Mycoplasma pneumoniae induces airway epithelial cell expression of MUC5AC in asthma

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ABSTRACT: As excess mucin expression can contribute to the exacerbation of asthma, the present authors hypothesised that Mycoplasma pneumoniae significantly induces MUC5AC (the major airway mucin) expression in airway epithelial cells isolated directly from asthmatic subjects.

A total of 11 subjects with asthma and six normal controls underwent bronchoscopy with airway brushing. Epithelial cells were cultured at an air–liquid interface and incubated with and without M. pneumoniae for 48 h, and in the presence and absence of nuclear factor (NF)-κB and a toll-like receptor (TLR)2 inhibitor. Quantitative PCR was performed for MUC5AC and TLR2 mRNA. MUC5AC protein and total protein were determined by ELISA.

M. pneumoniae exposure significantly increased MUC5AC mRNA and protein expression after 48 h in epithelial cells isolated from asthmatic but not from normal control subjects, at all concentrations as compared to unexposed cells. TLR2 mRNA expression was significantly increased in asthmatic epithelial cells at 4 h compared with unexposed cells. NF-κB and TLR2 inhibition reduced MUC5AC expression to the level of the unexposed control in both groups.

Mycoplasma pneumoniae exposure significantly increased MUC5AC mRNA and protein expression preferentially in airway epithelial cells isolated from asthmatic subjects. The toll-like receptor 2 pathway may be involved in this process.

KEYWORDS: Asthma, epithelial cell, MUC5AC, Mycoplasma pneumoniae, toll-like receptor 2
significant nonasthma pulmonary disease or other medical problems. All subjects provided consent to participate in this institutional review board-approved protocol.

**Bronchoscopy**
Subjects underwent bronchoscopy with endobronchial protected brushing to airway epithelial cells for culture, as previously described [11]. Supplemental oxygen was administered throughout the procedure along with monitoring of heart rate, blood pressure and oxygen saturation.

**Airway epithelial cell culture and exposure to M. pneumoniae**
Primary epithelial cells were cultured at an air–liquid interface in an identical fashion for asthmatic and normal subjects, as previously described [12]. Immunostaining using an antihuman pan cytokeratin antibody (Sigma, St. Louis, MO, USA) confirmed epithelial cell origin. Stock concentration of *M. pneumoniae* (ATCC 15331) was prepared as previously described [12] and was diluted with bronchiolar epithelial cell culture media to obtain designated concentrations of 10, 20 and 50 colony-forming units (cfu)-cell<sup>−1</sup>, respectively. *M. pneumoniae* was added to the apical surface in the exposed conditions and incubated for 4 h to determine TLR2 mRNA expression, and for 48 h for MUC5AC mRNA and protein determination. These time-points were chosen because Chu et al. [4] demonstrated maximal nuclear factor (NF)-κB activation at 4 h in cell lysates of lung extracts from BALB/c mice infected with *M. pneumoniae*, and maximal MUC5AC mRNA and protein expression at 48–72 h. An NF-κB inhibitor, caffeic acid phenylethyl ester (Sigma, 10 μM) and a TLR2 inhibitor (HM2064; Hycult Biotechnology, Uden, the Netherlands), the latter at 50 cfu-cell<sup>−1</sup> only, were added to a subset of cells separately, each at 10 μM 30 min before exposure to *M. pneumoniae* [13]. As an additional control, cells were exposed to a mutant *M. pneumoniae* (organism II-3) that contains a mutation at the P1 adhesion structure in the organism, and is therefore unable to adhere effectively to airway epithelial cells [14].

**Airway epithelial cell MUC5AC and TLR2 mRNA expression and MUC5AC protein expression**
Airway epithelial mRNA expression levels for MUC5AC and TLR2 were determined using reverse transcription, followed by real-time quantitative PCR, as previously reported [4]. The threshold cycle (Ct) was recorded for each sample and the comparative Ct method was used to represent the relative gene expression levels [15].

MUC5AC protein was determined in cell supernatants by direct ELISA, utilising the MUC5AC-specific 45M1 antibody, as previously described [16]. Total protein was determined using Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA). The detection range for the MUC5AC ELISA was 5–60 ng·mL<sup>−1</sup>.

**Statistical analysis**
MUC5AC mRNA, TLR2 mRNA and MUC5AC protein after exposure to *M. pneumoniae* at 10, 20 and 50 cfu-cell<sup>−1</sup> were first compared within groups as compared with the respective negative, unexposed control. This was followed by comparison of fold changes between asthma and normal groups from their respective negative, unexposed controls to compare group differences (asthma and normal control), time differences and mediator concentration differences employing a mixed effects model (repeated measures analysis). If the overall p-value for the model was significant, individual comparisons were performed to determine which individual concentrations, time-points and group differences achieved significance. Data are expressed as means±SEM and a p-value of <0.05 was considered statistically significant.

**RESULTS**

**Subject characteristics**
The characteristics of the subjects included in the study are shown in table 1.

**MUC5AC mRNA and protein expression**
Baseline MUC5AC mRNA expression, as expressed by Ct and adjusted for baseline glyceraldehyde phosphate dehydrogenase (GAPDH) expression, was not different between the asthma and control epithelial cells prior to *M. pneumoniae* exposure (ΔCt (MUC5AC Ct–GAPDH Ct) 10.57±1.50 versus 8.87±1.13 in the asthma and control groups, respectively; p=0.50). MUC5AC protein at baseline was significantly higher in the asthmatic cells compared with normal controls (optical density in asthma versus control cells 0.14±0.04 versus 0.04±0.009; p=0.03). Protein results are expressed as a ratio of unexposed cells to control for these baseline differences. Total protein was higher in the asthmatic group but the difference did not achieve statistical significance (130±19 versus 80±23 pg·mL<sup>−1</sup> in asthma versus control subjects; p=0.12).

Fold changes in MUC5AC mRNA and protein expression after exposure to *M. pneumoniae* for 48 h in the asthmatic and normal control cells are shown in figure 1. Addition of the NF-κB inhibitor, TLR2 inhibitor (data for 50 cfu-cell<sup>−1</sup> condition only) or use of the mutant *M. pneumoniae* organism significantly attenuated the effects of *M. pneumoniae* on MUC5AC expression in both asthmatic and normal airway epithelial cells (fig. 2).

| TABLE 1 Subject characteristics |
|---------------------------------|
|                                |
| **Subjects**                   |
| **Sex M/F**                    |
| **Age yrs**                    |
| **Medication**                 |
| **FEV<sub>1</sub> L**           |
| **FEV<sub>1</sub> % pred**      |
| **FEV<sub>1</sub>/FVC**         |
| **Methacholine PC<sub>20</sub> mg mL<sup>−1</sup>** |
| **Subjects n**                  |
| **4/6**                         |
| **33±7**                        |
| **Albuterol<sup>6</sup>**       |
| **2.8±0.3**                     |
| **FEV<sub>1</sub> % pred**      |
| **83±5**                        |
| **66±7**                        |
| **0.59±0.2**                    |
| **Normal**                     |
| **6**                           |
| **3/3**                         |
| **26±4**                        |
| **None**                        |
| **3.8±0.4**                     |
| **99±4**                        |
| **81±4**                        |
| **>25**                         |

Data are presented as mean±SD, unless otherwise stated. M: male; F: female; FEV<sub>1</sub>: forced expiratory volume in one second; % pred: % predicted; FVC: forced vital capacity; PC<sub>20</sub>: provocative concentration causing a 20% fall in FEV<sub>1</sub>. <sup>6</sup>: as needed.
with airway epithelial cells from normal subjects. The persistence of phenotypic group differences *ex vivo* in isolated airway epithelial cells suggests that asthmatics may have an inherently augmented epithelial response to infectious challenges, independent of adaptive immune mechanisms.

Several infectious agents have been shown to increase mucin gene expression, including rhinovirus, respiratory syncytial

### DISCUSSION

Results of the present investigation demonstrate, for the first time in humans, that gene and protein expression of the major mucin protein in human airways, MUC5AC, is increased in airway epithelial cells isolated directly from asthmatic subjects after exposure to the pathogen *M. pneumoniae*, as compared with airway epithelial cells isolated directly from normal control subjects. This effect was abrogated when a TLR2 inhibitor and an NF-κB inhibitor were added, respectively. TLR2 mRNA was also increased in asthmatic airway epithelial cells but not in normal airway epithelial cells 4 h after exposure. These data suggest that *M. pneumoniae* induces MUC5AC expression preferentially in asthma, as compared

### TLR2 mRNA expression

Baseline TLR2 mRNA expression was not different between asthma and control epithelial cells prior to *M. pneumoniae* exposure (ΔCt (TLR2 Ct–GAPDH Ct) 6.63±0.89 versus 5.2±0.35 in the asthma and control groups, respectively; p=0.40). Fold changes in TLR2 expression in asthmatic and normal control subjects are shown in figure 3.

### FIGURE 1. Fold changes in a) MUC5AC mRNA expression and b) MUC5AC protein after exposure to *Mycoplasma pneumoniae* for 48 h at 10 (MP10), 20 (MP20) and 50 (MP50) colony forming units (cfu)-cell⁻¹ in airway epithelial cells isolated from asthmatic subjects (●; n=11) and normal control subjects (□; n=6). #: p<0.05; #: p<0.05 between each respective pair.

### FIGURE 2. Fold changes in MUC5AC mRNA expression in airway epithelial cells from asthmatic (●; n=11) and normal control (□; n=6) subjects after exposure to *Mycoplasma pneumoniae* for 48 h at 10 (MP10), 20 (MP20) and 50 (MP50) colony forming units-per cell⁻¹, caffeic acid phenylethyl ester, a nuclear factor-κB inhibitor (NF-κB inh.), *M. pneumoniae* organism strain II-3 (II-3), which contains a mutation at the P1 adhesin and cannot bind airway epithelial cells, and a toll-like receptor 2 inhibitor (TLR2 inh.). #: p<0.05 between each respective pair; #: p<0.05 between *M. pneumoniae* alone versus *M. pneumoniae* plus each of the inhibitors (NF-κB inh., II-3 and TLR2 inh.).

### FIGURE 3. Fold changes in toll-like receptor (TLR)2 mRNA expression after exposure to *Mycoplasma pneumoniae* for 4 h at 10 (MP10), 20 (MP20) and 50 (MP50) colony forming units (cfu)-cell⁻¹ in airway epithelial cells isolated from asthmatic subjects (●; n=11) and normal control subjects (□; n=6). #: p<0.05.
virus and *H. influenzae*. [2, 3, 5, 17]. The present investigation is unique in that airway epithelial cells were isolated from well-characterised patients with asthma and normal controls, cultured at an air-liquid interface and exposed to *M. pneumoniae* in a dose–response manner. It is notable that the fold change in MUC5AC mRNA was higher than the fold change in MUC5AC protein. Secreted and not total MUC5AC protein was focused on; the former is potentially more relevant during an acute infection. In future studies, the present authors intend to evaluate the secreted and stored MUC5AC. The concentrations of *M. pneumoniae* used in the present study’s experiments were lower than those reported in acute infection resulting in pneumonia, where concentrations are generally 100–200 cfu-cell\(^{-1}\) (G. Cassell, University of Alabama, Birmingham, AL, USA; personal communication). The concentrations were therefore chosen for study to more closely mimic a subacute infection that could potentially exacerbate asthma.

The mechanism of this differential response in mucin expression to *M. pneumoniae* in asthma appears to involve the TLR2 receptor and NF-κB activation; the lack of MUC5AC expression after exposure to both a TLR2 inhibitor and an NF-κB inhibitor supports this assumption. Additional studies are needed that utilise more time-points and concentrations of *M. pneumoniae*, with a TLR2 agonist and antagonist to determine whether TLR2 is the main pathway employed by *M. pneumoniae*. The present study’s results support those of Chu et al. [4] in BALB/c mice infected with *M. pneumoniae*, where maximum TLR2 activation occurred at 4 h and maximum MUC5AC expression occurred at 48–72 h.

In summary, the present study has shown that *Mycoplasma pneumoniae* significantly enhances MUC5AC gene and protein expression in airway epithelial cells isolated directly from subjects with asthma. In contrast, minimal effect is seen with *Mycoplasma pneumoniae* exposure in airway epithelial cells isolated from normal subjects. Maintenance of the phenotype *ex vivo* suggests that asthmatic epithelial cells may be primed to respond vigorously to infectious agents.

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