

## MODELING THE AIRWAY EPITHELIUM IN ALLERGIC ASTHMA: INTERLEUKIN-13-INDUCED EFFECTS IN DIFFERENTIATED MURINE TRACHEAL EPITHELIAL CELLS

SUSAN M. LANKFORD, MARIANGELA MACCHIONE, ANNE L. CREWS, SHAUN A. MCKANE, NANCY J. AKLEY, AND LINDA D. MARTIN<sup>1</sup>

*College of Veterinary Medicine, North Carolina State University, Raleigh, North Carolina 27606*

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### SUMMARY

Mucous cells of the airway epithelium play a crucial role in the pathogenesis of human inflammatory airway diseases. Therefore, it is of importance to complement *in vivo* studies that use murine models of allergic asthma with *in vitro* mechanistic studies that use murine airway epithelial cells, including mucus-containing cells. In this study, we report the development and characterization of an *in vitro* culture system for primary murine tracheal epithelial (MTE) cells comprising ciliated cells and a substantial number of mucous cells. The increase in mucous cell number over that observed in the native murine airway, or in previously described murine cultures, creates a culture intermediate between the *in vivo* murine airway epithelium and *in vitro* cultures of human airway epithelial cells. To establish the usefulness of this culture system for the study of epithelial effects during inflammatory airway diseases, the cells were exposed to interleukin (IL)-13, a central inflammatory mediator in allergic asthma. The IL-13 induced two characteristic epithelial effects, proliferation and modulation of MUC5AC gene expression. There was a concentration dependence of these events, wherein high concentrations of IL-13 (10 ng/ml) induced proliferation, whereas lower concentrations (1 ng/ml) increased MUC5AC mRNA (where mRNA is messenger RNA). Interestingly, these effects occurred in an inverse manner, with the high concentration of IL-13 also provoking a significant decrease in MUC5AC gene expression. Thus, MTE cells cultured in this manner may provide an important link between experimental findings from animal models of allergic asthma and their application to human disease.

**Key words:** primary cell culture; mucous cell; mucus; proliferation; MUC5AC.

### INTRODUCTION

Allergic asthma affects 15 million people in the United States, with numbers continuing to rise (National Institutes of Health, 1999). Hallmarks of this disease include airway inflammation, hyperreactivity, and mucus overproduction (Arm et al., 1992; Bousquet et al., 2000), pathological elements shown to be inducible by the inflammatory cytokine interleukin (IL)-13 (Grunig et al., 1998; Wills-Karp et al., 1998; Zhu et al., 1999). The IL-13 is both necessary and sufficient for expression of an allergic asthmatic phenotype (Wills-Karp et al., 1998) and has been shown to have direct effects on airway epithelial cells in an *in vivo* mouse model (Kuperman et al., 2002). In addition, the concentration of IL-13 protein in bronchoalveolar lavage fluid has been shown to be significantly higher after segmental allergen challenge in asthmatic patients than in control subjects (Huang et al., 1995; Kroegel et al., 1996; Batra et al., 2004). To study allergic asthma, various whole-animal models (i.e., guinea pig [Pare et al., 1979], sheep [Abraham, 2000], mouse [Sarpong et al., 2003]) have been developed. In the mouse, asthma-like activity can be induced by allergens such as house dust mite (Sarpong et al., 2003) or ovalbumin (Hessel et al., 1995) or by the central asthmatic mediator, IL-13 (Grunig et al., 1998; Wills-Karp

et al., 1998; Zhu et al., 1999). With the availability of murine-specific reagents and the ability to create gene-altered individuals such as transgenic and knockout mice, the mouse has become a popular *in vivo* model of allergic asthma.

Much has been elucidated from work involving *in vivo* murine models of allergic asthma; however, studies of molecular mechanisms and signaling pathways are difficult to perform using whole animals. In addition, *in vivo* human studies to verify experimentation performed in mice are seldom feasible because of ethical and experimental considerations. Therefore, *in vitro* cell culture models that mimic the *in vivo* morphologic and functional characteristics of the airway epithelium have been established using airway epithelial cells from humans (Gray et al., 1996; Krunkosky et al., 2000) as well as other mammals including rat (Kaartinen et al., 1993), dog (Kondo et al., 1991), guinea pig (Adler et al., 1990), rabbit (Liedtke, 1988), hamster (Lee et al., 1984), horse (Sime et al., 1997), cow (Schumann et al., 1988), and manatee (Sweat et al., 2003). A distinct advantage of such *in vitro* systems is the ability to study the contribution of epithelial cells alone, excluding the influence of inflammatory or other cell types.

With the prevalent use of *in vivo* murine models of allergic asthma, it would seem that murine airway epithelial cells would also be well suited for *in vitro* studies. However, well-differentiated cultures of primary murine airway epithelial cells have been surprisingly difficult to establish and those that have been reported contain

<sup>1</sup> To whom correspondence should be addressed at E-mail: linda.martin@ncsu.edu

very few mucous cells (Robinson and Wu, 1991; Clarke et al., 1992; Kumar et al., 1997; Davidson et al., 2000; You et al., 2002). Although a paucity of mucous cells mimics their overall representation in the mouse airway in vivo, it poorly models the abundant mucous cell distribution found in the human airway. A murine airway epithelial cell culture with a mucous cell phenotype similar to that observed in the human airway epithelium could provide a needed bridge between in vivo murine and in vitro human epithelial cell models. Such a model would aid studies of the pathology of allergic asthma, including the development of mucous cell hyperplasia.

In this study, we report development of a differentiated murine tracheal epithelial (MTE) cell culture system that exhibits substantial numbers of ciliated and mucous cells. To establish the utility of these cultures as a model for the study of epithelial effects in allergic asthma, we investigated the effect of IL-13 on MTE cell proliferation and MUC5AC gene expression (a marker of mucous cell differentiation). We found that unique concentrations of this cytokine induce either proliferation or MUC5AC gene expression in these cells.

## MATERIALS AND METHODS

**Primary culture of mouse tracheal epithelial cells.** Mice [Cr:NIH(S), unless otherwise noted], 12–16 wks of age, were sacrificed by cervical dislocation. The tracheas were removed and immediately submerged in ice-cold Ham's F12 medium (Mediatech, Herndon, VA). Adherent muscle, vasculature, nerves, and connective tissue were removed and the tracheas were opened longitudinally, transferred to Ham's F12 media containing 0.1% protease (type XIV; Sigma Chemical Co., St. Louis, MO), and incubated at 37° C for 45 min. The protease activity was quenched with 3 ml of heat-inactivated fetal bovine serum (FBS; Hyclone Labs, Logan, UT). The tracheal epithelium was carefully brushed from the mucosal surface using the flat edge of a gel-loading pipette tip, and the cells were collected by centrifugation (100 × *g*, 4° C, 5 min). The cells were resuspended in 5 ml Versene (ethylenediamine-tetraacetic acid [EDTA] diluted 1:5000 in phosphate-buffered saline [PBS]; Invitrogen, Carlsbad, CA) for 15 min at 37° C followed by centrifugation and the addition of 5 ml of Complete Mouse Medium (Dulbecco's Modified Eagle's Medium: Ham's F12 as a 50:50 mixture [Mediatech]) containing amphotericin-B (250 ng/ml; Sigma), nystatin (44 IU/ml; Amresco, Solon, OH), gentamicin (100 µg/ml; Mediatech), bovine pituitary extract (104 µg/ml; Cambrex, Walkersville, MD), insulin (5 µg/ml; Cambrex), transferrin (5 µg/ml; Sigma), murine epidermal growth factor (EGF; 5 ng/ml; Sigma), dexamethasone (1 × 10<sup>-7</sup> M; Sigma), cholera toxin (20 ng/ml; List Biological Labs., Campbell, CA), and retinol (1 × 10<sup>-8</sup> M; Sigma) to which 10% FBS was added. The cells were again isolated by centrifugation (100 × *g*, 4° C, 5 min) and resuspended in 6 ml Complete Mouse Medium containing 10% FBS.

Transwell® clear polyester tissue culture inserts (12 mm, 0.4 µm pore size; Costar, Cambridge, MA) were coated with 200 µl of 300 µg/ml type I rat tail collagen (BD Biosciences, Bedford, MA) in 0.02 *N* acetic acid and allowed to equilibrate for 1 h at room temperature. The membranes were then washed with PBS and conditioned by adding medium to the basolateral compartment for at least 1 h at 37° C. The conditioning medium was aspirated from the basolateral compartment and replaced with Complete Mouse Medium containing 10% FBS. Cells were seeded onto the apical surface of the membranes in 500 µl of Complete Mouse Medium containing 10% FBS at a density of approximately 8 × 10<sup>4</sup> cells/cm<sup>2</sup> membrane (the number of cells routinely obtained from a 5-mm longitudinal length of trachea). Cells were allowed to grow in 5% CO<sub>2</sub> at 37° C, without changing the media, for 72 h. After this initial postseeding phase, the culture medium was changed every other day. When cells reached 80% confluence (approximately 5 to 7 d in culture), an air-liquid interface (ALI) was established by removing the apical medium and restricting feeding to the basolateral compartment. After establishment of the ALI, cultures were grown in serum-free, Complete Mouse Media refreshed every 24 h.

**Measurement of transepithelial resistance.** Apical and basolateral compartments of cultures to be tested were filled with serum-free, Complete Mouse Media and were allowed to equilibrate for 30 min in 5% CO<sub>2</sub> at 37° C.

Transepithelial resistance (*R*<sub>i</sub>) of the cultures was measured using an EVOM® Epithelial Voltammeter and an STX2 "chopstick" electrode (World Precision Instruments Inc., Sarasota, FL) according to the manufacturer's recommendations.

**Exposure to IL-13.** Cells were treated for 24 h with media ± 0.1 ng/ml, 1 or 10 ng/ml recombinant murine IL-13 (R&D Systems, Minneapolis, MN) in both apical and basolateral compartments.

**Thymidine incorporation assay.** Cells at ALI were grown in EGF-free media for 24 h. Subsequently, the media were replaced with EGF-free media containing <sup>3</sup>H-thymidine (1 µCi/ml) (Amersham Biosciences, Piscataway, NJ) with or without recombinant murine IL-13 in both apical and basolateral compartments. After 24 h exposure, the media were removed, and the cells were washed with ice-cold PBS, incubated twice with 0.5% trichloroacetic acid for 10 min on ice, and then washed with ice-cold water. The Transwell® membranes and attached cells were placed in scintillation fluid (ScintiSafe; Fisher Scientific, Pittsburgh, PA), and the incorporated <sup>3</sup>H-thymidine was measured on a LKB 1219 Rackbeta liquid scintillation counter.

**Histology.** Whole mount: cells at day 8 post-ALI establishment were washed with PBS, fixed with 10% normal buffered formalin (NBF), and stained with alcian blue–periodic acid–Schiff (PAS) to observe mucin in secretory cells. The entire Transwell® membrane was then mounted onto a slide. Cross sections: Transwell® membranes with adherent day 14 post-ALI MTE cells were fixed with 10% NBF, embedded in paraffin, and cross-sectioned. Cross sections (5 µm) were mounted and stained with either alcian blue–PAS or toluidine blue.

**Scanning electron microscopy.** Day 14 cells attached to Transwell® membranes were submerged in McDowell's and Trump's 4F:1G fixative (McDowell and Trump, 1976) for at least 1 h at 4° C. After two rinses in 0.1 *M* sodium phosphate buffer (pH 7.2), samples were dehydrated in a graded ethanol series to 100% ethanol at which time they were critical point dried with liquid CO<sub>2</sub>. Finally, they were mounted on specimen stubs with colloidal silver, sputter coated with gold–palladium, and examined with a JEOL JSM-6360LV scanning electron microscope.

**Western analysis.** Cytokeratin M5 and vimentin proteins were detected by Western blot analysis. Cells were scraped from the Transwell® membranes, mixed with lysis buffer containing protease inhibitors (50 mM Tris–HCl, pH 7.5, 10 mM NaCl, 1 mM EDTA, 1 mM benzamide, 10 µg/ml pepstatin A, 10 µg/ml leupeptin [all from Sigma], and 1% NP-40 [EMD Biosciences, La Jolla, CA]), and the supernatant was clarified by centrifugation. The concentration of total protein in the cell lysate was determined by Bradford (Bio-Rad, Hercules, CA) analysis, as per the manufacturer's instructions. Total protein from MTE cell lysate (50 µg) and control proteins (5 µg) were mixed with sample buffer (1% sodium dodecyl sulfate, 10% 2-mercaptoethanol, 10% glycerol, 0.001% bromophenol blue, and 0.125 *M* Tris–HCl, pH 6.8 [all from Sigma]) and incubated at 100° C for 5 min. Samples and protein controls were loaded onto a 4–15% polyacrylamide gradient gel (Bio-Rad) and separated by electrophoresis at 15 mA for 2 h. Proteins were transferred to 0.45-µm nitrocellulose membrane. Subsequently, the membrane was blocked for 1 h in Tris-buffered saline–Tween 20 (0.05 *M* Tris, 0.138 *M* NaCl, 0.0027 *M* KCl, pH 8, and 0.1% Tween 20) containing 5% nonfat dry milk. Membranes were incubated overnight at 4° C with the appropriate primary antibody in TBS-T containing 5% nonfat dry milk. Membranes were washed and incubated with the appropriate horseradish peroxidase (HRP)–conjugated secondary antibody, then developed using the ECL® chemiluminescent detection reagent (Pierce, Rockford, IL). For the cytokeratin blot, rabbit polyclonal antibody against murine keratin 5 primary antibody (1:2000; Covance, Richmond, CA) was used followed by goat anti-rabbit HRP-conjugated immunoglobulin G (IgG; 1:2000; Cell Signaling Technology, Beverly, MA) secondary antibody. Total protein lysate (5 µg) from A431 cells (human skin carcinoma; Upstate, Charlottesville, VA) was used as a positive control (molecular weight ~60 kDa). For the vimentin blot, goat polyclonal antibody against the carboxy terminus of vimentin (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA) was used as the primary antibody followed by donkey anti-goat HRP-conjugated IgG (1:2000; Santa Cruz) secondary antibody. Total protein from Human Intestinal Smooth Muscle (HISM) (jejunum smooth muscle; Santa Cruz) cell lysate (5 µg) was used as a positive control (molecular weight ~55 kDa). Primary antibodies used recognize both murine and human cytokeratin M5 or vimentin.

**The quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR).** The MTE cells were lysed by adding 250 µl TRI reagent (Sigma) to the Transwell® membrane followed by scraping. Total ribonucleic acid (RNA) was isolated according to the manufacturer's instructions. DNA-free® (Am-

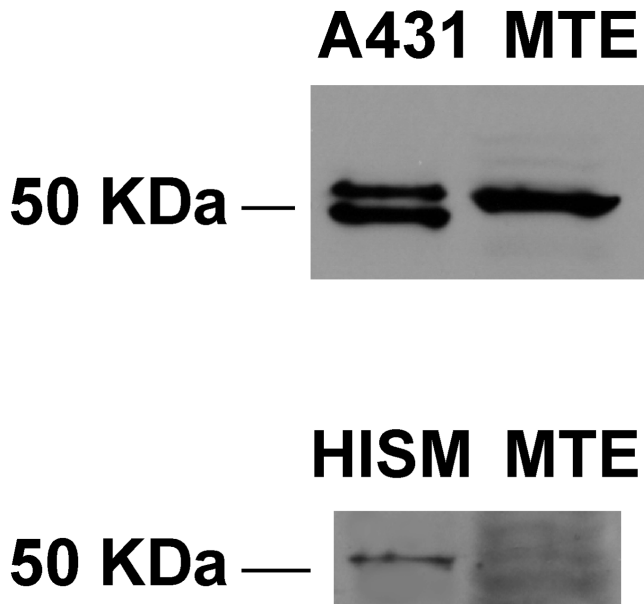


FIG. 1. Western analysis of total protein from MTE cell culture. Total protein isolated from day 8 post-ALI MTE cell lysate (50  $\mu$ g), A431 cell lysate (5  $\mu$ g, cytokeratin positive control), and HISM cell lysate (5  $\mu$ g, vimentin positive control) were separated by electrophoresis on a 4–15% polyacrylamide gel and transferred to nitrocellulose. The resulting blots were hybridized with (*top panel*) rabbit polyclonal antibody against murine keratin 5 and (*lower panel*) goat polyclonal antibody against vimentin. Western blot is representative of three separate experiments. MTE, murine tracheal epithelial; ALI, air–liquid interface; HISM, human intestinal smooth muscle.

bion Inc., Austin, TX) was used to remove any contaminating deoxyribonucleic acid (DNA) from the samples. The RNA was quantitated, and 1  $\mu$ g of total RNA was reverse transcribed using random primers and Superscript II (Invitrogen) according to the manufacturer's instructions.

Real-time polymerase chain reaction (PCR) was performed on a Bio-Rad iCycler iQ<sup>®</sup> PCR Detection System using iQ Supermix (Bio-Rad), gene-specific primers and dual-labeled Taqman probes. The equivalent of 100 ng RNA was used for the MUC5AC PCR reaction; the equivalent of 25 ng RNA was used for the 18S ribosomal RNA (rRNA) PCR reaction. All primers were designed to span an intron–exon junction, and the identities of the resulting PCR products were confirmed by sequencing. The murine MUC5AC primers (sense primer: 5'-ACGACACTTTTCAGTACCAATGAC-3', antisense primer: 5'-GCTTCCTTACAGATGCAGTCCT-3') amplify a product corresponding to position 1504–1634 of the murine MUC5AC cDNA (where cDNA is complementary DNA) sequence (GenBank accession number AJ010792). The MUC5AC probe corresponds to position 1534–1557 of the cDNA sequence and is dual labeled with 5-(and-6)-carboxyfluorescein (5[6]-FAM) and Black Hole Quencher: 5'/FAM/TGCGTGCCTTCCTGCCAATGGTGT/BHQ/-3'. The 18S rRNA primers and probe were obtained from Applied Biosystems (Foster City, CA). Samples were quantitated by the relative standard curve method using standard curves made from serial dilutions of mouse lung cDNA.

## RESULTS

**Morphological characterization of MTE cell culture.** To verify the epithelial nature of the MTE cell culture system, post-ALI cultures were assessed for cytokeratin expression by Western analysis (Fig. 1). Cytokeratins belong to a family of cytoskeletal protein filaments characteristic of epithelial cells (Moll et al., 1982), wherein a given epithelium expresses a specific set of cytokeratins. Cytokeratin 5 (58 kDa), expressed at high levels in tracheal epithelium (Reis-Filho et al., 2003), was detected in protein from day 8 post-ALI MTE cell cultures as well as in protein from positive control cells

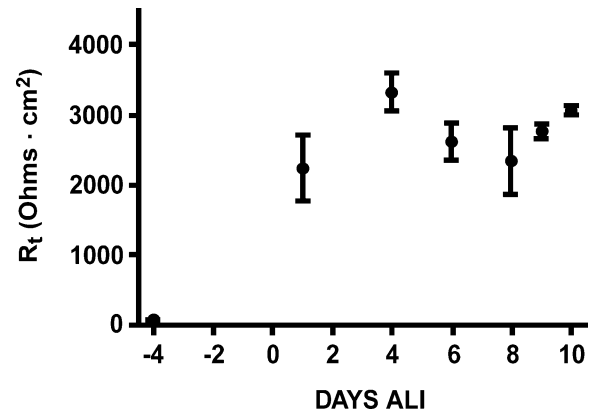


FIG. 2. Transepithelial resistance ( $R_t$ ) in murine tracheal epithelial cell cultures. Cells were grown on Transwell<sup>®</sup> tissue culture membranes submerged in Complete Mouse Medium containing 10% FBS. When cultures were 80% confluent, an air–liquid interface was established by restricting serum-free, Complete Mouse Medium to the basolateral compartment. Before making  $R_t$  measurements, cultures were submerged in serum-free, Complete Mouse Medium and equilibrated for 30 min at 37° C. The  $R_t$  measurements from three separate preparations were obtained using an EVOM<sup>®</sup> Epithelial Voltammeter and an STX2 “chopstick” electrode. Data reported as Mean  $\pm$  SD ( $n = 5$ –12 cultures/timepoint).

(A431; epidermoid carcinoma). A second, smaller protein band was also detected in the A431 cell lysate. This band is likely the result of nonspecific binding of the polyclonal antibody raised against murine keratin 5 to one of the other A431 cytokeratin proteins. Vimentin, an intermediate filament protein that is expressed at high levels in fibroblasts and other cells of mesenchymal origin (Isaacs et al., 1989), was not detected in the cultures, although it was readily detected in the positive control HISM protein. Thus, the expression of cytokeratin 5 and the lack of vimentin expression in the MTE cells indicate these cultures exhibit epithelial characteristics with little to no fibroblast contamination.

To further ascertain whether the MTE cultures exhibit properties of an intact epithelial layer,  $R_t$  was measured (Fig. 2). No significant  $R_t$  was measurable 4 d before establishment of the ALI, but by day 4 post-ALI,  $R_t$  increased to a high of  $3318 \pm 276.8 \Omega \cdot \text{cm}^2$ , with the  $R_t$  stabilizing around  $2342 \pm 475.8 \Omega \cdot \text{cm}^2$  by day 8 post-ALI. These  $R_t$  measurements are similar to those reported for other mouse (You et al., 2002) and human (Atherton et al., 2003) airway epithelial cell cultures. These results further confirm the epithelial nature of our newly established culture system.

Histological analysis was performed to evaluate the morphology of the MTE cell cultures. Samples from post-ALI MTE cell cultures were examined for general structure and the presence of mucus-containing cells using toluidine blue (Fig. 3A) and alcian blue–PAS (Figs. 3B and 4) stains. A culture cross section stained with toluidine blue highlights the presence of ciliated (*black arrow*) and mucus-producing (*red arrow*) cells (Fig. 3A). In an alcian blue–PAS–stained cross section (Fig. 3B), many PAS+ cells (stained pink) are present, indicating that cells in the culture contain stainable polysaccharides, possibly mucins. Some cells also exhibit dual alcian blue–PAS staining (*red arrow*), a pattern indicative of mucopolysaccharide-containing, or mucous, cells. An alcian blue–positive layer is also observed above the epithelial cell layer suggesting that mucin protein is being secreted from the MTE cells.

Alcian blue–PAS–stained whole mounts of post-ALI cultures (Fig.

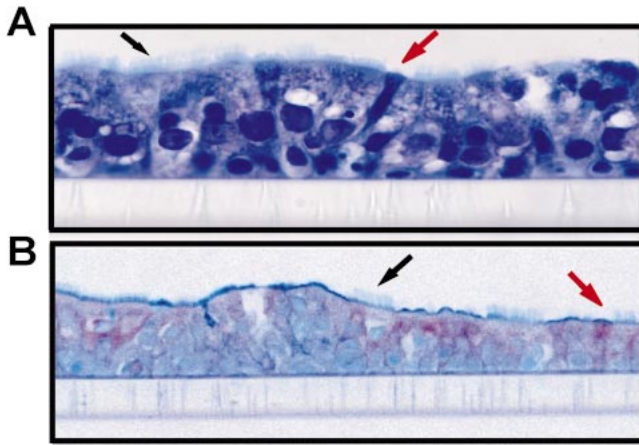


FIG. 3. Cross section of murine tracheal epithelial cell cultures. Day 14 post-ALI cells grown on Transwell® membranes were fixed with normal buffered formalin and embedded in paraffin. Cross sections (5  $\mu$ m) were mounted and stained with toluidine blue (A) or alcian blue-PAS (B). Ciliated cells (black arrows) and mucus-containing cells (red arrows) are visible in these cultures. Secreted mucus (stained blue) is observed on the cell surface (B). Magnification  $\times 400$ . ALI, air-liquid interface; PAS, periodic acid-Schiff.

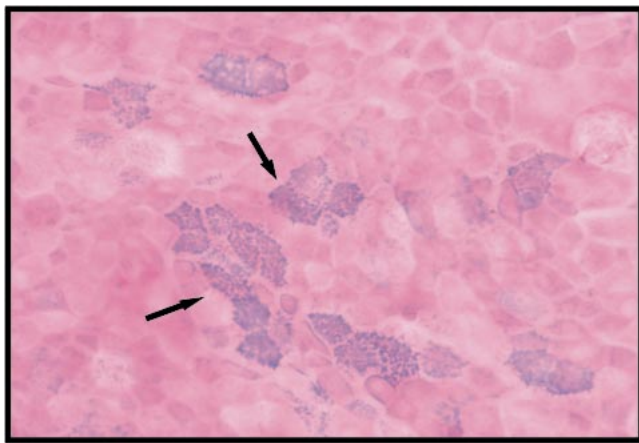


FIG. 4. Whole mount of MTE cell culture. Day 8 post-ALI MTE cells were washed with phosphate-buffered saline and fixed with 10% normal buffered formalin. The entire Transwell® membrane, with attached cells, was mounted onto a slide and stained with alcian blue-PAS. Note the hallmark cobblestone appearance of the epithelium with alcian blue-PAS-stained, (purple) mucus-containing cells. Arrows indicate putative mucus-containing cells. Magnification  $\times 400$ . MTE, murine tracheal epithelial; PAS, periodic acid-Schiff.

4) have a cobblestone appearance typical of epithelial cells. Clusters of alcian blue-PAS+ cells (arrows), indicative of mucus-containing cells, are interspersed throughout the cultures. The staining within these cells is punctate in nature, a pattern expected for cells containing mucin granules. Thus, on the basis of histological examination, the MTE cell cultures appear to retain the basic morphological characteristics of in vivo airway epithelium (You et al., 2002) including the presence of ciliated and mucus-producing cells.

To further confirm the cellular identity of the MTE cell cultures, electron microscopy was used. Scanning electron microscopy of fully differentiated, day 14 post-ALI cultures revealed clusters of ciliated cells surrounded by nonciliated cells (Fig. 5), a pattern similar

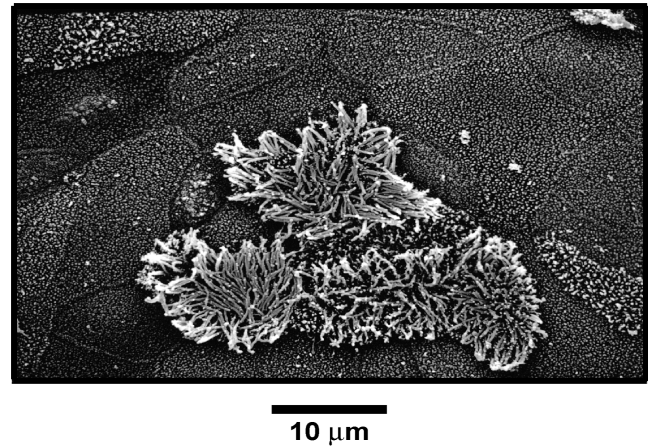


FIG. 5. Scanning electron micrograph of MTE cell culture. Day 14 post-ALI MTE cells attached to Transwell® membranes were prepared for scanning electron microscopy as described in Materials and Methods. A group of ciliated cells, surrounded by nonciliated epithelial cells, is observed at the center of the micrograph. MTE, murine tracheal epithelial; ALI, air-liquid interface.

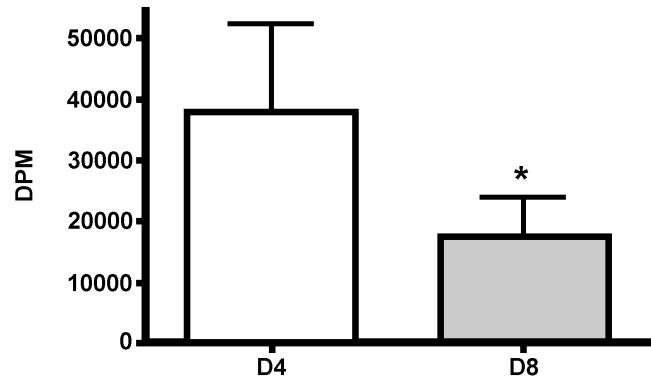


FIG. 6. Constitutive proliferation on different days in culture. The murine tracheal epithelial cells at day 3 and day 7 post-ALI were grown in epidermal growth factor-free media containing  $^3\text{H}$ -thymidine (1  $\mu\text{Ci/ml}$ ) in both apical and basolateral compartments for 24 h. On day 4 or day 8, Transwell® membranes and attached cells were placed in scintillation fluid, and the incorporated  $^3\text{H}$ -thymidine was measured. \* $P = 0.012$ , with SD shown,  $n = 5-6$ . ALI, air-liquid interface.

to that observed in the mouse trachea in vivo (Fig. 3B in You et al., 2002). Although the MTE cultures contain approximately 20% ciliated cells, this is less than the 36–39% ciliated cells reported to be in the native mouse trachea (Pack et al., 1980).

**Growth characteristics of MTE cell cultures.** To assess the constitutive proliferative capacity of the MTE cells in culture,  $^3\text{H}$ -thymidine incorporation was used as a measure of cellular proliferation on different days in culture. The proliferative capacity of MTE cells was found to vary over time in culture, with more constitutive proliferation observed in day 4 as compared with day 8 post-ALI culture (Fig. 6). A similar proliferation profile was observed in mouse tracheal epithelial cultures, as described by You et al., with BrdU incorporation decreasing over time in culture as cell number increased (You et al., 2002).

To characterize the differentiation state of the MTE cell cultures, MUC5AC mRNA (where mRNA is messenger RNA), a message

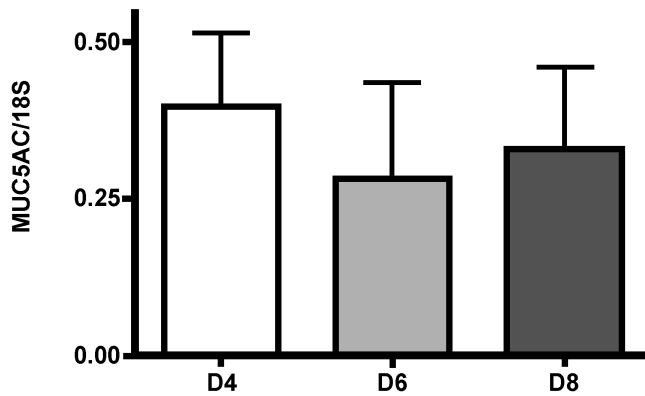


FIG. 7. Constitutive MUC5AC mRNA expression on different days in culture. Total RNA was isolated and reverse transcribed from day 4, day 6, and day 8 post-ALI MTE cells from CD1 mice. The resulting cDNA was subjected to real-time PCR analysis using primers and probes for MUC5AC mRNA and 18S rRNA. The MUC5AC and 18S levels were quantitated by the relative standard curve method. The 18S rRNA expression was not significantly different on different days. Data shown as MUC5AC normalized to 18S rRNA, including SD;  $n = 3-4$ . ALI, air-liquid interface; RNA, ribonucleic acid; mRNA, messenger RNA; rRNA, ribosomal RNA; PCR, polymerase chain reaction; MTE, murine tracheal epithelial; cDNA, complementary deoxyribonucleic acid.

expressed in mucous cells, was measured by real-time PCR. The MUC5AC mRNA was expressed in the cultures on all days tested, with no significant difference in MUC5AC gene expression observed on days 4, 6, or 8 post-ALI. Thus, in MTE cell culture, constitutive expression of the mucous cell marker, MUC5AC mRNA, is independent of the number of days in culture (Fig. 7).

*In vitro modeling of an asthmatic phenotype.* With the MTE cell culture system established, we wanted to determine whether it could be used to complement existing in vivo and in vitro models of allergic asthma. In vivo models of allergic asthma include the ovalbumin-sensitized and challenged mouse as well as the IL-13-instilled mouse (Wills-Karp et al., 1998). In these models, the percentage of mucus-producing cells, normally very low in the murine airway epithelium, is dramatically increased (Alimam et al., 2000) by a mechanism that appears to involve direct interaction of IL-13 with the epithelium (Kuperman et al., 2002). Similarly, in vitro models of normal human bronchial epithelial (NHBE) cells differentiated in ALI culture respond to exogenous IL-13 by a concentration-dependent increase in mucin5AC-expressing cells (Atherton et al., 2003) or by proliferating in response to IL-13 (Booth et al., 2001). We chose to examine the response of MTE cells to a central mediator of asthma, IL-13, by measuring cell proliferation and MUC5AC expression.

The MTE cell cultures (day 7 post-ALI) were exposed to different

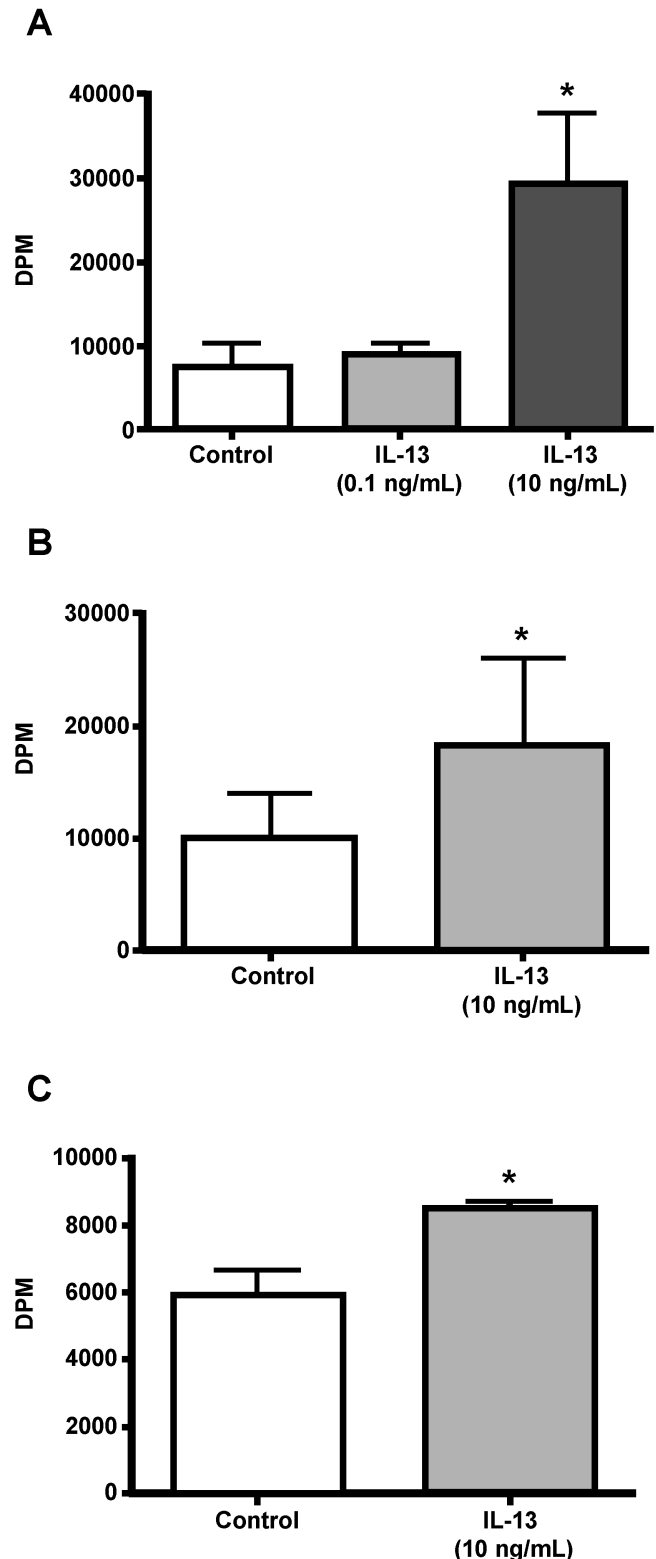


FIG. 8. Proliferation of MTE cells in response to IL-13. (A) Day 7 post-ALI MTE cells from an outbred mouse strain (Cr:NIH(S)) were grown in media containing  $^3\text{H}$ -thymidine ( $1 \mu\text{Ci/ml}$ )  $\pm 0.1$  or  $10 \text{ ng/ml}$  recombinant murine IL-13 in both apical and basolateral compartments. After 24 h (on day 8), the Transwell® membranes and attached cells were placed in scintillation fluid and the incorporated  $^3\text{H}$ -thymidine were measured.  $*P = 0.004$ , with SD shown,  $n = 4$ . Data shown are representative of three separate experiments. (B) Day 7 post-ALI MTE cells from an outbred mouse strain (Cr:NIH(S)) and (C) from an inbred mouse strain (C57BL/6) were grown in

media containing  $^3\text{H}$ -thymidine ( $1 \mu\text{Ci/ml}$ )  $\pm 10 \text{ ng/ml}$  recombinant murine IL-13 in both apical and basolateral compartments. After 24 h (on day 8), the Transwell® membranes and attached cells were placed in scintillation fluid and the incorporated  $^3\text{H}$ -thymidine was measured. (B)  $*P < 0.0001$ ,  $n = 31$ , with SD shown. Data pooled from seven individual experiments. (C)  $*P < 0.0001$ , with SD shown,  $n = 6$ . MTE, murine tracheal epithelial; ALI, air-liquid interface; IL, interleukin.

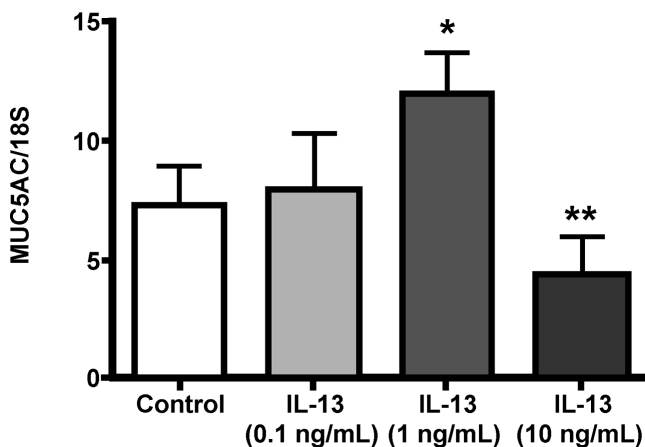


FIG. 9. The MUC5AC mRNA expression in response to IL-13. Day 7 post-ALI MTE cells from an outbred mouse strain (Cr:NIH(S)) were grown in media  $\pm$  0.1, 1, or 10 ng/ml recombinant murine IL-13 in both apical and basolateral compartments. After 24 h (on day 8), total RNA was isolated and reverse transcribed. The resulting cDNA was subjected to real-time PCR analysis using primers and probes for MUC5AC and 18S rRNA. The MUC5AC and 18S levels were quantitated by the relative standard curve method using standard curves made from serial dilutions of mouse lung cDNA. The 18S rRNA expression was not significantly different on different days. Data shown as MUC5AC mRNA normalized to 18S rRNA, including SD. The 1.0 and 10 ng/ml groups are significantly different from control, with  $*P = 0.029$  and  $**P = 0.072$ , respectively;  $n = 3-4$ . ALI, air-liquid interface; IL, interleukin; RNA, ribonucleic acid; mRNA, messenger RNA; rRNA, ribosomal RNA; cDNA, complementary deoxyribonucleic acid; PCR, polymerase chain reaction; MTE, murine tracheal epithelial.

concentrations of IL-13 for 24 h, and proliferation was assessed by a  $^3\text{H}$ -thymidine incorporation assay. No increase in proliferation was observed with exposure to 0.1 ng/ml IL-13; however, a significant proliferation was observed when cells were treated with 10 ng/ml IL-13 (Fig. 8A). This significant increase in proliferation was observed when day 7 post-ALI cells from an outbred mouse strain [Cr:NIH(S); Fig. 8B], or from an inbred mouse strain (C57BL/6; Fig. 8C), were exposed to 10 ng/ml IL-13 for 24 h. Proliferation of NHBE cells in vitro has also been shown to occur maximally in response to 10 ng/ml IL-13 in cultures of comparable age (Booth et al., 2001). These results indicate that MTE cells can proliferate in response to IL-13 in a manner that, although similar to NHBE cells, is independent of mouse strain.

The MTE cell cultures were also exposed to IL-13 to determine whether these cultures could be stimulated to differentiate, as measured by an increase in the mucous cell marker, MUC5AC mRNA. Varying doses of IL-13 were applied to the cultures for 24 h and the MUC5AC/18S levels assessed (Fig. 9). No significant change in this ratio was observed in cells treated with 0.1 ng/ml IL-13. In contrast, a significant increase in MUC5AC/18S was observed in day 8 post-ALI cells that had been exposed to 1 ng/ml IL-13, and a significant decrease in MUC5AC/18S was observed when these cells were exposed to 10 ng/ml IL-13. Although this response to IL-13 was more complex than anticipated, it shows clearly that MTE cell cultures can exhibit increased MUC5AC gene expression, as well as proliferation, in response to IL-13. These IL-13-induced responses are both concentration dependent, although each appears to require a different concentration profile. Having established that MTE cells respond to IL-13, this culture system can serve as an in vitro model of the airway epithelium in allergic asthma.

## DISCUSSION

In this study, we report the development of an in vitro culture system for MTE cells that maintains the differentiated characteristics of both ciliated and mucus-producing cells. Methods used to isolate the MTE cells result in serum-free, ALI cultures that are overwhelmingly epithelial in nature and the first such cultures to contain a substantial number of mucus-producing cells. Thus, these cultures would appear useful as an in vitro model to study epithelial cell changes, including mucous cell hyperplasia, that occur as a result of chronic airway inflammation. This use is supported by experiments that indicate IL-13, a central inflammatory mediator in allergic asthma, can induce both proliferation and the expression of MUC5AC mRNA in this in vitro model.

Under normal circumstances, murine conducting airways are lined with 40% ciliated and 60% nonciliated epithelial cells. Of the nonciliated cells, 95% are Clara cells, whereas less than 5% are mucous cells. However, the number of mucus-containing cells increases dramatically after allergen sensitization and aerosol challenge (Evans et al., 2004). In the untreated MTE cell cultures described in this study, about 15% of the cells are Alcian blue-PAS+, indicating a higher than expected number of mucus-containing cells. This increased number of mucus-containing cells is the main difference between these MTE cell cultures and differentiated cultures described previously (Davidson et al., 2000; You et al., 2002).

The culture method demonstrated in this study differs from other published methods in ways that may account for the increased number of mucous cells in the differentiated cultures. By using 12- to 16-wk-old mice, instead of 3- to 7-wk-old mice (Davidson et al., 2000; You et al., 2002), more tracheal cells can be obtained per mouse. You et al. (2002) digest the tracheae proteolytically using 1.4–1.5 ng/ml protease overnight at 4°C; our method involves protease (1.0 ng/ml) digestion for 45 min at 37°C. This decreased exposure to a lower concentration of protease may result in less cellular damage. Our technique of gently brushing the epithelial lining from the trachea, rather than passively dissociating the epithelium from the underlying tissue (Robinson and Wu, 1991; Davidson et al., 2000; You et al., 2002), may also lead to more available cells. We also use an EDTA step to further dissociate clumps of cells, which improves seeding efficiency. Before plating, 97% of the isolated cells are viable, as measured by a trypan blue-exclusion assay, with the majority present as single cells. Finally, our protocol lacks a culling step, where cells are allowed to adhere to a substrate as a means of separating smooth muscle cells and fibroblasts from epithelial cells. We find that the few fibroblasts co-isolated with the epithelial cells die once the ALI is established.

The cultures described in this study have a greater percentage of mucous cells than are observed either in the murine tracheal epithelium in vivo or in murine in vitro cultures described elsewhere (Davidson et al., 2000; You et al., 2002). However, the mucous cell number in our MTE cultures (estimated at 15–20%) is not vastly different from that described in airway epithelial cultures derived from nonmurine species. Approximately 25% mucous cells are noted in cultures derived from human bronchial epithelial cells (Krunkosky et al., 2000), and cultures of guinea pig tracheal epithelial cells contain about 35% secretory cells (Adler et al., 1990).

The complex ethics of in vivo human studies have necessitated development of animal models for the study of human disease. With the advent of technology to create specific genetic alterations in

mice, in vivo murine models have emerged as key tools in such research, including that targeting allergic asthma. Elucidation of complex signaling mechanisms that control intracellular disease-related events, however, still requires in vitro cell culture systems. Procurement of human tracheal tissue is very limited and expensive. Thus, the MTE cell culture system described in this study forms a bridge between in vivo murine models and human airway epithelial cell culture models, providing an intermediate phenotype with respect to mucous cell number. Because the method works for cells isolated from different strains of mice, it should be applicable to the development of cultures from a wide variety of genetically altered animals.

To further establish this MTE cell culture model as an appropriate intermediate for the study of allergic asthma, we investigated the response of these cells to IL-13. Previously, IL-13 had been shown to increase the number of mucus-containing cells in allergen-challenged mice (Grunig et al., 1998; Wills-Karp et al., 1998; Zhu et al., 1999). In human airway epithelial cell cultures, the effect of IL-13 on mucous cells appears to be concentration dependent. Atherton et al. (2003) reported that 1 ng/ml IL-13 caused a significant increase in goblet cell density in human bronchial epithelial cell cultures, whereas 10 ng/ml IL-13 caused a profound reduction in goblet cell density. Similar results were obtained in a separate study, wherein human bronchial epithelial cells were exposed to 10 ng/ml IL-13 with no change in *MUC5AC* expression observed (Chen et al., 2003). However, in this study, no lower IL-13 concentrations were investigated.

Mechanisms of IL-13-induced proliferation have also been characterized using the human cell culture system (Booth et al., 2001), and a recent study suggests such proliferation may also occur in the mouse in vivo (Evans et al., 2004). The study reported here confirms that IL-13 can similarly induce proliferation and modulation of *MUC5AC* gene expression in MTE cells in vitro. Interestingly, the observed concentration-dependent induction of these two IL-13-induced responses appears complex; proliferation is induced only at the highest concentration of IL-13 (10 ng/ml), whereas *MUC5AC* mRNA is decreased at this concentration when compared with control cells. In contrast, lower concentrations of IL-13 do not induce proliferation but do yield an increase in *MUC5AC* gene expression. Previous findings indicating that mucin5AC protein expression can be induced by IL-13 in a concentration-dependent manner in NHBE cells in vitro (Atherton et al., 2003) are thus extended by the current study, which indicates such a concentration-regulated event also occurs at the level of the mRNA. Our findings further suggest the possibility that IL-13-induced proliferation and *MUC5AC* gene expression are controlled by inversely related mechanisms.

This MTE cell culture method should prove useful for further exploration of the relationship between IL-13-induced epithelial cell proliferation and differentiation, because mechanistic studies can now be carried out in primary cells, with the ability to incorporate genetic manipulations afforded by knockout and transgenic animals. As such, this model can provide a unique bridge linking airway epithelial effects observed in murine models of allergic asthma to in vitro mechanistic studies carried out with human airway epithelial cells.

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