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IMPROVING THE DROMEDARY ANTIBODY RESPONSE: THE HUNT FOR THE IDEAL CAMEL ADJUVANT

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ABSTRACT

Research has highlighted the benefits of camels as a model of immunological disease and as a production source of single domain antibodies. However, even after decades of research, little investigation has been conducted into the most suitable vaccine adjuvant for camel antibody production. Through different modes of action, adjuvants enhance the immune response to a co-injected antigen, although often at the price of increased local and systemic adverse reactions. In this study, we sought to find the best adjuvant for camel vaccines, capable of inducing high vaccine immunogenicity but without excess reactogenicity. We tested 7 different adjuvants, with a viral (African Horse Sickness Virus; AHSV) or a bacterial (*Burkholderia mallei*) antigen. Antigen-specific antibody responses and measures of reactogenicity (inflammation, skin thickness and pyrogenicity) were assessed. As previously reported with other species, oil-based emulsion adjuvants such as Gerbu Vet and Montanide ISA enhanced antigen-specific antibody production but suffered from high reactogenicity. By contrast, two newer particulate adjuvants, the polysaccharide-based adjuvant, Advax HCXL™, and the Poly-gamma-glutamic acid adjuvant, Montanide PGA were well tolerated and non-reactogenic. Of all the adjuvants, Advax HCXL™, showed the most favourable overall response, enhancing high levels of specific antibody to both AHSV and *B. mallei* whereas Montanide PGA only induced antibodies to AHSV but not against *B. mallei*. This study identified at least one promising non-reactogenic vaccine adjuvant with high potential for future use in camels and related species.

Key words: Adjuvant, advax, antibody, camel, dromedary, gerbu and alum, inflammation, montanide

Immunological research on the camel has highlighted the benefits of this animal as a model for pathogenic diseases and as a potent source of antibody production (Abbas and Agab, 2002) including the development of novel antisera, serological testing methods, and even biomarkers (Deffar *et al,* 2009). Despite the utility of camel antibodies, little published research is available on the most suitable adjuvants for camel immunisation. This problem is not specific to this species alone but with adjuvant research in general, which lags behind other areas of vaccine research and development (Petrovsky and Aguilar, 2004).

With the use of an appropriate adjuvant it is possible to control and enhance the immune response to an antigen. Unfortunately, however, the immune-enhancing benefits of adjuvants are often outweighed by negatives including reactogenicity (local inflammatory side effects) and/or systemic toxicity (Petrovsky and Aguilar, 2004; Petrovsky, 2008).

In 1936, Freund developed the most potent known adjuvant - Freund's complete adjuvant (FCA), which comprises of a water and mineral oil emulsion containing killed mycobacteria (Freund *et al,* 1937; Stuart-Harris, 1969). Although FCA has long been used in medical and veterinary applications to maximise vaccine responses, its utility is limited by severe reactogenicity and toxicity (Chapel and August, 1976; Edelmen, 1980). Subsequent detuned modifications - Freund's incomplete adjuvant (FIA: water in oil emulsion without the added mycobacteria) is slightly better tolerated although still reactogenic (Gupta and Sibera, 1995).

At the Central Veterinary Research Laboratory (CVRL) in Dubai, both FCA and FIA had been previously used to enhance antibody production in the dromedary camel (Cook *et al,* 2010). Unfortunately, this use was associated with severe inflammatory reactions at the injection site, after sub-cutaneous inoculation with either FIA or FCA (U. Wernery, unpublished data).

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Subsequently, Gerbu Veterinary Adjuvant (Gerbu Vet) from Gerbu Biotechnik was tested in camels. Gerbu Vet similarly induced a severe inflammatory lesion in some camels, although by contrast with FCA or FIA this was largely acute and resolved within several months without abscess formation (U. Wernery, unpublished data). However, to maximise antibody production, a camel must be re-inoculated multiple times with up to 6 booster vaccine doses before a suitable antibody titre can be reached (Deckers *et al,* 2009). So far, the only adjuvant approved globally for human use is aluminium hydroxide (Alum) (Vogel and Powell, 1995). Alum is relatively well tolerated in most species although it can cause fibrosarcomas in dogs and cats (Hendrick *et al,* 1994) and there is concern that excess aluminium could be associated with neurodegenerative diseases such as Alzheimer's disease (Petrovsky and Aguilar, 2004).

In this paper we report the results of a study to identify a suitable non-reactogenic adjuvant for enhancement of vaccine-induced antibody production in the dromedary camel. The aim was to identify a suitable camel adjuvant able to stimulate high antigen-specific antibody production against both bacterial and viral antigens but without local injection site reactions or systemic toxicity.

Materials and Methods

Adjuvant Selection

Seven adjuvants were tested from 4 different companies specialised in adjuvant development. Modes of action for each adjuvant are discussed in the Analysis and Discussion.

1. Gerbu Vet from Gerbu Biotechnik, Germany was used as a comparison control, since its use and effects on the dromedary camel are already known. Gerbu Vet comprises biodegradable liposomes of cationised lipid (emulsifier WS160 without GMDP; Gerbu Product Catalogue).
2. Gerbu Pharma, also from GERBU Biotechnik Adjuvant consists of biodegradable cationised lipid nanoparticles, with lecithin as emulsifier and Glucosaminylmuramyl dipeptide (GMDP) as an immunostimulator (Gerbu Product Catalogue)
3. Montanide ISA 763 A VG (SEPPIC Ltd., France) is water in oil emulsion, which are continuously in oil phase. The oil used is not specified but is said to be a non-mineral, naturally occurring plant derivative (SEPPIC Product Catalogue).
4. Montanide IMS 3012 VG PR (SEPPIC) was designed especially for sensitive animals and is supposedly well tolerated (SEPPIC Product Catalogue).
5. Montanide Pet Gel A (PGA) (SEPPIC) is a water-based adjuvant specialised for use in companion animals such as dogs, cats and horses. It is claimed to be well tolerated and consists of a high molecular weight polyacrylic polymer in water (SEPPIC Product Catalogue).
6. Advax Horse and Camel Excel (Advax HCXLTM) adjuvant was developed by Vaxine Pty Ltd (Australia) and formulated especially for the use in horses and camels. It is an immunostimulatory particulate adjuvant made from nanocrystalline inulin, which is a polysaccharide particle consisting of linear chains of fructose and glucose (Petrovsky, 2006).
7. Sigma Aldrich Plc.'s Aluminium Hydroxide Gel was also included as a comparator adjuvant

Antigens

Two antigens were selected, one bacterial and the other viral. Both consisted of formalin-inactivated whole cell preparations. The bacterial antigen was derived from *Burkholderia mallei*, a zoonotic disease causing Glanders in equids and humans (Lehavi *et al,* 2002). The viral antigen was African Horse Sickness Virus strain 4 (AHSV4), a virus that causes a highly infectious and deadly disease of equids, spread by insect vectors.

Burkholderia mallei antigen preparation

Micro-bank vials of *B. mallei* (DXB Stain 7), stored at -20°C, were removed and pellets extracted. These were inoculated in 5ml brain heart infusion broth with 3% glycerol then incubated for 24hrs at 37°C. Using aseptic techniques, a single *B. mallei* colony was isolated from the agar onto a plastic loop and re-suspended in 1ml PBS with 0.9% NaCl. The suspension was vortexed until homogeneity was seen. One µl of 10% formalin was then added to the suspension and re-vortexed. The antigen for the first trial was stored at this stage for 4 years at -20°C, before its use in this trial; the antigen for the second trial was used straight after its preparation or stored for up to 6 weeks at 4°C. To ensure that the formalin had inactivated all bacteria, the suspension was inoculated on blood agar and confirmed to be free of any bacterial growth.

AHSV4 antigen preparation

AHS virus serotype 4 was isolated in 2007 from lung and spleen of a horse that died from

AHS in Kenya. The strain was confirmed and serotyped by IAH, Pirbright. The virus was cultured on BHK21 and Vero cells, and both tested positive by PCR. The AHSV was passageds five times in roller bottle cultures of BHK21 cells. This was then plaque-purified on Vero cells by selecting the largest plaque at terminal dilution. The final plaque material was passaged 4 times through Vero cell cultures and freeze dried in 2ml glass vials, making up the master seed virus. The master seed was checked for contamination using routine microbiological culturing methods. The inactivated vaccine was prepared according to House *et al* (1994) and the OIE Manual (2008).

Animals

A total of 18 dromedaries were used, stabled at CVRL (Table 1). Camels were checked for antibodies and had no prior immunity to either *B. mallei* or AHSV prior to this study. All were of different ages and genders, which could not be controlled due to a lack of numbers. Camels were kept in outdoor pens with shaded areas, fed twice a day on grain and hay and once a week on fresh alfalfa.

Inoculant Preparation

Adjuvants and antigen solutions were removed from their various storage conditions for inoculant preparation. Mixtures were prepared the afternoon before immunisation. Solutions were checked the day after for layers to guarantee proper mixing of the adjuvant and the antigen. Non-oily adjuvants and antigen solutions were measured out using P1000/P200 Gilson pipettes, however highly viscous adjuvants such as Alum and Gerbu Vet were measured out using a P5000 Gilson pipette. For the preparation of the mixtures containing adjuvants with antigen, the adjuvant was mixed with a ratio as specified by the supplying companies (Table 1). Most adjuvants, for instance, specified a 1:1 mix with the corresponding antigen but Montanide IMS required a 7:3 mix with the antigen (70% adjuvant). Adjuvants and antigens were mixed up in a volume of 2.4 ml to ensure that a 2ml immunisation dose could be easily drawn up and injected. After combining the adjuvant and antigen, the solution was thoroughly mixed by vortexing and then left overnight at 4°C before use the following day. Adjuvant-antigen mixtures were drawn into 10 ml injection syringes prior to inoculation.

Camel Immunisation

Eight camels (group 1) were injected with antigen-adjuvant mixtures on either side of the neck (AHSV antigen one side, *B. mallei* antigen the other side). Another 8 camels (group 2) were injected with pure adjuvant (without antigen) on one side. The last 2 camels (controls) were injected with antigen alone (without adjuvant) on one side (Table 1).

Individual camels were lead either into a camel box, or had their heads restrained and tied to the perimeter fence during the procedure. The side of the neck was shaved into an injection window. The temperature at the centre of the injection window was measured using a Terminator Multi-Thermo™ skin thermometer. Skin thickness was measured using a dial calliper, capable of measuring skin thickness to 1mm. After taking the measurements, the centre of the injection window was cleaned using 70% ethanol and circled with a marker pen to show the specific injection site (Fig 1). Camels were inoculated subcutaneously within the circled area at the centre of the shaved window.

Camels receiving adjuvanted-AHSV4 were given a single booster dose 3 weeks post-inoculation. This process was the same for the first *B. mallei* trial (Table 1). However, in the second *B. mallei* trial camels were boosted weekly to a total of 5 booster doses, from immunised camels at regular time points using standard blood collection procedures from the jugular vein into Vacutainer tubes.

Inflammatory Response Measurement

Camels were assessed for inflammatory responses every other day for a total of 2 months post-immunisation. They were tied to their pen's perimeter fence and their heads restrained. The injection site was thoroughly checked for inflammation at the centre and around the site of inoculation. If inflammation was observed, the width and length was measured with a digital calliper, and recorded. Skin thickness was measured at the injections site centre using a specialised dial calliper, and only if the skin was loose enough to measure. If the skin was too tight due to inflammation, then the camel was marked +++. If the skin was tight due to other reasons (such as angle of camel neck) then it was marked as ++. The skin temperature at the injection site centre was also measured. Blood was taken weekly for serological testing, to assess antibody production.

Antibody Detection

Two competitive ELISA's (cELISA) were used for the detection of antibodies produced to AHSV and *B. mallei* antigens. Sera samples taken from the camels during the trial were titrated to give dilution

Table 1. Inoculation regime showing the injection volume and the preparation of each adjuvant-antigen solution. These were injected into either the right of left side of the chosen camel. n/a stands for 'not added' and indicates that either the adjuvant or the antigen was not mixed. No. of injections indicates primary inoculation and the boosters that followed. In the case of Glanders, two trials were conducted, and therefore the number of injections for both trials are shown.

Group	No.	Pen	Camel ID	Side	Adjuvant	Antigen	Volume Prepared (ml)		Volume Injected (ml)		No. of
							Adjuvant	Antigen	Adjuvant	Antigen	injections
Group 1	1	6	MAX	Left	Gerbu Pharma 1	AHSV4	1.2	1.2	1.0	1.0	2
				Right	Montanide IMS	Glanders	1.68	0.72	1.4	0.6	2&5
	2	1	6B1	Left	Gerbu Pharma	Glanders	1.2	1.2	1.0	1.0	2
				Right	Montanide IMS	AHSV4	1.68	0.72	1.4	0.6	2
	3	6	601	Left	Gerbu Vet	Glanders	1.2	1.2	1.0	1.0	2
				Right	Montanide ISA	AHSV4	1.2	1.2	1.0	1.0	2
	4	1	5BF	Left	Gerbu Vet	AHSV4	1.2	1.2	1.0	1.0	2
				Right	Montanide ISA	Glanders	1.2	1.2	1.0	1.0	2
	5	3	F93	Left	Advax	Glanders	1.2	1.2	1.0	1.0	2&5
				Right	Alum	AHSV4	1.2	1.2	1.0	1.0	2
	6	2	973	Left	Advax	AHSV4	1.2	1.2	1.0	1.0	2
				Right	Alum	Glanders	1.2	1.2	1.0	1.0	2&5
	7	5	OCF	Left	Gerbu Pharma 2	Glanders	1.2	1.2	1.0	1.0	2&5
				Right	Montanide Pet Gel A	AHSV4	0.24	2.16	0.2	1.8	2
	8	6	782	Left	Gerbu Pharma 2	AHSV4	1.2	1.2	1.0	1.0	2
				Right	Montanide Pet Gel A	Glanders	0.24	2.16	0.2	1.8	2&5
Group 2	9	2	6A5	Right	Gerbu Pharma 1	n/a	1.2	0	1.0	n/a	2
	10	2	C6C	Right	Gerbu Pharma 2	n/a	1.2	0	1.0	n/a	2
	11	2	54A	Left	Gerbu Vet	n/a	1.2	0	1.0	n/a	2
	12	2	05E	Left	Montanide IMS	n/a	1.2	0	1.0	n/a	2
	13	2	91F	Left	Montanide ISA	n/a	1.2	0	1.0	n/a	2
	14	5	146	Right	ALum	n/a	1.2	0	1.0	n/a	2
	15	5	F7B	Left	Montanide Pet Gel A	n/a	1.2	0	1.0	n/a	2
	16	6	Brown	Left	Advax	n/a	1.2	0	1.0	n/a	2
	17	5	E2A	Right	n/a	AHSV4	n/a	1.2	n/a	1.0	2
Control	18	5	CDE	Right	n/a	Glanders	n/a	1.2	n/a	1.0	2&5

end points to determine the level of antibodies. Inhibition values of higher than 50% were regarded as positive. Once cELISA's yielded titration values for the samples, a percentage absorbance rate was measured using a spectrophotometer.

Results

Inflammatory Response

Major inflammation was observed for 5 out of 7 adjuvants, both in the AHSV4 trial (Fig 3A) and in the *B. mallei* trial (Fig 3B). The only adjuvants not associated with significant inflammation were Advax HCXL™ and Montanide PGA. In the control trial (Fig 3C) inflammation was observed for 4 out of 7 adjuvants injected without antigen, with Advax HCXL™, Alum and Montanide PGA again not being associated with inflammation. Most inflammation peaked between 12 and 20 days post-inoculation and resolved between 28 and 32 days. On average, *B. mallei* antigen induced a larger inflammation area than AHSV4. Control animals (antigen injected without adjuvant) were negative for inflammation over the course of the trial.

In the AHSV4 trial, Gerbu Vet and Montanide ISA induced the worst inflammatory reactions with average inflammation area peaking at 64 cm^2 and 68

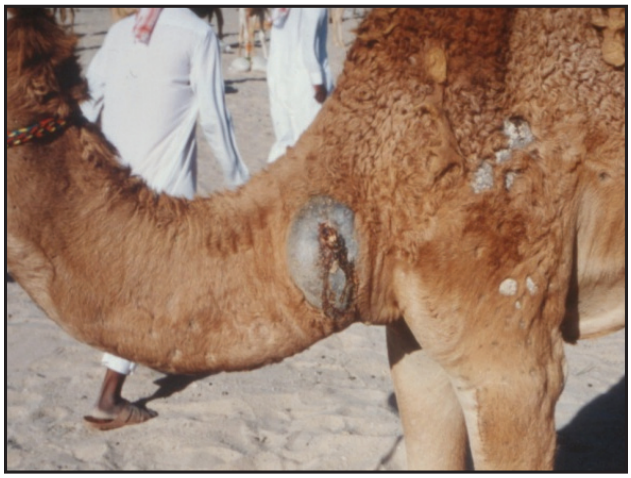

Fig 1. Picture showing a severe local reaction caused by Freund's Complete Adjuvant.

Fig 2. Picture showing the inoculation site prepared for the trial. Black circle indicates specifically where an adjuvant mixture was injected.

cm^2, respectively, soon after the 15th day. This was followed by Gerbu Pharma, which peaked at 54 cm^2 on the 14th day. Montanide IMS and Alum caused minor inflammation. Montanide PGA and Advax HCXLTM did not induce any inflammation of any kind.

In the *B. mallei* trial, Montanide ISA induced the worst reaction by far (Fig 4), with more than double the peak inflammatory area (158 cm^2) of the next most reactogenic adjuvant, Gerbu Vet (68 cm^2). The inflammation caused by Montanide ISA did not resolve even after 31 days. Gerbu Pharma and Gerbu Vet had similar average inflammatory responses peaking at 68 cm^2. Alum and Advax HCXL™ induced very little, and Montanide IMS and Montanide PGA were not associated with any, inflammation.

In the control trial (adjuvants without antigen), Montanide ISA induced the most severe inflammatory response with an average peak of 82 cm^2 followed by Gerbu Vet with an average peak of 60 cm^2. Montanide IMS and PGA produced only minor inflammatory responses and Gerbu Pharma, Alum and Advax HCXL™ produced no inflammation.

Comparing Adjuvant Inflammatory responses with Changes in Skin Thickness and Temperature

Gerbu Pharma, Gerbu Vet and Montanide ISA gave the highest average inflammatory responses when mixed with the AHSV4 antigen (Fig 5A) or the *B. mallei* antigen (Fig 5B). All 3 also induced a large increase in skin thickness, Gerbu Vet being the highest for AHSV4 antigen (90%) and Montanide ISA for *B. mallei* antigen (73%). Gerbu Pharma and Gerbu Vet induced a large average increase in temperature at the injection site compared to the control (antigen without adjuvant) in both trials. Montanide ISA induced a large temperature increase in the *B. mallei* trial but not in the AHSV trial. However, the highest average temperature increase was caused by Montanide IMS mixed with AHSV4 antigen although it was only associated with a small inflammatory response and increase in skin thickness.

Alum caused no major inflammatory response, when mixed with either AHSV4 antigen or *B. mallei* antigen, and only a small average increase in temperature compared with the control in the AHSV4 trial. In the *B. mallei* trial however, Alum caused a fairly large increase (53%) in skin temperature when compared to the control (Fig 5B). For both antigens, Advax HCXLTM and Montanide PGA induced no significant inflammatory response or increase in skin thickness. Both adjuvants induced only a small increase in temperature when mixed with *B. mallei* compared to the control. In the AHSV4 trial, Montanide PGA showed no inflammatory response, change in temperature or change in skin thickness.

A reactogenicity index (Fig 6) was constructed in order to summarise and compare the toxicity of each adjuvant. Maximum skin temperature, skin thickness and maximum area of inflammation were standardised to the same approximate level so that they each had an equal weight in the overall reactogenicity score. Gerbu Pharma and Montanide ISA were associated with the highest reactogenicity index. Alum, although it caused little change in inflammation and skin thickness, induced a high maximum temperature increase, which put

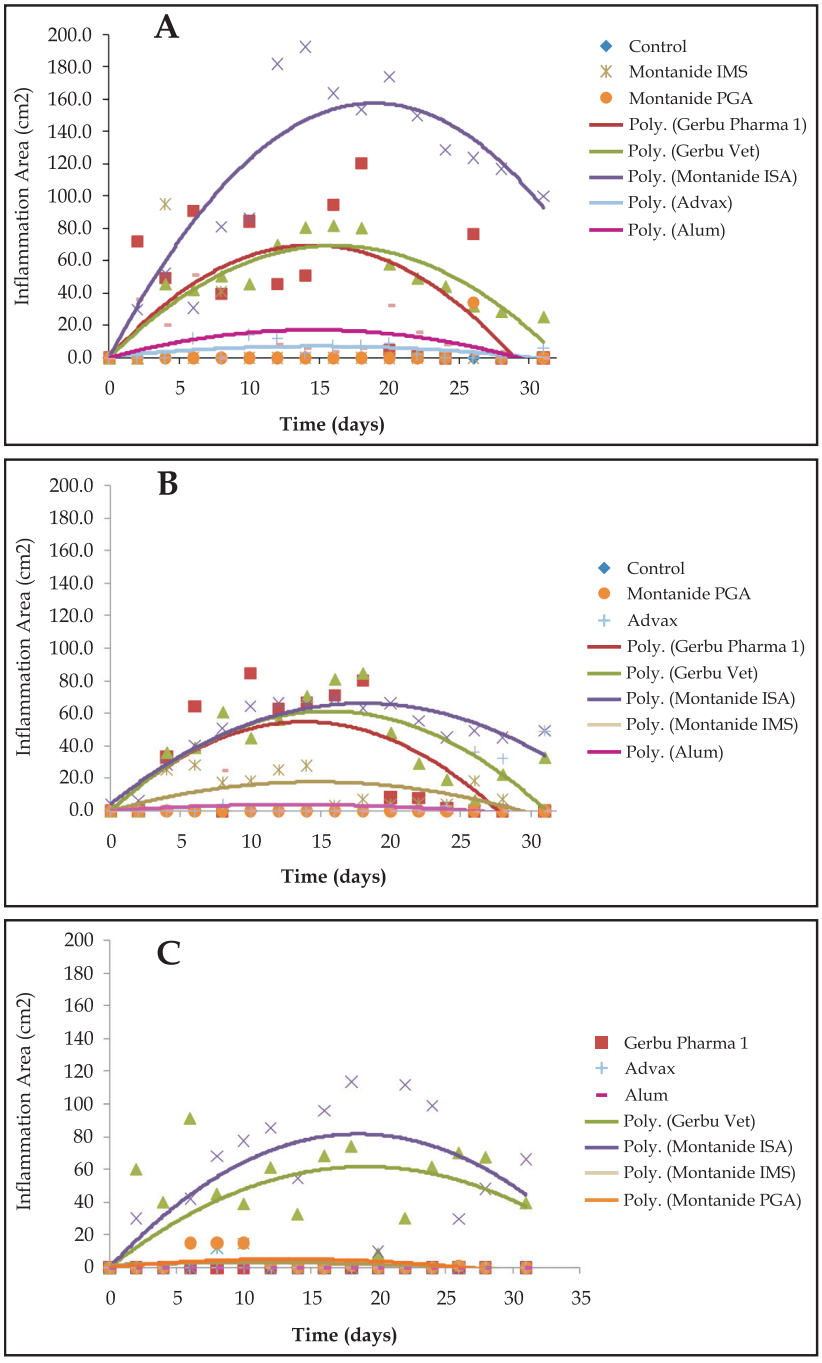

Fig 3. Line graphs showing the inflammatory response of different adjuvants mixed with (A) AHS4 antigen, (B) B. mallei antigen and (C) without any antigen added, over time. Inflammatory response was measured in terms of area (cm2) of inflammation observed every second day for 32 days. Trend lines indicate an average positive inflammatory response. Control refers to antigen without added adjuvant.

its reactogenicity above that of Advax HCXLTM, Montanide IMS and PGA. Advax HCXLTM was associated with a reactogenicity score well below that of Montanide PGA and equal to that of the control injection. This indicates Advax to be the best tolerated of all the adjuvants tested in the trial.

Comparing Adjuvant Inflammatory Responses and Antibody Production

Montanide ISA (Fig 7) induced the most severe inflammatory responses of all the adjuvants used: average inflammation area was 110.3 cm^2 for *B. mallei*, 47.0 cm^2 for AHSV4 and 58.1 cm^2 without antigen (control). However, it also gave the highest antibody response of all adjuvants to AHSV4 (77.8 % absorbance; Fig 8).

Gerbu Vet induced the second highest inflammatory response with an inflammation area of 45.7 cm2 for *B. mallei*, 39.5 cm^2 for AHSV4 and 49.3 cm^2 without antigen. It gave the third highest antibody response to AHSV4 of all the adjuvants tested with 64% absorbance.

Gerbu Pharma did not induce inflammation when injected without antigen, but induced a high inflammatory response (29.9 cm^2 for AHSV4 and 45.6 cm2 for *B. mallei*) when injected with the antigens. It gave a good average antibody output to AHSV (53% absorbance).

Montanide IMS, Montanide PGA, Alum and Advax HCXL™ all induced only very minor or no inflammatory responses in all three trials. However, for some adjuvants this may simply reflect a lack of adjuvant potency, as animals immunised with Montanide IMS, did not produce any detectable antibody response to either *B. mallei* or AHSV4.

The first *B. mallei* trial was unsuccessful in that no antibody production was seen with any of the initial adjuvants tested. This was either because the antigen used was too old and had degraded or because an insufficient number of booster doses were administered. Because of this, a second *B. mallei* trial was conducted with new *B. mallei* antigen, following the original CVRL protocol (Deckers *et al*, 2009) whereby one booster dose is given every week for 6 weeks instead of one dose every 3 weeks. As the unsuccessful *B. mallei* trial and AHSV trial had already identified particular

Fig 4. Picture showing a severe local reaction (circled black) induced by Montanide ISA mixed with *B. mallei* antigen, during this trial. Inflammation, pyrogenesis, alopecia, dermatitis and a large increase in skin thickness was recorded was observed.

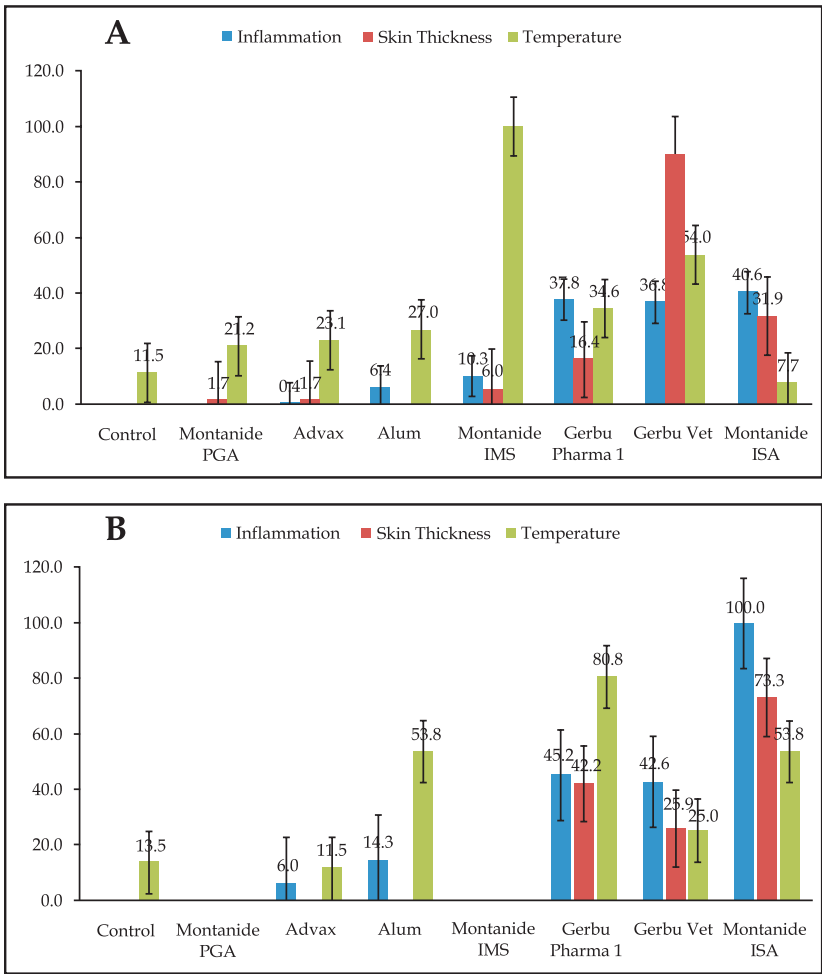

Fig 5. Bar graphs showing a comparison between the local inflammation area, increase in skin thickness and increase in local skin temperature caused by different adjuvants mixed with (A) AHSV4 antigen and (B) *B. mallei* antigen. All three parameters are measured as an arbitrary percentage where the highest value counts as 100%. This was done in order to compare small value changes such as skin thickness changes with large value changes such as inflammation area. Thus, for example, Montanide ISA with AHSV induced the greatest inflammation and was assigned a score of 100% whereas the area of inflammation induced by Gerbu Pharma 1 with *B. mallei* was 45.2% of the area induced by Montanide ISA.

adjuvants with excessive reactogenicity (Montanide ISA, Gerbu Vet and Gerbu Pharma) these adjuvants were not used in the second *B. mallei* trial to avoid such major adjuvant-associated inflammation. In particular it was feared that the reactogencity of these adjuvants would be further magnified by the planned six-booster regime and this would be too traumatic to the camels. Thus, only Montanide IMS, Montanide PGA, Alum, and Advax HCXLTM were used in the second *B. mallei* trial.

Out of the 4 adjuvants used in the second *B. mallei* trial, only Advax HCXLTM induced a measurable antibody response against *B. mallei* antigen. The Advax HCXLTM adjuvant gave an extremely high antibody response to *B. mallei* of 70.5% absorbance despite almost complete absence of inflammation or reactogenicity.

Analysis and Discussion

Modes of Action of Adjuvants

The adjuvants selected for the use in this trial have different chemical composition and modes of action, reflected in their differing immunogenicity and propensity to induce local reactions. Gerbu Vet comprises biodegradable liposomes of cationised lipid (emulsifier WS160 without GMDP; Gerbu Product Catalogue). Liposomes can augment both humoral and cellular immunity as well as extend the biological half-life of the antigen. They can also trap antigen and enhance the APC (Cox and Coulter, 1997). However, liposome formulations are well recognised to be associated with increased vaccine reactogenicity, as was observed in this study. Gerbu Pharma adjuvant has been used for human use and is claimed in its product catalogue to produce a good immune response without local reactions, including in "very sensitive animals such as horses and camels" (Gerbu Product Catalogue). The lipid nanoparticles are claimed to improve antigen presentation to APC and the GMDP additive is meant to modify endothelial and macrophage activity, and is thereby claimed to

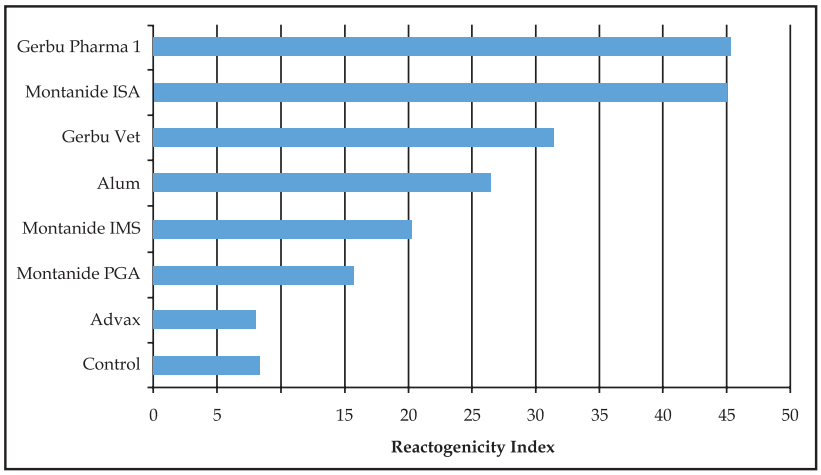

Fig 6. Bar graph showing adjuvants scored using a Reactogenicity Index. This index was derived using the following formula: 3(ΔTMax) + (IMax)/10 + (ΔSTMax) where Δ=change, TMax = Maximum Temperature, IMax = Maximum Inflammation and STMax = Maximum Skin Thickness.

promote a Th2 type immune response and increased antibody production (Bomford *et al,* 1992). Whilst the low reactogenicity of Gerbu Pharma adjuvant was confirmed when it was injected alone, this was not true when it was mixed with antigen. The high reactogenicity observed suggested an unfavourable interaction between the adjuvant and the antigens used.

Montanide ISA is an emulsified oil adjuvant that forms an antigen depot at the injection site, and is thereby claimed to promote a sustained immune response (Montanide Product Catalogue). Like liposomes, oil emulsion adjuvant formulations are also well recognised as being associated with increased reactogenicity. Montanide IMS is a water dispersal composition containing immunologically active organic compounds and emulsified liquid nanoparticles. This formulation is claimed to induce a fast onset of immunity and a balanced Th2/Th1 response (Montanide Product Catalogue). Montanide PGA is a water-based adjuvant, which consists of a high molecular weight polyacrylic polymer in water. It is claimed to be well tolerated in sensitive animals with induction of a sustained and strong immune response (Montanide Product Catalogue). Whilst this claimed lack of reactogenicity was found to be true in our study, it also proved to be an ineffective adjuvant for either AHSV4 or *B. mallei* vaccines.

Aluminium hydroxide has been the preferred adjuvant for animal and human vaccines for over 80 years. It has a high safety record in terms of local reactions, apart from in dogs and cats where it can cause fibrosarcomas. Through activation of NALP3, Alum induces a Th2 type immune response giving a good antibody output although it is very poor at inducing cellular immunity (Brewer *et al,* 1999).

Advax HCXLTM is a custom-designed horse and camel adjuvant based on delta inulin adjuvant with an immunostimulatory component which has an excellent safety and tolerability record in many animal species including horses (Lobigs *et al,* 2010) as well as being in development for human use (Petrovsky, 2006). AdvaxTM was designed to boost both antibody and cellular immune responses without reactogenicity and have been shown to enhance both Th1 (cellular) and Th2 (humoral) immune responses without the induction of IgE (Petrovsky and Aguilar, 2004).

Adjuvant inflammation: Induction to Resolution

The adjuvants tested produced a wide spectrum of inflammatory responses. Adjuvant-associated inflammation typically peaked between days 10 and 12 and in most, but not all, cases resolved by day 31. Inflammation is normally induced by activation of immune cells including macrophages, T cells and granulocytes migrating to the site of trauma and producing inflammatory cytokines such as interleukin (IL-1) and tumour necrosis factor (TNF)-α plus inflammatory free radicals (Serhan and Savill, 2005). The more immune cell and, in particular, granulocyte activation there is at the site of adjuvant inoculation, the higher the expected inflammatory response (Mosser, 2003). Oil-based adjuvants such as Montanide ISA and Gerbu Vet are highly viscous and are irritant to macrophages and neutrophils coming in contact with the oil droplets, thereby forming a long-term inflammatory depot at the inoculation site. This would explain why oil-based adjuvants like Montanide ISA and Gerbu Vet induced severe inflammation at the site of inoculation, even without the addition of an antigen.

Local inflammatory reactions to adjuvant-active compounds in the absence of antigen have been previously reported. Inoue *et al* (2005) found that diesel exhaust particles without antigen non-specifically induced IgE production. IgE induces inflammation via the activation and release of histamine and heparin from mast cells, in an allergic

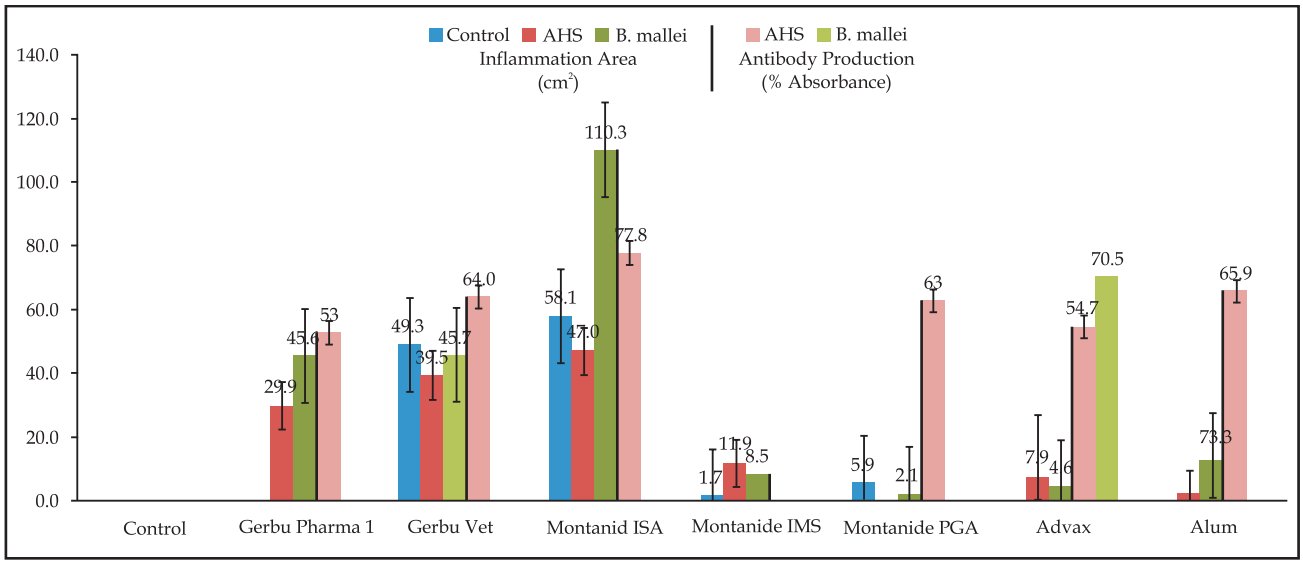

Fig 7. Bar graph showing the effect of different adjuvants on inflammation area and antigen-specific antibody production when mixed with AHSV4 antigen and *B. mallei* antigen, respectively. Inflammation caused by adjuvants without added antigen (control) is also included. Black line represents the partition between inflammation area and antibody production. Inflammation area was measured in cm^2 and antibody production was measured in percentage spectophotometric absorbance of ELISA sample.

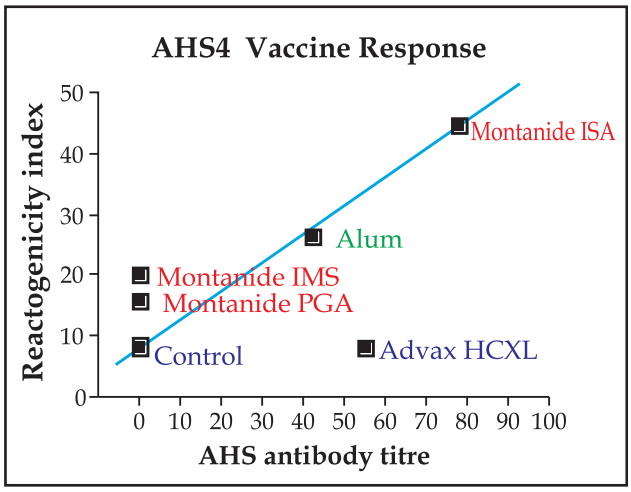

Fig 8. A plot of reactogenicity index against final AHSV4 antibody titres achieved at the end of the study demonstrates that Montanide ISA induces a strong antibody response but is equally strongly reactogenic. Alum, while less reactogenic than Montanide ISA is also less immunogenic and thereby sits on the same reactogenicity: immunogenicity line connecting the results for the control injection with those for Montanide ISA. Advax HCXL™ is the sole exception as it lies well to the right of the typical reactogenicity: immunogenicity line, being able to induce a strong antibody response to AHSV4 in the absence of any significant reactogenicity.

reaction (Inoue *et al,* 2005). It is possible, although not tested here, that Montanide ISA and Gerbu Vet might do the same. Given their high reactogenicity, oil emulsion adjuvants such as Montanide ISA or Gerbu Vet are relatively contraindicated as camel adjuvants, making identification of alternative better-tolerated adjuvants a major priority.

Correlations between Adjuvant-induced Inflammation, Skin Thickness and Pyrogenesis

Inoculation sites in control animals (antigen without adjuvant) showed a small increase in skin temperature with similar values for both *B. mallei* and AHSV antigen, even though no inflammation or change in skin thickness was observed. This small increase in skin temperature is most likely attributed to minor pyrogens such as cell wall lipopolysaccharide (LPS) in the antigen preparations. Therefore, increases in skin temperature caused by adjuvants were only counted if they were significantly above that of the control injections.

The oil emulsion adjuvants, Montanide ISA, Gerbu Vet and Gerbu Pharma, which induced severe inflammation, also caused an increase in skin thickness and temperature at the injection site in the case of both antigens. By contrast, Advax HCXLTM and Montanide PGA caused little or no inflammation, and were not associated with an increase in skin thickness or temperature. Surprisingly, Montanide IMS, which did not induce inflammation or an increase in skin thickness, caused the highest skin temperature increase of all adjuvants when mixed with AHSV4. This was not the case when it was mixed with *B. mallei*. Montanide IMS failed to induce any antibodies to *B. mallei* or AHSV4, which means it did not induce an immune response and thus the

elevation in temperature may not have been caused by the adjuvant, but rather by a coincidental infection.

If the results of Montanide IMS are discounted, a positive correlation between inflammation, skin pyrogenesis and the increase in skin thickness is apparent. The relationship between inflammation and skin temperature increases is not surprising and has been reported previously. During infection, pyrexia or pyrogenesis is a common defence mechanism, designed to inhibit the growth of the invading pathogen (Mackowiak, 1981). Increased skin thickness has mainly been reported during chronic rather than acute inflammation (Gaffney and Casley-Smith, 2005). It was hard to distinguish the difference between acute and chronic inflammation in this study, as some of the adjuvants caused major acute swelling that then lasted for up to one month post-inoculation. It is possible that during this prolonged period, the animal dermis responded to the inflammation by increasing its thickness. Since pyrogenesis and increased skin thickness were highly correlated with inflammation, the local skin temperature or skin thickness could potentially be used instead of measuring inflammation size to assess the reactogenicity of an adjuvant in future adjuvant assessment trials

Differences in Antigenic Effects on Inflammation

In general, it was observed that most adjuvants mixed with *B. mallei* produced greater inflammation than when mixed with AHSV4. Hence, not just the adjuvants but the particular antigens used in a vaccine formulation may contribute in an additive or synergistic fashion to vaccine reactogenicity.

B. mallei is the cause of the disease Glanders in equids and humans, with symptoms including nodular lesion in the lungs and ulceration of the mucous membranes (Drovak and Spickler, 2008). AHSV causes serious pulmonary oedema as its main symptom (Wolbach, 1912). *B. mallei* initiates an inflammatory response whereas AHSV causes lung congestion without initiating inflammation. This may explain why *B. mallei* antigen induced a larger inflammatory response than AHSV. This emphasises the importance of testing an adjuvant's activity with a variety of different antigens, to allow the proper assessment of its safety and reactogenicity.

Antigens injected without adjuvants did not induce any inflammatory responses or antibody production. This observation, similar to those that led to the discovery of adjuvants over 80 years ago (Ramon, 1925) is the reason why adjuvants are so important to a vaccine's ability to generate strong protective immunity.

Relationship between Adjuvant Reactogenicity and Antibody Production

Montanide ISA and Gerbu Pharma induced the most severe inflammation followed by Gerbu Vet. Montanide ISA also produced the highest antibody production to AHSV, followed by Gerbu Vet and Gerbu Pharma. Montanide IMS, which did not induce any inflammatory response to AHSV, also failed to produce antibodies to this antigen. There is a clear strong positive correlation between severity of adjuvant-induced inflammation and antibody production. Hence, in general, the more severe the local reaction caused by the adjuvant, the more antibodies were produced to the co-administered antigen. Similar observations have been used to support the so-called "danger" hypothesis, advanced by Polly Matzinger which proposes that the only means to induce an immune response is to create tissue damage and thereby danger signals which serve to alert and activate the innate immune system (Matzinger, 2002). The link between strong adjuvant potency and reactogenicity has been accepted since the time that adjuvants were first developed, leading to the presumption within the vaccine community that adjuvant potency is inseparable from inflammation and reactogenicity (Petrovsky *et al,* 2007; Petrovsky, 2008).

As shown in Figure 9 Alum fitted this reactogenicity: immunogenicity relationship, being weaker than the oil emulsion adjuvants on both counts. However, Advax HCXLTM adjuvant defied this trend as it significantly enhanced antibody production to AHSV4 and *B. mallei* while inducing almost no local inflammatory reaction. Thus Advax HCXLTM is a notable exception to the danger hypothesis, being able to induce high antibody responses to both viral and bacterial antigens without the need for inflammation or a danger signal. This finding suggests the existence of novel immune pathways whereby adaptive immune responses to vaccine antigens can in fact be enhanced without the need for inducing tissue damage or an immune danger signal (Petrovsky, 2008). Uncritical acceptance of the danger hypothesis by the immunology and vaccine adjuvant communities diverted focus towards identification of ever more reactogenic adjuvants in the search for greater vaccine potency, when the true goal should have been to look for better tolerated adjuvants, such as Advax HCXLTM, still able to

enhance vaccine immunogenicity without suffering the safety or tolerability problems of adjuvants that rely upon delivery of danger signals for their effectiveness.

Conclusion

The results show that a single adjuvant, Advax HCXLTM was able to deliver on both counts, inducing high antibody titre against both *B. mallei* and AHSV antigens in camels while not inducing any significant inflammatory reaction. While Montanide PGA and Alum both enhanced antibody titres against AHSV without excess inflammation, they failed to induce antibody against *B. mallei*. Montanide ISA, Gerbu Vet and Gerbu Pharma all suffered from excess reactogenicity causing severe inflammation at the injection site, although they did enhance antibody titres to AHSV4. Montanide IMS exhibited no adjuvant activity and did not enhance antibodies against either antigen. Advax CXLTM therefore proved in this study to be the best all-round, effective and well-tolerated camel adjuvant. This study emphasises the importance of the vaccine community reassessing the merits of the danger hypothesis particularly, if the full benefits for animals and humans of the discovery of safer and better-tolerated vaccine adjuvants are to be realised in the future.

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