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*J Immunol* 2007; 179:3196-3202; doi: 10.4049/jimmunol.179.5.3196

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

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Group B streptococcus (GBS)1 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 fulminant 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 as 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 bacterial viability, but is required for virulence in a neonatal rat sepsis infection model (11). Significantly, PBPs have been reported to secrete β-defensin 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 secretion of AMPs may also participate in limiting bacterial replication in the airway. The Journal of Immunology, 2007, 179: 3196–3202.

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Received for publication November 20, 2006. Accepted for publication June 26, 2007.

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1 This work was supported by Grant R01AI52299 from the National Institutes of Health (to A.L.J.).

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4 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; MOL, multiplicity of infection; PBP, penicillin-binding protein; TAMS, total AM survival; THA, Todd Hewitt agar; WT, wild type.

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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 8000 × g for 10 min, and the acellular lavage fluid filter was sterilized using a 0.2 μm filter. Approximately 106 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 (Mediatech) and counted, and their viability was assessed by trypan blue dye exclusion. Ninety-six-well flat-bottom plates (Corning Glass) were seeded with 1 × 104 AM. The plates were spun at 8000 × 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 survival (TAMS) assay. Bacteria were added to wells containing AM at a multiplicity of infection (MOI) of 5–10 per AM, and the plate was centrifuged at 8000 × g for 10 min to bring the bacteria in contact with the AM and then incubated at 37°C in 5% CO2. At timed intervals, the entire content of the well was collected, and serial dilutions were plated on THA. AM were examined for viability at each time point before lysis. In some assays, cytochalasin D (1 μg/ml; Sigma-Aldrich) was added for 2 h at 37°C to inhibit 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 bacteria 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 (8000 × g for 10 min) and high speed centrifugation (14,000 × g for 2 min), respectively. Affinity pure rabbit anti-BD-2 Ab was raised from Alpha Diagnostic. This Ab was generated 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; TTP) 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 phosphate–labeled secondary Ab (1:5,000) and para-nitrophenylphosphate substrate (Kirkegaard & Perry Laboratories). Detection of AMPs by ELISA was performed in triplicate and repeated three times. 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.

Results

PBP1a 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 andcpsE 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 cpsE 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 cpsE mutant, and 60% of the WT strain remained. The ponA mutant was cleared...
To evaluate whether the defect in survival of the factors killing the isogenic strains in the presence of AM by secreted 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 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 significant growth relative to the initial inoculum. The number of ponA viable, whereas only 20% of 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. 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 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 the 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 (p < 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; p < 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 (p < 0.05). As expected, more of the cpsE mutant bacteria were internalized compared with the WT strain (p < 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
eliminated (data not shown). These data suggested that the difference in TAMS that we observed for the 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 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 ~20% increase in the amount of rBD-2 released by AM and WT GBS following 4 h of incubation (190 ± 10 ng/ml for AM with WT GBS vs 156 ± 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 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 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 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.

PBP1a is critical for resisting airway defenses. In our in vivo studies using neonatal rat pups, PBP1a 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 PBP1a 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 rats. These initial assays measured the overall sensitivity of the strains to killing by extracellular factors secreted by the AM, phagocytosis, 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. As expected, the cpsE mutant strain was taken up by AM in greater amounts than the fully encapsulated WT strain. Although the number of bacteria internalized varied depending on the strain, intracellular killing occurred at a similar rate over the course of the assay. Our observations are consistent with those of Martin et al. (30, 31), who demonstrated that whereas acapsular mutant strains of GBS were more readily taken up by AM, intracellular killing occurred at the same rate as for isogenic strains with capsule.

One limitation of these studies is that we used AM from adult rats due to technical challenges associated with neonatal animals. A number of groups have compared the ability of adult and neonatal rat AM to take up and kill bacteria. In some studies, there was no significant difference in the phagocytic ability of neonatal AM compared with adult, although for some bacterial species the number of bacteria internalized by each neonatal AM was increased (42); other studies did report differences (43, 44). It should be noted, however, that adult rats are significantly more efficient at eliminating GBS from their lungs than neonates (31), which may suggest that we could have underestimated the differences in clearance between our strains by using AM from adult animals.

In our in vivo studies, we detected some clearance of the WT strain by 12 h postinoculation, and by 18 h postinoculation all of the strains had been efficiently cleared. Although the influx of neutrophils into neonatal rat lung may be delayed when compared with adult rats (42), the arrival of recruited neutrophils is most likely responsible for this clearance at these later time points. It is important to note that although this model is useful for examining the early interactions between GBS and the host, it does not represent a model of overt newborn pneumonia because the animals did not develop clinical signs of pneumonia or become bacteremic.

Significant progress has been made toward defining the AMPs present in the human airway; however, little is known about AMPs in the airway of the rat. Expression of mRNA for rat BD-1 and BD-2 and rCRAMP peptide has been detected in tissue homogenates of rat lungs using RT-PCR and Western blot analysis, respectively (45, 46). To our knowledge, AMP secretion by rat AM has not been specifically addressed; thus, our data represent the first report of secretion of BD and cathelicidin homologues by AM obtained from adult rats.

It has not yet been clearly established whether AMP expression by AM can be induced by exposure to inflammatory stimuli, or is constitutive similar to neutrophils (see Ref. 47 for review). Recent studies have implicated vitamin D in induction of cathelicidin expression by human macrophages (48, 49). In epithelial cells, HBD-1 is thought to be constitutively expressed, whereas HBD-2 expression is induced in response to proinflammatory stimuli or microorganisms. Less is known about the inducibility of HBD-2 in other cell types. Duits et al. (22) demonstrated induction of HBD-2 expression in AM upon exposure to inflammatory stimuli such as LPS and IFN-γ. However, other groups have failed to detect HBD-2 induction in AM under biologically relevant conditions (50). In our assays using freshly isolated AM, coincubation with WT GBS did not result in any detectable increase in AMP release. When the AM were cultured for 48 h before the assay, we did detect a 20–40% increase in the amount of rBD-2 release by AM incubated with bacteria for 4 h compared with AM incubated in medium alone. There was no detectable difference in rBD-2 release at earlier time points or in the release of rCRAMP at any time point. The biological relevance of this modest increase in rBD-2 concentration and whether it represents the onset of induction of expression requires further investigation.
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.

Using 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 required for bactericidal activity in our in vitro assays. This was not unexpected and is most likely explained by the fact that in the host, antimicrobial mechanisms are thought to act synergistically to kill bacteria. Synergistic bactericidal activity has been demonstrated experimentally in vitro between AMPs, and between AMPs and other innate immunity proteins such as lysozyme, which is one of the most abundant antimicrobial proteins in the airspaces of the lung (53–56). Future studies will characterize the sensitivity of the GBS ponA mutant to other host bactericidal factors that are present in the airway.

Our studies are the first to identify a role for a PBP promoting survival in the lung and in resistance to host AMPs in the BD family. However, PBP5s have been implicated in the virulence of a number of other bacterial pathogens, including mycobacterial, Gram-positive, and Gram-negative bacterial species (12–20). Resistance to AMPs has become increasingly recognized as a virulence mechanism for a number of bacterial pathogens and has been linked with pulmonary survival of GBS in a mouse model of infection (57). A GBS dltA mutant with increased sensitivity to AMPs was eliminated more rapidly from the lungs of 3-week-old mice inoculated intranasally and was unable to induce pneumonia. This mutant has an increase in negative surface charge due to reduced α-alanylation of the lipoteichoic acid, which results in increased binding of cationic AMPs relative to the WT strain (21). We have shown that the absence of PBP1a does not result in a change in the surface charge compared with the WT strain; thus, the mechanism by which PBP1a promotes resistance of GBS to AMPs in multiple classes remains to be determined and is being actively investigated. Understanding how PBP1a contributes to the ability of GBS to evade the innate mechanisms of the lung will inform our understanding of pathogenesis of neonatal pneumonia and may help us refine our strategies for prevention and treatment of early-onset disease.

Acknowledgment
We thank Marissa Braff for critical review of the manuscript.

Disclosures
The authors have no financial conflict of interest.

References

The Journal of Immunology
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