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Experimentally Induced Ocular Chlamydial Infection in Infant Pig-tailed Macaques

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Four Macaca nemestrina monkeys were inoculated in the conjunctiva with Chlamydia trachomatis (strain E) at 6 weeks of age. A fifth monkey was inoculated with HeLa cell materials only. Ten weeks later, all monkeys were reinoculated with either strain E or strain C. All inoculated monkeys were susceptible to infection with C. trachomatis as documented by fluorescent antibody staining of smears and reisolation of the organism from conjunctival and nasopharyngeal swab specimens. Rectal and vaginal swab specimens remained negative throughout the study. Three of four inoculated animals responded with IgM titers reaching a peak of 1:16 (M#3) and 1:32 (M#1, M#4) 2 weeks after the primary inoculation. IgG appeared in all inoculated animals and titers rose to peak levels of 1:64 (M#2), 1:128 (M#1, M#3), and 1:256 (M#4). Histopathology documented a dramatic difference in immunological response following secondary inoculation. Primary inoculation elicited a typical inflammatory response characterized by moderate stromal infiltration of polymorphonuclear and mononuclear leukocytes. Plasma cells appeared by week 3 postinoculation (pi). Following a secondary inoculation, classic follicle formation was evident by 1 week pi. Mononuclear markers identified a germinal center composed of B cells and a T cell cap. Epithelial thinning near the cap of the follicle was accompanied by a complete loss of goblet cells. This model may be useful for studying the immunopathology of infant chlamydial infections. Invest Ophthalmol Vis Sci 30:995–1003, 1989

Genital infections with Chlamydia trachomatis are approaching epidemic proportions in the United States. A newborn passing through an infected birth canal is susceptible to chlamydial infection of the conjunctiva, nasopharynx, rectum and vagina.1–3 In trachoma endemic areas, C. trachomatis has been identified as the leading infectious cause of preventable blindness. In hyperendemic areas, children are most often infected within their first year, almost all by 2 years of age.4 Activity of the disease is highest in children 4 months to 4 years of age.5

Nonhuman primates are the only susceptible animal in which diseases similar to humans can be produced. However, monkey experiments in the past have generally been limited to adult animals.6,7 In order to study the histopathology and immunopathogenesis of C. trachomatis conjunctivitis in infants, we have used the infant pig-tailed macaque (Macaca nemestrina). Weekly collection of biopsies provide a complete complement of histological changes for 4 weeks after primary and secondary inoculations with C. trachomatis. Clinical and serological observations are discussed, also.

Materials and Methods

Monkeys

Five pig-tailed macaques (Macaca nemestrina), less than 7 weeks old, were studied. Monkeys were caged individually in the Infant Regional Primate Research Center at the University of Washington in Seattle, Washington. Mothers of all animals were tested for serum antibody to C. trachomatis by the microimmunofluorescence method8 and were negative. Infants had neither chlamydial antibody nor apparent eye disease. Experiments with animals were done in conformance with the ARVO Resolution on the Use of Animals in Research.

Organisms

C. trachomatis serovars E/UW-5/Cx and C/TW-3/OT grown in HeLa 229 cell culture were used.9 The inocula contained 10^9 IFU/ml. Control inoculum consisted of HeLa cell materials.
Inoculation

Four monkeys were divided into two groups. Each group consisted of one male and one female. Both groups received a primary conjunctival inoculation with serovar E. Ten weeks after the primary inoculation, one group was reinoculated with the same serovar (homologous rechallenge) and one group with serovar C (heterologous rechallenge). A fifth monkey (female) was inoculated with the control inoculum at the time of the primary inoculation. Ten weeks later two monkeys received a homologous E rechallenge and two monkeys received heterologous C rechallenge. The HeLa control monkey received serovar C at this time. Con junctival biopsies were collected for 4 weeks after each inoculation. Each animal was biopsied a total of four times, twice from each eye.

Specimen Collection

Prior to infection, baseline blood, tear strips and swab specimens from the conjunctiva, nasopharynx, rectum and vagina were collected from all infant monkeys and tested for evidence of chlamydial infection. All specimens were negative.

Specimens were collected at weekly intervals following the primary (1°) and secondary (2°) inoculations. For each monkey, swab specimens (Medical Wire and Equipment Co. (Bath) Ltd. Corsham, Wiltshire, UK) were collected from the upper fornix and lower palpebral conjunctivae of both eyes, nasopharynges, rectum and vagina. Swabs for isolation were stored in chlamydia transport medium and frozen at −70°C until tested. Additional swabs from all sites were rolled onto slides and prepared for immunofluorescent detection of chlamydia organisms (Microtrak, Syva Corporation, Palo Alto, CA). Sera and tear specimens were tested for antibodies to serovars E and C, the infecting strains, by the microimmuno- fluorescence test. Filter paper strips (Whatman No. 41, 5 mm × 20 mm in size) were used for tear collection. A tear-saturated strip was eluted in 0.2 ml PBS, the eluate of which was considered to be 1:10 dilution of the original tear.

Biopsies were removed from the lower palpebral conjunctiva of all animals under Ketamine-Rompun-Atropine anesthesia at weeks 1, 2, 3, and 4 after each inoculation and placed in Methacarnoy’s fixative. Ophthalmic microsurgical scissors and forceps were used to remove a section of conjunctiva approximately 2 mm × 6 mm. Biopsies were scheduled to allow for adequate data collection with minimum trauma to each monkey. Each animal was biopsied a total of four times, twice from each eye. Each eye of each animal was biopsied at 10 week intervals and biopsies were removed from different sites to insure recovery of the conjunctiva (Fig. 1).

Clinical Examination

A Desmarres lid retractor was used to expose the tarsal plate during weekly examination of the upper lid. The lower conjunctiva was easily exposed by manual retraction of the lower lid. Inflammatory reaction, folliculosis and corneal involvement were noted during visual examination. Inflammatory reactions included conjunctival injection, appearance of papillae and vascular changes. Animals were examined by slit lamp at weeks 1, 2, 4 and 6 post-secondary inoculation by an ophthalmologist, T. Lindquist, to look for subepithelial fibrosis, pannus formation and stromal vascularization.

Ocular discharge and tearing were noted but not used as comparable clinical signs because tearing decreased when the monkeys received Atropine, and increased with manual manipulation of the eyelids during visual examination. The onset, duration and disappearance of photophobic reactions were noted. Each week, macrophotography of the lower conjunctiva of all monkeys was used to record clinical changes.

Chest x-rays were taken 2 weeks after the secondary inoculation to check for evidence of respiratory tract involvement.

Laboratory Tests

Isolation: Swabs for isolation were inoculated onto McCoy cells, grown in microtest wells in a CO2 incubator for 2 days, fixed with methanol and stained with a fluorescein-conjugated monoclonal antibody specific to C. trachomatis (Syva Corporation). Numbers of inclusions in positive cultures were recorded. Cultures were considered negative after two consecutive negative passes.
Fig. 2. (A) Conjunctival erythema after primary (1°) inoculation with C. trachomatis strain E/UW-5. Note edema of the eyelid margins. (B) Follicles appeared after secondary (2°) inoculation. The follicles covered the surface of the lower palpebral conjunctiva.

Direct fluorescent antibody (DFA) detection: Conjunctival smears were stained with FITC-conjugated monoclonal antibody specific to C. trachomatis (Microtrak, Syva Corporation), and read under a fluorescence microscope. Chlamydial elementary bodies were quantified and results compared to culture isolation findings.

Humoral and local antibody: Serum was tested for IgM and IgG antibodies and tears for IgMGA combined antibodies against serovars E and C organisms. Fluorescein conjugates of anti-human IgM, IgG or IgMGA combined used in the indirect fluorescent antibody staining were obtained from Hyland Laboratories (Los Angeles, CA).

Histology

Tissue biopsies were embedded in paraffin, sectioned to 4 μm and stained by standard hematoxylin and eosin. Parallel tissue sections were stained for the presence of C. trachomatis by indirect immunoperoxidase antibody markers (Dako, Santa Barbara, CA) or immunofluorescence using a C. trachomatis species-specific monoclonal antibody. Monoclonal antibodies specific to human lymphocyte antigens that are known to cross-react with monkey antigen were used to identify the inflammatory infiltrate. Antibodies included T200, a pan-lymphocyte marker against leukocyte common antigen (Dako) and L26, a B-lymphocyte marker (research antibody developed by Drs. Ishii Y, Takimi T, Yuasa H, Takei T, Kikushi K, Sapporo Medical College, Sapporo, Japan, 1984). Control tissues were stained in parallel. Vimentin (Dako), a marker of mesothelial cells, was used as an indicator of fibroblast activity.

Results

Clinical Manifestations of Primary Infection

Two days after a primary inoculation with C. trachomatis serovar E, the conjunctivae of infected animals were reddened and edematous. The outer margins of the eyelids were slightly swollen. Slight edema of the eyelid margins persisted from 1 week postinoculation (wk 1 pi) to wk 8 pi in all inoculated animals (Fig. 2A). Photophobia was demonstrated immediately following inoculation and lasted until wk 6 to wk 8 pi. Throughout the study, ocular and nasal discharge were noted occasionally in all infected monkeys.

Conjunctival injection increased markedly until wk 2 pi, subsided by wk 4 pi, and persisted at mild levels through wk 8 pi. In one infected animal (M#2), clinical pathology of the conjunctiva did not reach peak until wk 4 pi. In this animal, all symptoms persisted at mild levels until reinoculation at wk 10 pi. Small papillae began to appear on the conjunctiva of inoculated monkeys by wk 4 pi. The vascular pattern on the lower conjunctiva appeared disrupted (M#1,
Table 1. Reisolation of Chlamydia trachomatis after primary and secondary inoculations

<table>
<thead>
<tr>
<th>Monkey</th>
<th>Strain</th>
<th>d. 2 pi</th>
<th>wk. 1 pi</th>
<th>wk. 2 pi</th>
<th>wk. 3 pi</th>
<th>wk. 4 pi</th>
<th>wk. 5 pi</th>
<th>wk. 6 pi</th>
<th>wk. 7 pi</th>
<th>wk. 8 pi</th>
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<tr>
<td>M#1</td>
<td>E/UW-5</td>
<td>23/6*</td>
<td>120/432</td>
<td>750/1200</td>
<td>9000/4500</td>
<td>378/810</td>
<td>648/4500</td>
<td>4/3</td>
<td>-/7</td>
<td>324/306</td>
<td>45/102</td>
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<tr>
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<td>E/UW-5</td>
<td>1500/3000</td>
<td>324/1800</td>
<td>13500/2700</td>
<td>2700/2700</td>
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<td>82/10500</td>
<td>68/3600</td>
<td>-/7</td>
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<tr>
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<td>E/UW-5</td>
<td>1050/2700</td>
<td>1800/540</td>
<td>2250/10500</td>
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<td>M#4</td>
<td>E/UW-5</td>
<td>310/85</td>
<td>120/67</td>
<td>204/53</td>
<td>1350/162</td>
<td>22/45</td>
<td>33/4</td>
<td>1/1</td>
<td>-/1</td>
<td>4/3</td>
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<td>M#5</td>
<td>HeLa</td>
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Reisolation of C. trachomatis after secondary inoculation (IFUs/ml)

<table>
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<tr>
<th>Monkey</th>
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<th>wk. 2 pi</th>
<th>wk. 3 pi</th>
<th>wk. 4 pi</th>
<th>wk. 5 pi</th>
<th>wk. 6 pi</th>
<th>wk. 7 pi</th>
<th>wk. 8 pi</th>
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<td>E/UW-5</td>
<td>320/280</td>
<td>56/112</td>
<td>1080/66</td>
<td>22/45</td>
<td>18/270</td>
<td>-/8</td>
<td>-/-</td>
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<tr>
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<td>E/UW-5</td>
<td>648/324</td>
<td>224/112</td>
<td>4500/2250</td>
<td>1080/540</td>
<td>-/1</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
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<tr>
<td>M#3</td>
<td>C/TW-3</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
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<td>-/-</td>
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<tr>
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<td>C/TW-3</td>
<td>15/7</td>
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* Right/left conjunctiva.
† Right/left nasopharynx.

Laboratory Findings

Isolation: In the primary inoculation phase of the study, chlamydia was recovered by McCoy cell culture from the conjunctiva of three monkeys (M#2, M#3, M#4) until wk 8 pi. Chlamydia was recovered from one monkey (M#1) until the secondary inoculation at wk 10 pi.

Chlamydia was reisolated from the conjunctiva of two monkeys (M#2, M#4) by wk 1 pi, from a third monkey (M#3) by wk 2 pi, and from the fourth monkey (M#1) by wk 4 pi. Shedding of the organism lasted from 2 to 5 weeks (Table 1).

Following the secondary inoculation, chlamydia was recovered from the conjunctiva of animals that received homologous rechallenge (serovar E) for up to 6 weeks. Organisms were recovered from the conjunctiva and nasopharynx of heterologously rechallenged (serovar C) animals for only 2 weeks (Table 1). Infection was milder with heterologous rechallenge. However, serovar C also caused mild infection in the control animal after a single inoculation.

All cultures from the rectum and vagina in the infected animals and from any site in the monkey inoculated with HeLa cell materials were negative throughout the study.

Direct detection of organisms: Positive DFA was demonstrated in conjunctival and nasopharyngeal smears only. Eighty-three (90%) of 92 culture-positive specimens were DFA-positive. Thirteen DFA-
positive specimens were culture-negative. DFA scores generally paralleled IFU titers of culture reisolation. For a single specimen collection, higher numbers of elementary bodies counted by DFA corresponded to higher numbers of IFUs/ml by culture reisolation (DFA data not shown on Table 1).

Serology: All monkeys inoculated with serovar E developed a serum antibody response against E, but not against serovar C. IgM antibody was demonstrated in three of four monkeys with a peak titer of 1:16 (M#3) and 1:32 (M#l, M#4) 2 weeks after the primary inoculation. IgG antibody was demonstrated in all animals by wk 2 pi. The antibody levels peaked by wk 4 pi (IgG = 1:128 and 1:256) in two monkeys (M# 1 and M#4, respectively), remained at peak levels for 2 to 3 weeks, then gradually declined. A third monkey (M#3) demonstrated steady increase of IgG antibody for 9 weeks, reaching a peak titer of 1:128 (Table 2).

Animals rechallenged with strain E showed an increase in antibody titer to serovar E. Heterologous rechallenge (strain C) stimulated an antibody response to the primary inoculating strain E, in addition to a response to strain C (Table 2).

Tear antibody to serovar E, demonstrated from three of the four inoculated monkeys (M#1, M#3, M#4) was first detected by wk 2 pi and persisted throughout the duration of the study until wk 13 pi. Titers ranged from 1:10 to 1:40.

Histopathology

Primary infection: Cytological examination of conjunctival smears at day 2 pi revealed increased numbers of polymorphonuclear leukocytes (PMNs) characteristic of an acute inflammatory response (≥10 PMNs/×630 field). Polymorphonuclear leukocytes were not detected in smears from the control monkey.

Hematoxylin and eosin staining of tissue sections of conjunctival biopsies removed wk 1 pi showed a moderate inflammatory infiltrate consisting mainly of mononuclear leukocytes with occasional polymorphonuclear leukocytes in the subepithelial stroma (Fig. 3A, B). At wk 2 pi, mononuclear cells had formed small, loosely packed focal clusters in the stroma and appeared in patchy distribution near the epithelium. Cytochemical staining revealed both T and B cells in these focal patches. A progressive loss of goblet cells and patchy thinning of the epithelium was associated with the lymphocytic infiltrate. Capillaries were engorged.

At wk 3 pi, the number of mononuclear inflammatory cells in the stromal compartment had decreased, but the mononuclear cells were still visible within the epithelium. Some of the mononuclear cells had migrated into and through the epithelium and were seen at the epithelial surface. Plasma cells were identified in the stroma near the epithelium (Fig. 4A, B). At wk 4 pi, the inflammatory response had markedly subsided.

The histology of normal conjunctival tissue was characterized by stroma devoid of inflammatory infiltrate with an integrus layer of conjunctival epithelium five to six cell layers thick. Mucus-secreting goblet cells were evenly distributed throughout the epithelium. Monoclonal markers used to identify inflammatory cells were unreactive in control tissue.
Secondary infection: Histological findings following secondary inoculation were characterized by mononuclear infiltrate and lymphoid follicle formation. Findings were similar with homologous (serovar E) and heterologous (serovar C) rechallenge. By wk 1 pi, a heavy inflammatory infiltrate consisting of mononuclear leukocytes and plasma cells had begun to form germinal centers near the epithelium. By wk 2 pi, densely packed lymphoid follicles with distinct germinal centers were noted. Over the lymphoid follicle, a dramatic thinning of the normal multilayered epithelium was observed (Fig. 5A, B). Outside the follicles, lymphocytes were rarely seen in the stromal compartment. By wk 3 pi, the well established lymphoid follicles were capped by a single cell layer of conjunctival epithelium devoid of goblet cells (Fig. 6A, B).

Immunocytochemistry: Monoclonal markers identified B-lymphocytes in the center of the follicles and T cells in the cap region (Fig. 7A, B).

Vimentin staining of conjunctival biopsies indicated increased fibrosis in infected tissue, especially...
after secondary inoculation. However, in biopsies from infected monkeys, subepithelial fibrosis was indicated by markedly increased fibroblast activity and collagen content in the stroma, especially near the epithelium. A delicate network of connective tissue was characteristically found between the inflammatory cells within lymphoid follicles. Control biopsies contained a few fibroblasts, possibly due to the repeated biopsy procedures.

Chlamydial inclusions were not detected in any conjunctival sections by either avidin-biotin-complex immunoperoxidase staining or fluorescent antibody staining.

Discussion
In our study, infant pig-tailed macaques were found to be susceptible to acute self-limiting chlamydial conjunctivitis with genital or ocular strains of
C. trachomatis. The infection was confirmed by culture isolation of chlamydia and by direct fluorescent antibody staining of smears from the conjunctivae and nasopharynges of inoculated animals. In addition, an antibody response was elicited only from infected monkeys. Histopathology of conjunctival biopsies showed a diffuse and widespread inflammatory reaction following a primary infection. After a rechallenge, either homologous or heterologous, prominent lymphoid follicle formation was evident.

Clinical and immunopathological findings were similar to those reported by other workers using adult monkey models with one notable exception. Follicles did not appear in our monkeys until secondary inoculation.

Human infants with chlamydial conjunctivitis do not develop follicles unless the infection persists for more than 2 or 3 months because lymphoid tissue is deficient in the newborn conjunctiva. In our animals, follicles appearing after secondary inoculations, given at 4 months of age, are likely to be related to the development of lymphoid tissue also.
The control monkey, inoculated with strain C at 4 months of age, did not produce follicles. Strain C has been shown to cause milder effects than strain E in Taiwan monkeys, and this may account for our findings.

After reinoculation, chlamydia was reisolated more often from homologously rather than heterologously rechallenged animals. These observations may suggest that immune mechanisms have not fully developed in infant monkeys.

We have evaluated an animal model in which to study chlamydial infections in the infant pig-tailed macaque and have shown that this model can be applied for further studies on follicle development, immunity and pathogenesis of chlamydial infections in the infant.

Key words: chlamydial conjunctivitis, primate model, infant chlamydial infection, histopathology of conjunctivitis

References