Nutrient Medium from Rice Flour Hydrolysate for Cell Culturing and Evaluation of the Sensitivity to Influenza Viruses

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The nutrient medium on the basis of enzymatic hydrolysate of rice flour was used for culturing of MDCK and Vero(B) cells. Culturing of the vaccine line Vero(B) in this medium was not accompanied by changes in proliferative activity and sensitivity to influenza viruses A(H1N1) and A(H3N2).

Key Words: *cell cultures; nutrient medium; culturing; influenza virus*

The development of optimal conditions for cell activity outside the organism is essential for the use of cell cultures in various fields of biology, medicine, and veterinary. The main parameters of the cell culture remain practically unchanged under *in vitro* conditions. This dynamic system can be subjected to long-term passage. Therefore, the conditions for cell culturing should be maintained at a constant level. Standard nutrient media in biotechnology require the presence of bovine serum (BS) or FBS, which increases the risk of contamination with animal products. Much recent attention is paid to low-serum or serum-free media on the basis of enzymatic hydrolysates of plant proteins.

Low-serum nutrient media on the basis of hydrolysates of rice flour and soybean flour for cell culturing were developed at the State Research Center of Virology and Biotechnology "Vector" [3]. The optimal composition of the nutrient media includes a certain concentration of enzymatic hydrolysates from soybean or rice flour. The flours are dissolved in balanced Hanks solution and Earl's solution, respectively, which contain vitamins, microelements, and individual amino acids.

Growth-stimulating activity of nutrient media was studied by culturing MDCK cells (2 successive passages), Vero cells, and 4647 cells (5 successive passages). The proliferation index (PI) and cell morphology were taken into account [1,3]. PI of MDCK, Vero, and 4647 cells in hydrolysate-containing nutrient media was comparable with that in the control medium (Eagle's MEM). Cell morphology did not differ from that of normal cells. Continuous flat monolayer had no signs of degeneration. The optimal multiplicity of infection and reproduction of cold-adapted reassortant vaccine strains of influenza viruses A/H1N1, A/H3N2, and type B were evaluated on the culture of MDCK cells. Studying the reproduction of reassortant strains showed that they are easily reproduced under these conditions. The maximum titer of infection was 8.5-9.5 lg EID_{50}/cell (EID_{50}, embryo infectious dose; dose for infection of 50% infected embryos). The multiplicity was 0.001 EID_{50}/cell (in the presence of trypsin). The indexes are similar to those in control media (Eagle's MEM and Axcevir-MDCK).

The nutrient medium from rice flour hydrolysate was used for culturing of MDCK cells and vaccine line Vero(B) (R. Ya. Podchernyaeva *et al.*)

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[2]. Vero(B) cells are recommended for the manufacture of herpes virus vaccine and influenza virus vaccine [4]. The sensitivity of cells to reference strains of influenza viruses A(H1N1) and A(H3N3) (isolates of 1999 and 2005) was evaluated.

MATERIALS AND METHODS

Cultures of MDCK cells (cocker spaniel kidney cells) and vaccine Vero(B) cell strain (monkey kidney cells) were obtained from the Collection of Cell Cultures (D. I. Ivanovskii Institute of Virology, Russian Academy of Medical Sciences). These cells were grown in the nutrient medium on the basis of enzymatic hydrolysate of rice flour with 3, 4, and 5% FBS (PanEco). Eagle's nutrient medium with 3, 4, and 5% FBS and 10 mM L-glutamine served as the control. Inoculum concentration was 10^5 cells per 1 ml nutrient medium. The cells were incubated in 50-ml culture flasks (mattresses) at 37°C for 3-4 days. The cultures were subjected to 5 successive passages. The period of monolayer formation, proliferative activity (PI), viability, and morphology of the culture were evaluated. PI was calculated as the ratio of growing-to-inoculated cells. Cell culture viability (in percent) was evaluated by staining with 0.4% trypan blue. Unstained living cells were counted in a Goryaev chamber. Morphological study of samples was performed after staining with hematoxylin and eosin.

The cells were grown in 96-well plates (Nunc) with experimental or control nutrient media to study reproductive activity of influenza viruses. The cell suspension (inoculum concentration 2×10^5 cells/ml) was added to a well (100 µl). Incubation was performed in a thermostat at 5% CO_2 and 37°C.

Cell sensitivity to human influenza virus A was evaluated using reference strains A/New Caledonia/20/99(H1N1) and A/Wisconsin/67/05(H3N2). These strains were obtained by culturing on chick embryos. The hemagglutination titer of viruses was 512 hemagglutinating units. The study was performed on 2-day-old monolayer cultures. Cell cultures were grown in the control medium (Eagle's medium) and experimental medium with 3, 4, and 5% FBS. The growth medium was removed. The cells were infected with virus-containing allantoic fluid (10-fold dilutions). Infected cells were incubated in a thermostat at 5% CO_2 and 37°C. A serum-free medium with 2.5 µg/ml trypsin TPCK was used as the maintenance medium. The infectious titer of viruses is expressed as lg TCD_{50} (50% tissue cytopathic doses per 1 ml).

RESULTS

In series I we studied the effect of nutrient medium on the basis of enzymatic hydrolysate of rice flour on proliferation of Vero(B) and MDCK cells over 5 successive passages. Vero(B) cells formed a monolayer after 2-3-day culturing in control Eagle's medium with various concentrations of FBS. Proliferative activity remained high. PI was 5.3, 5.6, and 7.8 in the presence of FBS in concentrations of 3, 4, and 5%, respectively. Morphological characteristics of cells were typical of this line (Fig. 1). The culture was presented by polygonal and epithelioid cells with round or oval nuclei. Each nucleus had 1-2 large nucleoli. Binucleated or multinucleated cells were sometimes found. The cells included a small-granular cytoplasm. Atypical mitoses were revealed.

Vero(B) cell grown in rice medium also formed the monolayer on days 2-3. After addition of FBS in concentrations of 3 and 4%, growth-stimulating activity of this medium did not differ from the control (PI=5.2). PI was higher after addition of 5%

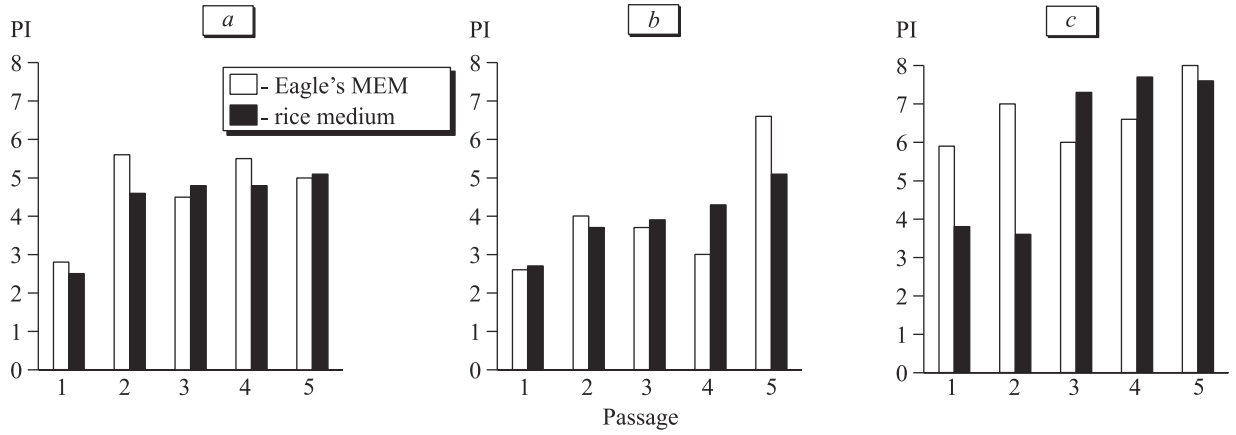

Fig. 1. PI of Vero(B) cells during culturing in various nutrient media over 5 passages (dependence on FBS concentration). Here and in Fig. 2: 3% FBS (*a*); 4% FBS (*b*); 5% FBS (*c*).

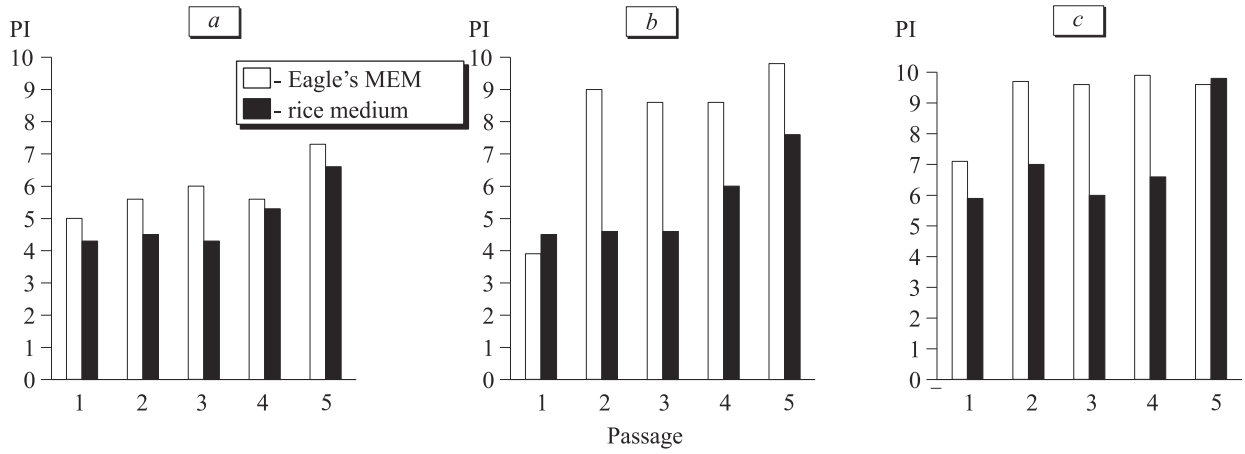

Fig. 2. PI of MDCK cells during culturing in various nutrient media over 5 passages (dependence on FBS concentration).

FBS to the medium (similarly to the control). After 3-5 passages, this index reached 7.3-7.8. Morphological characteristics of Vero(B) cells did not differ in the experimental and control medium. Cell culture viability during passage under control and experimental conditions was 92-98%.

Passage of MDCK cells in Eagle's medium was accompanied by monolayer formation (days 2-3). PI depended on FBS concentration in the culture medium (Fig. 2). After addition of 3 or 4% FBS, PI varied from 4.0 to 7.3-9.0 by the 4th-5th passage. PI reached 7.0-9.6 over 5 successive passages in the presence of 5% FBS.

The culture consisted of epithelioid cells with well-defined boundaries and large nuclei of different shape. Each nucleus had one or several large nucleoli. The cytoplasm looked like a cellular structure with inclusions. Abnormal mitoses were not found (Fig. 3, *a*).

It should be emphasized that culturing of MDCK cells in rice medium was accompanied by changes in cell morphology (Fig. 3, *b*). The cells gained an elongated (spindle-like) shape and formed a dense monolayer with superimposed layers. The cytoplasm was strongly vacuolated. Growth-stimulating activity was shown to decrease under these conditions. PI was below the control by 1.0-4.0 U. Cell viability remained unchanged over 5 passages. The ratio of living cells was 84-96%.

Our results indicate that the medium with rice flour hydrolysate and FBS (3, 4, and 5%) can be used for culturing of MDCK cell lines and vaccine Vero(B) strain. Proliferative activity of cells is maintained at a constant level over 5 successive passages.

In series II we evaluated the sensitivity of MDCK and Vero(B) cells to reproduction of reference strains of influenza viruses A(H1N1) and A(H3N2). The

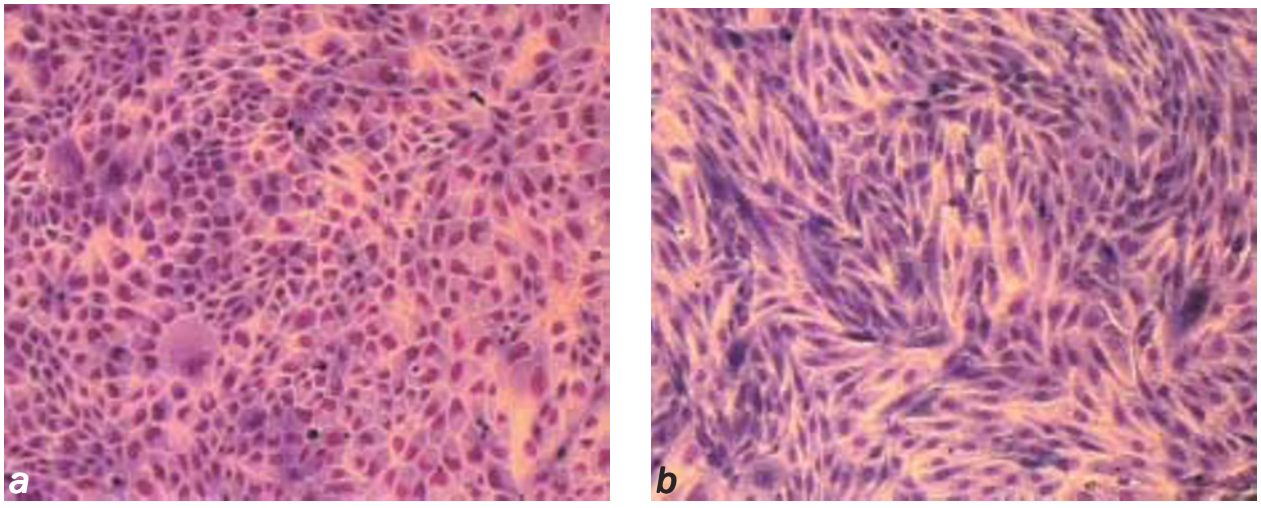

Fig. 3. Morphology of cultured MDCK cells from control Eagle's medium (passage 9, *a*) and rice medium (passage 4, *b*) after culturing in the presence of 5% FBS. Romanovsky staining (*400).

TABLE 1. Sensitivity of Cultured MDCK and Vero(B) Cells to Human Influenza Viruses A

Cells	Medium	FBS, %	TCD_{50} (lg) with reference strains of influenza viruses	
			A/New Caledonia/20/99(H1N1)	A/Wisconsin/67/05(H3N2)
MDCK	Eagle's	3	1.0	3.0
		4	1.0	3.0
		5	1.0	3.5
	Rice	3	1.0	2.0
		4	1.0	2.0
		5	1.0	2.0
Vero(B)	Eagle's	3	<1.0	4.0
		4	<1.0	3.5
		5	2.0	4.0
	Rice	3	2.0	3.5
		4	2.0	3.5
		5	2.0	3.5

cells were grown in control Eagle's medium or experimental rice medium with 3, 4, and 5% FBS and infected with allantoic culture of A/New Caledonia/20/99(H1N1) and A/Wisconsin/67/05(H3N2) viruses (successive 10-fold dilutions, Table 1). A/New Caledonia/20/99(H1N1) influenza virus was characterized by low-intensity reproduction (1.0 lg TCD_{50}) in MDCK cells during culturing with control Eagle's medium or experimental medium in the presence of trypsin and various concentrations of FBS. The degree of A/Wisconsin/67/05(H3N2) reproduction was greater in Eagle's medium (3.0-3.5 lg TCD_{50}) and experimental medium (2.0 lg TCD_{50}), which did not depend on FBS concentration.

Other results were obtained in studying the sensitivity of Vero(B) cells to these viruses. Reproduction of the strain A/New Caledonia/20/99(H1N1) did not occur in Eagle's medium with 3 or 4% FBS. However, TCD_{50} was 2.0 lg after addition of 5% FBS. The virus was reproduced in Vero(B) cells on rice medium with various concentrations of FBS (2.0 lg TCD_{50}). Reproduction of A/Wisconsin/67/05(H3N2) in Eagle's medium and rice medium (3.5-4.0 and 3.0 lg, respectively) was much greater than that of A(H1N1), which did not depend on FBS concentration.

We conclude that A/New Caledonia/20/99(H1N1) influenza virus strain is characterized by high-intensity reproduction in Vero(B) cells grown on rice medium. Reproduction of the strain A/Wisconsin/67/05(H3N2) is active in both cell lines and does not depend on the type of medium.

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