Secretion of lactoferrin and lysozyme by cultures of human airway epithelium

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Dubin, R. F., S. K. Robinson, and J. H. Widdicombe. Secretion of lactoferrin and lysozyme by cultures of human airway epithelium. Am J Physiol Lung Cell Mol Physiol 286: L750–L755, 2004; 10.1152/ajplung.00326.2003.—Lactoferrin and lysozyme are important antimicrobial compounds of airway surface liquid, derived predominantly from serous cells of submucosal glands but also from surface epithelium. Here we compared release of these compounds from the following human cell cultures: primary cultures of tracheal epithelium (HTE), Calu-3 cells (a lung adenocarcinoma cell line frequently used as a model of serous gland cells), 16HBE14o− cells (an SV40-transformed line from airway surface epithelium), T84 cells (a colon carcinoma cell line), and human foreskin fibroblasts (HFF). For lysozyme, baseline secretory rates were in the order Calu-3 carcinoma cell line), and human foreskin fibroblasts (HFF). Thus our data cast doubt on the utility of Calu-3 cells as a model of airway surface epithelium. However, the concentration of lysozyme in the forskolin-induced secretion from Calu-3 cells was much less than in airway gland secretions. Our data cast doubt on the utility of Calu-3 cells as a model of airway surface epithelium.

Lactoferrin and lysozyme are the two most abundant antibacterial proteins secreted into the airway lumen, being ~0.5 mg/ml in sputum (4, 20). In bronchial lavage they are typically ~10 μg/ml (32, 41). Given that ~1–4% of lavage is airway surface liquid (34, 44), their concentrations in the latter are 0.25–1 mg/ml. Because of the quantitative importance of lactoferrin and lysozyme as antimicrobials, we wanted to find cultures of human tracheal epithelium that released significant amounts of these compounds and then use these cultures for studies of mucin synthesis and release.

Earlier studies have localized both lactoferrin and lysozyme to the secretory granules of serous cells in the acini of human submucosal glands (2, 3, 13, 42). A human airway epithelial cell line, Calu-3, has been widely used as a model of serous gland cells (10, 26), mainly on the grounds that it has high levels of the cystic fibrosis transmembrane conductance regulator (CFTR) (38). In this study, we wanted to test further the suitability of Calu-3 cells as a serous cell model by comparing lactoferrin, lysozyme, and mucin release from Calu-3 cells and from primary cultures of human tracheal surface epithelium (HTE). We argued that the latter should release more mucins but less lactoferrin and lysozyme than the former. We also studied release from 16HBE14o− and T84 cells. The former is an SV40-transformed line from airway surface epithelium (7). The latter is a colon carcinoma cell line that resembles Calu-3 cells in having high levels of CFTR (12). We used human foreskin fibroblasts (HFF) as a negative control.

METHODS

For most studies, cells were grown on 12-mm opaque inserts (0.45 μm pore size; Corning, Acton, MA) coated with human placental collagen as described (5). HTE were obtained and cultured as previously (49). Cells were studied from 5 to 10 days after plating, at which time they had transepithelial resistance (Rt) >100 Ω/cm² and transepithelial potential difference (Vt) of >5 mV as determined with a “chopstick voltmeter” (Millicell ERS; Millipore, St. Louis, MO). The Calu-3 cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA) and grown as before (38). Cells were used at >10 days after plating, at which time they had Rt >50 Ω/cm² and Vt of >2 mV. T84 and 16HBE14o− cells were grown as previously (7, 12). HFF were obtained from ATCC and grown in T75 culture flasks according to ATCC recommendations. In the occasional instances when confluent epithelial cells in T75s were used, this is mentioned in the text.

We developed sandwich ELISAs to detect lysozyme and lactoferrin. Primary antibodies were applied in carbonate buffer (50 mM Na₂CO₃, pH 9.6) to Costar 96-well “high-binding” plates (Corning). Wash buffer consisted of 0.5 M NaCl, 3 mM KCl, 4 mM Na₂HPO₄, 4 mM KH₂PO₄, and 1% Triton X-100, pH 7.2. Blocking buffer was wash buffer plus 1% BSA. Samples, standards, and secondary and tertiary antibodies were all applied in blocking buffer. The following sequence was followed for both assays: incubation with primary antibody (100 μl; 1:1,000 for lactoferrin, 1:500 for lysozyme) overnight at 4°C, three washes, blocking buffer (200 μl, 1 h), application of standards and samples (100 μl, 24°C, 2 h), three washes, incubation with secondary antibody (100 μl, 1:500, 24°C, 4 h), three washes, tertiary antibody (100 μl; 1:500, 2 h, 24°C), three washes.

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Mucin ELISA was performed as previously described (27). Antibody 17B1 (200 μl; Babco, Berkeley, CA) at 1:500 dilution in 50 mM carbonate buffer (pH 9.6) was applied to Costar high-binding 96-well plates for 2 h at 37°C. Plates were washed five to six times with wash buffer (PBS + 0.05% Tween 20), following which samples and standards (purified human airway mucin from Dr. Reen Wu, University of California-Davis) were applied in wash buffer plus 0.05% 2-mercaptoethanol and incubated for 1–2 h at 37°C. After five to six washes, alkaline phosphatase-conjugated secondary antibody (17Q2, Babco; 1:500 dilution in wash buffer) was applied overnight at 4°C. Next, plates were washed again, and color reagent (NPP) added. Color was read at 405 nm in a Biotek El311 microplate reader.

To measure release across the apical membrane of cells on inserts, we added from 200 to 600 μl of culture medium to the cells’ mucosal surface and removed it to graduated 0.6-ml Eppendorf tubes after a set interval. From the level in the tube, final volume was estimated to the nearest 25 μl and used in calculating outputs of lactoferrin, lysozyme, and mucin. Generally, the volume recovered was within 10% of that added. In other experiments, aliquots of 50 μl were removed sequentially. In these experiments, we added FITC-dextran (50 μg/ml) to the mucosal medium, and the volumes at each time point and cumulative volume changes were calculated from measurements of FITC made with a fluorimeter (model 450; Turner Designs, Sunnyvale, CA). For the release of compounds from cells grown in T75 flasks, cells were rinsed three to five times with sterile PBS (pH 7.4). Five milliliters of the appropriate medium were then added to the flask, and subsequently 250 μl were removed at set times.

To study the effects of mediators on release, we exposed “test” cells to medium alone for a set period of 3 h and then exposed them to a mediator for a second period of the same duration. Control cells received two exposures to medium alone. Results were expressed as the secretory index (SI), which equals (T2/T1)/(C2/C1), where T1 and T2 are the release periods during the first and second exposure periods for the test cells, and C1 and C2 are the corresponding releases for the control cells. An SI >1 indicates stimulation of output, <1 shows inhibition.

Media of varying tonicity (50, 100, 200, and 400%) were made by dilution with water or addition of NaCl. Osmolarities of the test solutions, as determined with an Osmometer (Advanced Instruments, Norwood, MA), were (in mosmol/kgH2O) 109, 298, 581, and 1,111. To mechanically perturb the cells, ~95% of the mucosal medium (600 μl) was removed and rapidly squirited back on with an automatic pipettor 5, 10, or 20 times at intervals of ~5 s. Aliquots of the apical medium were collected 10 min and 1 h later. To obtain media of various pH, we added NaOH or HCl to HCO3−-free medium while continuously monitoring pH.

Samples of secretions from individual glands were provided by Dr. Jeffrey Wine (Stanford University). They had been collected in constant-bore capillary tubes as described (23). In brief, the luminal surfaces of pieces of human tracheal were covered with paraffin oil, and gland secretion was stimulated with methacholine. The resulting gland secretions welled up as beads below the oil and were aspirated (between columns of oil) in constant-bore micropipettes. Five tracheas were studied. For each trachea, samples from two to five glands were pooled in the same capillary tube. The average volume collected per trachea was 21 ± 15 μl (mean ± SD). The contents of each micropipette were blown out into PBS to which 5 mM dithiothreitol was added to disperse the mucus. Lactoferrin, lysozyme, and mucin were then measured in these samples by ELISA.

All drugs were used at a final concentration of 10−5 M except when specified. Data are presented as means ± SE.

RESULTS

Figure 2 shows the time courses of mucosal release of lysozyme, lactoferrin, and mucin from HTE. Clearly, the rates of release decline with time as product builds up in the mucosal medium. Accordingly, the points have been fitted with the best least-squares fits for exponential approaches to an asymptote: X(t) = X∞(1 − e−kt), where X∞ is the cumulative output of compound at any given time (t), and X∞ is the amount released after an infinite time. The product of k and X∞ equals the maximal rate of secretion at t = 0 (Jmax), and the rank order for Jmax was mucus >> lactoferrin > lysozyme (Table 1).

We could not detect lactoferrin release from Calu-3 cells, but time courses for release of mucin and lysozyme from these cells are shown in Fig. 3. The maximal rate of release of lysozyme from Calu-3 cells was ~15 times greater than from HTE (Table 1). By contrast, mucin release from Calu-3 cells was <1% that of HTE (Table 1).

HFF did not release detectable amounts of mucin, lactoferrin, or lysozyme. 16HBE14o− and T84 cells did not release detectable amounts of lactoferrin, and their release of mucins was comparatively trivial (Table 1). However, 16HBE14o− cells did release lysozyme at rates approaching those of Calu-3 cells, whereas lysozyme release from T84 cells was ~10% that of Calu-3 (Table 1).

Studies on the pharmacological regulation of lactoferrin and lysozyme release from either HTE or Calu-3 cells were performed on cells either on inserts or in T75 flasks, and similar results were obtained for both culture conditions. Output of lysozyme and lactoferrin was unaffected by methacholine,
epinephrine, neutrophil elastase (10^{-8} \text{ M}), or lipopolysaccharide (LPS, 5 \text{ \mu g/ml}) (\geq 3 \text{ trials per mediator per cell type}). Likewise, pHs of 4, 5, or 6, tonicities of 50, 200, or 400%, and repeated aspiration of medium (5, 10, or 20×) were without effect on release of either compound from either cell type (n=3 for each maneuver and cell). However, Calu-3 cells treated with forskolin showed volume secretion that averaged \sim 10 

\text{\mu L/cm}^2\cdot\text{h}^{-1} over an 8-h period (Fig. 4), and this volume secretion was associated with a threefold increase in lysozyme output (Fig. 5). The effects of forskolin on secretion of both lysozyme and fluid secretion were blocked by glibenclamide, an inhibitor of CFTR.

The concentrations of mucin, lactoferrin, and lysozyme in native gland secretions were 395 ± 190, 549 ± 97, and 231 ± 38 \text{ \mu g/ml}, respectively.

**DISCUSSION**

There were two aims to this study. First, we wished to determine if there were cultures of human airway epithelium that could be used to study the regulation of lactoferrin, lysozyme, and mucin release. Second, we wanted to see whether levels of lactoferrin and lysozyme release from Calu-3 cells were consistent with their widespread use as a model for serous gland cells.

### Table 1. Maximal rates of release of lactoferrin, lysozyme, and mucin from various cells

<table>
<thead>
<tr>
<th></th>
<th>HTE</th>
<th>Calu-3</th>
<th>16HBE14o⁻</th>
<th>T84</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mucin</td>
<td>32,500 ±9,600</td>
<td>83 ±15*</td>
<td>10 ± 2</td>
<td>20 ± 8</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>90 ± 67</td>
<td>0</td>
<td>-0</td>
<td>-0</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>20 ±6</td>
<td>348 ±117*</td>
<td>246 ± 9</td>
<td>32 ± 4</td>
</tr>
</tbody>
</table>

Values are means ± SE. \*Significant difference between human tracheal epithelium (HTE) and Calu-3 cells. The standard errors (SE) for maximal rates of secretion (J_{max}) were obtained from the standard relationship:

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\frac{SE_{J_{max}}}{J_{max}} = \sqrt{\frac{SE_{r}^2}{X_{t}} + \frac{SE_{k}^2}{k}},
\]

where X_{t} and k and their SEs were provided by the computerized best least squares fit to X_{t} = X_{0} (1 - e^{-k \times t}), where t is in hours and X is in pg/cm².

Serous gland cells have the highest levels of CFTR in the airways (15), and the use of Calu-3 cells as a model of serous gland cells (6, 19, 28, 39, 50) has been prompted mainly by their high levels of CFTR (21, 38). Calu-3 cells also show CFTR-dependent HCO_3⁻ secretion (10, 26), and HCO_3⁻ secretion underlies a significant fraction of airway gland secretion (1). However, Calu-3 cells lack the small electron-opaque granules of true serous cells (30, 42), instead containing large electron-opaque granules resembling those of mucous cells (38). They also stain with monoclonal antibodies specific for either goblet cells or serous gland cells (18).

In addition to their high levels of CFTR, serous gland cells constitute the predominant location of lactoferrin and lysozyme in the airways (2, 3, 13). Therefore, if Calu-3 cells are indeed a good model of serous gland cells, they should show greater release of these compounds than HTE. Conversely, although serous cells contain some mucins (37), levels are far lower than in mucous or goblet cells (8, 33). Therefore HTE should secrete more mucin than Calu-3 cells. We found these predictions to be true for mucus and lysozyme; Calu-3 cells secreted 15 times more lysozyme than HTE, whereas HTE secreted 400 times as much mucus as Calu-3. Surprisingly, however, no lactoferrin secretion was detectable from Calu-3 cells, although small amounts of lactoferrin were released from HTE.

Of the two other cell lines tested, T84 cells were chosen as they had levels of CFTR comparable to Calu-3 (38); they are probably derived from colonic crypts, a tissue that shows high levels of CFTR-dependent liquid secretion (11). However, they had much lower levels of lysozyme secretion than Calu-3 cells. The other cell line tested was the 16HBE14o⁻ cell line derived by transformation of primary cultures with SV40 large T antigen (7). Though polarized, these cells have a comparatively undifferentiated appearance, so the finding that their rates of mucin secretion were far lower than those of HTE was expected. Surprisingly, however, they showed robust secretion of lysozyme (at \sim \frac{1}{2} of the level of Calu-3 cells).

Duszyk (14) has shown that baseline secretion of lysozyme by Calu-3 cells is \sim 20 \text{ pg} \cdot \text{10}^6 \text{ cells}^{-1} \cdot \text{min}^{-1}. Here, we found baseline secretion of \sim 10 \text{ ng/cm}^2 over 8 h. Given that there are \sim 10^6 Calu-3 cells/cm² of insert (L. A. Sachs and J. H. Widdicombe, unpublished observations), this corresponds to

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Fig. 2. Release of mucus (A), lysozyme (B), and lactoferrin (C) from primary cultures of human tracheal epithelium. Means ± SE, n = 3–11, generally ~7.
approximately the same rate of secretion as reported by Duszyk. Interestingly, the rate of secretion of lysozyme by Calu-3 cells is similar to that of 30 pg·10⁶ cells⁻¹·min⁻¹ reported for primary cultures of human tracheal gland cells (29).

In agreement with Duszyk’s (14) results, we found lysozyme release to be increased by forskolin and to similar extents, 2.6-fold in his studies and 3.6-fold in ours. Duszyk also reported that lysozyme output was increased by the NO donor S-nitroso-glutathione, an effect believed to be due to elevation of [Ca²⁺]i (14). By contrast, we found no effect of methacholine (an agent that generally uses calcium as a second messenger) on lysozyme release. We note, however, that the effects of S-nitroso-glutathione on short-circuit current across Calu-3 cells are prolonged (14), whereas those of methacholine are transient (38).

In intact glands, cholinergic, α-adrenergic, and β-adrenergic agents all stimulate lysozyme secretion (42, 46), as do several other neurohumoral agents (9, 47). Furthermore, this stimulation is associated with serous cell degranulation (42). Therefore, we were surprised that, excepting forskolin, lysozyme secretion by Calu-3 cells was refractory to a large range of stimuli. One possible explanation for this is that the lysozyme released from glands by neurohumoral mediators represents material that has accumulated over time in the gland lumen, and the rate of secretion of lysozyme by the serous cells may not be regulated to any significant extent.

Forskolin-induced fluid secretion across Calu-3 cells was ~12.5 μl·cm⁻²·h⁻¹ over the first 8 h of exposure (Fig. 5). Forskolin-induced lysozyme secretion over the same period was ~3 ng·cm⁻²·h⁻¹. Thus the concentration of lysozyme in the induced secretions was ~0.24 μg/ml. Cholinergic agents are the most potent neurohumoral secretagogues of airway glands (17, 24). When applied to the ferret trachea in vitro, methacholine induced secretions containing ~200 μg/ml of lysozyme. Of course, fluid absorption by the surface epithelium (22, 45, 48) may have concentrated the primary gland secretions. However, the value for lysozyme in human gland secretions sampled directly, 549 ± 97 μg/ml, was similar to that estimated for the ferret.

These comparisons between intact trachea and Calu-3 cells strongly suggest that Calu-3 cells secrete much less lysozyme than gland serous cells. Primary cultures of human tracheal gland cells secrete the same amount of lysozyme as Calu-3 cells (29). We conclude, therefore, that at least as regards lysozyme secretion both types of cultured cell are poor models of serous gland function. In fact, the current primary gland cultures are notably dedifferentiated and of mixed seromucous phenotype (40, 43).
Airway secretions are rich in lactoferrin, reaching concentrations of ~0.5 mg/ml in sputum (4), and airway lactoferrin secretion (presumably from glands) can be stimulated by both adrenergic and cholinergic agents (31, 35). However, Calu-3 cells showed no detectable lactoferrin secretion.

HTE showed mucin secretion that was several orders of magnitude higher than in the other cell types studied. HTE grown under our culture conditions develop full mucociliary secretions (presumably from glands) can be stimulated by both concentrations of lysozyme and lactoferrin. Thus in cell sheets from one HTE culture, the 48-h releases of mucus secretion changes with time in culture, we do know that secretion of lactoferrin and lysozyme by HTE does not change. Thus in cell sheets from one HTE culture, the 48-h releases of lactoferrin and lysozyme were measured at 9, 16, 19, 31, 35, and 39 days after plating. For both compounds, the linear regressions the rate of release against time in culture were not significantly different from zero (data not shown).

Native surface epithelium of the airways contains mRNA for both lactoferrin and lysozyme (16). However, by immunocytochemistry, the corresponding proteins are undetectable but can be seen in primary cultures (16); the act of culturing the cells somehow induces the expression of the protein. Thus it was to be expected that HTE would also show both lactoferrin and lysozyme secretion, albeit at low levels. In fact, of the cells tested, only HTE showed significant levels of lactoferrin secretion, although this was at only ~0.3% that of mucin secretion by HTE and 25% that of lysozyme secretion by Calu-3 cells (see Table 1).

Neutrophil elastase, LPS, calcium ionophore, and ATP did not induce secretion of lysozyme or lactoferrin from either HTE or Calu-3 cells, although calcium ionophore and ATP did stimulate mucin secretion from HTE (data not shown). Mechanical, osmotic, and pH perturbations were also without effect on lactoferrin and lysozyme release. Mucin secretion by Calu-3 cells has earlier been shown to be refractory to a wide range of stimuli (18).

We draw several conclusions. First, Calu-3 cells resemble serous gland cells in showing higher levels of lysozyme release than other cell types. Nonetheless, the levels secreted are considerably less than those expected for gland serous cells. Calu-3 cells show no lactoferrin release. Thus in their handling of these antimicrobial compounds they bear little resemblance to serous gland cells. Second, release of lactoferrin (from HTE) and lysozyme (from HTE or Calu-3 cells) is refractory to most neurohumoral mediators and a variety of irritants. However, lysozyme release from Calu-3 cells is stimulated by forskolin. Third, consistent with their histology, HTE show high levels of mucin release and should prove suitable for studies on the regulation of this process.

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REFERENCES

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