# Measurement of cholesterol bidirectional flux between cells and lipoproteins

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**Abstract We developed an assay that quantitates bidirectional cholesterol flux between cells and lipoproteins. Incubating Fu5AH cells with increasing concentrations of human serum resulted in increased influx and efflux; however, influx was 2- to 3-fold greater at all serum concentrations. With apolipoprotein B (apoB)-depleted serum, the ratio of influx to efflux (I/E) was close to 1, indicating cholesterol exchange. The apoB fraction of serum induced influx and little efflux, with I/E > 1. Using block lipid transport-1 to block scavenger receptor class B type I (SR-BI)-mediated flux with different acceptors, we determined that 50% to 70% of efflux was via SR-BI. With HDL, 90% of influx was via SR-BI, whereas with LDL or serum, 20% of influx was SR-BI-mediated. Cholesterol-enriched hepatoma cells produced increased efflux without a change in influx, resulting in reduced I/E. The assay was applied to cholesterol-normal and -enriched mouse peritoneal macrophages exposed to serum or LDL. The enrichment enhanced efflux without shifts in influx. With cholesterol-enriched macrophages, HDL efflux was enhanced and influx was greatly reduced. With all lipoproteins, cholesterol enrichment of murine peritoneal macrophages led to a reduced I/E. We conclude that this assay can simultaneously and accurately quantitate cholesterol bidirectional flux and can be applied to a variety of cells exposed to isolated lipoproteins or serum.**—Zimetti, F., G. K. Weibel, M. Duong, and G. H. Rothblat. **Measurement of cholesterol bidirectional flux between cells and lipoproteins. *J. Lipid Res.* 2006. 47: 605–613.**

**Supplementary key words** efflux • influx • macrophages • hepatoma • ATP binding cassette transporter A1 • scavenger receptor class B type I • high density lipoprotein

The movement of cholesterol out of cells and onto extracellular lipoprotein acceptors represents the first step in the process of reverse cholesterol transport, a pathway by which excess cholesterol is removed from the peripheral tissues and delivered to the liver for excretion (1–3). Many studies have probed this cholesterol efflux step and established that a number of mechanisms can mediate the efflux of free cholesterol (FC). Among these mechanisms are unmediated aqueous diffusion (4) and pathways mediated by scavenger receptor class B type I (SR-BI) (5), ABCA1 (6), and other ABC transporters, such as the recently discovered ABCG1 and ABCG4 (7). Although there are multiple efflux mechanisms, the actual measurement of efflux is relatively simple and usually employs the quantitation of the release of radiolabeled FC from cells into the medium. However, because physiologically relevant acceptors, such as lipoproteins, contain both FC and cholesteryl ester (CE), there is a bidirectional movement of cholesterol between cells and lipoproteins. Whereas the measurement of cholesterol efflux is relatively simple, the quantitation of influx of lipoprotein cholesterol is much more complicated (8). Influx involves the uptake of both FC and CE and is mediated by several mechanisms, including the unmediated uptake of lipoprotein FC via aqueous diffusion, the internalization of intact lipoprotein particles delivering both FC and CE, and the SR-BI-enhanced flux pathways of lipoprotein FC and selective CE uptake. These flux pathways become even more complex when cells are exposed to whole serum, in which individual lipoproteins and apolipoproteins differentially contribute to both the efflux and influx of cholesterol. When cells are incubated with isolated lipoproteins or whole serum, the net movement of cholesterol mass can result in the accumulation of cell cholesterol (influx), the depletion of cell cholesterol (efflux), or the bidirectional flux of cholesterol without a net change in cholesterol in either extracellular or intracellular compartments (exchange). Net efflux, net influx, and exchange can be determined by several factors, such as the level of cholesterol in the cells, the expression of receptors on the cell surface, and the concentration and composition of the lipoproteins to which cells are exposed.

Although efflux studies have been very useful in identifying the different pathways involved in the movement of cholesterol out of cells, one criticism of this approach is that the assay does not allow the determination of bidirectional flux. In this study, we have developed an assay that, based on a combination of mass and isotopic determinations, permits the simultaneous quantitation of both cholesterol efflux and influx when cells are exposed to isolated lipoproteins or whole serum. With this

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approach, we have determined the contribution to cholesterol flux of different serum fractions and whole serum. Also, we have measured the bidirectional flux of cholesterol when either Fu5AH rat hepatoma cells or murine peritoneal macrophages (MPMs) are exposed to sera or isolated lipoproteins.

## EXPERIMENTAL PROCEDURES

### Materials

Tissue culture plasticware was obtained from Falcon (Lincoln, NJ). RPMI 1640 and MEM were purchased from Mediatech Cellgro (Herndon, VA). FBS, calf serum (CS), gentamycin, DNase I, sodium cholate, and the HMG-CoA reductase inhibitor Mevinolin were purchased from Sigma-Aldrich (St. Louis, MO). BSA was obtained from Serological Corp. (Norcross, GA). [1,2 $^3$H]cholesterol was purchased from Perkin-Elmer Analytical Sciences (Boston, MA). The ACAT inhibitor CP113,818 was kindly provided by Pfizer Pharmaceuticals (Groton, CT). The SR-BI inhibitor BLT-1 (for block lipid transport-1) was obtained from ChemBridge (San Diego, CA).

### Serum lipids and lipoprotein analysis

Human $HDL_3$ and LDL were obtained by sequential ultracentrifugation as described previously (for LDL, d = 1.019–1.063; for HDL, d = 1.063–1.21) (9). Reconstituted high density lipoprotein (rHDL) particles, containing 100:1 (mol/mol) 1-palmitoyl-2-oleoylphosphatidilcholine/apolipoprotein A-I were prepared using a cholate dialysis technique (10). Acetylated low density lipoprotein (acLDL) was obtained by modification of LDL with acetic anhydride, as described previously (11). The concentration of all of the lipoproteins used during the experiment was expressed as μg protein/ml. Human serum was collected with approved consent from healthy normolipidemic individuals and pooled. The apolipoprotein B (apoB)-depleted serum was obtained from the whole serum by precipitating the apoB-containing lipoproteins with a polyethylene glycol solution as described previously (12). Briefly, whole serum was incubated for 20 min with a 20% polyethylene glycol (PEG) 6000 solution. After spinning samples at 10,000 rpm, the supernatant was collected and stored at 4°C.

### Cell culture

Rat Fu5AH hepatoma cells were maintained in MEM containing 5% CS. Cells at 90% confluence were trypsinized and plated at a density of $6 \times 10^5$/well on 12-well plates. Cell number was determined using the Z1 Coulter Particle Counter (Hialeah, FL). MPMs were isolated from B6CF31 mice. Mice were injected intraperitoneally with 0.5 ml of a 10% solution of Brewer Thioglycollate Medium (Difco Laboratories, Detroit, MI). Three days later, macrophages were harvested by lavage of the peritoneal cavity with PBS. After spinning and resuspension in MEM-HEPES, cells were counted and plated in 10% RPMI at a density of $7 \times 10^5$ cells/well on 12-well plates. All media were supplemented with gentamycin.

### Preparation of BLT-1 solution

To prepare a stock solution, BLT-1 was dissolved in DMSO at a concentration of 10 mM. The day before the treatment with cells, BLT-1 was diluted in 4% BSA-containing medium at a concentration of 200 μM and stored at 4°C. Just before the addition to cells, BLT-1 was diluted in medium to a final concentration of 10 μM.

### Measurement of cholesterol bidirectional flux

The assay to measure cholesterol efflux and influx uses a combination of isotopic and mass determinations. Cells were labeled for 24 h with [$^3$H]cholesterol in medium supplemented with 2.5% CS. After an equilibration period in 0.2% BSA-containing medium, a set of cells was harvested before the incubation with cholesterol acceptors (time zero) and the cholesterol content of cell lysates was measured as described below. Total counts were determined as well, and the specific activity (SA) at time zero ($T_0$ SA) (cpm/μg cholesterol) was calculated. The remaining cells were incubated with either serum or isolated lipoproteins for 8 h, at the end of which cholesterol SA was again determined ($T_8$ SA). Because the medium volume can affect the quantitation of cholesterol influx, in all experiments, 0.5 ml of medium was used in the flux period. To inhibit de novo cholesterol synthesis, the HMG-CoA reductase inhibitor Mevinolin was added to the efflux medium at a concentration of 5 μg/ml (13). Furthermore, to prevent cholesterol esterification, 2 μg/ml of the ACAT inhibitor CP113,818 was added during labeling, equilibration, and the flux stages of the experiment. For the determination of efflux, the following parameters were established: *1*) $T_0$ cholesterol content; and *2*) fractional efflux. The efflux of cell cholesterol mass was calculated as fractional efflux × $T_0$ cholesterol content, as described previously (14). For influx determinations, the following terms were used: *1*) $T_0$ SA; *2*) $T_8$ SA; *3*) SA factor ($T_0$ SA/$T_8$ SA); *4*) theoretical mass ($T_0$ cholesterol content − the mass of cholesterol effluxed); and *5*) calculated mass (SA factor × theoretical mass). Influx was determined as the calculated mass − theoretical mass. Cholesterol efflux and influx are expressed as μg cholesterol/8 h/mg protein. The net cholesterol movement resulting from these two opposite fluxes are expressed as an influx-to-efflux ratio (I/E). I/E < 1 indicates net cholesterol efflux, whereas I/E > 1 reflects net cholesterol influx. Cholesterol exchange without significant net flux is reflected by I/E = 1.

### Protein and cholesterol determination

At the end of the experiment, cell monolayers were washed with MEM-HEPES medium and lysed in 0.5 ml of a 1% sodium cholate solution in water supplemented with 10 U/ml DNase. A total of 125 μl of a reaction buffer containing 0.5% Triton X-100, 0.5 M potassium phosphate (pH 7.4), 0.25 M NaCl, and 1% sodium cholate was added to the cell lysates, and plates were shaken for 30 min at room temperature. Care must be taken to ensure complete solubilization of the cell monolayer. After heating samples at 60°C for 30 min, to inactivate enzymes that could compete with the enzymatic cholesterol assay, cholesterol was measured fluorimetrically using the Amplex Red Cholesterol Assay Kit (Molecular Probes, Eugene, OR) as described by the manufacturer. Because the experiments were conducted in the presence of an ACAT inhibitor, the cholesterol present in the cells at $T_0$ was unesterified. However, the enzymatic kit assay for cholesterol included a cholesterol esterase and allowed the measurement of all cellular cholesterol. This permitted the quantitation of any CE incorporated from lipoproteins. The amount of cholesterol in each well was measured by comparison with a cholesterol standard curve included in each experiment. An aliquot of the cell lysates was also taken to measure cell protein by a modified Lowry method (15). The total [$^3$H]cholesterol present in cell lysates was measured by scintillation counting.

### Statistical analysis

All of the experiments were conducted in triplicate, and the data represent means of multiple experiments or multiple

TABLE 1. Cholesterol efflux, cholesterol influx, and I/E in Fu5AH cells exposed to lipoproteins or serum

| Parameter | rHDL, 25 μg/ml (n = 6) | $HDL_3$, 25 μg/ml (n = 9) | Human Serum, 2.5% (n = 9) | LDL, 100 μg/ml (n = 9) |
|---|---|---|---|---|
| Cholesterol efflux (μg/8 h/mg protein) | 5.29 ± 0.36 | 2.46 ± 0.45 | 5.76 ± 0.94 | 4.16 ± 0.37 |
| Cholesterol influx (μg/8 h/mg protein) | −0.42 ± 0.03 | 1.86 ± 0.73 | 12.32 ± 1.83 | 19.42 ± 2.67 |
| I/E | −0.08 ± 0.00 | 0.87 ± 0.52 | 2.15 ± 0.29 | 4.65 ± 0.24 |

I/E, influx-to-efflux ratio; rHDL, reconstituted high density lipoprotein. Cells were labeled with [$^3$H]cholesterol for 24 h in 2.5% serum-containing medium. After an equilibration period in 0.2% BSA-containing medium, apolipoprotein A-I/phospholipid rHDL (25 μg/ml), $HDL_3$ (25 μg/ml), human serum (2.5%), and LDL (100 μg/ml) were added for 8 h. ACAT inhibitor (2 μg/ml) was added during the entire experimental period, and 5 μg/ml Mevinolin was added during the flux period. Cholesterol efflux, influx, and I/E were calculated as described in Experimental Procedures. Data are expressed as averages ± SD.

assays. The error bars represent standard deviations. All statistical analyses were performed with GraphPad (San Diego, CA) Prism software.

## RESULTS

We first investigated whether the direction of net cholesterol flux depends on the type and composition of the extracellular acceptors. Fu5AH cells were exposed to isolated lipoproteins or serum for 8 h, and cholesterol efflux, cholesterol influx, and the I/E were measured. Rat Fu5AH hepatoma cells express high levels of SR-BI and thus can be viewed as a cellular system in which SR-BI-mediated bidirectional flux of cholesterol is of major importance (16). As shown in **Table 1**, when cells were exposed to rHDL, there was significant cholesterol efflux (5.29 ± 0.36 μg cholesterol/mg protein) in the absence of influx, as expected, because these particles contain no cholesterol. With $HDL_3$, cholesterol efflux and influx values were similar (2.14 ± 0.45 and 1.86 ± 0.73 μg cholesterol/mg protein, respectively), with the resulting I/E of 0.87 ± 0.52, indicating that cholesterol was exchanged between HDL and the hepatoma cells without resulting in net cholesterol flux. Furthermore, rHDL was a more efficient cholesterol acceptor than $HDL_3$ when the two particles were used at similar protein concentrations. Incubation of cells for 8 h with 100 μg/ml LDL resulted in influx being 4.7-fold greater than efflux, producing net cholesterol movement into cells (I/E of 4.65 ± 0.24). Net influx was also observed upon exposure of cells to 2.5% human serum, producing an I/E of 2.15 ± 0.29.

It has been proposed that SR-BI enhances aqueous diffusion, facilitating the bidirectional movement of FC, and this flux follows the concentration gradients between cell membranes and extracellular particles (17, 18). Thus, increasing cell cholesterol content should create a gradient that facilitates the movement of cholesterol out of cells. The initial cholesterol content in Fu5AH cells was increased by incubation with 100 μg/ml LDL added during the labeling period (**Fig. 1**). In a representative experiment, FC content was 20.4 ± 1.15 μg cholesterol/mg protein for cholesterol-normal cells and 36.5 ± 4.78 μg cholesterol/mg protein after cholesterol enrichment. $HDL_3$, LDL, and human serum were then added to the culture medium for 8 h, and cholesterol efflux and influx were measured. Cholesterol efflux and influx values for normal cells were similar to those presented in Table 1. The fractional release of cholesterol from enriched cells was similar to that obtained with cholesterol-normal cells. However, because the cellular FC pool was expanded by ~40%, the mass of FC released to all acceptors was increased, as has been described (19). Although the cholesterol mass released was increased, no significant change in cholesterol influx between normal and enriched cells was observed. Thus, the I/E obtained with enriched Fu5AH

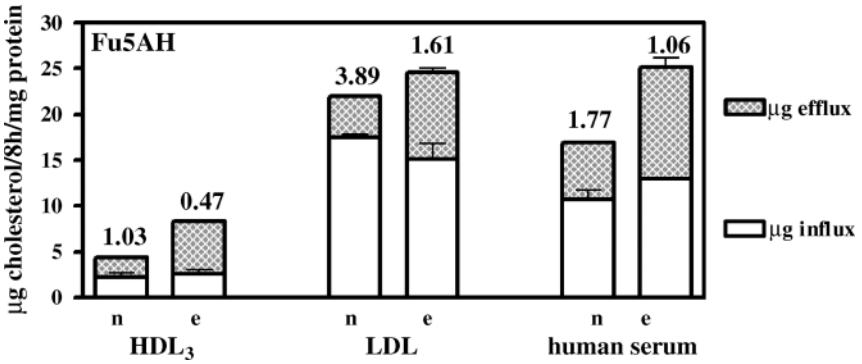


**Fig. 1.** Cholesterol efflux, cholesterol influx, and influx-to-efflux ratio (I/E) with cholesterol-normal (n) and cholesterol-enriched (e) Fu5AH cells. Cells were labeled for 24 h with [$^3$H]cholesterol in the absence (n) or presence (e) of 100 μg protein/ml LDL. After a 1 h equilibration period in 0.2% BSA-containing medium, $HDL_3$ (25 μg/ml), LDL (100 μg/ml), and human serum (2.5%) were added for 8 h. Experimental conditions were as described in Table 1. In a representative experiment, initial free cholesterol (FC) content in unloaded cells was 21.8 ± 1.1 μg/mg protein. After loading, FC content was 40.2 ± 4.8 μg/mg protein. Cholesterol efflux (shaded bars), influx (open bars), and I/E (numbers above the bars) were calculated as described in Experimental Procedures. Data are means ± SD (n = 3).

cells was reduced compared with that in cholesterol-normal cells.

In vivo, cells are exposed to a mixture of lipoproteins present in serum. For this reason, further experiments were performed to measure the bidirectional flux of cholesterol upon exposure of cells to serum. **Figure 2** shows how the mass of cholesterol undergoing efflux or influx, and the I/E, changed when Fu5AH cells were incubated with increasing concentrations of human serum, ranging from 2.5% to 12.5%. We also established the contribution of the apoB-depleted serum and the apoB-containing lipoproteins to both efflux and influx. To obtain the apoB-depleted serum, apoB-containing lipoproteins of whole serum were precipitated with PEG, as described in Experimental Procedures. The contribution of the apoB-containing lipoproteins was calculated by subtracting the flux obtained with PEG supernatant (i.e., HDL) from that obtained with serum. The cholesterol efflux to whole serum (Fig. 2A) increased in a dose-dependent manner, from 5.33 ± 0.16 μg cholesterol/mg protein with 2.5% serum to 11.23 ± 0.13 μg cholesterol/mg protein for the highest dose of serum (12.5%). Cholesterol influx from serum also increased in a dose-dependent manner, ranging from 11.15 ± 0.89 μg FC/mg protein with 2.5% serum to 27.24 ± 0.92 μg FC/mg protein at 12.5% serum. At every serum concentration, the mass of cholesterol incorporated into cells was greater than that released from cells, and this was reflected by I/E > 1, which increased linearly with increasing serum concentrations (Fig. 2B). After isolating the apoB-depleted fraction, the HDL- and apoB-containing lipoprotein contributions to efflux and influx were calculated. As shown in Fig. 2C, the apoB-depleted fraction was responsible for values ranging from 55% to 79% of the total amount of cholesterol released from cells (compare Fig. 2C with 2A). Furthermore, for every concentration of apoB-depleted serum, the I/E was somewhat <1. This indicated once again that HDL promoted either a small net efflux or exchange of cholesterol. By the difference between the whole serum and the apoB-depleted serum, we determined the ability of the apoB-containing lipoprotein component of serum to promote both efflux and influx. Figure 2E shows how this fraction made only a small contribution to cholesterol efflux, and the amount of cholesterol released was independent of the concentration of serum. As expected, the VLDL and LDL components of the serum were the major source of cholesterol for influx, with maximum values of 19.20 ± 1.38 μg cholesterol influx/mg protein with 12.5% serum. Moreover, apoB lipoproteins mediated 70–80% of the total cholesterol taken up by cells (compare Fig. 2E with 2A). Net cholesterol influx, expressed by the I/E, increased linearly with the serum concentration, as shown in Fig. 2F.

In the experiments described above, we used a flux period of 8 h to permit the quantitation of detectable cholesterol movement in and out of cells. To test whether cholesterol efflux, influx, and the resulting I/E changed

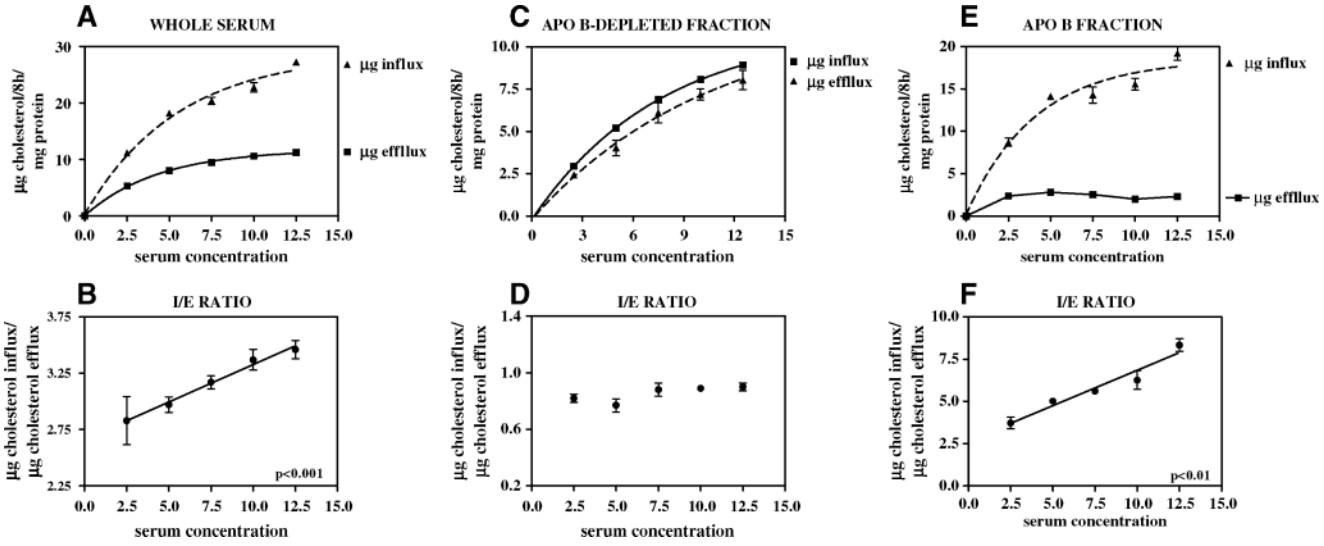


**Fig. 2.** A, B: Cholesterol efflux, cholesterol influx, and I/E with Fu5AH cells exposed to increasing concentrations of human serum. C–F: Contributions of the apolipoprotein B (apoB)-depleted serum (C, D) and the apoB-containing lipoprotein component of the serum (E, F) to cholesterol efflux, influx, and I/E. Cells were labeled with [$^3$H]cholesterol for 24 h in 2.5% serum-containing medium. After a 1 h equilibration period in 0.2% BSA-containing medium, increasing concentrations of human serum (from 2.5% to 12.5%) were added for 8 h. Experimental conditions were as described in Table 1. Cholesterol efflux, influx, and I/E were calculated as described in Experimental Procedures. Data are expressed as means ± SD (n = 3), and the values shown are representative of three independent experiments. A: Cholesterol efflux (solid line) and cholesterol influx (dashed line) when Fu5AH cells were exposed to whole serum. B: I/E when cells were exposed to whole serum. C: Cholesterol efflux (solid line) and cholesterol influx (dashed line) when Fu5AH cells were exposed to apoB-depleted serum. D: I/E when Fu5AH cells were exposed to apoB-depleted serum. E: Cholesterol efflux (solid line) and cholesterol influx (dashed line) promoted by the apoB-containing lipoprotein component of serum. The contribution was calculated by subtracting the apoB-depleted serum contribution from the whole serum contribution, as described in Experimental Procedures. F: I/E promoted by the apoB-containing lipoprotein fraction of the serum.

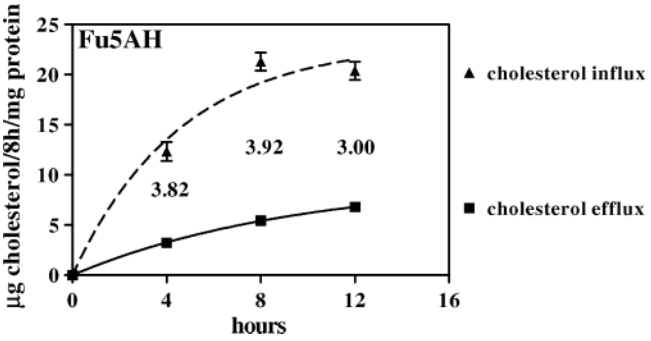


**Fig. 3.** Cholesterol efflux, cholesterol influx, and I/E with Fu5AH cells exposed to human serum for 4, 8, and 12 h. Cells were labeled with [$^3$H]cholesterol for 24 h in 2.5% serum-containing medium. After a 1 h equilibration period in 0.2% BSA-containing medium, 2.5% human serum was added for 4, 8, and 12 h. Experimental conditions were as described in Table 1. Cholesterol efflux (solid line), cholesterol influx (dashed line), and I/E (numbers) were calculated as described in Experimental Procedures. Data are expressed as means ± SD (n = 3).

with different flux times, we exposed Fu5AH cells to 2.5% whole serum for 4, 8, and 12 h, and at the end of the incubation times we measured both cholesterol efflux and influx. The results are shown in **Fig. 3**. For each time point, influx was greater than efflux and the I/E was >1, with ratios ranging from 3.00 to 3.92, indicating that time did not affect the direction of the net flux, at least over 12 h.

Cholesterol movement in Fu5AH cells is mainly attributable to a high expression of SR-BI; however, other pathways can be involved. To quantitate the contribution of SR-BI and other mechanisms to the bidirectional flux, we incubated Fu5AH cells with BLT-1, a low molecular weight compound capable of inhibiting lipid transport mediated by SR-BI (20). Fu5AH cells were treated with 10 μM BLT-1 for 2 h before cholesterol acceptors were added. The results of cholesterol efflux and influx, in the absence or presence of BLT-1, are shown in **Fig. 4A, B**. BLT-1 inhibited $HDL_3$-mediated efflux by 54%, indicating that approximately half of the efflux from Fu5AH cells to $HDL_3$ occurred via SR-BI (Fig. 4A). Cholesterol influx was inhibited by 87% in BLT-1-exposed cells (Fig. 4B). Exposing cells to LDL after BLT-1 treatment caused a reduction in cholesterol efflux of 67% (Fig. 4A), and the effect on influx was significant but modest (20%; Fig. 4B). In the presence of human serum as an acceptor, BLT-1 inhibition of efflux was 57%; the reduction in influx was only 20%. The apoB-depleted portion of the serum gave a flux pattern similar to that of isolated $HDL_3$, with ~50% of the efflux mediated by SR-BI and a much greater fraction of influx linked to SR-BI (~90%). I/E values in the presence or absence of BLT-1 are shown in **Table 2**. With isolated $HDL_3$ and apoB-depleted serum, inhibition of SR-BI reduced influx to a greater extent than efflux, resulting in a reduction in I/E. However, this pattern was reversed with serum and LDL, in which case the inhibition of SR-BI resulted in a greater reduction in efflux. This caused increases in I/E.

These bidirectional flux data were obtained with Fu5AH hepatoma cells expressing high levels of SR-BI, which mediates large amounts of cholesterol movement in and out of cells. We extended the assay to cholesterol-normal and cholesterol-enriched MPMs by incubation with 100 μg/ml acLDL (**Fig. 5**). The initial cholesterol content of cholesterol-normal cells was 21.84 ± 1.85 μg cholesterol/mg protein, and that in cholesterol-enriched cells was 40.20 ±

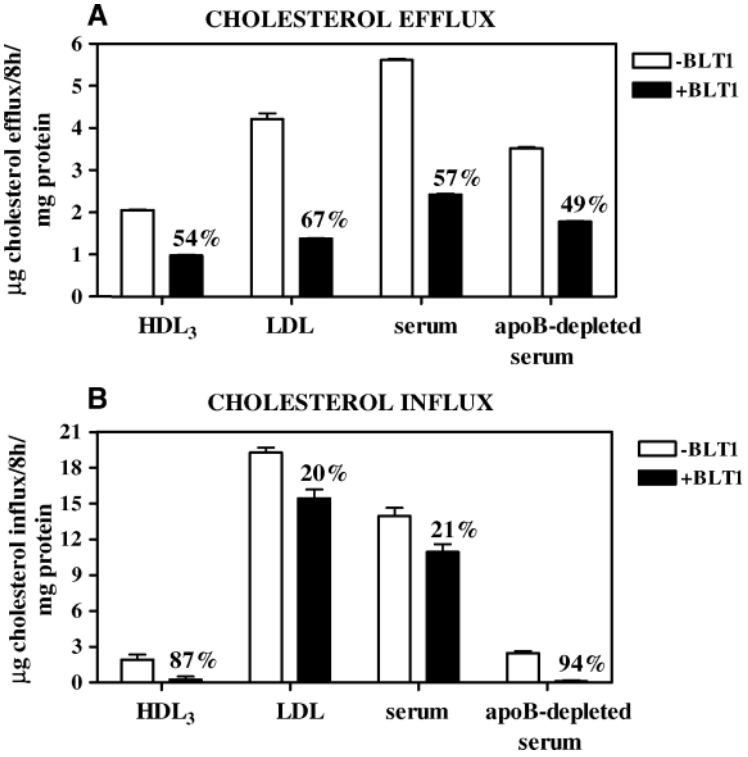


**Fig. 4.** Cholesterol efflux (A) and cholesterol influx (B) in BLT-1 (for block lipid transport-1)-pretreated Fu5AH cells exposed to different acceptors. Cells were labeled with [$^3$H]cholesterol for 24 h in 2.5% serum-containing medium. During a 2 h equilibration period in 0.2% BSA-containing medium, cells were incubated in the absence (open bars) or the presence (closed bars) of BLT-1 (10 μM). For the following 8 h, $HDL_3$ (25 μg/ml), LDL (100 μg/ml), human serum (2.5%), and its apoB-depleted fraction were added to the medium. Experimental conditions were as described in Table 1. Cholesterol efflux and influx were calculated as described in Experimental Procedures. The numbers above the bars indicate the percentage inhibition of efflux (A) or influx (B) after BLT-1 treatment. Data are expressed as means ± SD (n = 3).

TABLE 2. I/E with Fu5AH cells preincubated with BLT-1 and exposed to different acceptors

| Acceptor | −BLT-1 | +BLT-1 |
|---|---|---|
| $HDL_3$ | 0.93 ± 0.34 | 0.26 ± 0.44 |
| LDL | 4.59 ± 0.40 | 11.21 ± 0.85 |
| Human serum | 2.48 ± 0.22 | 4.52 ± 0.39 |
| Apolipoprotein B-depleted serum | 0.71 ± 0.09 | 0.06 ± 0.10 |

BLT-1, block lipid transport-1. Experimental conditions were as described for Fig. 4.

4.04 μg cholesterol/mg protein. Macrophages were incubated with $HDL_3$, human serum, LDL, and acLDL. The results obtained for efflux and influx were generally similar to those observed in Fu5AH cells, with an increase in efflux of cholesterol mass after enrichment and no significant changes in cholesterol influx upon cell loading. However, results obtained by incubation with $HDL_3$ were different from those with serum and LDL in that after loading, the I/E was dramatically reduced because of increased efflux and almost totally abolished influx. Using macrophages, we were also able to compare the difference between LDL and acLDL on flux. Because macrophages have high expression levels of the scavenger receptor SR-A, we anticipated enhanced influx with acLDL (21, 22). We observed that the amount of cholesterol released to acLDL was comparable to that released to the same concentration of LDL (Fig. 5). However, cholesterol influx promoted by acLDL was ~2-fold greater than influx promoted by LDL in both unloaded and loaded cells. Thus, as predicted, more efficient uptake occurred in the presence of acLDL. The I/E of 4.41 ± 0.25 in unloaded cells was reduced to 1.52 ± 0.30 after cholesterol enrichment of MPMs. This was exclusively attributable to a marked increase in efflux (63%), without any change in the amount of cholesterol delivered to cells.

## DISCUSSION

### Bidirectional flux assay

One aim of these studies was to develop a reliable assay that could quantitate the bidirectional flux of cholesterol between cells and lipoproteins and to design the assay so that it could be applied to a large number of samples with relative ease. Many studies have measured the efflux of cellular cholesterol from a number of different cells to a variety of extracellular acceptors (7, 16, 23, 24). In most of these studies, the efflux of cell cholesterol was quantitated through the release of radiolabeled cholesterol from cells prelabeled with either exogenous or endogenous cholesterol (8). Although cholesterol efflux can be mediated by a number of different mechanisms, the high-throughput measurement of isotopic release of cholesterol is accurate because only FC undergoes efflux. In contrast to efflux, the measurement of influx of cholesterol by cells from lipoproteins is more complicated, because both FC and CE are incorporated by a number of different mechanisms. Bidirectional flux of cholesterol has been measured in a limited number of studies, primarily using a double-labeled approach in which the cells are labeled with [$^3$H]FC and the lipoprotein is labeled with [$^{14}$C]FC (25). However, this approach measures only the flux of FC. The protocol we used in this study uses the release of labeled cell cholesterol to quantitate efflux and the dilution of cell cholesterol specific activity to assess cholesterol influx. This approach includes the uptake of both exogenous FC and CE and is independent of mechanism of influx, cell growth, or cell loss during the incubations. One of the features of the assay is that it includes the addition of the HMG-CoA reductase inhibitor to avoid any interference in the measurement related to de novo cholesterol synthesis. Even if we do not think that in 8 h there is substantial cholesterol synthesis, we choose to keep the inhibitor

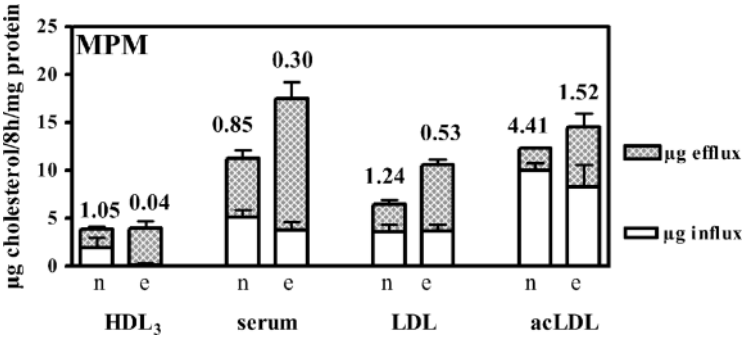


**Fig. 5.** Cholesterol efflux, cholesterol influx, and I/E in cholesterol-normal (n) and cholesterol-enriched (e) macrophages. Cells were labeled with [$^3$H]cholesterol for 24 h in the absence (n) or presence (e) of 100 μg protein/ml acetylated low density lipoprotein (acLDL). After a 1 h equilibration period in 0.2% BSA-containing medium, $HDL_3$ (25 μg/ml), 2.5% human serum, LDL (100 μg/ml), and acLDL (100 μg/ml) were added for 8 h. ACAT inhibitor (2 μg/ml) was added during the entire experimental period, and 5 μg/ml Mevinolin was added during the flux period. Initial FC content in unloaded cells was 21.8 ± 1.85 μg/mg protein; after loading, FC content was 40.2 ± 4.04 μg/mg protein. Cholesterol efflux (shaded bars), influx (open bars), and I/E (numbers above the bars) were calculated as described in Experimental Procedures. Data are expressed as means ± SD (n = 9).

present during the flux period to exclude any residual endogenous synthesis from the quantitation. The presence of the ACAT inhibitor during the experiment is more important. The inhibition of cholesterol esterification ensures that all of the intracellular cholesterol is free and thus the specific activity is uniform among the pools. Preliminary experiments omitting the ACAT inhibitor were performed, but interpretation of the data is difficult because of the complexity of the system. Changes in the assay protocol would have to be made to ensure similar cholesterol specific activity in both the free and esterified cholesterol pools. In addition, the rate-determining step could be FC efflux from the plasma membrane or CE hydrolysis, depending on the experimental conditions. With the protocol using ACAT inhibition, accurate measurement of cholesterol influx, efflux, and I/E can be readily obtained from a relatively large number of samples.

**Bidirectional flux with HDL**

The bidirectional flux assay was developed and validated using Fu5AH rat hepatoma cells, which have been used in cholesterol efflux experiments in a large number of studies (26–31). Fu5AH cells have high levels of SR-BI protein (32); thus, one of the major mechanisms for cholesterol flux is mediated by this receptor. In addition, Fu5AH cells express a mutated LDL receptor that is efficient in internalizing apoB-containing lipoproteins (33). Thus, influx of cholesterol would occur by FC exchange, lipoprotein internalization, and SR-BI-mediated selective uptake. In our study, we used purified $HDL_3$ and apoB-depleted human serum prepared by PEG precipitation. Both preparations gave essentially similar results. Incubation of Fu5AH cells containing basal levels of cholesterol with HDL resulted in similar movements of cholesterol influx and efflux, yielding I/E close to 1, consistent with cholesterol exchange (Tables 1, 2, Figs. 1, 2C). However, if the cells were enriched with cholesterol, the I/E shifted from ~1.0 to 0.5, indicating that net efflux occurred, as seen in Fig. 1. The shift to net efflux occurred because influx from the HDL was unaffected by cell cholesterol enrichment, whereas the mass of cholesterol released was increased. This result was consistent with earlier studies that demonstrated that increases of cell cholesterol did not change the fractional release of cholesterol but increased the size of the cell pool of cholesterol from which efflux occurred (19).

Using the SR-BI inhibitor BLT-1 (20, 34), we were able to determine the contribution of that receptor to the uptake and release of cholesterol from Fu5AH cells incubated with either $HDL_3$ or apoB-depleted serum (Fig. 4). Under the conditions of our assay, we observed that ~60% of the total efflux was inhibited by BLT-1 treatment, indicating that this was the contribution of SR-BI to total efflux. The mechanism for efflux of the remaining 40% has yet to be established. However, we have observed, using both fibroblasts and macrophages, that from 40% to 60% of the total efflux to HDL or serum is not mediated by either SR-BI or ABCA1 (M. Duong et al., unpublished data). Surprisingly, SR-BI contributed ~90% of the influx of HDL cholesterol.

**Bidirectional flux with LDL**

As with the HDL, we generated bidirectional flux values using both isolated human LDL and the apoB component of serum. The latter values were calculated as the difference in flux between whole serum and apoB-depleted serum. In both cases, there was large net influx (Tables 1, 2 Figs. 1, 2E). With Fu5AH cells, the I/E showed a linear increase with increasing concentrations of the apoB component of the serum, primarily because efflux was low and independent of concentration, whereas influx increased over the concentration range. The SR-BI contribution to both influx and efflux with LDL was again determined on the basis of the inhibition with BLT-1. SR-BI was responsible for ~60% of the total efflux from Fu5AH cells to LDL (Fig. 4A). SR-BI contributed much less to the influx of LDL (20%; Fig. 4B). Because Fu5AH cells express LDL receptors, the LDL internalization route of cholesterol incorporation probably has the greatest effect on cholesterol influx.

**Bidirectional flux with human serum**

With 2.5% human serum, there was net cholesterol influx, as indicated by an $I/E > 1$. Net cholesterol incorporation into cholesterol-normal hepatoma cells occurred at all serum concentrations used (2.5–12.5%; Fig. 2A). The direction of the flux was also not affected by using different incubation periods of cells with the serum, as shown in Fig. 3. The I/E increased linearly with the concentration of serum, reaching ratios as high as 3.5 at increased serum concentrations (Fig. 2B). This ratio, in the case of serum, was lower compared with the ratio obtained with the isolated apoB-containing lipoprotein fraction, reflecting the efflux contribution of the HDL present in the serum (compare Fig. 2B with 2F). Enriching the cells in cholesterol shifted the ratio from values reflecting net influx to values close to exchange (Fig. 1).

**Bidirectional flux with mouse peritoneal macrophages**

After using the Fu5AH hepatoma cells as a vehicle for developing and validating the bidirectional flux protocol, we then extended the studies to examine the flux of cholesterol between extracellular particles and elicited MPMs. Bidirectional flux was measured using cholesterol-normal cells and macrophages enriched with cholesterol by exposure to acLDL (Fig. 5). Human serum produced a small net efflux from cholesterol-normal cells, and this net efflux was greatly enhanced upon cell cholesterol enrichment. The impact of cell cholesterol enrichment was even more dramatic when the extracellular particle was LDL, in which case the I/E shifted from one describing net influx with cholesterol-normal cells to net efflux with cholesterol-loaded macrophages (Fig. 5). As with the hepatoma cells, the effect of FC loading was primarily to increase the movement of cholesterol out of cells, with a much more modest effect on movement into the cells. The shift in the I/E from net influx to net efflux, particularly with LDL, demonstrated how the cholesterol status of the cells can markedly influence net flux and may, at least in part,

explain the observation by Van Eck et al. (35) that SR-BI can play a dual role by promoting the deposition of cholesterol in early lesions, when macrophages would not yet contain large amounts of cholesterol, but can be atheroprotective at later stages of plaque formation, when macrophages would be cholesterol-loaded and net efflux could be elicited. When acLDL was the extracellular particle, there was a large increase in influx compared with control human LDL, resulting in increased net influx. This was particularly evident with the combination of cholesterol-normal macrophages exposed to acLDL and is consistent with the ability of modified LDL to enrich macrophages with excess cholesterol. Interestingly, in contrast to the other extracellular particles, in which cholesterol influx did not change after loading, cholesterol influx from $HDL_3$ was almost totally abolished in cholesterol-loaded cells. A recent publication by Yu et al. (36), as well as results from our research group, indicated that SR-BI levels decreased upon cholesterol loading of macrophages with acLDL. To enrich cells in our experiments, we used the same concentration of acLDL shown able to reduce SR-BI expression (36). As a consequence, no cholesterol influx from $HDL_3$ occurred. As observed in Fu5AH studies with BLT-1, this is consistent with cholesterol influx from HDL being largely mediated by SR-BI.

In conclusion, this study demonstrates that an assay is available that can simultaneously and accurately quantitate the bidirectional flux of cholesterol between cells and either serum or isolated lipoproteins. The assay can be applied to a variety of different cells, and by using inhibitors of cholesterol flux such as the SR-BI inhibitor BLT-1, it can provide a measurement of the role of different flux pathways in both cholesterol influx and efflux. In the future, this assay can be used as a tool to quantitate the ability of any sera to mediate the net flux of cholesterol mass or to test molecules that can modulate the first step of reverse cholesterol transport.

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