Available online at www.sciencedirect.com

ScienceDirect

Procedia in Vaccinology 2 (2010) 40–43

Procedia in Vaccinology

www.elsevier.com/locate/ procedia

3rd Vaccine Global Congress, Singapore 2009

Development of a Nano-vaccine against a Wild Bird H6N2 Avian Influenza Virus

Songhua Shan, Eddy Poinern, Trevor Ellis, Stan Fenwick, Xuan Le, John Edwards, Zhong-Tao Jiang

Murdoch University, Australia

Abstract

A DNA vaccine, pCAG-HAk, expressing the full-length haemagglutinin (HA) gene of avian influenza A/Eurasian coot/Western Australia/2727/1979 (H6N2) virus, together with a Kozak sequence, in a pCAGGS vector was formulated with a novel nanoparticle, Phema. Two intramuscular immunizations of layer chickens at 3-week intervals with different doses of a nano-vaccine as well as 100µg pCAG-HAk failed to induce detectable haemagglutination-inhibition (HI) antibody. Following virus challenge, the pCAG-HAk vaccinated group did not show a statistically significant difference in frequency of virus shedding in both oropharyngeal and cloacal swabs from the naive control group. However, in comparison with the naive control group, the 100µg nano-vaccine group did significantly reduce virus shedding from the oropharynx. Despite lack of a dose-sparing effect from the nano-vaccine, there was a dose-response effect. This pilot study indicated that Phema used as an adjuvant augmented the immune response induced by the pCAG-HAk construct.

Keywords: H6N2 subtype avian influenza, nanovaccine, adjuvant, chickens

1. Introduction

Genetic immunization is well documented to induce protective immunity to various infectious and non-infectious diseases in a range of animals (1). However, rapid degradation and poor cellular uptake of plasmid DNA has significantly impacted on the efficacy of 'naked' plasmid DNA vaccines (27). As adjuvants have historically played a major role in successful vaccine development (22), some chemical adjuvants such as Bupivacaine (26) or Marcaine, Ubenimex (19), monophosphoryl lipid A (MPL) (20), QS-21 saponin (21), levamisole(11) have been explored and have showed respectable facilitating effect on immune responses to DNA vaccines. Further investigation of novel adjuvants and their formulation technologies could improve the utility of plasmid DNA vaccination.

Nano-particles, varying in size from 10 to 500 nm, are solid particles made from inert materials (2). They are potentially biocompatible and biodegradable, are relatively stable *in vivo*, are relatively easy to link with immunogens, have straightforward delivery methods and show little or no side effects (3, 23). As early as 1995, solid inert beads adsorbed with antigen were reported to prime CD^{8+}T cell responses (7). Inert nanoparticles have subsequently been shown to induce strong immune responses to protein and peptide antigens in mice (8, 9), sheep (22), pigs (2) and cattle (2). In the context of DNA vaccines, a cationic nanoparticle formulated plasmid DNA encoding a reporter gene enhanced *in vitro* cell transfection efficiency and elicited 16-200-fold greater immune responses in mice than naked plasmid DNA alone following multiple delivery routes (3, 4, 5, 6). Co-administration of cholera toxin and lipid A with a nanoparticle-based plasmid DNA showed a synergistic effect and hence further enhanced immune responses (6). Thus, nanoparticles, as a novel class of adjuvants, have the potential to induce immune responses to protein or plasmid DNA immunogens without the side effects typically associated with local tissue damage caused by conventional chemical adjuvants. Though the microparticle formulated plasmid DNA encoding the NP gene of A/PR/8/34 (H1N1) virus was shown to enhance immune response in mice (10), there has been no reports on nanoparticle-based avian influenza DNA vaccines in chickens.

1877-282X/$–see front matter
doi:10.1016/j.provac.2010.03.008

For Australian biosecurity reasons, a low pathogenic avian influenza (LPAI) virus, A/Eurasian coot/Western Australia/2727/1979 (H6N2), isolated from a healthy Eurasian coot (*Fulica atra*) in Australia, was selected to perform this DNA vaccine adjuvant study. In the present study we investigated the immunologic adjuvant effect of a novel self-prepared Phema for DNA vaccination against avian influenza.

2. Formulation of plasmid DNA with Phema

The pCAG-HAk plasmid DNA expressing the complete haemagglutinin (HA) gene of avian influenza A/Eurasian coot/Western Australia/2727/1979 (H6N2) virus, together with a Kozak sequence, in a pCAGGS vector was used for a DNA vaccine as described previously (24).

A novel polymer (2-hydroxyethyl-methacrylate) (Phema), which formed a 150-300 nm hydrophilic nanoparticle suspension, was provided by the Nanotechnology Group, Murdoch University, Australia. Optimization of the solvent for mixing the Phema and DNA was determined using 100% dH_2O, dH_2O/ethanol (50:50) and 100% ethanol and calculation of the level of DNA binding. Then 2 mL of 1% Phema (w/v in ethanol) was sonicated for a few seconds followed by the addition of 1 mL of 1% polyvinyl alcohol (PVA) (w/v in dH_2O) and sonication for 1 min on ice. Subsequently 200 µL of plasmid DNA (9.3 µg/µL) was added and sonicated for 2 min on ice. The resultant solution was filtered through a 0.2 µm membrane to remove the surfactant. The flow-through was centrifuged at 15,000 g for 20 min at room temperature. The pellet was dissolved in PBS and the amount of DNA coated with nano-beads was calculated by subtracting the amount of DNA in the supernatant, measured by a Nanodrop spectrophotometer (Thermo Scientific, MA, USA), from the total DNA added.

3. Vaccination regime

Three-week old Hy-Line chickens (free of avian influenza) were housed in free-range pens with access to water and feed *ad libitum*. The experimental protocol used was shown in Table 8.1. Birds intramuscularly received two 0.2 mL injections (0.1 mL per leg) at 3 week intervals with 200 µL PBS (control), 100 µg pCAG-HAk, 10 µg, 100 µg, 200 µg Phema-adjuvanted pCAG-HAk. Sera were weekly collected to detect H6-specific antibody by haemagglutination-inhibition (HI) (16, 24).

Three weeks following the booster vaccination, each chicken received 0.5 mL H6N2 virus ($10^{6.5}$ EID_{50} /0.1 mL) by nasal instillation (0.1 mL), eyedrop (0.1 mL) and oral route (0.3 mL). Following challenge, in addition to daily observing all chickens, oropharyngeal or cloacal swabs were collected every second day over a seven day period. Virus isolation was performed as described (16).

4. Results and Discussion

Experiments with different ratios of alcohol and water showed that Phema adjuvant prepared with 100% ethanol as solvent gave the highest DNA binding rate. Thus, 100% alcohol was used for the preparation of the Phema adjuvant vaccine. Subsequently, the maximal plasmid DNA-Phema binding rate, determined using a constant Phema concentration and different DNA concentrations, was approximately 30%.

No H6 HI antibody titre was detected in all Hy-Line chickens following two immunizations. By 10 days post virus challenge, all birds sero-converted with significant difference in the geometric mean titre (GMT) of HI antibody between pre- and post-challenge using the paired-sample T test (Table 1).

Table 1. Antibody response prior to and post virus challenge.

	Naïve control	pCAG-HAk	Phema		
			200 µg	100 µg	10 µg
Pre-challenge	0	0	0	0	0
Post-challenge	6.0±2.0*	5.6±1.7	5.3±1.5	5.3±1.5	6.6±2.1

* Values represent geometric mean titre (GMT)± SD of each group (exponent index in $\log_2$).

As shown in Table 2, in comparison with the naive control group, the pCAG-HAk vaccinated group reduced the virus excretion rate from 70.8% to 45% via oropharynx post challenge and from 12.5% to 0 via cloaca. However, the pCAG-HAk vaccinated group was not significantly different from the naive control group in oropharyngeal and cloacal swabs. Possibly, the pCAG-HAk vaccine may have elicited some borderline level of protective immunity in the oropharynx (Chi square, P= 0.083).

In comparison with the naive control group, the 100µg Phema adjuvanted pCAG-HAk group showed significant decrease (Fisher's exact test, P=0.014) in virus shedding in oropharyngeal swabs but no significant difference in cloacal swabs. This suggested that Phema as adjuvant further augmented the immune response induced by the pCAG-HAk construct.

Table 2. Level of virus shedding in oropharyngeal and cloacal swabs in vaccinated chickens following H6N2 avian influenza virus challenge.

	Naïve control	pCAG-HAk	Phema		
			200 µg	100 µg	10 µg
Oropharyngeal swabs	17/24 (70.8)* [a]	9/20 (45) [ab]	8/16 (50) [ab]	3/12 (25) [bc]	9/20 (45) [ab]
Cloacal swabs	3/24 (12.5)	0/20 (0)	1/16 (6.3)	0/12 (0)	1/20 (5)

* Number of swabs positive for virus isolation/total number of swabs tested. Percentage rate is shown in parentheses.

For the oropharyngeal swab row, different lowercase superscript letters indicate statistical differences ($P < 0.05$) within the row using Chi square or Fisher's exact test. There were no significant differences between groups for the cloacal swabs.

The use of particulate carriers as novel vaccine delivery systems is an area currently receiving a high level of interest. Micro- and nano-size particle characteristics, such as size and surface properties, including surface charge and hydrophobicity, affect vaccine efficacy (28). A range of inert nanoparticles have been tested and shown to be effective delivery vehicles for protein and peptide antigens (3, 4, 5, 6, 12, 13, 14). Nevertheless, application of nanoparticles with DNA vaccines is only at the exploratory stage (6, 15). A biodegradable copolymer Phema has also been used in a drug delivery system (17, 18). However, there appears to be no previous reports using Phema for vaccine adjuvant purposes.

Due to the hydrophilicity of Phema, it is suspected that the ethanol solvent used in the current study may have imparted positive surface charges and allowed more absorption of the plasmid DNA than with water as a solvent. Plasmid DNA was possibly absorbed to the surface of Phema through electrostatic interaction or covalent binding and this did not appear to damage the DNA's biological functions. The interaction also appeared to be complex because the optimum effect was achieved with the 100µg dose group and the response was poorer with both the 10 and 200µg dose groups. The mechanisms behind the observed adjuvant effect have not yet been resolved. Possible mechanisms are that these nanocarriers prevent DNA degradation and facilitate targeted delivery to antigen presenting cells (12, 25).

The pCAG-HAk vaccines with or without adjuvants did not induce a measurable antibody response in the Hy-Line chickens, however, the Phema adjuvanted pCAG-HAk vaccines gave some enhanced reduction in virus shedding from the oropharynx. The reason why the pCAG-HAk induced antibody responses in SPF chickens (24), but lack of antibody response in Hy-Line chickens will require further investigation.

5. Acknowledgments

We thank Professor Hualan Chen for allowing us to prepare the pCAG-HAk construct at the Avian Influenza Reference Laboratory at Harbin Veterinary Research Institute, China.

6. References

1. **Abdulhaqq, S.A. and D.B.Weiner.** 2008. DNA vaccines: developing new strategies to enhance immune responses. Immunol Res. 42:219-232.
2. **Aucouturier, J., L.Dupuis, and V.Ganne.** 2001. Adjuvants designed for veterinary and human vaccines. Vaccine.19:2666-2672.
3. **Cui, Z. and R.J. Mumper**. 2002a. Genetic immunization using nanoparticles engineered from microemulsion precursors. Pharm Res.19:939-946.
4. **Cui, Z. and R.J. Mumper.** 2002b. Intranasal administration of plasmid DNA-coated nanoparticles results in enhanced immune responses. J Pharm Pharmacol.54:1195-1203.
5. **Cui, Z. and R.J. Mumper.** 2002c. Topical immunization using nanoengineered genetic vaccines. J Control Release.81:173-184.
6. **Cui, Z. and R.J. Mumper.** 2003. The effect of co-administration of adjuvants with a nanoparticle-based genetic vaccine delivery system on the resulting immune responses. Eur J Pharm Biopharm.55:11-18.
7. **Falo, L.D., M. Jr. Kovacsovics-Bankowski, K. Thompson, and K.L. Rock.** 1995. Targeting antigen into the phagocytic pathway in vivo induces protective tumour immunity. Nat Med.1:649-653.
8. **Fifis, T., A.Gamvrellis, B. Crimeen-Irwin, G.A. Pietersz, J.Li, P.L.Mottram, I.F. McKenzie, and M. Plebanski.** 2004a. Size-dependent immunogenicity: therapeutic and protective properties of nano-vaccines against tumors. J Immunol.173:3148-3154.
9. **Fifis, T., P. Mottram,V. Bogdanoska, J.Hanley, and M. Plebanski.** 2004b. Short peptide sequences containing MHC class I and/or class II epitopes linked to nano-beads induce strong immunity and inhibition of growth of antigen-specific tumour challenge in mice. Vaccine.23:258-266.
10. **Hartikka, J., A.Geall, V. Bozoukova, D. Kurniadi, D. Rusalov, J. Enas, J.H.Yi, A. Nanci, and A. Rolland.** 2008. Physical characterization and in vivo evaluation of poloxamer-based DNA vaccine formulations. J Gene Med.10:770-782.
11. **Jin, H., Y. Li, Z. Ma, F. Zhang, Q. Xie, D.Gu, and B.Wang.** 2004. Effect of chemical adjuvants on DNA vaccination. Vaccine.22: 2925-2935.
12. **Khatri, K., A.K. Goyal, and S.P.Vyas.** 2008. Potential of nanocarriers in genetic immunization. Recent Pat drug Deliv Formul.2:68-82.
13. **Li, G.P., Z.G. Liu, B. Liao, and N.S. Zhong.** 2009. Induction of Th1-type immune response by chitosan nanoparticles containing plasmid DNA encoding house dust mite allergen Der p 2 for oral vaccination in mice. Cell Mol Immunol.6: 45-50.
14. **Lori, F., S.A. Calarota, and J. Lisziewicz.** 2007. Nanochemistry-based immunotherapy for HIV-1. Curr Med Chem.14:1911-1919.
15. **Minigo, G., A. Scholzen, C.K. Tang, J.C. Hanley, M. Kalkanidis, G.A. Pietersz, V. Apostolopoulos, and M. Plebanski.** 2007. Poly-L-lysine-coated nanoparticles: a potent delivery system to enhance DNA vaccine efficacy. Vaccine.25:1316-1327.
16. O.i.d.é. 2008. Avian Influenza. In: Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2008. Office international des epizooties. http://www.oie.int/eng/normes/mmanual/2008/pdf/2.03.04_AI.pdf. [10Sept 2009, last date accessed.]
17. **Piotrowicz, A. and M.S. Shoichet.** 2006. Nerve guidance channels as drug delivery vehicles. Biomaterials.27:2018-2027.
18. **Rao, K.P.** 1998. New concepts in controlled drug delivery. Pure Appl Chem. 70: 1283-1287.
19. **Sasaki, S., J. Fukushima, K. Hamajima, N. Ishii, T. Tsuji, K.Q. Xin, H. Mohri, and K. Okuda.** 1998a. Adjuvant effect of Ubenimex on a DNA vaccine for HIV-1. Clin Exp Immunol.111:30-35.
20. **Sasaki, S., K. Hamajima, J. Fukushima, A. Ihata, N. Ishii, I. Gorai, F. Hirahara, H. Mohri, and K.Okuda**. 1998b. Comparison of intranasal and intramuscular immunization against human immunodeficiency virus type 1 with a DNA-monophosphoryl lipid A adjuvant vaccine. Infect Immun.66:823-826.
21. **Sasaki, S., K. Sumino, K. Hamajima, J. Fukushima, N. Ishii, S. Kawamoto, H. Mohri, C.R. Kensil, and K. Okuda.** 1998c. Induction of systemic and mucosal immune responses to human immunodeficiency virus type 1 by a DNA vaccine formulated with QS-21 saponin adjuvant via intramuscular and intranasal routes. J Virol.72:4931-4939.
22. **Scheerlinck, J.P., S. Gloster, A. Gamvrellis, P.L. Mottram and M. Plebanski.** 2006. Systemic immune responses in sheep, induced by a novel nano-bead adjuvant. Vaccine. 24:1124-1131.
23. **Scheerlinck, J.P. and D.L. Greenwood.** 2006. Particulate delivery systems for animal vaccines. Methods.40:118-124.
24. **Shan, S.H., Y.P. Jiang, Z.G. Bu, T. Ellis, X.Y. Zheng, J. Edwards, G.B. Tian, Y.B. Li, J.Y. Ge, H.L. Chen, S. Fenwick.** 2010. Investigation of approaches improving the efficacy of a H6 subtype avian influenza DNA vaccine in chickens (submitted for publication).
25. **Singh, M., M. Briones, G. Ott, and D. O'Hagan.** 2000. Cationic microparticles: A potent delivery system for DNA vaccines. Proc Natl Acad Sci USA.97: 811-816.
26. **Wang, B., K.E. Ugen, V. Srikantan, M.G. Agadjanyan, K. Dang, Y. Refaeli, A.I. Sato, J. Boyer, W.V. Williams, and D.B. Weiner.** 1993. Gene inoculation generates immune responses against human immunodeficiency virus type 1. Proc Natl Acad Sci USA. 90: 4156-4160.
27. **Wilson, K.D., S.D. de Jong, M. Kazem, R. Lall, M.J, Hope, P.R. Cullis, and Y.K. Tam.** 2009. The combination of stabilized plasmid lipid particles and lipid nanoparticle encapsulated CpG containing oligodeoxynucleotides as a systemic genetic vaccine. J Gene Med.11(1):14-25.
28. **Xiang, S.D., A. Scholzen, G. Minigo, C. David, V.** Apostolopoulos, P.L. Mottram, and M. Plebanski. 2006. Pathogen recognition and development of particulate vaccines: does size matter? Methods. 40:1-9.