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Progress in the Development of Pandemic Influenza Vaccines and Their Production Technologies

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Abstract—This article analyzes the current situation in the field of construction and production of pandemic influenza vaccines. The main task of protecting the population against influenza pandemics requires state-of-the-art approaches to the construction of influenza vaccines to be based on reassortment and genetic engineering techniques, including the analysis of primary structures of influenza viral genes, synthesis and cloning of the main viral genes, reverse genetics techniques, and banks of plasmids bearing basic viral genes. Reassortant technologies are now giving way to new approaches for objective reasons. The state-of-the-art technologies provide safety not only at the laboratories where vaccine viruses are constructed but also make the production process wholly safe. We are using the following approaches to the development of industrial production: use of nanoparticles and nanoemulsions as functional adjuvants, construction of totally-safe strains for live attenuated influenza vaccines with deletions of molecular determinants of pathogenicity, application of protein and chemical chaperones to provide self-assembly of haemagglutinin molecules of the H1N1v-2009 virus, and impregnation of whole-virion preparations with nanoparticles to enhance antigenicity.

Key words: adjuvants, antigens, vaccines, viral proteins, virus-like particles, live attenuated vaccines, replication-deficient vaccines.

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The level of protection against the avian flu epizootic and the influenza pandemic caused by the swine-lineage H1N1v virus can be characterized as insufficient for Russia's population. The experience in preventing an outbreak of SARS in the territory of Russia has evidenced that organized contraepidemic measures produce a temporary, albeit appreciable, effect. The WHO recognizes the functional priority of epidemiological services, including quarantine measures, in localizing epicenters of infections and preventing their cross-border spread to other countries and continents, that is, the very principles, on which the activities of public health services in our country have always been based. However, the most successful part of contraepidemic measures in preventing the spread of infections with respiratory transmission mechanism is vaccinoprophylaxis [1, 2].

Supply of vaccines critically depends on the level of technologies used in the preparation of vaccine strains and mass production of vaccines. The technologies now in use in Russia and abroad are those developed more than 15 years ago. However, this sphere has made significant progress over the last 5 years. The preparedness activities related to the influenza pandemic and its global outbreak in 2009 substantially increased the demand for vaccines. According to WHO's estimate, the annual global demand accounts for 2 to 3 billion doses. Russia's influenza vaccine production potential is higher than in other countries and sufficient for conducting annual vaccinations in more than 50% of the population. At the same time, the problems associated with the supply and quality of influenza vaccines remain the most important for Russia and the rest of the world and strongly depend on the promptness with which new technologies are adopted.

Abbreviations: WHO—World Health Organization, VLP—virus-like particles, LAIV—live attenuated influenza vaccine, IIV—inactivated influenza vaccine, RNP—ribonucleoproteide, WVP—whole-virion preparations, FDA—Food and Drug Administration, HA—haemagglutinin.

THE ROLE OF VACCINES IN INFLUENZA PROPHYLAXIS

Vaccination is an important part in the complex of contraactions organized by public health services against influenza pandemics. Like the Soviet Union used to be, the Russian Federation remains a leader in conducting mass prophylactic immunizations, in particular, antiinfluenza vaccinations. This must be marked as one of the most important achievements of the past. The country's complex of vaccine producers has demonstrated increased growth rates in recent years against the difficult situation in the pharmaceu-

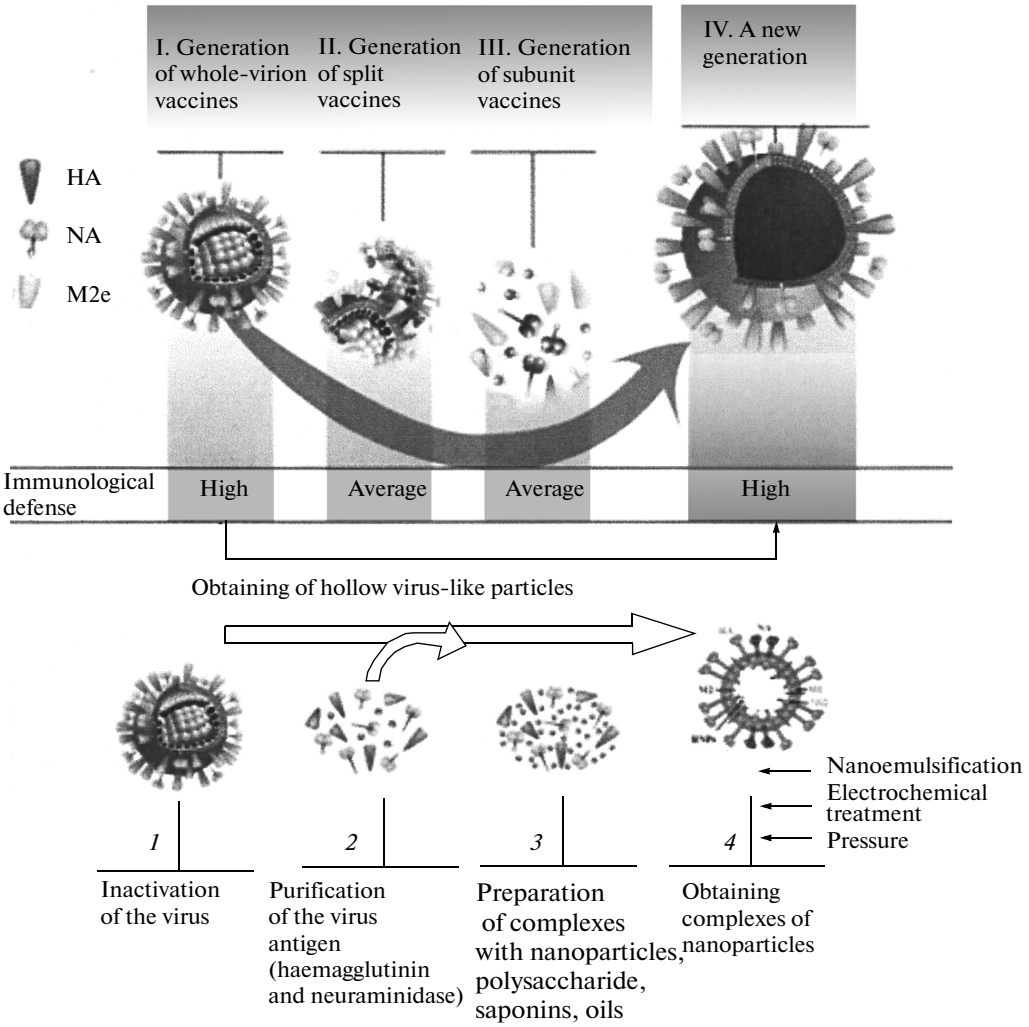


Fig. 1. Types of influenza vaccines: I–IV is four generations of vaccines; *1–4* is the phases of preparation. Data on the efficacy of the vaccines modified by nanoparticles have been obtained in the absence of adjuvants. HA, NA, and M2e are the H5N1 virus proteins (see Table 2).

tical industry. Despite certain technological lagging, our country remains a leading producer of practically a whole spectrum of important vaccines in the world's sector of this industry. In fact, from the late 1960s up to the late 1980s, all contemporary influenza vaccines were pioneered in the Soviet Union. Then basic technologies used to spread globally [1–7].

The initial impulse for scaling up the industrial supply came with the Hong Kong influenza pandemic of 1968 [5–8]. The avian flu epidemic also brought a serious challenge to the world community, but that was already the period when a necessity to employ novel biotechnological achievements in influenza vaccine production was realized [1].

Medical services have acquired much experience in the application of influenza vaccines. In the Russian Federation, the population has already been vaccinated using seasonal influenza trivalent and monovalent vaccines against the pandemic H1N1v-2009 virus.

In Russia and globally, two basic types of vaccines are used for prophylaxis [8–13]: inactivated influenza vaccines and live attenuated influenza vaccines.

The main types of vaccine preparations with their properties and compositions are given in Fig. 1 (on the colored inset). We give also novel types of vaccines; their properties and technology are described below; generation IV represents the contribution made by nanotechnologies playing a principal role in revolutionized vaccine production development.

Inactivated influenza vaccines contain influenza virus strains preliminarily subjected to UV and chemical inactivation or fragmentation with subsequent isolation of antigen-containing components. The efficacy of inactivated vaccines in preventing the disease onset accounts for 70–80% for healthy recipients under 65 years of age and 30–40% for patients over 65. On the contrary, the efficacy of inactivated vaccines in preventing influenza-related lethal outcomes accounts

for 80% even among such a risk group as patients over 65 years of age [7, 12].

By their production technologies inactivated vaccines can be subdivided into three groups: whole-virion vaccines (generation I), split vaccines (generation II) and subunit vaccines (generation III) (see Fig. 1).

Whole-virion vaccines consist of inactivated (chemical or ultraviolet inactivation) whole virions of influenza viruses and contain, apart from the required antigens for immunization, a considerable amount of ballast in the form of viral nucleocapsid. Their advantage is that the surface antigens in the composition of inactivated virions are in conformation close to the natural one.

Split vaccines are products of detergent fragmentation of virions' membranes with removal of all components of nucleoid and contain, along with surface antigens, internal antigens, such as protein M1. At the same time, they are real fragments of highly-purified membranes of viral particles (see Fig. 1, phase 2).

Subunit vaccines contain only surface protective antigens (haemagglutinin and neuraminidase, predominantly, the former) and are preparations with the highest degree of purification from influenza virus components. To the group of subunit vaccines, for example, belongs Grippol, a polymer, which cannot be characterized as an ideal preparation (see below). The main requirements for the vaccines and their production technologies were first specified under the WHO's special document [8, 13].

Live attenuated influenza vaccines, proposed at first by A.A. Smorodintsev in 1938, have successfully been applied in Russia for decades. In the United States, a live intranasal vaccine was approved by the FDA in June 2003 and permitted its use in age groups ranging from 5 to 49 years. Repetitive passages on chicken embryos in decreased-temperature conditions allowed researchers to obtain a weakened virus that does not replicate at high temperatures, characteristic of human lungs, but can propagate in the nasopharynx at a temperature of 34°C, causing a topical infection leading to the development of a secretory and generalized immune response [1, 14]. Since the LAIV vaccination resembles a naturally-occurring transient infection, it has a sufficient efficacy and is characterized, according to different sources, by 94%-seroconversion in children, while, in combination with an inactivated vaccine, it elicits a positive immune response in 68% of the elderly [1, 6].

The overwhelming majority of seasonal and pandemic viruses are products of reassortment[1] [1, 14]. First of all, this applies to the current H1N1v-2009 virus, the agent of the influenza pandemic. Therefore, application of LAIV for preventing the emergence of reassortants with novel properties is restricted on a rise of morbidity and the more so in the outbreak of an influenza pandemic.

The data on the age-related lethality from the pandemic H1N1v-2009 influenza has shown that the morbidity and mortality from influenza-related complications in the period from March to December 2009 were significantly lower among patients born after 1957. A relatively high level of antibodies to the swine-lineage H1N1viruses was revealed in patients over 50 to 55 years of age, which defines a sufficiently high level of protection against the current pandemic H1N1v-2009 influenza [12, 15, 16]. These data evidence in favor of conducting a total vaccination in relatively young groups of the population and children in the coming period prior to the second wave of the pandemic expected in 2010–2011.

Table 1 gives a group of 12 influenza vaccine preparations by domestic and foreign producers, which are now manufactured or registered in Russia.

NEW STRATEGIES IN VACCINE CONSTRUCTION

Studies on developing influenza vaccines and their production technologies have been extended in the most recent 5 years thanks to the deployed program on influenza pandemic preparedness. The WHO has adopted a special technology transfer program on developing influenza vaccine production in third world countries. Europe and the United States agreed to allot grants and worked out programs for developing novel technologies and vaccine preparations with improved qualities: a new generation of LAIV, that is, delNS1-vaccines with a limited replicative potential, LAIV with deletions of pathogenicity factors in genes, latest variations of subunit vaccines enhanced by adjuvants, and capsid nanovaccines and nanovaccines based on inactivated viruses and virus-like particles [14].

What are basic prospects opening before the vaccines constructed using state-of the-art genetic engineering techniques?

Construction of Strains for New Influenza Vaccines Using the Reverse Genetics Technique

The genome of influenza type A viruses consists of 8 segments encoding 11 genes (Table 2).

Vaccines against especially pathogenic strains of influenza viruses are particularly difficult to develop, since the conventional approaches, based on the reassortment of "wild" isolates (epidemiologically important viruses) and donors of attenuation, practically do not work for obtaining this type of candidate vaccine strains. Usually, in reassortment, the genes that encode surface antigens in the donor are replaced by HA and neuraminidase of the epidemic's virus. A candidate vaccine strain is unable to cause a disease but in immunization it supports the development of immu-

[1] Reassortment is an exchange of viral genome fragments between two or more viruses.

Table 1. Vaccines used for influenza prophylaxis in Russia [1, 2]

Vaccines		Preparation, producer	Groups of population	Number of vaccinations	Route of administration	Dose, ml
LAIV		LAIV (for children), JSC Immunopreparat, Irkutsk	3- to 14-year-old children	Two vaccinations with a 3–4-wk interval	Intranasally	0.5
		LAIV (for children and adults), Irkutsk	Children from 3 years of age	A single dose	The same	0.5
		LAIV purified (for adults and schoolchildren), enterprise St. Petersburg NIIVS	Teenagers from 16 years and adults	A single dose	"	0.5
IIV	Whole-virion	Inactivated influenza vaccine, JSC Immunopreparat, Ufa	Children from the age of 7	Two vaccinations with a 3–4-wk interval	Intranasally	0.5
			Adults from the age of 18	A single dose	Parenterally	0.5
		Inactivated influenza vaccine, enterprise of Research Institute of Vaccines and Sera (RIVS), St. Petersburg	Children from the age of 7	Two vaccinations with a 3–4-wk interval	Intranasally	0.5
			Adults from the age of 18	A single dose	Parenterally	0.5
				Two vaccinations with a 3–4-wk interval	Intranasally	0.5
				A single dose	Parenterally	0.5
	Split	Fluarix, SmithKline Beecham, Germany	Children from 1 year to 6 years of age	Two vaccinations with a 4–6-wk interval	Parenterally	0.25
			Children over 6 years of age and adults	A single dose	The same	0.5
		Vaccigrippe, Pasteur-Merieux, France	Children from 6 months to 3-years	Two vaccinations with one month apart	"	0.25
			Children from 3 years and adults	A single dose	"	0.5
		Begrivac, Chiron-Behring, Germany	Children from 6 months to 3-years	Two vaccinations with one month apart	"	0.25
			Children from 3 years and adults	A single dose	"	0.5
	Subunit	Influvac, Solvay Duphar, the Netherlands	Children from 6 months to 3-years	Two vaccinations with one month apart	"	0.25
			Children from 3 years and adults	A single dose	"	0.5
		Agrippal, Chiron S.p.A., Italy	Children from 6 months to 3-years	Two vaccinations with one month apart	"	0.25
			Children from 3 years and adults	A single dose	"	0.5
		Grippol, JSC Immunopreparat, Ufa	Children from 6 months to 3-years	Two vaccinations with one month apart	"	0.25
			Children from 3 years and adults	A single dose	"	0.5

Table 2. Segments of the influenza A virus genome and the functional role of products of expression in the virus's replicative cycle

Segment	Size of gene, bp	Code name of the corresponding polypeptide	Names and functions of proteins
I	2341	PB2	Component of a transcriptase complex: binding of 5'-end caps of mRNA
II	2341	PB1	Component of a transcriptase complex: elongation of RNA-synthesis
		PB1-F2	Viroporin—it causes pores-formation in mitochondria and induces apoptosis
III	2233	PA	Component of a transcriptase complex: endonuclease
IV	1778	HA	Haemagglutinin: identification and binding a receptor. Its native structure is trimer. Fusogenic peptides of HA2 form the attack complex
V	1565	NP	Nucleoprotein; the main component of a viral RNP, component of a transcriptase complex, it controls the nuclear-cytoplasmatic transport of RNA
VI	1413	NA	Neuraminidase; it splits residues of sialic acids, releases viruses from the receptor of plasmatic membranes, assists to gemmation
VII	1027	M1	The main component of viral membranes. It supports the processes of viral particles' self-assembly and gemmation
		M2	It forms the ionic channel—hydrogen pump
VIII	890	NS1	A nonstructural protein; localized in the nucleus and controls splicing and polyadenilation
		NS2 (NEP)	A nonstructural protein; controls nuclear-cytoplasmatic transport of mRNA

nity to the epidemically important virus. Influenza A (H5N1) viruses contain a few reliably-established genetic markers of pathogenicity. These markers are localized in the following genes of the influenza virus and below listed by their significance and pathogenic contribution:

(1) The HA molecule cleavage site.

(2) The open reading frame in the *PB1-PB1-F2* gene, which encodes the amphiphilic transmembrane protein with signaling peptide of mitochondrial localization.

(3) Mutations in the *PB2* gene (polymerase complex).

(4) Mutations in the gene encoding the nonstructural NS1protein—an antagonist of the interferon system of the first type.

(5) Mutations in the M2 protein gene that controls key stages of a reproductive cycle through the so called proton pump and drug resistance to Rimantadine.

As was mentioned, to construct a new vaccine, the surface antigens, primarily HA, should be replaced. Replacing HA in the donor of attenuation by the HA of the avian virus A (H5N1) will lead to a transference of the pathogenicity determinant. A candidate vaccine strain should contain at least one sign of pathogenicity. To obtain a safe vaccine, this determinant should be removed. However, this is impossible to realize at the RNA level. The viral genome should be converted to the DNA form. This technique was realized for the first time by D. Baltimore, a Nobel Prize laureate, for the polio virus RNA more than 20 years ago; relative to the RNA-containing influenza viruses, this approach was termed "reverse genetics" (in nature this process is realized by reverse transcriptase) and patented [14, 17–21]. Several research teams throughout the world construct viruses using the removal technique at the level of the DNA sequences encoding pathogenicity sites (the HA cleavage site in the case of avian influenza viruses). Six basic plasmid DNAs encode internal genes of a known donor of attenuation. Constructing plasmids is a specific process, since, in each plasmids, bidirectional transcription is provided for the virus-specific mRNA and the complimentary virion RNA with a negative polarity—a necessary factor for the synthesis of virus-specific proteins and accumulating genomic fragments when obtaining infectious viral progeny.

In 1973 researchers from the National Institute for Biological Standards and Control (NIBSC), London, Common Cold Research Unit (MRC, England, Salis-

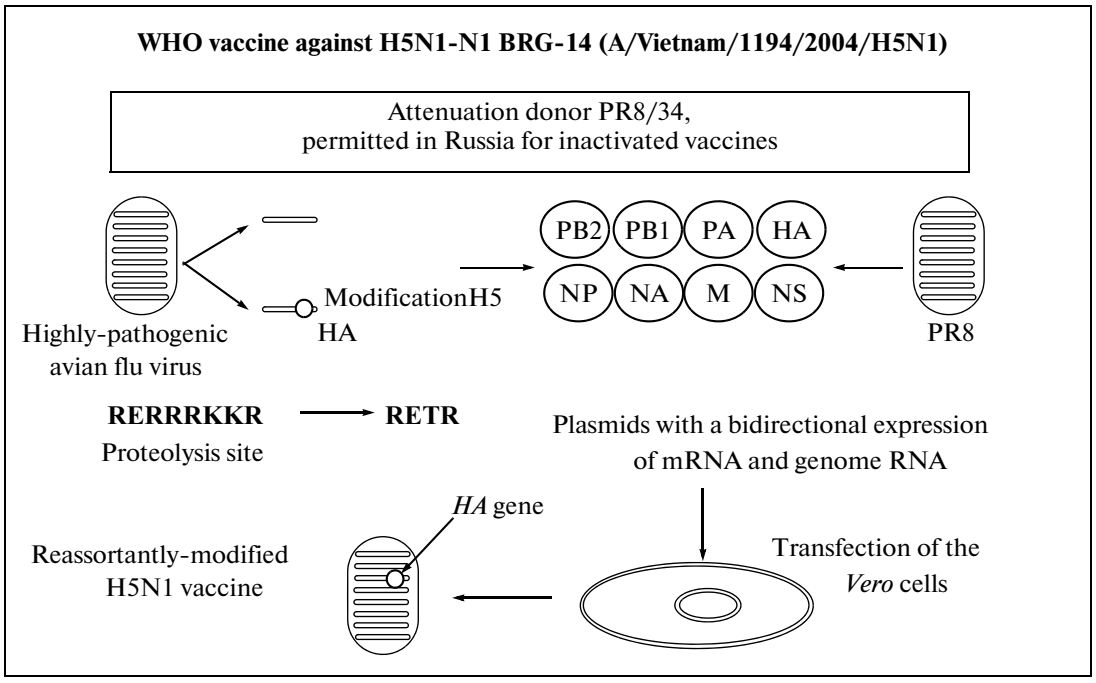


Fig. 2. A scheme for constructing the recombinant influenza H5N1 vaccine with modified HA based on individual DNA-plasmids (reverse genetic technique). See designations in Table 2; N1—neuraminidase 1; see explanations in the text.

bury), as well as from the RJC research and production therapeutic corporation, Rixensart, Belgium, offered the A/PR/8/34 strain as the master donor for constructing influenza vaccines. Eight reassortants of the influenza H1N1 and H3N2 virus serosubtypes were prepared on the basis of this donor and then thoroughly tested in sensitive subjects from different age groups at laboratories in the United States, West Europe, and Australia. The A/PR/8/34 strain was offered as a donor of attenuation, because the reassortants tested lacked reactogenicity and showed very high activity indicators in adults and children (3–4-fold excess over the generally accepted minimum protection level for humoral antibodies). The reverse genetics technique was successfully applied in constructing the candidate vaccine strains on the basis of the avian flu H5N1virus isolates having the most important pathogenicity determinant in the *HA* gene as a cluster of residues from lysine and arginine at the site of proteolysis with furine proteases. After the site was removed from the cloned gene its pathogenicity significantly decreased, being in conformity with the requirements for candidate vaccine strains.

The A/PR/8/34 virus is still used as a successful donor of high reproductive activity. This very strain served as the donor for constructing important viruses for inactivated, predominantly whole-virion vaccines at the Research Institute of Influenza (St. Petersburg). The vaccine passed through preliminary trials for efficacy, genetic stability, and antigenic homogeneity; the preparation is being prepared now for clinical trials.

Development of inactivated vaccines or vaccines with modified *NS1* and *PB2* genes, replication-deficient in the human body, is recommended for obvious reasons. Such vaccines are obtained by reverse genetics techniques mastered at the international laboratory of the Research Institute of Influenza, RAMS (Fig. 2).

A further attenuation of the virus through genetic modifications is possible by obtaining strains with lengthy deletions in the *NS1* gene encoding the antagonist of the first type interferons and the signaling domain PDZ localized at C-end of this viral protein [7, 13]. The level of this technology is so sophisticated that using the 8-plasmid technique complete copies of highly-pathogenic viral isolates can be constructed. In particular, within the European project FluVac, we obtained a complete copy of the Kurgan H5N1-2005 virus isolate.

The representative scheme (see Fig. 2) for the vaccine NI BRG-14 virus assembly shows the removal of the proteolysis site RERRRKKR and insertion of a new “attenuated” site RETR. The *NA* gene in this construction remained intact.

The vaccine strain on this base has specially been prepared for the use in the territory of the Russian Federation. Manipulations with the *NS1* gene were put into the basis of a new technology for obtaining influenza viruses with limited replication potential—replicatively-deficient strains [20, 21]—aimed to replace LAIV as more safe and efficacious. The safety of these vaccines is assessed primarily by their high sensitivity to the endogenous interferon. An influenza

virus is a strong inductor of interferon and, therefore, viruses deprived of the interferon antagonist—full-value protein NS1—are quickly eliminated from the body under the action of interferon I and II types after a short cycle of reproduction. This fact enables us to use the influenza virus as a safe vector. In our work the influenza virus was used as a vector for obtaining the strain expressing the ESAT-6 protein of the TB-causing bacteria [22–24]. The intranasally-administered modified virus manifested in animals the properties of an antitubercular vaccine [23].

Recombinant Subunit Vaccines—Selection of Protective Antigens

The arsenal of approaches to the technological development and solutions in constructing and manufacturing influenza vaccines has become considerably more substantial over recent years. The annual preparation of conventional vaccines (live attenuated, whole-virion, and split- and subunit vaccines) includes, in the first place, the phase of obtaining vaccine strains, which usually takes up to 6 months. Many technological techniques and innovations are oriented to the prompt preparation of antigen producers and the possibility to modify them in correspondence with the data on antigenic drifts of important seasonal or pandemic viruses. Thus, the vaccines now in use can be supplemented by the following list: DNA-vaccines, recombinant subunit vaccines, vector-based vaccines (their obtaining is connected with the use of one of the most attractive technologies for virus-like particles), and a universal vaccine based on highly-conservative antigens (M2, M1, and NP proteins).

In fact, the term recombinant vaccines can define any type of vaccine preparations obtained through genetic engineering. At the same time, considering the advance of these approaches to constructing influenza vaccines, it is more appropriate to specify recombinant vaccines by two types: recombinant influenza viruses used as vectors or deficient viruses for obtaining non-pathogenic vaccine strains and constructs bearing individual viral genes for obtaining different types of vaccines.

We considered above the first approach and now we consider the second approach.

In practice, the process of constructing individual recombinant vaccines comprises the following actions:

(1) Cloning and expression of individual viral genes in different vectors to obtain viral antigens in different expression systems as highly-purified vaccine preparations.

(2) Cloning and expression of individual viral antigens to obtain virus-like particles,

(3) Cloning and expression of viral antigens in eukaryotic vectors to obtain DNA-vaccines.

To achieve each of the above results, one is to make a well-grounded selection.

The following antigen proteins are localized on a viral particle's surface: haemagglutinin (HA), neuraminidase (NA), and the M2 protein. It is established that five antigenic sites are localized in the "head" of the apical part of the HA molecule and that the antibodies' virus-neutralizing activity is associated with the immune response to these sites. Antibodies to HA block the interaction of the virus with cellular receptors, that is, infecting the cells. Antibodies to NA block the secretion of viruses by the infected cells (i.e., propagation of infection). Antibodies to the M2 protein also considerably block the dissociation of infectious viral particles from the infected cells' membranes. We see further that, of the three main surface proteins, only HA is of a principal significance, the consideration of which the production technology for subunit vaccines predominantly containing HA is based. Immunity to NA is believed to be accessory. The M2 protein, considering its supreme conservativeness, became the model object for constructing a universal antiinfluenza A vaccine.

Thus, recombinant vaccines with individual components of influenza type A viruses are mainly oriented to the key and highly variable viral component—the HA molecule. Among the internal antigens of a viral particle, the NP, M1, and NS1 proteins are considered as material for obtaining vaccines.

Another, no less important problem consists in selecting a substrate for growing antigenic substances. Scientists have been discussing for many years the option of replacing chicken embryos with cell cultures. As previously, conventional technologies are oriented to growing viral material on chicken embryos. It applies to LAIV and IIV of the current generations. This technological process consumes millions of chicken embryos (in fact, one dose of a seasonal influenza vaccine is equivalent to one embryo). The scale of the problem is realized by everyone, if to consider that in the event of a pandemic only in Russia the demand for vaccines accounts for at least 100 million doses. This scale of mass production relying on the conventional substrate cannot be promptly deployed (by the coming of a season and more so by the outbreak of a pandemic).

The use of cell cultures in producing antigenic components of vaccines allows specialists to decide the above task rapidly and in sizable volumes. Vaccine production based on the *Vero* and *MDCK* cell cultures have already been licensed in the United States and European countries. Moreover, the use of this approach is in full correspondence with the complete technological chain for obtaining recombinant viruses using the 8-plasmid technique and, in fact, it allows producers to rapidly deploy mass production from the laboratory-scale capacity. In the last 7 or 8 years, candidate vaccine strains for a majority of seasonal viruses

have already been obtained using the so-called reverse genetics technique (see the above description). This approach corresponds also with the DNA-vaccine technology. Although DNA-vaccines have been under development for more than 15 years (their development continues) none of such vaccines are so far permitted for use.

A marked breakthrough in the construction of recombinant vaccines is related to the use of insect cells and the obtaining of virus-like particles (VLPs) based on baculovirus expression vectors. This technique deserves our consideration in detail because of the high technological and quality indicators demonstrated by vaccine preparations. Best solutions on the composition of vaccines, their technologies, high-immunogenicity, and safety are actually united under the project. In accordance with the classification presented, thanks to the absence of ballast components and preservation of the spatial structure of surface antigens, VLPs excel recombinant vaccines, DNA-vectors-based vaccines, and whole-virion vaccines grown on a cell substrate. A positive advantage is, evidently, that the sizes of VLPs and virions also correspond.

Systems of Products and Technologies

We will consider, one by one, projects and achievements in the field of new approaches to constructing influenza vaccines and the use of different systems for their production. We will discuss the projects that are now believed to be the most important for realizing a technological breakthrough in the mass production of safe influenza vaccines.

Use of vectors for constructing and producing virus-like particles as influenza vaccines. Adenoviruses have been used as a tool in model studies on gene therapy for many years. In recent years, specialists engaged in constructing recombinant vaccines have become interested in the advantages promised by adenovirus-based vectors. A particularly attractive feature of adenoviruses is their high productivity and efficiency in gene transfer and expression. Vectors for vaccines are constructed with deletions by early genes *E1* and *E2*.

In some research works, adenovirus vectors are used for the expression of individual viral antigens and, in particular, HA of influenza viruses. A serious disadvantage for these vectors is the presence in the human population of widespread immunity, which leads to a rapid limitation in the expression of target antigens. During epidemics, 15–25% of the human population suffers from adenoviral infections.

Hexon is the strongest antigen among adenoviral proteins. Antibodies to this protein are recorded in more than 75% of healthy subjects. Therefore, when planning the use of these vectors for some works one should consider, first of all, their population receptivity. It equally refers to gene therapy and recombinant vaccines intended for a wide application against mass infections, such as influenza. One of the projects of the Russian Corporation of Nanotechnologies is devoted to the use of adenoviruses as vectors for constructing influenza vaccines.

The vectors based on alpha-viruses are a relatively new tool for constructing recombinant vaccines. The Venezuelan equine encephalomyelitis virus, specifically pathogenic for horses, is especially attractive to virologists as an object of research. The author of this survey studied the basic problems of replication and expression of the RNA-containing virus genome in the early 1970s. The expression of this virus genome is supported by two operons: early and late genes. Foreign genes are inserted in the reading frames of the late genes, thus providing a high level of their expression, comparable with that of the surface major proteins of alpha-viruses. AlphaVax Inc. is the world's leader in using alpha-viruses as vectors for constructing vaccines. At the same time, we are underlining the safety of vectors based on alpha-viruses: they are nonpathogenic for humans, and the human population has no immunity to these viruses, since they have never circulated among people. Constructing recombinant vector influenza vaccines based on alpha-viruses is one of the independent and promising strategies in this field.

Use of insect cells for the expression of influenza virus proteins. The project offered by Novavax Inc. and Protein Science Inc. is one of the most prospective for constructing a new type influenza vaccines and organizing their production. These companies use various vectors for the expression of HA and other influenza virus proteins in insect cells. The latter grow at a temperature of approximately 22°C; the temperature regime for their growth is controlled by special equipment. At the same time, culturing insect cells is a simpler and less expensive process, compared to the growth of animal or human cells, and, at the same time, not associated with a risk of malignancy development. Human-safe vectors based on baculoviruses are used to express antigens and other foreign proteins in insect cells [25]. Baculoviruses express polyhedrin protein in considerable amounts under the control of a strong promoter. Thus, a polyhedrin promoter is used for the expression of foreign antigens (Fig. 3, on the colored insert). With the above constructs, a high level of expression is achieved in insect cells for HA of different subtypes.

At present, two approaches are applied for mass production of influenza vaccine components. One system (Protein Science) synthesizes HA, the main influenza virus component, for a vaccine. The other system (NovaVax) synthesizes simultaneously three proteins: HA, NA, and M1. Synthesis of individual HA allows using this antigen, purified, as the main component of a vaccine identical in its properties to a subunit vaccine. At the same time, it remains unclear if the protein folding with the formation of HA

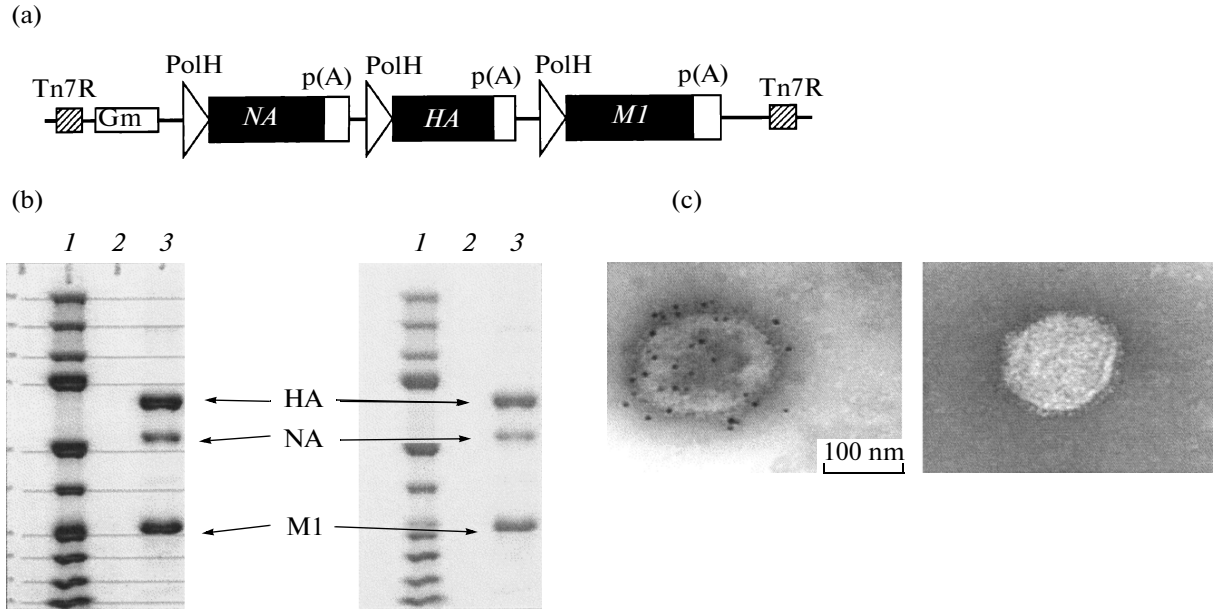


Fig. 3. Expression of VLP H5N1 in insect cells: a—construction of a baculovirus for the expression of VLPs of the avian influenza H5N1 virus strain A/Indonesia/05/2005. On the vector's map are places of the localization of polyhedrin promoters (PolH), polyadenylation signals (p(A)), the Tn7R regions, as well as *HA*, *MI*, and *NA* genes; b—electrophoresis of VLP components (HA, M1, and NA) in polyacrylamide gel: *1*—molecular mass markers; *2*—negative control; *3*—VLP-components; c—transmission electron microscopy of VLP with the identification of the surface HA using specific antibodies conjugated with colloidal gold (specific anti-HA antibodies are on the left, control markers are on the right) (the marker section is equal to 100 nm).

"rosettes" is used to prepare the vaccine, which is a very important process for increasing immunogenicity and ensuring the quality of the immune response, comparable to the native HA in the composition of viral particles. The simultaneous expression of viral proteins HA, NA, and M1 is accompanied by their self-assembly in insect cells. This process results in the formation of VLPs having high immunogenicity, allowing the antigenic burden to be reduced, compared to the individual HA.

The system of obtaining VLPs using the *Sf9* insect cell line with a baculoviral vector expressing three main proteins HA, NA, and M1 (see Fig. 3) was tested in the pandemic H5N1 and H9N2 viruses and the seasonal influenza H3N2 virus. In all cases, the VLPs had high immunogenicity in conformity with the requirements for vaccine preparations.

Use of plants for the expression of influenza virus proteins. For many reasons, including economic ones, the strategy of using systems producing vaccine components in plants is very attractive. Attempts are now being taken to obtain plant producers for influenza virus antigens [7, 14]. Russia is conducting such studies within a few projects. There are attempts jointly with Moscow State University and a group of European universities to optimize the expression of the genes encoding haemagglutins of the H5N1viruses and the pandemic H1N1v-2009 virus in various plant systems using viral vectors. Positive results have been obtained in optimizing the expression of the HA5 gene fragments (encoding immunodominant epitopes) in transgenic plants; a technology has been developed for culturing some species (*Lemna minor*) of the subfamily Lemnaceae in bioreactors; animal tests for the efficacy of the obtained edible vaccines against the avian flu H5N1 virus have already been completed.

It is natural that edible vaccines are important not only for controlling zoonozic pools of viruses and bacteria, but we believe that they are principally significant for early vaccination in babyhood when the immune system of the intestines in neonatals is still in the process of formation. Therefore, large-scale works on obtaining vaccine preparations based on rice cultivars are worthy of attention [26]. A progress in these studies may principally change our children's vaccination calendar and make vaccination in infancy completely safe.

UNIVERSAL VACCINE

Genetic properties of the influenza A viruses are characterized by extreme antigenic variability. The strains should be constantly monitored and studied as to their genetic structure and antigenic properties. A special commission of WHO considers, on an annual basis, data on the properties of isolates from all over the world and makes decisions on the strains in the composition of a next vaccine. At the same time, an outbreak of a pandemic brings a sharp change in the agent's antigenic structure or antigenic shift. In 2009,

Table 3. Comparative mortality data on influenza pandemics

Influenza	Pandemic's duration	Antigenic structure	Total morbidity	Mortality	
				Absolute values	%
Hong Kong flu	1968–1969	H3N2	Not available	1 million	<0.1
"Swine" flu	2009	H1N1sw1	620000	14.3 thousand	0.4 to 1.2

an antigenic shift occurred within a subtype of H1N1, and a new swine-lineage virus emerged with a principally different structure of the surface HA and NA antigens. The global population in its majority proved unprotected against this virus; its relatively high pathogenicity explains the increased mortality from the infection caused by this agent (Table 3).

On the other hand, for the majority of other RNA-containing viruses, researchers have obtained vaccine strains not requiring seasonal replacement. In particular, it refers to the agents of measles and poliomyelitis. The natural question has been raised for years: Is it possible to develop some universal anti-influenza A vaccine based on the agent's conservative proteins?

If we take surface antigens, only three can participate in the selection (see above). The M2 protein's principal difference from the other two is its high conservatism admitting only insignificant replacements in the gene structure. The surface of virions exposes approximately 20 copies of the M2 protein tetramer that performs the functions of ion channel—proton pump. The functional activity of the proton pump is important at the onset of infection for decapsidation of the virus in endosomes and, subsequently, under final phases for gemmation and release of viral particles. Thus, the M2 protein controls the process of infecting cells and propagation of infection. It has recently been established that the sign of contagiousness, or the virus spreading speed in human-to-human transmission, is associated with the M2 protein [27, 28].

According to other data, the M2 protein is significantly responsible for the development of oxidative stress accompanying influenza infections. Therefore, induction of an immune response to this surface protein is believed to be very important for the protection against influenza infections.

At present, the construction, preclinical trials and, in some cases, clinical trials have been completed on the following preparations:

(1) Recombinant preparation of the apical (extramembraneous) M2e domain in the capsid carrier of the hepatitis B protein particles.

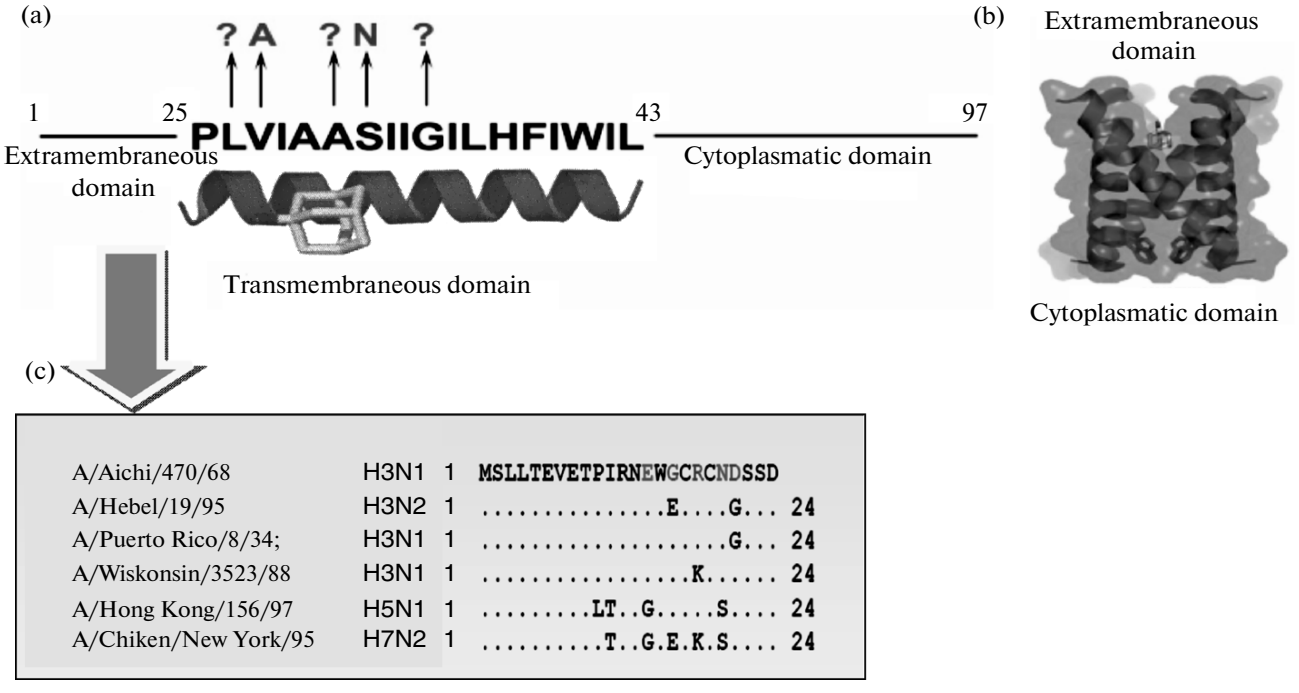


Fig. 4. Selection of the extramembraneous part of the M2 protein of influenza A viruses as an antigen for a universal influenza vaccine (see explanations in the text).

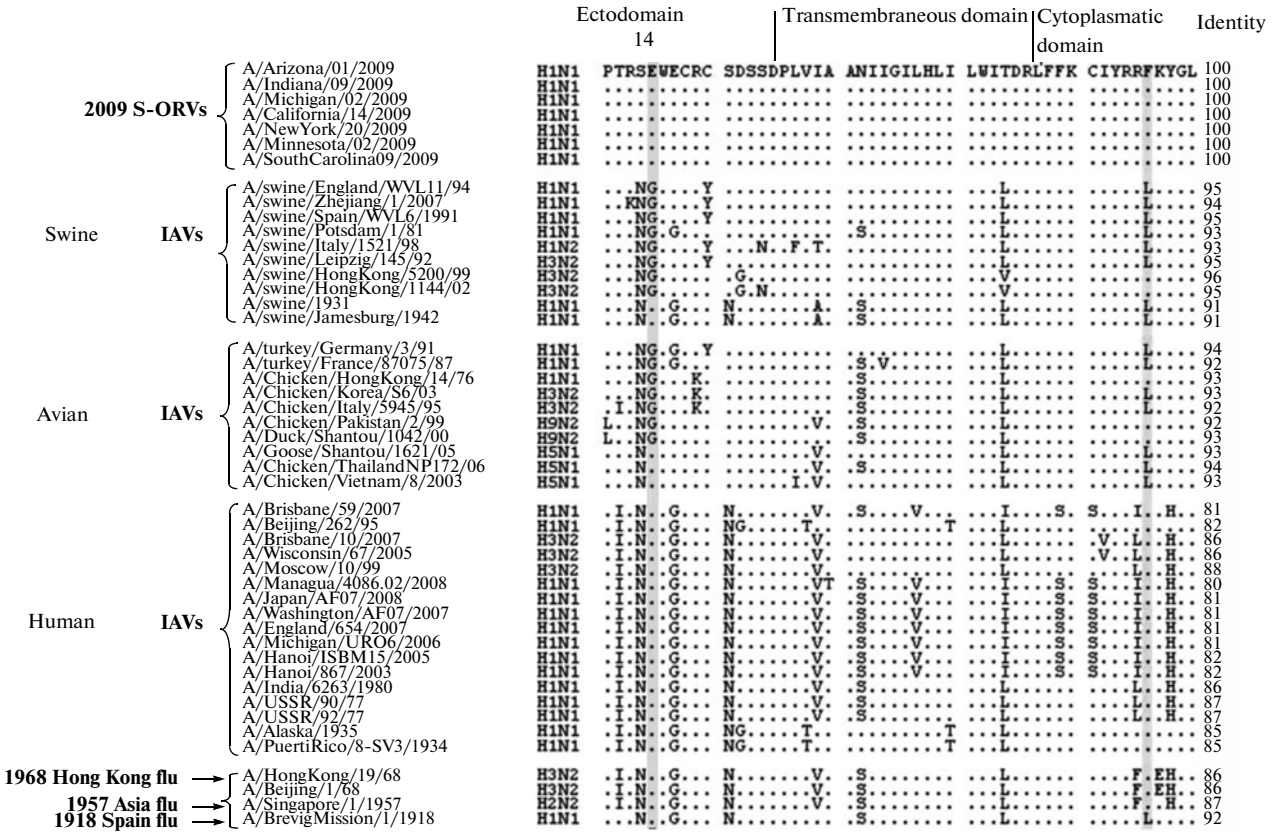


Fig. 5. Leveling of the M2 protein sequences for human, avian, and swine viruses. The block of sequences on the top—2009 S-OIVs—pandemic swine-lineage H1N1viruses; IAV—according to the international nomenclature, influenza A viruses.

(2) Recombinant preparation, including in-tandem repetitive copies of 24 amino acid residues of the M2 protein in the composition of *E. coli* flagellin.

(3) Recombinant fused protein M2e conjugated with a residue of cysteine at the 3-position and containing palmitine acid as an adjuvant.

In Fig. 4a (on the color insert), the M2 protein is represented by a physical map, where the transmembraneous domain is underlined by alpha-spiral (given is the most spread sequence with indicating amantadine- and rimantadine-resistant mutations; the amantadine molecule is designated in yellow in the region of adamant-binding site); given on the map's left is the extramembraneous domain at N-end (positions 1–25), the blue arrow points to variations of this domain's sequences in different subtypes of influenza A viruses (see Fig. 4c). On the top right is a tetramer model of the transmembraneous domain in a lipid membrane (see Fig. 4b).

Considering the first importance of projects on creating a universal vaccine, we give below the M2 protein sequences for important pandemic viruses characterized by a high level of conservativeness, a very valuable quality for constructing a universal vaccine (Fig. 5).

The high conservativeness of the main the M2 protein domains used in constructing recombinant vaccines allows us to rely on reaching a sufficient efficacy of the vaccines against any possible pandemic strain, including avian flu and swine-lineage H1N1 influenza. This type of vaccine has been created in Russia on the base of the Center Bioengineering, Russian Academy of Sciences [14].

THE TECHNOLOGY OF CONTEMPORARY VACCINES

Production of vaccines, as well as other biopharmaceutical processes, includes two main phases: preparation and main technological process.

The preparation phase consists in obtaining a sowing material—a virus in cell cultures—in a limited quantity, its subsequent cultivation inside voluminous reactors, and isolation of the virus from cells and main cellular components. As a result, a raw suspension of a viable virus is obtained, which is subjected to further purification.

The main technological process includes the following key phases of purification of the virus: concentration, chromatography, ultradiafiltration, isolation of

Fig. 6. The Uniflux system for the concentration of biomaterials, outer view.

separate components (for split or subunit vaccines), mixing with adjuvant (if the latter is included in the vaccine) and preparation of the final product for packaging.

Below we are discussing the current level and problems in the influenza vaccine production realization.

Production Cycle

The key phase of the preparatory technological process is fermentation. The development of wave bioreactors used for fermentation has become a turning point in the field of cell cultivation and has significantly increased cell-culture yields in growing viruses. A majority of companies trading in modern technological equipment recommends this type of reactors. The joint project of the Research Institute of Influenza of the RAMS with the GE Company (Sweden) includes a series of important solutions for the key part of the production cycle. The system of cell selection, concentration, and filtration with separation of the

Fig. 7. Chromatographic installation AKTA^{TM} for purifying viral suspensions on monolith columns.

fractions containing the end product is successfully realized using the UniFlux system developed within the above project (Fig. 6).

UniFlux systems can be used for concentrating virus suspensions, vaccine preparations, protein aggregates, monoclonal antibodies, and pure recombinant proteins. Concentration of virus suspensions is a necessary phase for preparing the material for chromatographic purification.

The use of standard monolith columns for the chromatography of viral particles is the most important technological solution (Fig. 7). Under a majority of technological processes, viral particles are subjected to purification through sucrose gradient ultracentrifugation. The chromatographically-purified vaccine was obtained in columns with macroporous glass. However, this vaccine contained hydrodynamically and mechanically destroyed virions. Exposing the viral nucleotide on the surface of the destroyed viral particles leads to the induction of immunity against the native viral ribonucleoproteid (RNP). This phenomenon is not so unsafe, since a cross-reaction of antibodies with RNP of the body's cells cannot be excluded, while this kind of phenomena may lead to autoimmune processes that may trigger systemic diseases.

Application of state-of-the-art techniques for chromatography of viral particles permits one to obtain highly-purified samples of both viruses and subunit preparations or VLPs. Chromatographic purification helps solve another, more important problem. Electron microscopy analysis of the current vaccines as to their structure reveals that antigens in the majority of vaccine preparations are in a denatured or heavily disorganized state (Fig. 8).

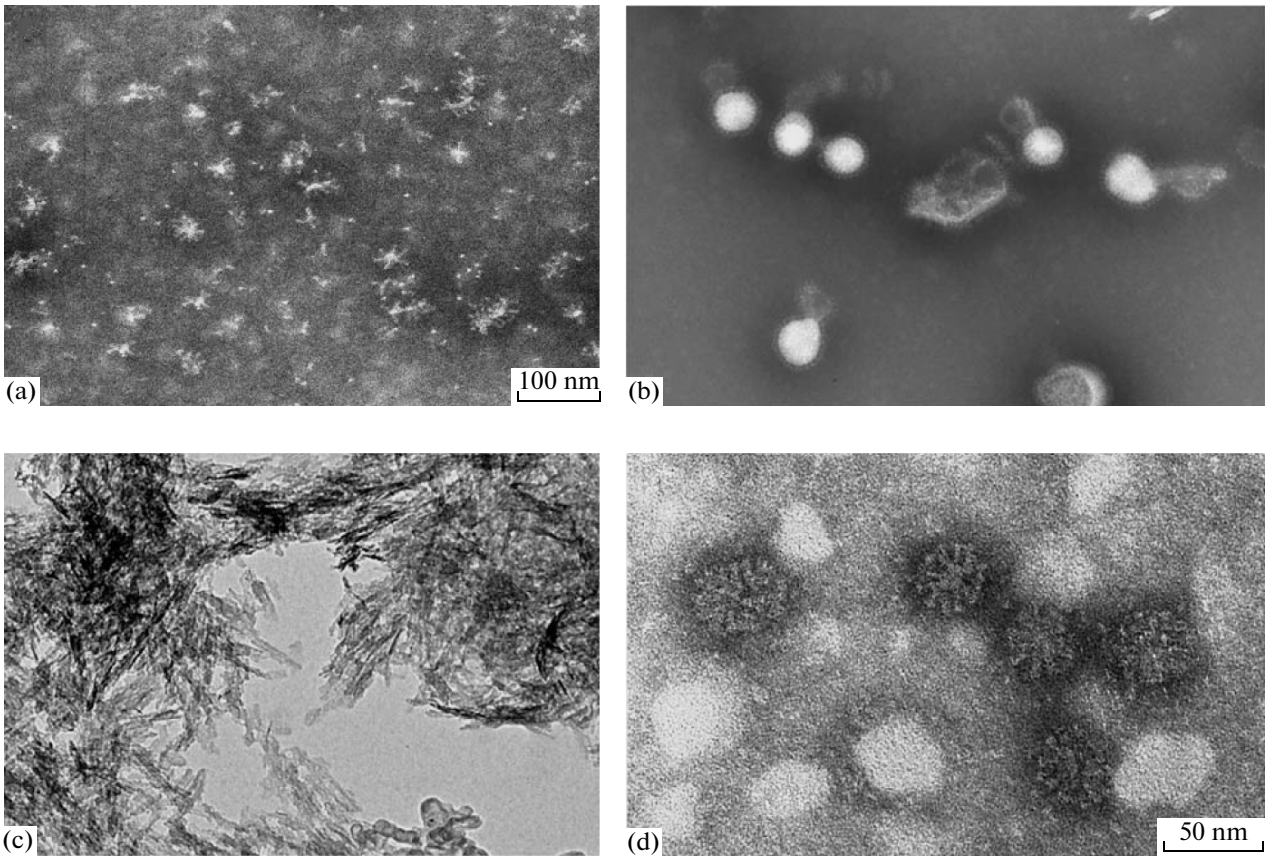


Fig. 8. Microphotography of influenza vaccine preparations: a—Grippol (individual rosettes); b—Grifor (seen are partially destroyed virions with signs of the nucleotide excess); c—Orniflu (subunit H5N1 vaccine with aluminum hydroxide) (seen are an excess of aluminum hydroxide and the absence of the rosette-arranged HA molecules); d—subunit-vaccine preparation obtained in laboratory conditions by controlled folding (seen are symmetrically-organized HA rosettes, heads of HA are turned to the surface, hydrophobic transmembraneous fragments form a hydrophobic "choir").

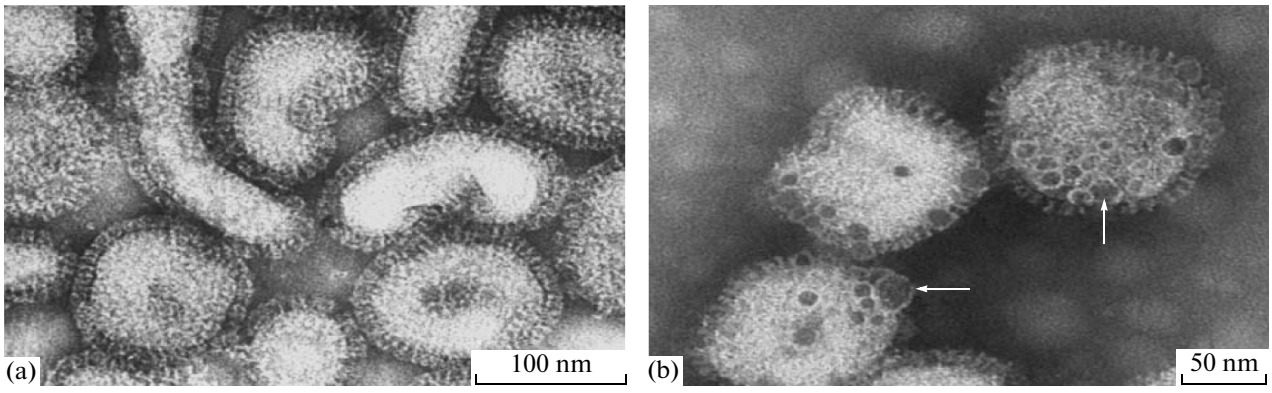


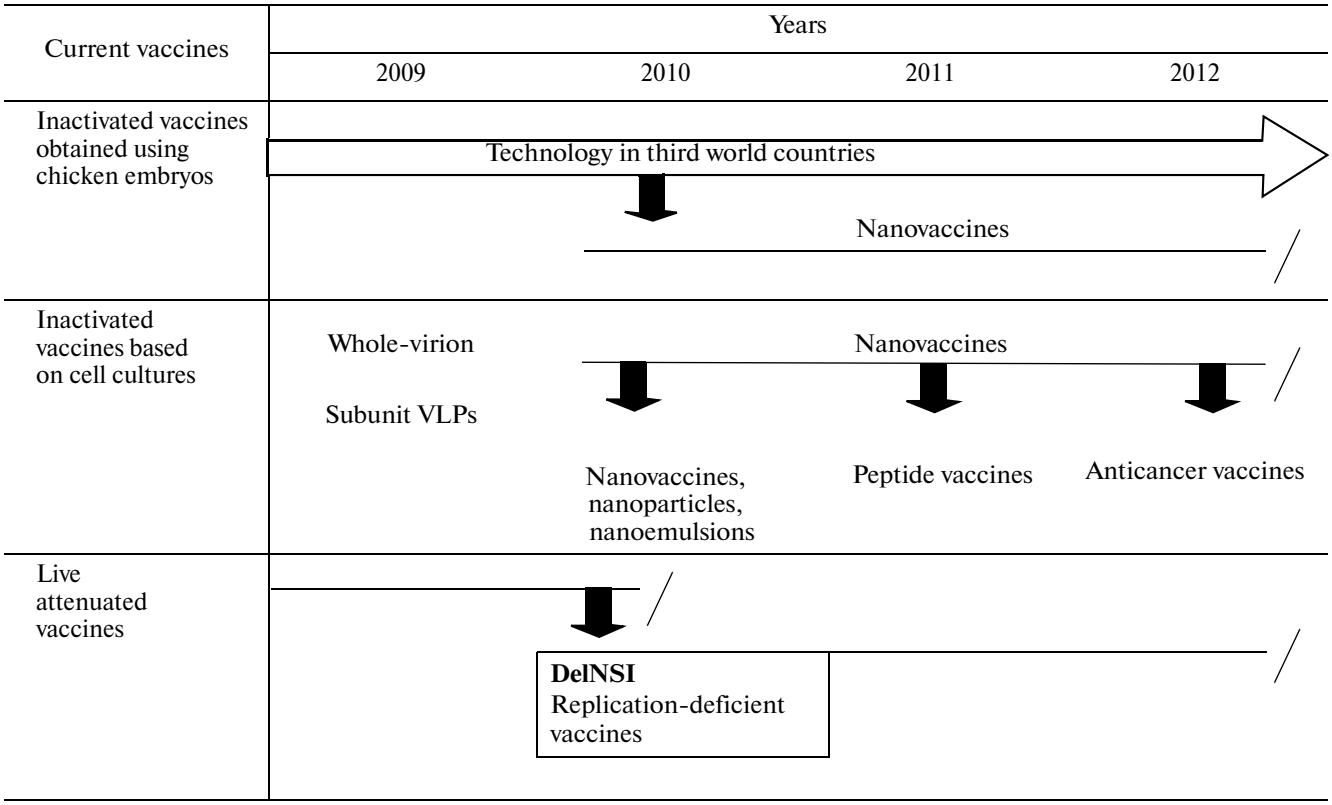
Fig. 9. Microphotography of influenza A virus virions (left) and inactivated viral particles impregnated with nanoparticles of aluminum oxide (indicated by arrows).

The Research Institute of Influenza of the RAMS is now developing, within some international projects, production technologies for a new generation of vaccine preparations:

(1) live replicatively-deficient vaccines Del-NS1 with additional genetic defects within a wide spectrum of molecular determinants of pathogenicity for optimizing their safety and preserving their high immunogenicity;

(2) protein nanovaccines based on the controlled folding technology;

Table 4. Approaches to the improvement of current vaccines (see explanations in the text)



(3) antigen and virion complexes with metalized nanoparticles (Fig. 9).

CONCLUSIONS

First of all, we should underline that the principal strategy in improving influenza vaccines has ever been their increased safety and immunogenicity. This explains the transition from whole-virion to split vaccines and from the latter to subunit vaccines. A high level of antigen purification generated the problem of decreased immunogenicity of vaccines and a search for immunoadjuvants. Approximately 15 years ago polyoxidonium was selected to perform this function. The polyoxidonium-based vaccines were termed polymer-subunit vaccines. However, such an approach has grown old now. A number of new adjuvants based on the known mechanism of stimulating the immune response through Toll-receptors have been tested in recent years [12, 29].

Discussions are still ongoing concerning the safety of LAIV. The current strategy is oriented to a search for inactivated analogues of many live vaccines. Firstly, this is connected with a probable reversibility of attenuated strains of viruses towards wild types, or reassortment of strains, having a fragmentary genome, with wild viruses. Using a live or even attenuated virus for mass vaccination, we, certainly, create a temporary circulatory background, considerably increasing a probability for the reassortment of viral genomes with viruses actively circulating in a current period of time in human population. Despite the fact that the phenomenon of reassortment between LAIV and wild viruses has not been confirmed and viruses with signs of pathogenicity have not been isolated in the majority of complicated vaccination cases, the application of LAIV is limited in the period when seasonal influenza is on the rise. Mass immunization with LAIV in the event of a pandemic should be conducted with maximum care.

At the same time, improvement of LAIV safety is possible through genetic techniques—in particular, we have achieved this in our studies within the European FluVac project. The team led by Professor A.Yu. Egorov has developed so-called DelNS1-vaccines. Vaccine strains carrying the *NSI* gene deletion can quickly be eliminated, since they are highly sensitive to the endogenous interferon (the latter is the antagonist of type I and type II interferons). Therefore, this type of live vaccine was classified as replicatively deficient. A probability of recombination or reassortment is ultimately low in this case.

Another approach to a genetically-controlled reduction in the LAIV reactogenicity consists in the removal of the reading frame in the *PB1* gene. The PB1-F2 reading frame encodes a short protein of 87 amino acids—the factor of macrophage apoptosis causing the death of cells during translocation in mito-

chondria. The PB1-F2 protein belongs to the amphiphilic proteins causing, similar to bacterial toxins, depolarization of membranes. It is probable that the main toxic effect of influenza infection is associated with hyperproduction of this protein. The PB1-F2 reading frame is completely preserved in the *PB1* gene of the LAIV attenuation donors. Its deletion by the reverse genetics techniques leads to essential changes in the phenotypic properties of the virus [12]. Thus, deletion of the key determinants of pathogenicity from vaccine viruses is a reliable strategy for raising the safety and harmlessness of LAIV. Table 4 presents the improvement strategies for the current vaccines for the next 3–4 years. Subunit vaccines with adjuvants, including on nanocarriers, are becoming obvious leaders among vaccine preparations. Serious competitors for LAIV are replicatively-deficient ΔNS1-vaccines. Reliable success is expected from a progress in the VLP technology.

The analysis of development strategies in vaccine production technology has shown that genetic engineering technologies are moving ever more to the forefront of this development. On the other hand, there is an understanding, initially absent in immunologists, that the tertiary structure of the antigen is equally important for adequate immunogenicity and the functioning of enzymes. Significantly improved instrumental provision of technological processes enables creating typical multipurpose vaccine productions, including vaccine preparations based on nanotechnologies.

The current discussions about the scale of mortality due to the H1N1sw1 pandemic are held with not quite correct argumentation. Even without very strict criteria, it is an emergency when a healthy young or middle-aged person dies from influenza. Vaccination and timely administration of efficacious antiinfluenza means, within a complex antiinfluenza therapy, offer good chances to avoid, in an overwhelming majority of cases, severe complications and, certainly, a lethal outcome. According to the latest statistics, the H1N1sw1-related mortality rate is reliably higher than the share of lethal outcomes in the 1968–1969 pandemic, while lower than during the 1957 Asiatic influenza. Of course, medical science has made a significant progress since 1957, continuously introducing from 1968 antiviral therapeutic means into clinical practice. Based on the above, we have to draw the following not very comfortable conclusion: high influenza-related mortality rates are, to a considerable degree, explained by our poor performance in organizing medical aid to patients. Russia is also no exclusion in this aspect. At the same time, availability of developed facilities for producing influenza vaccines allows Russia, in contrast to other countries, to promptly take decisions on a national vaccination program of scale.

Returning to the problem of genetic properties of pandemic viruses and the discussion about the molecular determinants of pathogenicity, we have to repeat that the 2009 influenza pandemic was caused by the mixed H1N1v-2009 virus with a genome that included genes of swine, avian, and human viruses. Based on the specific structure of its genome and pathogenicity level in animals (experimental models) and humans, we can characterize this etiological agent as a moderately pathogenic virus. In particular, HA of the H1N1v-2009 virus does not contain the proteolysis sites characterizing the highly-pathogenic H5N1 viruses. The PB1-F2 reading frame in the genome's second fragment (encoding the PB1 protein and the supplementary PB1-F2 protein resembling bacterial toxins by structure) is three times interrupted by stop-codons, which leads to the absence of protein synthesis in the infected cells. The absence of PB1-F2, as the most important factor of pathogenicity, in the influenza A viruses is an additional argument in support of specifying this virus as moderately pathogenic. In addition, this very genetic sign is a principal difference of the current pandemic H1N1v-2009 virus from the 1918 H1N1 virus that caused the Hispanic influenza pandemic.

Moreover, the pandemic H1N1v-2009 virus is deficient in the NS1 protein, the antagonist of I and II types interferons. In the NS1 protein from North America and the Russian Federation's isolates, researchers have found C-terminal deletion of an important regulatory site playing a key role in the activation of the signaling cell systems controlling the antiviral response of the body's cells (the deletion of so called PDZ-binding domain) in influenza infections. This deletion is probably related to the increased sensitivity of the pandemic H1N1v-2009 influenza to interferons and, in particular, to gamma-interferon.

Summarizing the above analysis of the functional domains of the H1N1v influenza virus protein, we have to state that the current pandemic influenza viruses are characterized by the following structural defects: the HA proteolysis site—PSIQSR/GLFGAI—is a substrate for the membrane-type serin protease TMP/SST, the presence of the three stop-codons in the PB1-F2 protein, and the presence of COOH-terminal deletion of the PDZ-binding domain of NS1.

The above genetic defects of pandemic viruses should additionally be characterized by the following supplementary properties:

(1) Resistance to the preparation Tamiflu, which is often manifested by seasonal H1N1 viruses and which is characteristic of a pandemic virus.

(2) Cooperative specific properties of the HA and NA proteolysis and their dependence on membrane-bound cellular proteases [12], a systemic induction of a "cytokine storm".

(3) NS1- and NS2-conditioned opposition of the interferon system and immunosuppression.

(4) Presence of the "weakened" consensus-sequence of the Ebola-like suppressive domain [12].

However, it should be underlined that the above defects may in part or fully be repaired in the process of active circulation of the H1N1sw viruses, carrying a risk of enhanced pathogenicity. Therefore, in constructing vaccines for the forthcoming pandemic seasons we shall have to foresee the constructive specific features associated with a probable emergence of viruses with modified properties.

As the most marked achievements in domestic science, we should note the obtaining of a full functional copy of the Kurgan isolates for the avian influenza H5N1 virus using the recursive PCR and reverse genetics techniques (8-plasmid system variation [12]) and the technology created for producing a universal vaccine based on the M2 protein [14].

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