Human plasma high-density lipoprotein-3 (HDL\textsubscript{3}) has been shown to bind to a variety of cells and tissues. To investigate the nature of HDL\textsubscript{3}-cell association, we studied the interaction of \textsuperscript{125}I-HDL\textsubscript{3} with porcine aortic endothelial cells, rabbit aortic smooth muscle cells, and normal human skin fibroblasts. At 37 °C, \textsuperscript{125}I-HDL\textsubscript{3} association with endothelial cells was nonsaturable. Furthermore, 60% protein digestion of HDL\textsubscript{3} by trypsin (T-HDL\textsubscript{3}) actually increased its ability, on a protein weight basis, to associate with endothelial cells and to displace \textsuperscript{125}I-HDL\textsubscript{3} from all three cell types. Synthetic phospholipid-cholesterol discs containing either apo-A-I or apo-A-II were equally effective in displacing \textsuperscript{125}I-HDL\textsubscript{3} from endothelial cells, and phospholipid-cholesterol vesicles containing no protein also displaced \textsuperscript{125}I-HDL\textsubscript{3} from endothelial cells. Neither lipid-free apo-HDL\textsubscript{3} nor apo-T-HDL\textsubscript{3} was able to competitively displace \textsuperscript{125}I-HDL\textsubscript{3}. The above competitive displacement data, when expressed on a protein weight basis, showed differences in the ability of the competitors to displace \textsuperscript{125}I-HDL\textsubscript{3} from cells in the following order of effectiveness: discs > T-HDL\textsubscript{3} > native HDL\textsubscript{3}. When these data were expressed on a surface lipid weight basis, all three competitors, as well as the lipid vesicles, were approximately normalized to a single competitive displacement curve. Studies on the nature of the cellular mediators of HDL\textsubscript{3}-cell association revealed that the cell surface sites were resistant to proteolytic treatment. Furthermore, both \textsuperscript{125}I-HDL\textsubscript{3} and \textsuperscript{125}I-T-HDL\textsubscript{3} association with fibroblasts preincubated with varying concentrations of cholesterol increased in parallel with the free cholesterol content of the cells; although cycloheximide blocked this increase in HDL\textsubscript{3}-cell association, cycloheximide also prevented the increase in cholesterol content of cholesterol-treated cells. We conclude that the association of HDL\textsubscript{3} with the cell types studied is not mediated by specific ligand and receptor proteins but rather involves the interaction of cellular surface lipids, possibly cholesterol, with the surface lipids of HDL\textsubscript{3}. and metabolism of HDL\textsubscript{3}, due, at least in part, to the epidemiologic evidence that plasma HDL levels are inversely correlated with coronary artery disease (1). The potential functional roles of HDL include removal of peripheral tissue cholesterol ("reverse cholesterol transport") (2), stimulation of prostaglandin synthesis (3), and support of endothelial cell growth (4). In addition, there is evidence that HDL may supply cholesterol to rat steroidogenic tissue for hormone synthesis.

Except for the apo-E-containing HDL\textsubscript{2} and HDL\textsubscript{subfractions}, which specifically bind to apo-E receptors (5), it is not known if HDL specifically binds to the tissues in question to subserv its roles. Several investigators have attempted to examine this relationship between binding and function, and there has recently appeared many published reports on HDL binding phenomena. The tissues and cells studied include human fibroblasts (6-8), endothelial cells (9), steroidogenic tissue and cells (10-12), hepatic tissue and cells (13, 14), smooth muscle cells (8), intestinal cells (15), and kidney membranes (16).

The observation of HDL binding to these tissues has led many workers to concur that there exist specific HDL receptors (10-18). However, neither a cellular receptor protein nor a specific HDL ligand has been definitively identified in any system. Therefore, we chose to characterize the interaction of HDL\textsubscript{3} with three different tissue culture cells, porcine aortic endothelial cells, rabbit smooth muscle cells, and human skin fibroblasts, with an emphasis on identifying those determinants on HDL\textsubscript{3} which might be important in this interaction. Our data strongly suggest that no specific protein ligand is involved in this interaction and also place in doubt the existence of a specific receptor protein in the cell lines studied. Rather, our results suggest that the surface lipids of both HDL\textsubscript{3} and the cell plasma membrane play an important role in the interaction.

**EXPERIMENTAL PROCEDURES**

**RESULTS**

Characterization of \textsuperscript{125}I-HDL\textsubscript{3} and Trypsinized HDL\textsubscript{3}-Cell Association—To characterize the interaction of \textsuperscript{125}I-HDL\textsubscript{3}.

1 The abbreviations used are: HDL, high-density lipoprotein; T-HDL\textsubscript{3}, trypsinized HDL\textsubscript{3}; LDL, low-density lipoprotein; apo, apoprotein; BSA, bovine serum albumin; DMEM, Dulbecco’s modified Eagle’s medium; SDS, sodium dodecyl sulfate.

2 The "Experimental Procedures" are presented in miniprint at the end of this paper. Miniprints are easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 84M-1177, cite the authors, and include a check or money order for $1.20 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
Mechanism of HDL₃-Cell Association

Fig. 1. Time and concentration dependence of ¹²⁵I-HDL₃ association with porcine aortic endothelial cells. Porcine aortic endothelial cells were incubated at 37 °C with 1 cc of serum-free DMEM containing either 50 μg/ml of ¹²⁵I-HDL₃ for the indicated time periods (left panel) or the indicated concentrations of ¹²⁵I-HDL₃ for 4 h (right panel). At the end of the incubation period the cells were assayed for total (○) and nonspecific (△) cell association as described under “Experimental Procedures.” Specific cell association (△) was calculated by subtracting the nonspecific value from the total value at each time or concentration point.

with cells, the time and concentration dependence of ¹²⁵I-HDL₃, porcine aortic endothelial-cell association were examined (Fig. 1, left and right, respectively). Both studies showed that a large percentage of the cell association was "specific" (defined as displaceability by excess cold HDL₃). Fig. 1 left shows that the cell association had an initial rapid phase and then increased more slowly. The concentration curve (Fig. 1 right) showed a steeper slope at lower HDL₃ concentrations (<50 μg/ml) than at higher concentrations. Neither the time nor concentration dependence data showed true saturability.

Camejo (35) has demonstrated that treatment of HDL with trypsin results in up to 70% hydrolysis of the HDL proteins and that the residual lipid-bound fragments contain no intact apoprotein. To determine whether the capacity of HDL₃ to associate with cells could be destroyed by such extensive apoprotein hydrolysis, we compared the ability of T-HDL₃ with that of native HDL₃ to associate with tissue culture cells. T-HDL₃ was prepared as described under "Experimental Procedures." HDL₃ and T-HDL₃ preparations were assayed for total (○) or ¹²⁵I-T-HDL₃ (△) cell association as described in the legend to Fig. 1.

Fig. 2. Sepharose CL-6B chromatography and sodium dodecyl sulfate-polyacrylamide gel electrophoresis of native HDL₃ and T-HDL₃. Native HDL₃ and T-HDL₃ were prepared as described under "Experimental Procedures." 1.3 mg of native HDL₃ (○) or 1.1 mg of T-HDL₃ (△) were loaded onto a column (1 × 112 cm) of Sepharose CL-2B equilibrated with lipoprotein buffer. 1.6-ml fractions were collected and the absorbance of each fraction at 280 nm was measured. The markers include blue dextran (Vₒ), native LDL, and phenol red (Vₗ). The inset shows nonreduced sodium dodecyl sulfate-polyacrylamide gel electrophoresis of delipidated native HDL₃ (29 μg of protein) and T-HDL₃ (20 and 80 μg of protein). See "Experimental Procedures" for details. The numbers to the right of the HDL₃ lane refer to the molecular weights of standard protein markers.

The ability of T-HDL₃ to associate with endothelial cells is demonstrated in Fig. 3. Both total and specific ¹²⁵I-T-HDL₃-cell association was greater than total and specific ¹²⁵I-HDL₃-cell association, respectively, when plotted as a function of protein concentration. To determine whether T-HDL₃ and HDL₃ shared common cell-association sites, T-HDL₃ was compared with HDL₃ in its ability to displace ¹²⁵I-HDL₃ from tissue culture cells. Fig. 4 shows that in three different cell lines, porcine endothelial cells (Fig. 4, top panel), rabbit smooth muscle cells (Fig. 4, middle panel), and human fibroblasts (Fig. 4, bottom panel), T-HDL₃ displaced ¹²⁵I-HDL₃ somewhat more effectively than native HDL₃ on a protein weight basis. Similar results were obtained whether relatively high ¹²⁵I-HDL₃ concentrations (50 μg/ml) or low concentrations (2–5 μg/ml) were used; thus, if there are separate high- and low-affinity sites for HDL₃, as suggested in earlier studies (8), both are displaced equally well by T-HDL₃. To investigate the possibility that T-HDL₃ interacts with cells in a nonspecific manner to broadly inhibit ligand-cellular interactions, the ability of T-HDL₃ to competitively inhibit the association of ¹²⁵I-LDL with cultured human fibroblasts was determined. The experiments showed no effect of T-HDL₃ on the cell association of ¹²⁵I-LDL.

Another interpretation of the above competitive displacement data is that T-HDL₃ exchanges apoproteins with ¹²⁵I-HDL₃, thus decreasing the specific activity of the latter. This...
would result in decreased cell association of $^{125}$I-HDL₃ radioactivity even if the same number of $^{125}$I-HDL₃ particles were in fact being bound. To test this possibility, T-HDL₃ and $^{125}$I-HDL₃ were preincubated for varying periods at 37 °C in the absence of cells and then exposed to cells for a relatively short time period (15 min). If there was significant apoprotein exchange, one would expect that with longer periods of preincubation, the specific activity and the apparent cell association of $^{125}$I-HDL₃ would decrease. However, as shown in Fig. 5, this was clearly not the case; the per cent inhibition of $^{125}$I-HDL₃ cell association remained constant and was independent of the time of preincubation. Although very rapid, apoprotein exchange (completed in less than 15 min) could give similar results, previous measurements of the kinetics of apoprotein exchange show that this is unlikely (36). (In the further competitive displacement experiments to be described below, employing HDL₃ analogues other than T-HDL₃, similar preincubation experiments were performed and gave the same result as that in Fig. 5.) Therefore, the data in Figs. 3 and 4 show that extensive trypsin treatment of HDL₃ actually enhances, on a protein weight basis, its ability to interact with HDL₃-cell association sites on tissue culture cells.

![Fig. 4. Displacement of $^{125}$I-HDL₃ from porcine endothelial cells, rabbit smooth muscle cells, and human fibroblasts by native HDL₃ and T-HDL₃.](image)

FIG. 4. Displacement of $^{125}$I-HDL₃ from porcine endothelial cells, rabbit smooth muscle cells, and human fibroblasts by native HDL₃ and T-HDL₃. The cells were incubated for 4 h at 37 °C with 1 cc of serum-free DMEM containing the indicated amounts of unlabelled native HDL₃ (•) or T-HDL₃ (○) plus the following amounts of $^{125}$I-HDL₃: 50 μg, endothelial cells (top panel); 25 μg, smooth muscle cells (middle panel); and, 2 μg, fibroblasts (bottom panel). $^{125}$I-HDL₃-cell association was determined as described under "Experimental Procedures" (except that $^{125}$I-HDL₃ specific activity for the fibroblast experiment was 500 cpm/ng) and expressed as per cent of control (i.e. $^{125}$I-HDL₃-cell association in the absence of unlabelled HDL₃ or T-HDL₃). Control $^{125}$I-HDL₃ cell association values are as follows: 164 ng/mg, endothelial cells; 127 ng/mg, smooth muscle cells; and 11.1 ng/mg, fibroblasts.

![Fig. 5. Effect of preincubation of T-HDL₃ with $^{125}$I-HDL₃ on the ability of T-HDL₃ to displace $^{125}$I-HDL₃ from porcine endothelial cells.](image)

FIG. 5. Effect of preincubation of T-HDL₃ with $^{125}$I-HDL₃ on the ability of T-HDL₃ to displace $^{125}$I-HDL₃ from porcine endothelial cells. Serum-free DMEM (1 cc) containing 50 μg of $^{125}$I-HDL₃ alone (control) or plus 200 μg of T-HDL₃ ("Experimental Procedures") was preincubated in the absence of cells at 37 °C for the indicated time periods and then exposed to endothelial cells for 15 min at 37 °C. $^{125}$I-HDL₃ cell association was determined as described under "Experimental Procedures." The data, expressed as per cent inhibition of $^{125}$I-HDL₃-cell association, were calculated for each preincubation time point as follows: (control value - experimental value)/control value) × 100. The control value, which did not vary by more than 10%, averaged 78.5 ng of cell-associated $^{125}$I-HDL₃/mg of cell protein.

![Fig. 6. Displacement of $^{125}$I-HDL₃ from porcine endothelial cells and rabbit smooth muscle cells by native HDL₃ and discs containing apo-A-I and apo-A-II.](image)

FIG. 6. Displacement of $^{125}$I-HDL₃ from porcine endothelial cells and rabbit smooth muscle cells by native HDL₃ and discs containing apo-A-I and apo-A-II. Endothelial cells (left panel) and smooth muscle cells (right panel) were incubated at 37 °C for 4 h with 1 cc of serum-free DMEM containing 25 μg $^{125}$I-HDL₃ alone (control) or plus the indicated amounts of unlabelled native HDL₃ (•), apo-A-I discs (○), or apo-A-II discs (△). The discs were prepared as described under "Experimental Procedures." $^{125}$I-HDL₃ cell association was determined and expressed as per cent of control cell association, which equaled 55.6 and 59.5 ng of cell-associated $^{125}$I-HDL₃/mg of cell protein for the endothelial cells and smooth muscle cells, respectively.

**Competitive Displacement of $^{125}$I-HDL₃ from Cells by Synthetic Discs and Vesicles**—The above data suggest a lack of ligand specificity in the interaction of HDL₃ with tissue culture cells. Further experiments were performed to determine if cell association of HDL₃ was mediated by specific apoproteins. Synthetic phospholipid-cholesterol discs containing either apo-A-I or apo-A-II were prepared as described under "Experimental Procedures" and their ability to displace $^{125}$I-HDL₃ from cells was tested (Fig. 6). In two different tissue culture lines, apo-A-I and apo-A-II containing discs caused similar displacement of $^{125}$I-HDL₃. Thus there is a lack of specificity in the ability of HDL₃ analogues to displace $^{125}$I-HDL₃.
vesicles were completely unable to displace lz5I-LDL from particles showed approximately equal ability to displace unlabeled HDL, or analogue rather than fold excess of protein by HDL, from porcine endothelial cells. The vesicles were almost equally effective and in their ability to displace lz5I-HDL, from the cells (Fig. 7, top panel). By contrast, the vesicles were completely unable to displace lz5I-LDL from human fibroblasts (not shown). To determine if the ability of T-HDL, and discs to displace lz5I-HDL, could be explained on the basis of lipid-lipid interactions, we replotted the data of Figs. 4 and 6 as fold excess of surface lipid weight of unlabeled HDL, or analogue rather than fold excess of protein weight (Fig. 7). Expressed in this way, all of the different particles showed approximately equal ability to displace native HDL. The considerable differences in displacement ability when expressed on a per protein basis were clearly reduced. The normalization of the data to a single displacement curve was suggested particularly strongly by the smooth muscle cell data (Fig. 7, bottom panel). Similar normalization was obtained when the direct cell association data of lz5I-HDL, and lz5I-T-HDL, (Fig. 3) was expressed on a per surface lipid weight basis (not illustrated). These results indicate that the lipids of HDL, or its analogues probably play a major role in determining cell association of HDL,.

**Cellular Determinants of HDL, Cell Association**—Having concluded that HDL, surface lipids and not a specific protein ligand mediate the interaction of HDL, with cells, we next sought to determine whether or not the cellular mediator of this interaction was a specific protein receptor. To initially address this issue, we determined if the cell surface binding sites were susceptible to proteolytic digestion. Porcine endothelial cells were treated with either trypsin or Pronase (at doses just below those found to detach the cells), and their ability to associate with lz5I-HDL, in comparison with untreated cells was examined (Fig. 8). The data show that treatment by neither enzyme affected lz5I-HDL, cell association (note that the three curves in Fig. 8 are partially superimposed). In the lz5I-LDL-fibroblast system, where a specific protein receptor-ligand interaction is known to occur, the lipoprotein-cell association is abolished by proteolytic treatment of the cells (37). Although the data in Fig. 8 do not rule out the existence of a specific HDL, receptor protein on porcine endothelial cells, since such a protein may simply be “hidden” from or resistant to the enzymes, they are consistent with the possibility that there is no HDL, receptor protein on these cells.

To further address the issue of the cellular determinants of HDL, cell association, we examined cells with “up-regulated” HDL, binding sites. Oram et al. (18) using human fibroblasts preincubated with nonlipoprotein cholesterol, and Tauber et al. (17) using bovine endothelial cells preincubated with 25-hydroxycholesterol, showed a severalfold increase in HDL, cell association with protease-treated porcine aortic endothelial cells. Endothelial cells were preincubated at 37°C for 20 min with either 1 cc of serum-free DMEM alone (control) or plus the indicated amounts of unlabeled HDL, (●) or phospholipid-cholesterol vesicles (▲; prepared as described under “Experimental Procedures”). lz5I-HDL, cell association was determined and expressed as per cent of control cell association, which equaled 106.7 ng of cell-associated lz5I-HDL;/mg of cell protein. The remaining data shown (○, T-HDL, △, apo-A-I discs; ■, apo-A-II discs) represent the data of Figs. 4 and 6 (top and middle panels) replotted on the basis of fold excess surface lipid mass. Surface lipid mass was calculated as phospholipid plus free cholesterol mass for HDL, T-HDL, and discs and as (% phospholipid + free cholesterol mass) for the small unilamellar vesicles (38) (see “Experimental Procedures” for compositional data). The control values are those indicated in the legends to Figs. 4 and 6.

**Fig. 7.** Displacement of lz5I-HDL, from porcine endothelial cells and rabbit smooth muscle cells by HDL, analogues and liposomes on the basis of surface lipid weight. Endothelial cells (top panel) were incubated at 37°C for 4 h with 1 cc of serum-free DMEM containing lz5I-HDL, (20 μg of surface lipid alone (control) or plus the indicated amounts of unlabeled native HDL, (●) or phospholipid-cholesterol vesicles (▲; prepared as described under “Experimental Procedures”). lz5I-HDL, cell association was determined and expressed as per cent of control cell association, which equaled 106.7 ng of cell-associated lz5I-HDL;/mg of cell protein. The remaining data shown (○, T-HDL, △, apo-A-I discs; ■, apo-A-II discs) represent the data of Figs. 4 and 6 (top and middle panels) replotted on the basis of fold excess surface lipid mass. Surface lipid mass was calculated as phospholipid plus free cholesterol mass for HDL, T-HDL, and discs and as (% phospholipid + free cholesterol mass) for the small unilamellar vesicles (38) (see “Experimental Procedures” for compositional data). The control values are those indicated in the legends to Figs. 4 and 6.

**Fig. 8.** Specific lz5I-HDL, association with protease-treated porcine aortic endothelial cells. Endothelial cells were preincubated at 37°C for 20 min with either 1 cc of serum-free DMEM alone (●—●), or 1 cc of medium containing 3 μg of Pronase (▲—▲), or 1 cc of DMEM containing 25 μg of trypsin (O—O). At the end of the preincubation period, the medium was removed and the cells were washed once with serum-containing DMEM and then twice with serum-free DMEM. The cells were then incubated with 1 cc of serum-free DMEM containing the indicated concentrations of lz5I-HDL, for 1 h. Values for total, nonspecific, and specific lz5I-HDL, cell association were derived as described under “Experimental Procedures,” only the specific cell association values are shown.
cellular association as compared with cells that had not been preincubated. We chose to examine the cholesterol-treated human fibroblasts because 25-hydroxycholesterol (50–100 µg/ml) was toxic to our endothelial cells. When the fibroblasts were preincubated for 24 h with nonlipoprotein cholesterol, 125I-HDL₃ cellular association was increased approximately 3-fold, as reported by Oram et al. (18). We examined the specificity of the interaction of the up-regulated cells with HDL₃ by testing the ability of these cells to interact with T-HDL₃. As shown in Fig. 9, T-HDL₃ was a better competitor than HDL₃ on a protein weight basis in its ability to displace 125I-HDL₃ from cholesterol-treated fibroblasts, similar to the results obtained with non-cholesterol-treated cells (see Fig. 4). In addition, progressive enrichment of fibroblasts with free cholesterol by incubation with increasing amounts of non-lipoprotein cholesterol led not only to an increase in 125I-HDL₃-cell association, as reported previously (18), but also to an increase in 125I-T-HDL₃ cell association (Fig. 10). Thus, up-regulated binding sites of cholesterol-treated fibroblasts show the same lack of specificity as the basal-state binding sites of nontreated cells and thus are probably not specific receptor proteins. In addition, the direct correlation between the free cholesterol content of fibroblasts, 90% of which is in the plasma membrane (39), and their ability to associate with 125I-HDL₃ and 125I-T-HDL₃ may indicate that cell surface cholesterol is an important determinant of HDL₃ cell interaction, direct interaction of HDL₃ with cell surface cholesterol may explain why cholesterol-treated cells bind more HDL₃.

Oram et al. (18) have shown that the protein synthesis inhibitor cycloheximide prevented the increased cell association of 125I-HDL₃ with cholesterol-treated fibroblasts. Since we have suggested that the mechanism of increased 125I-HDL₃ cell association with cholesterol-treated cells is related to cholesterol enrichment of the plasma membrane and not to up-regulation of a receptor protein, we sought to determine if the effect of cycloheximide might be due to prevention of the increase in free cholesterol content of cholesterol-treated cells. The data in Table I demonstrate this point; in parallel with cycloheximide's preventing the increased 125I-HDL₃-cell association with cholesterol-treated cells, cycloheximide also prevented the increase in the cells' free cholesterol content, which, as mentioned above, largely represents plasma membrane cholesterol (39). Thus, the prevention by cycloheximide of the increased 125I-HDL₃-cell association with cholesterol-treated fibroblasts does not necessarily indicate that synthesis of a protein receptor is necessary for up-regulation but rather suggests, together with the previous data, that the cell surface cholesterol content is a major determinant of HDL₃ cell interaction.

**Mechanism of HDL₃-Cell Association**

**Fig. 9.** Displacement of 125I-HDL₃ from cholesterol-treated human fibroblasts by native HDL₃ and T-HDL₃. Fibroblasts were preincubated at 37°C for 24 h in 2 cc of serum-free DMEM containing 100 µg of cholesterol (10 µl of a 10 mg/ml stock solution in ethanol). At the end of the preincubation period, the cells were washed three times in serum-free DMEM and then incubated at 37°C for 4 h with 1 cc of serum-free DMEM containing 2 µg of 125I-HDL₃ (500 cpm/ng) alone (control) or plus the indicated amounts of unlabeled HDL₃ (O) or T-HDL₃ (O). 125I-HDL₃-cell association was determined as described under "Experimental Procedures." The data is expressed as per cent of control cell association, which equaled 45.3 ng of cell-associated 125I-HDL₃/mg of cell protein.

**Fig. 10.** Free cholesterol content and 125I-HDL₃ and 125I-T-HDL₃ association with cholesterol-treated fibroblasts. Fibroblasts were preincubated for 24 h with the indicated concentrations of cholesterol as described in the legend to Fig. 7. Some plates (A) were then washed, lipid-extracted, and assayed for free cholesterol content as described under "Experimental Procedures." Under all conditions, the free cholesterol content of the cells represented greater than 90% of the total cholesterol content. Other plates were incubated for 1 h with either 5 µg/ml 125I-HDL₃ (O) or 125I-T-HDL₃ (O) and assayed for total cell association.

**DISCUSSION**

We have concluded by using both direct cell association and competitive displacement experiments with HDL₃, trypsinized HDL₃, synthetic discs, and lipid vesicles that the interaction of HDL₃ with certain tissue culture cells is mediated not by a specific protein ligand but rather by HDL₃ surface lipids which probably interact with cellular surface lipids.

The experiments in this study were performed at 37°C and therefore potentially measure both surface binding and internalization of HDL₃. However, other investigators, using similar cell lines to those studied here, report very low levels of HDL₃ internalization (6, 8, 9). For instance, both Tauber et al. (9) working with endothelial cells and Biesbroeck et al. (8) working with fibroblasts, found that less than 25% of cell-associated 125I-HDL₃ was internalized in 4–6 h at 37°C. Even if a significant fraction of the cell-associated 125I-HDL₃ were internalized, it is likely that the ability of a given analogue to competitively inhibit such internalization would be related to its ability to competitively inhibit the prior step of surface binding. These considerations indicate that valid conclusions
about surface binding can be drawn from our cell association studies.

Our data show that the interaction of HDL₃ with certain cultured cells probably does not involve the recognition of a specific ligand site. This conclusion was partially drawn from the T-HDL₃ data, which shows that T-HDL₃ was more effective, on a protein weight basis, than native HDL in associating with cells (Fig. 3) and in displacing ¹²⁵I-T-HDL₃ from cells (Figs. 4 and 10). If a specific HDL protein ligand did exist, it would very probably be destroyed or modified by the extensive trypsin treatment. Also, the equal efficiency of A-I and A-II discs in displacing HDL₃ indicates nonspecificity of the HDL ligand. It might be argued that a specific ligand site resides in the T-HDL₃ core peptides, perhaps a region of peptides with similar properties of A-I and A-II. However, since these core peptides probably represent the lipid-associated regions of the HDL₃ apoproteins (35), it seems unlikely that the binding site of a specific protein ligand would be buried in the hydrophobic domain of the HDL particle where it would be inaccessible to a cellular receptor. Our data showing that lipid vesicles without any protein can displace ¹²⁵I-HDL₃ from cells, whereas lipid-free apo-HDL₃ or apo-T-HDL₃ cannot displace ¹²⁵I-HDL₃ from cells, further support the conclusion that a specific ligand protein does not mediate HDL cellular interaction in our cell culture systems.

Rather than the HDL₃ apoproteins playing a major role in HDL₃ cellular interaction, the fact that our binding and competitive displacement data were approximately normalized when expressed on a per surface lipid basis provides positive evidence that the HDL₃ surface lipids subserve the major part of this role. The exact nature of the cell surface elements with which the HDL₃ surface lipids interact has not been definitively determined. The nonsaturability (Fig. 1) and the protease-resistance of the cellular sites (Fig. 8) suggest that a specific protein receptor is not involved. Our data (Fig. 9 and Table I) and that of Oram et al. (18), which show a direct correlation between the free cholesterol content of the cell (and thus of the plasma membrane (39)) and the ability of the cell to associate with HDL₃, may indicate that membrane cholesterol is an important determinant of HDL₃ cell association. HDL₃ may interact directly with cholesterol-rich patches in the cell membrane, or the plasma membrane cholesterol content may influence the arrangement of other cell surface lipids in a way to optimize their interaction with HDL₃ surface lipids.

The data from several previous studies also can be interpreted to show that specific HDL₃ receptor and ligand proteins do not exist. First, other studies, like ours, show nonsaturability of HDL₃ cellular association at 37 °C (9). Second, Miller et al. (6) demonstrated that HDL uptake in human fibroblasts could be accounted for solely by nonspecific adsorptive endocytosis and fluid pinocytosis without invoking specific receptor-mediated endocytosis. Third, although we were the first to show the protease-resistant nature of HDL₃ cellular interactions with endothelial cells, others have shown protease-resistant HDL association in a variety of other tissues (6, 7, 11, 13, 19, 20). Last, both Tauber et al. (9) working with HDL₃ and endothelial cells, and Jackson et al. (20), studying the interaction of apo-A-I and apo-A-II discs with ascites cells and erythrocytes, described cellular bound particles which were neither internalized nor removed by trypsin treatment. These data can be interpreted as suggesting non-protein-mediated cell surface binding.

Other investigations of HDL binding phenomena have led to the conclusion that specific HDL receptor and ligand proteins do exist. Tauber et al. (17) working with endothelial cells, and Oram et al. (18) working with fibroblasts, demonstrated that cycloheximide blocked the increase in ¹²⁵I-HDL₃-cell association that occurred with sterol treatment of the cells. Although the authors concluded from these data that up-regulation required the synthesis of a protein receptor, we believe these data indicate, in concert with our other data, that up-regulation requires cholesterol enrichment of the cell (specifically the cell membrane) since cycloheximide prevents the increase in the free cholesterol content of cholesterol-treated cells (Table I). Second, several investigators have demonstrated saturability of HDL₃ binding at 4 °C (8, 9, 13); however, the cell association became non-saturable at 37 °C (9). The only systems shown to have saturable ¹²⁵I-HDL₃-cell association at 37 °C are steroidogenic tissues (11); the interaction of HDL₃ with these tissues may involve different mediators from those in the cells we studied (see below).

There have been several abstract reports in which the authors have concluded that the HDL-tissue interaction involves specific HDL ligands. Brinton et al. (23) reported that albumin-containing liposomes competed less efficiently than native HDL₃ in displacing ¹²⁵I-HDL₃ from fibroblasts. However, they also found that apo-A-I and apo-A-II liposomes could both effectively displace ¹²⁵I-HDL₃ from cells. Their report that tetraniromethane treatment of HDL₃ or apo-A-I liposomes abolished their binding, whereas treatment with other protein-modifying agents did not, may be difficult to interpret since we have subsequently found that tetraniromethane causes apoprotein aggregation.⁴

In summary, we conclude that the nonsaturability, the protease resistance, and the nonspecificity of HDL₃ cell as-

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⁴ I. Tabas and A. R. Tall, unpublished observations.
association in our cell culture systems indicate that the mediators of this cell association do not include specific receptor and ligand proteins. Rather, based on data showing both excellent displacement of $^{125}$I-HDL$_3$ by lipid vesicles (and poor displacement by lipid-free apoproteins), equalization of competitive displacement abilities of HDL$_3$-analogues on a per surface lipid basis, and a positive correlation between cell cholesterol content and HDL$_3$-cell association, we conclude that the surface lipids of HDL$_3$, probably interacting with cell surface lipid, possibly cholesterol, are the major mediators of HDL$_3$-cellular association in our cell culture systems.

Our results do not exclude a relatively nonspecific role of the apoproteins in HDL$_3$-cellular interactions in addition to the role of the surface lipids. For example, the HDL apoproteins, known to have prominent hydrophobic regions and lipid-binding properties (for review, see Ref. 40), may directly interact with cell surface lipids. Similarly, it is possible that cell surface proteins may play a relatively minor, nonspecific role in interacting with the surface of HDL$_3$. These possibilities may explain previous data showing that trypsin treatment of cells to which HDL$_3$ has been bound release a portion of the HDL$_3$ (9, 18). That trypsin reassemblability of a bound lipoprotein does not necessarily indicate specific protein receptor-ligand interaction was demonstrated by Brown et al. (41) who showed that trypsin could release $^{125}$I-LDL, which had been bound to plastic Petri dishes that contained no cells.

The physiological significance of our conclusions about the mediators of HDL-cellular association remains to be determined. It is possible that lipid-lipid interactions mediate such proposed HDL-related processes as cellular cholesterol removal and HDL cholesterol ester delivery. In particular, Biersboeck et al. (8) have suggested that HDL$_3$ binding to cells is important in cellular cholesterol removal. Furthermore, Philips et al. (42) have provided evidence that the diffusion of cholesterol through the cell's large unstirred water layer to the acceptor may be a rate-limiting step in cellular cholesterol removal. Thus, the binding of cholesterol acceptors (such as HDL$_3$) to cells could increase the rate of cellular cholesterol removal by increasing the penetration of the acceptor into the cell's water layer. These workers also showed that the ability of different recombinant HDL particles to remove cholesterol from tissue culture cells could be normalized on the basis of external surface area (Fig. 6 in Ref. 43). A possible interpretation of this observation, in view of our data (Fig. 7), is that surface lipids of the particles mediate their interaction with the cells, and that this interaction, in turn, facilitates cellular cholesterol removal. If HDL$_3$ binding is, in fact, important in cellular cholesterol removal, and if HDL$_3$ interacts with cell surface cholesterol, as we have suggested (see above), then an important mechanism in reverse cholesterol transport from cholesterol-loaded cells may involve increased HDL$_3$ binding to the cholesterol-enriched surface of these cells. If lipid-lipid interactions were physiologically important in the case of HDL cholesterol or cholesterol ester delivery, one wonders how much specificity such a process would require and what, in the absence of a specific protein receptor-ligand system, would confer such specificity. Bamberger et al. (44) have shown that HDL cholesterol delivery to hepatoma cells is mediated by hepatic lipase, probably secondary to hydrolysis of HDL surface lipids. Therefore, while the surface lipids of HDL could potentially bind to the surface lipids of all cells, perhaps only those cells with a specific mediator molecule, such as a lipase, would subsequently interact with the bound HDL in a physiologically relevant manner. Such a molecule could therefore confer a certain degree of specificity to these types of HDL-tissue interactions.

Although we have examined in this study three different cell types from three different species, it is possible that the association of HDL with other cells and tissues is, in fact, mediated by a specific receptor-ligand protein system. In particular, several steroidogenic tissues show saturable HDL association at 37°C (11), hormone-mediated increases in HDL binding (10, 11, 19), relative ligand specificity (14), and selective HDL uptake (45). However, even in these systems no specific receptor or ligand proteins have been definitively identified, and in several cases the binding has been shown to be protease-resistant (11, 19). Thus, either a specific protein receptor-ligand system in steroidogenic tissues exists and has not yet been identified, or some mechanism other than a specific receptor and ligand confers selectivity to this particular HDL-tissue association. Interestingly, adrenal tissue has been shown to possess a lipase and thus may selectively interact with HDL in a similar manner to that proposed above.

The properties of HDL-cell association are markedly different from those of LDL-cell association, which is mediated by a specific ligand-receptor interaction: LDL-cell association shows saturability, specificity, and cellular protease-sensitivity (37). In addition, several investigators have tested the ability of trypsined LDL to interact with fibroblasts (46, 47). In these studies, trypsin removal only 20% of the LDL protein instead of the 60% removed from HDL in our study, and gel electrophoresis of the trypsined LDL revealed fragments larger than 70,000 daltons (46). Even with this comparatively limited proteolysis, the trypsined LDL still did not displace $^{125}$I-LDL from fibroblasts as well as native LDL on a protein weight basis (46) in contrast to our results where T-HDL$_3$ was a better competitive displacer than native HDL$_3$.

Much of our knowledge of lipoprotein metabolism has come from the studies of the LDL-fibroblast system; the identified cellular protein receptor has been the subject of numerous and fruitful investigations (37, 48) into the function and regulation of LDL cellular interactions. Our study has demonstrated that HDL-cellular association, in which surface lipids play a more important role than surface proteins, is fundamentally different from LDL-cellular association. Further identification of the mediators of HDL-cellular interaction may lead to a deeper understanding of the function and regulation of HDL metabolism.

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SUPPLEMENTARY MATERIAL TO
Mechanism of the Association of HDL with Endothelial Cells
Smooth Muscle Cells and Fibroblasts
Evidence Against the Role of Specific Intercellular and Receptor Protein

EXPERIMENTAL PROCEDURES

Materials — Human serum albumin (essentially fatty acid free), alpha-phosphatidylcholine, sodium sulfate, and sodium tungstate (reagent) were from Sigma. Cholesterol was obtained from Nakanishi Prep. Protein from Diplomatis Biological. BSA (99.95% purity) was purchased from Calbiochem. Trypsin (200 U/ml) and sterilizing filters (0.22 μm) were obtained from Millipore. Sepharose CL-4B was purchased from Pharmacia. Human serum albumin (Lot No. 79-5701-14) and murine endotoxin (Lot No. 79-5701-14) were purchased from New England Nuclear. Egg yolk phosphatidylcholine was obtained from Sigma. Human fibronectin (Type V) was obtained from Personale Biological. Colchicine, 1H,1′-bis(2-naphthylmethylene)tetraacetic acid (BMTA) and ATP were purchased from Calbiochem. Dextran (mol. wt 500,000) was obtained from Boehringer. HEPES (pH 7.4) was purchased from Sigma. Trypsin (200 U/ml) and sterilizing filters (0.22 μm) were obtained from Millipore.

Methods — Human platelets were obtained from nine healthy volunteers, and mononuclear cells were obtained 3 to 4 wk after apheresis.

EXPERIMENTAL PROCEDURES

Cell Cultures — Human platelet subcellular fractions and random smooth muscle cells (three passages), obtained and characterized as described previously [1,2], were kindly provided by Dr. Joseph Rabinowitz, Columbia University. Human normal foreskin fibroblasts (three passages) were kindly provided by Dr. Joseph Rabinowitz. Semliki Forest virus-infected fibroblasts were kindly provided by Dr. Joseph Rabinowitz. Columbia University. All cells were grown in liquid culture and thawed rapidly prior to use. For each experiment, the cells were suspended in MEM containing 10% fetal bovine serum, 100 μg/ml penicillin, 100 μg/ml streptomycin, 25 μg/ml G418, 50 μg/ml L-amino acids. The plates were incubated for 5 days at 37°C in an atmosphere containing 5% CO2/95% air.

Lipoproteins — Fibronectin (9B2, utilized here since this fraction of HDL contains very low apoB-100 and thus would not be expected to interact with the apoB-100 receptor) and LDL were obtained from fresh human plasma by preparative ultracentrifugation at 30,000 rpm, 3°C, to recover a fraction containing 0.13 μmol/L apoA-I and 0.3 μmol/L apoB-100. The concentration of HDL by absorbance was as follows: 1.8 mg protein, 2.5 μg phosphatidylcholine, 0.15 μg cholesterol, and 0.15 mg free cholesterol. Trypsinized HDL (T-HDL) was prepared by incubating 200 μg Trypsin (200 U/ml) with 250 μg Trypsin (200 U/ml) for 30 min at the Fracton of Specific Tension activity (1.2 g) was subsequently suspended in 102 ml of HEPES (pH 7.4) containing 108 mg of protein was isolated, and the HDL fractions were similarly prepared. The HDL fractions were collected and used for further experiments.

Characterization of T-HDL — The T-HDL fractions were collected and used for further experiments.

RESULTS — Human platelet subcellular fractions and random smooth muscle cells (three passages), obtained and characterized as described previously [1,2], were kindly provided by Dr. Joseph Rabinowitz, Columbia University. Human normal foreskin fibroblasts (three passages) were kindly provided by Dr. Joseph Rabinowitz. Semliki Forest virus-infected fibroblasts were kindly provided by Dr. Joseph Rabinowitz. Columbia University. All cells were grown in liquid culture and thawed rapidly prior to use. For each experiment, the cells were suspended in MEM containing 10% fetal bovine serum, 100 μg/ml penicillin, 100 μg/ml streptomycin, 25 μg/ml G418, 50 μg/ml L-amino acids. The plates were incubated for 5 days at 37°C in an atmosphere containing 5% CO2/95% air.

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Characterization of T-HDL — The T-HDL fractions were collected and used for further experiments.

DISCUSSION — The T-HDL fractions were collected and used for further experiments.

Supplementary Information

Mechanism of the Association of HDL with Endothelial Cells
Smooth Muscle Cells and Fibroblasts
Evidence Against the Role of Specific Intercellular and Receptor Protein

previously [38]. All lipoproteins were dialyzed extensively against 0.1M NaCl, 150 mM choline buffer, stirred through 0.05 M NaCl, and stored at −20°C. Lipoproteins were used within one month of preparation.

Trypsinization — Trypsinization of HDL was performed by incubating HDL with 200 U/ml of trypsin (Calbiochem) at 37°C for 30 min.

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