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Apolipoproteins in rat serum and renal lymph

(lipoprotein transport/peripheral lymph/very low density lipoproteins/low density lipoproteins/high density lipoproteins)

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ABSTRACT **The concentration of apolipoproteins was measured by quantitative immunoelectrophoresis in rat serum, in the lipoprotein-free ultracentrifugal fraction (density > 1.21) of serum, and in renal lymph. The A-IV and arginine-rich apolipoproteins were present in high concentrations (>18.5% of serum concentration) both in the fraction of density > 1.21 and in renal lymph, whereas the other apolipoproteins were found in low concentrations (<7% of serum concentration). The major apolipoproteins of renal lymph were similar to those found in the fraction of density > 1.21; however, the apolipoprotein composition of the renal lymph was very different from the apolipoprotein composition of serum lipoproteins. The presence of certain apolipoproteins in the fraction of density > 1.21 and in renal lymph suggests that these apolipoproteins might be present in the circulation as "free" apolipoproteins. The possible physiological importance of these specific apolipoproteins is postulated.**

The protein moieties of the various serum lipoproteins contain multiple heterogeneous proteins, the apolipoproteins (1, 2). In man, the very low density lipoprotein (VLDL) has been shown to contain three major groups of apolipoproteins: the large molecular weight protein, apo-B, which is also present as the major protein of the low density lipoprotein (LDL); an arginine-rich protein (ARP) of molecular weight 33,000; and a group of proteins of molecular weights ranging from 7,000 to 10,000, which have been designated C proteins. The human high density lipoprotein (HDL) contains as its major component a protein of molecular weight of 28,000 (A-I) and another component, A-II, of molecular weight 17,000. In addition, small amounts of the above-mentioned C proteins are also present (1, 2).

The apolipoproteins of the rat serum lipoproteins are analogous in many respects (3–6) to the human lipoproteins, with some exceptions: rat HDL contains not only A-I and the C apoproteins, but also an arginine-rich protein (3, 5) and an additional protein of molecular weight 46,000, which has been designated A-IV (5). Furthermore, the protein analogous to human A-II is present as a monomer, with a molecular weight of 8000 (5, 6).

Apolipoproteins have been found in the "lipoprotein-free" ultracentrifugal fraction of serum of density > 1.21 ($d > 1.21$ fraction) (7–10). Whether these apolipoproteins in the $d > 1.21$ fraction are normal constituents of native serum or are artifacts resulting from ultracentrifugation is not known.

In the present report, this question was examined by studying the apolipoprotein composition of peripheral lymph. Because the size of the "free" apolipoproteins is much smaller than that of the lipoproteins, it might be anticipated that they would preferentially be transferred into lymph from the serum. Renal lymph was studied, inasmuch as it flows spontaneously at a relatively high rate (11). Furthermore, the blood supply to the kidneys far exceeds the metabolic requirements of the organ, so that any newly synthesized proteins would be diluted and those found would largely reflect transfer of preformed apolipoproteins from plasma to lymph.

Lymph has been shown to contain lipoproteins (12); therefore, the finding of apolipoproteins would not necessarily prove that they had entered the lymph in the "free" form. However, if the apolipoprotein content of lymph differs markedly from that of the serum lipoproteins, and if it is similar to that of the $d > 1.21$ fraction of serum, then it can be reasonably inferred that they are transferred as free apolipoproteins.

MATERIALS AND METHODS

Renal lymph was collected (11) from Sprague-Dawley rats weighing 150–300 g (obtained from A. R. S. Schmidt Co., Madison, Wisc.). The animals were anesthetized with inactin-sodium (Promonta GMBH, Hamburg, West Germany), 120 mg/kg intraperitoneally. After cannulation of the femoral vein, the rats received an infusion of 0.5–4.0 ml of 0.9% saline solution in 3–4 hr. The left kidney was exposed by flank incision and gently freed from peritoneal attachments. The posterior surface of the hilum was observed under an operating stereo microscope and a hilar lymphatic vessel was cannulated with a polyethylene tubing 30–80 μm in diameter. Unless clear lymph flowed immediately and spontaneously from the cannula, the animals were not used. Lymph drained at a rate of 0.5–2.0 μl/min and was collected over a period of 3–4 hr in hematocrit capillary tubes; the ends of the tubes were flame-sealed and the samples stored at 4°. At the end of the experiment, blood was collected from the abdominal aorta.

The Quantitative Immunoelectrophoresis Method of Laurell was used (13) for determination of apolipoprotein concentration in serum and lymph. The agarose (Seakem, Marine Colloids, Inc., Roekland, Maine) was made up in a 0.8% solution in 0.025 M barbital buffer, pH 8.6, and poured on 200 × 100 × 2 mm plates. The antiserum was mixed with the agarose solution at 50°. During electrophoresis, precipitation occurred at the point of antigen-antibody equivalence, forming a rocket-shaped peak, the height of which is a function of the amount of antigen applied. Standard curves were obtained by dilution of rat serum with uninjected goat serum; the data are expressed as percentage of serum concentration of the antigen. The plates were stained for the detection of protein with Buffalo Black NBR (14) and for the detection of lipid with Oil Red-O (15).

The following antigens were used: apo-B, ARP, C-III, a mixture of C proteins, A-I, A-IV, and albumin. Apo-B was prepared by injecting low density lipoproteins of d 1.030–1.050 prepared by ultracentrifugation from normal rat serum. Arginine-rich apolipoproteins and the "C" proteins were prepared

Abbreviations: VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; VHDL, very high density lipoprotein; ARP, Arginine rich apoprotein; $d > 1.21$ fraction, infranatant fraction of serum after ultracentrifugation at density (d) 1.21.

Table 1. Apolipoprotein concentrations in renal lymph and the $d > 1.21$ fraction of serum*

	ARP	A-IV	A-I	C-III	Apo-B	Albumin
	(% of serum concentration)					
Lymph (10)†	18.5 ± 3.3‡	49.1 ± 6.0	<7	<3	<2	24.7 ± 1.9
$d > 1.21$ (5)	36.1 ± 2.0	30.5 ± 3.0	<7	<3	<2	98.7 ± 4.3

* Apolipoprotein concentrations were determined by quantitative immunoelectrophoresis (13). Samples were diluted 1:4 with uninjected goat serum, except for the determination of albumin, where the samples were diluted to 1:100; samples of 15 μl were applied. The electrophoresis was carried out overnight in a water-cooled chamber at 15°.
† No. of samples.
‡ Values are means ± SEM.

by Sephadex G-150 gel filtration of delipidated very low density lipoproteins ($d < 1.006$) (16). The ARP was eluted in the second Sephadex peak (VS-II) (1, 2) and the "C" proteins were eluted in the third peak of Sephadex (VS-III) (16). A-I apolipoprotein was obtained by Sephadex G-150 gel filtration of delipidated high density lipoproteins (d 1.063–1.21) and was present in the second Sephadex peak (HS-II). A-IV was prepared by preparative sodium dodecyl sulfate gel electrophoresis (5, 17). C-III was obtained by preparative isoelectric focusing (18). Albumin was prepared according to the method of Schwert (19). The various antisera were tested against whole serum and pure antigens by the double diffusion technique of Ouchterlony (20) and immunoelectrophoresis (21). Antisera were produced in goats according to the method of Vaitukaitis *et al.* (22).

Lipoprotein fractionation was performed in the Beckman L5-50 preparative ultracentrifuge according to the method of Havel, Eder, and Bragdon (23) using a 40.3 rotor. The centrifugation was carried out for 44 hr at 114,000 × g. The isolated lipoprotein fractions were washed by resuspension at the appropriate density and recentrifuged.

RESULTS

The comparison between the apolipoprotein concentration of renal lymph and the serum $d > 1.21$ fraction is shown in Table 1. Of the apolipoproteins measured, only ARP and A-IV were found in relatively high concentrations in both lymph and in the $d > 1.21$ fraction. The concentration of ARP was higher in the $d > 1.21$ fraction than in lymph, whereas this relationship was reversed for A-IV. The other apolipoproteins, A-I, C-III, and apo-B, were present in low concentrations (<7%) in both lymph and the $d > 1.21$ fraction. The concentrations of "C" apolipoprotein in lymph and in the $d > 1.21$ fraction were less than 3% of that in serum. All the albumin in serum was present in the $d > 1.21$ fraction, and the concentration in the renal lymph was 25% of that in serum.

Fig. 1 and Table 2 show the results of a representative experiment in which the whole serum, $d < 1.21$ and $d > 1.21$ fractions of serum, and renal lymph apolipoprotein concentrations were compared in the same animal. These findings are in agreement with the data shown in Table 1.

To exclude the possibility that the apolipoproteins present in the $d > 1.21$ fraction are constituents of the lipoprotein fraction designated $VHDL_1$ (d 1.21–1.25) (24), we adjusted the serum to different densities ranging from d 1.063 to 1.24 and determined the distribution of apolipoproteins in the infranatants. The apolipoprotein concentrations of the $d > 1.15$, $d > 1.21$, and $d > 1.24$ fractions were similar (Table 3).

DISCUSSION

These studies provide evidence that the apolipoproteins A-IV and ARP are found in relatively high concentrations in the $d > 1.21$ ultracentrifugal fraction of the serum. Furthermore, they demonstrate that the same apolipoproteins are also present in high concentrations in the renal lymph. Levy and Fredrickson have shown that as a result of ultracentrifugation apolipoproteins appear in the $d > 1.21$ fraction (8). Also, removal of lipids by ethanol-ether extraction from HDL results in the appearance of soluble apolipoproteins in the $d > 1.21$ fraction after ultracentrifugation. These soluble apolipoproteins readily combine with lipids and lipoproteins and upon recentrifugation float in densities similar to that of lipoproteins (25). Whether apolipoproteins found in the $d > 1.21$ fraction are present in the circulation as "free" apolipoproteins or as apolipoprotein complexes, or are artifacts due to ultracentrifugation (8), is not resolved. Because the molecular size of lipoproteins is much larger than that of free apolipoproteins, the presence of apolipoproteins in peripheral lymph would strengthen the suggestion that "free" apolipoproteins in the circulation are not artifacts and are derived from the serum apolipoprotein pool. The finding that only two apolipoproteins (A-IV and ARP) are present in relatively high concentrations in the $d > 1.21$ fraction and in renal lymph supports the conclusion that apolipoproteins or certain apolipoprotein complexes are present in the circulating plasma in a form other than lipoproteins. Were the apolipoproteins found in $d > 1.21$ fraction derived from alterations in serum lipoproteins, one would expect to find other apolipoproteins in high concentrations in this fraction. Thus, in the rat, HDL constitutes 80–90% of the total serum lipoproteins (26); of the HDL protein, about 50% is A-I, 10% is ARP, 10% is A-IV, and 20% are C proteins (5). In the $d > 1.21$ fraction, the relative concentrations of A-IV and ARP are severalfold higher than that of A-I. Were the $d > 1.21$ apolipoproteins derived from modification of the HDL during ultracentrifugation, it would be expected that A-I and other HDL apolipoproteins would be present in appreciable amounts.

Alaupovic *et al.* (24) have described in human serum a lipoprotein fraction of d 1.21–1.25 that they have designated $VHDL_1$, which contains apoprotein A as its major component. That the apolipoproteins we have found in the $d > 1.21$ fraction of rat serum are not constituents of $VHDL_1$ is demonstrated in

Table 2. Apolipoprotein concentrations in renal lymph, $d < 1.21$, and > 1.21 fractions*

	ARP	A-IV	Albumin
	(% of serum concentration)		
$d < 1.21$	75.0	70.0	0
$d > 1.21$	31.5	25.5	96.0
Lymph	13.5	49.5	28.5

* Same conditions as outlined in the legend to Table 1, except that all samples were obtained from the same animal.

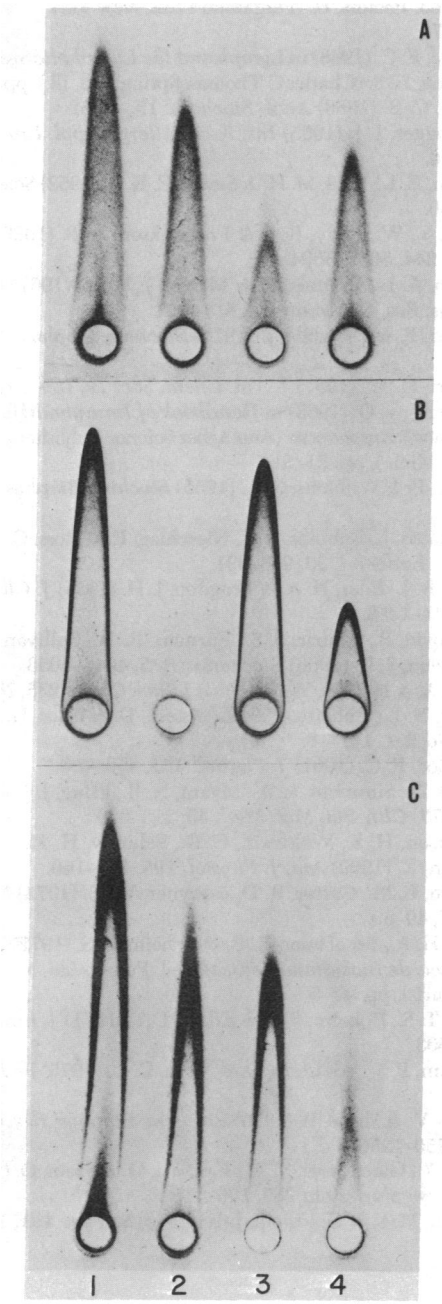

FIG. 1. Determination of (A) A-IV, (B) albumin, and (C) arginine-rich apolipoprotein by quantitative immunoelectrophoresis. Position 1, serum; position 2, $d < 1.21$ fraction; position 3, $d > 1.21$ fraction; position 4, renal lymph. For A-IV determinations, all samples were diluted 1:4 with uninjected goat serum. Albumin determinations were carried out in samples diluted 1:100. For ARP determinations, serum and the $d < 1.21$ fraction were diluted 1:4 and the $d > 1.21$ and lymph samples were diluted 1:2.

Table 3, which shows that most of the ARP and A-IV is present in the $d > 1.24$ fraction. Furthermore, their concentrations become constant at $d > 1.15$ and do not change as the density is increased.

The observation that A-IV and ARP are also the major apolipoproteins of the renal lymph, as well as the $d > 1.21$ fraction, suggests that these are derived from similar apolipoproteins in the serum that contain little or no lipid. It would seem likely that these specific apolipoproteins enter the lymph by transfer across the capillary membrane because they are present in the circulating plasma as relatively small particles. Although other apolipoproteins have lower molecular weights, e.g., the C proteins (about 10,000), it is probable that they and other apolipoproteins are present as constituents of lipoproteins of large particle size that do not readily cross the capillary membrane.

Table 3. Apolipoprotein concentrations* in infranatant fractions of $d > 1.063$†

	$d > 1.063$	$d > 1.10$	$d > 1.15$	$d > 1.21$	$d > 1.24$
	(% of serum concentration)				
A-IV	74	62	50	50	48
ARP	58	42	41	42	43
A-I	ND‡	15	7	6	5

* For apolipoprotein determinations, the same conditions were used as outlined in the legend to Table 1.

† Two pools of rat sera were used; each pool was obtained from 12 animals, 5 ml of sera were adjusted to different densities (23).

‡ ND, not determined.

Other studies have demonstrated the presence of lipoproteins in lymph. Courtice identified lipoproteins in peripheral lymph of cats by paper electrophoresis (27). Reichl *et al.* (28) obtained lymph from the dorsum of the foot and demonstrated the presence of lipoproteins by ultracentrifugal flotation. He reported that the lymph lipoproteins reacted with antisera to various lipoproteins. However, he noted that the distribution of the apolipoproteins among the various density lipoprotein fractions differed from that in the serum lipoproteins, and suggested that lipoproteins undergo modification on leaving the circulation, losing much of their lipid before reaching the peripheral lymph. Inasmuch as the individual apolipoproteins were not quantitated, it is difficult to relate his finding to those that we have reported.

It is possible that apolipoproteins enter the lymph as lipoproteins. Our data would seem more consistent with the hypothesis that the ARP and A-IV enter the lymph preferentially because they are also present in the plasma as "free" apolipoproteins and, thus, are able to traverse the capillary wall more readily than the high-molecular-weight lipoproteins. However, the finding of higher concentrations of A-IV in the lymph than in the $d > 1.21$ fraction suggests that mechanisms other than diffusion and filtration may be operative. Although experimental evidence is lacking, renal synthesis of apolipoproteins cannot be ruled out. It has been suggested that in addition to diffusion and filtration, vesicular transport across the cytoplasma of the endothelial cells may play a role in the transport of macromolecules through the capillary wall (29, 30). Apolipoproteins have a high affinity for lipids, including the lipids of the cell membrane. If by adsorption or other interactions specific apolipoproteins accumulate at the endothelial cell surface, apolipoproteins could be transported across the capillary wall in excess of that corresponding to their plasma concentration. Another possibility is that the "free" apolipoproteins are filtered at the glomerulus and reabsorbed by the tubules and appear in the pertibular lymph.

From these studies it cannot be determined whether the apolipoproteins found in the lymph are present as free apolipoproteins or as constituents of renal lymph lipoproteins; further analysis of larger quantities of renal lymph would answer this question. It could be postulated that free apolipoproteins are transferred through the capillary wall; however, they might be present in the lymph as components of lipoproteins. These apolipoproteins might provide the protein moiety for the interstitial transport of lipids.

The physiologic significance of the "free" apolipoproteins in plasma and lymph remains to be determined. Previous studies from this laboratory demonstrated the presence of protein in the $d > 1.21$ fraction, which combined with lipid to form VLDL when perfused through a rat liver (7). Subsequently, it was shown that this apoprotein appeared in the VS-II fraction (31), which is now known to consist largely of ARP. During sucrose-induced hyperlipidemia (32), reciprocal changes of A-IV concentrations in serum and $d > 1.21$ serum fraction were observed (33), i.e., serum concentration of A-IV increased, whereas its concentration in the $d > 1.21$ fraction decreased.

The presence of A-IV and ARP in the renal lymph could be of physiologic importance in the extravascular transport of lipids. Shore and Shore have shown that apolipoproteins, as well as lipoproteins, may affect cell membranes by altering Mg^{++} ATPase activity in the red cell (34). Because certain apolipoproteins are present in the lymph in significant amounts, it may be presumed that they are also present in the interstitial fluid where they could affect cell membranes, and may influence the cholesterol transport and metabolism of the cell (35, 36).

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