

## Rabbit Model for *Chlamydia pneumoniae* Infection

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Received 28 May 1996/Returned for modification 26 July 1996/Accepted 17 October 1996

A rabbit model was established for *Chlamydia pneumoniae* infection that may be helpful to understand the pathogenesis of disease in humans. Twelve, pathogen-free, 1-month-old New Zealand White rabbits were inoculated with  $1.0 \times 10^7$  to  $5.0 \times 10^7$  CFU of purified *C. pneumoniae* (ATCC strain VR 1310) via the nasopharynx (1 rabbit died immediately postinoculation, and 11 were available for study). Five controls were inoculated with the carrier buffer. Ten of the 11 study rabbits demonstrated serological evidence of acute infection (immunoglobulin G antibodies, 1:8 to >1:16), with the weakest response at 7 days and the strongest response at 28 days, whereas none of the controls showed any seroconversion. Study animals were sacrificed in batches of three, on days 7, 14, 21, and 28, but controls were sacrificed on days 7 and 28. Two-thirds of the animals demonstrated evidence of bronchiolitis and pneumonia on days 7 and 14 and resolution by day 21. Two study rabbits demonstrated, on histology, early and intermediate lesions of atherosclerosis: one animal (day 7) showed the accumulation of foamy macrophages (fatty streak) in the arch of the aorta, and the other animal (day 14) showed spindle cell proliferation of smooth muscle cells (intermediate lesion). Focal periaortitis was seen in the same animal (day 7). *C. pneumoniae* elementary bodies were demonstrated by immunocytochemical stain in the lungs ( $n = 2$ ), liver ( $n = 3$ ), spleen ( $n = 5$ ), and aorta ( $n = 2$ ), one of which corresponded to the intermediate lesion. *C. pneumoniae* was cultured from the lungs ( $n = 2$ ), liver ( $n = 2$ ), spleen ( $n = 2$ ), and aortic arch ( $n = 1$ ). All histopathological, immunocytochemical, and cultural studies were negative in the controls. Hence, the rabbit provides a useful animal model for the study of *C. pneumoniae* infection and its complications, particularly atherosclerosis.

*Chlamydia pneumoniae* is an intracellular bacterium that commonly causes respiratory disease in North America and Europe. It is probably the third most common cause of community-acquired pneumonia, accounting for 6 to 12% of pneumonias (6, 15) in young adults and children. It may also cause upper respiratory symptoms and sinusitis without pneumonia (6, 7). Recently, *C. pneumoniae* has been associated with coronary heart disease and myocardial infarction in several seroprevalence studies (4, 14, 16, 17, 19, 21, 25) and one prospective cohort study (22). This link has become more persuasive by the identification of *C. pneumoniae* elementary bodies in atherosclerotic plaques and fatty streaks in autopsy cases from the aorta and coronary arteries (12, 24), from coronary atherectomy (2), and from endarterectomy specimens from carotid arteries (3).

Although pneumonitis models in the mouse, monkey, and rabbit have been established for *C. pneumoniae*, there is no published data on atherosclerotic complications in these reports. The rabbit model may be more suitable for the study of the role of *C. pneumoniae* in the pathogenesis of atherosclerosis, because the cholesterol-fed rabbit is a well-established and commonly used model for aortic atherosclerosis.

In this study, we describe a rabbit model for *C. pneumoniae* respiratory tract infection and its possible role in the immunopathogenesis of atherosclerosis. Isolations of *C. pneumoniae* from the liver, spleen, and aortic arch indicate systemic infection or colonization as well as those of the lungs.

### MATERIALS AND METHODS

***C. pneumoniae* strain and inoculum.** TWAR ATCC strain VR 1310 was used in this study. Viable organisms were harvested from infected cultures of McCoy cells (12) by disrupting infected cells with glass beads and sonification. Organisms were partially purified by one cycle each of low- and high-speed centrifugation and resuspended in minimum essential medium and 10% fetal calf serum to provide a final inoculum of  $1.0 \times 10^7$  to  $5.0 \times 10^7$  inclusion-forming units per ml. Contamination of the inoculum by *Chlamydia trachomatis* and *Chlamydia psittaci* was excluded by staining with specific monoclonal antibodies and by analysis using PCR.

**Experimental animals.** Twelve 1-month-old male New Zealand White rabbits (pathogen free), fed standard rabbit chow, were inoculated with 1.0 ml of the purified strain of *C. pneumoniae* via the nasopharynx with a plastic catheter, under light halothane anesthesia to induce hyperventilation. No antibiotics were present in the rabbit chow.

Five control rabbits were inoculated in a similar manner with 1.0 ml of minimum essential medium with fetal calf serum without *C. pneumoniae*. In order to determine the sequence of inflammatory changes and persistence of the microorganism, animals were sacrificed in batches of three on days 7, 14, 21, and 28. Controls were sacrificed on days 7 ( $n = 2$ ) and 28 ( $n = 3$ ).

**Serology.** Antibodies (immunoglobulin G [IgG]) to *C. pneumoniae* were measured (26) by the micro-immunofluorescence test (MRL Diagnostics, Cypress, Calif.). The IgG serum antibody fractions were measured by using fluorescein-isothiocyanate-conjugated goat anti-rabbit IgG (Sigma Chemical Co., St. Louis, Mo.). Sera were tested at dilutions of 1:2 to 1:16. Blood was obtained from the earlobe veins at baseline before inoculation and on the day of sacrifice. IgM and IgA antibodies were not tested because anti-rabbit IgM and IgA conjugates were not available commercially.

**Culture of tissues.** Lungs, liver, spleen, and aortic arch samples (approximately  $1 \text{ cm}^2$ ) obtained by aseptic technique were weighed, minced, and homogenized with minimum essential medium containing 10% fetal calf serum. Tissue suspensions were centrifuged at  $500 \times g$  for 10 min to remove debris. Fresh tissue homogenates (refrigerated at  $4^\circ\text{C}$  for less than 2 h) were inoculated onto McCoy cells grown on 12-mm-diameter coverslips in flat-bottomed 1-dram (4 ml) shell vials. Inoculated cells were incubated at  $35^\circ\text{C}$  in a 5%  $\text{CO}_2$  incubator for 3 days and blindly passaged once for another 3 days. Monolayers were fixed with methanol and stained with a *Chlamydia* genus-specific monoclonal antibody (Pathfinder culture confirmation reagent; Kallestad, Chaska, Minn.) conjugated to fluorescein isothiocyanate (10). Inclusions were counted under a fluorescence microscope and expressed as inclusion-forming units per high-power field.

**Pathological investigations.** The lungs, liver, spleen, and aorta were removed from the animals. The aorta was cleared of any adhering fat, and the arch and the

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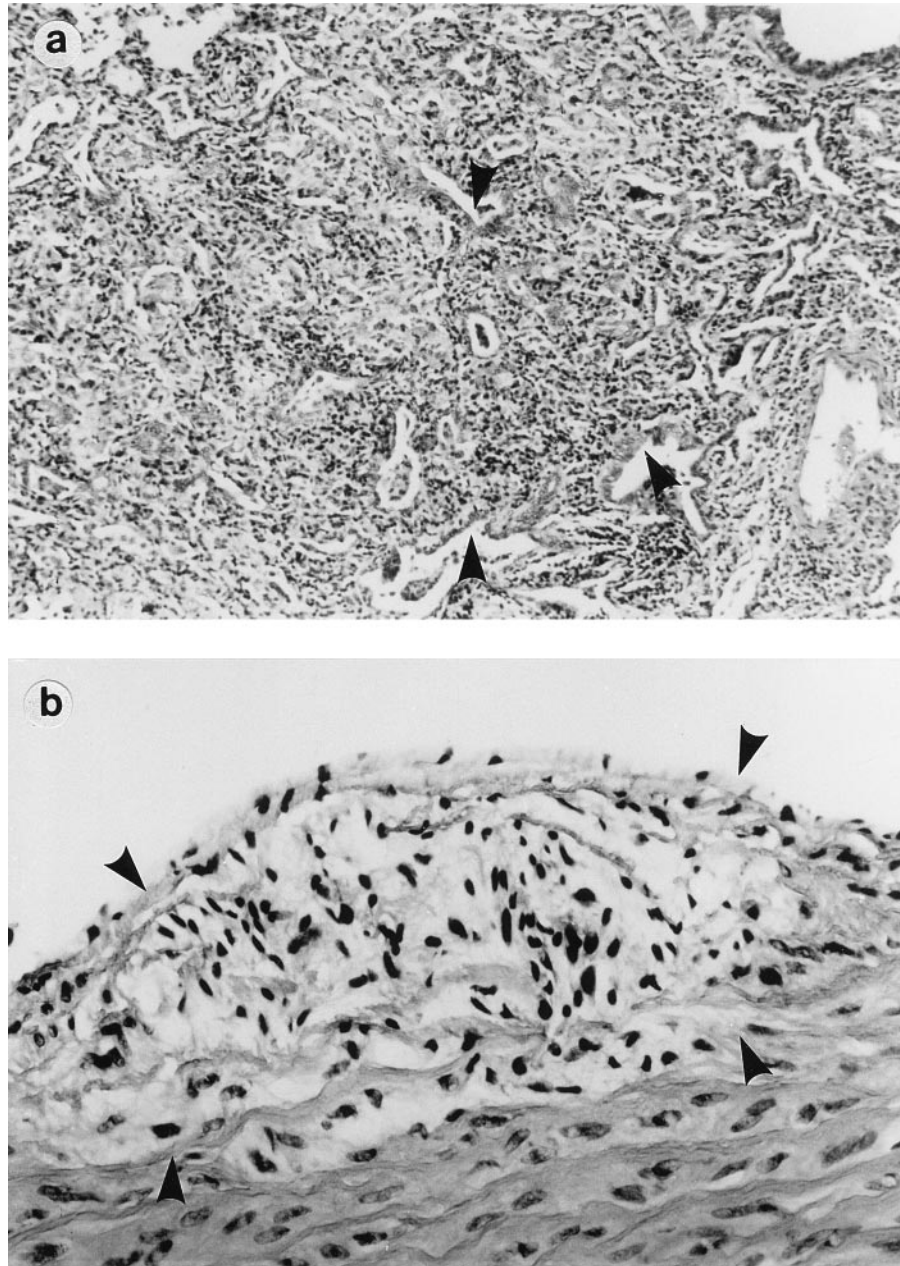


FIG. 1. (a) Histology of lung with mononuclear inflammatory cell infiltration of bronchioles, alveolar ducts, and alveoli with bronchiolar obliteration (arrowheads). Original magnification,  $\times 100$ . (b) Histology of aorta (day 7) with foam cell island (grade I, fatty streak, as indicated by arrowheads) in the intima and superficial media covered by endothelial cells. Hematoxylin and eosin stain was used. Original magnification,  $\times 250$ .

rest of the aorta were sectioned for processing. Pathological specimens were fixed in 10% buffered formalin, routinely processed, paraffin embedded, and sectioned for routine histological examination. Hematoxylin and eosin stain and hematoxylin-orcein-phloxine-saffron elastin stain were used to determine lesion composition. The atherosclerotic lesions were classified according to the work of Daley and associates (5) as follows: grade I, fatty streaks, defined as consisting of foamy macrophages in the intima; grade II, advanced fatty streaks, defined as consisting of approximately equal numbers of foam cells and spindle-shaped smooth muscle cells; grade III, fibrous plaque, defined as consisting of spindle-shaped smooth muscle cells; and grade IV, atheromatous lesion, defined as the presence of a core containing pools of extracellular lipid and/or necrotic debris with a fibrous cap. Immunohistochemical staining was performed on paraffin-embedded sections by the avidin-biotin-peroxidase method (11). A species-specific IgG-type monoclonal antibody to *Chlamydia* elementary body, Chlamydia-cel Pn (Cellabs, Brookvale, Australia), was used. The antibody was not diluted before use. Tissue samples with known immunoreactivity served as positive

control samples. Negative control samples consisted of omission of the primary antibody and substitution with diluted nonimmune serum from the species from the primary antibody obtained.

## RESULTS

One of the study rabbits died immediately after inoculation and anesthesia, leaving 11 study rabbits for analysis.

**Antibody response.** All baseline serum samples and control samples were negative for *C. pneumoniae* antibody at a 1:2 dilution. On day 7 (first sacrifice), two of three rabbits showed an antibody response at a 1:8 and 1:16 dilution. On day 14, all three rabbits sacrificed showed an antibody response at a titer

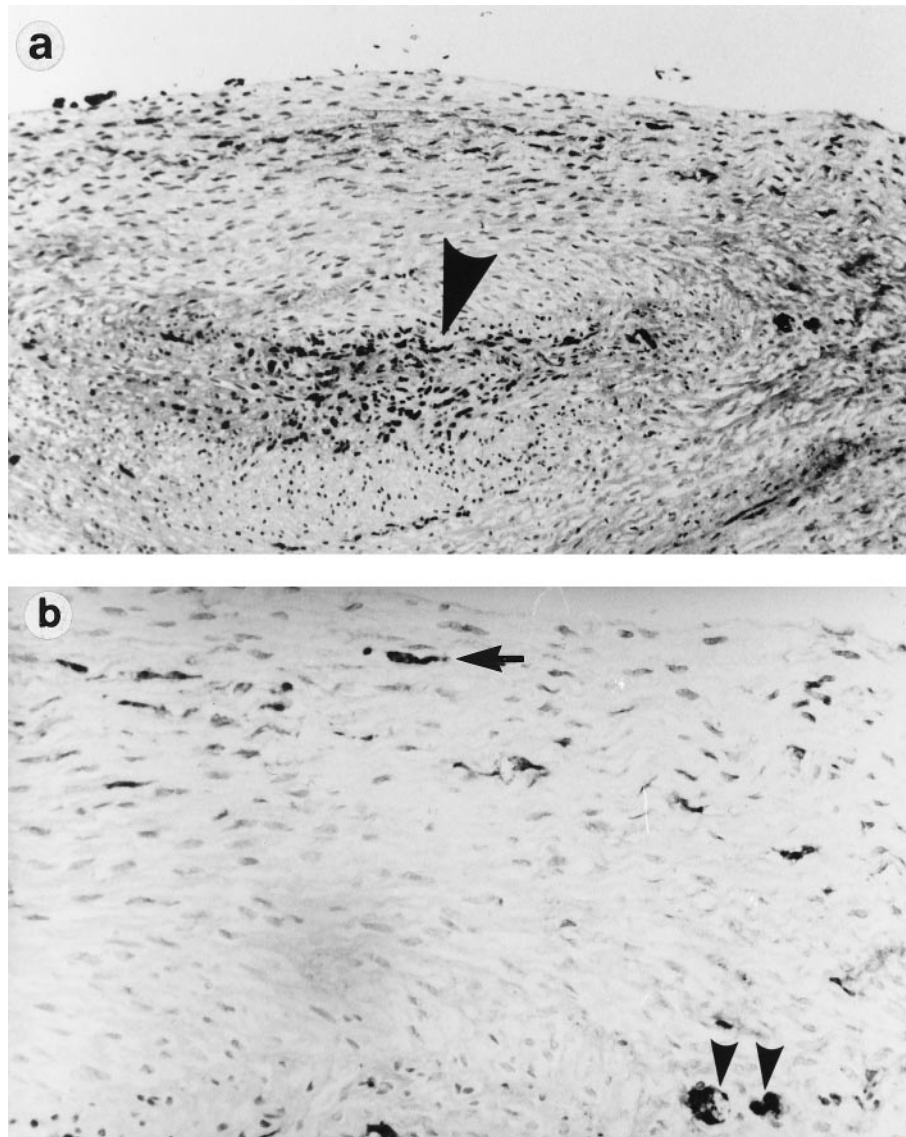


FIG. 2. (a) Day 14, avidin-biotin immunohistochemical stain with Chlamydia-cel Pn. Lesion is histologic grade III. Arrowhead indicates positivity in nodule of vascular smooth muscle cells. Hematoxylin was used for counterstaining. Original magnification,  $\times 100$ . (b) Higher magnification (original magnification,  $\times 250$ ) of panel a. Arrow indicates positivity in occasional smooth muscle cell, and arrowheads indicate two macrophages which are positive for Chlamydia-cel Pn. Immunohistochemical stain was used.

of 1:16. On days 21 and 28, all five rabbits demonstrated an antibody response of  $\geq 1:16$ , with the strongest immunofluorescence pattern at 28 days. Thus, 10 of the 11 inoculated rabbits demonstrated serological evidence of acute *C. pneumoniae* infection. All control rabbits' sera were negative for *C. pneumoniae* antibody ( $< 1:2$ ) at sacrifice.

**Histopathology and immunohistochemistry.** On day 7, two of three study animals demonstrated discrete, white patches on the surface of the lungs. Histologically, there were foci of bronchiolar infiltration with macrophages, lymphocytes, and plasma cells with the infiltrates extending into the alveolar ducts and alveolae (Fig. 1a). The adjacent interstitium was also infiltrated with lymphocytes and histiocytes and occasional histiocytic giant cells but no neutrophils. These findings are consistent with bronchiolitis and pneumonitis.

In one rabbit, a focal accumulation of foamy macrophages was found in the aortic arch, consistent with a fatty streak or

the early (histologic grade I) changes of atherosclerosis (Fig. 1b). In the same animal, focal periaortitis was seen in the abdominal aorta, consisting of lymphocytic infiltration in the adventitia. The immunohistochemical staining for *C. pneumoniae* was positive in the lungs and aorta of one rabbit and the spleen and liver of two animals. The positive stain in the aorta was in the same animal with the fatty streak.

On day 14, two of three animals sacrificed again showed inflammatory changes of mild bronchiolitis and pneumonitis. One rabbit showed a histologic grade III lesion of atherosclerosis in the aorta, containing a proliferation of spindle-shaped smooth muscle cells (Fig. 2a), and the immunohistochemical stain for *C. pneumoniae* was positive in the same aorta (Fig. 2b). The spleen in another rabbit was also positive on the immunocytochemical stain.

On day 21, the two animals sacrificed had normal lung histology except for occasional interstitial and peribronchiolar

TABLE 1. Summary of antibody, histology, immunocytochemistry, and culture results

| Sacrifice | No. | Positive IgG antibody (titer) | Positive histology   | Positive immunocytochemistry                          | Positive culture                   |
|-----------|-----|-------------------------------|--|---|------------------------------------|
| Day 7     | 3   | 2 (1:8-1:16)                  | Pneumonia ( $n = 2$ )<br>Fatty streak of aorta, periaortitis ( $n = 1$ ) | Lungs, aorta ( $n = 1$ )<br>Liver, spleen ( $n = 2$ ) | Lungs and spleen ( $n = 1$ )       |
| Day 14    | 3   | 3 (1:16)                      | Pneumonia ( $n = 2$ )<br>Grade III atherosclerosis of aorta ( $n = 1$ )  | Aortic plaque ( $n = 1$ )<br>Spleen ( $n = 1$ )       | Aorta, lung, and liver ( $n = 1$ ) |
| Day 21    | 2   | 2 ( $\geq 1:16$ )             | Peribronchial infiltration ( $n = 1$ )                                   | Liver ( $n = 1$ )<br>Spleen ( $n = 1$ )               | 0                                  |
| Day 28    | 3   | 3 ( $\geq 1:16$ )             | 0  | Spleen ( $n = 1$ )                                    | Spleen and liver ( $n = 1$ )       |
| Controls  | 5   | <1:2                          | 0  | 0   | 0                                  |

lymphocytic infiltration. The immunohistochemical stain for *C. pneumoniae* was positive in the liver and spleen of two separate rabbits. By day 28, all three rabbits had normal lung histology and *C. pneumoniae* was detected in the spleen of one rabbit. The five controls showed normal histology of the lungs and aorta, and none of the tissues stained positive for *C. pneumoniae*. A summary of the histopathological and immunocytochemical findings is shown in Table 1.

**Chlamydia culture.** Cultures for chlamydia were negative in most of the study animals and all of the controls. However, three rabbits did grow *C. pneumoniae* (one each on days 7, 14, and 28) from the following organs: lungs ( $n = 2$ ), liver ( $n = 2$ ), spleen ( $n = 2$ ), and aorta ( $n = 1$ ). The rabbit with recovery of *C. pneumoniae* in the aorta was the same animal which showed a grade III lesion of atherosclerosis and was positive on immunocytochemical stain for *C. pneumoniae*.

## DISCUSSION

The rabbit model for *C. pneumoniae* has demonstrated histological evidence of bronchiolitis and pneumonitis similar to the results of the mouse model (29, 30). However, there are some differences in these two models. Whereas the histological signs of bronchiolitis and pneumonitis resolve by 21 days in the rabbit, lung pathology may persist up to 60 days in Swiss Webster mice and *C. pneumoniae* has been isolated from the lungs up to 42 days in this model (28). Of interest is the evidence that previous infection in mice did not prevent inflammatory changes of pneumonia with subsequent challenge with *C. pneumoniae* (9). Dissemination of the microorganism to the spleen has been demonstrated in the murine model, similar to our findings, but we have found the bacterium in the liver and aorta as well (not previously described). We did not isolate chlamydias from most of our rabbits, but our culture method may not be as sensitive as that reported by Kuo and Grayston (12). The use of HL cells for isolation and propagation of *C. pneumoniae* rather than McCoy cells would likely have improved our culture results.

In the nonhuman primate models (baboons and cynomolgus monkeys), mild or asymptomatic chronic respiratory tract infection (mild interstitial pneumonia) developed after intranasal inoculation (1, 8). Recently, Moazed et al. (18) has described *C. pneumoniae* infections of the rabbit. Their lung findings were similar to ours with resolution of inflammation by day 28. However, they used a high-cholesterol diet-induced model. No inflammation was noted in the aorta of their animals, and cultures, immunocytochemical stain, and PCR were negative for *C. pneumoniae* in all aortic samples. The histological changes described in the rabbit model and the other animals are similar to that described in humans (23). Moreover, spontaneous resolution of respiratory tract infection or asymptomatic infection is probably common in humans as most people

with antibodies (>50% of the adult population (7) never reported previous episodes of pneumonia.

The role of *C. pneumoniae* in the pathogenesis of atherosclerosis is unknown, but the strong association merits further investigations. The fact that *C. pneumoniae* has been identified in various stages of atherosclerosis (fatty streaks and fibrous plaques) may suggest an intimate pathogenic role, but it could still be present as an innocent bystander (i.e., deposition of antigen-antibody complexes) without playing a causative role. Focal periaortitis in one animal at day 7 may suggest an immunological reaction. The fact that chlamydia particles are found inside lesions and not on the surface, however, suggests an active transport mechanism. In this respect, it is of major interest that in the rabbit model we can identify and isolate the bacterium from the aorta of a few animals and that in two rabbits there was evidence of early and intermediate changes of atherosclerosis in less than a month. The recovery of viable organisms may favor an active rather than a passive role in the pathogenesis of atherosclerosis. Previous studies in rabbits have shown that uninfected animals fed standard rabbit chow do not develop even the earliest changes of atherosclerosis when examined after 6 months (5, 20). Further experiments are planned both to confirm these preliminary findings and to elucidate the role of *C. pneumoniae* in the immunopathogenesis of atherosclerosis.

In conclusion, the rabbit is a useful and clinically relevant model for studying the pathogenesis of *C. pneumoniae* infection, particularly its role in the development of atherosclerosis.

## ACKNOWLEDGMENTS

We are grateful to Dayle Noda for her technical assistance, R. Bannatyne for his microbiological assistance, and Dawn Bajhan for her assistance in preparation of the manuscript.

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