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HISTOLOGY AND ULTRASTRUCTURE OF METAPLASIA OF ALVEOLAR EPITHELIUM FOLLOWING INFECTION OF MICE AND HAMSTERS WITH INFLUENZA VIRUS

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Summary.—Mice and hamsters were infected with the NWS strain of influenza virus and metaplastic changes which developed in the lungs during the healing phase were studied by histology and electron microscopy. Transformation of alveolar epithelium occurred in many animals, producing alveolar epithelialization. This was due to replacement of the normal alveolar epithelium by ciliated columnar and cuboidal cells of bronchiolar type. Squamous changes also developed in healing lungs during the 11–22 day period after infection. Since these lesions regressed later and the cells did not metastasize they were considered to be metaplastic rather than neoplastic in nature.

A NUMBER of descriptions have been published of metaplastic or neoplastic lesions produced in the lungs of rodents by infection with influenza virus and by exposure to a variety of noxious chemical agents (Kotin and Falk, 1956; Kotin, Falk and McCammon, 1958; Gross *et al.*, 1965; Harris and Negroni, 1967; Staemmler, Foitzik and Heydenreich, 1970). The changes exhibited in pulmonary tissue undergoing repair after pneumonia, including what is usually termed " alveolar epithelialization ", constitute a perplexing sphere for the diagnostic and experimental histopathologist, since it is frequently difficult to determine whether lesions in healing lungs represent hyperplasia, metaplasia or true neoplasia.

For many years there has been considerable controversy as to the type of cells involved in alveolar transformation. The use of the electron microscope in the study of pulmonary disease should ultimately clarify this situation, but as yet it has not been applied to a sufficient range of lesions. Pulmonary adenomata induced by various substances in rodents (Svoboda, 1962; Hattori, Matsuda and Wada, 1965; Brooks, 1968) and adenocarcinomata occurring naturally in man (Adamson, Senior and Merrill, 1969; Coalson *et al.*, 1970) have been examined by electron microscopy and shown in nearly all cases to consist of proliferating type II pneumonocytes. This cell has also been found to be responsible for alveolar epithelialization in chronic pneumonia in sheep (Alley and Manktelow, 1971; Nisbet *et al.*, 1971) and in pigs (Baskerville and Wright, 1973).

The alveolar epithelial changes which are found in the healing lung, however, have received little attention from electron microscopists, even though it is well recognized that in man carcinoma of the lung can occasionally develop in areas of fibrosis produced by previous tissue damage or infarction (Balo, Juhasz and Temes, 1956; Meyer and Liebow, 1965; Fraire and Greenberg, 1973). In a histological study of pneumonia produced by influenza virus in mice and hamsters changes were observed in the healing lungs which suggested that this system might provide

new and useful information on pulmonary metaplasia, and in particular on epithelialization. An ultrastructural investigation of the healing pneumonia was therefore undertaken, and in addition a selection of the histological material was processed for electron microscopy in order to correlate the histological appearance of the lesions with the identity and fine structure of their constituent cells.

MATERIALS AND METHODS

Four hundred female outbred Porton albino specific-pathogen-free mice, 3-weeks old at the time of infection, and 120 three-month old female golden hamsters were used in the infection experiments. A mouse-adapted NWS strain of influenza A virus was used for infection of all the animals and a pool of this virus was prepared by inoculating it into the allantoic cavity of 11-day old embryonated eggs and harvesting the fluid after 3 days' incubation at 37°. This stock virus was stored in aliquots of 10 ml at − 70° and had a titre of 6×10^5 egg infective units per 0·05 ml, which was the volume of virus suspension given to each animal.

All animals were infected by instilling 0·05 ml of virus pool suspension into the nostrils under light ether anaesthesia, care being taken to ensure that the entire volume of the drops was inhaled into the nostrils and retained. As control uninfected animals, 250 female mice of the same strain and age were given intranasally 0·05 ml of a formalin killed vaccine prepared from the NWS strain pool by the method recommended in the *British Pharmacopoeia* (1968). A further 50 mice and 25 hamsters were inoculated intranasally with 0·05 ml of sterile physiological saline.

A large number of the infected mice died in the early stages of the disease but groups of 5–10 animals from the survivors and from controls were killed with ether at each of the following stages after inoculation: 24 hours daily to 14 days, at 16 and 17 days, at weekly intervals from 3 to 8 weeks, and then at monthly intervals to 6 months.

At necropsy the lungs were removed from each animal, fixed by immersion in 5% formol saline and processed by standard histological methods. Paraffin sections cut at 5 μm were stained with haematoxylin and eosin, and selected sections were also stained by Gordon and Sweets' method for reticulin fibres. For electron microscopy of fresh lung tissue 1 mm cubes of areas showing lesions grossly were excised and fixed for 2 hours at 4° in 4% cacodylate buffered glutaraldehyde. After post-fixation in 1% osmium tetroxide for 2 hours the tissues were dehydrated and embedded in epoxy resin (Epon). Where necessary for the location of suitable areas, sections were cut at 1 μm and stained with Azure II. Ultra-thin sections were stained with uranyl acetate and lead citrate (Venable and Coggeshall, 1965).

Since the small residual late-stage lesions were not visible grossly, stained histological sections had to be used to provide material of this type for electron microscopy. The reclamation of paraffin sections for this purpose was carried out by the " open-face " embedding techniques described by Takeda (1969) and by Rossi, Luginbühl and Probst (1970).

Paraffin blocks were also subsequently used for electron microscopy and the method employed for their preparation was as follows: the required area was selected on the cut surface of the block with the aid of a section prepared from it, and an approximate 1 mm cube was excised with a scalpel. This was found more satisfactory than melting the paraffin, after which location of the desired area was often difficult or impossible. The block so obtained was next placed in 2 changes of xylene for a total of 1 hour and passed through the customary descending range of alcohols: for half an hour in each of 3 changes of absolute alcohol and half an hour each in 90%, 70% and 50% alcohol. The block was then immersed in cacodylate buffer for 1 hour, stained for 2 hours with 1% osmium tetroxide, dehydrated through an ascending series of alcohols and embedded in epoxy resin (Epon).

RESULTS

Gross findings

Areas of dark red consolidation were visible grossly in the lungs of most infected animals from 24 hours to 21 days after infection. The distribution of the lesions was random but no lobe remained regularly unaffected and the dorsal border of the left lung and the upper lobes of the right were those most consistently involved.

After the third week the lesions were pale purple-grey and occurred much less commonly than in the early stages of the disease. The surfaces of the lungs of all control animals appeared normal.

Histological findings

The changes which took place in the lungs of the mice and hamsters in the acute stage of the influenza infection from 24 hours to the 7th day were of a necrotizing and exudative nature. There was widespread necrosis of bronchial and bronchiolar epithelium and destruction of alveolar epithelium and interalveolar septa. This was accompanied by capillary congestion, exudation of oedema fluid and fibrin into alveoli, and even frank alveolar haemorrhage. There was also moderate localized infiltration of interalveolar and interlobular septa by polymorphonuclear leucocytes (PMN). The acute changes were thus the same as those described by earlier workers for infection with influenza A virus in man and experimental animals (Straub, 1937; Francis and Stuart-Harris, 1938; Hers, 1955).

Repair was first detectable in the lungs of both species on the 7th day. At this time there was an overlap with the acute process, and in some sections in which repair had commenced a few small foci of necrosis were still present. The healing phase was characterized by renewal of the epithelium of airways and by activation and proliferation of fibroblasts and macrophages in damaged airways and interalveolar septa. In addition, the distal lung tissue was infiltrated by moderate numbers of lymphocytes and plasma cells.

By the 11th day organization of affected regions had resulted in greatly thickened and fibrosed interalveolar septa (Fig. 1). Alveoli were irregular in size and contour, some appearing much smaller than normal whereas many others were greatly enlarged and distorted (Fig. 1). The enlarged airspaces were composite structures

EXPLANATION OF PLATES

FIG. 1.—Hamster lung 11 days after infection with NWS influenza virus. Interalveolar septa are thickened and alveoli distorted. Some alveoli have simple squamous epithelium but the majority are " epithelialized ". H. and E. × 200.

FIG. 2.—Mouse lung 12 days after infection. Many alveoli are filled by groups of squamous cells. H. and E. × 125.

FIG. 3.—Higher magnification of Fig. 2 showing infiltration of the tissue by squamous cells. Re-epithelialization of the small airway by a single layer of flat epithelial cells is taking place. H. and E. × 300.

FIG. 4.—Mouse lung at 14 days. There is squamous metaplasia of the epithelium of the lower right portion of the terminal bronchiolus and nests of squamous cells extend from this into adjacent alveoli. H. and E. × 300.

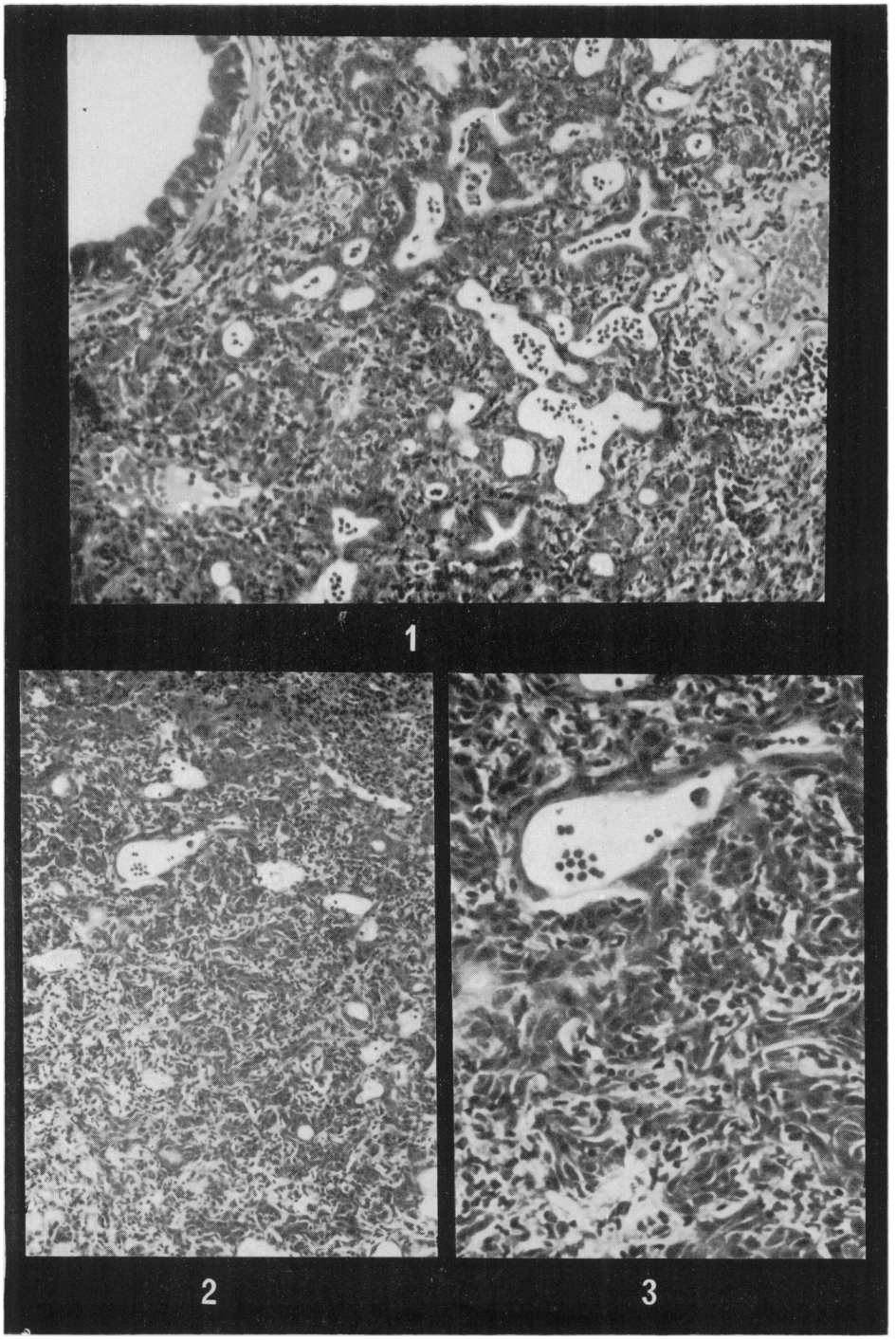
FIG. 5.—Hamster lung 5 weeks after infection showing residual peribronchiolar alveolar epithelialization. H. and E. × 150.

FIG. 6.—Higher magnification of Fig. 5. The alveoli opening directly into the respiratory bronchiolus (Rb) and those adjacent to it are lined by columnar and high cuboidal epithelium. H. and E. × 350.

FIG. 7.—Area of alveolar epithelialization in hamster lung at 6 weeks. The alveoli (a) are lined by high cuboidal and ciliated columnar epithelium. 1 μm Epon-embedded section. Azure II stain. × 500.

FIG. 8.—Electron micrograph of electron microscopical section prepared from the paraffin section shown in Fig. 5. The alveolar epithelium is of ciliated columnar type. Uranyl acetate and lead citrate. × 7200.

FIG. 9.—Group of squamous cells in mouse lung at 12 days. The cytoplasm contains tonofibrils. One cell is undergoing mitosis. Uranyl acetate and lead citrate. × 6000.



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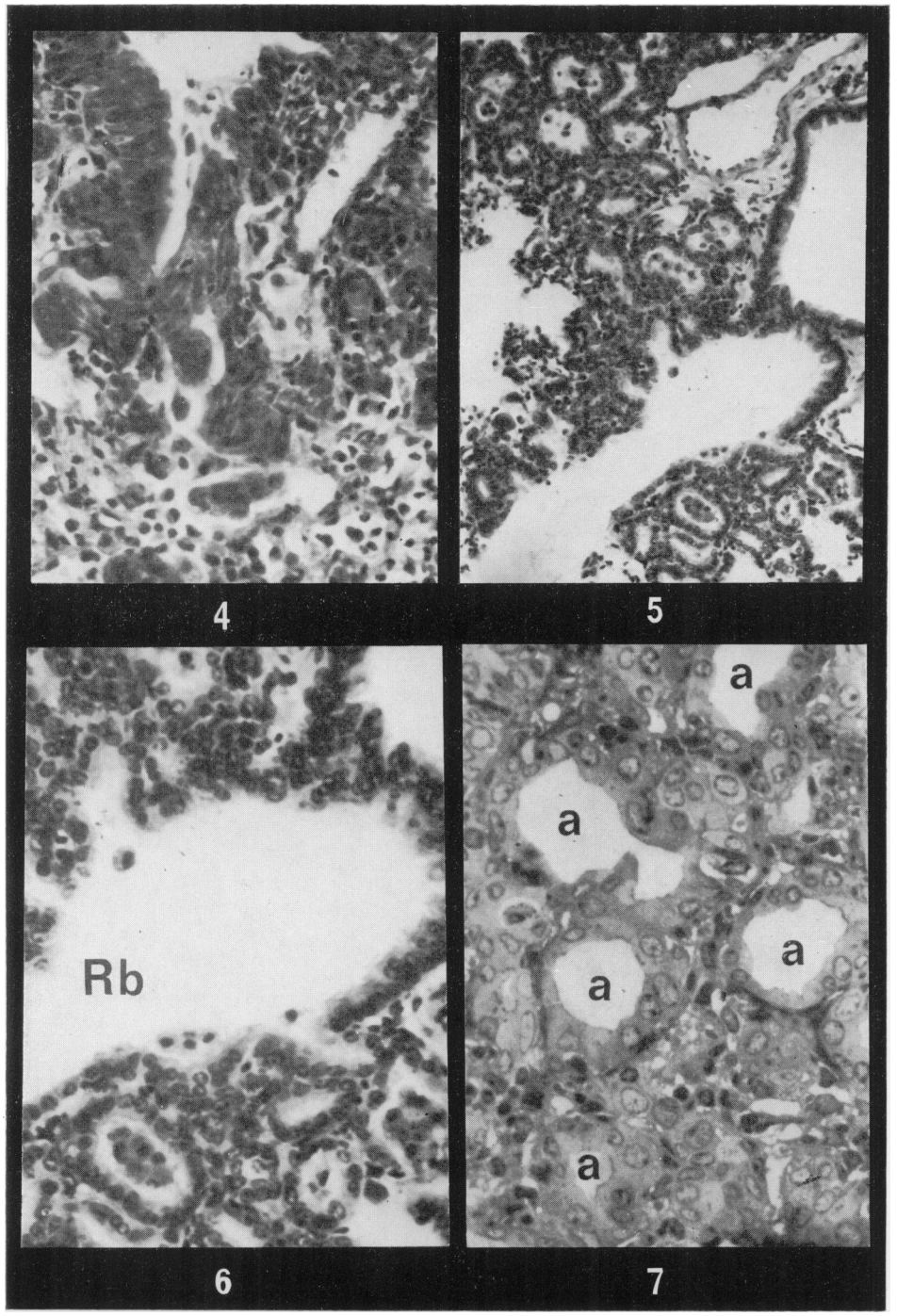

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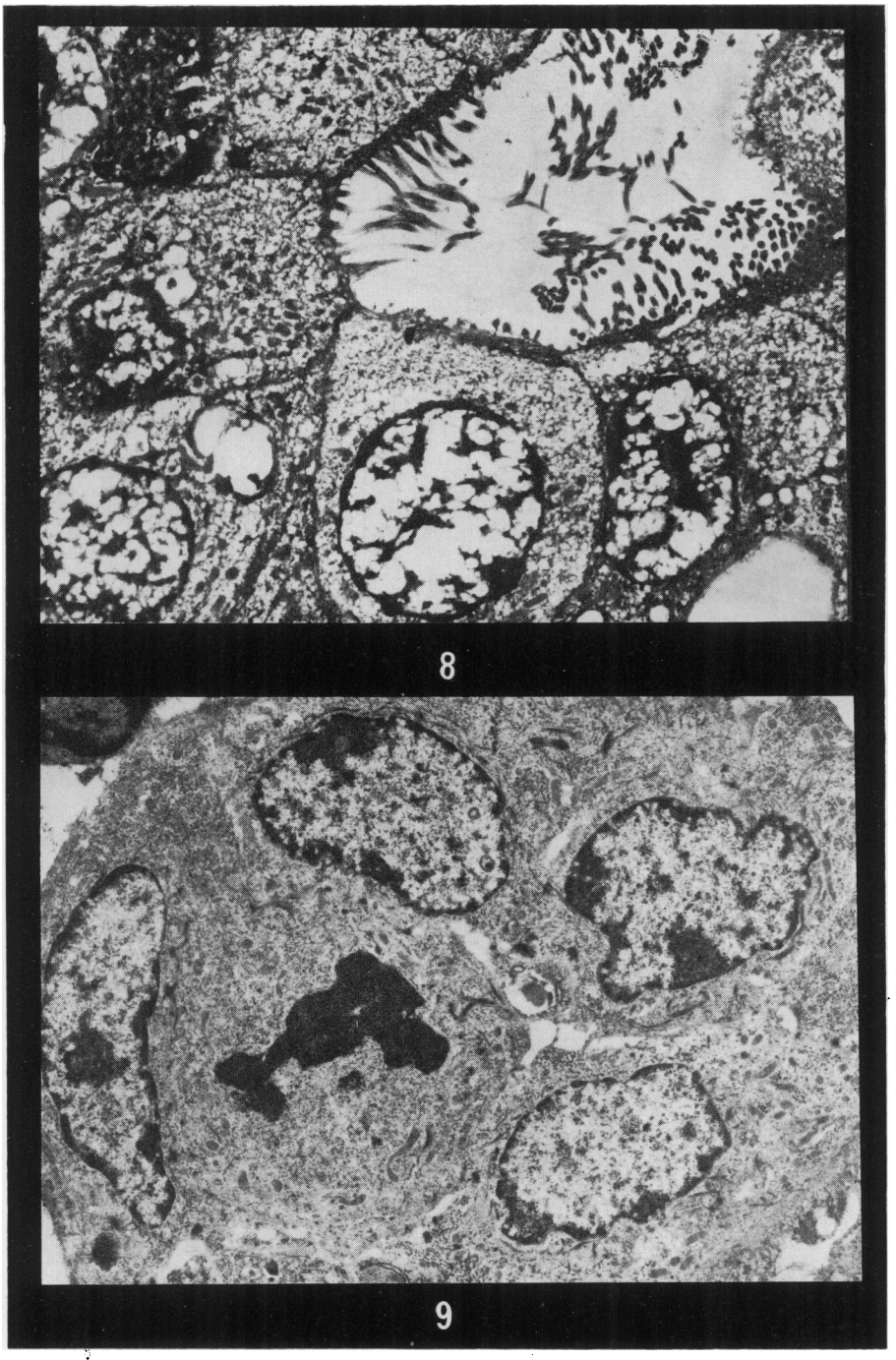

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probably formed by the disruption of alveolar walls. These could be distinguished from severely damaged large bronchioli by their lack of smooth muscle. Most alveoli and small airways were lined by flat cells with attenuated cytoplasm and elongated narrow nuclei (Fig. 1, 3). This pattern of lesions constituted the general appearance common to severely damaged lungs of animals killed between 9 days and 3 months after infection.

Two other types of cellular changes were superimposed on this in some areas. The more commonly occurring of these was a " glandular " transformation or epithelialization of alveoli. Many alveoli, of both normal and irregular shape, were lined entirely by high cuboidal or columnar epithelial cells so that they resembled bronchioli (Fig. 1). This was found in the lungs of 34 out of 75 (45·3%) mice and of 11 out of 45 (24·4%) hamsters killed between 11 days and 3 months after infection. Reticulin stains showed that these alveoli had a well developed basement membrane. At high magnification cilia could be seen at the apical surface of many of the columnar alveolar cells.

A less common change was the presence of nests of faintly basophilic, non-keratinizing squamous cells in alveoli and small airways, which in many cases completely occluded the airspace (Fig. 2, 3). A moderate number of mitoses was seen in these cell groups. This squamous metaplasia was found in the lungs of 11 out of 50 (22%) mice but in only 1 out of 15 (6·6%) hamsters killed between the 11th and the 22nd day. It did not occur outside this period. In some areas the clumps of squamous cells were situated in alveoli opening into respiratory bronchioli and were in close proximity to terminal bronchioli which exhibited squamous metaplasia (Fig. 4), indicating that the squamous cells may arise from the epithelium of terminal bronchioli.

The lengths of time for which the fibrosis persisted varied considerably and depended on the extent of the initial tissue damage. Smaller areas of damage resolved completely by the end of the 3rd week though larger ones remained as localized fibrosis of interalveolar septa at 6 months. At 4 weeks after infection most lungs were again normal but in some animals killed after this time small areas of well defined alveolar epithelialization were present and these were consistently situated in peribronchiolar tissue (Fig. 5). In such areas the epithelium of the respiratory bronchiolus was cuboidal or ciliated columnar in type as in the earlier lesions. This epithelium also lined the related alveolar duct and all adjacent alveoli, including those opening directly into the respiratory bronchiolus (Fig. 6).

Control lungs.—No abnormalities were detected in the lungs of animals used as controls.

Electron microscope findings

The electron microscope showed that in the repair phase of influenza from the 9th day the massive thickening of interalveolar septa was the result of infiltration by macrophages and lymphocytes, and of proliferation of fibroblasts, accompanied by deposition of large amounts of collagen. Renewal of epithelium in denuded areas and in the non-epithelialized newly formed enlarged airspaces was brought about by invasion of elongated cells with long attenuated cytoplasmic processes. These cells had the morphology of type I pneumonocytes and presumably migrated from areas of undamaged epithelium. In the earliest stage, and in later stages in severely damaged areas, this covering process was not precise and large spaces were

often visible between the new cell and the underlying tissue. Basement membrane was also lacking in the early stage in many areas and was formed only later.

Alveoli which by light microscopy were epithelialized (Fig. 7) were either lined completely by ciliated columnar epithelial cells of normal bronchiolar type, by cells with the same morphology as this but lacking only cilia, or by a mixture of these cell types and a small proportion of cuboidal cells having microvilli at the apical surface (Fig. 8). The nucleus and cytoplasmic organelles of all these cells were typical of ciliated bronchiolar epithelium of the normal rodent lung, the only slight difference being an apparent decrease in the quantity of rough surfaced endoplasmic reticulum and in the number of mitochondria present. There were no lamellated inclusions in the cytoplasm. Small clumps of glycogen, as found in the normal animal, were seen in a number of the epithelial cells. The lumina of a few alveoli contained small groups of degenerating inflammatory cells and cellular debris.

A striking feature was the complete absence of type II alveolar epithelial cells in damaged non-epithelialized areas. These cells had been destroyed along with other tissues in the necrotizing acute stage and they were not apparently replaced during the period studied. No precursor cell with an obvious relationship to the type II pneumonocyte was identified.

The squamous cells seen in some lungs were well differentiated and had the ultrastructural morphology of normal squamous cells. They were irregular in shape, with many cytoplasmic processes, numerous bundles of tonofibrils in the cytoplasm and a prominent nucleolus. These cells usually occurred in small groups (Fig. 9) and adjacent members were joined together by desmosomes formed at the point of contact of their cytoplasmic processes. All the squamous cells had numerous desmosomes. A number of the cells in the squamous nests were undergoing mitosis (Fig. 9).

DISCUSSION

The alveolar epithelialization which developed in rodent lungs after infection with influenza virus in these experiments was due to replacement of the normal epithelium by ciliated columnar cells of bronchiolar type. This is in contrast to the ultrastructure of epithelialization in chronic pneumonia in man (Okada and Genka, 1966), in sheep (Alley and Manktelow, 1971) and in pigs (Baskerville and Wright, 1973), in which the cuboidal metaplasia was shown to be caused by proliferation of type II pneumonocytes which completely lined the alveoli. Epithelialization is a nonspecific change induced by a wide variety of agents, but this difference in response may reflect the different nature of the exciting stimulus, since these latter conditions were long-standing and probably caused by relatively mild stimuli which produced little tissue destruction, whereas the lesions in this study developed early in the repair phase following an acute necrotizing virus infection. These results show that alveolar epithelialization is not always due to proliferation of type II cells, as has been increasingly assumed, but may also be caused by conversion of the respiratory epithelium to that of a conducting bronchiolar type. Before the advent of the electron microscope a number of workers who studied scarred lungs (Horning, 1950; King, 1954; Spencer and Raeburn, 1956) suggested that epithelialization was due to distal extension of bronchiolar epithelium, a fact which reflects the embryological relationship of the alveolar epithelium to that of the airway. Spencer and Raeburn also emphasized that in the adult the terminal

bronchioli retain their embryonic capacity for growth and proliferation, so that damage may stimulate them to produce ciliated or non-ciliated columnar cells or even squamous cells. The production of squamous cells and alveolar metaplasia by terminal bronchiolar epithelium in the present study illustrates this potential and extends the observation made by Meyer and Liebow (1965) on the close association of atypical acinar and squamous proliferation in scarred human lungs.

The nests of squamous cells which developed in the rodent lungs in our experiments were similar in appearance and distribution to those reported after influenza infection by other workers (Leuchtenberger and Leuchtenberger, 1966; Harris and Negroni, 1967; Staemmler *et al.*, 1970) and were present for a comparable period. Squamous changes and squamous carcinomata have also been induced in the lungs of rodents by exposure to various chemical agents, including urethane (Tannenbaum, 1966), cigarette smoke (Leuchtenberger and Leuchtenberger, 1966), dibenzanthracene (Gross *et al.*, 1965), and a range of hydrocarbons (Kotin and Wiseley, 1963). Most authors have stressed the difficulty of determining whether the squamous changes are metaplastic or neoplastic and recognize that many of the lesions may be interpreted in either way. Those induced by the chemical carcinogens are usually definitely carcinomatous, but the squamous cells which arise during the early repair phase after influenza infection were considered not to be neoplastic by Harris and Negroni (1967), who were unable to transplant them, and the present work confirms this. The lesions reported here did not form grossly visible tumours, did not metastasize and were not seen after the 22nd day. Although there are no reports of electron microscopy of the squamous changes in rodents for comparison, the ultrastructure of the cells described here was that of normal squamous cells, since they possessed numerous bundles of cytoplasmic tonofibrils and a large number of desmosome junctions with neighbouring cells, features which are lost by invasive neoplastic squamous cells.

The lack of type II epithelial cells after influenza infection is particularly interesting and appears not to have been recognized previously. It is similar to their absence from the lungs of pigs undergoing repair after widespread tissue destruction resulting from infection with another necrotizing virus, that of Aujeszky's disease (Baskerville, 1973) and may therefore be a common sequel to tissue destruction. If, as is generally held, the type II pneumonocytes are responsible for the production of pulmonary surfactant, then their absence during healing after pneumonia must result in serious impairment of aeration and stability of alveoli in large areas of the lungs.

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