THE HISTOLOGY OF "GREY LUNG VIRUS" LESIONS IN MICE AND COTTON-RATS.

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THE biological and physical characters of grey lung virus, a pneumotropic agent affecting mice, were briefly described by Andrewes and Glover (1945), and some account was given of the histopathology of the lesions in mouse lung. The virus has most unusual properties in that both it and the lesions it produces persist apparently indefinitely in infected animals. Though the virus is still present in large amounts and no immunity is demonstrable, the lesions remain almost static for many months and rarely lead to death. These facts, together with a later finding that grey lung virus is sensitive to some antibiotics, suggest that it may belong to a distinct virus group and call for a more detailed description of the histogenesis of the lung changes. This is all the more necessary as similar agents have been discovered by Dr. K. Goodner (1950, personal communication) in mouse stocks in Philadelphia ("Potash Virus"), in cotton rats by Dr. H. Rose (1950, personal communication) in New York, and also in normal cotton rats in this Institute (Andrewes and Niven, 1950).

METHODS.

Grey lung virus produces pathological changes only in the lungs, and in the more recent studies in mice and cotton rats, examination was limited to these organs.

The thoracic contents were removed *in toto*, the heart usually excised and the remainder immersed in fixative. The containers were placed immediately in a desiccator in which a partial vacuum (60 mm.) was obtained by means of a water pump. It is important not to increase the vacuum beyond this level, and when evacuation of air from the lungs is complete, air must be allowed to re-enter the desiccator slowly. In most instances, corrosive sublimate containing 4 per cent glacial acetic acid was used as the fixative: Bouin's fluid, Zenker-formol, Zenker's fluid + 4 per cent glacial acetic acid and formol-saline were occasionally employed. After the air had been exhausted, an oblique cut was made through both lungs and the tracheo-bronchial lymphatic glands with the object of having a plane of section through as many lobes and structures as possible. In a few cases, the skin was removed from the thorax and the whole animal immersed in formol-saline for four days (M. Straub, 1945, personal communication) after which the lungs were cut out and fixation was continued in corrosive sublimate alone for 24 hours. This allowed an accurate appreciation of the amount of fluid and air respectively in the lungs, but is, of course, an unsuitable technique for general histological purposes. Many staining methods have been employed—

haematoxylin and eosin, Mann's stain, phloxine-methylene blue, Giemsa's mixture, orcein for elastic tissue and various trichrome methods for collagen and reticulum fibres and a coelestin blue technique (W. Penny, 1948, personal communication) for mucin.

RESULTS.

The disease in mice: Histogenesis.

The interalveolar septa, 48 hours after inoculation, are more conspicuous owing partly to capillary congestion and also to enlargement of the septal cells. When the lungs are examined in their entirety, a few small areas of consolidation involving only two or three adjacent alveolar sacs can always be found. These tiny foci consist almost entirely of polymorphonuclear leucocytes, with only a small admixture of desquamated, slightly enlarged alveolar septal cells, and occur most commonly below the pleural surface, although they are occasionally seen more deeply in the lung substance close to the hilum (Fig. 1). After a further 48 hours, the prominence of the alveolar septa is yet more pronounced; they contain an excess of polymorphs, and the septal cells are not only enlarged and projecting into the alveolar spaces but are increasing slightly in numbers. The foci of consolidation have increased in number and size, and may be just visible macroscopically. By the sixth day, the areas of consolidation have enlarged considerably and are easily visible (Fig. 2). Although polymorphs still predominate, many septal cells now lie free amongst them in the alveolar spaces. At this stage the cells have an oval or round vesicular nucleus and fairly abundant sharply-defined eosinophilic cytoplasm; mitotic figures are quite numerous both *in situ* on the septal walls and in the alveolar spaces; phagocytic activity is also evident, polymorphs in all stages of disintegration being found within them. Almost all the alveolar spaces, whether or not they contain cells, show fluid exudate, and perivascular oedema of medium-sized and small arterioles is also apparent. Until this stage, no change has been found in the epithelial lining of the respiratory tract, but from the sixth to eighth day onwards, a slight

EXPLANATION OF PLATES.

FIG. 1.—Primary focus of intra-alveolar consolidation composed of polymorphonuclear leucocytes and desquamated septal cells: 48 hours after infection. × 180.

FIG. 2.—Six days after infection: large area of consolidation consisting of polymorphs and small numbers of septal cells. Bronchioles and alveolar ducts are patent. × 70.

FIG. 3.—Enlargement and detachment of alveolar septal cells which stain intensely and have sharply defined outlines: 8 days after infection.

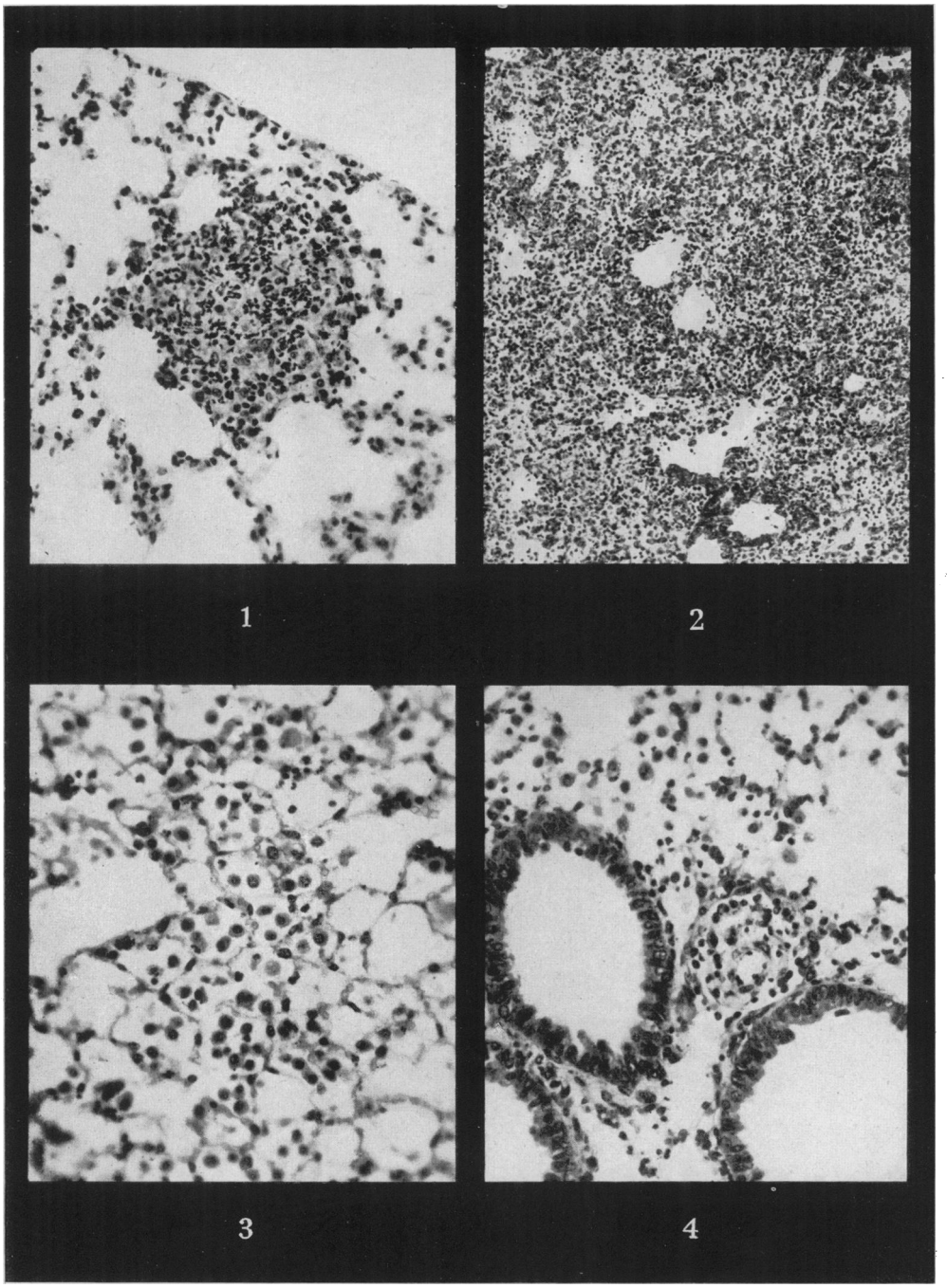
FIG. 4.—Hyperplasia of bronchiolar epithelium: an arteriole in the centre of the field shows marked perivascular oedema and a few infiltrating lymphocytes: 8 days after infection. ×245.

FIG. 5.—One month after infection: bronchi, bronchioles and alveolar ducts remain patent; alveolar sacs contain fluid exudate and varying numbers of cells. Mononuclear cells surround bronchi and blood vessels. × 55.

FIG. 6.—Six months after infection: dilated lymphatic channels amongst the plasma cells surrounding blood vessels. × 70.

FIG. 7.—Four months after infection: greatly enlarged and vacuolated alveolar cells. The interalveolar septa show increased cellularity. × 245.

FIG. 8.—Ten months after infection: the spaces in the lung substance have been formed by the disintegration of intra-alveolar cells and related septa. × 245.



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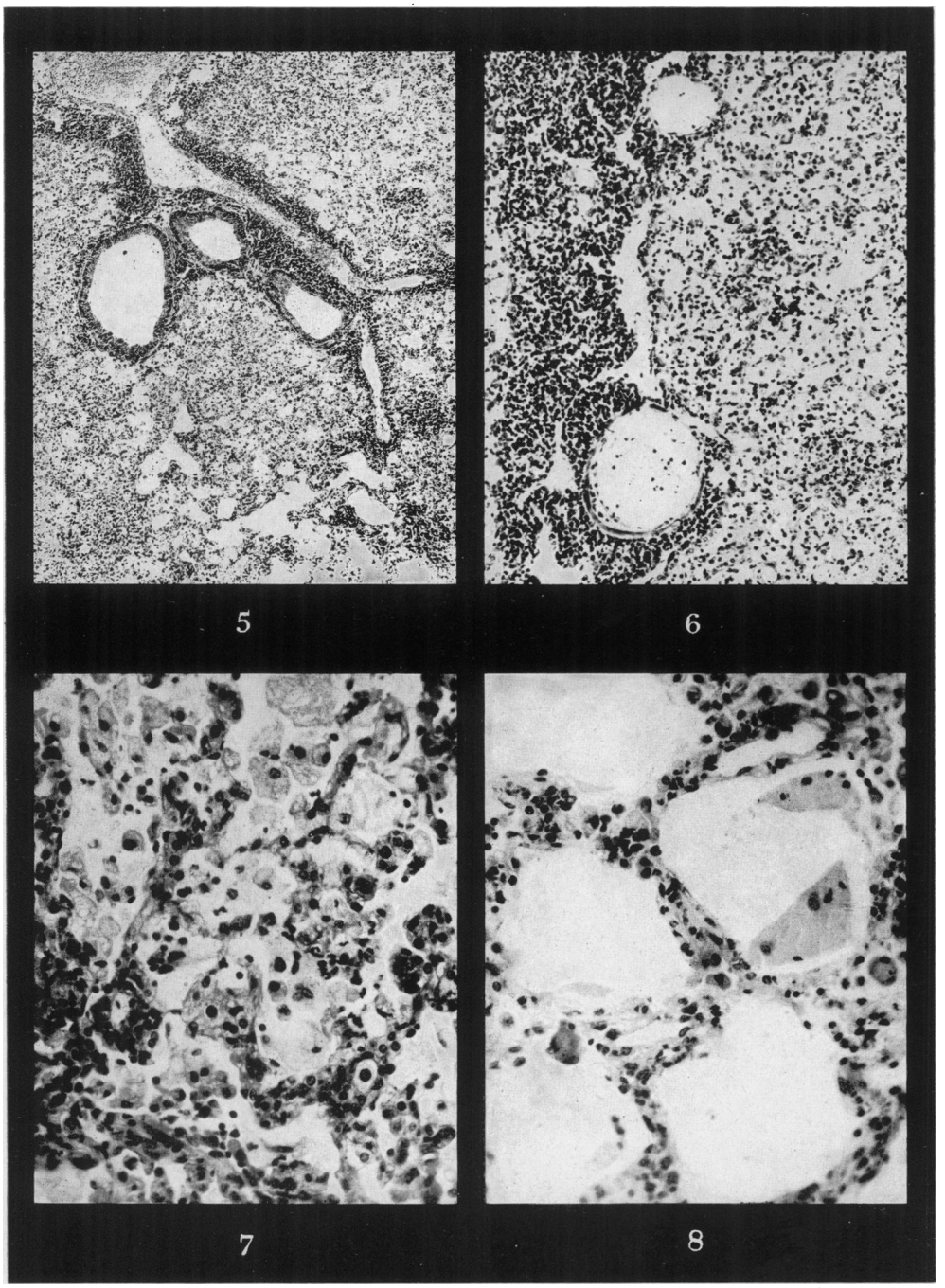

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but definite increased cellularity of medium-sized and smaller bronchi is obvious, mitotic figures being readily found. The newly-formed cells are packed closely together on the basement membrane, and the hyperplasia does not produce any fundamental alteration of the epithelium (Fig. 4). It must be emphasized at this point that these tissue alterations are not identical in extent either in each lung or in all the animals of a series inoculated at the same time, nor is there any localization in any particular lobe although the small azygos lobe does seem to be consistently involved at an early stage. Moreover, the rate of evolution of the lesions varies considerably; at the eighth day, when three out of six animals are showing considerable consolidation, the remainder may have only small subpleural foci, interstitial infiltration of polymorphs and early mobilization of the septal cells. The extent of the polymorph reaction is also another variable feature; it is primarily focal, and is the most conspicuous element of the initial reaction to inoculation, but mobilization of the septal cells is not everywhere in the lung preceded by an outpouring of polymorphs (Fig. 3). By the tenth day, a loose infiltration of mononuclear cells of lymphocyte type surrounds many of the arterioles and obscures the perivascular oedema. Many of the polymorphs have disappeared, partly by ingestion and partly by way of the lymphatics, degenerate forms being readily found in the sinuses and within the reticulo-endothelial cells of the tracheo-bronchial lymphatic glands. By the twelfth day, hyperplasia of the mesothelial lining of the pleura is evident, at first focal in relation to well-developed alveolar lesions and later generalized. Lymphocytes now occur around the bronchi and bronchioles, and the epithelial hyperplasia observed at an early stage is now evident throughout the entire bronchial tree.

Intra-alveolar oedema becomes generalized, and the subsequent evolution of the lung lesions is brought about by changes in the various cellular elements which have appeared during the first twelve days in response to the introduction of the causative agent. Macroscopically, the greyish colour of the lungs, the enlargement and the absence of collapse when the thorax is opened are the most striking findings observed. Although the cells in the alveolar sacs and the areas of round cell infiltration contribute to the enlargement, the most important factor is the exudate, for as fluid oozes out of the pulmonary tissue when it is incised, a marked diminution in the size of the lungs occurs. Hepatization, such as is found in a primary bacterial pneumonia, never takes place, except in late stages and in focal areas following secondary bacterial invasion. The small emphysematous patches which can sometimes be recognized macroscopically are either dilated alveolar ducts which always remain patent or groups of dilated alveoli whose walls remain normal or show only moderate swelling of the septal cells *in situ*.

The fundamental pattern of the disease is now established, and it is convenient to enumerate and describe separately the essential components. The polymorphonuclear leucocytes, so conspicuous in the first ten days following inoculation, do not come into that category; they represent an immediate reaction to the introduction of the causative agent and have disappeared as a rule within two weeks after inoculation (Fig. 5). At a later stage, when degenerative changes or secondary infection occur, they may reappear in considerable numbers. The large mononuclear cells in the alveoli, the fluid exudate, the epithelial and mesothelial hyperplasia and the perivascular and peribronchial

infiltration, on the other hand, form permanent features and persist although not necessarily unchanged throughout the course of the disease.

Alveolar cells.

Following disappearance of the polymorphs, the affected alveoli come to contain increasing numbers of cells derived from the alveolar walls. They originate from the " septal cells " ; this is a useful non-committal term for the inconspicuous cells located in the intercapillary spaces, and has the advantage of not implying either a mesenchymal or epithelial origin. Their enlargement *in situ* and separation from the septa can be readily seen, and they increase in numbers by mitotic division ; bi-nucleate forms are also frequently found. In the early stages of the condition (Fig. 3) up to about two months after infection, they have an intensely eosinophilic cytoplasm, but gradually vacuoles appear in increasing numbers, the cytoplasmic outlines become less sharply defined and the nucleus shrunken (Fig. 7). At a later stage, lipoid substances are deposited in the cells, sometimes in the form of doubly refractile rhomboidal plates, but this fatty change is a variable feature both in time of appearance and in extent. It has to be noted also that after the first two months, the appearance of the intra-alveolar cells varies in different parts of the lung ; in some areas considerable vacuolation may be present, while in others, they may still show a homogeneous eosinophilic cytoplasm and vesicular nuclei : it seems likely that the latter areas represent recently involved portions of the lung. In the early stages the cells show phagocytic activity and ingest polymorphs ; at a later stage, from about four months onwards, many contain iron pigment derived from phagocytosed erythrocytes. Although there is no fundamental alteration in the basic micro-anatomical structure of the lung, the presence of intra-alveolar exudate and cells must, at least focally, slow down the flow of blood through the capillaries and the occasional identification of small capillary thromboses supports this possibility. Small haemorrhages are then liable to occur, and the extravasated erythrocytes are dealt with in the usual way by the alveolar cells. As time passes, the alveolar cells continue to enlarge, the vacuolation increases, the nuclei become pyknotic and finally, the cells disintegrate. This can occur in individual cells in an alveolus, but more generally small groups of consolidated alveoli are affected simultaneously and the interalveolar septa disappear at the same time (Fig. 8). Polymorphs often invade the broken-down tissue, but quite often large fluid-containing spaces are the only end result of this degenerative process. As already stated, the mobilization of septal cells and their accumulation within the alveolar sacs is never generalized throughout the lungs and it would appear that about the second and third month after infection this reaction slows down and possibly stops. Thus, even at an advanced stage, one can always find areas of lung with an almost normal alveolar structure and showing only a moderate enlargement of septal cells as well as areas where the alveolar sacs contain only a few desquamated cells which still possess an eosinophilic non-vacuolated cytoplasm.

Fluid exudate.

Accumulation in the alveolar spaces of fluid in which fibrin is scanty or absent, is one of the contributory causes to the enlargement of the lungs which is such

a prominent feature of this condition; the abundant fluid which flows from the cut surfaces when the lungs are excised is truly remarkable. The presence of this exudate is probably responsible for the dilatation of the perivascular lymphatics, a constant finding three to four weeks after infection, and for the enlargement of the tracheo-bronchial glands. In the later stages of the disease, the products of alveolar cell disintegration are added to the fluid content of the lungs. Exudation of fluid follows the cellular reaction and seems, during the second month, to reach a maximum level which is maintained throughout the course of the disease.

Perivascular and peribronchial infiltration.

The formation of thick cuffs of mononuclear cells ensheathing the blood vessels and air passages, from their commencement in the lung at the hilum to their ultimate subdivisions at the level of capillary and alveolar duct respectively, plays a large part in determining the characteristic macroscopic appearances of the lungs; the actual bulk of tissue so formed can occupy a very considerable proportion of the total lung volume. Perivascular oedema which begins about the sixth day after infection around the small and medium-sized arterioles, precedes the onset of infiltration, but is soon obscured by increasing numbers of mononuclear cells. At first of small lymphocyte type with scanty cytoplasm and densely-staining nucleus, they soon acquire the characters of the medium-sized lymphocyte and proliferate actively. The infiltration is always more extensive round arteries and arterioles than around the bronchi, bronchioles and veins. Perivascular lymphatics are not obliterated and remain as conspicuous channels with intact endothelial lining (Fig. 6). Although occasionally isolated macrolymphocytes have been recognized in these cellular accumulations, no sharply defined cellular structures have ever been made out. In the later stages, the cells may come to acquire the characteristic morphology and tinctorial qualities of plasma cells and this, when it occurs, is always accompanied by a similar transformation in the tracheo-bronchial lymphatic glands. Although the process is progressive in character, the infiltrating cells do not invade the alveolar spaces, but adjacent alveoli become flattened out against the edge of the cellular areas. Moreover, the increasing bulk of this infiltration outside the respiratory epithelium tends gradually to narrow the lumen of the air passages, particularly of the terminal bronchioles, and may be an indirect factor favouring secondary bacterial invasion. Very occasionally in the terminal bronchioles in the late stages of infection mononuclear cells penetrate the basement membrane in small groups, but although the epithelium becomes flattened over them, ulceration has never been found.

Epithelial hyperplasia.

Most pneumotropic viruses attack directly bronchial and bronchiolar epithelial cells. The mouse pneumonia virus of Nigg (Nigg and Eaton, 1944) multiplies within the cytoplasm of individual cells which become stuffed with elementary bodies and eventually rupture. In experimental influenza in the mouse (Straub, 1937), considerable focal areas of epithelial necrosis, preceded by the appearance of eosinophilic granules in the cell cytoplasm, are produced at any part of the bronchial tree. In grey lung disease, however, no such host-

virus relationship is obvious, and the respiratory epithelium shows only a mild degree of orderly hyperplasia particularly of the ciliated cells ; this begins a few days after infection, has no obvious relationship to cell loss and persists throughout the course of the disease. Since the normal arrangement on the basement membrane is maintained, proliferation produces a degree of folding of the epithelium as a whole and the cells are moreover more columnar. In the later stages, goblet cells, usually present in small numbers only in the large bronchi, increase greatly in numbers and abundant mucin is produced.

Mesothelial hyperplasia.

Mesothelial hyperplasia is confined to the visceral pleura, varies very much in degree and may be generalized or focal. Mononuclear cells frequently accumulate in small groups and may give rise to small flat papillomatous projections : this is the only situation within the lung where their occurrence is unrelated to blood vessels or air passages.

Other changes.

Chronic bacterial infection of the lung of long duration is often accompanied by considerable overgrowth of connective tissue leading to permanent alteration of structure. Amongst virus infections, however, only influenza in the mouse produces irreversible changes, considerable areas of lung becoming epithelialized by the ingrowth of metaplastic epithelium from the terminal bronchioles into the alveoli (Straub, 1940). In grey lung infection, although no new formation of connective tissue disturbs the general micro-anatomical plan, the existing collagen and elastic fibrils in the interalveolar septa gradually become thicker and more numerous, particularly in consolidated areas and where the cellular content of the septa is increased, this change being most marked at the junction of alveolar ducts and air sacs. At a still later stage, however, the interalveolar framework within an area of consolidation in which the cells have become greatly enlarged and vacuolated eventually atrophies, and as the cells break down, the fibrils also disintegrate and disappear (Fig. 8). Abundant new formation of reticulum fibres occurs amongst the perivascular and peribronchial cuffs of mononuclear cases. In one case examined a year after infection, a perivascular cuff was replaced by dense hyaline connective tissue in which only a few mononuclear and plasma cells survived.

Secondary infection.

At one time the passage material was contaminated with the Nigg pneumonia virus, and bronchiolar and alveolar cells containing elementary bodies could be found, although never in very large numbers. During this period the primary lesions resembled uncomplicated Nigg pneumonia not only histologically, but also in their rapid development, and it was this similarity which prompted a careful day-to-day study. After treatment with sulphamerazine, the Nigg virus was eliminated and the usual histological course of the condition was re-established, the grey lung virus being quite unaffected by the drug. Gross bacterial infection sometimes occurs at a later stage of the disease, usually from 9 to 15 months after inoculation and the histological picture changes completely. The lungs become overrun with polymorphs, and small abscesses develop in

relation to terminal bronchioles. The larger bronchi are packed with polymorphs, the epithelium undergoes metaplasia and comes to consist of several layers of non-ciliated stratified cells. Around the abscesses and the bronchi considerable new formation of connective tissue takes place.

The disease in cotton rats.

Grey lung disease can be transmitted to cotton rats (Andrewes and Glover, 1945) and the infection follows the same course. Histologically a somewhat similar picture is produced but fluid exudate is scanty, pleural changes are slight and in the first series examined, the mobilized septal cells did not show the same tendency to vacuolation and disintegration nor were the mononuclear cell infiltrations so extensive as in the mouse. In a subsequent series, however, although fluid exudate remained minimal, the alveolar cells underwent similar cytological changes, and perivascular and peribronchial infiltrations were just as widespread as in the mouse. Epithelial hyperplasia, thickening of the framework of the interalveolar septa, prominence of lymphatic channels were also present. On histological grounds alone, it was found impossible to distinguish between grey lung disease in cotton rats and lesions in cotton rats produced by a virus recently isolated in that species (Andrewes and Niven, 1950).

DISCUSSION AND SUMMARY.

Differentiation of the grey lung agent from other pneumotropic viruses which have probably originated in mouse stocks, and from murine pleuropneumonia-like organisms was discussed by Andrewes and Glover (1945), and it is sufficient to recapitulate their main points. The absence of elementary bodies and lethal action distinguishes it from viruses of the Nigg pneumonitis group ; failure to provoke immunity, absence of antibodies and low pathogenicity differentiate it from the pneumonia virus of mice (P.V.M.) of Horsfall and Hahn (1940). An agent of the pleuropneumonia group cannot be excluded so definitely, but no new evidence has become available to support such a view. Histologically, grey lung virus behaves quite differently in mouse lung from the Nigg virus which causes lesions always related to the multiplication of elementary bodies within susceptible cells. In the case of P.V.M., histological differentiation is much less certain. Horsfall and Hahn describe fluid exudate and mononuclear cells in the alveolar spaces, an increased cellularity of the interalveolar septa and abundant perivascular and peribronchial infiltration. As already stated, however, the serological response in this disease is quite specific, and moreover, a haemagglutinating agent for murine erythrocytes can be extracted from infected lungs (Mills and Dochez, 1944). Infected animals succumb in 10 to 14 days following inoculation of material which has undergone a few mouse passages and resemblance is confined to the actual lung changes. Although the pulmonary tissue reactions to grey lung virus are thus not specific, it is unique in maintaining pathological changes apparently indefinitely. On histological grounds alone, it is impossible to decide which of these are due primarily to the virus and which are non-specific. Perivascular and peribronchial infiltration occur in response to many agents both in lethal and sublethal doses and belong to the latter category. The rapid disappearance of the alveolar cells

and repression of the epithelial hyperplasia following antibiotic therapy (Andrewes and Niven, 1950) do suggest, however, a close association with the causative agent. The oedema also disappears very quickly, and it cannot be denied that the virus may alter capillary permeability, the occasional haemorrhages being the result of a further degree of capillary dysfunction and not due to mechanical factors as suggested earlier. The chemotherapeutic experiments also show clearly that the alterations of lung structure unlike those sometimes produced by influenza virus (Straub, 1940) are not irreversible. The possibility that grey lung virus is an agent with neoplastic potentialities has also been considered, and with this in mind lungs from many animals 12 to 18 months after infection have been examined, without, however, finding any evidence of uncontrolled proliferation in the epithelium lining the bronchial tree, the most likely site of neoplasia. In two instances, both in animals inoculated 18 months previously, a single focus of fibroblastic proliferation, unrelated to secondary infection which was present elsewhere, was found near the tip of a lobe and close to the pleural surface ; in neither case were mitoses present, and the lesions could not be regarded as new growths within the framework of the lung.

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