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# Heterosubtypic protective immunity against influenza A virus induced by fusion peptide of the hemagglutinin in comparison to ectodomain of M2 protein

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**Summary. –** Several types of influenza vaccines are available, but due to the highly unpredictable variability of influenza virus surface antigens (hemagglutinin (HA) and neuraminidase) current vaccines are not sufficiently effective against broad spectrum of the influenza viruses. An innovative approach to extend the vaccine efficacy is based on the selection of conserved influenza proteins with a potential to induce inter-subtype protection against the influenza A viruses. A promising new candidate for the preparation of broadly protective vaccine may be a highly conserved N-terminal part of HA2 glycopolypeptide (HA2 gp) called fusion peptide. To study its capacity to induce a protective immune response, we immunized mice with the fusion peptide (aa 1–38 of HA2 gp). The protective ability of fusion peptide was compared with the ectodomain aa 2–23 of M2 protein (eM2) that is antigenically conserved and its immunogenic properties have already been well documented. Corresponding peptides (both derived from A/Mississippi/1/85 (H3N2) virus) were synthesized and conjugated to the keyhole limpet hemocyanin (KLH) and used for the immunization of mice. Both antigens induced a significant level of specific antibodies. Immunized mice were challenged with the lethal dose of homologous (H3N2) or heterologous A/PR/8/34 (H1N1) influenza A viruses. Immunization with the fusion peptide led to the 100% survival of mice infected with 1 $LD_{50}$ of homologous as well as heterologous virus. Survival rate decreased when infectious dose was raised to 2 $LD_{50}$. The immunization with eM2 induced effective cross-protection of mice infected even with 3 $LD_{50}$ of both challenge viruses. The lower, but still effective protection induced by the fusion peptide of HA2 gp suggested that besides ectodomain of M2, fusion peptide could also be considered as a part of cross-protective influenza vaccine. To our knowledge, this is the first report demonstrating that active immunization with the conjugated fusion peptide of HA2 gp provided the effective production of antibodies, what contributed to the cross-protection against influenza infection.

**Keywords:** influenza A virus; immunogenicity; cross-protection; fusion peptide of hemagglutinin; M2 protein

## Introduction

The influenza virus represents a highly contagious infectious agent whose unpredictable variability does not allow a reliable prophylaxis by the current seasonal vaccines. There is a need for the anti-influenza vaccine inducing a long-term cross-protection not only against seasonal drift variants, but also against occasionally emerging reassortant viruses with the pandemic potential. In recent years the pandemic danger appeared twice. Highly pathogenic H5N1 avian virus causing sporadic infections of humans since 1997 still represents a risk, since it may acquire an ability of transmission from human to human (Webster, 2006). Further threat is a new swine H1N1 (2009) virus with unpredictable evolution (Garten *et al*., 2009). Possibility of its mutation into the more virulent and dangerous form still

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**Abbreviations:** eM2 = ectodomain of M2 protein; KLH = keyhole limpet hemocyanin; HA = hemagglutinin; HA2 gp= HA2 glycopolypeptide; p.i. = post infection; PR8 = A/Puerto Rico/1/34 (H1N1); MISS = A/Mississippi/1/85 (H3N2)

persists (Hensley and Yewdell, 2009). The risk of emerging infections by influenza A viruses of such an HA subtype that did not occur in humans before, forces development of a new strategy for the vaccine development. A new approach consists of the selection of conserved influenza virus antigens that are able to induce heterosubtypic immunity, i.e. the immunity against influenza A viruses of various HA subtypes.

Most of the studies concern eM2 that is a part of proton channel protein playing an important role in the replication of influenza virus (Hay, 2000). The protective effect induced by eM2 have been described by several authors (Liu *et al.*, 2004; Fan *et al.*, 2004; Katz *et al.*, 2006; Tompkins *et al.*, 2007; De Filette *et al.*, 2008ab; Fiers *et al.*, 2009).

In this work, we focused on the conserved region of influenza virus HA, the fusion peptide that was selected as a promising inducer of protective immunity. Fusion peptide of HA2 gp is essential for the influenza infection (Cross *et al.*, 2009). In its native conformation, the peptide is embedded in the interspace between monomers of HA trimer. Under low pH, it is exposed and inserted into the target membrane, thus mediating the fusion of viral and endosomal membranes after virus endocytosis (Skehel and Wiley, 2000). Antibodies specific to HA stem comprising HA1 and HA2 glycopolypeptides inhibit the fusion of viral and endosomal membranes by preventing the conformation change of HA induced by low pH (Okuno *et al.*, 1993; Imai *et al.*, 1998; Vanlandschoot *et al.*, 1998; Throsby *et al.*, 2008; Sui *et al.*, 2009). Antibodies specific to the fusion peptide of HA2 gp were shown to reduce *in vitro* replication of influenza A viruses of various HA subtypes (Varečková *et al.*, 2003b; Lim *et al.*, 2008; Stropkovská *et al.*, 2009; Hashem *et al.*, 2010). These antibodies most probably inhibit the fusion of membranes by blocking an insertion of fusion peptide into the endosomal membrane (Varečková *et al.*, 2003a,b). Studies examining *in vivo* protection efficacy of monoclonal antibodies specific to the fusion peptide were published (Gocník *et al.*, 2007; Prabhu *et al.*, 2009), but no effective antiviral protection induced by active immunization with this peptide has been described till now.

In this work, the ability of HA2 fusion peptide to induce cross-protective immune response was compared with that of eM2.

## Materials and Methods

Viruses. A/Mississippi/1/85 (H3N2) – "MISS" and A/PR/8/34 (H1N1) – "PR8" were propagated in allantoic fluid of 10-day chicken embryos. Stocks of viruses were stored at -70°C. Viruses were obtained from the collection of viruses of Institute of Virology, Bratislava, Slovak Republic.

Mice. Six week-old female BALB/c mice (Faculty of Medicine, Masaryk University, Brno, Czech Republic) were used in animal experiments. In all experiments presented in this paper, animals were treated according to the European Union standards and the fundamental ethical principles including animal welfare requirements were respected.

Peptides and peptide conjugate.Synthetic peptides eM2 (H3 subtype) (93.94% purity) containing 23 aa (SLLTE VETPIRNEWGSRSNDSSD) with relative molecular mass Mr = 2,592.74 and the fusion peptide HA2 (H3 subtype) (94.8% purity) containing N-terminal 38 aa (GIFGAIAGFIENGWEGM VDGWYGFRHQNSEGTGQAADL) with relative molecular mass Mr = 4,059.44 were synthesized and supplied by ProImmune (USA). Consensus aa sequences of H3 subtype isolated in 1985 represented by A/Memphis/2/1985 (H3N2) (Acc. Nos. ABD61779 and ABD61777 for M2 and HA proteins, respectively) were used as a pattern for constructing synthetic peptides. Sequence of eM2 protein of H3 subtype was modified by substitution of C17, C19 to S17, S19, respectively, to avoid formation of disulphide bonds in the peptide.

Conjugation of synthetic peptides with KLH. Synthetic eM2 or fusion peptides were conjugated with KLH (Sigma) using glutaraldehyde. Briefly, synthetic eM2 or fusion peptide in PBS (2 mg/ml) were mixed with 2 mg/ml of KLH in PBS. Glutaraldehyde was added to the reaction mixture to a final concentration 2.5% and the mixture was incubated for 50 mins at room temperature. The reaction was stopped by addition of glycine (0.03 mol/l) and the mixture was dialyzed against PBS at 4 °C overnight. Aliquots of the conjugated peptides were stored at -20°C.

Immunization. Conjugated eM2 or fusion peptide of HA2 gp (30 μg/mouse/dose) emulsified in Freund´s adjuvant (1:2, v/v) were intraperitoneally administered to mice. Three doses of the antigen were given in 14 day-intervals: first dose with the complete and the second and third dose with incomplete Freund´s adjuvant. Blood samples were taken before the immunization and 14 days after each injection.

Infection of mice. Two weeks after the last injection, the mice were intranasally inoculated with the lethal dose of homologous MISS (H3N2) or heterologous PR8 (H1N1) virus in a volume of 40 μl. Survival and body weight of mice were recorded daily for 14 days post infection (p.i.). One experimental group comprised 8 animals unless stated otherwise. Results were evaluated statistically using Fisher's exact test. Differences were considered to be significant for $P \leq 0.05$.

ELISA. Fusion peptide (500 ng/100 μl) or eM2 (100 ng/100 μl) were used as antigens and adsorbed overnight at 4°C on 96-well plates. ELISA binding test was performed according to Varečková *et al.* (2003a). Titer of antibodies in serum was calculated as the reciprocal serum dilution at the point, where the regression line drawn through the three points of the titration curve crosses the cut-off value. Cut-off was estimated as the average value of 5 negative control samples plus 3-times standard deviation.

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>eM2: M2_2..24
>          2......10........20..24
H3(1985)SLLTEVETPIRNEWGCRCNDSSD
H1 PR8   SLLTEVETPIRNEWGCRCNGSSD

>Fusion peptide: HA2_1..38
>          1.......10........20.........30......38
H3(1985)GIFGAIAGFIENGWEGMVDGWYGFRHQNSEGTGQAADL
H1 PR8   GLFGAIAGFIEGGWTGMIDGWYGYHHQNEQGSGYAADQ
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**Fig.1**

**Comparison of amino acid sequences of eM2 or HA2 fusion peptide between H1 and H3 subtypes**

Amino acid sequence of the synthetic peptide eM2 (H3) aligned with M2 protein of A/Memphis/2/1985 (H3N2) (Acc. No. ABD61779) and PR8 (H1N1) (Acc. No. ABD77677) and amini acid sequence of the synthetic fusion peptide HA2 (H3) aligned with HA2 protein of A/Memphis/2/1985 (H3N2)) (Acc. No. ABD61777) and PR8 (H1N1) (Acc. No. ABD77675). The sequence of eM2 (H3) matches to A/Memphis/2/1985 (H3N2) except for the substitutions C17S and C19S. The sequence of the fusion peptide HA2 (H3) matches to A/Memphis/2/1985 (H3N2). Differences among synthetic peptides, A/Memphis/2/1985, and PR8 are shown.

## Results

To study the immune cross-protection against influenza infection with virus of H3 (MISS) and H1 (PR8) subtypes induced by either eM2 (H3) (aa 2–24) or fusion peptide (aa 1–38 of HA2), we selected peptide sequences derived from influenza virus of H3 subtype found in the NCBI Influenza Virus Sequence Database (Fig. 1). Since A/Mississippi/1/85 (H3N2) sequence of neither eM2 nor HA2 has been available at the GenBank Database, consensus aa sequences of H3 subtyte isolated in 1985, represented by A/Memphis/2/1985 (H3N2) (Acc. Nos. ABD61779 and ABD61777 for M2 and HA proteins, respectively) were used as pattern for constructing synthetic peptides. eM2 amino acid sequences of viruses of H1 and H3 subtypes selected from the database were conserved except one aa in position 21. The fusion peptide aa 1-38 displayed higher variability than eM2. While the region between aa 3–11 is fully conserved within all HA subtypes, there were several changes differing H3 and H1 subtypes in positions from aa 12–38 (Fig. 1).

*Antibody response to the fusion peptide (aa 1–38 of HA2 gp)*

A protective potential of antibodies induced with the conjugated fusion peptide was studied in mice immunized with the synthetic fusion peptide corresponding to the N-terminal part of HA2 gp of hemagglutinin of H3 subtype. A low level of antibodies was detectable after the first dose, which significantly increased after the second dose. The highest titer of antibodies specific to the fusion protein was obtained after the third immunization dose and reached the value of 26,500 (Table 1). Then, immunized mice were challenged with the influenza viruses of H1 or H3 subtypes and clinical symptoms of the disease were monitored.

*Cross-protection of mice immunized with the fusion peptide*

Mice immunized with the HA2 fusion peptide conjugated with KLH (n = 6) and control PBS-treated mice (n = 6) were infected with 1 $LD_{50}$ ( i.e. 5 x $10^3$ PFU) of mouse-adapted strain MISS of homologous subtype. Immunization with the conjugated fusion peptide improved survival rate of mice to 100%, while 50% of the control mice died (P = 0.0909) (Fig. 2a).

In the further experiment, infectious dose of MISS virus was increased to 2 $LD_{50}$ to find out the corresponding level of antiviral protection in immunized mice. Survival rate of immunized mice (n = 15) under these conditions was 46%, while all mice in control group treated with PBS died ($P \leq 0.05$) (Fig. 2c). Therefore, in cross-protective experiments the infectious dose 1 $LD_{50}$ (i.e. 65 PFU) of heterologous challenge virus was used.

The survival of immunized mice infected with the heterologous virus PR8 (H1N1) improved to 87.5% from 37.5% in control group (P = 0.0594) (Fig. 2b). The course of infection was milder in immunized than in control animals as evaluated in relation to the body weight change and other clinical symptoms like low activity and appearance of scrubby fur (Fig. 2d). It can be therefore concluded that the immunization of mice with the fusion peptide of HA2 gp conferred a significant protection against the

**Table 1. Titers of specific antibodies in sera of mice immunized with the HA2 fusion or eM2 peptides**

| Immunogen | ELISA antibody titer | | |
|---|---|---|---|
| | 1st dose | 2nd dose | 3rd dose |
| Fusion peptide | 199 | 25,700 | 26,500 |
| eM2 | 528 | 10,800 | 28,800 |

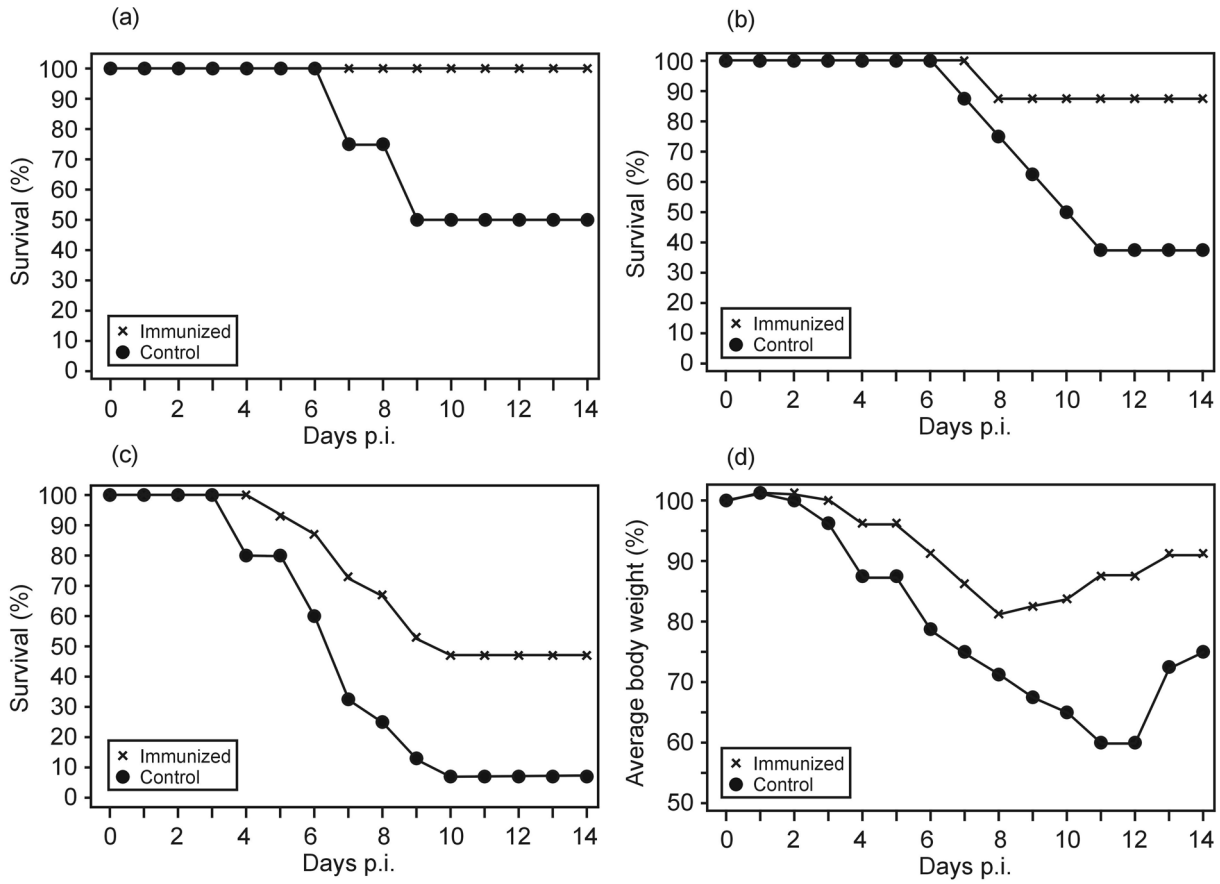


**Fig. 2**

**Effect of immunization with the fusion peptide on the course of influenza infection in mice**

Survival of BALB/c mice immunized with fusion peptide and then infected with MISS virus (H3N2) 1 $LD_{50}$ (a), 2 $LD_{50}$ (c) or infected with PR8 virus (H1N1) 1 $LD_{50}$ (b). Changes in body weight of mice infected with PR8 (H1N1) virus (d).

lethal infection with homologous as well as heterologous influenza virus.

*Antibody response to the eM2 peptide (aa 2–24) BALB/c*

mice were immunized with three doses of eM2 peptide conjugated to KLH. Levels of eM2-specific antibodies linearly increased after the first and second immunization dose. The third injection of peptide increased the antibody level to the titer of 28,800 (Table 1).

*Cross-protection of mice immunized with eM2*

To study the protection induced with conjugate KLH-eM2, the mice were immunized with three doses of conjugate and infected with homologous MISS (H3N2) or heterologous PR8 (H1N1) virus. All immunized mice survived the infection with both challenge viruses even with the dose 3 $LD_{50}$ ($P \leq 0.05$) (Fig. 3a, b). The immunized mice exhibited milder clinical symptoms and slighter body weight loss than the control mice (Fig. 3c, d). After the infection with PR8, no survival of mice in control group ($P \leq 0.05$) was observed, whilst in the control group infected with the same infectious dose of MISS virus one mouse survived ($P \leq 0.05$) (Fig. 3b). The survival as well as severity of clinical symptoms corresponded to the virulence of these two viruses described in our previous experiments evaluating MISS virus as medium virulent and PR8 virus as highly virulent (Fislová *et al.*, 2009).

## Discussion

There is a trend to broaden the protection efficacy of current anti-influenza vaccines in order to avoid the need of yearly vaccination against the seasonal influenza strains.

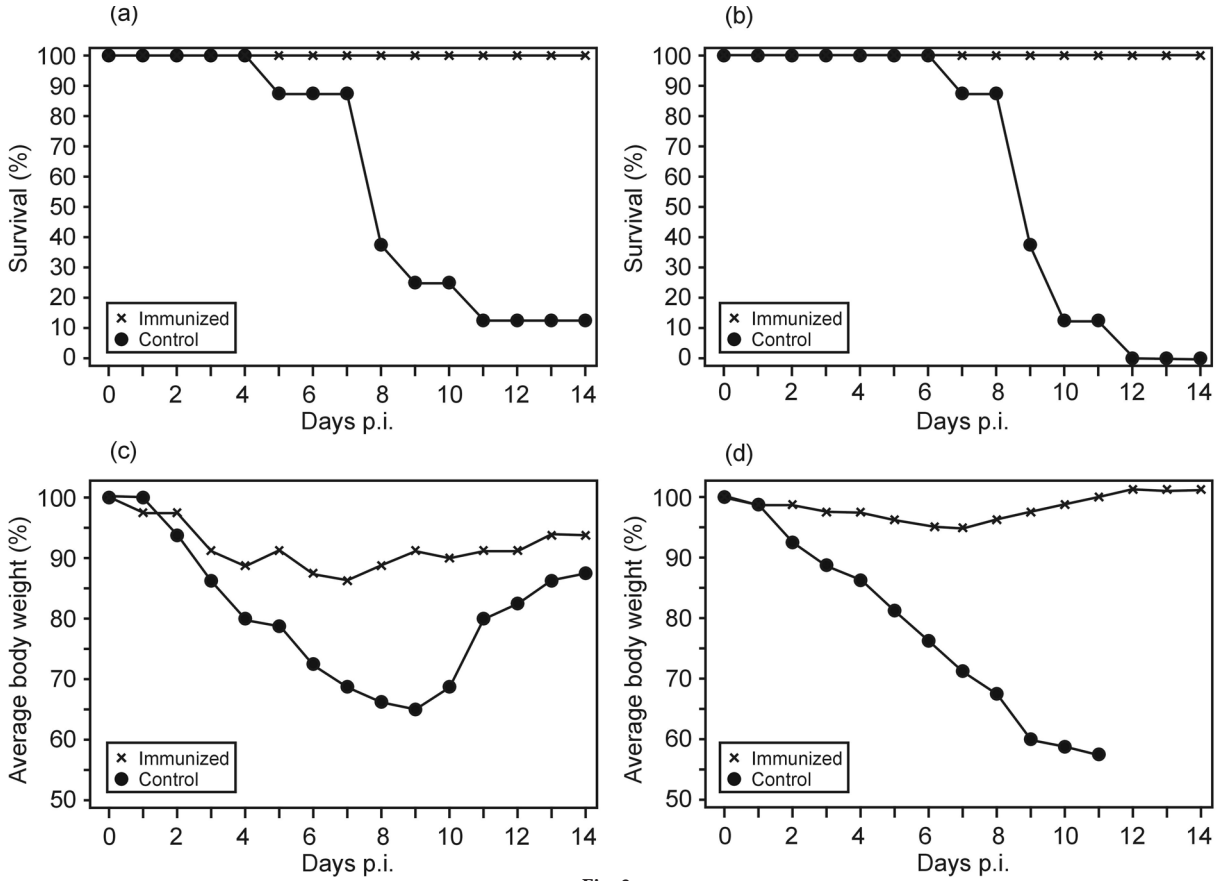


**Fig. 3**

**Effect of immunization with eM2 on the course of influenza infection in mice**

Survival (a, b) and changes in body weight (c, d) of BALB/c mice immunized with eM2 and then infected with 3 $LD_{50}$ of MISS (H3N2) (a, c) or 3 $LD_{50}$ of PR8 (H1N1) (b, d).

One approach to get closer to the cross-protective vaccine is to utilize conserved antigens capable of inducing heterosubtypic immunity. In this study, we focused on the N-terminal aa 1–38 of HA2 gp of influenza HA comprising the fusion peptide as a potential vaccine candidate (Cross *et al.*, 2009). The fusion peptide represents the conserved part of HA comprising aa 3–11 that are fully conserved among all HA subtypes of influenza A virus (Nobusawa *et al.*, 1991; Gerhard *et al.*, 2006). Until now, no effective protection utilizing fusion peptide of HA2 gp as an immunogen capable of eliciting polyclonal antibody response has been described. Horvath *et al.* (1998) immunized mice with the fusion peptide comprising N-terminal aa 1–13 of HA2 gp, but no effective antibody response has been achieved and no protection studies have been done. We previously proved that monoclonal antibody CF2 recognizing N-terminal aa 1–38 of HA2 gp is able to inhibit the hemolysis mediated by the influenza virus, fusion of cells expressing HA on their surfaces as well as virus-liposome fusion monitored by the resonance energy transfer method (Varečková *et al.*, 2003a). This antibody reduces the replication of influenza viruses of different HA subtypes as determined by a plaque assay on MDCK cells (Varečková *et al.*, 2003b; Stropkovská *et al.*, 2009). Monoclonal antibody CF2 confers the protection against lethal infection of mice within a given HA subtype (Gocník *et al.*, 2007). Based on these results, we supposed that the fusion peptide of HA2 gp itself could induce a protective antibody response against different subtypes of HA. In contrast to Horvath *et al.* (1998), we conjugated the fusion peptide aa 1–38 to KLH and immunized mice with three doses, what resulted in a significant antibody response in mice. Protective experiments revealed that the fusion peptide was able to induce cross-protective immune response, most probably mediated by specific antibodies.

The cross-protective potential of eM2 protein was used in this study for a comparison to the ability of the HA2 fusion peptide to induce antibody response and antiviral protection (Katz *et al.*, 2006; Fiers *et al.*, 2009).

The comparison of two peptides as immunogens showed that eM2 peptide is much more effective inducer of protec-

tive immunity than the fusion peptide. This conclusion was supported by the observation that animals immunized with eM2 were protected against the higher dose of infectious virus (at least 3 $LD_{50}$) in comparison to the mice immunized with the fusion peptide, which were protected against infection up to 2 $LD_{50}$. We believe that the protection induced by fusion peptide up to 2 $LD_{50}$ is sufficient for the vaccination purposes. Therefore, we suppose that the fusion peptide as well as eM2 could be useful for the induction of heterosubtypic protective immunity.

This report represents the first description of effective *in vivo* cross-protection induced by active immunization with the fusion peptide. Based on the fact that fusion peptide of HA2 gp is conserved across influenza viruses and that antibodies against this peptide may moderate the virus infection, a future vaccine design should take into account the enrichment of vaccine with the fusion peptide in properly immunogenic form. It would be of interest to see whether simultaneous application of eM2 and fusion peptides will enhance the cross-protection against influenza infection with viruses of various HA subtypes.

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