ance time of sheep cell agglutinins in mice was approximately 2 days, that of normal bactericidins, only a few minutes.

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Lipid Metabolism in Cultured Cells. II. Cholesterol Uptake from Serum of Normal and Atherosclerotic Human Adults.*† (26698)

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It has been shown that mammalian cells in culture take up considerable amounts of cholesterol from the serum used in the growth medium(1). It was found that the cholesterol content of cells was dependent both upon the type of cell cultured, and upon the type of serum used. Cholesterol uptake by cells was only indirectly related to cholesterol level in the medium, and appeared to be related more directly to the ratio of cholesterol to other unidentified components of the serum(2). The primary process in development of atherosclerotic lesions appears to be related to cellular deposition of cholesterol in the intima of the arterial walls(3). It was of interest therefore to determine if any differences could be found in deposition of cholesterol in cells cultured *in vitro* upon serum taken from normal subjects as compared with serum from atherosclerotic individuals.

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Materials and methods. Blood samples taken from normal and atherosclerotic subjects following overnight fasting were allowed to clot for 24 hours at 4° before centrifuging and collection of serum. Details of age, sex and clinical diagnosis of experimental subjects are given in Table I. Sterile conditions were maintained during collection, and serum samples were stored frozen at –40° until use. The strain of cells used in the studies reported here, the MB III strain of mouse lymphoblasts, was grown in roller tube cultures by methods described previously(1). Serum samples were deactivated by heating at 55° for 30 minutes before use. This heating procedure destroyed a toxic factor associated with most fresh serum samples. It did not affect the cholesterol content of cells grown on non-toxic serum.

Stock cultures of MB III cells in logarithmic growth phase, were harvested and washed and resuspended in Gey's balanced saline solution. Cell populations were determined by counting in a hemocytometer chamber, and aliquots of the suspension containing approximately 1 million cells were dispensed in duplicate into roller tubes containing the experimental serum samples. Gey's balanced salt solution was then added

TABLE I. Relationship between Serum Cholesterol, Age, and Cholesterol Content of Cells Grown on Serum in Normal and Atherosclerotic Human Subjects.

Normal adults				Atherosclerotics				
		Cholesterol					Cholesterol	
Sex	Age	In serum, mg %	In cells, %	Sex	Age	Diagnosis	In serum, mg %	In cells, %
♂	24	154	3.3	♂	77	M.I.	173	3.9
♂	26	170	3.2	♂	61	"	420	7.9
♂	29	164	3.0	♀	62	"	199	3.2
♂	29	213	3.2	♂	55	C	273	4.3
♂	29	142	3.3	♂	52	A	220	3.8
♂	30	128	2.8	♂	53	A	186	4.0
♂	30	146	3.0	♂	75	A	248	3.6
♂	31	—	3.1	♀	60	CVA	218	3.4
♂	31	206	3.7	♀	56	"	189	4.3
♂	31	207	4.3	♀	76	"	232	4.8
♀	32	189	3.3	♀	82	CBS	222	4.6
♂	37	225	3.6	♀	54	CVA	292	3.3
♂	40	—	4.0	♂	80	"	214	3.8
♀	41	195	3.6	♀	75	CBS	226	4.4
♂	41	162	3.0	♀	68	CVA	227	4.4
♀	45	219	3.7	♂	74	"	306	3.3
♀	48	257	4.8	♂	71	"	226	4.3
♀	57	324	4.6	♂	73	"	166	4.3
♂	58	197	4.3	♂	72	"	240	4.2
♀	58	280	4.2	♂	57	M.I.	181	3.3
				♀	80	CBS	226	3.5
Mean	37.35	198.8	3.60	♀	63	CVA	187	4.4
S.D.	±10.5	±49.2	±.575	♂	75	"	242	4.9
				♂	88	CBS	185	4.4
				♂	91	ASHD	167	3.8
				♂	83	CVA	186	3.7
				♀	73	CBS	256	3.5
				♂	82	"	240	3.3
				♀	78	"	298	4.0
				Mean	70.6		229.1	4.09
				S.D.	±34.2		±53	±.86

Stand. error of difference in cell cholesterol = .205.
Difference of mean = .49. $P < .01$.

Key to letter diagnosis: M.I., myocardial infarct; C, coronary occlusion; CVA, cerebro vascular accident; CBS, chronic brain syndrome; ASHD, arterio-sclerotic heart disease; A, angina pectoris.

so that final concentration of serum in the medium was 50%. Cells were grown for 4 days during which time the average population increase was 4-fold. The cells were harvested by centrifugation followed by washing with 2 successive portions of balanced saline to remove traces of medium. Cell lipids were extracted by refluxing with 5 ml of a 1:1 mixture of ethanol-ether for 30 minutes, and the extract was evaporated to dryness in a stream of nitrogen. The residue was then extracted twice with boiling petroleum ether (B.Pt. 30-60°) and cholesterol in the extract was determined by the method of Brown(4). The extracted cells were air dried at 110° for 1 hour and dry weight was determined by the colorimetric method of Bailey and Nejad(5). The cholesterol content of experimental serum samples was determined by using a similar double solvent extraction procedure.

In a second series of experiments a group of 6 rabbits were rendered atherosclerotic by feeding a diet containing 2% cholesterol in 5% lard for 3 months. A control group of 6 rabbits were maintained on a cholesterol-free diet. At the end of the 3 month period the animals were sacrificed and blood was obtained under sterile conditions by heart puncture. The cholesterol-fed group all had well developed plaques in the thoracic aorta, whereas the control group had none. Cells were grown on the serum obtained from both groups of animals and cholesterol content of

TABLE II. Cholesterol Content of Cells Grown on Normal and Atherosclerotic Rabbit Serum.

	No. in group	Mean serum cholesterol, mg %	Mean cell cholesterol, %
Control rabbits	6*	85.8 ± 27.1	7.80 ± 2.2
Atherosclerotic rabbits	6	2148 ±498	22.25 ± 2.89

* Results taken from ref. (2).

the harvested cells and the serum was determined as described above.

Results and discussion. The results of the experiments with cells grown on human serum samples are given in Table I. The mean serum cholesterol levels in the atherosclerotic group were significantly higher (13.6%) than those of the normal group. Cells grown on the atherosclerotic serum had a significantly higher cholesterol level than those grown on normal serum (4.09% *vs* 3.60%) and the mean elevation was again 13.6% suggesting a direct correlation between serum and cell cholesterol (see below). Examination of the group of normal sera shows a highly significant correlation between age of the donor and cell cholesterol level (correlation coefficient r = 0.73); however there was also a high correlation between serum cholesterol level and age of the donor, (r = 0.85) and also between serum cholesterol level and cell cholesterol (r = 0.86). In the atherosclerotic group there was a significant correlation between serum cholesterol and cell cholesterol (r = 0.5) but no significant correlation between age and cell cholesterol (r = 0.04) or age and serum cholesterol (r = 0.06). These results were analyzed by covariant analysis and the following conclusions were reached:—The increase in cell cholesterol with age of the donor in the normal group is entirely associated with the increase in serum cholesterol with age and the related correlation between serum cholesterol level and cell cholesterol. (r for cell cholesterol with age *independently* of serum cholesterol = 0; r for cell cholesterol with serum cholesterol *independently* of age = 0.67). The absence of significant correlation of serum cholesterol with age in the atherosclerotic group is to be expected in view of the high average age of this group (Table I). It has been shown that most of the increase in serum cholesterol occurs between 0 and 60 years of age(6).

The results for normal and atherosclerotic rabbits are given in Table II. The rabbits on the cholesterol diet showed a 20-25 fold increase in serum cholesterol over the 3-month period. Cells grown on the atherosclerotic rabbit serum had about 3 times the cholesterol content of those grown on normal rabbit serum. All rabbits in the cholesterol fed group developed 4+ plaques when graded according to the nomenclature of Wang(7). The interesting fact that the species from which the serum is derived is important in determining cell cholesterol levels, is apparent in these results. Normal rabbit serum has a lower cholesterol than normal human serum, yet grows cells with a higher cholesterol content. This apparent anomaly may be related to the ease with which this animal is rendered atherosclerotic by dietary means alone.

There is therefore no evidence in the experiments reported here for normal and atherosclerotic humans which suggests that factors other than the increase in serum cholesterol which occurs with ageing, are responsible for either the higher cholesterol content of cells grown on the atherosclerotic sera, or the increase in cell cholesterol with age of the donor in normal subjects. In view of the high average age of the atherosclerotic group (71 years) it may be questionable to consider this group as truly abnormal, since atherosclerosis is a fairly normal accompaniment of aging in this range. A more informative group of patients for experiments such as these might be atherosclerotics of lower age, so that comparative studies with young normals could be made with elimination of the age factor. The difficulty of obtaining sufficient numbers of patients in the latter category has prevented such a study from being carried out.

Summary. 1. The cholesterol content of cells grown on serum samples taken from normal and atherosclerotic humans and rabbits was determined. 2. Cells grown on atherosclerotic sera had significantly higher

cholesterol levels than those grown on normal sera. 3. In the group of normal human sera there was significant correlation between (a) age of the donor and cholesterol content of cells, (b) serum cholesterol level and cholesterol content of cells and (c) age of the donor and serum cholesterol level. 4. In the atherosclerotic human sera there was significant correlation between serum and cell cholesterol but none with age of the donor. 5. It appears from these results that in an older group of human atherosclerotics at least, there is no evidence that the higher cholesterol content of cells grown on the serum is due to factors other than the increase in serum cholesterol which occurs with aging.

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Increase of Plasma Lysozyme Activity Following Injections of Typhoid Vaccine.* (26699)

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Kerby has shown that white cells incubated *in vitro* with endotoxin from Gram-negative bacteria leak lysozyme into the fluid in which they are suspended(1). The studies reported here were undertaken to see whether injections of endotoxin into rabbits resulting in leukopenia were followed by an increase in plasma lysozyme activity.

Materials and methods. The lysozyme activity of rabbits' plasma was measured after intravenous injection of endotoxin or Newcastle disease virus (NDV). Blood was obtained from unanesthetized rabbits by repeated cardiac puncture using a syringe containing 0.3 ml of 5% sodium ethylene diamine tetraacetic acid/10 ml of blood.† Plasma was obtained free of cells by 2 centrifugations at 1000 rpm for 20 minutes at 4°C, and was stored at 4° until the assay of lysozyme activity was performed. Most of the assays were done within 8 hours after collection of blood, and none was delayed more than 24 hours. Pyrogen free glassware and syringes were used in preparation of plasma.

Lysozyme activity was measured by the rate at which plasma samples cleared turbid suspensions of *Micrococcus lysodeikticus*. Irradiated cell walls of *M. lysodeikticus* ("lysozyme substrate"—Difco Corp., Detroit, Mich.) were diluted in phosphate buffer pH 6.2 ("lysozyme buffer"—Difco) to a concentration of 0.5 mg/ml. Plasma was diluted in buffer 1/20, 1/40, or 1/80. Standard solutions containing 0.8 and 1.0 μg/ml of crystalline egg white lysozyme (Difco) were prepared in buffer.

Four ml of substrate and 4 ml of diluted plasma or standard solution were mixed in a cuvette and optical density was determined at 5 minute intervals in a Bausch and Lomb Spectronic 20 at 540 mμ wavelength. All tests were caried out at 32°C and the reactants were allowed to reach this temperature before mixing. The lysozyme concentration of plasma was determined by comparing the

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† Plasma of citrated blood had almost no detectible lysozyme activity.