

Experimental Infection with *Chlamydia pneumoniae* in Nonhuman Primates

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To serially examine the immunopathogenesis and histopathology of infection with *Chlamydia pneumoniae*, we inoculated two cynomolgus monkeys in the conjunctival sac, nose, and nasopharynx with *C. pneumoniae* TWAR. After inoculation, *C. pneumoniae* was isolated from the inoculation sites and the rectums of both monkeys for a period of 5 weeks. After a second inoculation, *C. pneumoniae* was recovered from the inoculation sites and the rectums of both monkeys for 20 weeks. A third inoculation with *C. pneumoniae* caused very little productive infection at any site. Prior *C. pneumoniae* infection did not prevent subsequent *C. trachomatis* serovar E (Bour strain) infection. Clinical and histopathologic ocular responses to *C. pneumoniae* infection were mild compared with those to infection with *C. trachomatis* serovar E. Rectal infection, demonstrated by culture isolation and immunohistopathology, occurred without direct experimental inoculation. Both immunofluorescent staining of mucosal smears with monoclonal antibodies and tissue culture were able to detect *C. pneumoniae* infection. Experimental nonhuman primate infection with *C. pneumoniae* appears to be clinically and histopathologically mild and can occur at extrapulmonary sites.

Chlamydia pneumoniae, previously known as TWAR, is emerging as an important human respiratory pathogen. It has been associated with pneumonia, bronchitis, and pharyngitis (4, 8). Epidemics of mild pneumonia and "ornithosis" have been linked serologically to *C. pneumoniae* (5, 9, 13). Acute lower respiratory tract infection in Filipino children has also been associated with acute *C. pneumoniae* infection (12). Serologic surveys indicate that *C. pneumoniae* exposure is worldwide and quite common in some groups (19).

TW-183, the first *C. pneumoniae* isolate, has been used previously for monkey inoculation (7). Of three animals exposed to a moderate inoculum, only one developed mild conjunctivitis. Reexposure to a larger inoculum resulted in mild conjunctivitis in only two of the three; the conjunctivitis was still more mild than in comparable *C. trachomatis*-inoculated monkeys. Baboon ocular and respiratory inoculation with AR-39, another *C. pneumoniae* isolate, resulted in no apparent disease despite culture recovery intermittently over 8 weeks (1). Baboon inoculation with IOL-207, an organism similar if not identical to *C. pneumoniae*, into the eye, urethra, and knee joint caused clinical inflammation at those sites (3). The organism was recovered from each site for up to 3 months. In view of the emerging importance of this agent, the mild clinical disease it seems to cause in animals, and its propensity for persistent infection, we have attempted to correlate the long-term clinical, serologic, microbiologic, and histopathologic responses after inoculation of cynomolgus monkeys with *C. pneumoniae*.

MATERIALS AND METHODS

Animals. Two female cynomolgus monkeys between 1.5 and 3 years of age were received from the Charles River Primate Co. (Port Washington, N.Y.) and kept in isolation for 6 weeks before experiments were begun. The monkeys were kept together in a P3 Horsefall isolation cage, an

airtight stainless steel cabinet with self-contained ventilation and filtration of entering and exiting air. Neither monkey had been previously experimentally exposed to *C. pneumoniae* or *C. trachomatis*. Both monkeys were negative for *Chlamydia* infection by culture, immunofluorescent cytology, and serology before inoculation.

Inoculation. *C. pneumoniae* TW-183 was obtained from Washington Research Center (Seattle, Wash.) and propagated in HeLa cells. Cultures were performed on HeLa cell monolayers in microdilution plates pretreated with 30 µg of DEAE-dextran per ml in phosphate-buffered saline with 0.01% CaCl₂ for 30 min at 37°C. Plates were centrifuged at 800 × g at 35°C and then incubated for 30 min at 37°C. Supernatant fluid was then aspirated, and 200 µl of RPMI 1640 medium with 10% fetal calf serum, 0.45% glucose, 1 µg of cycloheximide per ml, 2 µg of gentamicin per ml, 5 µg of vancomycin per ml, and 5 µg of nystatin per ml was added. Cultures were incubated at 37°C with 5% CO₂ for 48 to 72 h. For animal inoculation, the titer was increased by successive passages of *C. pneumoniae* onto fresh monolayers. Specimens for animal inoculation were harvested from flask cultures, purified on a Percoll density gradient, frozen at -70°C and thawed, and titers were determined. *C. trachomatis* serovar E (Bour strain) was passaged and prepared as described above using McCoy cells incubated at 35°C.

All examinations and inoculations were performed under ketamine hydrochloride anesthesia. The conjunctivae, nares, nasopharynx, and rectum were examined and specimens were cultured before inoculation. At week 0, 2 × 10⁴ infection-forming units (IFU) of *C. pneumoniae* in 20 µl was pipetted into each conjunctival sac and into each nostril, and 4 × 10⁴ IFU in 40 µl was instilled into the nasopharynx. We have previously shown that repeated inoculation with *C. trachomatis* can boost antibody and inflammatory responses (11, 15, 16). Therefore, in order to determine whether repeated inoculations with *C. pneumoniae* would elicit similar responses, reinoculation with 2 × 10⁵ IFU of *C. pneu-*

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moniae was done at weeks 15 and 38 in the same manner. Reinoculations were done after stable resolution of ocular clinical disease. To determine whether previous *C. pneumoniae* infection might influence subsequent infection with *C. trachomatis* serovar E (Bour strain) into each conjunctival sac was done at week 48.

Collection of clinical data and specimens. Clinical examination and specimen collection were done weekly or biweekly over 58 weeks. During anesthesia, the conjunctiva and pharynx were examined with a Kowa hand-held slit lamp (Parke, Davis & Co., Morris Plains, N.J.) for evidence of chlamydia infection. Photographs of the conjunctiva were taken with a 35-mm camera with macroscopic lenses, and pictures of the pharynx were taken with a Kowa hand-held fundus camera. The clinical response of each eye was scored for a number of signs that were combined as two simplified indices (17). Briefly, the follicular index quantitates the follicular response in the bulbar, limbal, superior tarsal, and superior fornix conjunctiva. The inflammatory index summarizes the nonspecific signs of inflammation, i.e., hyperemia or injection of the bulbar, superior tarsal, and superior fornix conjunctiva, and ocular discharge. Tears were collected from the medial canthus onto a dry, sterile cellulose sponge and kept in sterile polyethylene tubes at -70°C . Three milliliters of venous blood was collected at each examination for hematocrit and differential counts. Serum was separated and stored at -70°C .

Conjunctival and nasal cultures and smears for immunofluorescent staining were taken with sterile cotton swabs (type 1, flexible aluminum shaft; Spectrum Labs, Los Angeles, Calif.) rotated along the mucosa. Nasopharyngeal and rectal smears and cultures were taken with plastic shaft dacron swabs (SP Sterile Dacron; American Hospital Supply, McGaw Park, Ill.) rotated along the mucosa. Swabs were placed immediately into 1 ml of 0.2 M sucrose-phosphate medium with gentamicin (5 $\mu\text{g}/\text{ml}$), vancomycin (12.5 $\mu\text{g}/\text{ml}$), and nystatin (12.5 $\mu\text{g}/\text{ml}$) and stored at -70°C before being cultured. Nasal and nasopharyngeal results are reported together as nasopharyngeal.

Smears for immunofluorescent staining were taken from the lower fornix conjunctiva with a sterile platinum spatula. Nasal, nasopharyngeal, and rectal smears were made from swabs. All smears were fixed in absolute methanol for 10 min. Biopsies of the conjunctiva (weeks 19, 24, 42, and 56) and rectum (weeks 19, 26, and 42) were obtained under anesthesia and divided into Carnoy fixative, phosphate-buffered Formalin, and paraformaldehyde.

Chest radiographs were made 4 to 6 weeks after each inoculation.

Laboratory methods. All culture specimens were thawed and vortexed. Rectal specimens were sonicated for 5 to 10 s at moderate power (Ultrasonics, Plainview, N.J.). One hundred microliters of material was used in each of four wells (duplicate wells on duplicate plates). Culture was performed as described above. After 48 to 72 h of incubation, one well was passed to a secondary plate. Two other wells were fixed with absolute methanol for 10 min and stained for 30 min at 37°C with 30 μl of a fluorescein-conjugated genus-specific antichlamydia monoclonal antibody (Ortho Diagnostic Systems, Raritan, N.J., or Kallestad, Austin, Tex.). Culture plates were read by using an epifluorescence microscope (Zeiss, Oberkochen, Federal Republic of Germany) and were scored as positive if any inclusions were seen.

Mucosal smears were methanol fixed and stained for 30 min at 37°C with 30 μl of the same fluorescein-conjugated

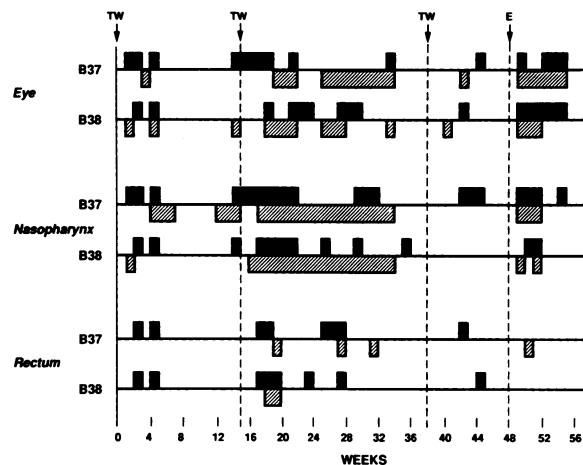


FIG. 1. Culture and immunofluorescence staining results. Symbols: ■, positive cultures; ▨, positive immunofluorescence staining. Blank spaces represent negative culture or immunofluorescence staining. TW, Week of *C. pneumoniae* inoculation; E, week of *C. trachomatis* serovar E (Bour strain) inoculation.

genus-specific reagent as for culture wells. The presence of three or more elementary bodies on epifluorescence microscopy was scored as positive.

Tear and systemic antibody titers were determined by the microimmunofluorescence assay using *C. pneumoniae* and *C. trachomatis* serovar L2 (LGV) as antigens through week 49 when *C. trachomatis* serovar E (Bour strain) was added (18). Fluorescein-conjugated goat anti-monkey antiserum (Cappel, Organon Teknika, West Chester, Pa.) was used as the indicator.

Histology. Biopsy specimens fixed in Carnoy fixative and phosphate-buffered Formalin were dehydrated through ethanol and paraffin embedded. Four-micrometer sections were stained with hematoxylin and eosin. Immunofluorescence staining was done on Carnoy-fixed, deparaffinized sections of conjunctiva and rectal mucosa by incubating tissue with anti-*C. pneumoniae* monoclonal antibody (Washington Research Center, Seattle, Wash.) for 30 min at 37°C , washing it, and then incubating it with a fluorescein-conjugated goat anti-mouse antiserum (Cappel, Organon Teknika). Slides were rinsed in distilled water, cover slipped, and read under epifluorescence. The presence of any inclusions was scored as positive.

RESULTS

Culture and immunofluorescence staining. The culture and immunofluorescent antibody (IFA) staining results from *C. pneumoniae* inoculation of the conjunctiva, nares, and nasopharynx and *C. trachomatis* serovar E inoculation of the conjunctiva are summarized in Fig. 1. Preinoculation cultures and IFA staining were negative in both monkeys. One week after inoculation with *C. pneumoniae*, one monkey developed positive cultures from the eye and nasopharynx, while the other had positive IFA staining of smears from these sites. By 2 weeks postinoculation, both animals were culture positive from eyes, nasopharynges, and rectums. Positive cultures from all sites persisted through week 4. One animal had positive nasopharyngeal IFA staining through week 6. After 9 weeks of negative cultures, both animals developed positive eye and nasopharyngeal cultures. After a second inoculation with *C. pneumoniae* at

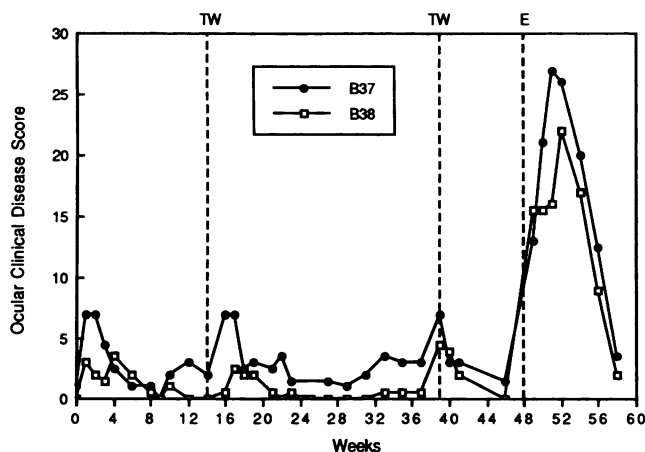


FIG. 2. Ocular clinical responses. TW and E indicate the weeks of *C. pneumoniae* and *C. trachomatis* serovar E inoculation, respectively.

week 15, culture and/or IFA staining was intermittently positive from all sites for 19 weeks. A third inoculation with *C. pneumoniae* at week 38 led to positive cultures from the nasopharynx of only one animal, and positive IFA staining from the conjunctivae of both animals. Overall, IFA staining and culture were positive with the same frequency from the eye and the nasopharynx. However, culture was positive more often than IFA for rectal *C. pneumoniae* infection.

Conjunctival sac inoculation at week 48 with *C. trachomatis* serovar E (Bour strain) led to intermittent recovery of organisms by culture and IFA staining over 8 weeks from the eye and the nasopharynx. Culture on McCoy cells for *C. trachomatis* serovar E yielded more inclusions than culture on HeLa cells. IFA staining of conjunctival scrapings showed quantitatively more EBs than seen with *C. pneumoniae* infection. There was no *C. trachomatis* culture isolation or IFA staining of specimens collected from the rectum.

Clinical responses. The clinical responses of both monkeys to *C. pneumoniae* and *C. trachomatis* infection are shown in Fig. 2. The ocular clinical response to *C. pneumoniae* infection was mild and brief. Initially, there was a mild nonspecific inflammatory response with the gradual formation of discrete follicles by weeks 3 and 4. By week 6 after the first inoculation, the clinical response had largely resolved. Again, after the second and third *C. pneumoniae* inoculations there was only a mild inflammatory response with a few follicles. Inoculation with *C. trachomatis* serovar E caused a much more severe clinical response in comparison with *C. pneumoniae* and induced an acute self-limited follicular conjunctivitis. These changes were not due to inoculation vehicle or repeated examination (14).

Pharyngeal examination results were consistently normal. Rectal temperatures were not elevated. Chest radiographs 4 to 6 weeks after inoculations showed no infiltrates. Anoscopy at weeks 19, 26, and 42 showed no evidence of proctitis.

Histology. Conjunctival biopsies from weeks 19, 24, 42, and 56 showed mild chronic inflammation on hematoxylin and eosin staining. *Chlamydia* inclusions were not seen by immunofluorescent staining.

The rectal biopsy from monkey B38 on week 19 showing a *Chlamydia* inclusion on immunohistochemical staining with anti-*C. pneumoniae* monoclonal antibody is shown in Fig. 3.

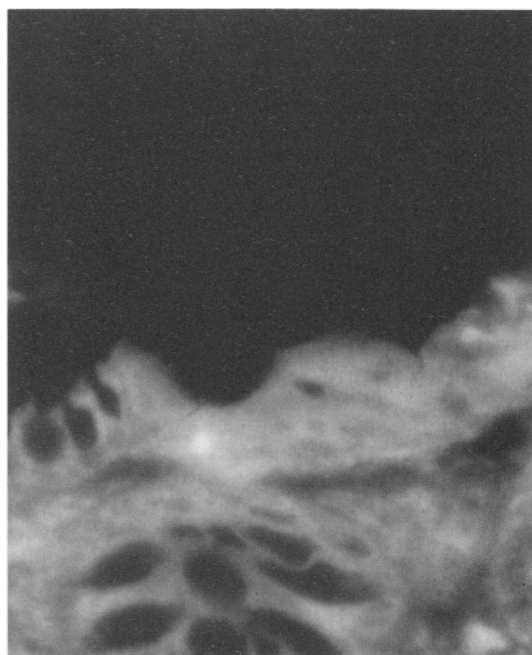


FIG. 3. Anti-*C. pneumoniae* monoclonal antibody immunohistochemical staining of a rectal biopsy from monkey B38 at week 19. The *Chlamydia* inclusion body can be seen between the base of the rectal crypt and the intestinal lumen.

The inflammatory response in the region of the inclusion was minimal. Specimens from weeks 26 and 42 showed mild scarring, fibrosis, and crypt distortion with an increased mitotic rate.

Serology. The immunoglobulin G (IgG) serologic responses of both animals are summarized in Fig. 4. Neither animal had detectable IgG, IgM, or tear IgA to *C. pneumoniae* or *C. trachomatis* before inoculation. Neither animal showed an IgM response to *C. pneumoniae*. Monkey B37 developed an IgG titer of 1:64 at week 8 after initial *C. pneumoniae* inoculation and again after the second *C. pneumoniae* inoculation, which peaked at 1:64 and declined rapidly. After the third *C. pneumoniae* inoculation, B37 had

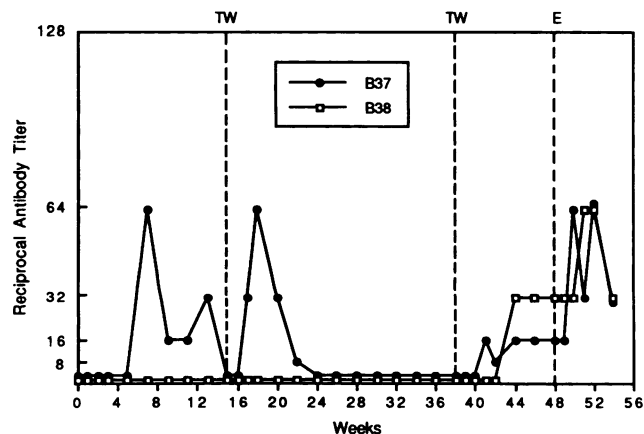


FIG. 4. Anti-*C. pneumoniae* IgG antibody response after inoculations with *C. pneumoniae* and *C. trachomatis* serovar E (TW and E, respectively). After *C. trachomatis* serovar E inoculation, anti-*C. pneumoniae* and anti-serovar E titers were identical.

a weaker *C. pneumoniae* antibody response which was boosted by subsequent inoculation with *C. trachomatis* serovar E.

Monkey B38 had no detectable antibody response to *C. pneumoniae* until 6 weeks after the third inoculation, when the anti-*C. pneumoniae* antibody titer rose to 1:32. This was boosted to 1:64 after inoculation with *C. trachomatis* serovar E.

DISCUSSION

Previous animal studies have shown *C. pneumoniae* to cause mild but persistent infections (1, 7). Bell et al. found low IgG titers and no IgM or tear antibodies over 8 weeks after baboon inoculation with a high titer of *C. pneumoniae* (1). Despite intratracheal instillation, no pulmonary infiltrates were seen on radiographs. Kuo et al. noted only mild conjunctival inflammation after two rounds of eye inoculation in Taiwan monkeys (7). Our study has confirmed these findings, extended the period of observation beyond 1 year, and added the rectum to the list of *C. pneumoniae*-infectable tissues.

Inoculation with *C. pneumoniae* caused infection in both animals as indicated by culture, immunofluorescence staining, and clinical and serologic responses. Both animals showed inflammatory reactions followed by follicular ocular pathology. There was no clinical evidence of pharyngeal, pulmonary, rectal, or systemic disease. The degree of clinical response was about the same after each *C. pneumoniae* inoculation but markedly less than the response to *C. trachomatis* serovar E infection.

Throughout the study we used an antilipoplysaccharide monoclonal antibody to detect *Chlamydia* inclusions and elementary bodies in specimens. As this antibody is genus specific, it cannot distinguish *C. pneumoniae* from other *Chlamydia* species. However, our animals were uninfected with *Chlamydia* before *C. pneumoniae* inoculation as determined by culture, IFA, staining, serology, and exam. Also, the chlamydia recovered after *C. pneumoniae* inoculation had growth characteristics more consistent with *C. pneumoniae* than *C. trachomatis*, such as enhanced growth in HeLa cells and eccentric inclusions (2, 6). These findings confirm that the organism recovered after *C. pneumoniae* inoculation was indeed *C. pneumoniae*. Conversely, after *C. trachomatis* serovar E inoculation, organisms recovered showed enhanced growth on McCoy cells and were stained with species-specific *C. trachomatis* monoclonal antibody. When monoclonal *C. pneumoniae* antibodies became available near the end of the study, representative samples were tested and confirmed the above findings. Therefore, immunofluorescence staining using a genus-specific antilipoplysaccharide antibody is able to detect *C. pneumoniae* in conjunctival and nasopharyngeal scrapings and cultures.

C. pneumoniae infected the rectums of both monkeys despite the absence of experimental inoculation at this site. In one animal, immunohistopathologic examination showed infection of the rectal mucosa. This finding confirmed that culture recovery from the rectum was not solely the result of ingested organisms which had survived gastrointestinal transit. Therefore, the rectal mucosa can be added to the growing list of tissues susceptible to *C. pneumoniae* infection.

Histopathology showed a generally mild inflammatory response. Conjunctival biopsies showed a follicle at week 19 in one animal, but *Chlamydia* inclusions were not detected in any conjunctival biopsy. Rectal specimens had mild crypt

distortion without active inflammation. As with the clinical responses, the histologic response was much less than that seen with *C. trachomatis* (10). A rectal *Chlamydia* inclusion was seen at week 19 without significant acute or chronic reaction. This is in distinction to the markedly inflammatory rectal histopathology seen in *C. trachomatis* L2 (LGV) proctitis but similar to that observed for non-LGV serovars of *C. trachomatis* (11).

The serologic responses of the two monkeys were unequal. Despite similar levels of culture and IFA recovery, only one animal showed an IgG response to the first two inocula. Therefore, productive infection can occur at several sites with or without detectable antibody response, and a systemic antibody response may not be required to clear the infection. Neither animal had detectable IgM titers despite serologic responses that were consistent with primary infection (8 weeks after the first inoculation in monkey B37 and 6 weeks after the third inoculation in monkey B38). In B37, antibody titers increased after subsequent inoculations with a time course consistent with a secondary response (3 weeks). Since IgG antibody titers were not high in these animals, it may be that the IgM response was below the level of detection or that it peaked and fell between two sampling times. Neither animal had detectable IgG or IgA antibodies to *C. pneumoniae* in tears. This may reflect that antibody was below the level of detection, that critical host factors for establishment of infection were absent (e.g., tissue ligands), or that antibody elaboration was not required for clearance of this organism from the conjunctiva.

Repeated infections with *C. pneumoniae* led to partial immunity, as evidenced by a diminished shedding after inoculations. Partial immunity has also been described with repeated *C. trachomatis* infection (15). This apparent *C. pneumoniae* immunity did not afford protection against serovar E infection. Interestingly, the anti-*C. pneumoniae* titers of both animals were boosted by infection with *C. trachomatis*, suggesting that *C. trachomatis* serovar E may carry a "senior" antigen to *C. pneumoniae* (18).

In summary, *C. pneumoniae* can cause infection of the conjunctiva, nasopharynx, and rectum of the cynomolgus monkey, but the resultant infection is clinically mild. It can be detected with a genus-specific monoclonal antibody, and it can be cultured. This animal model may be useful in further studies of the immunopathogenesis of *C. pneumoniae* infection.

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