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A Streptococcal Penicillin-Binding Protein Is Critical for Resisting Innate Airway Defenses in the Neonatal Lung

Amanda L. Jones,2,3* Robert H. Mertz,2† David J. Carl,* and Craig E. Rubens*

Group B streptococcus (GBS) is a major cause of neonatal pneumonia. The early interactions between innate airway defenses and this pathogen are likely to be a critical factor in determining the outcome for the host. The surface-localized penicillin-binding protein (PBP)1a, encoded by ponA, is known to be an important virulence trait in a sepsis model of GBS infection that promotes resistance to neutrophil killing and more specifically to neutrophil antimicrobial peptides (AMPs). In this study, we used an aerosolization model to explore the role of PBP1a in evasion of innate immune defenses in the neonatal lung. The ponA mutant strain was cleared more rapidly from the lungs of neonatal rat pups compared with the wild-type strain, which could be linked to a survival defect in the presence of alveolar macrophages (AM). Rat AM were found to secrete β-defensin and cathelicidin AMP homologues, and the GBS ponA mutant was more susceptible than the wild-type strain to killing by these peptides in vitro. Collectively, our observations suggest that PBP1a-mediated resistance to AM AMPs promotes the survival of GBS in the neonatal lung. Additionally, AM are traditionally thought to clear bacteria through phagocytic uptake; our data indicate that secretion of AMPs may also participate in limiting bacterial replication in the airway. The Journal of Immunology, 2007, 179: 3196–3202.

Group B streptococcus (GBS) is an important neonatal pathogen responsible for significant mortality and morbidity (1). Early-onset disease typically presents within the first 24 h of life as fulminating pneumonia following an ascending infection in the mother or after exposure to the bacteria during passage through the birth canal (reviewed in Ref. 2). The lung has been well documented as the portal of entry in neonatal GBS infections (3–5) and possesses a sophisticated array of innate immune mechanisms for defense against infection. These include mechanical barriers and mucociliary clearance, antimicrobial factors in the airway lining fluid, and resident alveolar macrophages (AM) (6–8).

Antimicrobial peptides (AMPs) are an integral component of innate immunity in the airway and at other sites in the host. These small (3–5 kDa) cationic peptides are stored in the cytoplasmic granules of phagocytes such as AM for release following phagocytosis. In the distal airways, secretory epithelial cells are also a source of defensin and cathelicidin AMPs, as well other antibacterial proteins such as lysozyme, lactoferrin, and secretory leukoprotease inhibitor (see Ref. 9 for review). AMPs have bactericidal activity against a broad spectrum of microorganisms as a result of their unique amphipathic structure and charge characteristics (10).

There is significant interest in identifying the bacterial factors that contribute to the development of pneumonia and allow GBS to resist clearance by the pulmonary innate immune system. Penicillin-binding proteins (PBPs) are surface-localized bacterial proteins that are best known for their role in cell wall biosynthesis. However, PBPs also appear to play an important role in virulence of GBS (11) and potentially other bacterial species (12–20). We have shown that GBS PBP1a encoded by ponA is not essential for bacterial viability, but is required for virulence in a neonatal rat sepsis infection model (11). Significantly, PBP1a was found to promote resistance to neutrophil α-defensins (21).

In this study, we describe the use of an aerosolization model and isogenic mutant strains to demonstrate that PBP1a also promotes the survival of GBS in the neonatal lung. AM are the resident phagocyte in the lung and the first line of defense against pathogens before the arrival of recruited neutrophils. In humans, AM have been reported to secrete β-defensin (BD) and cathelicidin AMP homologues (22–27), but AMP expression by rat AM has not been addressed. We demonstrate that AM from adult rats secrete the innate immune defense peptides BD and cathelicidin, and that the GBS ponA mutant is more sensitive to killing by these AMPs than the wild-type (WT) strain. This represents the first report describing a bacterial trait that facilitates persistence of GBS in the neonatal lung by promoting resistance to AMPs secreted by AM. Additionally, whereas AM are traditionally thought to participate in bacterial clearance through phagocytic uptake, our data indicate that the secretion of basal levels of AMPs by AM may also participate in limiting bacterial replication in the airway.

Materials and Methods

Bacterial strains

Three GBS strains were used as follows: A909, a serotype Ia WT strain (28); AJ3F6, a ponA transposon mutant (29); and A909ΔcpsE, an acapsular mutant in which the cpsE gene (glucosyltransferase) was deleted (a gift from L. Madoff, Harvard Medical School, Boston, MA). GBS strains were grown in Todd Hewitt broth at 37°C in 5% CO₂, to mid-logarithmic phase, washed, and used at a concentration of ~10⁶ CFU/ml for aerosolization assays, and at ~10⁵ CFU/ml for in vitro assays.

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A.L.J. and R.H.M. contributed equally to this work.

Address correspondence and reprint requests to Dr. Amanda L. Jones, 307 Westlake Avenue N., Suite 300, Seattle, WA 98109. E-mail address: amanda.jones@seattlechildrens.org

Abbreviations used in this paper: GBS, group B streptococcus; AM, alveolar macrophage; AMP, antimicrobial peptide; BAL, bronchoalveolar lavage; BD, β-defensin; rCRAMP, rat cathelin-related antimicrobial peptide; HBD, human BD; MOI, multiplicity of infection; PBP, penicillin-binding protein; TAMS, total AM survival; THA, Todd Hewitt agar; WT, wild type.

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Neonatal rat aerosolization studies

Timed-pregnant female Sprague-Dawley rats were obtained from Charles River Laboratories. All rat pups used were <24 h old. Aerosolization experiments were performed using a modification of a previously described method (30) using a chamber equipped with two standard nebulizers. After dispersion of the inoculum (~45 min), five pups were sacrificed for quantitation of bacterial deposition. The lungs were aseptically removed and homogenized in PBS, and serial dilutions were plated on Todd Hewitt agar (THA) plates. The efficiency of deposition of the bacterial strains was not affected by the physical location of the pups within the chamber (data not shown). All experiments were approved by the Children’s Hospital and Regional Medical Center Institutional Animal Care and Use Committee.

Bronchoalveolar lavage (BAL) and AM assays

BAL using 0.9% NaCl was performed on adult female Sprague-Dawley rats, as previously described (31). BAL fluid was pooled from three to five animals for each experiment. AM were pelleted by centrifugation at 800 × g for 10 min, and the acellular lavage fluid filter was sterilized using a 0.2 μM filter. Approximately 108 bacteria in 0.9% NaCl were added to the pooled airway lavage fluid in a final volume of 1 ml. The number of bacteria surviving over a 3-h incubation at 37°C was quantified. The assay was performed in triplicate and repeated three times. For AM assays, the cells were resuspended in RPMI 1640 medium containing 10% FBS (Me-tab) and counted, and their viability was assessed by trypan blue dye exclusion. Ninety-six-well flat-bottom plates (Corning Glass) were seeded with 1 × 105 AM. The plates were spun at 800 × g for 10 min to bring the cells in contact with the plate, and then the AM were allowed to adhere for 2 h at 37°C in 5% CO2. Before use in the assays, the AM were washed twice in RPMI 1640/10% FBS to remove any contaminating RBC.

Bacterial survival in the presence of AM was assessed using the total AM phagocytosis (32). To evaluate intracellular survival, the isogenic strains were added to AM prepared as described above, and phagocytosis was allowed to proceed for 2 h. The supernatants were removed, the AM were washed twice, and then medium containing antibiotics (100 μg/ml gentamicin and 5 μg/ml penicillin) was added for 2 h to kill the extracellular bacteria, as described (32). At timed intervals, AM were lysed and intracellular bacteria were quantified, as described above.

Detection of AMPs by ELISA

FBS was omitted from these assays to avoid interference with subsequent ELISA; this did not noticeably affect the viability of the AM during the incubation period (data not shown). Bacteria resuspended in RPMI 1640 medium were added to wells containing AM at a MOI of 5–10 per AM. RPMI 1640 alone was added to the AM as a control. AMPs secreted into the medium were quantified by ELISA (33) following removal of AM and bacteria by low speed (800 × g for 10 min) and high speed centrifugation (14,000 × g for 2 min), respectively. Affinity pure rabbit anti-BD-2 Ab (from Alpha Diagnostics) was added against a mixture of BD-2 peptide fragments and detects rat, mouse, and human BD (HBD-2). Rat cathelin-related antimicrobial peptide (rCRAMP) and rabbit anti-rCRAMP Ab were generously provided by B. Agerberth, Karolinska Institute, Stockholm, Sweden. Wells of high-binding ELISA plates (Immunomaxi; TPP) were coated with AM supernatants overnight at 4°C and blocked with PBS containing 1% BSA for 3 h at room temperature. Rat BD-2 was detected using a 1/10,000 dilution of rabbit anti BD-2 Ab, followed by a goat anti-rabbit HRP-labeled secondary Ab (1:10,000) and SureBlue tetramethylbenzidine peroxidase substrate (Kirkegaard & Perry Laboratories) used according to the manufacturer’s instructions. rCRAMP was detected using a 1/10,000 dilution of rabbit anti-rCRAMP Ab, followed by a goat anti-rabbit alkaline phosphatase-labeled secondary Ab (1:5,000) and para-nitrophenylphosphate substrate (Kirkegaard & Perry Laboratories) used according to the manufacturer’s instructions. Standard curves of synthetic BD-2 peptide mixture and rCRAMP were generated and used to estimate the concentration of each peptide in the AM supernatant samples.

AMP sensitivity assays

The sensitivity of the WT and ponA mutant strain to BD-2 and rCRAMP was assessed using previously described bactericidal assays (34). HBD-2, obtained from Peptides International, was used as a representative of the BD class because active, full-length rat BD-2 is not commercially available. HBD-2 was resuspended in 0.01% acetic acid, and CRAMP was resuspended in 0.1% trifluoroacetic acid. The bacterial strains were resuspended in 10 mM NaPO4 containing 0.1% Todd Hewitt broth (pH 7.4) at 105 CFU/ml and incubated with HBD-2 or rCRAMP for 2 h at 37°C. The number of surviving bacteria was determined by plating serial dilutions on THA.

Statistical analyses

Statistical significance was assessed using two-tailed, unpaired Student’s t test. Value of p < 0.05 was considered to be statistically significant.

Results

PB14a promotes survival of GBS in the lung

Aspiration of GBS into the airway represents the main route of entry into the neonate. Thus, we used an aerosolization model to evaluate clearance of our WT and isogenic mutant GBS strains from the lungs of neonatal rat pups. In initial optimization studies to determine the maximum deposition of bacteria that could be achieved in the lungs, we aerosolized a range of doses of WT GBS (105–1011 total CFU). The animals were sacrificed immediately following aerosolization, the lungs were removed, and the number of bacteria deposited was quantified. Maximum deposition of ~1 × 106 bacteria was achieved at a dose of 1010 CFU of aerosolized GBS. There was no increase in deposition at higher doses; thus, we selected an inoculum of 1010 CFU for subsequent experiments. Using this dose, deposition of the two mutant strains was also ~1 × 106 bacteria/lungs, indicating that there was no difference in the efficiency of nebulization or deposition between the strains. We then compared the clearance of the ponA mutant strain with the WT parental strain and cphE mutant control strain over time. As seen in Fig. 1, at each time point, both of the mutants were cleared significantly faster than the WT strain (p < 0.05). By 6 h postinfection, only 25% of the initial inoculum of the ponA mutant bacteria and 60% of the cphE mutant bacteria remained. In contrast, the WT strain had grown to ~180% of the initial inoculum, an observation consistent with previous studies demonstrating that at early time points before the arrival of neutrophils, neonatal rat pups were unable to clear WT GBS strains following either aerosol or intratracheal inoculation (30, 31). By 12 h postinfection, ~5% of the ponA mutant bacteria, 14% of cphE mutant, and 60% of the WT strain remained. The ponA mutant was cleared

FIGURE 1. Clearance of the GBS ponA mutant strain from the lungs of neonatal rat pups following aerosolization. The bacterial load in the lungs of five neonatal rat pups was determined at each time point following inoculation. Data are presented as the ratio of the mean percentage of survival of the inoculum relative to the initial deposition ± SEM. This experiment was repeated six times. *, p < 0.05 compared with WT; **, p < 0.05 compared with cphE mutant strain.
To evaluate whether the defect in survival of the factors viable up to the 2-h time point, but not as well as the WT strain. Greater than 80% of the AM were due to a factor released into the medium by the AM, we repeated ever, it grew significantly better than the known antiphagocytic role of GBS capsular polysaccharide. How- AM at the 30-min time point, an observation consistent with the mutant bacteria was initially reduced by icant growth relative to the initial inoculum. The number of significant differences in survival between the WT and ponA mutant strain, we performed assays to evaluate the contribution of AM to the clearance of the airway lavage fluid. We next performed assays to evaluate the contribution of AM to the clearance of the airway lavage fluid collected from adult rats. Data represent the percentage of the initial inoculum relative to the input CFU ± SD. *, ρ < 0.05.

Survival of strains in airway lavage fluid

To investigate the difference in pulmonary clearance between the WT and ponA mutant strain, we performed in vitro studies comparing the survival of the isogenic strains in airway lavage fluid. Due to technical limitations associated with collecting a sufficient quantity of fluid and AM from neonatal rat pups, we collected airway lavage fluid from adult rats. The AM were removed by centrifugation, and the survival of the ponA mutant and WT strain was assessed in the resulting fluid. After 30 min of incubation, the WT strain had undergone a limited amount of growth; however, the ponA mutant had been effectively killed and only 50% of the initial inoculum survived (Fig. 2). This statistically significant difference in survival was evident for the duration of the assay. These data suggested that the ponA mutant was more sensitive to a secreted factor present in the airway lavage fluid.

Survival of strains in the presence of adult rat AM

We next performed assays to evaluate the contribution of AM to the clearance of the ponA mutant strain that we observed in vivo. TAMS assays were used to assess the overall sensitivity of the strains to killing by extracellular factors secreted by the AM, phagocytic uptake, and intracellular killing. The strains were added to wells containing AM at a MOI of 10 bacteria:1 AM, and the number of surviving bacteria in the well was quantified over time by plating aliquots on THA. As seen in Fig. 3a, after 30 min of exposure to the AM, ~90% of the WT bacteria in the well were viable, whereas only 20% of ponA mutant bacteria had survived. After 2 h of incubation, the WT strain had overcome any inhibitory effect and grown to 800% of the initial inoculum. In contrast, the ponA mutant strain remained static and did not exhibit any significant growth relative to the initial inoculum. The number of cpsE mutant bacteria was initially reduced by ~80% in the presence of AM at the 30-min time point, an observation consistent with the known antiphagocytic role of GBS capsular polysaccharide. However, it grew significantly better than the ponA mutant (ρ < 0.05), but not as well as the WT strain. Greater than 80% of the AM were viable up to the 2-h time point.

Killing of the isogenic strains in the presence of AM by secreted factors

To evaluate whether the defect in survival of the ponA mutant was due to a factor released into the medium by the AM, we repeated the TAMS assay with the inclusion of 1 μg/ml cytochalasin D to inhibit phagocytosis. Consistent with previous reports (35), this concentration of cytochalasin D was found to effectively prevent phagocytosis of all strains by the AM (data not shown); thus, any killing that we observed should have occurred in the extracellular milieu. The addition of cytochalasin D at this concentration did not affect the growth of either of the GBS strains in the extracellular milieu in this medium (data not shown). Under these conditions, growth of the ponA mutant strain was still inhibited relative to the WT strain (Fig. 3b; ρ < 0.05), suggesting that this strain was killed external to the AM, presumably by a secreted factor. As expected, with the inclusion of cytochalasin D, the cpsE mutant survived and grew as well as the WT strain (Fig. 3b), an observation consistent with the known antiphagocytic role of GBS capsular polysaccharide (30–32, 36–38).

Uptake and intracellular survival in adult rat AM

To further dissect the differences in extracellular vs intracellular killing that we had observed for the ponA and cpsE mutant GBS strains, we assessed the initial uptake and intracellular survival over time. Initial uptake was determined following a 2-h incubation with the AM to allow phagocytosis and a subsequent 2-h incubation in medium containing antibiotics to kill extracellular bacteria. As shown in Fig. 4, we detected fewer ponA mutant GBS inside the AM at the initial time point compared with the WT strain (ρ < 0.05). As expected, more of the cpsE mutant bacteria were internalized compared with the WT strain (ρ < 0.05). We also assessed the survival of the internalized bacteria over an 8-h period. Although the number of bacteria internalized varied depending on the strain, once inside the AM, the strains were killed at a similar rate. At longer time points, all strains were effectively significant.
eliminated (data not shown). These data suggested that the difference in TAMS that we observed for the \( \text{ponA} \) mutant was due to higher levels of extracellular killing, because once inside the AM, all of the strains were killed at an equivalent rate.

**Secretion of AMPs by adult rat AM**

As seen in Fig. 3b, our data suggested that during the course of a 2-h incubation with AM, the \( \text{ponA} \) mutant strain was preferentially killed relative to the WT strain, by a factor secreted by the AM. Although AMP expression by rat AM has not been described, AM from a variety of other mammalian species have been reported to secrete homologues of BD-1 and BD-2 and cathelicidin AMPs (27). Thus, we assayed for the rat homologue of BD-2, and the rat cathelicidin homologue rCRAMP by ELISA in supernatants from AM incubated in the absence or presence of WT GBS. As seen in Fig. 5, we were able to detect release of both rBD-2 and rCRAMP peptides by the AM into the medium. Using freshly isolated AM, there was no significant difference in the amount of either peptide detected when the AM were incubated in the presence of WT GBS for up to 4 h compared with the control. When AM that had been cultured for 48 h were used for the assay, incubation with WT GBS did not affect the amount of rCRAMP released at either time point (data not shown). We also did not detect any significant difference in rBD-2 release following 2 h of incubation with the WT GBS strain compared with control AM. Following 4 h of incubation, we did detect a \( \sim 20\% \) increase in the amount of rBD-2 released by AM and WT GBS following 4 h of incubation (190 \( \pm \) 10 ng/ml for AM with WT GBS vs 156 \( \pm \) 12 ng/ml for control AM in a representative experiment; \( p < 0.05 \)). We were not able to assay for the rat homologue of BD-1 because reagents are not yet commercially available. No AMPs were detected in RMPI 1640 medium in the absence of AM.

**Susceptibility of GBS strains to killing by AMPs**

Because we had detected release of BD-2 and rCRAMP by the adult rat AM, we compared the susceptibility of the WT and \( \text{ponA} \) mutant strain to killing by these peptides. Survival of the two strains in the presence of a range of concentrations of HBD-2 and rCRAMP was assessed using standard in vitro bactericidal assays. Using the concentrations of AMPs shown in Fig. 6, the \( \text{ponA} \) mutant was significantly more sensitive to killing by both AMPs than the WT strain (\( p < 0.05 \)). At higher concentrations, both strains were effectively killed. This increased sensitivity to killing by AMPs may explain in part the decreased TAMS and increased lung clearance that we observed for the \( \text{ponA} \) mutant strain.

**Discussion**

The lung is the major focus of infection in early-onset GBS infections. It possesses a sophisticated array of innate immune defense
mechanisms. These defenses do not require previous exposure to pathogens, are relatively nonspecific, and are able to rapidly kill invading microbes (6). Following aspiration into the lung, GBS are initially exposed to resident tissue AM, which are the first line of defense against bacteria in the distal airways before the arrival of neutrophils. Despite the considerable defenses, serious infections still occur, suggesting that pathogens such as GBS possess strategies that allow for evasion of these defenses.

PBPs are best known for their role in cell wall biosynthesis. However, we have recently shown that PBPa also plays a role in the virulence of GBS. GBS PBPa is a member of the class A high m.w. PBPs, which are bifunctional proteins responsible for polymerization of the glycan chains (glycosyltransferase activity) and for the cross-linking of the interpeptide bridges (transpeptidase activity). Bacterial species express multiple PBPs, which often have redundant functions in the biosynthesis of cell wall peptidoglycan (see Ref. 39 for review). Expression of PBPa was found to be critical for virulence of GBS in a sepsis model of infection and to promote resistance to neutrophil AMPs (11, 21).

There are several classes of AMPs, including defensins and cathelicidins. The defensins are subdivided into α and β groups based on sequence and the position of three disulfide bonds. The majority of AMPs found in neutrophils are members of the α-defensin family. In contrast, the majority of AMPs present in the airways are BD. Cathelicidins are structurally distinct from the defensins and are present in the granules of neutrophils and in the airway (see Ref. 40 for review). In humans, four BD have been identified, with HBD-1 and HBD-2 being the best studied (see Ref. 41 for review).

In our present in vivo studies using neonatal rat pups, PBPa expression was a critical factor for survival in the lung as the ponA mutant. In contrast to the WT strain, the ponA mutant was significantly killed in acellular lavage fluid, suggesting the presence of a secreted factor to which this mutant was sensitive. Based on our previous observation that PBPa promotes resistance to neutrophil α-defensin AMPs (21), we hypothesized that the ponA mutant strain was cleared more effectively from the lungs of the neonatal rat pups in these studies due to an increased sensitivity to AMPs present in the lung.

The most significant difference in survival between the ponA mutant and WT strains was evident during the first 6 h following inoculation of the pups. At this early time point before the arrival of significant numbers of neutrophils or induction of new synthesis by airway epithelial cells, the main source of AMPs in the airway is AM. Thus, we first evaluated the contribution of AM to the clearance of the GBS mutant strains by first comparing the overall survival of the strains in the presence of AM collected from adult neonatal rats. These initial assays measured the overall sensitivity of the strains to killing by extracellular factors secreted by the AM, phagocytic uptake, and intracellular killing. The ponA mutant bacteria were killed rapidly in the first 30 min after incubation with AM, and their growth was inhibited at later time points. In contrast, the WT, and to a lesser extent, the acapsular strain, were able to overcome the inhibition and were actively replicating by 1–2 h of incubation. The inclusion of cytochalasin D to prevent phagocytosis restored the growth of the cpsE mutant to WT levels; however, the growth of the ponA mutant strain was still inhibited relative to the WT strain. These data indicated that the ponA mutant was killed in the extracellular milieu by a factor secreted by the AM.

In intracellular survival assays, significantly fewer ponA mutant bacteria were recovered inside the AM at the initial time point compared with the WT strain, suggesting that they had been killed by antimicrobial factors secreted by the AM before phagocytosis.
AMs are traditionally thought to participate in bacterial clearance through phagocytic uptake and intracellular killing following phagosome-lysosome fusion (see Ref. 51 for review). Our observations suggest that the secretion of AMPs by AMs may also participate in limiting bacterial replication in the airway. This may represent an important mechanism of AMP-mediated defense before the arrival of neutrophils carrying α-defensins and de novo synthesis of AMPs by airway epithelial cells, which would be activated over time following exposure to pathogens or inflammatory stimuli.

In vitro bactericidal assays, we demonstrated that a GBS ponA mutant was significantly more susceptible than the WT strain to killing by BD and cathelicidin AMPs. Although we were not able to specifically assess the bactericidal activity of the rat BD-2 homologue against our GBS strains, these two AMPs are highly homologous (53% identity) (52) (see Ref. 40 for review); thus, it seems likely that PBP1a also promotes to rBD-2. Taken together, our data suggest that PBP1a-mediated resistance to the AMPs secreted by AM is a contributing factor in the ability of GBS to resist pulmonary clearance. In future studies, we will adapt our studies characterizing the ponA GBS mutant to infection models for which transgenic animals that lack one or more AMPs are available. This would allow us to definitively link AMPs with the increased clearance of the ponA mutant.

The concentration of rBD-2 and rCRAMP that we detected in medium from the cultured AM was significantly lower than that of early-onset disease.

References

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