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Two distinct regions of HA2 glycopolypeptide of influenza virus hemagglutinin elicit cross-protective immunity against influenza

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Summary. – Currently, a new trend in development of vaccines against influenza with broader spectrum of efficacy is focused on conserved antigens of influenza virus. The HA2 glycopolypeptide (HA2 gp) is one of conserved antigens, potentially suitable as immunogens inducing cross-protection against influenza. We selected two distinct domains of HA2 gp originating from influenza A virus (IAV) of H3 subtype for induction of antiviral immune response: the ectodomain (EHA2) comprising aa 23–185 and the fusion peptide (FP) comprising N-terminal aa 1–38. BALB/c mice were immunized with three doses of EHA2 and FP, respectively, and subsequently challenged with 2 LD_{50} of IAV of homologous (H3) or heterologous (H7) HA subtype. Both peptides induced significant antibody response and protected mice against the lethal infection. The most efficient protection was achieved with EHA2 against homologous virus.

Keywords: influenza A virus; cross-protection; HA2 glycopolypeptide; HA2 ectodomain; fusion peptide; mice; vaccine

Introduction

IAVs cause acute respiratory diseases in humans, which can often be accompanied by health complications with a severe course and even a fatal end. The control of spread of infection can be achieved by vaccination or therapy with antiviral substances, however, both have limited efficacy. The yearly repetition of influenza epidemics is the consequence of high and unpredictable variability of IAVs. The low fidelity of viral RNA-polymerase, the RNA segmented genome as well as the wide host specificity of IAVs, are the main factors contributing to their mutational rate. Currently used vaccines induce virus-neutralizing antibodies, targeted to HA1 gp localized on the globular part of HA, which is variable. Owing to the rapid antigenic drift of IAVs, these vaccines become ineffective against newly emerging IAVs, which infect people lacking specific immunity. Therefore, based on influenza surveillance worldwide, vaccination strains must be annually updated. Moreover, preparation of actual vaccines takes time, which can be critical in case a new pandemic virus is emerging. A highly pathogenic IAV (H5N1) that has recently infected humans in several continents, represents a pandemic threat. It can become transmissible from person to person and cause a severe infection by cumulative drift changes or by reassortment. Therefore, new approaches to prevention providing a broad cross-protective immunity to influenza are focused on the conserved antigens of IAVs. A passive immunization with cross-protective antibodies has been successfully applied to several experimental models (Gocník *et al*., 2007; Throsby *et al*., 2008; Prabhu *et al*., 2009; Yoshida *et al*., 2009; Wang *et al*, 2010; Corti *et al*., 2011; Király *et al*., 2011). However, the passive immunization is limited to therapeutic purposes only and for effective prevention a new strategy of active immunization is required.

Many studies have been focused on the M2 protein (Okuda *et al*., 2001; Fan *et al*., 2004; Liu *et al*., 2004; DeFilette *et al*., 2006; Hoelscher *et al*., 2006; DeFilette *et al*., 2008a,b; Huleatt

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Abbreviations*:* A/Chicken/Germany/34 (H7N1) virus, Rostock strain; A/Miss = A/Mississippi/1/85 (H3N2) virus; A/Rostock = avian HA = hemagglutinin; EHA2 = ectodomain of HA2 gp; FA = Freund´s adjuvant; FP = fusion peptide; HA0 = HA precursor; HA1 = HA1 glycopolypeptide; HA2 = HA2 glycopolypeptide; IAV(s) = influenza A virus(es); MAb = monoclonal antibody; vRNA = viral RNA; p.i. = post infection

et al., 2008; Zhang *et al.*, 2009; Li *et al.*, 2011; Park *et al.*, 2011; Staneková *et al.*, 2011), but recently also the conserved part of HA, HA2 gp, was used as an inductor of cross-protective immunity (Okuno *et al.*, 1993; Horváth *et al.*, 1998; Gocník *et al.*, 2008; Sui *et al.*, 2009; Bommakanti *et al.*, 2010; Steel *et al.*, 2010; Wang *et al.*, 2010; Staneková *et al.*, 2011). The latter was the subject of our interest for several years. HA2 gp plays an important role in the virus entry into the cell. IAV HA is synthesized as a precursor (HA0), which is subsequently glycosylated, trimerized and proteolytically cleaved into two parts, HA1 and HA2. After cleavage they remain connected by disulphide bond. The attachment of virus to cell surface is mediated by receptor-binding site on HA1 gp and virus is then endocyted. In endosomes, acidic pH induces major conformational changes in the HA molecule, leading to exposure of FP at the N-terminus of HA2 and subsequent fusion of viral and endosomal membranes. The virus genome is released into the cytoplasm and transported to the site of virus replication, the cell nucleus.

HA2 representing the major part of the HA stem is highly conserved across IAVs within a subtype (Nobusawa *et al.*, 1991; Gerhard *et al.*, 2006). Moreover, there are some regions of HA2 gp, which are conserved also among various subtypes (Varečková *et al.*, 2002, 2008). As we have shown previously, HA2 gp is immunogenic and able to induce a protective antibody response in mice (Varečková *et al.*, 2003a; Gocník *et al.*, 2008). The most conserved part of HA2 gp is its N-terminus, FP. Its sequence analysis revealed that aa 3–11 exhibit even an 100% identity among all influenza subtypes (Nobusawa *et al.*, 1991). Though HA2-specific antibodies are induced also during a natural IAV infection of humans (Styk *et al.*, 1979), their level is low and not effective enough to protect against the infection, as epitopes of HA2 gp are weak natural immunogens (Staneková *et al.*, 2012). These findings led us to assume that an effective antiviral protection could be achieved by targeted immunization with HA2 gp.

Therefore, in this work, we examined the *in vivo* cross-protective potential of immunization by two peptides originating from HA2 gp of H3 subtype, EHA2 comprising aa 23–185 and N-terminal aa 1–38, FP. BALB/c mice, lethally infected with IAVs of H3 or H7 subtype served as an experimental model, in which main parameters of infection were monitored.

Materials and Methods

Viruses. Influenza virus A/Mississippi/1/85 (H3N2) (abbreviated as A/Miss) and avian A/Chicken/Germany/34 (H7N1) virus, Rostock strain (abbreviated as A/Rostock) originated from the collection of viruses of Institute of Virology, Slovak Academy of Sciences. Viruses were propagated in fertilized chicken eggs and purified by differential sucrose density gradient centrifugation (Russ *et al.*, 1974).

EHA2 was expressed in *Escherichia coli* BL21 transformed with pLM-1 plasmid encoding the region of aa 23–185 of HA2 gp of recombinant X-31 virus. The latter originated from A/Aichi/2/68 (H3N2) virus, kindly provided by Drs D.C. Wiley and J. Chen, Harvard University, Boston, USA. The expressed EHA2 was purified as described, the final step consisting of affinity chromatography on CNBr Sepharose (Sigma) coupled with HA2-specific monoclonal antibody (MAb) IIF4 (Chen *et al.*, 1995, 1999; Varečková *et al.*, 1993).

FP containing 38 N-terminal amino acids of HA2 gp of H3 subtype (1-GIFGAIAGFIENGWEGMVDGWYGFRHQNSEG TGQAADL-38) with M_r of 4059.44 (94.8% purity) was supplied by ProImmune (Oxford, UK). Its sequence was based on that of IAV (H3N2) 1985 strain (Acc. No. ABD61777). Low immunogenicity of FP as a small hydrophobic molecule was enhanced by conjugation with KLH. Briefly, 2 mg/ml FP was mixed with 2 mg/ml KLH, both in PBS. Glutaraldehyde was added to final concentration of 2.5% and the mixture was incubated for 50 mins at RT. The reaction was stopped with glycine added to final concentration of 0.03 mol/l and the mixture was dialyzed against PBS at 4°C overnight. Aliquots of the conjugated peptide were stored at -20°C.

Adaptation of viruses to mice. Six-week-old BALB/c mice were infected intranasally under light anesthesia with 40 µl of allantoic fluid containing infectious A/Miss or A/Rostock. After 2 days the mice were sacrificed, the lungs were homogenized with 1 ml of PBS, cell debris were pelleted and 40 µl of the obtained supernatant was used for further infection of mice. After 6 subsequent mouse passages the virus was propagated in 9-day-old chicken embryos. Virus titer was determined by standard micro-haemagglutination test using guinea pig erythrocytes.

Immunization and challenge. Six-week-old BALB/c mice were immunized intraperitoneally with three doses of EHA2 and FP, respectively, supplemented with inulin and FA as adjuvants, in two-week intervals. Since FP is a small hydrophobic molecule of low immunogenicity, the latter was enhanced by conjugation of FP to KLH. Single immunization dose contained EHA2 (10 µg/mouse) or FP conjugate (30 µg/mouse), inulin (100 µg/mouse) (Cooper and Steele, 1988; Petrovsky, 2006) and FA (v/v FA: antigen 1:2) in a total volume of 400 µl. Control mice were given PBS. Two weeks after the third dose, mice were challenged intranasally under light anaesthesia with 2 LD_{50} (40 µl) of A/Miss or A/Rostock. The course of the infection including survival, body weight change, the presence of infectious virus and viral RNA (vRNA) in the lungs, was monitored for 14 days post infection (p.i.).

Rapid culture assay (RCA). To determine infectious virus in the lungs two mice per group in two-day intervals were sacrificed, the lungs were homogenized with 1 ml of PBS, cell debris were pelleted and the supernatant was assayed for infectious virus as described (Tkáčová *et al.*, 1997). Briefly, MDCK cells were cultured in DMEM with 5% FBS in 96-well plates for 24 hrs to a density of $5x10^4$ cells/well. Two-fold dilutions of the supernatant in PBS were added to wells with cells for 45 min at 37°C. There-

after the inoculum was removed and a serum-free Ultra-MDCK medium was added. After 18 hr-incubation at 37°C and 5% CO_2 cells were fixed with methanol and the virus was detected using MAb 107L specific to IAV nucleoprotein (Varečková *et al.*, 1995) (1.5 μg/ml in PBS with 5% dry milk). After the incubation with HRP-conjugated goat anti-mouse IgG (90 min r.t.) positive reaction was visualized by addition of substrate solution containing 3-amino 9-ethyl-carbazole with 0,03% H_2O_2. Differentiated red colored cells, identified by light microscopy, were considered as positive.

RT-PCR. To detect vRNA in the lungs 200 μl of lung cell homogenate was mixed with 500 μl of Instapure™ System (Eurogentec). Total RNA was extracted by phenol-chloroform and examined for vRNA by RT-PCR. The RT step with random heptamer primers (Invitrogen) was followed by PCR amplification of specific virus nucleoprotein gene segment as described (Varečková *et al.*, 2006).

ELISA. To detect specific antibodies in mouse sera blood samples were collected from *sinus orbitalis* in two-week intervals before immunization and after each immunization dose. The obtained sera were tested for the presence of specific antibodies by sandwich ELISA. Briefly, EHA2 (80 ng/100 μl /well) or FP (500 ng/100 μl /well) was adsorbed onto 96-well plates for 24 hrs, the wells were blocked with 0.5 % ovalbumine for 1 hr at RT, washed and incubated with 2-fold dilutions of sera (100 μl/well) in PBS with 0.5% ovalbumine for 90 min. Specific antibodies were detected by a HRP-conjugated goat anti-mouse IgG after 90 min incubation and addition of the substrate solution containing ortho-phenylendiamine with 0,03 % H_2O_2. The reaction was stopped with 1M HCl and A_{495} was read.

Results

We examined the effect of immunization with conserved parts of IAV HA2 gp of H3 subtype, EHA2 comprising aa 23–185 and FP consisting of N-terminal aa 1–38 on the course of lethal influenza infection of mice with homologous IAV of H3 subtype of human origin (A/Mississippi/1/85(H3N2)) and heterologous, highly pathogenic avian IAV of H7 subtype (A/Chicken/Germany/34 (H7N1), Rostock strain).

Antibody response of mice to two fragments of HA2 gp

First we analyzed the antibody response elicited by immunization of mice with three doses of EHA2 and FP, respectively, by ELISA.

A noticeable production of HA2-specific antibodies, in particular an antibody titer of 5,140 was obtained already after the first dose of EHA2. The second dose raised the antibody titer to 1,030x10^3. No further increase of HA2-specific antibodies was registered after the third immunization step (Fig. 1a).

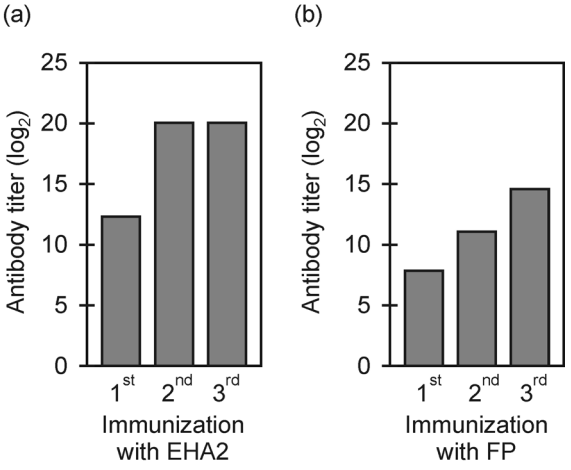

Fig. 1

Antibody response of mice to immunization with EHA2 (a) and FP (b)

The immunization with another part of HA2 gp, FP, resulted in a stepwise increase of the antibody titer up to 26,800 after the third dose (Fig. 1b).

Cross-protective effect of immunization with EHA2 on lethal influenza infection of mice

The course of infection in mice immunized with EHA2 and challenged with homologous influenza virus

Immunization of mice (n = 13) with three doses of EHA2 significantly increased the survival of mice after challenge with 2LD_{50} of homologous A/Miss (H3N2) virus from 27% in non-immunized controls (n = 15) up to 100% (p = 0.0001) (Fig. 2). Control mice exhibited clinical symptoms of influenza such as scrubby fur, loss

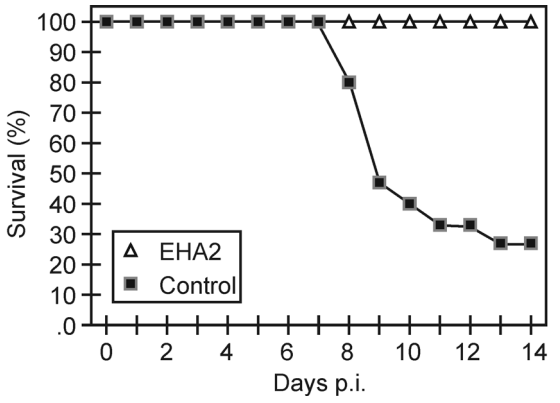

Fig. 2

Survival of mice immunized with EHA2 and challenged with homologous influenza virus

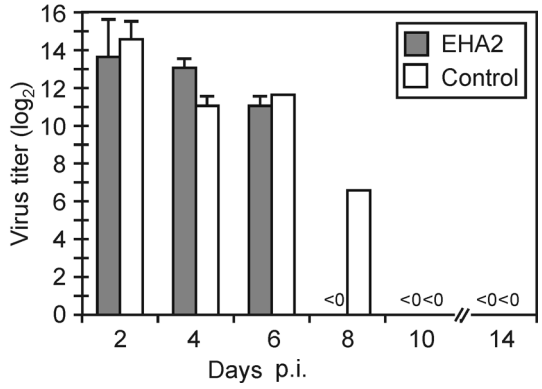

Fig. 3

Infectious virus in lungs of mice immunized with EHA2 and challenged with homologous influenza virus

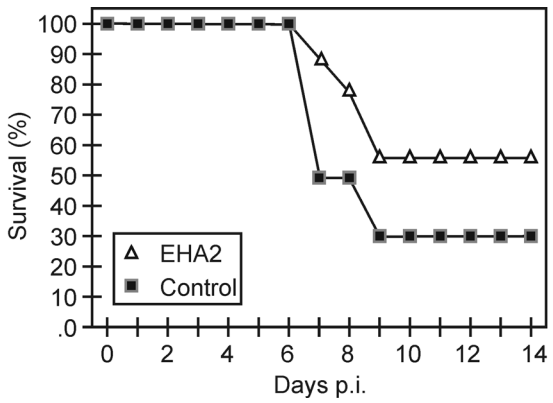

Fig. 4

Survival of mice immunized with EHA2 and challenged with heterologous influenza virus

of body weight and low activity and died on days 8–13 p.i. (Fig. 2).

The virus titer in lungs of both immunized and control mice reached a maximum on day 2 p.i. (12,800 *vs.* 25,600, respectively). The virus titer in lungs decreased more rapidly in immunized mice compared to control mice. Infectious virus was eliminated from lungs of immunized mice by two days earlier (day 8 p.i.) compared to control mice (day 10 p.i.) (Fig. 3). Similarly, vRNA was also eliminated from lungs of immunized mice earlier (day 8 p.i.) compared to control mice (day 14 p.i.) (Table 1).

Table 1. Detection of vRNA in lungs of mice immunized with EHA2 and challenged with homologous virus

Immunogen	Days p.i.					
	2	4	6	8	10	14
EHA2	+/+	+/+	+/+	−/−	−	−
Control	+/+	+/+	ND/+	−/+	+	−

The course of infection in mice immunized with EHA2 and challenged with heterologous influenza virus

To assess the cross-protective potential of EHA2 immunization, a heterologous, highly pathogenic avian A/Rostock (H7N1) virus was used for challenge. The immunization of mice (n = 9) with three doses of EHA2 slightly increased their survival from 30% in control mice (n = 10) to 56% (p = 0.3698) (Fig. 4). During the first days after the challenge, the course of infection was relatively mild until day 7 p.i. Thereafter, clinical symptoms worsened and mice started to die. The elimination of vRNA from lungs of immunized mice was observed by 4 days earlier compared to control mice (Table 2).

We can therefore conclude that the immunization with EHA2 gp conferred to mice a significant and complete protection against IAV of homologous subtype and a partial protection against a heterologous, highly pathogenic avian IAV.

Table 2. Detection of vRNA in lungs of mice immunized with EHA2 and challenged with heterologous virus

Immunogen	Days p.i.					
	2	4	6	8	10	14
EHA2	−	+/−	+/−	+/+	−	−
Control	+	+/+	+/−	+/+	+	−

Cross-protective effect of immunization with FP on lethal influenza infection of mice

The course of infection in mice immunized with FP and challenged with homologous influenza virus

Our previous data showed that immunization of mice with three doses of FP conjugated to KLH significantly protected them against a lethal infection with 1 LD_{50} of homologous virus A/Miss (Staneková *et al.*, 2011). Here, even though the infective dose was increased to 2 LD_{50}, the protection induced by immunization was still observable. The immunization of mice (n = 15) with the FP conjugate significantly increased their survival from 7% in non-immunized control (n = 15) to 47% (p = 0.0352) (Fig. 5). Infectious virus titer in lungs reached a maximum on day 2 and decreased to undetectable levels on day 8 p.i. in both immunized and control mice (Fig. 6). However, a significantly lower titer (800) was observed on day 6

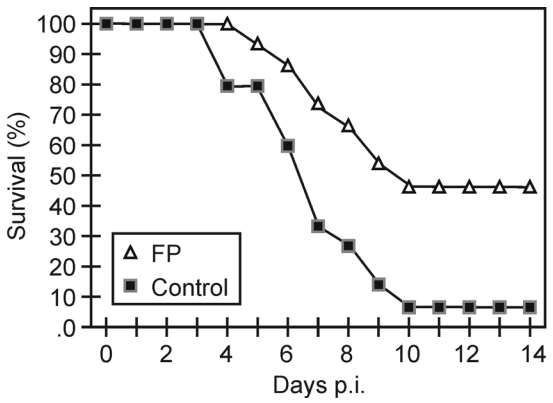

Fig. 5

Survival of mice immunized with FP and challenged with homologous influenza virus

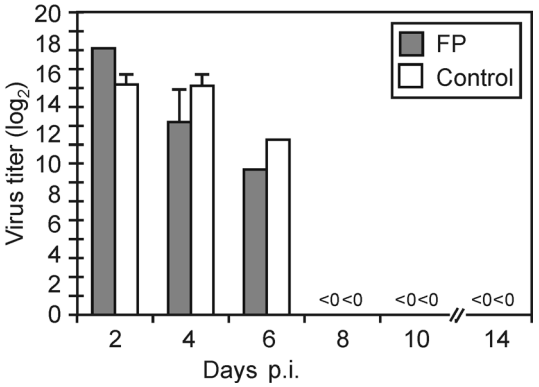

Fig. 6

Infectious virus in lungs of mice immunized with FP and challenged with homologous influenza virus

p.i. in immunized mice compared to that in non-immunized control (3,200). A clear elimination of infectious virus from lungs was observed by 2 days earlier in immunized mice compared to control mice (days 6 *vs.* 8) (Table 3).

Table 3. Detection of vRNA in lungs of mice immunized with FP and challenged with homologous virus

Immunogen	Days p.i.					
	2	4	6	8	10	14
FP	+	+/–	+/–	–	–	–
Control	+	+	+	+	–	–

The course of infection in mice immunized with FP and challenged with heterologous influenza virus

The cross-protective potential of immunization with FP was examined similarly as described above except A/Rostock, a heterologous virus was used for challenge.

First clinical symptoms appeared on day 6 and mice died until day 12 p.i. in both immunized and control groups. However, a higher survival was observed in immunized mice compared to non-immunized control (71% *vs.* 30%, p = 0.1534) (Fig. 7).

From these results we can conclude that although FP is a weaker immunogen than EHA2, following potentiation of its immunogenicity by conjugation with KLH and adjuvanting with inulin and FA, it confers at least a partial protection against influenza infection.

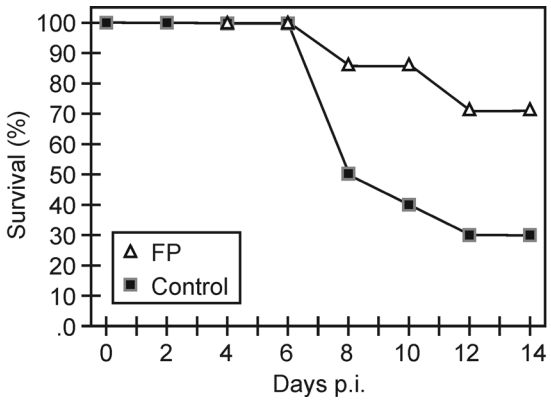

Fig. 7

Survival of mice immunized with FP and challenged with heterologous influenza virus

Discussion

HA2 gp plays an important role in the virus entry into the cell. In our previous studies we have shown that MAbs specific to HA2 gp, which are cross-reactive with different virus subtypes, inhibit the fusion activity of HA and reduce virus replication *in vitro* (Varečková *et al.*, 2002, 2003a,b; Stropkovská *et al.*, 2009). These MAbs, when passively applied, protected mice against lethal infection with IAVs of homologous or heterologous HA subtype (Gocník *et al.*, 2007; Janulíková, unpublished data).

In this study, we used two fragments of HA2 gp as immunogens, namely EHA2 and FP, the most conserved part of HA. Analysis of the specific antibody response induced by three immunization doses showed that EHA2 is a stronger immunogen than FP. The efficacy of *in vivo* protection against the lethal infection with 2 LD_{50} of homologous virus of H3 subtype was higher for EHA2 compared to FP (100% *vs* 45%). However, in cotrast to the EHA2 immunization, the FP immunization increased survival of mice challenged with homologous or heterologous virus to a similar extent.

These results are in accordance with previous data on protective immunity induced by immunization with HA2, which was expressed by recombinant vaccinia viruses (Gocník *et al.*, 2008) or *E. coli* (Bommakanti *et al.*, 2010). In this work, we purified EHA2 according to Chen *et al.* (1995). It was shown by crystalographic analysis that EHA2 purified in this way exhibits a low pH conformation (Chen *et al.*, 1999). The antibodies induced by such an immunogen were cross-reactive with different virus subtypes (Janulíková, unpublished data) and were cross-protective, as demonstrated by the above results.

We can conclude that both peptides, EHA2 and FP can be used as inducers of heterosubtypic immunity. After their insertion into a suitable vector their antigenicity and cross-protectivity could be significantly enhanced (Staneková *et al.*, in press) and widened. Based on our previous results, particular HA2 epitopes express different immunogenicity. In further protective studies, those of them which are more immunogenic and protective can be preferred (Staneková *et al.*, 2012).

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