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Organ Cultures of Respiratory Epithelium Infected with Rhinovirus or Parainfluenza Virus Studied in a Scanning Electron Microscope

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Organ cultures of bovine tracheal epithelium were infected with a rhinovirus or a strain of parainfluenza type 3 virus, and the epithelial surfaces were studied by scanning electron microscopy. When washed free from mucus, normal control cultures showed a thick carpet of normal cilia, whereas the two viruses each produced specific morphological abnormalities. In rhinovirus-infected cultures, degenerating ciliated and nonciliated cells with finely granular surfaces were rapidly extruded from the epithelium. The denuded epithelial surface was relatively smooth, and showed some evidence of squamous metaplasia. By contrast, in cultures infected with parainfluenza type 3 virus, damage developed more slowly and the epithelial surface was ultimately covered with a profuse array of short microvillous projections. In thin sections, some of these were shown to be the sites of viral maturation.

Methods for the study of soft tissue surfaces in a scanning electron microscope have recently been developed, and normal ciliated respiratory epithelium has already been studied (2). It has also previously been shown that bovine tracheal tissue maintained in organ culture will support the growth of a number of bovine respiratory viruses (12). We now report the use of scanning electron microscopy in studies of the morphological changes occurring in bovine tracheal epithelium infected with a rhinovirus or a parainfluenza virus.

MATERIALS AND METHODS

Organ cultures. Cultures were prepared from tracheas of calves less than 3 weeks old (12). The tracheal wall was cut into fragments which consisted of an epithelial surface measuring about 3 by 4 mm, supported by underlying submucosa and cartilage. Four such fragments were placed in each plastic petri dish of 50-mm diameter, with the epithelial surface of each fragment uppermost. Eagle's medium containing penicillin (100 units/ml) and streptomycin (100 μg/ml) was added until the fluid just covered the epithelial surfaces. Cultures were incubated in a stationary state at 33 C in a humidified atmosphere of 5% CO_2 in air. Before inoculation, the cultures were washed four times in Hanks saline, and the medium was changed to Eagle's medium with 0.1% bovine albumin.

Viruses. The Sd-1 strain of bovine rhinovirus (3) and a bovine strain of parainfluenza 3 virus, PI-3 (1), were grown in primary cultures of calf kidney cells. Diluted tissue culture fluids were used to inoculate the organ cultures.

Titration of viruses. Ten-fold dilutions of fluid from infected organ cultures were titrated in tubes containing monolayers of calf kidney cells, and the end point of infectivity was calculated by the Karber method.

Ciliary activity. The epithelial surface of the cultures was observed by low-power microscopy with the use of reflected light. Ciliary activity was graded as normal or as slightly, moderately, or markedly impaired.

Virus infection of organ cultures. Cultures were inoculated by dropping 0.2 ml of tissue culture fluid containing 10^4 $TCID_{50}$ of rhinovirus or PI-3 onto the surface of the fragments. The medium was changed on the day after inoculation, and twice weekly thereafter. Samples of medium were also stored for titration of virus infectivity. Observations of ciliary activity were made, and at intervals fragments of tissue from infected and control cultures were fixed for scanning electron microscopy.

Preparation of cultures for scanning electron microscopy. Vigorous washing procedures were necessary to free the normal ciliated surfaces from mucus. In most cases, cultures were washed four times with Hanks saline, which was expelled through a 21-gauge needle at the rate of 0.3 to 0.5 ml/sec, so that the jet

of fluid impinged on the epithelial surface at an angle of 30° from the horizontal. For cultures in the later stages of infection, more gentle washing with a Pasteur pipette was adequate. Cultures were fixed at room temperature in Karnowsky's fixative (M. J. Karnowsky, J. Cell Biol. Abstr. **270**:137A, 1965) for 24 hr or longer. After fixation, the epithelial layer was sliced off from the underlying cartilage, and, after being washed in distilled water, the specimens were quenched in dichlorodifluoromethane cooled with liquid nitrogen at −150 C. They were then freeze-dried at −65 C, glued to 3.2-mm aluminum rivets, given conducting coatings of carbon and gold by vacuum evaporation, and examined in a Cambridge Stereoscan scanning electron microscope operated at 10 kv as described by Boyde and Wood (4).

Preparation of cultures for transmission electron microscopy. The tissue fragments were rinsed in Hanks saline and fixed by the method of Hirsch and Fedorko (6). The epithelium was sliced away from the cartilage and cut into smaller fragments before being embedded in araldite. Ultrathin silver-gray sections (60 to 90 nm thick) were cut with a Dupont diamond knife, stained in uranyl acetate and lead citrate, and examined in an AEI EM6B electron microscope.

RESULTS

Uninfected control cultures. The uninfected control cultures maintained good ciliary activity for the duration of the experiments. A few control cultures over 2 weeks old showed slight diminution of activity, and most cultures over 3 weeks old showed slightly diminished activity. Unless the control cultures were vigorously washed before fixation, examination by scanning electron microscopy showed that the ciliated surface was obscured to a variable extent by mucus. This appeared as extensive sheets, as rounded globules of very variable size, or as fine strands and filaments which partially or completely obscured the cilia. The diameter of the mucus strands was often about 100 nm or less.

In normal cultures observed at low magnification (50 to 500 times) in a scanning electron microscope, the longitudinal ridges of the normal tracheal epithelium and the openings of the submucous glands were well seen. At somewhat higher magnifications, in well-washed cultures (Fig. 1), the cilia appeared as a thick carpet of fine projections. These measured 250 to 300 nm in diameter. In controls fixed late in the course of the experiment, the carpet of cilia was again apparent, but a search of the surface of these cultures sometimes revealed small patches in which cilia were less densely packed. In these areas, it was possible to focus down between the cilia to the cell surface, which showed rather scanty, fine, fingerlike microvillous projections measuring 150 to 200 nm in diameter.

Cultures infected with rhinovirus. When an inoculum of about 10^4 infectious particles of rhinovirus per dish was used, ciliary activity became diminished by the fourth day of the infection and was almost completely absent by the eighth day. The titer of infectious virus in the medium reached a maximum of $10^{4.25}$ $TCID_{50}$ per ml on the fourth day and declined slowly during the succeeding days.

Infected and control cultures fixed on the sixth day after infection were examined by scanning electron microscopy. Figure 2 shows that a striking feature of this infection was the large number of cells seen extruding from the epithelium. These were mainly ciliated cells, many of which remained attached by a narrow neck, or a fine thread. Occasionally, goblet cells were also seen extruding from the surfaces. The extruding cells were examined under higher magnification (Fig. 3). The surface of these cells was generally finely granular; the clump of cilia at one pole of the cell remained intact, and there were only a few microvilli. Occasional perforations, such as those shown in Fig. 3, were considered to be "ice crystal artifacts" produced during the preparation of the specimen. They were not found in specimens freeze-dried from a saturated solution of chloroform in water as described by Boyde and Wood (4).

Figure 4 shows another specimen from a culture infected with rhinovirus 6 days previously. In this area, only a few tufts of cilia remained, and some degenerate ciliated cells were still attached to the surface. The exposed nonciliated surface was generally fairly smooth, with few microvilli (Fig. 5). There was also some evidence of squamous metaplasia, in the form of large, smooth-surfaced flattened cells from which cytoplasmic processes extended outwards over the deciliated surfaces.

In cultures fixed 8 days after infection with rhinovirus, appearances were generally similar to those found earlier. There was no clear evidence of regeneration of cilia. In view of the findings in PI-3-infected cultures (see below), a search was made in rhinovirus-infected cultures for microvilli. Small areas and individual cells were found in which the surface was formed of very low projections, 150 to 200 nm in diameter. These areas were much less common than in PI-3-infected cultures.

Cultures infected with parainfluenza virus. Diminution of ciliary activity occurred much more slowly in cultures inoculated with PI-3 than in those inoculated with rhinovirus. In the PI-3-infected cultures ciliary activity was slightly decreased by the seventh day after infection, and a marked decrease was noted by the eleventh day.

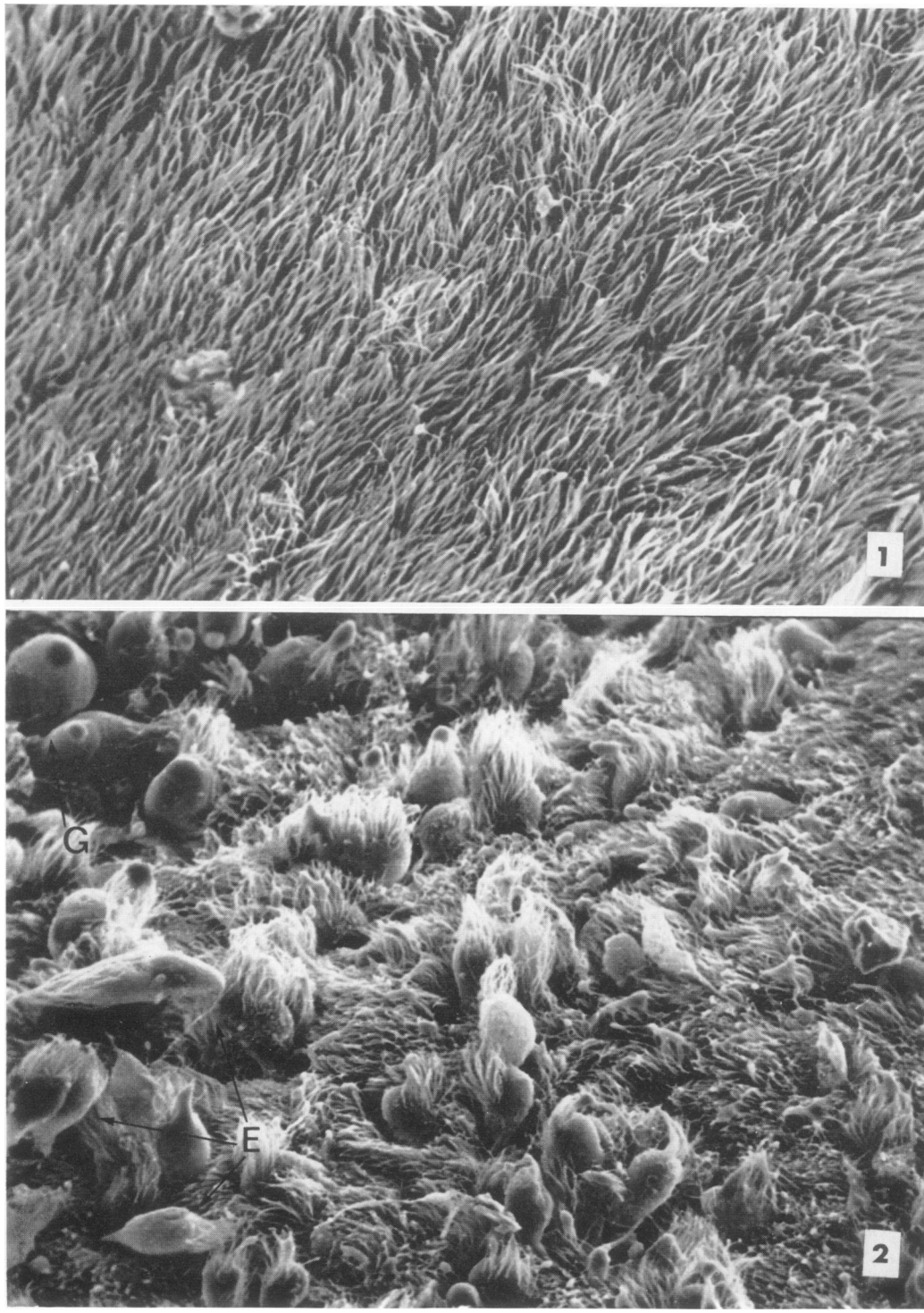

FIG. 1. *Normal control cultures. Field width, 100 μm.*

FIG. 2. *Culture infected with rhinovirus, fixed 6 days after inoculation. E, extruding ciliated cells, attached to epithelium by cytoplasmic strands. G, extruding goblet cells. Field width, 180 μm.*

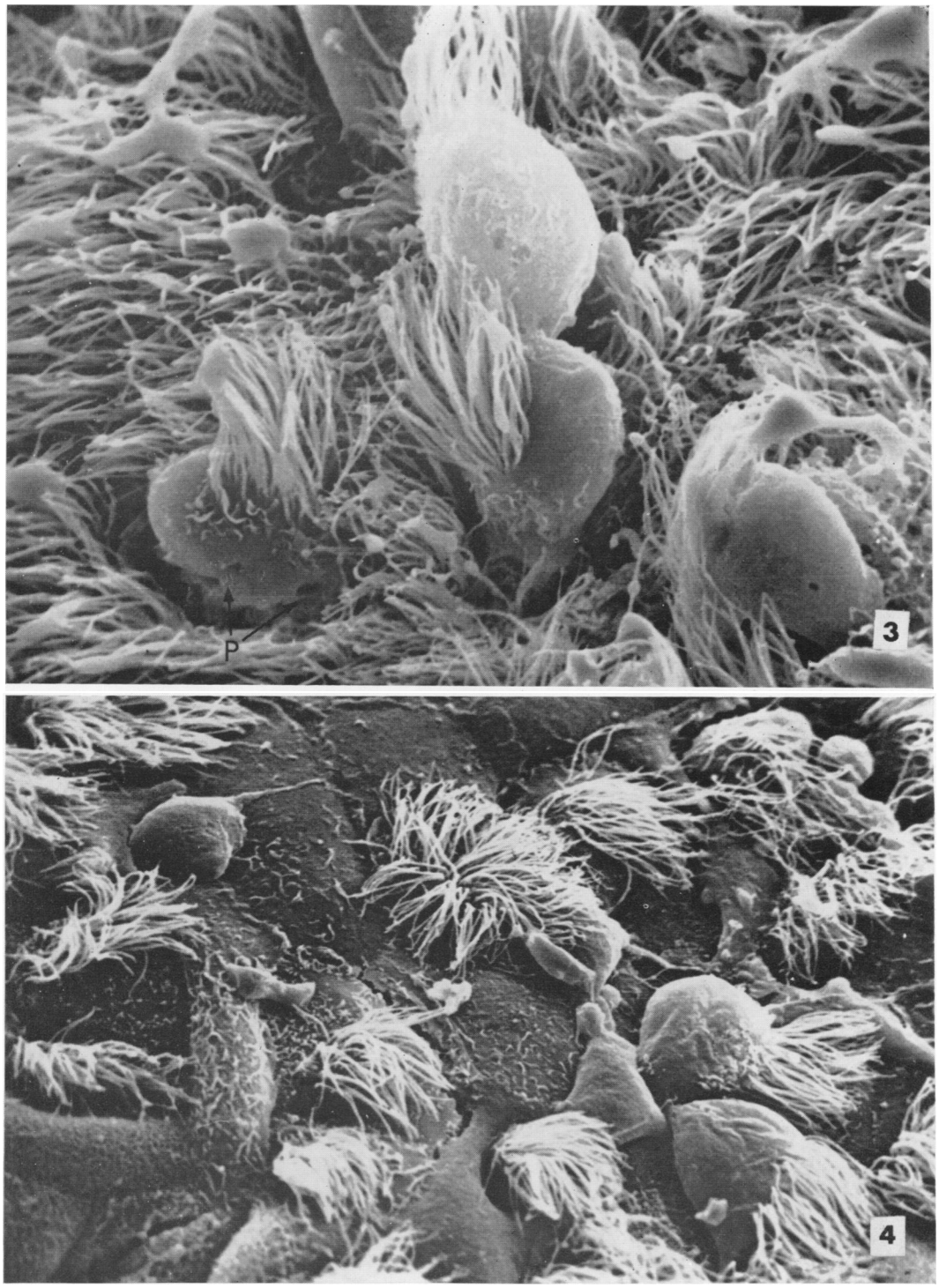

FIG. 3. *Higher magnification of part of Fig. 2. P, perforations. Field width, 55 μm.*
FIG. 4. *Rhinovirus-infected cultures fixed 6 days after inoculation. Field width, 100 μm.*

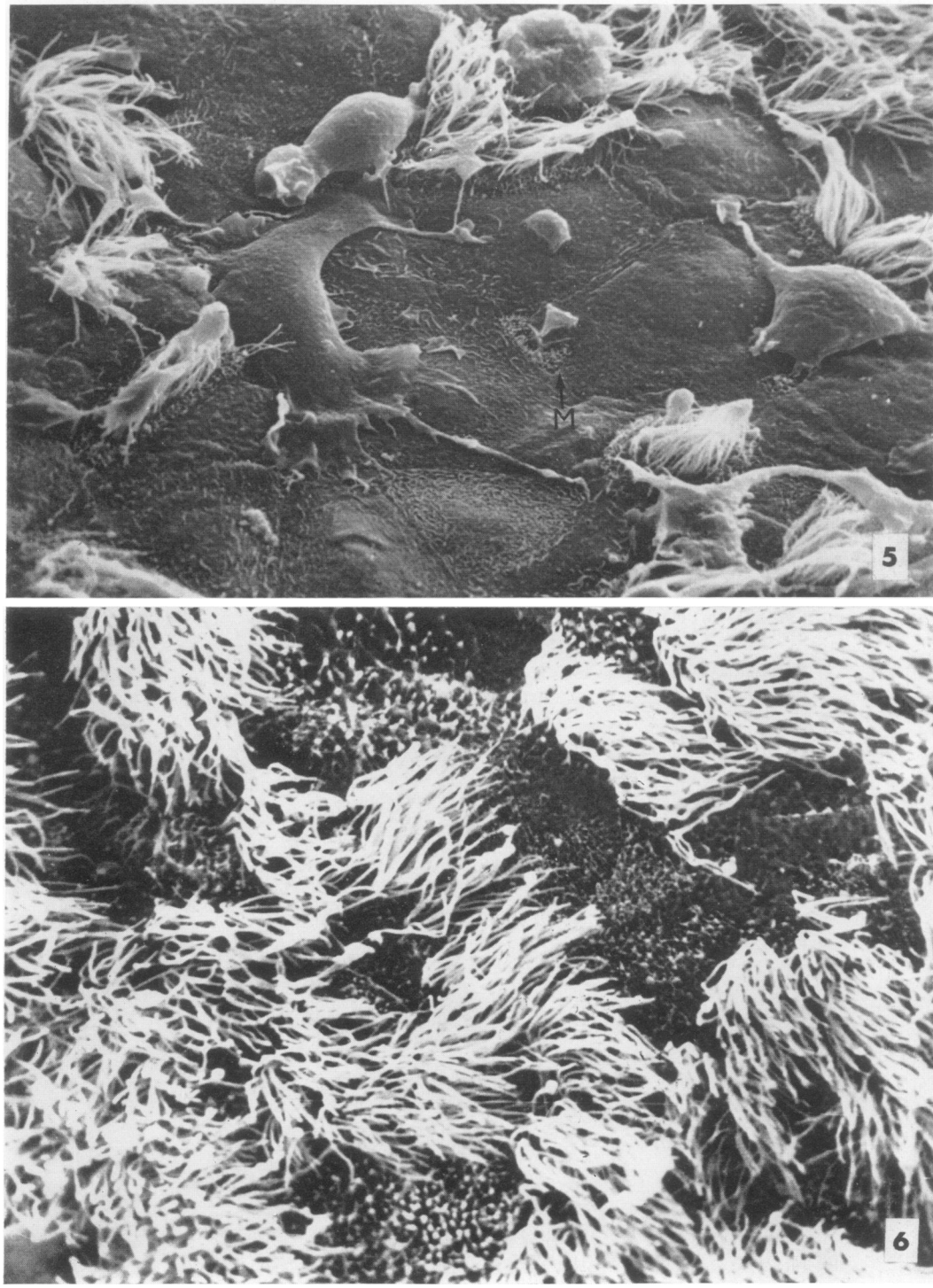

FIG. 5. *Rhinovirus-infected cultures fixed 6 days after inoculation. M, microvilli. Field width, 100 μm.*
FIG. 6. *Cultures infected with PI-3, fixed 10 days after inoculation. Field width, 45 μm.*

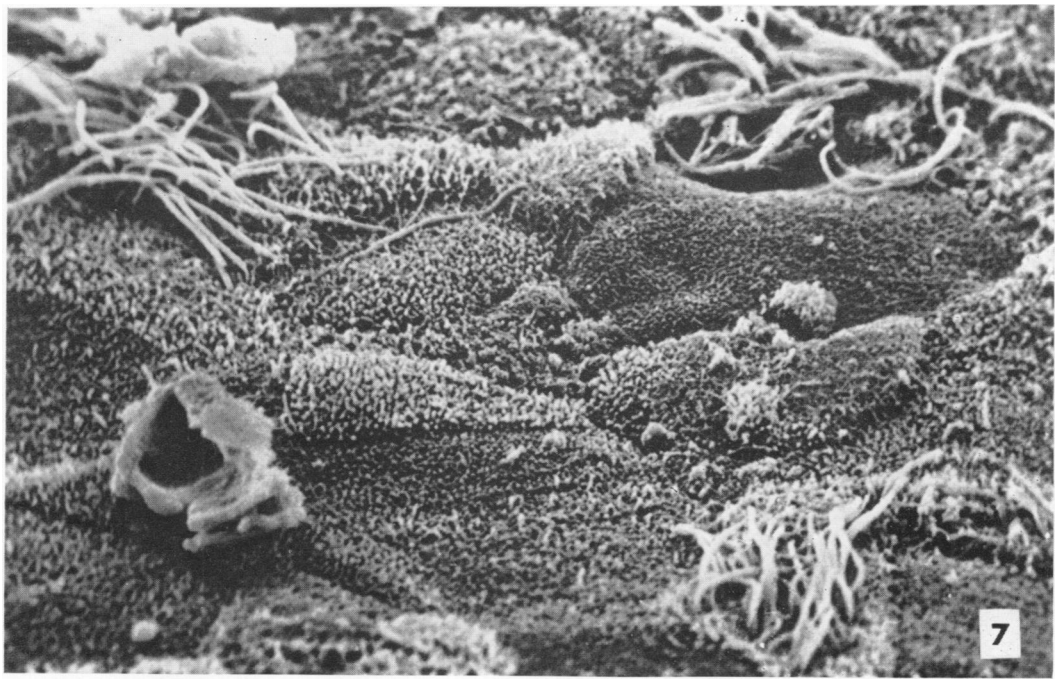

FIG. 7. *Cultures infected with PI-3, fixed 11 days after inoculation. Field width, 50 μm.*

The titer of virus in the medium reached $10^{5.5}$ $TCID_{50}$ per ml on the fourth day, and rose to a maximum of $10^{6.25}$ $TCID_{50}$ per ml on the ninth day.

By scanning electron microscopy, slight abnormalities were found in cultures fixed on the sixth day of the infection, and these changes occurred increasingly commonly in tissue fixed on succeeding days to the twelfth day. The early changes consisted of patchy thinning of the normal layer of cilia. This presumably occurred by slow loss of ciliated cells, but cells were only rarely seen in process of extrusion. As the thinning of the ciliary layer became more marked, the surface of the culture between the remaining cilia could be examined, and the exposed nonciliated surface was found to be profusely covered with short projections (Fig. 6). These were sometimes partly overlaid with a network or layer of mucus, and in some specimens this mucus layer adhered around the bases of the remaining cilia in a way which suggested that the normal mechanisms for transportation of mucus by ciliary action might be inefficient.

Figure 7 shows the appearance of the microvillous projections found replacing the normal ciliated surface in the later stages of infection with PI-3. In these cultures, both ciliated and nonciliated cells were occasionally seen extruding from the epithelium, but no specimens were found in which the extrusion process was seen as frequently as in the rhinovirus-infected cultures. Figure 8 shows a ciliated cell in process of extrusion from a culture infected with PI-3. Its surface, like that of the denuded epithelium, was

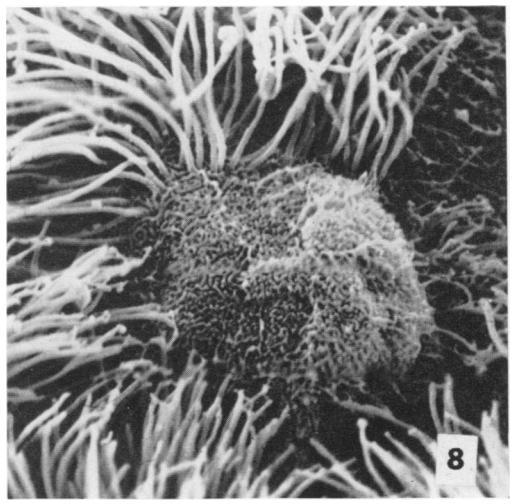

FIG. 8. *Cultures infected with PI-3, fixed 10 days after inoculation. Field width, 18 μm.*

covered with microvillous projections, which in this case measured 180 to 200 nm in diameter. Figure 9 shows at higher magnification a typical area of the surface of a culture infected with PI-3. The projections on these surfaces were short and rounded; their diameter varied slightly from cell to cell, but always fell within the range of 150 to 250 nm. The diameter of normal cilia was usually about 300 nm, and it seemed unlikely that the projections were stumps of cilia. Control cultures and rhinovirus-infected cultures were also examined for the presence of a similar surface, and small areas of microvillous projections similar to those found in PI-3-infected cultures were occasionally seen, especially near the cut edges of the tissue fragments. However, it was only in PI-3-infected cultures that the microvilli covered very large areas of the epithelial surface. Similarly, it was noted that the cells extruding from PI-3-infected cultures frequently had a microvillous surface, whereas those shed from rhinovirus-infected cultures did not.

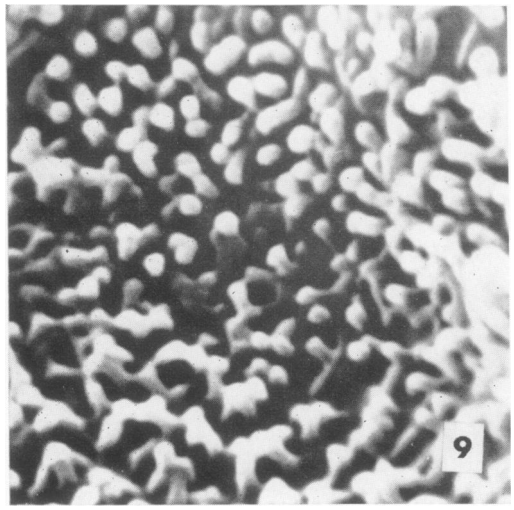

FIG. 9. *Cultures infected with PI-3, fixed 9 days after inoculation. Field width, 5 μm.*

It was considered that at least some of the projections on the surface of cultures infected with PI-3 probably represented budding parainfluenza virions, although it was not possible to identify this process specifically. Consequently, cultures which had been infected with PI-3 8 days previously, and which showed moderate or marked diminution of ciliary activity, were fixed for transmission electron microscopy. Sections of these preparations showed that the majority of the projections examined were bounded by a "unit membrane." However, some areas were also found in which the structure of the membrane was altered in a way that indicated viral maturation. In these areas, viral internal component

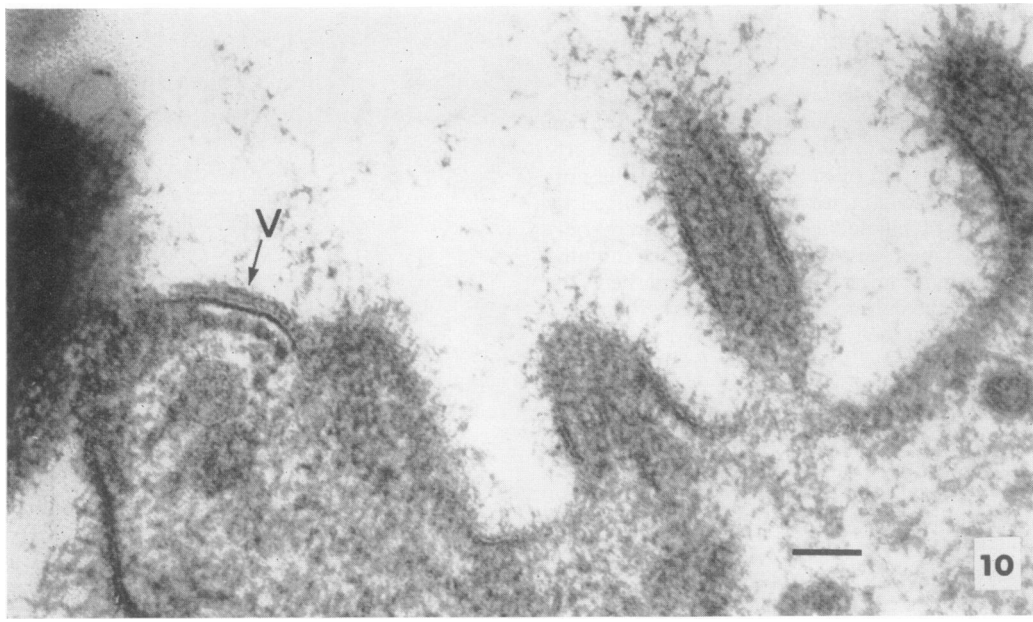

FIG. 10. *Thin sections of cultures infected with PI-3, fixed 8 days after inoculation. V, site of virus maturation. Bar = 100 nm.*

was present beneath a thickened membrane bearing an outer layer of spikes (Fig. 10). In some of the epithelial cells, cytoplasmic inclusions, consisting of accumulations of viral ribonucleoprotein, were also found, which were similar to those described by Compans et al. (5) in cells infected with parainfluenza virus SV5.

DISCUSSION

The scanning electron microscope can readily provide information about virus-infected surfaces which would otherwise be obtainable only by examination of very large numbers of thin sections by light microscopy and transmission electron microscopy. At low magnifications, the extent and distribution of the lesions produced by virus infection can be assessed, and at higher magnifications the detailed surface structure of virus-infected cells can be examined.

Examination of unwashed organ cultures of normal bovine tracheal epithelium in a scanning electron microscope showed only the layer of mucus which blanketed the normal ciliated surface, but, after the cultures had been thoroughly washed, the densely packed cilia were revealed. In contrast, specific changes followed infection with rhinovirus or PI-3. An input dose of 10^4 $TCID_{50}$ of rhinovirus established a rapidly destructive infection of the ciliated epithelium, from which many cells were shed. The histological appearance of cells ballooning from the surface of human nasal organ cultures infected with human rhinovirus were originally described by Hoorn and Tyrrell (8). Our studies in bovine cultures demonstrated that infection with the bovine rhinovirus resulted in extrusion of ciliated cells and goblet cells from large areas of the epithelium. These cells, which presumably were virus-infected and in process of degeneration, had a finely granular surface. It seemed likely that rhinovirus infection produced changes in the cell surface which rapidly led to detachment of the infected cell from its neighbors, and its elimination from the epithelium. The epithelial surface of cultures denuded by rhinovirus infection was relatively smooth and showed only a few microvilli.

In cultures inoculated with the same input dose of PI-3, epithelial damage was not detected until the sixth day, although moderately high titers of virus were present in the medium in the first few days of the infection. The reason for this is not clear. Possibly, virus was being replicated in some part of the culture other than the epithelial surface. It is also possible that in its early stages the epithelial infection was a moderate one, which involved minimal cell damage.

Histological studies of bovine tracheal organ cultures infected with PI-3 have shown abnormalities in all layers of the epithelium, with partial loss of ciliated cells (12). In the scanning electron microscope, the most striking feature of similar cultures was the mass of microvilli covering the exposed surfaces, including the surfaces of cells which were extruding from the epithelium. The appearance contrasted with the finely granular surface of cells in rhinovirus-infected cultures, and this may reflect the different ways in which the two viruses cause cell damage. Bovine PI-3, like other paramyxoviruses, buds from the surface of infected tissue culture cells (9, 11). Our own findings show that in infection of respiratory epithelium the virus buds from cell surfaces which are profusely covered with microvilli. It has been suggested that the behavior of the cell membrane is relevant to production of either a lytic or a moderate infection with parainfluenza virus SV5; in a lytic infection, this virus matured from a microvillous cell surface (5, 7). PI-3 eventually largely destroyed the epithelium of our organ cultures by a lytic infection, and the sites of viral maturation could be identified among the microvilli at the time of maximal release of infectious virus into the medium. The ability of the epithelial cells to form microvilli may perhaps be a prerequisite for, or a consequence of, lytic infection with this virus. The formation of microvilli has also been noted to be related to the development of polykaryocytes in virus-infected cultures (10). However, PI-3 did not form polykaryocytes in our organ cultures, although it did so in primary cultures of bovine kidney cells.

Although the scanning technique clearly showed the abundance of microvilli on the surface of the cultures infected with PI-3, the viral buds were distinguishable only by examination of thin sections. Methods of specifically identifying virus at the cell surface by use of a scanning electron microscope are being investigated.

ACKNOWLEDGMENTS

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