Evaluation of anti-influenza efficiency of polyclonal IgG antibodies specific to the ectodomain of M2 protein of influenza A virus by passive immunization of mice

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Summary. – We attempted to quantify the protective potential of polyclonal IgG antibodies specific to the ectodomain of M2 protein (eM2) of influenza A virus (IAV) against lethal influenza infection of mice. For this purpose, eM2 conjugated with keyhole limpet hemocyanin (KLH) or KLH alone were administered with Freund's adjuvant intraperitoneally (i.p.) to BALB/c mice. IgG antibodies specific to the KLH-eM2 conjugate (anti-KLH-eM2 IgGs) and KLH (anti-KLH IgGs), respectively, were purified from ascitic fluids. Analysis of the preparation of anti-KLH-eM2 IgGs by ELISA revealed that it contained about 25% of anti-eM2 IgGs and 75% of anti-KLH IgGs. Taking into account this finding mice were passively immunized by intravenous route with 320, 160, 80, and 40 μg of anti-eM2 IgGs per mouse, respectively, while 320 μg of anti-KLH IgGs were used in control. Following subsequent infection with 3 LD_{50} IAV the survival of mice was determined. An absolute protection (100% survival) was obtained with 320 μg of anti-eM2 IgGs, and a relatively strong significant protection (~80% survival, p = 0.024) with 160 μg. The amount 160 μg of IgGs represents approx. 100 μg IgGs per 1 ml of blood.

Keywords: influenza; M2 protein; eM2-specific IgG concentration; protection

Introduction

There are current efforts to avoid every year vaccination against influenza with a vaccine containing seasonally actualized hemaglutinin (HA) and neuraminidase (NA) antigens by use of a vaccine based on a conserved antigen(s). Such a vaccine should evoke a long-lasting immune response efficiently suppressing the infection with various antigenic variants or even subtypes. The main candidate molecule is the M2 protein of IAV, particularly its 23 aa-long ectodomain

(eM2) that is characteristic by an outstanding antigenic conservativity and is abundantly expressed in the membrane of infected cells (Neirynck *et al.*, 1999; Palese and Garcia-Sastre, 2002; Lamb, 1985; Zebedee and Lamb, 1988). Nevertheless, its immunogenicity during the natural influenza infection is very weak and short-term (Feng *et al.*, 2006). These inconvenient properties of eM2 represent the main issues to be solved. A number of various approaches for preparation of eM2-based vaccine focused on increasing its immunogenicity have been published (Slepuskin *et al.*, 1995; Neirynck *et al.*, 1999; Wynne *et al.*, 1999; Okuda *et al.*, 2001; Liu *et al.*, 2004; Ben-Yedidia and Arnon, 2005; Huleatt *et al.*, 2008; DeFilette *et al.*, 2008). The mechanism of the eM2-associated biological action was described as the antibody-dependent cell-mediated cytotoxicity (ADCC), in which NK cells with their low-affinity Fc gamma receptors bind IgG antibodies already bound to the M2 protein expressed on the surface of infected cells and mediate their lysis (Jagerlehner *et al.*, 2004). This hypothesis explains why anti-eM2 IgGs, when present in sufficiently high

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concentration, only attenuate the infection while neutralizing antibodies to HA that directly inhibit the binding of virus to the cell receptor provide a very effective instant protection.

Despite the lower efficiency of eM2-specific antibodies, the idea of utilization of eM2 in future influenza vaccine remains actual and promising as this molecule has a potential to elicit a a broad cross-protective response (Sui *et al.*, 2010; Stanekova *et al.*, 2011). In addition to the eM2 immunogenicity, the concentration of eM2-specific antibodies remains an important characteristic to be followed that can also serve as an indicator of effectivity of particular eM2-based vaccine preparation.

In this study, we atttempted to quantify the protective potential of polyclonal IgG antibodies specific to eM2 IAV against lethal influenza infection of mice by determining the dependence of survival of infected mice on actual concentration of these antbodies in their blood following passive immunization.

Materials and Methods

Virus. A/Mississippi/1/85 (H3N2) was propagated in allantoic fluid of 10-day chicken embryos and stored at -70°C.

Mice. Six-week-old female BALB/c mice purchased from the Faculty of Medicine, Masaryk University, Brno, Czech Republic, were used. The animals were treated according to the European Union standards and fundamental ethical principles including animal welfare requirements.

eM2 peptide and KLH-eM2 conjugate. A 23-aa-long synthetic eM2 peptide of IAV (H3 subtype) of the sequence SLLTEVET-PIRNEWGSRSNDSSD, M_r of 2,592.74 and 93.94% purity was purchased from ProImmune (USA). The peptide contained substitutions C17S and C19S to avoid formation of disulphide bonds in

the peptide. The conjugation of eM2 with KLH (Sigma) was done using glutaraldehyde as described by Staneková *et al.* (2011).

Immunization of mice. BALB/c mice were immunized i.p. with three doses of KLH-eM2 (30 μg of eM2 per mouse) or KLH (30 μg pre mouse), respectively, supplemented with Freund's adjuvant, in 14 day intervals Staneková *et al.* (2011).

Polyclonal antibodies were purified from ascitic fluids by affinity chromatography on Protein A-Sepharose columns (Ey *et al.*, 1978).

Passive immunization of mice. BALB/c mice – 5 animals per group – were administered i.v. anti-eM2 IgGs in 200 μl doses of 320, 160, 80, and 40 μg per mouse, respectively, while control mice obtained 320 μg of anti-KLH IgGs and 200 μl of PBS, respectively.

Infection of mice. Two hrs after passive immunization the mice were intranasally (i.n.) inoculated with 3 LD_{50} of A/Mississippi/1/85 (H3N2) in 40 μl. Survival of mice was recorded daily for 14 days. Statistical significance of survival was evaluated by Fisher exact test.

ELISA. eM2- or KLH-specific antibodies were assayed by a binding test according to Varečková *et al.* (2003a). Wells of 96-well plates were coated overnight with 100 ng of eM2 or KLH as antigens in 100 μl at 4°C. The antibody titer was calculated as the reciprocal of sample dilution at the point where the regression line drawn through the titration curve crossed the cut-off line. The latter value was the mean from 5 negative control samples plus 3 SD.

Results and Discussion

Antibody response in mice to immunization with eM2

In this work, we used a simple model of the eM2-KLH conjugate as immunogen supplemented with the Fre-
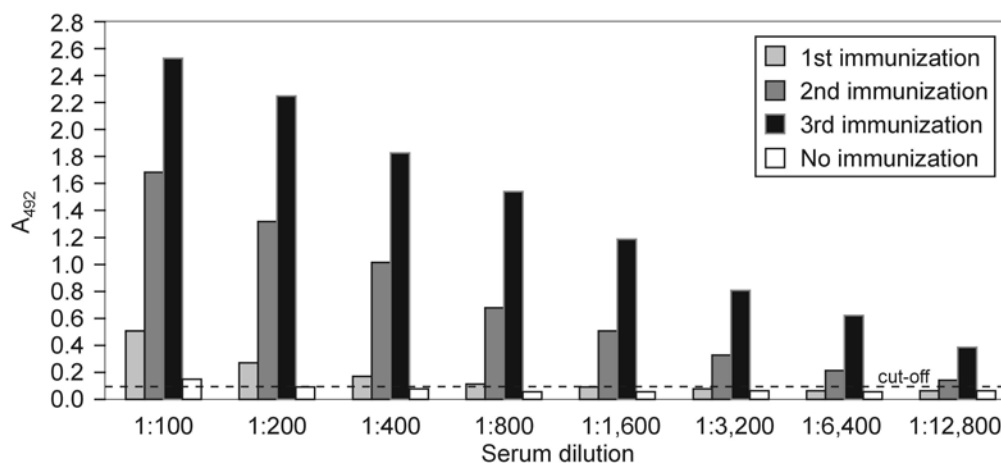

Fig. 1

Antibody response in mice to immunization with KLH-eM2

Mice were i.p. immunized with 3 doses of KLH-eM2 and their sera were assayed for eM2-specific IgG antibodies by ELISA.
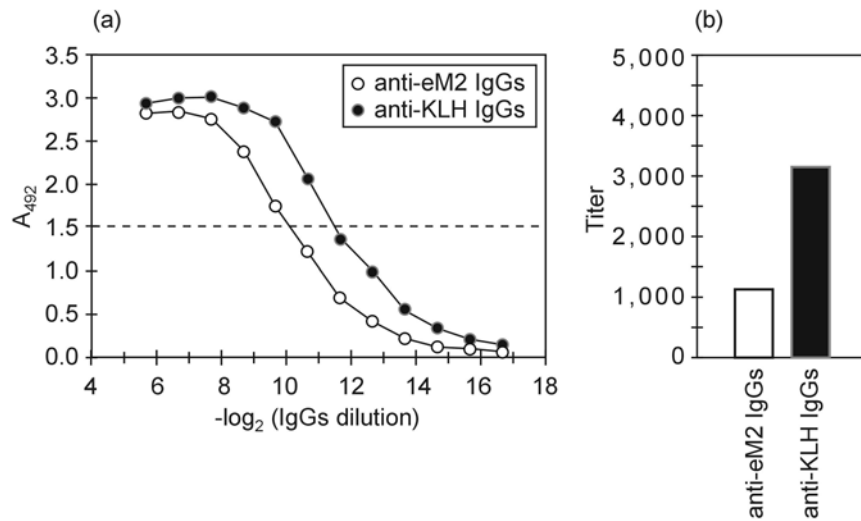
Fig. 2

Proportions of eM2- and KLH-specific antibodies in IgGs purified from ascitic fluid of mice immunized with KLH-eM2

Titration curves of anti-eM2 and anti-KLH IgGs (a) and titers of anti-eM2 and anti-KLH IgGs at A_{492} of 1.5 (b) in ELISA.

und's adjuvant to induce an antibody response in mice. Control mice were given KLH alone. Following three immunizations the majority of mice developed ascites due to administration of the adjuvant. The antibody response to eM2 gradually increased, corresponding to serum titers of 528, 10,800, and 28,800, respectively (Fig. 1). Ascitic fluids obtained after the last immunization served for purification of polyclonal IgG antibodies.

Proportions of eM2- and KLH-specific antibodies in IgGs purified from ascitic fluid from mice immunized with KLH-eM2

Proportions of IgG antibodies specific to eM2 and KLH, present in IgGs purified from mice immunized with KLH-eM2, were assayed by ELISA (Fig. 2). The distance of titration curves at A_{492} of 1.5 was estimated at 1.49 $\log_2$ units and corresponding titers of anti-eM2 and anti-KLH IgGs were 1120 and 3149, respectively. This means that provided equal numbers of accessible eM2 epitopes specific to anti-eM2 IgGs and KLH epitopes specific to anti-KLH IgGs adsorbed onto respective wells, the ratio of anti-eM2 and anti-KLH IgGs was approximately 1:3.

Effective anti-eM2 IgG concentration required for the protection to influenza infection

Taking into account the anti-eM2 IgGs content of the anti-KLH-eM2 IgGs purificate, groups of mice were given i.v. 320, 160, 80, and 40 µg of anti-eM2 IgGs per animal. Control mice were administered 320 µg of anti-KLH IgGs

and 200 µl of PBS, respectively. Two hrs later the mice were infected with 3 LD_{50} IAV and observed for survival. An absolute protection (100% survival) was obtained with 320 µg, a relatively strong significant protection (~80% survival) with 160 µg ($p = 0.024$), and a weak protection (~20% survival) with 80 and 40 µg of anti-eM2 IgGs (Fig. 3). Control mice scored a 100% mortality.

Estimating total blood volume in mouse at 1.5 ml, the applied doses 320 µg and 160 µg of anti-eM2 IgGs resulting in significant protection corresponded to the concentrations of 213 and 107 µg/ml anti-eM2 IgGs, respectively, in the blood.

These results roughly agree with those of Fu *et al.* (2009), who also evaluated the anti-eM2 protective response, however, by use of MAbs. They found that two of four tested MAbs at doses of 0.2–2.0 mg per mouse ensured a high survival, while 20 µg resulted in a low survival. Another group of authors (Beerli *et al.*, 2009) found that a most effective anti-eM2 MAb exhibited a fair protection against infection with 4 LD_{50} of virus at a dose of 20 µg per mouse, but only a weak one at a dose of 6 µg. Such a high efficiency of this MAb as compared with our observations as well as those of Fu *et al.* (2009) can be most probably ascribed to a high affinity ($K_d = 4$ nmol/l) of that particular MAb.

In conclusion, we assume that results of this study contribute to a recently accepted presumption that the eM2 molecule can be an effective and cross-protective immunogen. Its immunogenicity can be enhanced by applying it in appropriate form and/or with an optimally selected adjuvant. Its cross-reactivity can be ensured mainly by polyclonal character of the resulting antibody response. Moreover, the
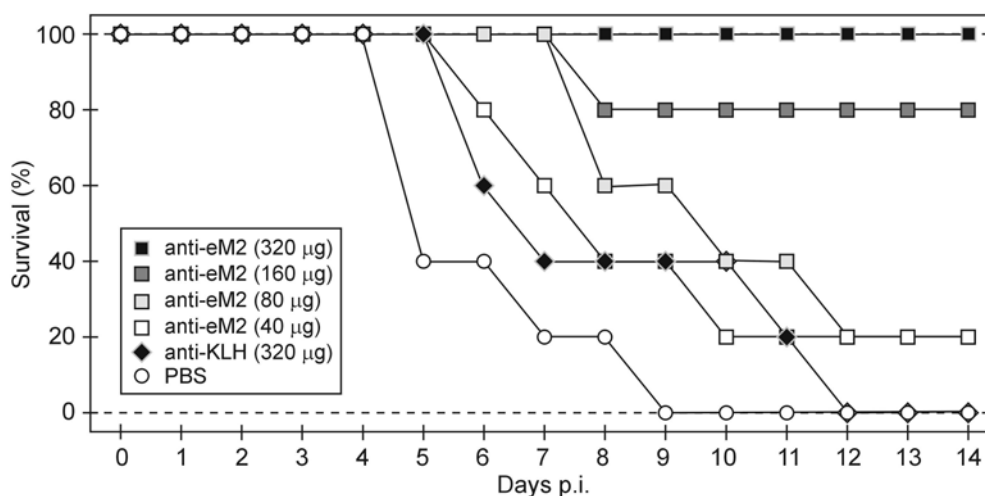
Fig. 3

Survival of mice passively immunized with purified anti-KLH-eM2 IgGs and infected with IAV

In immunization, the purified anti-KLH-eM2 IgGs were applied in amounts corresponding to 320, 160, 80, and 40 µg of anti-eM2 IgGs. Control mice obtained 320 µg of purified anti-KLH IgGs and PBS, respectively.

latter may contribute to the prevention of the appearance of antibody-escape mutants during natural infection (Zharikova *et al.*, 2005).

References

Beerli RR, Bauer M, Schmitz N, Buser RB, Gwerder M, Muntwiler S, Renner WA, Saudan P, Bachmann MF (2009): Prophylactic and therapeutic activity of fully human monoclonal antibodies directed against influenza A M2 protein. Virol. J. 6, 224. doi.org/10.1186/1743–422X–6–224

Ben-Yedidia T, Arnon R (2006): Flagella as a Platform for Epitope–Based Vaccines. Isr. Med. Assoc. J. 8, 316–318 8

De Filette M, Martens W, Roose K, Deroo T, Vervalle F, Bentahir M, Vandekerckhove J, Fiers W, Saelens X (2008): An influenza A vaccine based on tetrameric ectodomain of matrix protein 2. J. Biol. Chem. 283, 11382–11387. doi.org/10.1074/jbc.M800650200

Ey PL, Prowse SJ, Jenkin CR (1978): Isolation of pure IgG1, IgG2a and IgG2b immunoglobulins from mouse serum using Protein A-Sepharose. Immunochemistry 15, 429–436. doi.org/10.1016/0161–5890(78)90070–6

Feng J, Zhang M, Mozdzanowska K, Zharikova D, Hoff H, Wunner W, Couch RB, Gerhard W (2006): Influenza A virus infection engenders a poor antibody response against the ectodomain of matrix protein 2. Virol. J. 3, 102. doi.org/10.1186/1743–422X–3–102

Fu TM, Freed DC, Horton MS, Fan J, Citron MP, Joyce JG, Garsky VM, Casimiro DR, Zhao Q, Shiver JW, Liang X (2009): Characterizations of four monoclonal antibodies against M2 protein ectodomain of influenza A virus. Virology 385, 218–226. doi.org/10.1016/j.virol.2008.11.035

Huleatt JW, Nakaar V, Desai P, Huang Y, Hewitt D, Jacobs A, Tang J, Mcdonald W, Song L, Evans RK, Umlauf S, Tussey L, Powell TJ (2008): Potent immunogenicity and efficacy of a universal influenza vaccine candidate comprising a recombinant fusion protein linking influenza M2e to the TLR5 ligand flagellin. Vaccine 26, 201–214. doi.org/10.1016/j.vaccine.2007.10.062

Jegerlehner A, Schmitz N, Storni T, Bachmann MF (2004): Influenza A vaccine based on the extracellular domain of M2: weak protection mediated via antibodydependent NK cell activity. J. Immunol. 172, 5598–5605.

Lamb RA, Zebedee SL, Richardson CD (1985): Influenza virus M2 protein is an integral membrane protein expressed on the infected-cell surface. Cell 40, 627–633. doi.org/10.1016/0092–8674(85)90211–9

Liu W, Peng Z, Liu Z, Lu Y, Ding J, Chen YH (2004): High epitope density in a single recombinant protein molecule of the extracellular domain of influenza A virus M2 protein significantly enhances protective immunity. Vaccine 23, 366–371. doi.org/10.1016/j.vaccine.2004.05.028

Neirynck S, Deroo T, Saelens X, Vanlandschoot P, Jou WM, Fiers W (1999): A universal influenza A vaccine based on the extracellular domain of the M2 protein. Nat. Med. 5, 1157–1163. doi.org/10.1038/13484

Okuda K, Ihata A, Watabe S, Okada E, Yamakawa T, Hamajima K, Yang J, Ishii N, Nakazawa M, Okuda K, Ohnari K, Nakajima K, Xin KQ (2001): Protective immunity against influ-

enza A virus induced by immunization with DNA plasmid containing influenza M gene. Vaccine 19, 3681–3691. doi. org/10.1016/S0264–410X(01)00078–0

Palese P, Garcia-Sastre A (2002): Influenza vaccines: present and future. J. Clin. Invest. 110, 9–13.

Slepushkin VA, Katz JM, Black RA, Gamble WC, Rota PA, Cox NJ (1995): Protection of mice against influenza A virus challenge by vaccination with baculovirusexpressed M2 protein. Vaccine 13, 1399–1402. doi.org/10.1016/0264–410X(95)92777–Y

Staneková Z, Király J, Stropkovská A, Mikušková T, Mucha V, Kostolanský F, Varečková E (2011): Heterosubtypic protective immunity against influenza A virus induced by fusion peptide of the hemagglutinin in comparison to ectodomain of M2 protein. Acta Virol. 55, 61–67. doi. org/10.4149/av-2011-01-61

Sui Z, Chen Q, Wu R, Zhang H, Zheng M, Wang H, Chen Z (2010): Cross-protection against influenza virus infection by intranasal administration of M2-based vaccine with chitosan as an adjuvant. Arch. Virol. 155, 535–544. doi. org/10.1007/s00705–010–0621–4

Varečková E, Mucha V, Wharton SA, Kostolanský F (2003a): Inhibition of fusion activity of influenza A hemagglutinin mediated by HA2–specific monoclonal antibodies. Arch. Virol. 148, 469–486. doi.org/10.1007/s00705–002–0932–1

Wynne SA, Crowther RA, Leslie AG (1999): The crystal structure of the human hepatitis B virus capsid. Mol. Cell. 3, 771–780. doi.org/10.1016/S1097–2765(01)80009–5

Zebedee SL, Lamb RA (1988): Influenza A virus M2 protein: monoclonal antibody restriction of virus growth and detection of M2 in virions. J. Virol. 62, 2762–2772.

Zharikova D, Mozdzanowska K, Feng J, Zhang M, Gerhard W (2005): Influenza type A virus escape mutants emerge in vivo in the presence of antibodies to the ectodomain of matrix protein 2. J. Virol. 79, 6644–6654.