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Growth of Influenza A Virus in Primary, Differentiated Epithelial Cells Derived from Adenoids

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Epithelial cells of adenoid origin were grown in tissue culture to examine viral replication in cells that are the primary target of many human pathogens. These cells remained highly differentiated, with subpopulations of cells which retained active ciliary motility and others which demonstrated specialized secretory functions. The epithelial cells were permissive for growth of influenza A virus. Primary respiratory epithelial cells provide a model system for examining virulence, cell tropism, and receptors for viruses which replicate in the nasopharynx.

Remarkably little is known about the replication and cytopathology of respiratory viruses in nasopharyngeal epithelium (9, 17). Respiratory epithelium has not been readily accessible for in vivo study beyond quantitation of virus in secretions or for in vitro study in tissue or organ culture. Under standard tissue culture conditions, epithelial cells are overgrown by fibroblasts and sustained viability of adenoid human organ culture has been difficult to achieve (5). Human fetal tissue was used in early experiments to examine virus replication in tracheal organ rings; however, this tissue is no longer available (11). Growth of rhinovirus and coronavirus has been demonstrated in outgrowths of epithelium around bits of respiratory mucosa (15).

Many viruses undergo replication solely in the nasopharynx. Viral replication commonly occurs for 7 to 14 days (4, 6–8), with primary infection accompanied by epithelial cell destruction, increased mucin production, ciliary dysfunction (3), and occasional secondary bacterial invasion. For other viruses, e.g., measles and varicella viruses, the epithelium is an initial site of amplification before systemic spread. Finally, some viruses, e.g., adenovirus and Epstein-Barr virus, are recovered for long periods from a respiratory site in individuals with a latent or persistent infection.

The factors that determine the efficient, organ-specific, but time-limited replication of many viruses in the respiratory mucosa are not well understood. The present experiments focus on the growth in primary adenoid epithelial cells of influenza A virus, which in humans causes an acute infection limited to the respiratory epithelium.

Adenoids and tonsils were obtained at the time of adenoidectomy and tonsillectomy under a protocol approved by the Vanderbilt Institutional Review Board. The approach to the derivation and successful culturing of respiratory epithelial cells was based on the experience of Wu et al. (16).

Establishment of cell growth. Optimal recovery of viable epithelial cells was obtained by placing the whole tissue in minimal essential medium (Gibco Laboratories, Grand Island, N.Y.) with 0.1% pronase type 14 (Sigma Chemicals, St. Louis, Mo.) antibiotics and rocking overnight at 4°C. The superficial layers of cells were further dispersed by pipetting and cells were placed in medium containing 10% fetal calf serum to inactivate the pronase. The cells were then centrifuged and resuspended in 50% Ham's F-12 medium (Cellgro; Mediatech)–50% Eagle's minimal essential medium with the following supplements: insulin, 5 μg/ml; transferrin, 5 μg/ml; epidermal growth factor, 10 ng/ml; cholera toxin, 10 ng/ml; hydrocortisone, 10^{-6} M; bovine hypothalamic extract, 15 μg/ml; *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer, 0.015 M; retinol, 10^{-7} M, gentamicin, 50 μg/ml; penicillin G, 15 U/ml; streptomycin, 15 U/ml; fetal calf serum, 0.5%; and, for the first 5 days, immune globulin (Gamimune N; Cutter Biological, Elkhart, Ind.), 3.3 mg/ml. The cells were incubated at 37°C under 5% CO_2.

Essential for the growth of the cells was an underlying collagen matrix of Vitrogen 100 (Celtrix Laboratories, Palo Alto, Calif.), 5× minimal essential medium, 75 mM HEPES, and NaOH to bring the pH to 7.0.

Histologic and immunohistochemical characterization. Use of a well insert (Transwell; Costar, Cambridge, Mass.) coated with Vitrogen 100 was the optimal way of preparing cells for histochemistry. Routine histology and immunohistochemistry were performed to document the epithelial morphology and degree of differentiation of the epithelial cells in culture. Following fixation with 10% buffered formalin, the insert containing cells and collagen matrix was lifted from the well. Paraffin-embedded sections through the cells were stained with hematoxylin and eosin and with periodic acid-Schiff stain. Additional sections were stained by an immunoperoxidase technique (13) with diaminobenzidine as a substrate and with antibodies to secretory component (DAKO, Carpenteria, Calif.), Clara cell 10-kDa protein (CC10; a gift from G. Singh and S. L. Katyal), a marker for dendritic cells (S100 DAKO), a marker for epithelial cells (cytokeratin 14 [CAM 5.2]; Becton-Dickinson, Mountain View, Calif.), a marker for mesenchymal cells (vimentin; Boehringer-Mannheim, Indianapolis, Ind.), a marker for dividing cells (proliferating-cell nuclear antigen [DAKO]), and leukocyte common antigen (DAKO).

Initially, the predominant cells in the tissue culture were lymphocytes, but these nonadherent cells were removed from the culture with changes of medium. The cells reached confluency in 10 to 14 days, remaining largely as a monolayer but with some mounding of cells observed. Evidence of differentiation was present in most cultures of discrete patches of

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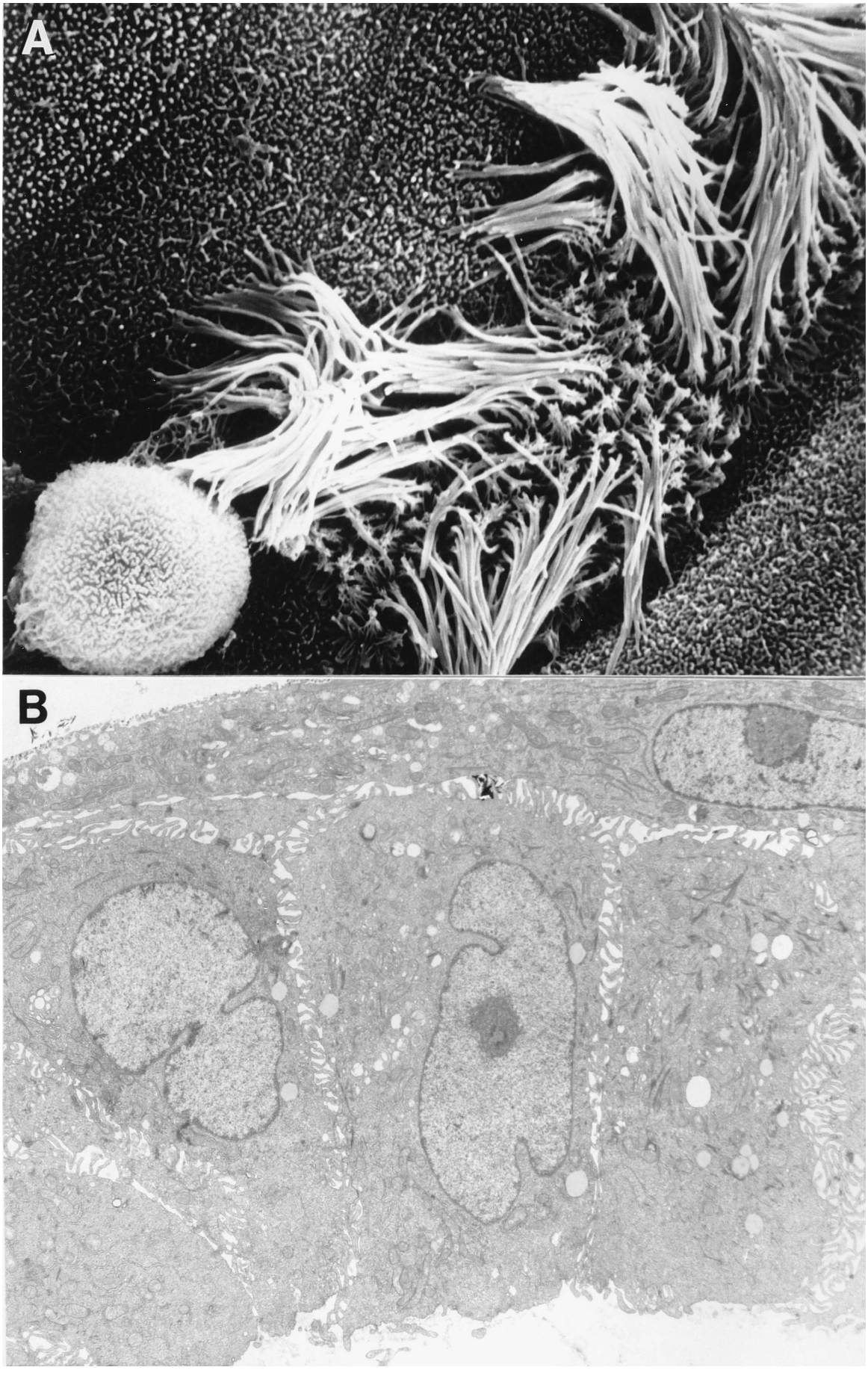
A
B

FIG. 1. (A) Scanning electron micrograph of epithelial cells after 9 days in culture. Magnification, ×6,125. (B) Transmission electron micrograph of epithelial cells after 7 days in culture. Magnification, ×5,000.

ciliated cells, which maintained regular ciliary activity for 1 to 3 weeks after reaching confluency.

Epithelial cells in culture were compared with epithelial cells in the intact adenoid in terms of differentiation and distribution. Routine histologic staining of the epithelial cells demonstrated a relatively homogeneous population of epithelial cells. With the periodic acid-Schiff stain, discrete cells with mucin production, which resembled those in the intact adenoid, were evident in culture. Immunocytochemical staining showed cells in both intact adenoid and tissue culture preparations to have secretory component, the immunoglobulin A transport protein which serves as a marker for epithelial cells. Finally, in both intact adenoid and epithelial cell culture, there were individual cells staining for the 10-kDa protein that is a marker for Clara cells.

Ultrastructural studies. Scanning electron microscopy was performed on uninfected cells fixed in 3% glutaraldehyde in 0.12 M sodium cacodylate buffer (pH 7.3). Samples were post-fixed for 1 h in 1% osmium tetroxide (in cacodylate buffer), rinsed, and dehydrated in an ethanol series. Cells were examined in a Hitachi S-500 scanning electron microscope at 20 keV. Transmission electron microscopy was performed on uninfected cells that were fixed by immersion in 2.5% glutaraldehyde in phosphate buffer. After being rinsed in 7.5% sucrose in phosphate buffer (pH 7.3 to 7.4), tissues were fixed in Millonig's osmium tetroxide for 2 h, dehydrated by Luft's procedure, and embedded in Spurr resin. Sections were stained with uranyl acetate and lead citrate and examined in a Philips 300 electron microscope.

The appearance of the cells in scanning electron microscopy (Fig. 1A) and transmission electron microscopy (Fig. 1B) was determined. Scanning electron microscopy confirmed that a subpopulation of cells were ciliated. The transmission electron microscopy showed intact cells with typical epithelial-cell morphology.

Viral replication. Between 1×10^7 and 3×10^7 epithelial cells could typically be recovered from an adenoid (average weight, 0.8 g), and 3×10^5 to 5×10^5 cells were seeded into each Vitrogen-coated well of 35-mm six-well plates (Costar). As described previously, adenovirus was recovered frequently from cultures of adenoid tissue (12). To diminish adenovirus contamination, for the first 5 days an immune globulin preparation was added to the medium. On reaching confluence, the monolayers of epithelial cells averaged 10^6 cells per 35-mm plate. Growth of epithelial cells from adenoids was consistently better than that from tonsils in spite of a much larger volume of tonsillar tissue (average weight, 4.3 g). The success rate in reaching confluency was 83 of 106 (78%) of adenoid cell samples cultured. By growing adenoid cells in 10% serum, a fibroblast culture was obtained and was used for comparative studies of viral growth. The growth of an influenza A virus strain designated A/Nashville/47/91 (isolated from a sick child) which antigenically resembled A/Beijing/H3N2 was examined. The standard input of virus was 10^3 (0.001 infectious virus particle per cell). Viral replication was measured by determining titers in supernatants obtained 1, 6, 24, 48, and 72 h after infection of the epithelial cells, using Madin-Darby canine kidney (MDCK) cells, a cell line susceptible to influenza A virus infection.

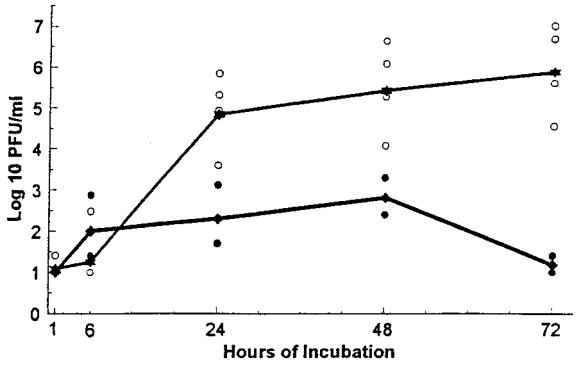

FIG. 2. Growth of wild-type influenza viruses in cells derived from adenoid tissue. The top curve shows growth of influenza A/Nashville/47/91 virus in epithelial cells (mean and individual values of four replicate experiments) (●). The bottom curve shows growth of influenza A/Nashville/47/91 virus in fibroblasts (mean and individual values of two replicate experiments (○).

Influenza A/Nashville/47/91 virus at input multiplicities of 0.001 to 1.0 showed progressive increases in titer over 48 to 72 h. Progressive cell destruction and interruption of ciliary activity were seen. A peak viral titer of 10^6 PFU/ml of supernatant was typically achieved with a multiplicity of infection of 0.001 (Fig. 2). Efficient growth of influenza A/Nashville/47/91 virus was maintained through three serial passages in epithelial cells (data not shown).

To demonstrate the dependence of influenza A/Nashville/47/91 virus replication on differentiated epithelial cells, growth was assessed in an adenoid-derived fibroblast line. The fibroblast and epithelial-cell lines were prepared in an identical way, with the exception of the addition of 10% serum to the fibroblasts. Even with a multiplicity of infection of 1, the adenoid-derived fibroblasts did not support the growth of influenza virus (Fig. 2). There was a significant difference in viral growth between the epithelial and fibroblast cells ($P = 0.008$) with repeated analysis of variance.

Cleavage of influenza virus hemagglutinin. Influenza A/Nashville/47/91 virus was labeled with 0.1 mCi of [^{35}S]methionine per 35-mm well in methionine-free medium 4.5 h after initiation of infection in the epithelial cells. As a control, labeled virus was grown in MDCK cells with or without trypsin. After 18 h of incubation, the supernatant was clarified and pelleted onto 30% sucrose. The pellet was lysed with 0.5% Triton X-100. The influenza virus hemagglutinin was conjugated to monoclonal antibodies against A/Bangkok (courtesy of Yoshihiro Kawaoka) and immunoprecipitated with rabbit anti-mouse coated Sepharose beads. The samples were then boiled, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on the isolated hemagglutinin, followed by autoradiography, as previously described (14).

Effective cleavage of the influenza A/Nashville/47/91 virus hemagglutinin precursor HA0 to its subunits by the adenoid epithelial cells was demonstrated. Virus grown in epithelial cells has the same pattern of cleavage into HA1 and HA2 hemagglutinin subunits as does virus grown in MDCK cells with added trypsin (Fig. 3). MDCK cells without the addition of trypsin did not mediate cleavage of the hemagglutinin.

Conclusions. The epithelial cells of adenoid origin remain highly differentiated as judged by ciliary activity and immunohistochemical evidence of mucin synthesis, Clara cells, and the

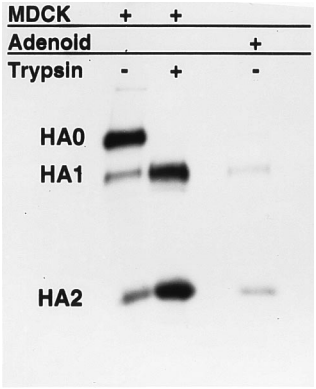

FIG. 3. SDS-PAGE of [^{35}S]methionine-labeled influenza A/Nashville/47/91 virus hemagglutinin grown in MDCK cells without and with trypsin and in epithelial cells.

presence of secretory component. The epithelial cells support the efficient replication of influenza A virus to high titer. The level of virus replication was highly consistent from adenoid to adenoid. Thus, the cells appear to be an excellent model for study of virus–epithelial-cell interactions.

Epithelial cells are the primary target tissue in acute viral respiratory infection. The in situ pathologic lesions that are described are due to desquamation; they are replaced by a stratified epithelium and rapid loss of ciliated cells with accompanying loss of ciliary function (3). These pathologic observations are modeled in the epithelial-cell monolayers.

A property of viruses in the orthomyxovirus family is their dependence on proteolytic cleavage of their surface glycoproteins for successful replication. The ease with which a particular hemagglutinin is cleaved is a determinant of the virulence and tropism of influenza virus (2, 14). The capability to initiate cleavage of influenza virus surface glycoproteins has been demonstrated by using nasal secretions (1). In that complex fluid, cleavage may be attributable to bacterial or leukocyte proteases, transudated serum proteases, or proteases produced by specialized cells in the respiratory tract, such as mast cells.

The adenoid epithelial cells join a very limited list of cells, namely, primary cultures of primate cells of renal origin, cells lining the allantoic and amniotic cavities of embryonated eggs, and fetal tracheal ring organ cultures, that will support the growth of influenza virus without an exogenous protease such as trypsin. Fibroblasts grown from the same adenoidal tissue did not sustain replication of influenza virus. The ability of influenza virus to grow in isolated respiratory epithelial cells and the demonstration that the virus released in the supernatant fluid has a fully cleaved hemagglutinin imply that a protease that mediates glycoprotein cleavage is present in respiratory epithelium.

One candidate for a protease-producing cell is the Clara cell. Clara cells are present in large numbers in the intact adenoid respiratory epithelium and were preserved in our adenoid cell cultures. In the rat lung, Clara cells produce a protease that cleaves the influenza virus hemagglutinin (10). The hypothesis can be advanced that the protease produced by Clara cells is secreted and acts in *trans* to mediate cleavage of the hemagglutinin as influenza virus is assembled and buds at the cell surface or, alternatively, that these protease-producing cells are the cells in which influenza virus replicates. This system should allow testing of the postulate that restriction of influenza virus growth to the respiratory tract may be determined by the presence of specific proteolytic activity. A newly described primary epithelial-cell system appears to maintain characteristics and functional capabilities of epithelial cells in the upper respiratory tract. As such, it provides a model in which many aspects of replication of viruses whose pathogenesis depends on growth in respiratory epithelium can be examined.

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