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Study of the immunogenicity of hepatitis B surface antigen synthesized in transgenic potato plants with increased biosafety

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ABSTRACT

Oral immunogenicity of the hepatitis B surface antigen (HBsAg) synthesized in the tubers of marker-free potato plants has been demonstrated. Experiments were performed in the two groups of outbred NMRI mice. At the beginning of investigations, the mice of experimental group were fed the tubers of transgenic potato synthesizing the HBsAg three times. The mice of control group were fed nontransgenic potato. Intraperitoneal injection of the commercial vaccine against hepatitis B (0.5 μg/mouse) was made on day 71 of the experiment. Enzyme-linked immunoassay (ELISA) of the serum of immunized animals showed an increase in the level of HBsAg antibodies significantly above the protective value, which was maintained for 1 year after the immunization. In 1 year, the experimental group of mice underwent additional oral immunization with HBsAg-containing potato tubers. As a result, the level of antibodies against the HBsAg increased and remained at a high protective level for several months. The findings show the possibility of using transgenic plants as a substance for obtaining a safe edible vaccine against hepatitis B.

1. Introduction

Viral hepatitis B is a widespread human infection with bad after-effects, including chronic liver lesions that lead to cirrhosis and liver cancer. According to the data of the World Health Organization, about two billion people are infected with the hepatitis B virus worldwide (Hwang and Cheung, 2011). The most efficient method of viral infection control is vaccination. However, for a variety of reasons, the application of vaccines in many countries is not as widespread as required. Plants are the most convenient, safe and inexpensive alternative for production of various therapeutic proteins, vaccines and antibodies, compared to the expressing systems based on microorganisms, animal cell cultures, or transgenic animals. The plants obtained in recent decades synthesize a great number of valuable proteins such as human serum proteins, growth regulators, antibodies, vaccines, industrial enzymes, biopolymers, and reagents for molecular biology and biochemistry. Plant cells possess enzymatic systems of posttranslational modification, which are necessary for the assembly of synthesized monomeric proteins of vaccine into immunogenic multimers. The target antigens causing active immune response can be also synthesized in plant cells (Mason and Arntzen, 1995). Edible vaccines currently in development are based on fruits, leaves and seeds of transgenic plants. Such vaccines are prepared without expensive purification of antigens, which is commonly required for parenterally administered vaccines (Lugade et al., 2010). Various subunit vaccines are expressed in plants; many of them were shown to be immunogenic after oral immunization of people and animals (Haq et al., 1995; Yusibov et al., 1997; Tacket et al., 1998; Yu and Langridge, 2001; Lamphear et al., 2004; Thanavala et al., 2005; Alvarez et al., 2006). Plant cell walls protect the synthesized antigens from proteolysis when they travel through the digestive system, and antigenic proteins can be easily delivered to the cells of intestinal mucosa for immune response.

The surface hepatitis B antigen (HBsAg) synthesized in plants does not differ from the antigen obtained from yeast cells in its physicochemical and immunological properties and stimulates the formation of specific IgG immunoglobulins when introduced into organism (Thanavala et al., 1995). The HBs-antigen of transgenic plants is more effective when administered perorally compared to the yeast one. Since HBsAg is assembled in plant cells into multimeric particles accumulating inside the membrane vesicles, such

Abbreviations: HBsAg, hepatitis B surface antigen; ELISA, enzyme-linked immunosorbent assay.

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natural "bioincapsulation" protects the antigen from aggressive impacts in the digestive system until it contacts the effector cells of the immune system on the mucous surface of the intestines (Richter et al., 2000). The application of marker-free transgenic plants without the selective genes of antibiotic and herbicide resistance as an edible vaccine increases their biosafety. This study was aimed at testing the immunogenicity of an edible vaccine based on marker-free transgenic potato plants synthesizing the surface hepatitis B antigen (HBsAg) in mice.

2. Materials and methods

2.1. Plant material

Transgenic potato marker-free plants *Solanum tuberosum* L. variety Desiree, synthesizing HBsAg, were used in our experiments. HBsAg of the ayw serotype was used in the study. These plants were obtained using the previously constructed plasmid pBM-Ag in the *Agrobacterium tumefaciens* strain LBA4404 (pAL4404) (Rukavtsova et al., 2009). Agrobacterial transformation of leaf potato explants was performed as described in (Rocha-Sosa et al., 1989). The obtained regenerants were grown on Murashige and Skoog medium (Murashige and Skoog, 1962) containing 250 mg/l cefotaxime and 0.1 mg/l indolyl butyric acid. Six hundred potato regenerants were initially divided into groups of 20–30 seedlings. The mixture of explants of each group was grounded and the extracts were assayed by ELISA with the antibodies against HBsAg. Several such groups displayed the presence of HBsAg. Further analysis of individual seedlings makes it possible to find the transformants synthesizing HBsAg. Transgenic potato plants were cultivated under greenhouse conditions at the Biotron artificial climate station.

2.2. Plant DNA isolation and the screening of transgenic plants for the HBsAg gene

The transformed potato plants were screened by PCR for the *HBsAg* gene (681 bp) using the following primer pairs: forward, 5′-CGGGATCCATGGAAAACATTACTTC-3′; reverse, 5′-CGGGATCCCTATCATTAAATGTAAACC-3′. Plant DNA was extracted according to the method (Edwards et al., 1991).

2.3. Quantification of HBsAg levels in transgenic potato plants

The leaves and tubers of analyzed plants (100–200 mg) were homogenized in 0.3 ml of extraction buffer (0.05 M Na–phosphate buffer, pH 7.5, 0.15 M NaCl, 1 mM EDTA, 0.3% Tween 20, 0.0004 M PMSF, 1% sodium ascorbate, 0.2 mg/ml leupeptin). The resulting lysates were centrifuged for 10 min at 12,000 rpm and stored on ice. Monoclonal enzyme-linked immunosorbent assay (ELISA) was used to quantify antigenically reactive HBsAg. The Vektogep B-HBs-antigen test system (VectorBest, Russia) was employed according to the manufacturer's instructions. The recombinant HBsAg obtained from yeast cells was used as a positive control (Combiotech, Russia). The content of soluble protein was determined according to Bradford (1976).

2.4. Immunoprecipitation and Western blot analysis of tuber-derived HBsAg

HBsAg from the analyzed tuber protein extracts was immunoprecipitated using mouse monoclonal antigens against native HBsAg on protein A-Sepharose™ CL-4B (GE Healthcare, USA). The pellet was dissolved in sample buffer (62.5 mM Tris–HCl, pH 6.8, 10% glycerol (by volume), 2% SDS, 2% dithiothreitol, and 0.05% bromophenol blue) and boiled for 5 min. Then the proteins were fractionated by electrophoresis in 15% polyacrylamide gel and transferred onto Immobilon™ PVDF membrane (Millipore, USA) by electroblotting, using a MiniTrans-blot® apparatus (Bio-Rad, USA) in the buffer containing 0.025 M Tris–HCl, 0.193 M glycine, and 20% ethanol for 2 h at 100 V. The membrane was rinsed in TBS-T buffer (0.01 M Tris–HCl, pH 7.5, 9% NaCl, 0.1% Tween-20); the blocking was performed in the same buffer with 5% skim milk powder for 12 h. The membrane was treated with rabbit polyclonal antibodies against HBsAg monomer (1:5000, Combiotech, Russia). Goat antibodies (1:10,000) conjugated with horseradish peroxidase (Pierce, USA) were used as secondary antibodies. Membrane development was performed using the ECL chemiluminescence system (Pierce).

2.5. Gel filtration of HBsAg

Potato tuber cell-free extract (3 ml) was applied on a 1.5 cm × 20 cm Sephadex G-25 column (LKB Pharmacia, Sweden) equilibrated with 0.05 M sodium phosphate buffer (pH 7.5) containing 0.15 M NaCl and eluted with the same buffer. The void volume fraction was concentrated 3-fold in a vacuum lyophilizer and used for high-performance gel filtration. For this purpose, 100 μl of the fraction was applied on a Superose-6 (HR10/30) HPLC column (LKB Pharmacia) equilibrated with 0.05 M sodium phosphate buffer (pH 7.5) containing 0.15 M NaCl, and eluted with the same buffer for 1 h at 0.4 ml/min flow rate. The eluted material was detected by measuring absorbance at 280 nm. Fractions of eluted material (0.8 ml) were collected; the quantitative content of HBsAg antigen was determined by ELISA. Dextran blue D2000 (2000 kD), thyroglobulin (669 kD), and ferritin (443 kD) were used as molecular weight standards. Gel filtration was performed on the column under the same conditions.

2.6. Immunization of mice

Two groups of 10 outbred NMRI mice (23–25 g) were used to study the immunogenicity of the HBsAg synthesized in marker-free potato tubers. All mice were maintained under standard conventional conditions, with food and water provided ad libitum. The animals from the experimental group were fed transgenic potato tubers on day 1, 7, and 14 of the experiment (20 g of tubers containing 20 μg of HBsAg per mouse). The control group was fed untransformed potato tubers. All mice were fed a standard diet throughout the experiment. The mice had starved for 12 h before being fed the potato tubers. On day 71 of the experiment, the mice from the experimental group were injected intraperitoneally with 0.5 μg of the recombinant yeast hepatitis B vaccine (Combiotech, Russia). In 320 days, the animals from the experimental group were fed the tubers of transgenic potatoes three times with an interval of 1 week for re-immunization. Blood samples from each mouse in the groups were analyzed separately. Blood from the caudal vein of mice was collected every 10–14 days in order to assess the immunity against hepatitis B. The obtained blood serum was frozen and stored at −20 °C. All samples were analyzed for the presence of antibodies against HBsAg by ELISA using a D-0562 test system (VectorBest, Russia) according to the manufacturer's recommendations. All the experiments were carried out in triplicate and standard errors were calculated.

3. Results and discussion

The selective genes of antibiotic and herbicide resistance were extensively used to select transgenic plant systems expressing HBsAg (Mason et al., 1992; Rukavtsova et al., 2003). However, such transgenic plants have considerable potential biological and environmental hazards. There are various methods for removal of these selective genes by recombination systems (Rukavtsova et al., 2013),

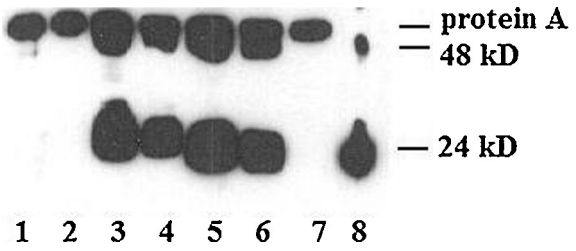

Fig. 1. Western blot analysis of potato-derived HBsAg. The antigen was precipitated from protein extracts by immunoprecipitation with protein A-Sepharose CL-4B sorbent. 1, 2 – The protein extract from untransformed potato leaves; 3, 4 – the extract from transgenic potato leaves; 5, 6 – the extract from recombinant yeast producing HBsAg; 7 – negative control (protein A-Sepharose CL-4B); 8 – positive control (yeast-derived HBsAg without immunoprecipitation). Polyclonal antibodies against HBsAg monomer diluted 1:5000 were used. Antibody binding was visualized using an ECL kit (Pierce).

but it is a long process, which ultimately slows down the production of transgenic plants. We have developed a new method for construction of marker-free transgenic plants. In order to obtain transgenic plants containing the *HBsAg* gene without any additional selective or reporter genes, we constructed the marker-free vector pBM and inserted the target gene in this vector (Rukavtsova et al., 2009). The transgenic potato plants carrying the *HBsAg* gene under the control of the CaMV 35SS promoter were selected by ELISA with the antibodies against HBsAg. Our method allows HBsAg detection in the extracts of transgenic seedlings even after multifold dilution by nontransformed plant extracts. We assay seedling explants of approximately similar weight (about 200 mg) for the presence of HBsAg. In the performed experiments, the transformation efficiency was 2%. As a result, several marker-free potato lines synthesizing antigen at a level up to 0.05% of total soluble protein in the cell were selected. PCR analysis was carried out to show the presence of transgene of expected size (681 bp) in the genomic DNA of tested plants (data not shown). HBsAg content in the tubers of various potato lines was up to 1 μg/g of wet weight.

Western blot analysis of plant-produced HBsAg was performed after its immunoaffinity purification (Fig. 1). Eleven lines of transgenic marker-free plants were obtained. All lines of transgenic potatoes were used in a Western blot analysis and demonstrated positive signals (data not shown). Fig. 1 shows the analysis of the best HBsAg producers. The molecular weight of the purified potato-derived HBsAg is approximately 24 kD, which corresponds to the molecular weight of the major envelope protein of the hepatitis B virus. The part of the antigen after denaturation remained as a dimer of approximately 48 kD. The antigenicity of HBsAg is highly dependent on the degree of disulfide binding and multimerization (Vyas et al., 1972; Huovila et al., 1992). The existence of multimeric form of HBsAg in transgenic potato plants was demonstrated in the experiments on gel filtration of HBsAg. HBsAg was detected in a narrow elution zone of high molecular weight compounds of approximately 2000 kD (Fig. 2). Considering that the monomeric form of HBsAg is 24 kD, it may be concluded that the *HBsAg* gene expression product in transgenic plant cells forms multimers of no less than 70–80 monomers. Our data on the gel filtration of HBsAg synthesized by transgenic potato cells correspond to sedimentation and electron microscopy characteristics of the HBsAg extracted from transgenic tobacco cells by other authors (Mason et al., 1992; Smith et al., 2003). Under gel filtration, HBsAg is eluted in the zone free from the most of the proteins. It makes the technique of gel filtration an efficient stage in large-scale production of hepatitis B vaccine based on transgenic plants. The multimeric form of HBsAg has significantly higher immunogenic and antigenic activities compared to the original monomeric form and a potential to be used as a substance for hepatitis B vaccine production. Thus, in the cells of transgenic plants, as well as in the cells of recombinant strains of yeast-producing HBsAg, the monomeric forms of HBsAg are assembled into immunogenic multimeric aggregates that can be used as a substance to produce a vaccine against hepatitis B virus.

The immunogenicity of HBsAg synthesized by potato tubers was studied in two groups of 10 outbred NMRI mice. The potato tubers of one line with the highest synthesis of HBsAg were used in the analysis. The animals of the experimental group were fed transgenic potato tubers on day 1, 7 and 14 of the experiment. The

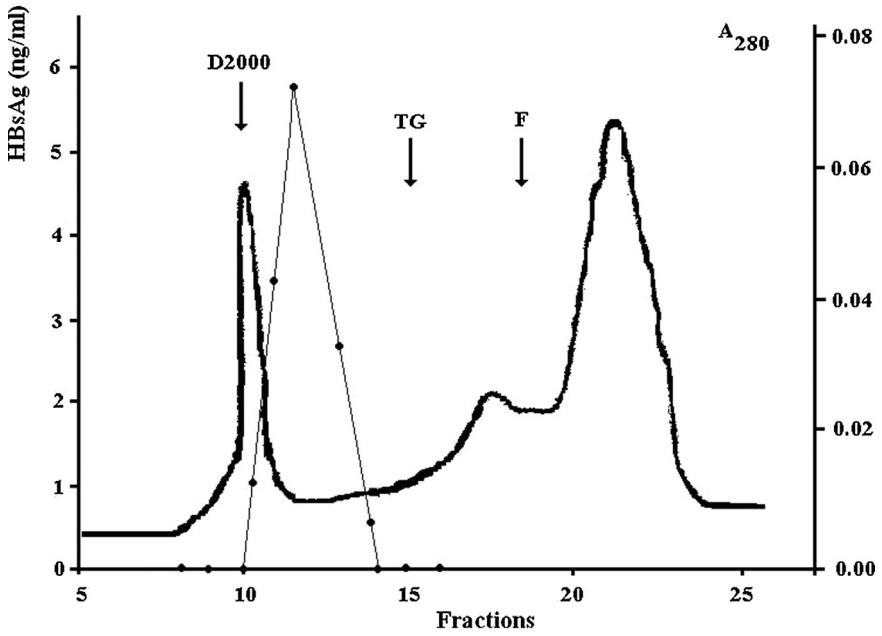

Fig. 2. High performance gel filtration of HBsAg extracted from transgenic potato tubers on a Superose-6 column (HR 10/30). Elution with 0.05 M Na–phosphate buffer (pH 7.5) containing 0.15 M NaCl for 60 min at 0.4 ml/min flow rate. The thick line indicates absorbance, and the thin line shows HBsAg detection. D2000 – dextran blue (2000 kD); TG – thyroglobulin (669 kD); F – ferritin (443 kD).

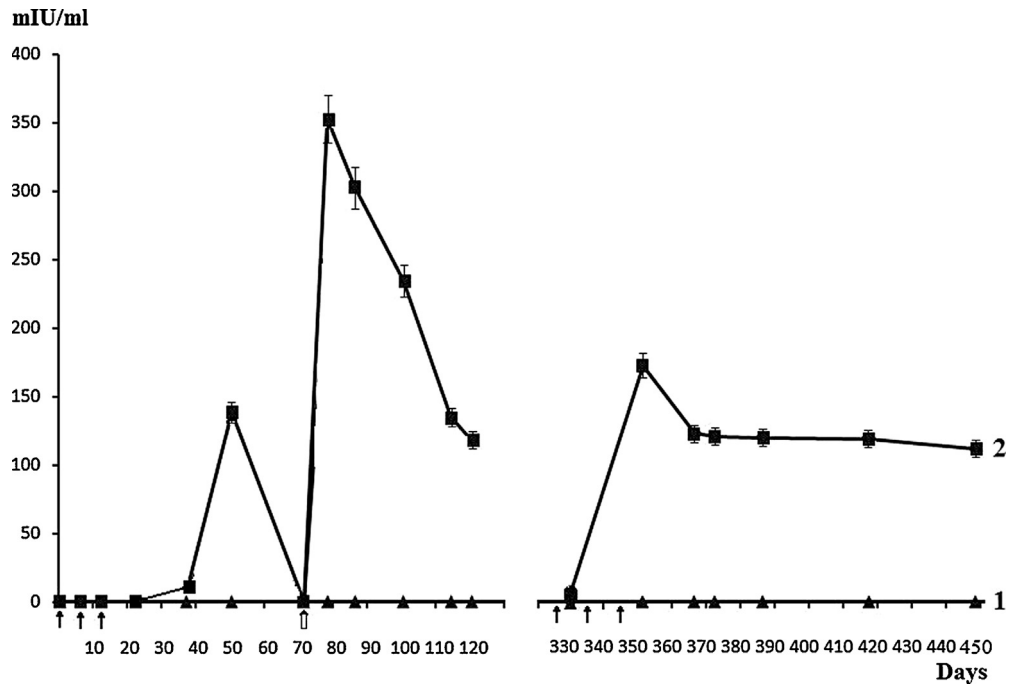

Fig. 3. Dynamics of HBsAg antibody content in blood serum of mice. 1 – the control group was fed untransformed potato; 2 – the experimental group was fed transgenic potato. The black thin and white thick arrows indicate the time of feeding and the time of recombinant yeast vaccine injection, respectively. All the experiments were carried out in triplicate and standard errors were calculated.

control group was fed untransformed potato tubers. Blood from the caudal vein was taken in each group of mice every 10–14 days in order to assess the immunity against hepatitis B. Blood serum was analyzed by ELISA for the presence of antibodies against HBsAg. The level of antibodies against HBsAg in the blood serum of experimental mice began to increase on day 36–50 after the first feeding (Fig. 3). The content of antibodies against HBsAg was up to 170 mIU/ml on day 50 from the beginning of the experiment. The blood of mice from the control group was free from anti-HBsAg antibodies.

The mice of experimental group were injected intraperitoneally with 0.5 μg of recombinant yeast hepatitis B vaccine (Combiotech, Russia) on day 71 of the experiment. The antibody content increased in mice a week later and reached a maximum within 15–43 days after the injection (up to 350 mIU/ml). On day 120 after the start of the experiment, the level of antibodies against HBs-antigen in the experimental group of mice was significantly higher than the minimum protective level (up to 100 mIU/ml).

In 320 days after the beginning of the experiment, the mice of the experimental group were fed transgenic potato tubers three times. In the mice that showed an immune response to the hepatitis B virus in the previous experiment, the level of the HBsAg antibody in the blood serum increased to 185 mIU/ml. The level of protective antibodies was maintained for several months (Fig. 3). Accordingly, booster vaccination was performed to enhance the postimmune defense, and it was confirmed that the animals preserved stable immunological memory of the infection. Up to now, no experiments of such duration with transgenic plants used as an edible vaccine against the hepatitis B virus have been performed. Similar experiments of other authors were limited to 24–38 weeks (Richter et al., 2000; Kong et al., 2001); our own experiments continued about 65 weeks. Our data indicate that transgenic marker-free plants are promising substances for the production of edible vaccines against the hepatitis B virus.

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