The Use of Components of Plant Origin in the Development of Production Technology for Live Cold-Adapted Cultural Influenza Vaccine

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Translated from *Kletochnye Tehnologii v Biologii i Medicine*, No. 3, pp. 157-160, August, 2008
Original article submitted April 3, 2008

The impact of culturing conditions (multiplicity of cell culture infection with influenza virus, composition of growth and maintenance nutrient media) for the efficiency of multiplication of cold-adapted reassortant vaccine strains of influenza A and B viruses was evaluated. Soybean hydrolysate protein-based biological additive to nutrient medium provided effective reproduction of influenza A virus in MDCK cells in the presence of 2 μg/ml trypsin. The use of soybean peptone-based stabilizer provided retention of infectious titer of influenza virus grown in MDCK culture after its lyophilization to a level of 8.5 lg EID_{50}/ml.

Key Words: *influenza virus; MDCK cells; plant additives; live cultural vaccine*

Influenza remains a serious problem for the majority of countries for a long time [13]. The threat of appearance of new pandemic strains of influenza is permanent, which is seen from the events of recent years: appearance of strains with "avian" hemagglutinins H5 and H9 in the human population, which caused lethal respiratory infection in humans. It is probable that some other HA subtypes, in addition to H5 and H9, can render the virus virulence for humans under conditions of an appropriate combination of the rest genes.

It is therefore impossible to develop and produce a vaccine protecting from future pandemic beforehand. The development of cell culture technologies permitting production of vaccine based on the pandemic virus strain directly after its appearance can help to solve this problem. Intensive studies in this direction are carried out in many countries [3,4,7-10].

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In Russia, the development of production technology for live cold-adapted attenuated influenza vaccine on MDCK cells is realized since 2001 at Vector Institute of Virology and Biotechnology in collaboration with I. I. Mechnikov Institute of Vaccines and Sera, Russian Academy of Medical Sciences [2,6]. Due to the use of MDCK continuous culture for virus multiplication, it is possible to produce the virus within short time and to provide the retention of antigenic characteristics of the epidemic strain in the virus produced on MDCK cells [11,12,14]. It is desirable to remove any products of animal origin in biotechnology of cells intended for production of cultural vaccines for humans and replace them by substances of plant origin, therefore the state agencies, controlling the manufacture of pharmaceutical products in Europe (the European Medicine Evaluation Agency; EMEA) and in the USA (the Food and Drug Administration; FDA) called on the leading pharmaceutical companies to rule out or limit the use of components of animal origin in creation of vaccines [5].

The aim of this study was to develop a production technology for live vaccine on the basis of

0007-4888/08/1461-0144

cold-adapted reassortant vaccine strains of influenza viruses A and B, cultured in MDCK cells in serum-free medium with components of plant origin (plant additive and stabilizer).

MATERIALS AND METHODS

Influenza virus strains. Cold-adapted reassortant (CR) strains of influenza virus: A/47/Shandon/93/1 (H1N1), A/47/Johannesburg/94/2 (H3N2), and B/60/Petersburg/95/20 were obtained from I. I. Mechnikov Institute of Vaccines and Sera.

Cell culture. The study was carried out on MDCK (adult female cocker spaniel kidneys) cells from collection of Vector Institute. The cells were cultured in Eagle's MEM with 5% fetal calf serum (Gibco). The cells were infected with influenza virus CR strains grown on developing chicken embryos (DCE) at different multiplicity of infection. Infected cells were incubated at 34°C for 60 min, after which Axcevir-MDCK serum-free maintenance medium with 2 μg/ml trypsin was added, and incubated at 34°C. Plant additive was added into the maintenance medium or medium without additive was used. Virus samples were taken on days 1-5. Infective titers were tested by infecting 11-day DCE.

Evaluation of thermodegradation of lyophilized influenza virus CR strains. The shelf life of dry sterile vaccine strains was evaluated by the rapid aging method at high temperatures [1]. Freeze dried specimens of three CR strains were exposed to 37.0±0.5°C for 7 days, after which specimens of vaccine strains were dissolved in sterile distilled water and titered on DCE for evaluation of infective titers.

The results were processed using common methods of variation statistics. The significance of differences in the means was evaluated by Student's *t* test.

RESULTS

Multiplication of CR influenza virus strains in a monolayer culture of MDCK cells. Study of the virus multiplication in MDCK cell culture at different multiplicity of infection showed that the maximum titer at 0.005 EID_{50}/cell multiplicity of infection was attained on day 3 for strain A/H3N2, on day 4 for strain A/H1N1, and on day 5 for influenza B virus (Table 1). At multiplicity of infection of 0.001 and 0.0005 EID_{50}/cell, the maximum titers of all three strains, which were a little lower than at multiplicity of 0.005 EID_{50}/cell, were observed on day 4 for strain A/H3N2 and A/H1N1 and on day 5 for influenza B virus strain.

TABLE 1. Dynamics of Influenza Virus Accumulation in MDCK Cell Culture at Different Multiplicity of Infection (*M*±*m*)

Multiplicity of infection, EID_{50}/cell	Titers of cold-adapted reassortant strains of influenza virus, lg EID_{50}													
	H1N1, days					H3N2, days					B, days				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
0.0001	<1.0	1.0±0.1	6.5±0.1	8.0±0.1	7.5±0.1	N.d.	6.4±0.1	8.0±0.1	8.3±0.1	8.0±0.1	N.d.	N.d.	5.7±0.1	7.3±0.1	8.0±0.1
0.0005	2.0±0.1	4.5±0.1	7.0±0.1	9.1±0.1	9.0±0.1	N.d.	5.5±0.1	8.5±0.1	9.2±0.1	9.0±0.1	N.d.	5.5±0.1	7.2±0.1	8.0±0.1	8.5±0.1
0.001	2.5±0.1	5.0±0.1	7.5±0.1	9.0±0.1	8.9±0.1	5.0±0.1	8.2±0.1	8.7±0.1	9.3±0.1	8.5±0.1	4.0±0.1	5.8±0.1	7.5±0.1	8.5±0.1	8.8±0.1
0.005	4.5±0.1	7.0±0.1	8.5±0.1	9.5±0.1*	8.0±0.1	N.d.	6.8±0.1	9.5±0.1*	9.0±0.1	8.4±0.1	N.d.	5.5±0.1	7.0±0.1	8.7±0.1	9.2±0.1*

Note. N.d.: not determined. *$p<0.05$ *vs.* the parameter at multiplicities of 0.001, 0.0005, and 0.0001.

TABLE 2. Reproduction of Cold-Adapted Influenza Virus Strains in MDCK Cells with Plant Additive (*M*±*m*)

Strain	No additive	Influenza virus titers (lg EID_{50}/ml) after 3 days in the presence of plant additive in different concentrations			
		0.02	0.5	0.8	1.0
A/47/Shandon/93/1	7.5±0.1	8.7±0.1*	8.5±0.1*	8.5±0.1*	8.7±0.1*
A/47/Johannesburg/94/2	8.7±0.1	8.5±0.1	9.3±0.1*	8.5±0.1	8.5±0.1
B/60/Petersburg/95/20	7.5±0.1	7.0±0.1*	6.0±0.1*	6.7±0.1*	7.0±0.1*

Note. *$p<0.05$ compared to value without additive.

Effects of soybean hydrolysate proteins on the reproduction of influenza virus CR strains in MDCK cell culture. The effects of soybean hydrolysate proteins on specific activity of CR strains were studied under conditions for their multiplication at which none of these strains reached the maximum titer. Virus strain multiplication after addition of soybean hydrolysate proteins into the medium was evaluated 3 days after infection at multiplicity of 0.001 EID_{50}/cell. Soybean hydrolysate proteins (Sigma) were added into Axcevir-MDCK maintenance nutrient medium to the concentrations of 0.02 to 1.0%. Addition of soybean hydrolysate proteins into nutrient medium increased specific activity of influenza A CR strains (Table 2). For strain A/47/Johannesburg/94/2 (H3N2) this increase constituted 0.6 lg after addition of 0.05% soybean hydrolysate proteins into nutrient medium. For strain A/47/Shandon/93/1 (H1N1) specific activity increased by 1.0-1.2 lg at all studied concentrations of plant proteins. The plant additive was ineffective for influenza B strain.

Effects of soybean hydrolysate proteins on retention of specific activity of influenza CR strains cultured in MDCK cells. For this study, we replaced traditional gelatose or human serum albumin, used as stabilizers in influenza vaccines, with soybean peptone solution (Sigma; International application PCT/Ru/03/00263 of June 23, 2003). This stabilizer was added into virus-containing culture fluid in concentrations of 1-10% In order to evaluate the retention of specific activity of influenza virus CR strains cultured in MDCK cells during lyophilization and storage, this stabilizer was added to concentrations of 1-10%. This was followed by lyophilization of vaccines (virus samples containing the stabilizers). The shelf life of lyophilized semi-finished vaccines was evaluated by the method of rapid aging at high temperatures [1]. Specific activities of virus-containing fluid, liquid and semi-finished vaccine preparations were evaluated before and after thermal processing by parallel titration of the preparations in DCE. The results indicate that soybean hydrolysate-based stabilizer promoted the retention of specific activity of influenza virus CR strains during lyophilization and storage at high temperature (the data for influenza A H1N1 strain are presented; Table 3). The sta-

TABLE 3. Effects of Plant Protein Hydrolysate on Retention of Specific Activity of A/H1N1 Virus Cultured in MDCK Cells (*M*±*m*)

Plant protein hydrolysate concentration, %	Influenza A/H1N1 virus titer, lg EID_{50}/ml			
	virus-containing fluid	liquid semi-finished vaccine	lyophilization semi-finished vaccine	lyophilization semi-finished vaccine after thermodegradation at 37°C for 7 days
0	9.0±0.1	8.8±0.1	8.0±0.1*	6.3±0.1
0.01	9.5±0.1	8.5±0.1	7.5±0.1*	6.3±0.1
0.02	8.7±0.1	8.7±0.1	8.5±0.1	6.3±0.1
0.5	9.1±0.1	8.5±0.1	8.5±0.1	7.4±0.1
0.8	8.8±0.1	8.5±0.1	7.5±0.1*	6.3±0.1
1.0	9.5±0.1	8.7±0.1	7.7±0.1*	7.3±0.1

Note. *$p<0.001$ compared to the value for liquid semi-finished vaccine at the same concentration of plant protein hydrolysate.

bilizer concentration of 0.5% proved to be optimal; specific activity (8.5 lg EID_{50}/ml) of semi-finished liquid monovaccine based on A/H1N1 strain did not decrease during lyophilization and decreased by 1.1 lg during thermal processing. In control samples (no stabilizer) lyophilization and thermodegradation led to a more significant reduction of specific activity (by 0.8 and 1.7 lg, respectively). Increasing the stabilizer concentration to 1.0% led to reduction of monovaccine specific activity during lyophilization, similarly as in the control specimen (by 1.0 lg), and during thermodegradation (by 0.4 lg; 1.3 lg less than in the control; Table 3).

Hence, addition of soybean proteins to maintenance nutrient medium increases reproduction of influenza A virus cultured in MDCK cells, while the use of a stabilizer of plant origin provides the retention of specific activity of monovaccine during lyophilization and thermodegradation. Our new technology for the production of live cultural influenza vaccine consisting in replacement of components of animal origin (animal serum, gelatose, or human serum albumin as stabilizers) with products of plant origin will eliminate allergic reactions caused by possible contamination of influenza vaccines with other foreign viruses, Mycoplasma, or prions.

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