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Plant-Produced Recombinant Influenza Vaccine Based on Virus-Like HBc Particles Carrying an Extracellular Domain of M2 Protein

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Abstract—Conventional influenza vaccines are based on a virus obtained in chicken embryos or its components. The high variability of the surface proteins of influenza virus, hemagglutinin and neuraminidase, requires strain-specific vaccines matching the antigenic specificity of newly emerging virus strains to be developed. A recombinant vaccine based on a highly conservative influenza virus protein M2 fused to a nanosized carrier particle can be an attractive alternative to traditional vaccines. We have constructed a recombinant viral vector based on potato X virus that provides for expression in the *Nicotiana benthamiana* plants of a hybrid protein M2eHBc consisting of an extracellular domain of influenza virus M2 protein (M2e) fused to hepatitis B core antigen (HBc). This vector was introduced into plant cells by infiltrating leaves with agrobacteria carrying the viral vector. The hybrid protein M2eHBc was synthesized in the infected *N. benthamiana* plants in an amount reaching 1-2% of the total soluble protein and formed virus-like particles with the M2e peptide presented on the surface. Methods of isolation and purification of M2eHBc particles from plant producers were elaborated. Experiments on mice have shown a high immunogenicity of the plant-produced M2eHBc particles and their protective effect against lethal influenza challenge. The developed transient expression system can be used for production of M2e-based candidate influenza vaccine in plants.

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Key words: influenza, vaccine, plant biofactories, M2 protein, HBc antigen, viral vector, virus-like particle

Influenza is a widely distributed viral infection of humans and animals. Conventional influenza vaccines are based on reassortants of epidemically current influenza viruses and standard donors of genes encoding internal proteins produced in chick embryos or in cell cultures [1]. A high variability of surface viral proteins, hemagglutinin and neuraminidase, results in appearance of a new epidemical strain every one-two years [2], and this requires the vaccines to be renewed at the same frequency. Moreover, a recombination can appear between human and animal influenza viruses and result in production of viruses with new antigenic features not recognizable by the human immune system and thus potentially pandemic. Recent examples are the influenza A virus of the H1N1 subtype of swine origin that caused the 2009-2010 pandemic and highly pathogenic strains of the avian influenza A(H5N1) that caused hundreds of cases of illness and lethal outcomes in countries of Southeast Asia and the Near East. The creation and production of a conventional vaccine specific for a new strain usually takes six to eight months, during which the infection can be lethal for many humans [3].

A promising solution to this problem is recombinant vaccines that can be rapidly produced in standard expression systems. This not only abolishes the production

Abbreviations: GFP, green fluorescent protein; HBc, hepatitis B virus nuclear antigen; LD_{50}, the dose corresponding to 50% lethality; M2e, M2 protein extracellular domain of the influenza virus; PBS, phosphate-buffered saline; PXV, potato X virus; 35S promoter, 35S RNA promoter of the cauliflower mosaic virus; 35S terminator, 35S RNA terminator of the cauliflower mosaic virus.

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dependence on chick embryos and solves general problems of safety of vaccines based on a whole pathogen [4] but opens a possibility for creating vaccines capable of "overlapping" antigenic properties of various current influenza strains. An extracellular domain of the influenza virus transmembrane protein M2, M2e, is promising for creation of a recombinant vaccine [5]. The length of M2e is only 23 amino acid residues, and it is quite conservative, which allows to create vaccines effective against a wide spectrum of strains. Thus, this M2e sequence is virtually unchanged in all influenza viruses isolated from humans beginning from 1933 [6-8], and in strains of "avian" and "swine" influenza it is different only in a few amino acids. But creation of vaccines based on M2e is hindered by the low immunogenicity of this protein, and immune response against it is virtually not activated during the infection [9]. This problem can be solved by fusion of M2e to a virus-like carrier particle either genetically by creation of a hybrid protein or through chemical cross-linking. Hepatitis B nuclear antigen producing virus-like particles with ~30 nm in size can be used as such carrier protein [10]. The insertion of M2e into certain regions of the HBc antigen results in production of recombinant particles with the epitope presented on the surface [8, 11]. Immunization of mice with recombinant M2eHBc particles produced in *Escherichia coli* demonstrated protection against lethal influenza infection [8]. Similar M2eHBc particles carrying the M2e peptide of the "swine" influenza virus A/California/04/2009(H1N1) protected mice against infection with the corresponding virus strain [12]. In addition to HBc, virus-like particles based on human papilloma virus [13], bacteriophage Qβ [14], papaya mosaic virus [15], and cow pea mosaic virus [16] can also be used as M2e carrier particles.

Recombinant proteins including those for medical purposes can be produced in different expression systems, including bacteria, yeast, plants, and mammalian cells. Plants as biofactories are advantageous due to simple cultivation resulting in a low final price of the product and also due to absence of common human and plant pathogens that ensures the complete safety of plant proteins. Recombinant proteins can be produced in plants by creation of a transgenic plant producer, but creation of such plants requires a long time and, and the expression level of the desired protein is usually low, leading to high cost of protein purification and of the resulting product [17]. The use of self-replicating viral vectors is an alternative approach providing sufficient amounts of the desired protein [18]. To create such vectors, the tobacco mosaic virus or the potato X virus are usually used. Their genomes are small and contain the plus RNA chain. The viral vectors can be introduced into plant cells by infiltration of the plant leaf tissue with agrobacteria carrying the viral vector within tDNA transferred into the plant cell during the agroinfection [19, 20]. Viral vectors can provide during a few days the expression in plants of the desired proteins at the level of 20-30% of the total soluble protein, about 1-2 g/kg plant biomass [20]. Expression systems based on plant viruses have been used for production of different medical proteins, including antibodies and vaccine proteins [21, 22], in particular, influenza virus hemagglutinin [23, 24].

Up to now M2eHBc particles were produced using expression systems in *E. coli* cells. In the present work we have shown that M2eHBc particles can be produced in *N. benthamiana* plants using a viral expression system. Immunization of mice with the M2eHBc particles produced in plants induced the immune response and ensured formation of protective immunity against the lethal influenza infection.

MATERIALS AND METHODS

Media, reagents, synthetic gene, and vectors for expression of target proteins in plants. Bacteria were grown in LB broth or on plates with LB agar at 37°C (*E. coli*) or at 30°C (*Agrobacterium tumefaciens*). If necessary, the media were supplemented with antibiotics: ampicillin (100 μg/ml), kanamycin (50 μg/ml), rifampicin (50 μg/ml), or gentamycin (25 μg/ml).

An artificial *M2eHBc* gene encoding the hybrid protein containing the influenza virus peptide M2e on the N-terminus of the HBc antigen was synthesized by Evrogen (Russia) within pGEM-T plasmid. Nucleotide sequences of this gene and of the corresponding protein are presented in Fig. 1a.

Recombinant proteins were expressed in plants using the pA7248GFP vector based on the potato virus X genome [25]. This vector is a binary one and can be maintained in both *E. coli* and *A. tumefaciens*. The gene encoding the desired protein was cloned in pA7248GFP at the *Asc*I and *Sma*I sites, and the resulting recombinant vector pA7248amvM2epHBc was transferred from *E. coli* into the agrobacterial strain GV3101.

The plasmid pBIN_P19 is a binary vector with the *p19* gene of the tomato bushy stunt virus cloned under the control of the 35S RNA promoter of cauliflower mosaic virus.

Agroinfiltration of plants. Agrobacteria carrying recombinant binary vectors were grown for 12 h in a shaker at 30°C. The cells (1.5 ml) were precipitated by centrifugation for 5 min at 4000*g*, and the precipitate was resuspended in 1.5 ml of buffer containing 10 mM MES (pH 5.5) and 10 mM $MgSO_4$. Leaves of *N. benthamiana* plants were injected with suspension of agrobacteria (optical absorption of the solution A_{600} was 0.2) using a syringe without a needle.

Isolation of proteins from plant tissue. The leaf material (10 mg) was ground to prepare a homogenous suspension in an extracting buffer (0.4 M sucrose, 50 mM Tris (pH 8.0), 5 mM $MgCl_2$, 10% glycerol, 5 mM β-mercap-

a

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1   GGC GCG CCG GAT CCT TTT TAT TTT TAA TTT TCT TTC AAA TAC TTC CAT CAA ATG TCT CTT
      (AscI)                                                             M   S   L

61  CTG ACT GAA GTG GAA ACT CCA ACT CGT AAC GGA TGG GAA TCT AAA TCT TCA GAT AGC TCT
      L   T   E   V   E   T   P   T   R   N   G   W   E   S   K   S   S   D   S   S

121 GAC ATG GAC ATT GAC CCT TAC AAA GAA TTT GGA GCT ACT GTG GAG CTT CTC AGC TTT TTG
      D   M   D   I   D   P   Y   K   E   F   G   A   T   V   E   L   L   S   F   L

181 CCT TCT GAC TTC TTT CCT TCT GTC AGG GAT CTC CTT GAC ACT GCC TCA GCT CTT TAT AGG
      P   S   D   F   F   P   S   V   R   D   L   L   D   T   A   S   A   L   Y   R

241 GAA GCC TTG GAG TCT CCT GAG CAT TGC TCA CCT CAC CAT ACT GCA CTC AGG CAA GCC ATT
      E   A   L   E   S   P   E   H   C   S   P   H   H   T   A   L   R   Q   A   I

301 CTC TGC TGG GGA GAA TTG ATG ACT CTT GCT ACC TGG GTG GGT AAC AAT CTA GAG GAC CCA
      L   C   W   G   E   L   M   T   L   A   T   W   V   G   N   N   L   E   D   P

361 GCA TCC AGA GAT CTT GTT GTT AAC TAT GTT AAT ACT AAT GTG GGT TTG AAG ATC AGG CAA
      A   S   R   D   L   V   V   N   Y   V   N   T   N   V   G   L   K   I   R   Q

421 CTC TTG TGG TTT CAT ATA TCT TGC CTT ACT TTT GGA AGA GAG ACT GTA CTT GAA TAT TTG
      L   L   W   F   H   I   S   C   L   T   F   G   R   E   T   V   L   E   Y   L

481 GTC TCT TTT GGA GTG TGG ATT AGA ACT CCT CCA GCC TAT AGA CCA CCA AAT GCC CCT ATC
      V   S   F   G   V   W   I   R   T   P   P   A   Y   R   P   P   N   A   P   I

541 TTG TCG ACT CTT CCA GAA ACT ACT GTT GTT CGA AGA AGG GAC AGG GGC AGA TCC CCT AGA
      L   S   T   L   P   E   T   T   V   V   R   R   R   D   R   G   R   S   P   R

601 CGT AGA ACT CCC AGC CCT AGA AGA AGG AGA TCC CCA TCT CCT AGG AGG AGA AGG TCT CAA
      R   R   T   P   S   P   R   R   R   R   S   P   S   P   R   R   R   R   S   Q

661 TCT AGG GAA TCT CAA TGT TAA CCC GGG
      S   R   E   S   Q   C   -  (SmaI)
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b

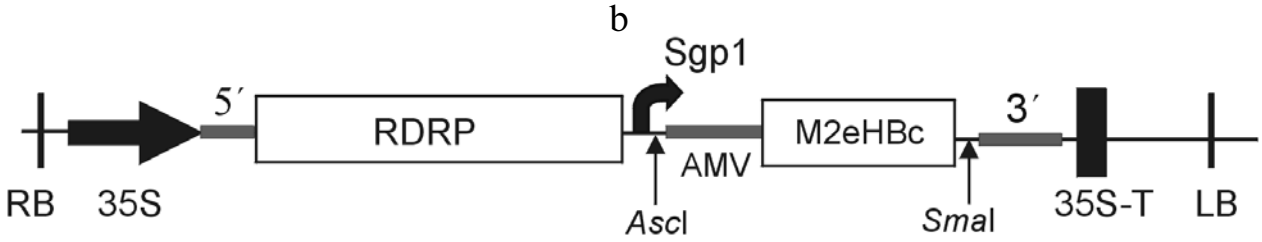

Fig. 1. a) Nucleotide sequence of the artificial gene *M2eHBc*. Recognition sites of restriction enzymes *Asc*I and *Sma*I are italicized, the nucleotide sequence AMV is underlined. The M2e peptide coding sequence is shown in gray. b) Scheme of the viral vector pA7248amvM2epHBc. The tDNA region of the binary vector is shown. 5′, the 5′-nontranslated region of PXV; 3′, the 3′-nontranslated region of PXV; RDRP, RNA-dependent RNA polymerase of PXV; Sgp1, subgenomic promoter of the PXV transport protein 25K; AMV, the leader sequence of RNA 4 of the alfalfa mosaic virus; M2eHBc, the sequence coding the hybrid protein M2eHBc; 35S, promoter; 35S-T, terminator of the cauliflower mosaic virus RNA; LB and RB are the left and right borders of tDNA.

toethanol). The resulting mixture was centrifuged at 14,000*g* for 15 min, and the protein-containing supernatant was taken. The protein contents were determined by Bradford's method.

Western-blotting of protein preparations. The proteins were subjected to SDS-PAGE in a 10% gel. The proteins were transferred from the gel onto a Hybond-P membrane (GE Healthcare, USA) by electroblotting. To prevent nonspecific binding of antibodies with the membrane, it was treated with 5% solution of dry milk in TBS-T buffer. Then the membrane was hybridized with mouse monoclonal antibodies to the M2e peptide of the A/Duck/Potsdam/1402-6/1986(H5N2) strain and then hybridization was performed with secondary antibodies

conjugated with peroxidase. Specific protein complexes were detected using a Western Blot ECL Plus kit (GE Healthcare).

Purification of M2eHBc particles. Virus-like M2eHBc particles were purified from the plant tissue by precipitation with ammonium sulfate. A solution of the protein isolated from leaves of the plant producer was supplemented with 0.5 volume of saturated solution of ammonium sulfate, and the mixture was incubated at 4°C for 60 min. The proteins were precipitated by centrifugation at 13,000*g* for 10 min. The resulting protein precipitate was dissolved in 1 ml of buffer consisting of 50 mM Tris-HCl (pH 8.0) containing 0.5 M NaCl, 15 mM EDTA, and 20% sucrose. Then the procedure of precipitation with ammonium sulfate was repeated once more, and finally the preparation was dissolved in 50 mM Tris-HCl buffer (pH 8.0) containing 0.5 M NaCl, 15 mM EDTA, and 20% sucrose (to the concentration of 5 mg/ml) and stored at –20°C. At this stage the desired protein M2eHBc constituted about 15% in the preparation.

The particles were additionally purified by centrifugation in sucrose and cesium chloride gradients [26]. A specimen was placed onto the gradient and centrifuged for 20 h at 26,000 rpm (Avanti J-30 I centrifuge, SW41 rotor; Beckman Coulter, USA). The gradient fractions taken upon the centrifugation were dialyzed against STE buffer (pH 8.0) for 48 h using a Mini-Dialysis kit (GE Healthcare) and examined for presence of protein M2eHBc by SDS-PAGE in 12% gel and by Western blotting. The M2eHBc-containing fractions were combined. The purity of the desired protein was no less than 90%.

Electron microscopy. Specimens were prepared by routine negative contrasting using 1% uranyl acetate solution, examined with a JEM-1011 transmission electron microscope (JEOL, Japan), and photographed with a Morada digital camera (Olympus-SIS) at the magnification calibrated by the diffraction lattice.

Atomic force microscopy. An INTEGRA Prima atomic force microscope (NT-MDT, Russia) was used, and measurements were performed in semi-contact regimen with probe head thickness of 10 nm. The M2eHBc particle preparation was 50 times diluted with water. The samples were precipitated and dried on a sapphire substructure, and measurements were performed in air.

Immunization of mice. To study the immunogenicity and protective action of the candidate vaccine, mice were immunized thrice intraperitoneally at two-week intervals; for the first immunization Sigma system adjuvant (Sigma, USA) was used, and the subsequent immunizations were performed with incomplete Freund adjuvant (Sigma) according to the supplier's instructions. The dose of the preparation was 20 μg/mouse at the first immunization and 50 μg/mouse at the subsequent ones.

Sera were taken two weeks after each immunization, and antibody titers were determined in two or three pools of mouse sera (three or four mice per pool) of all experimental and control groups. Sera of non-immunized mice were used as a negative control, and monoclonal antibodies to M2e peptide of the A/Duck/Potsdam/1402-6/1986 (H5N2) strain provided by P. G. Sveshnikov (Russian Research Center for Molecular Diagnostics and Therapy (RCMDT)) were used as a positive control.

Synthetic peptides and antibodies. Antibodies against M2e were determined with synthetic peptides G-11-1 (SLLTEVETPTRNEWECRCSDSSD corresponding to M2e of the strain A/Chicken/Kurgan/05/2005) and G19 (SLLTEVETPTRNGWECKCSDSSD corresponding to M2e of the strain A/Duck/ Potsdam1402-6/1986) used as standards. Monoclonal mouse antibodies to M2e peptide G19 (the D2 clone) were provided by P. G. Sveshnikov (RCMDT).

Enzyme-linked immunoabsorbent assay (ELISA) for determination of specific antibody titer. To perform ELISA, 96-well plates with a high sorption ability (Greiner, Germany) were coated by synthetic peptides G-11-1 or G19 (5 μg/ml) in carbonate buffer (pH 9.5-9.6) and kept overnight at 4°C. The plates were treated with an inhibiting buffer (0.01 M phosphate-buffered saline (PBS, pH 7.2-7.4) supplemented with 5% fetal calf serum) for 1 h at room temperature and washed thrice using PBS with Tween. The plate wells were filled with 100 μl of twofold dilutions of the sera (starting from 1 : 200) in an inhibiting buffer and incubated for 1 h at room temperature. As a conjugate, rat monoclonal anti-mouse IgG1 and IgG2 (BD Biosciences, USA) diluted 1 : 500 and streptavidin-peroxidase (BD Bioscience) diluted 1 : 1000 were used for 30 min. Tetramethylbenzidine as the substrate was reacted for 5 min. The reaction was recorded at 450 nm. The greatest serum dilution resulting in the optical density of at least twofold higher than the sera of non-immunized mice at the same dilution was taken as the titer.

Virus and infection of mice. Animals were infected with the influenza virus A/Chicken/Kurgan/05/2005(H5N1) adapted to mice. The virus in the dose 1 LD_{50} was injected intranasally, 50 μl/mouse under slight ether anesthesia. The animals were observed daily for 14 days after the infection. The protective effect of the preparation was assessed by changes in two parameters: body weight and survival.

RESULTS AND DISCUSSION

Expression in plants of hybrid protein coding HBc with fused M2e peptide. To prepare virus-like particles, we constructed an artificial gene *M2eHBc* encoding a hybrid protein containing on the N-terminus the M2e peptide of the influenza virus. The M2e sequence corresponded to the strain A/Duck/Potsdam/1402-6/1986(H5N2) of the avian influenza virus. To improve the solubility of the protein, two cysteine residues in positions 17 and 19 of the M2e peptide responsible for formation of disulfide bonds

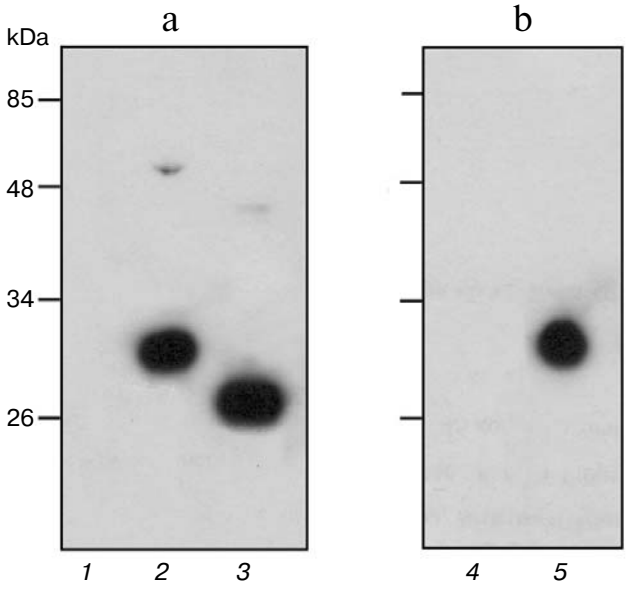

Fig. 2. Western blotting of protein preparations isolated from plant producers. a) Detection with polyclonal antibodies to the HBc antigen; b) detection with monoclonal antibodies to the M2e peptide. *1*, *4*) Preparation of soluble proteins from non-inoculated leaves of *N. benthamiana*; *2*, *5*) preparation of soluble proteins from *N. benthamiana* leaves inoculated with the viral vector pA7248amvM2epHBc; *3*) standard preparation of the HBc antigen.

were replaced by serine residues, which did not influence the immunological characteristics of M2e [11]. The M2e was followed by HBc antigen nucleotide sequence optimized for expression in plants [27]. Before the initiation codon a translation enhancer was placed represented by a nucleotide sequence corresponding to the 5′-nontranslated region of RNA of the alfalfa mosaic virus (AMV). When using viral vectors, this sequence increased several-fold the expression level of the desired gene in plants [25].

To express the hybrid protein in the *N. benthamiana* plants, the viral vector pA7248GFP [25, 28] based on the potato X virus (PXV) genome was used. This vector includes the 5′-nontranslated region of the PXV genome, the polymerase gene, the subgenomic RNA first promoter, the translation enhancer represented by the AMV nucleotide sequence, the *GFP* gene flanked by unique restriction sites *Asc*I and *Sma*I, the last 60 nucleotides of the coat protein gene, and the 3′-nontranslated region of the PXV genome. This construction is inserted between the 35S promoter and 35S terminator and cloned in the binary vector pBIN19. During the agroinfiltration of the leaves tDNA is transferred from the *Agrobacterium* cell into the plant cell. The mRNA transcription from the 35S promoter results in synthesis of the viral vector RNA, the viral vector is replicated in the infected cells, the subgenomic RNA encoding the desired gene is synthesized, and the protein product is expressed at a high level. To create the viral vector responsible for production of the M2eHBc protein, a sequence of the synthetic gene *M2eHBc* was cloned in pA7248GFP between the *Asc*I and *Sma*I sites, which resulted in construction of a recombinant vector pA7248amvM2epHBc (Fig. 1b).

To express the protein M2eHBc, the vector was inserted into *A. tumefaciens* strain GV3101, which was used for agroinfiltration of leaves of *N. benthamiana* plants. To suppress virus-induced gene silencing, the plant leaves were concurrently infiltrated with agrobacteria carrying the vector pBIN_P19, which was a producer of the suppressor protein P19 [29] of the tomato bushy stunt virus. The plants were grown for 4-6 days, then the leaves were collected, and the desired protein preparations were isolated.

The resulting preparations were analyzed by Western blotting using antibodies to the M2e peptide and to the HBc antigen. Figure 2 shows that M2eHBc is effectively expressed in *N. benthamiana* cells and is soluble. Western blotting of different solutions of the protein preparations from the producer plants and of standard amounts of HBc revealed that the yield of M2eHBc was 1-2% of the soluble protein fraction.

Formation of virus-like particles. Virus-like M2eHBc particles were isolated and purified in two stages. In the first stage the particles were precipitated with ammonium sulfate according to the earlier developed method [12]. The particles were additionally purified by zonal centrifugation in sucrose and cesium chloride density gradients.

The resulting preparation was analyzed by SDS-PAGE and Western blotting (Fig. 3). The buoyant density of the protein complex produced by M2eHBc is different from the density of the soluble plant proteins, and this

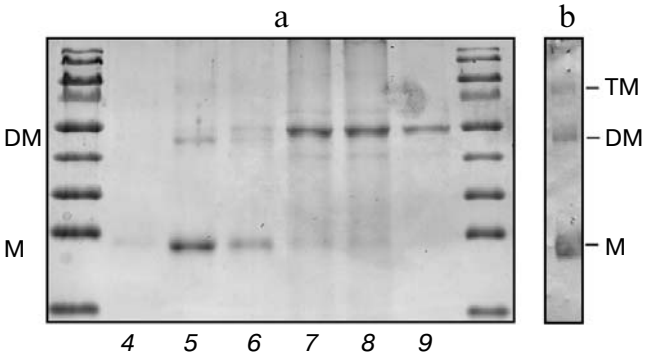

Fig. 3. a) SDS-PAGE of M2eHBc protein preparations fractionated by ultracentrifugation. The bands represent preparations from different fractions of the density gradient, from the maximal (*4*) to the minimal (*9*) one. Positions of the M2eHBc monomer (M) and dimer (DM) are indicated; the highest weight band in fractions *7-9* corresponds to plant ribulose biphosphate carboxylase. b) Western blotting of the protein preparation from fraction *5*, detection with monoclonal antibodies to the M2e peptide. Positions of the M2eHBc monomer (M), dimer (DM), and trimer (TM) are indicated.

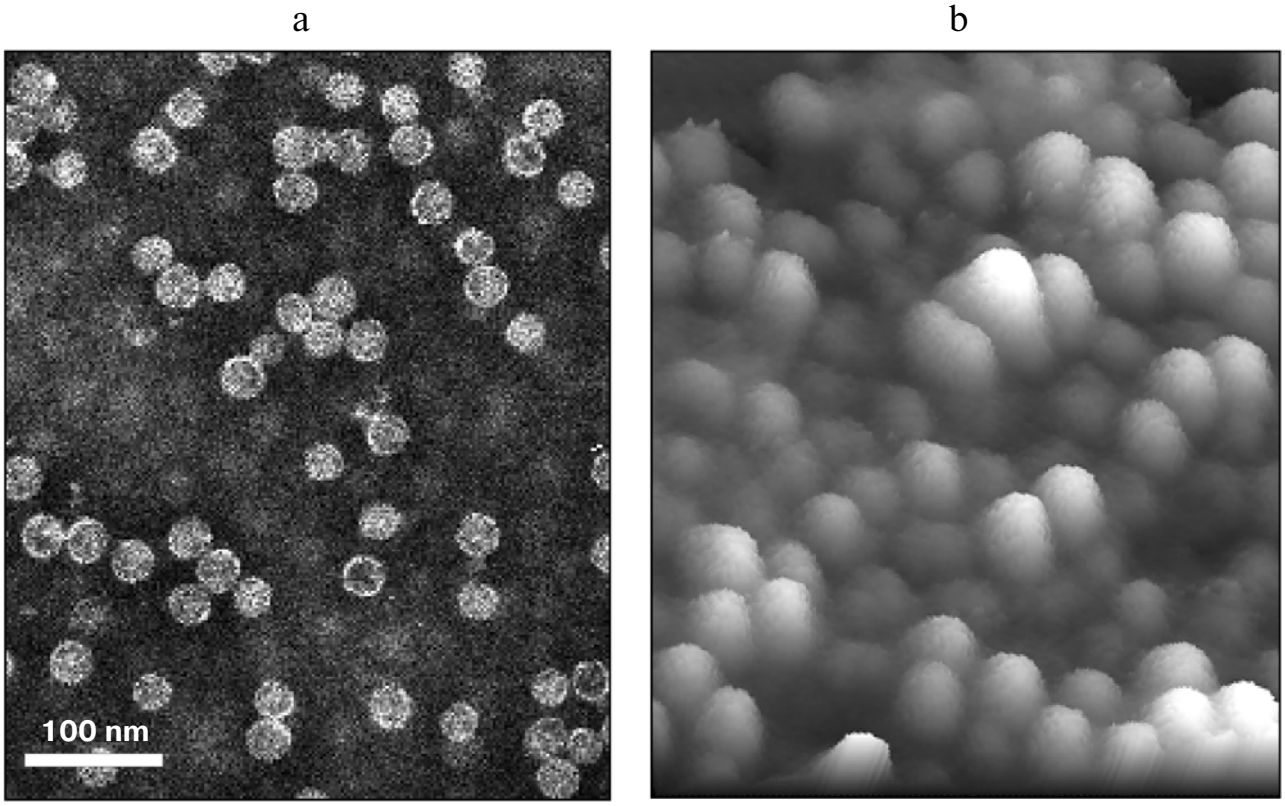

Fig. 4. Analysis by electron (a) and atomic force microscopy (b) of structure of the M2eHBc particles purified by ultracentrifugation.

complex is mainly present in "heavy" fractions of the gradient, which suggests the M2eHBc is isolated from the producer plants as virus-like particles (Fig. 3a). The purification degree of the desired protein is no less than 90%. According to Western blotting, M2eHBc specifically binds as both a monomer and multimers with monoclonal antibodies to the M2e peptide (Fig. 3b). The assembly of the hybrid protein in nanosized virus-like particles with diameter of ~30 nm was also confirmed by electron microscopy (Fig. 4a). The spherical particles were of uniform size. Analysis of the preparation by atomic force microscopy gave similar results (Fig. 4b).

Immunogenicity and protective action of the "plant-produced" M2eHBc particles. To characterize the immunogenicity and protective action, mice were immunized with the plant-produced preparation of M2eHBc particles. The experimental animals were immunized intraperitoneally at two-week intervals using Sigma system Adjuvant (Sigma) for the first vaccination and incomplete Freund adjuvant (Sigma) for the two subsequent vaccinations.

To evaluate the immunogenicity of the candidate vaccine, the mouse sera were studied two weeks after the second and after the third immunizations, and antibody titers were determined in three pools of the sera (three or four mice in each pool) from mice of the experimental group. ELISA was performed using synthetic peptides G-11-1 and G19, which possess sequences corresponding to M2e of the avian influenza strains A/Chicken/Kurgan/05/2005 and A/Duck/Potsdam1402-6/1986. The results (table) indicate that the three-fold immunization induced the production of high titers of IgG isotype serum antibodies binding with both the synthetic polypeptide G19 (its sequence corresponded to M2eHBc used for the immunization) and the peptide G-11-1 (its sequence corresponded to M2e of the heterologous strain of influenza virus). The titer of the IgG2a subtype antibodies was significantly higher than the titer of the IgG1 subtype antibodies, and this suggested a predominant induction of the Tx-1 type immune response mainly responsible for the cell response.

To assess the protective effect of the vaccine, experimental and control mice were challenged with the mouse-adapted influenza strain A/Chicken/Kurgan/05/2005(H5N1). The virus was injected intranasally at the dose of 1 LD_{50}.

Figure 5a shows the decrease in the body weight of the animals upon the infection that indicates severity of the disease course. Upon infection the body weight of the immunized animals decreased significantly less than in the control mice (to 90 and to 70% of the initial weight, respectively). These results indicate that immunization

Titers of IgG antibodies to M2e synthetic peptides

Specimens of sera	G-11-1		G-19	
	IgG1	IgG2a	IgG1	IgG2a
Mice after the 2nd immunization				
pool 1	1600	12 800	800	12 800
pool 2	3200	25 600	1600	12 800
pool 3	1600	12 800	200	6400
Mice after the 3rd immunization				
pool 1	25 600	51 200	6400	51 200
pool 2	25 600	51 200	6400	51 200
pool 3	25 600	51 200	1600	25 600
Negative control (sera of non-immunized mice)	<200	<200	<200	<200

with the candidate vaccine significantly relieves the course of the illness. The lethality of mice challenged with influenza virus is presented in Fig. 5b, and the data show the 90% protective effect of M2eHBc. In the control group of mice only half of the animals survived under the conditions of the infection.

Prospects of using the virus expression system in plants for production of recombinant anti-influenza vaccines. The purpose of the present work was to produce in plants a candidate recombinant vaccine against influenza based on the highly conservative M2 protein. To increase the immunogenicity of the M2e peptide, it was expressed as a component of a hybrid protein including the HBc antigen of the hepatitis B virus. Different hosts can be used as producers for expression of HBc: *E. coli* [30], yeasts [31], insect cells cultures [32], transgenic plants [33]. For transient expression of HBc in plants viral vectors can be used [27, 34, 35].

We have created a viral system of expression in plants of the HBc antigen with the fused M2e peptide. Using a viral vector based on the potato X virus, the M2eHBc was expressed in *N. benthamiana* leaves at the level of 1-2% of the total soluble protein, and the synthesized hybrid protein was assembled as virus-like particles. Immunization of mice with the M2eHBc particles induced an effective immune response against M2e and was responsible for development of protective immunity against the lethal influenza infection. Thus, this work shows that production of virus-like M2eHBc particles in plants by agroinfiltration with a recombinant viral vector is a promising approach for production of recombinant vaccines against influenza.

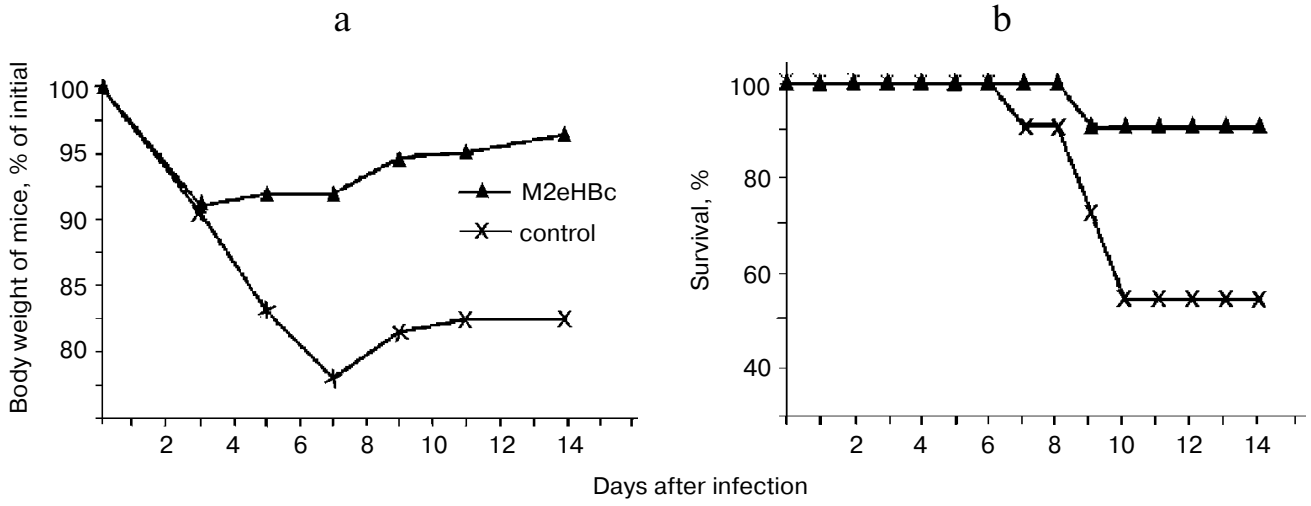

Fig. 5. Protective effect of the M2eHBc particle preparation. Changes in the mean body weight of the surviving mice (a) and the lethality of mice (b) challenged with 1 LD_{50} of the influenza virus A/Chicken/Kurgan/05/2005(H5N1).

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