ORIGIN OF INTESTINAL LYMPH CHOLESTEROL
IN RATS: CONTRIBUTION FROM LUMINAL
ABSORPTION, MUCOSAL SYNTHESIS AND
FILTRATION FROM PLASMA

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ABSTRACT

Measurement of cholesterol transport from plasma to intestinal lymph based on
i.v. labeling with radioactive β-sitosterol was validated by the simultaneous i.v.
administration of 4-14C-β-sitosterol and of 1,2-3H-cholesterol to two rats with bile
duct, intestinal lymph, duodenum and jugular vein cannulations. In 11 other rats
undergoing intestinal lymphduct cannulation, each potential source of lymph cho-
lesterol was determined 2-3 weeks after i.v. pulse administration of 1,2-3H-β-sitosterol
and 4-14C-cholesterol. For this purpose, lymph fat, after an intragastric infusion of
cottonseed oil (1900mg), was used as a marker for total cholesterol mass transport-
ed into intestinal lymph. In these two experimental groups of rats, namely, in the ab-

ence and in the presence of supplemental dietary cholesterol, filtration of cholesterol
from plasma to lymph and absorption of cholesterol derived from bile did not
change in the presence of exogenous cho-

In other words, absorption of cho-

Plasma cholesterol appearing in intes-

Studies in human chyluria suggest that
the bulk of intestinal lymph cholesterol
derives from plasma (1). In monkeys with
cannulation of the intestinal lymph duct,
lymph cholesterol is not accounted for
solely by mucosal synthesis or absorption
from the intestinal lumen (2). Other ex-
periments suggest that part of the fatty
acyl moiety of lymph cholesterol esters is
derived from plasma (3). Since intestinal
lymph lipoproteins are rich in apoproteins
produced primarily in the liver (4,5), it is
conceivable that significant amounts of
plasma lipoproteins are present in lymph
(4,6).

In human chyluria, the transudation of
plasma cholesterol into lymph has been
evaluated by means of intravenous pulse
labeling with radioactive β-sitosterol (1),
a marker chosen based on the following
criteria: a) simultaneous intravenously
infused radioactive β-sitosterol and cho-
lesterol distribute evenly among plasma
lipoproteins (7); b) β-sitosterol is poorly
absorbed by the intestine; thus little or no
significant intestinal reabsorption of ra-
dioactivity excreted in bile is expected (8). Because of ongoing shifts in cholesterol and its metabolites from one body fluid to another or from intestinal absorption, a simple test of isotopic exchange rate between blood and tissues may be misleading to assess plasma transudation of cholesterol and its metabolites. However, rat intestinal lymph contains cholesterol absorbed from the bile and diet, in addition to the locally synthesized mucosal cholesterol (9), plus cholesterol from an unidentifable origin (2,6), and therefore, the sources of intestinal lymph cholesterol ought to be measurable in rat intestinal lymph using radioactive steroids according to the chyluria protocol (1). Accordingly, we validated in multicannulated rats, the intravenous use of labeled β-sitosterol to quantify the plasma to lymph transfer of cholesterol; these rats were studied using radioactive labeling of both β-sitosterol and cholesterol administered simultaneously by intravenous infusion. Furthermore, other rats with isolated intestinal lymph duct cannulae were used to determine each source of lymph cholesterol after an intravenous pulse infusion of both radioactive steroids in two separate experimental groups: 1) after a single intragastric instillation of cholesterol-free fat; and 2) a gastric instillation of cholesterol containing fat.

The distribution of plasma-derived and of absorbed cholesterol in the different lymph lipoproteins was also examined in the multicannulated rats based on the concurrent duodenal and intravenous infusion of radioactive cholesterol labeled with different isotopes.

MATERIALS AND METHODS

Radioactive sterols 1,2-3H and 4-14C cholesterol as well as 4-14C and 22,23-3H-β-sitosterol (New England Nuclear, Boston, MA and Amersham, Buckinghamshire, England), were purified on Florisil thin layer chromatography after developing with solvent system ethyl ether: heptane (55:45, v/v) (10). After drying under nitrogen, all radioactive material was dissolved in approximately 50μl of ethanol admixed to 0.4ml of saline for intravenous administration, or to 0.8ml of pooled donor rat bile for duodenal gavage.

Experimental protocol

Operative procedures

Wistar male rats weighing about 360g were anesthetized intraperitoneally with sodium pentobarbital (50mg/kg body weight) and gavaged with approximately 0.5g of cottonseed oil. Plastic catheters were placed in the intestinal lymph duct, bile duct, duodenum and jugular vein before restraining in a Bollman cage. Cannulation of the bile duct interrupted the enterohepatic circulation of cholesterol, whereas nutrients and radiotopes were chronically infused intravenously and via the duodenum. Infusions of nutrients were done by pumps (model 940 Harvard Apparatus, South Natick, MA) at the rate of 1ml per hour:

- intravenous infusion: bovine albumin (2.5g), 50% glucose (20 ml), 19.1% KCl (3.2ml) in 180ml saline.
- duodenal infusion: pooled bile from donor rats (30ml) plus glucose (9.6g) in 120ml water. Additional pulses every 4 hours of 1ml Intralipid (Darrow Laboratories, Rio de Janeiro, Brazil) containing soybean oil (10g), lecithin (1.2g), and glycerol (2.5g) as emulsion in 100ml water, were also supplied to maximize high volumes of lymph output.

The rats were subjected to two experimental protocols starting 1-2 hours after the surgery:

Protocol 1 (2 rats)

Simultaneous intravenous pulse infusion of 1,2-3H cholesterol (30μCi) and 4-14C β-sitosterol (10μCi) was utilized to validate use of the latter isotope as a method of quantifying the fraction of lymph cholesterol originating from plasma filtration.

Lymph samples containing at least 1ml/hour were collected on ice for 21 and 24 hours and blood was drawn at
4-hour intervals from the rat tail. The experiment was terminated by aortic exsanguination.

Protocol 2 (22 rats)

Simultaneous pulse infusion of $^{14}$C-cholesterol (10$\mu$Ci) admixed to 0.8ml of bile was instilled into the duodenum and 1,2-3H cholesterol (30$\mu$Ci) in 0.1ml saline was administered intravenously. The enteric isotope was aimed at identifying in lymph lipoprotein fractions the absorbed cholesterol and then measuring its free and esterified proportions. The intravenous infusion of radioactive cholesterol, on the other hand, was used to identify in lymph the amount of cholesterol derived solely from plasma (i.e., transudation). The contribution by duodenal absorption of $^{14}$C-cholesterol was able to be ascertained through the simultaneous infusion of bile. Random samples of lymph (at least 1ml) were collected during 1-2 hours over 6 to 10 hours of experiments after starting the infusions.

Analytical procedures

Lymph samples were submitted to preparative ultracentrifugation. Chylomicrons and VLDL (d<1.006) were separated from LDL plus HDL (d=1.006-1.21) as previously described (11). Resultant fractions were lyophilized, extracted with chloroform and the free and esterified cholesterol bands eluted with ethyl ether from T.L.C. on silica gel H (E. Merck, Darmstadt, Germany) after developing with ethyl ether/heptane (55:45, v/v).

Total cholesterol was separated by T.L.C. (10) modified with a solvent [petrol ether/diethyl ether/acetic acid (90:10:1, v/v)] (12) and determined by gas-liquid chromatography (10).

In separating free and esterified cholesterol from lymph, digitonin precipitation (13) was used and mass measured by an enzymatic assay (14). Serum free and esterified cholesterol levels were determined as described for lymph.

Aliquots were utilized for measurement of radioactivity in toluene phosphor solution in a beta liquid scintillation counter (Beckman, model LS 100).

Calculations for Protocol 1

a) Percent of lymph cholesterol originating from plasma filtration = radioactivity in d.p.m./mg of cholesterol in lymph/plasma x 100.

b) Percent of total plasma cholesterol that filters into the lymph/hour = (filtered cholesterol µg/h/total plasma cholesterol mass in µg) x 100, where filtered cholesterol (µg/h) = (d.p.m./mg of cholesterol in lymph/plasma) x lymph cholesterol in µg/hour. In calculating the total plasma cholesterol, the plasma volume of the rats was taken as 3.88% of body weight (15).

Protocol 3–isolated lymph duct cannulation studies (11 rats)

Operative procedures

Male rats of Wistar origin weighing about 300g were used. A mixture of 4-$^{14}$C-cholesterol (10$\mu$Ci) and 22,23-3H $\beta$-sitosterol (30$\mu$Ci) was pulse administered intravenously and rats were fed ad libitum a cholesterol-free commercial pellet diet for two to three weeks prior to operative manipulation. After a 12-hour overnight fasting period, rats received exactly 1900mg of cottonseed oil by gavage utilizing Hamilton syringe according to two experimental groups:

1) controls received cottonseed oil only (6 rats).

2) cholesterol-fed groups (5 rats) received 80mg of cholesterol (E. Merck, Darmstadt, Germany) added to the cottonseed oil and cholesterol absorption in lymph measured simultaneously by mass and by an isotopic procedure based on the intravenous administration of the radioactive cholesterol.

The infused oil was a marker for total lymph cholesterol transported based on the completeness of absorption and exclusive transport through the intestinal lymph system (16,17). Two hours after intragastric infusion, rats underwent intestinal lymph duct cannulation (18) under
light ethyl ether anesthesia and restrained in Bollman type cages (19). Intestinal lymph was collected over ~ 3 hours and thereafter the rats were exsanguinated by aortic puncture. At least 2ml of lymph were obtained.

**Analytical procedures**

Lymph and serum cholesterol were measured by the combined T.L.C. and G.L.C. procedures (10). Lymph fat was measured gravimetrically (1).

**Calculations**

a) Total lymph cholesterol mass transported in mg was derived from \( C = Ch \times G/g \) where \( Ch \) is the lymph cholesterol mass, \( G \) the intragastrically infused oil (1900mg) and \( g \) the lymph fat mass.

b) Lymph cholesterol mass derived from plasma by filtration (F) stems from the negligible intestinal absorption of the intravenously infused labeled \( \beta \)-sitosterol: \( F = C \times 3\text{H}-\beta\text{-sitosterol} \) in d.p.m./mg cholesterol in lymph/plasma.

c) Lymph cholesterol mass derived from de novo mucosal synthesis (S) = \((1-\text{C}-\text{cholsterol in d.p.m.}/\text{mg cholesterol in lymph/plasma}) \times C\). This value represents synthesis in control rats only because absorption of the intragastrically administered unlabeled cholesterol in the cholesterol-fed group further dilutes the lymph cholesterol specific activity in relation to plasma.

d) Absorption of endogenous cholesterol, namely biliary cholesterol \( (A_{end}) = C - (F + S) \) in the control group. S in the cholesterol-fed group includes the absorption of cholesterol dissolved in fat \( (A_{d}) \).

e) Absorption of fed cholesterol \( (A_{f}) \) can be derived from two methods: either as the C or the S value difference between cholesterol-fed and the control group. Isotopic calculations are based on the premise that cholesterol specific activity in bile and plasma are nearly identical when several days have elapsed after the intravenous infusion of radioactive cholesterol. Therefore, in the control groups, \( C = F + S + A_{end} \) whereas in the cholesterol-fed rats, \( C = F + S + A_{end} + A_{d} \).

Absorption by lymph balance represents the true mass of cholesterol from dietary origin that is found in lymph and is measured as \( C_{chol} - C_{control} \). It can also be estimated isotopeically as \( S_{chol} - S_{control} \) provided that the isotopic cholesterol exchange among plasma, intestinal mucosa and lumen pools is negligible during the fat absorption experimental phase.

**RESULTS**

Results of two multicanulated rats pulse infused intravenously with \( ^{3}\text{H}-\text{cholesterol} \) plus \( ^{14}\text{C}-\beta\text{-sitosterol} \) are shown in Table 1. (In these bile fistula rats, lymph radioactivity must necessarily come from plasma.) Mean filtration values in the two rats were 51.1% by the \( ^{3}\text{H}-\text{cholesterol} \) and 51.8% by the \( ^{14}\text{C}-\beta\text{-sitosterol} \) measurements.

Data from isolated lymph duct cannulation experiments are shown in Table 2. The amount of cholesterol derived from plasma based upon \( ^{3}\text{H}-\beta\text{-sitosterol} \) was 56% of the total intestinal lymph cholesterol in controls, which was remarkably close to 51% found in the multicannulated rats; however, it was only 18% in the cholesterol-fed rats.

Absorption of the fed cholesterol \( (A_{f}) \) by straightforward lymph cholesterol mass measurement (C cholesterol fed - C control) was similar to that determined by the isotopic procedure (S cholesterol fed - S control), 13.9mg and 13.4mg, respectively.

De novo mucosal cholesterol synthesis was about 20% of the total cholesterol mass transported in lymph in the control group.

Further experiments in 22 multicannulated rats (Protocol 2) aimed at identifying which lymph lipoprotein (chylomicron + VLDL as compared to LDL + HDL) carried the absorbed and the plasma-derived cholesterol and how these components were distributed between the free and the ester fractions are shown in Fig. 1. Most of the cholesterol
Fig. 1. Percent distribution of sterols in plasma and lymph as free □ and ester □ fractions in separate experiments in 22 multicannulated rats (Protocol 2): radioactive sterols were admixed to 0.8ml of bile for duodenal infusion and to 0.1ml of saline I.V. administration. Number of determinations at the bottom of each figure in brackets. Panel A: distribution of the cholesterol mass in lymph and plasma; Panel B: lymph distribution of labeled sterols after simultaneous intravenous pulse labeling with radioactive cholesterol and β-sitosterol; Panel C: labeled cholesterol in lymph lipoproteins after duodenal radioactive cholesterol infusion; Panel D: cholesterol mass in lymph lipoproteins; Panels E and F: distribution in lymph lipoproteins of the labeled sterols that transude from plasma after simultaneous intravenous pulse dose of radioactive cholesterol and β-sitosterol.
Table 1
Lymph cholesterol filtration from plasma measured in two multicannulated rats (Protocol 1). After the surgical procedure, rats were pulse infused I.V. simultaneously with 4-\textsuperscript{14}C-8-sitosterol and 1,2-\textsuperscript{3}H cholesterol, and lymph collected sequentially. Lymph collections started 21 hours after labeling rat #1 and 24 hours after labeling rat #2. A plateau of plasma and lymph radioactivity was attained during the whole length of the study.

<table>
<thead>
<tr>
<th>Rat</th>
<th>Sequential collection Periods in hours</th>
<th>Type of Label</th>
<th>Percent lymph Cholesterol originating from plasma filtration</th>
<th>Percent of total plasma cholesterol mass filtered into the lymph per hour</th>
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<tr>
<td>1</td>
<td>2:45</td>
<td>\textsuperscript{3}H</td>
<td>38.87</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td></td>
<td>\textsuperscript{14}C</td>
<td>37.88</td>
<td>0.40</td>
</tr>
<tr>
<td></td>
<td>5:30</td>
<td>\textsuperscript{3}H</td>
<td>35.56</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>\textsuperscript{14}C</td>
<td>33.15</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td>14:40</td>
<td>\textsuperscript{3}H</td>
<td>66.58</td>
<td>0.62</td>
</tr>
<tr>
<td></td>
<td></td>
<td>\textsuperscript{14}C</td>
<td>76.39</td>
<td>0.71</td>
</tr>
<tr>
<td></td>
<td>5:20</td>
<td>\textsuperscript{3}H</td>
<td>46.47</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td></td>
<td>\textsuperscript{14}C</td>
<td>45.10</td>
<td>0.40</td>
</tr>
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<td>2</td>
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<td></td>
<td></td>
<td>\textsuperscript{14}C</td>
<td>45.90</td>
<td>0.53</td>
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<tr>
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<td>\textsuperscript{3}H</td>
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<td>0.62</td>
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<td></td>
<td></td>
<td>\textsuperscript{14}C</td>
<td>66.75</td>
<td>0.59</td>
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<td></td>
<td>4:00</td>
<td>\textsuperscript{3}H</td>
<td>49.68</td>
<td>0.60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>\textsuperscript{14}C</td>
<td>48.01</td>
<td>0.58</td>
</tr>
<tr>
<td></td>
<td>10:00</td>
<td>\textsuperscript{3}H</td>
<td>60.76</td>
<td>0.24</td>
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<tr>
<td></td>
<td></td>
<td>\textsuperscript{14}C</td>
<td>61.20</td>
<td>0.24</td>
</tr>
</tbody>
</table>

mass in plasma was esterified as expected, but not in the intestinal lymph (Fig. 1a).

The radioactive free cholesterol that transferred from plasma to lymph was mostly in the nonesterified fraction (Fig. 1b). Almost 50% of the free radioactive cholesterol administered into the duodenum was transported in lymph as ester (Fig. 1c), whereas the lymph cholesterol mass was predominantly nonesterified (Fig. 1d).

Chylomicrons and VLDL, as opposed to lipoproteins of higher density, are the major carriers of lymph cholesterol, namely absorbed radioactive cholesterol (Fig. 1c), total cholesterol mass (Fig. 1d) and radioactive sterols that derive from plasma transudation (Figs. 1e and 1f). The ratio free/ester of the cholesterol mass is much higher in chylomicrons and VLDL that are made entirely by the mucosa--as compared to that in LDL+HDL (Fig. 1d). In addition, the same higher free/ester ratio of the filtered component is found in chylomicrons and VLDL (Fig. 1e and 1f).

DISCUSSION

Although the largest share of intestinal lymph cholesterol was accounted for by filtration from plasma (Table 1), its mass represented only a small portion of the whole plasma cholesterol content, that is, 11% of the plasma cholesterol mass filtered into intestinal lymph in 24 hours. The plasma derived cholesterol mass was independent of the luminal cholesterol content; that is, it did not vary with cholesterol feeding (Table 2). Consequently, it represents a passive process whereby plasma lipoproteins percolate into the intestinal mucosal lacteals.

The similarity of dietary cholesterol absorption by the direct mass measurement in lymph and by the isotopic method strengthens the conclusion that isotopic cholesterol exchange between plasma and the intestinal wall or the luminal content is negligible. These data confirm other experimental reports utilizing radioactive cholesterol administered intraduodenally (16,20).
Table 2

<table>
<thead>
<tr>
<th>Experimental Groups (No. of animals)</th>
<th>Controls (6)</th>
<th>Cholesterol (5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean mg±SD</td>
<td>Mean mg±SD</td>
</tr>
<tr>
<td>Total Lymph</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol (C)</td>
<td>6.69±1.43</td>
<td>20.60±5.42</td>
</tr>
<tr>
<td>Filtration (F)</td>
<td>3.78±0.72</td>
<td>3.68±1.42</td>
</tr>
<tr>
<td>Synthesis</td>
<td>1.56±1.33</td>
<td>15.01±3.16</td>
</tr>
<tr>
<td>Endogenous absorption (A&lt;sub&gt;endo&lt;/sub&gt;)</td>
<td>1.36±0.91</td>
<td>1.91±1.01</td>
</tr>
<tr>
<td>Dietary absorption (A&lt;sub&gt;d&lt;/sub&gt;)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymph balance</td>
<td>13.91</td>
<td></td>
</tr>
<tr>
<td>Isotopic procedure</td>
<td>13.45</td>
<td></td>
</tr>
<tr>
<td>Lymph fat (G)</td>
<td>64.85±27.47</td>
<td>44.09±28.40</td>
</tr>
</tbody>
</table>

a: each animal means one lymph sample collected; b: represented as percent of the total lymph cholesterol mass (C); p< N.S.; c: S includes absorbed unlabeled dietary cholesterol and de novo mucosal synthesis, thus their independent contribution cannot be known. d: p<.001; e: fat was measured during 3 hours of complete lymph collection and utilized for the calculation of the value C as shown in calculations section.

Intestinal mucosal synthesis accounted for roughly 1/5 of the lymph cholesterol mass which closely agreed with earlier reports (21,22). Another source of cholesterol in lymph is via absorption of the biliary component. Although relying upon indirect measurement methods, endogenously absorbed cholesterol was able to be measured in lymph and accounted for 1/5 of the whole lymph mass with a cholesterol-free diet.

Since most of the intestinal lymph cholesterol is non-esterified it is reasonable to conclude that in rats the cholesterol contribution from plasma and mucosal synthesis is predominantly in this form. Probably at the level of the intestinal submucosa, free-cholesterol is preferentially transferred to predominantly lighter lymph lipoproteins in accordance with the well known fast exchange rate of free-cholesterol with lipoproteins (22-24). We also found that less than 50% of the absorbed radioactive cholesterol is esterified in contrast to other reports of 50 to 85% esterification (20,25,26). This discrepancy may be attributable to: 1) the large duodenal mass of fat administered (Intralipid) fortuitously stimulated local mucosal synthesis of cholesterol which preferentially was transported in the non-esterified form (22); 2) in our experiments the duodenal infusion contained cholesterol of bile origin only, whereas in earlier reports, a larger cholesterol mass had been administered (20,25,26). Therefore, contrary to these previous reports (20,25,27), esterification of cholesterol is not required for efficient absorption by the intestine into lymph. Although the present study did not further pursue the mechanisms involved, it nevertheless suggests that cholesterol esterification depends primarily on the mass ratio of cholesterol/fat in the diet (28-30).
In conclusion, plasma cholesterol filtration contributed substantially to the intestinal lymph cholesterol in the rat and the accuracy of measurement was corroborated in multicanulated rats by the intravenous infusion of radioactive $\beta$-sitosterol.

The contribution to the intestinal lymph of cholesterol from other sources was also determined by combining intravenous pulse infusion of radioactive cholesterol to the measurement of cholesterol mass in lymph and plasma. Most of the plasma-originated cholesterol in intestinal lymph binds to chylomicrons, which is the fraction known to transport the bulk of the absorbed luminal cholesterol. Radioactive cholesterol and $\beta$-sitosterol in intestinal lymph derived from plasma sterols were predominantly in the non-esterified form and signify that under our experimental conditions (intravenous constant infusion of radioactive free sterols), most of the plasma radioactive sterols was in the free fraction.

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