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Patterns of binding of aluminum-containing adjuvants to *Haemophilus influenzae* type b and meningococcal group C conjugate vaccines and components

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ABSTRACT

The basis of *Haemophilus influenzae* type b (Hib) and *Neisseria meningitidis* serogroup C (MenC) glycoconjugates binding to aluminum-containing adjuvants was studied. By measuring the amount of polysaccharide and protein in the non-adsorbed supernatant, the adjuvant, aluminum phosphate, $AlPO_4$, was found to be less efficient than aluminum hydroxide, $Al(OH)_3$ at binding to the conjugates, at concentrations relevant to licensed vaccine formulations and when equimolar. At neutral pH, binding of TT conjugates to $AlPO_4$ was facilitated through the carrier protein, with only weak binding of $AlPO_4$ to CRM_{197} being observed. There was slightly higher binding of either adjuvant to tetanus toxoid conjugates, than to CRM_{197} conjugates. This was verified in $AlPO_4$ formulations containing DTwP–Hib, where the adsorption of TT-conjugated Hib was higher than CRM_{197}-conjugated Hib. At neutral pH, the anionic Hib and MenC polysaccharides did not appreciably bind to $AlPO_4$, but did bind to $Al(OH)_3$, due to electrostatic interactions. Phosphate ions reduced the binding of the conjugates to the adjuvants. These patterns of adjuvant adsorption can form the basis for future formulation studies with individual and combination vaccines containing saccharide-protein conjugates.

1. Introduction

Aluminum-containing adjuvants have been successfully used since the 1920s to stimulate the immune response to the relatively more purified diphtheria toxoid (DT) and tetanus toxoid (TT) vaccine antigens (Ags) being prepared at that time [1–4]. An understanding of the mechanism(s) of action and the physical nature of the adjuvant-Ag interactions leading to an enhanced immune response following vaccine administration have only more recently been recognized [5–9].

The most commonly used aluminum-containing adjuvants in vaccines have been aluminum phosphate, $AlPO_4$, and aluminum hydroxide, $Al(OH)_3$, which have very different structural properties in terms of their size, molecular organization, colloidal properties, and solubility [6,10–12]. The binding or adsorption of protein antigens to aluminum adjuvants occurs principally through electrostatic interactions (involving the Al^{3+} ion or negatively-charged counter ion) [12] as well as metal ion coordination and hydrogen bonding with water molecules and hydroxyl groups [10,11,13]; there is also evidence that hydrophobic interactions are involved in some cases [14]. The large surface area of these colloidal gel adjuvants, and size of the particles (1–100 μm in diameter) also contributes to their adsorption [6,12,15]. Aluminum hydroxide has an isoelectric point (pI) of >7.3 to 11.4 [6,12], while $AlPO_4$ with a pI of ≅4, binds primarily to positively charged proteins and molecules [12].

Large aluminum adjuvant-Ag complexes of >0.2 μm shown to be efficiently phagocytosed by antigen presenting cells [5,16], act as slow-releasing local depots for a longer term re-exposure to Ag [17,18], and lead to innate signaling that causes low-grade inflammation to

Abbreviations: Ag, antigen; CRM, cross-reacting material; Hib, Haemophilus influenzae type b; MenC, Meningococcal serogroup C; PS, polysaccharide; TT, tetanus toxoid.

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stimulate and recruit Th-1 and Th-2-type immune cells [19,20]; all of which are separate means of stimulating T-cell help in the overall production of neutralizing and protective antibodies. Studies have shown that immunopotentiation can occur without adsorption or depot formation at the inoculation site [8,21].

The modern development of adjuvant-containing vaccines involves identifying the adjuvant benefit in preclinical immunogenicity studies, assessing its safety profile in non-clinical toxicity studies and assuring that the adjuvant formulation is stable with its adsorption to Ag consistently controlled throughout the shelf-life of the product [22–24]. Product-specific minimum and maximum limits of adjuvant adsorption should be established based on the level of adsorption of the vaccines used in clinical trials. Minimum adsorption levels applicable to all vaccines are no longer applicable, as complete adsorption of the Ags to adjuvant may not be considered necessary or ideal [25–28], and partial adsorption or association may be preferable [8,9]. The stability of the adjuvant formulation is also important with respect to maintaining a consistent adsorption level throughout its shelf-life, and avoiding any adverse effects on the vaccine [9,29,30]. The lot release of vaccines by manufacturers, as well as control laboratories, in some cases, involves assessing the adjuvant material in context of the vaccine Ag(s) [25,27]. This can involve quality control tests of the bulk adjuvant material, such as purity, content, pH, and adsorptive capacity, as well as a measure of the adsorption of Ag to adjuvant in formulated vaccine.

In glycoconjugate vaccines, the bacterial oligo- or polysaccharide target Ag is covalently coupled to a carrier protein such as TT, diphtheria toxoid or a non-toxic genetic mutant of diphtheria toxin, CRM_{197} [31], recombinant exotoxin A from *Pseudomonas aeruginosa*, recombinant Protein D from non-typeable *Haemophilus influenzae* or outer membrane protein complex from *Neisseria meningitidis* group B. While the carrier protein already acts as an 'intrinsic' adjuvant to provide T-cell help in the immune response to the weaker T-independent polysaccharide epitope [18], there can be additional benefits from the inclusion of aluminum adjuvants. When a glycoconjugate is administered simultaneously or in combination with more immunodominant Ags which may potentially interfere with the protective response to the bacterial polysaccharide capsule, an adjuvant may provide the immune stimulus to aid the production of high levels of circulating antibodies and the formation of long-lived CD27+ IgG+ memory cell pools.

Although the first licensed monovalent *H. influenzae* type b (Hib) conjugate vaccines were not formulated with aluminum adjuvants, adjuvants were added to subsequent pneumococcal and meningococcal group C conjugate (MenC) vaccines to boost the production of polysaccharide-capsule specific bactericidal antibodies (Table 1). Current DTP combinations that include Hib conjugates contain aluminum adjuvants which may adsorb the Hib conjugate component through ionic bonding. In clinical and post-licensure studies, the immune response to some conjugate vaccines has been found to be less efficacious in the presence of other more dominant Ags present in combination or concurrently-administered vaccines [32,33]. Limited persistence of serum levels of bactericidal antibodies to Hib and serogroup C meningococcus [36–38] has led to the introduction of a booster dose of Hib and MenC conjugates at 12 months in the U.K. The interactions of saccharide-protein conjugates with adjuvants remain an important but rather poorly understood area despite their common and widespread use.

The aims of this study were to characterize the binding of the individual saccharide and protein components of Hib and MenC vaccines to aluminum adjuvants at physiological pH and ionic strength, and under the formulation conditions of commercial vaccines routinely given to infants in Europe and other parts of the world. The effect of carrier protein and buffer salts, in particular, phosphate ions, was studied. The stability of MenC conjugate vaccines in context of adjuvant adsorption was also evaluated.

Table 1
Aluminum adjuvant concentrations in licensed conjugate vaccines.

Vaccine[a]	Adjuvant	[Adjuvant], μg Al^{3+}/ml[b]
Hib-CRM_{197} B[c,d]	$AlPO_4$	0.6
MenA-TT	$AlPO_4$	0.6
MenC-CRM_{197} B	$AlPO_4$	0.25
Pneumo-CRM_{197}	$AlPO_4$	0.25
Pneumo-TT	$AlPO_4$	1.0
DTaP5Hib	$AlPO_4$	0.66
DTwP (HepB)Hib[e]	$AlPO_4$	0.6–0.7
Hib-OMPC	$Al(OH)_3$	0.44
MenC-CRM_{197} A	$Al(OH)_3$	0.7
MenC-TT	$Al(OH)_3$	1.0
DTaP3Hib	$Al(OH)_3$	1.0
DTwPHib	$Al(OH)_3$	0.8

[a] The following vaccines do not contain any aluminum adjuvant: Hib-CRM_{197} A, Hib-TT, Men ACWY-CRM_{197}, MenACWY-DT, MenACWY-TT. Hib-CRM_{197} and MenC-CRM_{197} letter code designations are according to [39] and [40], respectively.
[b] Adjuvant concentrations were calculated from dose equivalent values given in the publically accessible *Summary of Product Characteristics* for each product. For lyophilised vaccines, the concentration is that obtained following reconstitution in its diluent.
[c] A dash (-) sign is used between PS and carrier protein in conjugate vaccines.
[d] Those vaccines in bold font have been used in this study.
[e] According to the list of WHO Pre-qualified vaccines [69].

2. Materials and methods

2.1. Vaccines and components

The Hib conjugate and MenC bulk conjugate vaccine components used in this study consisted of a capsular oligo- or polysaccharide conjugated to either CRM_{197}, the diphtheria toxin mutant protein, or to TT, tetanus toxoid, as protein carrier. They were received as bulk conjugates and were stored frozen at −20 °C or at 4 °C, according to the manufacturers' recommendations. Hib-CRM_{197} and MenC-CRM_{197} were supplied from the same manufacturer (and correspond to Hib-CRM_{197}-B from Ref. [39] and MenC-CRM_{197}-A from Ref. [40]). Hib-TT corresponds to Hib-TT-B from Ref. [41]. Vaccine types used in the study are indicated in Table 1.

Both Hib bulk conjugates and MenC-CRM_{197} were extensively dialyzed at 4 °C with three changes of 154 mM NaCl, pH 6.0–6.4 (saline), using SpectraPor 7 membrane with a designated pore size of 10 kDa. The bulk vaccine MenC-TT was supplied in saline.

The corresponding carrier proteins, CRM_{197}, stored at −20 °C, and TT, stored at 4 °C, were obtained from the manufacturers of the corresponding conjugates and were also dialyzed in saline. The Hib polyribosyl ribitol phosphate (PRP) polysaccharide used was the WHO 1st International Standard (NIBSC, 02/208) [42]. MenC α2-9-linked polysialic acid was that routinely used as an in-house reference preparation for the quantitation of MenC PS. PS stock solutions (10 mg/ml) were stored frozen at −20 °C and were diluted in the appropriate buffers prior to use.

Final fill MenC-CRM_{197} from two manufacturers and Hib-TT and MenC-TT monovalent vaccines were also used and were stored at 4 °C. Diphtheria-Tetanus-Whole-cell pertussis (DTwP)-Hib combination vaccines from four manufacturers containing 0.06–2.5 mg Al^{3+}/ml as $AlPO_4$ were stored at 4 °C.

PS concentrations of the bulk conjugates prior to adsorption were calculated based on the PS/protein ratios supplied by the manufacturers and determined protein concentrations (see section

2.4). The content of the PS stocks were determined from their dry weights.

2.2. Adjuvant adsorption and separation

Prior to adsorption to adjuvant, the bulk conjugates in saline were diluted in a 2 x stock saline-based buffer or solution to give a concentration of 40 μg saccharide/ml. These samples were then mixed with an equal volume of 2 mg/ml adjuvant to achieve 20 μg saccharide/ml and adjuvant at 1 mg/ml, similar to that found in the final products unless quoted. The final buffer concentrations used for adsorption study were 5 mM sodium phosphate, pH 7.2; 50 mM sodium phosphate, 154 mM NaCl, pH 7.2 (PBS); or, 55 mM 3-(N-Morpholino)-propanesulfonic acid, 154 mM NaCl, pH 7.2 (MOPS-saline). Hib-TT was used additionally in 154 mM NaCl. In the combination vaccine experiments, pre-mixing of the Hib and MenC conjugates was performed prior to adjuvant adsorption.

The adjuvants used were aluminum hydroxide, $Al(OH)_3$ (supplied by Brennag Biosector as Alhydrogel, 2%) and aluminum phosphate, $AlPO_4$, which was manufactured by mixing aluminum chloride with tribasic sodium phosphate [15]. These were stored at 2–8 °C as stocks of at least 10 mg/ml. Adjuvants were added to the vaccine components at room temperature to give 0.1 mg or 1 mg Al^{3+}/ml of aluminum hydroxide, or 0.1 or 1 mg/ml $AlPO_4$ (equivalent to 0.025 or 0.25 mg Al^{3+}/ml). Mixing was carried out in 2 ml sterile screw-capped eppendorfs for ~16–18 h on a roller at room temperature, to give complete adsorption. The concentrations chosen were typical of those in licensed MenC conjugate vaccines or were 1/10 of that allow for measurement of their partial binding, as vaccine formulations have been developed that promote maximal binding.

To determine the % adsorption of the vaccines and components to adjuvant, the amount of non-adjuvanted PS or protein was measured. Adsorbed samples were first centrifuged at 8500× *g* in a table-top microcentrifuge for 15 min at 4 °C. Non-adsorbed supernatants were carefully taken up in a pipette to avoid mixing of the adjuvant-containing pellet and transferred to new eppendorf tubes, and then re-centrifuged and transferred to minimize any turbidity due to residual adjuvant, which may interfere with colorimetry.

Protein and/or PS concentrations in the supernatants and in control samples without adjuvant were analyzed immediately, or following storage of the samples at 4 °C for up to 1 week. The % recovered non-adsorbed protein and/or saccharide were expressed relative to controls without adjuvant, as % Adsorbed = 100% − % Non-adsorbed.

2.3. Saccharide determination

The concentration of non-adsorbed Hib PRP PS in the supernatants was determined using the orcinol assay according to Kabat and Mayer [43], with slight modifications. Standards were prepared in triplicate and samples were prepared in duplicate or triplicate to a volume of 500 μL in glass vials in the same buffer as with the standards. Reagents were added and well mixed after each addition as follows: 500 μL $FeCl_3$ (1.8 mM in 36% HCl), followed by 50 μL of an orcinol solution (693 mM orcinol in 100% ethanol). Vials were closed with Teflon seals and incubated for 20 min at 100 °C in a heating block. Samples were equilibrated to room temperature prior to measuring their absorbance at 670 nm, using a Perkin Elmer Lambda 800 UV–Vis spectrophotometer. Ribose standards (Sigma R7500) or samples containing between 1 and 25 μg/ml ribose gave $A_{670\ nm}$ values of ~0.1–0.8. Ribose concentrations (in μg/ml) were converted to PRP concentrations by multiplying by a conversion factor of 2.448 g PRP/g ribose [42]. The combined standard uncertainty for the orcinol assay was determined to be 3.3%, based on between assay variability of 2.3% for each determination.

Hib PRP saccharide not bound to aluminum phosphate adjuvant was also measured by the HPAEC-PAD method developed for DPT combination vaccines by Bardotti et al. [44]. Manufacturing lots of DTwP–Hib ± HepB were mixed at room temperature with 1 M sodium phosphate, pH 6.8 and 1 M NaCl to a final concentration of 10 mM sodium phosphate, pH 6.8, 50 mM NaCl for 1 h, and centrifuged for 30 min at 18,400× *g*. These '10 mM desorbed' supernatants were used for the quantification of PRP content, relative to untreated, samples, which were not desorbed or spun. Standards (0.5–27 μg PRP/ml) were prepared using the 1st WHO International Standard for Hib polysaccharide (NIBSC code 02/208). Samples and standards were hydrolyzed in 0.3 M HCl at 100 °C for 2 h, and after cooling, were neutralized with 0.3 M NaOH and filtered using a 100 kDa MWCO Microcon ultrafilter. An injection of 100 μl of fitrate was made onto the CarboPac MA-1 analytical column in combination with a MA-1 guard column and the samples were eluted with 580 mM NaOH for 35 min, with a 1 M NaOH column re-generation step between samples, if required. A combined standard uncertainty of 10.2% for the HPAEC-PAD assay on combination vaccines was derived from inter-assay variability of 2.2% for the desorbed sample and 10.6% for the untreated samples.

MenC-containing samples were analyzed for N-acetyl neuraminic acid, or NANA, using a resorcinol assay based on the method of Svennerholm [45]. To 500 μL standard or sample, 500 μL of the resorcinol-$CuSO_4$-HCl reagent (containing 18.2 mM resorcinol, 0.4 mM $CuSO_4$ in 30% HCl) was added and well mixed. The glass vials were sealed with Teflon seals and incubated at 110 °C for 15 min in a heating block. Absorbance at 564 nm was read. A standard curve of 1–30 μg/ml NANA (Sigma A-2388) gave $A_{564\ nm}$ in the range of 0.02–0.45. The average absorbance of the blank was subtracted from all standards and samples. The combined standard uncertainty for the resorcinol-determined values was determined to be 7.5%, based on an intermediate precision of 5.3% for each determination.

The addition of 6.4–8 mM NaOH to the sample prior to orcinol or resorcinol-acid reagent was initially performed to aid in the dissolution of the aluminum adjuvant, but this was later deemed to be unnecessary. It was also found that the presence of non-specific formulation sugars use in the vaccine samples, that is non-pentose or -sialic acid – containing sugars, did not interfere with the determination of the PRP or NANA, respectively. Likewise, there was no apparent interference from the other specific saccharide (Hib or MenC) during analysis of the combination vaccine mixtures.

2.4. Protein determination

Protein concentrations were determined by UV spectroscopy ($A_{280\ nm}$–$A_{450\ nm}$) using a Perkin–Elmer Lambda 800 UV–Vis spectrophotometer. The following molar absorption coefficients: $A_{280,\ 0.1\%} = 1.229\ cm^{-1}\ mg^{-1}$ ml for TT (based on amino acid content) and $0.757\ cm^{-1}\ mg^{-1}$ ml for CRM_{197} (based on the manufacturer's determined value), to determine the mg/ml concentrations. The lower limit of quantitation was 4 μg/ml TT or 7 μg/ml CRM_{197}. The combined standard uncertainty of measurement of protein concentration in the adjuvant mixtures was determined to be ±6.6% for TT and 5.4% for CRM_{197} based on spectrophotometric accuracy.

2.5. pH determination

A Jenway model 3305 pH meter was calibrated with pH 4, 7 and 10 buffers at room temperature. Samples containing the adsorbed components or non-adsorbed supernatants were equilibrated to

room temperature prior to pH measurement. The pH values were accurate to ±0.05 pH units.

3. Results

3.1. Bulk conjugate adsorption studies

The adsorption of individual or combined Hib-CRM_{197} and MenC-CRM_{197} conjugates to aluminum-containing adjuvants was measured in 5 mM sodium phosphate, pH 7.2 by measuring the % recovery of the carrier protein, CRM_{197}, or PS (Hib PRP or MenC polysialic acid) present in the non-adsorbed supernatant. The vaccine component concentration was equivalent to that of licensed MenC conjugate vaccines, while the adjuvant concentration was 1/10 that, to be able to study the effects of buffer salts, and polysaccharide and carrier protein type on binding. Both CRM_{197} conjugates were individually shown to adsorb weakly or not at all to aluminum phosphate (0–11% for Hib-CRM_{197} and 0% for MenC-CRM_{197}), and, to bind more tightly to aluminum hydroxide (88–100% to Hib-CRM_{197} and 90–100% to MenC-CRM_{197}) as shown in Table 2. The combination of Hib-CRM_{197} and MenC-CRM_{197} did not affect the adsorption of the conjugates to either of the adjuvants.

A discrepancy of up to 21% was observed between % adsorption levels determined by protein or saccharide assay, due mainly to their combined uncertainty of measurement, but in part to the occasional turbidity of residual adjuvant, which could interfere with the protein estimations, at final fill concentrations. The pH of the MenC-CRM_{197} conjugate remained relatively stable (pH 7.0), but the pH of the Hib-CRM_{197} in aluminum hydroxide decreased to pH 6.4 compared to its control or aluminum phosphate solution (pH 7.0), suggesting that the 5 mM phosphate solution was not adequately buffered.

Because of the differences observed in the binding of the CRM_{197} conjugates to the adjuvants in a low ionic strength phosphate buffer, the influence of the phosphate ion was studied in an adequately buffered solution at a physiological saline concentration. Phosphate ions have been found to have a number of different effects on adjuvant, such as promoting Ag-adjuvant adsorption, competing with Ag adsorption by binding, or exchanging ligands with adjuvant [8,13,14,21,28,46–48].

Hib and MenC PS conjugated to TT were used in addition to the CRM_{197} conjugates and were prepared in more strongly buffered solutions: either PBS (50 mM sodium phosphate, 154 mM NaCl, pH 7.2) or a non-phosphate buffered saline (55 mM MOPS, 154 mM NaCl, pH 7.2) of equivalent ionic strength and pH. The pKa of MOPS (7.2) allowed for a direct comparison with PBS.

As with the weakly buffered phosphate solution (5 mM sodium phosphate, pH 7.2), the adsorption of the Hib-CRM_{197} and MenC-CRM_{197} to aluminum phosphate was negligible. In contrast to the significant binding of CRM_{197} conjugates to aluminum hydroxide at low phosphate-containing buffer, there was only low binding of these conjugates to aluminum hydroxide in PBS. Negligible binding of Hib-TT and MenC-TT conjugates to either adjuvant in full-strength PBS was also observed (Table 3A).

If MOPS-saline, pH 7.2 was used, measurable adsorption of the conjugates to the aluminum adjuvants was observed, with higher adsorption (88–97% adsorption) to aluminum hydroxide than to aluminum phosphate (up to 31%) as shown in Table 3B.

The pre-mixture of CRM-conjugates or TT-conjugates prior to adsorption did not alter this binding pattern. Lower adsorption of the conjugates to aluminum phosphate (5–26%) than to the 'hydroxide' form (59–100%) of the adjuvant was observed (Table 3C). It was notable that at pH 7.2, the TT-conjugated vaccines bound aluminum adjuvants to a higher degree than did the CRM_{197} conjugates.

3.2. Individual component adsorption analysis

To explore the basis for the adjuvant–conjugate association, the adsorption of the individual PS or carrier protein alone (non-conjugated) was studied, in addition to bulk conjugate binding to adjuvant, at final vaccine concentrations. Low-to-negligible binding of the Hib PRP and MenC poly-sialic acid to aluminum phosphate was seen. In contrast there was high binding of the PS to aluminum hydroxide (Table 4). There were clear differences between the carrier proteins in their adjuvant binding properties. CRM_{197} did

Table 2
Adsorption of Hib- and MenC-CRM_{197} conjugates to aluminum adjuvant.

Vaccine	Adjuvant[a]	pH	% Adsorption[b]		
			Protein	Hib PS	MenC PS
Hib-CRM	$AlPO_4$	7.0	11 ± 5	0 ± 3	
Hib-CRM	$Al(OH)_3$	6.4	88 ± 5	100 ± 3	
MenC-CRM	$AlPO_4$	7.1	0 ± 5		0 ± 7.5
MenC-CRM	$Al(OH)_3$	7.1	100 ± 5		90 ± 7
Combined[c]	$AlPO_4$	n.d.	0 ± 5	21 ± 2	1 ± 7
Combined	$Al(OH)_3$	n.d.	91 ± 5	96 ± 3	78 ± 6

[a] Conjugates formulated to 20 μg PS/ml and adjuvant at 0.1 mg Al^{3+}/ml for $Al(OH)_3$ or 0.025 mg Al^{3+}/ml for $AlPO_4$ (equivalent to 0.1 mg $AlPO_4$/mL) were incubated in 5 mM sodium phosphate, pH 7.2 for 16–18 h at room temperature prior to determining the % of the non-adsorbed protein or polysaccharide component remaining in the supernatant.
[b] Statistical intervals were derived from applying standard combined uncertainty to the measured values.
[c] Hib-CRM and MenC-CRM were premixed prior to adjuvant addition. The results were obtained from a single experiment.

Table 3
Effect of buffer on the adsorption of conjugates to aluminum adjuvants.

Vaccine	Adjuvant[a]		% Adsorption[b,c]	
	Type	Mg Al^{3+}/ml	Hib PS	MenC PS
A. In PBS buffer				
Hib-CRM	$AlPO_4$	0.025	2 ± 3	
Hib-CRM	$Al(OH)_3$	0.1	2 ± 3	
Hib-TT	$AlPO_4$	0.025	0 ± 3	
Hib-TT	$Al(OH)_3$	0.1	4 ± 3	
MenC-CRM	$AlPO_4$	0.025		0 ± 8
MenC-CRM	$Al(OH)_3$	0.1		0 ± 8
MenC-TT	$AlPO_4$	0.025		0 ± 8
MenC-TT	$Al(OH)_3$	0.1		0 ± 8
B. In MOPS-saline Buffer				
Hib-CRM	$AlPO_4$	0.025	2 ± 3	
Hib-CRM	$Al(OH)_3$	0.1	88 ± 3	
Hib-TT	$AlPO_4$	0.025	31 ± 3	
Hib-TT	$Al(OH)_3$	0.1	94 ± 3	
MenC-CRM	$AlPO_4$	0.025		11 ± 7
MenC-CRM	$Al(OH)_3$	0.1		96 ± 7
MenC-TT	$AlPO_4$	0.025		35 ± 5
MenC-TT	$Al(OH)_3$	0.1		97 ± 7
C. Combined in MOPS-saline buffer				
Hib/MenC-CRM	$AlPO_4$	0.025	5 ± 3	19 ± 6
Hib/MenC-CRM	$Al(OH)_3$	0.1	75 ± 3	59 ± 4
Hib/MenC-CRM	$Al(OH)_3$	1	93 ± 3	100 ± 8
Hib/MenC-TT	$AlPO_4$	0.025	26 ± 2	10 ± 7
Hib/MenC-TT	$Al(OH)_3$	0.1	84 ± 3	100 ± 8
Hib/MenC-TT	$Al(OH)_3$	1	92 ± 3	99 ± 8

[a] Adjuvants used were $AlPO_4$ at 0.025 (panels A–C), and $Al(OH)_3$ at 0.1 and 1 mg Al^{3+}/ml as indicated.
[b] Buffers used were PBS (50 mM sodium phosphate, 154 mM NaCl, pH 7.2) or MOPS-saline (55 mM MOPS, 154 NaCl, pH 7.2). In panel (C) conjugates were pre-mixed prior to adjuvant adsorption. The results from panels A–C were obtained from three consecutive, single experiments.
[c] Statistical intervals were calculated with the combined uncertainty on the determined values.

Table 4
Adsorption of individual PS and protein components to aluminum adjuvants.

Component	Vaccine type	% Adsorption[a] to $AlPO_4$	% Adsorption to $Al(OH)_3$
PS	PRP	1	98
	MenC	6	100
Protein	CRM_{197}	0	100
	TT	37	100
Conjugate[a]	MenC-CRM	5	100
	MenC-TT	50	83
	Hib-TT	48	91

[a] The % adsorption values of the PS and the protein components were calculated based in the recoveries of the PS and protein in the non-adsorbed supernatants relative to controls without adjuvant. The individual components and MenC conjugates were in MOPS-saline buffer, pH 7.2, while the Hib-TT conjugate was in saline. The adjuvants were at concentrations of 0.25 mg Al^{3+}/ml for $AlPO_4$ and 1 mg Al^{3+}/ml for $Al(OH)_3$, typical of final product. The concentrations of vaccine components were close to that expected in the final product, with 15–20 μg saccharide/ml and 35–50 μg protein/ml. The % adsorption values for the conjugates are an average of those determined for the protein and PS moieties.

not bind to aluminum phosphate, but was completely adsorbed to aluminum hydroxide. TT bound partially to aluminum phosphate and completely to aluminum hydroxide.

3.3. Comparison of aluminum concentration

The concentrations of the adjuvants used in the binding studies described in section 3.1 were only one-tenth those used in commercial vaccines to allow for a comparison of binding at sub-maximal absorption conditions. Because the Al^{3+} ion concentration in the aluminum phosphate-adsorbed vaccines or components is much lower than that in the aluminum hydroxide samples, adsorption was measured over an equivalent Al ion concentration range.

Fig. 1 shows the adsorption of the MenC-CRM_{197}, MenC-TT and Hib-TT bulk conjugates to aluminum phosphate and aluminum hydroxide in a non-phosphate-containing saline solution at concentrations ranging from 0.06 to 2 mg Al^{3+}/ml. The MenC-CRM_{197} showed a clear difference in binding to aluminum phosphate, with only 20–40% adsorption of the protein component up to 0.5 mg Al^{3+}/ml, compared to 100% absorption to aluminum hydroxide at the same concentration (panel A). A differential in the adsorption of the two aluminum salts to TT conjugates was not as obvious. It was notable that although the adsorption of both of the TT conjugates (panel B) was titratable at lower concentrations, their adsorption was always greater than 20% even at the lowest concentration used (0.125 mg Al^{3+}/ml).

As the binding of conjugates to aluminum phosphate at the pH of this study is mainly through the protein rather than PS moiety, the amount of protein bound/0.5 mg Al^{3+} was calculated. For MenC conjugates, 8.4 μg CRM_{197} adsorbed to 0.5 mg Al^{3+} in aluminum phosphate compared with 21.5 μg CRM_{197} adsorbed to the same amount of Al^{3+} in aluminum hydroxide, showing the higher strength of binding of aluminum hydroxide to CRM_{197}.

3.4. Adsorption in monovalent MenC conjugate vaccines

The adsorption of three final fill monovalent MenC conjugate vaccines from different manufacturers were measured in their own formulations after storage for 1 month at 4 °C and 37 °C. MenC-CRM_{197} A was lyophilized in a sodium phosphate buffered solution with mannitol. MenC-CRM_{197} B and MenC-TT were in saline formulations. All three vaccines were found to be >98% adsorbed to their adjuvant whether measured by PS or protein found in the non-adsorbed supernatant (Table 5). Compared with the minimal binding of conjugates to aluminum phosphate at pH 7.2, the low pH saline formulation of (pH 6.1) MenC-CRM_{197} B, appeared to promote adsorption. After 1 month at 37 °C, the adsorption remained at 100%. The pHs of the MenC-CRM_{197} vaccines were stable, but the pH of the MenC-TT had decreased by 1 pH unit. Although in an unbuffered saline formulation, it remained within specification; long-term stability studies have also demonstrated its stability in $Al(OH)_3$ [49,50].

3.5. Effect of carrier protein on adsorption of Hib conjugate in combination vaccines

The adsorption of the Hib component of DTwP combination vaccines was evaluated for two CRM_{197} and TT-based conjugates in aluminum phosphate formulations, pH 6.0–6.5. There was considerable adsorption of the TT-conjugates to adjuvant

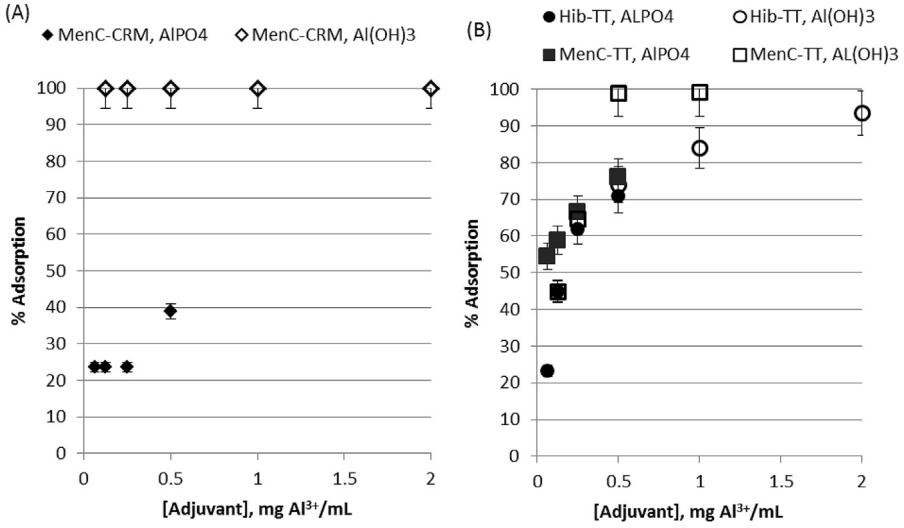

Fig. 1. Effect of adjuvant concentration on binding of CRM_{197} and TT conjugates. The adsorption of (A) MenC-CRM_{197} and (B) Hib-TT and MenC-TT to aluminum phosphate and aluminum hydroxide were studied as a function of adjuvant concentration. The % adsorption was calculated by measuring the recovery of non-adsorbed protein in the supernatant, relative to controls without adjuvant. The MenC vaccines were in MOPS-saline buffer, pH 7.2 and the Hib-TT was in saline. The target concentration was 20 μg PS/ml. The error bars represent the combined standard uncertainty.

Table 5
Adjuvant adsorption of licensed monovalent MenC conjugate vaccines.

Vaccine[a]	Adjuvant	[Adjuvant] mg Al^{3+}/ml	% Adsorption[b]		pH	
			4 °C	37 °C	4 °C	37 °C
MenC-CRM-A	$Al(OH)_3$	0.7	99	100	7.2	7.2
MenC-CRM-B	$AlPO_4$	0.25	98	98	6.1	6.1
MenC-TT	$Al(OH)_3$	1	100	100	6.9	5.8

[a] Commercial monovalent MenC conjugate vaccines were used with their formulated adjuvants. The vaccines were incubated at the indicated temperature for 1 month prior to the measurement of their adjuvant adsorption.
[b] Values given for % adsorption to adjuvant were arithmetic averages from the individual adsorptions of the corresponding protein and saccharide components.

(25–65%), with vaccine D being relatively more adsorbent than vaccine C (Fig. 2). Saydam et al. also found variable adsorption of similar vaccines by ELISA [51]. Only slight adsorption (up to 10%) of the CRM_{197}-conjugates was found.

4. Discussion

A clear pattern of adsorption of the highly negatively-charged Hib and MenC conjugate vaccines to aluminum adjuvant was observed in this study. At pH 7.0–7.2, aluminum hydroxide is positively charged (pI = 7.4 to 11.4) [6,10] and bound the carrier proteins, tetanus toxoid (pI ≪ 5.95) and CRM_{197} (pI = 5.85) [31,52] and the anionic capsular PS of Hib (poly-ribosylribitol phosphate) and serogroup C meningococcus (partially *O*-acetylated or completely de-*O*-acetylated polysialic acid) more avidly and to a higher degree than did aluminum phosphate (pI ≅ 4) [12]. The binding of the PS-TT conjugates to aluminum phosphate adjuvant was primarily facilitated through the carrier protein rather than through the oligo- or polysaccharide at neutral pH. The patterns of adsorption were predominated by electrostatic interactions, in line with that observed from model protein systems [12,53].

Besides the charge of the adjuvant, a second factor favoring the relatively higher binding of the conjugates to the aluminum hydroxide could have been the higher Al^{3+} concentration of aluminum hydroxide used in with conjugate vaccines, as compared with aluminum phosphate (Table 1). Since the binding or adsorption of protein Ags to aluminum adjuvants occurs principally through electrostatic interactions involving Al^{3+} metal coordination, this could explain the higher adsorption of aluminum hydroxide to the vaccine conjugates at vaccine relevant concentrations. For both CRM_{197} and TT-conjugates, it was clear, however, that at equimolar amounts of aluminum ion, a higher amount of conjugate is bound to aluminum hydroxide than to aluminum phosphate at pH 7.2.

A third factor, specific to Hib, is the presence of phosphoryl groups on the repeating units, which could potentially repel the phosphorylated adjuvant.

On this basis, other saccharide-based conjugate vaccines, utilizing negatively-charged PS, such as meningococcal serogroups A, B, W, X and Y; pneumococcal serotypes 1, 6A, 6B, 18C, 19A, 19F and 23F; and, Group B Streptococci type III would be expected to have similar adjuvant binding behavior under similar conditions, provided similarly-charged carrier proteins were used.

With respect to the contribution of the carrier proteins, Coombes et al. [54,59] reported near-complete binding of diphtheria toxoid (DT) (pI ≅ 4.1–4.6 [55]) and TT, to aluminum hydroxide in combination vaccines at neutral pH; lesser adsorption to aluminum phosphate was found, corroborating the results found here. In a separate study, higher adsorption of DT to aluminum phosphate was found to be possible through lowering the pH [56], as was also borne out with MenC-CRM_{197} B in its own vaccine formulation (Table 5).

The notable higher binding of the TT carrier protein compared to CRM_{197} to aluminum adjuvant from the composite or combination vaccines cannot be explained on the basis of isoelectric point alone. They bind to the adjuvants with different adsorption mechanisms; being different in structure, with unique surface side-chain charge densities, hydrophobic regions and hydrogen bonding propensity. Current methodological approaches that rely on the measurement of conjugate vaccine potency from non-adsorbed supernatants, assuming equal interactions for CRM_{197} and TT conjugates, could give misleading results, and product-specific approaches need to be considered [51].

Charged excipients also play a role in adjuvant binding patterns. As the ionic strength of the buffer increases so too does the likelihood of the interference with the surface charges of the species in the medium, as also explored by others [47,57,58]. In this study, phosphate ions inhibited the binding of all four types of conjugates to both adjuvants. As little as 5 mM phosphate, 0.9% NaCl, pH 7.4 has been observed to reduce adsorption of proteins to aluminum adjuvants [46]. Similar inhibition has been observed for diphtheria and tetanus toxoids in combination vaccines [48] and with a monovalent MenC-TT [50] or MenA-TT vaccine (Tiengwe, Mattick & Bolgiano, unpublished). With a higher strength PBS buffer, containing 10 times more phosphate ion, negligible binding to the adjuvants was found irrespective of the aluminum salt used.

Opposite charge-effects have been found when using basic proteins, such as the Hc domain of botulinum toxins, as would be predicted [47], and in formulations dominated by the low pH of aluminum phosphate, there was a lack of effect of phosphate on the adsorption of pneumococcal 9 V which was 94% bound to adjuvant [15]. Hem & HogenEsch have described the competing (and sometimes enhancing) effects from phosphate as due to ligand exchange or substitution with hydroxyl groups at the surface of the adjuvant [6].

The predominantly charged nature of these interactions means that vaccine formulation studies, involving pH, ionic strength and type of buffer salt can be used to fine-tune the adsorption and amount of aluminum required. By changing only the buffer anion, variations in adsorption, particularly for aluminum phosphate, can be achieved. It is interesting to note that all three licensed monovalent MenC conjugate vaccines used in this study were completely bound to adjuvant, including that of a MenC-CRM_{197} B to aluminum phosphate at its lowered isoelectric point in unbuffered saline. Phosphate ions, pH and elevated temperatures are often, utilized in

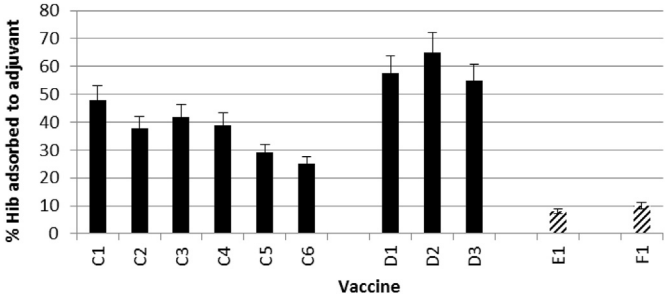

Fig. 2. Adsorption of Hib conjugate in DTwP-combination vaccines. The adsorption of Hib conjugates to aluminum phosphate in DTwP combination vaccines was measured in TT conjugates (C and D series), and CRM_{197} conjugates (E and F series). The numeral in the code represents different manufacturing lots of the same vaccine. The % adsorbed values were determined from measure of PRP remaining in the supernatant of spun-desorbed samples relative to that in untreated vaccine. The error bars represent the combined standard uncertainty of the assay.

'desorption' protocols [7,14,41,49,54] and factors affecting adsorption are also thought to affect the natural elution of Ags from adjuvants post-administration.

In this study, the vaccine components in the non-adsorbed fraction were measured by spectrophotometry and anion-exchange chromatography. The protein assay was the least sensitive and most variable, and measured protein adsorption was often lower than polysaccharide adsorption, also seen by the manufacturer of a MenC-TT vaccine [50]. ELISA assays for carrier protein [54,59] or PS [50,51], which was used directly on the adjuvant-precipitate, offer other alternatives, as does HPAEC-PAD [44,60].

The stability of vaccines adsorbed to adjuvant depends on consistent manufacturing, and stable adjuvant-Ag interaction throughout shelf-life, formulation and storage temperature [22,30,61–63], avoiding effects as seen with metal ion-catalyzed depolymerisation of Hib PS arising from an aluminum adjuvant [29] or possible Ag-adjuvant displacement. The possibility of Ag desorption from adjuvant could conceivably occur, for example, following depolymerization of the labile phosphodiester bonds in Hib, MenA, MenX, and Pneumo types 6B, 18C, 19F and 23F, from the formation of phosphate ions. In this study, there was no evidence of a physical interaction between Hib and MenC conjugates when combined adding to previous findings from a size-exclusion chromatography study of CRM_{197} conjugates [64]. The stability of the adsorption of three licensed MenC conjugates in this study was demonstrated at 4 °C and 37 °C for one month.

Because the effect of adjuvants are demonstrated during the non-clinical evaluation of vaccines [23], there are few published examples that link adjuvant adsorption, composition or dose with clinical immunogenicity, efficacy or reactogenicity in humans [65–67]. Prior to the introduction of a booster dose of conjugate vaccines at 12 mo in the U.K., the effect of combining Hib conjugates with diphtheria-tetanus-acellular pertussis vaccines gave lower than expected immunogenicity and protection due to lower antibody quality [34,35]. Specific adjuvant adsorption to the Hib component has been considered as a possible cause [68]. However, a separate clinical study of Hib-CRM_{197} conjugates in phosphate buffered saline and equivalent aluminum and Ag dose levels administered to infants with DTaP vaccine, showed no significant difference in anti-PRP responses [65]. Despite some clinical and post-marketing findings of potential interference in the immunogenicity and protection from conjugate vaccines, immunization programs depend on the co-administration and combination of a large number of pediatric vaccines. Complex vaccine formulations should be optimized in terms of stability of adsorption, and Ag-adjuvant dose to maximize the benefit of saccharide-protein conjugate components when aluminum adjuvants are necessary.

Conflict of interest statement

The authors have no financial interest in any vaccine manufacturer, including any arising from employment, consultancies, honoraria, stock ownership, patents, grants or royalties.

Contributors

RBDO and SEA performed adsorption studies as partial fulfillment of a MSC in Applied Biomolecular Technology at the University of Nottingham, and were supervised by DTC and BB. KB performed the DTwP analysis. All authors were involved in the preparation of the manuscript.

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