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

J. KIRÁLY, E. VAREČKOVÁ, V. MUCHA, F. KOSTOLANSKÝ

Department of Orthomyxoviruses, Institute of Virology, Slovak Academy of Sciences, Dúbravská cesta 9, 845 05 Bratislava, Slovak Republic

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 LD50 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 hemagglutinin (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

Corresponding author. E-mail: virufkos@savba.sk; fax: +421-2-54114284.

Abbreviations: IAV = influenza A virus; eM2 = ectodomain of M2 protein; HA = hemagglutinin; anti-KLH-eM2 IgGs = IgG antibodies to KLH-eM2; anti-KLH IgGs = IgG antibodies to KLH; i.n. = intranasally; i.p. = intraperitoneally; i.v. = intravenously; KLH = keyhole limpet hemocyanin; MAb(s) = monoclonal antibody(ies); NA = neuraminidase
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 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 effectiveness of particular eM2-based vaccine preparation.

In this study, we attempted 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 antibodies 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, Mr 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 Stanekova 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 per mouse), respectively, supplemented with Freund’s adjuvant, in 14 day intervals Stanekova et al. (2011).

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

**Materials and Methods**

**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-
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 of 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 A492 of 1.5 was estimated at 1.49 log2 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 LD50 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 LD50 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 (Kd = 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
latter may contribute to the prevention of the appearance of antibody-escape mutants during natural infection (Zharikova et al., 2005).

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