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# Mediators of Neutrophil Function in Children With Protracted Bacterial Bronchitis

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**BACKGROUND:** Protracted bacterial bronchitis (PBB) is a common and treatable cause of chronic wet cough in children in which the mechanisms are not understood. This study investigates the IL-1 pathway and a neutrophil gene expression signature in PBB.

**METHODS:** BAL was collected from children in an experimental cohort (n = 21, PBB; n = 33, control subjects), and a second validation cohort (n = 36, PBB; n = 11, control subjects). IL-1β, IL-1 receptor antagonist (IL-1RA), and α-defensins 1-3 were assayed by enzyme-linked immunosorbent assay, western blot, and quantitative real-time polymerase chain reaction, together with selected IL-1 pathway members and neutrophil-related molecules.

**RESULTS:** In the experimental cohort, children with symptomatic PBB had significantly higher levels of IL-1β and α-defensin gene and protein expression. Expression of the neutrophil chemokine receptor C-X-C motif receptor 2 was also higher in PBB. IL-1RA protein was higher, however, the IL-1RA:IL-1β ratio was lower in children with PBB than control subjects. In the validation cohort, protein and gene expression of IL-1β and α-defensins 1-3 were confirmed higher, as was gene expression of IL-1 pathway members and C-X-C motif receptor 2. IL-1β and α-defensin 1-3 levels lowered when PBB was treated and resolved. In children with recurrent PBB, gene expression of the IL-1β signaling molecules pellino-1 and IL-1 receptor-associated kinase 2 was significantly higher. IL-1β protein levels correlated with BAL neutrophilia and the duration and severity of cough symptoms. IL-1β and α-defensin 1-3 levels were highly correlated.

**CONCLUSIONS:** PBB is characterized by increased IL-1β pathway activation. IL-1β and related mediators were associated with BAL neutrophils, cough symptoms, and disease recurrence, providing insight into PBB pathogenesis. CHEST 2014; 146(4):1013-1020

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**ABBREVIATIONS:** CXCL = C-X-C motif ligand; CXCR = C-X-C motif receptor; IL-1RA = IL-1 receptor antagonist; IRAK = IL-1 receptor-associated kinase; NF-κB = nuclear factor-κB; PBB = protracted bacterial bronchitis; PELI1 = pellino-1; Q = quartile; TLR = Toll-like receptor; TNF = tumor necrosis factor

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Protracted bacterial bronchitis (PBB) is an important and common cause of chronic wet cough in children.[1] Once correctly diagnosed, the child's cough resolves with prolonged antibiotic therapy.[2] PBB is now internationally accepted as a diagnostic entity[3] and has been incorporated into national[4] and international[5] pediatric guidelines for cough management. However, despite this increased clinical recognition, the underlying mechanisms of PBB remain to be elucidated.

Prior studies have shown that bacterial colonization and airway neutrophilia are present in children with PBB.[2,6] This was associated with upregulation of the Toll-like receptors (TLRs) TLR2 and TLR4 in the BAL of children with PBB.[6] This implicates persistent neutrophilic inflammation in the pathogenesis of PBB and suggests that neutrophil pathway mediators such as IL-1β may play an important role in pathogenesis. In adults with neutrophilic asthma, using gene expression profiling we have implicated the IL-1 and tumor necrosis factor (TNF)-α/nuclear factor-κB (NF-κB) pathways in sputum[7] and a blood gene expression signature involving neutrophil defensins and proteases.[8] Since neutrophils play a role in both PBB and neutrophilic asthma, there may be common mechanisms involved. Therefore, this study evaluated these pathways and mediators in two cohorts of PBB and control children. We hypothesized that IL-1β and the neutrophil gene expression signature would be elevated in PBB and related to symptoms and recurrence.

## Materials and Methods

### *Subject Recruitment and Sampling*

Inflammatory mediators were evaluated in two cohorts. The experimental cohort (n = 54) comprised subjects and BAL samples collected in previous studies.[6,9] The validation cohort (n = 47) was obtained from a second cohort with purposive matching of control subjects.[2] The selection of samples for analysis was based on the availability of specimens and clinical diagnosis; details of enrollment of children is described previously.[2,6,9] A clinical history was obtained on the day of the bronchoscopy, and parents were provided with a cough diary card[10] used to document response to antibiotics, defined as absence of cough or >75% reduction in score (for ≥3 days) within 2 weeks of antibiotic use (amoxycillin-clauvanate 45 mg/kg/d in two doses for 14 days[2]) postbronchoscopy. In the validation cohort, children with PBB were contacted at monthly intervals to document recurrence.

PBB was defined as the presence of a history of chronic (>4 weeks) wet cough and a response to antibiotic treatment with resolution of the cough within 2 weeks in the absence of signs and symptoms of other diseases. Symptomatic PBB was defined as children with PBB who were coughing when bronchoscopy was undertaken. Resolved PBB was defined as children who previously had a chronic wet cough that responded to 2 weeks of antibiotics and who were cough-free at the time when bronchoscopy was undertaken. Recurrent PBB was defined prospectively as more than three episodes of wet cough responding to antibiotic treatment within 12 months following the initial diagnosis, and nonrecurrent PBB as those with fewer than three episodes in the same timeframe.

Experimental cohort control was a convenient sample of children undergoing gastroscopy, whereas in the validation cohort control subjects were age-matched and obtained opportunistically from children undergoing bronchoscopy for assessment of the airways (eg, stridor) with no history of chronic cough and no respiratory infection in the preceding 2 weeks. Informed consent was obtained, and the studies were approved by the Ethics Committees of the Royal Children's Hospital and University of Queensland (HREC/03/QRCH/17).

### *Target Selection and Gene Expression*

Inflammatory gene expression was determined in RNA extracted from BAL cell pellets using real-time quantitative polymerase chain reaction and standardized TaqMan methods as described in detail in e-Appendix 1. Genes tested include those previously identified as increased in sputum in neutrophilic asthma and include IL-1β (*IL1B*), IL-1 receptor 2 (*IL1R2*), IL-1 receptor antagonist (*IL1RN*), pellino-1 (*PELI1*), and IL-1 receptor-associated kinase 2 (*IRAK2*); TNF-α/NF-κB pathway members TNF receptor superfamily member 1B (*TNFRSF1B*) and NF-κ light polypeptide gene enhancer in B cells 2 (*NFKB2*); and the chemoattractant receptor C-X-C motif receptor 2 (*CXCR2*).[7] Also tested was a blood neutrophil gene expression signature including the α-defensins (*DEFA1-3* and *DEFA4*), protease elastase (*ELANE*), and cathepsin G (*CTSG*).[8]

### *Protein Measurements*

IL-1β (undiluted) and IL-1 receptor antagonist (IL-1RA) (one-fifth dilution) protein levels were measured in BAL supernatant using the DuoSet enzyme-linked immunosorbent assay as per the manufacturer's instructions (R&D Systems, Inc). α-Defensins 1-3 (also known as the human neutrophil peptides 1-3) were measured in BAL supernatant (undiluted) using the Human HNP1-3 enzyme-linked immunosorbent assay kit as per the manufacturer's instructions (HK317; Hycult Biotech). Western blot was performed on undiluted BAL from a subset of subjects in the experimental cohort as described in e-Appendix 1.

### *Statistical Analysis*

Data were analyzed using Stata 11 (StataCorp LP) and reported as median (quartile [Q]1, Q3). Statistical comparisons were performed using the two-sample Wilcoxon rank sum (Mann-Whitney) test for nonparametric data with $P < .05$ considered significant. Spearman rank correlations were used to test relationships.

## Results

### *Clinical Characteristics*

The children with PBB in both the experimental (n = 21) (Table 1) and validation (n = 36) (Table 1) cohorts comprised mainly infants and young children with similar profiles of moderate cough severity and a mean symptom duration of >20 weeks. Lung inflammation was present with increased BAL cellularity including neutrophils. The validation cohort tended to have more males and less intense airway neutrophilia than the experimental cohort. The control subjects were older in the