Study of immunogenicity of recombinant proteins based on hemagglutinin and neuraminidase conservative epitopes of Influenza A virus

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Background: Recombinant hemagglutinin (rHA) and neuraminidase (rNA) developed in our investigation are amino acid sequence consensus variants of H1N1 2009 subtype influenza virus strain, also including immunogenic epitopes typical for other influenza virus subtypes (H3N1 and H5N1). Substitutions were made: typical for Russian virus isolates (in HA – S220T, NA – D248N) and in active centers of molecules – R118L, R293L, R368L; C92S, C417S to increase recombinant proteins stability in E. coli. The aim of the present work was to study immunogenicity of the obtained rHA and rNA.

Material/Methods: Fragments aa 83–469 of NA and aa 61–287 of HA were chosen because they include the main B-cell epitopes and are the minimal structures for correct folding of target proteins. The designed nucleotide sequences were synthesized and purified and the expression of rNA and rNA were analyzed. For immunization and virus challenge we used influenza viruses A/California/04/2009 (H1N1), A/PR/8/34 (H1N1), A/Perth/16/2009 (H3N2), A/Chicken/Kurgan/05/2005 R.G. (H5N1), and B/Florida/04/2006. Specific IgG levels were determined by ELISA in 96-well ELISA plates. Significant differences of survival in mouse groups were analyzed by Mantel-Cox (log-rank) and Gehan-Breslow-Wilcoxon tests.

Results: The obtained results demonstrate the high immunogenicity and ability of indicated proteins mixture to provide similar cross-protection against influenza viruses of the H1N1 subtype.

Conclusions: The data obtained suggest efficient pluripotent vaccine creation based on HA and NA conservative regions.

Key words: conservative epitopes • immunogenicity • influenza virus • recombinant haemagglutinin • recombinant neuraminidase

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Background

According to WHO data, 250 000 to 500 000 people (most of them older than 65 years) die each year from of all variants of the virus during seasonal epidemics in the world; in some years the number of deaths could reach a million [1,2]. Many researchers around the world are working on a universal flu vaccine [3,4]. However, existing vaccines only have effectiveness against certain strains of influenza virus.

A common focus for creating a universal vaccine is the development of substances that affect the virus structure, which is conservative and highly homologous among the different types of viruses. At the moment, the flu vaccine should be created every year because the virus is constantly mutating, forming new antigenic determinants, and every time the disease is caused by a new type of virus.

Influenza viruses belong to the Orthomyxoviridae family, which includes the genera influenza A, B, and C, which are defined depending on presence of 2 internal virion proteins (M1 and NP). Further division is carried out in accordance with subtypes (serotypes) of the surface proteins hemagglutinin (HA) and neuraminidase (NA) [5,6]. Currently, 16 HA and 9 NA subtypes are known.

It is of epidemiological importance that human have viruses containing 3 subtypes of hemagglutinin (H1, H2, and H3) and 2 subtypes of neuraminidase (N1, N2). The major virion antigenic components of influenza A and B is the NA and HA; the C virus does not contain neuraminidase. Antibodies produced in response to the hemagglutinin constitute the basis of immunity against a specific subtype of influenza virus [7–9].

The immune system usually does not respond to the “stem” part of hemagglutinin, which is difficult to reach, but instead it rapidly produces antibodies to surface determinants, which are more accessible. However, their high variability allows the virus to remain unrecognized. Even in case of administration of specially isolated conserved regions of hemagglutinin, the immune system will respond with the formation of a wide spectrum of antibodies, most of which will not be able to recognize a live virus because this area is deeply hidden [10].

Our study was conducted in an attempt to create 2 recombinant short conserved regions of viral surface proteins – hemagglutinin and neuraminidase integrated epitopes – against which both T- and B-cells are active.

NA- and HA-coding genes were obtained and expressed in E. coli during the decade of the 1980s to study their immunological properties [11–13]. The first hemagglutinin subunit (HA1) amino acids (aa) 193–199 forming QNPTTYI epitope remained conservative among influenza H5N1 virus strains recently in circulation and could be recognized by monoclonal antibodies to HA1 of influenza virus A/Vietnam/1203/04 (H5N1). HA1 region aa 42–75 (HA1 N-terminus) in H5N1 viruses, aa 262–295 in H1N1 viruses, aa 57–90 in H3N2 viruses, and aa 28–61 in H7N7 viruses were shown to be highly conserved [14,15]. HA conservative epitope sequences of influenza H3N2 virus (aa 173–181, 227–239) and antibodies to these epitopes neutralizing strains of this subtype were demonstrated [16].

Highly homologous HA areas among H1, H3, and H5 subtypes of influenza A virus were detected. Nine HA1 sites have similar secondary structure (aa: 31-37, 89-96, 118-130, 200-208, 238-246, 272-277, 304-312, 317-334, 341-348) among H1, H2, H3, and H5 subtypes [7,17]. Similar structure regions were identified among influenza H1N1 and H5N2 virus subtypes, probably being the main epitope inducing formation of neutralizing antibodies to a vast variety of influenza virus strains [16]. These regions located behind the receptor-binding HA sites are conservative among H3N2 subtype of influenza virus strains and highly conservative in H1N1 and H5N1.

Nucleotide sequences coding rHA and rNA of influenza A and B virus and antigenic determinants with codon optimization for expression in E. coli are unknown.

The aim of the present work was to study immunogenicity of obtained rHA and rNA.

Fragments aa 83–469 of NA and aa 61–287 of HA were chosen because they include the main B-cell epitopes and are the minimal structures for correct folding of target proteins.

Material and Methods

Gene synthesis

Designed nucleotide sequences were synthesized as described by Young [18].

Vector pUC57 was used for cloning. Fragments were cut from the vector and amplified using the polymerase chain reaction (PCR) method. Nucleotide sequences coding HA and NA were synthesized by overlapping of elongation of oligonucleotides [19] and cloned into plasmid pET151/D-TOPO. For gene engineering work, DH10B/R E. coli cells (Gibko BRL, USA) were used.

rHA and rNA expression and purification.

BL21(DE3) E. coli cells were transformed by electroporation. Induction of rHA and rNA expression was performed by 0.2%
lactose in induction medium (1% peptone, 0.5% yeast extract, 50 mM NaH₂PO₄, 50 mM K₂HPO₄, 25 mM (NH₄)₂SO₄, 2 mM MgSO₄, 0.5% glycerol, 0.05% glucose, 0.2% lactose).

Fermentation was performed in induction medium containing ampicillin (100 µg/ml) in thermostatic rotor shaker (37°C, 250 rpm, 16–20 hours).

Disc-electrophoresis in PAAG of cell lysate of BL21(DE3)pPVNA and BL21(DE3)pPVHA E. coli strains was performed for densitometric measurement of rHA and rNA nucleotide sequences expression level in E. coli.

Proteins were purified according to the Ni-NTA Purification System protocol (Invitrogen, Catalog Number K950-01).

**Immunization and virus challenge**

Balb/c mice (16 per each group, 16–18 g) were immunized by 20 µg rHA and rNA in the ratio 1:1 with 0.5 mg of Alhydrogel. Two intraperitoneal immunizations were conducted 2 weeks apart. Control mice received PBS (priming) and vaccine filling agent (boosting). Blood samples (from 5–6 mice) were collected from the ventral vein 2 weeks after the second immunization.

Influenza viruses A/California/04/2009 (H1N1), A/PR/8/34 (H1N1), A/Perth/16/2009 (H3N2), A/Chicken/Kurgan/05/2005 R.G. (H5N1), and B/Florida/04/2006 were used. Immunized mice were challenged intranasally with 1LD₅₀ of A/California/04/2009 (H1N1) and A/PR/8/34 (H1N1) or 5LD₅₀ of A/California/04/2009 strain (50 µl per mouse). Animals were monitored daily for 2 weeks for survival and weight loss.

**Proteins denaturation**

Tween-20 was added to the sample – the final concentration 1% (w/v), followed by water bath incubation (1 h, 37°C) with further centrifugation (1 h, 20°C, 2000 g). Supernatant containing viral envelope proteins of influenza viruses A/PR/8/34 and A/Chicken/Kurgan/05/2005 R.G. was taken. Detergent was removed using the Detergent-OUT™Micro Kit (Millipore) and the sample was concentrated on SpeedVac by achieving initial volume. The preparation of additional elaboration was held with 8M carbamide in presence of DDT (0.02 M) with further night dialysis versus carbonate buffer (pH 8.5).

**Serum antibody determination**

Specific IgG levels were determined by ELISA in 96-well ELISA plates (“Greiner”, Germany) coated overnight at 4°C with 100 µl/well of rHA, rNA (3 µg/ml) and influenza strains A/California/04/2009, A/PR/8/34, A/Perth/16/2006, A/Chicken/Kurgan/05/2005 R.G., B/Florida/04/2006 (2 µg/ml) resuspended in carbonate coating buffer. Plates were blocked with blocking buffer (PBS with 5% FCS) – 300 µl/well, 1 h at room temperature. Sera were serially diluted. The diluted sample (100 µl) was added to duplicate wells (incubated 1 h at room temperature). HRP-labeled rabbit anti-mouse IgG antibodies (Abcam, UK) and goat anti-rabbit IgG antibodies (Sigma-Aldrich) diluted in blocking buffer were added (100 µl/well) (incubation at room temperature for 1 h). Plates were developed with TMB substrate (BD Bioscience) according to the manufacturer’s instructions. ELISA endpoint titers were defined as inverse value of the highest dilution that yielded an OD 450 nm value above 2 times the mean of negative control wells.

**Statistical analysis**

The life length evaluation was done with instantaneous sampling (Kaplan-Meyer). Significant differences in survival in mouse groups were analyzed by Mantel-Cox (log-rank) and Gehan-Breslow-Wilcoxon tests.

**Results**

**rHA and rNA design and expression**

Alignment of 837 aa rNA and rHA sequences of influenza A and B virus strains (NCBI, 2009) resulted in aa sequence creation representing NA and HA consensus aa variants. Fragments including the main A (H1N1, H3N2, H5N1) and A influenza virus B-cell epitopes were chosen. Substitutions in an active center of the NA molecule and those typical for Russian virus isolates were made. Corresponding nucleotide gene sequences were codon-optimised for E. coli.

The obtained expression of pPVNA and pPVHA plasmids provide rNA and rHA synthesis in E. coli under specific promoter induction (Figures 1 and 2). Sequenation has shown the absence of mistakes in the gene structure.

**rHA and rNA separation and purification**

NA accumulation assessed with densitometry was 23% of total cell protein amounts in BL21(DE3)pPVNA E. coli cells and 43% in BL21(DE3)pPVHA E. coli cells. Expression levels did not change during 6 passages, which confirms the expression stability. After purification, rHA was obtained with 96% purity and rNA with 95% purity.

**rHA+rNA immunogenicity in mice**

The specific serum IgG antibodies to rHA, rHA+rNA, A/California/04/2009, A/PR/8/34, A/Perth/1620/09, and A/Chicken/
Kurgan/05/2005 R.G were determined by ELISA. Immunization induced high titers of antibodies to rHA and rNA and titers were 2 times lower to influenza viruses surface glycoproteins (Table 1).

After denaturation of influenza virus surface glycoproteins, the antibody titers increased by 4 times to A/PR/8/34 and by 8 times to A/Chicken/Kurgan/05/2005 R.G. (Table 2).

| Mice groups                  | Fixed antigens |
|-----------------------------|----------------|
|                             | rHA | rHA+ rNA | HA A/California/04/2009 | A/California/04/2009 | A/PR/8/34 | A/Perth/16/2009 |
| Mice immunized with rHA+rNA | 409 600 | 819 200 | 6 400 | 51 200 | 12 800 | 3200 |
| Control mice                | 200 | 200 | 200 | 200 | 200 | 200 |

| Mice groups      | Fixed antigens |
|------------------|----------------|
|                  | A/PR/8/34 | A/PR/8/34 Tw | A/Kurgan/05/2005 | A/Kurgan/05/2005 Tw |
| Mice immunized with rHA+rNA | 12 800 | 51 200 | 200 | 1 600 |
| Control mice     | 200 | 200 | 200 | 200 |
Anti-HA and anti-NA serum IgG induced by immunization with inactivated A/California/04/2009 were shown to be incapable of binding to rHA and rNA of this virus. Two months following immunization of mice, IgG titers to influenza virus A/California/04/2009 were 102,400, titers to rHA were 12,800, and titers to rNA were 3,200. Serum of rabbits immunized with influenza B virus was shown to be capable of binding to both rHA and influenza virus B/Florida/04/2006. The serum IgG titers were 409,600 and 204,800, respectively.

Mice protection from influenza challenge

We evaluated the ability of mice to resist a lethal dose after immunization with rHA+rNA containing virus. Immunized mice challenged with 1LD₅₀ of influenza virus A/California/04/2009 showed 100% survival compared to 43% (p<0.05) survival in the control group (Figure 3). Immunized mice lost less body weight and started rapidly gaining weight compared to the control group (p<0.05). These findings indicate a recovery process starting 6 days after viral challenge. Administration of 5LD₅₀ of influenza virus A/California/04/2009 resulted in 57% survival level among immunized mice compared to 14% among control animals (Figure 3) and also resulted in less body weight loss than in unvaccinated controls. Inoculation with 1LD₅₀ of A/PR/8/34 showed 100% survival in the group of immunized mice (Figure 4) and 53% in the control group (p<0.05). Animals of the control group had more pronounced body weight loss compared with immunized mice (p<0.05).

Discussion

Nowadays recombinant proteins based on the conservative epitopes of influenza virus HA, M2, and NP genes obtained in E. coli are used for the development of influenza vaccines, including the so-called “universal” vaccines requiring little seasonal modifications and designed with gene engineering methods representing minimal outlay [4,20–22].

Figure 3. Survival (A) and body weight dynamics (B) after challenge with 1LD₅₀ and 5LD₅₀ A/California/04/09 (H1N1).

Figure 4. Survival (A) and body weight dynamics (B) after challenge with 1LD₅₀ A/PR/8/34 (H1N1).
Antibodies to conservative HA regions can induce a broad cross-reactive immune response to new influenza A virus strains [16], hence HA1-conservative regions could become potential targets for universal influenza vaccines. Strain-specific antibodies to HA are considered to have a weak cross-activity with other HA types. Antibodies to definite epitopes having cross-reactive neutralizing activity also exist. Furthermore, H1N1-induced infection stimulates the production of protective cross-reacting antibodies to H5N1 [6], which are supposed to serve as the basis for development of universal vaccines against flu and therapeutically immunobiological medications [23,24]. Descriptive studies have been conducted of antibodies interacting with different influenza virus subtypes, as well as corresponding antigen determinants [25–28]. Synthetic immunogens based on such epitopes inducing the formation of antibodies displaying high cross-reactivity with H1N1, H3N2, H2N2, H5N1, H7N2, H7N3, H7N7, and H9N2 virus antigens were described and their protective immune response were shown [29]. Recombinant protein consisting of 6 HA epitopes highly protective against influenza virus A strains H1N1, H2N2, and H3N2 was characterized [3]. According to the obtained data, the synthesis of neutralizing antibodies to influenza H1N1 virus strains that emerged during the last 73 years was induced by H1 HA-based immunogens [30].

The originality of the suggested approach is that HA and NA (inducing immunogenic reactivity spectrum in vaccine composition) antigen determinants of 2 influenza virus types (A and B) were used for the first time and sequences presenting combination of B- and T-cell conservative epitopes (HA – 61–287 aa, NA – 83–469 aa) are codon-optimized for increase of rHA and rNA nucleotide sequences expression level. The obtained rHA and rNA are influenza A (H1N1) virus aa sequences consensus variants also containing immunogenic epitopes typical for other influenza virus strains (H3N1 and H5N1).

Conclusions

Most current efforts to create universal vaccines hinge on the idea of generating antibodies against a portion of the virus that is relatively unchanged year-to-year. A vaccine, based on using antibodies simultaneously to two conservative and highly homologous virus structures serves to increase the degree of defence from different types of viruses.

The results obtained for rHA and rNA immunogenicity and protective efficacy demonstrate the possibility and necessity of this approach for development of a universal pandemic influenza vaccine.

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