Antibody-Mediated Complement C3b/iC3b Binding to Group B Streptococcus in Paired Mother and Baby Serum Samples in a Refugee Population on the Thailand-Myanmar Border

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Streptococcus agalactiae (group B streptococcus [GBS]) is the leading cause of neonatal sepsis and meningitis. In this study, we determined antibody-mediated deposition of complement C3b/iC3b onto the bacterial cell surface of GBS serotypes Ia, Ib, II, III, and V. This was determined for 520 mother and umbilical cord serum sample pairs obtained at the time of birth from a population on the Thailand-Myanmar border. Antibody-mediated deposition of complement C3b/iC3b was detected to at least one serotype in 91% of mothers, despite a known carriage rate in this population of only 12%. Antibody-mediated C3b/iC3b deposition corresponded to known carriage rates, with the highest levels of complement deposition observed onto the most prevalent serotype (serotype II) followed by serotypes Ia, III, V, and Ib. Finally, neonates born to mothers carrying serotype II GBS at the time of birth showed higher antibody-mediated C3b/iC3b deposition against serotype II GBS than neonates born to mothers with no serotype II carriage. Assessment of antibody-mediated C3b/iC3b deposition against GBS may provide insights into the seroepidemiology of anti-GBS antibodies in mothers and infants in different populations.

Group B streptococcus (GBS) (Streptococcus agalactiae) is a natural commensal of the vagina and rectum and is normally carried asymptomatically (1). However, it is also the leading cause of neonatal sepsis and meningitis in developed countries and is associated with a high mortality rate with early-onset disease (0 to 6 days postbirth) and high morbidity in survivors of late-onset GBS meningitis (2, 3). Prophylactic treatment with antibiotics has been introduced in the United States and Australia, greatly reducing the incidence of early-onset disease in those countries (4, 5). However, the incidence of late-onset disease (defined as occurring between 7 and 90 days after birth) has not decreased (5). In late-onset neonatal sepsis (LONS), GBS may be acquired horizontally from the community, making it harder to target the source of infection (6, 7).

Natural antibodies to GBS are transferred from mother to baby in utero beginning at 17 weeks (passive transport) and by active transport during the third trimester of pregnancy, and they are thought to protect the neonate from disease. Several clinical factors affect the likelihood of developing neonatal sepsis, including prematurity (8, 9), as premature babies have reduced specific antibodies to GBS (10). Furthermore, effective vaccination will reduce the long-term costs of treatment of infected neonates; the health care costs of infants that acquire neonatal GBS infection are estimated to be roughly double those of control neonates matched for age and birth weight (10). Functional immunity to GBS has been demonstrated in an opsonophagocytosis killing assay (OPKA) that measures the antibody and complement-dependent uptake and killing of bacteria by human phagocytes (12, 13). Human vaccine sera with positive OPKA activity have also been shown to passively protect mice in a maternal passive protection/infant challenge model (14). OPKA assays are generally laborious to perform and require large volumes of test sera. Reliance on human donor phagocytes introduces an extra variable, although this has been addressed with the use of the human phagocytic cell line HL60 (15). Additionally, this HL60-based OPKA has been adapted to measure the uptake of fluorescently labeled bacteria by flow cytometry, thus removing the extra plating and counting required for assay analysis (16). A fluorescence opsonophagocytosis assay (fOPA) was developed that measures both the uptake of bacteria and the functional respiratory activity of the HL60 cells in a 96-well format (17). However, OPKA and fOPA require considerable resources to perform.

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large studies, and minimizing the sample volume required is important, particularly for infant studies.

We evaluated the antibody-mediated deposition of complement C3b/iC3b onto bacteria representing five GBS serotypes with 534 serum sample pairs from mother and cord blood samples from a population located on the Thailand-Myanmar border (18). This was evaluated using flow cytometry, and as opsonization of bacteria by antibody and complement is required for uptake and killing by phagocytic cells, this may be a surrogate for opsonophagocytosis.

MATERIALS AND METHODS

Sample collection. The study population was described by Turner et al. (18). In brief, mothers enrolled in the study were residents of the Maeda refugee camp located on the Thailand-Myanmar border. Inhabitants were mostly Karen refugees, with a total population of approximately 43,000. Around 1,500 deliveries occur in the camp every year, and all antenatal care is provided by the Shoklo Malaria Research Unit. A total of 549 women were enrolled in the study at 28 to 30 weeks’ gestation. Venous blood samples were collected from the mothers and umbilical cords at the time of birth. Of the sera collected, a total of 543 serum samples from mothers and 525 serum samples from umbilical cords were available for this study. Data were also collected on the GBS carriage status of the mother at the time of birth, which was assessed by culture of vaginal and rectal swabs (18).

Study ethics. All women gave informed consent to participate in the study. Ethical approval was granted by the ethics committee of the Faculty of Tropical Medicine, Mahidol University, Thailand (MUTM 2009-011-03) and the Oxford Tropical Research Ethics Committee, Oxford University, United Kingdom (48 08).

GBS isolates and growth conditions. Group B streptococcus isolates used in this study were geographically diverse United Kingdom clinical isolates from neonatal blood cultures. H092040676 (serotype Ia), H0908020125 (serotype Ib), H090320548 (serotype II), H092120162 (serotype III), and H091780506 (serotype V) were kindly provided by Androulla Efstratiou, Public Health England, Colindale. Throughout the article, strains are referred to by their serotype.

GBS isolates used in the complement deposition assay were grown in Todd-Hewitt broth at 37°C with shaking (200 rpm). Once an optical density at 600 nm (OD600) of 1.0 was reached, cultures were centrifuged at 3,060 × g for 5 min to pellet the bacteria. The pellet was resuspended in the same volume of phosphate-buffered saline (PBS, pH 7.4; Severn Biotech, United Kingdom) containing 2% formaldehyde and incubated at room temperature for 1 h. Bacteria were then washed in PBS by 3 rounds of centrifugation at 3,060 × g for 5 min and resuspended. The final cell pellet was resuspended in 1 ml PBS, which was used to store at 4°C before use.

Production of IgG-depleted human complement. Human complement used in the complement deposition assay was produced as described previously (19). Briefly, blood was collected from volunteers into containers with 50 µg/ml lepirudin (Movianto, United Kingdom). Plasma was then removed, pooled, and depleted of IgG by protein G Sepharose affinity chromatography. Aliquots were stored at −70°C until use.

Antibody-mediated complement C3b/iC3b deposition assay. Complement deposition assays were performed in 96-well plates, and each test serum sample was analyzed at a single dilution. To each well, 5 µl sera (10% final concentration) was added, followed by 10 µl IgG-depleted human complement (20% final concentration) and 35 µl fixed bacteria (1.8 × 10^7 CFU/well). The bacteria were resuspended in blocking buffer (BB) (PBS containing 1% bovine serum albumin [BSA]; Sigma, United Kingdom). Controls included samples of bacteria only and bacteria and complement only, which were made to the same volume using BB. Pre-activated zymosan (1.8 × 10^7 particles/well; CompTech, USA) was also used as a control to normalize for day-to-day variation. The plate was incubated for 7.5 min at 37°C with shaking at 900 rpm. Plates were then centrifuged at 3,060 × g for 5 min, and the supernatant was removed, ensuring that the bacterial pellet was not disturbed. Pellets were washed with 200 µl BB and then centrifuged at 3,060 × g for 5 min, and the supernatant was removed, ensuring that the bacterial pellet was not disturbed. Each pellet was then resuspended in 200 µl BB containing rabbit anti-human C3c conjugated to fluorescein isothiocyanate (FITC) at 1/500 (Abcam, United Kingdom), which binds to both C3b and iC3b, and the plate was incubated at 4°C for 20 min. The plate was centrifuged at 3,060 × g for 5 min, and the bacterial pellet was washed twice in BB, as performed previously. Finally, the pellet was resuspended in 200 µl BB and kept at 4°C until analysis by flow cytometry.

Data collection and analysis. Samples were analyzed on a CyAn ADP flow cytometer (Beckman Coulter, Inc.) with an automated microsampler system (Cytek Development, Inc.). Data were acquired in a Summit 4.3 (Beckman Coulter, Inc.), and 5,000 events were obtained for each sample. This was performed by setting a gate that contained 10% of the events detected with the control containing bacteria and complement only. A fluorescence index (FI), which was calculated by multiplying the number of events in the gate noted above and the mean fluorescence intensity, was assigned to each sample. For each sample, the FI of the control containing bacteria and complement only was subtracted, giving a final fluorescence index minus complement-only control (FI − CI) value for each serum sample, which was used as a measure of antibody-dependent complement deposition on the bacterial surface. Antibody-independent C3b/iC3b deposition was similar between strains except for the serotype II isolate, which showed greater FI values. Data from each day were normalized to the C3b/iC3b bound to the preactivated zymosan control to account for day-to-day variation within the assay.

Each serum sample was analyzed in the antibody-mediated complement deposition assay (CDA) against each of the five GBS serotypes chosen. Each serum sample was analyzed in duplicate, and if the coefficient of variance (%CV) between the two replicates was >35%, then the serum was analyzed again. If the repeat FI − complement values were not within 50% of each other, then the serum was subjected to a further repeat analysis. If the repeats were within 50% of each other, the two C values were averaged; this was also the case if the analysis was repeated a third time. If all three repeats were not within 50% of each other, then the sample was given a not determined (ND) value for FI − CI. Graphs include all data except the samples that were assigned ND, unless otherwise stated.

Clinical data analysis and statistics. Clinical data were obtained from the mothers enrolled in the study and the neonates (18). These data included GBS carriage status of the mother, ethnic group, past obstetric history, recent antibiotic exposure, and any complications occurring during birth. Infants were also visited at 7 and 28 days postbirth and at unplanned sick visits, and the health of the neonate was checked specifically for signs of neonatal sepsis. All graphs were drawn in GraphPad Prism 6 (GraphPad Software, Inc.). Reverse cumulative distribution curves for CDA data were prepared as described by Reed et al. (20). The effects of clinical parameters on antibody-mediated C3b/iC3b deposition were evaluated using one-way analysis of variance (ANOVA).

RESULTS

Complement deposition assay. The CDA was optimized for incubation times, blocking buffer formulation, IgG-depleted human plasma concentration, shaking speed, bacterial concentration, and serum dilution. Reproducibility of the CDA was measured using a panel of 1 rabbit and 13 human sera against a serotype II strain. Intra-assay precision (three assays performed on 1 day) and interassay precision (one assay performed on 3 separate days) returned mean coefficients of variance of 22% and 26%, respectively. Interassay and complement variations over time were controlled by normalizing all data to the fluorescence index obtained with activated zymosan-complement controls, which were included in each assay. Over 67 assays, the mean standard deviation of FI for the zymosan controls was 8,830 (coefficient of variance, 24%).
cient of variance, 31.6%). Linearity of the assay was assessed using positive-control human sera (kind gift from Carol Baker), and it was shown to be highly linear, with a mean $R^2$ of 0.96 for the five serotypes tested.

**Antibody-mediated C3b/iC3b deposition by paired mother and cord serum samples.** Antibody-mediated C3b/iC3b deposition onto GBS isolates of serotypes Ia, Ib, II, III, and V for each serum sample was evaluated using high-throughput flow cytometry. A total of 1,045 serum samples were analyzed: 520 from mothers and 525 associated cord samples (5 sets of twins). Carriage and clinical data relating to the mothers and neonates are reported in Turner et al. (18). The GBS isolates were selected to represent the two most prevalent serotypes (II and Ia), two serotypes of medium prevalence (III and V), and one serotype of low prevalence (Ib) in this region. A value (fluorescence index obtained with the test serum minus complement-only control value [FI–C’]) of C3b/iC3b deposition was assigned to each serum sample against each serotype tested. Sera with C3b/iC3b deposition values above or below the lower limit of detection (FI–C’ < 2,000) or not determined due to a variable FI–complement value obtained in repeat analyses were compared when geometric mean C3b/iC3b depositions were compared for the mother and cord data sets for each serotype (Fig. 2).

Although GBS carriage was only detected in 12% of mothers, antibody-mediated C3b/iC3b deposition was above the lower limit of detection in 97% of cord samples and 92% of mother samples to at least one serotype tested, as shown in Fig. 3. Moreover, 7% of cord samples and 4% of mother samples contained antibodies that mediated C3b/iC3b deposition to all five serotypes tested. The percentages of individuals with antibody-mediated C3b/iC3b deposition to ≥1 strain with FI–C’ values of >5,000, >10,000, and >20,000 are also presented, with fewer individuals possessing CDA activity at these higher levels. However, there is a greater divergence between values obtained for mother and cord samples as the cutoff value is increased.

**Association of clinical data with antibody-mediated C3b/iC3b deposition.** In this cohort, no neonates were found to have early-onset disease (EOD) due to group B streptococcal infection. Two neonates born to carriage-positive mothers showed signs of EOD, but GBS was not identified as the causative agent. Thus, it was not possible to evaluate the level of protective antibody-mediated complement deposition required to prevent disease.

The effects of a number of other clinical parameters on levels of antibody-mediated C3b/iC3b deposition were evaluated by one-way ANOVA. These included the mother’s age, number of previous pregnancies, neonate’s gestational age, and use of antibiotics. None of these parameters had a significant effect ($P > 0.05$) on the functional antibody levels in mother and cord samples in this study (data not shown). Sera from babies born at <37 weeks’ gestation did not show lower functional antibody levels to any of the five GBS serotypes tested (data not shown).

GBS carriage was determined for each mother at the time of birth by culture of vaginal and rectal swabs (18). No carriage data were obtained from the neonate. For each serotype, antibody-mediated C3b/iC3b deposition in samples from mothers that carried GBS at the time of birth and their neonates was compared to that of mothers and neonates where GBS carriage was not detected. The antibody-mediated complement deposition in the cord samples from neonates with mothers carrying GBS serotype II was significantly higher ($P < 0.001$) than that determined in cord samples taken from neonates born to mothers that did not carry serotype II GBS (Fig. 4). A similar trend was observed when comparing sera from mothers who carried serotype Ia GBS and their neonates with samples from noncarriage mothers, shown in Fig. 5. This did not reach statistical significance due to the low number of mothers carrying this serotype. No significant differences were determined between mother and cord serum samples against serotypes III, V, and Ib, possibly due to the small number of mothers carrying these serotypes (18).

**DISCUSSION**

We developed a flow cytometry assay that measures antibody-mediated deposition of complement components (C3b/iC3b) that are required for opsonophagocytosis onto the surface of GBS serotypes Ia, Ib, II, III, and V. Maternal antibodies to GBS capsular polysaccharides have been shown to protect infants from early-onset GBS disease (21, 22), with opsonophagocytic killing the likely effector mechanism (15, 23). Opsonophagocytosis assays were developed for a range of bacteria (24–27) and have been widely used to assess immune responses to pneumococcal vac-

### TABLE 1 Antibody-mediated C3b/iC3b deposition values above or below the lower limit of detection (FI–C’ < 2,000) or not determined due to a variable FI–complement value obtained in repeat analyses

| Serotype | FI–C’ > 2,000 (% [n]) | FI–C’ < 2,000 (% [n]) | FI–C’ not determined (% [n]) |
|----------|-----------------------|-----------------------|-----------------------------|
| Ia       | 56.6 (605)            | 34.6 (370)            | 8.7 (93)                    |
| Ib       | 26.7 (285)            | 72.2 (771)            | 1.1 (12)                    |
| II       | 71.2 (760)            | 22.8 (244)            | 6.0 (64)                    |
| III      | 38.7 (413)            | 53.4 (570)            | 8.0 (85)                    |
| V        | 42.6 (455)            | 53.6 (572)            | 3.8 (41)                    |

$n = number of samples analysed. FI–C’ is the fluorescence index value obtained with the test serum minus the C3b/iC3b fluorescence obtained with the control containing bacteria and complement only.
FIG 1 (A) Reverse cumulative distribution (RCD) curves show distribution of antibody-mediated C3b/iC3b deposition levels against GBS serotypes Ia (n = 975), Ib (n = 1,059), II (n = 1,007), III (n = 986), and V (n = 1,030) in serum samples taken from mothers and umbilical cords. (B) RCD curves show antibody-mediated C3b/iC3b deposition levels in mother and cord samples separately against GBS serotypes Ia (mother, n = 497; cord, n = 478), Ib (mother, n = 535; cord, n = 521), II (mother, n = 505; cord, n = 499), III (mother, n = 500; cord, n = 483), and V (mother, n = 518; cord, n = 509). Fl-C', fluorescence index minus complement only.
cines where a role in protection was demonstrated (28–30). However, these assays rely on a source of phagocytic cells, such as fresh human peripheral blood mononuclear cells (25, 27, 31) or differentiated HL60 cells (29, 30), and day-to-day variability in these cells can affect assay performance (32). The interaction of complement proteins with a number of pathogens, including Neisseria meningitidis (33) and Haemophilus influenzae (34), has been studied in detail and is key in protecting against disease. Complement interactions with Streptococcus pneumoniae are thought to be important, with individuals deficient in classic pathway components having increased susceptibility to disease (35) and serotypes with resistance to C3b/iC3b opsonization demonstrating greater invasive capacity (36). The potential role of opsonophagocytosis in protection from GBS disease was demonstrated using viable count assays that use log reduction in CFU determination as a primary readout (37–39).

FIG 2 Geometric means of antibody-mediated C3b/iC3b deposition levels in mother and cord samples against serotypes Ia, Ib, II, III, and V GBS (n = the number of samples included in each group). Data do not include the values that were not assigned an FI−C value. Bars represent 95% confidence intervals.

FIG 3 Percentages of mother and cord samples that show antibody-mediated C3b/iC3b binding against ≥1 GBS strain tested using a cutoff FI−C value of >2,000 (A), >5,000 (B), >10,000 (C), and >20,000 (D). Samples that were assigned a not-determined value were classified as not having measurable functional antibody levels along with the samples assigned an FI−C value of 1,000. Cord, n = 525; mother, n = 543.

FIG 4 Box plot shows the effect of serotype II carriage on antibody-mediated C3b/iC3b binding to serotype II GBS in sera from mother and cord pairs taken from mothers who were carriage positive (serotype II only) versus those from mothers who were carriage negative. **, P < 0.001.
In this study, a complement deposition assay was utilized as a possible surrogate for OPA. This flow cytometric assay measures the antibody-dependent deposition of the complement opsonins C3b/iC3b onto the surface of whole bacteria. This assay uses smaller volumes of test sera than OPKA and fOPA yet measures the total antibody-dependent complement deposition mediated by all anti-polysaccharide and anti-protein antibodies, including immunoglobulins other than IgG. Use of this single-dilution assay enables the screening of a large number of samples in a relatively short time. The assay uses fixed bacteria, which allowed a single batch of bacteria for each serotype to be used for the study, thus avoiding assay variability caused by growth-to-growth variation.

A total of 1,068 sera were analyzed against five GBS serotypes using the CDA, and antibody-mediated C3b/iC3b deposition was determined in mother and cord pairs from samples taken at the time of birth. Within this cohort of 549 mothers, none of the neonates developed GBS disease; therefore, it was not possible to determine the level of antibody-mediated C3b/iC3b deposition required to prevent disease. However, sera from neonates born to GBS serotype II carriage-positive mothers had significantly higher antibody-mediated complement deposition levels against serotype II GBS than sera from those born to mothers who were not carriers of serotype II. Coupled with the absence of invasive disease in this population, this may indicate that neonates at risk (born to carrier mothers) had adequate levels of antibodies to prevent disease. Greater antibody-mediated C3b/iC3b deposition in neonates born to carrier mothers was also observed for serotype Ia and was not observed for the other three serotypes tested likely due to the small numbers of carrier mothers. This phenomenon was observed previously, with infants born to GBS-colonized mothers having antibody concentrations to a GBS surface protein (Sip) that were higher than those found in infants born to mothers who had no colonization (40).

Antibody-mediated C3b/iC3b deposition above the lower limit of detection to at least one serotype was observed in 97% of cord samples and 92% of mother samples, with 7% of cord samples and 4% of mother samples containing antibodies that mediate C3b/iC3b deposition to all five serotypes tested. As only 12% of mothers were found to be carriers of GBS at the time of birth, this prevalence is greater than expected and may indicate that broad immunity is generated by repeated transient episodes of carriage of different GBS serotypes. No studies have evaluated the length of carriage of GBS, which may vary depending on the geographic region and ethnic population. The high proportion of individuals with functional anti-GBS antibodies observed in this population may explain the low incidence of GBS neonatal disease in this geographic region, with Southeast Asia showing some of the lowest rates of neonatal GBS disease (18, 41, 42). This requires further study of invasive disease epidemiology and antibody prevalence.

As only 32/525 infants in the study were <37 weeks’ gestation, we expected consistently higher geometric mean antibody-mediated C3b/iC3b deposition levels in the cord samples than in samples from mothers due to the active transfer of IgG in the third trimester (43, 44). For the two most prevalent serotypes, the cord samples indeed showed greater complement deposition than the samples from mothers. However, in the serum samples from mothers, IgM may also contribute to the complement deposition observed and would not have been transferred to the neonates, which may explain why higher neonatal/maternal complement deposition ratios were not seen for the three least-carried serotypes.

Serum samples from premature and term neonates were compared in this study, but no difference was seen in complement deposition mediated by samples from the two groups. Prematurity was previously shown to affect antibody levels that are transferred from mother to baby (43). However, in this cohort, no neonates were born severely premature, since mothers were enrolled when already 28 to 30 weeks pregnant, and the low number of neonates born within this group (n = 32) may lack statistical power. In addition, antibody transfer occurs in the third trimester (≥27 weeks’ gestation), which may be why we saw no difference between the neonates born prematurely (<37 weeks) and those born at term.

The antibody-mediated complement deposition assay is a very useful tool for assessing large numbers of sera against multiple serotypes/strains. It could potentially be used to assess why there are different incidences of disease in different ethnic groups and geographic regions. For instance, in the United States, a 3-fold-higher risk of GBS infection (late onset) was observed in the African American community, with an incidence of 0.42/1,000 live births versus an incidence of 0.11/1,000 live births in the nonblack community (6), despite presumably having the same circulating GBS strains. This has also been observed in southern Israel, where the Bedouin Arab population has a GBS EOD incidence of 0.07/1,000 live births, whereas the Jewish population has an incidence of 0.35/1,000 live births (45). It may be that in these higher-incidence populations, lower concentrations of functional antibodies are present in mothers, resulting in higher neonatal disease rates. Disease rates also vary in different geographic regions, with Africa thought to have the highest incidence of disease, followed by the Americas, Europe, and Southeast Asia (46). Geographic differences were also observed in England (0.72/1,000 live births), Scotland (0.42/1,000 live births), and Northern Ireland (0.9/1,000 live births) (9). Currently, little information is available to help understand these differences and why they occur to aid the rational introduction of a GBS vaccine or other therapy.

This study has opened up the potential to evaluate the levels of
antibody-mediated complement deposition against different GBS serotypes in large-scale seroepidemiological studies. Although the goal of evaluating the antibody-mediated complement deposition required to prevent disease was not attained in this study, this research has provided a baseline for future studies. This assay will enable some of the key unanswered questions, for instance, why different populations show different incidences of disease, to be studied. Use of this and other immunoassays in efficacy trials of new GBS vaccines may identify a correlate of protection that will facilitate vaccine development and evaluation.

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