Human lymphocytes respond optimally to mitogenic stimulation when cultured in serum-free medium supplemented with transferrin if fatty acids necessary for maximal proliferation are provided. Either lipoproteins or exogenous fatty acids support optimal lymphocyte responses. The current studies examined the role of cell surface receptors for low density lipoprotein (LDL) in the enhancement of lymphocyte proliferation. Support of lymphocyte growth by limiting concentrations of LDL was found to involve interaction of the lipoprotein with LDL receptors. Thus, modification of LDL by reductive methylation so as to inhibit receptor-mediated interactions markedly decreased the capacity of LDL to enhance lymphocyte proliferation. Moreover, growth of lymphocytes obtained from patients with LDL receptor-negative homozygous familial hypercholesterolemia was minimal when cultures were supplemented with low concentrations of LDL (<10 μg cholesterol/ml). LDL also enhanced lymphocyte proliferation by a receptor-independent mechanism since high concentrations (≥50 μg cholesterol/ml) supported growth of both normal and familial hypercholesterolemia lymphocytes. In contrast, support of lymphocyte proliferation by high density lipoprotein (HDL) subclass 3 was completely independent of LDL receptors. Thus, HDL enhanced responses of both normal and familial hypercholesterolemia lymphocytes in an equivalent concentration-dependent manner; this effect was not altered by reductive methylation of HDL₃.

One function of lipoproteins in this system may be the provision of fatty acids since oleic and linoleic acids enhanced DNA synthesis by both normal and familial hypercholesterolemia lymphocytes in the absence of lipoproteins. These results indicate that lipoproteins may provide fatty acids necessary for optimal proliferation of human lymphocytes by both LDL receptor-mediated and LDL receptor-independent interactions.

The importance of plasma lipoproteins in cellular lipid metabolism has been well documented. Low density lipoprotein (LDL) transports cholesterol in the plasma and is the major extracellular source of cholesterol (1-4). Following binding of LDL to specific cell surface receptors, the lipoprotein is internalized by the process of receptor-mediated endocytosis (1-4). After lysosomal degradation, the cholesterol is released from cholesteryl esters in the core and made available for membrane synthesis (1-4). Other lipoproteins, including very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL) and high density lipoprotein (HDL) may also provide cholesterol in a similar manner, if they are able to bind to LDL receptors (4). Receptor-mediated endocytosis of LDL and other lipoproteins also results in the delivery of phospholipid and triglyceride to the cell, as well as cholesterol. Like cholesterol, the phospholipids and triglycerides of the lipoprotein particle are transported to lysosomes in the cell. However, the intracellular fate of these non-sterol lipids is less well defined.

In previous studies, we found that both LDL and HDL were able to support optimal proliferation of lymphocytes cultured in serum-free medium supplemented with transferrin (5). Various proteins, cholesterol, and cholesteryl esters were ineffective at promoting lymphocyte growth, whereas LDL depleted of cholesteryl esters and other neutral lipids by heptane extraction was able to enhance lymphocyte responses (5). Thus, a component of lipoproteins other than cholesterol or neutral lipid appeared to be responsible for the capacity of these particles to support lymphocyte growth. Phospholipid, triglyceride, and the non-esterified fatty acids, oleic acid and linoleic acid, were each able to replace lipoproteins and enhance DNA synthesis of mitogen-stimulated lymphocytes cultured in transferrin-supplemented serum-free medium, thereby suggesting that these lipid components of lipoproteins accounted for the growth promoting activity (5).

The current studies were undertaken to examine the requirement for specific LDL receptors in the enhancement of lymphocyte proliferation by lipoproteins. Previous experiments had established that the provision of cholesterol to proliferating lymphocytes by lipoproteins was absolutely dependent on LDL receptors (6-8). The results of the present experiments demonstrate that receptor-mediated interaction with LDL may also provide fatty acids necessary for human lymphocyte proliferation. However, unlike the provision of cholesterol, non-receptor-mediated mechanisms appear to be capable of promoting lymphocyte growth. Although LDL receptors enhance the effect of lipoproteins, such receptor-mediated interactions are not required. In vivo, lipoproteins may play an important role in providing lipids necessary for cellular proliferation by LDL receptor-dependent and independent mechanisms.

**MATERIALS AND METHODS**

Lipoprotein Isolation—The following lipoprotein fractions were isolated and characterized as detailed previously (5): VLDL + IDL, HDL, LDL, VLDL, IDL, intermediate density protein; BSA, bovine serum albumin; PBMC, peripheral blood mononuclear cells; PHA, phytohemagglutinin.
LDL Receptor-dependent and -independent Provision of Fatty Acids

(d < 1.029 g/ml), LDL (d = 1.029-1.050 g/ml), and HDL subclass 3 (HDLs, d = 1.125-1.230 g/ml) Purify of lipoprotein fractions was confirmed by electron microscopy of negatively stained preparations and by analysis of apolipoprotein composition as previously described (8). Total and non-esterified fatty acid contents of lipoprotein preparations were measured by titrimetric assay, and individual fatty acid compositions were examined by gas-liquid chromatography (Mayo Medical Laboratories, Rochester, MN). For some experiments, lipoproteins were modified by methylation to alter LDL receptor-mediated recognition of apolipoprotein B-100, as described (9).

Regents—Transferrin, fatty acid-free and fatty acid-containing bovine serum albumin (BSA) and fatty acid-containing human serum albumin were purchased from Sigma and Boehringer Mannheim. Non-esterified fatty acids contents of albumin preparations were measured by titrimetric assay (Smith Kline BioSciences Laboratories, Van Nuys, CA and Mayo Medical Laboratories, Rochester, MN). Fatty acids were obtained from Calbiochem-Behring Corp., LaJolla, CA, dissolved in ethanol and added to cultures as indicated in results. These cultures were also supplemented with BSA to prevent nonspecific inhibitory effects of fatty acids. BSA concentrations of 7.5 μM were sufficient to prevent inhibitory effects of 5-10 μM nonesterified fatty acids, whereas 75 μM BSA was required to prevent inhibition by 0.1-1 μM nonesterified fatty acids. The BSA was used to prevent inhibition in these experiments contained insufficient free fatty acid to support lymphocyte growth and proliferation alone. Lovastatin, a specific inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase, the rate-limiting enzyme for cholesterol synthesis, was provided by the Upjohn Co., Kalamazoo, MI. Bovine serum albumin (BSA) was required to prevent inhibition of lymphocyte growth and proliferation after methylation (8, 9). Methyl-LDL was found to be markedly less effective than native LDL at enhancing mitogen-stimulated lymphocyte DNA synthesis and proliferation in transferrin-supplemented cultures (Table I and Fig. 1). However, at higher concentrations, methyl-LDL was able to support some lymphocyte responsiveness. These results suggest that blocking the interaction between LDL and cell surface LDL receptors by reductive methylation significantly reduced but did not abolish the capacity of LDL to enhance lymphocyte proliferation.

To confirm that methylation had altered receptor-mediated recognition of LDL particles, experiments were carried out in which mitogen-induced lymphocyte proliferation was made dependent on LDL receptor-mediated delivery of cholesterol. This was accomplished by culturing PBMC in cholesterol-depleted medium and blocking endogenous sterol synthesis withLovastatin (6). In the absence of an extracellular cholesterol source,Lovastatin suppressed lymphocyte DNA synthesis by 94% (Table II). The addition of native LDL (5 μg of cholesterol/ml) provided sufficient exogenous cholesterol to restore normal DNA synthesis by lovatatin-blocked lymphocytes. In contrast, methyl-LDL was only minimally able to provide the cholesterol required for lymphocyte responses. Lovastatin also blocked mitogen-induced proliferation of normal lymphocytes cultured in lipoprotein-deficient medium (control = 104,100 ± 7,000 cells/well;Lovastatin 0.5 μM = 19,700 ± 400 cells/well). LDL but not methyl-LDL restored proliferation (LDL 5 μg of cholesterol/ml = 118,800 ± 7,800 cells/well, methyl-LDL 5 μg of cholesterol/ml = 22,500 ± 2,800 cells/well, 50 μg cholesterol/ml = 38,100 ± 800 cells/well).

Variable Requirement for LDL Receptors in Promotion of Lymphocyte Proliferation by Lipoproteins—The requirement for LDL receptors in facilitating enhancement of lymphocyte responses by other lipoproteins was also examined. Reductive methylation of lipoproteins was used to differentiate LDL receptor-dependent and receptor-independent enhancement of lymphocyte growth. Preparations of VLDL + IDL were similar to LDL in their support of lymphocyte proliferation. Thus, as shown in Fig. 2, VLDL + IDL enhanced mitogen-induced lymphocyte growth, after methylation there was a significant diminution in this capacity (p < 0.01). Similar results were obtained with VLDL + IDL fractions isolated from three individual donors (data not shown). All VLDL + IDL preparations were also able to supply cholesterol to lovatatin-blocked lymphocytes; this was prevented by methylation (Table III). The requirement for LDL receptors in the enhancement of lymphocyte proliferation by HDL was also examined. Methylation did not alter the function of HDL.
**TABLE I**

**LDL-mediated enhancement of lymphocyte responses: effect of reductive methylation**

PBM were incubated in serum-free medium, with or without PHA, transferrin, and lipoproteins in varying concentrations as indicated. After 4 days, lymphocyte DNA synthesis was measured by the incorporation of \(^{[3]H}\) thymidine. Unstimulated cultures incorporated <400 cpm. Results are mean ± S.E. of triplicate determinations. Optimal stimulation observed in cultures supported by 1% human serum was a mean (± S.E.) cpm of 243,900 ± 16,700 (n = 6).

| Lipoprotein | PHA-induced lymphocyte | [3H]Thymidine incorporation |
|-------------|------------------------|-----------------------------|
|             | Experiment 1 | Experiment 2 |
|             | Medium | Transferrin | Medium | Transferrin |
| μg cholesterol/ml | | | | |
| Nil | | | | |
| LDL | 6.8 ± 0.7 | 64.0 ± 4.3 | 9.5 ± 0.3 | 90.0 ± 3.4 |
| 50 | 8.6 ± 0.3 | 150.7 ± 3.6 | 10.7 ± 0.5 | 138.5 ± 3.5 |
| 100 | 10.5 ± 0.9 | 176.0 ± 11.6 | 12.3 ± 1.9 | 252.4 ± 39.0 |
| 5 | 9.5 ± 0.1 | 238.9 ± 3.4 | 12.9 ± 0.9 | 348.0 ± 2.8 |
| 10 | 9.8 ± 0.5 | 235.2 ± 9.7 | 13.9 ± 1.4 | 338.1 ± 2.6 |
| 50 | 6.4 ± 0.4 | 64.8 ± 2.1 | 7.1 ± 0.3 | 73.0 ± 2.4 |
| 100 | 6.7 ± 0.2 | 70.9 ± 2.3 | 12.3 ± 0.5 | 72.3 ± 3.4 |
| 5 | 6.9 ± 0.1 | 121.7 ± 17.8 | 9.4 ± 0.1 | 140.9 ± 8.2 |
| 10 | 138.1 ± 13.0 | 4.8 ± 0.7 | 215.4 ± 14.7 |

**TABLE II**

**Provision of cholesterol by LDL: inhibition by reductive methylation**

PBM were incubated in medium supplemented with 1% lipoprotein-poor plasma and with or without PHA, lovastatin (0.5 μM), and lipoproteins in varying concentrations as indicated. After 4 days, lymphocyte DNA synthesis was measured by the incorporation of \(^{[3]H}\) thymidine. Unstimulated cultures incorporated <400 cpm. Results are mean ± S.E. of triplicate determinations.

| Lipoprotein | μg cholesterol/ml | PHA-induced lymphocyte | [3H] Thymidine incorporation |
|-------------|-------------------|------------------------|-----------------------------|
| Nil         | 150.0 ± 2.8       | 9.2 ± 0.6             (94) |
| LDL         | 5 158.1 ± 2.3     | 154.3 ± 2.7           (2)  |
| Methyl-LDL  | 133.3 ± 8.3       | 23.7 ± 2.1             (82) |
| 50          | 127.8 ± 0.9       | 26.0 ± 2.6             (80) |
| 100         | 140.3 ± 1.2       | 28.9 ± 1.1             (79) |
| 100         | 149.8 ± 2.0       | 31.2 ± 0.9             (79) |

**TABLE III**

**Provision of cholesterol by VLDL + IDL: inhibition by reductive methylation**

PBM were incubated in medium supplemented with 1% lipoprotein-poor plasma and with or without PHA, lovastatin (0.5 μM) and lipoproteins (50 μg cholesterol/ml) as indicated. After 4 days, lymphocyte DNA synthesis was measured by the incorporation of \(^{[3]H}\) thymidine. Unstimulated cultures incorporated <300 cpm. Results are mean ± S.E. of triplicate determinations.

| Lipoprotein | PHA-induced lymphocyte | [3H] Thymidine incorporation |
|-------------|------------------------|-----------------------------|
| Nil         | 119.1 ± 9.4           | 11.7 ± 0.2                 (90) |
| VLDL + IDL  | 109.1 ± 6.3           | 130.0 ± 2.2                (6)  |
| Methyl-VLDL + IDL | 106.5 ± 1.6 | 18.9 ± 0.8                (82) |
In serum-free medium supplemented with transferrin and lipoproteins as indicated. After 7 days, lymphocyte proliferation was quantitated. Unstimulated cultures contained 11,700 ± 100 cells (HDL₃) after the 7 day incubation. Results are mean ± S.E. of triplicate determinations.

**Table IV**
Variable regulation of sterol synthesis by different lipoprotein preparations

PBM were cultured in medium with or without PHA and lipoproteins (50 μg cholesterol/ml) as indicated for 24 h before measurement of rates of sterol synthesis by the incorporation of [1-14C]acetate into digitonin-precipitable sterols. Results are mean ± S.E. of triplicate determinations. ND = not done.

| Stimulus | Lipoprotein | Experiment 1 | Experiment 2 | Experiment 3 |
|----------|-------------|--------------|--------------|--------------|
| Nil      | Nil         | 4.6 ± 0.6    | 11.8 ± 0.3   | 8.7 ± 1.3    |
| PHA      | VLDL        | 290.2 ± 9.3  | 301.7 ± 10.2 | 202.7 ± 8.5  |
|          | LDL + IDL   | 138.2 ± 0.6  | ND           | ND           |
|          | LDL         | 105.2 ± 1.8  | 141.3 ± 5.2  | 51.8 ± 6.5   |
|          | HDL₃        | ND           | ND           | 272.0 ± 12.5 |

(Fig. 3). HDL₃, unlike other lipoprotein fractions, was unable to provide cholesterol to lovastatin-blocked lymphocytes (8 and data not shown). These observations suggested that lipoproteins could support lymphocyte proliferation by both LDL receptor-dependent and independent mechanisms. Moreover, the data obtained with HDL₃ supported the conclusion that an interaction with LDL receptors was not essential for this function.

In order to confirm that HDL₃ was unable to interact with lymphocyte LDL receptors, the ability of this lipoprotein subfraction to regulate lymphocyte sterol synthesis in serum-free cultures was examined. As shown in Table IV, rates of sterol synthesis in unstimulated PBM cultured in serum-free medium were low and increased markedly with PHA stimulation. When LDL was added to PHA-stimulated cells, rates of sterol synthesis were decreased by 64 ± 6% (mean ± S.E., n = 3). VLDL + IDL preparations were also able to regulate cholesterol synthesis, HDL₃, in contrast, did not inhibit sterol biosynthetic rates. These results confirm that HDL₃ could not interact with LDL receptors and thereby regulate sterol synthesis, although HDL₃ was able to support lymphocyte proliferation in serum-free medium.

**HD₃ and High Concentrations of LDL Promote Growth of LDL Receptor-negative Lymphocytes—**The next experiments examined the requirement for functional LDL receptors in lipoprotein-mediated promotion of lymphocyte growth by utilization of cells isolated from patients with LDL receptor-negative familial hypercholesterolemia (1, 3). In the absence of lipoproteins, mitogen-stimulated proliferation of normal and familial hypercholesterolemia lymphocytes cultured in medium supplemented with transferrin was minimal (Table V). The addition of low concentrations of LDL (5–10 μg of cholesterol/ml) markedly enhanced proliferation of normal lymphocytes, whereas growth of familial hypercholesterolemia lymphocytes was not altered. Similar results were obtained with three different LDL preparations (data not shown). In contrast, the addition of low concentrations of HDL₃ promoted the growth of both normal and familial hypercholesterolemia lymphocytes comparably. When higher concentrations of LDL were added, proliferation of familial hypercholesterolemia lymphocytes was also observed. However, even at high concentrations, enhancement of familial hypercholesterolemia lymphocyte proliferation by LDL was significantly less than that observed with HDL₃. These results indicate that LDL receptors markedly facilitate the enhancement of mitogen-induced lymphocyte proliferation by low concentrations of LDL but are not necessary for growth promotion by HDL₃ and high concentrations of LDL.

**Fatty Acids Enhance DNA Synthesis of Both Normal and Familial Hypercholesterolemia Lymphocytes—**Previous experiments have indicated that the non-sterol lipids provided by lipoproteins are fatty acid(s) (5). To examine the specificity of the fatty acids, various ones were evaluated for the capacity to support lymphocyte responses. When added as individual fatty acids, both oleic (18:1) and linoleic (18:2) acids (5 μM) enhanced lymphocyte DNA synthesis measured after 4 days, whereas identical concentrations of linolenic (18:3) and stearic (18:0) acids were much less effective (Experiment 1, Table VI). Similar results were obtained when lymphocyte proliferation was measured after 7 days (data not shown). In other experiments, palmitic acid (16:0) was found to be equivalent to stearic acid and provided only minimal growth support, whereas arachidonic acid (20:4) inhibited responses (data not shown). Thus, non-esterified fatty acids differed markedly in their ability to enhance mitogen-stimulated lymphocyte proliferation, with oleic and linoleic acids being the most effective. To determine whether oleic and/or linoleic acid could also support the growth of familial hypercholesteroliemia lymphocytes, preparations of LDL were incubated in medium with cholesterol (50,000/microwell) were incubated in serum-free medium supplemented with transferrin and lipoproteins as indicated. After 7 days, lymphocyte proliferation was quantitated. Unstimulated cultures contained 11,700 ± 100 cells (HDL₃) after the 7 day incubation. Results are mean ± S.E. of triplicate determinations.

**Table V**
HDL₃ promotes growth of LDL receptor-negative lymphocytes

PBM (50,000 cells/microwell) from normal controls or patients with LDL receptor-negative homozygous familial hypercholesterolemia (FH) were incubated in serum-free medium with transferrin and with or without PHA and lipoproteins in varying concentrations as indicated. Mitogen-induced lymphocyte proliferation was measured after 7 days by counting cells. Unstimulated cultures always contained <30,000 cells after 7 days. Results are mean ± S.E. of triplicate determinations.
Bovine serum albumin (7.5 e200 cDm. Results are mean

Experiment 2). ND = not done.

| Fatty acid source | Non-esterified fatty acid conc. | PHA-induced lymphocyte DNA synthesis |
|-------------------|--------------------------------|-------------------------------------|
|                   | µM         | Medium | Transferrin |
| Experiment 1      |            |        |             |
| Nil               | 8.6 ± 2.0 | 81.3 ± 13.7 |
| Oleic acid        | 5          | 13.1 ± 1.9 | 150.9 ± 11.7 |
| Linolenic acid (18:2) | 5      | 13.5 ± 2.3 | 128.6 ± 13.3 |
| Linolenic acid (18:3) | 5      | 8.6 ± 1.1 | 93.4 ± 6.6 |
| Stearic acid (18:0) | 5        | 4.2 ± 1.2 | 87.3 ± 13.8 |
| Experiment 2      |            |        |             |
| Nil               |           | ND     | 46.6 ± 2.6 |
| Oleic acid        | 5          | ND     | 114.7 ± 6.2 |
| LDL (50 µg cholesterol/ml) | 0.6 | ND     | 188.9 ± 4.4 |
| LDL + oleic acid  | 0.6 + 5    | ND     | 222.4 ± 4.5 |
| LDL + oleic acid  | 0.6 + 50   | ND     | 234.4 ± 11.9 |
| LDL (100 µg cholesterol/ml) | 1.2 | ND     | 232.9 ± 12.3 |

TABLE VII

Oleic and linoleic acid promote responsiveness of LDL receptor-negative lymphocytes

PBM were incubated in serum-free medium with or without PHA and transferrin as indicated. All cultures were supplemented with bovine serum albumin (7.5 µM), the fatty acid content of which was insufficient to support lymphocyte DNA synthesis. Fatty acids (5 µM) or LDL (50 µg cholesterol/ml) were added directly to cultures. After 4 days, mitogen-induced lymphocyte DNA synthesis was measured by [%H]thymidine incorporation. Unstimulated cultures incorporated <200 cpm. Results are mean ± SE of triplicate determinations. FH, familial hypercholesterolemia.

| Addition          | PHA-induced lymphocyte DNA Synthesis |
|-------------------|-------------------------------------|
|                   | Normal Cells | PBM Cells |
|                   | Control | Transferrin | Control | Transferrin |
|                   | cpm x 10^3 |             |         |             |
| Nil               | 2.6 ± 0.2 | 87.9 ± 3.3 | 2.8 ± 0.1 | 86.6 ± 2.5 |
| LDL               | 4.1 ± 0.1 | 272.1 ± 21.3 | 2.9 ± 0.1 | 113.2 ± 4.1 |
| Linoleic acid     | 3.8 ± 0.2 | 236.8 ± 9.7 | 4.0 ± 0.2 | 253.2 ± 6.4 |
| Oleic acid        | 3.5 ± 0.1 | 262.5 ± 3.7 | 4.3 ± 0.2 | 285.8 ± 37.1 |

Bovine serum albumin (BSA, 7.5 and 75 µM) was added directly to all cultures in experiments 1 (7.5 µM) and 2 (75 µM) contained insufficient fatty acids to support lymphocyte DNA synthesis. After 4 days, lymphocyte DNA synthesis was measured by the incorporation of [%H]thymidine. Results are mean ± S.E. of six separate assays (experiment 1) and of triplicate determinations (experiment 2). ND = not done.

More Effectively

| Fatty acid source | Non-esterified fatty acid conc. | PHA-induced lymphocyte DNA synthesis |
|-------------------|--------------------------------|-------------------------------------|
|                   | µM         | Medium | Transferrin |
| Experiment 1      |            |        |             |
| Nil               | 10.6 ± 1.2 | 18.4 ± 1.9 |
| LDL               | 0.6        | 25.3 ± 2.2 | 340.2 ± 4.2 |
| BSA 7.5 µM        | 6.0        | 18.3 ± 1.8 | 80.3 ± 6.7 |
| BSA 75 µM         | 60.0       | 22.2 ± 1.6 | 126.4 ± 15.9 |
| Experiment 2      |            |        |             |
| Nil               | 22.0 ± 3.7 | 19.3 ± 2.9 |
| VLDL + IDL        | 3.0        | 203.5 ± 18.1 |
| HDLz              | 8.0        | 215.7 ± 13.0 |
| BSA 7.5 µM        | 6.0        | 12.5 ± 0.4 | 53.7 ± 7.2 |
| BSA 75 µM         | 60.0       | 11.3 ± 0.7 | 107.3 ± 12.1 |
| Experiment 3      |            |        |             |
| Nil               | 9.0 ± 1.1  | 13.8 ± 2.1 |
| VLDL + IDL        | 3.0        | 178.5 ± 11.5 |
| BSA 7.5 µM        | 36.0       | 22.4 ± 1.1 | 85.0 ± 7.5 |
| BSA 75 µM         | 360.0      | 141.5 ± 5.0 | 161.9 ± 10.7 |

- Lipoproteins enhance lymphocyte proliferation more effectively than albumin

PBM were incubated in serum-free medium with or without PHA, transferrin, lipoproteins (50 µg of cholesterol/ml) and bovine serum albumin (BSA, 7.5 and 75 µM) as indicated. Fatty acid/albumin molar ratios were 0.8:1 (experiments 1 and 2) and 4.8:1 (experiment 3). The oleic acid/albumin molar ratio was 0.35:1 (experiment 3). After 7 days, lymphocyte proliferation was quantified. Unstimulated cultures contained 34,000 ± 1,700 (experiment 1), 21,600 ± 3,000 (experiment 2), and 17,800 ± 800 (experiment 3) cells/well after the 7 day incubation. Results are mean ± S.E. of triplicate determinations.

| Fatty acid source | Non-esterified fatty acid conc. | PHA-stimulated lymphocyte proliferation |
|-------------------|--------------------------------|----------------------------------------|
|                   | µM         | Control | Transferrin |
| Experiment 1      |            |         |             |
| Nil               | 10.6 ± 1.2 | 18.4 ± 1.9 |
| LDL               | 0.6        | 25.3 ± 2.2 | 340.2 ± 4.2 |
| BSA 7.5 µM        | 6.0        | 18.3 ± 1.8 | 80.3 ± 6.7 |
| BSA 75 µM         | 60.0       | 22.2 ± 1.6 | 126.4 ± 15.9 |
| Experiment 2      |            |         |             |
| Nil               | 22.0 ± 3.7 | 19.3 ± 2.9 |
| VLDL + IDL        | 3.0        | 203.5 ± 18.1 |
| HDLz              | 8.0        | 215.7 ± 13.0 |
| BSA 7.5 µM        | 6.0        | 12.5 ± 0.4 | 53.7 ± 7.2 |
| BSA 75 µM         | 60.0       | 11.3 ± 0.7 | 107.3 ± 12.1 |
| Experiment 3      |            |         |             |
| Nil               | 9.0 ± 1.1  | 13.8 ± 2.1 |
| VLDL + IDL        | 3.0        | 178.5 ± 11.5 |
| BSA 7.5 µM        | 36.0       | 22.4 ± 1.1 | 85.0 ± 7.5 |
| BSA 75 µM         | 360.0      | 141.5 ± 5.0 | 161.9 ± 10.7 |

- Lipoproteins enhance lymphocyte proliferation more effectively than albumin

Whether fatty acids necessary for optimal lymphocyte proliferation are likely to be provided by albumin or by lipoproteins was next examined. For these experiments, commercial preparations of bovine serum albumin containing physiological and supraphysiological molar ratios of fatty acid/albumin (0.8:1 and 4.8:1) were employed (15). Both fatty acid-containing albumin and lipoproteins were able to increase lymphocyte DNA synthesis (data not shown) and proliferation (Table VIII). However, when compared on a molar basis, non-esterified fatty acids bound to albumin were less effective than fatty acids in lipoproteins at enhancing lymphocyte responses. Similar results were obtained when fatty acid-containing human serum albumin was compared with lipoproteins (data not shown). The content of individual fatty acids in BSA was measured to ensure that the lack of effect of the commercial BSA preparation was not the result of a low concentration of oleic and linoleic acid. The BSA lot used in Experiment 3, Table VIII contained both oleic acid (0.35:1 oleic acid/albumin molar ratio) and linoleic acid (0.8:1 molar ratio). These findings support the conclusion that lipoproteins are more effective than fatty acid-containing albumin at promoting growth of lymphocytes, even when the albumin contains more than physiologically attainable concentrations of fatty acid.

DISCUSSION

Optimal proliferation of normal human lymphocytes stimulated by mitogens can be obtained when cells are cultured in...
serum-free medium supplemented with transferrin and lipoproteins (5). Transferrin, the iron-transporting serum glycoprotein, is necessary for DNA synthesis and thus required for continuing proliferation (5, 12, 13). When added to transferrin-containing medium, lipoproteins completely replace the requirement for serum and support optimal lymphocyte responses (5). Neither exogenous cholesterol nor cholesteryl ester is able to enhance lymphocyte responses, whereas phospholipid, triglyceride, and the fatty acids, oleic and linoleic, promote maximal mitogen-induced lymphocyte DNA synthesis (5). Furthermore, LDL depleted of cholesteryl ester by heptane extraction retains the capacity to support growth (5). Thus, the growth-promoting activity of lipoproteins does not result from the provision of protein or cholesterol but rather may result from the ability of lipoproteins to provide fatty acids (5).

Normal peripheral blood lymphocytes have been shown to express functional receptors for LDL (6), but the requirement for these receptors in the support of optimal lymphocyte responses by lipoproteins has not been established. In the current studies, two different methods were used to investigate the requirement for LDL receptors. The first approach was that of chemical modification of the apolipoprotein in order to prevent recognition by and binding to the LDL receptor (9). The studies reported here confirm the previous finding that modification of apolipoproteins by reductive methylation interferes with LDL receptor-mediated provision of cholesterol to lymphocytes by lipoproteins (8). The modest increase in DNA synthesis by lovastatin-blocked normal lymphocytes with the addition of methyl-LDL was equivalent to that observed in cultures of lovastatin-blocked LDL receptor-negative familial hypercholesterolemia lymphocytes upon the addition of native LDL (6). Therefore, the minimal increase in [3H]thymidine incorporation observed is secondary to nonreceptor-mediated delivery of cholesterol. Similarly, continuing proliferation of lovastatin-blocked normal lymphocytes, measured after prolonged culture, was not supported by methyl-LDL. The small increase in the number of cultured lymphocytes present after a 7-day incubation with the highest concentration of methyl-LDL could be accounted for by LDL receptor-independent provision of cholesterol, since it was equal to that observed with the addition of native LDL to lovastatin-blocked LDL receptor-negative familial hypercholesterolemia lymphocytes (6). These findings indicate that reductive methylation completely prevented both LDL receptor-dependent lymphocyte DNA synthesis and LDL receptor-dependent lymphocyte proliferation. Methylation of LDL, thus effectively blocked LDL receptor-dependent uptake of lipoproteins and thereby provided a means to assess the role of LDL receptors in the provision of fatty acids to lymphocytes.

When LDL apolipoproteins were modified by reductive methylation, enhancement of lymphocyte proliferation in serum-free cultures was significantly decreased, indicating that support of lymphocyte growth was similar to provision of cholesterol in that it was dependent on LDL receptor-mediated processes. The acceptance of this conclusion is dependent on the demonstration that methylation completely prevented receptor-mediated uptake, as noted above, and that the lack of effectiveness of methyl-LDL in this system did not result from a nonspecific alteration of the lipoprotein but rather was related to blocking an interaction with the LDL receptor. Indirect evidence indicates that nonspecific alteration of the LDL did not account for the findings. Thus, methyl-LDL had no significant effect on DNA synthesis and proliferation of mitogen-stimulated lymphocytes cultured in lipoprotein-poor plasma without lovastatin. Furthermore, methyl-LDL did not inhibit responses supported by unmodified LDL (data not shown). Moreover, methylation of other lipoproteins such as HDL did not alter their capacity to enhance lymphocyte proliferation, additionally indicating that the process of reductive methylation did not result in nonspecific inhibitory effects. The second experimental approach used to examine the role of LDL receptors avoided any potential nonspecific effects of methylation by comparing normal and LDL receptor-negative familial hypercholesterolemia lymphocytes. Low concentrations of both LDL and HDL substantially increased the proliferation of normal lymphocytes, whereas only HDL and high concentrations of LDL were able to enhance the growth of mitogen-stimulated LDL receptor-negative familial hypercholesterolemia lymphocytes. The conclusion arising from this series of experiments is that lipoproteins are able to promote lymphocyte proliferation by both LDL receptor-mediated and receptor-independent mechanisms.

The mechanism of LDL receptor-mediated provision of cholesterol is well established (1-4). The metabolic fate of the other lipid components of the lipoprotein particle, including phospholipids, triglyceride, and fatty acids, is not well defined. The current studies suggest that mitogen-stimulated lymphocytes cultured in serum-free medium internalize lipoproteins and release lipids from the core, since LDL regulated endogenous sterol synthesis. These experiments also suggest that following LDL receptor-mediated internalization, fatty acids in lipoproteins may become available to enhance lymphocyte responses. Alternatively, internalization of lipoproteins via the LDL receptor pathway may increase endogenous synthesis of necessary fatty acids or may allow lymphocyte growth to occur by a mechanism unrelated to fatty acids. Since LDL receptor activity increases with activation and proliferation of lymphocytes (6, 16), the potential capacity of lipoproteins to provide both fatty acids and cholesterol is also increased. The need for LDL receptors to provide cholesterol to proliferating lymphocytes may be relatively minor compared with their role in processes dependent on fatty acids, since endogenous synthesis of cholesterol is sufficient to support lymphocyte activation and proliferation when cells are cultured in medium depleted of exogenous cholesterol (6, 10, 11).

Lipoproteins also enhanced lymphocyte growth by LDL receptor-independent mechanisms. Thus, large concentrations of methyl-LDL could support the growth of normal lymphocytes, whereas large concentrations of normal LDL supported proliferation of familial hypercholesterolemia lymphocytes. Moreover, HDL supported proliferation of both normal and familial hypercholesterolemia lymphocytes. One possible mechanism by which HDL and other lipoproteins may enhance lymphocyte proliferation is by providing fatty acids or fatty acid-containing moieties by an LDL receptor-independent mechanism. Lipoprotein phospholipids may exchange with cell membrane phospholipids or be transferred to the cell membrane, depending on the relative cholesterol-phospholipid ratios of the lipoprotein and membrane, and thereby deliver fatty acids (17). Recent studies have suggested that the association of HDL with cells is not mediated by a specific receptor or unique apolipoproteins but rather involves interaction of cell membrane lipids with the surface lipids of HDL (18). Such an association may then promote transfer of lipids. It is also possible that a lipid transfer protein plays a role in the transfer of cholesteryl esters or triglycerides from lipoproteins to lymphocytes. Lipid transfer protein activity is secreted by human monocyte-derived macrophages (19). This activity may also be secreted by the mononuclear phagocytes
present in the cultures used in these studies. Supporting this possibility is the finding of mRNA for the major plasma cholesteryl ester transfer protein in spleen (20), suggesting the presence of this protein and its lipid transfer activity in activated lymphocytes or mononuclear phagocytes. Lymphocytes are known to transfer fatty acids from triglycerides to phospholipids by a process that is increased by mitogenic stimulation (21). A similar process may allow transfer of fatty acid-containing lipids from lipoproteins to lymphocytes. Thus, there exists at least two potential mechanisms for the non-receptor-mediated transfer of fatty acids in this system, either dependent on the activity of a specific lipid transfer protein or independent of such an effect. Alternatively, lipoproteins may function to support lymphocyte growth by an LDL receptor-independent mechanism that is not related to the provision of fatty acids, as discussed above for LDL.

Fatty acids circulate in plasma in a non-esterified form bound to albumin or esterified in the phospholipid, triglyceride, and cholesteryl ester of lipoproteins. The current studies demonstrate that both fatty acid-containing albumin and lipoproteins can enhance in vitro lymphocyte responses. A number of pieces of evidence indicate that the action of lipoproteins in supporting lymphocyte growth may be of physiologic relevance and more important than the effect of fatty acid-containing albumin in vivo. Thus, the lipoprotein concentrations that enhanced mitogen-induced lymphocyte proliferation in vitro are in the physiologic range. Interstitial fluid and peripheral lymph concentrations of lipoproteins have been estimated to range between 10 and 30% of the corresponding serum values (22–27). Thus, for example, the concentration of LDL in peripheral lymph is 10% of the serum concentration (23), whereas the concentrations of LDL used in these experiments are <5% of normal serum concentrations (28). Therefore, physiologically relevant concentrations of lipoproteins support optimal lymphocyte proliferation. The data also support the conclusion that fatty acids in lipoproteins may play a more important role than non-esterified fatty acids bound to albumin in enhancing lymphocyte responses in vivo. Thus, lipoproteins enhanced mitogen-induced lymphocyte proliferation more effectively than did albumin-bound fatty acids. This was particularly noteworthy when the molar concentrations of non-esterified fatty acid in lipoprotein and albumin were compared. Only at fatty acid/albumin molar ratios exceeding the maximal physiological level of 2:1 (29), was albumin-bound fatty acid able to support optimal lymphocyte responses. Increasing the albumin concentration to that observed in interstitial fluid, which is estimated to be 30% of serum levels (24), did not alter the findings (data not shown). The greater effectiveness of lipoproteins was not the result of a higher concentration of oleic acid. Thus, the concentration of oleic acid associated with 50 μg of lipoprotein cholesterol/ml was 1.1–1.5 μM, whereas that associated with 75 μM BSA was approximately 25 μM. Clearly, lipoprotein-associated oleic acid was more effective than albumin-bound oleic acid at supporting lymphocyte proliferation. Although it is difficult to relate in vitro observations directly to in vivo conditions, the data support the conclusion that lipoproteins are a more effective source of the exogenous fatty acid necessary for lymphocyte growth than albumin.

In summary, the data presented here have demonstrated that LDL, receptor-mediated interaction with lipoproteins may result in provision of fatty acids necessary for optimal proliferation of mitogen-stimulated normal lymphocytes. Lipoproteins not interacting with LDL receptors may also provide lipids other than cholesterol to both normal and LDL receptor-negative familial hypercholesterolemia lymphocytes and thereby promote growth in transferrin-containing medium. The data, therefore, indicate that both LDL receptor-mediated and LDL receptor-independent lipoprotein-cell interactions may provide fatty acids necessary for optimal lymphocyte proliferation.

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