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The Growth Characteristics of Novikoff Hepatoma Cells in the Presence of Different Fatty Acid:Albumin Ratios[1] (39817)

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Steele and Jenkin (1) grew Novikoff hepatoma cells *in vitro* in the presence of various concentrations of stearic (18:0), petroselinic (6-18:1) or oleic (9-18:1) acids, and observed that the best rate of growth occurred when the growth medium contained 9-18:1 and the poorest when it contained 18:0, with medium containing 6-18:1 giving an intermediate rate of growth. Spector and Steinberg (2) have found that for any given fatty acid:albumin ratio, absorption of fatty acids by cells cultured *in vitro* increases as the chain length of the fatty acid increases but decreases with unsaturation. The melting point of fatty acids increases with chain length but decreases with unsaturation. Since the melting point of 6-18:1 is intermediate between that of 18:0 and 9-18:1, it is tempting to speculate that it is the physical characteristics of each fatty acid which determine its effect on growth rate of cells.

Davis and Dubos (3) have demonstrated that serum albumin acts as a protective growth factor for the tubercle bacillus by binding traces of fatty acid in the growth medium. They also found that albumin could protect erythrocytes against hemolysis in the presence of 9-18:1. If the mechanism whereby fatty acids affect the integrity of the tubercle bacillus or erythrocytes is the same as that for Novikoff hepatoma cells, then it should be possible to prevent 18:0 or 6-18:1 from damaging these cells by increasing the concentration of albumin in the growth medium. Further, if these assumptions are correct, more albumin per unit weight of 18:0 would be necessary to reverse the cytotoxic effect on cells than of 6-18:1. A series of investigations has been carried out to test this hypothesis and the results are now presented.

Materials and methods. Novikoff hepatoma cells (NISI-67) were a gift from Dr. P. G. Plagemann, University of Minnesota (4). The cells were grown as described in a previous publication (5). At the beginning of each investigation in this series of experiments, all flasks were seeded with 2×10^5 cells/ml taken from a population in the log phase of growth. All incubations were carried out at 37° in a water-bath shaker. Each treatment was carried out in triplicate and a minimum of two independent experiments was performed using each treatment. Cell numbers were determined with the aid of a hemocytometer (6).

The fatty acids used in these experiments were obtained from the Lipids Preparation Laboratory, The Hormel Institute, Austin, Minnesota, or Nu-Chek Preparations, Elysian, Minnesota. The purity of these compounds was greater than 99% as determined by thin-layer chromatography (TLC) and gas-liquid chromatography (GLC). The preparation of the sodium salts and the method of their dispersion in the growth medium using bovine serum albumin as a carrier have been previously reported (5). The bovine serum albumin was Fraction V, fatty acid free, obtained from Pentex, Inc., Miles Laboratories, Kankakee, Illinois.

The growth medium contained 5% fetal calf serum which resulted in an inherent concentration of 0.025 m*M* albumin. Since the fetal calf serum contained very low concentrations of free fatty acid and the bovine serum albumin was virtually fatty acid free, the concentration of total free fatty acid in the control medium was less than 0.005 m*M*.

The results were analyzed statistically by the methods outlined by Snedecor and Cochran (7).

Results. The effects of increasing the con-

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centration of bovine serum albumin (BSA) in the growth medium of Novikoff hepatoma cells are shown in Table I. When the concentration of BSA in the medium was increased there was a gradual reduction in the growth rate of the cells and, at a concentration of 0.22 m*M*, the maximum cell numbers were about 80% of those obtained in the unsupplemented medium.

In another series of experiments the concentration of BSA in the growth medium was kept at a constant level and the concentrations of each of three fatty acids, stearic (18:0), petroselinic (6–18:1), and oleic (9–18:1), were varied, and the resultant effects on the growth of the hepatoma cells were measured (Table II). When the ratio of 9–18:1 to BSA was increased above the normal physiological value of about 3, the growth characteristics of the cells were not significantly altered. However, at high fatty acid:albumin ratios the presence of both 6–18:1 and 18:0, especially the latter, significantly reduced the growth rate of the cells.

In an attempt to reverse the detrimental effects of high levels of these two fatty acids in the growth medium, the concentration of BSA was increased. In the presence of 6–18:1 (Table III) it was found that, with an increase in the amount of BSA to 0.1 m*M*, cells growing in medium containing 0.44 m*M* 6–18:1 had the same growth characteristics as those in medium containing no 6–18:1. However, before the growth rate of the cells in medium containing 0.71 m*M* 6–18:1 could be increased to the level of the controls, the concentration of albumin had to be increased to 0.18 m*M*. Further increases in the concentration of BSA above this amount resulted in a small reduction in cell numbers.

When the concentration of BSA was in-

TABLE I. GROWTH OF NOVIKOFF HEPATOMA CELLS IN SWIM'S G7-G MEDIUM CONTAINING VARIOUS CONCENTRATIONS OF BOVINE SERUM ALBUMIN.

	Bovine albumin (m*M*)			
Time (hr)	0.025	0.055	0.175	0.220
48	1.80 ± 0.21[a]	1.80 ± 0.22	1.50 ± 0.31	1.35 ± 0.27
60	2.40 ± 0.29	2.40 ± 0.28	2.05 ± 0.33	1.75 ± 0.31
72	2.25 ± 0.31	2.20 ± 0.33	2.00 ± 0.38	1.55 ± 0.33
96	1.75 ± 0.40	1.70 ± 0.39	1.45 ± 0.33	1.15 ± 0.38

[a] Cell count × 10^6/ml ± SE. The cell population at 0 hr was 2 × 10^5/ml.

TABLE II. GROWTH OF NOVIKOFF HEPATOMA CELLS IN SWIM'S 67-G MEDIUM CONTAINING 0.044 m*M* BOVINE SERUM ALBUMIN (BSA) IN THE PRESENCE OF MOLAR RATIOS OF VARIOUS FATTY ACIDS:BSA.

	Ratio of fatty acid:bovine albumin				
Time (hr)	0	1.5	3.0	6.0	9.0
			6–18:1 (Petroselinic acid)		
48	2.30 ± 0.25[a]	2.50 ± 0.31	2.20 ± 0.35	1.45 ± 0.21	0.50 ± 0.20
60	2.80 ± 0.31	3.50 ± 0.28	2.70 ± 0.38	1.60 ± 0.28	0.70 ± 0.21
72	2.50 ± 0.42	3.00 ± 0.29	2.00 ± 0.40	1.50 ± 0.30	0.90 ± 0.31
96	1.20 ± 0.41	1.50 ± 0.39	0.65 ± 0.21	0.60 ± 0.22	0.85 ± 0.30
			9–18:1 (Oleic acid)		
48		2.90 ± 0.25	2.70 ± 0.30	2.30 ± 0.31	2.00 ± 0.30
60	See 6–18:1	3.60 ± 0.18	2.90 ± 0.35	2.40 ± 0.35	2.25 ± 0.35
72		3.10 ± 0.22	2.60 ± 0.28	2.10 ± 0.40	2.00 ± 0.38
96		1.70 ± 0.31	1.30 ± 0.30	0.95 ± 0.28	0.90 ± 0.29
			18:0 (Stearic acid)		
48	See 6–18:1	2.10 ± 0.31	1.70 ± 0.21	0.40 ± 0.30	0.20 ± 0.15
60		2.60 ± 0.35	2.00 ± 0.22	0.50 ± 0.25	0.15 ± 0.10
72		2.20 ± 0.30	1.60 ± 0.25	0.90 ± 0.25	0.10 ± 0.09
96		0.90 ± 0.20	0.80 ± 0.25	1.00 ± 0.30	<0.01

[a] Cell count × 10^6/ml ± SE. The cell population at 0 hr was 2 × 10^5 cells/ml.

creased in the medium in the presence of different amounts of 18:0, it was found that the number of cells in medium containing 0.35 m*M* of 18:0 did not reach the same number as those cells in the control medium even at the lowest fatty acid:albumin ratios (Table IV). It is of interest to note that the number of cells in 0.35 m*M* 18:0 was always less than those growing in 0.70 m*M* 6-18:1 or 9-18:1, irrespective of the fatty acid: albumin ratio.

In each observation where cell numbers were drastically reduced by high ratios of fatty acid:albumin, the cell size was very much increased.

Discussion. The results of the present series of experiments support the hypothesis that it is possible to eliminate the toxic effects of certain long-chain fatty acids on Novikoff hepatoma cells by increasing the amount of albumin in the growth medium, and that more albumin per unit weight is required to do this in the presence of 18:0 than in the presence of 6-18:1. However, these investigations also showed that there is a limit to the amount of albumin that can be added to the medium before there is an adverse effect on growth rate of the cells.

The blood serum of most species contains about 3-4% albumin, yet in the present experiments when the amount of albumin in the growth medium was increased to 1.5% (0.22 m*M*) the growth rate of the hepatoma cells was reduced. The two most likely explanations for this are that the albumin was toxic to the rat cells because it was obtained from a different species (bovine) or that the albumin concentrations of interstitial fluid

TABLE III. GROWTH OF NOVIKOFF HEPATOMA CELLS IN MEDIUM CONTAINING DIFFERENT MOLAR AMOUNTS OF 6-18:1 (PETROSELINIC ACID) AND BOVINE SERUM ALBUMIN (BSA).

BSA (m*M*)	0.025		0.055			0.110	
6-18:1 (m*M*)	0.00	0.00	0.45	0.71	0.00	0.45	0.71
Time (hr)							
48	1.70 ± 0.30	1.75 ± 0.21[a]	0.25 ± 0.20	<0.10	1.60 ± 0.22	1.50 ± 0.40	0.40 ± 0.15
60	2.35 ± 0.31	2.40 ± 0.33	0.35 ± 0.15	<0.10	2.20 ± 0.25	2.00 ± 0.30	0.65 ± 0.20
72	2.20 ± 0.26	2.10 ± 0.35	0.30 ± 0.20	<0.10	2.00 ± 0.25	1.90 ± 0.31	0.70 ± 0.25
96	1.80 ± 0.31	1.70 ± 0.40	0.10 ± 0.10	<0.10	1.55 ± 0.28	1.60 ± 0.21	0.55 ± 0.25
		0.175			0.200		
	0.00	0.45	0.71		0.00	0.45	0.71
48	1.40 ± 0.30	1.50 ± 0.30	1.35 ± 0.29		1.30 ± 0.29	1.20 ± 0.33	1.10 ± 0.29
60	2.00 ± 0.33	2.00 ± 0.25	1.80 ± 0.31		1.70 ± 0.30	1.65 ± 0.28	1.45 ± 0.30
72	1.90 ± 0.35	1.95 ± 0.31	1.85 ± 0.29		1.45 ± 0.30	1.35 ± 0.28	1.30 ± 0.31
96	1.40 ± 0.40	1.40 ± 0.25	1.35 ± 0.31		1.05 ± 0.25	1.00 ± 0.30	0.90 ± 0.33

[a] Cell count × 10^6/ml ± SE. The cell population at 0 hr was 2 × 10^5 cells/ml.

TABLE IV. GROWTH OF NOVIKOFF HEPATOMA CELLS IN SWIM'S 67-G MEDIUM CONTAINING VARIOUS MOLAR AMOUNTS OF 18:0 (STEARIC ACID) AND BOVINE SERUM ALBUMIN (BSA).

BSA (m*M*)	0.025		0.055			0.110	
18:0 (m*M*)	0.00	0.00	0.175	0.35	0.00	0.175	0.35
Time (hr)							
48	1.85 ± 0.30	1.85 ± 0.35[a]	1.70 ± 0.38	0.30 ± 0.20	1.70 ± 0.32	1.70 ± 0.22	1.10 ± 0.28
60	2.50 ± 0.30	2.55 ± 0.35	2.30 ± 0.30	0.35 ± 0.25	2.35 ± 0.29	2.40 ± 0.25	1.60 ± 0.31
72	2.30 ± 0.40	2.25 ± 0.39	2.00 ± 0.35	0.40 ± 0.20	2.15 ± 0.38	2.20 ± 0.20	1.70 ± 0.29
96	1.85 ± 0.41	1.80 ± 0.38	1.60 ± 0.33	0.25 ± 0.25	1.60 ± 0.29	1.80 ± 0.28	1.40 ± 0.31
		0.175			0.220		
	0.50	0.175	0.35		0.00	0.175	0.35
Time (hr)							
48	1.50 ± 0.29	1.40 ± 0.22	1.00 ± 0.30		1.35 ± 0.32	1.35 ± 0.35	0.80 ± 0.22
60	2.10 ± 0.40	2.00 ± 0.31	1.15 ± 0.21		1.80 ± 0.39	1.80 ± 0.35	1.00 ± 0.30
72	1.95 ± 0.40	1.80 ± 0.39	1.25 ± 0.35		1.60 ± 0.32	1.50 ± 0.34	1.20 ± 0.30
96	1.45 ± 0.42	1.40 ± 0.39	1.10 ± 0.39		1.10 ± 0.29	0.90 ± 0.22	0.80 ± 0.33

[a] Cell count × 10^6/ml ± SE. The cell population at 0 hr was 2 × 10^5 cells/ml.

are much lower than those of blood serum. Although it is difficult to determine the exact concentration of albumin in the interstitial fluid of tissues, there seems little doubt that the values for the albumin concentration in interstitial fluid are very much lower than those in blood serum irrespective of which method is used in the determination (8). It would therefore appear that the presence of high amounts of albumin in cell culture medium per se is toxic to the Novikoff hepatoma cells.

It has been noted in the present and in earlier experiments (9) that when the growth rate of the hepatoma cells is reduced by high concentrations of such acids as 18:0 and 6–18:1, the cell size is increased. Presumably this is induced as a mechanism whereby the cell presents as small a surface area as possible per unit weight to the toxic medium and can thereby reduce the absorption rate of toxic material.

Albumin binds 9–18:1 to a greater extent than it binds 18:0 (2). However, at any given fatty acid:albumin ratio, cells cultured *in vitro* adsorb 18:0 to a greater extent than 9–18:1 (6). It is thus difficult to know whether these values are a true reflection of fatty acid binding by the cells or whether the differential strength of the binding of individual fatty acids by the albumin results in a decrease of certain acids being released for uptake by the cells. In similar experiments Spector *et al.* (10, 11) also found that the longer-chain saturated fatty acids are more tightly bound by albumin than the shorter ones. In the present series of experiments, when the Novikoff hepatoma cells were incubated in medium which contained very high ratios of 9–18:1 albumin it was expected that a large amount of the fatty acid would only be weakly bound to the albumin (2). However, the rates of growth of the cells were not significantly different from those of the cells grown in the unsupplemented medium. Furthermore, when the cells were grown in the presence of small amounts of 18:0 the association between fatty acid and albumin was expected to be strong (10), yet the rates of growth of the cells were very much reduced compared to those of the cells grown in the control medium. These results therefore strongly support the conclusions that cells bind unsaturated fatty acids less strongly than they bind saturated fatty acids. It would therefore seem that the type of binding between fatty acids and albumin is different from the type of binding between fatty acids and cells. Although at the present time it is difficult to give the precise reason for this difference, it is, nevertheless, tempting to speculate that, since there is more likely to be an association between the individual molecules of 18:0 than between individual molecules of 9–18:1, fatty acids may adsorb onto cell surfaces in a nonspecific manner, whereas the geometric configuration of the fatty acid affects its association with albumin.

Summary. The growth characteristics of Novikoff hepatoma cells can be altered by varying the amount of albumin or by altering the ratio of certain long-chain fatty acids:albumin in the growth medium.

(i) When the concentration of albumin in the growth medium was increased to 0.22 m*M*, the rate of cell proliferation was reduced by about 20%.

(ii) The growth rate of the cells remained unaltered even when the concentration of oleic acid (9–18:1) was increased to 0.49 m*M* or when the 9–18:1:albumin ratio was increased to 9:1.

(iii) The rate of cell multiplication was significantly reduced when the concentration of petroselinic acid (6–18:1) in the medium was increased to 0.33 m*M* at a 6–18:1:albumin ratio of 6:1. Provided that the level of albumin in the growth medium was increased to 0.18 m*M*, the concentration of 6–18:1 could be increased to 0.71 m*M* without affecting cell production.

(iv) When the concentration of stearic acid (18:0) in the growth medium was increased to 0.35 m*M* and the ratio of 18:0:albumin was kept at 6:1, there was a large reduction in cell numbers which could be only partially reversed by increasing the concentration of albumin in the growth medium.

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