MANIPULATION OF FATTY ACID COMPOSITION IN ANIMAL CELLS WHICH REQUIRE SERUM

Robert E. Williams, Joseph K.-K. Li and C. Fred Fox

Department of Bacteriology and the Molecular Biology Institute,
University of California, Los Angeles, California 90024

Received December 4,1974

SUMMARY. Animal cells with obligate serum requirements for proliferation in culture have been propagated under conditions which permit controlled manipulation of fatty acid composition of the cellular phospholipids. Avian fibroblasts and mammalian cell lines such as BHK21, 3T3, SV40-transformed 3T3 and VA-2 utilize fatty acids supplied as Tweens when a salt fractionated serum preparation is used to promote cell proliferation. It was possible to increase the saturated fatty acid content of the cellular phospholipids by approximately 2-fold without rendering the cells incapable of serial propagation.

INTRODUCTION

Manipulation of the lipid composition of cells grown in culture has been an effective tool in studying the effects of lipid physical state on the function and assembly of membranes in bacterial and fungal systems (1-2). The application of this approach has recently been extended to an animal cell line, LM, which grows in chemically defined, serum-free medium (3-4). Our success in controlling the physical state of membrane lipids of LM cells (5) encouraged us to explore ways to manipulate the fatty acid composition of cells which exhibit a strict serum requirement. It is generally accepted that the lipids of animal cells grown in serum contain abundant quantities of unsaturated and polyunsaturated fatty acids derived from serum. If the fatty acid composition of cells is to be controlled, it is necessary to find a means of reducing the lipid content of serum without losing the factors required for cell proliferation.

A considerable body of knowledge exists on the biological activity of various growth supporting substances in serum, e.g., fetuin, insulin, and various globulins (6-8). In this paper we report the use of salt fractionated serum (SFS) which contains growth factors that support cell proliferation. Using SFS, the fatty acid composition can be manipulated by a simple

modification of the methods of Wisnieski et al. (3) and Williams et al. (4).

MATERIALS AND METHODS

Organisms.--Mouse fibroblast cells 3T3 C142 and SV40-3T3 C16 were provided by G.J. Todaro (N.I.H., Bethesda, Md.). Mouse LM cells and baby hamster kidney cells, BHK21-(C-13) were obtained from the American Type Culture Collection, and VA-2 cells from M. Edidin (Johns Hopkins Univ.).

Media and Culture Procedures.--The 3T3 and SV3T3 lines were grown in Dulbecco's modified Eagle's medium (DME, GIBCO) plus 10% calf serum (GIBCO). BHK21 was cultured in Eagle's minimal essential medium with Hank's salts (MEM, GIBCO) supplemented with 10% tryptose phosphate broth (Bacto-tryptose, 20 g/l; glucose, 10 g/l; NaCl, 5 g/l; and Na_2HPO_4, 2.5 g/l) and 5% calf serum. This medium was further modified to contain twice the normal glutamine and vitamin concentrations. Monolayer cultures were established in 250 ml Falcon flasks containing 15 ml of medium. Enrichment experiments were in 100 x 15 mm Falcon tissue culture dishes. At saturation density, LM cells were passed as previously described (3). BHK21 and 3T3 cell lines were passed as follows: Trypsin (Sigma: type 2, pancreatic; 1500 BAEE units/mg) 0.25% in Tris•saline (Tris, 3 g/l; NaCl, 8 g/l; and Na_2HPO_4, 0.1 g/l, adjusted to a pH of 7.4 with HCl) was used for 3T3 and SV3T3. BHK21 cells were passed using a trypsin concentration of 0.05%. BHK21 cells were passed at saturation density at a dilution of 1:30; SV3T3 cells at a dilution of 1:25 and 3T3 cells before saturation was achieved at a dilution of 1:10. All cell lines were incubated at 37° with humidified air and 6% CO_2.

Growth Factor Isolation.--Salt fractionated calf serum (SFS) was prepared by ammonium sulfate precipitation of calf serum using a modification of the gamma globulin fractionation procedure described by Campbell et al. (9). Ammonium sulfate solution (300 ml at 69.7 g/100 ml) was added to 500 ml of calf serum with stirring and left for 24 hr at 4°. The precipitate was collected by centrifugation at 10,400 x g for 20 min and dissolved in 500 ml of Tris•saline. Ammonium sulfate solution (300 ml) was again added

TABLE 1. The effects of SFS supplementation[a]

Cell line	Serum %	SFS %	Saturated fatty acids %	Polyenoic fatty acids[b] %	Doublings of cell number
LM	0.5[c]	0.0	28.0	<0.1	3.0
LM	0.0	15.0	25.5	3.7	3.2
BHK21	10.0	0.0	25.0	6.2	4.0
BHK21	0.0	15.0	21.5	10.2	3.1
3T3	10.0	0.0	35.3	23.5	3.9
3T3	0.0	15.0	31.8	11.5	2.6

[a]Compositional data is from fatty acid analysis of phosphatidylcholine plus phosphatidylethanolamine. Basal media (Materials and Methods) were supplemented with serum of SFS. Cultures were inoculated as follows and fatty acid anlysis determined after three days of growth: LM, 1.4 x 10^4 cell cm^{-2}; BHK21, 1.6 x 10^4 cell cm^{-2}; and 3T3, 1.9 x 10^4 cell cm^{-2}.

[b]Polyunsaturated fatty acids; e.g., 18:2, 18:3 and 20:4.

[c]Control LM cells were grown with 0.5% peptone in place of serum.

TABLE 2. Fatty acid composition alteration as a function of SFS concentration[a]

Cell line	SFS %	19:0 + 19:1 %	Odd chain %	Saturated %	Doublings of cell number
LM	1	34.7	39.7	47.9	1.5
	5	23.2	28.7	37.2	1.9
	10	26.9	30.6	39.4	2.2
	15	24.4	29.9	37.1	2.5
BHK21	5	35.5	44.4	48.0	0.2
	10	34.2	39.5	41.8	1.0
	15	26.7	30.9	39.0	2.2
	20	27.3	31.3	37.8	2.2
3T3	5	47.3	51.4	46.5	0.2
	10	39.2	42.5	43.6	1.2
	15	24.5	29.1	40.4	1.4
	20	24.5	28.5	38.6	1.3
SV3T3	5	65.9	72.6	86.5	-0-
	10	41.4	52.1	54.5	1.8
	15	33.2	41.8	47.9	2.5
	20	49.1	51.7	50.1	3.0

[a]Fatty acid composition was determined for phosphatidylcholine plus phosphatidylethanolamine. Basal media were supplemented with 8 µg/ml of Tween-19:0 plus the appropriate SFS concentration. Cultures were inoculated with the following cell densities: LM, 2.2 x 10^4 cells cm^{-2}; BHK21, 1.6 x 10^4 cells cm^{-2}; 3T3, 3.8 x 10^4 cells cm^{-2}; and SV3T3, 1.3 x 10^4 cells cm^{-2}. Fatty acid compositions were determined after three days of growth.

and the procedure repeated. The pellet was dissolved in 300 ml of Tris•saline and dialyzed 4 times against 4 liters of Tris•saline at 4°. The dialyzed fraction (SFS) was adjusted to 500 ml with MEM. Gentamicin (Schering Corp.) was added at a final concentration of 200 μg/ml, and the SFS was filtered and stored at -20°. Lipid analysis of total long chain fatty acids greater than lauric acid showed that serum contained greater than 600 μg/ml of fatty acid compared with less than 50 μg/ml for SFS.

Fatty Acid Supplements.--The fatty acid supplement, Tween-nonadecanoic acid (Tween-19:0) has been described (3-4). The procedure for enriching cultured animal cells with exogenously-supplied fatty acids was a modification of that used for LM cells (3-4), except for omission of biotin analogs.

Analysis of Cellular Growth and Viability.--Cell growth and viability were measured as previously described (4).

Lipid Analysis.--Phospholipids were extracted, and phosphatidylcholine (PC) and phosphatidylethanolamine (PE) isolated by thin-layer chromatography (3-4). Gas-liquid chromatography of methylated fatty acids employed stainless steel analytical columns (6 ft by 1/8 inch), and the following supports were used: 10% EGSS-X on Chromosorb W (DMCS), 100/120 mesh and 3% OV-1 on Chromosorb W (DMCS), 80/100 mesh, the latter to quantitate 19:0 and 18:2 which are poorly resolved on the former. Runs were temperature programmed from 135° to 200° at 4°/min, or alternatively done isothermally at 170°. Peak areas and retention times were calculated on an Infotronic digital integrator.

RESULTS AND DISCUSSION

Effects of Salt Fractionated Serum.--In our initial attempts to modify the fatty acid composition of cells grown in the presence of serum, the major problem encountered was the uptake of large quantities of polyunsaturated fatty acids. This was obviated by reducing the lipid content of serum (Materials and Methods). LM cells, which have no serum requirement, contained over 20% polyenoic fatty acids in phospholipids when grown with

TABLE 3. Fatty acid composition alteration as a function of Tween-19:0 concentration[a]

Cell line	Tween-19:0 µg/ml	19:0 + 19:1 %	Odd Chain %	Saturated %	Doublings of cell number
LM	0	-0-	1.0	25.5	
	4	21.3	23.9	40.4	2.5
	8	31.3	34.4	43.5	2.0
	12	38.5	43.7	46.1	1.7
	16	48.5	54.7	51.9	1.5
BHK21	0	-0-	1.8	21.5	
	4	21.9	27.3	37.9	2.4
	8	34.1	42.9	45.0	2.1
	12	43.1	53.8	57.3	0.5
	16	50.4	61.5	60.7	-0-
3T3	0	-0-	0.6	31.8	
	4	25.1	30.0	38.4	2.6
	8	39.7	47.8	40.9	2.2
	12	54.0	62.7	48.6	1.6
	16	43.0	52.5	52.1	-0-
SV3T3	4	26.4	29.3	42.1	2.5
	8	35.9	41.2	48.7	2.1
	12	44.0	53.6	51.6	1.0
	16	48.5	58.2	50.4	0.5

[a]Fatty acid composition was determined for phosphatidylcholine plus phosphatidylethanolamine. Basal media were supplemented with 15% SFS plus different concentrations of Tween-19:0. Cultures were inoculated at the following cell densities: LM, 3.1 x 10^4 cells cm^{-2}; BHK21, 1.1 x 10^4 cells cm^{-2}; 3T3, 1.6 x 10^4 cells cm^{-2}; and SV3T3, 4.0 x 10^4 cells cm^{-2}. Fatty acid compositions were determined after three days of growth.

10% calf serum (not shown). This was reduced to less than 4% (Table 1) when the cells were maintained in medium supplemented with 15% SFS. Substitution of SFS for serum sometimes led to little change in polyenoic fatty acid content in BHK21 cells. Most other lines (SV3T3, VA-2 and chick embryo secondary cultures) exhibited properties similar to those displayed by 3T3, where polyenoic fatty acid content was greatly reduced by substituting SFS for serum.

Effects of SFS and Tween-19:0 Concentrations on Uptake of 19:0 into Cellular Phospholipids.--The effects of varying SFS concentration on the extent of cell proliferation and fatty acid compositional modification are described in Table 2. Optimal cellular proliferation was obtained at 15%

SFS, and increasing SFS from 10 to 20% decreased uptake of odd chain acids only slightly. When SFS concentration was held constant at 15% and Tween-19:0 varied, concentrations greater than 8 μg/ml usually inhibited growth severely (Table 3). The optimal concentrations for SFS and Tween-19:0 were thus approximately 15% and 8 μg/ml, respectively.

Analysis of the human cell line VA-2 grown with 15% SFS plus 8 μg/ml of Tween-19:0 revealed 38% of 19:0 + 19:1, almost twice the enrichment observed in other cell lines (not shown), and high saturated fatty acid content (57%). Chick secondaries grown with 19:0 had 34% 19:0 + 19:1, but have a much greater tendancy to desaturate 19:0 than do the established mammalian lines.

Serial Propagation of Cells Maintained with SFS and Tween-19:0 Supplements.--LM cells, though they do not normally require serum for serial propagation, often deteriorate when propagated serially in the presence of Tween-19:0. This can be avoided by maintaining the cells in medium containing SFS (Table 4). Though LM cells normally have no serum requirement, they are more sensitive to manipulations on passage than are 19:0-modified BHK21 cells, and require passage at high density when maintained with Tween-19:0. Both the LM and BHK21 cells propagated through the two passages described in Table 4 were viable and formed confluent cultures when passed into medium containing SFS and Tween-19:0 at approximately the inoculum levels described for Pass II.

Using LM cells in which the fatty acid composition of lipids was modified during growth in serum free medium, we have demonstrated altered properties of the cell surface membrane (5,10-11). Horwitz et al. (12) have made similar observations using 3T3 and SV3T3 cells with lipid fatty acid composition modified during growth with solvent extracted serum, but they did not report if cells can be propagated serially under their conditions. In the companion paper, we report the use of the technique described here to study the critical temperature for irreversible adsorption and subsequent

TABLE 4. Fatty acid composition alteration as a function of continual maintenance[a]

Cell line	Fatty acid	Control	SFS Pass I	SFS Pass II	SFS + Tween-19:0[b] Pass I	SFS + Tween-19:0[b] Pass II
LM	≤15:0	2.6	1.9	1.0	0.7	0.8
	16:0	14.7	16.2	14.3	15.9	5.4
	16:1	9.1	9.1	5.7	5.2	2.9
	17:0	0.2	0.0	0.1	0.9	3.7
	17:1	0.2	0.6	0.6	2.1	5.2
	18:0	10.0	10.1	13.8	10.5	5.0
	18:1	59.5	54.8	61.6	45.3	28.0
	18:2	-0-	3.7	1.3	2.9	3.7
	19:0	-0-	-0-	-0-	10.5	35.5
	19:1	-0-	-0-	-0-	4.7	8.3
	Other[c]	3.7	3.6	1.6	1.3	1.5
	% Saturated	27.5	28.2	29.2	38.5	50.4
	Doublings of cell number	3.0	3.2	2.9	2.5	1.9
	Inoculation[d]	1.4	1.4	5.5[e]	1.4	7.0[f]
BHK21	≤15:0	4.0	0.5	0.8	1.3	0.7
	16:0	17.9	8.2	13.5	5.0	3.0
	16:1	15.7	4.5	11.7	1.0	2.0
	17:0	0.2	0	0	4.4	4.0
	17:1	0.4	1.9	1.5	3.7	3.5
	18:0	10.8	10.3	5.4	6.4	5.5
	18:1	43.6	63.5	52.4	30.3	13.4
	18:2	1.7	9.6	13.8	3.3	3.8
	19:0	-0-	-0-	-0-	37.9	56.1
	19:1	-0-	-0-	-0-	6.0	4.0
	Other[c]	5.7	1.5	0.9	0.7	4.0
	% Saturated	32.9	19.0	19.7	55.0	69.3
	Doublings of cell number	4.0	3.1	3.0	2.2	2.0
	Inoculation[d]	1.6	1.6	2.1[f]	1.6	1.1[f]

[a]Fatty acid composition was determined for phosphatidylcholine plus phosphatidylethanolamine. Control cells were grown with 0.5% peptone for LM and 10% calf serum plus 10% TPB for BHK21. SFS concentration was maintained at 15% throughout the entire procedure. LM and BHK21 cells were extracted for lipid analysis after 3 days of growth for Pass I, and after 6 days for Pass II. [b]Tween-19:0 concentration was 8 μg/ml. [c]Other: unsaturated fatty acids, e.g., 20:1, 20:4, 18:3. [d]Cells x 10^4 cm^{-2}. [e]High cell density not required. [f]High cell density required.

propagation of Newcastle disease virus in BHK21 cells with phospholipids of modified fatty acid composition (13).

ACKNOWLEDGMENTS. This work was supported by USPHS grants AI-10733 and GM-18233. CFF is the recipient of USPHS Career Development Award GM-42359.

LITERATURE CITED

1. Machtiger, N.A. and Fox, C.F., Ann. Rev. Biochem. 42, 575 (1973).
2. Fox, C.F., In Cell Walls and Membranes, MTP Biochemistry Series Vol. 2, ed. C.F. Fox, Medical and Technical Publishing Co. Ltd/Butterworth and Co., Oxford, England, in press (1974).
3. Wisnieski, B.J., Williams, R.E. and Fox, C.F., Proc. Nat. Acad. Sci. U.S. 70, 3669 (1973).
4. Williams, R.E., Wisnieski, B.J., Rittenhouse, H.G. and Fox, C.F., Biochemistry 13, 1969 (1974).
5. Rittenhouse, H.G., Williams, R.E., Wisnieski, B.J. and Fox, C.F., Biochem. Biophys. Res. Commun. 58, 222 (1974).
6. Temin, H.M., Pierson, R.W. Jr. and Dulak, N.C., In Growth, Nutrition and Metabolism of Cells in Culture, ed. G.H. Rothblat and V.J. Cristofalo, Academic Press, New York (1972).
7. Fisher, H.W., Puck, T.T. and Sato, G., Proc. Nat. Acad. Sci. U.S. 44, 4 (1958).
8. Waymouth, C. and Reed, D.E., Tex. Rep. Biol. Med. 23, suppl. 1, 413 (1965).
9. Campbell, D.H., Garvery, J.S., Cremer, N.E. and Sussdort, D.H., In Methods in Immunology, W.A. Benjamin, Inc., New York (1964).
10. Parkes, J.G., Rittenhouse, H.G., Wisnieski, B.J. and Fox, C.F., Proc. Can. Fed. Biol. Soc. 17, 148 (1974).
11. Fox, C.F., Abstracts 170th annual meeting, American Chemical Society (Division of Microbial Chemistry and Technology) (1974).
12. Horwitz, A.F., Hatten, M.E. and Burger, M.M., Proc. Nat. Acad. Sci. U.S. 71, in press (1974).
13. Li, J.K.-K., Williams, R.E. and Fox, C.F., Biochem. Biophys. Res. Commun., companion paper.