NUTRIENT COMPOSITION FOR CULTIVATION OF JAPANESE ENCEPHALITIS VIRUS *IN VITRO*

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Summary. – Effects of certain groups of nutrients such as glucose, essential amino acids (AA), non-essential AA, vitamins and trace nutrients on the multiplication of various strains of Japanese encephalitis virus (JEV) were studied with an aim to optimise the conditions for cultivation of the virus in porcine stable (PS) kidney cell cultures. Eagle's Minimal Essential Medium (MEM) was modified by addition of the nutrients in different concentrations and combinations. Glucose was found the most important single nutrient in promoting significantly the virus multiplication. Essential AA alone did not influence the virus yield, while in combination with glucose they caused its marked increase. Vitamins and other nutrients did not stimulate significantly virus multiplication. The study revealed that the extent of the glucose effect depends on the virus strain used.

Key words: Japanese encephalitis virus; PS cells; cultivation *in vitro*; optimal nutrient composition

Introduction

JEV is an important pathogen causing severe epidemics and outbreaks in South-East Asia since the last three decades (Rodrigues, 1984). The inactivated mouse brain vaccine (strain Nakayama-NIH) is highly effective (Oya, 1988) but requires multiple doses for the induction of an adequate immunity, and its preparation is relatively expensive. Therefore, we have been studying various aspects of cultivation of this virus in cell culture.

A variety of tissue culture media were designed and developed for the growth of particular cell lines (Morgan *et al.*, 1950). Thus, MEM was originally developed by Eagle (Eagle, 1959) as a modification of his Basal Medium for the growth of HeLa cells. The same media used for the growth and maintenance of cultured cells are often empolyed also for the multiplication of viruses in the same cells.

There are many reports showing that certain components of culture media play a specific role in the replication of animal viruses in cell cultures (Winter and Consigli, 1971). E.g., arginine was found to participate in the temporal regulation of protein synthesis during the replication of herpes simplex virus type 1 (Mikami *et al.*, 1974), in the assembly of adenovirus (Plaat and Weber, 1979), and in the maturation of Newcastle disease virus (Iinuma *et al.*, 1973). There are several reports on the AA requirements for the growth of togaviruses (Hotta, 1978). The growth of an alfavirus (Chikungunya) was inhibited to 60%, and of a flavivirus (dengue-1) to 50% of control, respectively, in complete absence of arginine (Matsumuratt *et al.*, 1975). Paranjape and Kadam (1985) have reported increased yields of alfaviruses in the presence of higher concentration of arginine. Thus, it appears that media which are optimal for cell growth are not necessarily optimal also for virus replication. This fact is often overlooked while using cell culture substrates for the multiplication of viruses.

The aim of this communication was the optimisation of the nutrient composition of medium for the cultivation of JEV in PS cell culture. The effects of glucose, essential and non-essential AA, vitamins, and other nutrients on the JEV growth are reported.

Materials and Methods

Virus. JEV strain Nakayama which underwent 52 mouse brain passages and 5 PS cell culture passages was empolyed in most experiments. It was plaque-purified in PS cells and its stock had

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Abbreviations: AA = amino acids; JEV = Japanese encephalitis virus; MEM = Eagle's Minimal Essential Medium; PS = porcine stable

Table 1. Effect of increased concentration of nutrients on JEV yields in PS cell cultures

Medium	Concentration of nutrients[1]					Virus yield[2] log $TCID_{50}$/ml
	E-AA	Glucose	Vitamins	NE-AA	Other n.[3]	
M-01	1x	1x	1x	–	–	6.80[a]
M-02	2x	1x	1x	–	–	7.14[ab]
M-03	4x	1x	1x	–	–	6.97[a]
M-04	1x	2x	1x	–	–	7.04[a]
M-05	1x	4x	1x	–	–	7.34[b]
M-06	1x	1x	2x	–	–	6.73[a]
M-07	1x	1x	4x	–	–	7.14[ab]
M-08	2x	4x	1x	–	–	8.23[c]
M-09	1X	4x	4x	–	–	7.48[b]
M-10	2x	4x	4x	–	–	8.30[c]
M-11	2x	4x	4x	+	+	8.50[c]
M-12	4x	6x	5x	+	+	8.43[c]

[1] Concentration corresponds to MEM (1x) or to its multiples (2x – 6x).

[2] Averages of 10 values. Averages not bearing common superscript differ significantly.

[3] Other nutrients: adenine sulfate, 5'-adenosine monophosphate, cholesterol, 2-deoxyribose, guanine hydrochloride, hypoxanthine, ribose, sodium acetate, thymine, Tween-80, uracil and xanthine in concentration corresponding to Medium 199.

a titer of $10^{7.8}$ PFU/ml. Also the following Indian JEV isolates from human brain were empolyed (place and year of isolation are indicated in brackets): P20778 (Vellore, 1958), P733913 (Banakura, 1973), 785730 (Gorakhpur, 1978), 821564 (Kolar, 1981), and 826309 (Goa, 1982).

Cells. PS cell line was grown in MEM supplemented with 10% goat serum. Subcultures were prepared by splitting the trypsinized monolayers in 1:4 ratio. Confluent monolayers were maintained in MEM without serum. Viable cell count was determined by the Trypan Blue exclusion method.

Experimental media. MEM with Earle's salts was taken as basal experimental medium (M-01) which was modified by increasing the concentration of glucose, essential AA, and vitamins (MEM constituents), and by adding non-essential AA and other nutrients (Medium 199 (Parker *et al.*, 1950) constituents). All the media contained Tween-80 (5 mg/l) and their pH was adjusted to 7.6. The osmolality of experimental media was checked by the method of freezing point depression.

Infection. Confluent PS cell monolayer cultures in milk dilution bottles were infected at a multiplicity of infection of 0.1 PFU per cell. After 30 mins of virus adsorption at 37 °C the respective experimental medium was added to cells, and the incubation at 37 °C continued for another 48 hrs. Virus yields ($TCID_{50}$/ml) were determined by titrating the extracellular virus-containing media. The significance of differences in virus yields was checked statistically by the Completely Randomized Design, and the Duncan's Multiple Range Test (Snedecor and Cochran, 1967; Cochran and Cox, 1957).

Virus titration was carried out on duplicate tube cultures of PS cells using serial two-fold dilutions of samples. The cytopathic effect of virus was read microscopically in a standard way and $TCID_{50}$ titers were calculated according to Reed and Muench (1938).

Results

MEM with Earle's salts (medium M-01, Table 1) served as basal experimental medium which was modified by increasing its content of glucose, essential AA, non-essential AA, vitamins and other nutrients from the values considered normal for MEM or Medium 199 to 2-fold, 4-fold or higher values (media M-02 – M-13, Table 1). The osmolality measurements showed that all these media were within the acceptable range of 280-300 mOsmol/kg.

The average virus yield obtained with normal MEM (M-01) was 6.80 log $TCID_{50}$/ml. An increase of the concentration of essential AA to 2-fold (M-02) and 4-fold (M-03) led to an insignificant rise of the virus yield (7.14 and 6.97, respectively). Similarly, the vitamin content increase up to the 4-fold (M-06, M-07) had no effect. On the other hand, the 4-fold glucose content (M-05) caused a significant rise of the virus yield (7.34; $p < 0.01$).

To look for a possible synergistic efffect of a combination of glucose with another nutrient, increased concentrations of both glucose and esseential AA were tested. A 4-fold glucose and 2-fold essential AA content (M-08) gave the highest rise of the virus yield (8.23; $p < 0.01$). The vitamins added at 4-fold concentration did not increase the stimulatory effect of the 4-fold glucose content (M-09). That the tested vitamins do not play an important role in JEV growth in PS cells was confirmed by adding the vitamins to the "optimal" medium (M-08); this medium (M-10) did not have any different effect (8.30 vs. 8.23). Similarly, non-essential AA and other nutrients exerted no effect (M-11, M-12). Thus, the optimal medium consisted of 4-fold glucose and 2-fold essential AA, and further increase of glucose (M-12) over this level did not have any effect.

The glucose stimulatory effect was tested also on other JEV strains. The JEV isolates P20778, 733913, 785730, 821564 and 826309 were grown on 4-fold glucose (M-05) in comparison with normal glucose content (M-01). In this experiment, the virus yields increased 2.1, 2.1, 1.5, 0.9 and 0.9 times, respectively, while the corresponding value for the reference Nakayama strain was 3.5. It can be seen that (a) there was a strain to strain variation in the stimulatory response to glucose, and (b) the earlier isolates (from years 1958, 1973) showed a greater growth stimulatory response than the later ones (from years 1981, 1982).

Discussion

The growth of most types of cells is restricted at high population density (e.g. in crowded monolayers) in any particular medium because of depletion of crucially important nutrients (e.g. glucose, AA). Thus, these nutrients become the limiting factors of cellular metabolism (growth). Glucose, which is the major source of readily available energy is utilized

quickly by cells in culture. Watanabe *et al. (1992)* have reported that hypoglycemia developed rapidly in cultures of human endothelial cells as a result of a precipitous drop of glucose content of medium. Blake and Mclean (1989) have observed an inability of bovine corneal endothelial cells to utilize glucose when its concentration in culture media fell below 0.54 mg/ml. There seems to be a minimum threshold concentration of glucose in the medium below which cells are unable to metabolize it. The simplest way how to avert such a depletion would be the addition of fresh medium or a more rational approach, the increase of the concentration of the respective nutrient (glucose).

The energy is required by cells also for their interaction with virus, namely for virus replication. In an earlier report (Eagle, 1959), glucose, glutamine and salts have been shown to be the only requirements for the growth of poliovirus in confluent monolayers of HeLa cells. It is possible that some AA are utilized also in metabolic pathways leading to energy production. Thus, it would be interesting to check the possibility that JEV-infected cells may have a higher gluconeogenesis than non-infected cells. The synergisitc effect of glucose and essential AA, observed in our experiments, indicates this possibility.

The differential growth stimulatory response of the JEV strains tested to the increased glucose concentration in medium is of special interest. Antigenic differences amomg various JEV strains have been reported (Okuno *et al.*, 1968; Cochran and Cox, 1957). In studies using monoclonal antibodies (Kobayashi *et al.*, 1984; Okuno *et al.*, 1987) the virus strains isolated more recently were demonstrated to have a different antigenicity from those isolated earlier. Our present results also indicate that the earlier JEV isolates (P20778, 733913) are more responsive to the stimulatory effect of glucose than the more recent isolates (821564, 826309).

It is known that poliovirus mutates during human intestinal passages *in vivo* (Evans *et al.*, 1985), while it is quite stable during tissue culture passages *in vitro*. In this regard it would be interesting to find out whether also JEV undergoes similar alterations during its transmission through man in natural conditions (human passages in epidemics). In the positive case it would mean a plasticity of the JEV genome, a property of epidemiological and taxonomic significance.

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