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Etiology of Chronic Pneumonia in Rats and a Study of the Experimental Disease in Mice

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The lungs of conventional rats with chronic pneumonia contained *Streptobacillus moniliformis* and *Mycoplasma pulmonis*. These organisms singly and in combination failed to produce lung disease when inoculated into specific pathogen-free rats. On the other hand, diseased lung homogenate not containing cultivable organisms caused a chronic pneumonia when inoculated into specific pathogen-free rats. The organism involved was seen by electron microscopy and is morphologically indistinguishable from the grey lung agent of Andrewes and Glover and Nelson's enzootic bronchiectasis "virus." All of these agents have morphological and biological properties which indicate close relationship to the mycoplasmas. However, we failed to culture them either in tissue cultures or on inanimate media and conclude that a group of highly fastidious mycoplasma-like agents are a cause of chronic pneumonia in rodents.

It is well known that laboratory rats kept under conventional conditions frequently develop chronic pneumonia, and numerous workers have isolated a variety of bacteria from the lungs of affected animals. However, bacteria are now generally believed to be secondary invaders of infected lungs rather than the primary cause of the condition. Nelson (16, 17) maintains that in laboratory rats chronic respiratory disease is a complex of two diseases, the primary cause of which can be either *Mycoplasma pulmonis* or a virus, but in a recent, comprehensive investigation and review of the disease Lindsey et al. (13) contended "that *M. pulmonis* is the primary pathogen responsible for chronic respiratory disease."

In the present investigation, attempts were made to isolate microorganisms from the lungs of conventional rats and to induce chronic pneumonia in specific pathogen-free (SPF) rats by inoculation of isolated organisms or lung material. We found that chronic pneumonia is indeed transmissible but present evidence that in our rat colony the primary cause was neither a virus nor *M. pulmonis*.

The causative organism of enzootic bronchiectasis (EBN) in rats isolated by Nelson (16) and the grey lung (GL) agent isolated by Andrewes and Glover (1) are compared with the rat pneumonia (RP) agent derived from rats in our laboratory.

MATERIALS AND METHODS

Animal techniques and evidence of lung infection. The maintenance of conventional and SPF rat colonies used in our experiments has been described by Wheeler (21).

Groups of rats aged from between 4 and 72 weeks were taken for investigation from the conventional and SPF colonies. Rats were killed with barbiturate, and sera were stored at −20 C without inactivation. The lungs were aseptically removed, the single left lobe fixed for histopathology, and the remaining lobes homogenized for the culture of microorganisms.

Lung homogenates and nasopharyngeal swabs were inoculated onto the agar medium described by Lemcke (11) and modified by the addition of thallium acetate (0.025%) and benzylpenicillin (100 units/ml) for primary isolation. All cultures were incubated aerobically at 37 C and examined daily under a low-power lens. Colonies were subcultured by using the "push-block" technique of Klieneberger-Nobel (10).

M. pulmonis isolates were identified by a complement fixation test by using an antiserum prepared in rabbits to the KON strain of *M. pulmonis* which was kindly supplied by Ruth Lemcke of the Lister Institute, London. *Streptobacillus moniliformis* was by microscopy examination and by serological cross-reaction in a complement fixation test with a standard strain.

The preparation of reagents and techniques used in complement fixation tests were as described by Card (2).

Transmission experiments in rats. To investigate the pathogenicity of lung homogenates and of or-

ganisms recovered from the lungs of rats with chronic pneumonia, groups of 10 neonatal SPF rats were challenged intranasally under light ether anesthesia with 0.2 ml of inoculum. The inocula were: (i) broth culture of *M. pulmonis*, containing 6.0×10^6 colony-forming units (CFU) per ml, isolated on agar from the lung of a diseased conventional rat and cloned twice on agar; (ii) broth culture of *S. moniliformis*, containing 1.8×10^6 CFU/ml, isolated on agar from diseased rat lung and cloned twice on agar; (iii) a mixture of *M. pulmonis* and *Streptobacillus* prepared as described above; (iv) sterile broth (Lemcke's medium); and (v) 20% lung homogenate from a conventional rat of 72 weeks of age with extensive macroscopic lung disease. (*M. pulmonis* and *Streptobacillus* were cultured from this inoculum.)

In separate experiments mice were inoculated intranasally with 0.2 ml or 20% lung homogenate from a 72-week-old SPF rat or from a 4-week-old conventional rat. No organisms were detected in either of these inocula.

Experiments in mice. A conventional closed colony of "virus-sensitive, bacteria-sensitive" mice were used throughout this study. Lung disease has not been detected in the colony. Serial passage of mouse lung or SPF rat lung homogenates by intranasal inoculation was undertaken on several occasions, but it consistently failed to produce lung disease.

Experimentally diseased mouse and rat lung was inoculated into a variety of inanimate media and tissue cultures. The inanimate media used were: Lemcke's medium (11); Her's medium (12); and Chanock's medium (3). To the above media in some experiments 10% human, rat, fetal calf, and horse serum or 10% aqueous extracts of mouse lung were added. In some cases lung cells from diseased mice were embedded in an agar overlay technique as described by House and Waddell (8). All cultures were incubated aerobically, anaerobically, and in the presence of 10% carbon dioxide at 33 and 37 C.

The tissue cultures used were mycoplasma-free HEp-2, BHK-21, and L cells grown on Eagle, Leibovitz, and Hanks lactalbumin hydrolysate and *N*-2-hydroxyethylpiperazine-*N'*-2'-ethanesulfonic acid tissue culture media with 10% fetal calf, pooled human, and Burroughs Wellcome no. 3 horse sera. Primary cell cultures of mouse embryo, mouse kidney, and mouse embryonic lung were grown on the same media.

All cultures apart from controls were inoculated with diseased mouse lung homogenates and incubated at both 33 and 37 C. Samples of the inoculated continuous lines were subcultured up to 10 times after inoculation, and primary cells were split between 3 and 10 times. "Ex-plant" cultures of infected mouse lung were grown by inoculating small trypsinized fragments onto established monolayers of mouse embryo cells. Tissue cultures were examined directly for cytopathic effects, and samples on coverslips were fixed in methanol and stained with 2% Giemsa stain for 24 hr at 37 C. They were then examined by using an oil immersion objective with phase contrast. Selected cultures were examined in thin section by electron microscopy.

Diseased mouse lung homogenates were also inoculated into 10-day-old fertile eggs by the amniotic, allantoic, and yolk sac routes and the eggs were incubated at 33 to 34 C. Embryos were examined by sampling every 3 days until the remaining eggs hatched. To test for the growth of organisms in tissue culture, broth, or embryonated eggs, material was inoculated into mice which were examined daily for the onset of respiratory signs (clearly audible chattering noises due to fluid in the trachea and bronchi). The lungs were examined macroscopically for lesions 21 days after inoculation.

Grey lung and enzootic bronchiectasis agents. An ampoule of lyophilized, infected mouse lung was kindly supplied by Janet Niven, M.R.C., Mill Hill, London. This was reconstituted in Eagle tissue culture medium and inoculated intranasally into mice. The lungs of mice developing chronic pneumonia were used to start a series of consecutive passages in mice. An ampoule of lyophilized, infected mouse lung was kindly supplied by J. B. Nelson of the Rockefeller University, New York. Reconstituted material was inoculated intranasally into Balb-C SPF mice. These mice were kept under strict isolation, and work with GL and RP was discontinued while experiments with EBN were in progress.

Necropsy procedure. One hundred and fourteen mice were used in the experiments. Three groups of 36 mice each were infected, respectively, with either the GL, RP, or EBN agents and six animals served as controls. Mice from each group were killed at 2, 4, 7, and 10 days, at 2, 3, 4, 5, and 12 weeks, and at 8 months after infection. All mice were killed by cervical dislocation. Immediately after death 2 ml of 10% buffered, neutral Formalin was infused into the trachea and lungs in situ by using a fine needle and syringe, and the trachea was then ligated. After 0.5 hr of fixation, the trachea and lungs were removed, and portions of tissue were taken and placed in 10% Formalin.

Histopathology. Paraffin sections (5 μm) from the lungs of all mice were stained with hematoxylin and eosin, and selected sections were also stained by the Van-Gieson method, by Gordon and Sweets method for reticulin, by the periodic acid-Schiff (PAS) reaction, by a combined PAS-Alcian Blue method, and also by an orcein technique for elastic tissue.

Electron microscopy. Mice were killed by inhalation of ether and the trachea and lungs were immediately removed. Small pieces of the lungs were immersed in cold 4% glutaraldehyde buffered at *p*H 7.2 with cacodylate. After postfixation in 1% osmium tetroxide the tissue was embedded in Araldite. Sections were cut on an LKB Ultratome III ultramicrotome, stained with uranyl acetate and lead citrate (20), and examined in an A.E.I. EM6B electron microscope. The techniques used for specimens of rat lung have been described by Gay (7).

RESULTS

Conventional rats: evidence of infection with S. moniliformis and M. pulmonis. Histological

examination occasionally revealed lesions in the lungs of 4-week-old rats. At this stage neither *S. moniliformis* nor *M. pulmonis* was detected in lung material, but *M. pulmonis* was isolated occasionally from the upper respiratory tract with nasopharyngeal swabs.

Sera from 4-week-old rats were tested for complement-fixing (CF) antibody to *M. pulmonis* and *S. moniliformis*. No antibody was detected. Sera from 12-week-old rats occasionally had detectable levels of antibody to *S. moniliformis* and *M. pulmonis*, and these organisms were intermittently isolated from the lungs of 12-week-old animals.

Conventional 28-week-old rats invariably had developed a chronic pneumonia. The pathology of the disease has been described in detail by Elmes and Bell (4) and it is similar to that described by Innes, McAdams, and Yevich (9). The severity and extent of the lung lesions increased with age and, with rats over 28 weeks old, both *M. pulmonis* and *S. moniliformis* could be recovered frequently from the lungs. A high proportion (90%) of older animals had antibody to both *M. pulmonis* and *S. moniliformis*.

These results closely parallel the earlier work of Klieneberger-Nobel (10) who also detected *M. pulmonis* and *S. moniliformis* infection in rats with chronic pneumonia by using both serological and cultural procedures.

SPF rats: microbiological and pathological examination of lungs. Lungs from SPF rats were examined periodically for evidence of chronic pneumonia. No lesions were detected. Repeated attempts were made to culture *S. moniliformis* and *M. pulmonis* from both the upper and lower respiratory tract of animals of varying ages, but these organisms were not detected. Serological studies of SPF rats by using the CF test also failed to detect antibody to either *S. moniliformis* or *M. pulmonis*.

Attempts to produce chronic pneumonia in SPF rats by inoculation with M. pulmonis and S. moniliformis. Five groups of 10 neonatal SPF rats each were inoculated intranasally (see Materials and Methods) with (i) *M. pulmonis*; (ii) *S. moniliformis;* (iii) *M. pulmonis* plus *S. moniliformis;* (iv) sterile broth; and (v) SPF rat lung homogenate.

All five groups of rats were examined at 36 weeks, and the lungs had developed no detectable chronic pneumonia. However, *M. pulmonis* and *S. moniliformis* were cultured from the nasopharynx of some of the animals inoculated with these organisms, and some animals developed CF antibody. No CF antibody to *M. pulmonis* or *S. moniliformis* was detected in groups four or five, nor could either organism be recovered from these groups.

Production of chronic pneumonia in SPF rats by inoculation of affected lung homogenate. Two groups of ten neonatal rats each were inoculated (see Materials and Methods) with (i) lung homogenate from an old rat with chronic pneumonia or (ii) lung homogenate from a 4-week-old conventional rat. All the rats of the first group had chronic pneumonia when killed and examined 36 weeks after inoculation.

Rats in the second group were killed 12 weeks after inoculation. Eight out of ten had lung lesions as shown by histopathological examination. Serological tests and cultural procedures failed to detect evidence of infection of these animals with either *M. pulmonis* or *S. moniliformis*.

Transmission of lung disease to mice using "sterile" diseased rat lung. As described in the previous section, lung material from 4-week-old conventional rats produced disease in neonatal SPF rats. The SPF rats were killed after 12 weeks, and 0.05 ml of a 20% suspension of lung from one of these animals was inoculated into mice from a colony which did not have chronic pneumonia. The inoculum was sterile in the sense that we failed to culture bacteria or mycoplasmas. The inoculated mice developed pneumonia which was detectable as early as 7 days after inoculation, and this developed into a chronic respiratory disease. The pathology of this condition is described in a subsequent section. Mice inoculated with lung material from SPF rats remained healthy.

The chronic pneumonia induced in mice was serially transmitted in mice for over 20 passages. Lung homogenate from mice at the 5th, 10th, and 20th passage was inoculated into SPF rats, and these were examined 20 weeks after inoculation. All had developed a chronic pneumonia which was histologically indistinguishable from the natural disease seen in conventional rats. Lung homogenate from uninoculated mice failed to produce chronic pneumonia in SPF rats.

Failure to culture RP agent in vitro. All attempts to culture an organism in cell-free media and in the wide range of tissue culture systems described were unsuccessful. Tissue cultures never developed a cytopathic effect and viruses or mycoplasmas were not detected, although the cell-free and tissue culture media described supported the growth of *M. pulmonis* in primary culture from rat lung. Furthermore, ex-plant cultures rapidly lost the ability to produce lung disease in mice by intranasal inoculation.

Histopathology of chronic pneumonia pro-

duced in mice by several agents. Nelson (16) found that chronic pneumonia could be transmitted in mice by inoculation of an agent originally derived from the lungs of laboratory rats with chronic lung disease. He could not culture either mycoplasmas or bacteria from the inoculum and believes that the causative agent, EBN, is a virus.

GL agent also produces a chronic pneumonia in mice (18). This agent is thought to be a mycoplasma but has not been cultured in vitro (5, 15).

To determine whether the chronic pneumonia, produced in mice by the agent derived from rats in our laboratory, is a unique pathological condition, a group of mice was inoculated with each of the three agents, and the lung from infected animals were examined at intervals from 2 days to 8 months after inoculation.

No gross lesions were present in the mice killed before the 7th day, when the surface of all lobes of the lungs exhibited regions of reddish-grey discoloration. Thereafter, the lungs of all infected mice had areas of variable size which were consolidated and greyish-purple, both superficially and in section. The lungs of six control mice inoculated with a 10% homogenate of uninfected mouse lung showed no lesions.

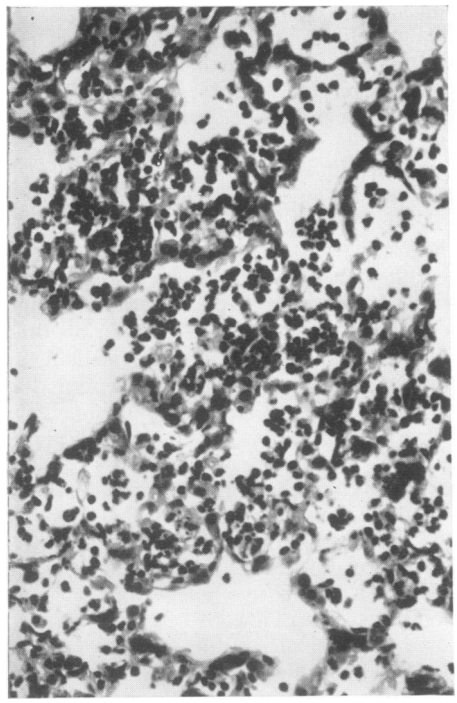

FIG. 1. *Typical lesions in mouse lung 7 days after infection with grey lung agent. Inflammatory cells in alveoli are predominantly polymorphs. Hematoxylin and eosin stain.* × *200.*

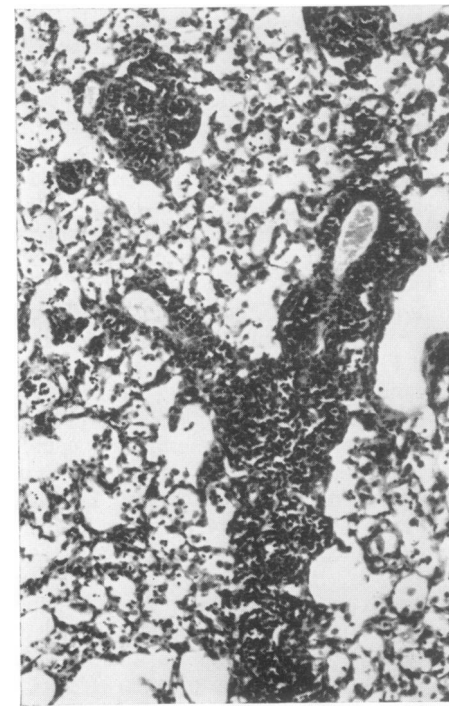

FIG. 2. *Mouse lung 10 days after infection with grey lung agent. Peribronchiolar lymphoid tissue has merged with that in the walls of blood vessels. Hematoxylin and eosin stain.* × *80.*

The changes found in the lungs of mice infected with the three agents were of the same type, extent, and distribution and, therefore, a single description of the lesions is given.

Lesions were not found in the lungs of any mice before the 4th day after inoculation. On the 4th day interalveolar septa were congested, and groups of polymorphonuclear leukocytes were present in a small number of alveoli, particularly in those surrounding terminal bronchioli. By the 7th day the reaction was much more extensive and involved the greater part of each lobe of the lungs. Aggregates of lymphoid tissue had accumulated in the peribronchiolar connective tissue and also in that of the adventitia of many arterioles and venules. The lumina of airways contained polymorphonuclear leuckocytes and a few largemononuclear cells, and in some cases the epithelium was hyperplastic. Many alveoli contained eosinophilic edema fluid and, in addition, those surrounding bronchioli contained more inflammatory cells than subpleural alveoli. The cellular exudate was predominantly polymorphonuclear with only isolated large mononuclear cells of macrophage type (Fig. 1). The cellular population of a few interalveolar septa in zones of intense in-

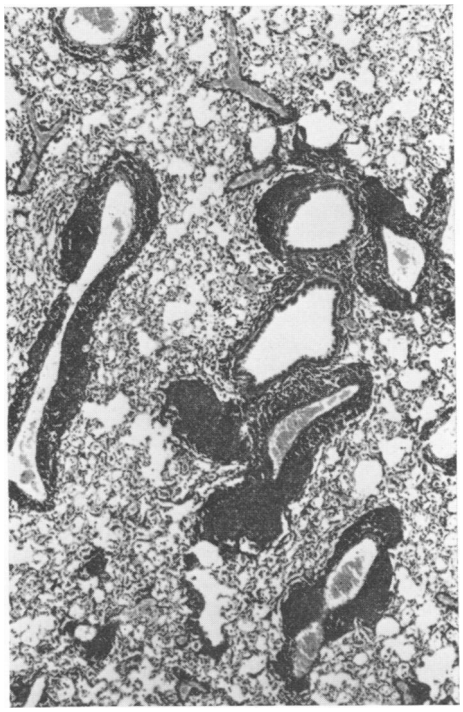

FIG. 3. *Nelson agent 21-day infection. There is extensive proliferation of lymphoid tissue around airways and blood vessels and many alveoli contain inflammatory cells. Hematoxylin and eosin stain.* × *30.*

flammatory change had apparently increased, causing slight thickening, but whether this was due to infiltration or proliferation of cells could not be determined. In the lungs of mice killed on the 10th day lymphoid hyperplasia had continued, and lymphoid tissue was found around most airways and blood vessels (Fig. 2). A number of cells in the larger masses were undergoing mitosis. The lesions were otherwise similar to those present earlier.

The accumulation of lymphoid tissue progressed gradually so that by the 21st day large aggregates were found around most large airways and blood vessels, and in some cases they formed a continuous coat (Fig. 3). Sections stained to demonstrate reticulin showed newly formed fibers throughout this lymphoid tissue. The epithelium of most airways was hyperplastic. On the 21st day fewer alveoli contained edema fluid, and macrophages were now found in them more commonly than polymorphonuclear leukocytes. In a few regions, nodules of macrophages, lymphocytes, and fibroblasts had formed. At 28 days after infection the proportion of plasma cells in the peribronchiolar lymphoid masses had increased. There were also numerous subpleural nodules, and these were now denser and more obviously fibrous. Many alveoli were filled with large mononuclear cells of macrophage type. These lesions had progressed by the 5th week to involve the majority of the sectional area of the lungs.

At 3 months the lymphoid masses of adjacent airways and blood vessels had merged and attained such a size as to cause stenosis of bronchiolar lumina and pressure collapse of surrounding alveoli. Alveoli not involved in granuloma formation contained large foamy macrophages, and frequently the cytoplasm of these had degenerated, filling the alveoli with floccular material which was faintly eosinophilic and PAS positive (Fig. 4).

Eight months after infection large areas of each lobe of the lungs were affected. The lymphoid aggregates were now the most extensive of the series, and networks of reticulin fibers were associated with them. Most alveoli were either involved in fibrous granulomata or contained groups of macrophages and isolated polymorphs. Many macrophages were degenerating and the alveoli were filled with masses of

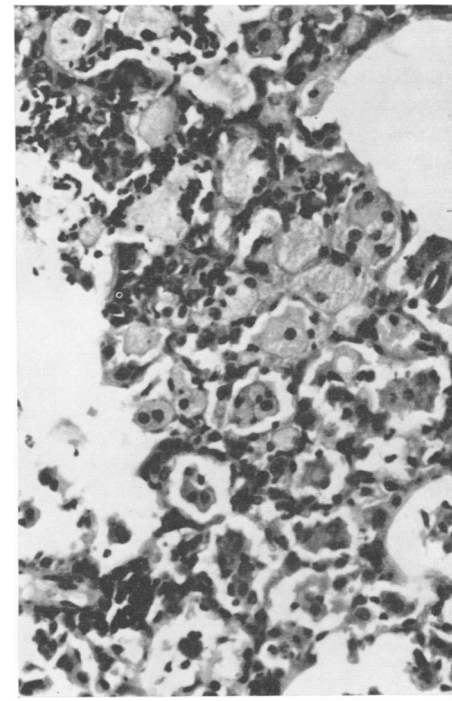

FIG. 4. *Three months after infection with enzootic bronchiectasis agent. Large macrophages with foamy cytoplasm are present in many alveoli. Some macrophages have degenerated, filling the alveoli with eosinophilic, floccular material. Hematoxylin and eosin stain.* × *200.*

eosinophilic, PAS-positive material in which cholesteral clefts were present.

Electron microscopy of mouse lung after EBN inoculation. Although the isolation and biological properties of the RP agent have not been documented previously, we have described its morphology as seen in thin sections and have further shown that it is indistinguishable in structure from the GL agent (5). Here we examine the structure of EBN agent and the ultrastructural changes which it produced in the lungs of mice 28 days after infection.

The most striking feature of many alveoli was the presence in them of a large number of mycoplasma-like organisms (Fig. 5). These were predominantly narrow, elongated organisms having a mean length of 580 nm and a mean thickness of 160 nm. The organisms most commonly occurred in parallel groups, but irregularly polygonal forms of similar dimensions were also seen. The structure of the organism was identical to that of the GL and RP agents and consisted of a limiting unit membrane of 11 nm separated by an 8-nm space from the inner cytoplasm. The cytoplasm contained a reticulum of 2- to 3-nm fibrils, between which were well-defined electron-lucent spaces. Ribosomes 11 to 12 nm in diameter were associated in groups with the network of fibrils. Many of the cells possessed an unbranched and frequently discontinuous peripheral fibril of 5 to 7 nm which bounded the internal cytoplasm. The organisms were never seen in contact with the cells of the alveolar epithelium, but they were frequently found in digestion vacuoles in polymorphs and macrophages.

The fundamental ultrastructural change at 28 days was the accumulation in alveoli of large numbers of macrophages, fewer polymorphonuclear leukocytes, and isolated plasma cells and lymphocytes. The macrophages had the typical morphology of pulmonary macrophages, with numerous long pseudopodia and a variety of cytoplasmic lysosomes and, additionally, there were large vacuoles containing unidentifiable cellular debris. A number of the macrophages had ringlike cytoplasmic inclusions of the type described by Gay (6). In cells having these inclusions, the cytoplasm also possessed numerous dense osmiophilic lysosomal bodies which appeared to be related to the ring structure. These small lysosomes were scattered throughout the cytoplasm and were made up of a darkly staining granular core bounded by a unit membrane. In many of these structures the granular material was separated from the limiting membrane by an electron-lucent zone.

Edema had formed in some alveoli and occasionally a few strands of fibrin were also present. Changes in the neighboring capillary endothelium in such regions were limited to swelling of the cytoplasm and the formation of small

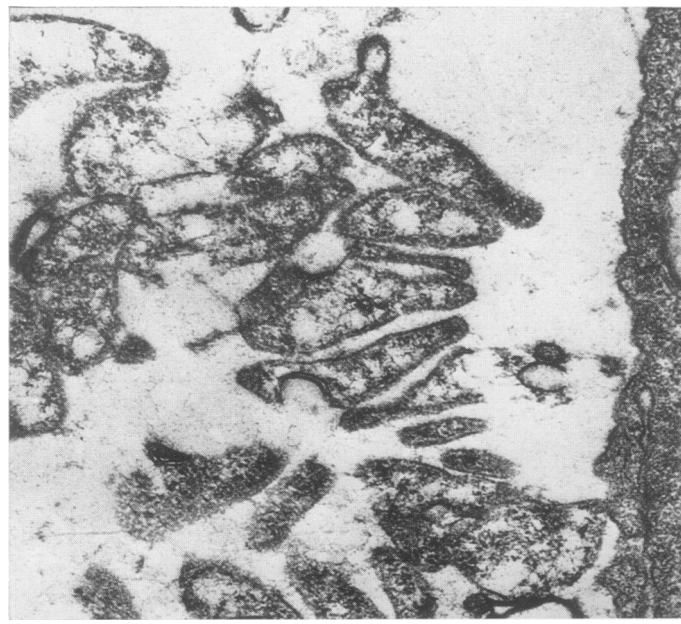

FIG. 5. *Electron micrograph of Nelson agent in alveolus of mouse lung at 28 days, showing a group of the mycoplasma-like organisms. The cytoplasm contains clumps of ribosomes and fine fibrils.* ×40,000.

vacuoles, and the alveolar epithelium and the respective basement membranes were intact and morphologically normal. The lumina of capillaries in areas of edema were congested and distended by erythrocytes and leukocytes. Debris consisting of degenerating polymorphonuclear leukocytes, macrophages, and other unidentified cells was found in some alveoli.

Interalveolar septa were thickened by an increased population of macrophages, plasma cells, and large and small lymphocytes. Type II alveolar epithelial cells were morphologically normal at this stage of infection. They did not undergo hyperplasia nor had any of them sloughed into alveoli. In many regions alveoli had been obliterated in a granulomatous reaction in which macrophages, lymphocytes, and fibroblasts were enmeshed in a mass of collagen fibers and an amorphous granular matrix. The fibroblasts in these nodules had the characteristic morphology of actively secreting cells, and the cisternae of the rough-surfaced endoplasmic reticulum were distended by floccular material of moderate electron density. Type II alveolar epithelial cells which had been incorporated into the granulomata could be identified in some cases.

Light microscopy of chronic pneumonia-producing agents. Giemsa-stained impression smears made from mouse lung infected with EBN contained large numbers of coccobacilli (Fig. 6) 0.3 to 1.0 μm in diameter. These structures are indistinguishable from those previously described in smears of lungs infected with GL and RP agents (5).

Effect of some drugs on the development of chronic pneumonia in mice inoculated with the RP agent. Persistent failure to culture an organism on inanimate media might suggest that the RP agent is a virus. However, structures resembling mycoplasmas in morphology were seen in infected lungs (but not in uninfected controls) in large numbers. No virus-like particles were seen in lungs infected with EBN, GL, or RP agents. Additional evidence supporting the conclusion that these agents are mycoplasmas was sought by examining the effects of chemotherapeutic substances on the development of chronic pneumonia in mice inoculated with the RP agent.

In all experiments, groups of weaned mice were inoculated with 0.1 ml of lung homogenate containing 10^3 to 10^5 ID_{50} of RP agent. Administration of the chemotherapeutic agents commenced on the following day. Animals were examined daily for signs of respiratory disease and the lungs were examined macroscopically 21 days after inoculation.

The results are recorded in Table 1.

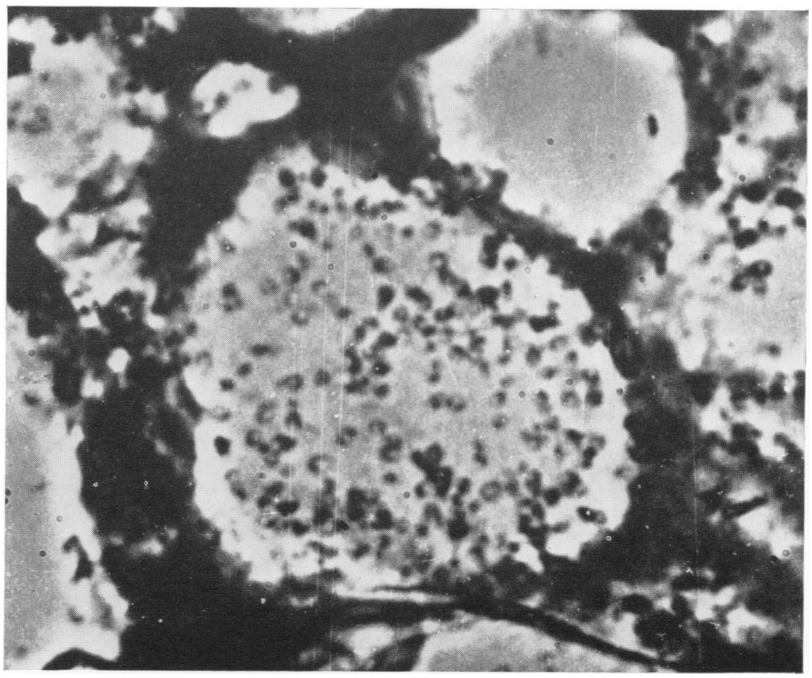

FIG. 6. *Giemsa-stained impression smear of lung infected with enzootic bronchiectasis agent. There are large numbers of coccobacilli and some have a "signet-ring" appearance. Oil immersion and phase optics. ×3,300.*

TABLE 1. *Effect of parenteral therapeutic agents on the development of pneumonia in mice inoculated with the rat pneumonia agent (RP)*

Drug	Dose (mg)	Frequency	Result[a]
Chlortetracycline	5	Daily	+
Oxytetracycline	5	Daily	+
Sodium aurothiomalate	1	3-Day intervals	+
Neoarsphenamine	1	3-Day intervals	±
Benzylpenicillin	5	Daily	−
Streptomycin sulfate	5	Daily	−
Chloramphenicol	5	Daily	−
Sulfadimidine	2	Daily	−
Cortisone	2.5	Daily	−

[a] Symbols: + = suppression (no macroscopic lesions); − = no effect; ± = partial suppression.

DISCUSSION

The most important observation in the present work is that chronic pneumonia could be transmitted to SPF rats or to laboratory mice by using diseased-lung homogenate from which no organisms could be isolated on the wide range of inanimate media and tissue cultures used. However, large numbers of coccobacilli were demonstrated in Giemsa-stained impression smears of the lungs of experimentally infected rats and mice. The disease in mice was suppressed by chemotherapeutic substances which are characteristically active against mycoplasmas. As previously reported (5), mouse lung infected with the RP agent and examined in thin section by electron microscopy contained organisms which closely resembled mycoplasmas in fine structure. Identical structures were seen in GL-infected mouse lung and also in the lungs of conventional rats (7). In the present study, mouse lung infected with EBN "virus" contained structures in stained impression smears and in thin sections which were identical to the RP and GL agents. In an attempt to distinguish the three agents, RP, GL, and EBN were inoculated into mice, and the lungs were examined macroscopically and by conventional histopathological techniques. No significant difference was detected in the lesions produced by the three agents and our description is in agreement with, but considerably extends, that in the classical work of Niven (18) with the GL agent. The initial response was one of accumulation of polymorphonuclear leukocytes in alveoli, followed by invasion by macrophages and proliferation of peribronchiolar and perivascular lymphoid tissue. Electron microscopy showed that numerous organisms were present in alveoli, and macrophages contained the unusual cytoplasmic lysosomal structures which have also been described in mice infected with GL, RP (5, 6), and *M. pulmonis* (19).

In the present study we were able to isolate *M. pulmonis* and *S. moniliformis* from a high proportion of old conventional rats with chronic pneumonia. However, we failed to reproduce the lung disease in SPF rats by inoculating pure cultures of these organisms either singly or in combination. Serological tests indicated that a proportion of these animals were indeed infected, and in some cases the organisms were recovered from the nasopharynx, but they were never recovered from the lungs. We conclude that in our conventional rats these organisms invade as opportunists after the lungs have become infected with the RP agent.

Lindsey et al. (13) have shown that most strains of *M. pulmonis* are capable of causing upper respiratory tract infection in experimentally inoculated SPF rats. This is in agreement with the contention of Nelson that "infectious catarrh" in conventional rat colonies is caused by *M. pulmonis*. We agree with Nelson that the lower respiratory tract disease can be caused by an agent other than *M. pulmonis* because the lungs of naturally infected rats become diseased and infectious before *M. pulmonis* can be recovered. Nelson has called the organism (EBN agent) causing the lower respiratory tract infection the "virus" of EBN. This agent so far has not been characterized (J. B. Nelson, *personal communication*), except that Nelson found its size to be approximately 200 to 250 nm by filtration. This is in reasonable agreement with our electron microscopy results. EBN causes a chronic pneumonia in mice, but the sensitivity of this disease to chemotherapeutic substances has not been tested. We conclude that EBN is not, in fact, a virus because it is indistinguishable in morphology and pathogenesis from the GL and RP agents. All three agents resemble mycoplasmas in fine structure, and the RP agent exhibits a range of sensitivities to chemotherapeutic substances (Table 1) characteristic of the mycoplasma group; thus, it is resistant to penicillin but sensitive to tetracyclines and to sodium aurothiomalate. The histopathology of EBN in mice, as determined in our laboratory, differs from that described by Nelson (16, 17). Although it is possible that another agent was activated in our mice by the use of Nelson's material, it is unlikely because continuous passage of normal mouse lung consistently failed to activate any disease. In general, we

conclude that an interesting and exceptionally fastidious group of mycoplasmas causes chronic pneumonia in the rat. It is clearly important to culture these organisms in vitro. Our failure to do so in a wide variety of tissue culture systems, including ex-plants of diseased lung, indicates that these organisms require special conditions for their culture. In spite of this we see no reason to suppose that they will not ultimately be cultivated on inanimate media, because they obviously grow in an extracellular location in the lungs.

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