Enhanced Expression of Mucin Genes in a Guinea Pig Model of Allergic Asthma

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The ovalbumin (OVA)-sensitized guinea pig is often used as an animal model of asthma and airway hyperreactivity. A characteristic lesion of asthma is excessive production of mucin in the airways. Mechanistic studies of this lesion in guinea pigs have been limited due to lack of mucin gene probes for this species. The aim of the present study was to clone the cDNAs encoding two major airway mucins (Muc2 and Muc5ac) from the guinea pig, and investigate mucin gene expression in lungs of sensitized animals in response to antigen challenge. We isolated and sequenced two cDNA fragments coding for the sequences located within the carboxyl-terminal cysteine-rich region of guinea pig Muc2 and Muc5ac mucins. Comparison of cloned cDNAs with those from other species revealed high degrees of sequence identity and conservation of all cysteine residues in deduced primary sequences. Based on the resultant sequence information, we also designed oligonucleotide primers for specific detection of guinea-pig Muc2 and Muc5ac steady-state mRNA levels via reverse transcriptase/polymerase chain reaction (RT-PCR). Levels of both Muc2 and Muc5ac mRNA in lungs of OVA-sensitized guinea pigs increased significantly by 30 min after an acute exposure to 0.3% OVA. In addition, levels of eotaxin mRNA also increased in these tissues, but the increases were not significant until 2 h after challenge. Correspondingly, the number of eosinophils in bronchoalveolar lavage fluid did not increase until 4 h postchallenge. Results of these studies suggest that the OVA-sensitized guinea pig responds to allergic challenge with enhanced expression of genes (e.g., eotaxin, Muc2, and Muc5ac) that likely play a role in increased airway inflammation and mucin overproduction, and enhanced mucin gene expression appears to occur before eosinophil infiltration.

Mucin glycoproteins (mucins) are the major structural components of mucus. To date, 12 different mucin genes have been identified, with 7 of them known to be expressed in the airways: MUC1, 2, 4, 5AC, 5B, 7, and 8 (reviewed in Ref. 1–4). Expression of MUC2 and MUC5AC is upregulated in inflamed airways (5–8) and may contribute to the pathogenesis of several airway diseases. Both MUC2 and MUC5AC are extremely large and highly glycosylated proteins. cDNAs encoding MUC2 and MUC5AC mucins have been isolated from a number of species including human (9–11), rat (12–14), and mouse (15, 16). Among these, human MUC2 is the only one for which a full-length cDNA has been cloned and sequenced (9).

The antigen-sensitized and -challenged guinea pig model of allergic pulmonary inflammation mimics many of the pathophysiologic components seen in humans with asthma, including airway hyperresponsiveness and eosinophilic inflammation (17–19). In addition, airways of these animals appear to undergo mucus cell hyperplasia and mucin hypersecretion (18–20). However, information related to mucin gene expression in guinea pig airways has not been forthcoming due to lack of mucin gene–specific probes for this species.

Here, we report cloning of two cDNA fragments coding for guinea pig Muc2 and Muc5ac mucins. Using a reverse transcriptase/polymerase chain reaction (RT-PCR) procedure based on the cloned cDNA sequences, we also evaluated acute temporal expression of Muc2 and Muc5ac genes in lungs of allergic guinea pigs following antigen challenge, and compared expression of these mucin genes with that of eotaxin (the eosinophil chemoattractant) gene in response to the challenge. Levels of steady-state mRNAs of Muc2 and Muc5ac, as well as eotaxin, were elevated in lungs of ovalbumin (OVA)-sensitized guinea pigs following acute OVA challenge. Enhanced expression of mucin genes preceded production of eotaxin and subsequent infiltration of inflammatory cells into the airways. In addition, because the levels of Muc2 and Muc5ac mRNAs peaked at 30 min after challenge, we examined whether histamine, an inflammatory mediator associated with the early phase asthmatic response (21), played a role in mucin gene expression in differentiated guinea pig tracheal epithelial (GPTE) cells *in vitro*. Results showed that histamine did not affect Muc2 mRNA levels at a concentration and time point that can elicit mucin hypersecretion from these cells (22). These studies suggest that upregulation of mucin gene expression in response to antigen challenge in the allergic guinea pig airways is not driven by eotaxin or histamine, and appears to be independent of additional inflammatory cell influx and subsequent airway inflammation.

Materials and Methods

Culture of GPTE Cells

Primary cultures of GPTE cells were established using the air–liquid interface procedure developed in this laboratory (23). Briefly, guinea pig tracheae were excised from killed animals. Epithelial cells were dissociated proteolytically, washed, and seeded in Transwell inserts (Corning Costar, Cambridge, MA) coated with rat tail collagen type I (Collaborative Research, Bedford, MA) at a density of 5×10^4 cells/cm^2 in Dulbecco's modified Eagle's medium (DMEM)/F12 supplemented with 5% fetal

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Abbreviations: Dulbecco's modified Eagle's medium, DMEM; fetal bovine serum, FBS; ovalbumin, OVA; reverse transcriptase/polymerase chain reaction, RT-PCR.

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bovine serum (FBS), 4 mM L-glutamine, 1% HL-1, 25 ng/ml human recombinant epidermal growth factor, 50 nM retinoic acid, 100 μg/ml gentamicin, 20 U/ml nystatin, and 0.5 μg/ml amphotericin-B. Cells were cultured at 37°C in an atmosphere of 3% CO_2/97% air. Medium was renewed every other day until the cells were 70–80% confluent (4–5 d), at which time medium was removed from the apical surfaces and cultures were fed basally with serum-free medium for an additional 7 d to allow full differentiation.

Cloning of Guinea Pig Muc2 and Muc5ac cDNAs

Guinea pig Muc2 and Muc5ac cDNAs were cloned from GPTE cultures and gastric mucosa, respectively. Briefly, total RNA was isolated from differentiated GPTE cells or guinea pig gastric mucosa using the one-step guanidinium-phenol-chloroform method of Chomczynski (24). Poly(A^+)-RNA was then purified using the Oligotex Combi Kit (Qiagen, Santa Clara, CA) according to the manufacturer's protocol. Reverse transcription (RT) was performed using ~ 50 ng of purified Poly(A^+)-RNA as template and random hexamers as primers, and 10% of the RT reaction mix was then used in a PCR. Primers for amplifying guinea pig Muc2 cDNA were two oligonucleotides corresponding to two octapeptide motifs that were conserved in both Muc2 and Muc5ac mucins (11, 14): forward primer 5′-GGCCAGTGCGGCACTTGCACC AAC; reverse primer 5′-GCCCTCCGGACAGAAGCAGCCTTC. The PCR was performed at 1.75 mM $MgCl_2$ for 30 cycles, each comprising 1 min of denaturation at 94°C, 1 min of annealing at 65°C, and 2 min of extension at 72°C. For PCR amplification of the guinea pig Muc5ac cDNA, two degenerate oligonucleotides corresponding to the two octapeptide motifs were designed and used as primers: forward primer 5′-GGXCARTGYGGXACXT GYACXAAYGA; reverse primer 5′-CCYTCXGGRCARAAR CAXCCYTC. The PCR was conducted under the following conditions: 1.8 mM $MgCl_2$; 10 cycles of each composed of 45 s of denaturation at 95°C, 30 s of annealing at 42°C, and 2 min of extension at 62°C; followed by an additional 30 cycles each composed of 45 s of denaturation at 95°C, 30 s of annealing at 50°C, and 90 s of extension at 72°C. The amplified cDNA fragments were purified and cloned into the pUC18 vector. Selected recombinant clones were analyzed by restriction enzyme digestion and inserts were sequenced using the T7 Sequenase Version 2.0 DNA Sequencing Kit and the Sequenase PCR Product Sequencing Kit (Amersham/USB, Piscataway, NJ) according to the manufacturer's instructions. The resultant sequences were compiled and analyzed using the Wisconsin Package GCG software (version 10; Accelrys, Madison, WI).

OVA-Sensitized Guinea Pigs

Experiments were performed under a protocol approved by the Animal Care and Use Committee of Schering-Plough Research Institute, in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, the Animal Welfare Act, and a program approved by the American Association for the Accreditation of Laboratory Animal Care. Specifically, on Day 0, male Hartley guinea pigs were sensitized with an intraperitoneal injection (0.5 ml) of a saline suspension containing alum (100 mg/ml) and OVA (100 μg/ml). One week later animals were boosted with a second intraperitoneal injection (0.5 ml) of the OVA suspension. Animals were returned to their cages and allowed food and water *ad libitum*. After 27 d, animals were exposed to an aerosol challenge of either saline or 0.3% OVA for 20 min. The aerosol was generated by an ultrasonic nebulizer (Model Ultra Neb99; DeVilbiss, Jackson, TN) and delivered to a large Plexiglas chamber. To prevent anaphylactic bronchospasm, animals received the H1 antagonist pyrilamine (15 mg/kg) intraperitoneally 30 min before antigen challenge.

Bronchoalveolar Lavage Cell Counts

OVA-sensitized animals underwent bronchoalveolar lavage (BAL) at 0, 0.5, 1, 2, 4, and 24 h after the challenge. Briefly, animals were anesthetized with ketamine/xylazine (10:1, i.e., 200 mg/kg ketamine and 20 mg/kg xylazine) at selected time points, then lavaged with 2×3 ml of phosphate-buffered saline (PBS, pH 7.2). Cells in the BAL fluid were harvested by centrifugation at $350 \times g$ for 10 min at 4°C. Collected cells were then washed once with PBS containing 10 μg/ml porcine pancreatic DNase I and resuspended in 1 ml PBS with 10 μg/ml DNase I and 5% heat-inactivated FBS. Cells were examined by cytospin and differential staining using the Hema3 staining system (Fischer Scientific, Pittsburgh, PA). Differential cell counts on a minimum of 200 cells were performed using standard histologic parameters. Total cell counts were performed using a Neubauer chamber.

Detection of Steady-State mRNA Levels by RT-PCR

Sensitized guinea pigs were killed at selected times after antigen challenge by an overdose injection of ketamine/xylazine. Lungs were surgically removed and separated from the trachea before freezing. Total RNA was isolated from random pieces of lung tissue cut from the frozen block using the RNeasy Mini Kit (Qiagen, Valencia, CA) following the manufacturer's protocol. To examine mucin gene expression in GPTE cells in response to histamine, cells were exposed to 100 μM histamine or to 15 ng/ml recombinant human tumor necrosis factor (TNF)-α (as a positive control) for either 1 or 4 h. Total RNA was then isolated from these treated cells as described above. All RNA preparations were examined for purity by UV spectrophotometry, and only those with A_{260}/A_{280} ratios in the 1.95 to 2.05 range were used in RT-PCR.

Primers for specific detection of guinea pig Muc2, Muc5ac, and eotaxin mRNAs were pairs of internal oligonucleotide sequences that would generate a 364-bp PCR product for Muc2, a 380-bp product for Muc5ac, and a 363-bp product for eotaxin (25), respectively. Muc2 forward primer: 5′-CTGTCCCCA CACTGACATCACTAC (position 123–146); Muc2 reverse primer: 5′-GGAGTTCTGTGAGGCACTTGACTG (position 463–486); Muc5ac forward primer: 5′-TCCCTACACTGGAG AGTGGACAAC (position 85–108); Muc5ac reverse primer: 5′-TGGTAGCAGTAGGATGGGTCTGAC (position 441–464); eotaxin forward primer: 5′-CAACCCAGAAACTATTGTCAC GCTG (position 22–46); eotaxin reverse primer: 5′-TGGTTT GTCATCTCAAGCACGATG (position 361–384).

To normalize each individual reaction, γ-actin was used as an internal control. Two oligonucleotide primers were synthesized according to the published cDNA sequences encoding γ-actins from other species (26–28): forward primer 5′-TGGCACCA CACCTTCTACAAC, reverse primer 5′-CCCATCAGGCA GCTCATAAC. A single RT-PCR product of 480 bp in size was amplified from guinea pig RNA using these primers. Sequence analysis showed that this 480-bp product had 100% sequence identity to γ-actin cDNAs from human, rat, and mouse (data not shown).

To detect steady-state mRNA levels of guinea pig Muc2 and Muc5ac, RT was performed in a total volume of 50 μl using 5 μg total RNA as template and 5 ng oligo$(dT)_{12\text{-}18}$ as primer. PCR amplification was performed for 30 cycles in 50 μl volume using 5 μl RT mix at the following conditions: 1.8 mM $MgCl_2$; 40 s of denaturation at 95°C, 30 s of annealing at 66°C, and 1 min of extension at 72°C. To examine eotaxin mRNA, the Superscript One-Step RT-PCR system (Gibco BRL, Bethesda, MD) was used according to the manufacturer's instructions with a 55°C annealing temperature. In addition, γ-actin was amplified using 1 μl RT mix at the following conditions: 1.8 mM $MgCl_2$; 25 cycles each composed of 40 s of denaturation at 95°C, 30 s of annealing at 56°C, and 1 min of extension at 72°C. Under these PCR conditions for

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        10                  30                  50                  70                  90
GGCCAGTGCGGCACTTGCACCAACAGCACGGCTGACGACTGTGTGCTGCCCAGTGGGGAGGTCACCTCCAACTGCGAACTGGCTGCTGAC
 G  Q  C  G  T  C  T  N  S  T  A  D  D  C  V  L  P  S  G  E  V  T  S  N  C  E  L  A  A  D

                110                 130                 150                 170
CAGTGGGTGGTGAATGACCCCTCTAAGCCACACTGTCCCCACACTGACATCACTACGGAGCGCCCGGTTATCAAGACAACTTTGCCCAAG
 Q  W  V  V  N  D  P  S  K  P  H  C  P  H  T  D  I  T  T  E  R  P  V  I  K  T  T  L  P  K

        190                 210                 230                 250                 270
AACTGCACTGTGTCTCCGCTCTGCCAGCTCATCAAGGACAGCTTGTTTTCCCAGTGCCACCCCTGGGTGCCGCCCCAGCACTACTACGAG
 N  C  T  V  S  P  L  C  Q  L  I  K  D  S  L  F  S  Q  C  H  P  W  V  P  P  Q  H  Y  Y  E

                290                 310                 330                 350
GCCTGCATATTCGACAGCTGCTTCGTGCCAAACTCTGGCATGGAGTGCGCCAGTGTGCAAGCCTACGCGGCCCTCTGCTCCCAGGAAAAG
 A  C  I  F  D  S  C  F  V  P  N  S  G  M  E  C  A  S  V  Q  A  Y  A  A  L  C  S  Q  E  K

        370                 390                 410                 430                 450
GTCTGTGTGGACTGGCGGTCGCACACTAACGGGTCCTGCGCTGTGACGTGTCCGGCTCATAGGCAGTACCAGCCCTGTGGGCCCGCAGAG
 V  C  V  D  W  R  S  H  T  N  G  S  C  A  V  T  C  P  A  H  R  Q  Y  Q  P  C  G  P  A  E

                470                 490                 510
GAGCCCACTTGCCAGTCAAGTGCCTCACAGAACTCCTCGGTCCTGGTGGAAGGCTGCTTCTGTCCGGAGGGC
 E  P  T  C  Q  S  S  A  S  Q  N  S  S  V  L  V  E  G  C  F  C  P  E  G
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Figure 1. Nucleotide sequence of guinea pig Muc2 cDNA fragment and its deduced primary sequence. The cDNA was cloned from cultured GPTE cells via RT-PCR as described in MATERIALS AND METHODS. Both strands of the cDNA were sequenced using the dideoxy chain termination method. The resultant sequence was compiled and analyzed by the Wisconsin Package GCG software (version 10). Octapeptide motifs are underscored.

each gene detection described above, all reactions generated one single product that was in the linear range of amplification and did not exceed the saturation point. Additional reactions that lacked either RT mix in the PCR or reverse transcriptase in the RT reaction were performed to ensure no contamination during the experimental procedures. The PCR products were analyzed by electrophoresis on a 1.5–2% agarose gel. Data were processed using NIH Image software or 1D Image Analysis software (Eastman Kodak, Rochester, NY). Levels of mRNA of each gene (Muc2, Muc5ac and eotaxin) were normalized to their own γ-actin internal control, and then converted to percentage value of their corresponding experimental (saline or medium) control.

Statistical Analysis

Data were analyzed for significance using one-way analysis of variance (ANOVA) with Bonferroni post-test corrections. Differences between treatments were considered significant at $P < 0.05$.

Results

Cloning of Guinea Pig Muc2 and Muc5ac cDNA Fragments

It was previously reported that there were two highly conserved octapeptide motifs in the carboxyl-terminal cysteine-rich regions of Muc2 and Muc5ac mucins from human and rat (11, 14). Thus, oligonucleotides with sequences corresponding to these two motifs were used as primers for PCR cloning of guinea pig Muc2 and Muc5ac cDNAs. Using RNA isolated from cultured GPTE cells as template, a dominant product of $\sim$ 500 bp in size was amplified by RT-PCR. Cloning and sequence analysis revealed that the actual length of this cDNA fragment was 522 bp (Figure 1), and it appeared to encode Muc2 mucin as it exhibited a high degree of identity to those cDNAs coding for human MUC2 (78% identity) and rat Muc2 (80% identity). Accordingly, the deduced amino acid sequence displayed a high degree of similarity to human and rat Muc2 mucins, and relatively low homology to Muc5ac mucins (Table 1). In addition, alignment of three Muc2 primary sequences (human, rat, and guinea pig) indicated conservation of all cysteine residues in the cloned region (Figure 2).

Muc5ac is also known as a major gastric mucin expressed by gastric mucosa in which Muc2 is not expressed (reviewed in Ref. 1, 2). Therefore, RNA isolated from guinea pig gastric mucosal tissue was used as template for RT-PCR cloning of Muc5ac cDNA. Using two degenerate primers corresponding to the two octapeptide motifs and a PCR procedure described in MATERIALS AND METHODS, a cDNA fragment with a size of 537 bp was amplified and cloned (Figure 3). Sequence comparison suggested that this cDNA fragment encoded Muc5ac mucin, and its sequence identity to human and rat Muc5ac cDNAs was 73% and 72%, respectively. The deduced amino acid se-

TABLE 1
Amino acid sequence similarity among the guinea pig, rat, and human Muc2 mucins

	Guinea pig Muc2	Human MUC2	Rat Muc2
Guinea pig Muc2	—	86%	88%
Human MUC5AC	56%	56%	55%
Rat Muc5ac	61%	56%	60%

Comparison was accomplished using the BestFit program in the GCG software package (version 10). Sequences used for comparison include the regions between two conserved octapeptide motifs in the Muc2 and Muc5ac mucins.

```
            1                                                    50
guinea pig  GQCGTCTNST ADDCVLPSGE VTSNCELAAD QWVVNDPSKP HCPHTDITTE
       rat  GQCGTCTNNT ADDCILPSGE IISNCEVAAD EWLVNDPSKP HCPHKGLTTK
     human  GQCGTCTNTT SDDCILPSGE IVSNCEAAAD QWLVNDPSKP HCPHSSSTTK

            51                                                  100
guinea pig  RPVIKT.... ..TLPKNCTV SPLCQLIKDS LFSQCHPWVP PQHYYEACIF
       rat  RPAITTPG.. ..PFPENCTV SPVCQLIMDS LFSQCHPFVP PKHYYEACLF
     human  RPAVTVPGGG KTTPHKDCTP SPLCQLIKDS LFAQCHALVP PQHYYDACVF

            101                                                 150
guinea pig  DSCFVPNSGM ECASVQAYAA LCSQEKVCVD WRSHTNGSCA VTCPAHRQYQ
       rat  DSCFVAGSGM ECASVQAYAA LCAQEGVCID WRNHTQGACA VTCPAHRQYQ
     human  DSCFMPGSSL ECASLQAYAA LCAQQNICLD WRNHTHGACL VECPSHREYQ

            151                          181
guinea pig  PCGPAEEPTC QSSAS.QNSS VLVEGCFCPE G
       rat  ACGPSEEPTC QSSSP.KNST LLVEGCFCPE G
     human  ACGPAEEPTC KSSSSQQNNT VLVEGCFCPE G
```

Figure 2. Conservation of guinea pig, rat, and human Muc2 mucins. Primary sequences between the two conserved octapeptide motifs of Muc2 mucins from different species were aligned using the Pileup program in the GCG software package (version 10). Cysteine residues are highlighted. Gaps inserted to optimize the alignment are indicated by *dots*.

```
         10                        30                        50                        70                        90
GGTCAGTGTGGGACGTGTACGAATGACAAGAAGGACGAGTGCCGGATGCCTGGGGGAGCAGTGGCCACCTCCTGCTCAGATATGTCCCTA
 G  Q  C  G  T  C  T  N  D  K  K  D  E  C  R  M  P  G  G  A  V  A  T  S  C  S  D  M  S  L

                   110                       130                       150                       170
CACTGGAGAGTGGACAACCCTGACGAGCTCTTCTGCCGTGGGCCACTCCCAACACCCACCTCTTTGGTGCCAACCCCCCCACCTACCGAA
 H  W  R  V  D  N  P  D  E  L  F  C  R  G  P  L  P  T  P  T  S  L  V  P  T  P  P  P  T  E

        190                       210                       230                       250                      270
TGCCCAGCATCGCCCATCTGCGAATTGATCCTGACTGATGTCTTCAAACCCTGCCACGCTGTGATCCCACCACTACCGTTCCATGAAGGC
 C  P  A  S  P  I  C  E  L  I  L  T  D  V  F  K  P  C  H  A  V  I  P  P  L  P  F  H  E  G

                   290                       310                       330                       350
TGTGTCTTTGACCACTGCCACAATATGGAGTTGGAGGTGGTGTGCTCTGGCCTGGAGCTCTATGCCTTGCTGTGTGCGGCCCAGGGCGTG
 C  V  F  D  H  C  H  N  M  E  L  E  V  V  C  S  G  L  E  L  Y  A  L  L  C  A  A  Q  G  V

        370                       390                       410                       430                      450
TGCGTGGACTGGAGGAGCTGGACCAACAACTCATGCTCCTTCCCCTGCCCTGAGGACAAAGTGTACCAGCCCTGCGGCCCGTCAGACCCA
 C  V  D  W  R  S  W  T  N  N  S  C  S  F  P  C  P  E  D  K  V  Y  Q  P  C  G  P  S  D  P

                   470                       490                       510                       530
TCCTACTGCTACCAGAGTGACAACGCCAGCTCCCTAGTCCTCCCCAAGGCCTACCGCATCACAGAGGGCTGCTTTTGCCCAGAGGGG
 S  Y  C  Y  Q  S  D  N  A  S  S  L  V  L  P  K  A  Y  R  I  T  E  G  C  F  C  P  E  G
```

Figure 3. Nucleotide sequence of guinea pig Muc5ac cDNA fragment and its deduced primary sequence. The cDNA was cloned from guinea pig gastric mucosa via RT-PCR as described in MATERIALS AND METHODS. Both strands of the cDNA were sequenced using the dideoxy chain termination method. The resultant sequence was compiled and analyzed by the Wisconsin Package GCG software (version 10). Octapeptide motifs are underscored.

quence showed a high degree of similarity to human and rat Muc5ac mucins, and relatively low homology to Muc2 mucins (Table 2). Sequence alignment of Muc5ac from different species indicated conservation of all cysteine residues in the cloned region (Figure 4).

Furthermore, a phylogenetic tree demonstrating the evolutionary distances between aligned mucin gene sequences was created using the GrowTree program in the GCG software package (Figure 5). The two cloned guinea pig cDNAs were allocated into two distinctive clusters named by their human orthologs, confirming that they were coding for Muc2 and Muc5ac, respectively.

Mucin and Eotaxin Gene Expression in Antigen-Challenged Guinea Pig Lungs

Both Muc2 and Muc5ac mRNA levels were elevated in the lungs of OVA-sensitized guinea pigs following an acute exposure to aerosolized OVA (0.3%) versus exposure to saline. The response was rapid and temporal, as the increases were elicited within 30 min postchallenge and then gradually returned to control levels or below by 4 h after challenge (Figure 6).

Expression of eotaxin was also examined by RT-PCR. As illustrated in Figure 7, the eotaxin mRNA level was increased in lungs of OVA-sensitized and -challenged animals. However, the increase was not as rapid as that for mucin genes, as eotaxin mRNA rose significantly above control level after 2 h postchallenge. In addition, unlike mucin genes, expression of the eotaxin gene became further enhanced up to 4 h after challenge.

TABLE 2

Amino acid sequence similarity among the guinea pig, rat, and human Muc5ac mucins

	Guinea pig Muc5ac	Human MUC5AC	Rat Muc5ac
Guinea pig Muc5ac	—	63%	78%
Human MUC2	49%	56%	56%
Rat Muc2	50%	55%	60%

Comparison was accomplished using the BestFit program in the GCG software package (version 10). Sequences used for comparison include the regions between two conserved octapeptide motifs in the Muc2 and Muc5ac mucins.

Eosinophil Infiltration in OVA-Sensitized Guinea Pig Airways

As illustrated in Figure 8A, OVA-challenged guinea pigs had increased inflammatory cell numbers in lavage (compared with saline-exposed animals) at 4 h postchallenge, which increased even more after 24 h. Similarly, eosinophilia, a hallmark of asthma, became apparent after 4 h postchallenge, as reflected by lavage eosinophil counts (Figure 8B).

Mucin Gene Expression in GPTE Cells in Response to Inflammatory Mediators

Histamine did not affect Muc2 mRNA level in GPTE cells after 1 or 4 h of exposure, as opposed to TNF-α, which caused a substantial increase in Muc2 mRNA (Figure 9). Muc5ac mRNA was not expressed at significant levels in these cultured cells, and neither TNF-α nor histamine upregulated its expression.

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             1                                                      50
guinea pig   GQCGTCTNDK KDECRMPGGA VATSCSDMSL HWRVDNPDEL FCRGPLPTP.
       rat   GQCGTCTNDK KDECRLPGGS IASSCSEMSL HWKV..PNQP SCQGPPPTP.
     human   GQCGTCTNDR KDECRTPRGT VVASCSEMSG LWNVSIPDQP ACHRPHPTPT

             51                                                    100
guinea pig   .......... .........T SLVPTPPPTE CPASPICELI LTDVFKPCHA
       rat   .......... .........T SMVPRSTPTP CSPSPLCQLI LSDVFKLCHD
     human   TVGPTTVGST TVGPTTVGST TVGPTTPPAP CLPSPICHLI LSKVFEPCHT

             101                                                   150
guinea pig   VIPPLPFHEG CVFDHCHNME LEVVCSGLEL YALLCAAQGV CVDWRSWTNN
       rat   IIPPLQFYEG CLFDYCHMLD LEVVCSGLEL YASLCAAQGV CIPWRSHTNN
     human   VIPPLLFYEG CVFDRCHMTD LDVVCSSLEL YARLCASHDI CIDWRGRT.R

             151                                                   200
guinea pig   SCSFPCPEDK VYQPCGPSDP SYCYQSDNAS .SLVLPKAYR ITEGCFCPEG
       rat   TCPFTCPENQ VYQPCGPSNP HYCYRNDDIS LSLAIQKAGP KSEGCFCPDD
     human   TCAHHLPSRQ GVPALRPSNP SYCYGNDSAS LG.ALREAGP ITEGCFCPEG
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Figure 4. Conservation of guinea pig, rat, and human Muc5ac mucins. Primary sequences between the two conserved octapeptide motifs of Muc5ac mucins from different species were aligned using the Pileup program in the GCG software package (version 10). Cysteine residues are highlighted. Gaps inserted to optimize the alignment are indicated by *dots*.

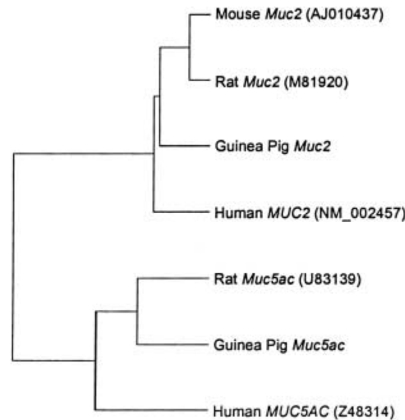

Figure 5. Phylogenetic tree of Muc2 and Muc5ac genes. The phylogenetic tree representing the evolutionary distances between mucin genes was created using the GrowTree program in the GCG software package (version 10). *Large brackets* indicate each orthologous gene cluster named by their human orthologs. Apparently, MUC2 and MUC5AC genes form two distinctive clusters. Guinea pig mucin genes are located close to their rodent homologs (mouse and rat) and relatively far away from their human homologs. The GenBank accession numbers for the sequences used in this comparison are shown in parentheses.

Discussion

The OVA-sensitized guinea pig has been utilized for years as an animal model of allergic asthma and airway hyperreactivity based on similarities in airway physiology and hypersensitivity upon challenges between the sensitized guinea pigs and human asthmatics. Studies demonstrating other aspects of the asthmatic response in this animal model, such as mucus cell hyperplasia and mucin overproduction, have been limited, due largely to a lack of appropriate molecular probes for guinea pig mucin genes and proteins. We have previously developed a series of monoclonal antibodies specific for guinea pig airway mucins (29) that were appropriate to measure mucin secretion *in vitro* (30). Here we report successful cloning of cDNA sequences coding for two airway mucins, Muc2 and Muc5ac, from the guinea pig. Our studies showed expression of both of these mucin genes was enhanced in the lungs of OVA-sensitized and -challenged guinea pigs. Steady-state mRNA level of eotaxin was also increased in these tissues, consistent with the observation reported in airways of humans with asthma (31).

Of particular interest in this study is the temporal sequence of upregulation of gene expression and influx of inflammatory cells in the airways in response to antigen challenge. The increase in steady-state mRNA levels of Muc2 and Muc5ac in the lungs of OVA-sensitized guinea pigs peaked at 30 min postchallenge, and then gradually decreased to control or lower levels afterwards. In contrast, mRNA for eotaxin started to increase between 1 and 2 h after challenge, and increased even more over time. Eotaxin is a potent chemoattractant for eosinophils in asthmatic airways. Accordingly, as illustrated in Figure 8, eosinophil influx into the airways was not detectable until 4 h after challenge. Thus, mucin gene upregulation appeared to occur before eotaxin production and inflammatory cell infiltration in the airways, suggesting that the enhanced mucin gene expression induced by acute antigen challenge was independent of eosinophil influx. Certainly, since a substantial amount of eosinophils and other inflammatory cells are already present in the airways of sensitized animals, possible involvement of these cells and their products (e.g., interleukin [IL]-4, IL-9, IL-13, TNF-α, proteases, etc.) in the mucin gene upregulation cannot be ruled out. In addition, the increase in mRNA levels of these mucins may be due to increased messenger stability,

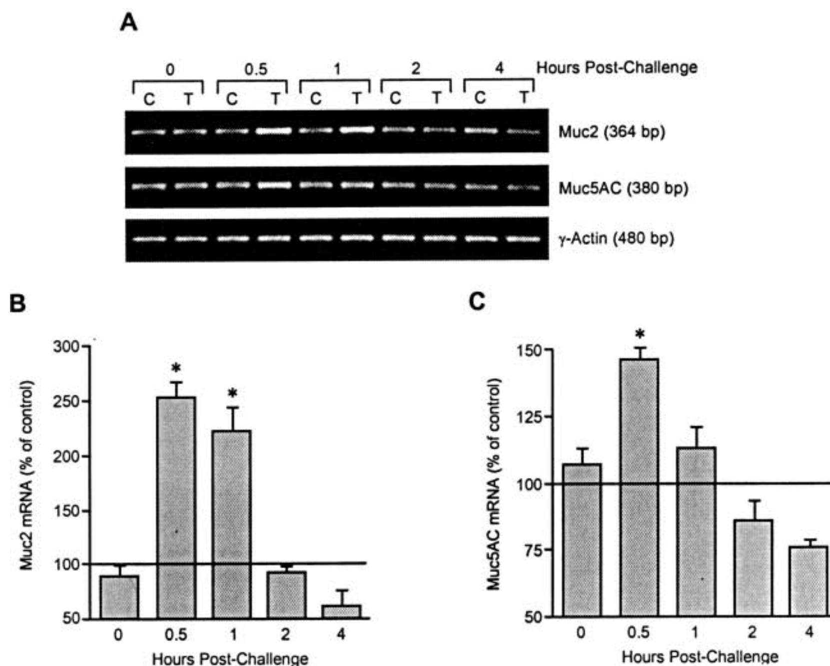

Figure 6. Upregulation of Muc2 and Muc5ac mRNA levels in lungs of OVA-sensitized and -challenged guinea pigs. OVA-sensitized animals were killed at selected times after challenge. Total RNA was isolated from lungs and Muc2 and Muc5ac mRNA levels were examined by RT-PCR as described in MATERIALS AND METHODS. (*A*) Analysis of RT-PCR products by 1.5% agarose gel electrophoresis. Data are representative of three separate experiments. C = Control (saline-challenged), T = Treatment (0.3% OVA-challenged). (*B* and *C*) Relative levels of Muc2 and Muc5ac mRNA in response to OVA challenge. The level of Muc mRNA is normalized to its own γ-actin internal control, and then presented as means $\pm$ SEM of the percentage value of its corresponding experimental (saline-challenged) control. *Significantly different from the saline control ($P < 0.05$).

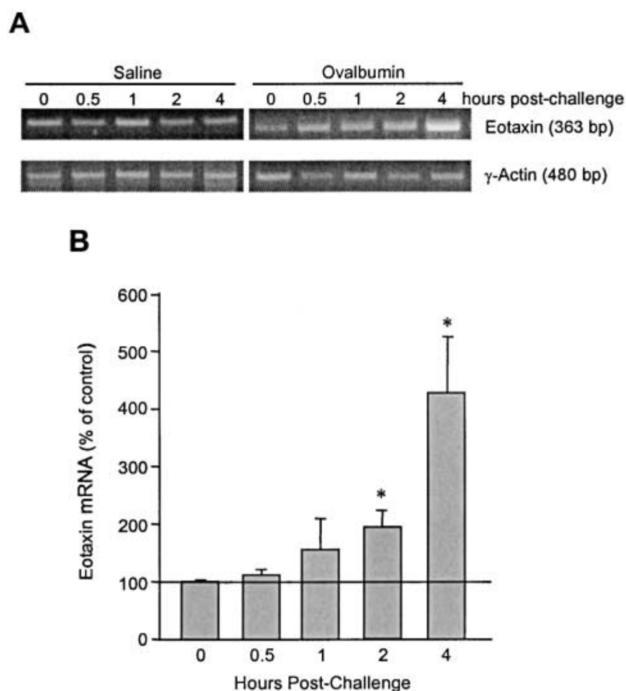

Figure 7. Upregulation of eotaxin mRNA levels in lungs of OVA-sensitized and -challenged guinea pigs. OVA-sensitized animals were killed at selected times after challenge. Total RNA was isolated from lungs and eotaxin mRNA levels were examined by RT-PCR as described in MATERIALS AND METHODS. (*A*) Analysis of RT-PCR products by 2% agarose gel electrophoresis. Data are shown from one representative experiment. (*B*) Relative levels of eotaxin mRNA in response to OVA challenge. The level of eotaxin mRNA is normalized to its own γ-actin internal control, and then presented as percentage value of the unchallenged (0 h) control. Data are means ± SEM of at least four separate experiments. *Significantly different from the unchallenged control ($P < 0.05$).

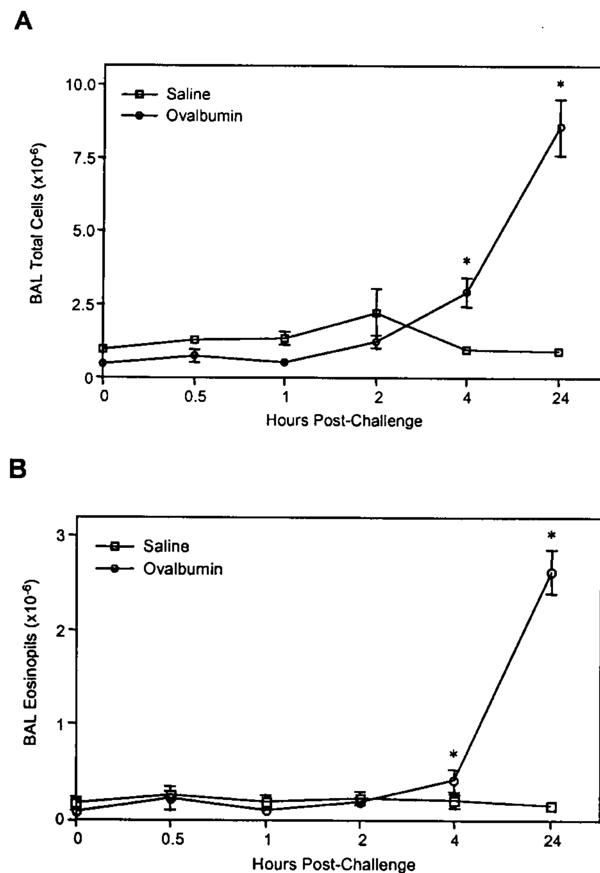

Figure 8. Infiltration of inflammatory cells in the allergic guinea pig airways in response to OVA challenge. Sensitized guinea pigs underwent bronchoalveolar lavage (BAL) at selected times after challenge. Cells collected from the BAL fluid were examined and counted following cytospin and differential staining. (*A*) Total inflammatory cells in BAL fluid after challenge. (*B*) Number of eosinophils in BAL fluid after challenge. $n = 6/7$ per group, values are means ± SEM. *Significantly different from the corresponding saline control ($P < 0.05$).

as has been reported for Muc5ac mRNA in human airway epithelial cells in response to elastase (32). This response is mediated by a mechanism involving intracellular oxidant species (33). Thus, one possible stimulus for enhanced Muc2 and/or Muc5ac expression in these allergic guinea pigs could be various oxidant species released by inflammatory cells in the airways of sensitized animals.

OVA-sensitized and -challenged mice have also been utilized as an *in vivo* model of allergic asthma despite the significant differences between murine and human airways. A recent study reported by Zuhdi Alimam and colleagues demonstrated expression of Muc5ac, but no detectable Muc2, in lungs of OVA-sensitized mice at 24 h after challenges, while neither Muc5ac nor Muc2 were detected in nonallergic (saline-treated) mice (34). It should be noted that the protocols used in that study were not entirely comparable to ours. The present study was designed to investigate mucin gene expression in sensitized guinea pigs in response to acute antigen challenge, with use of antigen-sensitized animals challenged by saline as controls, whereas Zuhdi Alimam and coworkers examined mucin gene expression in allergic mice versus nonallergic mice. Nevertheless, the results show some differences, particularly in Muc2 expression. Although Muc2 is apparently not a major mucin gene expressed in murine airways, it has been reported to be present in airways of human and rat (4, 8, 12), especially when those airways were infected or insulted. The simplest explanation for such divergence could be related to differential expression of mucin genes in different species.

Because histamine is associated with the early phase asthmatic response (21), we then examined its potential regulatory role in mucin gene expression. Our previous studies have shown that histamine can induce mucin hypersecretion from GPTE cells *in vitro* at a concentration of 100 μM and 30-min time point. Here, results indicated that the same concentration of histamine did not affect Muc2 mRNA levels in GPTE cells after 1 or 4 h of exposure. Although these results are based on *in vitro* data with a single dose at two time points, they suggest that histamine (at 100 μM) triggers airway epithelial cells to release preformed and stored mucin granules, as opposed to enhance mucin gene expression.

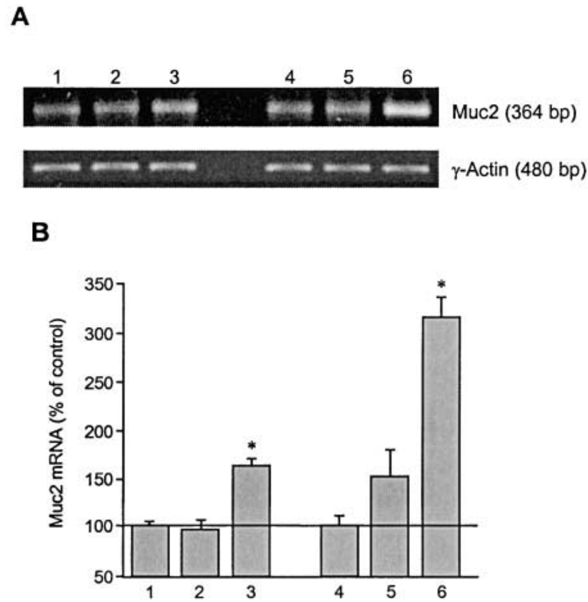

Figure 9. Effects of histamine and TNF-α on Muc2 mRNA levels in GPTE cells *in vitro*. GPTE cells were incubated with medium alone (*lanes 1* and *4*), 100 μM histamine (*lanes 2* and *5*), or 15 ng/ml TNF-α (*lanes 3* and *6*) for either 1 (*lanes 1–3*) or 4 h (*lanes 4–6*), then Muc2 mRNA levels were examined by RT-PCR as described in MATERIALS AND METHODS. (*A*) Analysis of PCR products by 1.5% agarose gel electrophoresis. Data are representative of three separate experiments. (*B*) Relative levels of Muc2 mRNA in response to histamine or TNF-α treatment. The level of Muc2 mRNA is normalized to its own γ-actin internal control, and then presented as percentage value of its corresponding medium control. Values are means ± SEM. *Significantly different from the medium control ($P < 0.05$).

Air–liquid interface culture of guinea pig tracheal epithelial cells has been used as an *in vitro* model for studying inflammatory responses of airway epithelium. Interestingly, results of this study showed Muc5ac mRNA was barely detectable in these cultures, even after exposure to TNF-α, which significantly elevated Muc2 mRNA levels (Figure 9). The reason for this low-level expression of Muc5ac in these cell cultures is not clear. Muc5ac gene expression in the guinea pig airways might be restricted to nonmucosal tissues *in vivo*, such as submucosal glands. Alternatively, some factors required for initiation of Muc5ac gene expression may be lacking in the cell culture system.

In summary, we have cloned two cDNA fragments coding for the guinea pig Muc2 and Muc5ac. Based on the sequence information, we have also examined steady-state mRNA levels of Muc2 and Muc5ac in the guinea pig allergic asthma model via RT-PCR. Enhanced expression of both Muc2 and Muc5ac were observed in the lungs of OVA-sensitized guinea pigs in response to acute OVA challenges. Upregulation of Muc2 and Muc5ac expression in these animals may relate to the goblet cell hyperplasia and mucin hypersecretion that characterize asthmatic airways. Enhanced mucin gene expression occurs rapidly after antigen challenge, and appears to be independent of additional eosinophil infiltration and subsequent airway inflammation.

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