

EXPERIMENTAL INFECTION WITH MYCOPLASMA
PNEUMONIAE (EATON'S AGENT)*

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(Received for publication, March 5, 1965)

Eaton's atypical pneumonia agent, *Mycoplasma pneumoniae*, has been established as a cause of human disease through efforts of a number of investigators over the past 25 years (1, 2). This work has provided knowledge concerning the natural history, diagnosis, and therapy of the disease. The pathogenesis of *M. pneumoniae* infections has been difficult to study, however, due to several factors: (a) research has been hampered by technical problems associated with identification and propagation of the agent; (b) human disease is a self-limiting, rarely fatal process, providing little pathologic material for examination; and (c) host systems for experimental infections have been limited in number and usefulness. Although growth of Eaton's agent in lifeless media (3) has facilitated many studies of the mycoplasma, the availability of human material for investigation will remain limited. A satisfactory animal host is thus needed to resolve problems that cannot be studied directly in man.

Eaton first demonstrated that pneumonia could be induced in cotton rats and hamsters inoculated with sputa from patients with the atypical pneumonia syndrome (4). The histopathology of this disease in the hamster and its relationship to *M. pneumoniae* were studied more extensively by Goodburn and Marmion (5). In the investigation to be reported, the hamster was further evaluated as a host for experimental *M. pneumoniae* infections. Confirmation of the earlier studies was accomplished using pure cultures of the mycoplasma for inoculation of the animals; the results were further extended by application of quantitative culture techniques, and special histopathologic methods per-

* This investigation was conducted under sponsorship of the Commission on Acute Respiratory Diseases, Armed Forces Epidemiological Board, and was supported by the Office of The Surgeon General, Department of the Army. A portion of this work was reported to the American Association of Immunologists, April 13, 1964.

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mitted localization of the organisms in the diseased host. The findings demonstrated the sensitivity of the hamster to *M. pneumoniae* and indicated the suitability of this animal as an experimental model.

Materials and Methods

Media.—Cultivation of *M. pneumoniae* was achieved using the medium devised by Hayflick (6) which contained 70 per cent Difco PPLO (pleuropneumonia-like organism) agar or broth base, 20 per cent unheated horse serum, and 10 per cent fresh aqueous extract of baker's yeast (Fleischmann, Standard Brands, Inc., New York) (7). Penicillin G, 1000 u./ml, was added to all PPLO media to inhibit bacterial growth.

Mycoplasma Strains.—Organisms used in the experiments were derived from two parent strains of *M. pneumoniae*, a recent isolate from this laboratory, and the Mac strain originally isolated by Eaton (4). The recent isolate, strain Bru (American Type Culture Collection 15377), was recovered on agar medium from sputum of a patient with atypical pneumonia whose case is described elsewhere (1). Single colonies were cut from the initial agar plate and placed in broth to produce a cloned subculture, designated Bru-A1B1 (agar 1, broth 1). This was used as inoculum for an additional broth passage, Bru-A1B2, which contained 10^6 colony-forming units (CFU)/ml of broth. For certain experiments, the pool was further subcultured 34 times in broth (Bru-A1B36) or once in hamsters (Bru-A1B2H1).

The Mac strain of *M. pneumoniae* was received from Dr. Chien Liu as the seventy-eighth passage in chick embryos (Mac-E78). Additional subcultures of several types were made: 5 times in chick embryos (Mac-E83); once in rhesus monkey kidney cell culture (Mac-E78MK1); and 21 times in PPLO agar followed by 11 times in PPLO broth (Mac-E78A21B11). Organisms from both Mac and Bru pools were identified as *M. pneumoniae* by use of techniques which have been described previously (7). The mycoplasma pools were divided into small aliquots which were stored at -65°C until needed for inoculation of the animals.

Hamsters.—Adult Syrian hamsters weighing approximately 80 gm were obtained from Hilltop Caviary, Scottdale, Pennsylvania. The animals were anesthetized with intraperitoneal pentobarbital sodium (nembutal, Abbott Laboratories, North Chicago, Illinois) since *M. pneumoniae* is rapidly killed by exposure to ether (8). The inocula were measured in tuberculin syringes, 0.1 ml being dropped into the nares of each animal through a 23 gauge needle. After inoculation, animals were housed in individual cages unless otherwise specified.

Mycoplasma Isolation Techniques.—In obtaining materials for culture the animals were sacrificed by exsanguination after pentobarbital anesthesia. The nasal turbinates were cultured *in situ* after the nasal passages were unroofed; lungs and tracheas were removed for culture. Turbinate, tracheal lumen, and cut lung surfaces were cultured using miniature Bradford swabs made from wisps of cotton and short lengths of 000 stainless steel suture wire. The lungs were separated by lobes, some being fixed or quick-frozen for pathologic study, while the remainder were aseptically ground with powdered aluminum in PPLO broth. The crude lung suspensions were centrifuged at 1000 *G* for 10 minutes to remove tissue debris, and the supernatant fraction was collected for quantitative culture. Additional details regarding culture techniques are described with the experiments concerned.

Agar medium inoculated with the harvested materials was incubated at 37°C in humidified air containing 5 per cent CO_2 . Plates were examined for characteristic *M. pneumoniae* colonies at intervals, and after 1 week an overlay of sheep blood agar was applied to the plates as previously described (9). Zones of clear hemolysis developing around the colonies in 18 to 24 hours' additional incubation aided recognition, identification, and enumeration of colonies. Cultures initiated in broth medium were incubated for 5 to 7 days and then subcultured to solid medium for processing in the same manner.

Histopathologic Techniques.—Tissues removed from the hamsters were fixed and stained by several different methods which are detailed in standard references (10). Fixative solutions employed were: 10 per cent USP formalin in distilled water, in which tissues remained 48 hours; Helly's fixative, applied for 6 hours; or Van de Grift's solution, in which tissues were left for 8 to 18 hours (critical). As the Van de Grift's fixative alone preserved the mycoplasma, its exact preparation is defined as follows: in the order stated were combined 95 per cent ethyl alcohol (80 ml), neutral formalin (12 ml), glacial acetic acid (4.5 ml), picric acid crystals (4 gm), mercuric chloride (0.2 gm), and urea (0.5 gm). Each solid was placed in solution before addition of the succeeding one. After fixation in one of these reagents, tissues were transferred to 70 per cent ethyl alcohol for 4 to 6 hours, embedded in paraffin, sectioned, and stained by several standard techniques (hematoxylin-eosin, Gram, Gomori, and Wright).

Special techniques included the intensified Giemsa method of Marmion and Goodburn (11), the Brown and Brenn stain (12), and the indirect fluorescent antibody reaction (13). Successful staining of *M. pneumoniae* was achieved in the present investigation through modification of the Brown and Brenn method, and the procedure was as follows. Sections 6 to 8 μ thick on slides were washed 10 minutes in tap water. In succession, with water washes between steps, were immersion in 1 per cent crystal violet (2 minutes), Lugol's iodine (1 minute), acetone (15 seconds), and 0.05 per cent basic fuchsin (5 minutes). Slides were blotted dry and washed for a few seconds each in acetone and 0.01 per cent picric acid in acetone. The sections were then passed through acetone-xylol (equal parts) and xylol, and were mounted permanently using Harleco synthetic resin (Hartman-Leddon Co., Philadelphia).

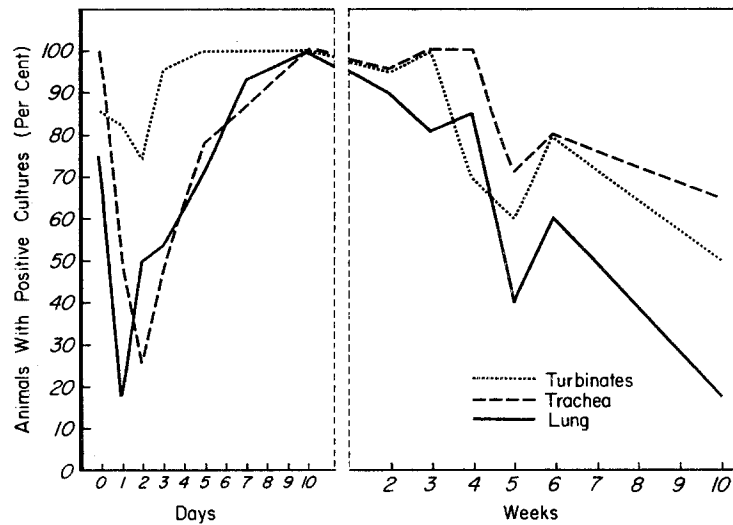
EXPERIMENTAL

Infection of the Hamster with M. pneumoniae.—It has been demonstrated that pneumonia can be produced in hamsters inoculated with sputa from certain patients with atypical pneumonia (4), and in hamsters given *M. pneumoniae* propagated in embryonated eggs (4, 5). Studies preliminary to the present investigation indicated that disease could also be produced by administration of *M. pneumoniae* cultivated in broth. Experiments were designed to determine the frequency with which hamsters could be infected, the pattern of growth of the mycoplasma *in vivo*, and the sensitivity of the hamster to the organism.

In the first experiments, the frequency with which the organisms could be recovered from various levels in the respiratory tracts of inoculated hamsters was studied. Groups of animals were inoculated intranasally with 10^6 CFU of strain Bru-A1B2, and were sacrificed at intervals for cultures of their nasal turbinates, tracheas, and lungs. Text-fig. 1 shows the results of several experiments performed over a 1 year period which have been combined because of high comparability. The percentage of specimens yielding *M. pneumoniae* at each interval represents the average of results obtained by study of 6 to 18 hamsters. Immediately following inoculation the organisms could be recovered from 75 per cent or more of the specimens, but a marked reduction occurred over the first 2 days in the number of positive lungs and tracheas. This was followed by a progressive increase in the percentages of samples positive, so that by 10 days all animals yielded organisms from all levels of their respiratory tracts. Beyond the tenth day there was a gradual decrease in the

number of positive samples, most marked in the lung specimens; however, the tracheas remained positive in 70 per cent of the animals for the duration of the experiments (10 weeks).

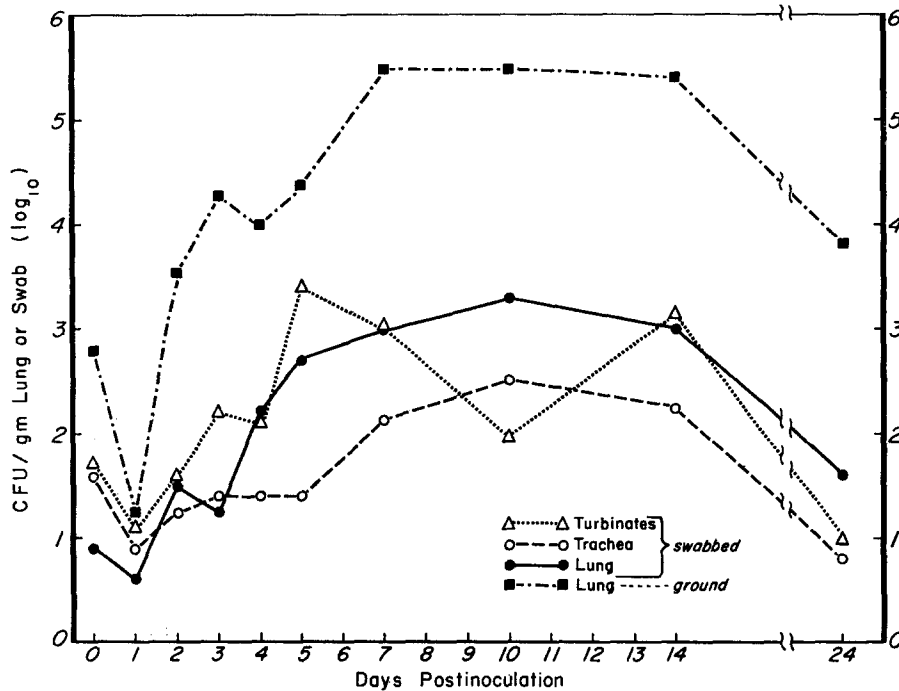
Additional experiments were performed using quantitative culture techniques to study growth of the organisms. For this purpose 78 hamsters were inoculated as before, and at intervals 6 to 8 animals were sacrificed. Samples of lung tissue were ground in sufficient PPLO broth to produce a 10 per cent w/v suspension, which was considered to be a 10^{-1} dilution of the material. Serial 10-fold dilu-



TEXT-FIG. 1. Recovery of *M. pneumoniae* from inoculated hamsters. Cultures were made of 3 sites in the hamster respiratory tract at intervals. Each point represents the average recovery frequency from study of 6 to 18 animals.

tions through 10^{-5} were prepared of the suspensions, and agar plates were inoculated in triplicate with 0.01 ml volumes of each dilution for colony counting. These data allowed calculation of the number of CFU per gram of lung tissue. Cultures were also obtained from the cut surfaces of lungs and from the nasal turbinates and tracheas by rubbing each area 10 times with miniature Bradford swabs of uniform size. The swabs were each rotated 20 times in 2 ml of PPLO broth, and the suspensions were considered to be undiluted as serial 10-fold dilutions were made for colony counting. The results of these studies, expressed as geometric mean titers for each point, are depicted in Text-fig. 2. With reference to the curve plotted for ground lung samples, $10^{2.75}$ CFU of *M. pneumoniae*/gm of lung tissue were recoverable immediately after inoculation, and a decrease of $10^{1.5}$ occurred during the first 24 hours. This was followed by an in-

crease to a maximum of $10^{5.5}$ CFU/gm of lung between the seventh and fourteenth days. A 10-fold decrease in the number of CFU occurred by the end of the experiments at 24 days. That a similar sequence occurred throughout the respiratory tract was suggested by the parallel relationships of curves plotted from values obtained by measurement of swabbed materials (Text-fig. 2). The



TEXT-FIG. 2. Quantitative recovery of *M. pneumoniae* from infected hamsters. Colony-forming units of mycoplasma present in the respiratory tract at various times following inoculation were calculated from colony counts of swab or tissue suspensions. Each point is a geometric mean value obtained by study of 6 to 8 animals.

quantitative discrepancy in the results from ground lungs and swabbed tissues was attributed to the techniques employed; the most comparable data were those for lungs cultured by the two methods. These studies indicated that growth of *M. pneumoniae*, rather than persistence of the inoculum, occurred in the hamster.

The foregoing experiments provided a basis for determination of the minimal infective dose of *M. pneumoniae* for the hamster. Serial 10-fold dilutions of organisms were prepared in PPLO broth to produce samples containing from 1 to 100,000 CFU/0.1 ml. Each dilution was used to inoculate 4 to 9 animals which were sacrificed 14 days later. The technique previously described was used

to determine the number of CFU per gram of lung tissue from each animal. As shown in Table I, all animals were infected following administration of 100 CFU or more. The quantity of organisms recovered from animals receiving 100 CFU varied from 10^3 to 10^5 CFU/gm of lung (geometric mean, $10^{3.8}$). Hamsters given 1000 CFU yielded more organisms, although further inoculum increases had no effect on the number recovered. Application of the Reed-Muench formula (14) revealed the 50 per cent infective dose to be 10 CFU, thus demonstrating the sensitivity of the Syrian hamster to experimental *M. pneumoniae* infection.

Pathology of Experimental M. pneumoniae Infection.—Since the nature of Eaton's agent has been determined, no fatal human infections have been reported. Autopsy findings on patients having atypical pneumonia have been

TABLE I
Effect of Inoculum Size on Recovery of M. pneumoniae from Hamster Lungs

Inoculum (CFU)*	Organisms recovered (CFU/gm Lung, log ₁₀)	
	Individual animals	Geometric mean
10 ⁵	10 ⁶ , 10 ⁶ , 10 ⁶ , 10 ⁶	10 ⁶
10 ⁴	10 ⁴ , 10 ⁵ , 10 ⁵ , 10 ⁵ , 10 ⁶ , 10 ⁶ , 10 ⁶	10 ^{5.3}
10 ³	10 ⁴ , 10 ⁵ , 10 ⁵ , 10 ⁵ , 10 ⁶ , 10 ⁶ , 10 ⁶ , 10 ⁶ , 10 ⁶	10 ^{5.4}
10 ²	10 ³ , 10 ³ , 10 ³ , 10 ³ , 10 ⁴ , 10 ⁴ , 10 ⁴ , 10 ⁵ , 10 ⁵	10 ^{3.8}
10 ¹ ‡	0, 0, 0, 0, 0, 10 ³ , 10 ³ , 10 ⁴ , 10 ⁵	10 ^{1.7}
10 ⁰	0, 0, 0, 0, 0, 10 ³	10 ^{0.5}
None§	0, 0, 0, 0	0

* Colony-forming units of mycoplasma given each animal, log₁₀.

‡ Fifty per cent infective dose, determined by Reed-Muench formula (see text).

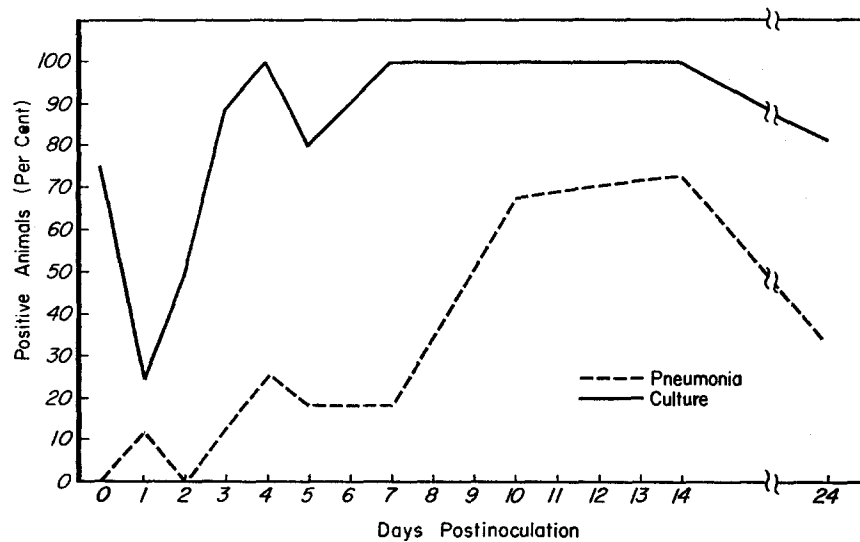
§ Inoculum of sterile PPLO broth.

published (15), but the exact etiology of these cases is difficult to establish in retrospect. The pathologic changes which accompany *M. pneumoniae* infection are therefore poorly understood, and the hamster provided a model in which the pathogenesis of disease could be studied.

Use was made of the animals sacrificed in the experiments described above for pathologic observations. No consistent pattern of gross pulmonary pathology was evident, in agreement with the studies of Goodburn and Marmion (5). Tissues from the animals were preserved in several fixative solutions and by quick-freezing with storage at -65°C for further study. Initial microscopic evaluation was made of tissues fixed in formalin, embedded and sectioned in paraffin, and stained with hematoxylin and eosin. Representative changes, which occurred only in mycoplasma-inoculated animals, are illustrated in Figs. 1 a to 1 d. The most frequent finding was an inflammatory infiltrate surrounding scattered bronchi and larger bronchioles, with preservation of normal archi-

texture in the lung parenchyma (Fig. 1 *a*). Less frequently intraluminal exudates were seen, which varied from a mild reaction (Fig. 1 *b*) to a severe response producing bronchial obstruction (Fig. 1 *c*). As shown in Fig. 1 *d*, the peribronchial infiltration was composed chiefly of mononuclear cells and occasional plasma cells; the intraluminal exudate consisted largely of polymorphonuclear leukocytes. Other findings seen rarely included small atelectatic segments with plasma cell infiltrates, and areas of alveolar edema or thickening.

The pathologic changes could be correlated with the results of cultures done previously on the same animals, as indicated in Text-fig. 3. On the day follow-



TEXT-FIG. 3. Correlation of *M. pneumoniae* isolation and pulmonary pathology in inoculated hamsters. Lung tissues from hamsters sacrificed in a previous experiment (Text-fig. 2) were examined microscopically, and results were related to cultures of the same specimens.

ing inoculation, before mycoplasma multiplication had been detected, a small percentage of animals were noted to have abnormal lungs. These changes, consisting chiefly of small atelectatic areas without inflammatory reaction, were thought to be related to inoculum aspiration since they did not persist beyond 2 days. The pneumonic process which has been described above and illustrated in Figs. 1 *a* to 1 *d* was present after the 3rd day, reached maximum intensity and frequency between the 10th and 14th days, and began to resolve by the 24th day. The development of the greatest degree of pulmonary pathology followed maximum replication of the mycoplasma by at least 3 days, as demonstrated in Text-fig. 3. The sequential nature of these observations suggested that the pathologic changes were induced by growth of the inoculated organisms. Control

animals, which had been given sterile PPLO broth and did not yield *M. pneumoniae* on culture, failed to develop any detectable pulmonary abnormalities. No structures suggesting bacterial forms or viral intracellular inclusions were found in any of the sections examined.

Previous studies have described the appearance of *M. pneumoniae* in infected chick embryos (11) and tissue cultures (16), but attempts to demonstrate the mycoplasma in experimentally infected animals have been unsuccessful (5, 17, 18). This may be attributable to use of techniques involving fixative solutions which do not preserve the integrity of the organisms (formalin, alcohols, osmium tetroxide) (5, 11); additionally, the *Mycoplasmataceae* strain poorly with basic dyes (19). In the present studies, use was made of the fluorescent antibody reaction on frozen lung sections fixed in acetone to localize *M. pneumoniae* antigens. This was followed by evaluation of various histopathologic techniques which might permit direct visualization of the organisms.

Indirect fluorescent antibody staining of hamster lung was performed using tissues sectioned at -25°C . Acetone-fixed sections of infected lungs were treated with human convalescent phase sera or *M. pneumoniae* rabbit antisera, followed by application of goat anti-human or anti-rabbit globulin labelled with fluorescein isothiocyanate. Specificity controls, which revealed no fluorescence, included infected lung sections treated with human acute phase or normal rabbit sera and normal lung sections to which the immune sera were applied. No specific fluorescence was seen in the lung tissue prior to the third day after inoculation, but beyond this time *M. pneumoniae* antigens were detected as small granular clumps on the bronchial epithelial surface as shown in Fig. 2 *a*. With progression of time after inoculation, the antigens increased in amount and were present in deeper layers of the bronchial mucosa (Fig. 2 *b*); at no time, however, was specific antigenic material found in the lung parenchyma. This configuration of the antigens suggested that the organisms were present as extracellular microcolonies, in the form that has been demonstrated in tissue cultures (16) and in preparations made from human sputum (20).

Successful localization of *M. pneumoniae* antigens in infected hamster lungs provided a basis for evaluation of conventional microscopic methods for demonstration of the organisms. Frozen lung sections adjacent to those positive by fluorescent antibody staining were first studied with the intensified Giemsa method described by Marmion and Goodburn (11). No pink coccal forms resembling those found in infected chick embryos were visualized by this means. No organisms could be seen in tissues fixed with formalin, Helly's, or Van de Grift's solutions followed by the stains of Wright, Gram, Giemsa, or Gomori. However, tissues fixed in Van de Grift's solution and stained by a modification of Brown and Brenn's method revealed pink coccobacillary bodies in exact correspondence to the form and localization of antigens demonstrable by fluorescent antibody staining. The appearance of these bodies and their relation to the

bronchial epithelium are demonstrated in Figs. 3 *a* to 3 *c*. As with the fluorescent antibody study, organisms were first seen on the surface of the bronchial epithelium 3 days after inoculation of the animals (Fig. 3 *a*), and later could be found in deeper layers of the bronchial mucosa (Fig. 3 *b*).

The duration of tissue fixation in Van de Griff's solution and the concentration of basic fuchsin in the Brown and Brenn staining procedure both proved to be critical for direct visualization of the mycoplasma. The similarity of this technique to that used by Nelson in work with poultry PPLO strains (21) can be cited.

Specificity and Communicability of Induced Disease.—The outcome of the experiments described above could be influenced by the presence of extraneous viruses, mycoplasma species, or pathogenic bacteria, or by the spread of experimentally-induced disease from one animal to another. The hamster has not previously been found to harbor PPLO (2, 19), nor were any species other than *M. pneumoniae* recovered in these studies. No bacteria were isolated when the tissues studied were cultured on sheep's blood agar or in trypticase-soy broth. While the material was not studied for the presence of viral agents, the inoculum was assumed to be free of viruses since it represented the third passage of *M. pneumoniae* in lifeless media. Gross or microscopic pneumonia was not produced in control animals inoculated with sterile broth, suggesting that possible activation of latent viruses did not result from the procedure.

These observations suggested that the induced disease resulted directly from inoculation. In view of the susceptibility of the hamster to small numbers of *M. pneumoniae*, and the large numbers of organisms recovered from infected animals, it was necessary to evaluate the possibility that the results of prior experiments had been influenced by spread of infection among animals receiving different inocula. In the experiments described previously, control animals inoculated with sterile PPLO broth were housed in the same room with animals given the mycoplasma. *M. pneumoniae* was not recovered from any of the control animals at the time of sacrifice (14 days). An additional experiment was designed to determine if spread among animals occurred after more prolonged contact or incubation periods. Four groups each consisting of 9 to 11 hamsters were placed into 4 isolated compartments. Several animals in each group were inoculated with 10^5 CFU of *M. pneumoniae*, while the remainder received sterile PPLO broth. As indicated in Table II, the groups of animals were sacrificed for culture at 14, 21, 28, and 35 days respectively following inoculation. While the majority of animals receiving the mycoplasma yielded positive cultures, none of the control hamsters were positive by culture nor was there evidence of pneumonia on pathologic study of the lungs. These data indicated that the communicability of *M. pneumoniae* infection among hamsters was of low degree, and that this factor would not influence the results of other experiments.

Effect of M. pneumoniae Strain Variations on Experimental Disease.—In studies

conducted with volunteers it has been shown that *M. pneumoniae* strains repeatedly subcultivated in artificial media are infectious for man but reduced in virulence (22). Most of the present experiments employed a recent isolate of *M.*

TABLE II
Communicability of M. pneumoniae Infection in Hamsters

Cage*	Days postinoculation	Mycoplasma recovery (No. positive/No. inoculated)	
		PPLO inoculated	Broth inoculated
A	14	7/7	0/4
B	21	6/6	0/4
C	28	7/7	0/3
D	35	5/7	0/2

* Animals inoculated with broth and with mycoplasma caged together for indicated period.

TABLE III
Effect of M. pneumoniae Strain Variations on the Response of Inoculated Hamsters

Inoculum		Response§	
Strain*	Size‡	Infection	Pneumonia
	(CFU)		
Bru			
A1B2.....	10 ⁵	5/5	5/5
A1B2H1.....	10 ⁴	5/5	3/5
A1B36.....	10 ⁵	5/5	2/5
Mac			
E83.....	10 ⁵	5/6	6/6
E78MK1.....	10 ²	2/4	0/4
E78A21B11.....	10 ⁵	5/5	0/5

* For source and derivation, see text. Numbers indicate subcultivations in chick embryos (E), monkey kidney cell culture (MK), agar (A), broth (B), or hamsters (H).

‡ Colony-forming units of mycoplasma given each animal, log₁₀.

§ Number of animals with positive cultures (infected) or microscopic pneumonia over number inoculated.

pneumoniae at the third passage level in artificial media (strain Bru-A1B2). The effect of further subcultivation in relation to the establishment and course of hamster infection was examined; additionally, comparison was made of results obtained with the recent isolate and a classic *M. pneumoniae* strain (Mac). The derivation of strains tested is indicated in Table III and described under Ma-

terials and Methods. Groups of hamsters were inoculated intranasally with each strain, and 14 days later the lungs were cultured and examined histologically. All strain derivatives used infected the animals equally well if allowance was made for inequality in the CFU of the inocula (Tables III and I); however, differences were detected in the frequency with which the inocula produced pneumonia. Successive passages of either strain Mac or strain Bru in artificial medium reduced the frequency with which pneumonia was induced, in comparison to material serially subcultured in chick embryos or freshly isolated on agar.

To investigate the possibility that the apparent attenuation of the organisms was a function of time required for growth or disease production rather than reduction of virulence, an additional experiment was performed. Two groups of hamsters were inoculated with strains Bru-A1B2 and Bru-A1B36 respectively, and animals from each group were sacrificed at intervals up to 28 days. The result confirmed that of the previous experiment, and suggested that passage of the mycoplasma in broth medium reduced the occurrence of pneumonia independent of the time at which the animals were examined.

DISCUSSION

The hamster was chosen for the present investigation in view of the work of Eaton (4) and Marmion and Goodburn (5, 11) which indicated susceptibility of this animal to *M. pneumoniae* infection. The nature of the agent, unknown earlier to Eaton, has been defined by growth of the organism in lifeless media; this accomplishment also aided performance of quantitative studies which were impractical previously. By use of these new techniques, the organisms were found to multiply throughout the hamsters' respiratory tract, although overt disease was detected only in the lungs. The appearance of pneumonia coincided with maximum growth of the mycoplasma, and resolution of disease was accompanied by disappearance of organisms from the lung. While the pathologic process thus appeared to be acute and self-limiting, chronic colonization of the upper respiratory passages with *M. pneumoniae* was observed. Recent studies of human disease indicate that the mycoplasma persists in the throat or naso-pharynx at least several weeks beyond clinical recovery (22-24). Furthermore, *M. pneumoniae* pneumonia in man is rarely associated with overt disease elsewhere in the respiratory system. These similarities between human illness and the experimental infections suggest that the hamster is an appropriate model for study of the pathogenesis of *M. pneumoniae* disease.

The infections induced were superficial in nature as indicated by localization of the majority of organisms on the pulmonary epithelial surfaces. While evidence has been produced of an intracellular phase of *M. pneumoniae* growth in one tissue culture system (25), this was not found in other systems (16) nor in the hamster as a reproducible or consistent phenomenon. An intimate relation-

ship between the organisms and the diseased areas was suggested by the results obtained; however, it should be noted that the infiltrative changes which represented the most frequent findings were peribronchial in location, while organisms were not seen peripheral to the muscularis layer of the bronchial mucosa by any technique. The possibility thus exists that the host response to *M. pneumoniae* infection may be mediated through the action of an extracellular product. It is known that *M. pneumoniae* produces a potent, diffusible hemolysin (9, 26, 2), and that other mycoplasma species pathogenic for animals possess extracellular toxins (27, 19).

Deficiencies in knowledge of human pathology prevent comparisons with these experimental data, although the possibility exists that human infection is also superficial in nature. This is suggested by observation that subclinical disease is frequent, pneumonia is mild and self-limited, and colonization with the mycoplasma can take place or persist in the presence of measurable antibody (22). Support for this hypothesis has been provided by Hers (20), who demonstrated that *M. pneumoniae* colonies parasitized the surface of respiratory epithelial cells recovered from human sputa. The induced hamster pathology may thus resemble that occurring naturally in man, by virtue of these similarities in the type of host-parasite relationship.

Study of the pathology of *M. pneumoniae* disease was augmented by demonstration of the organisms at the site of induced lesions. This has not been accomplished previously in a mammalian host. The mycoplasma was visualized by Marmion and Goodburn (11) in the infected chick embryo, although this host does not reveal evidence of disease (17). In the present study, certain technical points were defined which were critical for direct demonstration of the organisms. This suggests that studies of *M. pneumoniae* in other experimental models, where visualization of the organisms is important, would require careful attention to these factors. These technical implications extend to the study of *M. pneumoniae* disease specifically, and to mycoplasma infections generally, in the human host.

M. pneumoniae recovered recently from a living host (man, chick embryo, hamster, or tissue culture) was necessary for production of disease in the hamster. Attenuation of the organism which was produced by subcultivation in lifeless media implies loss of the factor(s) responsible for virulence; the nature of this alteration was not defined in the present investigation, although the virulent and attenuated strains of *M. pneumoniae* employed did not differ in basic biologic properties. Kim and Manire (28) have found recently, however, that antigenic deficits developed in broth cultivated mycoplasma strains compared to the same strains propagated in a tissue culture system. It has been demonstrated in volunteers that *M. pneumoniae* serially subcultivated in artificial media stimulated immune responses but did not produce disease. This finding may have implications for the production of vaccines (22). The effect on viru-

lence which could result from repetitive passage of *M. pneumoniae* through man or animals has not been determined.

SUMMARY

The pathogenesis of *Mycoplasma pneumoniae* infection was studied in the Syrian hamster with qualitative and quantitative culture methods and special histopathologic techniques. The animals were readily infected with the mycoplasma, which multiplied throughout the respiratory tract. Sensitivity of this experimental host to infection was indicated by the 50 per cent infective dose, which was 10 colony-forming units of the organism. Inoculation consistently resulted in the production of peribronchial pneumonitis which was induced by the mycoplasma. The organisms were visualized in a superficial location in the mucosa of involved bronchi, by means of indirect fluorescent antibody staining and by a modification of the Brown and Brenn technique. The data indicate applicability of the hamster to the study of problems concerned with *M. pneumoniae* disease which are impractical or impossible to resolve in the human host.

The authors are indebted to Dr. W. D. Huffines and Mrs. C. K. C. Taylor, Department of Pathology, University of North Carolina School of Medicine, for their invaluable advice and aid in conduct of the pathologic aspects of this investigation.

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EXPLANATION OF PLATES

PLATE 84

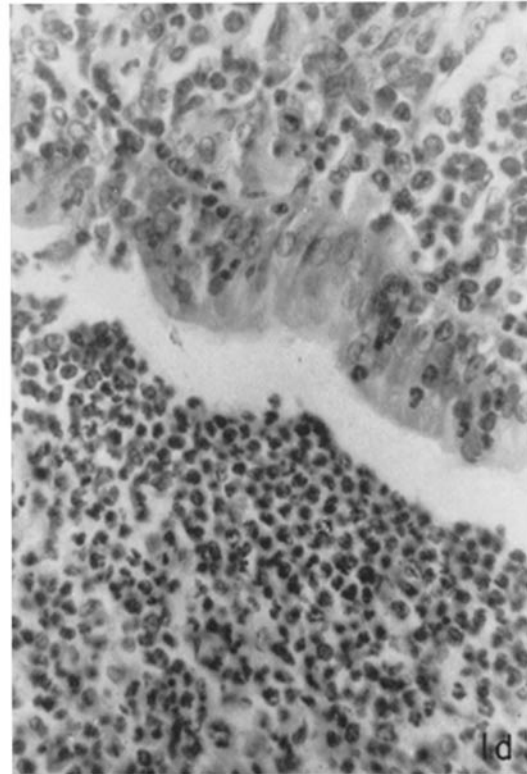
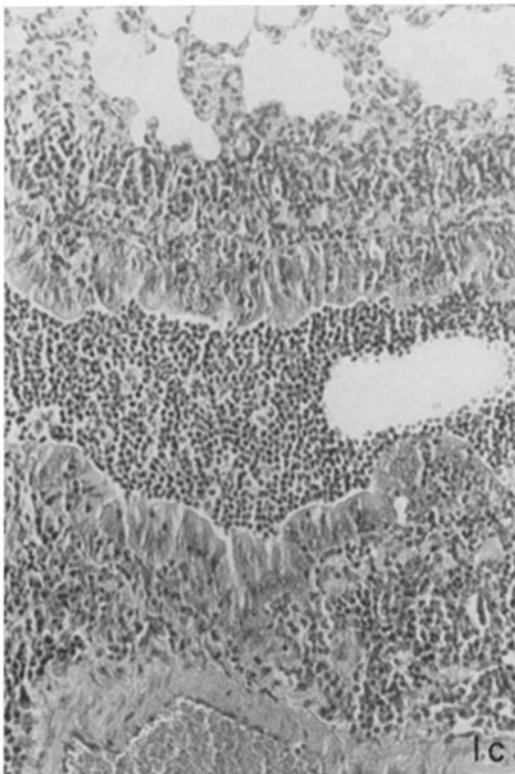
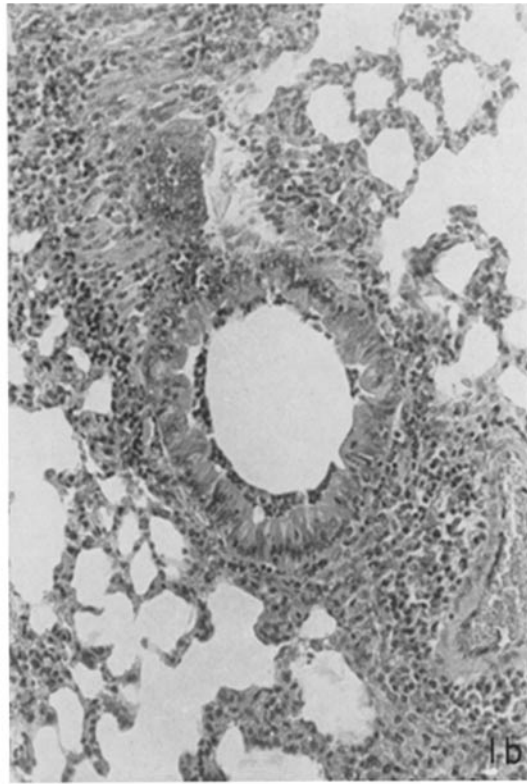
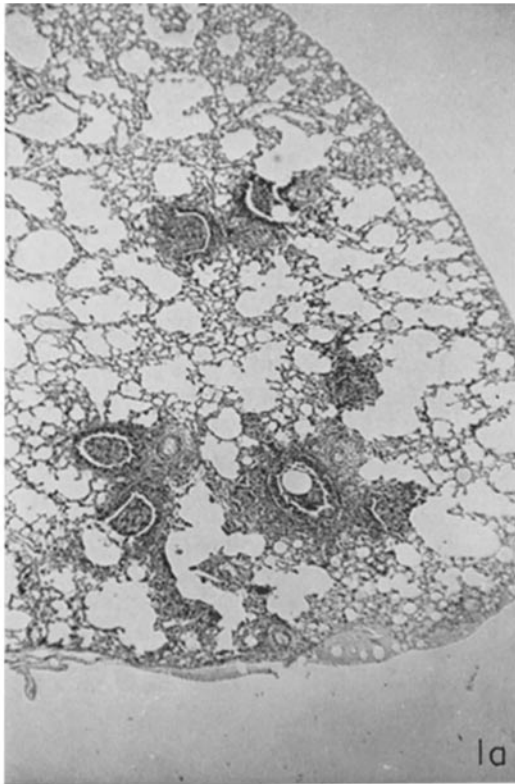
FIGS. 1 *a* to 1 *d*. Photomicrographs of lung tissue from hamsters inoculated 10 days previously with *M. pneumoniae*. Specimens were fixed in 10 per cent formalin and stained with hematoxylin-eosin.

FIG. 1 *a*. Peribronchial inflammatory infiltration is seen scattered through the section, with preservation of parenchymal lung architecture. $\times 8$.

FIG. 1 *b*. Cellular infiltrate surrounds a small bronchus, which also contains scant intraluminal exudate. $\times 48$.

FIG. 1 *c*. Inflammatory exudate completely obstructs a small bronchus sectioned longitudinally. $\times 48$.

FIG. 1 *d*. The inflammatory response is characterized by peribronchial mononuclear cell infiltration (upper right) and intraluminal polymorphonuclear exudate (lower left). $\times 120$.



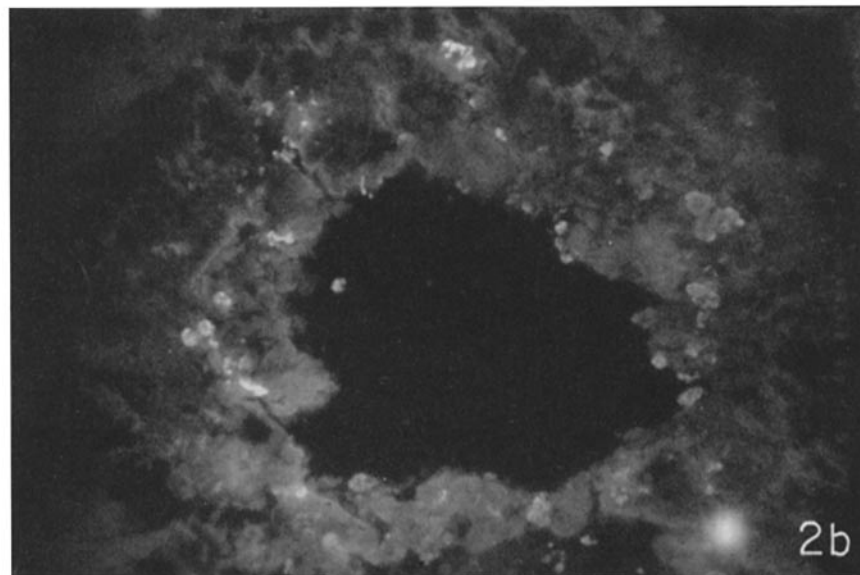
(Dajani *et al.*: *M. Pneumoniae* infection)

PLATE 85

FIGS. 2 *a* and 2 *b*. Acetone-fixed frozen lung sections of hamsters infected with *M. pneumoniae*, after indirect fluorescent antibody staining employing convalescent serum from a patient with atypical pneumonia and goat anti-human fluorescent globulin.

FIG. 2 *a*. Two fluorescing microcolonies are present on the bronchial epithelial surface 3 days after inoculation of the animal. $\times 60$.

FIG. 2 *b*. Appearance of *M. pneumoniae* antigens in a bronchus 7 days after inoculation of the hamster. The fluorescing material is more abundant than at 3 days, and is present throughout the bronchial mucosa. $\times 30$.



(Dajani *et al.*: *M. Pneumoniae* infection)

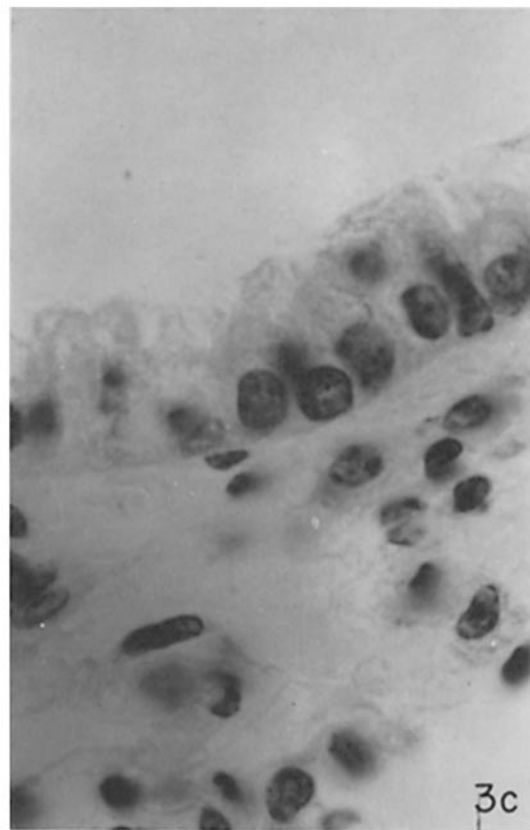
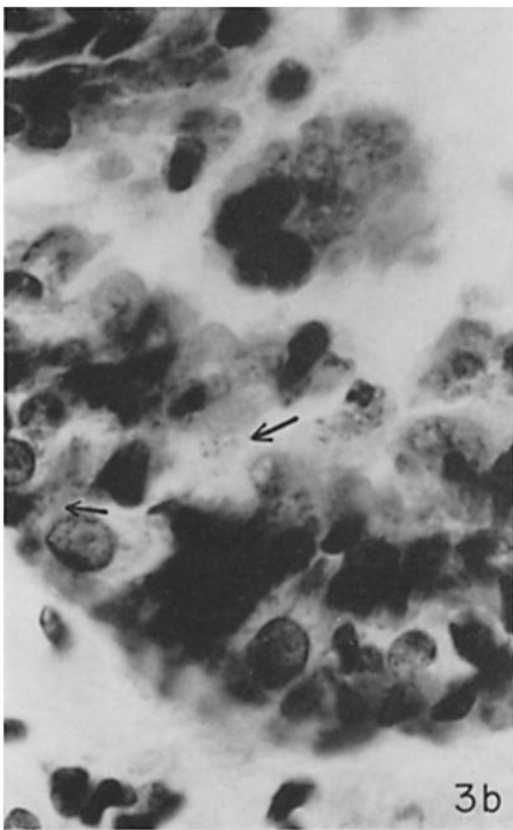
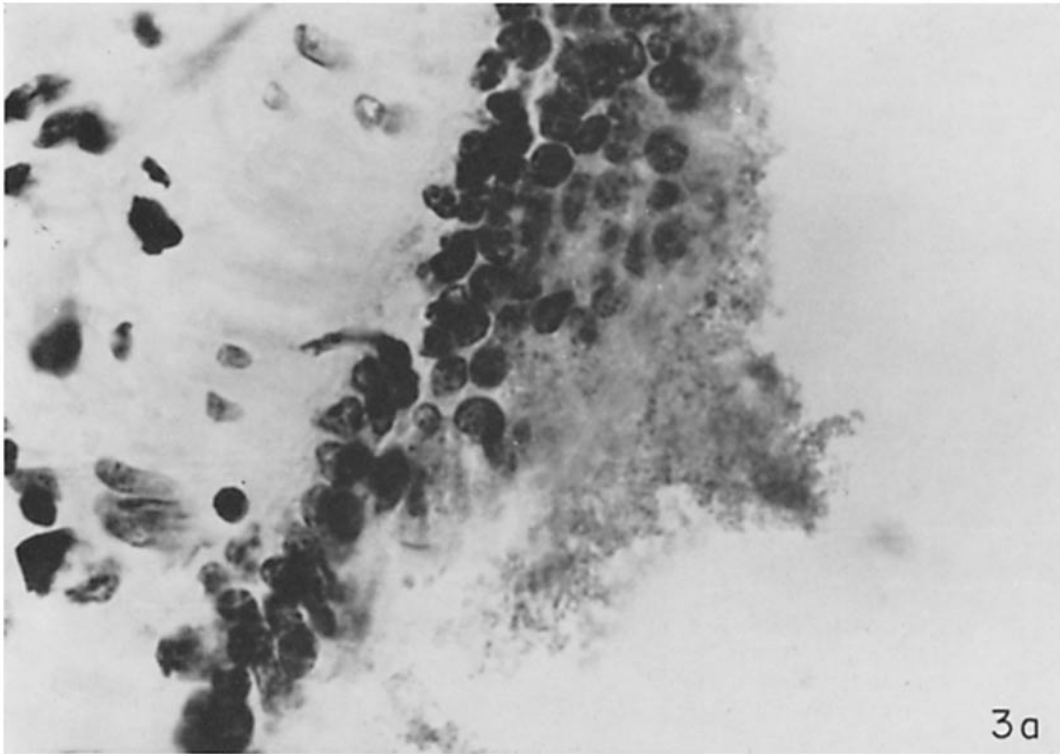
PLATE 86

FIGS. 3 *a* to 3 *c*. Sections from hamsters infected with *M. pneumoniae* were fixed in Van de Grift's solution and stained with a modified Brown and Brenn technique.

FIG. 3 *a*. Three days after inoculation of the animal, pink-staining clusters of minute cocci are seen on the surface of the bronchial epithelial. $\times 52$.

FIG. 3 *b*. On the 12th day after inoculation, the coccal forms are found scattered among the bronchial epithelial cells (arrows), and no organisms are present on the luminal border. Contrast in the photomicrograph has been photographically enhanced. $\times 348$.

FIG. 3 *c*. Section of bronchus from a hamster inoculated 12 days previously with sterile PPLO broth. No inflammatory reaction, extracellular microcolonies nor intracellular inclusions are present. $\times 348$.



(Dajani *et al.*: *M. Pneumoniae* infection)