Modification of the cholesterol efflux properties of human serum by enrichment with phospholipid

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Abstract To investigate the importance of phospholipid in promoting cholesterol efflux from cells, phospholipid multilamellar vesicles were incubated with normal human serum and the efflux ability of these lipid-modified sera was tested. When incubated under appropriate conditions, both dimyristoylphosphatidylcholine (DMPC) and bovine brain sphingomyelin (BBSM) were shown to combine with components of human serum to form new protein:lipid complexes and to markedly enhance the ability of serum to promote efflux of cholesterol from Fu5AH cells. In particular, the high density lipoprotein (HDL) particles were altered in their composition and electrophoretic properties and the α-migrating species, which were reactive with antibodies to apoA-I, were converted to larger, pre-β-migrating particles, similar in electrophoretic properties to $preβ_2$-HDL. DMPC, but not BBSM, also generated particles with mobility similar to $preβ_1$-HDL; these species were demonstrably different from the discoidal complexes formed by reaction of DMPC with purified apoA-I. However, no change in cholesterol efflux potential was observed when serum was mixed with phospholipids that failed to interact or when cells were incubated with phospholipid multilamellar vesicles alone. To further identify the components of serum that become altered in their efflux potential after reaction with phospholipid, isolated lipoprotein fractions were incubated with DMPC or BBSM and it was found that only interaction with HDL caused enhancement of cholesterol efflux. In summary, cholesterol removal from the Fu5AH cells by serum can be promoted by adding phospholipid under conditions where new HDL-like complexes can be formed between the phospholipid and serum components, most notably apolipoprotein A-I.—**Jian, B., M. de la Llera-Moya, L. Royer, G. Rothblat, O. Francone, and J.B. Swaney.** Modification of the cholesterol efflux properties of human serum by enrichment with phospholipid. *J. Lipid Res.* 1997. **38:** 734–744.

Supplementary key words sphingomyelin • reverse cholesterol transport • HDL subclasses • pre-β HDL

Cholesterol plays an important role in all cells, either as a membrane constituent or as an intermediate in the production of other bioeffector molecules. Cell cholesterol is derived from de novo synthesis and from receptor-mediated uptake of serum lipoproteins (1). As most cells cannot further metabolize this molecule, excess cholesterol is removed by a process known as reverse cholesterol transport (RCT) whereby it is transported to the liver for excretion in the form of bile acids (2). Efflux of cholesterol from peripheral cells to high density lipoprotein (HDL) is believed to be the first step in RCT.

Cholesterol efflux from cells has been extensively studied to determine the mechanism of cholesterol removal and the factors that influence the rate of this process. As the solubility of cholesterol in water is extremely low, acceptors must be present outside of the cell to pick up the cholesterol that desorbs from the plasma membrane. When whole serum is incubated with cells containing radiolabeled cholesterol, Castro and Fielding (3) and Francone, Fielding, and Fielding (4) have shown that several components in the HDL fraction acquire the majority of the labeled cholesterol. They have also shown that a small subfraction of the HDL, termed $preβ_1$ HDL based on electrophoretic mobility, is an initial acceptor of label and that, with time, the label shifts to larger preβ HDL and ultimately to the major HDL fraction with α-mobility. In our recent studies (5) of human serum and transgenic rat serum, we have found that the parameter that most highly correlates with the ability of serum to promote cholesterol efflux from cultured cells is HDL phospholipid. Presumably, the phospholipid layer on the lipoprotein surface serves as a region that can solubilize the cholesterol, and HDL plays

Abbreviations: BBSM, bovine brain sphingomyelin; CE, cholesteryl ester; DMPC, dimyristoyl phosphatidylcholine; DSPC, distearoyl phosphatidylcholine; POPC, palmitoyloleoyl phosphatidylcholine; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); DMS, dimethylsuberimidate; MEM, minimum essential medium; HDL, high density lipoprotein; LCAT, lecithin:cholesterol acyltransferase; LDL, low density lipoprotein; MLV, multilamellar vesicle; PL, phospholipid; RCT, reverse cholesterol transport; TG, triglyceride; Tm, phase transition temperature.

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an important role in efflux, being a small phospholipid-rich lipoprotein.

Intravenous infusion of HDL in animal models of atherosclerosis has been shown to cause the reversal of some of the characteristics of atherosclerosis, such as vessel wall thickening and lipid content (6). In fact, the therapeutic potential of phospholipid enrichment of serum to cause regression of atherosclerosis has been previously considered, especially as phospholiposomes are a potential vehicle for drug delivery (7, 8). However, while it is requisite for liposomes to retain their integrity when used as drug delivery systems, we have considered the opposite premise, that dissolution of the liposomes with formation of lipid–protein complexes might maximize the effect of such phospholipid in promoting cholesterol transport. The ability of liposomes to augment the PL content of lipoproteins and to enhance the antiatherogenic properties of serum has already been considered (8).

In our studies we have postulated that the capacity and/or efficiency of serum to promote cellular cholesterol efflux can be enhanced if exogenous phospholipid can be reacted with serum to enter the HDL fraction. Based upon the literature and our previous studies, we know that certain phospholipids are capable of reacting spontaneously with purified apolipoproteins or HDL at the phase transition temperature of the lipid, with the consequent formation of small protein–lipid complexes (9–11). Here we report that enrichment of the phospholipid content of serum by spontaneous reaction affects both the ability of serum to promote cholesterol efflux and the chemical and physical properties of the serum lipoproteins. DMPC and BBSM were used in our study; the reasons for using these two phospholipids are: *1*) DMPC has been well-studied in the past and its physical–chemical properties are well defined, *2*) sphingomyelin is the second most abundant phospholipid in mammalian plasma, and *3*) the content of sphingomyelin is elevated in the preβ HDL and apoE-containing γ-migrating particles which are early acceptors of cell-derived cholesterol (12, 13).

MATERIALS AND METHODS

Human serum was obtained from normolipemic volunteers. Lipoprotein fractions were isolated from serum by sequential ultracentrifugation (14). Dimyristoyl phosphatidylcholine (DMPC), bovine brain sphingomyelin (BBSM), 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC), and distearoyl phosphatidylcholine (DSPC) were purchased from Avanti Polar Lipids, Inc. 5,5′-Dithio-bis(2-nitrobenzoic acid) (DTNB) and dimethylsuberimidate (DMS) were purchased from Pierce Chemical Co. [^{14}C]dipalmitoyl phosphatidylcholine (DPPC) and [^{3}H]cholesterol were purchased from NEN Research Products (DuPont).

Preparation of phospholipid multilamellar vesicles (MLV)

DMPC or BBSM were dissolved in methanol and the solvent was evaporated with N_2 at room temperature; any remaining solvent was removed by vacuum at room temperature over a period of 2–18 h. Buffer, (0.01 M Tris, 0.15 M NaCl, pH 8.0), was added and the samples were warmed to 40–50°C. The lipids were then vortexed vigorously for 1 min, placed in a low power sonication bath for 10 min, and again vortexed for 30 s to obtain turbid multilamellar vesicle dispersions.

Lipid turbidity clearance studies

To measure the disruption of the phospholipid MLV, the phospholipid was maintained at the desired temperature in a thermostated cuvette and serum was added at a serum-to-lipid ratio of 1:2 (μl/μg). The absorbance change over time was monitored at 325 nm using a Hitachi 2000 spectrophotometer.

Modification of serum with phospholipids

Preparative amounts of serum were incubated with DMPC-MLV or BBSM-MLV at the phase transition temperature (Tm) of the lipid for various periods of time in the presence of 2 mM DTNB to inhibit lecithin:cholesterol acyltransferase (LCAT) activity. After incubation, the samples were tested for their ability to promote cholesterol efflux after dilution in minimal essential media (MEM) to the desired concentration. In some studies, a trace amount of [^{14}C]DPPC was mixed with DMPC to facilitate monitoring of the distribution of added phospholipid; after incubation with serum, the samples were filtered through a 0.45-micron filter to get rid of unreacted MLV and then applied to a gel filtration column (Superose 6B, Pharmacia). [^{14}C]DPPC was used as a marker for DMPC in these experiments because radiolabeled DMPC was not currently available. The effluent was monitored at 280 nm and fractions were collected and counted for radioactivity.

Cholesterol efflux assay

The cholesterol efflux ability of the lipid-modified whole serum or isolated serum fractions was evaluated by a 4 h cholesterol efflux assay described elsewhere (15). Briefly, serum or PL-modified serum was customarily diluted in MEM to 5% of the original concentration and incubated with [^{3}H]cholesterol-labeled Fu5AH rat hepatoma cells for 4 h at 37°C. The amount of radiolabeled cellular cholesterol released to the medium was expressed as the fraction of the total radioactive cholesterol present initially in the cells; all experi-

ments were performed in triplicate. The efflux potential of the individual samples was compared to the activity of the original serum sample. Dilution of serum was necessary in our assay system to prevent toxicity to the cells, although it is known that dilution can alter the size of certain HDL populations (16).

Two-dimensional nondenaturing electrophoresis and antibody blotting

To determine the distribution of apoA-I among HDL subclasses, plasma was first incubated with DMPC or BBSM at the Tm of the lipid for 2 h in the presence of 1.5 mM DTNB, after which the samples were electrophoresed in a 0.75% (w/v) agarose gel in 50 mM barbital buffer on Gelbond (FMC, Rockland, ME) and then placed on a 3–16% polyacrylamide gradient gel (Integrated Separation Systems, Natick, MA) in 25 mM Tris-glycine buffer (pH 8.3) (17). Electrophoresis was carried out for 4.5 h. After transfer to Nitro Plus transfer membranes (Micron Separation Incorporated, Westboro, MA), the samples were treated with a biotinylated goat polyclonal antibody to human apoA-I in 2% milk in 10 mM phosphate buffer, pH 7.0. ApoA-I-containing HDL species were visualized with ^{125}I-labeled streptavidin (Amersham, Arlington Heights, IL) and the nitrocellulose membranes were exposed to Fuji XLS film at −70°C.

Chemical compositions

Protein concentration was quantitated using the Markwell modification of the Lowry method (18). Total and free cholesterol were measured enzymatically and the mass of cholesteryl ester was computed from the differences between total and free cholesterol by utilizing a factor of 1.7. Total phospholipid was determined by the method of Sokoloff and Rothblat (19). The protein mass per particle was estimated by cross-linking the proteins with DMS (20). The hydrodynamic diameters of the particles were estimated by nondenaturing gradient gel electrophoresis (4–30%) using reference globular proteins (Pharmacia) (21).

RESULTS

Modification of serum by enrichment with phospholipid

Multilamellar dispersions of phospholipids are highly turbid due to light scattering of the large particles; when these dispersions are incubated with serum the turbidity decreases due to the disruption of the large particles and the formation of smaller complexes that do not scatter light. **Figure 1** shows the rate of turbidity clearance as a function of temperature when samples of either DMPC-MLV or BBSM-MLV were incubated with serum at the indicated temperatures. The rate of clearance was defined as the absolute change in absorbance at a wavelength of 325 nm (where no absorption of light takes place). The data demonstrate that the rate of clearance of DMPC is greatest at 24°C, which is the gel-to-liquid crystalline phase transition temperature (Tm) for this lipid (22); furthermore, the rate of clearance decreases rapidly at temperatures only slightly higher or lower than 24°C. The clearance of turbidity also occurred when serum was added to multilamellar dispersions of BBSM when the temperature was maintained close to its Tm phase. This temperature has been shown to depend upon the source of BBSM (23) and, similarly, we found by differential scanning calorimetry that BBSM obtained from Avanti Polar Lipids had a Tm of 37°C, while that obtained from Sigma Chemical Co. had a Tm of 27°C (data not shown). It also appears that the BBSM-MLV are cleared over a somewhat wider range of temperatures than DMPC-MLV; this may be a consequence of the more homogeneous nature of DMPC, which is a synthetic phospholipid, compared to BBSM, which contains a mixture of fatty acyl chains (principally stearic and nervonic). The studies reported here utilized material obtained from Avanti, although BBSM from both sources appeared equal in their ability to combine with serum and enhance cholesterol efflux. Other phospholipids, such as POPC and DSPC, were cleared by serum poorly or not at all, even at their phase transition temperature; we believe that this resistance to disruption of the vesicles is related to the cooperativ-

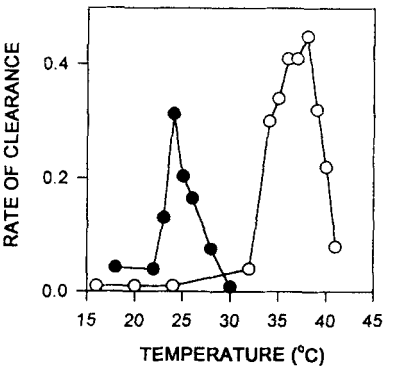

Fig. 1. Isothermal study of the effect of temperature on the clearance of DMPC-MLV turbidity by human serum. One ml DMPC-MLV (100 μg) was equilibrated in a thermostated cuvette at the indicated temperature and 50 μl of serum was added at T = 0. The absorbance at 325 nm was monitored for 1000 sec. The rate of clearance, defined as the absolute change in absorbance after 1000 sec, was plotted against the incubation temperature for multilamellar vesicles of DMPC (●) or BBSM (○).

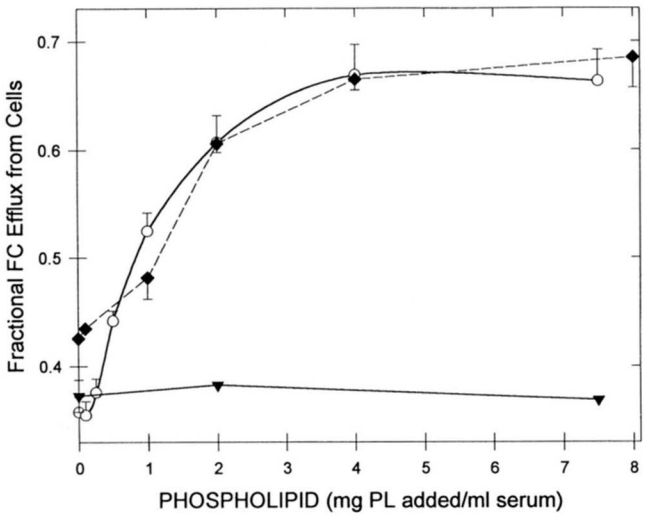

Fig. 2. The effect of preincubation of human serum with various amounts of phospholipid on the ability of the serum to promote cholesterol efflux from Fu5AH hepatoma cells. One hundred µl of serum was preincubated for 1 h with DMPC-MLV at 24°C (○), with POPC-MLV at 24°C (▼), or with BBSM-MLV at 37°C (◆) in the presence of 2 mM DTNB to inhibit LCAT. After this treatment the samples were diluted to 5% serum concentration with MEM and incubated with [^{3}H]-cholesterol-labeled Fu5AH cells for 4 h. Cholesterol efflux was determined by counting radioactivity in the culture medium, relative to the total counts in the cells + medium. Each data point represents the mean of three cell wells. The error bars represent 1 SD.

ity of the phase transition as described in our earlier publications (24, 25).

Effect of phospholipid enrichment of serum on cholesterol efflux

The effect of phospholipid enrichment on the ability of serum to promote cholesterol efflux from Fu5AH hepatoma cells was determined (**Fig. 2**). In these experiments, serum was pre-incubated with phospholipid-MLV at its phase transition temperature for 1 h and then held at 4°C until used for the efflux experiments. By varying the ratio of phospholipid to serum, we found that both DMPC and BBSM can increase the relative efflux from cells by about 50% compared to the control serum. The effect on efflux was saturable at high proportions of phospholipid. With DMPC, one half of the maximal enhancement of efflux was achieved at the ratio of 1 mg DMPC/ml serum; for reference, the phospholipid concentration in our samples of human serum was approximately 2 mg/ml.

When comparable amounts of phospholipid multilamellar vesicles alone were used in efflux experiments, minimal efflux was observed (<1% at 4 h), comparable to the efflux to medium alone (data not shown). Effectively no enhancement of cholesterol efflux was observed when serum was pre-incubated with POPC at 24°C, which is far from its Tm (Fig. 2). Data presented in **Fig. 3** show that the enhancement of efflux by PL-MLV was maximal at the phase transition temperature for each lipid, although some enhancement was observed at other incubation temperatures. However, incubation of serum with distearoylphosphatidylcholine (DSPC) at either 0°C or 37°C failed to enhance efflux, which suggests that failure to clear in the presence of serum correlates with a lack of effect on cholesterol efflux.

Correlation between reduction in turbidity and cholesterol efflux

While the reduction in turbidity of a phospholipid dispersion by serum is a useful indicator of serum-PL reaction, this is not a direct measure of complex formation. In order to define the extent to which clearance of turbidity was a useful predictor of changes in efflux potential, samples of DMPC and serum were mixed at 24°C and aliquots were removed over the time course of the clearance reaction for assay of cellular cholesterol efflux (**Fig. 4**). These data showed that the reduction in turbidity closely paralleled the increase in cholesterol efflux by an equivalent sample. Furthermore, a linear relationship was observed between the light scattering

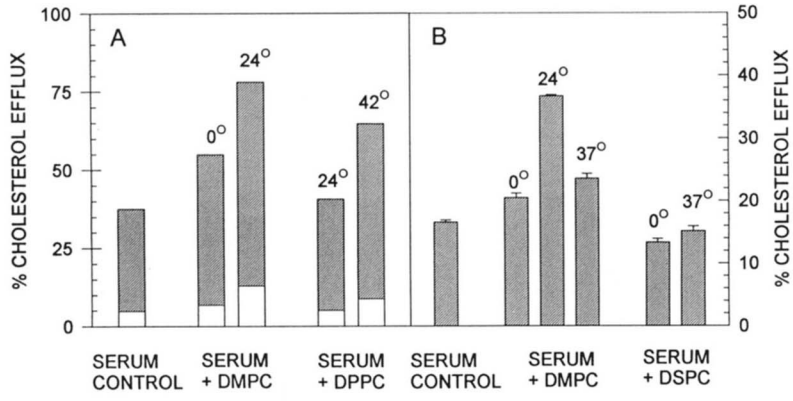

Fig. 3. The effect of preincubation of human serum with phospholipid at different temperatures on the ability of the serum to promote cholesterol efflux from Fu5AH cells. Serum was preincubated with PL-MLV for 1 h at the indicated temperature, then the samples were incubated with [^{3}H]-cholesterol-labeled Fu5AH cells for 30 min (open bar) or 4 h (hatched bar). Panel A: 100 µl of serum was preincubated for 1 h with 800 µg DMPC-MLV or DPPC-MLV, panel B: 49 µl of serum (containing 100 µg PL) was preincubated for 1 h with 200 µg DMPC-MLV or DSPC-MLV. Cholesterol efflux was determined using the method described in Fig. 2.

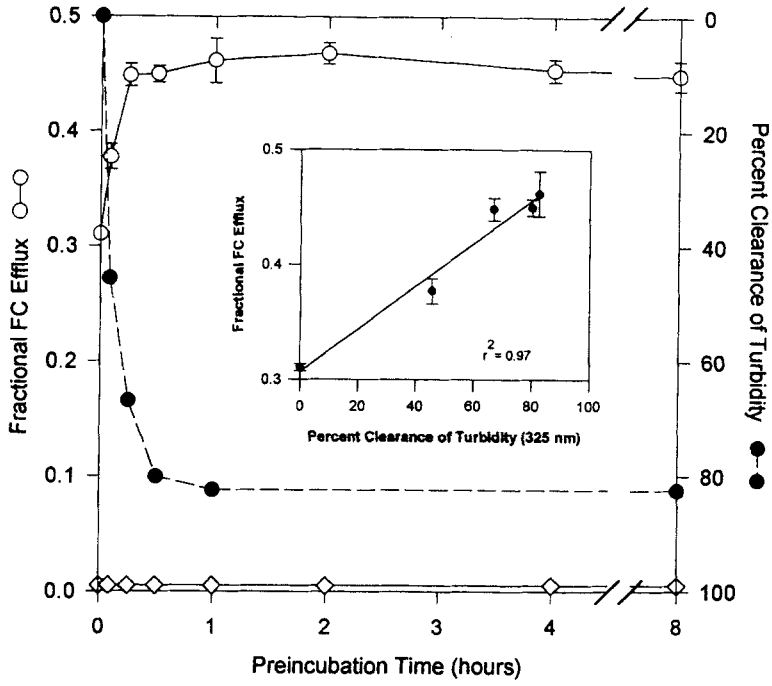

Fig. 4. Correlation between clearance of phospholipid turbidity by serum and cholesterol efflux. To a sample of DMPC-MLV equilibrated at 24°C in a spectophotometer cuvette was added an aliquot of serum at T = 0. Aliquots were removed at intervals and placed on ice for assay of cholesterol efflux from Fu5AH cells (○); the change in absorbance values was expressed as a percentage of the initial absorbance as a reflection of the clearance of turbidity (●). The open diamond (◇) represents the efflux mediated by DMPC-MLV alone.

measurements and cholesterol efflux with a correlation coefficient of 0.97 (Fig. 4, inset). Incubation of cells with DMPC-MLV alone showed no change in the amount of cholesterol removed over the 8-h period.

In order to ascertain the nature of the products formed in these studies, a parallel set of serum samples were incubated with DMPC containing a trace amount of [^{14}C]DPPC at 24°C and aliquots were removed from the incubation at different time points. The samples were filtered through a 0.45-micron filter and applied to a gel filtration column in order to determine the fraction of phospholipid actually incorporated into complexes (**Fig. 5**). At T = 0 less than 15% of the radioactivity could pass through the 0.45 micron filter, whereas by 2 h >99% of the radioactivity passed through the filter, suggesting that most of the DMPC was incorporated into lipoproteins or formed small, protein–lipid complexes. During the early time points, aliquots applied to the gel filtration column showed that for incubations lasting up to 1 h almost all the radioactive phospholipid was eluted in the region where HDL elutes, and the amount of radioactivity incorporated into protein–lipid complexes increased over this time interval. After 2 h of incubation the total amount of radioactivity eluting from the column remained constant but showed significant redistribution into higher molecular weight fractions, including the regions where LDL and VLDL elute (data not shown).

As the change in efflux reached its maximum level at the time points when phospholipid was maximally incorporated into HDL, we also evaluated the effect of incubation of phospholipid with individual lipoprotein fractions. The ability of individual lipoprotein fractions before and after lipid modification to promote cholesterol efflux are shown in **Table 1.** It should be noted that each sample contained the same amount of endogenous lipid, whereas samples incubated with DMPC or BBSM also contained exogenous lipid in unreacted MLV or as lipid–protein complexes. In the assay, lipoproteins were present at a concentration of 50 μg of PL/ml efflux medium while for the lipid-modified samples, 100 μg DMPC or BBSM was pre-incubated with the fractions (50 μg PL; final concentration: 150 μg PL/ml medium) for 1 h at 24°C or 37°C, respectively, prior to incubation with cells. The results show that, in the absence of added PL, similar efflux was achieved for all individual lipoprotein fractions except VLDL, based on the equal amount of phospholipid; VLDL yielded somewhat lower efflux values than the other fractions. Lipid modification did not stimulate efflux by the VLDL or LDL fractions; on the contrary, DMPC caused a significant decrease in efflux by the LDL fraction, possibly due to fusion of LDL particles as evidenced by increased turbidity and LDL size (data not shown). By contrast, efflux increased dramatically for the HDL fractions after modification by DMPC, while BBSM was able to enhance efflux by HDL_3 but not by HDL_2. We believe that BBSM is capable of modifying HDL_2 in whole serum, but fails to react with the isolated HDL_2 fraction. Both lipids were equally effective at enhancing cholesterol efflux by the d > 1.063 g/ml fraction and whole serum.

The contribution of the d > 1.21 g/ml fraction to

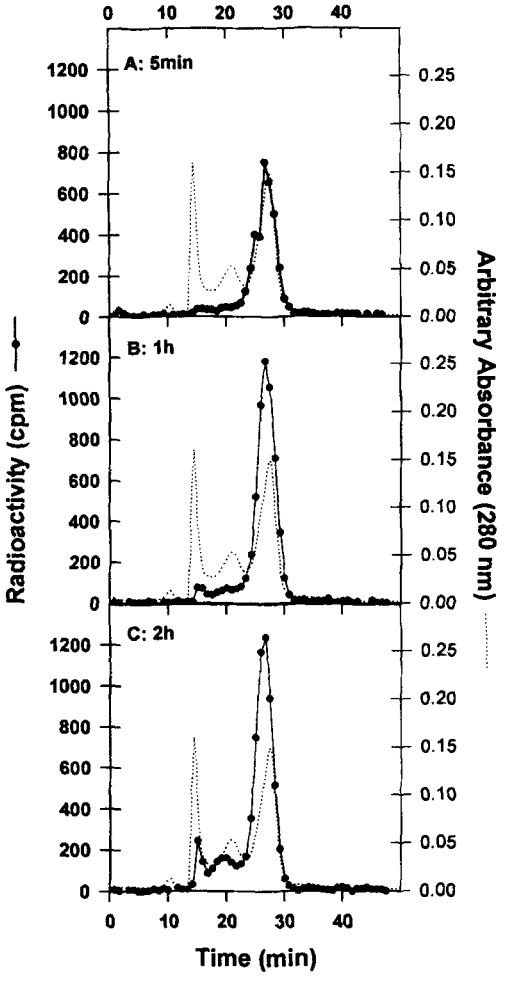

Fig. 5. Gel filtration on Superose 6 of serum after incubation with DMPC containing trace amount of [^{14}C]DPPC at 24°C for various periods of time (which is the same as in Fig. 4 clearance and efflux studies). Fractions from the column eluent were counted for radioactivity to determine the elution profile for the reacted phospholipid (solid circles). The dashed line in each panel shows the elution profile for the major lipoprotein classes (VLDL, LDL, and HDL, respectively) as taken from the elution of the d < 1.21 g/ml fraction from human serum. The samples shown are from the following times of incubation: A, 5 min; B, 1 h; and C, 2 h.

TABLE 2. Efflux ability of d > 1.21 g/ml and d < 1.21 g/ml fractions and the effect of DMPC modification on these fractions

Fraction	Fraction Alone (100 µl serum or equivalent)	Fraction + DMPC (1 mg DMPC/ml serum)
d < 1.21 g/ml	28.6 ± 1.37	49.7 ± 2.87
d > 1.21 g/ml	17.3 ± 0.29	44.6 ± 3.4
Serum	42.6 ± 0.96	58.3 ± 0.52

The d> 1.21 g/ml and d< 1.21 g/ml fractions were isolated by a single centrifugation step at a density of 1.21 g KBr/ml. Then serum or two fractions were preincubated with DMPC-MLV in a serum-to-DMPC ratio of 1:1 (ml/mg) at 24°C for 1 h. Four h efflux was performed as described in Methods. All values were means ± SD of triplicate wells.

the total cholesterol efflux was also investigated (**Table 2**). The d > 1.21 g/ml fraction was isolated by a single centrifugation step at a density of 1.21 g/ml. One hundred µl of serum or an equivalent volume of d > 1.21 g/ml and d < 1.21 g/ml fractions were diluted to 5% serum concentration (or its equivalent) and incubated with Fu5AH cells for 4 h. Cholesterol efflux mediated by the d > 1.21 g/ml fraction was approximately 40% of the total efflux by whole serum and efflux ability of both the d > 1.21 g/ml and d < 1.21 g/ml fractions increased dramatically after DMPC modification.

Effect of phospholipid enrichment on the properties of the serum lipoproteins

As the change in efflux by PL-enriched serum appeared to correlate with the specific incorporation of phospholipid into the HDL fractions, we attempted to characterize the changes that occurred in these lipoprotein fractions. **Table 3** shows the chemical compositions and diameters of HDL fractions with and without lipid modification. Whole serum was first reacted with DMPC or BBSM at the Tm of the lipid, then lipoprotein fractions were isolated by sequential ultracentrifugation and the purity of each fraction was checked by SDS-PAGE and GGE; HDL fractions were not "washed" by recentrifugation in order to prevent deterioration of these particles. It should be noted that the fraction isolated in the d 1.07–1.125 g/ml density interval may con-

TABLE 1. Effect of phospholipid enrichment on cholesterol efflux ability of individual lipoprotein fractions compared to the whole serum

	VLDL	LDL	HDL_2	HDL_3	d > 1.063 g/ml	Serum
Fraction alone	82 ± 0.6	119 ± 0.8	122 ± 0.3	121 ± 0.5	103.2 ± 0.6	100 ± 0.3
Fraction + DMPC	99 ± 0.4	32 ± 0.1	236 ± 0.7	255 ± 0.3	305 ± 1.53	221 ± 0.2
Fraction + BBSM	84 ± 0.3	96 ± 0.1	137 ± 0.1	201 ± 1.24	312 ± 1.11	225 ± 0.9

Serum or serum fractions were preincubated with PL-MLV in a serum-to-PL ratio of 1:2 (ml/mg) at the phase transition temperature of the lipid. Then all the samples were diluted in the efflux medium at a concentration of 50 µg of endogenous PL/ml efflux medium (for lipid-modified fractions, an extra 100 µg of exogenous lipid were added). The radioactivity released to the medium was expressed as the fraction of the total radioactive cholesterol present in the well and normalized by whole serum efflux. All values were means ± SD of triplicate wells.

TABLE 3. Chemical compositions and diameters of HDL with and without lipid modification

	Protein[a]	PL[b]	TG[c]	FC[d]	CE[d]	Protein Mass/Particle[e]	Diameter (PAGGE)[f]
			%			*kda*	*nm*
HDL_2							
Serum alone	43.1	24.7	8.5	3.1	20.6	89/119	8.8/9.8
Serum + DMPC	34.1	44.4	4.0	3.1	14.4	81	9.4
Serum + BBSM	34.0	47.2	4.1	4.1	10.6	84	9.6
HDL_3							
Serum alone	54.7	23.4	4.1	1.8	16.0	84	8.2
Serum + DMPC	44.2	38.8	4.1	1.9	11.0	56/76	8.1/9.3
Serum + BBSM	47.8	35.6	5.7	2.6	8.3	56/78	7.6/9.3

[a]Quantitated by the Markwell-Lowry method (18).
[b]Determined by the method of Sokoloff and Rothblat (19).
[c]Determined enzymatically using a kit prepared by Walco Pure Chemical Industries, Ltd.
[d]Total and free cholesterol were measured enzymatically and the mass of cholesteryl ester was computed from the differences between total and free cholesterol by utilizing a factor of 1.7.
[e]Estimated from SDS-polyacrylamide gel electrophoresis of samples after cross-linking with DMS, using cross-linked apoA-I as a standard (20).
[f]Estimated from nondenaturing gradient gel electrophoresis using reference globular proteins (21).

tain modified HDL_2, A-I/PL complexes, and/or fused HDL_3, which results from modification of HDL_3 by phospholipid (26). Results show that fractions isolated from both the HDL_2 and HDL_3 density intervals were modified by the phospholipid treatment; the phospholipid-to-protein ratio increased substantially in each lipoprotein fraction. Second, the sizes of HDL_2 and HDL_3 were found to be changed after lipid modification. Two HDL_2 bands, which correspond to HDL_{2a} and HDL_{2b}, were seen in the GGE gel for samples isolated from unmodified serum, while after lipid modification HDL_2 became more homogenous and only one band was seen on the GGE gel with a size between HDL_{2a} and HDL_{2b}. By contrast, one HDL_3 band was seen before lipid modification on GGE while two bands were visualized after lipid modification, one larger than the original HDL_3 while the other was smaller. Third, cross-linking studies indicated that the protein content per particle decreased, suggesting the removal of protein from HDL. Data in **Table 4** show that after phospholipid enrichment of serum there is a re-distribution of protein mass between the density intervals corresponding to HDL_2 and HDL_3 as well as a similar shift in the distribution of cholesteryl ester (CE). Total protein mass in the total HDL remains essentially unchanged as a result of PL modification.

The changes in size and mobility of apoA-I-containing particles were also investigated by two-dimensional gel electrophoresis (**Fig. 6**). In the experiments, aliquots of human serum were incubated with DMPC or BBSM at 24°C or 37°C, respectively, for 2 h at various proportions of phospholipid to serum, after which two-dimensional electrophoresis was performed (17). In control serum, two major zones with α-electrophoretic migration and molecular weights corresponding to HDL_2 and HDL_3 reacted with antibodies to apoA-I. Migrating slightly slower (with pre-β mobility) was a spot at low molecular weight ($preβ_1$ HDL) and three spots of lesser intensity with sizes greater than HDL_2 ($preβ_2$-HDL). A spot of higher molecular weight corresponding to $preβ_3$-HDL was observed in all samples, but was too faint to be photographically reproduced. After the titration of serum with DMPC, there was a marked increase in intensity of the spot corresponding to $preβ_1$-HDL and a gradual shifting of the higher molecular weight portion of the α-migrating zone to a more preβ

TABLE 4. Effect of phospholipid enrichment on the distribution of protein and cholesteryl ester between HDL_2 and HDL_3

	Serum Alone		Serum/DMPC		Serum/BBSM	
	HDL_2	HDL_3	HDL_2	HDL_3	HDL_2	HDL_3
Protein[a] (%)	18	82	44	54	68	32
Chol. ester[b] (%)	26	74	57	43	79	21
Protein[a] (mg)	0.96	4.39	2.45	3.11	3.9	1.84

[a]Quantitated by the Markwell-Lowry method. Protein mass was mg protein/5 ml serum.
[b]Total and free cholesterol were measured enzymatically and the mass of cholesteryl ester was computed from the differences between total and free cholesterol by utilizing a factor of 1.7.

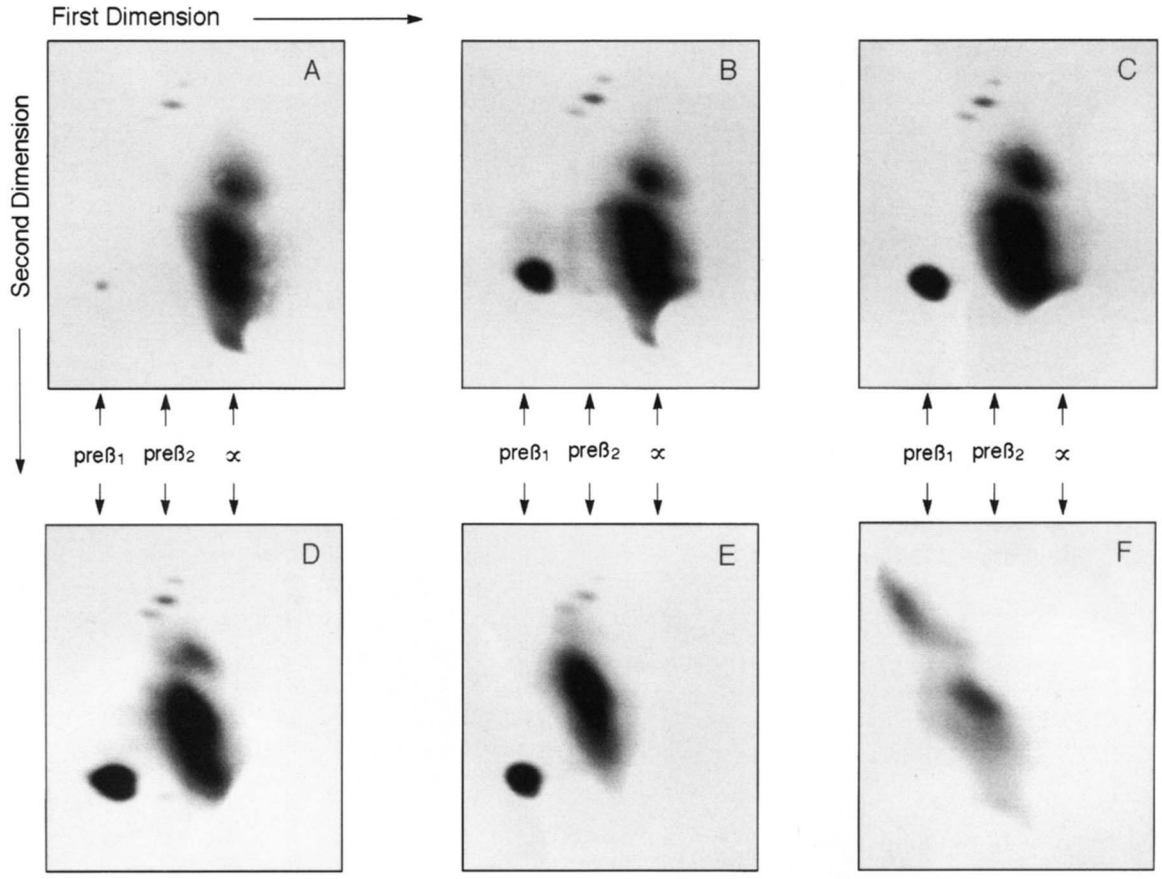

Fig. 6. Two-dimensional agarose-PAGGE electrophoresis of human serum reacted with DMPC for 2 h and immunodetected for apolipoprotein A-I by Western blotting. First dimension: agarose electropheresis; second dimension: polyacrylamide gradient gel electropheresis. The ratios of phospholipid: serum were: panel A, 0 mg/ml serum; panel B, 0.25 mg/ml serum, panel C, 0.5 mg/ml serum; panel D, 1.0 mg/ml serum; panel E, 2.0 mg/ml serum, and panel F, 8 mg/ml serum.

mobility. At high ratios of DMPC to serum (e.g., 8 mg/ml), the preβ$_1$-HDL spot disappeared and eventually almost all of the immunodetectable apoA-I was found in a preβ-migrating zone with a size equal to or larger than HDL$_2$ (e.g., 32 mg DMPC/ml serum, data not shown). The distribution of apoA-I was determined by scanning the film corresponding to Fig. 6 and plotting the percentage of apoA-I distributed in each zone (**Fig. 7**). These data suggest that apoA-I in the α-migrating region is transferred to the preβ$_1$-HDL region concurrent with reaction of serum with DMPC. Comparable studies of the effect of BBSM on the electrophoretic properties of the apoA-I-containing lipoproteins showed a similar shifting of α-HDL towards preβ-mobility, but with only minimal formation of preβ$_1$-HDL (data not shown).

DISCUSSION

Lipid deposition in the cells of vessel walls, which plays a role in atherogenesis, is believed to be reversible under some circumstances by the uptake of cell cholesterol by serum lipoproteins and its transport through the blood to the liver. Enhancement of the ability of serum to promote cholesterol removal from cells might prove beneficial, then, at retarding the progression of atherosclerotic disease. As we have found that efflux of labeled cell cholesterol as mediated by serum correlates best with HDL phospholipid, this suggests that enriching serum HDL-PL should promote cholesterol efflux from cells. To be effective at enhancing cholesterol efflux, it appears that phospholipid must form small complexes with proteins. Formation of such complexes with most lipids requires extreme conditions, such as high-powered sonication (27) or detergents (28, 29). However, some phospholipids, because of their unique physical properties, can spontaneously react with apolipoproteins at or near physiological temperatures, a fact that might find application for in vivo modification of serum.

Two such phospholipids are DMPC and BBSM, which we have shown to react spontaneously when incubated with serum at the phase transition temperature of the

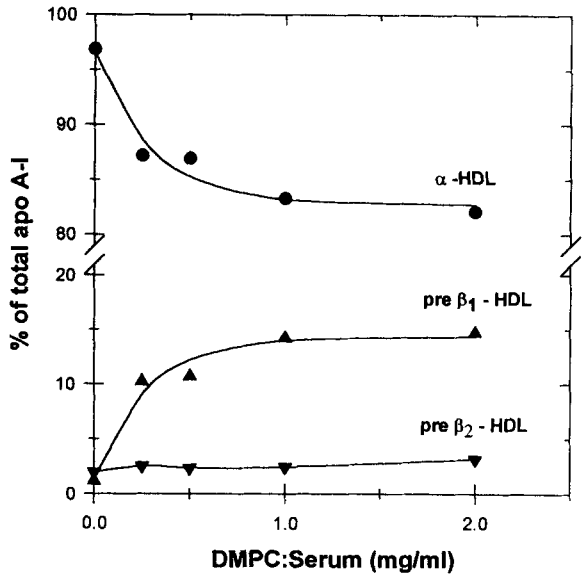

Fig. 7. Data from Fig. 6 were scanned and plotted to further illustrate the effect of phospholipid enrichment on the re-distribution of apoA-I within HDL fractions. The HDL fractions were: α-HDL (●), preβ$_1$HDL (▲), and preβ$_2$ HDL (▼).

lipid (Fig. 1). As a result of this interaction, the efflux ability of serum is increased by approximately 50% at saturating levels of phospholipid (Fig. 2). This efflux enhancement can be directly correlated with collapse of the PL-MLV, consistent with the finding that MLV alone cause no efflux enhancement (Fig. 4). Data from Table 1 suggest that both DMPC and BBSM showed a similar enhancement effect on whole serum efflux. Further, this increased efflux is achieved by enrichment of PL in HDL, but not in VLDL or LDL. On the contrary, efflux ability is decreased by enrichment of LDL with phospholipid; this can probably be attributed to aggregation of LDL induced by PL, with a decreased ability of these large particles to participate in cholesterol efflux (30).

Studies were performed that were aimed at characterizing the specific changes in the HDL fractions after reaction of whole serum with phospholipid. After lipid modification, both the phospholipid-to-protein ratio and the sizes of HDL$_2$ and HDL$_3$ increased (Table 3). The size of LDL was also found to be increased after DMPC modification but not following BBSM modification (data not shown). Decreases in protein mass per particle after lipid modification suggest that proteins are removed from the pre-existing HDL particles (Table 3). Also, after incubation with phospholipid, more protein mass is found in the HDL$_2$ region and this increase is accompanied by a corresponding decrease in HDL$_3$ protein (Table 4), indicating that HDL$_3$ particles are converted to HDL$_2$ by the addition of phospholipid; this hypothesis is supported by the finding of a comparable shift in cholesteryl ester from HDL$_3$ to HDL$_2$ (Table 4). Phospholipid/apoprotein complexes formed by interacting PL with apoprotein released from HDL may also be isolated in this density interval. Taken together, our results suggest that part of the exogenous phospholipid is directly inserted into pre-existing HDL and part of it forms new lipid–protein complexes with the apoproteins that dissociate from HDL.

Two-dimensional gel electrophoresis revealed that incubation of phospholipid with serum slows the migration of α-HDL toward to the preβ zone and increases the particle size (Fig. 6). Interestingly, the size and mobility of preβ$_1$-HDL do not change, but the intensity of the corresponding autoradiographic spot increased significantly at phospholipid-to-serum ratios less than or equal to 2 mg/ml (Fig. 6). The increased intensity in the preβ region is believed to be due to newly synthesized lipid/apoprotein particles. However, these newly formed preβ-migrating particles have markedly different electrophoretic properties compared to synthetic apoA-I/DMPC particles, which have preβ mobility but a larger size (data not shown).

Preβ-HDL is an initial acceptor of cholesterol and increased amounts of preβ-HDL are closely related to increased cholesterol efflux from cells (17). Preβ-HDL-like particles can be generated by incubation of free apoA-I and apoA-II with cholesterol-loaded macrophages (31). In addition, several proteins, such as hepatic lipase, phospholipid transfer protein (PLTP), and cholesteryl ester transfer protein (CETP), have been found to generate preβ-HDL by promoting the dissociation of apoA-I from α-HDL (17, 32, 33). In our studies, incubation of PL with serum at the Tm of PL also seems to generate preβ-HDL, especially preβ$_1$-HDL. The largest amount of preβ$_1$-HDL is generated at the PL-to-serum ratio of 2 mg PL/ml serum (Fig. 7). The production of preβ$_1$-HDL seems dependent upon the PL used, as there is only minimal formation of a preβ$_1$-HDL after serum-BBSM interaction, although BBSM is equally able to stimulate cholesterol efflux ability of serum. This suggests that efflux enhancement by BBSM modification of serum does not require the formation of preβ$_1$-HDL.

Unlike preβ$_1$-HDL, the intensity of preβ$_2$-HDL does not change as the PL-to-serum ratio increases (Fig. 6). This might suggest a different mechanism for formation of the preβ$_1$-HDL as opposed to the preβ$_2$- and preβ$_3$-HDL. However, at very high concentrations of PL, most of the α-migrating HDL shifts in size and mobility in the 2-D gel to approximate the preβ$_2$-HDL species.

The mechanism by which HDL is altered by phosphatidylcholine was investigated earlier by Tall et al. (26). By studying the incubation mixture of lecithin unilamellar or multilamellar liposomes and isolated HDL, they found that both HDL$_2$ and HDL$_3$ were modified

by phosphatidylcholine and transformed into lipoproteins of decreased density and increased size; the formation of discoidal PL/apoprotein complexes was identified by electron microscopy. These data suggested that PL was inserted into HDL and, at the same time, discoidal lipoproteins were formed as a result of interaction of liposomes with apoproteins released from HDL, similar results were obtained by Nichols et al. (34). We believe that in our studies new or modified HDL species are created similar to those described by Tall et al. (26) and Nichols et al. (34) and that some of these migrate with preβ-mobility.

It has been asserted for some time that sphingomyelin has a higher affinity for cholesterol than phosphatidylcholine, which may be attributed to greater intermolecular van der Waals interactions than are observed with phosphatidylcholine (35). Recently, it has been reported that the sphingomyelin content is elevated in the early cholesterol acceptors (pre-β HDL and γ-migrating apoE-containing lipoprotein) suggesting that sphingomyelin might enhance the ability of these particles to promote cholesterol efflux (12, 13). However, in our experiments we observed that, at all concentrations used, DMPC was at least as good as BBSM in enhancing the efflux ability of whole serum, which suggests that efflux is a function of multiple properties of the acceptors, the intrinsic affinity for cholesterol being only one of these properties.

Also paradoxical is the observation that some efflux enhancement is achieved in samples containing sera that were not incubated at the phase transition temperature of the phospholipids used, suggesting that phospholipid is incorporated into the serum by a non-spontaneous transfer (Fig 3). We also observed that enhancement of efflux by BBSM was more effective in whole serum than with isolated HDL (Table 1). These observations raise the possibility that some additional factor(s) in serum, such as PLTP or CETP, might play some role in the selective transfer of phospholipid from the MLV into serum components, such that reduced phospholipid uptake or efflux enhancement is observed with isolated HDL fractions. Furthermore, the efflux ability of the $d > 1.21$ g/ml fraction was also increased after DMPC modification (Table 2). Before DMPC modification, approximately 40% of efflux was observed which may be attributable to human serum albumin (36, 37). After DMPC modification, efflux ability of the $d > 1.21$ g/ml fraction increased about 157%. The increased efflux might be due to the formation of small particles between lipid-free or lipid-poor apolipoproteins, such as apoA-I or apoA-IV, and DMPC. Further studies need to be performed to test this hypothesis.

In conclusion, the ability of serum to promote cholesterol efflux can be markedly enhanced by the reaction of serum with suitable phospholipids. Enhancement of cellular cholesterol efflux is related to the specific enrichment of the HDL fraction by phospholipid. The mechanism of efflux enhancement by PL needs to be further investigated and the functions of other serum components, such as PLTP and CETP, in this lipid-modification and efflux enhancement process need to be further studies.

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