

# Blood Serum Atherogenicity Associated With Coronary Atherosclerosis

## Evidence for Nonlipid Factor Providing Atherogenicity of Low-Density Lipoproteins and an Approach to Its Elimination

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To reveal the presence of atherogenic potential in the blood serum obtained from patients with angiographically assessed coronary atherosclerosis we used primary cultures of subendothelial cells isolated by collagenase from unaffected human aortic intima. Earlier, we have demonstrated that such cultures are made up mostly of typical and modified smooth muscle cells. Within 24 hours of cultivation with a 40% sera of patients suffering from coronary atherosclerosis, the total intracellular cholesterol level increased twofold to fivefold. Cultivation with the sera of healthy subjects had no effect on the intracellular cholesterol level. The sera of patients were separated by ultracentrifugation into two fractions: total lipoprotein fraction containing the main classes of lipoproteins and a lipoprotein-deficient fraction. The former, but not the lipoprotein-deficient fraction, was characterized by atherogenicity (i.e., the ability to induce the accumulation of intracellular cholesterol). Lipoproteins of the patients' serum were separated into main classes: low density lipoproteins (LDL), very low density lipoproteins (VLDL), and high density lipoproteins (HDL<sub>2</sub> and HDL<sub>3</sub>). An atherogenic component of the serum capable of stimulating the deposition of intracellular cholesterol was represented by LDL and, in one case, by VLDL, but not by other classes of lipoproteins. LDL and other lipoproteins isolated from the blood serum of healthy subjects failed to raise the cholesterol content in cultured cells; that is, they were nonatherogenic. Simultaneous addition to the culture of a lipoprotein-deficient fraction from the patients' blood serum and the total lipoprotein fraction or LDL isolated from the serum of healthy subjects brought about the accumulation of intracellular cholesterol, which suggests that the patients' serum contains a nonlipoprotein component capable of imparting atherogenic properties to the initially nonatherogenic LDL. Atherogenicity of the patients' serum can be sharply reduced or even completely eliminated by passing it through a sorbent containing LDL immobilized on agarose. After this procedure, regeneration of the column by glycine buffer, pH 2.5, was performed. We failed to detect measurable amounts of cholesterol or apo B in the eluate, though pooling of the pH-2.5 eluate with the serum, which lost atherogenicity after passing through the LDL sorbent, partially restored its atherogenic potential. Simultaneous addition to the culture of the eluate derived from atherogenic serum of a patient and LDL obtained from the serum of a healthy donor caused a substantial accumulation of intracellular cholesterol, whereas LDL taken separately failed to alter the cholesterol level. (*Circulation Research* 1988;62:421-429)

The deposition of lipids in vascular wall cells plays an essential, if not the decisive, role in early manifestations of atherosclerosis at the cellular level. We have recently shown that the blood serum of patients with angiographically assessed coronary atherosclerosis can stimulate the accumulation of intracellular lipids (mostly free and esterified cholesterol) in primary culture of intimal smooth muscle cells of human aorta.<sup>1</sup> This property, termed atherogenicity, was absent in the sera of most healthy donors. Atherogenicity of the patients' serum was unrelated to the presence of cholesterol, low-density lipoprotein (LDL) or high-density lipoprotein (HDL). In this study,

we attempted to reveal the causes of serum atherogenicity and to find a way to eliminate it.

### Materials and Methods

#### Donors

Blood was drawn from the cubital vein into plastic tubes in the morning before meals. There were two groups of donors: the first was composed of 13 subjects free from any signs of coronary heart disease; the second included 16 patients who had coronary heart disease of II-IV functional classes according to the Canadian classification.<sup>2</sup> As determined by selective coronarography performed according to Judkins,<sup>3</sup> the extent of blockage of 1-3 major coronary arteries was 75% or higher. Blood was taken within the first days of patients' hospital stay prior to the beginning of drug therapy. Donors' sex, age, coronarography data, and other characteristics are presented in Table 1. The blood was incubated for 1 hour at 37° C and centrifuged for

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TABLE 1. Characteristics of Blood Sera Used for Experiments

Patients' characteristics					
No.	Sex	Age	Angina pectoris	Number of coronary arteries involved*	Atherogenicity (% control)†
1	M	52	+	3	205 ± 14‡
2	F	35	+	1	221 ± 32‡
3	M	56	+	3	309 ± 27‡
4	M	42	+	2	271 ± 15‡
5	M	38	+	1	187 ± 11‡
6	F	43	-	0	121 ± 17
7	M	42	-	0	124 ± 12
8	M	39	-	NI	107 ± 5
9	M	37	-	NI	102 ± 9
10	M	42	-	NI	105 ± 13
11	F	29	-	0	108 ± 4
12	M	37	-	NI	126 ± 10
13	M	56	+	3	260 ± 21‡
14	M	38	+	3	344 ± 20‡
15	M	41	+	1	193 ± 14‡
16	F	45	+	2	243 ± 17‡
17	M	41	+	3	228 ± 14‡
18	M	52	+	3	307 ± 14‡
19	M	31	-	NI	128 ± 15
20	M	42	-	NI	102 ± 13
21	M	45	+	3	453 ± 28‡
22	M	47	+	2	360 ± 11‡
23	M	48	+	3	367 ± 22‡
24	M	58	+	3	291 ± 21‡
25	M	48	+	3	418 ± 31‡
26	M	26	-	NI	113 ± 14
27	F	29	-	NI	104 ± 10
28	F	29	-	NI	102 ± 11
29	M	34	-	NI	123 ± 15

\*Coronarography data.

†Blood serum atherogenicity was determined by total cholesterol accumulation in cultured intimal cells as described earlier.<sup>1</sup> Cells of grossly normal human aortic intima were cultured for 24 hours in the media containing 40% of the respective serum. Total cholesterol concentration in control cells cultured with 10% lipoprotein-deficient serum of healthy donor was taken for a 100% value. In this series of experiments, cells from 18 isolations were used. The initial cholesterol content in cultures varied from 6.9 to 18.4  $\mu\text{g}/10^5$  cells. In each experiment performed on the cells of one batch, we revealed both atherogenic and nonatherogenic sera. Many sera were tested in several experiments on different cell batches, and the data on the sera's atherogenicity obtained in different experiments coincided. Also, a standard atherogenic and a standard nonatherogenic sera were used in each experiment in addition to control delipidated serum. Standard atherogenic serum caused a 190–236% rise in the intracellular cholesterol, whereas standard nonatherogenic serum never stimulated the accumulation of cholesterol (90–115% of the control).

NI, not investigated.

‡Significant differences from the control ( $p < 0.05$ ).

20 minutes at 3,000 rpm. The obtained sera were sterilized by filtration (pore size, 0.22  $\mu\text{m}$ ). A

lipoprotein-deficient serum was obtained by ultracentrifugation according to Lindgren.<sup>4</sup>

### Cell Cultures

Cells were obtained from the aorta of 40- to 60-year-old males and females within 1.5–3 hours after sudden death, which had ensued mainly from myocardial infarction. Subendothelial smooth muscle cells were isolated from a grossly normal intima by dispersion of aortic tissue with collagenase and cultured as previously described.<sup>5,6</sup> After mechanical separation of the adventitia, the vessels were incised longitudinally under sterile conditions, the vascular areas without visible signs of atherosclerotic lesions (grossly normal) were excised, and the excised loci were pooled. The intima and media were mechanically separated with forceps. The accuracy of separation was controlled microscopically. In the samples stained for Verhoeff's elastic, it was observed that the separation of the intima from the media went along the internal elastic lamina. The collected material from uninvolved intima was separated into fibers with forceps. Then, the minced intima was digested with 0.15% collagenase type II (Worthington Diagnostic System, Freehold, New Jersey) dissolved in the growth medium containing Medium 199, 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100  $\mu\text{g}/\text{ml}$  streptomycin, and 2.5  $\mu\text{g}/\text{ml}$  fungizone (all reagents from Grand Island Biological Company [GIBCO], Grand Island, New York), 10 ml enzyme mixture solution/g tissue. The incubation of tissue with the enzyme was carried out at 37° C with shaking at 50 rpm until almost complete dispersion took place, which required about 3–4 hours. A suspension of the isolated cells was filtered through a nylon mesh, centrifuged (200 g, 5 minutes), resuspended in the growth medium, and seeded into 24-well tissue culture plates with a density of 2–4  $\times 10^4$  cells/cm<sup>2</sup> of the growth area. The cells were cultured at 37° C in an atmosphere of 95% air-5% CO<sub>2</sub> in a humidified CO<sub>2</sub> incubator. For experiments, we used 7-day primary cultures of intimal cells. These cultures contained a mixed cell population composed primarily of typical and modified smooth muscle cells.<sup>6</sup> The medium was changed every other day. All additions diluted to the final concentration with Medium 199 were added to cultures on the 7th day.

On the day of experiment, the medium in all cultures was replaced with a fresh growth medium containing 40% serum under investigation or 10% lipoprotein-deficient human serum,<sup>4</sup> combined with LDL or other additions. Normally, 24 hours later, the cells were rinsed three times with phosphate-buffered saline (PBS), treated with 0.025% Trypsin-EDTA for 5 minutes and washed with PBS 5 times (all reagents were from GIBCO). The cells were removed from the substrate with 0.25% Trypsin-EDTA and washed twice by centrifugation (200 g, 10 minutes).

### Cholesterol Determination

Lipids were extracted from cells with a chloroform-methanol mixture (1:2, vol/vol) according to Bligh and

Dyer.<sup>7</sup> The total cholesterol content in the lipid extracts was determined using Boehringer Mannheim Monotest<sup>®</sup>, Cholesterol CHOD-PAP Method (catalog no. 236691, Boehringer Mannheim GmbH, Mannheim, Federal Republic of Germany). Cholesterol stock standard (stock no. 965-25, Sigma Chemical, St. Louis, Missouri) was used for standard preparation.

### Lipoproteins

Lipoproteins of different classes were isolated from the donor blood serum according to the conventional method of ultracentrifugation in a step-wise gradient of sodium bromide.<sup>4</sup> Ultracentrifugation was carried out on a Beckman SW-60 rotor (Beckman Instruments, Palo Alto, California). The fraction of very low density lipoproteins (VLDL) was isolated by centrifugation of the serum at 300,000g for 18 hours at 15° C at density 1.019 g/cm<sup>3</sup>. Then two thirds of the centrifuge tube volume was filled with the serum after adjusting its density to 1.050 g/cm<sup>3</sup>. Subsequently, one third of the volume of phosphate buffer having the same density was carefully applied to the serum, after which the second centrifugation stage was carried out for 24 hours. The floating fraction represented by LDLs was collected and recentrifuged for 24 hours through a layer of phosphate buffer with a density of 1.050 g/cm<sup>3</sup>. HDLs were obtained in a similar way by centrifuging the serum for 48 hours (gradient density 1.250 g/cm<sup>3</sup>). The total lipoprotein fraction and the lipoprotein-deficient fraction were obtained by centrifuging the serum at 300,000g (density 1.250 g/cm<sup>3</sup>) for 48 hours at +4° C. All lipoprotein preparations and the lipoprotein-deficient serum were dialyzed for 24 hours against 2,000 volumes of PBS, were sterilized by filtration, and were stored for not more than two weeks at +4° C. Usually, lipoproteins were utilized within 1–4 days after preparation. Protein content in the lipoprotein preparations was determined according to Lowry et al.<sup>8</sup> Concentration of apo B was measured by ELISA technique.<sup>9</sup>

### Sorbent

Immobilization of LDL on the BrCN-activated Sepharose CL 4B (Pharmacia Fine Chemicals AB, Uppsala, Sweden) was carried out according to the conventional methodology.<sup>10</sup> Sepharose was washed on a filter with a 15-fold volume of 10<sup>-3</sup> M HCl, after which the gel was transferred into a 1-ml column and the LDL preparation in 0.2 M borate buffer, pH 8.0, was passed through it by recycling for 2 hours. Nonreacting active groups of the sorbent were blocked with 1 M ethanolamine for 2 hours. To remove a nonspecifically bound protein, the sorbent with immobilized LDL was washed several times with a 0.2 M borate buffer, pH 8.0, and 0.2 acetate buffer, pH 4.0, containing 0.5N NaCl.

Atherogenic sera were passed through a 0.5 ml LDL-Sepharose gel suspension packed into the glass column. The sorbent was equilibrated with PBS, after which 5 ml serum was passed through it for 60 minutes in the recycling conditions at the rate of 20 ml/hr.

LDL-Sepharose was washed with PBS, and the adsorbed fraction was eluted with 10 ml 0.2 M glycine buffer, pH 2.5. The eluate obtained in this way was dialyzed for 24 hours against 200 volumes of PBS and concentrated to 0.5 ml by reverse dialysis in Ficoll 400 (Pharmacia). Protein content in the eluate was determined by spectrophotometry by measuring the optical density at 280 nm.

### Statistical Analysis

The significance of differences was evaluated by dispersion analysis methods using a BMDP statistical program package.<sup>11</sup>

### Results

The intracellular cholesterol level in the subendothelial cells of human aortic intima cultured with the serum of patients suffering from coronary atherosclerosis increases in the course of cultivation (Figure 1). A significant 2- to 2.5-fold increase in cholesterol versus the initial level was seen already at 24 hours. At 96 hours, we observed a fourfold to fivefold rise of cholesterol level. Within 48 to 72 hours, lipid inclusions inside the cells were easily distinguished in the form of grains and small droplets (Figure 2). At the same time (within 96 hours of cultivation), the sera of healthy donors showed no statistically significant alterations in the intracellular cholesterol level (Figure 1).

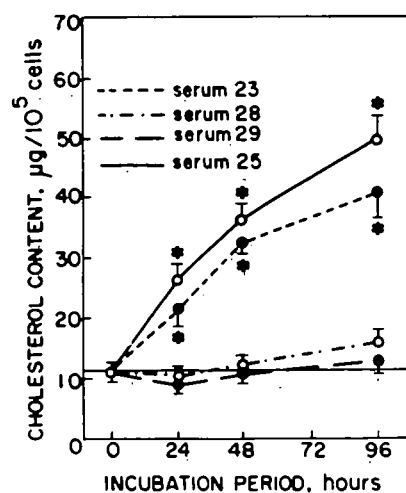


FIGURE 1. Time course of effects exerted by the sera of two healthy subjects and sera of two patients with coronary atherosclerosis on the total cholesterol level in cultured cells of human aortic intima. Shown are the results of 1 representative experiment out of 10 with 8 atherogenic and 6 nonatherogenic sera. Cells were cultured for the indicated period in the medium containing 40% serum of two healthy subjects (sera 28 and 29) and two patients with coronary atherosclerosis (sera 23 and 25). Baseline represents total intracellular cholesterol level in control cultures. Control cells were cultured in the media containing 10% lipoprotein-deficient nonatherogenic serum of a healthy subject. Values listed are mean  $\pm$  SEM of three determinations. From 0.5 to 5  $\mu$ g cholesterol was measured in each sample. \*Significant difference from the control,  $p < 0.05$ .

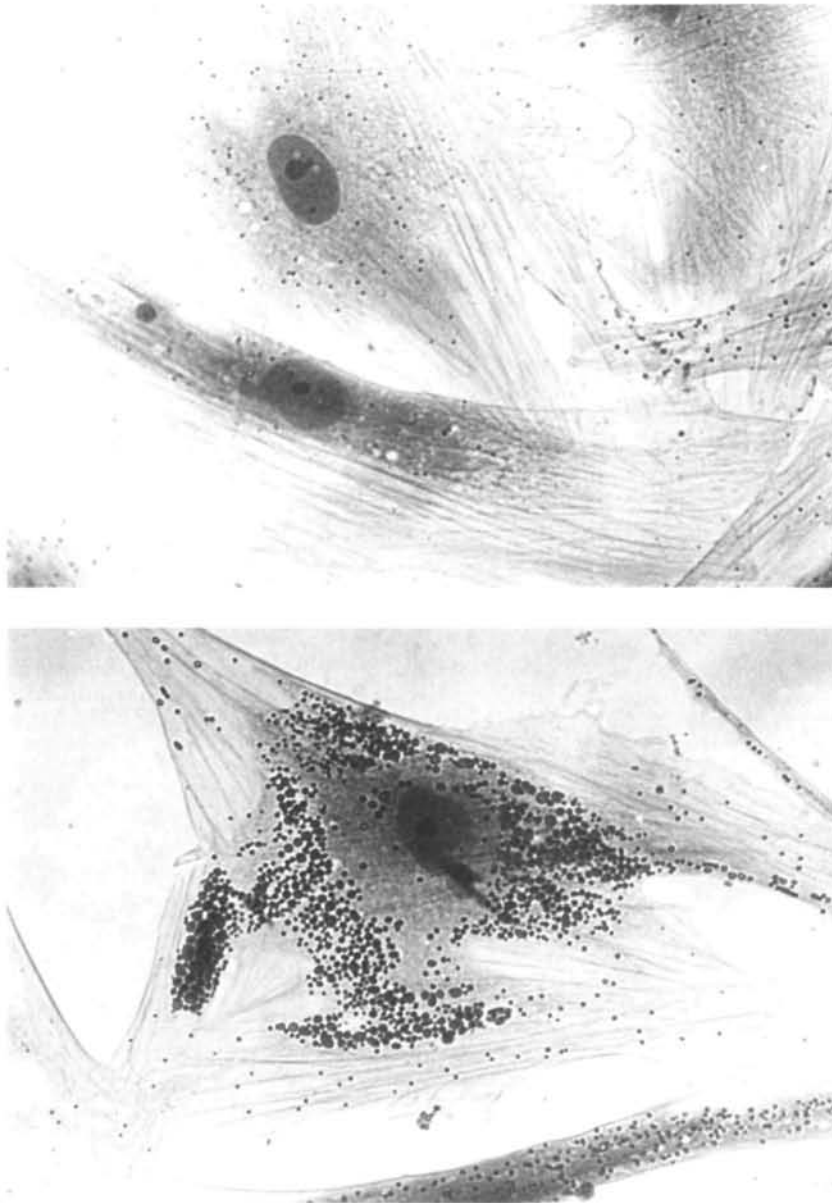


FIGURE 2. Cells cultured from the sub-endothelial intima of human aorta. A, Cells cultured for 72 hours with a 40% nonatherogenic serum; B, cells cultured for 72 hours with a 40% atherogenic serum. Staining with Oil Red O, hematoxylin,  $\times 400$ .

Table 1 shows the data on the ability of 29 sera to induce the accumulation of total intracellular cholesterol in cultured intimal subendothelial cells. In all cases, the sera of patients with angina and angiographically documented stenosis of 1–3 coronary arteries were atherogenic; that is, they were able to significantly increase the total cholesterol in cultured cells (twofold to fivefold) within 24 hours of incubation (sera 1–5, 13–18, 21–25). However, the sera of healthy donors and subjects with no record of coronary artery stenosis were nonatherogenic; that is, they failed to significantly alter the intracellular cholesterol within 24 hours of incubation (sera 6–12, 19, 20, 26–29). Table 1 shows the data on the sera specially selected for this study. As was shown before,<sup>1</sup> however, in a random sample most sera of the patients with coronary atherosclerosis are atherogenic while most sera of healthy subjects are not.

Five atherogenic sera and three nonatherogenic sera were separated by ultracentrifugation into the total

lipoprotein fraction containing all main classes of lipoproteins and a lipoprotein-deficient fraction. Lipoprotein-deficient fractions obtained from both atherogenic and nonatherogenic sera exhibited no atherogenicity in culture; that is, they failed to induce the accumulation of total cholesterol in cultured subendothelial cells of human aortic intima (Table 2, line 1). At the same time, total lipoprotein fractions isolated from atherogenic sera brought about a significant (1.5- to 3.5-fold) rise in the intracellular cholesterol (Table 2, column 1). The lipoproteins of nonatherogenic sera were devoid of atherogenicity. A combination of the total lipoprotein fraction with lipoprotein-deficient serum exhibited atherogenicity in culture if both fractions were derived from atherogenic sera, whereas their counterparts from initially nonatherogenic sera did not (Table 2, compare top left quadrant and bottom right quadrant). It should be pointed out that a combination of the total lipoprotein

**TABLE 2. Total Cholesterol Content in Subendothelial Cells of Human Aortic Intima Cultured With Total Lipoprotein Fraction (LP) and Lipoprotein-Deficient Serum (LDS)**

Lipoprotein fractions	Lipoprotein-deficient sera								
	None	Atherogenic					Nonatherogenic		
		LDS-1	LDS-2	LDS-3	LDS-4	LDS-5	LDS-6	LDS-7	LDS-8
None		74 ± 14	98 ± 1	109 ± 5	116 ± 10	91 ± 6	113 ± 12	89 ± 5	83 ± 12
<b>Atherogenic</b>									
LP-1	145 ± 9*	170 ± 11*		199 ± 22*			192 ± 9*		
LP-2	219 ± 12*	191 ± 24*	272 ± 16*	276 ± 28*				238 ± 9*	
LP-3	343 ± 33*			347 ± 45*					285 ± 36*
LP-4	185 ± 17*				260 ± 11*		206 ± 19*		
LP-5	167 ± 12*					179 ± 11*	164 ± 21		
<b>Nonatherogenic</b>									
LP-6	80 ± 12	111 ± 7					88 ± 8		
LP-7	95 ± 5	152 ± 6*	191 ± 5*					97 ± 10	93 ± 3
LP-8	104 ± 7	145 ± 15	205 ± 19*	185 ± 13*				101 ± 7	114 ± 5

Total lipoprotein fractions (LP-1–LP-8) and lipoprotein-deficient sera (LDS-1–LDS-8) were obtained from five atherogenic sera (sera 1–5) and three nonatherogenic sera (sera 6–8). LP and LDS from different sera were added to cultures of intimal cells in different combinations to final concentrations of 40% LDS and 40% LP (normalized to initial cholesterol). After 24 hours of cultivation under standard conditions, the total cholesterol content was determined in the cells. The values listed are means of four determinations ± SEM represented as the percentage of the control. The cholesterol content in control cultures was  $14.8 \pm 0.7 \mu\text{g}/10^5$  cells (eight determinations). \*Significant differences ( $p < 0.05$ ) from the intracellular cholesterol content in control cultures incubated with a 40% LDS of a healthy donor.

fraction with an autologous lipoprotein-deficient serum obtained from the initially atherogenic serum in all cases induces a somewhat more substantial accumulation of intracellular cholesterol as compared with a single lipoprotein fraction. This finding suggests that a nonlipid part of the serum, (i.e., the lipoprotein-deficient fraction) contributes to atherogenicity of the whole serum, which is nonatherogenic in itself. A combination of the lipoprotein-deficient fraction obtained from the initially atherogenic serum with a lipoprotein fraction from nonatherogenic serum caused the accumulation of cholesterol in cultured intimal cells, the cholesterol level in most cases being raised significantly (Table 2, bottom left quadrant). Combinations of initially nonatherogenic lipoproteins with the lipoprotein-deficient fractions obtained from the whole serum 1, which had a weak atherogenicity of its own, exhibited low atherogenicity in culture. (In two out of three cases, a rise in the intracellular cholesterol was nonsignificant.) The lipoprotein-deficient fractions obtained from initially nonatherogenic whole sera did not prevent the manifestation of atherogenic properties by total lipoprotein fractions derived from the initially atherogenic whole sera (Table 2, top right quadrant). Atherogenic total lipoprotein fractions mixed with nonautologous atherogenic lipoprotein-deficient sera retained their atherogenicity (Table 2, top left quadrant, columns 2 and 4). On the other hand, nonatherogenic lipoprotein fractions mixed with nonautologous nonatherogenic lipoprotein-deficient fractions did not acquire atherogenic properties (Table 2, bottom right quadrant, columns 8 and 9). Thus, atherogenicity of the sera of patients with coronary atherosclerosis is related to lipoproteins contained in these sera. Besides, one may assume that lipoprotein-deficient fractions of initially atherogenic whole sera from patients with coronary atherosclerosis contain a

factor(s) that cannot induce the accumulation of lipids in cells by itself but imparts atherogenic properties manifested in culture to the lipoprotein fraction derived from initially nonatherogenic whole serum of healthy subjects.

We decided to find out which class of lipoproteins constitutes an atherogenic component of the total lipoprotein fraction of the patients' blood serum. Four nonatherogenic and four atherogenic sera were used as a source of LDL, VLDL, and high-density lipoproteins of subclasses 2 and 3 (HDL<sub>2</sub> and HDL<sub>3</sub>, respectively). Neither of the lipoprotein classes derived from nonatherogenic sera significantly altered the intracellular cholesterol level; that is, they had no atherogenic effect on cultured intimal cells (Table 3). LDL isolated from atherogenic sera added to culture in the concentration of 250  $\mu\text{g}$  protein/ml brought about a twofold to

**TABLE 3. Effect of Whole Serum and Lipoprotein Fractions on Total Cholesterol Content of Subendothelial Intimal Cells Cultured From Human Aorta**

Patient	Cholesterol content (% control)				
	Serum	LDL	VLDL	HDL <sub>2</sub>	HDL <sub>3</sub>
9	102 ± 9	114 ± 5	113 ± 12	98 ± 9	106 ± 6
10	105 ± 13	98 ± 6	109 ± 12	133 ± 17	135 ± 6
11	108 ± 4	92 ± 4	133 ± 13	107 ± 25	114 ± 12
12	126 ± 10	120 ± 3	98 ± 8	95 ± 7	102 ± 7
13	260 ± 21*	332 ± 40*	123 ± 10	126 ± 12	115 ± 5
14	344 ± 20*	390 ± 41*	227 ± 18*	88 ± 10	111 ± 11
15	193 ± 14*	205 ± 12*	134 ± 11	115 ± 11	127 ± 11
16	243 ± 17*	240 ± 31*	148 ± 12	96 ± 11	129 ± 7

The initial control value of cholesterol was  $11.8 \pm 0.9 \mu\text{g}/10^5$  cells (15 determinations).

The serum and each lipoprotein fraction were added to culture in the concentration of 40% and 250  $\mu\text{g}$  protein/ml, respectively. Values listed are means of three determinations ± SEM.

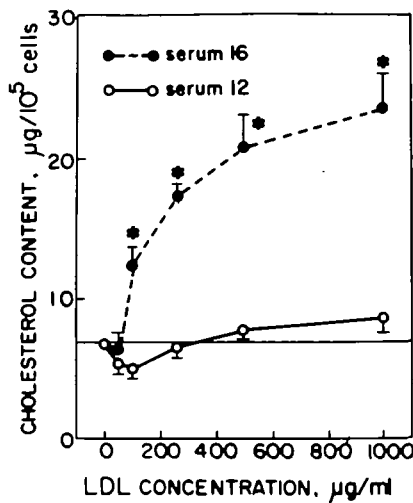


FIGURE 3. Dose dependence of effects exerted by LDL isolated from atherogenic and nonatherogenic serum on the total cholesterol level in cultured cells of human aortic intima. Shown are the results of one out of four representative experiments with four different pairs of LDL samples isolated from atherogenic and nonatherogenic sera. Cells were cultured for 24 hours in the medium containing 10% lipoprotein-deficient serum of a healthy subject and the indicated concentrations of LDL isolated from an atherogenic (serum 16) or nonatherogenic serum (serum 12). Other details are the same as for Figure 1.

fourfold increase in total cholesterol, the atherogenic properties of LDL correlating with the degree of atherogenicity of the initial serum. One of the four atherogenic sera (serum 14) contained VLDL possessing atherogenic properties manifested in culture, though VLDL from three other atherogenic sera failed to cause a statistically significant accumulation of cholesterol. HDL<sub>2</sub> and HDL<sub>3</sub> isolated from the initially atherogenic sera, which were added to culture in the same concentration as LDL (lipoprotein concentration was determined by the apoprotein content), proved to be nonatherogenic. Thus, an atherogenic component of the serum is represented by LDL and sometimes,

possibly, by VLDL, but not by other classes of lipoproteins.

We tested a wide range of LDL concentrations (from 50 to 1,000 µg protein/ml). It turned out that LDL isolated from atherogenic sera significantly increased the intracellular cholesterol starting from 100 µg protein/ml (Figure 3). At the same time, LDL from nonatherogenic sera exhibited no atherogenicity in culture even if taken in the concentration of 1,000 µg protein/ml. This observation indicates that atherogenicity manifested by LDL in vitro is related not to the concentration of lipoproteins in the culture medium but to certain qualitative differences in the properties of LDL from atherogenic sera.

LDL isolated from nonatherogenic serum used in the combination with a lipoprotein-deficient serum derived from initially atherogenic whole serum manifested atherogenic properties in culture (Table 4, bottom left quadrant, column 2). The lipoprotein-deficient serum obtained from initially nonatherogenic whole serum neither imparted atherogenic properties to nonatherogenic LDL (Table 4, bottom right quadrant, column 5) nor prevented the manifestation of atherogenicity by LDL isolated from an atherogenic serum (Table 4, top right quadrant). Thus, a hypothetical factor of atherogenicity present in a lipoprotein-deficient serum of patients with coronary atherosclerosis can impart atherogenic properties to initially nonatherogenic LDL isolated from the blood of healthy subjects.

We attempted to find out whether this atherogenic factor directly interacts with LDL. For this purpose, we prepared a sorbent on the basis of agarose with covalently coupled LDL. Initially atherogenic sera (sera 21–25) lost most of their atherogenic properties after passing through the sorbent (Table 5). However, nonatherogenic sera (sera 26 and 27) did not turn atherogenic after passing through the sorbent. The elements of atherogenic sera retained on this sorbent were eluted with 0.2 M glycine buffer, pH 2.5. The eluate did not contain measurable amounts of cholesterol or apo B (data not shown) and failed to exhibit

TABLE 4. Total Cholesterol Content in Subendothelial Cells of Human Aortic Intima Cultured With Low-Density Lipoprotein (LDL) and Lipoprotein-Deficient Serum

	None	Atherogenic		Nonatherogenic	
		LDS-17	LDS-18	LDS-19	LDS-20
None		115 ± 20	98 ± 10	105 ± 8	120 ± 14
Atherogenic					
LDL-17	187 ± 21*	252 ± 18*			203 ± 31*
LDL-18	261 ± 19*	290 ± 17*	294 ± 28*		250 ± 16*
Nonatherogenic					
LDL-19	125 ± 13	187 ± 14*		129 ± 12	127 ± 10
LDL-20	117 ± 8	201 ± 28*			114 ± 17

LDL (LDL-17–LDL-20) and lipoprotein-deficient serum (LDS-17–LDS-20) were obtained from two atherogenic (17 and 18) and two nonatherogenic sera (19 and 20). LDL and LDS from different sera were mixed in different combinations and added to intimal cell cultures in a 40% concentration. After 24 hours of cultivation under standard conditions, the intracellular content of total cholesterol was measured. Values listed are means of four determinations ± SEM represented as percentage of the control. The cholesterol content in control cultures was 18.4 ± 1.7 µg/10<sup>5</sup> cells (eight determinations).

\*Significant differences ( $p < 0.05$ ) from the intracellular cholesterol content in control cultures incubated with a 40% lipoprotein-deficient serum of a healthy donor.

**TABLE 5. Effect of Passing Through an LDL Column on Serum Atherogenicity**

Serum	Cellular cholesterol content (% control)			
	Serum before passing through the sorbent	Serum after passing through the sorbent	pH-2.5 eluate	Serum after passing through the sorbent + eluate
21	453 ± 28*	166 ± 21*	...	...
22	360 ± 11*	139 ± 4*	104 ± 12	218 ± 9*
23	367 ± 22*	117 ± 14	93 ± 8	137 ± 6
24	291 ± 21*	111 ± 6	113 ± 8	153 ± 8*
25	418 ± 31*	169 ± 7*	107 ± 17	224 ± 21*
26	113 ± 14	102 ± 12	...	...
27	104 ± 10	100 ± 15	...	...

The serum (1–3 ml) was applied to a column containing 0.5 ml LDL-Sepharose gel suspension. After passing through the sorbent, the serum was diluted with Medium 199 to a 40% concentration (serum concentration was determined by the volume and cholesterol content). The material retained on the sorbent was eluted with 0.2M glycine buffer, pH 2.5 (10 ml). The eluate was dialyzed and diluted with Medium 199 to the concentration corresponding to the content of the eluted substance in a 40% initial serum. The serum that passed through the column, the eluate, and initial serum in the concentration of 40% were added to cultures of intimal cells. The total intracellular cholesterol was measured in cultures 24 hours later. Values listed are means of three determinations ± SEM.

\*Significant differences from the control,  $p < 0.05$ .

The cholesterol level in control cultures (maintained in the presence of a 40% lipoprotein-deficient serum of a healthy donor) varied from 7.2 to 10.6  $\mu\text{g}/10^5$  cells.

atherogenicity in the absence of LDL (Table 5). In combination with the pH-2.5 eluate, the serum that lost its atherogenicity after passing through the LDL-sorbent partially recovered atherogenic potential (Figure 4, Table 5). Simultaneous addition of the eluate derived from atherogenic serum and LDL isolated from the blood of a healthy donor to culture of subendothelial intimal cells caused a substantial accumulation of intracellular cholesterol, whereas LDL applied separately did not alter the cholesterol level in cultured cells (Figure 5). Thus, the factor of atherogenicity present in the lipoprotein-deficient fraction of the patients' blood serum is able to associate with LDL, and this association apparently imparts atherogenic properties manifested in vitro to initially nonatherogenic LDL.

### Discussion

In this study, we have used cultures of subendothelial cells from human aortic intima that contained a mixed cell population made up mainly of typical and modified smooth muscle cells.<sup>6</sup> This model was chosen to investigate the mechanisms of lipidosis, since among the lipid-laden foam cells of an atherosclerotic lesion one can find, besides macrophages, cells of smooth muscle origin in which the cytoplasm is filled with grains and droplets of lipid.<sup>12</sup> According to our data, it is cells of smooth muscle origin, and not macrophages, that constitute the bulk of the lipid-laden intimal cell population in human aorta (Andreeva et al, to be published).

The accumulation of cholesteryl esters and other lipids in arterial wall cells is the most prominent manifestation of atherosclerosis at the cellular level.<sup>12</sup> Circulating LDLs, which are capable of penetrating the endothelial lining and being deposited within the subendothelial intima, are conventionally regarded as the source of cholesteryl esters.<sup>13–16</sup> However, the mechanism of the LDL-mediated accumulation of lipids in subendothelial cells remains obscure. Numerous attempts to induce the deposition of lipids in cultured cells by adding LDL to cultures failed to cause a substantial rise in intracellular lipids.<sup>17–20</sup> The LDL-mediated accumulation of lipids was registered in cultures of macrophages, but the LDL used in these experiments were chemically modified by acetylation, acetoacetylation, maleylation, or malondialdehyde derivatization.<sup>17, 18, 20–22</sup> However, the actual significance of this LDL modification in the pathophysiology of atherosclerosis is yet unclear.

We have demonstrated in this study that the LDL fraction of the serum obtained from patients with coronary atherosclerosis, but not healthy subjects, induces the accumulation of intracellular lipids. A hypothetical factor or factors present in the plasma of patients ensures LDL atherogenicity (i.e., their ability to stimulate cellular lipidosis). These factors impart atherogenic properties even to initially "normal" LDLs that are circulating in the blood of healthy subjects and are unable to induce the deposition of cholesterol in cultured cells. The nature of this factor or factors and the mechanisms of its interaction with LDLs whereby the latter acquire an atherogenic potential would be the object of our further investigations. So, at present, the discussion of this problem is limited to speculations and surmise.

Direct interaction of the serum atherogenic factor with LDL suggests that it might be represented by

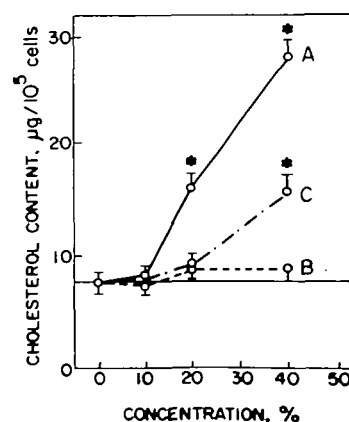


FIGURE 4. Dose dependence of effects upon the total cholesterol content in cultured cells of human aortic intima exerted by an atherogenic serum before and after passing through the sorbent with immobilized LDL as well as by reconstituted serum. Cells were cultured for 24 hours in the presence of the indicated concentrations of A, atherogenic serum (serum 23); B, the same serum subjected to passing through a sorbent with immobilized LDL; C, serum B combined with a pH 2.5-eluate. Other details are the same as in the notes to Table 5 and Figure 1.

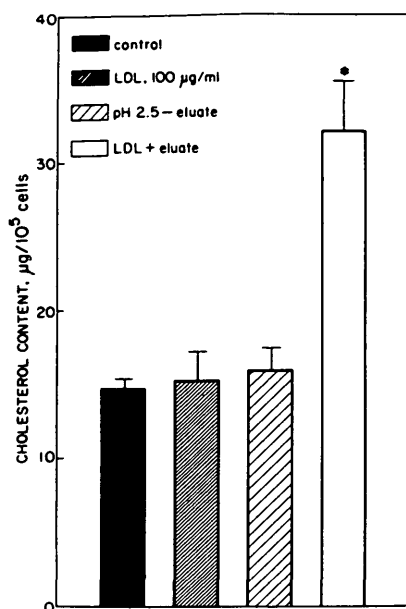


FIGURE 5. Effect of pH-2.5 eluate on the LDL-mediated total cholesterol accumulation in cultured cells of human aortic intima. Cells were cultured in the medium containing 10% lipoprotein-deficient serum of a healthy donor with the following additions: 100 µg/ml LDL isolated from the blood of a healthy donor; pH-2.5 eluate obtained from 1 ml atherogenic serum; LDL and eluate. Values are mean  $\pm$  SEM of three determinations. \*Significant difference from the control (i.e., cells cultured with 10% lipoprotein-deficient serum).

specific autoantibodies circulating in the plasma. The presence of high concentrations of circulating immune complexes, including those containing LDL, in the blood of patients with cardiovascular diseases and hyperlipidemias was demonstrated in many studies.<sup>23-29</sup> Immunoglobulins and other components of immune complexes were found in human atherosclerotic vessels and foam cells.<sup>30-32</sup> It was shown that the immune LDL-anti-apo B immunoglobulin G complex prepared in vitro brings about the accumulation of lipids in cultured macrophages and their subsequent transformation into foam cells.<sup>33,34</sup> It is possible that a circulating immune complex containing autoantibodies and LDL in vivo can just as well stimulate the deposition of intracellular lipids. In this case, the atherogenic factor removed from the serum of patients by passing it through a sorbent with immobilized LDL may be represented by autoantibodies to LDL.

It cannot be ruled out that the atherogenic factor is a serum component interacting with LDL that does not belong to immunoglobulins. As is known, LDLs are able to form insoluble complexes with certain substances and induce the accumulation of intracellular lipids after penetrating inside the cell within these complexes. It has been recently reported that the LDL-mediated accumulation of lipids in macrophages can occur when LDLs penetrate inside the cell within an insoluble complex with proteoglycans of aorta or a complex containing LDL, heparin, fibronectin, and denatured collagen.<sup>35-39</sup> Apparently, LDL complexes with proteoglycans, fibronectin, collagen, and other

connective tissue elements may be responsible for the deposition of intracellular lipids not only in culture but in vivo as well since such complexes are made up by the components present in the vessel wall. In our case, the factor imparting to LDL the ability to induce the accumulation of intracellular cholesterol is a serum component but not a part of the vessel wall extracellular matrix. However, a complex containing this factor and LDL can stimulate lipidosis in the same way as insoluble complexes composed of LDL and extracellular matrix components.

Whatever the nature of a nonlipid atherogenic factor may be, the fact of its discovery in the serum of patients with coronary atherosclerosis seems to be highly significant. Actually, its presence in the blood serves as a prerequisite for initial accumulation of lipids in the vessel wall cells.

It is hardly probable that atherogenicity of cardiovascular patients' serum is just a consequence of atherosclerosis. Earlier, we reported that about 20% of subjects without clinical signs of heart disease have an atherogenic serum, though it has a less prominent effect on cultured cells as compared with the serum of patients with coronary atherosclerosis (the amount of accumulated intracellular cholesterol is smaller).<sup>1</sup> Thus, blood of certain subjects who have no clinical manifestations of atherosclerosis possesses an atherogenic potential that might trigger the onset of fatty atherosclerotic lesions or facilitate the development of existing atheromas. Proceeding from these findings, one may assume that regular removal of the atherogenic factor from patients' blood may at least prevent the progression of atherosclerosis. The atherogenic factor can be removed by passing the blood plasma through a sorbent with immobilized LDL. Such a sorbent prepared on the basis of autologous patients' LDL can be used within a system of extracorporeal circulation to purify the plasma from the atherogenic factor. Clinical trials could establish the real role of this factor in the pathogenesis of the ailment basically manifested as atherosclerosis. It is possible that investigations in this direction would allow development of pioneer approaches to prevention and therapy of this ubiquitous disorder.

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