Introduction

*Streptococcus agalactiae* (Group B Streptococcus, GBS) is a human pathogen with particular importance in the perinatal and neonatal period. GBS is the leading cause of invasive diseases including pneumonia, meningitis and sepsis among newborns and of peripartum and postpartum infections among women [1]. In addition, there is a growing appreciation of the role of GBS as a pathogen of non-pregnant adults, particularly among the elderly and those with diabetes mellitus [2]. Due to the establishment of guidelines for screening and intrapartum antibiotic prophylaxis in pregnancy, the incidence of early-onset GBS diseases of neonates has decreased, but the burden of adult GBS diseases has remained substantial [3]. GBS is a cause of urinary tract infections (UTI) in adults, and clinical presentations may range from asymptomatic bacteriuria to cystitis, pyelonephritis, and urosepsis. In pregnant women, UTI caused by GBS is associated with high-level vaginorectal colonization and is a risk factor for complications including chorioamnionitis and neonatal GBS disease [4,5]. Despite its clinical importance, a detailed understanding of the pathogenesis of GBS-UTI remains elusive. In several studies employing murine models, GBS has been demonstrated to bind urothelium and activate a bladder cytokine response within 24 h of infection [6–8]. Such responses appear to be distinct from and relatively modest compared to those elicited by uropathogenic *Esherichia coli* [8]. Klein et al. found that capsular sialic acid was a critical component of GBS-mediated inflammation during infection [7], but the role of other virulence factors remains unclear.

In this study, we undertook a comprehensive analysis of the role of the pore-forming β-hemolysin/cytolysin (βH/C) in GBS UTI. Nearly all clinical GBS isolates produce βH/C, which is an important virulence factor involved in the invasion of human epithelial cells and release of pro-inflammatory cytokines [9]. βH/C is involved in many clinical sequelae associated with GBS infection such as meningitis and sepsis [10]. Consistent with prior reports [7], we noted that βH/C was not required for establishment of UTI in a murine model. However, we found that GBS induces proinflammatory cytokine production in a βH/C-dependent manner both in vitro and in vivo. Because of its effect on host inflammatory responses, GBS βH/C may be an important factor in UTI pathogenesis and sequelae.

Methods

Ethics Statement

This study was approved by the Columbia University Institutional Animal Care and Use Committee.

Bacterial strains, cell lines and reagents

GBS wild type (WT) strain CNCTC 10/84 (1169-NT1; ATCC 49447, serotype V) [11], the isogenic, βH/C-deficient *cat*
mutant (ΔcylE), and the trans-complemented strain ΔcylE ΔpfgC[10.E] [12] were generous gifts from Dr. Victor Nizet (University of California, San Diego). We generated a spontaneous streptomycin mutant of the WT strain for use in animal infection experiments. In order to prepare the frozen starter cultures, overnight cultures of GBS were diluted 1:40 in sterile tryptic soy broth, grown to OD600 = 0.6, and stored in 1 ml aliquots in overnight cultures of GBS were diluted 1:40 in sterile tryptic soy broth, grown to OD600 = 0.6, and stored in 1 ml aliquots in 80% ethanol at −80°C. On the day of the experiment, the frozen starters of GBS strains were thawed, washed in TS broth, and grown in fresh TS broth at 37°C without shaking to OD600 = 0.6–0.8. Bacterial density was confirmed by serial dilution with quantitative culture. Human bladder epithelial carcinoma cell line 5637 was purchased from ATCC (HTB-9) and maintained in RPMI (ATCC) with 10% fetal bovine serum at 37°C in a humidified atmosphere with 5% CO2.

Mouse model of ascending UTI

6–8 week old female C57Bl/6J mice were infected with 10³ colony-forming units (CFU) of GBS WT or ΔcylE in 50 μl volume instilled at a slow rate (10 μl/sec) by transurethral catheterization using a well-lubricated sterile soft polyethylene catheter. Mice were euthanized 1, or 5 days post-infection, and bladders, kidneys and urine samples were collected in a sterile fashion. Serial dilutions of organ homogenates or urine samples were cultured on TS agar plates in order to quantify bacterial load. Bladder or kidney halves were stored in RNAlater (Ambion) for RNA isolation using the RNasequeous-4-PCR (Ambion) kit according to the manufacturer’s instructions. For competition experiments, WT and KO bacteria were mixed in a 1:1 ratio prior to introduction into transurethral infection. At 1, or 5 days post-infection organs (bladder and kidneys) were harvested from each infected mouse. Each organ homogenate was plated on TS agar to determine total GBS density and on TS agar supplemented with 100 μg/ml streptomycin to determine specifically the density of the WT strain. Competitive index (CI) was calculated as [WT CFU recovered/WT CFU inoculated] ÷ [ΔcylE CFU recovered/ΔcylE CFU inoculated]]. A CI value of 1 indicates equivalent fitness; CI>1 indicates a WT fitness advantage, and CI<1 indicates a ΔcylE fitness advantage.

Epithelial cell adherence and cytokine transcription assays

Confluent ATCC 5637 cell monolayers in 24 well polystyrene plates were washed with serum-free RPMI medium. Cells were then infected with log-phase cultures of WT or ΔcylE strains (MOI = 10) and incubated at 37°C, 5% CO2 for 1 h. Bacterial inocula were enumerated by dilution plating on TS agar plates (Inoculum CFU; IC). Following the incubation, cell-free supernatants were collected for lactate dehydrogenase (LDH) assay to determine cell death (Roche). 5637 cells were washed three times to remove non-adherent bacteria and adherent CFUs determined (Adherent CFU; AC). % adhesion was determined as (AC – IC)/IC 120. RNA from washed 5637 cells was extracted as described below.

RNA extraction, reverse transcription and quantitative real-time PCR (qRT-PCR)

Total RNA from mouse organs or 5637 human bladder carcinoma cell line was extracted using the RNasequeous-4-PCR RNA extraction kit as above. RNA was reverse transcribed to cDNA using the high capacity cDNA reverse transcription kit (Applied Biosystems) qRT-PCR was carried out using Power SYBR-Green Master Mix in a StepOne Plus thermal cycler (Applied Biosystems). The list of primers used for qRT-PCR is shown in the Table 1. Relative quantification (RQ) values were calculated using a comparative threshold cycle (ΔΔCT) program on the StepOne software version 2.0.

Statistical Analysis

CFU/ml data from animal infections were compared using Mann-Whitney test, cytokine transcription and adherence frequency data were compared by t test or ANOVA using Prism 4 software (GraphPad). A P-value ≤0.05 was considered significant.

Results

βH/C-expressing GBS induce epithelial cell cytokine production

Using a human bladder carcinoma cell line, we observed that WT GBS induced significantly more transcription of interleukin (IL)-6, IL-8, and, to a lesser extent, IL-1α, than medium alone or the ΔcylE deletion mutant (Fig. 1). Importantly, the proinflammatory activity was restored to WT-like levels upon infection with trans-complemented ΔcylE strain (Fig. 1). At this time point (1 h) there were no significant differences in cytotoxicity induced by WT, ΔcylE deletion mutant, or the complemented strain as determined by LDH release assay, indicating that even at sublethal concentrations, βH/C may promote inflammation. WT bacteria caused discernably more lysis in bladder cells than ΔcylE only after 4 h of incubation (data not shown). We also noted that following 4 h incubation, equivalent numbers (CFU/ml) of WT and ΔcylE

Table 1. Primers used for qRT-PCR.

| Primer Name* | Reference |
|--------------|-----------|
| hIL-1α REV   | GAC TCA GCG TTA AGC TGC CA this study |
| hIL-1α FIF   | CCT TCC CGT TGG TTC CAT CT |
| hIL-6 REV    | AAG AGT AAC ATG TGT GAA AGC |
| hIL-6 FIF    | CAT CTC TCA AAT CTG TTC TGG |
| hIL-8 REV    | TTG GCA GCC TTC CTG ATT TC |
| hIL-8 FIF    | TAT GCA ATC ACA TCT AAG TCT TTT AG |
| hGAPDH FIF   | GGGCGGC CTG TTC ACC AGG GCT G |
| hGAPDH REV   | GGG GCC ATC CAC AGT CTT CTG |
| hIFNb FIF    | AAC TCC ACC AGC AAG CAG TG |
| hIFNb REV    | GTG GAG AGC ATG TGA GCA CA |
| hRANTES REV  | TCG TGC CCA CGT CAA GGAAGTT TT |
| hRANTES FIF  | TCT TCT CTG GTG TGG CAC ACA CTT |
| hMCP-1 FIF   | CGG CGC CTG TGG CCA ATG AGC TGC GC |
| hMCP-1 REV   | CTA GCA ATG AGT AGG CTG GAG AGC |
| hMCP-1 REV   | CAG AAG TGC TTG AGG TGG TG |
| hMCP-1 REV   | CAG AAG TGC TTG AGG TGG TG |
| hMCP-1 FIF   | GGT CCA CTA TAG CCA TTC CT |
| hMCP-1 REV   | TGA TGC ACT TCG AGA AAA CAA |
| hMCP-1 REV   | ATG AGC ACA GAG AGC ATG ATC |
| hMCP-1 REV   | TAC AGG CTT GTC ACT AGA ATT |
| hMCP-1 REV   | CGA AGA CTA CAG TTC TGC CAT T |
| hMCP-1 REV   | CGA AGA CTA CAG TTC TGC CAT T |
| hMCP-1 REV   | AGA AAC CAC AAC TGA AAC TGG |

* m and h denote mouse and human specificity of the primers.
bacteria had adhered to 5637 human bladder cell line (Fig. 2). These data indicate that βH/C is not involved in the adherence of GBS to bladder epithelial cells but may modulate host cell signaling.

**βH/C is dispensable for GBS UTI**

The proinflammatory nature of βH/C argues in favor of a role for this cytotoxin as an important virulence factor with the relevance to infection in the urinary tract. We hypothesized that in comparison to its WT counterpart, ΔcyIE GBS strain would be at a disadvantage in a mouse model of ascending urinary tract infection. However, when separately infected with 10^7 CFU/ml of WT or ΔcyIE strains for 24 h, we observe no significant differences by Mann-Whitney U test in number of bacteria recovered from the bladder (P = 0.72), kidney (P = 0.10), or urine (P = 0.96) of the animals (Fig. 3), indicating that production of βH/C is not required for establishment or maintenance of UTI.

In order to assess whether βH/C confers a subtle fitness advantage during UTI, we performed in vivo competition assays. On days 1 and 5 following infection, we found that CI values remain tightly clustered around 1, indicating that neither WT nor ΔcyIE has a fitness advantage in the mouse model of ascending UTI (Fig. 4).

Bacterial density and host response are both drivers of pathology during UTI in vivo, so we measured transcription of cytokine mRNA from murine bladders during single infection studies. Consistent with our in vitro observations using a bladder cell line, we noted significant transcriptional upregulation of proinflammatory cytokines such as IL-6, TNF-α and IL-1α (Fig. 5) in the setting of WT GBS infection, exceeding that induced by ΔcyIE, even in the setting of similar bacterial density. This finding implicates βH/C as a potential driver of host inflammation in the urinary tract.
However, findings from our in vitro and in vivo assays indicated a role in the establishment and maintenance of GBS UTI. We hypothesized that this factor would also have in a number of diseases including pneumonia, sepsis, and production, and neutrophil recruitment and is crucial for virulence cytotoxin that is involved in the induction of apoptosis, cytokine responses [17]. Thus, even in the absence of a direct effect on bacterial density during infection, ΒΗ/C may play an important role in UTI pathogenesis and is deserving of further investigation.

**Discussion**

The kidneys, bladder, and urine are sterile under normal conditions, but infection of these sites is exceedingly common and may be associated with substantial morbidity, especially in vulnerable patient populations. Understanding the pathogenesis of UTI is important to guide future preventive and therapeutic strategies, and a wealth of information has become available in recent years regarding both bacterial strategies for survival [13] and host detection of and defense against infection [14]. Most such studies have focused on E. coli and other enteric Gram-negative rods, as these are the most common pathogens in UTI in humans. However, it is also important to delineate the pathogenesis of less frequently isolated uropathogens, especially those such as GBS, which may affect disproportionately populations such as pregnant women and the elderly.

Clinically important GBS produce ΒΗ/C, a pore-forming cytotoxin that is involved in the induction of apoptosis, cytokine production, and neutrophil recruitment and is crucial for virulence in a number of diseases including pneumonia, sepsis, and meningitis [10]. We hypothesized that this factor would also have a role in the establishment and maintenance of GBS UTI. However, findings from our in vitro and in vivo assays indicated that ΒΗ/C is dispensable for epithelial cell adherence, bladder colonization, and ascending infection of the kidney. These findings were consistent with data described by Klein et al. [7]. In our experiments, competition assays designed to reveal subtle fitness defects supported these conclusions as well.

Induction of local cytokine responses is a crucial first response to bacterial infections, and epithelial signaling in the bladder recruits professional immune cells required for efficient clearance of infection [15]. We found that even in the setting of equivalent bacterial numbers, the ΒΗ/C-deficient mutant was severely attenuated in induction of inflammation in both of our models. Inflammation drives UTI-associated symptoms [16,17], and chronic infection and sequelae distant to the urinary tract (such as premature labor) are correlated with GBS UTI and may be dependent on the magnitude or duration of inflammatory responses [17]. Thus, even in the absence of a direct effect on bacterial density during infection, ΒΗ/C may play an important role in UTI pathogenesis and is deserving of further investigation.

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**Author Contributions**

Conceived and designed the experiments: RK TMR AJR. Performed the experiments: RK TMR SA AW FEA. Analyzed the data: RK TMR SA AW FEA AJR. Wrote the paper: RK TMR AJR.

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