose nitrate centrifuge tubes. Plasma (0.6–1.0 ml) was overlayered with saline, pH 7.4 (d = 1.006 g/ml) and centrifuged at 106,000 g for 18 h. A supernatant fraction (0.8 ml) and an infranatant fraction (1.2 ml) were quantitatively collected by tube slicing. The infranatant fraction was adjusted to d 1.063 and centrifuged at 106,000 g for 24 h to obtain low density lipoproteins (LDL) in the supernatant fraction. For studies involving protein analyses, LDL was washed once. The infranatant was adjusted to d = 1.21 g/ml and centrifuged for 42 h at 106,000 g to obtain high density lipoproteins (HDL).

The lipoproteins of d 1.006–1.063 were further resolved by hydroxylapatite column chromatography as described by Kostner et al. (14) for the isolation of lipoprotein X (Lp-X) from cholestatic patients. In these studies, columns were eluted at 0.1 M phosphate buffer (pH 6.8) to obtain Lp-X and at 0.65 M phosphate (pH 6.8) to obtain "residual LDL." Because initial studies demonstrated that the elution profiles monitored by optical density (280 nm) or cholesterol content were identical, optical density was routinely used.

Lipoprotein electrophoresis on agarose gel was carried out by the method of Maguire and Breckenridge (15), whereas electrophoresis on agar, for the detection of Lp-X, was performed as described by Seidel et al. (16). Apoproteins from various lipoproteins were analyzed by polyacrylamide gel electrophoresis after treatment of the lipoprotein with tetramethylurea (TMU) as described by Kane (17). Apoprotein that was insoluble in TMU was assumed to be apoprotein (apo)B. Apoprotein components were identified on polyacrylamide gels by comigration with purified apoproteins or by their relative electrophoretic mobility.

Apoproteins were also characterized by isoelectric focusing. Lyophilized lipoproteins were delipidated with three extractions of ethanol:ether 3:1 (vol/vol). The dried residue was taken up in Tris-HCl buffer (1 mM, pH 8) that contained 8 M urea and 1 mg dithiothreitol/100 μg protein. Isoelectric focusing was completed essentially according to the method of Pagnan et al. (18) with the following modifications. A linear gradient between pH 3 and 7 was achieved by using ampholytes composed of pH 3–5, 4–6, and 5–7 in a proportion of 1:2:2 (vol/vol per vol). Gels (0.5 × 12 cm) were prefocused for 1 h at 100 V. The samples (50- to 100-μg urea-soluble protein) were applied in 8 M urea and overlayered with an aqueous solution that contained 1% ampholyte mixture and 5% sucrose. The gels were focused for 250 V for 16 h and then stained as described elsewhere (19). For determination of the pH gradient a gel, lacking sample was subjected to focusing and then cut into 5-mm segments that were placed in distilled water (0.5 ml). The pH was recorded after 1 h. Apoproteins were identified by comparison to published values for their pI and by isoelectric focusing with purified apoproteins.

The various lipoprotein fractions were also assessed by double diffusion against anti-human albumin (Miles Laboratories Inc., Elkhart, Ind.) and anti-human LDL. The latter was prepared in rabbits by injection of human LDL (d = 1.030–1.050 g/ml) in an equal mixture with Freund's adjuvant.

Protein was estimated by the method of Lowry et al. (20) with bovine serum albumin as a standard. Lipoprotein fractions and standards were extracted with diethyl ether after color development. The protein content in TMU extracts was determined as described by Kane (17) except that TMU was used in the standards.

Lipid analysis by gas-liquid chromatography. Plasma or lipoprotein fractions were assayed for triglycerides, esterified cholesterol, free cholesterol, and phospholipids by an automated gas chromatographic procedure as previously described (21). For this purpose the plasma (50 μl) was digested under diethyl ether with phospholipase C (*Clostrid-*

ium welchii), which converted the lecithins and sphingo-myelins into the corresponding diglycerides and ceramides, and any lysolecithins into the monoglycerides. After extraction with chloroform-methanol, the mixed neutral lipids were treated with trimethylchlorosilane and hexamethyldisilazane, which converted any FFA into the trimethylsilyl esters, the free cholesterol, diglycerides, and ceramides into the corresponding trimethylsilyl ethers, and any monoglycerides into the ditrimethylsilyl ethers. The neutral lipid mixture was separated and quantitated in the presence of tridecanoin as internal standard by automated high-temperature gas-liquid chromatography with 20 × 1/16-in i.d. stainless steel tubes that contained 3% OV-1 (a methyl siloxane polymer) on Gas-Chrom Q (100–200 mesh) (Applied Science Laboratories Inc., State College, Pa.). The gas chromatograph was the Hewlett-Packard automated gas chromatographic system 5700 A (Hewlett-Packard Co., Palo Alto, Calif.), which included an automatic sampler, low mass oven with temperature programmer, electronic integrator, and a dual flame-ionization detector. The integrator output was simultaneously recorded on a strip chart recorder and a punched paper tape that encoded the peak retention times and areas in computer-compatible code. The tape was subsequently used for off-line data processing by means of computer programs (21). Appropriate calibration and conversion factors were used for differences in recovery, flame-ionization response, and the chemical form of analysis of the different plasma lipids. The precision of the methodology was 3–5% for the estimate of lipid classes, whereas the limit of detection of lipid classes was ~10 μg/dl.

A paired t test was used to compare lipid results in plasma before, during, and after Intralipid administration.

RESULTS

Initial observations (Fig. 1) in neonates receiving continuous Intralipid infusions revealed a marked rise in plasma free cholesterol that correlated significantly
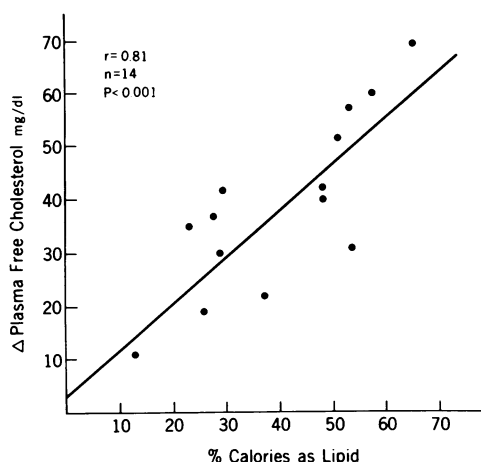

FIGURE 1   Effect of Intralipid load on plasma free cholesterol concentration in neonates ($n = 14$). All the infants received continuous Intralipid infusions (1–8 g/kg body wt) over a period of 20–24 h. The total nonfat calories were constant for any given subject and ranged from 28 to 76 kcal/kg body wt. The increase in plasma free cholesterol was linearly related ($P < 0.001$) to the amount of Intralipid given on a daily basis.

with the amount of Intralipid infused ($P < 0.001$) over the time period. These observations prompted more detailed time-related studies to assess the effect of Intralipid on plasma lipids and lipoproteins in infants requiring TPN.

Plasma lipids. In Fig. 2 the changes in total lipid profiles of an infant are shown before Intralipid infusion (panel A); after 24 h (panel B) and after 2.5 mo (panel C) on Intralipid infusion; as well as 3 mo after discontinuation of Intralipid (panel D). Before infusion the major plasma lipid constituents were free cholesterol (C27), phospholipids (C34–C40), and cholesterol esters (C43 and C45). After 24 h of Intralipid infusion there was a marked increase in free cholesterol and phospholipid concentration but no increase in triglycerides (C50–C54). By 2.5 mo plant sterols became evident as the amounts of campesterol (C28) and β-sitosterol (C29) were more obvious than at 24 h of infusion. In this individual there was a marked decrease in cholesterol ester, but this was not a systematic change because increases in cholesterol esters were observed in several short-term studies (7 d). It should be noted parenthetically that the Intralipid used in this study contained small amounts of plant sterols (2 mg/g Intralipid triglyceride) as well as cholesterol (4 mg/g Intralipid triglyceride). 3 mo after discontinuation of the Intralipid the proportions of free cholesterol, phospholipid, and cholesterol ester had returned to preinfusion levels.

A more extensive study of plasma lipid composition before and at 20–24 h of continuous Intralipid infusion was undertaken in 10 infants (Table I). A significant increase ($P < 0.01$) occurred in plasma free cholesterol and phospholipid concentrations, but little change in concentration was evident for esterified cholesterol and triglycerides. The plasma lipid values of the infants before Intralipid infusion were indistinguishable from a group of nine normal infants on formula feeds. The appearance of small amounts of plant sterols (campesterol and β-sitosterol) were observed in 5 of the 10 infants infused over 20–24 h.

To identify the early phases of free cholesterol and phospholipid accumulation, the plasma phospholipid and free cholesterol concentrations were followed (Fig. 3) over a 64-h period in a group of five infants receiving continuous Intralipid infusions. A rise in plasma phospholipid and free cholesterol was seen at very early times (4 h) after starting the infusion. It was apparent that during the initial 16 h the rise in phospholipid was greater than the rise in free cholesterol —but thereafter they rose in parallel. The increase in plasma free cholesterol concentration could not be attributed solely to the cholesterol in Intralipid, which contained 4 mg cholesterol/g triglyceride. A calculation (Fig. 3) of the increment in free cholesterol potentially attributable to the infused Intralipid was
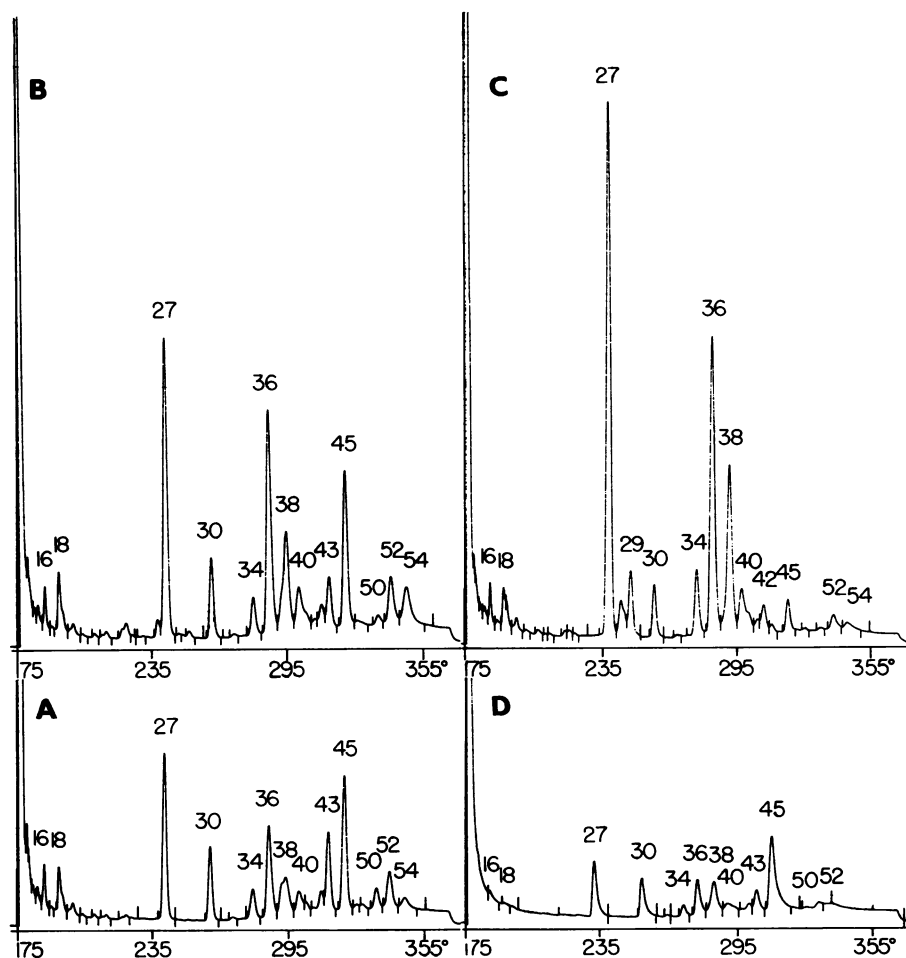
FIGURE 2 Total plasma lipid profiles of an infant. (Panel A) before Intralipid infusion; (Panel B) after 24 h and (Panel C) after 2.5 mo of Intralipid administration (2 g Intralipid triglyceride/kg body wt per 24 h); (panel D) 3 mo after discontinuation of TPN. Samples, 1 μl of approximately a 1% solution of neutral lipids in the silylation mixture; attenuation, 1/100 full sensitivity; carrier gas, nitrogen at 80 ml/min; detector, 350°C; column temperature programmed at 6°C/min. Other conditions are given in text. The numbering system refers to the number of carbons in each lipid moiety represented by each separate peak. Peak 30, tridecanoin is the internal standard. Peaks 16 and 18 are trimethylsilyl esters of C_{16} and C_{18} fatty acids. Peaks 27, 28 (unmarked between 27 and 29 in panel C), and 29 (panel C) are trimethylsilyl ethers of cholesterol, campesterol, and β-sitosterol, respectively. Peaks 30–40 are acyl carbons. Peaks 43–45 are cholesterol esters of C_{16}–C_{18} fatty acids. Peaks 50–54 are triglycerides containing 50–54 acyl carbons per glyceride molecule. A sharp increase in free cholesterol (peak 27) and phospholipids (peaks 36–40) in panels B and C during Intralipid infusion and its normalization 3 mo later in panel D is evident.

made assuming that all Intralipid cholesterol was distributed in the plasma and that the plasma volume was 50 ml/kg body wt. No more than 40% of the increment could have come from the exogenous source. Similar calculations were made for the increment in plasma phospholipids. The increment after 64 h represents only 16% of that potentially available from the total phospholipid administered via Intralipid. The changes in cholesterol ester were variable and showed no specific trend (Fig. 3).

Alterations in lipoprotein composition. The increments in plasma free cholesterol and phospholipid were confined almost entirely to an increase (sixfold) in the concentration of LDL ($d = 1.006-1.063$ g/ml as shown in Fig. 4). No significant changes occurred in absolute amounts of free cholesterol and phospholipids in HDL and very low density lipoproteins (VLDL). After cessation of Intralipid (5 wk) the lipoprotein composition and concentrations had returned to preinfusion values with the exception of HDL. The average

TABLE I
Effect of Continuous Intralipid Infusion on Plasma Lipid Concentrations in Infants*

			Component			
Experiment‡		Free cholesterol	Esterified cholesterol	Plant sterols§	Phospholipid	Triglyceride
	n			mg/100 ml		
A (a) Pre-Intralipid infusion	10	56.9±8.06	87.5±9.4	0	167±20	136±19
(b) 20–24 h of Intralipid infusion	10	95.5±8.2	82.8±5.3	3.82±1.6	295.2±21	171±30
B Formula fed (control)	9	45.4±4.9	89.2±17.2	0	161.1±14.4	127±18

* Total lipid profiles analyzed by gas-liquid chromatography as in legend of Fig. 2.

‡ (A) The 10 infants who were studied (a) before and (b) at 20–24 h of continuous Intralipid infusion of 2–4 g/kg per d had a gestational age of 34.5±1.2 wk (mean±SEM) and were 8±2 d old (mean±SEM). Five of the infants had received no feeds whereas the other five who had previously been commenced on formula feeds were fasting for at least 24 h before the study. (B) Nine fed infants receiving 5–10 g fat/kg per d from formula had a gestational age of 33.4±1.5 wk (mean±SEM) and were aged 11±2 d (mean±SEM). Significant differences $P < 0.01$ were obtained with paired t test for lipid values A (a) and (b). No significant differences were obtained between (B) formula-fed infants and (A) (a) pre-Intralipid infusion infants.

§ Plant sterols (campesterol and β-sitosterol) appeared in 5 of the 10 infants and the mean of the 5 is shown.

content of cholesterol ester in this lipoprotein was considerably higher than the amount observed before or during Intralipid infusion but there was extensive intraindividual variation. However, the ratio of free cholesterol:esterified cholesterol dropped from 3.48 ±0.51 during the infusion to 0.45±0.06 (mean±SEM) after the infusion. Before infusion this ratio was 4.4±1.38. It is noteworthy that the lipoprotein had an abnormally high free cholesterol esterified cholesterol ratio even before infusion.

The effect of Intralipid infusion on lipoprotein composition was further examined by agarose gel electrophoresis. In preinfusion plasma (Fig. 5A) β-lipoproteins (LDL), pre-β- (VLDL), and α- (HDL) bands were present and the origin was clear which indicates an absence of chylomicrons. After 64 h of Intralipid infusion (Fig. 5B) lipid-staining material was noted at the origin which indicates the presence of Intralipid. There was also a marked increase in the intensity of the β-band as well as a new, slow-migrating band just behind LDL. The appearance of the slow migrating band together with the increased concentrations of free cholesterol and phospholipid in the LDL fraction suggested the possible presence of Lp-X, which has been identified in the plasma of cholestatic patients (22). This suggestion was substantiated by agar electrophoresis where Lp-X migration is cathodal (Fig. 6). Fig. 6 also shows the appearance of Lp-X on agar electrophoresis coinciding with the accumulation of free cholesterol and phospholipid in plasma during a 64-h infusion of Intralipid. Lp-X starts to appear at 16 h along with the parallel increases in free cholesterol and phospholipid (Fig. 3), and accumulates progressively during the remainder of the study period as judged by the intensity of the Lp-X band. The
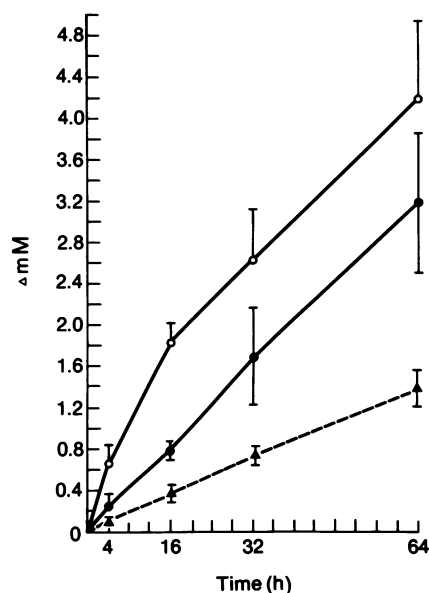

FIGURE 3 Changes in plasma free cholesterol and phospholipid during continuous intralipid infusions. Five neonates were given 1–4 g Intralipid triglyceride/kg body wt per 24 h. Results are shown as means and standard error of the mean. (○) Plasma phospholipids; (●) plasma free cholesterol; (▲) estimate of plasma cholesterol increment potentially available from cholesterol content of Intralipid (4 mg cholesterol/g triglyceride) and distributing only in the plasma compartment (plasma volume assumed to be 50 ml/kg body wt). The increment in phospholipid due to Intralipid phospholipid under the same assumption was not included because of the much greater scale. The change in plasma phospholipid in millimolars, if all Intralipid phospholipid had remained in plasma, would be: 4 h, 1.5; 16 h, 6.0; 32 h, 12.0; and 64 h, 24.0. Changes in cholesterol ester, which were variable and not significant, were as follows: (mean and range in parenthesis, mg/100 ml), 4 h, -5 (-2 to -5); 16 h, +1.3 (-14 to +16); 32 h, +6.2 (-5 to +16); and 64 h, +14.4 (-18 to +61).
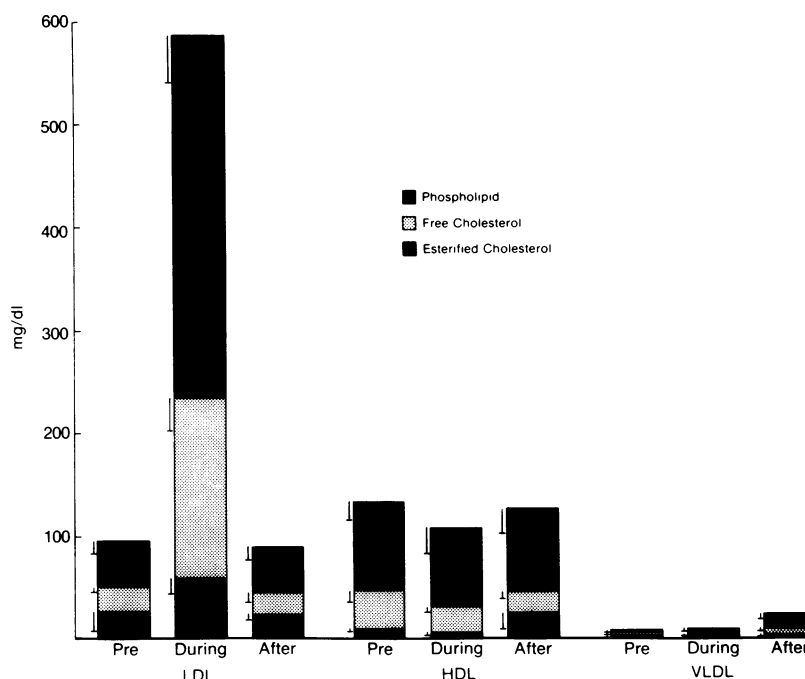
FIGURE 4 Composition of plasma lipoproteins ($n = 5$) before, during (7–10 d), and 6 wk after Intralipid infusion. The content of free cholesterol and phospholipid was significantly higher in LDL during Intralipid infusion ($P < 0.01$). The ratio of free cholesterol:esterified cholesterol was lower ($P < 0.02$) in HDL of neonates after infusion (0.45±0.09) than during infusion (3.48±0.51; mean±SEM).

presence of Lp-X before 16 h could not be demonstrated by the electrophoretic procedure.

Because the major accumulation of free cholesterol and phospholipid was in LDL fraction, and because Lp-X was apparent by agar electrophoresis, an attempt was made to isolate and characterize Lp-X using the technique of hydroxylapatite chromatography as described by Kostner et al. (14). After elution with 0.1 M

phosphate buffer a lipoprotein was isolated with a composition essentially equivalent to Lp-X isolated from a cholestatic patient (Table II). After 6 d of Intralipid infusion the free cholesterol of Lp-X increased 30±10 mg/dl (mean±SD) and represented ~25% of the total free cholesterol in LDL.

The small amount of protein (6%) in Lp-X was entirely soluble in TMU, indicating the absence of apoB. No apoB was detected immunochemically, but albumin was detected after delipidation of the lipoprotein (data not shown). Further analysis by isoelectric focusing of the urea-soluble proteins of Lp-X revealed (Fig. 7)
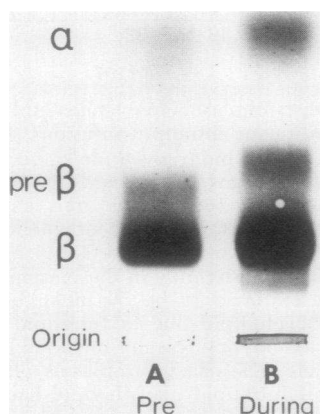

FIGURE 5 Agarose gel electrophoresis of plasma from a neonate. (A) Before and (B) at the end of 48 h of Intralipid infusion.
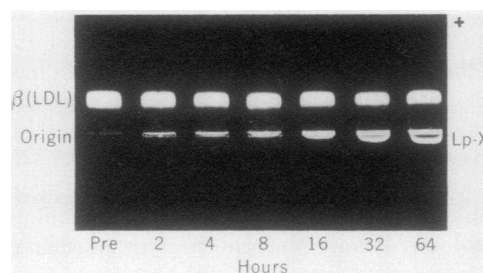

FIGURE 6 Agar gel electrophoresis of plasma of a neonate at various stages of Intralipid infusion (1 g Intralipid triglyceride/kg body wt per 24 h). The presence of cathodally migrating Lp-X can be seen by 16 h of continous Intralipid infusion.

TABLE II
Composition of Lipoproteins Isolated from LDL (d = 1.006–1.063) by
Hydroxylapatite Chromatography*

| | | | | | | Component§ | | | |
| | | | | | | | | Protein | |
Eluate from hydroxyapatite	Subject‡	FC	CE	TG	PL	Total	TMU soluble	TMU insoluble
0.1 M $PO_4^=$	Cholestatic	25.4	1.9	1.7	64.8	6.1	6.0	0
0.1 M $PO_4^=$	Intralipid infused	26.0	1.3	2.8	64.5	5.5	5.5	0
0.65 M $PO_4^=$	Intralipid infused	15.1	20.5	5.4	38.5	20.4	4.5	15.9
0.65 M $PO_4^=$	Normal LDL	6.3	42.5	5.1	22.5	23.5	1.1	22.3

* LP-X was isolated in 0.1 M phosphate buffer (pH 6.8) from total LDL by hydroxylapatite chromatography. Residual LDL was obtained by a subsequent elution with 0.65 phosphate buffer. ‡ Cholestatic sample was obtained from an adult subject with obstructive jaundice and plasma Lp-X. To obtain sufficient material for analysis the LDL from 0.5 ml of plasma, from three neonates infused with Intralipid (2–4 g/kg body wt per d) for 6 d, was first separated by hydroxylapatite chromatography. After the cholesterol was determined in the 0.1 M and 0.65 M phosphate buffer eluates, these fractions were pooled for complete lipid and protein analyses. The amount of total cholesterol in the 0.1 M $PO_4^=$ and 0.65 M $PO_4^=$ fractions was 30 ± 10 and 150 ± 20 mg/100 ml, respectively. Recovery of lipoprotein cholesterol after hydroxylapatite chromatography was $90\pm5\%$ (mean$\pm$SD). The amount of TMU-insoluble protein in LDL increased from 35.2 to 39.6 mg/100 ml in these subjects.
§ FC, free cholesterol; CE, cholesterol ester; TG, triglyceride; PL, phospholipid.

that a major protein focused at pI 6.0–6.2, which is characteristic for human albumin under these conditions. A series of bands focusing between a pI of 5.5 and 5.9 were identical to the pI of isomorphs of apoE from normal human VLDL. ApoCII and apoCIII were also identified. When expressed as a percentage of total, optical density on the gel albumin accounted for 50%, apoCII plus apoCIII for 30%, and apoE for 10%, with apoAI and unidentified peptides representing 5%. Small amounts of apoE and apoAI have been identified in Lp-X isolated by zonal ultracentrifugation from cholestatic plasma (23).

The residual lipoproteins of the LDL fraction eluted with 0.65 M phosphate buffer were abnormal (Table II). This fraction contained increased levels of free cholesterol, phospholipid, and TMU-soluble peptides, relative to normal LDL; but apoB, identified by immunodiffusion against anti-LDL and quantitated by the amount of protein insoluble in TMU, was still a major component. In a single analysis of LDL pooled from three subjects there was a 10% increase in apoB during an infusion for 6 d. Analyses of the urea-soluble proteins (Fig. 7) revealed that the TMU-soluble material was primarily composed of apoE (60%) and apoCII plus apoCIII (30%) with minimal amounts of albumin and unidentified peptides. Rechromatography on hydroxylapatite of the residual LDL fraction did not give Lp-X material that could be eluted with 0.1 M phosphate buffer (data not shown). A similar particle has been observed in the LDL fraction of plasma from rats infused with Intralipid (24).

Initial studies on plasma specimens from infants who had been discontinued from Intralipid for 5–6 wk (Fig. 4) indicated that the plasma lipoprotein concentration and composition had returned to essentially preinfusion concentrations. To study shorter time intervals, plasma specimens were obtained at daily intervals after cessation of Intralipid infusion. Both plasma free cholesterol and phospholipid began to decrease within 24 h of termination of the infusion. By 96 h the free cholesterol levels had almost returned to preinfusion levels and plant sterols had disappeared, whereas plasma phospholipid levels were still somewhat elevated (Fig. 8). No Lp-X could be detected by agar gel electrophoresis 72 h after Intralipid discontinuation.

DISCUSSION

Parenteral lipid is an important adjunct in the management of the infant who cannot be fed orally and requires 100–150 kcal/kg per d. Infants who are appropriate for gestational age and who have not lost excessive weight tolerate continuous Intralipid infusions, readily clearing 2–4 g of triglyceride/kg body wt per d (2–4) without developing hypertriglyceridemia. Despite a tolerance to the infused glyceride, hypercholesterolemia and hyperphospholipidemia develop as a function of the amount and duration of the Intralipid given, not only in infants, but also in adults (7–9) and experimental animals (24).

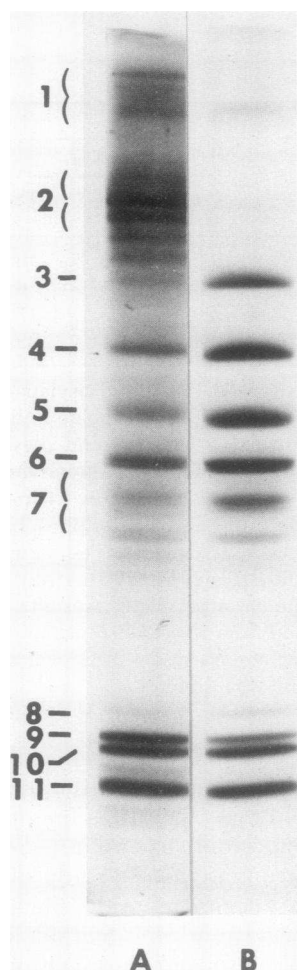On the basis of the cholesterol content of the Intra-

FIGURE 7   Isoelectric focusing of apoproteins in polyacryl-amide gels (10%) that contain 6 M urea. Preparation of apoproteins and isoelectric focusing are described in Methods. Columns A and B represent Lp-X and residual LDL, respectively, obtained by hydroxylapatite chromatography of LDL from a neonate who received 2 g Intralipid triglyceride/kg body wt per 24 h for 5 d. Identification of apoproteins was based on isoelectric focusing of isolated apoprotein preparations from normal lipoproteins. 1, unidentified; 2, albumin; 3, corresponds to the unidentified band in column B; 4, apoE$_3$; 5, apoE$_2$; 6, apoE$_1$; 7, apoAI isomorphs; 8, apo CIIIo + apoAII; 9, apoCII; 10, apoCIII$_1$; 11, apoCIII$_2$.

formation of Lp-X. In considering the mechanism of phospholipid clearance in the neonatal patients it is evident that the capacity was inadequate to remove the entire exogenous load at the infusion rates employed. Two enzymatic pathways involved in the removal of lecithin include (a) lecithin-cholesterol acyltransferase (LCAT), an enzyme equally important in the deacylation of phospholipid as in the esterification of cholesterol and (b) phospholipases that are heparin-releasable (25) and of hepatic origin (26). Although the quantitative importance of the phospholipases is unknown, the LCAT mechanism could theoretically be responsible for removal of ~12–90% of the infused phospholipid. The plasma LCAT activity in man of 60–130 μmol cholesterol esterified/liter of plasma per h (27–29) is considerably less than the rate of infusion of phospholipid in our study (145–580 μmol/liter of plasma per h). In view of the fact that LCAT activity in newborns is reduced (30) it is reasonable to conclude that the LCAT mechanism was saturated with the substrate load, thus favoring accumulation of phospholipid in plasma.

It is important to note that 10% of the phospholipid in Intralipid is in mesophase and 90% of the phospholipid is associated with large glyceride-rich par-
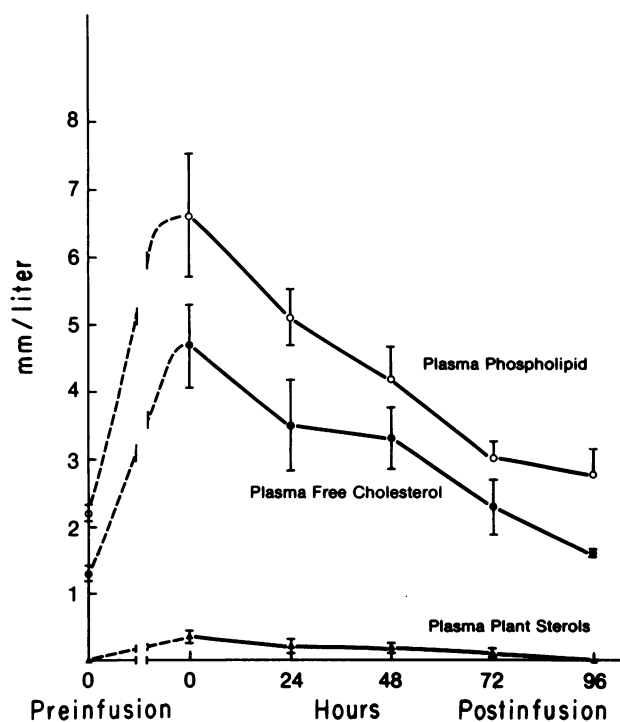
lipid and the amount infused, it was estimated that at least 50% of the free sterol that accumulated in plasma must have been derived from endogenous sources (Fig. 3). Similar calculations for the amount of phospholipid infused in Intralipid indicated that the bulk was cleared from plasma, but that the residual amount was sufficient to cause substantial increases in plasma phospholipids as a result of saturation of clearance pathways. The mechanism of phospholipid accumulation in plasma deserves comment because it may explain in part the hypercholesterolemia and the

FIGURE 8   Return toward normal of plasma lipid levels after discontinuation of Intralipid infusions. Infants (n = 5) had been on Intralipid infusion (2–4 g triglyceride/kg body wt) for 5–16 h. (○) Phospholipids; (●) free cholesterol; (▲) plant sterols.

ticles. The cholesterol:phospholipid ratio (0.063) of both fractions is much lower than that found in Lp-X (0.80). Because >10% of the infused phospholipid was retained in plasma, both mesophase phospholipid and phospholipids remaining after the clearance of triglyceride-rich particles can be implicated in acquiring cholesterol from available tissue sources. This notion is supported by the observations: (a) that the composition of phospholipids accumulating in plasma after Intralipid infusions is that of egg lecithin (31); (b) that the hypercholesterolemia after infusion of pure phospholipids in animals is a result of redistribution of cholesterol between tissue and plasma (32); and (c) that phospholipid liposomes can leach cholesterol from erythrocyte membranes (33), and that phospholipid apoprotein complexes promote efflux of sterols from cells in tissue culture (34). A portion of the cholesterol accumulating in plasma may have been newly synthesized because phospholipid infusions are associated with enhanced hepatic cholesterogenesis (35, 36), presumably a result of increased human hydroxymethylglutaryl-coenzyme A reductase activity (37).

In our study, Lp-X was discerned after 16 h of Intralipid infusion when the increase in plasma phospholipid was 50–70% above preinfusion levels (Fig. 6). Although Lp-X particles may be present but not detected at earlier times it is probable that the initial increment in phospholipid was accommodated by association with preexisting lipoproteins. This view is consistent with the data of Thompson et al. (8) who examined serum lipoproteins after a 4-h infusion of 50 g of Intralipid (containing 6.0 g of phospholipid) into healthy adults and observed no Lp-X formation. It is obvious that a much larger relative load of phospholipid must be administered and for longer periods of time before Lp-X appears; e.g., during TPN with Intralipid (6).

The exact origin of Lp-X is not established. Manzato et al. (38) suggested that in patients with cholestasis Lp-X was derived from bile lipoproteins that contained phospholipid, cholesterol, and albumin, which refluxed into the blood stream and acquired C-apopeptides. In familial LCAT deficiency the accumulation of Lp-X has been attributed to a failure to esterify cholesterol (39), which would be expected to lead to elevation of both free cholesterol and phospholipid levels. The extracellular formation of Lp-X has been reported in perfused livers in rats with cholestasis (40). Although the exact process responsible for Lp-X formation during Intralipid infusion cannot be discerned from this and previous (24) studies, an extracellular, physicochemical mechanism is proposed. As the level of plasma phospholipids exceeds the capacity for clearance, free cholesterol is mobilized by the lecithin-mesophase resulting in a stable phospholipid-cholesterol particle, which competes successfully with other lipoproteins for the apoproteins, and forming Lp-X. Alternatively,

the apopeptides may be mobilized by the lecithin mesophase particles before their equilibration with free cholesterol. This hypothesis requires experimental confirmation.

It is significant that the rate of clearance of Lp-X after cessation of Intralipid infusion was similar to the clearance of Lp-X in cholestatic patients after operations to correct bile duct obstruction (41) or after plasma transfusion to patients with LCAT deficiency (42). In these instances a 50% reduction in Lp-X occurred in 2–3 d. In our study over 95% of the Lp-X disappeared in 2–4 d. These findings suggest that clearance of Lp-X is LCAT dependent because a reduction in Lp-X precursor (Intralipid mesophase) load or increasing enzymatic activities Lp-X levels are reduced. Furthermore the fact that Lp-X occurred in conjunction with an absolute or relative deficiency in LCAT enzyme, whose function is entirely extracellular, supports the view that Lp-X occurs as an extracellular physicochemical event.

We have continued to use Intralipid (10%) in the management of infants who cannot be fed in the normal manner. The absence of significant hemolysis and of renal disease clinically and at postmortem during short-term infusions, suggests that tissue changes were not associated with the presence of Lp-X. The effect of long-term infusions are yet to be established and deserve attention in view of the nephropathy associated with the Lp-X accumulation of LCAT deficiency.

ACKNOWLEDGMENTS

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