Comparative Study of the Activity and Composition of HDL₃ in Russian and American Men

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Previous studies conducted within the framework of the Lipid Research Clinics Program showed a strong inverse correlation between high-density lipoprotein cholesterol (HDL-C) level and coronary heart disease (CHD) risk in American male populations, whereas in Russian populations such a correlation was less pronounced. It was assumed that HDL was less protective of CHD in Russian than in American males. This study compared the functional activity and lipid composition of HDL₃ isolated from the blood plasma of men with low, normal, and high HDL-C levels from Moscow (Russia) and Seattle (United States) populations. Results obtained showed that American HDL₃, irrespective of the plasma HDL-C level, had higher activity in stimulating both [³H]cholesterol and cholesterol mass efflux from cholesterol-loaded fibroblasts and in suppressing cellular cholesterol esterification when compared with Russian HDL₃. American HDL₃ remained more active than Russian HDL₃, even when apolipoprotein E-containing particles were removed from HDL₁ by heparin-Sepharose affinity chromatography. Russian and American ¹²⁵I-HDL₃ had similar binding to high-affinity cell-surface sites, but Russian HDL₃ had a higher nonspecific binding component compared with American HDL₃. This study demonstrates for the first time potential functional differences between HDL particles isolated from Russian and American populations. The lower activity of Russian HDL₃ in promoting cellular cholesterol efflux may partly explain the higher CHD risk in the Russian population compared with the American one. (Arterioscler Thromb. 1993;13:1770-1778.)

KEY WORDS • cholesterol efflux • HDL • cholesterol esterification • HDL binding • fibroblasts

According to World Health Organization data, the mortality from cardiovascular diseases associated with atherosclerosis, in particular from coronary heart disease (CHD), is 1.5 times higher in the USSR than in the United States.¹ The survey among random samples of men in Moscow and Leningrad in Russia, within the framework of the Lipid Research Clinics Program, showed a higher level of high-density lipoprotein cholesterol (HDL-C) in the Russian population than in the American.²³ According to the 8-year prospective population studies carried out in two Russian cities and in the United States, the American male population showed an inverse relation between HDL-C and CHD risk, whereas the Russian population did not show a significant relation.⁴ These data could indicate that HDL in the Russian population is a weaker protective factor for developing atherosclerosis and its related diseases compared with HDL in the American population. These discrepancies may be due to differences in HDL composition and/or in the determinants of HDL-C levels across the two cohorts.

This joint Russia-US study was undertaken to test the functional activity and lipid composition of HDL from comparable males to resolve the discrepancies in the HDL-C relation with CHD risk in the Russian and American male populations. We studied the functional activity of HDL₃ with respect to its ability to stimulate cholesterol efflux from extrahepatic cells, to inhibit cellular cholesterol esterification (ACAT activity), and to bind to specific cell-surface sites.

Methods

Subjects

Plasma samples from male subjects with normal (45 to 55 mg/dL), low (<35 mg/dL), and high (>60 mg/dL) HDL-C levels were used as a source for isolation of HDL₃. The subjects were recruited from Moscow (Russia) and Seattle (United States) populations and selected randomly for their blood lipid concentrations: HDL-C within the aforementioned criteria, total cholesterol (Ch) <250 mg/dL, and total triglycerides (Tg) <200 mg/dL. The mean age was 44.9±2.0 years in Moscow men (n=26) and 44.7±2.0 years in Seattle men (n=21). Diabetics were excluded from the study. None of the subjects was taking drugs affecting lipid metabolism. Other risk factors were not considered.
Plasma Lipid Analysis

Total Ch, total Tg, and HDL-C were determined enzymatically for Russian subjects in Moscow and for American subjects in Seattle. For Russian plasma samples, HDL-C was also measured in Seattle after storage at 0°C for 4 days. The differences in the HDL-C values obtained in Moscow and Seattle were no more than 2 mg/mL, without any influence on the mean HDL-C values in the three groups of subjects.

Lipoproteins

Blood was drawn from all Russian subjects, American subjects with normal HDL-C levels, and some of the American subjects with low and high HDL-C levels on the same day in Moscow and Seattle. Two American subjects with low HDL-C levels and four with high HDL-C levels had their blood drawn 8 to 9 days later. Blood was obtained by venipuncture after an overnight fast (12 to 14 hours) and was chilled on ice. The plasma was separated, and phenylmethylsulfonyl fluoride and 0.1 mg/mL gentamicin (Sigma Chemical Co, St Louis, Mo) were added to each plasma sample. Russian plasma samples were transported directly to the Seattle laboratory. To ensure that Russian plasma samples were as fresh as possible and were not modified during transit, (1) blood was drawn 24 hours before departure from Moscow, (2) plasma samples were transported carefully at constant temperature in a container with thawing ice, and (3) HDL₃ isolation procedures were started in Seattle 48 hours after blood was drawn. HDL₃ was isolated from 8 mL of plasma by two-step density ultracentrifugation: very-low-density lipoprotein + low-density lipoprotein (LDL)+HDL₂ were removed at d>1.125 g/mL and HDL₃ was isolated at d>1.21 g/mL. HDL₃ samples were stored at 4°C under gaseous nitrogen in 0.15 mol/L NaCl containing 1 mmol/L EDTA. All cell culture experiments were performed in Seattle within 9 weeks after isolation of HDL₃. Repeated experiments with representative Russian and American HDL₃ samples showed no change in the ability of these samples to promote cholesterol efflux from cells between 1 and 9 weeks of storage. For all experiments, Russian and American HDL₃ samples were used in parallel. For [³H]-HDL₃ binding experiments, eight Russian and eight American normal HDL₃ (HDL₃-norm; see “Results”) samples were mixed, subjected to heparin-agarose affinity chromatography to remove apolipoprotein (apo) E- and apo B-containing particles,6 and iodinated by the iodine monochloride method as described previously.7

Cells

Cultured human skin fibroblasts were grown and maintained in Dulbecco’s modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) as described previously.8-10 For [³H]-cholesterol efflux and cholesterol esterification experiments, fibroblasts were plated into 24-well multiwells; for cholesterol mass efflux and HDL binding experiments, cells were plated into 35-mm dishes and grown to confluency (7 to 9 days).

Cell Cholesterol Loading

In [³H]-cholesterol efflux experiments, fibroblasts were loaded with cholesterol by incubation for 48 hours with serum-free DMEM containing 2 mg/mL fatty acid-free bovine serum albumin (BSA) plus 50 µg/mL cholesterol added in ethanol (from 10 mg/mL cholesterol stock solution). Then cells were washed twice with phosphate-buffered saline (PBS) containing 1 mg/mL BSA (wash buffer) and incubated for 16 hours in N-hydroxysulfonic acid (HEPES)-buffered DMEM containing 1 mg/mL BSA to allow equilibration of cellular sterol pools. To enrich the plasma membranes of cholesterol-loaded fibroblasts with [³H]-cholesterol, cells were incubated at 37°C with HEPES-buffered DMEM containing 1 mg/mL BSA and 0.2 µCi/mL [³H]cholesterol added in ethanol. After 2 hours, cells were chilled on ice, washed five times with ice-cold buffer, and used in cholesterol efflux studies. This procedure specifically radiolabels the plasma membrane pool of cholesterol, since short-term incubation with trace quantities of [³H]cholesterol leads to incorporation of the isotope into plasma membranes without significant transfer to intracellular membranes.10-12 In those experiments in which cholesterol mass efflux was measured, fibroblasts were loaded with LDL (200 µg protein/mL medium) added to the cells in DMEM for 48 hours.

Sterol Efflux From Cells

Monolayers of cholesterol-loaded or radiolabeled fibroblasts were washed five times with wash buffer and incubated in the same medium containing the indicated amounts of HDL₃. At the indicated time, the efflux medium was collected and radioactivity counted. The cellular amount of [³H]-labeled unesterified cholesterol (UC) was measured after separation of sterol species by thin-layer chromatography (see below).

Intracellular Cholesterol Esterification

To assess the relative activity of ACAT after cholesterol efflux incubations, fibroblasts were washed two times with wash buffer and incubated for 1 hour at 37°C with serum-free medium containing [¹⁴C]oleate (20 µmol/L) bound to albumin (0.3 mg/mL). Cells were then chilled on ice, washed twice with cold wash buffer and twice more with cold PBS, and extracted in hexane/isopropanol. Lipid subclasses were separated by thin-layer chromatography, and incorporation of [¹⁴C]radioactivity into esterified cholesterol (EC) was measured as described below. Incorporation of the radiolabel into esterified sterol represents sterol esterification by ACAT.

Lipid Analysis

Washed fibroblast monolayers were extracted with hexane/isopropanol (3:2, vol/vol) as previously described.13 Briefly, sterol species were separated on silica gel H thin-layer chromatography plates (Analytech Inc, Newark, Del) developed in hexane/diethyl ether/acetone acid (130: 40:1.5 vol/vol) and detected with iodine.12 For isotope measurements, individual spots corresponding to cholesterol esters and UC were scraped into scintillation vials and counted. To determine UC mass, UC spots were scraped, extracted, and assayed by the cholesterol oxidase procedure as described previously.13 The content of UC and phospholipids (PL)14 in HDL was determined enzymatically. The amount of EC was
calculated as the difference between total HDL-C and HDL-UC.

**Sequential Competitive Binding**

For the sequential competitive-binding protocol, cells were exposed to competitor and ligand during sequential incubations as previously described. Briefly, cholesterol-loaded fibroblasts were washed twice at room temperature with PBS/BSA and then chilled on ice while exposed to a third wash. Cells were incubated at 0°C with carbonate-free DMEM containing 25 mmol/L HEPES (pH 7.4) containing 1 mg/mL BSA and the indicated concentrations of unlabeled competitor HDL3. After 3 hours, cells were rapidly washed three times with cold PBS/BSA and incubated for 1 hour at 0°C with HEPES-buffered DMEM containing 1 mg/mL BSA and 5 μg/mL of [125I]-HDL3. Cells were washed four times with cold PBS/BSA and three times with cold PBS. After digestion in 0.1N NaOH, cell-associated radioactivity and cell content were determined.

**Direct Binding**

Cholesterol-loaded fibroblasts were washed twice at room temperature and chilled on ice while exposed to a third wash. Cells were incubated at 0°C with HEPES/DMEM containing 1 mg/mL BSA and the indicated concentrations of [125I]-HDL3. After 3 hours, cells were washed and digested for radioactivity and protein measurements as described above.

**Other Methods**

Protein was measured by the method of Lowry et al with BSA as a standard. Cell viability, as tested by trypan blue exclusion, exceeded 95% in all experiments.

**Reagents**

Fatty acid-free BSA was purchased from Sigma. Cholesterol oxidase (Streptomyces, La Jolla, Calif. [7n]-[3H]cholesterol (50 Ci/mmol) and [1,4C]oleic acid (50 to 60 μCi/mmol) were obtained from Amersham Corp, Arlington Heights, Ill. Tissue culture media were from Flow Laboratories, McLean, Va, and FBS was from Whittaker Bioproducts, Walkersville, Md.

**Statistical Analysis**

Data are presented as the mean±SEM. Each value for the [3H]cholesterol efflux, cholesterol mass, and [14C]oleate incorporation assays represents the mean of triplicate incubations. Statistical analysis was done by Student’s t test.

**Results**

Men selected from Moscow and Seattle populations as donors of blood for isolation of HDL3 were assigned to three groups distinguished by plasma HDL-C levels. HDL samples isolated from the plasma of men with low, normal, and high HDL-C levels were referred to as HDL-hypo, HDL-norm, and HDL-hyper, respectively. Mean values for HDL-C were similar in groups of Russian and American men (Table 1).

Lipid composition analysis of the isolated HDL3 indicated that Russian HDL3-hypo and HDL3-norm had a higher UC/EC ratio compared with American HDL3 (Table 2). Also, Russian HDL3-hypo had a higher value for the ratio between the surface lipid components (UC+PL) and the major core component EC than in American ones.

The greater relative content of UC in Russian HDL3 may influence its ability to accept UC from the membranes of peripheral cells. Therefore, initially a comparative study of the functional activity of Russian and American HDL3 with respect to its ability to accelerate cholesterol efflux from peripheral cells and inhibit intracellular cholesterol esterification by the enzyme ACAT was conducted.

HDL3-mediated cholesterol efflux was assessed by measurement of [3H]cholesterol efflux into the incubation medium as well as by reduction of the cellular UC mass. It was necessary to choose the conditions for incubation of HDL3 with cells to provide optimum cholesterol efflux. [3H]cholesterol efflux was increased by addition to the medium of both Russian and American HDL3 (Fig 1). The profile of the curve showing removal of [3H]cholesterol from fibroblasts by HDL3 was found to have a tendency to saturate at HDL3 concentrations above 20 μg protein/mL. A similar [3H]cholesterol efflux pattern could be observed when

### Table 1. Plasma HDL-C Levels in Moscow and Seattle Men

<table>
<thead>
<tr>
<th>Groups of Subjects</th>
<th>HDL-Hypo, &lt;35 mg/dL</th>
<th>HDL-Norm, 40 to 50 mg/dL</th>
<th>HDL-Hyper, &gt;60 mg/dL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moscow</td>
<td>28.4±1.4</td>
<td>41.4±1.3</td>
<td>79.8±3.3</td>
</tr>
<tr>
<td>Seattle</td>
<td>33.4±1.5</td>
<td>41.6±1.7</td>
<td>67.8±2.1</td>
</tr>
</tbody>
</table>

HDL-C indicates high-density lipoprotein cholesterol and is reported in milligrams per deciliter.

### Table 2. Lipid Composition (Wt/Wt) of Russian and American HDL3

<table>
<thead>
<tr>
<th>Lipid Composition (Wt/Wt)</th>
<th>American HDL3</th>
<th>Russian HDL3</th>
<th>P&lt;sub&gt;Am vs Rus&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>UC/PL</td>
<td>0.067±0.002</td>
<td>0.064±0.003</td>
<td>NS</td>
</tr>
<tr>
<td>UC/EC</td>
<td>2.144±0.050</td>
<td>2.527±0.088</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>UC/EC</td>
<td>0.136±0.007*</td>
<td>0.150±0.004t</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>UC/PL</td>
<td>0.069±0.004</td>
<td>0.075±0.003</td>
<td>NS</td>
</tr>
<tr>
<td>UC/EC</td>
<td>2.133±0.060</td>
<td>2.253±0.107</td>
<td>NS</td>
</tr>
<tr>
<td>UC/EC</td>
<td>0.137±0.006*</td>
<td>0.155±0.006</td>
<td>=0.05</td>
</tr>
</tbody>
</table>

HDL3 indicates high-density lipoprotein; UC, unesterified cholesterol; PL, phospholipid; EC, esterified cholesterol; Am, American; and Rus, Russian.

*P<.05 differences versus HDL3-hyper.

tP<.05 differences versus HDL3-hyper. See text for definition of HDL3 groups.
Study of HDL From Russian and American Men

Shakhov et al

Fig 1. Line plots of effect of Russian (A) and American (B) high-density lipoprotein 3 (HDL 3) on [3H]cholesterol efflux from cholesterol-loaded fibroblasts. Cultured fibroblasts were loaded with cholesterol by pretreatment with serum-free medium plus cholesterol, then pulsed for 2 hours at 37°C with [3H]cholesterol, and chased with medium containing 0 to 40 μg protein/mL of HDL 3 for 6 hours at 37°C. HDL 3 samples were isolated from individuals with normal (•), low (△), and high (□) plasma HDL cholesterol levels. Results represent mean±SEM for radioactivity in the medium of triplicate incubations expressed as percent total radioactivity (medium plus cells) per milligram cell protein.

The line plots of the time course for HDL 3-mediated [3H]cholesterol efflux revealed that efflux of [3H]cholesterol was highest during the first 6 hours of incubation for both Russian and American HDL 3 (Fig 2). A similar [3H]cholesterol efflux pattern was seen in the presence of HDL 3 isolated from plasma samples with different HDL-C levels.

These results indicated that an HDL 3 protein concentration of 20 μg/mL and an incubation time of 6 hours provided optimum conditions for [3H]cholesterol efflux from the cells in the presence of HDL 3, regardless of the HDL-C level in the donor's plasma.

As seen in Figs 1 and 2, under these conditions American HDL 3 was more effective in promoting labeled cholesterol efflux compared with Russian HDL 3. This finding was supported by the experiments with several Russian and American HDL 3 samples isolated from plasma samples with normal, low, and high HDL-C levels (Fig 3). In two groups (HDL 3-norm and HDL 3-hyper), the mean values of the [3H]cholesterol efflux from fibroblasts were higher with American than with Russian HDL 3.

It is of interest that with either Russian or American HDL 3, HDL 3-hyper had a higher ability to stimulate labeled cholesterol efflux when compared with HDL 3-norm and HDL 3-hypo (P<.05) (Fig 3).

In studies of the HDL 3 effect on UC mass efflux from cells, the ability of American HDL 3 to promote cholesterol efflux was greater compared with Russian HDL 3.

Fig 2. Line plots of time course for removal of [3H]cholesterol from cholesterol-loaded fibroblasts by Russian (A) and American (B) high-density lipoprotein 3 (HDL 3). Experimental conditions are as described in the legend to Fig 1, except that HDL 3 was added at a concentration of 20 μg protein/mL. At indicated times, medium was collected and radioactivity was measured. HDL 3 samples were isolated from individuals with normal (•), low (△), and high (□) plasma HDL cholesterol levels. Results represent mean±SEM for radioactivity in the medium of triplicate incubations expressed as percent total radioactivity (medium plus cells) per milligram cell protein.

HDL 3-mediated UC mass efflux from cholesterol-loaded (preincubation with LDL, 200 μg/mL, 48 hours) fibroblasts was more marked in presence of American HDL 3 than in presence of Russian HDL 3, irrespective of the HDL-C level in the plasma that served as the source for isolated HDL 3 (Fig 4). Among Russian HDL 3 samples, the activity of HDL 3-hyper in terms of UC

Fig 3. Bar graph showing the mean values of [3H]cholesterol efflux catalyzed by Russian (○) and American (●) high-density lipoprotein 3 (HDL 3). Experimental conditions are as described in the legend to Fig 1, except that HDL 3 was added at a concentration of 20 μg protein/mL. Results represent mean±SEM for radioactivity in the medium of triplicate incubations of each HDL 3 sample expressed as percent total radioactivity (medium plus cells) per milligram cell protein. The number of tested HDL 3 samples is given in the bars. *P<.05, Russian HDL 3 vs American HDL 3. See text for definition of HDL 3 groups.
Hypo-HDL-C Normo-HDL-C Hyper-HDL-C

FIG 4. Bar graph showing the mean effect of Russian (a) and American (•) high-density lipoprotein 3 (HDL 3) on unesterified cholesterol (UC) mass efflux from fibroblasts. Cultured fibroblasts were loaded with cholesterol by pretreatment with serum-free medium plus 200 μg protein/mL low-density lipoprotein for 48 hours at 37°C and then incubated with medium containing 40 μg/mL HDL 3 for 6 hours at 37°C. Washed fibroblasts were extracted with hexane/isopropanol, and UC mass content was measured after thin-layer chromatography by the cholesterol oxidase procedure described in “Methods.” The data listed indicate the difference in cellular UC mass content before and after incubation with HDL3 and represent mean±SEM for triplicate incubations for each HDL 3 sample. The number of tested HDL 3 samples is given in the bars. *P<.05, Russian HDL 3 vs American HDL 3. See text for definition of HDL 3 groups.

Efflux stimulation was significantly greater than that for HDL 3-hypo and HDL 3-norm (P<.05), whereas among American HDL 3 samples, HDL 3-norm and HDL 3-hyper had similar activity, which was greater than that for HDL 3-hypo.

To confirm that American HDL 3 stimulated the depletion of cellular UC more strongly than Russian HDL 3, the effect of both types of HDL 3 on cholesterol esterification (ACAT activity) was studied, since ACAT activity has been shown to be regulated reciprocally by changes in the size of the intracellular cholesterol pool. Cholesterol esterification was assayed by pulse-incubating cells for 1 hour with [14C]oleate and by measuring the incorporation of the radiolabel into cholesterol esters (CE) (see “Methods”). When cholesterol-loaded fibroblasts were incubated with HDL 3, ACAT activity decreased substantially within 24 hours, indicating that HDL 3 depleted the intracellular sterol pool used as substrate for this enzyme (Fig 5).

HDL 3 samples isolated from blood plasma with different HDL-C levels and added to the cells had an approximately equal effect on cholesterol esterification: at concentrations of 20 μg/mL (Fig 6) and incubation times greater than 6 hours (Fig 5), there appeared to be saturation in the activity curves for both Russian and American HDL 3. It should also be noted that the inhibiting effect of American HDL 3 on cholesterol esterification under these conditions was more pronounced when compared with the effect of Russian HDL 3 (Figs 5 and 6). From the experiments with the available Russian and American HDL 3 samples (Fig 7), American HDL 3 inhibited cholesterol esterification more strongly when compared with Russian HDL 3. For both Russian and American HDL 3, the activity of HDL 3-hyper was greater than that for HDL 3-hypo and HDL 3-norm.

FIG 5. Time course of effect of Russian (A) and American (B) high-density lipoprotein 3 (HDL 3) on sterol esterification in cultured fibroblasts. Cultured fibroblasts were loaded with cholesterol by pretreatment with serum-free medium plus cholesterol and were incubated with the medium containing 20 μg/mL HDL 3 for indicated times, and then cells were pulse-labeled with [14C]oleate for 1 hour at 37°C. Amount of [14C]oleate incorporation into cholesterol esters was quantified (see “Methods”). HDL 3 samples tested were isolated from individuals with normal (●), low (●), and high (●) plasma HDL cholesterol. Results represent mean±SEM for cellular 14C radioactivity of triplicate incubations. Control, incubation without HDL 3.

FIG 6. Line plots of effect of Russian (A) and American (B) high-density lipoprotein 3 (HDL 3) on sterol esterification in cultured fibroblasts. Experimental conditions are as described in the legend to Fig 5, except for HDL 3 samples being added to the incubation media at concentrations of 0 to 40 μg protein/mL for 6 hours. HDL 3 samples tested were isolated from individuals with normal (●), low (●), and high (●) plasma HDL cholesterol levels. Results represent mean±SEM for cellular 14C radioactivity of triplicate incubations. Control, incubation without HDL 3.
Study of HDL From Russian and American Men

To be sure that the differences found in the effect of Russian and American HDL on cholesterol homeostasis in cultured fibroblasts were not due to different amounts of apo E in the HDL samples, the experiments were performed with Russian and American apo E–free HDL-norm preparations. American HDL-norm remained more active than Russian HDL in [3H]cholesterol efflux stimulation and in sterol esterification inhibition even when apo E–containing particles were removed from the HDL-norm by heparin-Sepharose affinity chromatography (Fig 8).

Studies done in the Seattle laboratory have suggested that HDL binding to high-affinity cell-surface sites is involved in the removal of excess cholesterol from intracellular pools. Therefore, experiments were conducted to determine and compare the affinity for binding of American and Russian HDL to cholesterol-loaded fibroblasts. For this purpose, we used a sequential competitive-binding assay wherein cells were exposed to competitor (unlabeled HDL) and ligand ([125I]-HDL) during the sequential incubations at 0°C. Since the rate of dissociation of HDL from high-affinity binding sites is slow at 0°C, a large proportion of binding sites that are occupied during the first incubation will remain occupied during the second incubation, thus blocking the interaction of [125I]-HDL with the same sites. This method detects mainly high-affinity binding sites for HDL and minimizes the artifact introduced during direct competitive binding caused by the exchange of apoproteins between [125I]-labeled and unlabeled particles.

When cholesterol-loaded fibroblasts were exposed to increasing concentrations of unlabeled HDL during the first incubation and then to [125I]-HDL (5 μg protein/mL) during the second incubation, binding of [125I]-HDL was inhibited by 20% to 45% (Fig 9). Two differences were observed between the Russian and American HDL samples. First, total cell-surface binding of pooled samples of Russian [125I]-HDL was greater than...
After 3 hours cells were washed, digested in NaOH, and assayed for cell-associated radioactivity. The table below represents the kinetic parameters of $^{125}$I-HDL₃ binding. Composition of Russian and American $^{125}$I-HDL₃ preparations is as described in Fig 9. HDL₃ indicates high-density lipoprotein.

![Fig 10. Direct binding of Russian (○) and American (△) $^{125}$I-HDL₃ to cholesterol-loaded fibroblasts. Cholesterol-loaded fibroblasts were incubated at 0°C with medium containing the indicated amount of $^{125}$I-HDL₃-norm. After 3 hours cells were washed, digested in NaOH, and assayed for cell-associated radioactivity. The table below represents the kinetic parameters of $^{125}$I-HDL₃ binding. Composition of Russian and American $^{125}$I-HDL₃ preparations is as described in Fig 9. HDL₃ indicates high-density lipoprotein.](image)

<table>
<thead>
<tr>
<th>HDL₃</th>
<th>Bₓmax</th>
<th>Km</th>
<th>Slope</th>
<th>non-specific</th>
</tr>
</thead>
<tbody>
<tr>
<td>American</td>
<td>540</td>
<td>4.07</td>
<td>5.0</td>
<td>9.09</td>
</tr>
<tr>
<td>Russian</td>
<td>590</td>
<td>3.50</td>
<td>4.07</td>
<td>9.09</td>
</tr>
</tbody>
</table>

that of pooled American $^{125}$I-HDL₃, regardless of the concentration of unlabeled HDL₃ preincubated with cells (Fig 9A). Second, expression of these results as a percent of control values showed that binding of American $^{125}$I-HDL₃ was inhibited to a greater extent by unlabeled American HDL₃ than binding of Russian $^{125}$I-HDL₃ by unlabeled Russian HDL₃ (Fig 9B). These results suggest that a lower percentage of Russian HDL₃ is bound to high-affinity binding sites when compared with American HDL₃.

Direct binding studies showed (Fig 10) that binding of Russian $^{125}$I-HDL₃ was less saturable when compared with American $^{125}$I-HDL₃ at concentrations of more than 20 μg protein/ml, which was previously shown to be the saturating dose of HDL₃ for high-affinity binding sites. It was estimated that the apparent $K_m$ and $B_{xmax}$ for binding of Russian and American $^{125}$I-HDL₃ to the high-affinity cell-surface sites were nearly identical. The main difference was found to be in the amount of low-affinity binding: Russian $^{125}$I-HDL₃ had a higher nonspecific binding component when compared with American $^{125}$I-HDL₃ (Fig 10).

Discussion

According to a current hypothesis, the physiological role of HDL is to promote reverse cholesterol transport, which includes its efflux from extrahepatic tissues and subsequent transport to the liver, where cholesterol is catabolized into bile acids. As shown by several epidemiological studies in the United States and other countries, there is an inverse relation between HDL-C and CHD. However, such a strong relation has not been demonstrated both in Moscow and Leningrad population studies. A study that examined HDL subclass profiles showed a significantly lower relative and absolute content of the denser HDL₃ subclass in Russian compared with American men, raising the possibility that Russian HDL may have a lesser ability than American HDL to remove cholesterol from tissues. These findings prompted us to conduct comparative studies of both early stages of HDL-mediated reverse cholesterol transport and of the lipid composition of HDL samples obtained from the plasma of male residents in Russia and the United States who had similar HDL-C levels.

Because the removal of UC from peripheral cells by HDL₃ is believed to be an initial step in reverse cholesterol transport, the ability of Russian and American HDL₃ samples to stimulate cholesterol transport from cholesterol-loaded cultured fibroblasts was compared. The experimental procedures used in this study provided an estimate of both the rate of unidirectional cholesterol flux from cells to HDL₃ particles and net transport of cholesterol from different cellular pools.

Promotion of cholesterol efflux from cells by HDL occurs by both passive and active processes. Cholesterol can passively desorb from plasma membranes and be readsorbed to HDL particles, which may be facilitated in some cases by the interaction of HDL apolipoproteins with plasma membrane lipids. When growth-arrested cells are overloaded with cholesterol, the number of high-affinity binding sites (receptors) for HDL increases on the cell surface, and the interaction of HDL apolipoproteins with their receptors appears to stimulate excretion of excess cholesterol by an active signaling process involving protein kinase C.

Results from this study showed that, in comparison with Russian HDL₃, all American HDL₃ samples were more active both in stimulating efflux of [³H]cholesterol from the plasma membrane and in cholesterol mass efflux from cholesterol-loaded fibroblasts. As an estimate of selective removal of excess intracellular cholesterol, HDL₃ samples were tested for their relative ability to deplete cells of cholesterol that was accessible to esterification by ACAT. Results again showed that American HDL₃ was more active in suppressing cellular esterification compared with Russian HDL₃. Thus, American HDL₃ had a greater ability than Russian HDL₃ to remove cholesterol from different cellular pools.

The most likely explanation for the current findings is that the American HDL₃ particles were better acceptors for cellular cholesterol than the Russian particles. It is less likely that the differences in cholesterol efflux-promoting activity between these groups of particles could be attributed solely to their effects on the cellular pathway that actively excretes excess cholesterol. If this were the case, American HDL₃ should have been better than Russian HDL₃ at removing cholesterol selectively from the substrate pool for ACAT, which depends on receptor binding, rather than from both this pool and the plasma membrane. The role of receptor-independent processes was indicated further by binding studies that showed no differences in the ability of American and Russian HDL₃ to interact with high-
affinity binding sites on cholesterol-loaded fibroblasts. Interestingly, Russian HDL₃ interacted with low-affinity binding sites to a greater extent than American HDL₃. The reason for this is unclear, but it could be related to differences in lipid composition between Russian and American HDL₃. Further studies are warranted to determine if this low-affinity binding influences transport of cholesterol between cells and HDL₃ particles. A lack of direct agreement between the effects produced by Russian and American HDL₃, HDL₃-hypo, and HDL₃-hyper on the [³H]cholesterol efflux and the transport of UC mass from fibroblasts was noted. This may be explained not only by the difference in the experimental conditions, in particular cholesterol loading of the cells (see "Methods"), but also by different mechanisms controlling these processes; i.e., efflux of plasma membrane cholesterol does not require interaction between HDL₃ and its receptors, whereas cholesterol mass transport is influenced by both receptor-dependent and receptor-independent processes.¹⁵,¹⁶,²⁷-³³

Russian HDL₃-hypo was isolated from plasma samples with relatively low HDL-C levels (mean value, 28.4±1.4 mg/dL, n=9). These HDL₃-hypo samples had modestly greater activity both in stimulating UC mass efflux and in suppressing cellular cholesterol esterification when compared with HDL₃-norm. Earlier, the authors from the Moscow laboratory reported that HDL isolated from the plasma of hypolalpholesterolemic subjects (HDL-C <35 mg/dL, n=11; mean value, 29.7±2.7 mg/dL) showed a reduced ability to stimulate [³H]cholesterol and UC mass efflux from cultured fibroblasts when compared with HDL from normolipidemic subjects (mean HDL-C value, 47.2±2.7 mg/dL, n=11).³² The difference between the experimental results obtained in previous studies and those presented here may be explained by the use of different HDL subfractions (total HDL in Moscow and HDL₃ in Seattle) and by the differences in experimental conditions: in Moscow cells were not preloaded with cholesterol, while in Seattle 48-hour preincubations with cholesterol (50 µg/mL) or LDL (200 µg protein/mL) were used (see "Methods").

In summary, the current study shows that Russian HDL₃ has less ability to stimulate cholesterol efflux from cholesterol-loaded fibroblasts than does American HDL₃, demonstrating for the first time potential functional differences between HDL particles isolated from Russian and American populations. It is unknown if these differences have any physiological relevance to reverse cholesterol transport or to protection against atherosclerosis, as the actual acceptor particles for tissue cholesterol may have markedly different properties than those of those found in the plasma HDL₃ subfraction. It is possible, however, that the concentration or composition of potential cholesterol acceptor particles in the HDL₃ density range may differ between Russian and American men. The lower cholesterol acceptor capacity of Russian HDL₃ is in accordance with the relatively higher proportion of UC in these samples.²⁷,³³ The relative content of UC in HDL is determined by multiple factors, including rates of transport of UC between tissues and other lipoproteins, the rate of UC esterification, the activity of cholesteryl ester transfer protein, and other processes that influence the surface properties of HDL particles.³⁴ Differences in these metabolic factors may explain why HDL in Russian men appears to be less antiatherogenic than HDL in American men. Identifying and characterizing these factors will be the subject of future study.

Acknowledgments

The study was performed thanks to the Russia-USA Inter-governmental Agreement for Cooperation in Public Health, partly supported by the National Institutes of Health (NIH) and by Edwards LIS Division of Baxter Health Corp. Additional support was provided by NIH grant HL-18645. We gratefully acknowledge Maria Culala and Ayo Bowen (University of Washington, Seattle), Irina Chadukova and Alexey Oflierov (National Research Center for Preventive Medicine, Moscow) for excellent technical assistance, and Olga Kuznetsova (Moscow) for the initial translation of the manuscript into English.

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and E L Bierman

doi: 10.1161/01.ATV.13.12.1770

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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