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STUDY ON THE GROWTH PROMOTION CAPACITY OF CALF SERUM FOR ANIMAL CELLS IN VITRO

I — Test of 56 lots of calf serum against several cell lines and primary cell cultures

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SUMMARY

Fifty-six different lots of calf serum were tested against eleven cell lines and two types of primary cell cultures in order to establish their efficiency in promoting cell multiplication and thus, the viability of their use in the routine serial propagation of animal cells **in vitro.** The study demonstrated that the efficiency of the sera tested varied from lot to lot. Sera rated "fair" or "poor" were unable to support the serial propagation of the majority of the cell lines for long periods of time although efficient for the multiplication of primary cells. Certain cell lines were more demanding in relation to the sera used. Even when a serum rated "good" was employed to supplement culture media, it was observed that certain cell lines could not be kept in active multiplication, which indicates that a great variability of nutritional needs prevails among cell lines.

INTRODUCTION

The discovery of ENDERS and co-workers [9,17] that polioviruses could be propagated in cell cultures of nonneural origin led to the widespread utilization of cell cultures in virus diagnosis, as well as in the production of vaccines. Until 1950, animal cells were cultivated using blood serum, plasma, body exdudates and extracts of various tissues and/or organs as culture media. They presented such variability and complexity that it was impossible to determine the substances actually taken up by the cells, either for survival or multiplication.

Mammalian cells in serial cultivation require certain proteins of high molecular weight, usually supplied in the form of serum, as well as many growth factors of low molecular weight [8,13,16]. Proteolytic degradation of serum proteins leads to low molecular weight products with a growth-promoting activity on these cells, similar to that of the intact proteins [8,18].

The role of animal serum in culture media is very controversial. However, the fact is that few cells grow in its absence and just for short periods of time. Even when growth does occur, its rate is always better when serum is added.

Proteins of the medium may play a protective role for the cells, perhaps by adsorbing to their surface. It is known that when complex components such as serum, tissue extracts etc, are diminished or eliminated from the medium, the capacity of the cells to adjust to the new environment decreases progressively.

In many countries, tissue culture media, animal sera and various biological buffered solutions can be easily purchased from commer-

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cial sources which provide well standardized products. In Brazil, where Virology is in expansion, the specific media needed for the cultivation of the cells depend either on their expensive importation, or on their local preparation. The consistency of quality of such media is challenged by — a) the constant change of the brands of reagents and, consequently, of their purity and quality; b) the quality of the water used; c) the sterilization system adopted. Where animal sera are concerned, the situation is even worse. A great variation of their growth-promotion capacity can be observed among different lots when tested against different types of cells.

This first paper deals with the preparation and testing of 56 lots of calf serum used in the serial cultivation of animal cells.

MATERIALS AND METHODS

Culture medium

Eagle's medium[3] supplemented with 10% calf serum, 50 μg Streptomycin and 200 U.O. Penicillin per milliliter, was used in all experiments.

Calf sera

Young fasting calves were aseptically bled by jugular punction, 500 to 1,000 ml of blood being collected from each animal in sterile glass flasks. After coagulation at room temperature (± 23°C), the flasks containing individual harvests had their coagulum cut with a sterile spatula and were kept overnight at + 4°C to improve serum separation. The flasks were then centrifugated at 2,000 rpm for 40 minutes, under refrigeration. The supernatants were decanted, pooled and spun again as described.

Each of the 56 serum pools obtained, consisting of 7-8 liters, was then inactivated in a water-bath (+ 56°C) for 30 minutes with periodic agitation, immediately clarified through a Seitz pad, type K-5 (31 cm diameter) prewashed with 4 liters of deionized water, and sterilized by filtration through a Seitz pad, type EKS. The filtrate was dispensed in glass bottles, tested for sterility and immediately frozen at —20°C, until use.

Sterility tests

Throughout the experiment, all solutions and media employed were checked for sterility in thioglycollate, soybean-casein digest medium and liquid Sabouraud. Tubes containing 15 ml of the sterility media received and inoculum of 0.4-0.5 ml of each solution tested. The incubation period was 15 days, thioglycollate being kept at 36°C, while Sabouraud and soybean-casein digest medium remained at room temperature (± 23°C).

Cell cultures

Eleven cell lines originally obtained in U.S.A. laboratories and belonging to the cell culture bank of the Tissue Culture and Control Laboratory of the Instituto Butantan, were selected to check the growth-promoting efficiency of each serum lot:

- HEp_2-C : Epidermoid carcinoma of human larynx, Cincinnati Strain.
- Vero : Kidney of African Green Monkey, **Cercopithecus aethiops.**
- BHK-21 : Kidney of Syrian or Golden Hamster, **Mesocricetus auratus.**
- $LLC\text{-}MK_2$: Kidney of Rhesus Monkey, **Macaca mulatta.**
- BS-C-1 : Kidney of African Green Monkey, **Cercopithecus aethiops.**
- Sirc : Staatens Seruminstitut Rabbit Cornea, **Oryctolagus cuniculus.**
- KB : Epidermoid carcinoma, oral, human.
- HK : Human kidney.
- AV_3 : Human amnion.
- RK-13 : Rabbit kidney, **Oryctolagus cuniculus.**
- HeLa : Epidermoid carcinoma of human cervix.

  Two primary cell cultures were also tested.
- RK : Rabbit Kidney, **Oryctolagus cuniculus.**
- CE : Chick embryo fibroblasts.

Subcultivation of cells

Cell subcultivation was performed every 3-4 days, when confluency of the cell sheet was observed. The detachment of cells was carried out with ATV (association of trypsin-versene), pH 7.3 — 7.4, containing 0.2% trypsin Difco

(1:250 or 1:300) and 0.02% Versene (Titriplex III) in a balanced salt solution containing 1o/oo dextrose. Five milliliters of ATV were dispensed in each bottle to wash the confluent cell sheet and eliminated 2-3 minutes later, with gentle rotation. After complete detachment, cells were resuspended in growth medium, pipetted into new culture bottles and incubated at 36.5°C for multiplication. Split ratios of 1:2 or 1:3 were adopted, according to the characteristics of the cell line under test.

Testing the multiplication promotion efficiency

Cell suspension contained in glass ampoules kept frozen in liquid nitrogen were quickly thawed in a 37°C water-bath and seeded into glass culture bottles, after resuspension in Eagle's medium supplemented with 10% calf serum of a known standard batch, with high multiplication promotion capacity. Usually the contents of one ampoule was seeded in one bottle of 45 cm^2 surface. After 24-48 hours, the bottles had their medium changed and were re-incubated, until full confluency of the monolayer.

In the first passage, when the original bottle was split in two, one of the new bottles continued to be serially propagated in the same medium described above, and the second was seeded with medium supplemented with 10% of the test serum. From then on, every 3-4 days both cultures, "control" and "test", were submitted up to 8 — 9 subcultivations. As the behaviour of the test and control cultures had been registered before each passage, the sera quality could be definitely established as "good", "fair" or "poor", as evaluated by the morphological aspect of the monolayer and the multiplication rate.

Criteria for the evaluation of the growth promoting capacity of the sera tested

A serum lot was rated "good" when the corresponding test cells maintained their normal morphology and formed confluent monolayers in 3-4 days. When cell cultures presented normal morphology but slower multiplication, the serum was considered "fair". A serum was rated "poor" when changes of the cell morphology or granulation were observed, with gradual degeneration of the culture after 3-4 subcultures. For primary cells, the efficiency was rated according to the quality of the first confluent monolayer.

RESULTS

Data obtained during the study of the growth promoting capacity of 56 lots of calf serum for thirteen types of animal cells, are shown in Table I.

DISCUSSION

The variability of serum as growth promoting component was observed by CHANG[7] when working with long term, continuous subcultures of epithelial-like cells from human tissue: human inactivated sera obtained from different individuals exhibited different growing capacities.

In the present study, fifty-six pools of sera collected from a large number of young calves were tested against several cell lines and two types of primary cultures. It was observed that the individual variability of sera reported by CHANG[7] in relation to human cells does also occur in the pools, and that cell lines originally obtained from different species responded differently to each serum lot. The efficiency of each serum lot in promoting the multiplication of the cell lines or primary cells, was evaluated as described above in tests consisting of 8 — 9 serial subcultures of each of the chosen cell lines. For primary cells, the efficiency was rated according to the quality of the first confluent monolayer.

According to Table I, for cell lines such as BHK-21, SIRC and KB, only 50.0 — 54.0% of the tested sera rated as "good", whereas for AV_3, RK-13 and HK, 62.0 — 69.0% exibited the same performance. In the conditions of the experiment, BHK-21 cells behaved as the most demanding in relation to serum efficiency: the lowest percentage of "good" sera (50.0%), as well as the highest of "poor" sera (38.0%) were scored for these cells. With BS-C-1, HEp_2-C, $LLC\text{-}MK_2$, HeLa and VERO cells, 70.0 — 82.0% of the serum lots were found "good". VERO cells seemed to be the easiest to cultivate "in vitro": 82.0% of the sera rated "good" for them, whereas only 9.0% were considered "poor".

TABLE I

Efficiency of calf serum in promoting "**in vitro**" multiplication of different types of cells

Cell		Number of serum lots tested	Efficiency in promoting cell multiplication (%)		
			Good (*)	Fair	Poor
Primary	Chicken Embryo	18	94.0	6.0	0.0
	Rabbit Kidney	15	87.0	7.0	6.0
Line	VERO	55	82.0	9.0	9.0
	HeLa	42	74.0	12.0	14.0
	LLC-MK_2	30	73.0	17.0	10.0
	HEp_2-C	52	73.0	17.0	10.0
	BS-C-1	10	70.0	0.0	30.0
	HK	42	69.0	17.0	14.0
	RK-13	18	67.0	17.0	16.0
	AV_3	40	62.0	20.0	18.0
	KB	24	54.0	21.0	25.0
	SIRC	17	53.0	29.0	18.0
	BHK-21	34	50.0	12.0	38.0

(*) **Growth promoting efficiency**
Good: Cells presenting normal morphology and forming confluent monolayers in 3-4 days
Fair: Cells presenting normal morphology but slower multiplication
Poor: Cells presenting altered morphology or granulation, and inability to form confluent monolayers

With primary cultures of rabbit kidney and chicken embryo the sera under study presented 87.0 and 94.0% of efficiency, respectively. The latter was the only culture for which no serum tested rated "poor". Comparing both types of cells, it can be said that chick embryo cells are less fastidious than rabbit kidney cells, and that both require lower growth-promoting efficiency of the serum than the eleven cell lines studied.

Results reported here show that each cell line has its own needs and are valid for the experiment described. They may not necessarily apply to different experimental conditions using different cells, culture media and lots of calf serum, as the ability of serum to act as a growth promoting component for the cultivation of cells "in vitro" is dependent upon several factors.

The growth-promoting activity of serum fractions is likely to be linked to albumin and alpha-globulins [1,2,4,5,6,10,11,14,15]. Activity on cell multiplication, attachment and spreading of cells to constitute monolayers is exhibited by alpha-globulins [12,14] but the nutritional function of these serum proteins has not been elucidated yet [8,18].

Toxic properties of the serum seem to be related to one or more factors, such as hemolysis, age of donor, and fat contents [16]. Toxic or inhibitory factors induce clumping or intense granulation of cells. Heating serum at 56°C for 30 minutes may destroy this inhibitory effect and increase the serum growth potential [3]. Thus, all the fifty-six sera tested in this study were inactivated by heat but nevertheless, sera rated as "poor" continued to determine signs of intoxication, like cytoplasmic granulation.

The fact that only certain lots of serum worked with certain cell lines clearly shows that all lots of serum prepared should be routinely pre-tested with each of the cell lines used in a tissue culture laboratory. As result, by previously scanning the "affinities" existing between a cell line and a certain lot of serum, one could work safely, with no risks of loosing cells that should be kept in active multiplication.

In a follow-up project, a number of the serum lots tested in the present report, as well

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as some new lots, will be electrophoretically studied, in an attempt to establish the factors determining such variability in the growth-promoting capacity of calf serum.

RESUMO

Estudo da capacidade do soro de vitelo, de estimular a multiplicação de células animais mantidas in vitro. I — Pesquisa de 56 lotes de soro contra diversas células de linhagem contínua e primárias

Cinqüenta e seis lotes de soros de vitelo foram testados contra 11 linhagens celulares e a dois tipos de culturas de células primárias, com o objetivo de estabelecer sua eficiência na multiplicação celular e assim, a viabilidade do seu uso na manutenção de rotina de células animais, **in vitro.** O estudo demonstrou que a eficiência dos soros testados variou de lote para lote. Soros considerados "regulares" ou "pobres" foram incapazes de manter a propagação em série da maioria das linhagens celulares por períodos longos de tempo, embora se mostrassem eficientes na multiplicação de células primárias. Certas linhagens foram mais exigentes do que outras em relação ao soro usado. Mesmo quando soros considerados "bons" foram empregados para suplementar meios de cultura que observou-se, certas linhagens celulares não puderam ser mantidas em multiplicação ativa indicando que, entre elas predomina grande variabilidade quanto às necessidades nutritivas.

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REFERENCES

1. BAKER, L. E. & CARREL, A. — Lipoids as the growth-inhibiting factor in serum. **J. Exp. Med.** 42: 143-154, 1925.

2. BAKER, L. E. & CARREL, A. — Effect of age on serum lipoids and proteins. **J. Exp. Med.** 45: 305-318, 1927.

3. CAILLEAU, R. & KIRK, P. — Some factors affecting the growth-promoting properties of serum in tissue culture. **Tex. Rep. Biol. Med.** 15: 237-249, 1957.

4. CARREL, A. & EBELING, A. H. — Heterogenic serum, age and multiplication of fibroblasts. **J. Exp. Med.** 35: 17-38, 1922a.

5. CARREL, A. & EBELING, A. H. — Heat and growth-inhibiting action of serum. **J. Exp. Med.** 35: 647-656, 1922b.

6. CARREL, A. & EBELING, A. H. — Antagonistic growth-activating and growth-inhibiting principles in serum. **J. Exp. Med.** 37: 653-658, 1923a.

7. CHANG, R. S. — Continuous subcultivation of epithelial-like cells from normal human tissues. **Proc. Soc. Exp. Biol. Med.** 87: 440-443, 1954.

8. EAGLE, H. — Nutrition needs of mammalian cells in tissue culture. **Science** 122: 501-504, 1955.

9. ENDERS, J. F.; WELLER, T. H. & ROBBINS, F. C. — Cultivation of the Lansing strain of poliomyelitis virus in cultures of various human embryonic tissues. **Science** 109: 85-87, 1949.

10. FISHER, H. W.; PUCK, T. T. & SANTO, G. — Molecular growth requirements of single mammalian cells. III — Quantitative colonial growth of single 53 cells in a medium containing synthetic small molecular constituents and two purified protein fractions. **J. Exp. Med.** 109: 649-660, 1959.

11. HOLMES, R. & WOLFE, S. W. — Serum fractionation and the effects of bovine serum fractions on human cells growth in a chemically defined medium. **J. Biophys. Biochem. Cytol.** 10: 389-401, 1961.

12. LIEBERMAN, J. & OVE, P. — Purification of a serum protein required by a mammalian cell in tissue culture. **Biochem. Biophys. Acta** 25: 449-450, 1957.

13. McQUILKIN, W. T.; EVANS, V. J. & EARLE, W. R. — The adaptation of additional lines to NCTC clone 929 (Strain L) cells to chemically defined protein free medium NCTC 109. **J. Natl. Cancer Inst.** 19: 885-896, 1957.

14. MICHL, J. — Metabolism of cell in tissue culture in vitro. **Exp. Cell Res.** 23: 324-334, 1961.

15. MICHL, J. — Metabolism of cell in tissue culture in vitro. II — Long term cultivation of cell strains and cells isolated directly from animals in a stationary culture. **Exp. Cell Res.** 26: 129-135, 1962.

16. PUMPER, R. W. — Adaptation of tissue culture cells to a serum-free medium. **Science** 128: 363-366, 1958.

17. ROBBINS, F. C.; ENDERS, J. F. & WELLER, T. H. — Cytopathogenic effect of poliomyelitis viruses in vitro on human embryonic tissues. **Proc. Soc. Exp. Med.** 75: 370-374, 1950.

18. SCHAER, J. C. & SCHINDLER, R. — Requirement of cell cultures for serum proteins. **Biochem. Biophys. Acta** 147: 154-161, 1967.

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