

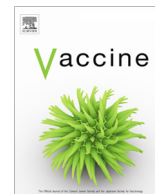
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## Carbohydrate fatty acid monosulphate esters are safe and effective adjuvants for humoral responses



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### ABSTRACT

Carbohydrate fatty acid sulphate esters (CFASEs) formulated in a squalane-in-water emulsion are effective adjuvants for humoral responses to a wide range of antigens in various animal species but rise in body temperature and local reactions albeit mild or minimal hampers application in humans. In rabbits, body temperature increased 1 °C one day after intramuscular (IM) injection, which returned to normal during the next day. The effect increased with increasing dose of CFASE but not with the number of injections (up to 5). Antigen enhanced the rise in body temperature after booster immunization ( $P < 0.01$ ) but not after priming. Synthetic CFASEs are mixtures of derivatives containing no sulphate, one or multiple sulphate groups and the monosulphate derivatives (CMS) were isolated, incorporated in a squalane in-water emulsion and investigated. In contrast to CFASE, CMS adjuvant did not generate rise in body temperature or local reactions in rabbits immunized with a purified, recombinant malaria chimeric antigen R0.10C. In comparison to alum, CMS adjuvant revealed approximately 30-fold higher antibody titres after the first and >100-fold after the second immunization. In ferrets immunized with 7.5 µg of inactivated influenza virus A/H7N9, CMS adjuvant gave 100-fold increase in HAI antibody titres after the first and 25-fold after the second immunisation, which were 10–20-fold higher than with the MF59-like AddaVax adjuvant. In both models, a single immunisation with CMS adjuvant revealed similar or higher titres than two immunisations with either benchmark, without detectable systemic and local adverse effects. Despite striking chemical similarities with monophospholipid A (MPL), CMS adjuvant did not activate human TLR4 expressed on HEK cells. We concluded that the synthetic CMS adjuvant is a promising candidate for poor immunogens and single-shot vaccines and that rise in body temperature, local reactions or activation of TLR4 is not a pre-requisite for high adjuvanticity.

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### 1. Introduction

Novel adjuvants are needed for the new generation of well-defined antigens to improve the level and duration of immunity and to reduce the number of non-responders. Of particular interest is their use in emergency vaccines thereby reducing the dose of antigen and the number of injections. Avoiding the need for a booster reduces costs, time-to-immunity and complexity of vaccination campaigns, which are critical factors in regions with poor infrastructure and in emergency situations. Furthermore, one instead

of multiple vaccinations results in lower overall risks of adverse events associated with vaccination.

Synthetic carbohydrate fatty acid sulphate esters (CFASEs)-based adjuvants are known for several years to effectively enhance humoral responses to a wide range of antigens [1–8] in different animal species. Multiple cycles of lead-finding and optimisation focused on efficacy/toxicity (E/T)-ratio and structure-response-relationship in large, non-rodent species, resulted in a series of CFASE adjuvants. Incorporated into a squalane-in-water emulsion, CFASEs exerted strong synergy [1–4] and outperformed oily adjuvants with respect to efficacy, safety and quality [4,9–13], which is unprecedented for an aqueous adjuvant formulation.

The first generation of CFASE adjuvants consisted of polysaccharide derivatives and is exploited in single-shot vaccines for veteri-

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nary purposes [3] and investigated in a cancer vaccine [14,15]. The second generation based on a disaccharide known as 'CoVaccine HT™ [4–6] reached phase IIa clinical trial with two therapeutic, peptide-protein vaccines [16,17]. Drawbacks of these CFASEs are rise in body temperature one day after injection and local reactions at the site of injection albeit mostly mild or minimal. To annihilate these adverse effects while retaining adjuvanticity, we examined several factors influencing E/T-ratio including dose of adjuvant and antigen, number of injections and chemical structure of the active molecule. Results on a third generation of carbohydrate derivatives are reported here.

## 2. Materials and methods

### 2.1. Antigens

Three different antigens were used to assess *in vivo* performance of the adjuvants. A peptide-protein conjugate (gonadotropin-releasing hormone peptide coupled to keyhole limpet haemocyanin; 'GnRH-KLH') prepared for nonclinical GLP toxicity studies as described by Turkstra et al. [13] was used at 400 and 800 µg of protein per dose. A chimeric recombinant malaria antigen R0.10C composed of a fragment of *Plasmodium falciparum* glutamate rich protein fused with a correctly folded fragment of Pfs48/45, produced in *Lactococcus lactis* and immune-purified ([18]; Radboud UMCN, Nijmegen, The Netherlands) was used at 10 µg protein per dose. Whole virion A/H5N1 and A/H7N9 grown on cell culture, inactivated and purified, were kindly provided by National Health Research Institute of Taiwan and Medigen Vaccine Biologicals Corp. (Taipei, Taiwan) and used at 7.5 µg HA per dose.

### 2.2. Adjuvants

CFASE was prepared as described by Blom and Hilgers [4]. Briefly, maltose dissolved in pyridine plus N,N-dimethylformamide was contacted with one equivalent of SO<sub>3</sub>pyridine and 8 equivalents of decanoylchloride. Monosulphate derivatives (CMS) and polysulphate derivatives (CPS) were isolated from the CFASE by preparative liquid chromatography on silica with 12 v/v% triethylamine and 12 v/v% 2-isopropanol in n-heptane (all from Sigma-Aldrich, St. Louis, MO) as eluent. Fractions containing either CMS or CPS as determined by thin layer chromatography were pooled and dried and purity was determined by LC-MS.

Adjuvant formulations were prepared by passing mixtures of 40 g of CFASE or CMS or CPS, 80 g of squalane (Bayer, Germany), 40 g of Polysorbate 80 (Montanox 80 PPI; Seppic, Paris, France) and 840 g phosphate buffered saline (PBS; Fisher Scientific) three times through a high-pressure emulsifier (Microfluidizer Y110, Microfluidics, Newton, USA). The submicron emulsions obtained were passed through a 0.2 µm filter (PES Supor EKV, Pall Life Sciences, Portsmouth, UK), stored in sterile containers at 4 °C and tested as described below.

Alhydrogel (Brenntag Biosector, Frederikssund, Denmark) and the MF59-like adjuvant AddaVax (InvivoGen, Toulouse, France) were used as benchmarks.

Vaccines were prepared by mixing antigen, adjuvant and PBS at appropriate volume ratio one day before administration. Prefilled 1-mL syringes with 0.5 mL (plus 0.1–0.2 mL excess) of either vaccine, equipped with 25G needles, labelled with animal ID, study ID and date were supplied to the animal facility.

The dose of adjuvant was 22 mg of CFASE or 8 mg of CMS or 8 mg of CPS (22 mg CFASE contains approximately 8 mg CMS and 8 mg of CPS), 0.2 w/v% Alhydrogel or 50 v/v% of AddaVax.

### 2.3. Animals

SPF Female rabbits (New Zealand White, Harlan Laboratories, UK), 3–6 months of age were housed individually with supply of tap water *ad libitum* and 100 g food per animal per day.

Male ferrets (undisclosed provider in Denmark), 6–12 months of age and negative for relevant (circulating and vaccine) influenza strains (A/H7N9, A/H1N1, A/H3N2 and B) and Aleutian Disease virus, were housed in standard group cages with *ad libitum* supply of tap water and food (Hope Farms, Ferret super).

The animals were randomly assigned to treatment groups using a generalized randomized block design on the base of body weight. Animals were checked daily for overt signs of disease and treatment-related adverse events.

### 2.4. Ethical statement

The use of animals was performed in accordance with the regulations set forth by the relevant national and/or local Ethical Committee for Animal Experimentation and in accordance with European Community Directive 86/609. All the techniques and procedures were refined to provide for minimal discomfort and stress to the animals.

### 2.5. Immunization and blood collection

Test items in 0.5 mL were injected IM two times alternating in the left and right thigh muscle at 3-week interval, unless stated otherwise. At different time intervals before and after each immunization blood samples of 1–5 mL were collected and rectal body temperature was measured.

### 2.6. Antibody titres to malaria in rabbits

Antibody titres in serum samples were measured by Radboud UMCN (Nijmegen, the Netherlands) by ELISAs with purified R0.10C or gametocyte extract as coating antigens. Microtiter plates (Sterilin® ELISA plates, Netherlands) were coated overnight, blocked with 5% milk in PBS, incubated with serial dilutions of serum samples in PBS-0.05% Tween 20 (PBST) for 4 h at room temperature, incubated with anti-rabbit IgG-HRP (H + L) (DAKO, Denmark) and developed with tetramethyl benzidine (TMB) substrate solution for 20 min at room temperature. The colour reaction was stopped with 0.2 N H<sub>2</sub>SO<sub>4</sub> and the optical density was read at 450 nm in a Microplate Reader (Labtec BV, Germany). After each incubation step plates were washed extensively with PBST – 0.5 M NaCl. A positive and negative control serum were included in each test to confirm validity of the test. The antibody concentration was expressed as the EC50-value calculated by using GraphPad Prism (GraphPad Software, USA).

### 2.7. Antibody titres to H7N9 in ferrets

Antibody responses to H7N9 in ferrets were determined by Viroclinics Biosciences BV (Rotterdam, the Netherlands) as described elsewhere [19]. Briefly, serum samples were pre-treated with chelera filtrate resulting in a pre-dilution of 1:6 and serially two-fold dilutions were tested in the HAI test using 2:6 reassortant (A/Anhui/1/2013 H7N9 and A/PR/8/34) as described elsewhere [20].

### 2.8. Safety assessment in rabbits

Rectal body temperature of rabbits was measured 6 h before and 3 or 6, 24, 48 and 96 h after each immunization. Local reactions were determined by palpation and visual inspection during

the life phase and by necropsy 7 days after the second immunization (i.e. 28 days after the first immunization) as described elsewhere [21]. The local reaction score at necropsy takes into account the size and nature of the reaction as well as the time interval between treatment and necropsy.

### 2.9. *In vitro* activation of human TLR4 expressed on HEK cells

CMS adjuvant was tested for its potential to activate human TLR4 as described elsewhere [22,23]. Briefly, human endothelial kidney (HEK293) cells transfected with TLR4-encoding construct (InvivoGen, San Diego CA) and with reporter vector expressing luciferase under the control of an NF- $\kappa$ B-responsive promoter (InvivoGen) were plated in flat-bottom 96-wells plates at  $1 \times 10^5$  cells/well. After incubation for 16 h at 37 °C with test substance in triplicate, cells were lysed in Steady Glo luciferase buffer (Promega Benelux, Leiden, The Netherlands) and bioluminescence was measured using a Packard 9600 Topcount Microplate Scintillation & Luminescence Counter (Packard Instrument, Meriden, CT). As positive control for NF- $\kappa$ B-mediated activation 25 ng/ml TNF- $\alpha$  (PeprroTech, London, U.K.) was used. As positive control for TLR4 activation, 1 and 0.01  $\mu$ g/ml of LPS (Sigma-Aldrich, St. Louis, MO) was included. Mean values, SDs and variances were calculated for each compound tested in triplicate.

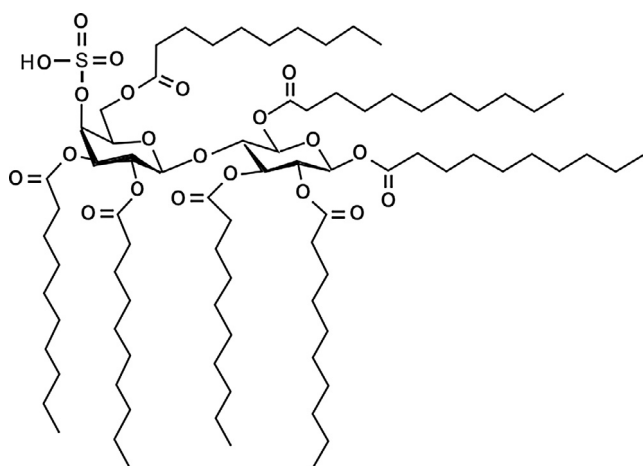
### 2.10. Data processing and statistical analysis

The animal studies were conducted as blind, benchmark-controlled, comparative studies and treatments of individual animals were disclosed only after collection and quality approval of the data. For each group the geometric mean antibody titre (GMT) of the 2Log-values of individual results, standard deviation (SD), antilog-value (2 power GMT) and factor of increase were calculated. Two-tailed Students' *t* test at  $P$ -value  $\leq 0.05$  was selected to establish statistical difference between groups.

## 3. Results

### 3.1. Effect of CFASE on body temperature in rabbits

In a repeated-dose study in rabbits, groups of 6 animals were injected IM five times at 2-week intervals with 0, 20 or 40 mg of CFASE adjuvant ('CoVaccine HT') plus 0, 400 or 800  $\mu$ g of GnRH-KLH as model antigen. After each injection, body temperature was monitored (Fig. 2 and Table 1).



**Fig. 1.** Chemical structure of CMS molecule maltose 4'-monosulphate 1,2,3,6,2',3',6'-heptadecanoic acid ester.

Three h post-treatment, none of the groups exhibited significantly increased body temperature. Twenty-four h post-treatment, animals injected with CFASE adjuvant with or without antigen demonstrated an overall increase in body temperature of 0.9 °C ( $P < 0.01$ ), which disappeared during the next day. Forty mg of CFASE gave 0.1 °C higher body temperatures than 20 mg ( $P < 0.01$ ). CFASE adjuvant plus antigen induced on average 0.4 °C higher body temperature than CFASE adjuvant alone after the second up to the fifth ( $P < 0.01$ ) but not after the first injection ( $P > 0.14$ ). Rises in body temperature after the 5 consecutive treatments remained constant.

### 3.2. Fractionation of CFASE and *in vivo* pilot study

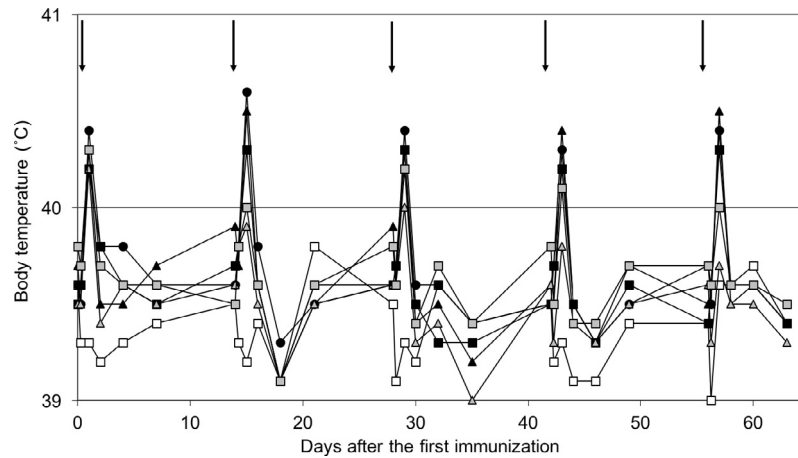
CFASE of maltose was fractionated into derivatives with no sulphate, one sulphate per disaccharide (maltose fatty acid monosulphate esters; 'CMS') or two or more sulphates per disaccharide (maltose fatty acid polysulphate esters; 'CPS'). CMS and CPS were 96% pure as measured by LC-MS. In a pilot study with inactivated H5N1 in rabbits CFASE, CMS and CPS formulated in squalane-in-water emulsions gave comparable increases in HAI antibody titres. H5N1 alone already gave rise in body temperature of 0.5–1.0 °C one day post-vaccination, which was not further increased by either adjuvant (data not shown). One week after the second immunization, local reaction score (i.e. the product of the volume of abnormal muscular tissue multiplied by an arbitrary value of 1 for mild reactions such as discoloration, loss of structure and oedema, 3 for moderate reactions such granuloma, mild inflammation and connective tissue, or 9 for severe reactions such as necrosis and abscess, see reference 21) with CPS adjuvant was >100-fold higher than with CFASE adjuvant, CMS adjuvant or alum (data not shown). *In vitro* CPS adjuvant but not CMS adjuvant exerted strong haemolytic activity (data not shown) and therefore, we decided to continue with CMS.

### 3.3. Adjuvant activity of CFASE, CMS and alum to a recombinant malaria chimeric antigen in rabbits

A safety and immunogenicity study was conducted with a recombinant, chimeric malaria recombinant protein. Effects of CFASE adjuvant (CoVaccine HT™), CMS adjuvant and alum on the response to R0.10C and gametocyte extract were compared. After the first immunization with no adjuvant or alum, no significant or low ELISA antibody titres to R0.10C and gametocyte extract were observed. CFASE and CMS adjuvant gave 31 and 20 fold increases in antibody titres to R0.10C (Fig. 3a) and 2 and 5 fold to gametocyte extract (Fig. 3b), respectively. After the second immunization, responses increased significantly in all groups. CFASE and CMS adjuvant generated >100-fold higher antibody titres to both R0.10C and gametocyte extract as compared to antigen alone and 50-fold higher as compared to alum. Responses with CMS and CFASE adjuvant were similar and were significantly higher than with alum ( $P < 0.001$ ) or without adjuvant ( $P < 0.0001$ ). No significant increase in body temperature was noted with CMS adjuvant ( $P > 0.50$ ) or alum ( $P > 0.14$ ) after the either immunization (Fig. 3c). CFASE adjuvant gave increases of about 0.5 °C after both the first and second immunization, which were significantly higher than without adjuvant ( $P < 0.003$  and  $P < 0.004$ , respectively) or with CMS adjuvant ( $P < 0.034$  and  $P < 0.006$ , respectively).

### 3.4. Effects of CMS and AddaVax to inactivated whole-virion H7N9 in ferrets

In addition to a subunit, CMS adjuvant was tested in combination with inactivated whole virus. Groups of 6 ferrets were injected



**Fig. 2.** Rectal temperature in rabbits after five injections at 2-week intervals with combinations of 0, 20 or 40 mg CFASE adjuvant plus 0, 400 or 800  $\mu$ g of GnRH-KLH. Each point represents the mean of a group of 6 animals. The arrows indicate the moment of IM injection. Group 1: 0 mg CFASE adjuvant + 0  $\mu$ g GnRH-KLH (open squares); Group 2: 20 mg CFASE adjuvant + 0  $\mu$ g GnRH-KLH (grey triangle); Group 3: 40 mg CFASE adjuvant + 0  $\mu$ g GnRH-KLH (grey square); Group 4: 20 mg CFASE adjuvant + 400  $\mu$ g GnRH-KLH (black triangle); Group 5: 20 mg CFASE adjuvant + 800  $\mu$ g GnRH-KLH (black square); Group 6: 40 mg CFASE adjuvant + 800  $\mu$ g GnRH-KLH (black circle).

**Table 1**

Summary of the effects of different combinations of CFASE adjuvant and GnRH-KLH antigen on body temperature 24 h post injection in rabbits represented in Fig. 2.

		Mean body temperature ( $^{\circ}$ C) $\pm$ SD 24 h post immunization 1–5 (group number is indicated between parentheses)			
		Dose of GnRH-KLH antigen			
		0 $\mu$ g	400 $\mu$ g	800 $\mu$ g	Total
Dose of CFASE	0 mg	39.34 $\pm$ 0.15 (1)			39.34 $\pm$ 0.15
	20 mg	39.92 $\pm$ 0.19 (2)	40.40 $\pm$ 0.10 (4)		40.16 $\pm$ 0.29
	40 mg	40.12 $\pm$ 0.13 (3)	40.26 $\pm$ 0.05 (5)	40.42 $\pm$ 0.11 (6)	40.27 $\pm$ 0.16
	Total	39.79 $\pm$ 0.37	40.33 $\pm$ 0.11	40.42 $\pm$ 0.11	

Groups of 6 female rabbits were injected intramuscularly five times 2 weeks apart with 0, 20 or 40 mg CFASE adjuvant plus 0, 400 or 800  $\mu$ g GnRH-KLH antigen. Body temperature ( $^{\circ}$ C) was measured 24 h after each of the 5 injections and arithmetic mean values  $\pm$  SDs of in total 30 observations per group are represented.

IM at Day 0 and 21 with 7.5  $\mu$ g of inactivated, whole-virion influenza virus strain A/H7N9 with or without adjuvant. At the start of the study, none of the animals had detectable antibodies to H7N9 in serum pre-diluted 5-fold. Three weeks after the first immunization animals developed substantial levels of anti-H7N9 antibodies in serum (Fig. 4). Immunization without adjuvant resulted in a GMT of 6 (mean  $^2$ log-value of  $2.7 \pm 0.8$ ). AddaVax gave 5-fold ( $P < 0.004$ ) and CMS 100-fold ( $P < 0.001$ ) higher HAI antibody titres.

The second immunization increased the antibody titres in all but 2 animals by a factor of at least 2. One animal in the group without adjuvant and one in the AddaVax adjuvant group did not demonstrate increased HAI titres. Three weeks after the second immunization, GMT without adjuvant was 96 (mean  $^2$ log of  $6.6 \pm 2.3$ ) with AddaVax 335 (mean  $^2$ log-value of  $8.4 \pm 1.1$ ) and with CMS adjuvant 2429 (mean  $^2$ log-value of  $11.3 \pm 0.3$ ). CMS adjuvant but not AddaVax generated significantly higher titres than antigen alone ( $P < 0.0001$  and  $P = 0.116$ , respectively). Responses with CMS adjuvant were significantly higher than with AddaVax ( $P < 0.001$ ). In comparison to antibody levels prior to the second immunization, boosts with no adjuvant, AddaVax or CMS adjuvant resulted in 15, 11, or 4-fold increases in GMT, respectively.

To assess the persistence of circulating antibodies, anti-H7N9 HAI antibody titres were measured again 21 weeks after the first immunization (Day 147). GMTs without adjuvant, with AddaVax or CMS adjuvant were 23, 108 and 532, respectively. The response to CMS adjuvant was significantly higher than to AddaVax ( $P < 0.001$ ) and the latter was significantly higher than to antigen alone ( $P < 0.01$ ). The calculated half-life of the HAI antibodies in the period from day 42 to day 147 varied between 6 and 8 weeks.

Interestingly and observed also with the malaria subunit in rabbits, a single immunization with CMS adjuvant gave similar or higher titres than two immunizations with the benchmark or without adjuvant.

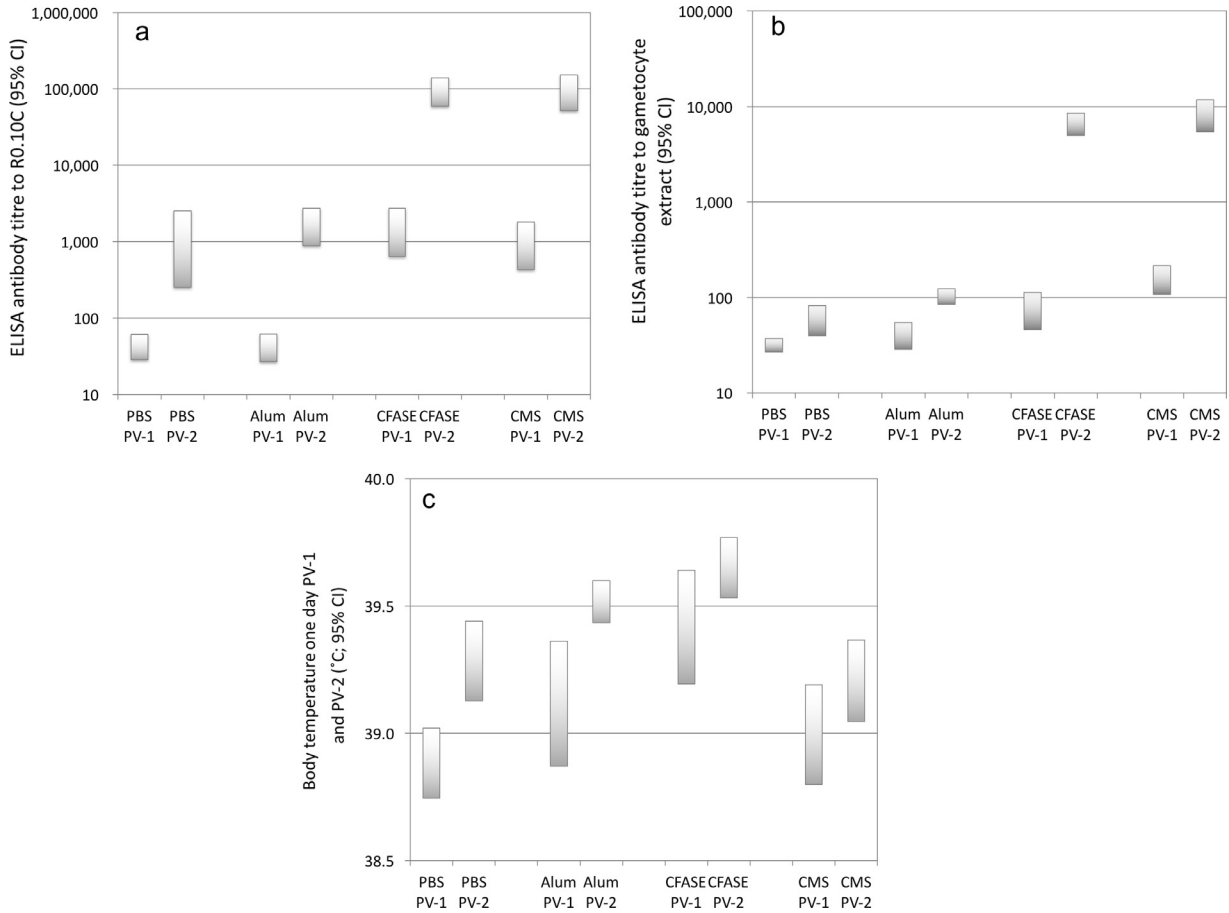
The study was not designed to determine systemic and local adverse events other than general performance and health conditions. No adverse effects were observed with either vaccine.

### 3.5. *In vitro* activation of human TLRs expressed on HEK cells

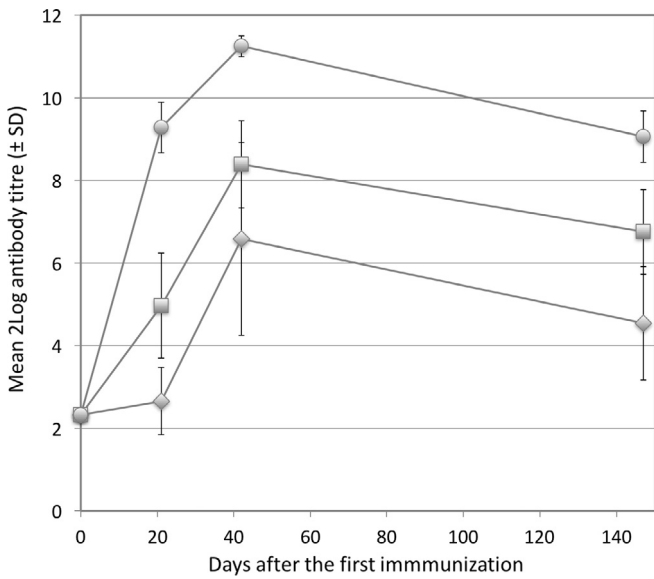
CMS was tested for its potential to activate human TLR4 expressed on HEK cells *in vitro*. TLR4 was not activated by CMS adjuvant at concentrations of 100 or 1000  $\mu$ g CMS per mL while LPS at concentrations of 1.0 or 0.01  $\mu$ g/mL caused 400–600-fold increase in bioluminescence of the TLR4-transfected HEK cells (Fig. 5). Lack of activation by CMS adjuvant was not the result of cytotoxic effects as viability of the cells was not affected and cells retained the ability to respond to LPS (J. Bajramovic, personal communication).

## 4. Discussion

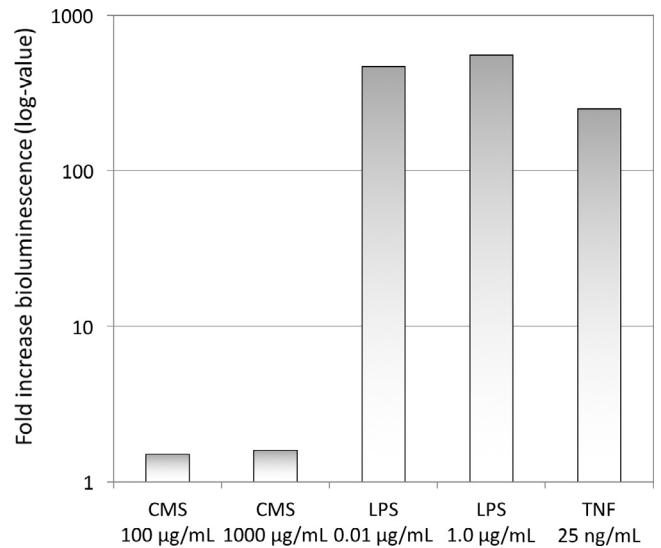
Despite several novel vaccine adjuvants (MF59, AS03, AS04, AF01) and products in advanced stage of development (GLA-SE, CAF01, IC31), there is still a need for more effective adjuvants for a variety of applications. The successful development of a new generation of vaccines of well-defined antigens depends on more effective adjuvants that are sufficiently safe for a particular use. In addition, better adjuvants open possibilities for lower doses of



**Fig. 3.** Effect of immunization of rabbits with recombinant malaria chimeric antigen R0.10C alone (‘PBS) or with alum, CFASE adjuvant or CMS adjuvant on (a) the ELISA antibody response to R0.10C, (b) the ELISA antibody response to *Plasmodium falciparum* gametocyte extract 21 days after the first (PV-1) and second vaccination (PV-2) and (c) the body temperature one day after each immunization. Results are expressed as 95% confidence interval (‘95% CI’).



**Fig. 4.** GMT HAI antibody responses in ferrets at various time intervals after immunization with 7.5 µg HA of inactivated, whole-virion influenza virus A/H7N9 with no adjuvant (diamonds), MF59-like AddaVax (squares) or CMS adjuvant (circles).



**Fig. 5.** CMS adjuvant does not activate of TLR4 on HEK cells. Each test item was analysed in triplicate and means are expressed as log values of the bioluminescence measured. Variance (not indicated) was between 50 and 100% for CMS adjuvant and less than 10% for the positive controls.

antigen and fewer injections. Therefore, we investigated a novel series of adjuvants based on synthetic carbohydrate esters. The novel concept is the outcome of the stepwise optimization of E/

T-ratio of synthetic carbohydrate derivatives through analysis of structure-activity relationship in larger, non-rodent species such as pigs or rabbits. Efficacy was determined by immune responses

after one or two immunizations with a purified, recombinant protein and toxicity was assessed by necropsy of local reactions after two IM injections [19]. Advantages of larger non-rodent species compared to small rodents, include that local reactions can be quantified precisely and in an objective manner, projected human doses of vaccine can be administered, and extrapolation to performance in humans is thought to be more adequate.

CFASE adjuvants such as CoVaccine HT™ have been proven to be highly effective to many types of antigens. Other research groups reported that these adjuvants revealed similar or even higher immune responses than oily adjuvants such as Freund's Adjuvants and ISA51 in nonhuman primates, pigs, rabbits and sheep [4,7–10,13]. A recent paper reported that not only the quantity but also the quality of the antibodies induced by CFASE adjuvant was improved as compared to those elicited by Freund's Complete or Incomplete Adjuvant as demonstrated by stronger affinity and enhanced functional potency [10]. This is unprecedented for an aqueous product. Although CoVaccine HT™-adjuvanted vaccines passed formal preclinical toxicity assessment, rise in body temperature one day after injection hampered further clinical development. Therefore, we aimed at a novel generation of CFASE-based adjuvants lacking this adverse effect while retaining high efficacy.

One approach was the separation of CFASE into fractions with no sulphate or one (CMS), two or more sulphate (CPS) groups per carbohydrate backbone and determination of E/T-ratio thereof. CMS (Fig. 1) consists of monosulphate isomers and its purity already offered important advantages in the characterization and quality control of the product. In a feasibility study in rabbits, CPS but not CMS adjuvant elicited significant local reactions. Strong *in vitro* haemolytic activity of CPS but not CMS adjuvant confirmed high surface-activity of derivatives with multiple sulphate and multiple fatty acid esters.

The CMS adjuvant comprises of a submicron emulsion of squalane-in-water, stabilized by Polysorbate 80 and is comparable with known products such as MF59, SE, and CoVaccine HT™. Passage through 0.2 µm filter resulted in a sterile, ready-for-use formulation of 4 well-defined chemicals. Based on 2-year stability of lab batches of CMS adjuvant and >10-year stability of its predecessors, we anticipate long-lasting chemical stability of ingredients and physical stability of the formulation. The manufacturing process is similar to the procedures established earlier for the predecessors except that a purification step by liquid chromatography is included.

For lead finding and optimization considering local reactions, the pig was very useful. To examine effects of body temperature, however, we decided to employ the rabbit as it is the standard species for pyrogenicity testing of medicinal products. A repeated-dose study revealed that CFASE with or without antigen induced a significant but transient, dose-dependent increase in body temperature of about 1 °C one day after IM injection (Fig. 2). The spikes remained constant for five consecutive treatments indicating that no tolerance or increased sensitivity occurred. The addition of a high-molecular peptide-protein conjugate as model antigen did not increase the body temperature after the first injection. After booster immunization, there was a slight increase in body temperature, which was statistically significant when results with different vaccines were taken together. Formation of antigen-antibody complexes and/or activation of immunological memory may be responsible for this additional effect.

Further analysis of immunogenicity and safety was conducted with a small, well-defined subunit and inactivated whole virus at much lower doses. The immuno-affinity purified, malaria chimeric glycoprotein R0.10C with molecular weight of 90 kD produced in *L. lactis* at a dose of 10 µg [24] was selected as it is a poor immunogen. Two or three injections with adjuvant are needed for detectable immune responses in small rodents [22]. In the rabbit study

reported here, CFASE and CMS elicited similarly high antibody responses after one and two injections and CFASE but not CMS elicited fever in at least some animals (Fig. 3).

In a qualified model for influenza vaccines in ferrets, we examined the effects of CMS on the immune response to inactivated, whole-virion A/H7N9 influenza virus. CMS gave 100-fold higher HAI antibody titres after the first and 25-fold after the second immunization, which were 20- and 8-fold higher than with AddaVax (Fig. 4). Body temperature or local reactions were not analysed in this study but daily monitoring of the animals for general behaviour and frequent palpation of the injection site did not exhibit any adverse effects. A/H7N9 was used at 7.5 µg HA per injection and as we demonstrated previously that 4 µg HA of H5N1 plus CFASE elicited high responses in ferrets and macaques [6,7], the antigen dose could be lowered further.

Remarkably, a single immunization with CMS adjuvant generated comparable or higher antibody titres than two immunizations either with benchmark or without adjuvant. This is in accordance with immunogenicity data on CFASE adjuvant but unique relative to existing adjuvants. A recent comparative study revealed that none of on existing adjuvants administered once reached the anti-flu HAI antibody titre obtained by two immunizations without adjuvant [25]. This interesting capacity of CMS adjuvant opens possibilities of single-shot vaccines, which may result in decreased costs of vaccination campaigns (goods, logistics and staffing), reduced time-to-immunity, improved adherence and reduced overall risks of vaccination.

The mechanisms underlying CMS adjuvant action is not yet understood. CFASE adjuvant has been shown to induce fever *in vivo* and to promote the maturation of human dendritic cells *in vitro*, which could be blocked by anti-TLR4 antibodies [26]. As CMS adjuvant did not cause fever or activate human TLR4 expressed on HEK cells (Fig. 5), we hypothesized that other components of CFASE are responsible for these actions. These could be derivatives with two or more sulphate groups present in CFASE and CPS therefrom. Indeed, CPS but not CMS adjuvant elicited strong local reactions at the site of injection and this could be linked to fever and TLR4 activation. In this respect, there might be some analogy with Lipid A and monophospholipid A where removal of one phosphate group led to a distinct, more favourable activity profile. But the striking difference between monophospholipid A and CMS is their ability to activate TLR4 and further investigations are needed to solve this discrepancy. Furthermore, detailed *in vivo* and *in vitro* analysis will contribute to our insights in structure-response relationships of carbohydrate derivatives with one or two sulphate groups instead of one or two phosphate groups.

We concluded that CFASEs consist of different carbohydrate derivatives with different profiles, that CMS is not responsible for increased body temperature, local reactions and TLR4 activation and that these (side-)effects are no pre-requisites for high adjuvant activity. CMS adjuvant is a promising candidate for poor immunogens and single-shot vaccines and further research is needed to confirm its potentials and to elucidate the immunological mechanisms involved.

#### Additional information

Competing financial interests: LH, PP and HvdB are co-inventors on a patent application covering the use of CMS as vaccine adjuvants. All rights have been assigned to LiteVax BV. LH, PP and HvdB hold shares in LiteVax BV. JB and LV are employees of Biomedical Primate Research Centre, a Dutch non-profit institute. RS and WR are employees of Radboud University Nijmegen Medical Centre. MP, GvA and KS, are employees of Viroclinics Biosciences BV.

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