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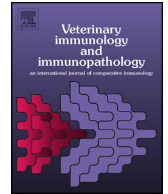

Research paper

Characterization of the *in situ* immunological responses to vaccine adjuvants

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ABSTRACT

Adjuvants are included with many inactivated and some modified live vaccines to enhance immune responses to specific antigens. While early vaccines relied exclusively upon aluminum salts, still the major adjuvant used in human vaccines, other adjuvant products are used in veterinary medicine. In addition to enhancing antigen presentation, adjuvants can also enhance the development of specific immune responses. Thus, alum adjuvants often preferentially stimulate humoral immune responses. By contrast, lipid-based adjuvants are often more effective at stimulating cell-mediated immune responses. Metastim® is a lipid-based adjuvant reported to elicit both humoral and cellular immune responses, though the mechanism responsible for this activity remains unclear. In this study, we compared the ability of equine influenza virus vaccines containing either saline or Metastim® or an aluminum phosphate adjuvant to stimulate antigen presenting cell function *in vivo*. Six ponies were intradermally inoculated with inactivated equine influenza (KY97) mixed with either adjuvant or saline. Multiple sites were injected so that biopsies could be collected at different times post injection. The 4 mm punch biopsies were formalin-fixed, paraffin-embedded, and stained with hematoxylin and eosin (H&E). Total RNA was isolated from 2 mm punch biopsies for the determination of gene expression by real-time PCR. H&E staining revealed a variety of cells recruited to the injection sites, including lymphocytes, neutrophils, eosinophils and macrophages. Real-time PCR analysis of the injection site confirmed this cellular infiltration and identified increased expression of activation markers. Both vaccines also stimulated gene expressions of pro-inflammatory cytokines. The vaccine containing Metastim® elicited significantly higher gene expression of interferon-γ, IL-12, CD4and CD83 compared to alum ($p < 0.05$). While the greater induction of IFNγ-related gene expression indicates that Metastim® can elicit Th-1 immune responses more effectively than the aluminum salt, there was also evidence of Th2 cytokine induction.

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1. Introduction

Immunological protection against intracellular pathogens, including viruses and some bacteria, involves both cellular and humoral immune responses (Minke et al., 2004). The stimulation of an effective immune response requires both antigen presentation and other accessory signals (Ahlers et al., 2003). These accessory signals include both cytokine production and the expression of cell surface antigens on the antigen presenting cell (Mbow et al., 2010). Recent improvements in the overall efficacy of killed vaccines are the result of the ability of newer

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adjuvants to potentiate immune responses (Patel and Heldens, 2009). These adjuvants enhance both humoral and cellular immune responses to the vaccine by increasing antigen processing and presentation (Spickler and Roth, 2003).

Some adjuvants possess stimulatory properties that can influence either T helper1 (Th1), Th2 or regulatory (Treg) cell responses (Mills, 2009). Vaccines containing aluminum salt based adjuvants and are noted for inducing strong Th2 responses (Cox and Coulter, 1997). By contrast, the incorporation of lipid moieties such as monophosphoryl lipid A (MPLA) favor the induction of Th1 responses (Dubensky and Reed, 2010). Liposomes can also serve as effective vehicles for delivering conjugated or unconjugated peptides and proteins to antigen presenting cells for presentation *via* MHC class I and class II pathways (Alving et al., 1995). This is thought to be due in part to enhanced phagocytosis of lipid embedded particles by macrophages and other antigen presenting cells (Copland et al., 2005; Wilson et al., 2009). Whether this also leads to increased expression of accessory molecules and cytokines remains unclear. Metastim® is a lipid-based adjuvant shown to stimulate strong serological and cellular immune responses to various antigens (Wallace, 1996; West et al., 1999). Here, we assessed the ability of vaccine containing Metastim to stimulate both cytokine and accessory antigen expression at the site of injection and compared these results to a similar vaccine using alum (potassium aluminum sulfate) as the adjuvant. We focused on those cytokines involved in pro- (IL1, IL6, TNFα, and monocyte chemoattractant protein-1 (MCP-1)) and anti-inflammatory (IL-10) responses, as well as those involved in Th1- (IL-12, IL-18, IFN-γ) and Th2-induction (thymic stromal lymphopoietin (TSLP)). We also measured mRNA expression of cell surface (CD4, CD8β, Cd163) and activation antigens (CD83, CD86, and ELA-II (equine MHC-II)).

2. Materials and methods

2.1. Horses

Six seronegative yearling ponies of mixed sex that were reared on the Department of Veterinary Science Farm were used in this study. All of the ponies had no prior equine influenza exposure. The ponies were pre-screened for serum antibodies to equine influenza virus by ELISA and hemagglutination–inhibition (HI) assay and confirmed to be seronegative.

2.2. Virus and vaccines

Equine influenza A/equine-2/KY/07 was used as the vaccine antigen in this study. The first vaccine contained Metastim® as the adjuvant (Fluvax Innovator, Zoetis, Inc.). The second vaccine, also provided by Zoetis, contained the same amount of virus and included aluminum phosphate (AlPO4) as the adjuvant. The third vaccine contained the same amount of inactivated virus in saline. The vaccines were identical in appearance. The investigators were blinded as to the vaccine composition until the conclusion of study

2.3. Inoculations and sampling

In order to access the *in situ* response to the vaccines, all three preparations were administered as intradermal injections (100 μl) into the neck of six ponies. The individual injection sites were staggered and 10 cm apart on both sides of the neck to minimize the possibility of lymphatic drainage from one site into another. An additional saline only control was also included for use as a calibrator (see below). Biopsy samples were collected from the different injection sites at 6, 24, 48 and 72 h post inoculation using 2 mm and 4 mm punch biopsy needles. Tissues retrieved from the 2 mm biopsies were placed in RNAlater® (Ambion, Life Technologies, Grand Island, NY) and stored at −20 °C until subsequent isolation of total RNA. Tissue samples collected from the 4 mm biopsies were stored in formalin at room temperature until later processing for histological staining (Liu et al., 2012).

2.4. Tissue homogenization

Total RNA was isolated from the 2 mm biopsy samples as described (Liu et al., 2012). Briefly, each sample was suspended in 500 μl for RNA Stat 60 (Tel Test, Friendswood, TX) and homogenized for three cycles of three minutes duration at 30 Hz/s using a Retsch MM301 (Glenn Mills Inc., Clifton, NJ) tissue homogenizer. Samples were placed on ice for one minute following each cycle. The supernatant was then transferred to a microcentrifuge tube and an additional 500 μl of RNA Stat 60 was added. Samples were stored at −80 °C until further processing.

2.5. RNA Isolation and reverse transcription

Total RNA was isolated from the RNA STAT 60 samples by phenol chloroform extraction (Chomczynski and Sacchi, 1987). Isopropanol was used to precipitate the RNA followed by a 75% ethanol wash after which the RNA pellet was air dried. The RNA pellet was suspended in RNAase free water and sample purity and RNA content was determined spectrophotometrically (Biophotometer, Eppendorf). One microgram of RNA from each sample was reverse transcribed into cDNA using the AMV Reverse Transcriptase (Promega, Madision, Wisconsin). The reactions were incubated at 42 °C for 15 min and then at 95 °C for 5 min using a thermocycler (Biorad Laboratories, Hercules, CA). The cDNA was stored at −20 °C until real-time PCR was performed.

2.6. Primer/probe sequences and RT-PCR

Relative cytokine gene expression was determined using equine specific, intron-spanning primer probes (Life Technologies) for the following: IL1, IL-5, IL6, IL-10, IL-12, IL-13, IL-18 IFN-γ, MCP-1, TNFα and TSLP. Relative expression of the following cell surface antigens was also performed: CD4, CD8β, CD83, CD86, Cd163, and ELA-II. As

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