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Immunologic evaluation of 10 different adjuvants for use in vaccines for chickens against highly pathogenic avian influenza virus

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ABSTRACT

Avian influenza viruses (AIV) are a threat to poultry production worldwide. Vaccination is utilized as a component of control programs for both high pathogenicity (HP) and low pathogenicity (LP) AIV. Over 95% of all AIV vaccine used in poultry are inactivated, adjuvanted products. To identify the best formulations for chickens, vaccines were prepared with beta-propiolactone (BPL) inactivated A/British Columbia/314514-1/2004 H7N3 LP AIV using ten commercially available or experimental adjuvants. Each vaccine formulation was evaluated for immunogenicity in chickens. Challenge studies with an antigenically homologous strain of HPAIV were conducted to compare protection against mortality and measure reductions in virus levels in oral swabs. The four best adjuvants from the studies with BPL inactivated antigen were selected and tested identically, but with vaccines prepared from formalin inactivated virus. Mineral and vegetable oil based adjuvants generally induced the highest antibody titers with 100% seroconversion by 3 weeks post vaccination. Chitosan induced positive antibody titers in 100% of the chickens, but the titers were significantly lower than those of most of the oil based adjuvants. Antibody levels from calcium phosphate and alginate adjuvanted groups were similar to those of non-adjuvanted virus. All groups that received adjuvanted vaccines induced similar levels of protection against mortality (0–20%) except the groups vaccinated with calcium phosphate adjuvanted vaccines, where mortality was similar (70%) to groups that received non-adjuvanted inactivated virus or no vaccine (60–100% mortality). Virus shedding in oral swabs was variable among the treatment groups. Formalin inactivated vaccine induced similar antibody titers and protection against challenge compared to BPL inactivated vaccine groups. These studies support the use of oil adjuvanted vaccines for use in the poultry industry for control for AIV.

1. Introduction

Avian influenza (AI) is a highly consequential disease of poultry resulting in significant economic losses worldwide due to mortality, morbidity, culling of birds, and lost trade markets. Vaccination is used to help control AI virus (AIV) and limit losses in areas where the virus is endemic. Although vectored vaccines are available and licensed in some countries, 95.5% of the AIV vaccine used for poultry, by dose, is oil emulsion, inactivated whole virus vaccine [1]. Despite the disadvantage that this type of vaccine must be applied to each bird individually, inactivated vaccines are safe, effective, and relatively inexpensive, therefore will remain highly utilized for AIV in poultry particularly in areas where labor costs are low. Individual inoculation does have the advantage that it can ensure high coverage within the vaccinated populated.

Optimal formulations of inactivated vaccines need an appropriate antigen to match the field challenge virus. However, even highly immunogenic AIV strains require adjuvants to elicit a sufficient immune response. Vaccine adjuvants are chemical substances, microbial components or proteins, that enhance the

Abbreviations: 70VG, Montanide ISA 70VG adjuvant; 71VG, Montanide ISA 71VG adjuvant; 760VG, Montanide ISA 760VG adjuvant; 780VG, Montanide ISA 780VG adjuvant; AI, avian influenza; AIV, avian influenza virus; BHI, brain heart infusion buffer; BPL, beta-propiolactone; CAP, calcium phosphate adjuvant; DPC, days post-challenge; ECE, embryonating chicken eggs; EID_{50}, 50% egg infectious doses; ELISA, enzyme linked immunosorbent assay; GEL01, Montanide GEL01 adjuvant; GMT, geometric mean titer; HA, hemagglutinin protein; HAU, hemagglutinating units; HI, hemagglutination inhibition; HP, highly pathogenic; IFA, Freunds' incomplete adjuvant; IM, intra-muscular; IV, intra-venous; LP, low pathogenic; OP, oropharyngeal; NAIV, non-adjuvanted inactivated virus; NP, nucleoprotein; SPF, specific pathogen free; SQ, sub-cutaneous; WPV, weeks post vaccination.

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immune response to inactivated vaccines. An ideal adjuvant should be stable and environmentally safe, should not cause an inflammatory reaction at the injection site and must be cost effective. Numerous commercial and experimental adjuvants that fulfill most of these criteria are available, but with a few exceptions [2,3] data for adjuvants with AIV vaccines in poultry are generally lacking.

The inactivation process can contribute to producing a vaccine that induces antibody to protective epitopes by affecting protein structure. Chemical treatment is the most common method of AIV inactivation for vaccine production. Formalin and beta-propiolactone (BPL) are the most commonly utilized chemicals, but both can decrease the hemagglutination (HA) titer and reduce antigenicity of influenza virus *in vitro* because of cross-linking [4,5]. Also, BPL has been shown to affect influenza HA2 protein in a manner that inhibits fusion [6]. Since the protective epitopes for influenza A neutralization reside on the HA protein this suggests that the antigenic structure could be affected. Formalin may maintain the epitopes better [5], but residues in vaccines may reduce egg production [7]. To our knowledge there is no data demonstrating the relative effects of each of these chemicals on immunogenicity with birds *in vivo*.

The goal of this study was to identify optimal adjuvants for AIV vaccines for chickens and to compare the two most common chemical inactivation methods. To accomplish this we compared the antibody responses of birds vaccinated with the same dose of different formulations of vaccines and evaluated protection (mortality and oral virus shed) against challenge with a homologous strain of highly pathogenic (HP) AIV.

2. Materials and methods

2.1. Virus

The A/chicken/British Columbia/314514-1/2004 H7N3 low pathogenic (LP) AIV isolate was used to produce the vaccines, and a related highly pathogenic (HP) AIV isolate (antigenically homologous isolate) was used for challenge: A/chicken/British Columbia/314514-2/2004 H7N3 [8]. These isolates were selected so the vaccine could be prepared with an LP strain for safety and the challenge could be conducted with an antigenically identical but highly virulent (i.e. HP) isolate. Additionally, previous studies with these isolates have shown that they are moderately immunogenic, therefore should better discriminate between adjuvants than isolates at the low or high extremes of immunogenicity [9]. Using standard methods in specific pathogen free (SPF) embryonating chicken eggs (ECE) [10] each isolate was propagated and titrated for use as vaccine antigen, antigen for hemagglutination inhibition (HI) assay, and challenge virus.

2.2. BPL inactivation

The LPAIV (infectious allantoic fluid from embryonating chicken eggs) was inactivated by treatment with 0.1% BPL with incubation at ambient temperatures (approximately 20–23 °C) for 4–6 h with constant mixing, then was incubated overnight at 4 °C [11]. Prior to testing the antigenic content by hemagglutination assay (HA) the pH was adjusted to approximately 7.0 with sodium bicarbonate solution. The HA assay was conducted using standard procedures [12].

2.3. Formalin inactivation

The LPAIV was inactivated by treatment with 0.02% formalin with incubation at 37 °C for 18–24 h [4]. The antigenic content was quantified by standard HA assay [12].

2.4. Vaccine preparation

Each vaccine that was prepared with a commercial adjuvant was made in accordance with the manufacturers recommendations. Commercial oil based (water-in-oil) adjuvants included: Montanide ISA 70VG (70VG) (mineral oil based) (SEPPIC, Inc., Fairfield, NJ), Montanide ISA 71VG (71VG) (SEPPIC), Montanide ISA 760VG (760VG) (synthetic polymer and ester based) (SEPPIC), Montanide 780 VG (780VG) (vegetable oil based) (SEPPIC) and Montanide GEL01 (GEL01) (synthetic polymer based) (SEPPIC) (Table 1). A mineral oil adjuvant that was developed in-house was prepared as described by Stone et al. [13] (Stone adjuvant). Incomplete Freund's adjuvant (IFA) was prepared with a commercial product (Sigma-Aldrich Co, St. Louis, MO). Calcium phosphate (CAP), alginate, and chitosan adjuvanted vaccines were prepared as reported previously [14,15]. Because potency has been shown to vary between 160 and 512 HAU among different AIV isolates [9,16], we used the maximum uniform dose we could achieve taking into account the dilution effect with each adjuvant. Therefore all vaccine formulations were standardized to contain 384 hemagglutinating units (HAU) per dose.

Table 1
Vaccine formulations, routes of administration and treatment group sizes.

Adjuvant	Abbreviation	Adjuvant type	Emulsion type	Route of inoculation[b]	Number of chickens	
					BPL[c] inactivated	Formalin inactivated
Montanide ISA 70 VG	70 VG	Mineral oil	Water-in-oil	SQ	10	NA
Montanide ISA 71 VG	71 VG	Mineral oil	Water-in-oil	SQ	10	20
Montanide ISA 760 VG	760 VG	Synthetic lipophilic polymer/ester	Water-in-polymer	SQ	10	20
Montanide ISA 780 VG	780 VG	Vegetable oil	Water-in-oil	SQ	10	20
Montanide GEL 01	GEL01	Synthetic polyacrylic polymer	None, aqueous	SQ	10	NA
Stone adjuvant	Stone	Mineral oil	Water-in-oil	SQ	10	20
Incomplete Freund's	IFA	Mineral oil	Water-in-oil	SQ	10	NA
Chitosan	NA[a]	Carbohydrate (deacylated chitin)	None	SQ	10	NA
Ca Phosphate	CAP	Mineral nanoparticle	None	SQ	10	NA
Alginate	NA	Seaweed derived	None	SQ	10	NA
Non-adjuvanted virus	NA	NA[a]	NA	IM	10	NA
Non-adjuvanted virus	NA	NA	NA	IV	10	NA
Non-adjuvanted virus	NA	NA	NA	SQ	10	20
Non-vaccinated	NA	NA	NA	NA	10	20

[a] NA = not applicable.
[b] IM = intramuscular; IV = intravenous; SQ = subcutaneous.
[c] BPL = beta propiolactone.