

Human Airway Epithelial Cell Culture

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1. Introduction

Development of methods to culture airway epithelial cells has been needed to carry out research into various lung diseases, such as cancer, cystic fibrosis, and bronchial asthma. However, the culture of airway epithelial cells remained difficult. We have improved the culture conditions of these cells, so that these cells can now be used to better understand the mechanisms underlying cystic fibrosis (*1–4*), for characterizing viral infections (*5–7*), and for advancing our knowledge of airway inflammation.

In order to improve the conditions under which cultured human tracheal epithelial cells can retain their ion transport properties and ultrastructure of the original tissue, we have developed the following protocol. Briefly, human tracheal epithelial cells are isolated by digestion with protease overnight (*1,2,8,9*). The isolated epithelial cells are plated on vitrogen gel-coated porous-bottomed inserts in media containing Ultrosor G serum substitute (USG). Cells are grown with an air interface (i.e., no medium added to the mucosal surface). These culture conditions, the vitrogen gel, USG-supplemented medium, and the air interface, lead to the appearance of cilia, an increase in the depth of the cell sheets (50 μm), longer and more frequent apical microvilli, and increased interdigitations of the basolateral membrane (**Fig. 1**). Protein and DNA content are also significantly increased. Secretory granules are present, which stain with antibody to goblet cells, but serous or mucous gland cells are not seen (**Fig. 2**) (*1*).

Acini of human tracheal submucosal glands are isolated by digestion with various enzymes (*5–7,10*). The isolated gland acini are incubated in flasks coated with human placental collagen in media containing USG and a variety of growth factors. The attached gland acini make confluent cell sheets after

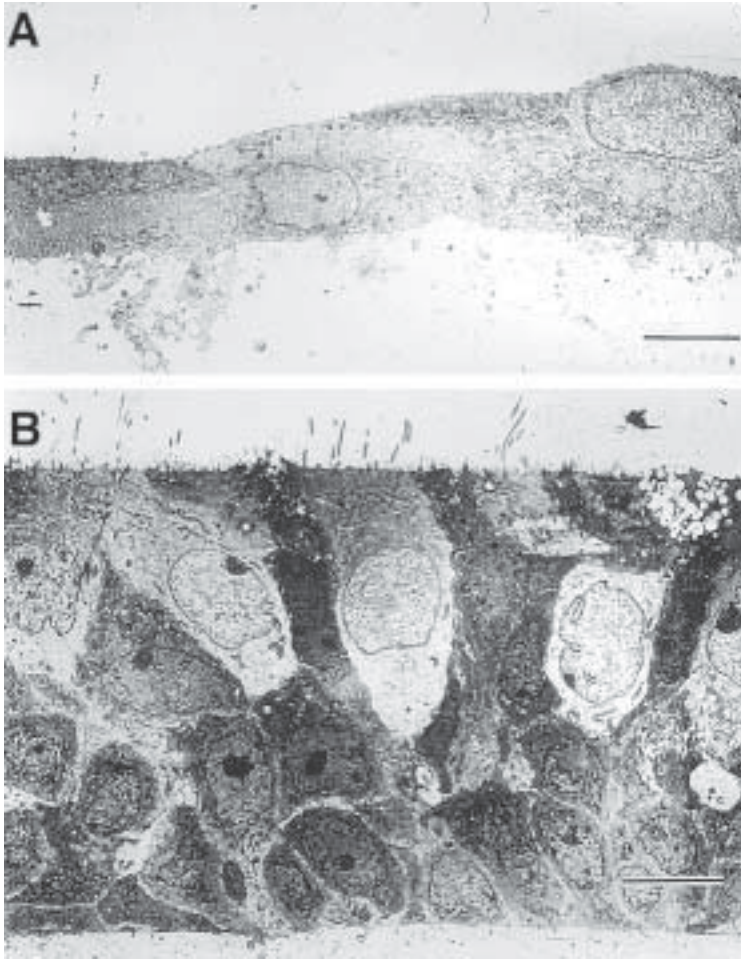


Fig. 1. Low power electron micrographs of cultured human tracheal epithelial cells. (A) human placental collagen, FCS-medium, immersed feeding. (B) vitrogen gel, USG medium, air interface feeding. Cells are multilayered, and the luminal surface contains cilia and secretory granules. Scale bars = 10 μ m.

14–21 d (5–7,10). The cells are then isolated by trypsinization and replated in media containing USG and growth factors on porous-bottomed inserts coated with human placental collagen and grown with an air interface (5–7). Cells cultured under these conditions have high transepithelial electrical resistance and high short-circuit current. The human tracheal epithelial cells and gland cells can secrete chloride ions in response to bradykinin, α - and β -adrenergic and cholinergic agents, and ATP.

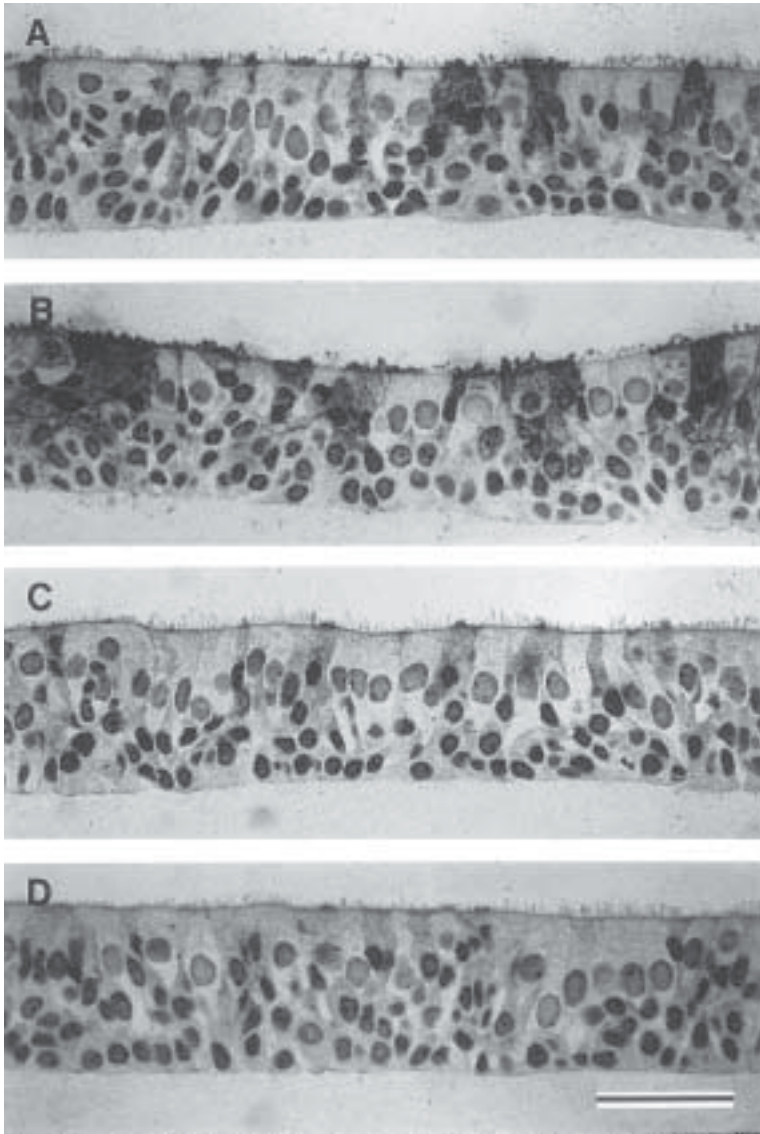


Fig. 2. Expression of goblet cell antigens by cultured human tracheal epithelial cells. Glycomethacrylate secretions were incubated with monoclonal antibodies and stained using an avidin-biotin-peroxidase procedure. (A) antibody A3G11 and (B) antibody B6E8. Both antibodies recognize tracheobronchial goblet epithelial and mucous gland cell antigens and stain cells throughout the cultured tracheal epithelial cells. (C) antibody A8E4. This antibody recognizes a tracheobronchial mucous gland cell antigen. Staining is absent. (D) antibody B1D8. This antibody recognizes a tracheobronchial serous gland cell antigen. Staining is absent. Scale bar = 50 μ m.

Human tracheal epithelial cells and submucosal gland cells can be cultured in glass tubes, coverslips, slide glasses, and culture dishes as well as filter membranes. Cells cultured under these conditions can be used for studies on ion transport, intracellular calcium concentration, epithelial permeability, repair of epithelial cells after injury, and production of various enzymes and proteins, such as cytokines and intercellular adhesion molecules (1–10).

2. Materials

2.1. Coating of Culture Vessels

2.1.1. Vitrogen Gels

1. Minimal essential media (MEM) (GIBCO BRL Life Technologies).
2. 0.1 mol/L Sodium hydroxide (NaOH).
3. Vitrogen solution (Collagen).
4. Millicell-CM or Millicell-HM inserts (Millipore): 0.45 μm pore size, 0.6 cm^2 area.

2.1.2. Collagen

1. Human placental collagen (Sigma).
2. 0.2% Glacial acetic acid in double-distilled water.
3. 12-well Tissue culture plates (Falcon).
4. Millipore-CM inserts with 0.45 μm pore size, 0.6 cm^2 area.
5. Transwell inserts (Corning Costar): 0.4 μm pore size.
6. Phosphate-buffered saline (PBS) (GIBCO BRL Life Technologies) supplemented with 10^5 U/L penicillin, 100 mg/L streptomycin, 50 mg/L gentamicin, and 2.5 mg/L amphotericin B (all from Sigma).

2.2. Human Tracheal Epithelial Cell Culture

1. Dissection kit.
2. Dissection tray.
3. PBS.
4. 5 mol/L Dithiothreitol (DTT) (Sigma) in PBS.
5. Protease solution: 0.4 mg/mL protease Sigma type XIV (Sigma), 10^5 U/L penicillin, 100 mg/L streptomycin, 50 mg/L gentamicin, and 2.5 mg/L amphotericin B in PBS.

Dissolve 20 mg protease in 40 mL PBS, which already contains the penicillin, streptomycin, and gentamicin, in a 50-mL tube. Shake by hand until dissolved. Sterilize by passing through a 0.45- μm filter and then add the amphotericin B.

6. Fetal calf serum (FCS) (GIBCO BRL Life Technologies).
7. F-12-DMEM-FCS Mix I: Dulbecco's modified Eagle's medium (DMEM) (GIBCO BRL Life Technologies) mixed 1:1 with Ham's F-12 medium (GIBCO BRL Life Technologies) and supplemented with 5% FCS.
8. 0.4% Trypan blue (Sigma).

9. Ultrosor G serum substitute (USG) medium: 1:1 DMEM:Ham's F12 supplemented with 2% USG (BioSeptra), 10^5 U/L penicillin, 100 mg/L streptomycin, 50 mg/L gentamicin, and 2.5 mg/L amphotericin B.

Dissolve the USG in distilled water to make a stock solution according to the manufacturer's instructions. Mix 10 mL of the USG stock solution with 480 mL DMEM:Ham's F-12, to give a final concentration of 2% USG. The medium should then be supplemented with the antibiotics (**I**).

10. Vitrogen gel-coated Millicell inserts.
11. Collagen-coated Transwell inserts.
12. Millipore-CM inserts with 0.45 μ m pore size, 0.6 cm² area.
13. Hemocytometer.
14. 50-mL Conical centrifuge tubes (Corning Costar).
15. T₂₅ Tissue culture flasks (Corning Costar).
16. Glass tubes with round bottoms, 15 mm in diameter, 105 mm long (Iwaki Glass), coated with human placental collagen. To coat the tubes, add 1 mL of collagen working stock solution to the tubes. Keep the tubes stationary at a slant of 5° and incubate for at least 2 h. Remove the collagen solution and air-dry the tubes.
17. Roller culture incubator (HDR-6-T; Hirasawa, Tokyo, Japan).

2.3. Human Tracheal Submucosal Gland Culture

1. PBS.
2. Enzyme Solution: Hanks' buffered salt solution (HBSS) (GIBCO BRL Life Technologies) supplemented with 20 mM HEPES buffer, pH 7.4 (Sigma), 500 U/mL collagenase type IV (Sigma), 6 U/mL pancreatic porcine elastase (Sigma), 200 U/mL hyaluronidase (Sigma), 10 U/mL deoxyribonuclease (Sigma).

Dissolve collagenase, pancreatic porcine elastase, hyaluronidase and deoxyribonuclease in 50 mL HBSS, containing 20 mM HEPES, penicillin, streptomycin and gentamicin. Sterilize by passing through a 0.45- μ m filter and then add the amphotericin B (**5**).

3. F-12-DMEM-FCS Mix II: 40% Ham's F12, 40% DMEM, 20% FCS.
4. Growth medium: 1:1 DMEM:Ham's F12 supplemented with 0.1% USG, 10 μ g/mL insulin (Becton Dickinson), 5 μ g/mL transferrin (Becton Dickinson), 20 ng/mL triiodothyronine (Becton Dickinson), 0.36 μ g/mL hydrocortisone (water soluble) (Sigma), 7.5 μ g/mL endothelial cell growth supplement (Becton Dickinson), 25 ng/mL epidermal growth factor (Becton Dickinson), 0.1 mol/L retinoic acid (Sigma), 20 ng/mL cholera toxin (Sigma), 10^5 U/L penicillin, 100 mg/L streptomycin, 50 mg/L gentamicin, and 2.5 mg/L amphotericin B.

Stock solutions of growth factors:

- a. Insulin: 20 mg in 4 mL distilled water.
- b. Transferrin: 10 mg in 4 mL distilled water.
- c. Triiodothyronine: 20 mg in 10 mL distilled water.
- d. Hydrocortisone: 10 mg in 10 mL distilled water.
- e. Endothelial cell growth supplement: 15 mg in 4 mL distilled water.

- f. Epidermal growth factor: 100 μg in 10 mL distilled water.
- g. Retinoic acid: Dissolve in 100% ethanol to give a stock solution of 1 mM. Dilute to 10 μM in distilled water.
- h. Cholera toxin: 0.5 mg in 5 mL distilled water.

To make up growth medium (500 mL): mix 484 mL 1:1 DMEM:Ham's F12 (242 mL of each), 1 mL insulin stock solution, 1 mL distilled water containing 5 μL triiodothyronine stock solution, 1 mL distilled water containing 35 μL hydrocortisone stock solution, 1.25 mL epidermal growth factor stock solution, 5 mL 10 μM retinoic acid. Sterilize by passing the solution through a 0.45- μm filter. Then add 1 mL transferrin stock solution, 1 mL endothelial cell growth supplement stock solution. Add 10^5 U/L penicillin, 100 mg/L streptomycin, 50 mg/L gentamicin, and 2.5 mg/L amphotericin B, and 0.5 mL USG stock solution.

Because retinoic acid is photosensitive, the growth medium and stock solution of retinoic acid should be made up in the dark, as much as possible. Wrap the bottle of growth factor medium in foil and store at 4°C.

5. 0.25% trypsin-EDTA solution (Sigma).

3. Methods

3.1. Coating of Culture Vessels

3.1.1. Vitrogen Gels

1. Mix 10% 10X MEM with 10% 0.1 M NaOH and 80% vitrogen solution, at 4°C (v/v/v).
2. The solution will be yellow.
3. Add 0.1 M NaOH until the color changes to red.
4. Add 0.15 mL/cm² of this solution to the Millicell inserts.
5. Place these at 37°C for 1 h.
6. Use within 2 h of manufacture.

3.1.2. Collagen

1. Make a stock solution of human placental collagen by dissolving 50 mg collagen in 100 mL 0.2% glacial acetic acid, using a magnetic stirrer.
2. Sterilize by passing the solution through a 0.45- μm filter.
3. Dilute the stock solution 1:5 with double-distilled water. This will give a working concentration of 20 μg of collagen per cm² of surface area, when added to the culture vessels.
4. Coat 35-mm dishes, wells, or coverslips in 6-well plates with 2 mL of working stock solution, 12-well plates with 1 mL/well, Millicell or Transwell inserts with 0.5 mL or glass tubes with 1 mL.
5. Incubate for at least 2 h, or preferably overnight, at room temperature.
6. Remove the collagen solution and allow to air-dry.
7. Prior to use, rinse dishes, plates or inserts with PBS containing antibiotics, and allow to dry.

3.2. Human Tracheal Epithelial Cell Culture

1. Open tracheas for cell culture longitudinally along the anterior surface.
2. Mount in a stretched position, with the epithelium uppermost, in a dissection tray.
3. Score the surface of the epithelium in longitudinal strips.
4. Clamp the end of one of these mucosal strips and pull off the entire length from the submucosa (*I,II*).
5. Rinse the tissue strips 4× in 5 mM DTT in PBS. The DTT is important to prevent the formation of mucus globs.
6. Rinse the strips twice in PBS alone.
7. Incubate at 4°C overnight in 40 mL of protease solution in a 50 mL conical centrifuge tube.
8. The following day, add FCS to a final concentration of 2.5%, to stop the action of the protease solution.
9. Remove 20 mL of the solution and add the same volume of F-12-DMEM-FCS Mix I.
10. Dislodge the smaller sheets of cells from the epithelial strips by vigorous agitation.
11. Remove the denuded strips.
12. Disperse the remaining sheets of cells by repeated aspiration using a 10-mL pipet.
13. Pellet cells at 200g for 10 min.
14. Resuspend the pellet in F-12-DMEM-FCS.
15. Count the cells using a hemocytometer and estimate viability using trypan blue, counting cells with blue-stained nuclei as dead (*I,II*).

3.2.1. Preparation of Cells for Further Analyses

1. To measure the electrical properties or enzyme production of epithelial cell sheets or transepithelial permeability, plate cells at 10^6 viable cells/cm² onto Millicell-CM or Millicell-HA inserts coated with Vitrogen gel (*see Subheading 3.1.*) (*I,2*).
2. One day after plating the cells out, replace medium with USG medium.
3. The cells should be grown with an air interface by removal of fluid on the mucosal side.
4. Culture cells at 37°C in 5% CO₂-95% air incubator, and change the media every day for the first 7 d, and then every 2 d thereafter.
5. The epithelial cells will form a confluent cell sheet about 5 d after plating. At this point, the cells can be used for experiments.
6. To measure the intracellular calcium concentrations [Ca²⁺]_i of the human tracheal epithelial cells, plate cells onto collagen-coated membranes and culture as above (*see Subheading 3.2.*) (*2*).
7. To examine the repair and proliferation of epithelial cells, plate cells onto Millicell-CM inserts at 10^6 viable cells/cm², or plate onto 6-well culture dishes or T₂₅ flasks at 1 to 2×10^5 viable cells/cm², and culture as above (*3*).
8. To examine the repair of epithelial cells in T₂₅ flasks touch the epithelial cells with a pipet tip to introduce defects in focal contacts.
9. Observe cell growth every day.

10. To examine cell proliferation, culture cells in medium supplemented with [^3H] thymidine for 24 h and then measure radioactivity.
11. To examine the effects of virus infection on the production of inflammatory cytokines and intercellular adhesion molecules by the human tracheal epithelial cells, plate cells at 5×10^5 viable cells/mL (2×10^5 cells/cm 2) in glass tubes with round bottoms coated with human placental collagen (8,9).
12. Seal the glass tubes with rubber plugs, keep stationary at a slant of approximately 5° , and culture at 37°C .
13. When the epithelial cells have formed confluent sheets, infect the cells with rhinovirus, and culture at 33°C in a roller culture incubator.

3.3. Human Tracheal Submucosal Gland Cell Culture

1. Score the tracheal surface epithelium in longitudinal strips and pull away from the submucosa.
2. Dissect the gland-rich submucosal tissue away from the cartilage and the adventitia.
3. Immerse in fresh PBS.
4. Rinse the submucosal tissue 4 times in PBS.
5. Mince with scissors.
6. Centrifuge the tissue fragments at 200g for 10 min.
7. Resuspend the fragments in enzyme solution (5,6,10).
8. Place in a flask on an orbital shake (set to 240 rpm) and leave to disaggregate for 12–16 h at room temperature.
9. Decant the fluid, which should contain the disaggregated tissue.
10. Centrifuge at 200g for 10 min.
11. Wash once in a mixture of F-12-DMEM-FCS Mix II.
12. Wash twice in PBS.
13. Resuspend the disaggregated tissue in F-12-DMEM-FCS Mix II.
14. Plate acini out onto two T_{25} tissue culture flasks and incubate for 24 h at 37°C in 5% CO_2 -95% air. These are both the attached and the unattached acini.
15. The fragments of submucosal tissue remaining in the trypsinizing flask should be exposed again to enzymatic digestion as above.
16. The cells collected from the second digestion are dispersed gland acini.
17. Combine both the unattached acini from the two T_{25} flasks with the dispersed gland acini.
18. Spin the combined acini from these two sources at 200g for 10 min.
19. Resuspend in fresh F-12-DMEM-FCS Mix II and plate in the two T_{25} flasks containing the attached acini from the first plating.
20. The following morning, replace the medium with growth medium (5–7,10).
21. It takes 14–21 d to achieve confluency, at which point the cells should be collected by trypsinization.
22. To trypsinize, wash twice with PBS and then add 5 mL trypsin.
23. Incubate for 10–20 min or until all the cells have detached.
24. Pellet the cells at 200g for 10 min.
25. Resuspended in F-12-DMEM-FCS Mix II.

26. Count cells using a hemocytometer and estimate the viability with trypan blue.

3.3.1. Preparation of Cells for Further Analyses

1. To measure the electrical properties of the human tracheal submucosal gland cells, plate the cells out in F12-DMEM-FCS Mix II at 10^6 cells/cm² on Millicell-CM inserts.
2. Cells will appear confluent after 24 h and should then be grown in growth media.
3. Culture the cells for 7–9 d, at which point they can be used in experiments.
4. Cells should be grown with an air interface; so no media is added to the mucosal surface.
5. To measure the intracellular calcium concentrations $[Ca^{2+}]_i$ of the human tracheal submucosal gland cells, plate cells onto either collagen-coated Transwell membranes (2) or collagen-coated coverslips (*see Subheading 3.1.*) (7).
6. Culture the cells for 7–10 d before measurement of $[Ca^{2+}]_i$.
7. To examine the effects of virus infection on the production of inflammatory cytokines and intercellular adhesion molecules by the human tracheal submucosal gland cells, plate the cells at 5×10^5 viable cells/mL (2×10^5 cells/cm²) in glass tubes with round bottoms coated with human placental collagen (10).
8. When the submucosal gland cells have formed confluent sheets, infect the cells with rhinovirus, and culture at 33°C in a roller culture incubator.

Acknowledgments

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References

1. Yamaya, M., Finkbeiner, W. E., Chun, S. Y., and Widdicombe, J. H. (1992) Differentiated structure and function of cultures from human tracheal epithelium. *Am. J. Physiol.* **262**, L713–L724.
2. Yamaya, M., Ohru, T., Finkbeiner, W. E., and Widdicombe, J. H. (1993) Calcium-dependent chloride secretion across cultures of human tracheal surface epithelium and glands. *Am. J. Physiol.* **265**, L170–L177.
3. Yamaya, M., Sekizawa, K., Masuda, T., Morikawa, M., Sawai, T., and Sasaki, H. (1995) Oxidants affect permeability and repair of the cultured human tracheal epithelium. *Am. J. Physiol.* **268**, L284–L293.
4. Yamaya, M., Sekizawa, K., Yamauchi, K., Hoshi, H., Sawai, T., and Sasaki, H. (1995) Epithelial modulation of leukotriene-C4-induced human tracheal smooth muscle contraction. *Am. J. Respir. Crit. Care Med.* **151**, 892–894.
5. Yamaya, M., Finkbeiner, W. E., and Widdicombe, J. H. (1991) Ion transport by cultures of human tracheobronchial submucosal glands. *Am. J. Physiol.* **261**, L485–L490.
6. Yamaya, M., Finkbeiner, W. E., and Widdicombe, J. H. (1991) Altered ion transport by tracheal glands in cystic fibrosis. *Am. J. Physiol.* **261**, L491–L494.
7. Yamaya, M., Sekizawa, K., Kakuta, Y., Ohru, T., Sawai, T., and Sasaki, H. (1996) P2u-purinoreceptor regulation of chloride secretion in cultured human tracheal submucosal glands. *Am. J. Physiol.* **270**, L979–L984.

8. Terajima, M., Yamaya, M., Sekizawa, K., et al. (1997) Rhinovirus infection of primary cultures of human tracheal epithelium: role of ICAM-1 and IL-1 β . *Am. J. Physiol.* **273**, L749–L759.
9. Suzuki, T., Yamaya, M., Sekizawa, K., et al. (2000) Effects of dexamethasone on rhinovirus infection in cultured human tracheal epithelial cells. *Am. J. Physiol.* **278**, L560–L571.
10. Yamaya, M., Sekizawa, K., Suzuki, T., et al. (1999) Infection of human respiratory submucosal glands with rhinovirus: effects on cytokine and ICAM-1 production. *Am. J. Physiol.* **277**, L362–L371.
11. Widdicombe, J. H. (1988) Culture of tracheal epithelial cells, p. 291–302, in *Methods in bronchial mucology* (Braga P. C. and Allegra, L., eds.), Raven Press, New York.



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