

## Salivary antibodies following parenteral immunization of infants with a meningococcal serogroup A and C conjugated vaccine

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### SUMMARY

Bacterial and viral salivary antibody testing is proving sensitive and specific, useful for epidemiological studies, and is simple and non-invasive. Salivary serogroup C polysaccharide-specific (SC PS-S) IgA and IgG were determined as a proportion of total salivary IgA and IgG in a group of UK infants who were recipients of a conjugated A/C meningococcal PS vaccine. Geometric mean concentrations (GMCs) of salivary SC PS-S IgG per mg of total IgG ( $\mu\text{g}/\text{mg}$ ) were 0.1 pre-vaccination, rising to 8.2 post first, 16.1 post second and 29.3 post third dose of vaccine. For IgA, the corresponding GMCs in ng/mg were 0.1, 82.8, 69.6 and 91.2. Significant correlations ( $P < 0.0001$ ) were found between serum Ig and salivary IgG SC PS-S antibody for pre-vaccine and 1 month post each dose of vaccine suggesting that SC PS-S IgG in saliva was largely derived from serum. Of the five infants whose sera were analysed for isotype-specific responses, only traces of IgM and IgA were measurable suggesting that the SC PS-S IgA was locally produced. These findings suggest that the widespread use of meningococcal conjugate vaccines is likely to reduce nasopharyngeal carriage and may thereby induce herd immunity in the vaccinated population.

### INTRODUCTION

Meningococcal infections are an important cause of morbidity and mortality worldwide, with serogroup B and C strains responsible for most cases in the United Kingdom. In England and Wales, notifications of meningococcal disease rose from 1129 cases in 1994 to 2330 cases in 1996 with serogroup C cases accounting for 40% of recent laboratory-confirmed cases [1]. *Neisseria meningitidis* is carried in the nasopharynx and is transmitted from person to person by air-borne droplets or contact with respiratory secretions. Nasopharyngeal carriage of *N. meningitidis* is normally asymptomatic but is occasionally followed by invasive

disease. An ideal meningococcal vaccine should protect from disease but in addition should prevent carriage of meningococci, thereby preventing transmission.

Serological assays are used currently for the detection and quantitation of antibodies to meningococcal serogroup A and/or C polysaccharide and to measure responses to polysaccharide-protein conjugate vaccines [2, 3], thereby providing an indirect estimate of protection against invasive meningococcal infection. However, collection of sera for large scale epidemiological studies is difficult and expensive. Salivary antibody testing is now developing into a useful alternative to serological assays for the detection of antibodies against various bacterial and

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viral diseases and for evaluation of vaccine-induced immunity. Collection of saliva is simple and non-invasive, has the potential for blood-free home and workplace collection of patient samples (with avoidance of inadvertent transmission of blood-borne pathogens), and is easier to use in paediatric and elderly populations. Salivary antibody testing is sensitive and specific and has proved to be a feasible addition or alternative to serology for diseases such as, rubella [4], measles [5], mumps [6], hepatitis A [7] and HIV [8].

*Haemophilus influenzae* type b (Hib) conjugate vaccines have proved to be safe, immunogenic and protective against Hib infection [9–13] and meningococcal serogroup A and C oligosaccharide–protein conjugate vaccines are similarly proving safe and immunogenic in young infants [14, 15]. Hib conjugate vaccines have been shown to reduce carriage of Hib [16–20] and the large scale use of Hib conjugate vaccines in several countries has resulted in a reduced incidence even in those age groups who were not included in the vaccination programme. This decreased incidence is thought to be due to a reduction in Hib carriage with consequent reduced exposure to Hib amongst unimmunized individuals [21]. Reduction of Hib carriage suggests that parenterally administered conjugate vaccines may induce local immunity in the nasopharynx. An effect of serogroup C conjugate vaccine on nasopharyngeal carriage of the homologous meningococcal serogroup has yet to be demonstrated.

To investigate whether mucosal antibodies were produced following parenteral vaccination with a meningococcal oligosaccharide–protein conjugate vaccine, we measured SC PS-S IgG and IgA antibodies in saliva collected from infants prior to, and following vaccination. Salivary antibody levels were compared with serum immunoglobulin antibody levels, which had been determined previously [15].

## METHODS

### The study population

The study population consisted of 58 infants resident in the Gloucestershire health district, aged 8–12 weeks, who were eligible for routine primary immunization with diphtheria, tetanus, pertussis (DTP), Hib and polio vaccines, between January and March 1995. Study nurses obtained written informed consent from parents and participants were immunized according

to the standard UK schedule at 2, 3 and 4 months of age.

### Vaccines and immunization

All infants were given combined Hib oligosaccharide conjugated to diphtheria CRM<sub>197</sub> (HibTITER<sup>™</sup>, Wyeth–Lederle Vaccines and Pediatrics, Pearl River, USA) and Evans/Medeva (Leatherhead, UK) DPT whole cell vaccine with 0.6 mg of aluminum hydroxide adjuvant (Al(OH)<sub>3</sub>) in the right thigh. These vaccines were mixed in the same syringe at the time of immunization according to the manufacturers' instructions. Each infant also received oral polio vaccine at each visit.

Infants received the meningococcal serogroup A/C conjugate vaccine by intramuscular vaccination in the left thigh. This included 0.25 ml of meningococcal vaccine (lot 090991/2) and 0.25 ml of Al(OH)<sub>3</sub> (lot 250693) (Biocine Sclavo). Each vaccine dose contained 11.2 µg of serogroup A, 11.7 µg of serogroup C oligosaccharide and 48.7 µg of CRM<sub>197</sub>. The production and physicochemical properties of this vaccine have been detailed elsewhere [22].

Blood and saliva samples were obtained from each infant before the first vaccine dose, and then 4 weeks after each dose of vaccine.

### Serum SC Ig PS-S antibody levels

Serogroup C PS-S total immunoglobulin levels in serum had been determined previously [15] by a standardized ELISA method [2].

### Serum SC PS-S IgG, IgM and IgA isotype antibody levels

Serum SC PS-S IgG, IgM and IgA isotype antibody levels were measured in five subjects for pre-vaccination, post first, second and third doses of vaccine. The methodology utilized was described above for the serum SC PS-S Ig except individual isotype anti-human IgG Fc PAN monoclonal (mab), anti-human IgM Fc PAN mab or anti-human IgA Fd PAN mab conjugated to peroxidase were used (Stratech Scientific Ltd, Luton, Bedfordshire, UK). The substrate utilized was 3,3',5,5'-tetramethylbenzidine dihydrochloride (TMB) (Sigma–Aldrich Co. Ltd, Poole, Dorset, UK) and reactions were stopped with 2.5 M sulphuric acid and absorbances read at 450 nm. The

values assigned for IgG, IgM and IgA for the standard serum CDC 1992 were 24.1, 2 and 5.9  $\mu\text{g/ml}$ , respectively.

### Saliva collection

Oral test kits (Malvern Medical Developments, Blackpole Trading Estate West, Worcester, UK) were utilized for saliva collection. Briefly, the foam swab was removed from the collection device and rubbed over the infants' gums until saturated with saliva. The foam swab was then replaced in the tube, labelled and posted to the Meningococcal Reference Unit (MRU), Manchester Public Health Laboratory (MPHL).

### Extraction of saliva samples from foam swabs

Transport medium (10% foetal calf serum, 0.2% Tween 20, PBS pH 7.4) (1 ml) was added to the tube containing the swab and vortexed for 20 s to ensure foaming of the transport medium. The swab was removed using sterile forceps in a twisting motion to extract as much liquid as possible. The swab was then inverted and replaced in the tube so that the pink foam was at the top. The cap was replaced and the tube centrifuged at 2000 *g* for 5 min. The inverted swab was removed and discarded and the extracted saliva recovered from the tube, divided into aliquots and stored at  $-80^\circ\text{C}$ .

### Quantitation of total IgG and IgA immunoglobulins in human saliva by ELISA

Immulon 2 plates (Dynex Technologies, Billingshurst, West Sussex, UK) were coated with either goat anti-human IgG or IgA (Dako, A/S, DK-2600 Glostrup, Denmark) at a dilution of 1/1000 in 10 mM PBS, pH 7.4. Plates were sealed with pressure-sensitive film and incubated at  $4^\circ\text{C}$  overnight. After overnight incubation the well contents were aspirated and washed five times with PBS, pH 7.4 containing 0.1% polyoxyethylene lauryl ether (Brij-35) following by addition of 200  $\mu\text{l}$  of 10 mM PBS, pH 7.4 containing 5% newborn bovine serum (NBBS) to each well. Following incubation at room temperature for 1 h, the buffer was decanted from the plate wells and the plates were blotted vigorously. Serum/conjugate buffer (10 mM PBS, pH 7.4 containing 5% NBBS and 0.1% Brij-35) (100  $\mu\text{l}$ ) was added to wells of columns 1, 2 and 3 which were used for the standards which

comprised immunoglobulins IgG (5.7 mg/ml) (Sigma–Aldrich Co. Ltd) and IgA (2.1 mg/ml) (Sigma–Aldrich). Twofold pre-dilutions of both immunoglobulin isotypes were made in serum/conjugate buffer at a dilution of 1:5000 and 1:2000 for IgG and IgA respectively. Dilutions of the twofold series (100  $\mu\text{l}$ ) were added to wells A1, A2 and A3, the contents mixed five times and double diluted through to wells H1, H2 and H3 where 100  $\mu\text{l}$  was discarded so that the final volume was 100  $\mu\text{l}$ . High (1:200), middle (1:400) and low (1:800) internal controls were added (100  $\mu\text{l}$ ) to plate wells A12–B12 (high), C12–D12 (middle), and E12–F12 (low). These internal control dilutions were made from saliva collected from a member of staff recently vaccinated with a licensed meningococcal A/C vaccine. As a conjugate control blank, 100  $\mu\text{l}$  of serum/conjugate buffer was added to plate wells G12–H12. The study group saliva samples, diluted 1:100 and 1:200, in serum/conjugate buffer were added in duplicate to the remaining wells of the microtitre plate with 16 saliva samples assayed per plate. Plates were sealed and incubated overnight at  $+4^\circ\text{C}$ .

Appropriate amounts of goat anti-human IgA and IgG conjugate peroxidase (Sigma–Aldrich) at a final dilution of 1:1000 for each isotype in serum/conjugate buffer were prepared. Following five washes, 100  $\mu\text{l}$  of these conjugates were added to all wells of the plate and incubated for 2.5 h at room temperature. Following a further five washes, 100  $\mu\text{l}$  of freshly prepared substrate, *o*-phenylenediamine dihydrochloride (OPD) (Sigma–Aldrich) was added to all wells. Plates were incubated at room temperature for 10 min and the reaction stopped with 2.5 M sulphuric acid. The optical density (OD) of each well was read at 492 nm and blanked on each plate on the average of the ODs of plate wells G12 and H12.

Data were analysed using a four-parameter logistic curve model with ELISA software provided by CDC, Atlanta, USA [23]. The calibration value for IgA was 5700  $\mu\text{g/ml}$  and for IgG was 2100  $\mu\text{g/ml}$ . Results were calculated as  $\mu\text{g}$  of total IgA or IgG antibody per ml of saliva.

### Quantitation of SC PS-S antibodies (IgG and IgA) in saliva

The methodology for salivary IgA and IgG antibody testing was a modification of the serological assay of Gheesling and colleagues [2]. Immulon 2 plates were

coated with a mixture of methylated human serum albumin (mHSA) at a final concentration of 5 µg/ml and meningococcal serogroup C polysaccharide at a final concentration of 5 µg/ml in 10 mM PBS, pH 7.4. Plates were sealed and incubated at 4 °C overnight. After overnight incubation the well contents were aspirated and wells washed five times with 10 mM PBS, pH 7.4 containing 0.1% Brij 35. Wells were then filled with 200 µl of 10 mM PBS (pH 7.4) containing 5% NBBS.

Following incubation at room temperature for 1 h, serum/conjugate buffer (100 µl) was added to columns 1, 2 and 3. Twofold pre-dilutions of the standard reference serum (*N. meningitidis*) human serum pool CDC1992 were made, 1:75 for IgG and 1:50 for IgA, in serum/conjugate buffer and 100 µl of these dilutions were added to wells A1, A2 and A3. The contents of the wells were mixed and double diluted through to wells H1, H2 and H3 where 100 µl was then discarded.

The saliva samples to be quantitated, 32 specimens per plate, in duplicate, were dispensed undiluted into the microtitre plate. High (1:4), middle (1:8) and low (1:16) internal controls were added (100 µl) to plate wells A12–B12 (high), C12–D12 (middle), and E12–F12 (low). These control dilutions were made from saliva collected from a member of staff recently vaccinated with a licensed meningococcal A/C vaccine (AC Vax, SmithKline, Rixenstat, Belgium). As a conjugate control blank, 100 µl of serum/conjugate buffer was added to plate wells G12–H12. Plates were sealed and incubated overnight.

Following five washes with 10 mM PBS, pH 7.4 containing Brij 35, goat anti-human IgA and IgG peroxidase conjugates (Sigma) were prepared separately at a final dilution of 1:1000 for each isotype in serum/conjugate buffer. Diluted conjugate (100 µl) was added to each well, plates were sealed and incubated for 2.5 h at room temperature. The substrate (OPD) was prepared, added as above and the plates read at 492 nm. Data were analysed using the ELISA software with calibration factors of 24.1 and 5.9 µg/ml for IgG and IgA respectively. Results were calculated as ng of SC PS-S IgA or IgG isotype antibody per ml of saliva.

### Statistics

Antibody levels were log-transformed and GMCs with 95% confidence intervals (CIs) were calculated. The Mann–Whitney *U* test was used to establish if

Table 1. Serogroup C-specific IgG, IgM and IgA antibodies for five infants' sera including pre-vaccination, post one, post two and post three doses of serogroup A/C conjugate vaccine

Subject no.	Bleed*	Serum serogroup C-specific (µg/ml)		
		IgG	IgM	IgA
1	A	1.8	nd†	nd
	B	10.3	0.1	nd
	C	7.8	nd	nd
	D	14.5	0.1	nd
2	A	0.6	0.2	nd
	B	8.2	0.1	0.2
	C	41.8	0.2	0.5
	D	47.1	0.3	0.4
3	A	0.4	nd	nd
	B	7.1	0.2	nd
	C	17.4	0.3	nd
	D	16.9	0.4	0.1
4	A	0.3	nd	nd
	B	14.4	0.2	0.4
	C	35.5	nd	0.4
	D	32.3	0.2	0.4
5	A	1.4	nd	nd
	B	2.3	nd	nd
	C	40.2	nd	1.0
	D	22.6	nd	0.7

\* A, Pre-vaccination; B, Post one dose; C, Post two doses; D, Post three doses.

† nd, non detected.

differences in salivary antibody concentrations between different vaccine doses were significant. The relationship between serum and salivary SC PS-S antibody levels was measured using Pearson's correlation coefficient.

## RESULTS

### Compliance with sampling

Of the 58 infants in the study cohort, both serum and saliva samples were available for testing from 51 infants pre-vaccination, 48 infants post first dose, 49 infants post second dose and 45 infants post third dose. Serum SC PS-S isotype responses were measured retrospectively and thus complete sets of sera were only available for five infants.

### Serum SC PS-S IgG, IgM and IgA levels

The serum SC PS-S isotype responses for five infants are given in Table 1. Low or non-detectable levels of

serum SC PS-S IgM and IgA were found with the principal isotype antibody response being IgG.

#### Total salivary IgA and IgG immunoglobulin levels

The GMCs with 95% CIs of the total salivary IgA and IgG immunoglobulin levels are shown in Table 2. IgA was detectable in all saliva samples analysed but IgG was not detected in 6/51 (11.8%) of pre-vaccine and 6/49 (12.2%) of post second vaccine dose saliva samples. There were no significant differences between the GMCs of total salivary IgA or IgG of saliva samples collected pre or post each dose of vaccine.

#### Salivary IgA and IgG SC PS-S antibodies

The GMCs with 95% CIs for IgA and IgG SC PS-S antibodies in saliva samples are also shown in Table 2. A significant rise in both salivary SC PS-S IgA and IgG ( $P < 0.001$ ) was observed between the pre-vaccination and the post first dose samples. However, no significant differences were observed between antibody levels after any further doses of vaccine. Pre-vaccine salivary SC PS-S IgA or IgG was detectable in 7/51 (13.7%) and 2/51 (3.9%) of infants, respectively. After one dose of vaccine this rose to 44/48 (91.7%) and 46/48 (95.8%) for IgA and IgG respectively. For IgA and IgG respectively, the proportions remained similar after two doses (44/49, 91.7%, and 46/49, 93.9%) and three doses of vaccine (42/45, 93.3%, and 48/48, 100%).

#### Amount of SC PS isotype antibody per mg of total salivary antibody

The GMCs with 95% CIs for IgA and IgG SC PS-S antibodies per mg of total salivary IgA or IgG antibody are shown in Table 2. A significant rise in both SC PS-S IgA and IgG per mg of salivary antibody ( $P < 0.001$ ) was observed between the pre-vaccine bleed and the post first dose bleed.

#### Correlation between serum Ig and salivary IgA and IgG SC PS-specific antibodies

Significant correlations ( $P < 0.0001$ ) between salivary SC PS-S IgG per mg of total salivary IgG and serum SC PS-S Ig were found for samples collected pre-vaccination ( $r = 0.943$ ), post first dose ( $r = 0.738$ ), post second dose ( $r = 0.440$ ) and post third dose ( $r = 0.862$ ). The corresponding correlations for IgA were

found to be 0.131 ( $P = 0.009$ ), 0.318 ( $P < 0.0001$ ), 0.053 ( $P = 0.112$ ) and 0.002 ( $P = 0.918$ ).

#### DISCUSSION

The amount of salivary SC PS-S IgG and IgA was quantified per mg of the total salivary IgG and IgA due to the variability of saliva collection. Salivary flow rates are known to vary significantly among individuals as well as in the same individual under different conditions [24]. The oral test kit was utilized since these devices have been designed to collect crevicular fluid-rich saliva in which immunoglobulin concentrations are much higher than in salivary gland secretions [25].

After a single dose of meningococcal conjugate vaccine, both IgA and IgG SC PS-S antibody were detected in infants' saliva samples. Levels of salivary SC PS-S IgG antibodies correlated with serum SC PS-S-antibodies, though levels of salivary IgA did not. This finding has also been noted in relation to Hib [26] and pneumococcal conjugate vaccines [27]. These IgG antibodies detected in saliva may have been derived from serum. Serum-derived fluid enters the saliva from the gingival crevice and across mucosal surfaces and is rich in immunoglobulins [28]. The significant increase in salivary SC PS-S IgA antibody suggests that this meningococcal conjugate vaccine although administered parenterally, induced local production of IgA antibody. This is consistent with the current view that virtually all IgA in saliva is of local origin and is produced independently of the systemic response [29].

To the authors' knowledge, no previous studies have been performed on salivary antibody responses following immunization with meningococcal serogroup A and/or C oligosaccharide-protein conjugate vaccines. However, antibody-secreting cells (ASC) and serum antibody responses have been measured in adult volunteers immunized subcutaneously with a serogroup A/C meningococcal PS vaccine [30]. The vaccine was found to induce an IgA-dominated response directed against the homologous polysaccharides suggesting activation of mucosa-committed B cells.

The ability to reduce the carriage rate of Hib appears to be a property of Hib conjugate vaccines [16–20] whereas non-conjugated Hib polysaccharide vaccines have little effect on carriage or colonization [31]. Although the suggestion has been made that a reduction in carriage of serogroup C meningococci

Table 2. Geometric mean concentrations (GMCs) and 95% confidence intervals (CI) for serum and salivary antibody levels in the cohort of infants who had received three doses of serogroup A/C conjugate vaccine and from whom both serum and saliva samples were collected

	GMCs of serum serogroup C specific Ig $\mu\text{g/ml}$ (95% CI)	GMCs of salivary total IgA $\mu\text{g/ml}$ (95% CI)	GMCs of salivary total IgG ng/ml (95% CI)	GMCs of salivary serogroup C specific IgA ng/ml (95% CI)	GMCs of salivary serogroup C specific IgG ng/ml (95% CI)	GMCs of ng of salivary serogroup C specific IgA per mg of total IgA ng/mg (95% CI)	GMCs of $\mu\text{g}$ of salivary serogroup C specific IgG per mg of total IgG $\mu\text{g/mg}$ (95% CI)
Pre-vaccine	0.6 (0–1.4)	30.1 (22.2–38)	432.4 (314.0–550.8)	0.1 (0–2.1)	0.6 (0–0.4)	0.1 (0–21.7)	0.1 (0–0.7)
Post one dose	11.8 (7.1–16.5)	28.3 (19.7–37)	552.5 (407.9–697.1)	3.5 (2.0–4.8)	4.6 (2.5–6.7)	82.8 (34.9–130.7)	8.2 (4.1–12.3)
Post two doses	40.0 (27.8–52.1)	24.5 (17.9–31.2)	404.4 (289.5–519.4)	2.9 (0–6.2)	9.2 (2.9–15.5)	69.6 (25.1–114.1)	16.1 (8.0–24.3)
Post three doses	37.1 (23–51.2)	21.9 (15.5–28.3)	426.6 (347.7–505.5)	2.8 (1.6–4.0)	12.6 (7.9–17.3)	91.2 (7.7–174.6)	29.3 (16.2–42.4)

may follow immunization with meningococcal plain PS [32] it is generally considered that, as for Hib PS vaccines, nasopharyngeal carriage is not affected. As Hib conjugate vaccines reduce Hib carriage rates it might be assumed that meningococcal serogroup C conjugate vaccines would also reduce carriage of serogroup C meningococci.

The reasons why Hib PS vaccines do not reduce carriage rates whereas conjugated vaccines do, may be due to the higher levels of serum anti-capsular antibodies induced by conjugated vaccines than by PS vaccines, an immune response that is likely to be reflected on the mucosal surface. Conjugated vaccines may also induce mucosal immunological memory. This could be brought about by stimulation of B cells in lymph nodes near the vaccine injection site followed by migration to the mucosa to secrete antibodies [33, 34].

Studies on serum antibody responses clearly show that although meningococcal PS vaccines induce T-independent responses [35] conjugate vaccines are able to induce T cell-dependent immunological memory [36, 37]. The development of mucosal memory could result in a rapid mucosal immune response after contact with meningococci and in the elimination of the bacteria. Evidence for the elimination of meningococcal serogroup C carriage will be difficult to demonstrate due to the low carriage rate of serogroup C meningococci [38, 39].

Salivary antibody testing is now used increasingly due to the inherent advantages of this cost-effective technology, especially for large-scale epidemiological studies. Indeed the impact of a mass vaccination campaign against measles and rubella in England and Wales has been assessed using a saliva test for measles-specific antibody [40] and the first Zambian population-based HIV survey showed that saliva-based testing was accurate and acceptable [41].

Our results suggest that after immunization with conjugated meningococcal serogroup C vaccine, SC PS-S IgG in saliva was derived from serum whereas, due to the low levels of serum SC PS-S IgA antibodies, salivary IgA of the same specificity implied activation of mucosa-committed B cells. As with Hib carriage following vaccination with the Hib conjugate vaccine, the induction of SC PS-S antibodies on the nasopharyngeal mucosa by parenteral immunization with the homologous conjugated vaccine are likely to lead to a reduction of meningococcal serogroup C carriage in due course, thereby offering protection through herd immunity to susceptible individuals.

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