Selective removal of apolipoprotein B-containing serum lipoproteins from blood plasma

(extracorporeal blood filtration/monospecific anti-low density lipoprotein-Sepharose/plasma low density lipoprotein immunoadsorption/ kinetics of low density lipoprotein replenishment/low density lipoprotein adsorption by heparin-Sepharose 4B)

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ABSTRACT   Studies were undertaken to determine the applicability and effectiveness of immunoadsorption chromatography on anti-low density lipoprotein (LDL) columns as a plasma-cholesterol-lowering procedure. Mass production and isolation of monospecific antibodies against swine LDL ($\rho = 1.006$–1.063 g/ml) was carried out by immunization of sheep with swine LDL and selective antibody adsorption from their antisera by chromatography on LDL-Sepharose. The isolated LDL antibodies were then covalently linked to Sepharose CL-4B. Pig plasma LDL was effectively removed *in vitro* by the anti-LDL-Sepharose beads. *In vivo* studies were performed in pigs with (*i*) a plasma-separator membrane permeable to solutes below M_r 2,000,000 or (*ii*) a blood centrifuge interposed into an arteriovenous shunt to separate the corpuscular elements of blood from the plasma. In either case, the plasma was passed through the anti-LDL-Sepharose column and recirculated into the venous part of the shunt. Plasma cholesterol levels were reduced 70–80% by this procedure and rebounded 3–4 days after the extracorporeal immunoadsorption procedure. This continuous plasma separation–immunoadsorption procedure may have broad applicability for the elimination of any plasma component with antigenic properties.

Familial hypercholesterolemia (type II hyperbetalipoproteinemia) (1) is characterized by increased plasma cholesterol concentrations and increased plasma low density lipoprotein (LDL) levels. It has been established that this form of hypercholesterolemia is due to a reduced fractional catabolic rate and to a 2- to 3-fold overproduction of LDL (1–3); as a result, there is premature development of atherosclerosis.

Three independent studies have been reported (4–6) that were designed to reduce the plasma cholesterol concentrations in patients with familial hypercholesterolemia by nonsurgical methods: plasmapheresis techniques (4, 6) and extracorporeal removal of LDL by means of heparin-agarose beads in blood venous packs (5). These approaches have produced transient reductions in plasma LDL levels.

We report a procedure that results in specific removal of apolipoprotein B (apo B)-containing lipoproteins (VLDL and LDL) by immunoadsorption. This study was undertaken to evaluate (*i*) the extent to which plasma LDL can be removed *in vitro* by an anti-LDL-Sepharose immunoadsorbent and (*ii*) whether and to what extent LDL (and VLDL) can be removed by continuous extracorporeal immunoadsorption in swine. Monospecific LDL antibodies were prepared from the serum of sheep immunized against pig LDL and were covalently linked to Sepharose. When swine plasma was passed through a column of anti-LDL-Sepharose, LDL was absorbed from the plasma. *In vivo* studies in pigs were performed by shunting blood from the arterial branch between the common carotid artery and internal jugular vein, separating the blood into a cell concentrate and a plasma fraction by means of a selective plasma-separator membrane technique or a continuous-flow blood cell-separator centrifuge. The plasma fraction was passed over an anti-LDL-Sepharose column, and the LDL-depleted plasma was recycled into the cell concentrate and returned to the animal through the venous branch of the shunt. A comparison of continuous immunoadsorption and continuous heparin-Sepharose adsorption of LDL was also performed.

MATERIALS AND METHODS

Animals. Swine from a local farm and Hanford minipigs (provided by Nattermann & Cie, Köln, Federal Republic of Germany) were used for *in vivo* studies. Pig blood was obtained from a local slaughterhouse.

Isolation of Low Density Lipoprotein. Plasma from heparin- or EDTA-treated pig blood (1 mg/ml) was separated by centrifugation, and the LDL was isolated by ultracentrifugation in a 60 Ti rotor of a Beckman L5-65B centrifuge (7, 8). For the first centrifugation, a density of 1.006 g/ml was achieved with KBr. The supernatant was removed, and the density of the infranatant was adjusted to 1.063 g/ml with KBr. After ultracentrifugation, the upernatant containing LDL with density 1.006–1.063 g/ml was collected, sedimented at density 1.006, washed once at density 1.063, and used immediately for coupling to Sepharose for immunization of sheep. LDL values given represent the total weight, with protein content given in parentheses.

Preparation of LDL-Sepharose CL-4B. Sepharose CL-4B (30 ml) was washed with water and suspended in 90 ml of ice-cold 1M Na_2CO_3. CNBr (6 g in 3 ml of acetonitrile) was added with stirring, and the activated Sepharose beads were collected on a fritted-glass funnel after 2 min. The Sepharose cake was washed with 5 vol of ice-cold 0.2 M bicarbonate buffer (pH 9.5) and 5 vol of 0.5 M bicarbonate buffer (pH 8.5) and was resuspended immediately in a solution of 300 mg of LDL (60 mg of apo B) in 60 ml of 0.5 M bicarbonate buffer (pH 8.5). The incubation was continued for 20 hr at 4°C, the suspension was centrifuged, the supernatant was decanted, and the sediment was resuspended in 30 ml of phosphate-buffered saline/2M glycine for 12 hr at room temperature. Finally, the LDL-Sepharose beads were thoroughly washed with 20 vol of phosphate-buffered saline. Between 5.6 and 9.5 mg of LDL (1–2 mg of apo B per ml of wet Sepharose beads) was covalently linked, as determined from the unbound protein and the cholesterol in the supernatant and from the cholesterol content in chloroform/methanol extracts of aliquots of LDL-Sepharose 4B.

Immunization and Isolation of Sheep Anti-Pig LDL. Sheep were immunized by three subcutaneous injections at intervals

Abbreviations: LDL, low density lipoprotein; VLDL, very low density lipoprotein; HDL, high density lipoprotein; apo B, apolipoprotein B of LDL.

of 1 week with 5 mg of LDL (1 mg of apo B) in 0.5 ml of phosphate-buffered saline suspended in 0.5 ml of complete Freund's adjuvant. The increase in antibody concentration was followed by quantitative single radial immunodiffusion (9, 10) performed every other day. The maximum concentration (≈25 mg of antibodies per ml of plasma) was reached after 4 wk. Pig LDL-antibody protein was isolated by recycling 50 ml of heparin- or EDTA-treated sheep plasma (1 mg/ml of blood) diluted with the same volume of saline over a LDL-Sepharose CL-4B column (bed volume, 250 ml) for 4–12 hr. Unbound proteins were eluted with saline (1200 ml), and adsorbed antibody protein was eluted as a single band with a 0.2 M glycine·HCl buffer (pH 3.0). Approximately 4 g of sheep LDL-antibody protein was collected by repeating the immunoadsorption.

Preparation of Anti-LDL-Sepharose CL-4B. Anti-pig LDL [390 mg in 100 ml of 0.2 M bicarbonate buffer (pH 9.3)] was added to 50 ml of CNBr-activated Sepharose CL-4B and treated as described above. As determined by Lowry protein measurements (11) of the supernatant, 350 mg (90%) of LDL-antibody protein was covalently linked.

Preparation of Heparin-Sepharose CL-4B. Heparin-Sepharose CL-4B was prepared as described (12).

LDL Depletion of Pig Plasma by Immunadsorption *in Vitro*. Heparin-treated pig plasma (100 ml) was passed through an anti-pig-LDL-Sepharose column (bed volume, 20 ml; flow rate, 3 ml/min). Plasma proteins were eluted with 150 ml of saline, and the bound LDL was desorbed with 250 ml of 0.2 M glycine·HCl buffer (pH 3.0).

LDL Adsorption from Pig Plasma *in Vivo*. An arteriovenous shunt was constructed between the common carotid artery and the internal jugular vein of pigs and was tunneled subcutaneously to the neck region. Surgical procedures and *in vivo* studies were performed in pigs under combined anesthesia [Stresnil (Azaperon) and Metomidat (Hypnodil), Janssen GmbH,
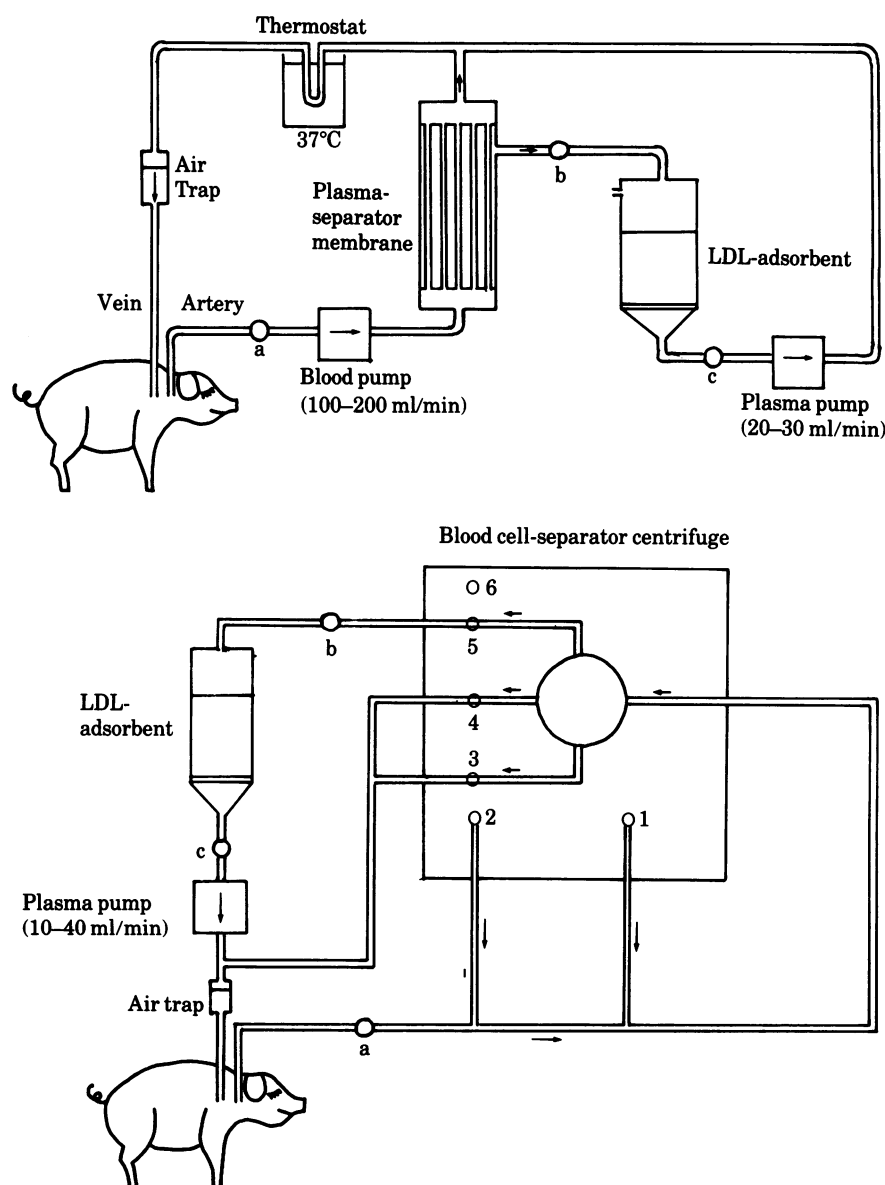

FIG. 1. Arrangement of the plasma separator membrane system and LDL adsorbent (*Upper*) and the continuous-flow blood cell-separator centrifuge and immunoadsorbent column (*Lower*) in the arteriovenous shunt. Samples were derived from the flow system at a, b, and c in time intervals of 40 min (see Fig. 2). Pumps of the blood cell separator centrifuge (*Lower*): 1, citrate anticoagulant (1–3 ml/min); 2, heparin (1 ml/min, 80,000 units/liter of saline); 3, leukocytes; 4, erythrocytes (10–40 ml/min); 5, plasma (10–40 ml/min); 6, lubrication (1 ml/min, 2000 units of heparin per liter of saline).

Düsseldorf, Federal Republic of Germany (13)]. The arterial blood stream of the shunt was pumped with a masterflex pump at a flow rate between 60 and 200 ml/min over a plasma separator (Plasma-flo membrane, Asahi Medical, Tokyo) (Fig. 1 *Upper*). The resulting plasma flow was transported by a second masterflex pump over the anti-LDL-Sepharose CL-4B column (bed volume, 340 ml). Our recent studies utilized a continuous-flow blood cell separator centrifuge (Aminco, Silver Spring, MD) which allowed VLDL to remain in the plasma fraction (Fig. 1 *Lower*).

Analytical Methods. Protein was determined by the Lowry procedure (11). Cholesterol and cholesterol esters were determined enzymatically with the Preciset cholesterol assay (Boehringer, Mannheim, Federal Republic of Germany). LDL concentrations were calculated from the difference between the concentration of total plasma cholesterol and the concentration of HDL cholesterol in the supernatant after Mg^{2+}/phosphotungstate precipitation of LDL (14).

RESULTS

The aim of these studies was to determine the effectiveness of immunoadsorption for the specific removal of the apo B-containing plasma lipoproteins VLDL and LDL *in vitro* and *in vivo*.

In Vitro **LDL Depletion of Swine Plasma.** *Anti-LDL Sepharose immunoadsorption.* Increasing volumes of swine plasma (750, 1250, and 2950 ml) with LDL concentrations of 1.7 mg/ml of plasma were chromatographed over an anti-LDL-Sepharose column (350-ml bed volume, 6.5-cm inside diameter), and the effluent was analyzed for LDL by agarose electrophoresis of the concentrated eluate and for LDL cholesterol. The LDL concentration bound by immunoadsorption also was determined. Plasma proteins were eluted with saline, and LDL was desorbed with glycine·HCl buffer (pH 3.0). For all three volumes tested, plasma cholesterol decreased from 112 to 40 mg/dl, and triglycerides decreased from 58 to 12 mg/dl; however, the total serum protein concentration remained unchanged. Total LDL bound equaled 117 ± 3 mg for each 100 ml of plasma passed through the anti-LDL-Sepharose column, representing a 64% reduction in LDL content.

Heparin-Sepharose CL-4B. The LDL-binding capacity of heparin-Sepharose was titrated as described above. A 450-ml heparin-Sepharose column was able to bind a maximum of 1550 mg of LDL (3.4 mg of LDL bound per ml of column volume), as compared to the binding of 3520 mg of LDL by the anti-LDL-Sepharose column (10.1 mg of LDL bound per ml of column volume). The total plasma protein concentration was diminished to about 70% by the heparin column.

In Vivo **LDL Removal by Combined Plasma Separation–Immunoadsorption.** The first masterflex pump transported arterial blood (50–120 ml/min) to the plasma-separator membrane which delivered about 25–30 ml of plasma per min to the anti-LDL-Sepharose column. The eluting plasma deprived of apo B-containing lipoproteins was returned to the concentrated blood by a second masterflex pump before entering the venous part of the shunt. This combined plasma–immunoadsorption procedure was performed over periods of 2–4 hr under light anesthesia. Aliquots of the arterial plasma before the plasma separator (Fig. 2, curve a) and of the plasma before (Fig. 2, curve b) and after (Fig. 2, curve c) the anti-LDL-Sepharose immunoadsorbent were taken for the determination of LDL cholesterol. Plasma LDL decreased by 50% within 20 min and reached 25% of the initial concentration during 2–3 hr of shunting (Fig. 2, curve a). Extremely low LDL concentrations in the column effluent remained constant, demonstrating that the immunoadsorption of LDL did not exhaust the capacity of the bed
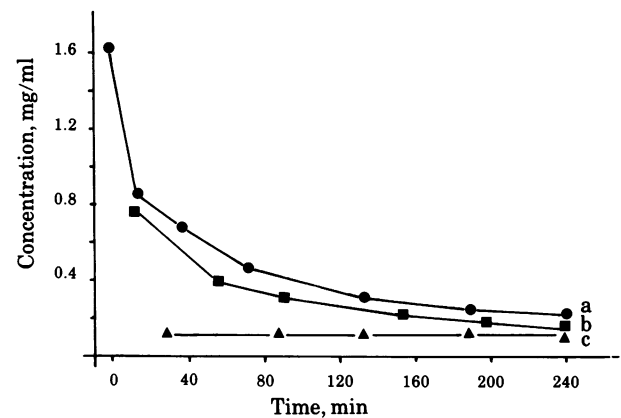

FIG. 2. Kinetics of LDL removal from pig plasma *in vivo* by adsorption to anti-LDL-Sepharose CL-4B, showing plasma LDL concentration before plasma separation (curve a) and LDL concentration before (curve b) and after (curve c) LDL adsorption.

volume of the immunoadsorbent during the duration of the experiment (Fig. 2, curve c).

The anti-LDL-Sepharose column was regenerated by desorption of LDL with glycine buffer (pH 3.0) and immediate equilibration with phosphate-buffered saline. The anti-LDL-Sepharose column was stored at 4°C under saline containing 0.01% NaN₃. Prior to each experiment, the column was washed with sterile saline (≈10 liters). Under these conditions, no loss of LDL-binding capacity was observed over a period of 6 mo in more than 20 *in vitro* and *in vivo* experiments.

Table 1 summarizes the results of four plasma separator–anti-LDL-Sepharose immunoadsorption experiments in pigs. With increasing volumes of blood passing through the shunt, there was a progressive decrease in the plasma LDL concentration. In experiment 4, the LDL concentration was decreased by 84%; the total plasma cholesterol was decreased from 115 to 35 mg/dl, and triglycerides were decreased from 60 to 10 mg/dl. Electrolytes, total protein, glucose, uric acid, bilirubin, and serum enzyme concentrations (serum glutamic-oxaloacetic transaminase, serum glutamic-pyruvic transaminase, γ-glutamyl transpeptidase, alkaline phosphate) remained unaltered during these experiments. The heparin-Sepharose column also effectively removed LDL from the plasma, with a 72–79% reduction in LDL. Thus, the data indicate that the column performance *in vivo* was similar to that reported for *in vitro* studies and that a

Table 1. LDL removal *in vivo* by plasma separation–anti-LDL-Sepharose immunoadsorption and plasma separation–heparin-Sepharose chromatography

Exp.	Duration, min	Blood volume passing shunt, ml/min	LDL removed, mg	Initial plasma LDL remaining,* %
		Anti-LDL-Sepharose		
1	105	60	660	36
2	150	110	670	33
3	145	230	1560	27
4	230	220	1830	16
		Heparin-Sepharose		
5	150	200	1710	21
6	240	200	1620	28

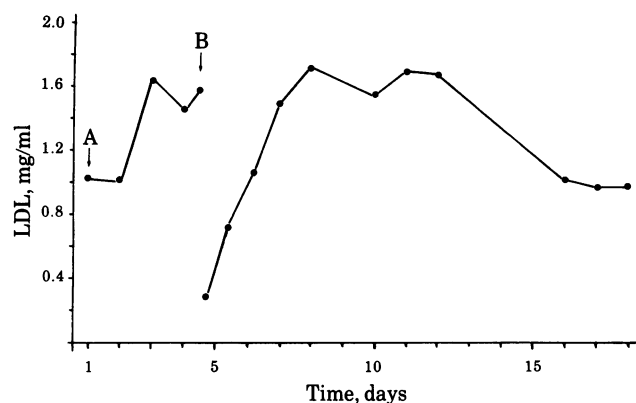* Initial concentration ranged from 1.5 to 1.8 mg of LDL per ml of plasma.

FIG. 3. Changes in LDL concentration after surgical introduction of an arteriovenous shunt (at A) and after LDL depletion (at B) by combined plasma separation and immunoadsorption on a column of anti-LDL-Sepharose.

maximal binding capacity of 1600–1700 mg of LDL was observed.

Kinetics of the Blood LDL Replenishment. Blood LDL concentrations were measured daily because of the easy access to the shunt in the neck region of the experimental animal. Fig. 3 shows that the institution of the arteriovenous shunt caused an increase in LDL concentration from 1.0 to 1.8 mg/ml, and the plasma separation–immunoadsorption lowered LDL to 0.4 mg/ml. The concentration of plasma HDL (1.6 mg/ml) remained constant during the immunoadsorption, and agarose electrophoresis of the lipoproteins desorbed from the column failed to reveal the presence of HDL. Three to 4 days later, the LDL concentration rebounded to 2.0 mg/ml (which exceeded the starting preoperative plasma LDL concentration by 50%), remained constant for 3–5 days, and then decreased to the initial LDL concentration of 1.0 mg/ml of plasma.

LDL-Removal by Plasma Separation with Heparin-Sepharose. The kinetics of blood LDL replenishment following plasma separation by heparin-Sepharose chromatography is shown in Fig. 4. Institution of the arteriovenous shunt caused a transient increase in plasma LDL, which returned to preoperative levels 6 days after surgery. After LDL depletion from the plasma, the kinetics of LDL replenishment exhibited a pattern similar to that observed in pigs subjected to immunoadsorption: an overshoot of plasma LDL concentration followed by gradual return to preoperative control concentrations.

DISCUSSION

Plasma filtrates with all components of M_r <2,000,000 can be obtained with plasma-separator membranes; thus, both LDL and HDL completely pass these membranes. Therefore, a combination of the plasma separator and a high-capacity immunoadsorbent column specific for plasma apo B-containing lipoproteins would be expected to remove LDL from plasma exclusively. In order to test this, Sepharose was crosslinked with monospecific swine LDL antibodies and was tested *in vitro* and *in vivo* for its ability to remove apo B-containing lipoproteins from plasma. When the plasma separator and immunoadsorbent were interposed in series in an arteriovenous shunt of swine, the plasma LDL concentration was reduced by 70–80% in a 2–3 hr experiment. The physical status of the experimental animal remained unimpaired. This experimental approach has unique potential for the study of the biochemical basis of a number of problems [e.g., the replenishment of the plasma LDL level within a rather short period of time, turnover studies of LDL apoproteins (15–18), cholesterol distribution between blood
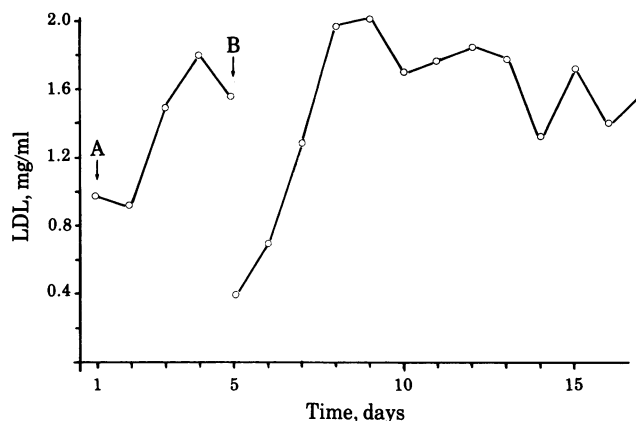

FIG. 4. Changes in LDL concentrations after surgical introduction of an arteriovenous shunt (at A) and after LDL adsorption (at B) by a heparin-Sepharose column.

plasma and organs and between serum lipoproteins *in vivo*, and the role of LDL in atherogenesis either in cholesterol-fed swine with elevated LDL and HDL_{Chol}* concentrations (19) or in patients with familial hypercholesterolemia].

Table 1 shows approximately the same removal of LDL by anti-LDL-Sepharose and by heparin-Sepharose columns. However, the advantages of the immunoadsorption procedure as compared to LDL binding by heparin-Sepharose are significant: numerous plasma proteins are known to have high binding affinity to immobilized heparin [e.g., antithrombin III, Factors VII, IX, XI, XII, and XIIa, and thrombin as components of the coagulation cascade (20–25); components of the complement system (26); lipoprotein lipase (27); and hepatic triglyceride lipase (28)]. Of potentially greater importance is the observation that heparin columns also remove the HDL (29) that are considered to be protective against the development of arteriosclerosis (30). The anti-LDL-Sepharose column appears to remove only apo B-containing lipoproteins, as judged by the fact that plasma HDL levels remain unchanged during immunoadsorption and that double immunodiffusion tests of the desorbed material from the column yield only one precipitin line with anti-pig plasma antibodies from rabbits.

In principle, this combination of plasma separation and immunoadsorption in an extracorporeal circulation should be applicable to the selective removal of any low molecular weight plasma component of endogenous or exogenous origin, provided that the component has antigenic properties.

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* Cholesterol-induced high density lipoprotein with α-mobility.

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Medical Sciences: Stoffel and Demant

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