Group B *Streptococcus* β-hemolysin/Cytolysin Breaches Maternal-Fetal Barriers to Cause Preterm Birth and Intrauterine Fetal Demise in Vivo

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**Background.** Maternal vaginal colonization with *Streptococcus agalactiae* (Group B *Streptococcus* [GBS]) is a precursor to chorioamnionitis, fetal infection, and neonatal sepsis, but the understanding of specific factors in the pathogenesis of ascending infection remains limited.

**Methods.** We used a new murine model to evaluate the contribution of the pore-forming GBS β-hemolysin/cytolysin (βH/C) to vaginal colonization, ascension, and fetal infection.

**Results.** Competition assays demonstrated a marked advantage to βH/C-expressing GBS during colonization. Intrauterine fetal demise and/or preterm birth were observed in 54% of pregnant mice colonized with wild-type (WT) GBS and 0% of those colonized with the toxin-deficient *cylE* knockout strain, despite efficient colonization and ascension by both strains. Robust placental inflammation, disruption of maternal-fetal barriers, and fetal infection were more frequent in animals colonized with WT bacteria. Histopathologic examination revealed bacterial tropism for fetal lung and liver.

**Conclusions.** Preterm birth and fetal demise are likely the direct result of toxin-induced damage and inflammation rather than differences in efficiency of ascension into the upper genital tract. These data demonstrate a distinct contribution of βH/C to GBS chorioamnionitis and subsequent fetal infection in vivo and showcase a model for this most proximal step in GBS pathogenesis.

**Keywords.** *Streptococcus agalactiae*; toxin; chorioamnionitis; perinatal infection.

*Streptococcus agalactiae* (Group B *Streptococcus* [GBS]) is a significant neonatal pathogen and the leading infectious cause of morbidity and mortality among infants in the United States [1]. Colonization of the maternal genital tract is the primary risk factor for neonatal disease [2, 3]. Transmission to the fetus or newborn occurs through direct exposure during parturition or via ascension of the organism from the vagina to the placenta and amniotic fluid [4, 5]. Efforts to prevent vertical transmission of GBS, including universal maternal screening for vaginal-rectal colonization and intrapartum antibiotic prophylaxis, have led to a nearly 80% reduction in the incidence of early-onset disease [6]. However, the unintended consequences of widespread antimicrobial use during pregnancy, including expense, possible maternal allergic reactions, the potential for emergence of resistant organisms, and disruption of the normal vaginal microbiota, are significant concerns [7, 8]. In addition, such strategies are ineffective in preventing ascending infection (chorioamnionitis) prior to labor and have not reduced rates of late-onset invasive GBS disease [9].

GBS produces β-hemolysin/cytolysin (βH/C), a surface-associated, pore-forming toxin that is cytolytic for a broad range of eukaryotic cells [10]. Production
of βH/C and the GBS ornithine rhamnolipolyene pigment (granadaene) are encoded by the genes of the cly operon [11, 12], and both factors are under the control of the CovR/S (also called CsrR/S) 2-component system in GBS [13, 14]. Some data suggest that granadaene may itself be the active agent of pore formation [15, 16]. In addition to its pore-forming activity, βH/C induces apoptosis, recruits neutrophils, stimulates cytokine release, and enhances bacterial intracellular invasion [10]. In vivo studies reveal an important role for βH/C in invasive neonatal diseases including sepsis, pneumonia, and meningitis [17–19]. In one published study, the specific contribution of this toxin to the establishment and maintenance of colonization remained unclear, as the percentage of mice successfully colonized following a given intravaginal inoculum was significantly higher for wild-type (WT) GBS than its isogenic βH/C mutant, yet among successfully colonized animals the bacterial colony-forming units (CFU) recovered over time were similar [20]. Importantly, the role of the βH/C in promoting GBS ascension to the upper genitourinary tract and vertical transmission to the fetus has not yet been explored. In vitro and ex vivo experimental data suggest that GBS induces placental trophoblast death [21] and invades human amniotic epithelial cells thereby disrupting the maternal-fetal barrier [15] in a βH/C-dependent manner.

Using a series of staggered and simultaneous co-colonization models, we delve deeper to demonstrate that expression of the βH/C toxin confers an advantage during vaginal colonization in vivo. Furthermore, we have developed a novel model of ascending GBS infection in pregnant dams—allowing for the first time in vivo exploration of the distinct contribution of specific GBS virulence factors to adverse pregnancy outcomes following maternal vaginal colonization. Concordant with previous studies of human placental explants [15], we demonstrate a crucial role for βH/C in disrupting maternal-fetal barriers and subsequent vertical transmission of GBS to the fetus in vivo.

METHODS

Bacterial Strains and Growth Conditions
GBS wild type (WT) strain NCTC 10/84 (1169-NT1; ATCC 49447, serotype V) [22] and the isogenic, βH/C-deficient, in-frame clyExcat mutant (referred to as clyE KO) [11] were used. The WT NCTC 10/84 strain is hyperhemolytic in comparison to other GBS strains, including strain 2603V/R, which is also serotype V [23]. The clyE KO strain is nonhemolytic, lacks production of the granadaene pigment, and is in the NCTC 10/84 genetic background. Spontaneous streptomycin resistant mutants were generated from these strains and used for animal colonization experiments. All bacteria were grown at 37°C in trypticase soy (TS) broth and plated on TS agar supplemented with streptomycin (100 μg/mL) or RambaCHROM StrepB agar (Gibson Laboratories).

Vaginal Colonization
All experimental procedures were reviewed and approved by the Columbia University Institutional Animal Care and Use Committee. Female C57BL6/J mice were purchased from Jackson Laboratories (Bar Harbor, Maine). At 8–12 weeks of age, animals were subcutaneously injected with 10 μg of water-soluble 17β-estradiol (Sigma) at 48 and 24 hours prior to colonization with GBS in order to synchronize the estrous cycle. For the mono-infection model with either WT or clyE KO strains, bacterial cultures were grown overnight to stationary phase, centrifuged, and resuspended in a 1:1 mixture of TS broth and sterile 10% gelatin to a final concentration of 107 CFU/mL. Animals were anesthetized with 3–5% isoflurane (Baxter), and 50 μL of the GBS-gelatin suspension was administered intravaginally using a sterile pipette. Upon recovery from anesthesia, animals were housed in separate cages for the remainder of the experimental procedures. Serial vaginal swab specimens were collected using a sterile, calcium alginate-tipped swab that was vigorously shaken into 200 μL of TS broth. Serial dilutions were plated for enumeration of CFUs. For the coinfection and staggered infection models, mice were simultaneously or serially infected with both WT and clyE KO strains as indicated. Serial vaginal swabs were obtained as above, and the competitive index [(WT CFU recovered/WT CFU inoculated)/(clyE KO CFU recovered/clyE KO CFU inoculated)] was calculated.

Ascending GBS Infection
Timed-pregnant C57BL6/J mice were purchased from Jackson Laboratories (Bar Harbor, Maine) and given 5 days to acclimate to new surroundings prior to experimental procedures. On pregnancy day 13 (E13), dams were anesthetized and colonized with WT GBS or clyE KO as above. A sham-infected group was similarly inoculated with a 1:1 mixture of TS broth and sterile 10% gelatin. Upon recovery from anesthesia, animals were housed in separate cages and monitored twice daily for the remainder of the experimental procedures to document weight gain, general wellness, and preterm delivery. All dams were killed on E17, and a laparotomy performed immediately under sterile conditions for gross and histopathological inspection of placentas and fetuses. Maternal and fetal blood cultures (a single fetus from each litter, most proximal to cervix, left side) were obtained via intracardiac puncture. Placental tissue (a single placenta from each litter, most proximal to cervix, left side) was homogenized and plated on appropriate media to assess for bacterial invasion. The composite outcome of preterm birth (on or before E17) or any intrauterine fetal demise as noted on uterine inspection was compared between groups.

Microscopy and Staining
Nonpregnant animals were anesthetized and killed 5 days postcolonization with WT GBS. The lower genital tract was removed, fixed in 4% paraformaldehyde, embedded in paraffin,
and serially sectioned. Pregnant animals were killed on day E17, and the entire fetal-placental unit (most proximal to cervix, right side) was removed, separated, and fixed in 4% paraformaldehyde and embedded in paraffin wax. Hematoxylin and eosin staining was performed as per standard protocols. Immunofluorescent labeling of GBS was performed following deparaffinization and rehydration of sectioned tissue. Heat-induced epitope retrieval was performed as per manufacturer’s recommendations (Abcam). Nonspecific binding sites were blocked with 10% normal goat serum and 1% bovine serum albumin (Sigma). Rabbit anti-GBS polyclonal antibody (Abcam ab53584, 1:200 dilution) was applied, and slides were incubated at 4°C overnight. Following serial washes with phosphate-buffered saline (PBS) + 0.025% triton X-100, Alexa Fluor 488 or Alexa Fluor 647 goat anti-rabbit immunoglobulin G (IgG; Invitrogen; 1:500 dilution) was added for 30 minutes in the dark with gentle shaking. Slides were counterstained using Hoechst 33342 (Invitrogen). Cover slips were mounted with Vectashield Hardset mounting medium (Vector Laboratories), and slides were stored at 4°C. Slides to which no primary antibody or no secondary antibody were added served as negative controls. Images were acquired on a Zeiss AxioObserver Z1 inverted microscope.

**Histopathological Scoring**

A pathologist blinded to study group assignment examined hematoxylin and eosin stained-placental sections. Scores (0–3) were assigned based on the maternal inflammatory response scoring system as previously published by von Chamier et al [24].

**Figure 1.** Expression of the βH/C toxin confers a competitive colonization advantage to GBS in the murine vaginal mucosa. A, GBS visualized adhering to the stratum corneum of the murine vaginal epithelium using GBS-specific immunofluorescent staining (white arrow). Epithelial cell nuclei are stained with DAPI. B, Competition assay: Animals were simultaneously infected with both WT and cylE KO GBS and serially swabbed for enumeration of CFU/mL recovered for each strain. Median competitive indices [95% confidence interval] of $5.2 \times 10^3 \ [2 \times 10^2, 8.7 \times 10^4]$, $2.5 \times 10^3 \ [1.0, 1.2 \times 10^4]$, and $15.4 \ [3, 7.5 \times 10^2]$ were observed on days 1, 5 and 7 respectively. C, Separate cohorts were serially infected with either the cylE KO strain followed 24 hours later by WT strain (median competitive indices [95% confidence interval] of $0.8 \ [0.5, 1.7]$ and $0.6 \ [0.2, 5.0]$ at days 3 and 5 respectively) or (D) with the WT strain followed by cylE KO strain (median competitive index [95% confidence interval] of $25.1 \ [1.9, 1.8 \times 10^5]$ and $2.3 \ [1.9, 5.1]$ at 3 and 5 days respectively). Competitive index >1 indicates WT advantage. Abbreviations: GBS, Group B Streptococcus; KO, knockout; WT, wild type.
**RESULTS**

**βH/C-expressing GBS Have a Significant Competitive Advantage During Co-colonization**

Although GBS is not a commensal organism in mice, sustained colonization is induced following vaginal inoculation. Five days following colonization, GBS may be visualized adhering to the stratum corneum of the murine vaginal epithelium (Figure 1A). We observed that βH/C-deficient (cylE KO) GBS colonizes as effectively as the WT strain in a mono-infection model, with no significant difference in the number of CFU/mL recovered or duration of colonization (data not shown). To more carefully probe the impact of the βH/C toxin upon the establishment or maintenance of GBS vaginal colonization, we employed a series of bacterial competition models to compare the 2 strains. First, mice were simultaneously inoculated with similar concentrations ($10^7$ CFU/mL) of both WT and cylE KO strains of GBS. Median competitive indices of 5200, 2500, and 15.4 were observed on days 1, 5 and 7 respectively in animals coinfected with both strains, indicating a marked colonization advantage for the βH/C-expressing WT strain (Figure 1B). In order to exclude the effect of subtle early growth differences on colonization outcome, we used staggered co-infection models to determine whether WT GBS could disrupt existing colonization with the cylE KO strain and vice versa. Following synchronization of the estrus cycle, animals were inoculated with the cylE KO strain. Twenty-four hours after colonization, vaginal swabs were collected to document established colonization. Animals were then infected with a similar concentration of WT GBS. Vaginal swabs were collected 3 and 5 days after colonization. The median competitive indices were noted to be 0.8 and 0.6 at days 3 and 5, respectively, indicating that WT GBS can effectively colonize the murine vaginal mucosa even in the setting of previously established colonization with the cylE KO strain.

Figure 2. βH/C contributes to adverse pregnancy outcomes in a novel murine model. A, Experimental protocol to assess role of βH/C toxin in ascending GBS infection in pregnant mice. B, Total maternal weight gain for each dam following vaginal colonization (days E13-E17). * $P < .05$; Dunn multiple comparisons test. C, Kinetic assessment of maternal weight following vaginal colonization. D, Percentage of positive placental cultures obtained in WT and cylE KO colonized study groups. $P = .13$; Fisher exact test. E, Percentage of dams with the composite outcome of IUFD or preterm delivery in WT and cylE KO colonized study groups. **$P = .01$; Fisher exact test. Abbreviations: GBS, Group B Streptococcus; IUFD, intrauterine fetal demise; KO, knockout; WT, wild type.
strain (Figure 1C). The reverse experiment was conducted in a separate cohort of animals, infecting first with WT GBS, followed by introduction of the cylE KO strain 24 hours later. The median competitive indices were 25.1 and 2.3 at 3 and 5 days, respectively, indicating that the cylE deficient strain is impaired in the setting of previously established colonization with WT GBS (Figure 1D).

### The βH/C Toxin Contributes to Adverse Pregnancy Outcomes

We developed a standardized protocol for vaginal colonization and monitoring of timed-pregnant mice with WT or cylE KO strains of GBS (Figure 2A). Overall maternal weight gain was significantly reduced in the dams colonized with WT GBS compared to those in the cylE KO or sham colonized groups ($P < .05$, Figure 2B), and examination of weight trends in individual mice demonstrated an effect on kinetics as early as 48 hours following colonization with the WT strain (Figure 2C). Although both WT and cylE-deficient GBS ascended into the upper genital tract, as evidenced by positive placental cultures (88% and 44%, respectively; Figure 2D), the composite outcome of preterm delivery or intrauterine fetal demise (IUFD) was observed only in those mice colonized with the WT GBS strain ($P = .01$, Figure 2E).

### βH/C Induces Robust Placental Inflammation and Promotes Fetal Invasion

Examination of placentas obtained from colonized dams revealed large collections of bacteria located primarily along the yolk sac (Figure 3). Such collections were noted in both WT and cylE-KO GBS colonized animals. However, histologic evidence of bacterial disruption of Reichert’s membrane (separating the fetal trophoblast cells of the labyrinth from the yolk sac) was more extensive in the WT-infected dams (Figures 3, 4A, 4B) than in animals colonized with the cylE-KO. Invasion of the labyrinth by WT bacteria was accompanied by a significant inflammatory response that in some cases progressed to diffuse necrosis (Figure 4C). In order to more rigorously assess histopathologic changes, we used a previously validated scoring system [24]. Placentas obtained from cylE-KO-infected dams exhibited less inflammation, with histopathological scores that

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**Figure 3.** Placental histopathology. Representative images of hematoxylin and eosin-stained placental sections. Numerous bacteria are located along the yolk sac (black arrows) and Reichert’s membrane (white arrows). Scale bars indicate 500 µm for 4x, 100 µm for 10x and 40x, and 20 µm for 100x images.
were not statistically different from the sham-infected animals. (Figure 4D). Notably, maternal bacteremia was documented in 50% of WT-infected and 33% of cylE-KO-infected dams (Figure 5A and 5C). Similar dissemination was not observed following vaginal colonization of nonpregnant animals (0/7 positive blood cultures). Positive fetal blood cultures were
observed more commonly in those animals infected with the WT vs cylE KO strains (43% and 11%, respectively), though this difference was not statistically significant (Figure 5B). Fetuses extracted from WT-infected dams were frequently necrotic in appearance, indicating intrauterine fetal demise. Histopathologic examination of affected fetuses revealed bacterial infiltration of fetal lung and liver (Figure 6).

**DISCUSSION**

Maternal vaginal colonization with GBS is the critical first step in the pathogenesis of invasive neonatal disease. Using a murine model of vaginal colonization, we observed a significant competitive colonization advantage for GBS strains expressing βH/C. These experimental results, taken together with previous epidemiologic investigations demonstrating that the vast majority of GBS strains recovered from pregnant women are hemolytic [25, 26], suggest a potential role for this toxin in the establishment or maintenance of vaginal colonization. The precise mechanism by which the toxin provides a relative advantage during colonization is unclear; however, we speculate that early modulation of and relative resistance to innate immune responses by the βH/C-expressing bacteria may account for this observation. For example, GBS βH/C accelerates the apoptotic cell death of host macrophages, whereas the phenotypically linked pigment helps the bacteria neutralize reactive oxygen species, together promoting phagocyte resistance [27]. GBS βH/C also modulates mitogen-activated protein kinase pathways to promote release of interleukin 10, blunting host innate immune responses [28]. Alternative models by which βH/C might contribute to GBS colonization include: (1) the toxin could have a direct role in docking of GBS to host cells, or (2) toxin-induced injury to host cell membranes at the vaginal mucosal surface could unmask ligands for other GBS adhesins. Surface-expressed proteins FbsA/B, ScpB, Srr1, pili, BibA, LTA, and ACP have all been implicated in adherence to eukaryotic cells or extracellular matrix components [29], as has the more recently identified GBS adhesin BsaB [30], and are candidates to participate in such a mechanism. Using a similar animal model of colonization, Sheen et al recently noted that both pili and serine-rich repeat proteins promote GBS adherence to the murine vaginal mucosa in vivo [31].

We present here direct in vivo evidence that the βH/C toxin induces adverse pregnancy outcomes including IUFD, preterm birth, and fetal infection. Our findings extend previous hypotheses generated through in vitro and ex vivo investigations implicating this pore-forming toxin in placental tissue invasion and trophoblast destruction [15, 21], providing validation that the toxin indeed disrupts critical maternal-fetal barriers during pregnancy. Importantly, preterm delivery and IUFD appear to be the direct result of toxin-induced tissue damage and subsequent inflammatory changes rather than inherent differences in the ability of these GBS strains to ascend into the upper genital tract, as cylE-deficient bacteria were recovered from nearly 50% of the placentas in the KO-infected study group.

We describe a novel murine model of chorioamnionitis that closely mimics the human condition, in which intrauterine infection most frequently results from ascension of bacteria that first colonize the vaginal mucosa. The majority of previously published animal models of chorioamnionitis require inoculation of bacterial or microbial products directly into the intrauterine or intra-amniotic space [32, 33]. Prior models of true ascending bacterial infection during pregnancy are limited to rabbits, where intracervical or upper vaginal inoculation of bacteria have led to preterm delivery [33, 34] and one previously published murine model that relies upon endoscopic intracervical injection of bacteria [35]. Our model offers several potential advantages. There is no need for invasive manipulation of the pregnant dam, thereby avoiding stimulation of inflammatory pathways that may impact parturition. Furthermore, it allows for the examination of both host (via the availability of genetically manipulated mouse strains) and bacterial factors that may promote or prevent ascension of vaginal bacteria into the upper genital tract. Finally, this model does not uniformly produce fetal loss or preterm delivery, consistent with findings in humans. Rather, there is variability in terms of pregnancy...
outcome, even with documented recovery bacteria from the intrauterine space, enabling future exploration of potential therapeutic or preventative strategies.

There are limitations to using animal models to explore potential determinants of human GBS vaginal colonization and intrauterine infection. Unlike the human vaginal mucosa, the superficial layers of the murine vaginal epithelium are highly keratinized [36], and therefore the specific interactions underlying bacterial adherence may differ. Disparities in vaginal pH, hormonal cycling, and the composition of the local microbiota must also be considered. The placenta itself is a very morphologically diverse organ across mammalian species. Notably, the placental circulation in mice is similar to that found in humans in that maternal blood comes into direct contact with fetal membranes (hemochorial placentation), although maternal-fetal interdigitation in rodents is labyrinthine rather than villous [36]. Despite these limitations, comparable animal models of vaginal and intrauterine infection have provided significant insight into bacterial virulence factors that promote vaginal colonization and affect pregnancy outcomes [37, 38]. Similarly, our findings provide justification for further exploration of BH/C and perhaps other bacterial pore-forming toxins as potential targets to disrupt or inhibit vaginal colonization, bacterial invasion of the intra-amniotic space, and vertical transmission to the fetus.

Notes

Financial support. This work was supported by the National Institutes of Health [R01 AI092743, R21 AI089654 to A. J. R.; K23 HD065844, UL1 TR000040 (formerly UL1 UC024156) to T. M. R.]. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

Potential conflicts of interest. All authors: No reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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23. Dramsi S, Morello E, Poyart C, Trieu-Cuot P. Epidemiologically and genetically diverse organ across mammalian species. Notably, the placental circulation in mice is similar to that found in humans in that maternal blood comes into direct contact with fetal membranes (hemochorial placentation), although maternal-fetal interdigitation in rodents is labyrinthine rather than villous [36]. Despite these limitations, comparable animal models of vaginal and intrauterine infection have provided significant insight into bacterial virulence factors that promote vaginal colonization and affect pregnancy outcomes [37, 38]. Similarly, our findings provide justification for further exploration of BH/C and perhaps other bacterial pore-forming toxins as potential targets to disrupt or inhibit vaginal colonization, bacterial invasion of the intra-amniotic space, and vertical transmission to the fetus.