

Chlamydial Pneumonitis Induced in Newborn Guinea Pigs

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One- to three-day-old guinea pigs were inoculated intranasally with the chlamydial agent of guinea pig inclusion conjunctivitis. Physical signs of infection included a marked increase in respiration rate on days 5 to 10 of infection and radiographic evidence of pneumonia on day 6. When animals were killed at various times after infection and lung tissue was examined by histopathology, evidence of pneumonia was found beginning on day 4 and lasting as long as day 12, with maximal pathological changes on days 6 to 8. The pneumonia was generally unilateral and consisted of an acute inflammatory component in the bronchioles with granulocytes in both the lumen and the wall of the bronchioles and an interstitial and intra-alveolar mononuclear infiltrate in the parenchyma of the lung. Chlamydial antigen was detected in the bronchial epithelial cells by immunoperoxidase staining, and the guinea pig inclusion conjunctivitis organism was isolated from lung tissue on days 6 to 9. No other significant bacteria were isolated from lung tissue or seen on gram stains of lung sections. Both immunoglobulin M and immunoglobulin G serum antibodies to the guinea pig inclusion conjunctivitis agent were detected as early as day 8 and reached peak levels on day 12. The infection was apparently self-limiting. This model presents the opportunity to investigate pathophysiological and immunological aspects of chlamydial respiratory infections in a neonatal animal.

Chlamydia trachomatis has become recognized as a frequent cause of pneumonia in the newborn (3). The clinical aspects of this infection have been well described (2, 3, 6, 17); however, very little is known about the nature of the pathophysiological and immunological mechanisms involved. Obviously much of this information cannot be acquired from human studies; thus an appropriate animal model is necessary. Several mouse models with adult mice have been reported in which oculogenital strains of *C. trachomatis* (7, 8) or the agent of mouse pneumonitis, a *C. trachomatis* biovar, (19) has been used. In the former, infection has been of short duration (7, 8), and in the latter, a lethal pneumonia was occasionally encountered (19). Perhaps the best model has been that described by Harrison et al. (5), in which a pneumonia was produced, similar in many aspects to that seen in humans, in infant baboons (10 to 24 days of age) with *C. trachomatis*. Unfortunately, studies with this system have been limited because of the expense involved.

The *Chlamydia psittaci* agent of guinea pig inclusion conjunctivitis (GPIC) has been shown to produce conjunctival (12) and genital (1, 10, 11) infections in guinea pigs. Not only can the agent be transmitted sexually in guinea pigs (11), but guinea pig newborns have been shown to develop conjunctival infections by passage through an infected birth canal similar to human newborns (10). In the present study, we report that intranasal inoculation of newborn guinea pigs with GPIC can result in a chlamydial pneumonia which may serve as a model for human neonatal chlamydial respiratory infection caused by *C. trachomatis*.

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MATERIALS AND METHODS

Experimental animals. Pregnant Hartley strain female guinea pigs were obtained at 40 to 50 days of gestation from Simonson Laboratories, Gilroy, Calif. This stock has been found to be free of the GPIC agent. Females were housed individually, and upon birth, litters were allowed to remain with their mothers.

Experimental design. Newborn guinea pigs of both sexes were inoculated with GPIC on either day 1 or 3 after birth (Table 1). An entire litter was inoculated at the same time with the same inoculum. Respiratory rates were recorded daily in certain litters. Individual animals were killed at various times after infection by pentobarbital sodium overdose. The lungs were removed, and one lung was processed for histopathology, whereas the other was frozen at -80°C in 2-SP (20) containing gentamicin (50 $\mu\text{g/ml}$), vancomycin (100 $\mu\text{g/ml}$), and amphotericin B (2.5 $\mu\text{g/ml}$) at -80°C for recovery of GPIC. In the cases in which gross pathology was observed, those tissues were divided for histopathology and recovery of GPIC. Blood was removed by cardiac puncture, and the serum was retained for antibody assays.

Infection of guinea pigs. Two different preparations of GPIC were used for infection purposes (Table 1). In the first, a suspension was prepared from infected yolk sacs in sucrose-potassium-glutamate buffer (pH 7.2) containing gentamicin (50 $\mu\text{g/ml}$) and vancomycin (100 $\mu\text{g/ml}$) as described previously (13). Because of concern over the large amount of lipid in the yolk sac preparation, some animals were inoculated with GPIC grown in HeLa cells which was prepared as follows. GPIC stock was diluted in Eagle minimal essential medium containing 5% glucose, 0.5 μg of cycloheximide per ml, 10% fetal calf serum, 50 μg of gentamicin per ml, 100 μg of vancomycin per ml, and 2.5 μg of amphotericin B per ml and was added to HeLa cell monolayers for 3 h at 37°C with frequent agitation. Additional medium was added, and the cultures were incubated for 72 h. Before inoculation the cells

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TABLE 1. Summary of inoculated animals

Days on which animals were killed after inoculation	Total no. of animals	Age inoculated (days)	Inoculum ^a
1,6,8,9,12	6	1	GPIC-YS
4,6,10,12,17,23	7	3	GPIC-YS
2,3,6,9,12	9	1	GPIC-HeLa
7	5	3	GPIC-HeLa
6,9	2	1	YS
4,6,8,12	4	3	YS
9	2	1	HeLa

^a YS, Yolk sac-derived material; HeLa, HeLa cell-derived material.

were sonicated (E. B. Moses, E. J. Peck, Jr., and A. L. Barron, *Curt. Microbiol.*, in press).

Newborn guinea pigs were anesthetized with methoxyflurane or ether and inoculated with 0.05 ml of GPIC intranasally by placing five 0.005-ml droplets in each naris with an automatic dispensing syringe. Initially, each animal received 1×10^6 50% egg lethal doses of the yolk sac suspension or 2.6×10^7 inclusion forming units of the HeLa cell preparation. When a similar yolk sac preparation was quantitated by both methods, it was found to contain 7.9×10^6 50% egg lethal doses or 1.35×10^6 inclusion forming units. Control animals were inoculated with normal yolk sac or uninfected HeLa cells prepared similarly to the GPIC-infected material.

Histopathology. At necropsy, the lungs were removed, and one lung was injected via the bronchus with 10% buffered Formalin (pH 7.2) and stored in Formalin before processing. Tissues were embedded in plastic as described previously (18). Sections were stained with either hematoxylin and eosin or toluidine blue (pH 4.0).

Tissue for immunoperoxidase staining was fixed as described but mounted in paraffin. Sections were treated with reagents in the following order: (i) normal rabbit serum (1:5) to saturate nonspecific protein binding sites; (ii) anti-GPIC guinea pig serum pool (1:20) titered at 640 by indirect immunofluorescence, obtained from convalescent animals; (iii) rabbit anti-guinea pig immunoglobulin G (IgG) (H and L chain specific) horseradish peroxidase conjugate (1:40) (Miles Laboratories, Inc., Elkhart, Ind.); and (iv) 3-amino-9-ethyl-carbazole (Sigma Chemical Co., St. Louis, Mo.) with 0.01% H_2O_2 .

Detection of serum antibodies to GPIC. IgG antibody titers to GPIC were determined by indirect immunofluorescence with fluorescein-conjugated rabbit anti-guinea pig IgG (H and L chain specific) (Miles Laboratories) (13). IgM antibody titers were measured similarly except rabbit anti-guinea pig IgM (μ chain specific) was used, followed by fluorescein-conjugated goat anti-rabbit IgG (H and L chain specific).

Recovery of GPIC from lung homogenates. Upon thawing, lung sections were homogenized in sucrose-potassium-glutamate buffer so that 9 ml was added for each gram of lung tissue. Ten-fold and hundred-fold dilutions were prepared, and 0.2 ml of each dilution was inoculated into the yolk sacs of five embryonated eggs per dilution. The presence of chlamydiae was confirmed in the yolk sacs of dead eggs by staining with Gimenez stain.

Respiratory rates and radiographs. To obtain respiratory rates, neonates were allowed to remain with their mothers and were observed in an undisturbed environment. Each

animal was observed until the number of inspirations could be counted over a 30- to 60-s interval.

Routine chest roentgenographs were obtained by securing the animals in a prone position to polystyrene holders with tape (postero-anterior view). The animal was allowed to adapt to the restraints in the dark, and the roentgenogram was taken with standard equipment. Individual films were processed before the removal of restraints in case a second exposure was necessary due to motion artifact.

Bacteriological studies. One litter of guinea pigs, inoculated with GPIC grown in HeLa cells, was sacrificed by pentobarbital sodium overdose on day 6, and the chest cavity was opened by the aseptic technique. The lung was removed and placed in a sterile 60-mm petri dish. A portion of the lung was removed and homogenized in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) in a tissue grinder. Samples were inoculated onto blood, chocolate, and MacConkey agar plates which were incubated aerobically at 37°C. The remaining suspension was added to thioglycolate broth. The plates were examined for growth at 24 and 48 h, and the thioglycolate tube was examined daily for 1 week. Positive broths were Gram stained and subcultured onto blood, chocolate, and MacConkey agar plates.

Additional portions of lung tissue were homogenized in 1% Casamino Acids (Difco Laboratories, Detroit, Mich.), inoculated onto charcoal blood agar, and examined daily for 1 week for the growth of *Bordetella* species.

RESULTS

Physical Findings. In the period after inoculation with GPIC, newborn guinea pigs appeared to be healthy. However, when respiratory rates were measured, obvious differences were observed between infected and control animals (Fig. 1). Although the mean respiratory rate in control animals was generally lower than that in infected animals, the rates varied little until day 5. At this point, the respiratory rates of the infected animals began to increase, reaching a peak rate of 81 respirations per min on day 6. This was significantly greater than that of the controls on day 6 according to a one-tailed *t* test ($P < 0.001$). Furthermore, the respiration rate of the infected animals on day 6 also was significantly greater than the day 1 rates ($P < 0.001$). By day

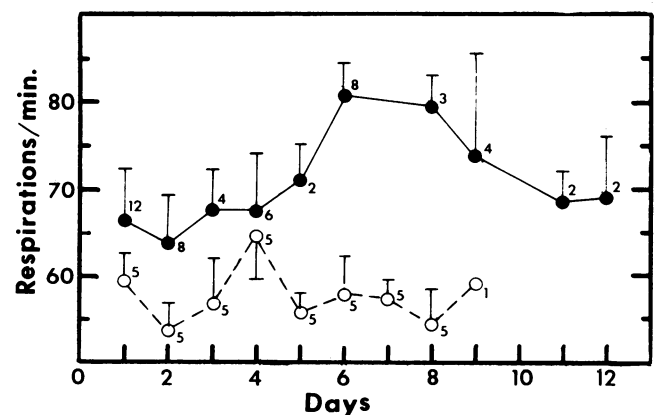


FIG. 1. Respiratory rates in neonatal guinea pigs inoculated with GPIC (●) or control (○) suspensions. Both groups included animals inoculated with either yolk sac or cell culture material. The bars represent one standard deviation. The numbers of each point represent the number of animals defined by the mean.

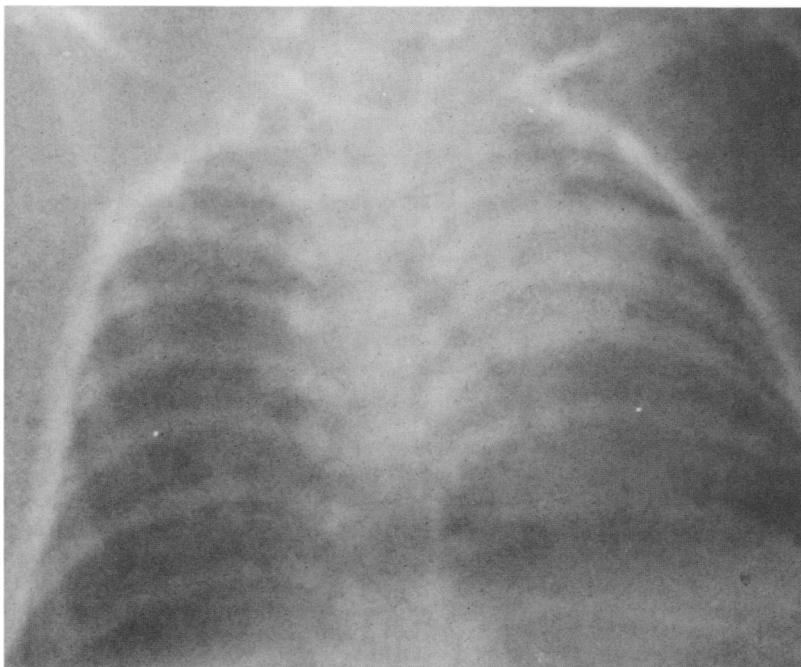


FIG. 2. Chest X ray of neonatal guinea pig 6 days after inoculation with GPIC grown in yolk sac. Note the evidence for severe left upper-lobe consolidation typical of pneumonia. An air bronchogram is present.

9 the rates began to descend, and they reached near normal levels by days 11 to 12. Additional evidence of pneumonia was also detected when roentgenographs of certain infected guinea pigs were taken on day 6. Characteristically, severe pneumonia was noted in a portion of the lung but never in the entire lung (Fig. 2). It was common to find a confluent pneumonia in one lobe adjacent to a totally normal lobe. The pneumonia was generally unilateral.

Histopathology. When lung tissue was examined on days 6 to 8 of infection, a severe confluent pneumonia was observed which consisted of both bronchial and parenchymal components (Fig. 3A). The bronchial component was composed of an acute granulocytic inflammatory reaction in both the lumen and epithelial lining of the bronchioles (Fig. 3B). No damage to the bronchiolar epithelium was apparent; however, there were increased numbers of mitotic epithelial cells as compared to those in the control. In contrast, the parenchymal component consisted of a predominantly mononuclear infiltrate in both the alveoli and the interstitium (Fig. 3C) with only some occasional granulocytes. Characteristically, the pneumonia was localized, with normal lung very sharply demarcated from consolidated lung in some areas, suggesting limited invasive potential. Varying degrees of the above response were seen before and after days 6 to 8 of infection. In no case were chlamydial inclusions detected by hematoxylin and eosin staining or by toluidine blue staining. Gram stains of lung sections did not reveal any other bacteria. There was no apparent difference between the pathological changes elicited by either yolk sac- or cell culture-derived GPIC despite the difference in inoculating dose. There also was no apparent difference between animals inoculated on day 1 or 3 after birth.

When sections of lung from animals inoculated with either uninfected yolk sac or cell culture material were examined, no differences from normal neonatal guinea pig lung were noted (Fig. 3D).

To detect the presence of GPIC, sections of lung from infected animals were stained by the immunoperoxidase technique. Occasional chlamydial inclusions were identified in bronchiolar epithelial cells (Fig. 4). Only day 6 specimens were positive for antigen. The number of cells stained was quite low (0 to 2 inclusions per bronchiole). No antigen was found in the parenchyma of the lung. Moreover, GPIC localization in the bronchioles was always associated with an acute inflammatory reaction. Nonspecific staining was excluded by staining sequential sections of lung with normal guinea pig serum. These were routinely negative. Sections of lung from control uninfected animals were also negative for GPIC antigens with immune and control guinea pig serum.

Course of infections. A total of 27 neonatal guinea pigs were examined at various times after intranasal inoculation with GPIC. Pathological changes were first noted on day 4, were maximal on days 6 to 8, and were still evident on day 12 but at greatly diminished levels (Fig. 5). GPIC was only isolated from lung homogenates on days 6 to 9.

Both IgM and IgG antibodies to GPIC were first detectable in the serum on day 8 and reached peak levels on day 12. IgG remained elevated in each of two animals on days 17 and 23, whereas IgM decreased markedly.

Bacteriological Findings. In one experiment, sections of lung obtained from five infected animals on day 6 were processed and cultured for spurious bacteria and GPIC. Three of the guinea pigs had consolidation of portions of the lung consistent with pneumonia. In these cases, a portion of the area of gross pathological change was removed for bacteriological culture and chlamydial isolation. Four of five animals were positive for GPIC upon culture. The direct culture from only one of the animals with gross pathology had slight growth which was identified as viridans streptococci. The direct culture from one other animal also had a few colonies of this organism. The thioglycolate cultures from all of the animals were positive. Identification of the

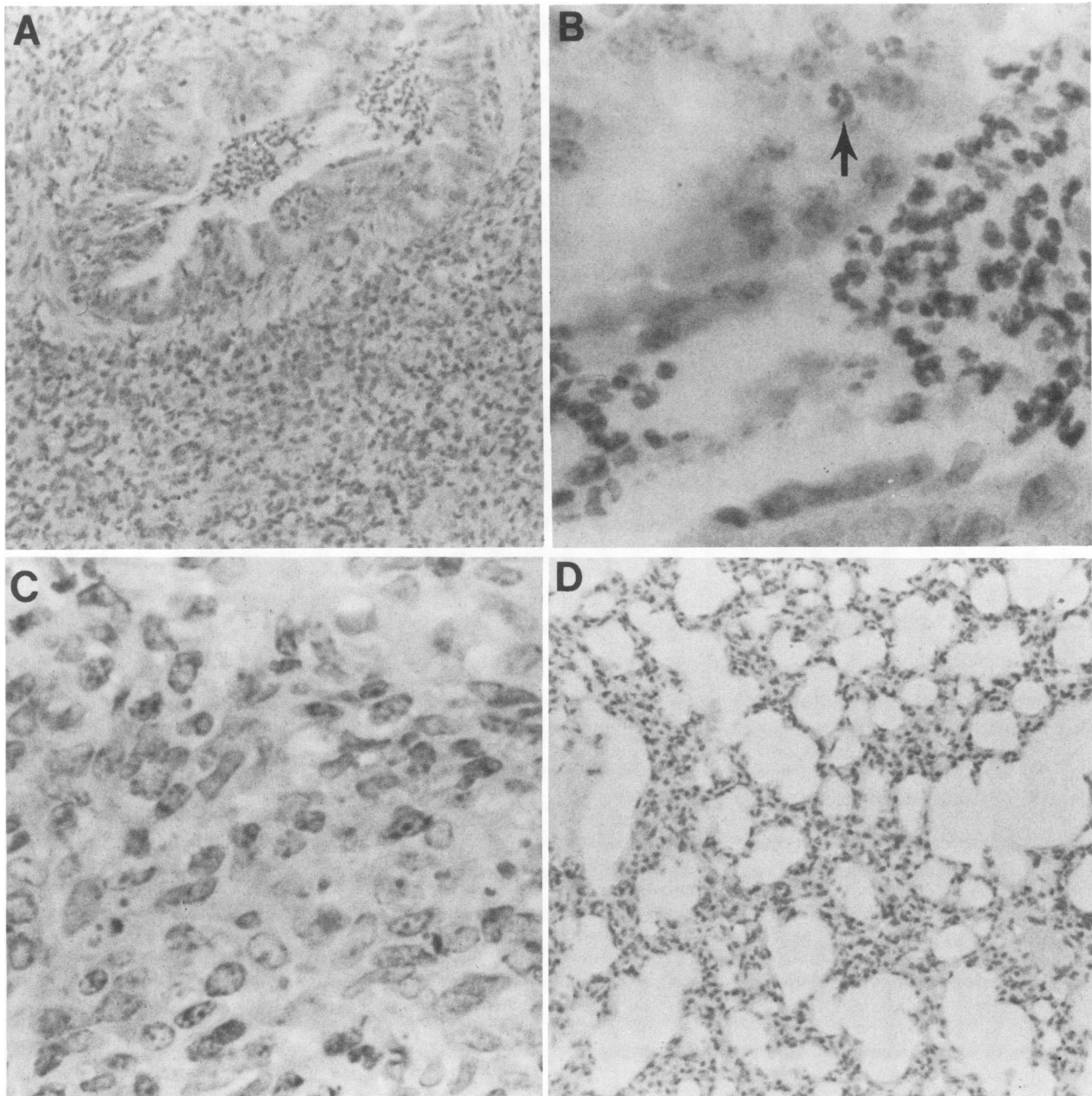


FIG. 3. Photomicrographs of lung after inoculation with GPIC (A, B, and C) or control material (D). (A) Six days postinoculation. Note the severe confluent pneumonia with both bronchial and parenchymal components (hematoxylin and eosin; magnification, $\times 100$). (B) Higher magnification demonstrating bronchial component of pneumonia. Note the acute inflammatory infiltrate of granulocytes in both the lumen and wall (arrow) (hematoxylin and eosin; magnification, $\times 450$). (C) Higher magnification of lung parenchyma from (A) showing mononuclear infiltrate in both interstitium and within the alveolar lumina (hematoxylin and eosin; magnification, $\times 450$). (D) Lung from control animal 6 days after inoculation with normal yolk sac (hematoxylin and eosin; magnification, $\times 100$).

organisms included viridans streptococci, *Escherichia coli*, coagulase-negative staphylococci, and diphtheroids. No one organism was found consistently in each culture. All cultures for *Bordetella* species were negative.

DISCUSSION

In this study, we have demonstrated that chlamydial pneumonia can be produced in newborn guinea pigs by the intranasal inoculation of GPIC. The infection was characterized by a noninvasive confluent mononuclear interstitial pneumonia with an acute granulocytic component associ-

ated with the bronchioles. The results of histopathology in the guinea pig model are consistent with the results of biopsies in humans (2) and necropsy material from *C. trachomatis*-infected infant baboons (5). In the latter study, a marked interstitial lymphoid hyperplasia accompanied by granulocytic inflammatory exudates in the bronchioles and small bronchi was noted. Chlamydial inclusions were also detected in that study in endothelial cells associated with the bronchioles. This finding was also supported in the GPIC-guinea pig model in which chlamydial inclusions were restricted to the bronchiolar epithelium and associated with an

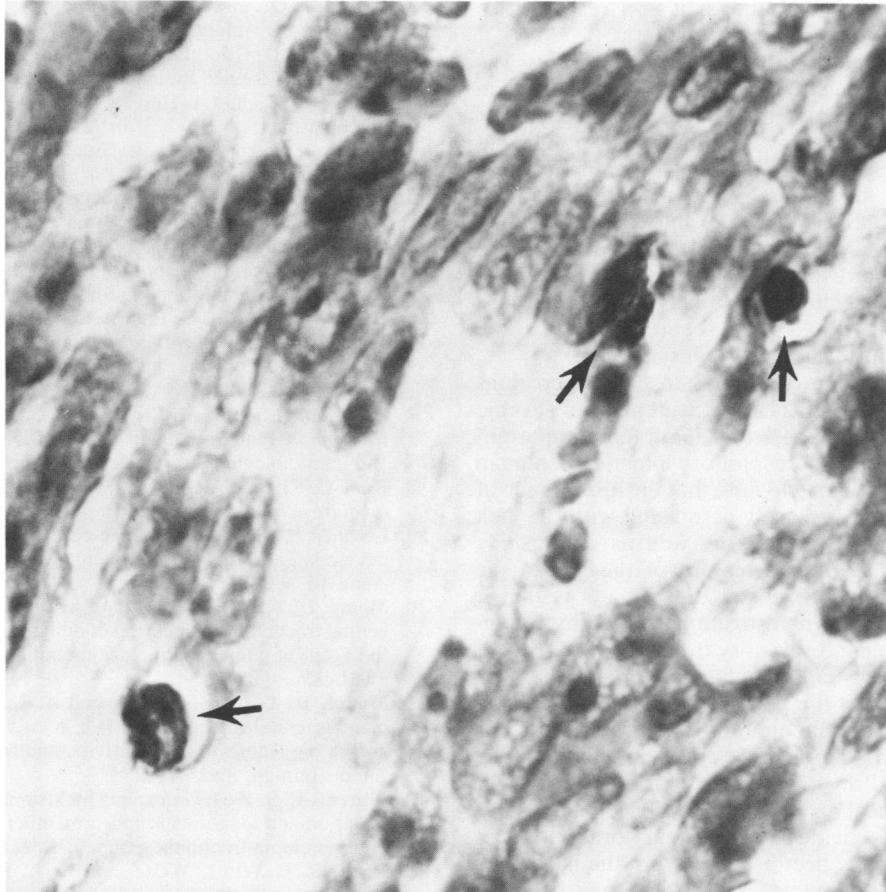


FIG. 4. Bronchiole showing chlamydial inclusion within bronchiolar epithelial cell (immunoperoxidase; magnification, $\times 1,000$).

acute inflammatory reaction. This association with epithelial cells and with an acute inflammatory response has been routinely observed in all aspects of the guinea pig model including the male (14) and female (1, 18) genital tracts and the eye (12). Whether chlamydiae are found only in bronchiolar epithelium in humans is not known at this point, as inclusions have not been detected in biopsy material.

However, in contrast to infection of human neonates, the pneumonia in newborn guinea pigs was restricted to only certain lobes of the lung and never spread throughout the entire lung. Also, there was variance in the degree of pathology during the course of infection (Fig. 5). This may have been related to the positioning of the animal during anesthesia and inoculation as well as to variation in the inoculating dose of chlamydiae given to the different litters. Furthermore, it was difficult to standardize the size of the inoculum actually gaining access to the respiratory tree when giving it via the intranasal route, and the large droplets introduced in this manner may have limited the distribution of the material in the lung.

The histopathology caused by chlamydiae in newborn guinea pigs supports the physical signs of infection. A marked tachypnea on days 6 to 8 was noted and could have been the result of partial obstruction of the airways by the acute inflammatory reaction. Although radiological signs of the infection were seen in our model, they were somewhat different from those seen in humans and baboons in which chest X rays showed a diffuse, symmetrically distributed interstitial pulmonary infiltrate with hyperinflation (3, 5). Neonatal guinea pigs also had evidence of a severe intersti-

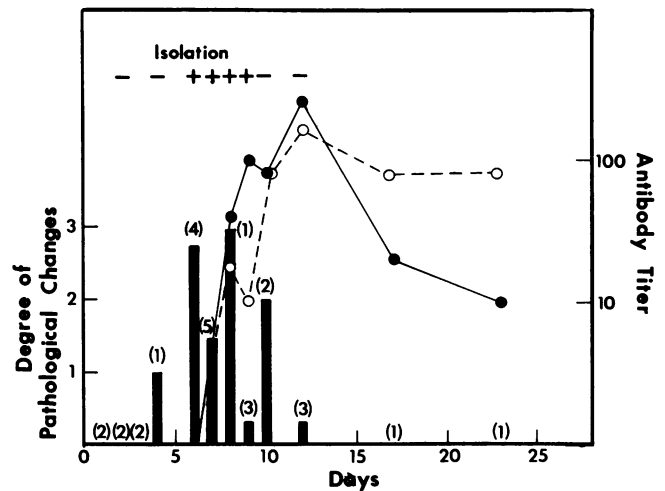


FIG. 5. Kinetics of respiratory infection after intranasal inoculation of GPIC. The figure is a summary of all animals included in the study. The numbers within parentheses represent the number of animals assessed at each time point. Pathological changes range from 0 to 3+ and are scored as follows: 1+, diffuse interstitial and bronchial infiltrate; 2+, moderate interstitial and bronchial infiltrate which is focal in nature; 3+, confluent pneumonia, heavy interstitial and bronchial infiltrate. Symbols: ●, IgM antibody to GPIC, ○, IgG antibody to GPIC. Sera from day 7 animals were not assessed for antibody. Successful isolation of GPIC in tissue culture from lung homogenates is indicated by a + .

tial infiltrate, but as described above, it was restricted to only certain areas and generally was unilateral.

The course of the infection in newborn guinea pigs was indeed shorter and more acute than that observed in human neonates in which the infection may run a chronic course of about 40 days (3). It is quite possible that susceptibility to chlamydial infection is related to the relative maturity of the respiratory tract and the immune system. Although these systems of neonatal guinea pigs are still immature, they develop at a comparatively faster rate than do those of humans. Moreover, the respiratory and immune systems of guinea pigs at birth are relatively more advanced than those of humans (9, 16). It would thus seem logical that the infection should be shorter in guinea pigs.

Both IgM and IgG serum antibodies to GPIC were produced in newborn guinea pigs. The measurement of specific IgM antibodies to chlamydiae is considered to be important in the diagnosis of chlamydial respiratory infection in human newborns (15). It is interesting to note that the appearance of high titers of antibody was closely associated with the lack of GPIC isolation from the lungs as well as diminishing pathological changes. A unique feature of the guinea pig model for chlamydial pneumonia of the neonate is that it presents the opportunity to investigate both the physiological effects of a developing respiratory tract and the effects of a developing immune system on chlamydial infection.

There was concern that the pneumonia in this model may have resulted from a concomitant bacterial, viral, or mycoplasma infection. Although we could not eliminate the possibility of viral and mycoplasma infections, control animals had no pathological changes, and bacteriological studies of infected animals yielded only normal flora. Although mycoplasma species have been isolated from the nasopharynx and genital tract of guinea pigs, there has been no evidence for a mycoplasma pneumonia (4). The isolation of GPIC from lung tissue was closely associated with the peak of the infection as determined by pathological changes. Thus, it is unlikely that another agent might be associated with GPIC in producing the described pathological changes.

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