Comparative Analysis of Reproduction of Influenza Virus Strains in Cell Lines Perspective for the Creation of Cultural Vaccines Grown on Nutrient Medium on the Basis of Rise Flour Protein Hydrolysate

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We studied vaccine strains of influenza viruses A and B during their culturing in MDCK and Vero cells grown in Eagle's MEM medium and in a medium on the basis of enzyme hydrolysate of rise flour proteins with reduced (2%) content of fetal calf serum. Optimal conditions for cell culturing and reproduction of influenza virus strains in these cells were studied. Culturing of vaccine strains of influenza viruses in MDCK and Vero cells grown in nutrient media on the basis of rise flour protein hydrolysate yielded high infection titers, which suggests that this medium can be used for the development of cultural influenza vaccine.

Key Words: *influenza virus; cell cultures; nutrient media; enzyme hydrolysate*

The fact that vaccine prophylactics is the most effective method of preventing influenza in the population is now beyond doubts [1]. The majority of live and inactivated vaccines are prepared on developing chick embryos (DCE). In 1995, WHO recommended to use cultured cells complying with WHO requirements instead of DCE as the substrate for the production of influenza vaccines [15]. Culture-derived vaccines have some advantages over the embryonic vaccines.

First, chick embryos are a nonstandard substrate, whereas standard properties of cell strains are easy to maintain. MDCK or Vero cell strains are usually used as the substrates. In contrast to DCE, culturing of influenza A and B viruses in MDCK cells does not lead to selection of hemagglutinin (HA) variants differing by the amino acid sequence from that in viruses circulating under natural conditions and causing diseases in humans [14]. Vero cells are certified by WHO for the production of viral vaccines for humans and is promising for the production of influenza vaccines. Viruses grown in Vero cells are genetically stable and are identical to viruses grown in MDCK cells by the amino acid sequence of HA1 subunit; moreover, they retain antigenic properties of natural viruses [12].

Second, culture-derived vaccine produced in serum-free medium contains no proteins, allergens, which can be present in embryonic vaccine. In various countries including Russia, the development of production technologies for culture-derived influenza vaccines is based on the use of serum-free media of foreign companies [2,3,7,8,10].

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Researchers of Vektor Center of Virology and Biotechnology created nutrient media on the basis of enzyme hydrolysates of rise flour proteins, which can be used for culturing cells at low serum concentrations [4,5].

Here we compared reproduction of vaccine strains of influenza A and B viruses in MDCK and Vero cell cultures grown in Eagle's MEM medium and a nutrient medium on the basis of enzyme hydrolysate of rise flour proteins with reduced concentration of fetal calf serum (2%).

MATERIALS AND METHODS

Influenza virus strains. Vaccine strains A/Solomon Islands/03/06 (H1N1) and B/Malaysia/2506/2004 obtained from Microgen Research-and-Production Company were used in the study. The strains were maintained in 10-11-day DCE. Infected embryos were incubated for 48 h at 34°C (influenza A virus) or 32°C (influenza B virus).

Cell cultures. The following cells were used in the study: MDCK (Madin-Darby canine kidney cells) and Vero (African green monkey kidney cells) from the cell culture collection of Vektor Research Center of Virology and Biotechnology. Cells of both strains were grown in Eagle's MEM (control) or in a medium on the basis of enzyme hydrolysate of rise flour proteins (experiment). Experimental and control media were supplemented with 5 or 2% fetal calf serum (Gibco). Seeding concentration was 2×10^5 cells per 1 ml nutrient medium, time of incubation was 1-2 days at 37°C. The cells were harvested from the glass with 0.25% trypsin and 0.2% EDTA (1:1). For evaluation of reproductive activity of vaccine strains of influenza A and B viruses, MDCK and Vero cells were grown in experimental or control nutrient media in 96-well plates (Costar): 1 ml cell suspension with a seeding concentration of 2×10^5 cells was transferred to wells in a volume of 100 μl/well.

Nutrient media. Eagle's MEM medium (M. P. Chumakov Institute of Poliomyelitis and Viral Encephalites, Russian Academy of Medical Sciences) containing 5 or 2% fetal calf serum (Gibco) was used as the control for culturing MDCK and Vero cells. The composition of experimental medium and preparation of the enzyme hydrolysate of rise flour proteins were descried previously [5].

Reproduction of vaccine strains of influenza A and B viruses in confluent MDCK and Vero cell cultures. Strains of influenza A virus (A/Solomon Islands/03/06) and influenza B virus (B/Malaysia/2506/2004) preliminary adapted to MDCK cells (1 passage) or Vero cells (3 consecutive passages) were used in experiments. We also used 1-2-day monolayer cultures of MDCK or Vero cells grown in control Eagle's MEM medium with 5 or 2% fetal calf serum or in experimental medium containing the same serum concentrations. After 1-h incubation of MDCK or Vero cells infected with influenza A or B viruses at 34 or 32°C, respectively, control or experimental serum-free medium containing 2 μg/ml trypsin was added, and the cultures were incubated for 3 days at 34 or 32°C for influenza A or B viruses, respectively. Infection titer of the virus was determined using MDCK cell culture in samples taken after 3 days and was expressed in lg TCD_{50}/ml (50% tissue cytopathic doses per 1 ml).

The data were processed by standard methods of variation statistics. The significance of differences was evaluated using Student *t* test.

RESULTS

Comparative analysis of reproductive activity of influenza A and B viruses not adapted and adapted to MDCK cells. Cultured MDCK cells were infected with adapted and nonadapted to these cells (1 passage) strains of influenza virus. For both nonadapted strains the titers on DCE were 8.0 lg EID_{50}/0.2 ml (EID_{50} is embryonic inducing dose) and 1:128 in the reaction of hemagglutination. After adaptation during 1 passage to MDCK cells, the titers on DCE for strains A/Solomon Islands/03/06 and B/Malaysia/2506/2004 were 9.5±0.1 and 9.0±0.1 lg EID_{50}/ml ($p<0.05$), respectively; in the reaction of hemagglutination the titer for both strains was 1:512. Reproductive activity of adapted and nonadapted to MDCK strains of influenza A and B viruses in control and experimental cell cultures was studied after similar multiple infection (1.0 EID_{50}/cell). Three days after infection of MDCK cells grown on control and experimental media with adapted strain A/Solomon Islands/03/06, the infection titers were higher by 2 orders of magnitude than the titers of nonadapted strain (Table 1). Similar results were obtained for B/Malaysia/2506/2004 strain. For adapted strain B, the infection titers on MDCK cells grown in both media were by 2.5 lg higher than for nonadapted strain in the same media (Table 1). Similar results were obtained when studying reproduction of influenza virus A/Solomon Islands/03/06 and B/Malaysia/2506/2004 adapted and not adapted to Vero cells during 3 passages. In further experiments we used only influenza A and B viruses adapted to MDCK and Vero cells.

Evaluation of A/Solomon Islands/03/06 strain reproduction in MDCK and Vero cells. Repro-

TABLE 1. Reproduction of Influenza A and B Virus Strains Adapted and Not Adapted to MDCK Cells on Day 3 after Infection of MDCK Cells (*M*±*m*; *n*=5)

Strain	lg TCD_{50}/ml	
	grown on Eagle's MEM medium+2% FCS	grown on rise hydrolysate medium+2% FCS
A/Solomon Islands/03/06		
adapted during 1 passage	9.5±0.2*	9.5±0.1*
nonadapted	7.3±0.1	7.7±0.1
B/Malaysia/2506/2004		
adapted during 1 passage	8.7±0.1*	9.0±0.1*
nonadapted	6.2±0.1	6.5±0.1

Note. FCS: fetal calf serum (Gibco). *p<0.001 compared to nonadapted strain.

ductive activity of A/Solomon Islands/03/06 strain in MDCK and Vero cells grown in experimental and control media containing 2 or 5% fetal calf serum (Gibco) was evaluated at infection multiplicity of 1.0, 0.1 and 0.01 EID_{50}/cell. The optimal dose was 1.0 EID_{50}/cell (Table 2). Reproductive activity of A/Solomon Islands/03/06 strain was higher on MDCK cells and on both cell cultures grown on

TABLE 2. Reproduction of Vaccine Strain A/Solomon Islands/03/06 on MDCK and Vero Cell Cultures on Day 3 after Infection (*M*±*m*; *n*=5)

Cell culture	Growth medium	lg TCD_{50}/ml at EID_{50}/cell		
		1.0	0.1	0.01
MDCK	Eagle's MEM+5% FCS	8.5±0.1*	8.0±0.1^{x}	N.d.
	Rise hydrolysate+5% FCS	8.7±0.1*	7.9±0.1^{x}	N.d.
	Eagle's MEM+2% FCS	9.1±0.1*$^{+}$	8.4±0.2^{x}	N.d.
	Rise hydrolysate+2% FCS	9.5±0.1*$^{+o}$	8.5±0.1^{x}	N.d.
Vero	Eagle's MEM+5% FCS	8.0±0.1	7.8±0.1	5.0±0.1
	Rise hydrolysate+5% FCS	8.1±0.1	7.5±0.1^{x}	4.5±0.1
	Eagle's MEM+2% FCS	8.5±0.1^{+}	8.0±0.1^{x}	5.5±0.1
	Rise hydrolysate+2% FCS	9.0±0.1^{+o}	8.2±0.1^{x}	4.9±0.1

Note. Here and in Table 3: N.d.: not determined, p<0.01 compared to: *Vero cells, $^{+}$5% FCS, ^{x}infection multiplicity 1.0 EID_{50}/cell; ^{o}p<0.05 compared to Eagle's MEM medium.

TABLE 3. Reproduction of Vaccine Strain B/Malaysia/2506/2004 on MDCK and Vero Cell Cultures on Day 3 after Infection (*M*±*m*; *n*=5)

Cell culture	Growth medium	lg TCD_{50}/ml at EID_{50}/cell		
		1.0	0.1	0.01
MDCK	Eagle's MEM+5% FCS	8.50±0.12*	8.0±0.1^{x}	4.8±0.1
	Rise hydrolysate+5% FCS	N.d.	N.d.	N.d.
	Eagle's MEM+2% FCS	8.3±0.1*	7.6±0.1	5.2±0.1
	Rise hydrolysate+5% FCS	9.0±0.1*^{o}	8.0±0.1^{x}	4.7±0.1
Vero	Eagle's MEM+5% FCS	8.0±0.1	7.4±0.1^{x}	4.5±0.1
	Rise hydrolysate+5% FCS	8.0±0.1	7.1±0.1^{x}	3.8±0.1
	Eagle's MEM+2% FCS	7.7±0.1	7.0±0.1^{x}	4.7±0.1
	Rise hydrolysate+5% FCS	8.5±0.1^{+o}	7.5±0.1^{x}	4.2±0.1

experimental medium compared to control medium. Reproduction of this virus on both cell lines in experimental or control medium in the presence of 2% fetal calf serum was higher than in the presence of 5% this serum. The increase in the infection titers of influenza virus A/Solomon Islands/03/06 in the culture of MDCK cells in experimental or control medium in the presence of 2% serum was 0.7 and 0.8 lg, respectively. The corresponding increase in infection titers in Vero cells was 0.5 and 0.9 lg for control and experimental grown medium, respectively (Table 2).

Reproduction of B/Malaysia/2506/2004 strain in MDCK and Vero cell cultures. Reproduction of B/Malaysia/2506/2004 virus strain was studied under conditions similar to those for A/Solomon Islands/03/06 strain. Reproductive activity of influenza B virus was lower by 0.5 lg compared to A/Solomon Islands/03/06 strain in both cell cultures (Table 3). Similarly to influenza A virus, B/Malaysia/2506/2004 strain attained higher infection titers in MDCK cells compared to Vero cells. Moreover, reproductive activity of B/Malaysia/2506/2004 in infection multiplicity of 1.0 EID_{50}/cell was higher in MDCK and Vero cells in the experimental medium containing 2% fetal calf serum (Table 3).

Reproductive activity of influenza A and B virus strains used in our experiments was higher in MDCK cells compared to Vero cells. Lower reproductive activity of viruses in Vero cells compared to MDCK cells can be explained by the fact that Vero cells secrete a factor rapidly inactivating exogenous trypsin [9]. Under optimal culturing conditions for Vero cells, including repeated addition of trypsin into the medium, vaccine strains of influenza A and B viruses attain higher infection titers. Despite the fact that optimal culturing conditions for reproduction of vaccine strains can be chosen for both MDCK and Vero cells, MDCK cell culture according to published data is more perspective for the production of culture-derived influenza vaccines. It was demonstrated that maximum production of the virus on MDCK cells can be attained at shorter terms after infection than on BHK and Vero cells [11]. In 1996, seeding and working MDCK cell banks were created and certified according to WHO requirements [15]. Preclinical and clinical testing of influenza vaccines obtained on MDCK cells showed that culture-derived vaccines are safe and meet the immunogenicity criteria for influenza vaccines [6,13].

The results of our experiments showed that culturing of MDCK and Vero cells in grown medium with low (2%) concentration of fetal calf serum compared to usual (5%) concentration ensured higher reproductive activity of influenza A and B virus vaccine strains. The yields of these strains on MDCK and Vero cells cultured on enzyme hydrolysate of rise flour proteins were comparable or surpassed the corresponding parameters obtained on Eagle's MEM medium.

Thus, the nutrient medium based on enzyme hydrolysate of rise flour proteins developed by us and containing low concentration of fetal calf serum can be used for culturing MDCK and Vero cells promising for the production of culture-derived influenza vaccines. MSCK cells grown in this medium ensure higher yields of vaccine strains of influenza A and B viruses at shorter terms compared to Vero cell culture. Now we develop serum-free nutrient media on the basis of plant raw materials, enzyme hydrolysate of rise and soya flour proteins for culturing of MDCK and Vero cells, the main substrates for culture-derived influenza vaccines.

REFERENCES

1. T. A. Bektimirov, *Bulletin. Vaccination*, **3**, No. 27, 1-5 (2003).
2. Yu. Z. Gendon, *Vopr. Virol*., **46**, No. 3, 5-12 (2001).
3. N. A. Mazurkova, T. Yu. Sen'kina, and T. M. Sviridenko, *et al., Biotekhnologiya*, No. 3, 58-64 (2003).
4. G. G. Onishchenko, N. A. Mazurkova, G. P. Troshkova, *et al*., *Ibid*., No. 3, 31-37 (2007).
5. G. P. Troshkova, L. D. Martynets, E. V. Kirova, *et al., Ibid*., No. 4, 74-78 (2006).
6. R. Brands, A. M. Palache, and G. J. M. van Scharrenburg, *Options for the Control of Influenza III*, Eds. L. E. Broun *et al.*, Cairns (1996), pp. 683-693.
7. P. Bruhl, A. Kerschbaum, O. Kistner, *et al., Vaccine,* **19**, 1149-1158 (2001).
8. Y. Z. Gendon, S. G. Markushin, I. I. Akopova, *et al., Ibid*., **23**, 4678-4684 (2005).
9. N. V. Kaverin and R. G. Webster, *J. Virol*., **69**, No. 4, 2700-2703 (1995).
10. O. Kistner, P. N. Barrett, W. Mundt, *et al., Vaccine*, **16**, Nos. 9-10, 960-968 (1998).
11. O. W. Merten, J. C. Manuguerra, C. Hannoun, and S. van der Werf, *Dev. Biol. Stand*, **98**, 23-37 (1999).
12. B. J. Montagnon, B. Fanget, and J. C. Vincent-Falquet, *Rev. Infect. Dis.*, Suppl. 2, 341-344, (1984).
13. A. M. Palache, R. Brands, G. J. M. van Scharrenburg, *J. Infect. Dis.*, **176**, Suppl. 1, 520-523 (1997).
14. J. Robertson, *Virology,* **3**, 97-106 (1993).
15. WHO Cell Culture as a Substrate for the Production of Influenza Vaccine: Memorandum from a WHO Meeting. *Bull. Wld Hlth Org.*, **73**, 431-435 (1995).