Development and ELISA-based detection of anti-M2e IgY antibodies using an encoding plasmid for M2e-Hsp70 C-terminal gene

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Abstract:

BACKGROUND: The use of IgYs in a variety of methods in different areas of research, diagnostics, medical application and biotechnology should be considered widely. **OBJECTIVES:** Development of antibodies against extra cellular domain of influenza M2 (M2e) protein in egg yolk of laying hens. **METHODS:** A Fusion construct harboring C-terminal of bovine heat shock protein 70 (Hsp70) and influenza M2e coding genes was injected to laying hens. Serum and egg yolk antibodies were screened for the presence of anti-M2e antibodies by indirect enzyme-linked immunosorbent assay (ELISA). **RESULTS:** Anti-M2e antibodies were detected in egg yolks and sera of injected hens from 13 and 7 days post injection (PI), with the peak titer detected on 41 and 35 days PI, respectively. **CONCLUSIONS:** Anti-M2e IgY titers could be an index for expression potential of pcDNA3.1-M2e-HspC-terminal construct in laying hens. This construct could be considered as a promising tool in production of anti-M2e polyclonal, monospecific IgY antibodies. Such anti-M2e antibodies could be exploited for influenza diagnostic and therapeutic measures.

Introduction

Influenza is a segmented, negative stranded and enveloped RNA virus of the Orthomyxoviridae family (Nayak et al., 2009). Influenza A viruses can infect both human and a wide range of animals e.g. pigs, horses, wild mammals, and birds (Neumann and Kawaoka, 2006; Jourdain et al., 2010). There is a potential threat of transmission of some subtypes of avian influenza viruses e.g. H5N1, H7N7 and H9N2 into humans (Perdue and Swayne, 2005; Ebrahimi, and Tebianian, 2010a). The spread of endemic influenza infections in poultry flocks around the world and the consequent risk for occurrence of influenza pandemics and epidemics highlights the necessity to take some reliable prophylactic, therapeutic and immunodiagnostic measures suitable for different influenza virus strains.

One of the most highly conserved proteins in influenza virus is a transmembrane integral protein, which is called M2 .The 23-amino acid extracellular domain of M2, known as M2e, presents on the surface of influenza virus alongside Hemagglutinin and Neuraminidase (Nemchinov and Natilla, 2007). Unlike HA and NA, M2e is highly conserved among different subtypes of influenza A viruses. This makes M2e as an attractive target for development of specific antibodies (Fan et al., 2004; Grandea et al., 2010).

Since M2e peptide sequence contains only 23

amino acids, there is some doubts about the immunogenicity of this short peptide. However, highly conserved heat shock proteins have been considered as immunologic adjuvants (Ebrahimi and Tebianian, 2010b). Hsp70 is a member of the heat shock protein family (Hsps). Hsp70 role as an adjuvant in the induction of immune response is highly related to its capacity to present the exogenous antigens through the MHC class I pathway, a mechanism known as cross-priming and elicits cytotoxic T cell (CTL) response (Wang et al., 2002).

Linkage of M2e peptide to an appropriate carrier such as C-terminal domain of *Mycobacterium tuberculosis* Hsp70 (Hsp70 359-610 or Hsp70 C-terminal) could enhance its immunogenicity and improve the potency of M2e-based influenza vaccines (Ebrahimi et al. unpublished data).

IgY is a major specific serum immunoglobulin in chickens. Specific and biologically active IgY antibodies are transmitted vertically from serum into the egg yolk, so IgY could be extracted easily from the egg yolk. We employed DNA immunization to obtain anti-M2e IgY antibodies from laying hens. Production of antibodies based on DNA-designed avian IgY technology is followed by genetic immunization of hens with a plasmid encoding a given antigen. This is a useful method for direct generation of antibodies and eliminates the use of costly and laborious antigen preparation and purification in the conventional immunization methods (Rollier et al.,2000; Sun et al.,2001; Cova, 2005). IgYs have been used successfully in a variety of methods in different areas of research, diagnostics, medical application and biotechnology(Cova, 2005).

The aim of this study was to determine whether eukaryotic expression plasmid of M2e-Hsp70 C-terminal, administered via IM route, could elicit production of anti-M2e antibodies in laying hens.

Materials and Methods

1. Eukaryotic expression M2e-Hsp70 359-610 plasmid: Recombinant pcDNA 3.1 (+) plasmid bearing M2e-Hsp70 C-terminal coding sequence (pcDNA 3.1-M2e-Hsp70C-terminal) was kindly provided by Dr. Mahmoud Ebrahimi (Baqiyatallah Medical Sciences University, Tehran, Iran). M2e gene has 100% identity with the M2 gene (1-72 bp) of avian influenza A virus (influenza virus A/ chicken/ Iran/ 101/98, H9N2, GenBank accession number, EU477375). The Hsp 359-610 gene sequence also has a 100% identity with *Mycobacterium tuberculosis* H37Rv Hsp70 gene (1075-1888 bp) (GenBank accession number, BX842573).

Chemically competent *E. coli* strain DH5α was transformed with this vector. Making chemically competent bacteria and transformation were done according to the protocol explained by Sambrook and Russell (2001). Positive transformants were selected on LB-agar plates containing 100 mg/ml ampicillin. In the selected single colonies, the presence of plasmids was analyzed by colony PCR using BGH and T7 universal primers. PCR was performed under the following conditions; 4 min at 95°C, 30 cycles of 94°C for 60 s, 62°C for 60 s and 72°C for 60 s, with a terminal step of 10 min at 72°C.

Purification of pc DNA3.1-m2e-Hsp 70C-terminal and pcDNA3.1 was carried out using endotoxin free plasmid Midi Kit (Qiagen, Germany) prior to injection.

2. Immunization of hens: Thirty-week-old white leghorn laying hens were obtained from a local breeding unit, the laboratory animal resources of Amin Abad (Tehran, Iran). These hens received an inactivated H9N2 influenza vaccine according to the farm's vaccination schedule. Hens were intramuscularly (IM) injected with pcDNA3.1-m2e-Hsp70C-terminal. The purified pcDNA3.1 was used for the negative control group.

The Plasmids (300 µg in 200 µl of PBS) were injected in the breast muscle of hens and booster doses were given 4 and 6 weeks after first injection. Eggs were collected weekly after first immunization. Egg yolk antibody was extracted as described elsewhere (Nikbakht Brujeni et al., 2011) and blood samples obtained weekly. The separated sera were stored at -20^{o}C until use for ELISA.

3. Indirect enzyme-linked immunosorbent assay (ELISA): The M2e peptide, used for ELISA assay, was synthesized by solid phase technology (GL Biochem, China) with purity above 95%. For titration of anti-M2e antibodies in the serum and egg yolk samples, ELISA test was optimized by checkerboard titration method. 96-well microplate (Maxisorp, Nunc, Denmark) was coated with 100µl of 4 different concentrations, 1, 0.5, 0.25 and 0.125

µg/ml of synthetic M2e peptide solution in PBS (pH 7.4) and the plate was incubated overnight at 4°C. The unbound antigens were then removed by washing with washing solution (1X PBS, 0.05% Tween-20). Blocking was performed by loading 200 µl of blocking buffer (1X PBS, 0.05% Tween-20 and 5% BSA) in each well. Volumes of 100 µl of different dilutions of serum samples or egg yolk extracted antibodies were then added to the wells coated with different concentrations of M2e. Serum or egg yolk extracted antibodies were prepared as fourfold serial dilutions, starting from 1:40 and ending at 1:2560. After 30 min incubation the plate was washed and 100 µl HRP-labeled goat anti-chicken IgG (IDEXX laboratories, USA) was loaded in each well and left for 30 min at 37 °C. After washing steps, 100 µl of TMB (3, 3', 5, 5'-Tetramethylbenzidine) substrate (IDEXX laboratories, USA) was added, and incubated for 15 min at room temperature. The reaction was stopped by the addition of 100 µl/well stop solution (2M H_2SO_4). Absorbance was measured at wavelength of 450 nm using a microplate reader (Stat Fax 2000, Awareness Technology, Inc., USA).

Sera and egg yolk antibodies from the hens injected with control plasmid and samples obtained prior to injections, were included as controls.

Results

ELISA was used to investigate the production of antibodies in serum and egg yolk samples. The checker board ELISA titration method has been used to optimize ELISA conditions and obtain the appropriate concentration of M2e and also dilution of sera and egg yolk IgYs. Data is shown in figures 3.1 and 3.2. Values for which absorbance is < 0.4 are not acceptable. Among acceptable values and considering the ratio of nonspecific binding we chose dilution of 1:40 for both serum and egg yolk IgY samples. The best M2e concentration for serum ELISA was 0.25 µg/ml while that for egg yolk IgY was 0.5 µg/ml.

Anti-M2e antibody titers in serum and egg yolk samples were also evaluated. Changes of anti-M2e antibody titers in two groups of laying hens have been shown in figures 3.3 and 3.4. These antibodies were detected in egg yolks and sera of injected hens from 13 and 7 days post injection, with the peak titer at 41 and 35 days PI respectively. No considerable change was observed in serotiters yolk titers of control group. Compared to the serum IgY titers, the level of egg yolk IgY was relatively lower.

Discussion

Influenza M2e has been considered as an appropriate target for development of antibodies; although some strategies should be employed to improve the immunogenicity of M2e-based constructs. C-terminal domain of mycobacterial Hsp70 could act as an effective natural adjuvant (Nikbakht Brujeni et al., 2011; Wang et al., 2008; Wang et al., 2009). Ebrahimi et al. genetically fused influenza M2e to $Hsp70_{C\text{-terminal}}$ and designed pcDNA3.1-M2e-$Hsp70_{C\text{-terminal}}$ expression vector. They showed a Hsp70 based influenza M2e vaccine could effectively induce immune response against PR8 infection in mice (Ebrahimi et al. unpublished data).

According to several studies antibodies produced against M2e are able to limit influenza virus replication and reduce morbidity and mortality rates of influenza infections (Zharikova et al., 2005). Fan et al. showed that passive transfer of sera from monkeys immunized with M2e conjugate to mice, could protect them against lethal viral challenge (Fan Liang et al., 2004). Several studies proved that passive delivery of anti M2e antibodies induced immunity against lethal challenge of influenza viruses in mice (Wang et al., 2008; Liu et al., 2004). Therefore, M2e-specific antibodies could be considered as potential candidate in prophylactic and therapeutic measures against influenza A virus infections (Wang et al., 2009). Some individuals such as elderly, young children and immunocompromised patients might poorly respond to conventional influenza vaccines; besides in some cases a rapid induction of protection is crucial, in all these cases passive immunization is highly recommended (Beerli et al., 2009). From diagnostic point of view M2e-specific antibodies have applications in some tests like Western blot and immune dot blot.

The present study aimed towards assessing the ability of DNA-based immunization with pcDNA 3.1-M2e-Hsp70 $_{C\text{-termina}}$l plasmid in production of anti-M2e IgY antibodies in laying hens.

Bollen and Hau showed that after immunization of chickens with human IgG the concentrations of

immunospecific egg yolk or serum IgYs are similar. The serum antibody appears prior to the corresponding egg yolk IgY, because the serum antibody should reach to a specific level before it could transfer to the egg yolk (Bollen and Hau, 1997).

The concentration of IgY in egg yolk reflects levels of IgY in serum sample of 6-7 days behind. This gap is due to the time needed for oocyte growth and development (Hau and Hendriksen, 2005).

The use of laying hens to produce antibodies has so many advantages over the conventional methods of immunization using mammalian species. Concentration of egg yolk antibodies could reach 100 mg of IgY per egg (Mine and Kovacs-Nolan, 2002; Lee et al., 2004; Hau and Hendriksen, 2005). Thus the capacity of a laying hen to produce IgY is approximately 10 times greater than the rabbit's IgG productivity. This means, by simply replacing of mammals with hens no need for restraining and blood sampling resulting in the reducing of stress level in mammals plus saving the time (Hau and Hendriksen, 2005). Beside passive immunotherapy, IgY could be used for different laboratory techniques such as protein purification in affinity columns, and also as an immunodiagnostic tool in Western blot analysis, immunoelectrophoresis, immunoprecipitation, ELISA, and other Abbased assays (Cova, 2005).

However, the antibody response elicited by plasmid immunization could be affected by multiple factors such as hen's age (Bollen and Hau, 1999) and so warranted further studies particularly on the adjuvant characteristics that can improve the immunogenicity of recombinant proteins.

In conclusion, expression of recombinant M2e-$Hsp70_{C\text{-}terminal}$ peptide is applicable in laying hens and this peptide is capable of eliciting anti-M2e IgY antibodies in serum and egg yolks. This promising tool for induction of anti-M2e polyclonal, monospecific IgY antibodies could be nominated as a potent DNA vaccine candidate against avian influenza in future studies.

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References

1. Beerli, R.R., Bauer, M., Schmitz, N., Buser, R.B., Gwerder, M., Muntwiler, S., et al. (2009) Prophylactic and therapeutic activity of fully human monoclonal antibodies directed against influenza a m2 protein. Virol. J. 6: 224-226.
2. Bollen, L.S., Hau, J. (1999) Comparison of immunospecific antibody response in young and old chickens immunized with human IgG. Lab. Anim. 33: 71-76.
3. Bollen, L.S., Hau, J. (1997) Immunoglobulin g in the developing oocytes of the domestic hen and immunospecific antibody response in serum and corresponding egg yolk. In Vivo. 11: 395-398.
4. Choi, K.S., Lee, E.K., Jeon, W.J., Park, M.J., Yoo, Y. N., Kwon, J.H. (2010) Diagnostic utility of egg yolk for the detection of avian metapneumovirus antibodies in laying hens. Avian Dis. 54: 1230-1236.
5. Cova, L. (2005) DNA-designed avian igy antibodies: Novel tools for research, diagnostics and therapy. J. Clin. Virol. 34: 570-574.
6. Ebrahimi, S.M., Tebianian, M. (2010) Heterologous expression, purification and characterization of the influenza a virus m2e gene fused to *Mycobacterium tuberculosis* hsp70 (359-610) in prokaryotic system as a fusion protein. Mol. Biol. Rep. 37: 2877-2883.
7. Ebrahimi, S.M., Tebianian, M. (2010) Influenza a viruses: Why focusing on m2e-based universal vaccines. Virus Genes. 42: 1-8.
8. Fan, J., Liang, X., Horton, M.S., Perry, H.C., Citron, M.P., Heidecker, G.J., et al. (2004) Preclinical study of influenza virus a m2 peptide conjugate vaccines in mice, ferrets, and rhesus monkeys. Vaccine. 22: 2993-3003.
9. Grandea, A.G., 3rd, Olsen, O.A., Cox, T.C., Renshaw,

M., Hammond, P.W., Chan-Hui, P.Y., et al. (2010) Human antibodies reveal a protective epitope that is highly conserved among human and nonhuman influenza a viruses. Proceedings of the National Academy of Sciences of the United States of America. 107: 12658-12663.

10. Hamal, K.R., Burgess, S.C., Pevzner, I.Y., Erf, G.F. (2006) Maternal antibody transfer from dams to their egg yolks, egg whites, and chicks in meat lines of chickens. Poult Sci. 85: 1364-1372.
11. Hau, J., Hendriksen, C.F. (2005) Refinement of polyclonal antibody production by combining oral immunization of chickens with harvest of antibodies from the egg yolk. ILAR journal / National Research Council, Institute of Laboratory Animal Resources. 46: 294-299.
12. Holsinger, L.J., Shaughnessy, M.A., Micko, A., Pinto, L.H., Lamb, R.A. (1995) Analysis of the posttranslational modifications of the influenza virus m2 protein. J. Virol. 69: 1219-1225.
13. Jourdain, E., Gunnarsson, G., Wahlgren, J., Latorre-Margalef, N., Brojer, C., Sahlin, S., et al. (2010) Influenza virus in a natural host, the mallard: Experimental infection data. PloS ONE. 5: e8935.
14. Lee, D.Y., Cho, Y.W., Kang, S.G., Shin, S.J., Yoo, H.S. (2004) Development of a novel antigen capture-elisa using igy against porcine interleukin-6 and its application. J. Vet. Sci. 5: 337-343.
15. Liu, W., Zou, P., Chen, Y.H. (2004) Monoclonal antibodies recognizing evetpirn epitope of influenza a virus m2 protein could protect mice from lethal influenza a virus challenge. Immunol. lett. 93: 131-136.
16. Mine, Y., Kovacs-Nolan, J. (2002) Chicken egg yolk antibodies as therapeutics in enteric infectious disease: A review. J. Med. Food. 5: 159-169.
17. Nayak, D.P., Balogun, R.A., Yamada, H., Zhou, Z.H., Barman, S. (2009) Influenza virus morphogenesis and budding. Virus Res. 143: 147-161.
18. Nemchinov, L.G., Natilla, A. (2007) Transient expression of the ectodomain of matrix protein 2 (m2e) of avian influenza a virus in plants. Protein Expr. Purif. 56: 153-159.
19. Neumann, G., Kawaoka, Y. (2006) Host range restriction and pathogenicity in the context of influenza pandemic. Emerg. Infect. Dis. 12: 881-886.
20. Nikbakht Brujeni, G., Jalali, S.A., Koohi, M.K. (2011) Development of DNA-designed avian igy antibodies for quantitative determination of bovine interferon-gamma. Appl. Biochem. Biotechnol. 163: 338-345.
21. Perdue, M.L., Swayne, D.E. (2005) Public health risk from avian influenza viruses. Avian Dis. 49: 317-327.
22. Rollier, C., Charollois, C., Jamard, C., Trepo, C., Cova, L. (2000) Maternally transferred antibodies from DNA-immunized avians protect offspring against hepadnavirus infection. J. Virol. 74: 4908-4911.
23. Sambrook, J., Russell, D.W. (2001) Molecular cloning: A laboratory manual. (3rd ed.) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NewYork, USA.
24. Sun, S., Mo, W., Ji, Y., Liu, S. (2001) Preparation and mass spectrometric study of egg yolk antibody (igy) against rabies virus. Rapid Commun. Mass Spectrom. 15: 708-712.
25. Wang, R., Song, A., Levin, J., Dennis, D., Zhang, N.J., Yoshida, H., et al. (2008) Therapeutic potential of a fully human monoclonal antibody against influenza a virus m2 protein. Antiviral Res. 80: 168-177.
26. Wang, Y., Kelly, C.G., Singh, M., McGowan, E.G., Carrara, A.S., Bergmeier, L.A., et al. (2002) Stimulation of th1-polarizing cytokines, c-c chemokines, maturation of dendritic cells, and adjuvant function by the peptide binding fragment of heat shock protein 70. J. Immunol. 169: 2422-2429.
27. Wang, Y., Zhou, L., Shi, H., Xu, H., Yao, H., Xi, X.G., et al. (2009) Monoclonal antibody recognizing slltevet epitope of m2 protein potently inhibited the replication of influenza a viruses in mdck cells. Biochem. Biophy. Res. Commun. 385: 118-122.
28. 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.

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تهیه آنتی بادی های زرده تخم مرغ بر ضد آنتی ژن M2e آنفولانزای طیور با استفاده از ژن M2e-Hsp70 C-terminal و تشخیص آن با روش الیزا

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چکیده

زمینه مطالعه: استفاده از آنتی بادی های زرده (IgY) به جهت کاربردهای متنوع تشخیصی، درمانی و همچنین تحقیقاتی و بیوتکنولوژی مورد توجه روز افزون قرار گرفته است. **هدف:** در این مطالعه تولید آنتی بادی ضد پپتید M2e آنفلوانزای طیور در زرده تخم مرغ بررسی گردید. **روش کار:** پلاسمید حاوی قطعه کدکننده M2e و بخش انتهایی کربوکسیل پروتئین شوک حرارتی ۷۰ به عضله سینه مرغ های تخم گذار تزریق شد. سرم و زرده تخم مرغ از نظر وجود آنتی بادی های ضد M2e توسط آزمون الایزای غیر مستقیم مورد بررسی قرار گرفتند. **نتایج:** حضور آنتی بادی های ضد M2e در نمونه های زرده و سرم مرغ ها به ترتیب از روز ۱۳ و ۷ بعد از تزریق مورد سنجش قرار گرفتند و بیشترین مقدار آنتی بادی به ترتیب در روزهای ۴۱ و ۳۵ مشاهده شد. **نتیجه گیری نهایی:** تولید آنتی بادی های ضد M2e در مرغ های تخم گذار امکان استفاده از ساختار پلاسمیدی M2e-HspC-terminal جهت ایمن سازی رانشان می دهد و ابزار مناسبی برای تهیه آنتی بادی های پلی کلنال و اختصاصی بر علیه این آنتی ژن است. این آنتی بادی ها برای اهداف تشخیصی و درمانی آنفولانزا کاربرد دارند.

واژه های کلیدی: آنفولانزای طیور، M2e، پروتئین شوک حرارتی ۷۰، آنتی بادی زرده، الیزا.

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