The Role of Beta-Lipoprotein, Cholesterol, and Various Sera in Tissue Culture Intracellular Lipidosis (35287)

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Previous studies have indicated that rooster serum can induce the production of sudanophilic cytoplasmic inclusions within mouse fibroblasts (1). Similar lipidosis has also been observed within cultured cells by others (2–5) while Rutstein *et al.* (5) found that cholesterol, either in microcystalline or protein-bound form, was responsible for this inclusion formation. This suggestion as to the possible etiology for the observed inclusions, led to a study of the role played by cholesterol and its primary carrier, the serum β-lipoprotein, in cytoplasmic inclusion formation.

Materials and Methods. "Low line" mouse fibroblasts (NCTC No. 2445) were used as described in earlier reports (1). β-Lipoprotein was separated from serum either by ultracentrifugation (7, 8), or by precipitation with low molecular weight (60,000–90,000) dextran sulfate from Sigma (6). After resuspending the precipitate in 20% NaCl–0.1 *M* NaC_2O_4 solution, a majority of the dextran sulfate was removed with protamine sulfate (6) and the remainder with Sephadex-50. This preparation was then dialyzed twice against distilled water for a total of 3 hr, diluted with an equal volume of double strength Waymouth medium and sterilized by Millipore filtration. From β-lipoprotein-free serum, the α-lipoprotein and other serum proteins were salted out with $(NH_4)_2SO_4$ after which the precipitate was redissolved in water and prepared for cell culture as above. The purity of the β-lipoprotein preparation was checked electrophoretically and no α-lipoprotein or other serum protein contaminants were found to be present. Conversely, the α-lipoprotein suspension was found free of β-lipoprotein.

Cholesterol analysis was carried out by a method of Sperry and Webb (9) utilizing KOH hydrolysis of the ester and digitonin precipitation for the unesterified cholesterol.

Paper electrophoresis, utilizing a Veronal buffer at 8.6, was performed on a Beckman apparatus which included Analytrol disc integration for quantitation.

Protein-bound cholesterol was prepared by "jetting" 0.1 ml of a 1% cholesterol solution in 95% alcohol into 10 ml of sterile medium containing 1% bovine albumin (Sigma). By paper electrophoresis, this preparation was found to be free of lipoprotein and protein fractions other than albumin. After incubation at 37° for 24 hr, the unbound crystals were removed by passage through a Millipore filter. Microscrystalline suspensions of cholesterol were prepared in a similar manner except that unfiltered medium contained no protein.

The serum end-point titer was defined as the last dilution of test serum still capable of producing inclusions in cell cultures exposed to that dilution for 48 hr.

Eight- to 12-month-old Leghorn roosters served as experimental animals. Before blood was drawn, these roosters were subjected to one of four conditions as follows: (a) cage run with regular diet, (b) stress with regular diet, (c) stress with cholesterol diet, and (d) cage run with cholesterol diet. A high cholesterol diet was one to which 1% cholesterol was added to the regular diet. Stress conditions consisted of a 105°F temperature environment and 60% relative humidity for 24 hr, other conditions being unchanged. "Cage run" roosters were kept in an average sized cage with food and water continuously available and served as a negative control.

Results. Sera from various sources were found to vary in their capability for producing sudanophilic cytoplasmic inclusions (Table I), with human serum having the greatest

TABLE I. End-Point Titer of Serum from Various Sources.

Serum	No. of serum sources	Titer (mean)[a]
Human	6	1:255
Nonstressed rooster		
Regular diet (group a)	5	1:27
Cholesterol fed (young bird)	5	1:95
Cholesterol fed (old bird) (group d)	5	1:320
Calf	2	1:30
Fetal calf	2	1:50
Horse	2	1:5

[a] The last serum dilution producing intracellular inclusions.

potential and horse serum the least. Cholesterol feeding increased the ability of rooster serum to produce inclusions. This increase in end-point titer appeared to be directly related to serum cholesterol and β-lipoprotein content (Tables II, III). Furthermore, it was found that the titers paralleled the percentage of unesterified but not the percentage of esterified cholesterol (Table II), suggesting that it is the β-lipoprotein-bound unesterified cholesterol within the serum which is directly involved in inclusion formation.

β-Lipoprotein from either rooster, calf, or human serum in concentrations equivalent to that found in a 10% serum medium was found to elicit numerous inclusions (Fig. 1), which were identical to those produced by serum from stressed animals as reported earlier (1). Both S_f 0–20 and 20–400 fractions, isolated from rooster and human serum, produced inclusions. The α-lipoprotein preparation in equivalent concentrations did not induce inclusion formation; however, if the concentration of this lipoprotein was increased 3-fold, a few sudanophilic droplets did appear. β-Lipoproteins isolated from rooster, calf, human, and horse serum were all found to be active, but to varying degrees, paralleling the activity of the serum from which the lipoprotein was isolated. The linear relationship between end-point titer of the serum and its β-lipoprotein content, but not α-lipoprotein content, further serves to demonstrate that beta- and not alpha-lipoprotein is the major active serum protein factor (Table III).

Effect of bound cholesterol and cholesterol suspension in inclusion formation. Microcystalline suspensions of unesterified cholesterol (4 mg/100 ml) were found to elicit essentially no inclusions. However, if unesterified cholesterol bound to bovine albumin (3 mg/100 ml) was added, many sudanophilic droplets identical to those produced by β-lipoprotein appeared within the cells. Substitution of cholesterol acetate for unesterified cholesterol resulted in no formation of sudanophilic inclusions.

Discussion. The ability of protein-bound unesterified cholesterol to form typical sudanophilic inclusions and the direct relationship between unesterified cholesterol and end-point titer, suggests that bound or solubilized unesterified cholesterol is a major fac-

TABLE II. Relationship Between Serum Cholesterol and End-Point Titer of Rooster Serum Containing Various Cholesterol Levels.

Test conditions[a] (group)	Serum cholesterol					Titer[b] (mean of 5 birds)
	Total (mg/100 ml)	Esterified		Unesterified		
		(mg/100 ml)	% of total	(mg/100 ml)	% of total	
a	40.0	34.28	85.6	5.75	14.4	1:43
b	46.2	36.14	78.2	10.05	21.8	1:64
c	75.38	48.43	64.2	26.95	35.8	1:105
d	108.92	49.85	45.7	59.11	54.3	1:124

[a] Test conditions explained in Methods and Materials.

[b] The last serum dilution producing intracellular inclusions.

TABLE III. The Relationship Between Lipoprotein Concentrations and the End-Point Titer of Rooster Serum Containing Various Cholesterol Levels.

Test conditions[a] (group)	Lipoproteins (cm^2)		Titer[b] (mean of 5 birds)
	α	β	
a	9.2	12.7	1:60
b	9.0	10.0	1:72
c	3.1	36.0	1:124
d	3.8	44.8	1:148

[a] Test conditions explained in Methods and Materials.

[b] The last serum dilution producing intracellular inclusions.

tor responsible in inclusion formation by β-lipoprotein. If, however, this was the sole factor responsible, then one might expect media containing similar end-point titers of β-lipoprotein to have similar concentrations of lipoprotein-bound unesterified cholesterol regardless of the lipoprotein source. This appears not to be the case since these values range from 0.14 mg/100 ml for fetal calf to 0.68 mg/100 ml for rooster β-lipoprotein (Table IV). Moreover, one would expect the product of the end-point titer and the unesterified cholesterol concentration of the serum from roosters exposed to various environmental conditions to be approximately constant. These products are not constant however, but vary from 1.4, 1.6, and 2.6 to 4.8 for conditions a, b, c, and d, respectively. Possible explanations for these results include the positioning of the cholesterol on the β-lipoprotein, type or degree of charge on the complex, or other lipid components contained with the β-lipoprotein complex. One of the latter components, the esterified cholesterol, appears not to be directly involved in inclusion formation since bound esterified cholesterol is inversely related to end-point titer and does not induce inclusions when bound to bovine albumin. However, this does

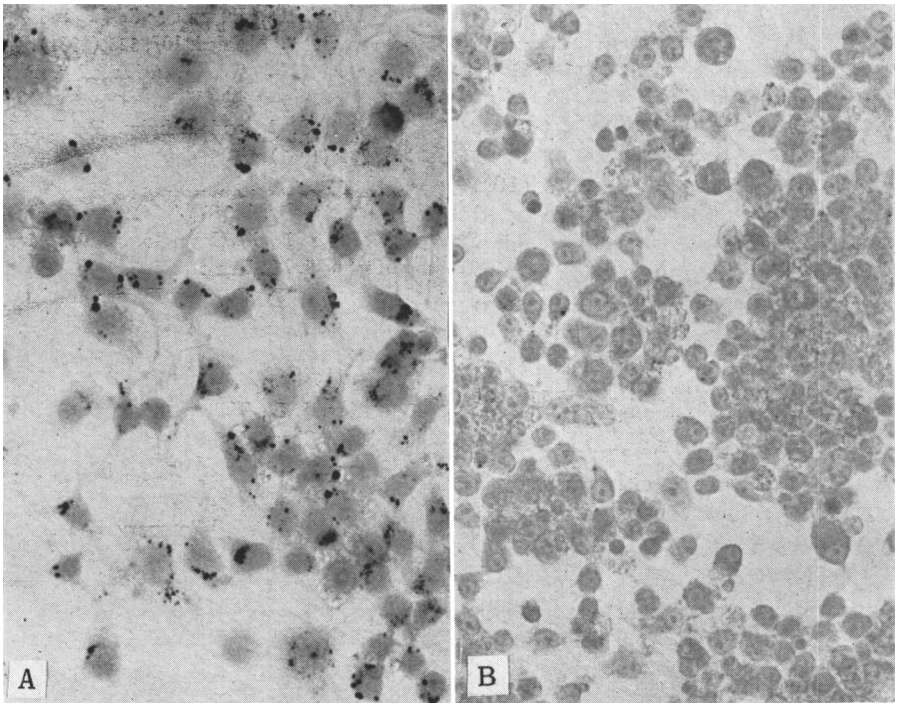

FIG 1 A. Mouse fibroblasts after 48-hr growth in medium containing human β-lipoprotein. The multiple dark staining, circular, sudanophilic inclusions within the cytoplasm of these cells are lipid material; ×125. (B) Mouse fibroblasts after 48-hr growth in medium containing human serum minus its β-lipoprotein. Note the absence of the dark staining cytoplasmic inclusions as shown in (A); 125×.

TABLE IV. The Variation in the Amount of β-Lipoprotein Bound Unesterified Cholesterol in Media with Equal Inclusion-Forming Potential, but with Different β-Lipoprotein Source.

Serum	No. of individual serum samples	Unesterified cholesterol bound to β-lipoprotein at end-titer dilution[a] (mg/100)
Human	10	0.18
Rooster		
Regular diet	5	0.68
Cholesterol fed	5	0.65
Calf	2	0.33
Fetal calf	2	0.14
Horse	5	0.30

[a] The last serum dilution producing intracellular inclusions.

not imply that esterified cholesterol does not influence indirectly the uptake or possibly the esterification of cholesterol in some unidentified way. Other lipid components of the lipoprotein have not been investigated and their roles are unknown.

The finding that β-lipoprotein is the factor responsible for inclusion formation is of particular interest since this serum component has been incriminated in atherogenesis (10). Whether the *in vitro* phenomenon reported here is similar to the mechanism of cholesterol deposition in atherosclerosis is unknown. However, if such a relationship should exist, then this *in vitro* system acting as a biological counterpart, may provide another means for studying the etiology of that disease.

Summary. Serum from human, calf, fetal calf, horse, and roosters fed either a regular or high cholesterol diet produced sundanophilic cytoplasmic inclusions within cloned mouse fibroblasts ("Low line" NCTC No. 2445) in tissue culture. Serum from roosters either fed a 1.0% cholesterol diet or subjected to heat stress exhibited an increased ability to produce cytoplasmic inclusion. The serum protein factor responsible for these inclusions proved to be the low density, β-lipoprotein. α-Lipoprotein produced inclusions only in high concentrations. Besides varying with different sera, the amount of lipidosis was found to be directly proportional to total cholesterol and to the percentage of β-lipoprotein-bound unesterified cholesterol, but inversely proportional to the percentage of bound esterified cholesterol. Furthermore, bovine albumin-bound cholesterol, only in the unesterified form, produced similar appearing sudanophilic, cytoplasmic inclusions. These observations suggest that the protein-bound, and more specifically, the β-lipoprotein-bound, unesterified cholesterol plays a significant role in inclusion formation, although other factors are porbably involved in this process.

1. Rose, K. D., Fuenning, S. I., and Maca, R., Proc. Soc. Exp. Biol. Med. **107**, 525 (1961).

2. Branwood, A. W., J. Atheroscler. Res. **1**, 358 (1961).

3. Pollak, O. J., and Kakubu, T., J. Atheroscler. Res. **1**, 229 (1961).

4. Myasnikov, A. L., Block, Y. E., and Pavlov, V. M., J. Atheroscler. **6**, 224 (1966).

5. Rutstein, D. D., Ingenito, E. F., Craig, J. M., and Martinelli, M., Lancet **274**, 545 (1958).

6. Cornwell, D. G., and Kruger, F. A., J. Lipid Res. **2**, 126 (1961).

7. Burstein, M., and Somaille, J., J. Physiol. (Paris) **49**, 83 (1957).

8. Oncley, J. L., Walton, K. W., and Cornwell, D. G., J. Amer. Chem. Soc. **79**, 4666 (1957).

9. Sperry, W. M., and Webb, M., J. Biol. Chem. **187**, 97 (1950).

10. Gofman, J. W., "Coronary Heart Disease," p. 353. Thomas, Springfield, Ill. (1959).

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