

Respiratory Syncytial Virus in Allergic Lung Inflammation Increases Muc5ac and Gob-5

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Respiratory syncytial virus (RSV) is associated with wheezing and childhood asthma. We previously reported that RSV infection prolongs methacholine-induced airway hyperresponsiveness in ovalbumin (OVA)-sensitized mice. In addition, allergically sensitized RSV-infected (OVA/RSV) mice had more abundant airway epithelial mucus production compared with OVA mice 14 days after infection, whereas there was almost no mucus in mice that were only RSV infected. We hypothesized that this increased mucus was associated with mucosal expression of Muc5ac, a mucus gene expression in airways, and gob-5, a member of the Ca^{2+} -activated chloride channel family. By histochemical analysis, we found that there was significantly increased staining for gob-5 and Muc5ac in the airways of OVA/RSV mice compared with either OVA mice or allergically sensitized mice that were challenged with inactivated RSV, and virtually no detectable staining in the RSV group. These findings were confirmed by Western blot analysis. The increased mucus expression in the OVA/RSV group was associated with increased lung levels of interleukin-17, a factor known to stimulate airway mucin gene expression. The impact of virus infection combined with allergic inflammation on mucus production may partially explain the more severe disease and airway hyperresponsiveness associated with RSV in the setting of atopy.

Keywords: mucin; MUC5ac; gob-5; respiratory syncytial virus

A great majority of wheezing illnesses during childhood is linked to viral infection (1). Respiratory syncytial virus (RSV) infection in particular is associated with wheezing and childhood asthma (2). RSV is the most common viral cause of severe lower respiratory tract disease in childhood (3). The mechanisms by which viral infection in the setting of an allergic airway condition induces airway hyperresponsiveness (AHR) are not fully elucidated. However, RSV infection in mice during allergic sensitization with ovalbumin (OVA/RSV) prolonged AHR when compared with mice that were allergically sensitized alone (OVA) (4). This increase in AHR was associated with amplified airway epithelial mucus production and was not related to an augmented production of type 2 cytokines in the OVA/RSV mice (5, 6). Heightened mucus production in murine models of RSV infection during allergic airway inflammation has also been demonstrated by other groups (7–9).

The mucus layer provides a protective barrier against pathogenic and noxious agents and participates in the mucosal response to inflammation and infection (10). Increased mucus production apparently follows increased expression of mucin genes (11). To date, at least eight MUC genes, MUC1, 2, 4, 5AC, 5B, 7, 8, and 13, are expressed as mRNA in the normal human adult lung (10). The MUC5AC gene product is a major component of respiratory secretions (12, 13), and MUC5AC mRNA levels are increased in bronchial tissue of patients with asthma (14). *In vitro* studies suggest that MUC5AC expression is controlled, at least in part, by interleukin (IL)-17, a proinflammatory cytokine secreted by activated T cells (15, 16). IL-17 has recently been reported to stimulate MUC5AC expression in primary human tracheobronchial epithelial cells, whereas the type 2 cytokines IL-4, IL-9, and IL-13 did not directly upregulate MUC5AC (15).

Mouse gob-5 (mCLCA3) is a member of the Ca^{2+} -dependent chloride channel (CLCA) family (originally termed CaCC family) (17), and has a selective expression in airway goblet cells of allergically sensitized mice (18). *In vitro* transfection of an hCLCA1 (human counterpart to gob-5) or gob-5 expression vector into the human mucoepidermoid cell line, NCI-H292, increased mucus production and induces the MUC5AC gene (18, 19), suggesting that gob-5 regulates MUC5AC expression.

Based on previous studies showing that RSV infection augmented mucus production in the setting of allergic lung inflammation, we hypothesized that RSV infection upregulates IL-17 and gob-5 expression, which then increases Muc5ac (murine MUC5AC homolog) production, possibly mediating prolongation of AHR in OVA/RSV mice. To test this hypothesis, we used our well characterized murine model of prolonged AHR induced by RSV in the setting of allergen sensitization with OVA.

METHODS

Mice

Pathogen-free 8–10-week-old female BALB/c mice were purchased from Jackson Laboratory (Bar Harbor, ME).

Cells and Virus

The A2 strain of RSV was originally provided by Dr. Robert Chanock, National Institutes of Health. Master stocks and working stocks of RSV were prepared as previously described (20).

Allergen Sensitization Protocol

Mice were injected intraperitoneally with 0.1 ml (10 μg) of OVA (chicken OVA, grade V; Sigma, St. Louis, MO) complexed with 20 mg of $\text{Al}(\text{OH})_3$ on Day –16 (Figure 1). Mock-sensitized mice were injected intraperitoneally with 20 mg of $\text{Al}(\text{OH})_3$ on Day –16. On Days –2 to 5, the mice were placed in an acrylic box and exposed to aerosols of 1% OVA diluted in sterile phosphate-buffered saline, using an ultrasonic nebulizer (Ultraneb 99; DeVilbiss, Somerset, PA) for 40 minutes each day.

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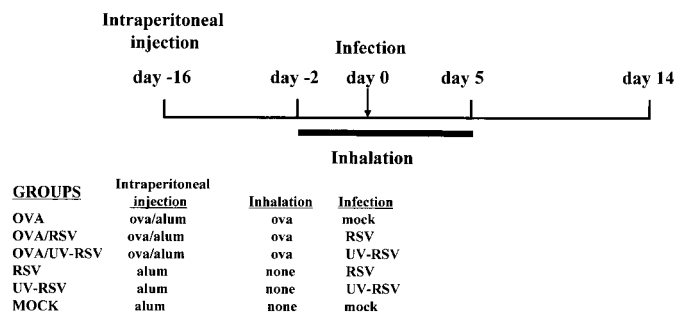


Figure 1. Timeline of experimental protocol. Mice were sensitized by intraperitoneal injection on Day -16, infected on Day 0, and exposed to inhalation treatments from Day -2 to Day 5. Mice were killed on Days 0, 2, 4, 6, 10, and 14 to collect samples for Western blot; on Days 0, 2, 6, 10, and 14 to collect samples for cytokine protein, and on Day 14 for histopathology. Alum = aluminum hydroxide; UV = ultraviolet light.

Mouse Infection

On Day 0, mice were infected with 1×10^7 plaque-forming units of RSV intranasally, as described elsewhere (20). Mock-infected mice were inoculated with a working stock for mock infection by the same procedure as RSV infected mice on Day 0.

Study Protocol

The study protocol is outlined in Figure 1. The mice were divided into six groups. The “MOCK” group was mock sensitized and mock infected. The “OVA” group was OVA sensitized and mock infected. The “RSV” group was mock sensitized and RSV infected. The “OVA/RSV” group was OVA sensitized and RSV infected. The “UV-RSV” group was mock sensitized and intranasally challenged with ultraviolet (UV) light-inactivated RSV. The “OVA/UV-RSV” group was OVA-sensitized and intranasally challenged with UV light-inactivated RSV.

Western Blotting Analyses for Muc5ac

The electrophoresis of mucin proteins was performed as previously described (21, 22). Electrophoresis of secretions for Muc5ac was performed on 1.0% (wt/vol) agarose (molecular biology grade; GIBCO BRL, Grand Island, NY) gels including 0.1% sodium dodecyl sulfate. One hundred micrograms of the protein in each sample was solubilized in electrophoresis sample buffer.

For Western blot analysis, the membranes were blocked and incubated with HO8 antibody (23), anti-Muc5ac specific antibody, diluted 1:2,000 in dilution buffer overnight with agitation at 4°C. Membranes were incubated with peroxidase-conjugated rabbit anti-chicken/turkey

IgG (H + L) (Zymed, San Francisco, CA) at a dilution of 1:10,000 with dilution buffer for 1 hour, and the signal was developed with ECL-Plus (Amersham Life Science, Heights, IL).

Western Blotting Analyses for Gob-5

The procedure for preparing samples was the same as that for Muc5ac except that a 4.75% stacking/10% separating sodium dodecyl sulfate-polyacrylamide gel electrophoresis was used. The mouse anti-gob-5 antibody was generously provided by Takeda Chemical Industries (Tsukuba, Japan). The second step, horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin (Ig) was purchased from Amersham Life Science. Peroxidase activity was visualized by ECL kit (Amersham Life Science) using standard procedures.

Measurement of Cytokines in Lung Tissues

Cytokine proteins in lung tissues of the mice groups were measured using commercially available ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions, as previously described (5).

Protocol for Examining Lung Sections

Mice were killed by cervical dislocation on Day 14, and the lung block was removed and embedded in paraffin, as previously described (5). The immunohistochemical analyses for gob-5 and Muc5ac were performed with the anti-gob-5 and anti-Muc5ac antibodies that were the same as the antibodies for Western blot. Slides were examined by one observer in a blinded fashion.

Statistical Analysis

Results are expressed as means $\pm$ SEM. Measurements of histopathology and cytokines were analyzed by one-way analysis of variance with Fisher’s least significant difference as a *post hoc* analysis. Differences were considered to be significant if *p* was less than 0.05.

RESULTS

Histochemical Analysis of Lung Tissue Sections Stained with Anti-gob-5 and Anti-Muc5ac Antibodies

Mice from the OVA, OVA/RSV, OVA/UV-RSV, RSV, and UV-RSV groups were killed on Day 14 and the lungs were harvested. Sections from each mouse were stained with both anti-gob-5 and anti-Muc5ac antibodies and then scored by a pathologist who was blinded to the groups. Figure 2 shows the results of staining for gob-5 and Muc5ac in each group of mice. The OVA/RSV group had greater staining for gob-5 and Muc5ac than any other group ($p < 0.05$). There was no difference in either gob-5 or Muc5ac staining for the OVA or OVA/UV-RSV groups, although both were significantly greater than the RSV

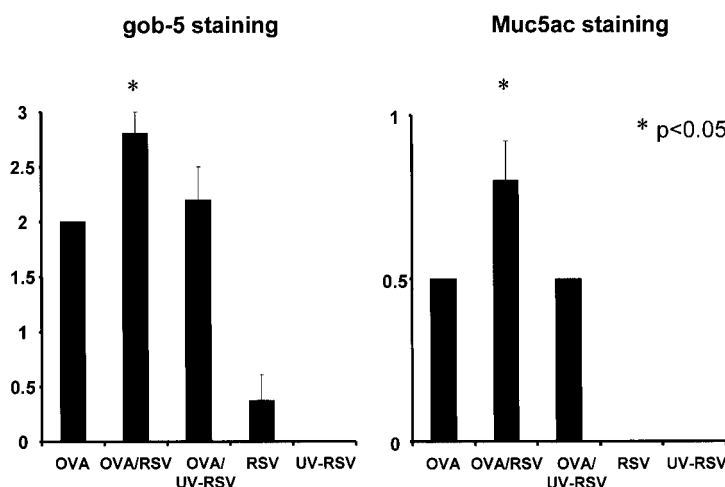


Figure 2. Histochemical analysis of gob-5 and Muc5ac expression in the airways. Mice from the ovalbumin (OVA)-sensitized ($n = 5$), OVA/respiratory syncytial virus (RSV) ($n = 5$), OVA/UV-RSV ($n = 5$), RSV ($n = 3$), and UV-RSV ($n = 4$) groups were killed on Day 14 and the lungs were harvested. Lung sections from each mouse were stained with both anti-gob-5 (left panel) and anti-Muc5ac (right panel) antibodies. Scoring was performed by a pathologist who was blinded to the groups. Error bars represent standard error of the mean. * $p < 0.05$ compared with all other groups.

or UV-RSV groups ($p < 0.001$). There was no difference in either gob-5 or Muc5ac staining for the RSV and UV-RSV groups. The location of gob-5 and Muc5ac expression within the airway epithelium is shown in Figure 3. Therefore, RSV infection in the setting of allergic inflammation in the lung results in increased expression of both gob-5 and Muc5ac.

Muc5ac Expression by Western Blot—General Comments

Western blot analysis for lung expression of Muc5ac using the well characterized antibody HO8 has not previously been reported. Figure 4 shows the results of expression of Muc5ac by Western blot in allergically sensitized lung and stomach tissues. Muc5ac bands A and D are expressed in stomach but not in murine lung, whereas bands B and C are present in murine lung tissue from an allergically sensitized mouse but not in murine stomach, suggesting that there are tissue-specific isoforms or post-translational modifications of Muc5ac.

Comparison of Expression of Muc5ac in OVA/RSV and OVA Mice

We performed a time course experiment on Days 0, 2, 4, 6, 10, and 14 of Muc5ac expression by Western blot (Figure 5A). Only

Western blot analysis for OVA and OVA/RSV groups are shown as there was no difference in either gob-5 or Muc5ac histochemical staining for the OVA and OVA/UV-RSV groups or the RSV and UV-RSV groups, respectively. In OVA mice, lung Muc5ac was detectable 4 days after starting OVA inhalation (Day 2 of the protocol), with maximal induction of Muc5ac on Day 6, the day after the last OVA inhalation. After discontinuing OVA inhalation, Muc5ac protein expression decreased. In OVA/RSV mice, the time course of induction of Muc5ac was similar to that in the OVA group, but in OVA/RSV mice Muc5ac expression was prolonged as late as Day 14. This finding was confirmed when multiple samples of OVA and OVA/RSV groups were assayed on Day 14 (Figure 5B). Next, we examined whether RSV infection alone could induce Muc5ac expression. We performed Western blot for Muc5ac on Days 6, 10, and 14 in MOCK, OVA, RSV, and OVA/RSV mice (Figure 5C). In the MOCK group, there was no expression of Muc5ac on any day. Although RSV infection prolonged Muc5ac expression in OVA/RSV mice, RSV infection alone caused no detectable Muc5ac expression, results similar to those obtained in the MOCK group.

Comparison of Induction of gob-5 in OVA/RSV and OVA Mice

As gob-5 may be involved in bronchial hyperreactivity and mucus overproduction in a murine model of allergic asthma (22), we examined induction of gob-5 in our model by Western blot. At first, we assessed the time course of gob-5 induction in OVA and OVA/RSV mice. Low levels of expression were observed on Day 0 (the day of infection and 2 days after starting OVA inhalation), indicating induction of gob-5 (data not shown). Increased expression of gob-5 was detected in both the OVA and OVA/RSV groups on Days 2 and 6 (data not shown). As illustrated in Figure 6, there was no detectable gob-5 protein expression in either the MOCK or RSV group. By Day 14, gob-5 expression was decreased from Day 10 in the OVA group, whereas gob-5 expression remained high in the OVA/RSV group.

Comparison of Expression of Lung Cytokines in OVA/RSV and OVA Mice

As IL-17 induces Muc5ac (15), we hypothesized that the OVA/RSV group would have increased IL-17 protein expression as measured by ELISA when compared with the OVA mice. As shown in Figure 7, we found that there were significantly greater

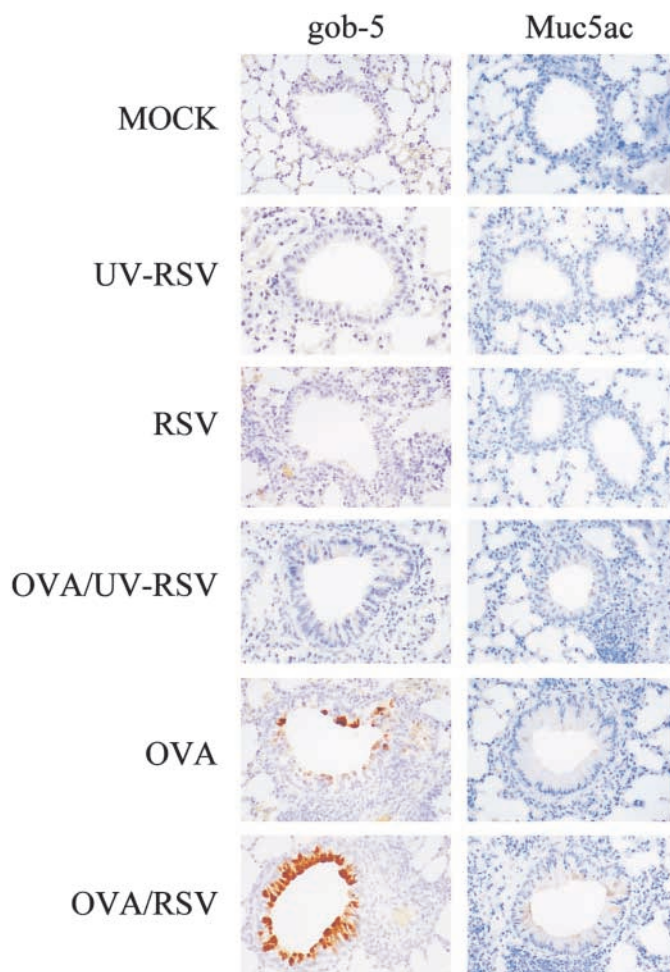


Figure 3. Expression of gob-5 and Muc5ac in the airway goblet cells of lung tissues. Paraffin sections of lung tissues in the OVA, OVA/RSV, OVA/UV-RSV, RSV, UV-RSV, and MOCK groups on Day 14 were immunohistochemically stained with anti-gob-5 (left panel) and anti-Muc5ac (right panel) antibodies. MOCK = mock sensitized and mock infected.

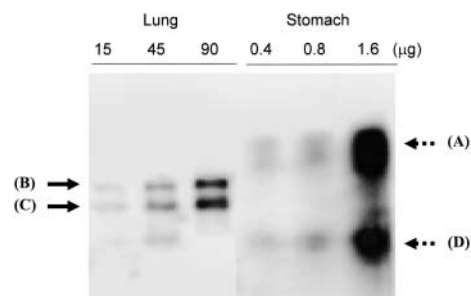


Figure 4. Assessing Muc5ac expression in the lung by Western Blot. Samples were made from mouse lung or mouse stomach mucosa in OVA group on Day 5. The samples were subjected to electrophoresis in 1% (weight/volume) agarose gel, as described in METHODS. The proteins were transferred to nitrocellulose and probed with the anti-Muc5ac antibody, HO8. Lung Muc5ac (left panel) is shown as (B) and (C) (solid-line arrows), and stomach Muc5ac (right panel) is shown as (A) and (D) (dotted-line arrows).

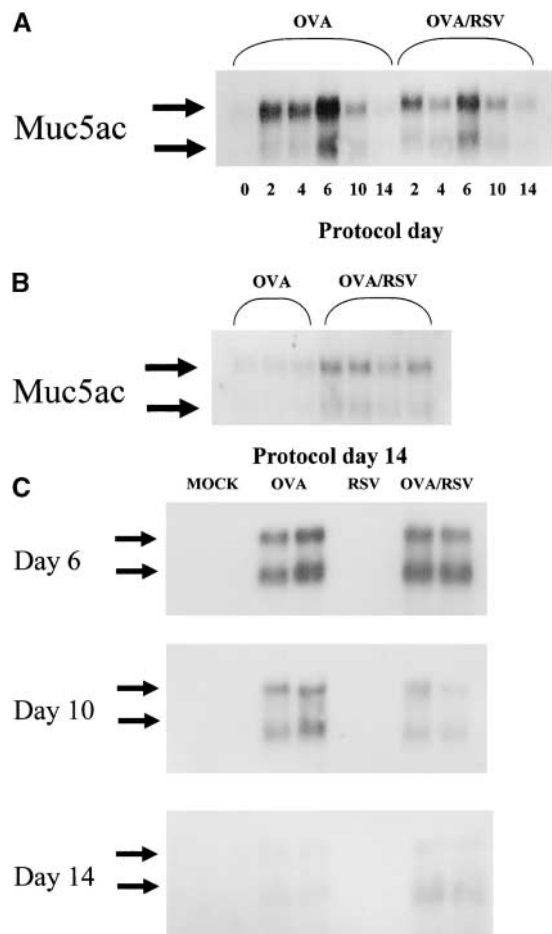


Figure 5. Muc5ac protein expression over time in MOCK, OVA, RSV, and OVA/RSV groups. (A) Time course of Muc5ac protein expression from Day 0 to Day 14. (B) Comparison of multiple samples on Day 14 between OVA ($n = 3$) and OVA/RSV ($n = 4$). (C) Comparison of Muc5ac protein expression among MOCK, OVA, RSV, and OVA/RSV groups—two mice in the groups were killed for Western blot analysis of Muc5ac on Days 6, 10, and 14. Proteins from each mouse lung were separated on 1% (wt/vol) agarose gel and transferred onto a nitrocellulose membrane. One hundred micrograms of protein were used for each sample. The membrane was treated with chicken antibody HO8 against mouse Muc5ac. Arrows indicate the Muc5ac protein bands.

levels of IL-17 in the lung supernatants of the OVA/RSV group compared with the OVA group both at Day 6 (92 ± 14 vs. 38 ± 1 pg/ml; $p < 0.01$) and at Day 10 (29 ± 2 vs. 16 ± 5 pg/ml; $p < 0.05$). IL-17 was undetectable in both the OVA/RSV and OVA groups at Day 14. Although RSV infection increased IL-17 levels in the OVA/RSV group, RSV infection by itself did not result in detectable levels of IL-17. We also measured expression of other cytokines that have been implicated in mucus production. The OVA mice had increased IL-13 protein levels compared with the OVA/RSV mice both on both Day 2 (212 ± 59 vs. 78 ± 31 pg/ml; $p < 0.05$) and at Day 6 (119 ± 3 vs. 61 ± 5 pg/ml; $p < 0.01$). There was no difference between the OVA and OVA/RSV groups at Day 10, and neither group had detectable levels at Day 14. Thus, in these experiments, RSV infection in the setting of allergic lung inflammation decreased lung levels of IL-13, confirming our earlier work (5). There was no detectable IL-13 in the RSV group at any time point. IL-6 has also been reported to induce Muc5ac expression,

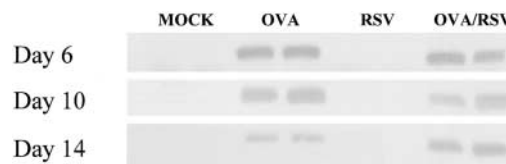


Figure 6. Comparison of lung gob-5 protein expression over time from MOCK, OVA, RSV, and OVA/RSV groups. Two mice in the MOCK, OVA, RSV, and OVA/RSV groups were killed for Western blot analysis of gob-5 on Days 6, 10, and 14. One hundred micrograms of protein were used for each sample. Proteins from each mouse were separated on a 4.75% stacking/10% separating polyacrylamide gel and transferred onto a nitrocellulose membrane. The membrane was treated with rabbit antibody against mouse gob-5.

as well as to be induced by IL-17 (15). We found detectable levels of IL-6 only in the RSV group at Day 6 (132 ± 18 pg/ml), whereas there were no detectable levels in any other group at any time point. Likewise, IL-10 was detectable in supernatants only in the RSV group on Day 6 (48 ± 6 pg/ml). Finally, we measured IFN- γ levels in supernatants and found that there were significantly increased levels of this cytokine in the RSV group compared with the OVA/RSV group ($3,520 \pm 914$ vs. 375 ± 144 pg/ml; $p < 0.01$). IFN- γ was not detectable at any other time point in any group.

DISCUSSION

In children with underlying asthma, 80–85% of asthma exacerbations are associated with viral respiratory infections (1). Approximately 80% of children who have asthma are allergic, so that it is very important to understand the mechanisms by which viral infections lead to asthma exacerbations. Airway mucus overproduction is one of the hallmarks of asthma (1). Viral infections cause asthma exacerbations and are frequently associated with increased mucus production. We and others have previously reported that RSV infection in the setting of allergic inflammation in the lung results in augmented mucus production (5, 7–9). We therefore sought to study the mechanisms by which this increase in airway mucus production is regulated. In this study, we found that RSV infection in allergically sensitized mice prolongs expression of two key factors in mucus production, Muc5ac and gob-5, whereas RSV infection alone does not lead to expression of either Muc5ac or gob-5.

MUC5AC expression is generally restricted to surface goblet cells in the upper and lower respiratory tract (24–26). Hyperplasia of goblet cells with mucus overproduction is a feature of asthma (27). Furthermore, Muc5ac mRNA and protein expression, as well as goblet cell metaplasia, are induced in murine models of asthma (28).

Another mucin gene upregulated in allergic airway inflammation is gob-5, a member of the CLCA family that is widely distributed in human secretory organs (29). Recently, gob-5 was demonstrated in asthmatic airway goblet cells as well as in parts of the digestive tract in a murine model of allergic asthma (18). This study also demonstrated that intratracheal administration of adenovirus-expressing antisense gob-5 RNA into AHR model mice efficiently suppressed the asthma phenotype, including AHR and mucus overproduction. In contrast, asthma phenotype is exacerbated by the adenoviral vector-induced overexpression of gob-5 in airway epithelium (18). In human samples, expression of hCLCA1 in bronchial tissues was significantly upregulated in patients with bronchial asthma compared with control subjects. *In situ* hybridization and immunohistochemical analysis

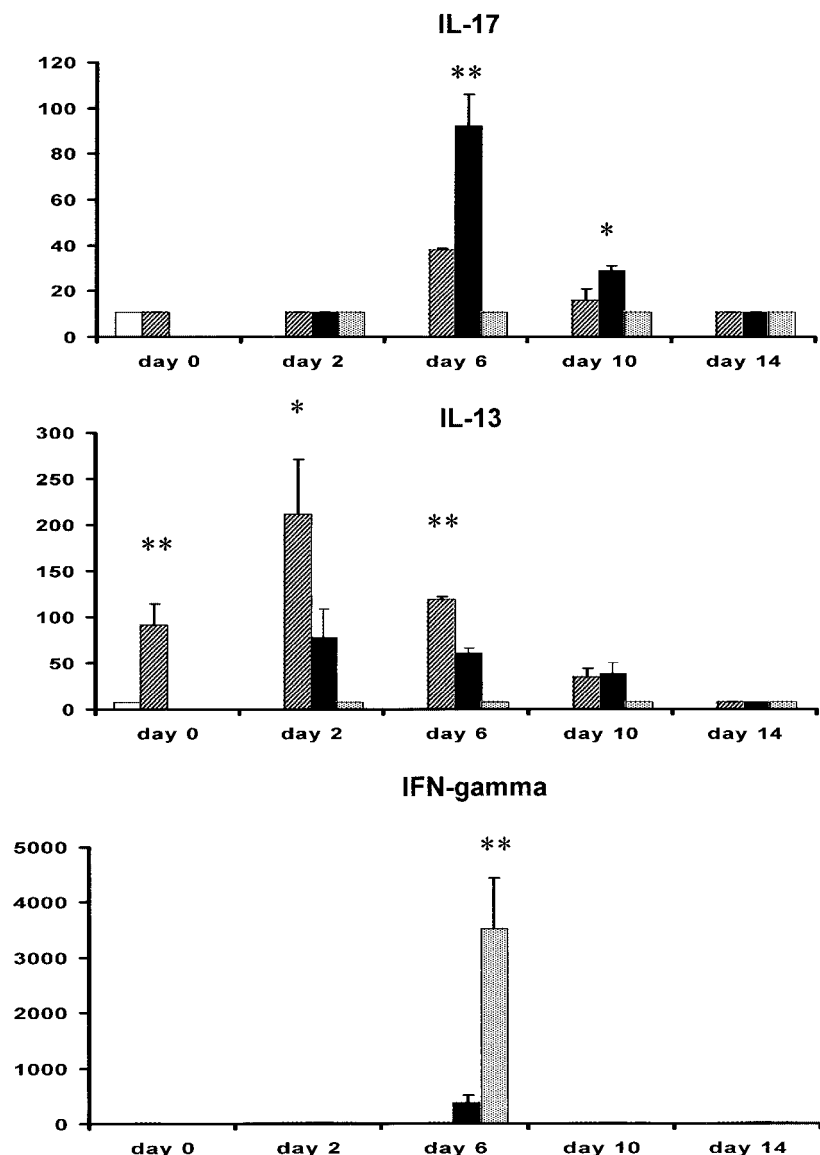


Figure 7. Lung concentrations of interleukin (IL)-17, IL-13, and IFN- γ proteins. The concentrations of IL-17 (upper panel), IL-13 (middle panel), and IFN- γ (lower panel) proteins in the lung supernatants from mice on Days 0, 2, 6, 10, and 14 ($n = 3$ for each group at each time point). Data shown are representative of two separate experiments. MOCK, white bars; OVA, hatched bars; OVA/RSV, black bars; RSV, gray bars; * $p < 0.05$ compared with all other groups; ** $p < 0.01$ compared with all other groups.

demonstrated that hCLCA1 is located in the bronchial epithelium, especially in mucus-producing goblet cells (19).

Type 2 cytokines, such as IL-4, IL-9, and IL-13, mediate goblet cell metaplasia and induce murine Muc5ac mRNA (28, 30–34). Expression of gob-5 has been induced by intratracheal administration of IL-9, IL-4, and IL-13, but not by IFN- γ in murine experiments (35). In our model, we found that RSV infection actually decreased lung levels of IL-13 in the OVA/RSV group in comparison to the OVA group. Thus, lung levels of IL-13 did not correlate with increased mucus production in this model of RSV infection and allergic lung inflammation. IL-10 is another type 2 cytokine that is a candidate to induce AHR and mucin overproduction. The role of IL-10 in asthma remains controversial. Some studies found that IL-10 expression is increased in subjects with asthma compared with nonasthmatic controls (36–38), a finding not confirmed by others (39–42). However, IL-10 may be important in the development of airway hyperresponsiveness in a murine model of allergic sensitization (43, 44). In transgenic mice overexpressing IL-10, periodic acid-Schiff-staining cells were prominent in the airway as compared with wild type mice. RSV-infected IL-10-overexpressing mice had increased levels of mRNA encoding Muc5ac, Muc4, and Muc2, and also

prominently induced gob-5 mRNA (45). In our experiments, we found detectable IL-10 only in the RSV group on Day 6. We did not find detectable IL-10 protein in the lung in either the OVA or OVA/RSV group; thus, IL-10 did not correlate with increased mucus production.

Although we did not find an increase in type 2 cytokine production in the OVA/RSV mice in comparison to the OVA group, we did find that the increased gob-5 and Muc5ac expression in the OVA/RSV mice was associated with a significant increase in lung levels of IL-17. IL-17 is produced solely by activated T lymphocytes and, binding of IL-17 to its receptor activates ERK, JNK, and p38 MAP kinase pathways, resulting in upregulation of IL-6, IL-1, and NF- κ B (16). In human, monkey, and mouse tracheobronchial epithelial cells, IL-17 and IL-6 stimulated MUC5AC expression after 16 hours of culture, whereas the Th2 cytokines IL-4, IL-9, and IL-13 did not (15). The correlation of the increased mucus production with augmented IL-17 levels in the OVA/RSV group is certainly suggestive of a pathogenic role of this cytokine in the heightened mucus production in this group. However, as we cannot block IL-17 activity *in vivo*, we cannot rule out the possibility that an IL-17-independent pathway is responsible for the increased mucus

production in the OVA/RSV group. It is important to note that IL-17 levels were undetectable in the RSV group, a finding that correlated with the lack of mucus expression in these mice. However, RSV infection led to a doubling or greater of the IL-17 production in the OVA/RSV group in comparison to the OVA group on both Days 6 and 10. We are currently exploring the mechanism by which RSV infection increases IL-17 in allergically inflamed lungs. In this regard, IL-17 induces IL-6 expression *in vitro* (14), but we have not seen increased IL-6 in the lung supernatants of the OVA/RSV group.

Our data showing that RSV infection does not increase mucus production is contrary to a report that RSV infection alone increases airway Muc5ac expression in a murine model (46). Possible explanations for these differences include the dose of virus given and potential differences in the strains of virus employed in the studies. The likelihood of a different strain of RSV accounting for the difference in mucus production is heightened by the fact that this same group has reported that primary RSV infection induces IL-13 production in the lung (47, 48), whereas we have not found detectable levels of lung IL-13 either by ELISA or RNase protection assay. Given their results in primary RSV infection, increased mucus production would be expected in this model as others have also reported that IL-13 induces mucus expression. At this point, the differences between our results and those of Miller and colleagues remain unexplained.

In conclusion, RSV infection during allergic sensitization prolongs both expression of Muc5ac and gob-5. The differences in mucus production were not apparent during the acute infection, and were restricted to the recovery phase. This would seem to suggest that allergic individuals who contract RSV infection might not have more severe acute manifestations, but could have prolonged recovery.

Conflict of Interest Statement: K.H. does not have a financial relationship with a commercial entity that has an interest in the subject of this article; B.S.G. does not have a financial relationship with a commercial entity that has an interest in the subject of this article; S.B.H. does not have a financial relationship with a commercial entity that has an interest in the subject of this article; K.B.A. received \$3,000 in 2002 and \$5,000 in 2003 from Sepracor Corp. for lecture fees/honoraria and a research grant in 2002 from Sepracor Corp. to study effects of Albuterol isomers on airway epithelial cells, is a member of the Scientific Advisory Board of BioMarck Corp., and serves as a consultant to AstraZeneca; R.D.C. does not have a financial relationship with a commercial entity that has an interest in the subject of this article; S.J.O. does not have a financial relationship with a commercial entity that has an interest in the subject of this article; W.Z. does not have a financial relationship with a commercial entity that has an interest in the subject of this article; T.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this article; P.W.J. does not have a financial relationship with a commercial entity that has an interest in the subject of this article; K.G. does not have a financial relationship with a commercial entity that has an interest in the subject of this article; J.F.O. does not have a financial relationship with a commercial entity that has an interest in the subject of this article; R.S.P. received \$10,500 for serving as a member of Merck's Pediatric Advisory Board in 2003, received \$10,000 for giving scientific talks at universities as a member of Merck speaker's bureau in 2003, and also received a \$50,000 investigator-initiated research project from Merck in 2001–2002.

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