Respiratory disease in a colony of rats

II. Isolation of *Mycoplasma pulmonis* from the natural disease, and the experimental disease induced with a cloned culture of this organism

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**SUMMARY**

*Mycoplasma pulmonis* was isolated from the pneumonic lung of a rat. Two groups of mycoplasma-free rats were inoculated, one with a culture of the *M. pulmonis* strain which had been cloned four times (group A) and the other with a lung homogenate of the rat from which the strain had been isolated (group B). A third group (C) consisted of uninoculated control animals. Each group was kept in strict isolation and allowed to breed so that the progeny was naturally exposed to any pathogens present in the inoculated animals. After different periods of exposure, rats were autopsied, respiratory tracts and inner ears were cultured for mycoplasmas and bacteria, and sera were tested for complement-fixing antibodies to murine mycoplasmas.

In group-A rats, *M. pulmonis* was consistently isolated from the inner ears or lungs from 50 to 715 days after exposure. Complement-fixing antibody to *M. pulmonis* was detected 20 days after inoculation, but in the naturally exposed progeny antibody took longer than 50 days to develop. Antibodies to the other known mycoplasmas of murine origin, *M. arthritidis* and *M. neurolyticum*, were never found. Purulent otitis interna was consistently found from day 55 onwards, while lung lesions were first observed at 85 days and persisted to 715 days. Pulmonary lesions developed more slowly in inoculated parents than in exposed progeny. Similar results were found in group-B rats, which were examined up to 441 days after inoculation. Uninoculated group-C rats were examined up to 768 days of age, but *M. pulmonis* was not recovered; of the 54 animals whose serum was tested all were negative to the three species of mycoplasmas, except one which had a titre of 16 with *M. pulmonis*. Pneumonia, bronchiectasis or lymphoreticular hyperplasia were not seen in any of these control rats. Bacterial respiratory pathogens were not isolated from rats in any of the groups, nor was antibody to Sendai virus detected.

The results suggest that *M. pulmonis* alone can cause pneumonia and bronchiec-...
tasis in rats since mechanical carry-over of another pathogen with the initial cloned inoculum is very unlikely and there was no evidence for the participation of any other rat pathogen. The respiratory disease induced by the cloned culture was comparable with that induced by the lung homogenate, and with the well-known syndrome of chronic respiratory disease and bronchiectasis in the rat.

INTRODUCTION

This paper is concerned with the role of *Mycoplasma pulmonis* in the production of pulmonary disease in the rat. Reviews of the many possible aetiological agents involved in chronic murine pneumonia have been published by Joshi, Blackwood & Dale (1961) and Brennan, Fritz & Flynn (1969). The terminology and wider significance of chronic respiratory disease in rats and its association with *M. pulmonis* has recently been reviewed by Lindsey et al. (1971).

Although *M. pulmonis* is the organism that has been isolated most consistently from pneumonia of the rat, and although it is generally accepted that this organism is frequently the cause of infectious catarrh of the upper respiratory tract, there is doubt whether *M. pulmonis* alone causes pneumonia in the rat. Klieneberger & Stebben (1940) demonstrated a close connexion between the presence of the L3 organism (later known as *M. pulmonis*) and the bronchiectatic pneumonia of laboratory rats, but they could not produce experimental lung infection with the organism. Lemeke (1961) found that *M. pulmonis* was established in the nasopharynx of young rats in the first few weeks of life, but they did not develop lung lesions until they were much older, the incidence of pneumonic lesions increasing with age. Klieneberger-Nobel & Cheng (1955) and Klieneberger-Nobel (1962), however, showed that pneumonia and bronchiectasis associated with *M. pulmonis* could be induced in younger rats by bronchial ligation, intubation for anaesthesia or exposure to low temperatures. Nelson (1940, 1967) showed an association between infection with *M. pulmonis* (described in the early paper as ‘coccobacilliform bodies’) and the development of infectious nasal catarrh, otitis media and possibly pneumonia in rats. However, in an assessment of almost 30 years work, Nelson (1967) came to the conclusion that pulmonary lesions were not caused by *M. pulmonis* alone but that a ‘virus’ was also involved; this latter agent does not appear to have been characterized sufficiently to enable later workers to test his hypothesis. Gay (1967a) provided evidence that *M. pulmonis*, although colonizing the upper respiratory tract of the rat, did not invade the normal lung. He considered that the organism was probably an opportunist following chronic inflammation caused by another agent. Gay also showed that a chronic rat pneumonia could be induced with inocula free from cultivable *M. pulmonis* or other known mycoplasmas; he suggested that his agent might nevertheless be a mycoplasma because of its morphology in electron micrographs, and its antibiotic sensitivity when studied in mice. Vrolijk, Verlinde & Braams (1957) may well have been dealing with the same agent. Bell (1967) also considered *M. pulmonis* to be merely a secondary organism in chronic respiratory disease of the rat. This view was supported by later work (Bell & Elmes, 1969) in which only minor lung changes
were induced in rats by the intranasal inoculation of either *M. pulmonis* alone or *M. pulmonis* in combination with *Streptobacillus moniliformis*. Nevertheless, lung lesions developed in rats inoculated intranasally with lung homogenates from rats with chronic respiratory disease; the responsible pneumonia-inducing agent was not identified.

Kohn & Kirk (1969) induced gross lung lesions in rats inoculated repeatedly with a once-cloned culture of *M. pulmonis*. The disease was more severe in some rats kept in adjacent cages than it was in the inoculated rats. Although Lindsey et al. (1971) induced pneumonia with various inocula containing unpurified *M. pulmonis*, their cloned strain (N) did not produce even microscopic lung lesions. A few studies of chronic respiratory disease in rats have included tests for serum antibodies; the plate-agglutination test was used by Kohn & Kirk (1969), who detected antibody to *M. pulmonis* in most of the gnotobiotic rats exposed to this organism but not in unexposed controls. Complement-fixing antibody to *M. pulmonis* was demonstrated in the sera of naturally and experimentally infected rats (Lemcke, 1961); its presence correlated with the demonstration of the mycoplasma by cultural methods. However, Bell & Elmes (1969) were unable to correlate titres of specific complement-fixing antibody with the presence of *M. pulmonis* in conventional rats with chronic respiratory disease or in specific pathogen-free rats inoculated with lung homogenates from rats with chronic respiratory disease. Only one animal with chronic respiratory disease gave a titre as high as 16 and most were less than 4. Similar results had been reported from the same laboratory by Gay (1967a).

Thus evidence on the role of *M. pulmonis* in the production of pneumonia in the rat is conflicting. The present work was therefore undertaken to investigate whether a recent isolate of *M. pulmonis* from a case of pneumonia in a naturally occurring outbreak of severe respiratory disease in rats (Lane-Petter, Olds, Hacking & Lane-Petter, 1970) would induce a similar respiratory disease syndrome experimentally, and to study the serological response to experimentally-induced infection.

**MATERIALS AND METHODS**

*Sources of materials for transmission experiments*

Seven rats which were affected with respiratory disease in a natural outbreak at Alconbury (Lane-Petter *et al.* 1970) were removed to an isolation cubicle at the School of Veterinary Medicine, Cambridge, where they were held for a few days to allow clearance of the previously administered oxytetracycline before they were killed. The lungs of two of the rats, 3102 and 3103, were quantitatively cultured for mycoplasmas and were examined bacteriologically. A lung suspension from rat 3102 and a mycoplasma isolated from the same lung and subsequently cloned, were used as inocula in the transmission experiments.

*Cultivation of mycoplasmas*

The media used for isolating and cloning mycoplasmas and for preparing rat inocula were designated A26. These were the liquid and solid Hartley's broth-based media described by Whittlestone (1969), except that the bicarbonate was
not included. All constituents and containers were prepared and maintained at tissue-culture standard. Constituents were sterilized either by autoclaving or Millipore filtration to avoid Seitz filtration.

Solid medium was poured into 50 mm. plastic Petri dishes with non-sealing lids which were incubated at 37°C in a humid atmosphere of 5–10% CO₂ in air. Liquid media were sealed and incubated at 37°C.

**Titres of mycoplasmas in cultures or tissues**

The numbers of mycoplasmas were expressed either as colony-forming units (CFU) on agar medium or colour-changing units (CCU) in liquid medium. The number of CFU were calculated from the colony counts obtained from 0.02 ml. drops of at least three log₁₀ dilutions after about 6 days incubation, after which no new colonies appeared. In liquid medium the titre of CCU was expressed as the reciprocal of the highest dilution at which the phenol-red indicator showed a drop in pH from 7.4 to 6.8 after 14–21 days incubation. In some instances – for example, with the rat inocula – the titrations were made in triplicate to obtain a more accurate estimate of the titre of mycoplasmas.

**Purification of cultures**

*Mycoplasma 3102* was purified by four serial single-colony subcultures on solid medium as follows. An agar block bearing colonies was put into 1 ml. of liquid medium and the colonies emulsified. Tenfold dilutions were made and 0.02 ml. volumes plated on solid medium. After 6 days incubation an agar block bearing a single well-separated colony was cut out and the colony emulsified in 1 ml. of liquid medium. The dilution, inoculation, incubation and cloning procedure was repeated three more times serially before the culture of mycoplasma 3102 was used for preparing the rat inoculum and for serological identification.

**Rat inoculations**

Group-A rats received the serially cloned mycoplasma derived from lung 3102; the inoculum was a subculture in liquid medium of colonies from the final cloning and contained ca. 10⁵ CCU/ml.; on agar the inoculum yielded 0.5 x 10⁵ CFU/ml.

Group-B rats were inoculated with a suspension of pneumonic lung from rat 3102. This material, which had been kept frozen at −60°C since harvesting, was thawed rapidly and ground with horse serum to give a 10⁻¹ suspension. A further ten-fold dilution in mycoplasma medium was used as inoculum and contained ca. 10⁷ CCU/ml. (ca. 10⁶ CFU/ml.). Rats were anaesthetized with CO₂ gas and their nostrils immersed in about 1 ml. of culture or 0.5 ml. of lung suspension. Each rat inhaled 0.1–0.25 ml. of fluid.

**Experimental animals and accommodation**

The rats used in the transmission experiments were derived from a small colony of rats established in December 1968; the young foundation stock of four females and two males were primary hysterectomy-derived CFE rats from Carworth,
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U.S.A. From this barrier-maintained colony two litters born on adjacent days were divided into separate cubicles as follows: 8 rats which were inoculated with the cloned mycoplasma when 45 days old (group A); 7 which were inoculated with the pneumonic lung suspension when 52 days old (group B); 4 which were retained in the uninoculated control colony (group C). Some limited breeding was allowed in each colony so that the total number of rats in groups A, B and C reached 88, 52 and 109 respectively.

The cubicles in which the three colonies were housed were in a specially designed building provided with filtered air at positive pressure; each cubicle was entered via an ante-room in which protective clothing was put on. Before introducing rats into a new cubicle, all the equipment was either steamed or autoclaved, and the whole cubicle with its equipment then fumigated with formalin. Food was autoclaved and the drinking water acidified (Lane-Petter et al. 1970). Rats were kept in plastic boxes with wire-mesh tops and were provided with sawdust bedding and wood shavings.

For the first few months of the experiments different attendants looked after the normal and experimental rats. Later, all the rats were cared for over a period of several months by one attendant, who took a shower after attending infected rats and did not enter another cubicle for at least several hours. The order of attending the groups was C, A, and then B.

Post-mortem examinations

Harvesting tissues and exudates

Rats were anaesthetized with CO₂ gas and bled out for serum from an incision in the axilla. The skin was reflected from the thoracic cavity, the sternum removed, and the lung tissue harvested aseptically.

To collect fluid from the middle ear the external pinna was severed close to the tympanic membrane, which was pierced with a fine Pasteur pipette. The inner-ear samples were collected from the cochlea and vestibule, which were opened ventrally, after exposing the overlying bony bulla and searing it with a hot piece of shaped aluminium foil.

Harvested specimens and cultures were stored at — 60° C. or below and sera at — 24° C. Rats dying on days when it was impracticable to make a detailed examination were stored at — 24° C. in plastic bags and thawed under running cold water before examination.

Histological methods and assessments

For Giemsa-stained touch preparations the method of Whittlestone (1967) was used.

After aseptic collection of lung specimens, the larynx, trachea and lungs were removed, a few blocks were fixed in acetic Zenker and the remainder of the lung usually fixed in 10% neutral formalin before preparing blocks by section at various levels.

Sections were paraffin embedded and stained with haematoxylin and eosin. The degree of bronchiectasis was assessed in the histological preparations, by measuring
the internal diameters of transverse sections of bronchioles of rats from the infected colonies and then subtracting the equivalent figure for rats of approximately the same age from the control colony. The degree of bronchiolar distension thus assessed was recorded numerically in 0.1 mm. units.

The degree of lymphoreticular hyperplasia was assessed similarly and expressed in the same units.

Recording of gross lesions

The extent of pneumonia was recorded as an approximate percentage of the lung surface affected, by using the following scoring system: the right apical, cardiac and intermediate lobes were each allocated 10 points, the right azygos lobe 20 points, and the left lung 50 points if completely pneumatic. The most extensive case of pneumonia in this series thus scored 80 out of a theoretical 100 points for a completely pneumatic lung.

Culture of tissue for mycoplasmas and bacteria

Lung tissue was ground to produce an approximate 10^–1 suspension in mycoplasma medium or 10% horse serum broth and further ten-fold dilutions were made in appropriate liquid media before incubation or inoculation onto agar. The cultivation of mycoplasmas is described above. For bacterial isolation a drop of lung suspension was sown on pairs of blood-agar, 'chocolate'-agar and MacConkey-agar plates, and on Loeffler’s serum slopes. One sample of each pair was incubated anaerobically and the other in air with 5% CO_2. A cooked-meat medium was also sown and incubated in air. Bacteriological media were prepared by the methods of Cruickshank (1965).

The routine cultural examinations of the inner ear, nasal mucosa and trachea were made by preparing approximately 10^-2 dilutions of the surface exudates in appropriate broth, which were then diluted, inoculated and incubated as described for the lung suspensions. In some instances exudates were inoculated directly on to solid medium.

Serological methods

Reference cultures of mycoplasmas

In all, 18 species were used. The murine mycoplasmas M. pulmonis (Kon, M1, MB, and Sabin’s type C) M. arthritidis (PG27 and Jasmin) and M. neurolyticum (KSA) were those described by Lomcké (1961, 1964) and Lomcké, Forshaw & Fallon (1969). Other non-murine mycoplasmas were M. hominis (SC4), M. pneumoniae (FH), M. salivarium (B3), M. fermentans (G2), M. orale type 1 (837), M. mycoides var. mycoides (Gladysdale), M. bovigenitalium (PG11), M. gallisepticum (X95), M. gallinarum (Fowl), M. iners (M), M. primatum (Naval), M. suipneumoniae (J), Acholeplasma laidlawii (A), avian strain A 30 and Dinter’s porcine strain B3 (Lomcké et al. 1969; Goodwin, Pomeroy & Whittlestone, 1967).

Media

The basal medium used in the preparation of antigens was that described by Hollingdale & Lomcké (1969), but supplemented with pooled, inactivated human
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serum (20%, v/v) instead of the human plasma. Difco PPLO agar with unheated horse serum (Burrough’s Wellcome No. 3) was used for the growth-inhibition tests.

Growth inhibition (GI)

Tests were made by the method of Clyde (1964).

Complement fixation (CF)

Antigens were prepared and the method carried out as described by Hollingdale & Lemcke (1970). Anticomplementary rabbit or rat sera were diluted with normal guinea-pig serum (1/10) and held at 4° C. for 24 hr. before inactivation at 56°C. for 30 min.

Antisera

Antisera to M. suipneumoniae and Dinter’s porcine mycoplasma B3 were previously described by Goodwin et al. (1967). Sera against the other 16 species were those prepared by Lemcke (1964, 1965), Hollingdale & Lemcke (1969) and Lemcke, Forshaw & Fallon (1969), and all inhibited growth of the homologous species.

Antisera against strain 3102 were prepared in two rabbits. Antiserum for growth inhibition, where antibody to foreign serum proteins was unimportant, was prepared in Rabbit 187 by intravenous inoculation of mycoplasma cells from 20 hr. cultures in the human-serum liquid medium referred to above. In all, the rabbit received washed cells from 1 l. of culture; six injections were given at intervals of 3 days, and 3 weeks later a second similar series was given. For CF tests, antiserum was prepared in Rabbit 188 against 3102 grown for 48–72 hr. in liquid medium containing unheated rabbit serum (15–20%, v/v). The strain was subcultured seven times on agar or in broth containing rabbit serum before the inoculum was prepared. Cultures were harvested when the pH reached 7.0–7.2, washed twice in saline and stored at −30°C. Growth from 250 ml. of culture was emulsified in adjuvant (9 parts of Esso Markol 52 to 1 part of Arlacel) and given in two subcutaneous injections, separated by an interval of 3 weeks. Three weeks after the second inoculation, a saline suspension from another 250 ml. of culture was given in six intravenous injections. Rabbits were bled 1 week after the last injection.

Haemagglutination-inhibition (HI) test for Sendai virus (parainfluenza 1)

For the tests done at Pfizer Laboratories the Sendai virus used was an isolate from Carworth mice and was used at eight haemagglutinating (HA) units. Sera were titrated with and without prior heating at 56°C. for 30 min. Virus-serum mixtures were held at 4°C, for 1 hr., then 1% chicken red cells were added, followed by a further hour at 4°C. before reading the end-point as the highest dilution showing complete inhibition. As a control, a rabbit antiserum with a titre of 128 was run in parallel.

For the Sendai antibody tests made at the Clinical Research Centre Laboratories the method used was that described by Tyrrell & Coid (1970).
RESULTS

Isolation of mycoplasmas from the natural disease

During the 3–6 days in which the seven rats from the natural outbreak were held in isolation before killing, their clinical condition progressively improved, although they were receiving an antibiotic-free diet.

When killed, five showed extensive pneumonia (scores 18, 20, 30, 35 and 52) with copious catarrhal exudate in the trachea and major bronchi while the other two had small pneumonic lesions. Histologically the group showed purulent or chronic bronchitis and bronchiolitis, peribronchiolar mononuclear cell accumulations, massive lymphoreticular hyperplasia and organization. Perivascular mononuclear cell accumulations were common.

The lungs of two rats, 3102 and 3103, were examined for mycoplasmas; touch preparations showed many small rod-shaped organisms of the mycoplasma type associated with the cilia of the epithelial cells (Pl. 1, fig. 1), while on cultural examination each lung yielded approximately $10^8$ mycoplasma colonies/g.

After storage in the deep freeze, the lung tissue of rat 3102 was cultured for bacteria using a sample of the suspension prepared for inoculation of the group-B rats. No eubacteria were isolated after seeding 0.025 ml. samples on the media already described as well as into Albini brucella broth.

Cultural characteristics of the isolates from rats 3102 and 3103

After 2 days incubation on A20 solid mycoplasma medium, minute colonies could be detected under the microscope ($\times 30$ magnification); with dilute inocula the mycoplasma could just be seen by naked-eye examination after 4–5 days incubation. Crowded colonies were usually of the ‘fried egg’ type whereas in sparse cultures, the colony form was generally convex. There was usually no increase in colony numbers and little increase in colony size after the sixth day of incubation, by which time colonies were over 0.5 mm. in diameter. At this age colonies still could be subcultured readily. Touch preparations of colonies contained pleomorphic elements of the mycoplasma type.

Colonies formation was not inhibited by penicillin (200 units/ml.) or thallium acetate (1/80,000), i.e. the concentrations used in the normal growth medium. Both strains (3102 and 3103) were sensitive to the tetracyclines, e.g. in tests with disks containing 5 μg. of tetracycline, the nearest mycoplasma colonies developed 14 mm. away.

Strain 3102 was passaged on solid medium without penicillin or thallium acetate, immediately after primary isolation. It retained its usual colonial appearance during these passes.

In liquid medium both strains produced acid, the pH of the medium changing from 7.5 to 6.8 within 24 hr. if 0.01 ml. of culture was seeded into 1 ml. A26 medium. Cultures that had just reached a pH of 6.8 contained $10^8$ to $10^9$ CCU but no turbidity was observed. Where the same material was titrated in parallel in liquid and on solid medium, the liquid media gave a titre of CCU about 1 log higher than the titre of CFU on solid medium.
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Table 1. Reactions of mycoplasma 3102 with antisera to mycoplasmas of murine origin

<table>
<thead>
<tr>
<th>Antiserum against mycoplasma</th>
<th>Homologous 3102</th>
<th>Width of growth inhibition zone (mm.)</th>
<th>Reciprocal of CF titres with antigens</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. pulmonis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kon</td>
<td>5-6</td>
<td>1280</td>
<td>640-1280</td>
</tr>
<tr>
<td>M1</td>
<td>6-7</td>
<td>5120</td>
<td>2560-5120</td>
</tr>
<tr>
<td>MB</td>
<td>5-5</td>
<td>1280-2560</td>
<td>1280</td>
</tr>
<tr>
<td>Type C</td>
<td>5-5</td>
<td>1280-2560</td>
<td>1280</td>
</tr>
<tr>
<td>M. arthritidis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PG27</td>
<td>2-0</td>
<td>20480</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>Jasmin</td>
<td>NT</td>
<td>20480</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>M. neurolyticum (KSA)</td>
<td>4-0</td>
<td>10240</td>
<td>&lt; 10</td>
</tr>
</tbody>
</table>

NT = not tested.
Bold type represents homologous reactions.
Pre-immunization sera corresponding to all sera except M1 and Jasmin were available and gave CF titres of < 10 with both homologous and 3102 antigens.

Serological identification of mycoplasma 3102

Testing of 3102 against antisera to various mycoplasmas

In growth-inhibition tests strain 3102 was inhibited by antisera to four strains of M. pulmonis, namely Kon, M1, MB and Sabin's type C (Table 1). These antisera were judged to be specific as they did not inhibit any of the other mycoplasmas listed in Materials and Methods. Pre-immunization sera from the rabbits subsequently inoculated with Kon, MB and type C did not inhibit 3102. Pre-immunization serum corresponding to M1 antiserum was not available. Mycoplasma 3102 was not inhibited by antiserum to the other 17 species.

With antisera to M. pulmonis strains Kon, M1, MB and type C, 3102 gave complement-fixing (CF) titres comparable with those given by the homologous antigens (Table 1). It did not react with antiserum to the other murine mycoplasmas M. arthritidis or M. neurolyticum (Table 1).

Testing of antiserum to 3102 against various mycoplasmas

Both the antiserum prepared against 3102 in rabbits 187 and 188 inhibited the growth on agar of the homologous strain and M. pulmonis strains Kon, M1, MB and type C. Zones of inhibition were 5-6 mm. in width with an inoculum of 10^4-10^5 CFU/5 cm. plate. The other mycoplasmas listed above were not inhibited by the antiserum.

In CF tests, antiserum prepared in rabbit 188 reacted with Kon, M1, MB and type C as well as with the homologous antigen, but did not react at 1 in 10 with antigens of M. arthritidis and M. neurolyticum (Table 2).

Mycoplasma 3102 was therefore identified as a strain of M. pulmonis. It is closely
Table 2. **CF reactions of antiserum 188* with mycoplasmas of murine origin**

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Reciprocal of CF titres with sera</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Homologous antiserum 188</td>
</tr>
<tr>
<td>3102</td>
<td></td>
</tr>
<tr>
<td><em>M. pulmonis</em></td>
<td></td>
</tr>
<tr>
<td>Kon</td>
<td>2560</td>
</tr>
<tr>
<td>M1</td>
<td>1280</td>
</tr>
<tr>
<td>MB</td>
<td>1280–2560</td>
</tr>
<tr>
<td>Typo C</td>
<td>1280–2560</td>
</tr>
<tr>
<td><em>M. arthritidis</em></td>
<td></td>
</tr>
<tr>
<td>PG27</td>
<td>20480</td>
</tr>
<tr>
<td>Jasmin</td>
<td>20480</td>
</tr>
<tr>
<td><em>M. neurolyticum</em> (KSA)</td>
<td>10240</td>
</tr>
</tbody>
</table>

* Prepared against Mycoplasma 3102. Bold type represents homologous reactions.

related by both CF and GI tests to four strains of *M. pulmonis*, but not to *M. arthritidis* or *M. neurolyticum*, the other recognized mycoplasma species of murine origin. No relationship was found by the GI test between mycoplasma 3102 and 17 other species of mycoplasmas or acholoplasmas derived from a wide variety of sources.

**Clinical signs in the group-A rats**

No clinical abnormalities were detected in the group-A rats for many weeks after they were inoculated with cloned *M. pulmonis* strain 3102; but sneezing was noticed in some of their progeny before they were weaned. It had been noted earlier that the clinical condition of the natural cases of the disease improved when they were moved to a similar isolation cubicle; this suggested that improved environmental conditions could reduce the severity of the disease. Because of the absence of overt signs in the group-A rats, an attempt was made to create conditions in the isolation cubicle that were more likely to favour the development of clinical respiratory disease. From the 79th day after the experiment started, the rat cages were cleaned out only once weekly instead of twice. Following this, an ammoniacal smell was constantly present in the isolation cubicle. The density of rats was also increased by further breeding.

The first case of obvious respiratory disease, which developed when the experiment had been running for 118 days, was in rat 3145 born in the cubicle 78 days previously. This rat had distinct râles when handled, a sign which persisted until it was killed at 85 days of age. Subsequently the number of similarly affected rats increased, so that by about 200 days after the beginning of the experiment nearly all the rats in three-quarters of the cages were affected. The general picture in the colony was one of chronic respiratory infection. However, when the experiment had been running about 450 and 640 days, transient exacerbations occurred.
Pathological findings in the group-A rats

The most consistent finding in the group-A rats was purulent exudate in the bullae of the inner ears. Such exudate was already present in the inoculated rats killed 55 days after infection and in the naturally exposed rat killed at 85 days of age. Thereafter the internal ears of practically every rat were examined and all but two showed copious pus in one or usually both bullae. Very often the bullae were grossly enlarged and the surface bone showed rarefaction so that the creamy-yellow pus could be seen through the bone before the bulla was opened. The older rats (550–715 days) were at least as severely affected as the younger. In one rat (3281) which showed locomotor disturbance for a day before euthanasia, pus had tracked centrally from one bulla through the bone and into the central nervous system.

Table 3 summarizes the pathological findings in the lungs of the group-A rats after different periods of exposure to infection.

The earliest sign of lung involvement in the eight inoculated rats was in one killed at 125 days; this animal showed considerable lymphoreticular hyperplasia with some degree of bronchiolar dilatation. The rat which died at 155 days had quite extensive pneumonia with moderate lymphoreticular hyperplasia and bronchiolar dilatation; the one dying at 573 days had very extensive pneumonia with marked bronchiectasis.

In the rats exposed to natural infection from the inoculated animals, neither gross nor histological pneumonic lesions were detected in the animals killed at 50 days, but at 85 and 108 days quite extensive pneumonia was seen. Thereafter, out of the 40 rats examined up to 715 days of age, 30 showed gross pneumonia. The pneumonic lesions did not appear to become either more or less extensive with increasing age but there were more old animals without pneumonia.

The main histological features seen in the lungs of the group-A rats were associated with the bronchial tree; the earliest cases showed migration of polymorphonuclear neutrophils into the lumina, hyperplasia of the epithelium and an increase in the lymphoid cells surrounding the branches of the bronchial tree.

The later cases showed hyperplasia of the peribronchial glands and accumulation of purulent exudate in the bronchial tree which became grossly distended to form bronchiectatic cavities (Pl. 1, fig. 2). Bronchiectasis was first seen in a naturally exposed rat killed at 85 days and subsequently was obvious during the infection range 291–678 days, but only about half the rats were severely affected. Bronchiolar distension was greatest in animals exposed for 530–570 days, but of 13 rats examined later than this, only three showed any obvious bronchiectasis.

The development of bronchiectasis was associated with squamoid metaplasia of the bronchial and bronchiolar epithelium, and in some long-standing cases much of the alveolar tissue was replaced by multiple bronchiectatic cavities with folded walls (Pl. 2, fig. 3). The bronchioles containing exudate often had lost the cilia from the epithelium. Some of the bronchioles which were distended with purulent exudate showed partial or complete loss of epithelium, the purulent
Table 3. Summary of results from rats (group A) from the colony infected with *M. pulmonis* 3102

<table>
<thead>
<tr>
<th>Rats</th>
<th>Exposure (days)</th>
<th>Extent of pneumatic lesions*</th>
<th>Degree of bronchiolar distension†</th>
<th>Degree of lympho-reticular hyperplasia‡</th>
<th><em>M. pulmonis</em> CF titre in lung (reciprocal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3122</td>
<td>20</td>
<td>0</td>
<td>0</td>
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* M. pulmonis* in lung – N

 AUTHORED BY P. WHITTLESTONE AND OTHERS
Mycoplasma pulmonis pneumonia in rats

Table 3 (cont.)

<table>
<thead>
<tr>
<th>Rats</th>
<th>Exposure (days)</th>
<th>Extent of pneumatic lesions*</th>
<th>Degree of bronchiolar distension†</th>
<th>Degree of lympho-reticular hyperplasia‡</th>
<th>M. pulmonis in lung</th>
<th>M. pulmonis CF titre of serum (reciprocal)</th>
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<tr>
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The first eight rats were inoculated intranasally with cloned M. pulmonis when 45 days old. All other rats were born to those rats or their progeny and thus the length of possible exposure is the same as the age at death.

D = died. N = material not examined. + = isolation of M. pulmonis. (+) = isolation of colonies with the morphology of M. pulmonis.

* 0 = no pneumonia, 100 = entire surface of lung pneumatic.
† Distension of lumen of bronchioles in 0.1 mm. units
‡ Enlargement of peribronchiolar lymphoid tissue in 0.1 mm. units.

The cultural examinations made on bacteriological media resulted in the regular

Microbiology of the group-A rats

The results of the cultural examination of lungs for mycoplasmas are presented in Table 3. It will be noted that the first isolation of mycoplasmas from the inoculated group was from one of the 125-day rats, whereas in the naturally exposed rats one of the lungs was already positive by day 50.

The lungs of 21 rats with pneumonia were examined culturally and 14 yielded colonies of the M. pulmonis morphology. Nine of these isolates were checked serologically and all were specifically identified as M. pulmonis. This series of examinations included the three oldest animals with substantial areas of pneumonia (3341, 3347 and 3348) which had been exposed to infection for 78, 712 and 715 days; all yielded M. pulmonis, the titres being 10⁸, 10⁶ and 10⁴ CCU/g, respectively.

The inner-ear bullae of 26 rats were cultured for mycoplasmas. Of these, 21, including the earliest case (50 days) and the latest case (715 days) so examined, yielded colonies of the M. pulmonis morphology. One isolate was checked serologically and identified as M. pulmonis.

In addition, various cultures were occasionally prepared from other sites, particularly the nasal and tracheal mucosal surface. Practically all these checks were positive for M. pulmonis-type colonies, which were obtained from the nasal cavity up to 379 days and from the trachea up to 631 days after primary exposure. All the mycoplasmas isolated and judged to be M. pulmonis on colonial morphology proved to be this organism when checked serologically.

The cultural examinations made on bacteriological media resulted in the regular
isolation of mycoplasma colonies; these results being comparable with those on mycoplasma media. In contrast, bacterial colonies were obtained infrequently and no species was regularly associated with disease. Thus of 31 lungs cultured bacteriologically, only seven yielded bacteria and from three of these only single bacterial colonies were obtained from 0.02 ml. of a $10^{-1}$ suspension. The only organisms isolated in appreciable numbers were *Pseudomonas pyocyanea* (three lungs) and *Citrobacter freundii* (one lung), but the last rat died and the citrobacter could well have been a post-mortem invader.

The other bacteria isolated from the lungs, inner ears or nasal mucosae in order of descending frequency were *Proteus mirabilis* (from the lung of one rat and from the inner ears of five), *Staphylococcus albus*, *Enterobacter aerogenes*, *Micrococcus spp.*, *Staphylococcus aureus*, *Escherichia coli* and *Proteus morgani*.

It will noted be that no bacteria considered to be respiratory pathogens of the rat were isolated.

*Antibody status of the group-A rats*

Sera from 33 group-A rats were examined by the CF test. No antibody to *M. arthritidis* or *M. neurolyticum* was detected in any serum sample, even at 1/8, the lowest dilution tested. The CF titres obtained with *M. pulmonis* (3102) antigen are presented in Table 3. The rats inoculated and killed after 20 days already showed antibody and those killed after 125 days showed similar antibody titres. Their progeny, which were exposed naturally to infection, were negative at 1/8 at 50 days but positive after 85 days. Thereafter, every serum sample showed a titre of $\geq 32$, the antibody persisting at about the same level (usually 64–512) irrespective of the duration of the infection. The oldest rat killed at 715 days had a titre of 128. Sera from 11 group-A rats were examined for HI antibodies to Sendai virus. All were negative at the lowest dilution tested (1/2 or 1/5). The sera were evenly distributed from the 20th to the 713th day of the experiment.

*Comparison of results from group-B rats with those from group A*

The group-B rats were included in the experiment to find whether an inoculum of pneumonic tissue would induce disease comparable with that observed at Alconbury. The disease which developed in the group-B rats was similar to that in group A, and in each group the disease was milder for the first few months than in the Alconbury outbreak.

Since the group which received the cloned culture developed the full respiratory disease syndrome, group B was not examined in such detail and the results are therefore summarized only briefly.

The rats inoculated were killed between 13 and 441 days later, and the progeny of these animals were killed between 42 and 401 days of age.

Twenty-eight rats were examined post mortem; otitis media was first detected in a rat killed 49 days after inoculation and almost every rat examined later had copious purulent exudate in the bullae of the internal ear. Almost half the rats killed later than 40 days had gross pneumonia, the two most extensive cases scoring 70 and 60 after being exposed to infection for 168 and 401 days respectively.
Catarrhal tracheitis occurred both in pneumonic rats and in some of the rats without gross pneumonia.

The histopathological changes in the pneumonic lungs, including the degree of bronchiectasis and lymphoreticular hyperplasia, were comparable with those occurring in the group A rats.

Sera from 36 group-B rats were examined for CF antibodies against the three known species of mycoplasmas of murine origin; six of the sera were from rats inoculated with the pneumonic lung suspension, the rest from their offspring. Antibody to \textit{M. pulmonis} was detected as early as 13 days after exposure (titre 32–04) in rats directly inoculated and was still present after 441 days, when the last rat was killed. The naturally infected progeny, like those in group A, took longer to develop a positive titre; antibody was not detectable at 42 days of age, although present at 168 days and thereafter up to 401 days, the age of the oldest rat examined. Titres were of the same order as those in group A, namely 32–256. Antibody to \textit{M. arthritidis} or \textit{M. neurolyticum} was not detected. Sera from six group-B rats were examined for HI antibodies to Sendai virus; all were negative at a dilution of 1/2, the lowest dilution tested.

\textit{Absence of diseases in control rats (group C)}

The group-C colony remained in good health until completion of the experiment in September 1971; only seven animals died from accident, tumour or old age.

Post-mortem examinations were made on 58 rats, starting 20 days before the group A and B rats were inoculated. The oldest control rats so examined were more than 700 days old when killed. Apart from the five rats with tumours, virtually no macroscopic abnormalities were detected and only rat 3239 had gross consolidated areas in the lung; these consisted of tumour tissue.

Ten of the 57 lungs examined histologically had tiny discrete areas in which foamy macrophages had accumulated in the subpleural alveolar spaces. The condition was morphologically identical with that described by Beaver, Ashburn, McDaniel & Brown (1963). Otherwise the lungs were histologically normal with no exudates in the bronchial tree; the lymphoid nodules associated with the bronchiolar tree were very small, usually being 0·3–0·4 mm. in diameter, but reaching 0·7 mm. in some rats. These dimensions together with the measured diameters of the bronchiolar lumina were taken as normal in assessing the degree of lymphoreticular hyperplasia and bronchiectasis in the infected rats.

The sera of 54 group-C rats were examined for CF antibody to the three murine strains of mycoplasma. Antibody to \textit{M. arthritidis} or \textit{M. neurolyticum} was never detected. Only one rat had a titre of 16 with \textit{M. pulmonis}; this animal had a mandibular neoplasm.

\textbf{DISCUSSION}

The organism isolated from the pneumonic lung of rat 3102 from the Alconbury outbreak of severe respiratory disease was identified as a mycoplasma on the basis of its morphological and cultural characteristics and its antibiotic sensitivity, and it was identified serologically as \textit{M. pulmonis}. That \textit{M. pulmonis} was estab-
lished in the Alconbury colony (Lane-Potter et al. 1970) was confirmed by the regular isolation of mycoplasmas from affected rats – those isolates checked serologically all proved to be *M. pulmonis* – and by the detection of antibody in several other rats (R. J. Olds and R. M. Lemcke, unpublished observations).

All the evidence supports the view that the culture inoculated into the group-A rats contained only *M. pulmonis*. The culture had been purified by four serial single-colony passages on solid medium, and the inoculum yielded only mycoplasma colonies of one morphological type which were identified serologically as *M. pulmonis*. The possibility of mechanical carry-over of a hypothetical virus from the natural disease to the rat inoculum would seem to be remote, since the cumulative dilution of the original pneumonic lesions in the rat inoculum was at least $10^{-25}$.

The disease in rats of group A directly inoculated with *M. pulmonis* was characterized first by inner-ear involvement and only later by pathological changes in the lung. Although histological abnormalities were not seen in the lungs of the rats killed at 55 days, CF antibody was already present at 20 days. In the progeny of the inoculated rats, lung involvement and CF antibody were first detected after 85 days exposure, so that lung lesions appeared more rapidly but CF antibody more slowly than in the rats directly inoculated.

The slower development of CF antibody in the exposed progeny may have resulted from their smaller infecting dose, or their younger age at exposure, when they were immunologically less efficient and when they probably had passively acquired antibody. The earlier isolation of the organism from the naturally exposed progeny and the more rapid development of pneumonia in these animals could be related to various factors, such as the organism's adaptation to the natural host, its association with natural exudates, and the size of the inhaled particles. Clearly more work to investigate the possible role of these factors is needed.

An analysis of the results in the naturally infected rats in group A showed that there was a correlation between the extent of pneumonia and the CF titre to *M. pulmonis* at the time the rat was killed. If the rats were divided into two groups on the basis of CF titre, the group with CF titres of 64 and less had an average pneumonic score of 5.0 whereas the group with CF titres of 128 and above had an average pneumonic score of 36.3.

That the long-standing extensive pneumonias consistently yielded *M. pulmonis*, while the lungs with small or no lesions were culturally negative, suggests that there is a close correlation between the continued activity of *M. pulmonis* and both the CF titres and extent of pneumonia.

In other species, pneumonias caused by mycoplasmas resolve slowly; for example, the diseases in man and the pig caused by *M. pneumoniae* and *M. suipneumoniae* eventually resolve. Similarly, in our experiments with *M. pulmonis* there was evidence for eventual resolution of the pneumonia. Although some group-A rats still had pneumonia after 2 years' exposure, the rats killed more than 500 days after exposure more frequently had low pneumonic scores than had rats infected for shorter periods (Table 3).
The fact that both pneumonia and *M. pulmonis* persisted in the presence of high titres of circulating CF antibody suggests that the latter does not play a major part in protection or recovery from the mycoplasma infection. A similar lack of correlation between the presence of circulating antibody and the immune status has been reported in two other pneumonic diseases caused by mycoplasmas—bovine contagious pleuropneumonia (Davies & Hudson, 1968) and enzootic pneumonia of swine (Goodwin, Hodgson, Whittlestone & Woodhams, 1969). CF antibody can nevertheless be a useful specific indicator of infection.

The group-B rats inoculated with a pneumonic lung suspension and their progeny showed a similar pattern of diseases and antibody development to that seen in group A. Group-B rats were no more severely affected than those in group A and there was therefore no indication that the pneumonic lung suspension contained pathogens in addition to *M. pulmonis*.

In the uninoculated group-C rats the absence of histological abnormalities correlated with the consistent failure to isolate *M. pulmonis* and to detect CF antibody to the mycoplasma.

The respiratory disease in the experimental animals of groups A and B was less acute than that observed during the natural outbreak at Alconbury described by Lane-Petter et al. (1970), in that it took a long time to become severe and cause deaths. This might indicate that in the Alconbury outbreak an agent in addition to *M. pulmonis* was involved, and that this was not present in the material used to inoculate the experimental rats. On the other hand, if *M. pulmonis* was the prime pathogen in the natural disease, differences between the environments of the experimental and of the Alconbury rats could account for the disease presenting differently. Environmental factors such as degree of crowding, concentration of ammonia in the air and ventilation and humidity are known to influence the establishment of lower respiratory disease in the rat (Giddens, Whitehair & Carter, 1971). Indeed, seven severely affected rats removed from the Alconbury colony to an isolation cubicle similar to those in which the transmission experiments were carried out, improved clinically. The Alconbury workers considered that these rats would probably have died soon if they had been left in the colony.

That more overt respiratory disease developed in groups A and B when the density of the population was allowed to increase, and cages were cleaned out only once a week, also indicates that environmental conditions can affect the course of respiratory disease. If the colony had been of a size comparable to the one at Alconbury a more acute type of infection might well have developed.

*M. pulmonis* was the only pathogen consistently isolated post mortem from rats of groups A and B. All the isolates were of the *M. pulmonis* colony type, which is distinct from that of *M. arthritidis* or *M. neurolyticum*, and all the isolates tested serologically proved to be *M. pulmonis*. Similarly, CF antibody was detected only to *M. pulmonis* but not to *M. arthritidis* or *M. neurolyticum*. That the *M. arthritidis* antigen used to monitor the rat sera in these experiments was satisfactory was shown by its ability to detect specific antibody in another stock of rats from which *M. arthritidis* was isolated (R. M. Lomcke, unpublished observations). The negative results with *M. neurolyticum* are consistent with the fact that this
mycoplasma has never been reported to occur naturally in rats. Although the isolation of urea-metabolizing T-strains was not attempted, these mycoplasmas have never been reported in rodents. There was no evidence, therefore, that mycoplasmas other than *M. pulmonis* were involved in the experimental disease. Similarly, bacterial respiratory pathogens such as *Pasteurella pneumotropica*, *Streptobacillus moniliformis*, *Bordetella bronchiseptica* and *Diplococcus pneumoniae* were not found in the experimental cases.

The possibility of Sendai virus being involved in the experimentally-produced disease was considered since it was present in mice in the Alconbury colony (Lane-Petter *et al.*, 1970) shortly before the rat material used in this study was collected. This virus has been shown to cause acute respiratory infection in rats, coupled with high antibody titres within three weeks of inoculation (Tyrrell & Coid, 1970). It is not known how long such antibody persists in rats, but in mice it appears to last for life (Parker & Reynolds, 1968; C. R. McDonald, personal communication). Thus the failure to find Sendai antibody in any of the experimental rat sera is probably significant.

Rat coronavirus induces a fatal pneumonitis in newborn rats (Parker, Cross & Rowe, 1970). It seems unlikely from the clinical picture in our rats that this virus was active, but specific serological checks should be made for it in future studies.

The possible presence of unidentified agents (e.g. the 'virus' of Nelson, 1967; the grey-lung agent, or the similar non-cultivable mycoplasma-like organism described by Gay, 1967a, b; 1969) cannot be excluded in the absence of definitive tests for such agents. The dilution of the inoculum of the cloned mycoplasma renders the carry-over of any non-cultivable agent unlikely. The possibility arises that the primary barrier-maintained stock used for the experiments were infected with some pneumonia-inducing agent which was exacerbated by intranasal inoculation. All the evidence seems to be against this since the progeny in groups A and B developed respiratory infection merely by being in contact with the inoculated animals. Moreover the histopathology of the uninoculated control animals from the same stock over a period of 2 years (group C) did not suggest the presence of an infective agent.

The progressive development of pulmonary lesions in this series of experiments was similar to that described by Lindsey *et al.* (1971). It should be noted, however, that in their work frank pulmonary lesions resulted only from the inoculation of pneumonic lung or of inocula containing unpurified cultures of *M. pulmonis*. Their only cloned strain (N) did not produce otitis media, tracheitis or even microscopic lung lesions; nor were the tracheal cultures of rats inoculated with this strain positive for *M. pulmonis*. This would suggest either that their cloned strain had lost its pathogenicity or that their experiments were not of sufficient duration for the organism's pathogenicity to manifest itself. If we had killed all of our rats by 60 days (as did Lindsey *et al.* with their cloned *M. pulmonis* group) we would have seen virtually no evidence that our cloned strain was pathogenic for the lower respiratory tract.

Although considerable lymphoroticular hyperplasia developed in a proportion of the group-A rats, this feature did not progress as the duration of infection in-
Mycoplasma pulmonis pneumonia in rats

creased, and it did not reach the proportions often seen in rat colonies naturally affected with chronic respiratory disease. This suggests that factors in addition to \textit{M. pulmonis} might be involved in progressive lymphoreticular hyperplasia. On the other hand it is also possible that under different circumstances \textit{M. pulmonis} alone might induce this change.

We noted that, in addition to the otitis media frequently associated with \textit{M. pulmonis} infection, this organism also invaded the cochlea and vestibule of the inner ear and caused a persistent purulent otitis interna.

Thus, our experiments showed that pneumonia and bronchiectasis as well as inner-ear infection can develop in a barrier-maintained rat stock infected with a serially cloned culture of \textit{M. pulmonis} in the absence of any other demonstrable pathogen. Only Kohn & Kirk (1969) have obtained similar results in gnotobiotic rats with their once-cloned \textit{M. pulmonis}. The cloned strain of Lindsey et al. (1971) appeared not to be pathogenic by the intranasal route. Bell & Elmes (1969) did not produce chronic respiratory disease or CF antibody response using intranasal infection of rats; they called their organism \textit{M. pulmonis}, but did not provide evidence of its identity. The unidentified mycoplasmas inoculated into rats by Pankevicius, Wilson & Farber (1957) and Joshi, Dale & Blackwood (1965) also failed to produce pathological changes in the lungs.

These apparently conflicting results might be explained by differences in the experimental procedures. For example, in most experiments, a single inoculum of the mycoplasma was used, whereas the rats of Kohn & Kirk (1969) were repeatedly inoculated or naturally exposed. Similarly, in our experiments, the group-A progeny in which pneumonia was most severe were also repeatedly exposed to infection by being in direct contact with the rats which had been inoculated. Further, pneumonia developed only slowly in our rats, and some of the experiments described by others may not have been run long enough to allow the development of lung lesions. The environmental conditions under which the rats were kept in the various experiments probably affected the rapidity with which pulmonary infection developed; it is likely that different strains of rats vary in their susceptibility to \textit{M. pulmonis} infection. This obtains in the development of both pneumonia (Brennan & Feinstein, 1969) and arthritis (Hannan, 1971) following the inoculation of \textit{M. pulmonis} into different strains of mice. Moreover the strains of \textit{M. pulmonis} used by different workers probably differed in their pathogenicity. Virulence may be lost on passage in artificial media as it is with \textit{M. pneumoniae} (Couch, Cate & Chanock, 1964) and the type of medium upon which the organism is passed may influence the rate of loss of virulence. The medium used in our experiments for the isolation, cloning and propagation of \textit{M. pulmonis} 3102 was that developed for the isolation of \textit{M. suis pneumoniae} and, as it differs in several respects from the formulae generally used for mycoplasmas, it may favour the maintenance of pathogenicity.

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ADDENDUM

The culture of *Mycoplasma pulmonis* (3102) purified by four serial single-colony subcultures has been deposited at the Mycoplasma Reference Laboratory, Central Public Health Laboratory, Colindale Avenue, London, N.W. 9

REFERENCES


Fig. 1

Fig. 2

P. WHITTLESTONE, RUTH M. LEMCKE AND R. J. OLDS  
(Facing p. 406)
Mycoplasma pulmonis pneumonia in rats


EXPLANATION OF PLATES

PLATE 1

Fig. 1. Touch preparation of pneumonic lung of rat 3102 showing large numbers of rod-shaped and coccal organisms of the mycoplasma type in the ciliary area of bronchiolar epithelial cells. Giomsa, × 1000.

Fig. 2. Section of lung of rat 3341 exposed to cloned Mycoplasma pulmonis for 678 days. Marked distension of the bronchial tree due to accumulation of purulent exudate. H & E, × 40.

PLATE 2

Fig. 3. Section of lung of rat 3291 exposed to cloned Mycoplasma pulmonis for 631 days. The alveolar tissue is replaced by multiple bronchiectatic cavities with folded walls. H & E, × 40.