Vaccine xxx (xxxx) xxx

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Sublingual immunisation with GBS serotype III capsular polysaccharide-tetanus toxoid conjugate vaccine induces systemic and mucosal antibody responses which are opsonophagocytic and inhibit GBS colonisation of vaginal epithelial cells

Mohamed Deifallah Yousif [a,b], Arif Felek [a], Manolya Saydam [a], Seanette Wilson [c], Sudaxshina Murdan [b,*], Fatme Mawas [a,*]

[a] Vaccines Division, Scientific Research & Innovation Group, MHRA, Potters Bar, UK
[b] Department of Pharmaceutics, UCL School of Pharmacy, University College London, London, UK
[c] The Biovac Institute, Cape Town, 7405, South Africa

ARTICLE INFO

Article history:
Received 15 July 2022
Received in revised form 24 August 2022
Accepted 26 August 2022
Available online xxxx

Keywords:
Group B streptococcus
Conjugate vaccine
Mucosal immunity
Opsonophagocytosis
Sublingual
Vaginal colonisation

ABSTRACT

No vaccines are currently licensed against Group B streptococcus (GBS), an important cause of morbidity and mortality in babies and adults. Using a mouse model, and *in vitro* opsonophagocytosis and colonisation assays, we evaluated the potential of a sublingually-administered polysaccharide-conjugate vaccine against GBS serotype III. Sublingual immunisation of mice with 10 μg of GBS conjugate vaccine once a week for 5 weeks induced a substantial systemic IgG anti-polysaccharide response which was similar to the level induced by subcutaneous immunsation. In addition, sublingual immunisation also induced mucosal (IgA) antibody responses in the mouth, intestines and vagina. Immune sera and intestinal washes were functionally active at mediating killing of the homologous GBS serotype III in an opsonophagocytosis assay. In addition, intestinal and vaginal washes inhibited the colonisation of mouse vaginal epithelial cells by the vaccine homologous strain. These results suggest that, in addition to the induction of high levels of IgG antibodies that could be transduced from the immunised mother to the foetus to protect the newborn against GBS infection, sublingual immunisation can elicit a substantial mucosal antibody response which might play an important role in the prevention of GBS colonisation in immunised women, thereby eliminating the risk of GBS transmission from the mother to the baby during pregnancy or at birth.

1. Introduction

Group B streptococcus (GBS) is a leading cause of severe bacterial infection in the first 3 months of life and of septic morbidity amongst mothers [1–3]. It is also an important cause of morbidity and mortality among non-pregnant adults, particularly older people, individuals of black ethnicity and adults with underlying medical conditions [4–6]. GBS is also likely to be an important cause of stillbirth, especially in Africa [7].

GBS-infected infants generally acquire the organism intrapartum from their rectovaginally-colonised mothers, and the proportion of women colonized with GBS during pregnancy ranges from 10 to 40 % worldwide [8–10]. Newborns colonized with GBS may develop sepsis, pneumonia, or meningitis. Early-onset disease (EOD) appears during the first week of life and accounts for 60–80% of GBS disease, while late-onset disease (LOD) appears between 8 and 90 days of age and accounts for 20–40% of GBS disease [11–15]. Current rates of EOD disease are estimated to range from 0.2 – 4 cases/1000 live births [16–19]. In EOD, bacteraemia is present in 27–87% and meningitis is documented in 6–17% of cases [18]. Neurological sequelae occur in 15–50% of survivors of both early and late-onset meningitis.

All GBS strains possess a capsular polysaccharide (CPS) on their surface which is a major virulence factor. Ten different CPS serotypes have been characterized (Ia, Ib, II, III, IV, V, VI, VII, VIII and IX), of which six (Ia, Ib, II, III, IV and V) are responsible for the vast

* Corresponding authors.
E-mail address: fatme.mawas@nibsc.org (F. Mawas).

https://doi.org/10.1016/j.vaccine.2022.08.064
0264-410X/

majority of disease in North America and Europe [17,20–24], whereas serotypes V, VI, VII and IX, are prevalent in Africa [25] and serotypes Ia, Ib, V, VI and VIII predominate in Japan [26–28].

Antibodies against the CPS confer protection against GBS; therefore, the risk of the infant acquiring the infection from a colonized mother will depend on whether or not the newborn has acquired protective levels of serotype-specific maternal antibodies. Major risk factors for developing EOD include maternal GBS carriage, prematurity, low birth weight, prolonged rupture of membranes, intrapartum fever, young maternal age, black ethnicity, previous delivery of an affected baby and low levels of anti-CPS antibodies [1]. LOD can be acquired through either vertical, nosocomial or community sources [29].

The current strategy for treatment of GBS diseases is the use of intrapartum antimicrobial prophylaxis in the mother (IAP) to prevent infection in newborns. This prevention strategy involves administration of antibiotics to mothers who have certain risk factors for GBS (17% of all women) [30] or those with positive GBS culture [31]. However, this approach has many complications and limitations, and it does not prevent all EOD because vertical transmission might occur at any stage up to and during labour. IAP has no effect on invasive maternal disease, stillbirth, preterm delivery and LOD and increases the risk of emergence of antibiotic-resistant strains [32,33]. Concerns have also been raised about the potential negative impact of the overuse of antibiotics during labour on the development of the neonatal microbiome, which may lead to health consequences later in life [34].

An alternative strategy to IAP would be the administration of GBS vaccines to child-bearing age/pregnant women to enable subsequent passive transfer of vaccine-induced antibodies from the mother to the foetus/infant. Monovalent, bivalent and trivalent GBS conjugate vaccines [prepared from CPS conjugated to tetanus toxoid (TTxd) carrier protein or non-toxic cross-reacting material from *Corynebacterium diphtheria*; CRM197 protein] have been developed and evaluated in Phase 1 and II clinical trials in adult volunteers, including pregnant women at 30–32 weeks of gestation and shown to be well tolerated and immunogenic [35–40]. Other vaccines in Phase I/II clinical trials include a CRM-197 conjugate vaccine against the 6 predominant serotypes: Ia, Ib, II, III, IV and V [41] and a protein-based vaccine made of the N-terminal of GBS alpha and rib proteins (all GBS strains express either one or both of the proteins) [42]. All these vaccines are for parenteral administration to mothers during the 3rd trimester with the aim of inducing systemic IgG which can cross the placenta to protect the foetus or newborn against GBS infection.

In contrast to these injectable vaccines which mainly induce systemic IgG, we are proposing mucosal, specifically sublingual, administration of the vaccine to the mother. Mucosal immunisation via routes such as sublingual, nasal, rectal and topical is known to elicit systemic as well as mucosal immunity which can provide the first line of defence against a pathogen [43]. Given that the main source of GBS infection in the infant is from the maternal rectovaginal surfaces during vaginal birth, induction of effective immunity at these mucosal surfaces might be able to reduce or eliminate GBS colonisation of the mother and transmission to the baby. One way of achieving mucosal immune responses in the vagina is by vaccine administration via the sublingual route [44–47]. In addition, sublingual immunisation also gives rise to systemic immunity enabling transplacental transfer of maternal IgG antibodies to the foetus and protection against GBS infection.

The aim of this study was to investigate the potential of a sublingually-adminstered GBS conjugate vaccine candidate against serotype III to induce mucosal and systemic immunity that can prevent colonisation with GBS. Experiments were conducted in a mouse model, using Cholera Toxin (CTX) or Cholera Toxin B subunit (CTB) as adjuvants.

2. Materials and methods

2.1. Materials

GBS serotype III conjugate vaccine candidate (prepared from GBS serotype III CPS conjugated to TTxd) was supplied by The Biovac Institute (South Africa) in 100 mM phosphate buffer, pH 7.2 and stored at 4 °C. CPS-biotin conjugate for ELISA was prepared in our laboratory, as described below. Cholera Toxin from Vibrio cholerae (CTX) and the non-toxic Cholera Toxin B subunit (CTB) were purchased from Sigma Aldrich. All chemicals and reagents used in this study were of analytical grade, unless stated otherwise.

2.2. Animals and immunisation

Six to eight week old female BALB/c mice (Charles River, UK) were immunised sublingually (SL) as follows: mice were anesthetised in an isoflurane chamber. Once anaesthetised, the tongue is lifted with a pair of dressing forceps placed under the tongue to expose the underside. The immunogen (20 µl) is deposited slowly and carefully onto the underside of the tongue using a micropipette. Once the entire dose is dispensed, the mouse is returned to the induction chamber and placed in a ventral dorsal position and head straightened to ensure no airways are obstructed. The mouse remains under anaesthesia for a further 30 min before being allowed to recover. Mice were immunised once a week for five weeks, with 20 µl of GBS conjugate (containing 2 or 10 µg of CPS) in the presence or absence of 10 µg of CTX or CTB as adjuvants. The vaccine dose was chosen based on previous experience in SL with ovalbumin (as a model antigen) and CTX as an adjuvant. Subcutaneous (SC) immunisation (once a week for five weeks) with 200 µl of the conjugate (containing 2 µg of CPS, but no adjuvant) served as the positive control for induction of serum IgG response. Blood and faecal pellets were collected on Day 0, one week after the third and last immunisations. At the end of the experiment, the mice were terminally bled by cardiac puncture under general anaesthesia. Mucosal secretions (intestinal, colonic, vaginal and mouth washes) were collected in protease inhibitor buffer (Fischer Scientific UK), supplemented with 2% Foetal calf serum (FCS). All animal procedures were conducted in accordance with the Home Office (Scientific Procedures) Act 1986.

2.3. ELISA for anti-CPS III IgG and IgA antibodies

2.3.1. Preparation of CPS-biotin conjugate

Biotin-CPS conjugates were prepared as previously described by Buffi *et al.* [45]. In summary, carboxylic acid residues on GBS CPS type III were activated using EDC [1-ethyl-3-(3-dimethylaminopropyl) carbodiimide] in MES buffer (pH 5) at a ratio of 1:0.5 (w/w). Biotin hydrazide was added to the reaction at a ratio of 1:0.5 (w/w). The components were allowed to react for 16 h at RT while gently stirred. The conjugated polysaccharide was purified using the Vivaspin centrifugal filter devices fitted with 10 kDa molecular weight cut off filters (Satorius, UK).

2.3.2. ELISA

An *in house* ELISA assay was developed to determine the level of CPS serotype III-specific IgG or IgA antibodies. 96 well microtitre plates (Nunc-Immuno Maxisorp™) were coated overnight at 4 °C with 100 µl/well of the prepared CPS-biotin conjugate at a CPS concentration of 0.5 µg/ml in carbonate buffer (pH 9.6). Serum samples or mucosal secretions from individual mice diluted in assay buffer (phosphate buffered saline, 1% bovine serum albumin, 0.1 % Tween 20) were added to the first column of the plate. Two fold serial dilutions were carried out in the plates which were then

incubated for 2 h at RT. The presence of GBS serotype III- specific IgG or IgA antibodies was detected with rabbit anti-mouse IgG or IgA conjugated to horseradish peroxidase (HRP) (1:2000; Sigma Aldrich), followed by the TMB substrate (Tetramethylbenzidine; Sigma Aldrich). The reaction was stopped with 3 M HCl after 20 min and the absorbance was measured at 450 nm using a Multiskan MS plate reader (Lab systems; ThermoLife Sciences). Data are presented as antibody titre (the last dilution giving OD of $\geq$ 0.5) for IgG and endpoint titre (the last dilution giving OD of $\geq$ mean Blank OD + 3SD) for IgA.

2.4. Opsonophagocytosis assay

2.4.1. Differentiation of HL-60 cells

HL-60 cells (CCL-240; ATCC) were grown in RPMI 1640 (Sigma Aldrich) with 10% heat-inactivated FCS (Gibco) in upright flasks at 37 °C, 5% CO_2. Once confluent, the cells were harvested by centrifugation at 1000g for 5 min and re-suspended to give a final cell density of 6×10^5 cells/ml in the growth medium. Cells were then differentiated into granulocyte-like cells by the addition of 0.8 % N, N-Dimethylformamide (Sigma Aldrich) and incubated for 5 days at 37 °C, 5% CO_2.

2.4.2. Bacteria preparation

A clinical isolate of GBS serotype III (obtained from NCTC) was grown in Brain Heart Infusion broth to mid-exponential phase. Small aliquots in 15% glycerol were prepared (for single use in opsonophagocytosis assay), snap-frozen in liquid nitrogen and stored at −80 °C. The CFU content of the frozen aliquots was estimated from a pre-determined standard curve for viable count vs optical density. On the day of the assay, an aliquot was thawed and resuspended in assay buffer (Hank's Balanced Salt Solution (HBSS) supplemented with 10% heat-inactivated FCS; both from Gibco) to give a concentration of $2.5x10^5$ CFU/ml.

2.4.3. Opsonophagocytsis assay (OPA)

An OPA assay developed *in house* was used to measure the functional activity of the CPS serotype III-specific mouse antibodies: pooled animal sera or mucosal washes from each group of mice were serially diluted in 10 µl volumes (in tripicates) in a flat-bottom 96-well microtitre plate (Falcon) using assay buffer (HBSS, 10% FCS). Bacteria, prepared as described above, were added at 20 µl/well and the plate was incubated at 37 °C for 30 min at 200 rpm. Differentiated HL60 cells (washed and resuspended to give a final concentration of $6.6x10^7$ cells/ml), mixed 1:1 with a mixture of baby rabbit complement (20% v/v; Mast Group; US) and assay buffer (30% v/v) were added in 60 µl volumes to all wells (this will give a bacteria: HL60 cell ratio of 1:400).

Control wells contained effector cells, complement, and bacteria but no immune serum. The reaction mixtures were incubated for 45 min (200 rpm) in a shaking incubator at 37 °C. At the end of the incubation period, 10 µl samples were removed from each well, diluted and then plated on blood agar plates. The plates were incubated overnight at 37 °C, the number of colonies were counted and the means CFU from the triplicate wells at each serum dilution were used to generate the OPA titration curve. Results were expressed as OPA titre being the serum dilution giving 50 % killing.

2.5. Epithelial cell colonisation

Mouse vaginal epithelial cells (CRL-2616, ATCC) were grown to confluence in 24 well plates. One ml of GBS culture, prepared as for the OPA assay ($5x10^6$ CFU/ml), was then added to the vaginal epithelial cells monolayer and the co-culture was incubated for one h at 37 °C. Subsequently, any non-bound bacteria were removed by washing the wells with warm wash buffer (HBSS, 10 % FC S). Cells were detached with 200 µl/ well of 0.25% trypsin-EDTA (10 min) and then lysed with 800 µl / well of 0.025% Triton X-100 in assay diluent. The reaction was then neutralised with one ml of assay diluent. 10 µl aliquots were spotted on blood agar plates and incubated overnight at 37 °C to count the total number of bound bacteria. To measure the level of colonisation inhibition, bacteria were pre-incubated (30 min at RT) with immune serum or mucosal secretion before addition to the epithelial cell cultures in the assay and results were expressed as % colonisation inhibition in the presence of immune serum/mucosal secretion.

2.6. Statistical analysis

The presence/absence of differences (at 95% significance level) between two different groups was determined using the Mann-Whitney test for the immune responses and the Student's *t*-test for % inhibition.

3. Results

3.1. Immunogenicity of the GBS conjugate

3.1.1. Systemic IgG response

Sublingual immunisation of mice with the CPS serotype III conjugate (once a week for 5 weeks) in the presence of CTX adjuvant induced a substantial level of systemic anti-CPS IgG response (GMT 7,352; $p < 0.01$ compared to pre-immune serum). The sublingually-induced IgG response was slightly but not significantly higher than the IgG response following subcutaneous immunisation with the conjugate alone (GMT 4,579; $p > 0.05$ SL vs SC; Fig. 1A). Equally, high IgG response was also obtained in another experiment where the sublingual immunisation was con-

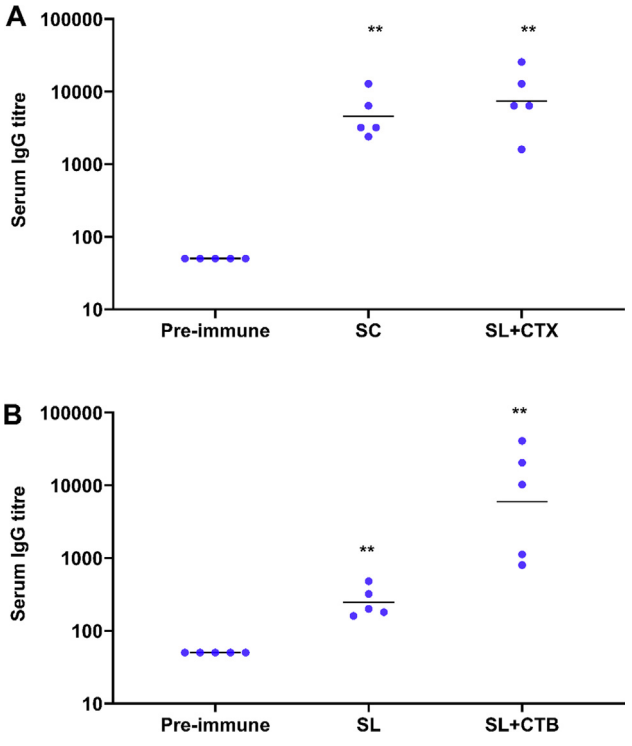

Fig. 1. Serum IgG response to GBS conjugate vaccine, *Mice were immunised SC with 2 µg of GBS conjugate or SL with 10 µg of GBS conjugate in the presence or absence of 10 µg CTX (A) or CTB (B) adjuvant. Sera were tested for anti-CPS response by ELISA. Data is presented as serum titre for individual mice (scatter) and the horizontal bar is the GMT of the group.** p < 0.01, in comparison with pre-immune antibody level.*

ducted in the presence of CTB adjuvant (GMT 5,988; $p < 0.01$; Fig. 1B). Sublingual immunisation with the conjugate without adjuvant induced a weak response (GMT 245) which, however, was still significantly higher than the background IgG level (GMT 50; $p < 0.01$), measured in pre-immunisation mice sera.

3.1.2. Mucosal IgA response

The level of anti-CPS IgA antibodies was determined in secretions from several mucosal tissues and surfaces (mouth, intestines, colon and vagina) and in faecal pellets. Subcutaneous immunisation induced a very low level of CPS-specific IgA antibodies at the different mucosal surfaces ($p > 0.05$). In contrast, mice immunised sublingually with the conjugate in the presence of CTX or CTB adjuvants had substantially higher levels of IgA in the mouth, small intestines, colon, vagina and faecal pellets, compared to pre-immune faecal IgA level (Fig. 2). The GMT of IgA in mice immunised with the conjugate and CTX adjuvant was 128 in the mouth ($p > 0.05$ compared to pre-immune faecal IgA levels, due to one non-responding mouse in the group); 1,549 in the small intestines ($p < 0.01$); 1,176 in the colon ($p < 0.01$), 388 in the vagina ($p < 0.01$) and 2,040 in faecal pellets ($p < 0.01$). A similar good response was also achieved when CTB was used as an adjuvant at all mucosal surfaces, suggesting that the non-toxic CTB is a good choice of mucosal adjuvant. The conjugate alone (i.e. in the absence of adjuvant) was not able to induce any substantial IgA response following subcutaneous or sublingual immunisation (Fig. 2), which was similar to the background IgA level in pre-immune faecal pellets.

3.1.3. Immunogenicity of three vs five doses of GBS conjugate

To investigate the effect of number of immunisations on the the systemic and mucosal responses, serum IgG and faecal IgA (as an indicator of mucosal antibody response) were evaluated after three

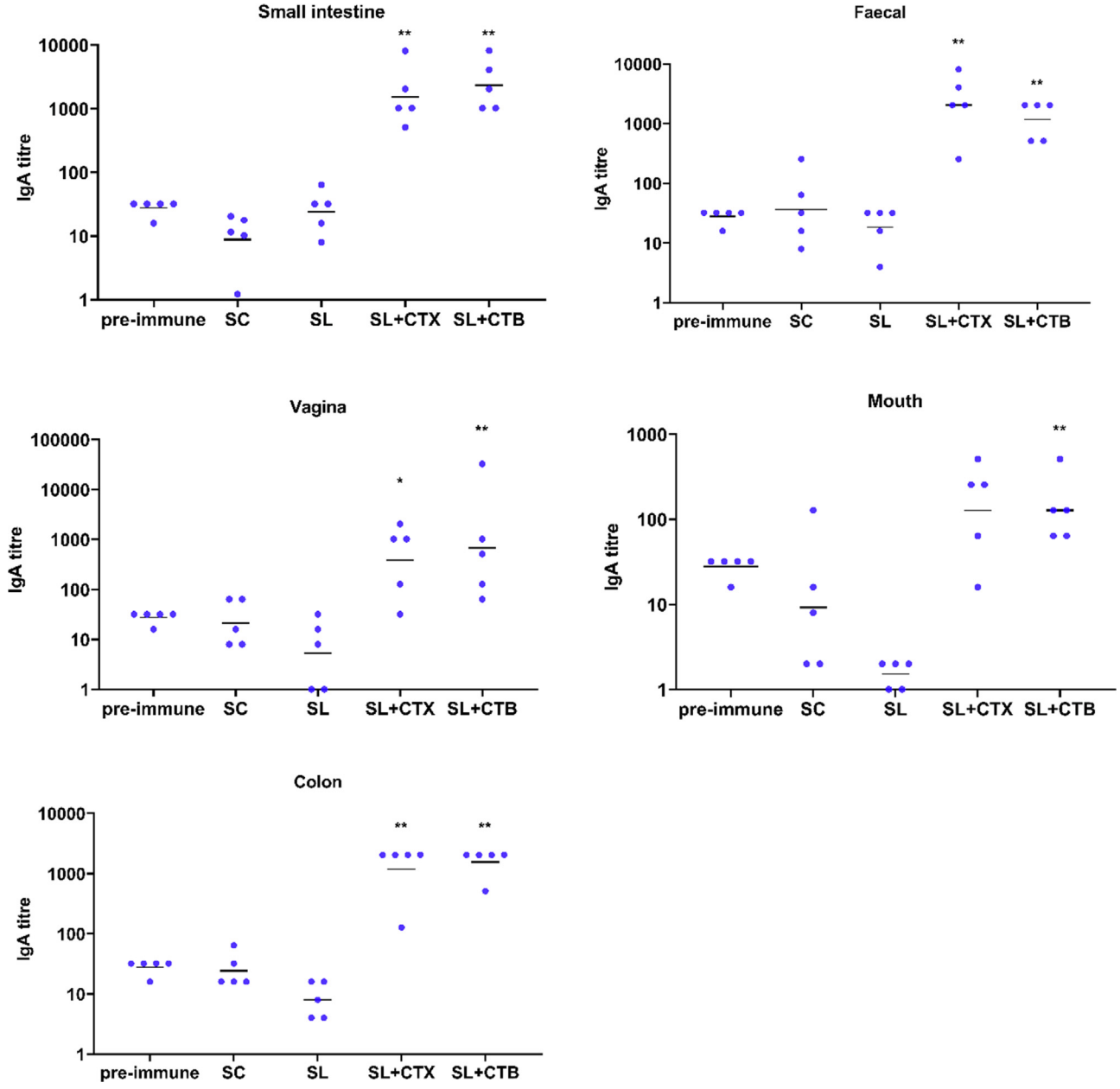

Fig. 2. Mucosal immune response, *Mice were immunised subcutaneously with* 2 μg *of GBS conjugate or sublingually with* 10 μg *of the conjugate in the presence or absence of CTX or CTB adjuvant. Faecal supernatant and mucosal secretions were tested for anti-CPS IgA response by ELISA. Data is presented as IgA titre for individual mice (scatter) and the horizontal bar is the GMT of the group. *p < 0.05; ** p < 0.01 in comparison with pre-immune IgA antibody level measured in day 0 faecal pellets.*

and five immunisations with GBS conjugate. Three subcutaneous immunisations induced a substantial serum IgG response (GMT 2,649; $p < 0.01$;Fig. 3) which was slightly, but not significantly, increased after an additional two immunisations (GMT 4,579; $p > 0.05$). A smaller IgG response was obtained following three sublingual immunisations (GMT 942) which significantly increased by 8-fold after a further two immunisations (GMT 7,352; $p < 0.01$).

The impact of the number of immunisations on the mucosal IgA response was very different. A very low level of mucosal IgA response was measured following three sublingual immunisations (GMT 97), which was not much different from the pre-immunisation level ($p > 0.05$). However, a further two (i.e. a total of five immunisations) resulted in a statistically significant 21-fold increase in the faecal IgA response (GMT 2,040; $p < 0.01$), indicating that the generation of a substantial mucosal IgA response required five sublingual immunisations. In contrast, in the subcutaneously-immunised mice, the mucosal IgA levels were low after three immunisations and remained the same after two further immunisations (GMT 98 and 103, respectively).

3.2. Effect of conjugate dose on the systemic and mucosal immune responses

As the dose of the GBS conjugate was five times lower in the subcutaneous compared to the sublingual route, it was decided to investigate whether a reduced dose of the conjugate could still induce a substantial immune response. Mice were immunised sublingually with 2 or 10 μg of the GBS conjugate in the presence of CTB and the serum IgG and mucosal IgA levels were determined and compared to the response following subcutaneous immunisation. Results showed that the systemic IgG and mucosal IgA responses, at the different mucosal sites, were dose-dependent (Fig. 4). Sublingual immunisation with 2 μg of the GBS conjugate and CTB induced substantial serum IgG response (GMT 2,703) which was lower than, but not significantly, the response following subcutaneous immunisation (4,579; $p > 0.05$). Although this response was at least doubled (GMT 5,989) when the 10 μg antigen dose and CTB was used, this increase was not statistically significant ($p > 0.05$). A similar trend was seen in the mucosal IgA levels at the different surfaces, where after immunisation with the low dose of the conjugate a significant response ($p < 0.05$) was detected at the intestinal (GMT 365), colonic (GMT 338), vaginal (GMT 223) surfaces and in faecal pellets (GMT 169). The IgA response was low in mouth washes (GMT 74) and was not significantly higher than the background ($p > 0.05$). Immunisation with the 10 μg dose induced a two to six-fold increase in the IgA response at the different mucosal surfaces which were all significantly higher than background levels ($p < 0.01$).

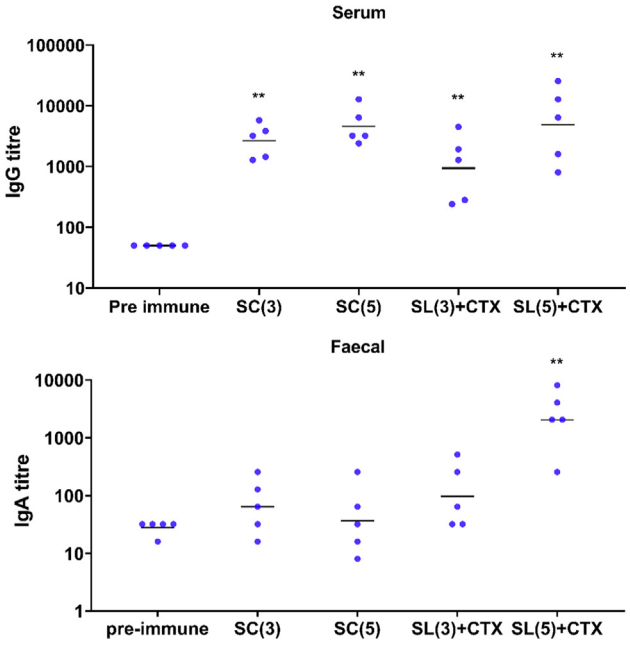

Fig. 3. Kinetics of the systemic and mucosal antibody response, *Mice were immunised subcutaneously or sublingually with 3 or 5 doses of the conjugates (with/without CTX adjuvant) and levels of anti-CPS serum IgG and faecal IgA antibodies were determined. ** $p < 0.01$ in comparison with pre-immune serum IgG or Faecal IgA.*

3.3. Opsonophagocytic activity of the systemic and mucosal antibodies

The opsonophagocytic activity of the immune sera and intestinal washes following sublingual or subcutaneous immunisation was evaluated using an *in-house* developed opsonophagocytosis assay (OPA). Immune sera from mice immunised sublingually or subcutaneously with the GBS conjugate were equally effective at mediating the killing of GBS serotype III by HL60 effector cells with a serum OPA titre of 1,280 while there was no non-specific killing using the pre-immune serum (Table 1). The OPA titre of the intestinal washes from the same groups of animals was 15 for sublingually-immunised animals, while washes from mice immunised subcutaneously had no killing activity at all (OPA titre < 5), which was similar to day 0 faecal supernatant.

3.4. Inhibition of vaginal epithelial cell colonisation by anti-CPS antibodies

To determine whether the induced mucosal responses could play a role in preventing GBS colonisation at mucosal surfaces, a GBS-vaginal cell binding assay was performed to determine the level of GBS III colonisation and the ability of intestinal and vaginal mucosal antibodies to prevent/reduce GBS colonisation (Fig. 5). GBS serotype III was capable of binding to the vaginal cell line ($2x10^4$ CFU/ cell monolayer gave 100% binding). Pre-incubation of the bacteria with vaginal washes from mice sublingually-immunised with 10 μg or 2 μg of the conjugate and CTB reduced bacterial binding by ~ 75% ($p < 0.001$, when compared to Day 0 faecal supernatant), while vaginal washes from control mice sublingually-immunised with GBS conjugate in the absence of CTB reduced the GBS binding by only 32% ($p > 0.05$), a level similar to that achieved by pre-immunisation faecal supernatant. Intestinal washes from the same mice (i.e. sublingually immunised with the conjugate + adjuvant) reduced GBS colonisation by about 50% ($p < 0.001$), while intestinal washes from mice sublingually-immunised with the GBS conjugate alone failed to reduce GBS colonisation compared to the pre-immunisation control ($p > 0.05$).

4. Discussion

Monovalent and multivalent GBS conjugate vaccines (using mainly tetanus toxoid as a carrier protein) against the 5 main serotypes in North America and Europe (Ia, Ib, II, III and V) have been prepared and shown to be safe and induce protective antibody levels in several preclinical [48–51] and clinical studies in healthy adult volunteers, including pregnant women [52]. Other vaccines based on selected conserved surface-exposed proteins have also been developed and shown, in animal models, to confer protection against GBS challenge or induce opsonophagocytic antibodies against several GBS strains and some are currently in clinical trials [53–55]. However, none of these vaccines have approached licensing so far, because of unresolved issues with regulatory authorities on the target group, safety concerns with regards to immunising

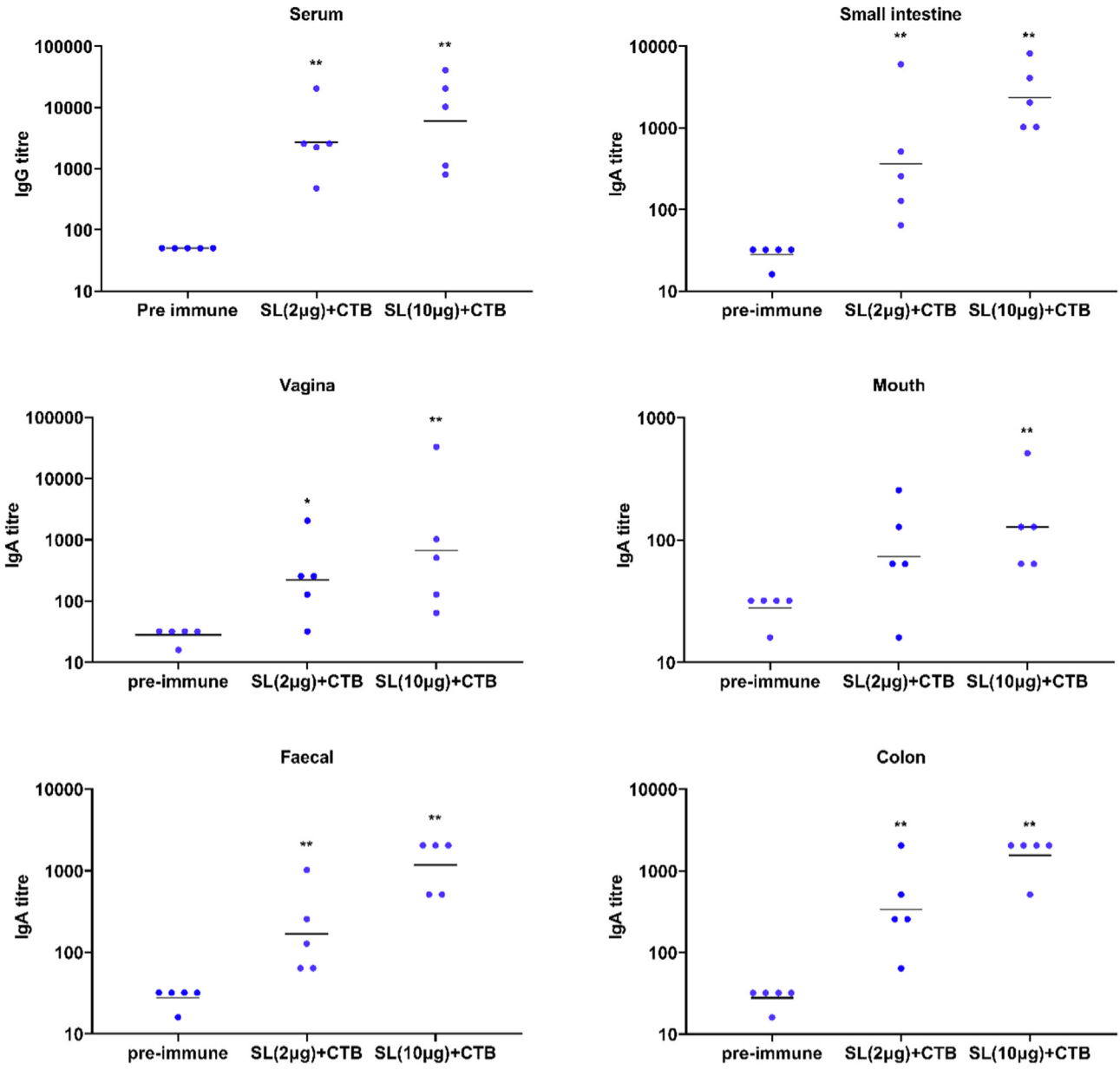

Fig. 4. Effect of conjugate dose on the systemic and mucosal immune responses. *Mice were immunised sublingually with* 2 µg *or* 10 µg *of the conjugates and levels of anti-CPS serum IgG and faecal IgA antibodies were determined.* * $p < 0.05$, ** $p < 0.01$.

Table 1
Opsonophagocytosis of GBS III with immune serum and mucosal secretions.

Sample tested	GBS Conjugate dose (µg)	Delivery route	Adjuvant	OPA titre
Serum	10	SL	CTX	1280
Serum	2	SC	None	1280
Pre-immune serum	None	None	None	< 5
Intestinal washes	10	SL	CTB	15
Intestinal washes	2	SC	None	< 5
Pre-immune faecal supernatant	None	None	None	< 5

pregnant women and agreement on surrogates of protection to replace efficacy trials. A Phase III efficacy trial of a GBS vaccine to prevent neonatal disease is likely to be very difficult, due to ethical issues and the widespread use of antibiotics to treat at-risk deliveries. An alternative approach to assess a GBS vaccine efficacy would be to use the serological correlate of protection, measurement of specific antibodies and OPA that measures the ability of serum antibodies to opsonise GBS for killing by effector cells in the presence of complement [56,57].

In this study, we used these *in vitro* correlates of immunity/protection to evaluate the potential of the sublingual route for delivery of a GBS III conjugate vaccine candidate to induce protective systemic and mucosal antibody responses. The sublingual route is non-invasive and more akin to how pathogens usually enter the body i.e. via mucosal surfaces, compared to the injection route of immunisation.

We showed that sublingual immunisation elicited a substantial serotype-specific systemic IgG antibody response at a similar level to that induced by subcutaneous immunisation. The IgG antibodies from sublingual and subcutaneous immunisations were equally opsonically active *in vitro* against the homologous GBS serotype.

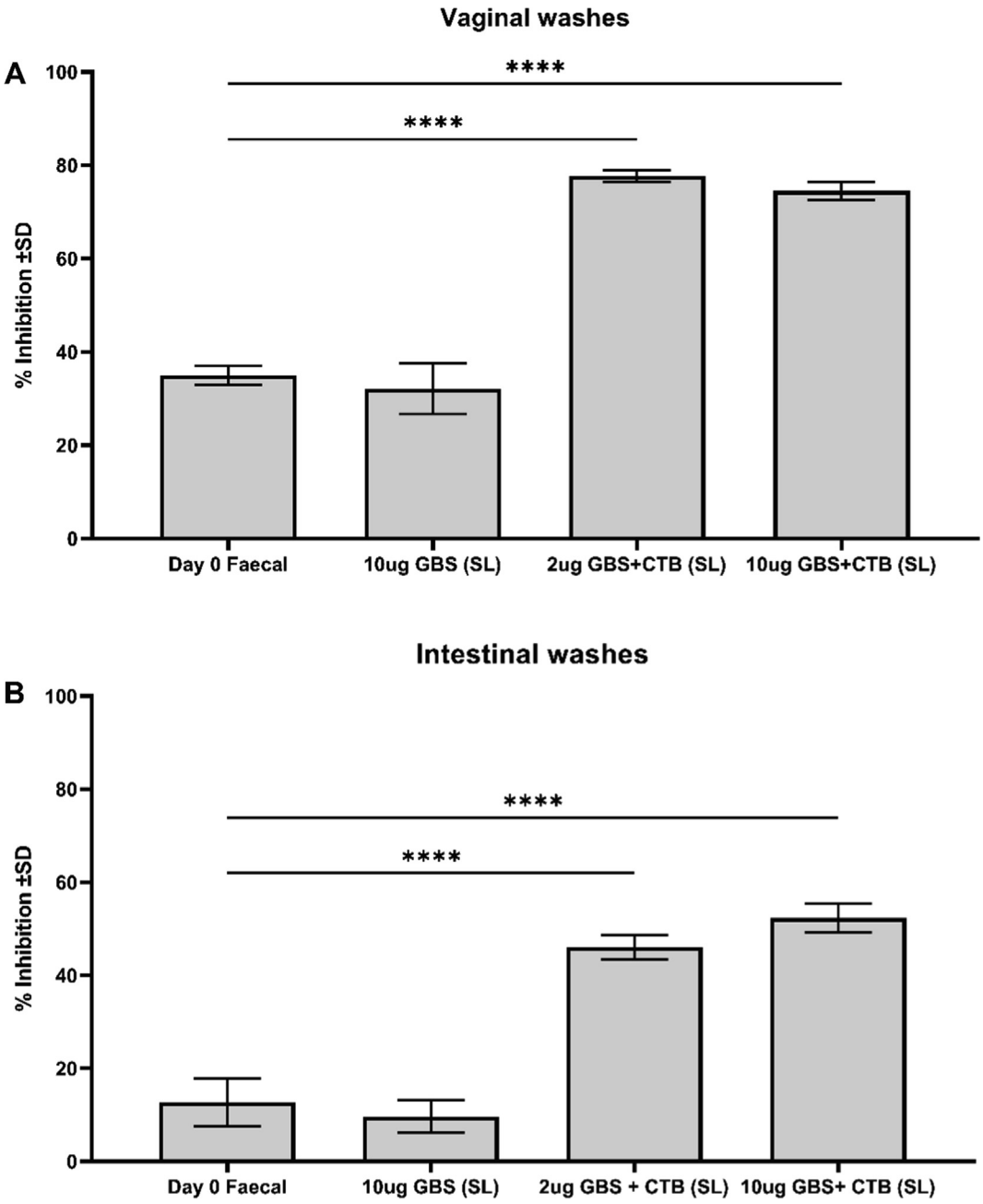

Fig. 5. Inhibition of epithelial cell binding by CPS antibodies. *Confluent monolayers of vaginal epithelial cells were co-cultured with GBS serotype III. Non-bound bacteria were removed, and the total number of bound bacteria was determined after detaching and lysing the cells. To demonstrate inhibition of binding, bacteria were pre-incubated with immune vaginal (A) or intestinal (B) washes prior to adding them to the epithelial cell monolayer. The assay was conducted in triplicates and data are presented as % inhibition, calculated relative to the total number of bound bacteria ± SD.*

The induction of serotype-specific functional systemic IgG by the sublingual route is very important as it is the only antibody isotype that is placentally-transferred from the pregnant mother to the foetus [58].

We also showed that the sublingual route of immunisation has the potential to induce a high level of mucosal IgA antibodies at different mucosal surfaces, including the intestines, colon and vagina, while subcutaneous immunisation only induced systemic response. The induced mucosal response had the ability to substantially reduce adherence of GBS to vaginal epithelial cells, an important step in GBS colonisation and acquisition by the foetus and newborn from colonised mother. A vaccination strategy that could prevent colonisation in pregnant women would effectively eliminate the risk of vertical transmission of GBS from the mother to the foetus/newborn and could also prevent the horizontal spread of infection.

We also showed that the systemic IgG response following sublingual immunisation was efficient at killing the bacteria in the OPA assay and that the mucosal secretions (vaginal and intestinal) were capable of reducing vaginal epithelial cell colonisation by GBS. Similar findings were reported in previous preclinical vaccine studies which showed that parenteral immunisation with a serotype III GBS conjugate vaccine reduces but does not eliminate vaginal colonisation [59]. Our results confirm the potential of this needle-free, easy to administer, route to be an effective immunisation route to induce protective systemic and mucosal antibody

responses. Interestingly, both the systemic and mucosal immune responses following sublingual delivery were dependent on the use of the potent mucosal adjuvants CTX and CTB, and sublingual immunisation in the absence of these adjuvants completely abrogated the antibody responses.

Secretory immunoglobulin (sIgA) plays an important role as a first line of defence against micro-organisms that infect via mucosal surfaces [60], thus an important requirement in the prevention of GBS transmission to the newborn would be the development of vaccines that induce local production of GBS-specific sIgA antibodies in the rectovaginal tract. This supports the importance of our findings that sublingual immunisation induces IgA responses at all the mucosal surfaces investigated. The antibody response was dose-dependent: immunisation with 2 μg of the conjugates induced a substantial response which was further increased when 10 μg was used, although this increase was not statistically significant, suggesting that the sublingual route allows for the use of a small amount of the antigen in the presence of a potent adjuvant. This is advantageous over the oral route which requires a large dose of the antigen to be able to induce systemic and some mucosal response, even in the presence of a potent adjuvant due to enzyme-catalyzed hydrolysis and pH-fluctuations in the gastrointestinal tract that affect the stability of oral vaccines [61]. Sublingual immunisation is also advantageous over other mucosal routes, namely rectal and vaginal which are less user-acceptable, and the nasal route which presents some adverse effects concerns and formulation challenges [61]. A previous study on mucosal immunisation with heat killed cholera vaccines and CTB, using the oral, rectal or vaginal routes showed that induction of mucosal immunity in the rectum and genital mucosae of women may require local immunisation [62], whereas our data showed the ability of the subingual route to elicit both systemic and mucosal immunity at local and distal mucosal sites.

Although it is not possible to directly compare findings from animal and human studies, our results suggest that the sublingual route is superior to the oral, rectal and vaginal route in its ability to elicit functional antibodies in the circulation and at the mucosal surfaces investigated, as demonstrated by the ability of intestinal washes to mediate the killing of GBS in OPA and of vaginal washes to interfere with vaginal epithelial cell colonisation, suggesting that maternal immunisation by the sublingual route could ensure transplacental IgG transmission to the foetus/newborn and would also reduce GBS colonisation at the vaginal mucosae.

To the best of our knowledge, all previous sublingual immunisation studies used either heat-killed pathogens or protein subunit vaccine candidates [44–47,63]. Our study is the first to show that sublingual immunisation is capable of inducing protective systemic and mucosal anti-polysaccharide responses at various mucosal surfaces: mouth, intestines, colon and vaginal. Our results suggest that this route could be successfully applied for other polysaccharide-based conjugate vaccines, where systemic and mucosal immunity are important in the interference of pathogen acquisition at mucosal surfaces and to induce a systemic protective response. Further studies are ongoing in our lab to investigate nasal and pulmonary immunity following sublingual immunisation and comparing it to intranasal delivery of polysaccharide conjugate vaccines.

In our study we demonstrated the requirement for a potent adjuvant (CTX or CTB) to stimulate induction of systemic and mucosal immune responses after subligual immunisation. The use of cholera-based adjuvants in humans, and pregnant women, in particular, may raise safety concerns. However, data from a mass vaccination campaign with Dukoral (a heat inactivated-whole cell oral cholera vaccine) in Zanzibar, where 196 pregnant women had received at least one dose of Dukoral demonstrated no statistically significant indication of a harmful effect of Dukoral exposure during pregnancy [64].

Although it is difficult to extrapolate to the human situation, our data suggest that sublingual immunisation could probably be sufficient to generate anamnestic protective IgG and IgA responses. The needle-free, easy to administer, convenient, patient-acceptable sublingual route would eliminate the risk of needle-stick injuries and contamination, reduce the need for and cost of training personnel for vaccine administration, and is expected to enhance compliance with immunisation programmes. However, it remains important to remember that despite the differences between human and rodent sublingual mucosae (the human sublingual epithelium is non-keratinised while rodents have a sublingual mucosa lined by a keratinised layer), rodent models and especially mice can nonetheless be chosen as the most pertinent animal model for evaluation of drug biodistribution, safety or efficacy and still represent a largely used model for sublingual vaccination [65]. Due to its thinness, the sublingual mucosa is considered as a privileged site for rapid drug adsorption. Protein antigens usually need 15–20 min to cross mucus and sublingual mucosa and reach antigen-presenting cells (APCs) which undergo maturation and migrate to draining LNs within 2 to 6 h where the adaptive immune response can be initiated through antigen presentation to T and B lymphocytes. After a day, the antigen-specific immune response can be disseminated to other LNs and to distant mucosae either through the lymphatic system as antigen-bearing DCs or through blood vessels by plasma cells [45,46].

In summary, this study showed that a GBS conjugate vaccine against CPS serotype III was very immunogenic when administered sublingually in the mouse model and induced high levels of functionally active IgG and IgA antibodies that mediated the killing of GBS and was capable of reducing GBS colonisation of vaginal epithelial cells. This data supports the need for further investigations into the suitability of this delivery method in clinical trials.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper: [Dr Sudaxshina Murdan reports financial support was provided by BactiVac].

Acknowledgements

This work was supported by a grant from BactiVac, an MRC and BBSRC funded GCRF supported Vaccine Network (MR/R005974/1). Grateful thanks also to the Biological Services Division, NIBSC for excellent husbandry. We appreciate our on-going collaborations with the vaccine manufacture Biovac for providing the GBS conjugate vaccine.

References

[1] Le Doare K, O'Driscoll M, Turner K, et al. Intrapartum Antibiotic Chemoprophylaxis Policies for the Prevention of Group B Streptococcal disease worldwide: systematic review. clinical. Infectious dis 2017;65(Suppl 2):S143–51.
[2] Baker CJ. Group B streptococcal infections. Clin Perinatol 1997;24(1):59–70.
[3] Say L, Chou D, Gemmill A, et al. Global causes of maternal death: a WHO systematic analysis. Lancet Glob Health 2014;2(6):e323–33.
[4] Schwartz B, Schuchat A, Oxtoby MJ, Cochi SL, Hightower A, Broome CV. Invasive group B streptococcal disease in adults. A population-based study in metropolitan Atlanta. JAMA 1991;266(8):1112–4.
[5] Edwards MS, Baker CJ. Group B streptococcal infections in elderly adults. Clin Infect Dis 2005;41(6):839–47.
[6] Skoff TH, Farley MM, Petit S, et al. Increasing burden of invasive group B streptococcal disease in nonpregnant adults, 1990–2007. Clin Infect Dis 2009;49(1):85–92.

[7] Seale AC, Blencowe H, Bianchi-Jassir F, et al. Stillbirth With Group B Streptococcus Disease Worldwide: Systematic Review and Meta-analyses. Clin Infect Dis 2017;65(suppl_2):S125–32.
[8] Russell NJ, Seale AC, O'Driscoll M, et al. Maternal Colonization With Group B Streptococcus and Serotype Distribution Worldwide: Systematic Review and Meta-analyses. Clin Infect Dis 2017;65(suppl_2):S100–11.
[9] Kwatra G, Cunnington MC, Merrall E, et al. Prevalence of maternal colonisation with group B streptococcus: a systematic review and meta-analysis. Lancet Infect Dis 2016;16(9):1076–84.
[10] Barcaite E, Bartusevicius A, Tameliene R, Kliucinskas M, Maleckiene L, Nadisauskiene R. Prevalence of maternal group B streptococcal colonisation in European countries. Acta Obstet Gynecol Scand 2008;87(3):260–71.
[11] Heath PT, Balfour G, Weisner AM, et al. Group B streptococcal disease in UK and Irish infants younger than 90 days. Lancet 2004;363(9405):292–4.
[12] Neto MT. Group B streptococcal disease in Portuguese infants younger than 90 days. Arch Dis Child Fetal Neonatal Ed 2008;93(2):F90–3.
[13] Berardi A, Lugli L, Baronciani D, et al. Group B streptococcal infections in a northern region of Italy. Pediatrics 2007;120(3):e487–93.
[14] Yagupsky P, Menegus MA, Powell KR. The changing spectrum of group B streptococcal disease in infants: an eleven-year experience in a tertiary care hospital. Pediatr Infect Dis J 1991;10(11):801–8.
[15] Fluegge K, Siedler A, Heinrich B, et al. Incidence and clinical presentation of invasive neonatal group B streptococcal infections in Germany. Pediatrics 2006;117(6):e1139–45.
[16] Grimwood K, Darlow BA, Gosling IA, et al. Early-onset neonatal group B streptococcal infections in New Zealand 1998–1999. J Paediatr Child Health 2002;38(3):272–7.
[17] Bergseng H, Rygg M, Bevanger L, Bergh K. Invasive group B streptococcus (GBS) disease in Norway 1996–2006. Eur J Clin Microbiol Infect Dis 2008;27(12):1193–9.
[18] Kalliola S, Vuopio-Varkila J, Takala AK, Eskola J. Neonatal group B streptococcal disease in Finland: a ten-year nationwide study. Pediatr Infect Dis J 1999;18(9):806–10.
[19] Vergnano S, Embleton ND, Collinson A, Menson E, Bedford Russell AR, Heath PT. Missed opportunities for preventing GBS infections. Arch Dis Child Fetal Neonatal Ed 2010;95(1):F72–3.
[20] Phares CR, Lynfield R, Farley MM, et al. Epidemiology of invasive group B streptococcal disease in the United States, 1999–2005. JAMA 2008;299(17):2056–65.
[21] Davies HD, Raj S, Adair C, Robinson J, McGeer A. Population-based active surveillance for neonatal group B streptococcal infections in Alberta, Canada: implications for vaccine formulation. Pediatr Infect Dis J 2001;20(9):879–84.
[22] Weisner AM, Johnson AP, Lamagni TL, et al. Characterization of group B streptococci recovered from infants with invasive disease in England and Wales. Clin Infect Dis 2004;38(9):1203–8.
[23] Persson E, Berg S, Trollfors B, et al. Serotypes and clinical manifestations of invasive group B streptococcal infections in western Sweden 1998–2001. Clin Microbiol Infect 2004;10(9):791–6.
[24] von Both U, John A, Fluegge K, Siedler A, Berner R. Molecular epidemiology of invasive neonatal Streptococcus agalactiae isolates in Germany. Pediatr Infect Dis J 2008;27(10):903–6.
[25] Shabayek S, Ferrieri P, Spellerberg B. Group B streptococcal colonization in african countries: prevalence, capsular serotypes, and molecular sequence types. Pathogens Pathogens 2021;10:1606.
[26] Lachenauer CS, Kasper DL, Shimada J, et al. Serotypes VI and VIII predominate among group B streptococci isolated from pregnant Japanese women. J Infect Dis 1999;179(4):1030–3.
[27] N, Hirai, K, Kasahara, R, Nakano, et al. Clinical characteristics and molecular epidemiology of invasive Streptococcus agalactiae infections between 2007 and 2016 in Nara, Japan . PLoS.One. 15(10):e0240590.
[28] Kimura K, Matsubara K, Yamamoto G, Shibayama K, Arakawa Y. Active screening of group B streptococci with reduced penicillin susceptibility and altered serotype distribution isolated from pregnant women in kobe. Japan Japanese j Infectious Dis 2013;66(2):158–60.
[29] Trager JD, Martin JM, Barbadora K, Green M, Wald ER. Probable community acquisition of group B Streptococcus in an infant with late-onset disease: demonstration using field inversion gel electrophoresis. Arch Pediatr Adolesc Med 1996;150(7):766–8.
[30] Heath PT, Feldman RG. Vaccination against Group B streptococcus. Expert Rev Vaccines 2005;4(2):207–18.
[31] Schrag S, Schuchat A. Prevention of neonatal sepsis. Clin Perinatol 2005;32(3):601–15.
[32] Schuchat A. Neonatal group B streptococcal disease:screening and prevention. N Engl J Med 2000;343:209–10.
[33] Ledger W, Blaser M. Are we using too many antiboitoics during -pregnancy?. BJOC: on the intestinal microbiota of infants: a systemic review. An Int J Obstetrics & Gynael 2013;120:1450–2.
[34] Zimmermann P, Curtis N. Effect of intrapartum antibiotics on the intestinal microbiota of infants. a systemic review. Arch Dis Child Fetal Neonatal Ed 2020;105:201–8.
[35] Baker CJ, Rench MA, Fernandez M, Paoletti LC, Kasper DL, Edwards MS. Safety and immunogenicity of a bivalent group B streptococcal conjugate vaccine for serotypes II and III. J Infect Dis 2003;188(1):66–73.
[36] Baker CJ, Paoletti LC, Rench MA, et al. Use of capsular polysaccharide-tetanus toxoid conjugate vaccine for type II group B Streptococcus in healthy women. J Infect Dis 2000;182(4):1129–38.
[37] Baker CJ, Rench MA, McInnes P. Immunization of pregnant women with group B streptococcal type III capsular polysaccharide-tetanus toxoid conjugate vaccine. Vaccine 2003 Jul 28;21(24):3468–72.
[38] Baker CJ, Paoletti LC, Wessels MR, et al. Safety and immunogenicity of capsular polysaccharide-tetanus toxoid conjugate vaccines for group B streptococcal types Ia and Ib. J Infect Dis 1999;179(1):142–50.
[39] Kasper DL, Paoletti LC, Wessels MR, et al. Immune response to type III group B streptococcal polysaccharide-tetanus toxoid conjugate vaccine. J Clin Invest 1996;98(10):2308–14.
[40] Baker CJ, Rench MA, Paoletti LC, Edwards MS. Dose-response to type V group B streptococcal polysaccharide-tetanus toxoid conjugate vaccine in healthy adults. Vaccine 2007;25(1):55–63.
[41] Absalon J, Simon R, Radley D, et al. Advances towards licensure of a maternal vaccine for the prevention of invasive group B streptococcus disease in infants: a discussion of different approaches. Hum Vaccin Immunother 2022::1–16.
[42] Fischer P, Pawlowski A, Cao D, et al. Safety and immunogenicity of a prototype recombinant alpha-like protein subunit vaccine (GBS-NN) against Group B Streptococcus in a randomised placebo-controlled double-blind phase 1 trial in healthy adult women. Vaccine 2021;39(32):4489–99.
[43] Lavelle EC, Ward RW. Mucosal vaccines - fortifying the frontiers. Nat Rev Immunol 2022;22(4):236–50.
[44] Kweon MN. Sublingual mucosa: a new vaccination route for systemic and mucosal immunity. Cytokine 2011;54(1):1–5.
[45] Paris AL, Colomb E, Verrier B, Anjuere F, Monge C. Sublingual vaccination and delivery systems. J Controlled Release 2021;332:553–62.
[46] Kraan H, Vrieling H, Czerkinsky C, Jiskoot W, Kersten G, Amorij JP. Buccal and sublingual vaccine delivery. J Controlled Release 2014;190:580–92.
[47] Monge C, Verrier B. Sublingual antigen delivery: a solution for needle-free HIV vaccination. Expert rev vaccines 2021;20(9):1047–50.
[48] Buffi G, Galletti B, Stella M, Proietti D, Balducci E, Romano MR, et al. Novel Multiplex Immunoassays for Quantification of IgG against Group B Streptococcus Capsular Polysaccharides in Human SeramSphere 2019;4(4):e00273–e319.
[49] Paoletti LC, Pinel J, Kennedy RC, Kasper DL. Maternal antibody transfer in baboons and mice vaccinated with a group B streptococcal polysaccharide conjugate. J Infect Dis 2000;181(2):653–8.
[50] Lancaster L, Saydam M, Markey K, Ho MM, Mawas F. Immunogenicity and physico-chemical characterisation of a candidate conjugate vaccine against group B streptococcus serotypes Ia. Ib and III Vaccine 2011;29(17):3213–21.
[51] Shen X, Lagergard T, Yang Y, Lindblad M, Fredriksson M, Holmgren J. Preparation and preclinical evaluation of experimental group B streptococcus type III polysaccharide-cholera toxin B subunit conjugate vaccine for intranasal immunization. Vaccine 2000;19(7–8):850–61.
[52] Baker CJ, Rench MA, Edwards MS, Carpenter RJ, Hays BM, Kasper DL. Immunization of pregnant women with a polysaccharide vaccine of group B streptococcus. N Engl J Med 1988;319(18):1180–5.
[53] Margarit I, Rinaudo CD, Galeotti CL, et al. Preventing bacterial infections with pilus-based vaccines: the group B streptococcus paradigm. J Infect Dis 2009;199(1):108–15.
[54] Maione D, Margarit I, Rinaudo CD, et al. Identification of a universal Group B streptococcus vaccine by multiple genome screen. Science 2005;309(5731):148–50.
[55] Xue G, Yu L, Li S, Shen X. Intranasal immunization with GBS surface protein Sip and ScpB induces specific mucosal and systemic immune responses in mice. FEMS Immunol Med Microbiol 2010;58(2):202–10.
[56] Guttormsen HK, Mascuch SJ, West JC, Paoletti LC. A fluorescence-based opsonophagocytosis assay to measure the functional activity of antibody to group B Streptococcus. Hum Vaccin 2009;5(7):461–6.
[57] Guttormsen HK, Liu Y, Paoletti LC. Functional activity of antisera to group B streptococcal conjugate vaccines measured with an opsonophagocytosis assay and HL-60 effector cells. Hum Vaccin 2008;4(5):370–4.
[58] Hanson LA, Korotkova M, Lundin S, et al. The transfer of immunity from mother to child. Ann NYAcad Sci 2003;987:199–206.
[59] Chiarot E, Spagnuolo A, Maccari A, et al. Protective effect of group B streptococus type-III polysaccharide conjugates against maternal colonisation, ascending infection and neonatal transmission. Sci Rep 2018;8:2593.
[60] Corthesy B. Secretory immunoglobulin a: well beyond immune exclusion at mucosal surfaces. Immunopharmacol Immunotoxicol 2009;31(2):174–9.
[61] Kang SH, Hong SJ, Lee YK, Cho S. Oral vaccine delivery for intestinal immunity-biological basis, barriers, delivery system, and m cell targeting. Polymers (Basel) 2018;10(9):948.
[62] Kozlowski PA, Cu-Uvin S, Neutra MR, Flangan TP. Comparison of the oral, rectal, and vaginal immunisation routes for induction of antibodies in rectal and genital tract secretions of women. Infec Immun 1997;65(4):1387–94.
[63] Czerkinsky C, Holmgren J. Mucosal delivery routes for optimal immunization: targeting immunity to the right tissues. Current Topics in Microbiol Immunol 2012;354:1–18.
[64] Hashim R, Khatib AM, Enwere G, et al. Safety of the Recombinant Cholera Toxin B Subunit, Killed Whole-Cell (rBS-WC) Oral Cholera Vaccine in pregnancy. PLoSNegl Trop Dis 2012;6(7):e1743.
[65] C, Thirion-Delalande, F, Gervais, C, Fisch, et al. Comparative analysis of the oral mucosae from rodents and non-rodents: Application to the non clinical evaluation of sublingual immunotherapy products. PLoS ONE 12(9):e0183398.