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VIROLOGY

Composition of the Stabilizer and Conditions of Lyophilization for Preserving Infectious Activity of Influenza Virus

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For stabilization of vaccine preparations, they are lyophilized. The composition of the protective medium is an important parameter affecting the quality of the vaccine after drying. In view of the risk of spreading prion diseases, the use of media containing animal proteins is not recommended. In this study, protective media containing no animal proteins and lyophilization regimen were determined. The optimum lyophilization regimen consisted of three stages: freezing at -70°C, main stage at -35°C, and drying at 24°C. Protective medium containing 4% trehalose or protective medium with 10% sucrose and 5% soy peptone ensured highest stability of the lyophilized vaccine preparation in temperature range of 4-24°C. This can help to overcome possible break in the cold chain, which is important during transporting or storage of vaccine preparations.

Key Words: *influenza viruses; stabilizers; live influenza vaccine; lyophilization*

Influenza is an infectious disease in which continuous changes of the virus plays primary importance for annual epidemics, and sometimes pandemics. Over the last hundred years, the world has faced five pandemics: in 1918, 1957, 1968, 1977 and 2009 [6]. Influenza epidemics can negatively affect the economy and create excessive burden for healthcare service.

Vaccination is the most effective method of protection against influenza. The use of live cold-adapted reassortant influenza vaccine is an effective way to protect susceptible population from not only seasonal epidemics, but also global influenza pandemics.

Live vaccines are based on attenuated strains of the virus that retains limited ability to reproduce in the human body, but is capable of inducing immune response. In order to ensure high immunogenicity, a live vaccine should have high reproductive activity (high infectious titer) [7].

To maintain a high infectious titer, vaccine strains are subjected to lyophilization. Prior to lyophilization, the biological product is dissolved in a protective medium. This technological stage stabilizes the properties of the biopreparation and considerably increases shelf life of the product.

At the A.A. Smorodintsev Department of Virology, Institute of Experimental Medicine, 13% peptone (product of animal origin) was traditionally used as a supplement for drying of influenza viruses for subsequent storage. However, the use of substances that contain animal proteins is now not recommended due to the risk of transmission of prion diseases and more stringent requirements to the composition of the preparations are imposed [4].

Our aim was to choose optimal conditions for lyophilization of vaccine samples and to select optimal protective medium containing no proteins of animal origin.

MATERIALS AND METHODS

We used attenuation donor A/Leningrad/134/17/57 (H2N2) and vaccine strains A/17/Bolivia/2013/6585 (H1N1)pdm09, A/17/Switzerland/2011/1 (H3N2),

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TABLE 1. Composition of the Stabilizers

Code	Composition	Manufacturer	References
Stabilizer No. 1	Soy peptone 5%	Himedia (India)	[2]
	Sucrose 10%	Vecton (Russia)	
Stabilizer No. 2	Mannitol 4%	PanReac AppliChem (Spain)	Materials provided by Dr. Friede Martin Howell (personal communication)
	Sucrose 4%	Vecton (Russia)	
Stabilizer No. 3	Mannitol 8%	PanReac AppliChem (Spain)	Materials provided by Dr. Friede Martin Howell (personal communication)
Stabilizer No. 4	Trehalose 4%	PanReac AppliChem (Spain)	Materials provided by Dr. Friede Martin Howell (personal communication)
Control medium	Peptone of the animal origin 13%	Merck & Co, Inc (USA)	Protocol that used in A. A. Smorodintsev Department of Virology, Institute of Experimental Medicine

and A/17/Anui/2013/61 (H7N9) prepared on the basis of this donor at the A. A. Smorodintsev Department of Virology by classical reassortment with epidemic viruses A/Bolivia/559/2013 (H1N1)pdm09, A/Switzerland/9715293/2013 (H3N2), and A/Anui/1/2013 (H7N9) in accordance with WHO recommendations on the composition of influenza vaccines for the 2015-2016 epidemic season [8]. Vaccine samples were accumulated in 10-day developing chick embryos (DCE) at 32°C for 48 h.

Four compositions of protective medium and one control medium (13% peptone) were added to the vaccine strains as stabilizers (Table 1).

Lyophilization of vaccine strains was conducted in the Labconco FreeZone Stoppering Freeze dryer 7948030 (Labconco). The virus-containing liquid of each vaccine strain was mixed with one of the 4 stabilizers so that the final concentrations of the protective medium components corresponded to values specified in Table 1. The material was transferred into 0.5 ml vials. The vials with the material were placed on a stand and were kept at -70°C for 4-5 h; then, they were placed in a sublimator at -35°C, and after the vacuum was attained, main drying was conducted for 24 h followed by additional drying at a positive temperature for 18-20 h. Then, the vials were sealed under vacuum and were rolled.

Biological activity of the samples was determined by titration on a MDCK cell culture at 33°C for 6 days and in DCE at 32°C for 48 h and expressed in $\log TCD_{50}$/ml (50% tissue cytopathic dose) and in $\log EID_{50}$/ml (50% embryonic infectious dose).

RESULTS

Stabilization of biological preparations is very important problem. Wide use of vaccines requires preservation of their biological activity. One of traditional methods is lyophilization that includes the steps of freezing and drying. The absence of unified technology of lyophilization of biological preparations dictates the need in conducting studies on stabilization of each specific biological object.

For unification of the technique of lyophilization effectiveness monitoring, preliminary experiments were conducted aimed at selection of optimal system and model virus for titration.

As DCE serves as the substrate for production of live influenza vaccine, virus titration is also performed in this system. It should be noted that a great variety of substances-stabilizer are used in the screening part of the study and virus titration in the DCE system becomes problematic due to the need of a large number of chick embryos.

In series I, parallel titration of several viruses was performed in two systems, in DCE and MDCK cell culture, to select the model virus that replicates equally well in chicken embryos and in cell culture.

Titers of viruses A/17/Bolivia/2013/6585 (H1N1) pdm09, A/17Switzerland/2011/1 (H3N2), and A/Leningrad/134/17/57 (H2N2) in the test systems differed significantly, while for virus A/17/Anui/2013/61 (H7N9), the differences in titers were negligible (Table 2). Therefore, strain A/17/Anui/2013/61 (H7N9) was chosen for further experiments.

The process of lyophilization consisted of three steps: freezing, primary drying and final drying. During primary drying, water crystals are eliminated from the frozen product bypassing the liquid phase. Final drying starts from temperature increase to remove absorbed water from semidried product. It should be noted that the stage of freezing poses the greatest risk for virus preservation. Thus, it was necessary to choose protective medium and parameters of lyophi-

TABLE 2. Results of Virus Titration in Two Different Substrates

Virus	Culturing system	
	DCE, log EID_{50}/ml	MDCK, log TCD_{50}/ml
A/17/Bolivia/2013/6585 (H1N1)pdm09	4.53±0.15	3.03±0.09
A/17/Switzerland/2011/1 (H3N2)	7.23±0.12	2.47±0.19
A/Leningrad/134/17/57 (H2N2)	8.57±0.07	4.23±0.18
A/17/Anui/2013/61 (H7N9)	7.23±0.18	7.17±0.12

lization ensuring virus protection from damage [5]. To this end, experiments were conducted to study the basic parameters of lyophilization, such as the temperature and freezing time of drug.

In lyophilization of viral preparations, sucrose, lactose, trehalose, mannitol, and other substances are widely used as protective media [1]. At A. A. Smorodintsev Department of Virology, 13% peptone was used for a long time as a stabilizer. Therefore, we used the same parameters of lyophilization for stabilizers as for 13% peptone: freezing temperature -70°C, primary drying at -35°C, and final drying at 4°C (protocol No. 1).

After lyophilization, biopreparation with stabilizer No. 1 (sucrose and soy peptone) had vitreous state. According to [3], drying temperature for biological preparations with sucrose-based protective medium should be increased. We increased it from 4°C to 22°C (drying regimen 2) (Fig. 1). The data indicate that for protective peptone-based medium containing sucrose, trehalose, or mannitol as the second component, the temperature of drying should exceed 4°C. We determined optimal temperature as 22°C. This regimen was used in further experiments (protocol No. 2).

Comparison of the infectious titers of virus-containing samples prepared with use of stabilizers Nos. 1 and 4 showed that their infectious activity was

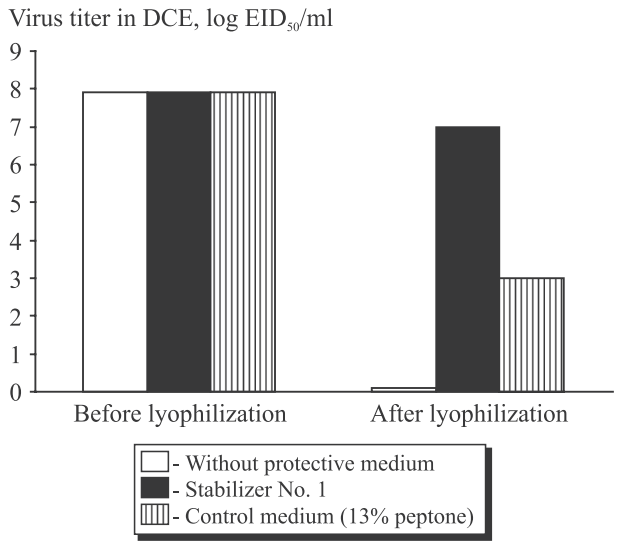

Fig. 1. Titer of virus A/17/Anui/2013/61 (H7N9) before and after lyophilization using the first regimen of drying.

preserved better than in the control. Thus, drying of the material in the presence of 10% sucrose and 5% soy peptone was accompanied by minimal loss that did not exceed 0.2-0.5 log EID_{50}/ml. It was shown that stabilizer No. 4 with 4% trehalose exhibited the best protective properties for all studied vaccine samples. No decrease in virus titer were observed in samples containing this stabilizer immediately after lyophilization and over 2 weeks after it (Table 3).

The obtained dry vaccine samples were stored at 4±2°C for 3 months. Samples were taken at certain intervals to determine the infectious titers. The loss of infectious activity during storage for preparations protected with stabilizer No. 1 (sucrose and soy peptone) and stabilizer No. 4 (trehalose) was insignificant (Fig. 2).

In addition, thermostability of vaccine preparations stored at high and low temperatures for 1 month was analyzed (Fig. 3). Comparison of the infectious titer of lyophilized preparations stored at different temperatures showed that the optimal temperatures of storage are -70°C and 4°C. At these temperatures, the infectious titer of the virus decreased by no more 23%.

TABLE 3. Infectious Titer of Virus A/17/Anui/2013/61 (H7N9) before and after Lyophilization with the Test Stabilizers

Time of titration	Virus titer in MDCK culture, log TCD_{50}/ml				
	stabilizer No. 1	stabilizer No. 2	stabilizer No. 3	stabilizer No. 4	control medium (13% peptone)
Before lyophilization	6.1	6.1	6.1	6.1	6.1
After lyophilization	5.9	5.3	5.3	6.1	5.6
1 week	5.9	5.3	5.3	6.1	5.6
2 weeks	5.6	5.3	5.3	6.2	5.6

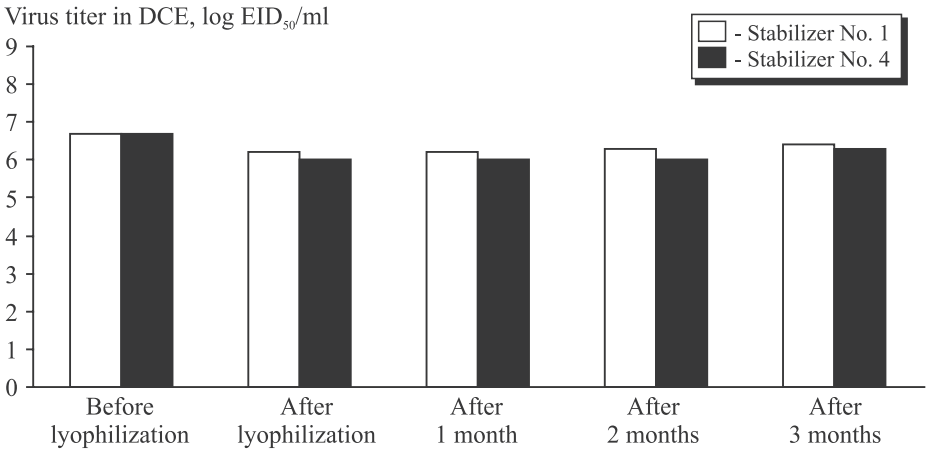

Fig. 2. Titer of the virus A/17/Anui/2013/61 (H7N9) before and after lyophilization, as well as at regimen storage.

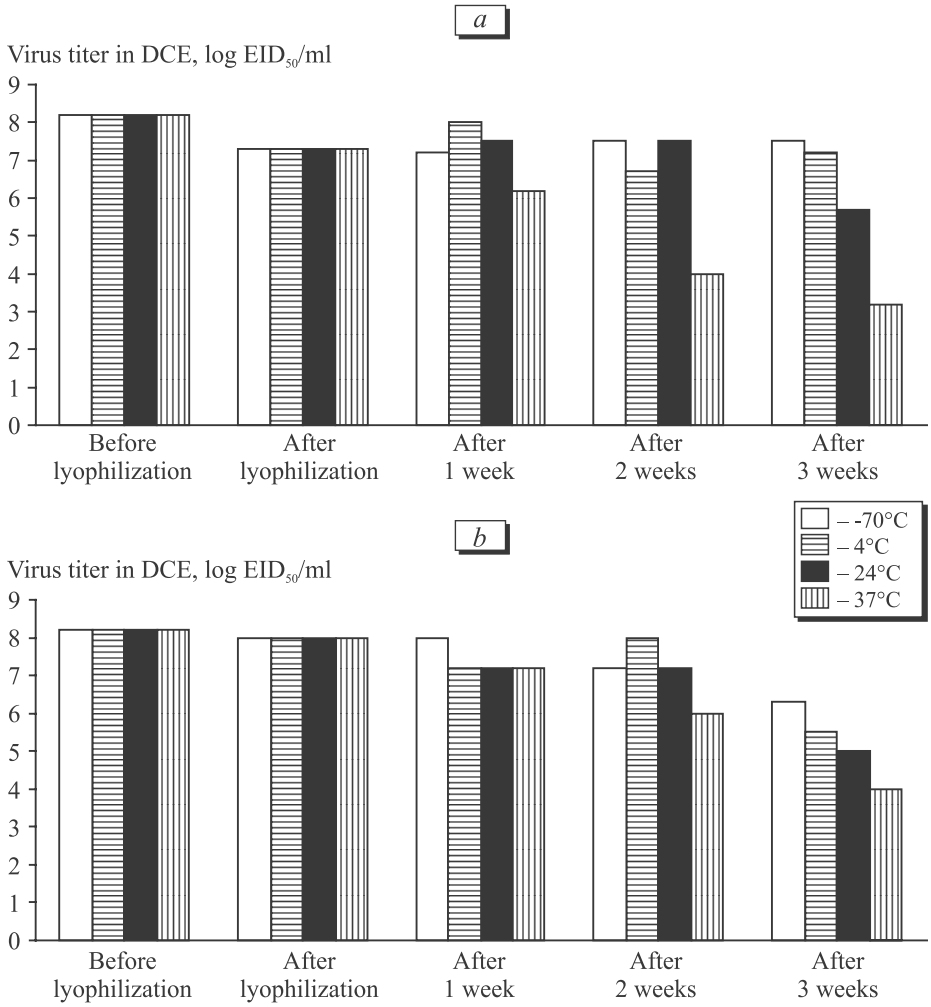

Fig. 3. Infectious titer of virus A/17/Anui/2013/61 (H7N9) during storage with stabilizer No. 1 (*a*), infectious titer of vaccine strain during storage with stabilizer No. 4 (*b*).

Thus, the optimum regimen of lyophilization in the presence of the proposed stabilizers not containing animal proteins consists of the following steps: freezing at -70°C, main drying at -35°C, and final drying at 24°C. The decrease in the infectious titer of the virus was minimum when the drug was stored at -70°C and

4°C. Moreover, short-term fluctuations of temperature up to 24°C did not critically reduced the infectious titer of the preparation. The optimum protective medium is stabilizer No. 4 that contains 4% trehalose and stabilizer No. 1 that contains 10% sucrose and 5% soy peptone. The proven stability of the lyophilized vaccine preparation at temperatures from 4°C to 24°C will help to overcome possible cold chain break, which is very important for transportation and storage of the vaccine preparation.

REFERENCES

1. Arshinova OY, Sanarova EV, Lantsova AV, Oborotova NA. Drug synthesis methods and manufacturing technology: Lyophilization of liposomal drug forms (review). Pharm. Chem. J. 2012;46(4):228-234.
2. Mazurkova NA, Skarnovich MO, Troshkova GP, Shishkina LN. Optimization of freeze-drying process for cultured cold-adapted vaccine samples of influenza A/H5N2 virus. Mezhdunar. Zh. Priklad. Fundament. Issled. 2011;(5):98-100. Russian.
3. Gel'fand AS, Bryzgalova SI, Mel'nikov SJ. Patent RU No. 2290205. Method for preparing live vaccine for influenza prophylaxis. Bull. No. 36. Published December 27, 2006.
4. EMEA 2001. Questions and answers on bovine spongiform encephalopathies and vaccine. URL: http://www.ema.europa.eu/docs/en_GB/document_library/Other/2009/09/WC500003715.pdf.
5. Heikal A, Box K, Rothnie A, Storm J, Callaghan R, Allen M. The stabilisation of purified, reconstituted P-glycoprotein by freeze drying with disaccharides. Cryobiology. 2009;58(1):37-44.
6. Taubenberger JK, Morens DM. Influenza: the once and future pandemic. Public Health Rep. 2010;125(Suppl. 3):15-26.
7. WHO. Expert committee on biological standardization. Geneva, 19 to 23 October 2009. WHO recommendations to assure the quality, safety, and efficacy of influenza vaccines (human, live attenuated) for intranasal administration. URL: http://www.who.int/biologicals/areas/vaccines/influenza/Influenza_vaccines_final_14MAY_2010.pdf.
8. WHO. Recommended composition of influenza virus vaccines for use in the 2015-2016 northern hemisphere influenza season, 26 February 2015. URL: http://www.who.int/influenza/vaccines/virus/recommendations/2015_16_north/en/.