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Induction of mucin secretion from human bronchial tissue and epithelial cells by rhinovirus and lipopolysaccharide[1]

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KEY WORDS rhinovirus; lipopolysaccharides; dolichos biflorus agglutinin; mucins; MUC5AC mucin; bronchi; epithelium; human

ABSTRACT

AIM: To examine the effects of rhinovirus and lipopolysaccharide (LPS) on mucin secretion from bronchial tissue and epithelial cells *in vitro*. **METHODS:** Human small bronchial tissue fragments (HSBTF) and human bronchial epithelial cells (HBEC) were cultured with rhinovirus 16 and LPS, respectively and culture supernatants were collected for mucin measurement. To determine mucin levels in the culture supernatants, a MUC5AC enzyme linked immunosorbent assay and an enzyme linked lectin assay procedure with dolichos biflorus agglutinin (DBA) were developed, and mucin release was expressed as percentage increased (or decreased) secretion over baseline level. **RESULTS:** A concentration-dependent release of DBA mucin and MUC5AC mucin were observed when HSBTF were infected with various concentrations of rhinovirus 16 at 37 ºC. The maximum-induced DBA mucin and MUC5AC mucin release were approximately 258 % and 83 % over baseline. The response of HSBTF to rhinovirus was completely abolished by metabolic inhibitors. Rhinovirus was also able to induce a concentration-dependent release of DBA mucin and MUC5AC mucin from primarily cultured HBEC. LPS 100 mg/L was able to provoke up to approximately 19 % and 54 % increase in DBA and MUC5AC mucin release over baseline, respectively from HSBTF, and 3.1 % and 57 % increase from HBEC at 20 h. Soybean trypsin inhibitor (SBTI) 30 mg/L was able to inhibit LPS-induced mucin release from HSBTF and HBEC. **CONCLUSION:** Rhinovirus is able to induce mucin secretion from human bronchial tissue and bronchial epithelial cells *in vitro.* LPS can induce MUC5AC mucin release from HSBTF and HBEC.

INTRODUCTION

Mucus hypersecretion was often a marked feature of patients with asthma[1] and chronic obstructive pulmonary disease (COPD)[2], caused by airway remodeling with goblet cell metaplasia and submucosal gland hyperplasia. To date, a total of 11 human mucin genes including MUC1-4, MUC5AC, MUC5B, MUC6-8, and MUC11-12 had been identified[3]. Of these, MUC5AC and MUC5B proteins had been isolated from human airway secretions and were considered to be major constituents of the mucus gel[4,5]. Increased level of MUC5AC protein had been observed in the airways of the subjects with asthma and COPD alone with goblet cell hyperplasia and increased stored mucin, indicating that it might contribute to chronic airway blocking in asthma[6] and COPD[7].

Rhinovirus infection was the major cause of asthma

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morbidity in both adults[8] and children[9]. It resulted in not only upper airway symptoms, but also lower airway symptoms. In fact, symptoms of lower airway associated with rhinovirus infection was significantly more severe and longer-lasting in asthmatics than in healthy subjects[10]. This might result from that rhinovirus was able to infect bronchial epithelial cells of inflamed asthmatic airways, and induce production of pro-inflammatory cytokines interleukin-6, -8, and -16 and RANTES from these cells[11]. Rhinovirus infection was also observed in COPD, in which increased sputum production was strongly associated with the presence of rhinovirus[12]. However, the direct effect of rhinovirus on mucin secretion from bronchial tissue and epithelial cells remained unknown though the evidence above implicate that rhinovirus should be able to infect human small airways and cause mucin hypersecretion from bronchial tissues.

Recently, lipopolysaccharide (LPS) was reported to be able to up-regulate MUC2 mRNA expression[13] and MUC2 and MUC5AC mRNA expression[14] in human airway epithelial cells, and MUC5AC and MUC5B mucin secretion and mRNA expression in goblet cell line[15]. However, little was known of ability of LPS in induction of MUC5AC mucin secretion from human bronchial tissue or epithelial cells. In the current study, the potential effects of LPS and rhinovirus on MUC5AC mucin and dolichos biflorus agglutinin (DBA) mucin secretion from small bronchial tissue and primary bronchial epithelial cells were examined.

MATERIALS AND METHODS

Human small bronchial tissue fragments (HSBTF) culture Human bronchi with a diameter less than 3 mm were dissected from the parenchyma of dissected lung specimens. Only macroscopically normal tissue, which possesses intact epithelial lining and distant from tumour, was used for experiment. Following washing three times (2000 r/min, 20 ºC for 6 min) with small airway growth medium (SAGM, Clonetics, UK), the tissue was chopped into 1 to 2 mm^2 fragments. The homogenate tissue suspension in SAGM was then washed for further three times before being placed into a 24-well culture plate, 0.5 mL per well. The plates were incubated at 37 ºC in a humidified incubator (5 % CO_2) for about 48 h before challenge.

HSBTF challenge To establish the baseline mucin (BM) release, the tissue fragments were washed before and after incubation with the fresh medium. After a 3-h or 20-h period, the supernatant from each well was collected and stored in an appendoff at -20 ºC until use. After further washing, the tissue fragments were challenged with Rhinovirus 16 (a present from Prof Sebastian L JOHNSTON, Imperial College, London, UK) or LPS (Sigma) for 3 h or 20 h. The supernatant from each well was collected and stored in an appendoff at -20 ºC until use. Where added, metabolic inhibitors 2-deoxy-*D*-glucose (10 g/L) and antimycin A (1 μmol/L) were incubated with HSBTF for 40 min at 37 ºC before addition of rhinovirus.

Primary culture of human bronchial epithelial cells (HBEC) Samples of human bronchial epithelium were obtained from bronchi removed from 4 subjects undergoing lobectomy. In all cases specimens were taken from the bronchi distant from the cancer area and macroscopically normal (with intact epithelial lining). Cells in primary cultures were grown from explants of bronchial epithelium that had been micro-dissected away from underlying connective tissue of bronchial specimens. The explants sized approximately 4-6 mm^2 were plated onto Greiner 24-well tissue culture plates (Greiner) and cultured for 4-6 weeks in a mixture of bronchial epithelium growth medium (BEGM, Clonetics, UK) and modified M199 medium (Gibco) (1:1, vol/vol) with retinoic acid 50 nmol/L (Sigma), at 37 ºC in a humidified incubator (5 % CO_2). During this time, epithelial cells grew to form a confluent monolayer that was approximately 2 cm in diameter around each explant tissue. All cells were grown to around 90 % confluence, before being challenged with chymase or control compounds. All human tissues were obtained from Pathology Department, the Second Affiliated Hospital of Shantou University Medical College, and the study had approved by the Ethics Committee of Shantou University Medical College.

Stimulation of mucin release from HBEC On the day of challenge, HBEC were first washed twice and then incubated with fresh medium for 3 h and 20 h period, respectively before the supernatant being collected for determination of basal mucin release. Immediately after washing, HBEC were incubated with rhinovirus or LPS in fresh medium for 3 h or 20 h before supernatant from each well being collected for determination of stimulated mucin release. All supernatants collected were stored in appendoffs at -20 ºC until use.

Development of MUC5AC enzyme linked immunosorbent assay (ELISA) An ELISA procedure

was developed for measuring human mucin in culture supernatants. Mucin standards or samples (50 μL in each well) were coated to a 96-well NUNC Maxisorp plate (Gibco) overnight at 4 ºC. After washing the plate with PBS+0.1 % Tween-20 (PBST), 250 μL of 5 % skimmed milk powder (Marvel) in PBS was added to each well for 60 min at 37 ºC in order to block non-specific protein binding sites. MUC5AC mucin specific antibody (diluted 1:2000 in PBS, Pfizer) was added to the plate and incubated at 37 ºC for 60 min. Biotinylated sheep anti-rabbit IgG (Sigma) was used as secondary antibody, and this was followed by addition of Extr-Avidin peroxidase (Sigma) into each well. The colour was developed with OPD substrate and the plate was read at 490 nm. The sensitivity of the assay was from 10 μg/L, and interassay variation was less than 5 %.

Development of enzyme linked lectin assay (ELLA) An ELLA procedure was developed for measuring human mucin in culture supernatants. A 96-well NUNC Maxisorp plate was coated with lectin, dolichos biflorus agglutinin (DBA, 6 mg/L, Sigma) overnight at 4 ºC, 60 μL each well. After washing with high salt PBS (PBS+NaCl 0.5 mol/L+0.1 % Tween-20), 50 μL of mucin sample or standard was added to each well and the plate was incubated at 37 ºC for 40 min. This was followed by addition of peroxidase conjugated DBA (1 mg/L, Sigma) at 37 ºC for 40 min. The reactions in the plate were visualized by addition of 100 μL OPD substrate and the plate was read at 490 nm. The sensitivity of the assay was from 30 μg/L, and interassay variation was less than 5 %.

Calculation of the mucin release The stimulated (or inhibited) mucin release (SM) was expressed as percentage increase (or decrease) over baseline [% increase (or decrease)=(SM-BM)/BM×100 %]. The positive data represent more mucin released over baseline (a stimulatory effect), and negative data mean less mucin released than baseline (an inhibitory effect).

Statistics Statistical analyses were performed using SPSS software (version 10.0). Data were shown as the mean±SD for the number of experiments indicated. Where analysis of variance indicated significant differences between groups, for the preplanned comparisons of interest, paired *t*-test was applied. For all analysis, $P<0.05$ was taken as significant.

RESULTS

Baseline DBA mucin release from HSBTF and HBEC Following 3-h incubation period, accumulated baseline DBA mucin release from HSBTF was 19 μg/g tissue fragments (median value from four donors) with a range from 12 to 31 μg/g. DBA mucin release from HBEC was 1100 ng/well (median value from four donors) with a range from 660 to 1800 ng/well in a 24-well plate.

Baseline MUC5AC mucin release from HSBTF and HBEC Following 3-h incubation period, accumulated baseline MUC5AC mucin release from HSBTF was 11 μg/g tissue fragments (median value from four donors) with a range from 4 to 32 μg/g, whereas the baseline MUC5AC mucin release from primarily cultured HBEC was 820 ng/well (median value from 4 donors) with a range from 630 to 1450 ng/well in a 24-well plate.

Rhinovirus-induced DBA mucin release from HSBTF A concentration-dependent release of DBA mucin was observed when HSBTF were infected with various concentrations of rhinovirus 16 at 37 ºC for 3 h or 20 h. The maximum-induced DBA mucin release was approximately 258 % over baseline stimulated by 200 $TCID_{50}$ virus at 20 h after infection. At 3 h after infection, however only up to approximately 63 % DBA mucin release over baseline was observed. When HSBTF were pretreated with metabolic inhibitors, rhinovirus was not able to stimulate DBA mucin release from them. Interestingly, DBA mucin release from HSBTF was even less than baseline level following the treatment with metabolic inhibitors (Fig 1).

Rhinovirus-induced MUC5AC mucin release from HSBTF Rhinovirus was also able to induce MUC5AC mucin release from HSBTF in a concentration-dependent manner. The maximum rhinovirus-induced MUC5AC mucin release was approximately 83 % over baseline after 20 h infection. At 3 h following infection, only up to approximately 40 % MUC5AC mucin release over baseline was achieved with 200 $TCID_{50}$ virus. Similarly, the metabolic inhibitors not only inhibited rhinovirus-induced MUC5AC mucin release, but also reduced the baseline MUC5AC mucin release from HSBTF (Fig 2).

Rhinovirus-induced mucin release from HBEC Rhinovirus was able to induce a concentration-dependent release of DBA mucin and MUC5AC mucin from primarily cultured HBEC when the cells were infected with the virus at 37 ºC for 3 h. The maximum-induced DBA mucin and MUC5AC mucin release were approximately 41 % and 40 % over baseline, respectively stimulated with 200 $TCID_{50}$ virus (Fig 3).

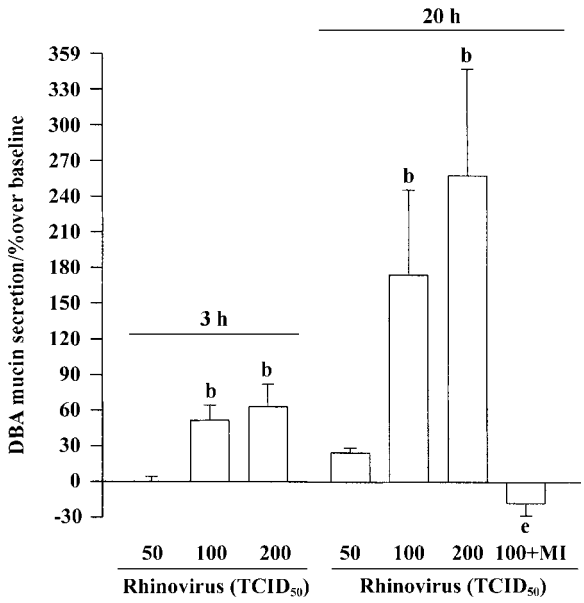

Fig 1. Induction of DBA mucin secretion from HSBTF by rhinovirus following 3 h and 20 h incubation periods. *n*=four to five separate experiments performed in duplicate. Mean± SD. $^{b}P<0.05$ compared with the baseline. $^{e}P<0.05$ compared with the uninhibited response to rhinovirus. MI=metabolic inhibitors.

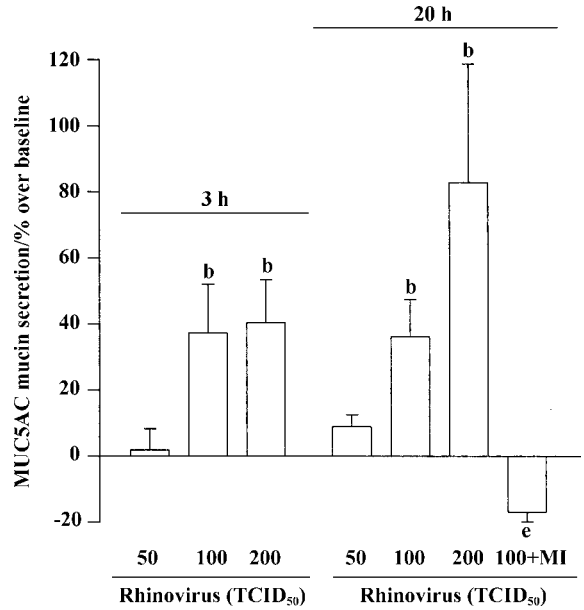

Fig 2. Induction of MUC5AC mucin secretion from HSBTF by rhinovirus following 3 h and 20 h incubation periods. *n*=four to five separate experiments performed in duplicate. Mean±SD. $^{b}P<0.05$ compared with the baseline. $^{e}P<0.05$ compared with the uninhibited response to rhinovirus. MI=metabolic inhibitors.

LPS-induced DBA mucin release from HSBTF and HBEC LPS at a concentration of 100 mg/L was able to induce approximately 8.9 % and 19 % DBA mucin release over baseline from HSBTF after 3 h and 20 h incubation period, respectively. The same concentration of LPS stimulated also 3.4 % and 3.1 % DBA mucin release from HBEC after 3 h and 20 h incubation period, respectively. SBTI an inhibitor of mast cell chymase and trypsin at a concentration of 30 mg/L significantly inhibited LPS-induced DBA mucin release from HSBTF and HBEC at 20 h (Tab 1).

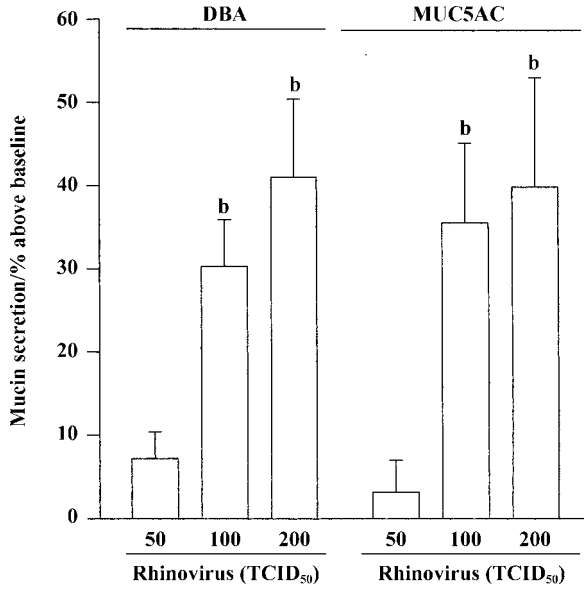

Fig 3. Induction of DBA and MUC5AC mucin secretion from HBEC by rhinovirus following 3 h incubation period. *n*=four separate experiments performed in duplicate. Mean±SD. $^{b}P<0.05$ compared with the baseline.

Tab 1. Effect of LPS on DBA mucin secretion from human small bronchial tissue fragments (HSBTF) and human bronchial epithelial cells (HBEC) following 3 h and 20 h incubation periods. *n*=four to five separate experiments performed in duplicate. Mean±SD. $^{b}P<0.05$ compared with baseline. $^{e}P<0.05$ compared with the uninhibited response to LPS.

Compound /mg·L^{-1}	DBA mucin secretion (% over baseline)			
	HSBTF		HBEC	
	3 h	20 h	3 h	20 h
LPS 100	8.9±2.4^{b}	19±6^{b}	3.4±1.0^{b}	3.1±1.0^{b}
LPS 100+SBTI 30	7±4^{e}	10.0±2.0^{e}	2.7±1.8^{e}	2.9±0.7^{e}

LPS-induced MUC5AC mucin release from HSBTF and HBEC Similarly, LPS at a concentration of 100 mg/L provoked up to approximately 54 % and

57 % increase in MUC5AC mucin release over baseline at 20 h, respectively from HSBTF and HBEC. It appeared that the incubation periods (3 h and 20 h) had little effect on LPS-induced MUC5AC mucin release from HSBTF and HBEC. SBTI 30 mg/L was able to inhibit LPS-induced MUC5AC mucin release from HSBTF and HBEC (Tab 2).

Tab 2. Effect of LPS on MUC5AC mucin secretion from human small bronchial tissue fragments (HSBTF) and human bronchial epithelial cells (HBEC) following 3 h and 20 h incubation periods. *n*=four to five separate experiments performed in duplicate. Mean±SD. $^{b}P<0.05$ compared with baseline. $^{e}P<0.05$ compared with the uninhibited response to LPS.

Compound /mg·L^{-1}	MUC5AC mucin secretion (% over baseline)			
	HSBTF		HBEC	
	3 h	20 h	3 h	20 h
LPS 100	52±12^{b}	54±12^{b}	50±12^{b}	57±14^{b}
LPS 100+SBTI 30	20±6^{e}	20±7^{e}	20±5^{e}	19±5^{e}

DISCUSSION

It was demonstrated rhinovirus was able to induce mucin secretion from human bronchial tissue and bronchial epithelial cells *in vitro*, which implicated that rhinovirus was not only able to replicate in bronchial tissue and bronchial epithelial cells at 37 ºC, but also able to directly induce mucin hypersecretion from them. Since mucus overproduction was a characteristic feature of COPD, virus pneumonia, and severe asthma our current finding strongly supported the view that rhinovirus caused or exacerbate these diseases by induction of mucus hypersecretion.

Rhinovirus dramatically induced approximately 258 % DBA mucin and 83 % MUC5AC mucin release over baseline indicated that the virus was one of the most potent known stimulus of mucin release from small bronchus though it was rather difficult to compare the potency of different stimuli with different experimental systems. The response of HSBTF to rhinovirus was completely abolished by metabolic inhibitors indicated that the mucin release process from bronchial tissue was non-cytotoxic and most likely to be stimulated directly by rhinovirus through its receptor intercellular adhesion molecule-1[16]. Since rhinovirus infection was reported to be associated with increased levels of IL-8[17], IL-6[17], IL-1β[18], and IL-12[19] in airways, we could not exclude that the action of rhinovirus on mucin release from bronchial tissue was partially through an indirect mechanism by induction of the above cytokine release. The temperature of 37 ºC was chosen as the standard temperature throughout the study because it was the optimal temperature for growth of human bronchial tissue and bronchial epithelial cells, and rhinovirus was reported to be able to effectively replicate at this temperature[20].

It was reported that MUC5AC mucin to be confined to goblet cells[21], whereas DBA was observed to specifically recognize *N*-acetylgalactosamine[22], one of the linking sugars of airway mucin[23]. Approximately 41 % DBA mucin and 40 % MUC5AC mucin release over baseline from primarily cultured HBEC after 3-h incubation suggested that the mucin released from these cells was almost entirely MUC5AC mucin, which took approximately 60 % DBA mucin released from bronchial tissue fragments at 3-h incubation period. However, the big difference between the quantities of DBA mucin and MUC5AC mucin release from bronchial tissue fragments at 20-h incubation period implicated that the large proportion of mucin released at this time point was not MUC5AC mucin, and most likely to be secreted from submucosal glandular cells.

It was also the first observation that LPS could induce MUC5AC and DBA mucin release from HSBTF and HBEC. LPS induced mucin release was almost exclusively from airway goblet cells as similar degrees of mucin release from HSBTF and HBEC were observed. Since similar quantities of MUC5AC and DBA mucin were released from HSBTF and HBEC in response to LPS stimulation, it was likely that the majority of LPS-induced mucin release was MUC5AC mucin.

In conclusion, rhinovirus was demonstrated to be able to induce mucin secretion from human bronchial tissue and bronchial epithelial cells *in vitro*, which added some novel information to the understanding of mechanisms by which virus infection could cause exacerbation of asthma and COPD. Induction of mucin hypersecretion by LPS would help to understand the occurrence of airway mucus hypersecretion during bacterial infection.

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