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Examination of the effects of virus inactivation methods on the induction of antibody- and cell-mediated immune responses against whole inactivated H9N2 avian influenza virus vaccines in chickens

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ABSTRACT

Several types of avian influenza virus (AIV) vaccines exist, including live-attenuated, vectored, and whole inactivated virus (WIV) vaccines. Inactivated vaccines offer some advantages compared to other types of vaccines, including ease of production and lack of ability to revert to a virulent state. However, WIV are poorly immunogenic, especially when these vaccines are delivered to mucosal surfaces. There are several factors that contribute to the immunogenicity of vaccines, one of which is the method used to inactivate viruses. Several methods exist for producing influenza WIVs, including formaldehyde, a chemical that affects protein structures leading to virus inactivation. Other methods include treatment with beta-propiolactone (BPL) and the application of gamma radiation, both of which have less effects on protein structures compared to formaldehyde, and instead alter nucleic acids in the virion. Here, we sought to determine the effect of the above inactivation methods on immunogenicity of AIV vaccines. To this end, chickens were vaccinated with three different H9N2 WIVs using formaldehyde, BPL, and gamma radiation for inactivation. In addition to administering these three WIVs alone as vaccines, we also included CpG ODN 2007, a synthetic ligand recognized by Toll-like receptor (TLR)21 in chickens, as an adjuvant for each WIV. Subsequently, antibody- and cell-mediated immune responses were measured following vaccination. Antibody-mediated immune responses were increased in chickens that received the BPL and Gamma WIVs compared to the formaldehyde WIV. CpG ODN 2007 was found to significantly increase antibody responses for each WIV compared to WIV alone. Furthermore, we observed the presence of cell-mediated immune responses in chickens that received the BPL WIV combined with CpG ODN 2007. Based on these results, the BPL WIV + CpG ODN 2007 combination was the most effective vaccine at inducing adaptive immune responses against H9N2 AIV. Future studies should characterize mucosal adaptive immune responses to these vaccines.

1. Introduction

Low pathogenic avian influenza viruses (LPAIV) are a class of avian influenza viruses (AIV) that commonly infect wild birds without causing any signs of clinical disease [1]. H9N2 AIV is a LPAIV that has become a concern for economical and health related reasons. H9N2 virus infection in chickens can decrease egg production, and co-infection with other infectious pathogens can result in immunosuppression and pathological changes [2,3]. There is strong evidence of human exposure to the H9N2 virus among poultry workers in certain regions of the world including China and India [4,5], although not many cases of infection are reported. H9N2 AIV is also responsible for contributing its internal genes to a reassortant H7N9 virus which has demonstrated 40% mortality in human cases in China [6].

Vaccination is a potential strategy that can be used to control H9N2 infection in poultry. Many types of influenza vaccines exist, including live-attenuated vaccines, subunit vaccines, and

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inactivated vaccines. Whole-inactivated virus (WIV) vaccines for influenza were first explored in the 1940s, when formaldehyde and other compounds were used to inactivate influenza viruses [7]. Since then, many different ways to produce influenza WIV vaccines have emerged, including other chemical treatments or the application of different forms of electromagnetic radiation [8].

Two common chemicals used to produce influenza WIVs include formaldehyde and beta-propiolactone (BPL). As an aldehyde, formaldehyde reacts with protein structures, crosslinking various amino acids. This in turn affects the function of proteins and can lead to a loss of virus infectivity [9]. Altering protein structures is an effective way to induce virus inactivation, however, this can change the outcome of the elicited immune response following vaccination due to changes on epitopes of immunogenic proteins, such as the influenza hemagglutinin (HA) protein [9]. BPL is an electrophilic compound that reacts with nucleic acids, mainly with the nucleotides guanosine and adenosine [8]. These reactions alter the structure of nucleic acids inducing changes such as strand breaks and improper linkages between nucleic acids and protein, and between nucleic acids and other nucleic acids [9]. Although nucleic acids are the main target of BPL, there is evidence demonstrating that BPL affects protein structures of influenza virus [10]. Also, it has been shown that BPL inactivated influenza viruses have a diminished ability to fuse with lipid membranes due to changes in the HA protein structure [11]. Nevertheless, formaldehyde inactivated influenza viruses have also been shown to lack the ability to fuse with lipid membranes [12].

Another method used to inactivate viruses is by the application of various forms of electromagnetic radiation. Inactivation is achieved through direct effects on nucleic acids, resulting in strand breaks, linkages, and nucleotide damage [13]. Initial reports on using gamma radiation to inactivate pathogens stated that a complete loss of influenza virus infectivity can be achieved after application of 0.65 kiloGrays (kGy) of gamma radiation, and this must be increased to 200 kGy before the HA protein structure is compromised [14]. This makes gamma radiation an effective way to inactivate influenza viruses without altering potential immunogenic proteins in the process. In the present study, we chose to compare three different methods for the inactivation of H9N2 AIV, namely formaldehyde, BPL, and gamma radiation. Immunogenicity of these preparations was assessed in a series of *in vivo* studies. In addition to the type of vaccine administered, vaccine adjuvants can also affect immune responses after vaccination. CpG ODN 2007 is a synthetic oligodeoxynucleotide recognized by TLR21 in chickens that has been shown to be an effective adjuvant when added to inactivated influenza virus chicken vaccines [15,16]. For this reason, CpG ODN 2007 was also combined with each of the H9N2 WIVs (formaldehyde, BPL and gamma radiation) to examine its effects on antibody- and cell-mediated immune responses following vaccination in chickens with different H9N2 WIVs.

2. Material and methods

2.1. Chickens

One-hundred and thirty, one-day-old specific pathogen free (SPF) chicks were purchased from the Canadian Food Inspection agency (Ottawa, Canada). The chickens were kept in the isolation facility at the Ontario Veterinary College, University of Guelph, Ontario. All sampling and treatment protocols were approved by the University of Guelph Animal Care Committee and were conducted with compliance to the guidelines provided by the Canadian Council on Animal Care.

2.2. Inactivation of H9N2 AIV

H9N2 AIV (A/Turkey/Wisconsin/1/66) was propagated as previously described [17]. Inactivation with BPL and formaldehyde was performed as described previously [9,15]. Briefly, H9N2 virus was mixed with formaldehyde or BPL for 72 h at 37 °C (0.02%), or 30 min at 4 °C (0.1%), respectively. For gamma radiation of H9N2, concentrated H9N2 was lyophilized and subjected to 12.5 kGy of gamma radiation at the Southern Ontario Centre for Atmospheric Aerosol Research at the University of Toronto. Total protein concentration was determined for each WIV using a Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL) following the manufacturer's recommendations.

2.3. CpG ODN 2007

CpG ODN 2007 was purchased from InvivoGen (San Diego, California, USA) and was resuspended in sterile phosphate buffered saline (PBS).

2.4. Vaccine formulation and experimental outline

Chickens were divided into 10 groups summarized in Table 1. The abbreviated group names in Table 1 will be used to describe the vaccine which that group of chickens received. On day 7 post-hatch all chickens were vaccinated via intramuscular injection in the thigh muscle with 15 μg of one of the three WIVs. WIVs were administered alone, or in combination with an oil-emulsion adjuvant (AddaVax™) or 2 μg of CpG ODN 2007. On day 21 post-hatch the chickens received a second vaccine dose. One group received PBS and served as a negative control group. Serum samples were collected weekly and spleens were harvested 10 days after the second vaccination.

2.5. Hemagglutination inhibition (HI) and enzyme-linked immunosorbent assay (ELISA)

The HI assay was carried out as described previously [15]. For the ELISA, 96-well Maxisorp flat bottom plates were coated

Table 1
Vaccination groups. All birds were vaccinated on day 7 and 21 post-hatch.

Group	Inactivated Virus	Adjuvant
1. PBS	None	None
2. Form	15 ug formaldehyde inactivated H9N2	None
3. Form + Add	15 ug formaldehyde inactivated H9N2	AddaVax™
4. Form + CpG	15 ug formaldehyde inactivated H9N2	2 ug CpG ODN 2007
5. BPL	15 ug beta-propiolactone inactivated (BPL) H9N2	None
6. BPL + Add	15 ug BPL inactivated H9N2	AddaVax™
7. BPL + CpG	15 ug BPL inactivated H9N2	2 ug CpG ODN 2007
8. Gamma	15 ug gamma radiation inactivated H9N2	None
9. Gamma + Add	15 ug gamma radiation inactivated H9N2	AddaVax™
10. Gamma + CpG	15 ug gamma radiation inactivated H9N2	2 ug CpG ODN 2007

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