The Potentiality of Vero Cell Growth in a Serum Diminished Medium and Its Possible Use in Propagation of Attenuated PPR Virus

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Abstract

Through three series of thirteen consecutive subculture passages, vero cells could be weaned from growing on a 10% serum-containing Eagle's MEM to grow on a 2% serum-containing one. The serum replacement solution was an aqueous mixture of 3% of each of lactalbumin hydrolysate, tryptose phosphate broth and tryptic soy broth powders. The first 2 series were constituted of 3 consecutive subculture passages each, supplemented with a serum percentage of 7 and 4, respectively. The balance of 10 % was the serum replacement solution. The third series was done in the same manner using 2 % serum, for seven consecutive subculture passages. Modulated vero cells were found to be of normal architecture in sheet morphology as well as growth pattern. Responsiveness to the attenuated virus of Peste des Petits Ruminants remained classic as for microscopic consequences of cytopathic effect as well as $TCID_{50}$ determination.

Introduction

Cell culture technology is used in virtually all fields of biomedical research and testing. The manufacture of viral vaccines has historically been accomplished using animal products such as chicken eggs, or cell cultures using foetal bovine serum (**4**). Since the early days of cell and tissue culture development, animal serum has been added to the culture media as a source of nutrients and other ill-defined factors, a practice that exists to this day, despite technical disadvantages to its inclusion, its high cost, and the increasing availability of alternatives. Increasing concerns about animal suffering inflicted during serum collection add an ethical imperative to a move away from the large scale use of serum wherever possible (**14**).

The Vero cell line, isolated from the kidney of a normal adult African Green Monkey (Cercopithecus aethiops), has been well

characterized and is instrumental in the biotechnology sector for virus replication studies, viral plaque assays, $TCID_{50}$ determinations and production of viral vaccines.

The present work was aiming at developing a serum-diminished medium that could support growth of Vero cells as well as replication of the attenuated Peste des Petits Ruminants virus (PPRV) on such growth-modulated cells.

Material and Methods

1- Cells:

Vero, Cercopithecus aethiops (monkey, African green), American Type Culture Collection, ATCC, Number CCL-81, originally established by (**16**), were used in the present study. Stock cultures of these cells were obtained from Navy American Medical Research Unit-3 (NAMRU-3), Abbasia, Cairo, Egypt and maintained at the Rinderpest Research Department, Veterinary Serum and Vaccine Research Institute (VSVRI), Abbasia, Cairo. The methods described by Butler (2004) were essentially those used for growth, maintenance, storage and passage of Vero cells. Vero cells were the substrate of concern that was subjected to a modulation trial to be grown and maintained on a minimal serum percentage containing medium. They were also used for PPRV $TCID_{50}$ determinations (**10**).

2- Serum replacement solution (SRS):

It was composed of an aqueous solution of 3 % of each of lactalbumin hydrolysate (LH) (DIFCO, USA), tryptose phosphate broth (TPB), (BD, France) and tryptic soy broth (TSB) (BD, France). The SRS was used to replace a certain percentage of the newborn calf serum (NCS) through a predetermined indicated series of consecutive passages of Vero cells.

3- Weaning of Vero cells from a 10% serum containing medium to that of a minimal percentage:

Vero cells grown on Eagle's minimum essential medium (MEM) with Hank's salts and L-glutamine (SIGMA, USA) supplemented with

10% newborn calf serum (NCS) (GIBCO, New Zealand) were consecutively passaged in 3 series. The first series was 3 serial passages on the same Eagle's MEM supplemented with 7% NCS and 3% of SRS. The second series was conducted in the same manner but with 4% NCS and 6% of the SRS. The third series was done with 2% NCS and 8% of the SRS. The third series of 3 consecutive passages was subsequently continued up to 7th serial one. Vero cell subculture passages were done every 3 days at a split ratio of 2. The process of weaning is tabulated below:

Table 1. Weaning process of vero cells from a 10 % serum containing medium to that of a minimal percentage.

Series No.	No. of passages	% of NCS	% of supplement (SRS)
	Initiation	10	0
1st	3	7	3
2nd	3	4	6
3rd	7	2	8

NCS: Newborn Calf Serum.
SRS: Serum Replacement Solution.

4- Vero cell viability measurement:

Determination of the concentration of viable Vero cells at each of the 13 consecutive passages was carried out on representative suspension samples, drawn up at the 48th hour post seeding (PS) of cells, by haemocytometer counting (**2**).

5- Virus responsiveness:

The attenuated Vero cell-adapted Peste des Petits Ruminants virus (PPRV) (**7; 9; 12**), was used to test the responsiveness of growth modulated vero cells to the virus. Criteria of responsiveness at a multiplicity of infection (MOI) of 0.01 $TCID_{50}$/cell were both microscopic recognition of specific cytopathic effect (CPE) and PPRV $TCID_{50}$ determinations done on the virus harvest, 5 days post infection (PI) (**10**).

Responsiveness testing was carried out at the level of the 13 Vero cell respective passages on 36 hours old cultures using NCS/SRS-free Eagle's MEM as a maintenance medium.

Results

1- Vero cell-weaning process from a 10 % NCS-containing Eagle's MEM down to a 2% - containing one:

Results of these procedures in terms of microscopic appearance of cells and percentage of viable are detailed in table (1).

2- Responsiveness of growth-modulated Vero cells to PPRV:

Results of these tests are shown in Table 2.

Table 2. Results of Vero cell-weaning process from a 10% NCS-containing Eagle's MEM down to a 2%-containing one.

No. of series	No. of passages	Serial No.*	% of		Microscopic appearance of cells	% of viable cells **
			NCS	SRS		
Starting culture			10	0	normal architecture of cells in sheets	97
		1				95
I	3	2	7	3		97
		3				96
		4				98
II	3	5	4	6		96
		6				97
		7				96
		8				95
		9				97
III	7	10	2	8		97
		11				98
		12				96
		13				97

NCS: Newborn Calf Serum. SRS: Serum Replacement Solution.

* Subculture consecutive passages were done every 3 days in a split ratio of 2.

**As determined by haemocytometer counting on representative samples drawn up 48 hours post cell seeding.

N.B.

Control subculture consecutive passages were done using the conventional 10% ncs in correspondence of which mean result is represented at the term: starting culture.

Table 3. Results of growth-modulated Vero cell responsiveness to the attenuated PPRV.

No. of series	No. of passages	Serial No.*	% of		Log_{10} $TCID_{50}$ titres/ml **
			NCS	SRS	
Starting culture			10	0	6.4
		1			6.3
I	3	2	7	3	6.5
		3			6.3
		4			6.5
II	3	5	4	6	6.4
		6			6.5
		7			6.3
		8			6.6
		9			6.5
III	7	10	2	8	6.4
		11			6.3
		12			6.5
		13			6.4

NCS: Newborn Calf Serum. SRS: Serum Replacement Solution.

* as followed throughout a 5-days period post inoculation of 0.01 TCID50 of PPRV/Vero cell, done on 36 hours old cultures just released from growth media.

** as determined on the 5th day PI-virus harvest.

Discussion

The vero cell line initiated from the African green monkey is an excellent cell line for the production of animal and human prophylactic viral vaccines (**8**). Animal serum is routinely added to culture media as a source of nutrients and other ill-defined factors (**5**). Technical disadvantages to using serum include the undefined nature of serum, batch-to-batch variability in composition, and the risk of contamination (**4**). Serum is a largely undefined, complex mixture of many and various constituents, some 200 of which have been identified so far (**14**). The effect of many of these disadvantages on cultured cells remains unclear, and there is some evidence to suggest that there are cytotoxins in serum that have a detrimental effect on both primary and established cell lines (**17**).

Hence, the work presented was aiming at exceedingly minimizing the serum percentage conventionally added to the growth media for vero cells. This was carried out through a vero cell weaning process in which a starting vero cell culture grown on a 10% NCS-containing Eagle's MEM was subjected to 3 consecutive series of subculture passages (Table 1). Thirteen consecutive subculture serial passages were conducted starting from an initial vero cell culture passage that was grown on a 10% NCS-containing Eagle's MEM. A gradual minimization of the NCS-percentage was effectuated down to 2, paralleled with maximization of the SRS one up to 8 (Table 1). A result of an attractive interest was the normal microscopic appearance of architecture of vero cells in sheets throughout the 13 passages performed. Moreover, viable vero cells remained above 95% throughout these passages. These two criteria are well characterized for monitoring the status of growing cells. The SRS is not far from the use in biological work. The enzymatic hydrolysate of lactalbumin (LA) was used for decades dissolved in Hank's balanced salt solution (HBSS) as a nutrient for growing of so many animal cell cultures (**11**).

Tryptose phosphate broth (TPB) being composed of tryptose, dextrose, sodium chloride and disodium phosphate is a well known

medium that enhances the growth of fastidious types of bacteria and is also used for blood culture work (**15**). Tryptic soy broth which is also termed soybean-casein digest medium is constituted of pancreatic digest of casein, enzymatic digest of soybean meal, dextrose, sodium chloride and dipotassium phosphate; is a well characterized rich medium for growing fastidious microorganism as well (Singh et al., 1967). It was of interest to find out that such a SRS when incorporated into the Eagle's MEM with Hank's salts could replenish the high percent of NCS (8%) removed from the growth medium (Table 1). Such a result was reproducible for a 7 consecutive subculture passages. It is suggestive that Eagle's MEM with Hank's salts, itself is of a crucial role in supplying the essential amino acids, vitamins and other elements that are quite instrumental components of a nutrient medium, since a number of 29 elements are composing this medium. Nevertheless, it should be borne in mind that whatever being the nutrient medium, there is a finite lifespan intrinsic to the genetic make-up of all normal animal cells (**1**). The success in the method of weaning lies in the gradual process of adaptation of vero cells subcultured in successive media that contain reduced levels of serum down to that of 2%. An attribute, in this respect, might be changes in cellular metabolism and induction of specific cellular growth factors (**6**). A result of much interest was that modulated vero cells responded typically to PPRV-inoculation as evidenced by the microscopic appearance of CPE as well as the resultant virus titres (Table 2). Both criteria were found to be comparable to corresponding control.

It could be concluded that vero cells could be grown and maintained on an only 2% serum-containing Eagle's MEM using a very cheap serum replacement solution, thus saving more than 90% of the cost of serum. Moreover, accounting for reduction of much of the disadvantages to which serum is vulnerable. In addition, such modulation of vero cells was not interfering with the classic response to PPRV.

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فاعلية نمو خلايا كلي القرد الاخضر الافريقي في وسط قليل المصل واستخدامه المحتمل في تكاثر فيروس PPR المضعف

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أجريت هذه الدراسة لمحاولة تطويع خلايا كلي القرد الأخضر الأفريقى (Vero) لكى تنمو فى وسط غذائى (Eagle's MEM) يحتوى على أقل نسبة ممكنة من مصل العجول حديثة الولادة (Newborn calf serum) وقد أمكن تحقيق ذلك من خلال عملية فصال (weaning) تدريجية للخلايا النامية على الوسط الغذائى المحتوى على ١٠% مصل تضمنت مرحلة أولى فى ثلاث تمريرات متتالية للخلايا على وسط غذائى يحتوى على (٧% مصل، ٣% محلول بديل) ويتكون المحلول البديل من مزيج مائى مركب من ٣% من كل من: Lactalbumin hydrolysate powder, tryptose phosphate broth powder and tryptic soy broth powder وتلى ذلك مرحلة ثانية فى ثلاث تمريرات متعاقبة للخلايا على وسط غذائى (MEM) يحتوى على (٤% مصل، ٦% محلول بديل) وأجريت بالتالى مرحلة ثالثة باستخدام (٢% مصل، ٨% محلول بديل) لعدد سبع تمريرات متتالية.

وقد ثبت باستقراء النتائج على خلفية المعايير المعروفة أن:

أولا: إنتفاء حدوث تغير ظاهرى أو سلوكى على نموى للخلايا المطوعة على مدى ١٣ تمريرة متتالية مقارنة بالضوابط.

ثانيا: إنتفاء حدوث تغير (أو إنحراف) فى المظاهر الكلاسيكية المعروفة (CPE) نتيجة حقن الخلايا المطوعة بفيروس اللقاح الحى لطاعون المجترات الصغيرة.

ثالثا: استجابة خلايا فيرو المطوعة لفيروس اللقاح الحى لطاعون المجترات الصغيرة بمعيار نتاج فيروسى لا يختلف عن النتاج التقليدى وذلك مقارنة بالضوابط.

يستخلص من نتائج هذه البحث أن تقليص كمية مصل العجول حديثة الولادة – المضاف إلى الوسط الغذائى لتنمية خلايا فيرو – إلى ٢ % مع إضافة نسبة ٨ % من المحلول البديل المشار إليه ضمن سياق البحث – لم يؤثر سلباً على نمو الخلايا أو استجابتها للفيروس – الأمر الذى يمكن معه إحتساب تحجيم كلفة اقتصادية قدرها ٩٠ % تقريباً.