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Research paper

CoVaccine HT™ adjuvant is superior to Freund's adjuvants in eliciting antibodies against the endogenous alarmin HMGB1

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ABSTRACT

Adjuvants are used to enhance the immune response against specific antigens for the production of antibodies, with the choice of adjuvant most critical for poorly immunogenic and self-antigens. This study quantitatively and qualitatively evaluated CoVaccine HT™ and Freund's adjuvants for eliciting therapeutic ovine polyclonal antibodies targeting the endogenous alarmin, high mobility group box-1 (HMGB1). Sheep were immunised with HMGB1 protein in CoVaccine HT™ or Freund's adjuvants, with injection site reactions and antibody titres periodically assessed. The binding affinity of antibodies for HMGB1 and their neutralisation activity was determined *in-vitro*, with *in vivo* activity confirmed using a murine model of endotoxemia. Results indicated that CoVaccine HT™ elicited significantly higher antibody tires with stronger affinity and more functional potency than antibodies induced with Freund's adjuvants. These studies provide evidence that CoVaccine HT™ is superior to Freund's adjuvants for the production of antibodies to antigens with low immunogenicity and supports the use of this alternative adjuvant for clinical and experimental use antibodies.

1. Introduction

The passive administration of antibodies (Abs) has been effective in rapidly targeting foreign antigens and inflammatory mediators to limit the progression of potentially life threatening and/or debilitating infections and diseases. This practice has extended to direct pathogen and toxin neutralisation, reversal of drug toxicity, depletion of immune effector cells or molecules as well as the inhibition of host cell function in cancer therapy (Tolstrup et al., 2006; Casadevall, 1996, 1999; Tallman, 2002; de Cassan et al., 2011; Buonaguro et al., 2011). This approach is also widely applicable to complex disease scenarios such as autoimmune disease (Harrison, 2008; Keijzer et al., 2013), inflammatory diseases (Kazatchkine & Kaveri, 2001), neurodegenerative disorders (Brody & Holtzman, 2008; Schwartz & Kipnis, 2001; Fisher et al., 2001) as well as traumatic stress or injury (Schwartz & Kipnis, 2001; Hauben et al., 2001; Hauben & Schwartz, 2003) in which therapies are targeted to self-antigens.

Antibodies are generated by the immune system's T helper and B cells responding to the presentation of normally foreign antigen epitopes which causes B cell maturation, antigen-specific antibody (Ab) production and the induction of memory cells. This classical immune response enables an enhanced reaction upon rechallenge with the same foreign antigen and is a mechanism exploited for the rapid production of Abs for clinical or experimental purposes. However, the generation of Abs against self-antigens is much more difficult as exposure of the immune system to self-antigens instigates immune tolerance, a response which operates to prevent auto-Ab production and autoimmune disease in the host. These tolerogenic mechanisms need to be interrupted to be able to produce Abs for passive immunotherapeutic approaches (Bachmann et al., 1993).

Adjuvants are commonly used in vaccine production to enhance the immune response to the often weakly immunogenic immunising antigen (Jennings, 1995; Fraser et al., 2007), which include self-antigens, but are also used to increase the overall yield (Petrovsky & Aguilar, 2004). The use of Freund's adjuvants (FA) for experimental purposes has remained the 'gold standard' for producing potent Abs against soluble antigens. Complete Freund's adjuvant (CFA), commonly used for the first immunisation, consists of heat killed *mycobacterium tuberculosis* and mannose monooleates in paraffin oil. This elicits a Th-1 dominated inflammatory response (Billiau & Matthys, 2001) and often results in granuloma formation at the site of administration. Incomplete Freund's adjuvant (IFA), which lacks the mycobacterial components, is the adjuvant of choice for subsequent immunisations as it induces a Th-2

Abbreviations: CV, CoVaccine^{HT}; FA, Freund's adjuvant; CFA, Complete Freund's adjuvant; IFA, Incomplete Freund's adjuvant; SFASE, sucrose fatty acid sulphate ester; HMGB1, high mobility group box-1; Ab, antibody; Abs, antibodies.

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dominated response. The intense inflammatory response and localised granuloma formation of FA have been associated with the formation of distant sub-pleural hepatic and renal granulomas (Broderson, 1989; Johnston et al., 1991; Leenaars et al., 1994), necrotising-dermatitis (Steiner et al., 1960), as well as spinal cord compression from an injection site granuloma (Kleinman et al., 1993). The extent of inflammation, necrosis, pain and distress associated with FA limits its use to experimental purposes only (Petrovsky & Aguilar, 2004; Bodewes et al., 2010). Consequently, the use of alternative adjuvants is highly encouraged, particularly those that can improve the welfare of animals used for immunisation regimens (Petrovsky & Aguilar, 2004). One such adjuvant is CoVaccine HT™ (CV), an oil-in-water adjuvant which consists of sucrose fatty acid sulphate ester (SFASE) immobilised on oil droplets of a submicron emulsion of squalane-in-water (Bodewes et al., 2010). The effectiveness of the aqueous formulation is similar to water-in-oil based adjuvants with no severe or persistent side effects at the site of injection, thus making it safe for even human use. Indeed, we and others have shown that CV employed in production of Abs against viral antigens is strongly immunogenic (Stevens et al., 2013) and safe (Kusi et al., 2011) while it also produces cell mediated and intense humoral immunity to poor immunogens (de Cassan et al., 2011) in large, non-rodent species (Stevens et al., 2013; Blom & Hilgers, 2004; Draper et al., 2010; Hilgers & Blom, 2006).

In this study the use of CV and FA were evaluated for the production of therapeutic polyclonal Abs targeting the endogenous alarmin, High Mobility Group Box-1 (HMGB1) protein. High Mobility Group Box-1 is a highly conserved nuclear protein involved in DNA replication, transcription, and tissue repair (Diener et al., 2013; Landsman & Bustin, 1993), however it has also emerged as a key mediator in the regulation of immune responses to infection and injury (Schaper et al., 2014). Furthermore HMGB1 influences the development of auto-immune disorders (Tang et al., 1799), chronic inflammatory diseases (Abdulahad et al., 2011) and cellular processes that can dictate disease progression in cancer (Tang et al., 1799; Sims et al., 2010). As extracellular HMGB1 has recently been implicated as a key late inflammatory mediator in sepsis, where it regulates multiple pathways associated with pathogenesis of the disease, it is therefore considered an important potential therapeutic target for clinical evaluation (Diener et al., 2013; Yang et al., 2006). In this report, we show that CV induced significantly higher Ab tires, with a stronger affinity than Abs induced with FA. These Abs also proved to provide potent protection in a murine model of endotoxemia. Most importantly, the negative side effects of immunisation were minimal, thus increasing the welfare of host animals without compromising Ab yield. Therefore these results provide evidence that CV is superior to FA for the production of Abs against poor immunogen rHMGB1, highlighting the prospective advantages of using CV in the production of Abs for experimental and/or clinical use.

2. Materials and Methods

2.1. Recombinant HMGB1 production

The HMGB1 mRNA sequence was cloned from cDNA prepared from healthy human donor PBMC's using custom primers with appropriate restriction sites and a C-terminal 6× His-tag. The sequence was cloned into the pFastBac™1 vector from the Bac-to-Bac expression system (Invitrogen) and the construct used to produce recombinant baculovirus according to the manufacturer's instructions. Briefly; insect cells (sf21) were maintained in sf900 SFM III (Invitrogen) supplemented with L-Glutamine (100 μg/mL), penicillin (100 U/mL), gentamycin (100 μg/ml) and HEPES (10 mM, pH 7.2) in roller bottle flasks at 27 °C. Insect cells at 2×10^6 cells/mL were infected for 96 h prior to protein harvest. Cultures were supplemented with L-glutamine 100 μg/mL 24 h prior to protein harvest to enable maximum protein yield. Expressed protein was purified from cell lysate using Ni-NTA agarose beads (Qiagen) according to the manufacturer's instructions before dialysis in PBS. Protein purity was analysed by SDS-PAGE and anti-HMGB1 western blot. Concentration was determined by BCA assay (Thermo Fisher Scientific).

2.2. Immunisation and sample collection

For FA administration purified recombinant HMGB1 (rHMGB1) antigen (200 μg) was diluted in PBS and emulsified with an equal volume of CFA (Sigma.) for prime immunisation and IFA (Sigma.) for boost immunisation 14 and 28 days later. A sample of the resulting emulsion (10ul) was extruded into PBS and stored at room temperature to assess stability of the emulsion. The droplet was inspected daily for signs of dissolution. Groups of 2 year old Border Leister x Merino ewes were immunised with the 2 mL emulsion subcutaneously, at 4 axillary sites (500 μL at each site). The CFA and IFA used for the immunisations were commercially sourced from Sigma Aldrich (catalogue number: F5881 and F5506 respectively), and are comprised of 15% mannide monooleate in paraffin oil with the addition of 1 mg/mL heat-killed and dried Mycobacterium tuberculosis (ATCC 25177) for complete Freund's adjuvant. For CV (kindly provided by BTG Plc (UK)) administration 200 μg or 20 μg rHMGB1 in PBS was gently mixed with an equal quantity of CV (40 mg/mL) and sheep were immunised with 2 mL emulsions subcutaneously at 4 axillary sites (500 μL at each site). Sheep were administered a prime immunisation and boosted every 14 days for a total of 2 boosts at sites adjacent to prime immunisations. Serum was taken prior to each immunisation and stored at −20 °C for later analysis. Sheep that received either 200 μg or 20 μg of rHMGB1 in CV were routinely monitored for immunisation site reactions by trained veterinary staff for bleeding and ulceration consistent with animal welfare requirements and in addition, size of the local reaction measured with callipers and site surface temperature was measured with a laser thermometer (FLUKE IR thermometer 561). At the end of the immunisation period sheep were anaesthetised with 5% Isoflurane in oxygen, exsanguinated via the jugular vein, then after the right atrium/anterior vena cava stops bleeding, sheep are humanely killed under anaesthesia with 35 ml of 4 M KCl and serum collected stored at −20 °C for IgG purification.

2.3. Endpoint anti-HMGB1 Ab ELISA

Stored serum samples from immunised sheep were used for Ab endpoint analysis as previously desscribed (Frey et al., 1998). Briefly, high-binding EIA/RIA ELISA plates (Costar) were coated with100μL rHMGB1 (5 μg/mL) overnight at 4 °C in a bicarbonate buffer (pH 9). Plates were washed (3 times) with PBS, blocked with 2% (*w*/*v*) BSA (Sigma) in PBS for 1 h at 37 °C, and then washed with PBS-T (PBS + 0.05% Tween). Thawed pre-immune and hyper-immune serum was diluted 1:50,000 and 100 μL added to duplicate wells and further serially diluted 1:4, with plates subsequently incubated for 2 h at 37 °C. Plates were washed 3 times with PBS-T before bound ovine Abs were detected with HRP conjugated anti-ovine IgG (Sigma); 1:10,000 at 37 °C for 1 h. Plates were washed with PBS-T three times and developed using SIGMAFAST ™ OPD substrate (Sigma: A3415-1ML). The reaction was stopped with 3 M HCL and the OD read at 490 nm by absorbance spectroscopy. The endpoint titre of the samples are the reciprocal of the highest dilution that has a reading above the cut off which is calculated as mean + 2 standard deviations of negative serum samples.

2.4. Purification of ovine polyclonal anti-HMGB1 from serum

Stored serum from CV and FA immunised sheep was thawed and filtered through glass wool. The Abs were precipitated by addition of saturated ammonium sulfate solution to 45% (*v*/*v*). After centrifugation (20 min, 10,000 x g) pelleted protein precipitate was dissolved in milliQ water and dialysed into PBS before application to a Protein G agarose column (Thermo Fisher Scientific). Bound IgG was eluted with 0.1 M