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Isolation, Propagation, and Characterization of a Newly Recognized Pathogen, Cilia-Associated Respiratory Bacillus of Rats, an Etiological Agent of Chronic Respiratory Disease

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A Gram-negative, filamentous, rod-shaped bacillus which failed to grow in cell-free media was isolated in apparently pure culture from the bronchial scraping and washing of a laboratory rat suffering from chronic respiratory disease by inoculating embryonated chicken eggs via the allantoic route. None of the embryos died during 20 serial passages at weekly intervals. The bacillus was reisolated in embryonated eggs from cesarean-derived barrier-maintained N:SD(SD) rats 8 and 12 weeks after intranasal inoculation with 10th-passage allantoic fluid. The inoculated rats were housed in Horsfall-type units and remained free from other known respiratory pathogens, including mycoplasmas and murine viruses, throughout the study. The bacillus colonized the ciliated epithelial cells of the respiratory tract and caused a marked peribronchial infiltration and hyperplasia of mononuclear cells which progressed with time. The bacillus, ca. 0.2 μm wide by 4 to 6 μm long, stained very poorly with basic aniline dyes but was readily demonstrated with the Warthin-Starry silver technique. It was heat labile (56°C for 30 min); spore forms were not observed. It withstood freeze-thawing and was successfully stored at −70°C. Although no visible means of locomotion was observed with the electron microscope, a slow gliding motility, sometimes with bending and flexing of bacilli apparently adherent to the glass surface, was observed with phase microscopy. As an etiological agent of chronic respiratory disease of rats, this cilia-associated respiratory bacillus (tentatively designated the CAR bacillus) may be the first recognized gliding bacterium known to cause disease in a warm-blooded vertebrate.

Chronic respiratory disease (CRD) of laboratory rats complicates, compromises, and often nullifies long-term studies, especially those involving the respiratory tract. It is the major cause of morbidity and mortality in conventionally maintained colonies.

The etiology of CRD of rats, a subject of investigation for more than 4 decades, remains unresolved. As a result of an early and extensive effort, Nelson (18) proposed an etiology consisting of two elements: *Mycoplasma pulmonis* which primarily colonized the upper respiratory tract ("infectious catarrh"), and a viral agent (Nelson's rodent enzootic bronchiectasis virus) which primarily affected the lower respiratory tract. However, subsequent attempts to confirm the presence of the viral agent in rats suffering from CRD failed (3, 6, 10–12, 23). Consequently, *M. pulmonis* was proposed as the singular or primary etiological agent of murine CRD (2, 10–12, 23). After extensive studies, Lindsey et al. (14) concluded that all previous descriptions and terms relating to murine CRD were indistinguishable clinically, pathologically, and etiologically, and therefore were descriptive of one and the same disease. Furthermore, as Koch's postulates had been fulfilled with the use of pure cultures of *M. pulmonis* in pathogen-free mice (13, 16) and rats (10–12, 23), Lindsey et al. (14) proposed the term murine respiratory mycoplasmosis to encompass all previous descriptions and terms for the CRD syndrome of mice and rats.

The recent description of an explosive and devastating epizootic of CRD in a large rat colony at the Toegepast Natuurwetenschappelijk Onderzoek in the Netherlands by van Zwieten et al. (21, 22) provided the stimulus for the present study. Though there was serological evidence of Sendai virus and *M. pulmonis* infection, they consistently found a filamentous bacterium parallel to and among the cilia of the respiratory tract upon light and electron microscopic (EM) examination of infected tissues. The etiological significance of this bacillus could not be confirmed as attempts to isolate the bacillus failed. A morphologically similar bacterium was subsequently observed in wild rats; also in *Mystromys albicaudatus*, rabbits, and a mouse (17). Again, the etiological significance of the filamentous bacterium could not be established as mycoplasmas were present in the wild rats and isolation attempts failed.

This paper describes the isolation, propagation, and preliminary characterization of a filamentous bacterium with properties similar to those described by van Zwieten et al. (21, 22) and MacKenzie et al. (17). Cilia-associated respiratory (CAR) bacillus has been suggested as a descriptive term until such time as the bacterium is properly classified (J. R. Ganaway, T. H. Spencer, T. D. Moore, and A. M. Allen, abstr. no 88, Lab. Anim. Sci. 33:502–503, 1983).

MATERIALS AND METHODS

Experimental design. Heretofore, the criterion for the determination of CAR bacillus infection was based solely upon morphological descriptions from light microscopic examination of silver-stained tissue sections and EM examination of thin tissue sections (17, 22). Initially, this criterion was employed to identify the CAR bacillus-infected rats used in this study. The rats were obtained from a colony known to be affected by CRD. They were used to establish a small breeding colony to provide animals for these studies. A source of rats free of CAR bacillus infection was similarly determined.

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The status of the infected and noninfected source rats was subsequently confirmed by serological procedures described herein. The high probability of encountering a variety of pulmonary pathogens other than the CAR bacillus in bronchial scraping-washings of CRD-affected rats (3, 5) was considered a major obstacle to isolation and propagation of the CAR bacillus in vitro. We were able to demonstrate that the CAR bacillus was readily transmitted to noninfected rats by the intranasal inoculation of bronchial scraping-washings of CAR bacillus-infected rats. We therefore felt that with trial and error, we would eventually examine a CAR bacillus-infected rat that would be free of infection with other known respiratory pathogens including viruses, bacteria, and mycoplasmas. This was accomplished by serological testing for viral infections, culturing for common bacteria in cell-free media, and testing for *M. pulmonis* infection by culture and serology (enzyme-linked immunosorbent assay [ELISA]). We also felt that initial attempts to grow the CAR bacillus in cell-free media would probably not be successful due to numerous previous failures to culture such an agent (3, 5, 12, 17, 18, 21, 22). Thus, initial attempts to isolate the CAR bacillus were restricted to inoculation of embryonated chicken eggs. Special attention was directed toward detection of *M. pulmonis* infection because of its known association with the CRD syndrome in rats. Indeed, we were simultaneously studying the fall of maternal antibody and the rise of IgG antibody to *M. pulmonis* infection in newborn litters of naturally infected dams. During a weekly bleeding procedure, the dam of a 3-week-old litter died and the litter was isolated in a Horsfall unit (see below). Fortunately, for reasons unknown, the titer to *M. pulmonis* in individual littermates fell to an insignificant level at 9 weeks of age and failed to rise through a 27-week observation period. Cultural attempts at necropsy to isolate *M. pulmonis* from several loci of the respiratory tract of each of four rats of the litter failed; EM studies showed that the rats were infected with the CAR bacillus but not with *M. pulmonis*. An intensive effort was therefore made to isolate the CAR bacillus from the three remaining 27-week-old littermates by inoculating embryonated eggs via the allantoic route with lower trachea and bronchial scraping-washings of each rat.

Animals. During the normal course of investigating intercurrent disease problems of laboratory animals used in the intramural research programs at the National Institutes of Health (NIH), a source of CAR bacillus-infected Sprague-Dawley rats was discovered. Fifteen (10 female and 5 male) 3- to 6-month-old rats were obtained from the infected colony for further study. They were housed in polycarbonate cages (19 by 10.5 by 8 in. [48.3 by 26.7 by 20.3 cm]) containing pine wood shavings and were offered NIH rat and mouse ration (open formula) and water ad libitum. The cages were held in Horsfall-type units with filtered air under slight negative pressure. N:SD(SD) male and female rats, 4 to 6 weeks old, were obtained from the Small Animal Section (Veterinary Resources Branch, Division of Research Services, NIH), and were housed and maintained as described above. This cesarean-derived, barrier-maintained colony of rats was previously described (4) and has been routinely monitored for microbial pathogens and health status during the past 17 years. The colony has remained free of respiratory disease; culture and serology (ELISA) for *M. pulmonis* infection has been negative, and serology for selected murine viruses has been negative (Sendai, Reo3, PVM, RCV, KRV, H-I, and LCM; performed by Microbiological Associates, Bethesda, Md.). N:NIH(S) weanling male and female mice were obtained from the Small Animal Section, (NIH), and were housed and maintained in Horsfall-type units in a manner similar to that described above for rats. This mouse colony was cesarean-derived, barrier-maintained, and remained free of respiratory disease; culture and serology (ELISA) for *M. pulmonis* infection were negative and serology for selected murine viruses was negative (Sendai, MVM, Reo3, PVM, MHV, Ectromelia, GDVII, mouse adeno, polyoma, K, and LCM; performed by Microbiological Associates), except for MHV in one of three rooms. All mice and rats were housed and maintained in accordance with the Guide for the Care and Use of Laboratory Animals (U.S. Department of Health and Human Services, NIH publication no. 80-23, 1980).

Embryonated eggs. Embryonated White Leghorn chicken eggs (Truslow Farms, Inc., Chestertown, Md.) were inoculated by the allantoic route, 0.1 ml per egg, and allantoic fluid was harvested by standard procedures (7). The eggs were incubated at 37°C in the upright stationary position. Viable embryos were determined before inoculation and daily thereafter by candling. The initial inoculum was obtained from the washing and scraping of the lower trachea and major bronchus of a CAR-bacillus infected rat in 2 ml of sterile phosphate-buffered saline (PBS) (0.85% NaCl, pH 7.2). Harvested allantoic fluid was serially passaged at weekly intervals. Control eggs were inoculated with sterile PBS, and harvested allantoic fluid was similarly passaged at weekly intervals. Infected and control allantoic fluids were frozen and stored at −70°C.

Bacteriology. All inocula for embryonated eggs and animals were examined by conventional aerobic and anaerobic cultural methods as previously described (1, 4). Harvested and pooled 10th-passage allantoic fluid containing the CAR bacillus was examined for growth in the following media: Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.); Trypticase soy broth plus normal chicken embryo allantoic fluid (10% final); fluid thioglycollate with and without $CaCO_3$; Schaedler broth; 5% sheep erythrocytes (SRBC)–Columbia agar base; 5% SRBC-Trypticase soy agar base plus vitamin K and hemin; Trypticase soy agar (3%) with and without 5% SRBC; Casitone agar with and without 5% SRBC; tryptone agar with and without 5% SRBC; charcoal yeast extract agar; brucella agar plus cysteine, sonicated HEp-2 cells, and 5% SRBC; Feeley-Gorman agar; Mueller-Hinton agar plus sonicated HEp-2 cells and IsoVitaleX; Casitone agar plus cysteine and ferric pyrophosphate. All media were incubated at 37°C and examined for growth daily through 2 weeks. Triplicate plates of each agar medium were inoculated, and one plate was incubated under each of the following atmospheric conditions: normal atmosphere, 5% CO_2 in air, and in an anaerobic chamber containing 85% N_2, 10% H_2, and 5% CO_2.

Serology. Murine viral serology was performed commercially (Microbiological Associates). The microtiter quantitative ELISA was used for detection of antibody to *M. pulmonis* as a modification of the method of Horowitz and Cassell (8). Akaline phosphatase-labeled, affinity-purified goat anti-mouse immunoglobulin G (IgG) and anti-rat IgG antibody (Kirkegaard and Perry Lab, Inc., Gaithersburg, Md.) were used at a 1:200 dilution in PBS (pH 7.4). Serum samples to be tested were diluted 1:100 in PBS (pH 7.4). Optical density was determined spectrophotometrically at 405 nm (Titertek Multiskan, type 3100, Flow Laboratories, Inc., Rockville, Md.). All samples were tested in duplicate. The quantitative micro-ELISA procedure was adapted for the determination of CAR-bacillus antibody. Allantoic fluid (10th passage) containing the CAR bacillus was harvested

and pooled from four embryonated eggs and centrifuged (40 × *g* for 10 min) to sediment coarse particles and erythrocytes. The supernatant fluid was collected by aspiration, and the CAR bacillus was sedimented (4,000 × *g* for 30 min) and similarly washed three times by suspending the sediment in 15 ml of sterile PBS (pH 7.2). The sediment was then suspended in 5 ml of carbonate-bicarbonate coating buffer (pH 9.6) and sonicated (Branson Sonic Power Co., Danbury, Conn.) at peak intensity for 2 min while being cooled in an ice bath. Portions of this antigen were stored at −70°C. Microtiter plates (Falcon no. 3912 Microtest III, 96 flat-bottom wells; Becton Dickinson and Co., Oxnard, Calif.) were sensitized with a 1:200 dilution (determined by block titration) of CAR-bacillus antigen in coating buffer, 100 μl per well, overnight at 4°C. Alternate rows of 12 wells were sensitized with control antigen (normal chicken embryo allantoic fluid similarly diluted in coating buffer). With both the *M. pulmonis* and CAR bacillus ELISA, a serum sample was considered positive when its optical density value exceeded the calculated value of two standard deviations above the mean value of normal sera (i.e., from 20 germfree and from 30 known pathogen-free, cesarean-derived, barrier-maintained rats or mice).

Transmission experiments. Initially, to establish the transmissibility of CAR bacillus infection, 12 N:SD(SD) weanling rats free of pulmonary pathogens (see above) were inoculated intranasally under light ether anesthesia with 0.05 ml of bronchial scraping-washing of CAR bacillus-infected rats in sterile PBS; four control rats were similarly inoculated with sterile PBS. At 2-week intervals, four rats (three inoculated with CAR bacillus and one control) were killed with chloroform in a fume hood. Culture for bacteria and mycoplasmas was performed on a portion of the lower trachea of each rat, individual sera were collected for viral and *M. pulmonis* serology, and tissues from the respiratory tract were fixed in 10% unbuffered Formalin. Tissues were processed and sections were stained by standard procedures (15). A similar transmission experiment was performed in 16 N:SD(SD) weanling rats and 16 N:NIH(S) weanling mice, using the 10th passage of the CAR bacillus in chicken embryo allantoic fluid. Control rats and mice were inoculated with normal allantoic fluid. An additional group of 10 N:SD(SD) weanling rats was inoculated intranasally with the 10th passage of the CAR bacillus in allantoic fluid. They were held for 12 weeks in separate Horsfall units and bled at weekly intervals. Individual sera were collected and stored at −20°C.

RESULTS

Lesions, typical of CRD in rats (5, 9, 12, 19), were present throughout the respiratory tract of each of the naturally infected rats obtained from the laboratory of an investigator. The most characteristic and consistently observed lesion was massive accumulations of mononuclear lymphoid cells around the bronchus (Fig. 1) and bronchioles. Various degrees of suppurative pneumonia with polymorphonuclear exudate and debris in the bronchial passages, loss of columnar epithelium and squamoid changes in the bronchus, atelectasis, emphysema, and bronchiectasis were also observed. The ciliated epithelium was heavily colonized with filamentous bacteria (Fig. 2). The CAR bacillus stained poorly with basic aniline dyes but was readily demonstrated by the Warthin-Starry silver impregnation technique (Fig. 3). The bacillus was transmitted from the nursing infected dam to her offspring at 1 to 2 weeks of age (demonstrable in upper respiratory passages) and to each of 12 N:SD(SD) rats after intranasal inoculation (2-week through 3-month observation period) with bronchial scraping-washing from infected rats. Comparable N:SD(SD) rats inoculated intranasally with sterile PBS and held in a separate Horsfall unit remained free of pulmonary disease and recognized pathogens throughout the 3-month observation period. The infected source rats had antibody to PVM, RCV, and Sendai viruses, and to *M. pulmonis*. However, they apparently were no longer shedding virus, as these viruses were not transmitted to their offspring or to the inoculated N:SD(SD) rats. *M. pulmonis* was transmitted erratically to offspring (see above) but readily to N:SD(SD) rats inoculated intranasally with bronchial scraping-washing from natural CAR bacillus-infected rats. Small numbers of bacteria (non-hemolytic *Streptococcus* sp. and coliforms) were isolated occasionally from the bronchial washings of natural CAR bacillus-infected rats.

Having determined previously (see above) that an isolated litter of rats, naturally infected with CAR bacillus, was free of infection with known murine viruses and *M. pulmonis*, a 27-week-old rat was killed, and the bronchial scraping-washing was harvested in PBS and used to inoculate embryonated eggs via the allantoic route. All embryos died of bacterial growth (enterococcus). The bronchial washing of another littermate was used in a second attempt. None of the embryos of 10 inoculated eggs died during 7 days of observation or throughout 20 subsequent serial passages of allantoic fluid at weekly intervals. Neither bacteria nor mycoplasmas could be cultured from the 10th-passage allan-

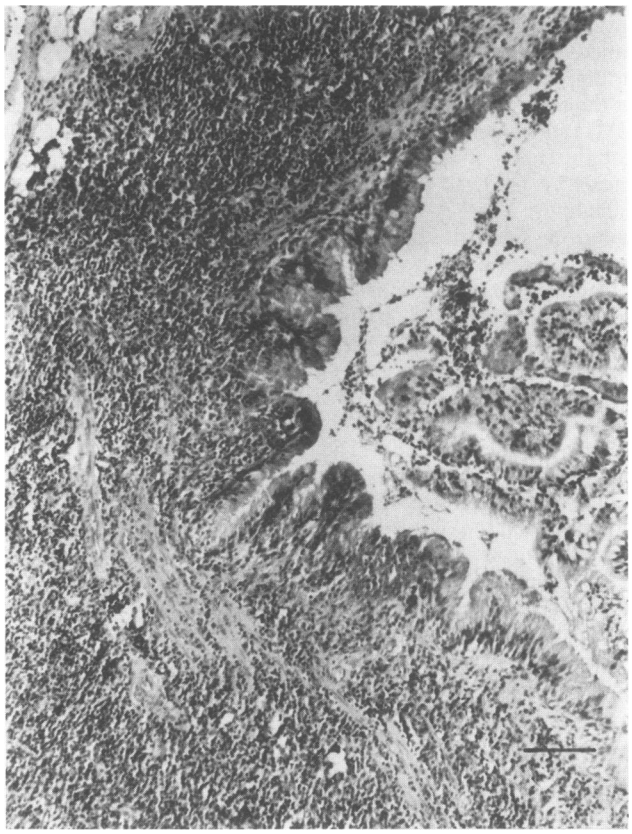

FIG. 1. Lung of 27-week-old Sprague-Dawley rat from which the CAR bacillus was isolated. Note the marked peribronchial infiltrate and hyperplasia of mononuclear cells. The bacillus is not demonstrated with this hematoxylin and eosin stain. Bar = 100 μm.

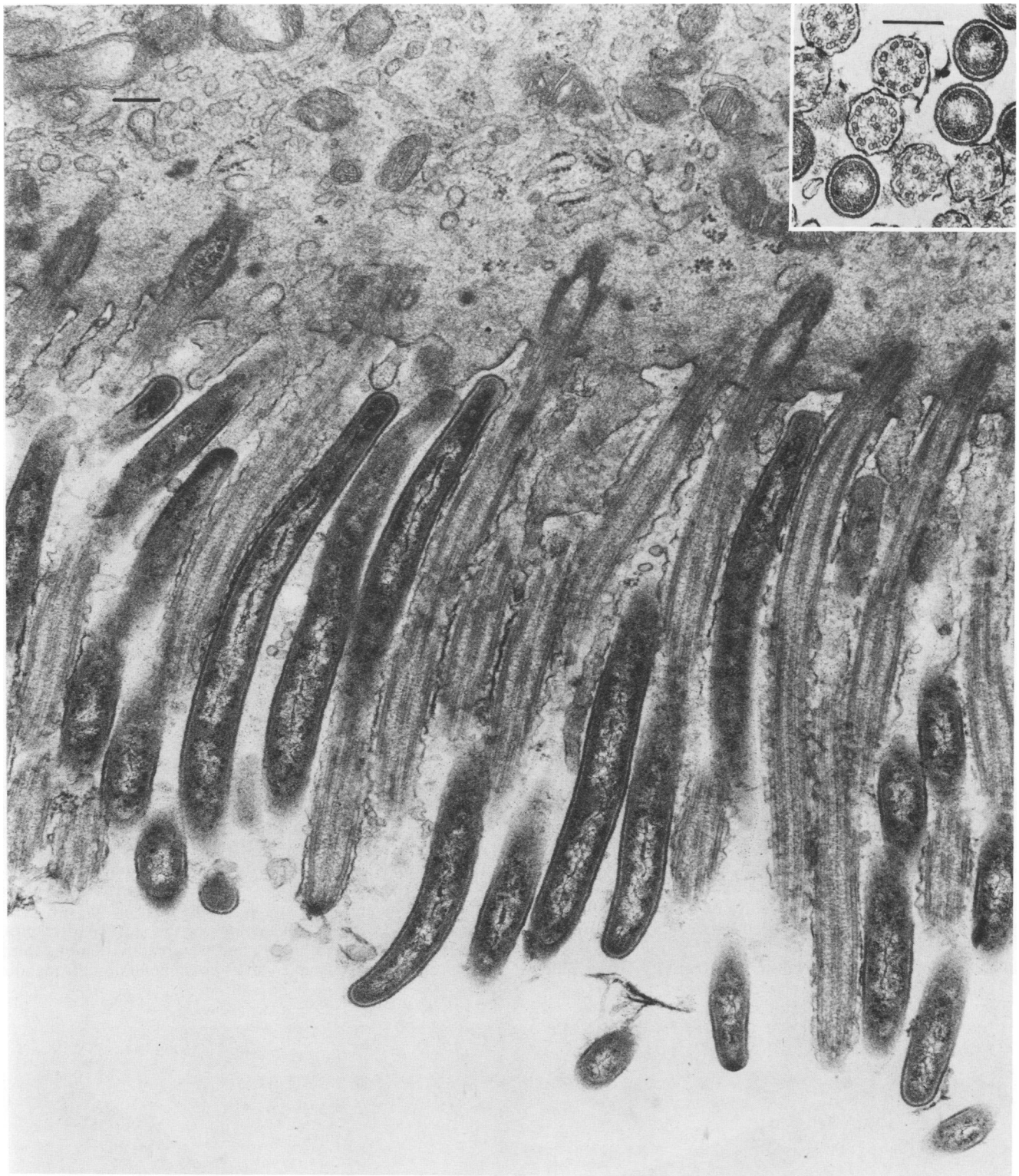

FIG. 2. The ciliated bronchial epithelium of rat lung shown in Fig. 1 is heavily colonized with filamentous bacteria which are parallel to and among the cilia. Note the knob-like end of the bacillus adjacent to the microvillus. Inset: Cross-section of bacilli and cilia. Uranyl acetate and lead citrate. Bar = 0.2 μm.

toic fluid on a wide variety of cell-free media incubated in various atmospheres. A nonflagellated filamentous bacterium (Fig. 4) morphologically similar to that observed among the cilia of infected rats was demonstrated in the 3rd-passage allantoic fluid by transmission EM. Warthin-Starry-stained sections of chorio-allantoic membranes (Fig. 5) from inoculated eggs demonstrated the CAR bacillus in the allantoic sac only; hematoxylin- and eosin-stained sections failed to dem-

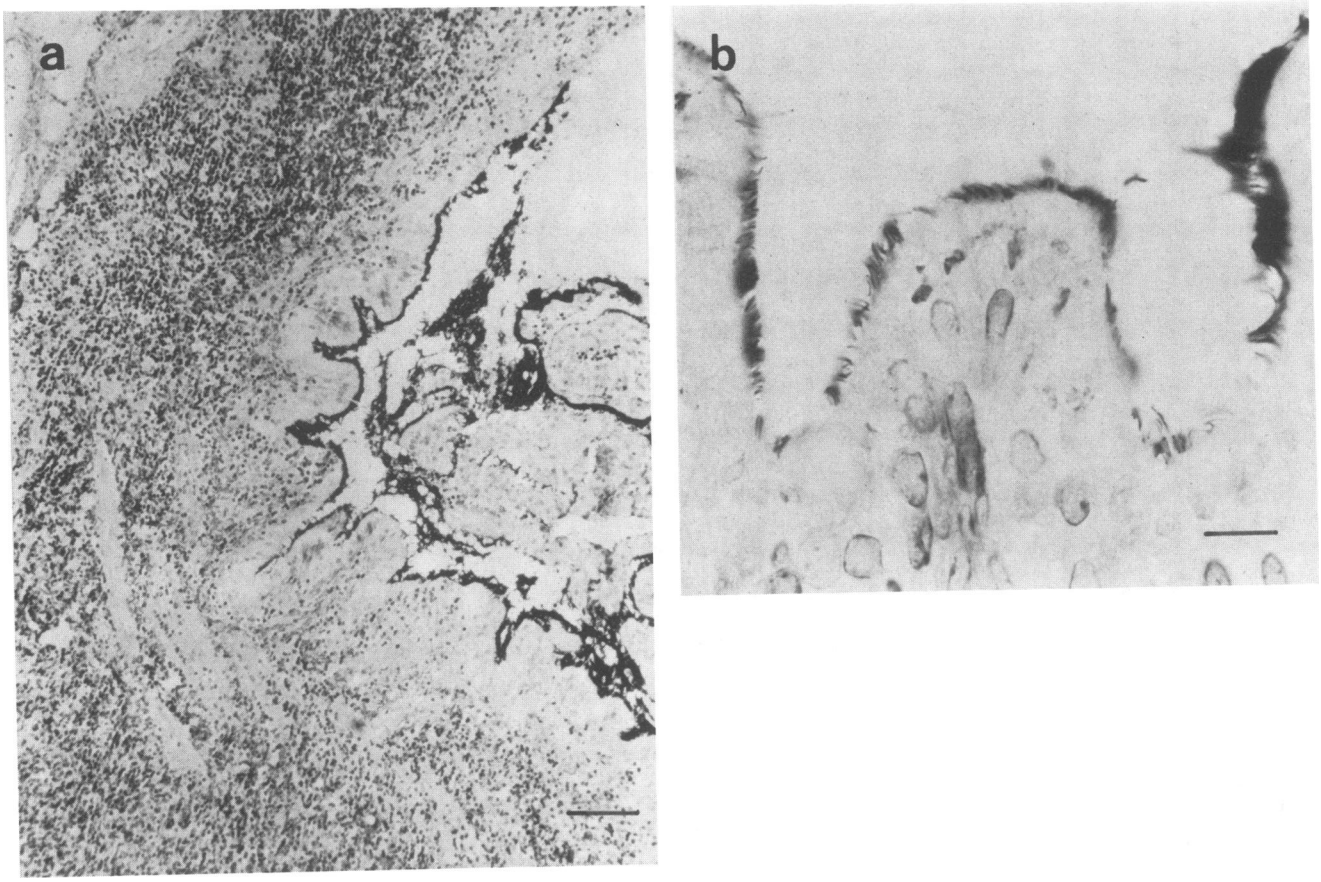

FIG. 3. (a) Same rat lung as that shown in Fig. 1. A heavy mat of silver-stained material, not readily recognizable as bacteria, is visible on the surface of the ciliated bronchial epithelium. (b) Individual bacilli are discernible among the bronchial cilia of this 5-week-old N:SD(SD) rat 2 weeks postinoculation intranasally with the 10th egg passage of the CAR bacillus. Warthin-Starry stain. Bars: (a) 100 μm; (b) 10 μm.

onstrate the bacillus. Examination of the 10th and subsequent passage allantoic fluids by phase microscopy revealed typical CAR bacilli which were motile by a "gliding" movement. Not all bacteria in a field of vision (500×) were motile. Motility appeared to be dependent upon adherence to a solid phase. Flexing movements (bending) were commonly observed, particularly of the free end of a bacterium, the other end of which appeared to be adherent to the glass slide. Length of the bacilli varied between 4 and 6 μm.

Heat treatment, 56°C for 30 min, of the 10th-passage allantoic fluid appeared to destroy infectivity. After inoculation of eggs with such heated fluid, the CAR bacillus could not be demonstrated by phase microscopy in allantoic fluid harvested 1 week later. The bacillus was infective after standing for 1 week at 23°C in allantoic fluid. It readily withstood freeze-thawing and storage at −70°C.

Lesions typical of CRD in rats were observed in all N:SD(SD) rats (Fig. 6) and N:NIH(S) mice (Fig. 7) after intranasal inoculation with the 10th passage of the CAR bacillus. The CAR bacillus colonized the surface of respiratory tract ciliated epithelium and was readily demonstrated in all rats and mice from 2 weeks postinoculation throughout

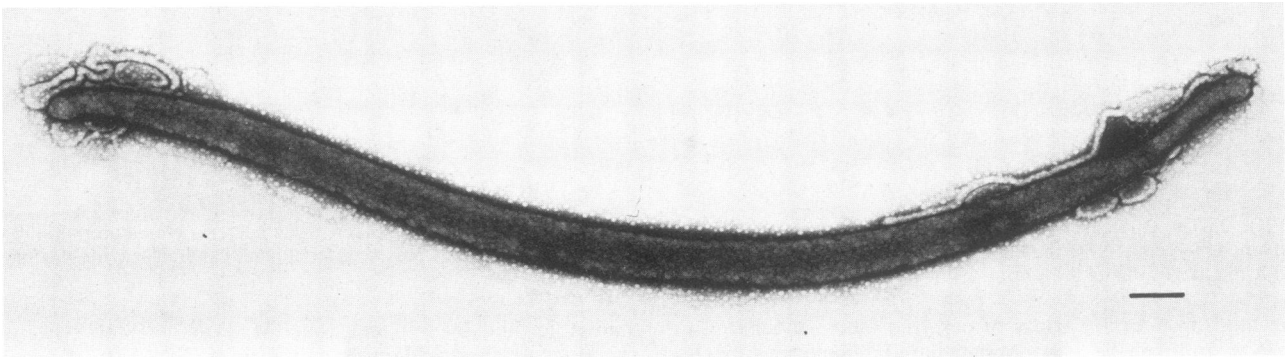

FIG. 4. CAR bacillus, propagated in embryonated chicken eggs inoculated via the allantoic route. Note the absence of flagella. The bacillus was negatively stained with phosphotungstate (pH 7.1). Bar = 0.2 μm.

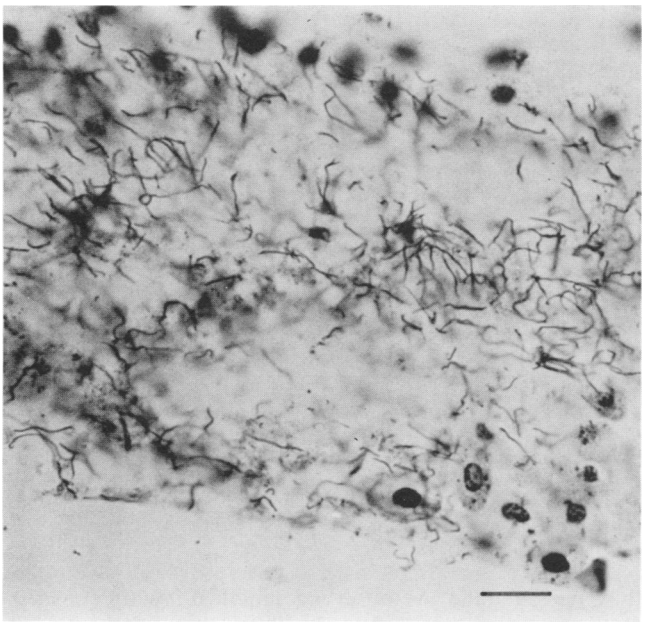

FIG. 5. Filamentous bacteria are demonstrated within the allantoic sac of an embryonated chicken egg, 7 days postinoculation with CAR bacillus via the allantoic route. Warthin-Starry stain. Bar = 10 μm.

the 3-month observation period. The extent of peribronchial mononuclear cell infiltration and hyperplasia appeared to increase with the passage of time. The CAR bacillus was reisolated in embryonated eggs from the bronchial washing of rats at 8 and 12 weeks postinoculation. Comparable rats and mice inoculated intranasally with normal allantoic fluid did not develop lesions of CRD, and the CAR bacillus could not be demonstrated in their respiratory tracts. None of the inoculated N:NIH(S) mice or N:SD(SD) rats developed antibody to any of the known murine viruses or to *M. pulmonis*, and neither mycoplasmas nor recognized bacterial pathogens were isolated from bronchial washings of inoculated N:NIH(S) mice or N:SD(SD) rats.

Weaned littermates of a dam naturally infected with the CAR bacillus were bled at weekly intervals and tested for IgG antibody to CAR bacillus antigens by the ELISA technique (Fig. 8). High levels of antibody (maternal) were detected at 3 weeks of age which dropped to a low level at 6 to 8 weeks of age and sharply increased thereafter, apparently in response to natural infection acquired from the dam. The CAR bacillus was isolated from the bronchial washings of these rats, and morphologically similar bacilli were demonstrated in tissue sections by use of the Warthin-Starry stain. Individual serum samples, collected at weekly intervals from 10 N:SD(SD) rats inoculated intranasally with the 10th passage of CAR bacillus and tested for IgG antibody by the ELISA technique, revealed a uniform seroconversion at 4 weeks postinoculation; all were negative at 2 weeks and all were positive at 6 weeks. Similarly tested sera of germfree rats (20 samples) and cesarean-derived, barrier-maintained N:SD(SD) rats (30 samples) did not have anti-CAR IgG antibody. The serum of each rat (15 samples) obtained from a conventional colony (source rats for the CAR bacillus isolation) contained high levels of IgG antibody to the CAR bacillus (optical density range, 0.8 to 1.7; significant optical density was ≥0.08).

During the preparation of the antigens to be used in the ELISA, a casual observation was made which may have a bearing on the eventual classification of the CAR bacillus. When the allantoic fluids were removed from the refrigerated centrifuge, the fluid containing the CAR bacillus appeared quite milky, whereas the control allantoic fluid remained clear. The milky character of the fluid cleared when it was warmed to 37°C.

DISCUSSION

A summary of the characteristics of the CAR bacillus is shown in Table 1. The elusive nature of the CAR bacillus poses many questions. The CAR bacillus may be the primary etiological agent of CRD of rats. If this were true, present concepts of the etiology of murine CRD (2, 14) would need modification. Historically, the failure to demonstrate the CAR bacillus in tissue sections of CRD-affected rats until quite recently (17, 21, 22), and the failure of the bacillus to grow in cell-free media, are features which could explain the previously unconfirmed conclusion of a viral etiology of murine CRD (18). Furthermore, the sensitivity of the CAR bacillus to sulfonamide therapy (unpublished observation) would explain previous reports of the prophylactic effect of sulfonamides in preventing the development of lesions of CRD in treated rats (3, 6). The question of whether the CAR bacillus could have been the rodent bronchiectasis

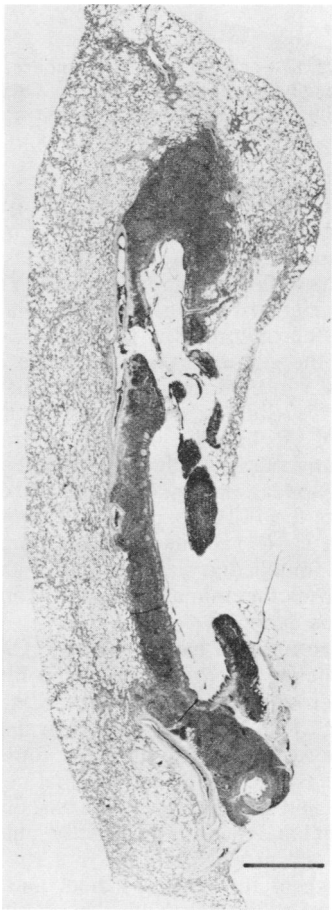

FIG. 6. Lung of 10-week-old N:SD(SD) rat, 6 weeks postinoculation intranasally with 10th egg passage of CAR bacillus. Note marked accumulation of mononuclear lymphoid cells around the bronchus. Hematoxylin and eosin stain. Bar = 1,428 μm.

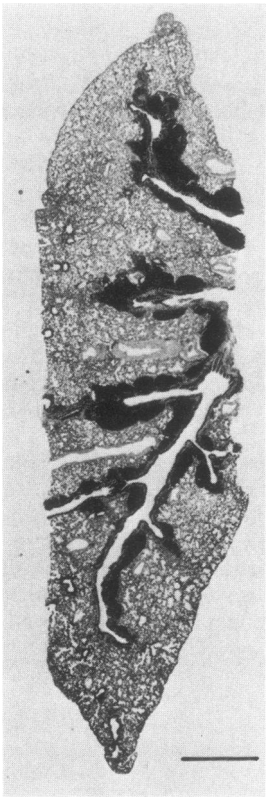

FIG. 7. Lung of 12-week-old N:NIH(S) mouse, 8 weeks post-inoculation intranasally with 10th egg passage of CAR bacillus. Note marked peribronchial mononuclear cell response. Hematoxylin and eosin stain. Bar = 1,000 μm.

virus of Nelson (18) must remain unresolved since all tissues, infective suspensions, and sera have been destroyed (personal communications to John B. Nelson; reply by Dennis Stark, Rockefeller University, New York, N.Y.). A morphologically similar bacillus has been demonstrated in a teaching slide of chronic respiratory mycoplasmosis from a rat received at the Armed Forces Institute of Pathology, Washington, D.C., in 1958, and such a bacillus is reported to be widespread (17). Studies are needed to determine prevalence rates of CAR bacillus infection in rats and other animals by means of serological procedures such as the ELISA, which appears promising, and to determine the pathology of long-term CAR bacillus infection alone and the extent to which CAR bacillus infection compromises the normal defense (mucociliary apparatus) of the respiratory tract or predisposes the animal to insult by other infectious and noninfectious agents.

Our decision to attempt isolation of the CAR bacillus in embryonated chicken eggs was influenced by the reported failure of numerous investigators (including one of the present authors, J.R.G.) to culture such an agent in cell-free media from diseased rats. The allantoic route was chosen because the sac is lined with endodermal cells associated with respiration and numerous viruses associated with respiratory disease have been successfully cultured by this method. Successful propagation was not readily apparent, however, as none of the embryos died, and we were not initially impressed sufficiently with the staining qualities of the bacillus. We initially used Gram, Giemsa, and methylene blue stains to detect the presence of the bacillus in harvested allantoic fluid. In Gram-stained smears of the second and

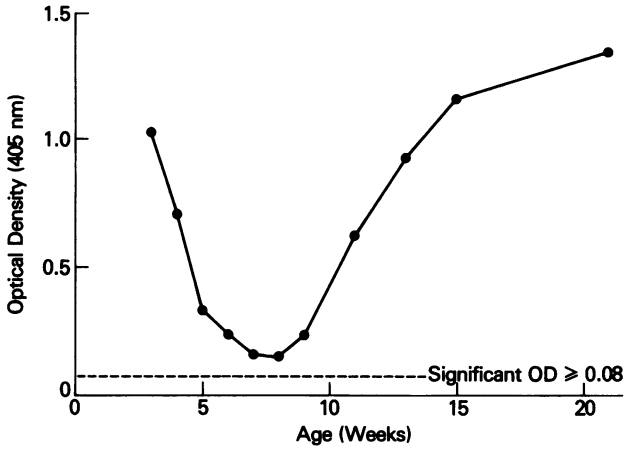

FIG. 8. Serum antibody to CAR bacillus in weanling littermate Sprague-Dawley rats as determined by ELISA. Each point represents the arithmetic mean of three rat serum determinations.

third passage fluid, we observed a modest few (in consideration of a possible replicating bacillus), very faintly stained, gram-negative, filamentous organisms. The possibility that these were detached cilia of cultured bronchial epithelial cells was considered since cilia are of similar size and shape and they also stain poorly with these dyes. The EM demonstration of the bacillus in the fluid of the third blind passage provided stimulating evidence of propagation and emphasized the importance of using the Warthin-Starry silver stain in lieu of basic aniline dyes.

Antigens of the CAR bacillus appear to be unrelated to antigens that are a part of the normal microflora of rats. Colonization of the respiratory tract occurs readily and profoundly stimulates antibody production, as evidenced by the uniform peribronchial mononuclear response and the production of IgG antibody. The absence of anti-CAR bacillus IgG antibody in the sera of normal noninfected rats which have an established gastrointestinal microflora, and the uniform presence of antibody in sera of known CAR bacillus-infected rats, suggest that the CAR bacillus ELISA is a specific as well as sensitive serological procedure.

The CAR bacillus grown in embryonated eggs measured 0.2 μm wide by 4 to 6 μm long. Previously reported measurements of the bacillus in rat tissue sections wre 0.12 to 0.2 μm wide by 6 to 8 μm long (17) and 0.21 μm wide by 8 μm (range, 4 to 12 μm) long (22). The width of the CAR

TABLE 1. Characteristics of the rat CAR bacillus

Parameter	Characteristic in CAR bacillus
Gram stain	Gram negative (very poor stain with basic aniline dyes)
Size	0.2 μm wide by 4 to 6 μm long
Spores	None; infectivity destroyed by heat (56°C for 30 min)
Acid fastness	Non-acid-fast
PAS	Negative
Stability	Good: withstands freeze-thawing, storage at −70°C, and 23°C for at least 7 days in allantoic fluid
Growth in cell-free media	None
Growth in chicken eggs	Slow growth in embryonated chicken eggs inoculated via the allantoic route
Motility	Motile without visible means of locomotion

bacillus grown in embryonated eggs agrees well with the width of the filamentous bacteria observed among the respiratory tract cilia of diseased rats. Further studies are needed to explain the reported differences in the length of the bacillus.

The motility of the CAR bacillus (observed with phase microscopy) in the absence of a demonstrable means of locomotion suggests that it belongs to that large group of microorganisms known as the "gliding bacteria." The milky appearance of harvested allantoic fluid from CAR bacillus-inoculated eggs could be due to a product of CAR bacillus metabolism such as slime which is produced by all gliding bacteria (20). According to Reichenbach and Dworkin (20), an intricate association of gliding bacteria with higher organisms is relatively rare; they have been demonstrated in the mouth and intestinal tract of warm-blooded animals including humans, they may contribute to certain disorders of the teeth, and several are considered dangerous fish pathogens. CAR bacillus infection of the rat may be the first proven instance of a gliding bacterium causing disease in a warm-blooded vertebrate.

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