An animal model for trachoma

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An animal model of cicatrizing trachoma was developed in cynomolgus monkeys. This model is consistent with our hypothesis that repeated ocular inoculation of Chlamydia trachomatis, BOUR strain, mimics the repeated reinfection that occurs naturally in endemic human trachoma. A chronic follicular conjunctivitis developed, and scarring later appeared in the superior tarsal conjunctiva. The organism was reisolated after the infection and was also demonstrated cytopathologically. Specific antichlamydial antibodies of both the IgM and IgG types appeared in the sera of the monkeys. Histopathologic examination of conjunctiva showed a marked lymphocytic response and the presence of germinal centers; areas of conjunctival scar tissue were also examined. Efforts to produce a similar model in rhesus monkeys were less successful.

Key words: Chlamydia trachomatis, trachoma, cynomolgus monkey, rhesus monkey, lymphocytic germinal centers, follicular conjunctivitis, conjunctival scarring

Trachoma is a chlamydial infection of the conjunctival epithelium, producing chronic inflammation in the subconjunctival tissue. The causative organism, Chlamydia trachomatis, was first isolated in 1957 and subsequently was shown to have a number of serotypes. Serotypes A, B, BA, and C are commonly associated with endemic trachoma. In man the disease shows a number of phases of progression. Initially the infection produces a conjunctivitis of moderate severity, associated with a mild to moderate amount of discharge. Follicles (subepithelial germinal centers) develop, especially in the superior tarsal conjunctiva. The chronic inflammation leads to a conjunctival "papillary" response, which again is mainly on the conjunctiva of the upper tarsal plate. Later, scarring appears in the upper tarsal conjunctiva. At first this is seen as a few fine bands that progress to form a fine basket weave network. Ultimately the scarring may form strong consolidated bands of tissue, which with time can cause buckling of the tarsal plate. This distortion of the tarsal plate leads to entropion and trichiasis.

In association with those on the tarsus, follicles may appear at the limbus. When these resolve, small depressions are left at their site (Herbert's pits). An epithelial keratitis commonly occurs at the superior limbus, and this is usually followed by corneal stromal opacification and neovascularization, which are clinically evident as trachomatous pannus. The pannus itself usually does not sig-
nificantly affect vision, although it may occasionally progress to cover the visual axis. Trachomatosus blindness is usually a result of the cicatricial distortion of the tarsal plate and subsequent trichiasis.

Of considerable importance in some areas, especially in Northern Africa and the Middle East, is the effect of superimposed acute bacterial conjunctivitis on eyes already chronically inflamed with trachoma. The devastating synergistic effect produced by the combined infections has been termed "communicable ophthalmia." Trachoma occurs mainly in areas where there is poor personal and community hygiene. The importance of the spread of infectious human ocular secretions by flies, direct human contact, including finger-to-eye contact, and by clothing and bedding is now generally recognized, and the importance of repeated episodes of reinfection in the establishment of endemic trachoma is apparent. It is well established that follicles may reappear in eyes that already have the structural sequelae of previous trachomatous inflammation.

Although the clinical picture of trachoma is well known, and the epidemiology of the infection has been partially elucidated, very little is understood about the pathogenesis of trachoma or the role of the host's immune response. Some have suggested that trachoma is basically an immunologic disease process, with the persistence of the infectious agent (or antigen) in the conjunctival epithelium stimulating an immunologic response in the subepithelial tissues. Although much effort has been devoted to studying the immunopathogenesis, progress has been hampered by the lack of an appropriate animal model. Most previous attempts at establishing animal models of trachoma used a single inoculant and, by and large, were unsuccessful in mimicking chronic cicatricial disease. There have been a few outstanding exceptions to this, and the appearance of pannus or conjunctival scarring has been reported occasionally. Because repeated reinfection is thought to be important in the development of human disease, it seemed logical to pursue this concept in establishing an animal model. This article outlines the development of such a model in which significant tarsal conjunctival scarring occurred.

Methods

**Animals.** The animals used in these studies were four male rhesus monkeys, 5 to 6 months old at the start of the studies, and four cynomolgus monkeys, two males 12 and 36 months old and two females 18 and 42 months old. All eight monkeys had been reared in the animal colony at The Johns Hopkins Medical Institutions and had normal ocular findings before the experimental infection. They were housed in isolation cages (P3; Horsefall), which are airtight, stainless steel cabinets with self-contained ventilation and filtration of both the entering and exiting air.

Two of the rhesus monkeys were housed together in the same cage for the duration of the experiment. The other two rhesus monkeys were housed together for the first 2 months but then had to be separated. The two younger cynomolgus monkeys were housed together for the duration of the experiment, but the two older cynomolgus monkeys were housed separately.

**Examination and specimen collection**

**Examination.** Under systemically administered ketamine hydrochloride anesthesia, each eye of a monkey was examined with a Kowa hand-held slit lamp (Parke, Davis & Co., Morris Plains, N.J.). The cornea, limbus, bulbar conjunctiva, and the superior tarsal and superior fornix conjunctiva were examined in detail. The findings were recorded by the protocol given in Table I.

**Tear antibodies.** Samples of tears were collected from both eyes with sterile, dry cellulose sponges and were placed in sterile, dry polyethylene tubes. These tubes were stored at −70°C. Antibody levels were determined by microimmunofluorescent assay using homologous antigen.

**Cytology.** The upper tarsal conjunctiva of each eye was gently scraped with a sterile platinum spatula, and the cells were collected, smeared on Fluoro glass slides (Curtin Matheson Scientific, Inc., Washington, D. C.), fixed in acetone (99.5%) at 4°C, and stored at −70°C. These smears were examined by an indirect immunofluorescent antibody technique. Two smears were also taken for cytology (on precleaned glass slides), and were air-dried and fixed in methanol at room temperature before being stained by the standard Giemsa method; they were then examined for presence of
Table I. Grading protocol for signs of trachoma

<table>
<thead>
<tr>
<th>Sign</th>
<th>Severity</th>
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<tbody>
<tr>
<td>Fornix follicles</td>
<td>Just present Grade I</td>
</tr>
<tr>
<td>Tarsal follicles</td>
<td>Obviously present Grade 2</td>
</tr>
<tr>
<td>Papillae</td>
<td>Grossly present Grade 3</td>
</tr>
<tr>
<td>Chemosis</td>
<td></td>
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<tr>
<td>Conjunctival injection</td>
<td></td>
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<tr>
<td>Discharge</td>
<td></td>
</tr>
<tr>
<td>Tarsal scarring</td>
<td>Just present Grade I</td>
</tr>
<tr>
<td></td>
<td>Obviously present Grade 2</td>
</tr>
<tr>
<td></td>
<td>Grossly present with distortion of tarsus and displacement of Meibomian gland openings Grade 3</td>
</tr>
<tr>
<td>Pannus</td>
<td>1-2 mm Grade 1</td>
</tr>
<tr>
<td></td>
<td>2-3 mm Grade 2</td>
</tr>
<tr>
<td></td>
<td>3-4 mm Grade 3</td>
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<tr>
<td></td>
<td>4-5 mm, etc. Grade 4, etc.</td>
</tr>
<tr>
<td>Limbal follicles</td>
<td>1 to 3 Grade 1</td>
</tr>
<tr>
<td>Bulbar follicles</td>
<td>4 to 7 Grade 2</td>
</tr>
<tr>
<td>Herbert’s pits</td>
<td>8 or more Grade 3</td>
</tr>
<tr>
<td>Trichiasis</td>
<td>Less than 1/3 of lid Grade 1</td>
</tr>
<tr>
<td></td>
<td>Less than 2/3 of lid Grade 2</td>
</tr>
<tr>
<td></td>
<td>More than 2/3 of lid Grade 3</td>
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</tbody>
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the characteristic chlamydial intracytoplasmic inclusions.

Isolation of organism. The upper tarsal plate of each eye was swabbed with a sterile alginate swab (Calgi swab; Inolex Corp., Glenwood, Ill.); the swab was placed in 1 ml of tissue-culture transport medium and stored at −70°C. Isolations were performed with the use of 5-iodo-2-deoxyuridine–treated McCoy cells.23 Iodine staining was used to identify cytoplasmic inclusions.

Serology. Two milliliters of venous blood were obtained by venipuncture at the initial preinfection examination and every 4 weeks thereafter. The blood was placed in sterile plastic tubes, and the separated serum was stored at −70°C. At intervening examinations blood was obtained by finger-prick and was collected in heparinized capillary tubes. These were sealed and centrifuged, and the portion of the tube containing the plasma was separated, sealed at both ends, and stored in dry, sterile polyethylene tubes at −70°C. Antibody levels were determined by microimmunoﬂuorescent techniques.23

Infection protocol. After examination and specimen collection the animals were inoculated with a suspension of elementary bodies of C. trachomatis, BOUR strain (serotype E), which had been cultured in egg yolk and diluted to 10^3.4 egg lethal dose, 50% (ELD50) in phosphate-buffered saline. Prior to use the vials of infectious agent were stored at −70°C. The monkeys received 20 µl of this suspension, pipetted into each conjunctival sac, on day 1; thereafter 20 µl were instilled into the right conjunctival sac at weekly intervals. Clean sterilized instruments were used for each animal. Investigators washed their gloved hands with alcohol after examining and inoculating each animal.

Histology. Biopsy material was obtained from the bulbar conjunctiva and the superior fornix of the left eye by simple conjunctival excision. A 2 mm trephine biopsy of the superior tarsal conjunctiva and the underlying tarsal plate was also obtained from the left eye. The biopsy specimens were fixed in alcohol formaldehyde for 48 hr, subsequently dehydrated through ascending concentrations of ethanol, and finally embedded in JP-4 epoxy medium (Polysciences, Inc., Warrington, Pa.). Two-micrometer sections were cut and stained with Lee’s methylene-blue–basic-fuchsia stain (which approximates hematoxylin-eosin) or with Diff-Quik Giemsa Stain (Harleco, Gibbstown, N.J.).

Results

Cynomolgus monkeys

Clinical course. One week after the initial infection all four monkeys showed evidence of acute follicular conjunctivitis with the presence of a mucopurulent discharge, conjunctival injection, a papillary response, and
the appearance of follicles in the upper fornix. Follicles first appeared in the upper tarsal conjunctiva at 3 weeks in one animal and at 5 weeks in two others (Fig. 1). Small limbal follicles and bulbar conjunctival follicles were seen in three animals 3 to 5 weeks after the initial infection. These follicles tended to be transient, often lasting for only 1 or 2 weeks, and were followed by the appearance of new limbal or bulbar follicles.

An inflammatory scoring system was devised, which was the aggregate score for the severity of papillae, bulbar conjunctival injection, chemosis, and ocular discharge. This score was ascertained separately for each eye of each animal, and the mean was determined for the right eyes and the left eyes of the four monkeys. The score was a measure of the general nonspecific inflammatory response, which had reached a moderate intensity by 1 week (Fig. 2). Although the intensity of inflammation lessened somewhat with time, it persisted throughout the course of the study (120 weeks at time of writing).

An aggregate score also was constructed for the follicular response for each eye of each animal. This score was the aggregate of the grading of tarsal, bulbar, and limbal follicles. The follicular response developed more slowly than the inflammatory response (Fig. 2), reaching a plateau by 4 to 6 weeks, and it showed only a slight decline over the next 9 months. It should be remembered that each animal was reinfected only in the right eye, although both eyes showed a similar inflammatory and follicular response.

Fine linear conjunctival scars developed in three monkeys from 3 to 6 weeks after initial infection. Fine basket weave subconjunctival scarring, as is typically seen in clinical trachoma, developed in one monkey at 7 weeks and in all four by 21 weeks. This classic trachomatous scarring of the upper tarsal plate continued to progress to a moderate severity throughout the 54-week course of these experiments (Fig. 3). In all four monkeys the conjunctival scarring was bilateral and approximately symmetric in appearance and development.

Dilation of limbal blood vessels and early pannus development were seen at about 6 weeks. These failed to progress centrally beyond 2 mm and almost completely resolved during the next 4 to 5 weeks. An interesting finding at 16 weeks was the development of midstromal haze of the peripheral cornea in three monkeys, which was associated with the opacification of corneal nerves for the first 2 to 3 mm of their corneal course. This was seen especially in the inferior cornea. Herbert's pits did not develop in the sites occupied by the limbal follicles.
At 54 weeks the conjunctival scarring continued to progress, although none of these animals had as yet developed distortion of the tarsal plate or trichiasis.

Cytology. Chlamydial inclusions were identified rather infrequently. This was true for the Giemsa-stained specimens and for the fluorescent antibody technique. Inclusions were found in both eyes of two of the four cynomolgus monkeys but were not seen in either eye of the other two animals, despite the development of similar clinical disease. All the inclusions that were identified were in specimens obtained during the first 8 weeks of the study. Subsequent cytologic examination failed to show further inclusions.

Reisolation. BOUR strain chlamydial organisms were reisolated from the eyes of three of the monkeys. Most of the reisolations were from specimens taken during the first 2 weeks of the study, and all reisolations were within the first 6 weeks. Since then, despite continued reinfection, repeated cultures taken 1 week after inoculation have been negative.

Serologic response. None of the animals had circulating antichlamydial antibodies detected by microimmunofluorescence before commencement of infection. After infection all the cynomolgus monkeys showed a marked development of specific antichlamydial IgM in their sera (Fig. 4). In three of these animals the titers were specific for chlamydial serotypes E and F, whereas in the fourth animal a broader response was seen. At 4 weeks the IgM titers had risen from zero to 1:250, and titers of up to 1:1028 were detected in the serum antichlamydial IgG levels. The peak titers of IgG were found for serotypes D, E, and L1-L2.

Histologic results. Histologic examinations of normal bulbar, superior tarsal, and superior fornix conjunctiva of uninfected cynomolgus monkeys showed a well-stratified normal epithelium with many goblet cells. A few mast
Fig. 3. Fine network of basket weave scarring of the superior tarsal conjunctiva seen at 35 weeks in the same eye as shown in Fig. 1.

Fig. 4. Presence of specific antichlamydial IgG and IgM in the serum of four cynomolgus and four rhesus monkeys.

cells were seen in the subconjunctiva, and only an occasional mononuclear cell or polymorphonuclear leukocyte (PMN) was seen within the subepithelial connective tissue. Four weeks after the initial infection, however, conjunctival biopsy specimens showed a marked reduction in the number of goblet cells and thinning of the epithelium with disruption of the epithelium by PMN cells. By 8 weeks there was a preponderance of mononuclear cells (small lymphocytes and plasma cells) in the epithelial infiltrate, although numerous PMN cells were also present (Fig. 5). On several occasions, intracytoplasmic inclusions were seen in epithelial cells. The subconjunctiva showed a marked inflammatory-cell infiltrate, including lymphocytes, plasma cells, and larger mononuclear cells.
Follicles with well-developed germinal centers, including lymphoblasts and macrophages, were identified (Fig. 6). These germinal centers were surrounded by many plasma cells, and in other areas the subconjunctival tissue was diffusely infiltrated with plasma cells and other mononuclear cells. Some follicles were surrounded by elongated spindle cells, which were presumed to be fibroblasts. Whether this represented a proliferation of fibroblasts or their displacement by the development of the germinal center is uncertain. Follicles were also seen in the superior tarsal conjunctiva. The epithelium overlying these follicles was heavily infiltrated with mononuclear cells (Fig. 7, A). Later, resolving tarsal follicles were seen that had necrotic centers and were surrounded by elongated spindle cells and increased dense extracellular material (Fig. 7, B). In an area of clinically apparent scarring of the superior tarsal conjunctiva, a considerable increase in the thickness of the subepithelial connective tissue was observed (Fig. 7, C). This increase in tissue resulted from an accumulation of long spindle cells resembling fibroblasts. There were relatively few inflammatory cells in this area of presumed scar tissue.

**Rhesus monkeys.** Overall the response seen in the four rhesus monkeys was much milder than that seen in the cynomolgus. Each rhesus monkey developed a mild conjunctivitis with a follicular response confined to the upper fornix, but no tarsal or limbal follicles developed in these animals, and no animal developed tarsal conjunctival scarring.

Three of the four rhesus monkeys had intracellular inclusions demonstrated at some time in conjunctiva of both eyes, and chlamydiae were also reisolated from three of the four animals.

The serologic response of the rhesus monkeys was generally similar to that seen in the cynomolgus; however, one rhesus did not...
demonstrate a rise in IgM titer, although antichlamydial IgG antibodies were detected (Fig. 4).

Because of the lesser response seen in the rhesus, the reinfection schedule was discontinued at 12 weeks. The monkeys then received intramuscular injections of sulfamethoxazole-trimethoprim (Hoffman-LaRoche, Inc., Nutley, N. J.) (10 and 2 mg/kg, respectively) for 10 days and were not used further.

Discussion

The ability to culture the chlamydial organism prompted renewed interest in the study of trachoma, and many attempts to establish animal models have been made in the past in an effort to understand the pathogenesis of trachoma and to develop vaccines.

Subhuman primates are known to be susceptible to infection with the trachoma organism, and the initial demonstration of intracytoplasmic inclusions was made in conjunctival scrapings from infected orangutans as early as 1907. In the early 1960s a number of reports appeared that confirmed the susceptibility of other primates to acute chlamydial infection. The animals used in such studies included rhesus and cynomolgus monkeys,14 baboons,77 Taiwan rock monkeys,15 grivets,7 stump-tailed monkeys,14 owl monkeys and marmosets,17 and African green monkeys.28

Considerable work has also been carried out with inclusion conjunctivitis of the guinea pig as a model of ocular chlamydial infections.18, 26, 30 This agent causes a naturally occurring acute epidemic follicular conjunctivitis of guinea pigs. Although this model has much to recommend it, the agent is a member of the Chlamydia psittacosis group.

Most of the early animal studies were confined to a single inoculation of the infectious agent. In most of the species used, an acute follicular conjunctivitis resulted from the single infection, and the follicles that developed were usually confined to the fornix.31 Classic trachoma follicles of the tarsal conjunctiva were not seen, nor were conjunctival scarring and corneal pannus, which are
the hallmarks of the human clinical disease. In apes and Old World monkeys the acute conjunctivitis usually resolved spontaneously within 1 or 2 months.\(^{14, 19, 27}\) The response to inoculation in New World monkeys (e.g., owl monkeys), though much more florid, was also short-lived, resolving spontaneously in 2 to 3 weeks.\(^{16}\)

Although most of the early experiments were confined to the use of a single inoculation, there were a few reports in which the animals were inoculated on more than one
occasion. These studies were aimed at developing vaccines to prevent trachoma, and the repeated infections were used to study the influence of immunization. The first of those reinfection studies was performed in cynomolgus monkeys, and Thygeson et al. reported that the response to a second infection seemed to be the same as to the primary infection. Mordhorst, also using cynomolgus monkeys, found a slightly decreased clinical response with the third episode of reinfection. A similar decrease was also reported in owl monkeys. Of particular interest therefore was the finding by Collier and Blyth of an increased response to reinfection observed in three baboons after attempted vaccination with inadequate vaccines. Wang and Grayston also reported the very interesting observation that heightened responses to inoculation occurred in some animals that had been infected or vaccinated previously. Wang et al. further reported the development of pannus of more than 1 mm in about 5% of those Taiwanese rock monkeys. In most cases, however, the pannus regressed spontaneously after several months. In a subsequent report, two of the Taiwanese monkeys were reported to have developed scarring and trichiasis 10 years after their initial challenge with the chlamydial agent.

The BOUR strain of *C. trachomatis* has been used extensively in experimental work. This organism was isolated from a patient with ocular disease that was indistinguishable from trachoma, although he came from a nonendemic area. That organism was later shown to belong to the E serotype, which is usually associated with inclusion conjunctivitis. Thygeson et al. used this strain to infect monkeys, and in comparative studies it was found to produce more severe disease than other chlamydial strains. In Taiwanese monkeys the BOUR strain was more likely to induce pannus formation than were other strains, and in a few of those reinoculated animals it led to scarring of sufficient severity to produce trichiasis.

The major difference between our model system and previous attempts is that our animals were repeatedly reinfeeted at weekly intervals. This approach was used because it simulates the continual reinfection that occurs in areas of endemic human trachoma, where reinfection is considered an important factor in the development of the disease.

Several interesting observations were made during this study. First, the use of repeated reinfection did produce an animal model of cicatrizing trachoma. This gives experimental confirmation to the clinical impression, obtained from field work, of the importance of reinfection in endemic trachoma. The disease that was produced in cynomolgus monkeys closely mimics that seen in humans with endemic trachoma. After an initial "nonspecific" conjunctivitis, follicles appeared in the superior tarsal conjunctiva and elsewhere. Despite repeated reinfection the nonspecific inflammation slowly waned, although the follicles persisted and gradually conjunctival scarring appeared. These changes in the conjunctiva have been confirmed histologically.

Second, both the rhesus and cynomolgus monkeys showed a similar serologic response to infection and had a similar frequency of positive cytologic findings and reisolations, although the clinical response of the cynomolgus monkeys was much more marked than that of the rhesus. Further, the overall response of the monkeys within each group was fairly uniform, suggesting that individual variation was not the cause of the observed difference in response between the groups. Thus the difference between the clinical response of the rhesus and cynomolgus monkeys probably represents a true species difference rather than one that is attributable to other factors such as the younger ages of the rhesus monkeys (Dawson and Schachter, unpublished data).

Third, although both eyes were inoculated directly on day 1 and only the right eye was inoculated subsequently, the clinical response and the rate of identification of chlamydial inclusions in cytologic smears were the same for both eyes. This would suggest that there was a significant transfer of organisms, and hence infection, from the repeatedly reinfeeted right eye to the nonreinfeeted left eye.

Further support for the importance of re-
peated reinfection comes from recent preliminary experiments. Six cynomolgus monkeys have been infected in each eye with a single inoculum of C. trachomatis (BOUR strain). They developed an acute, self-limited follicular conjunctivitis within 3 days of inoculation, which had essentially resolved by 8 weeks. Organisms were reisolated from all animals during the first 2 weeks. However, chlamydiae could not be cultured more than 6 weeks after inoculation, despite repeated attempts. These findings support the importance of reinfection in two respects. First, they show that repeated reinfection is a requisite for the creation of the model of chronic disease in cynomolgus monkeys. Second, they suggest the likelihood of cross-infection in the model we have described, where after initial bilateral infection the subsequent reinfection of only one eye led to a symmetric, chronic bilateral disease.

A report has appeared recently that discusses the development of conjunctival scarring in guinea pigs reinfected five to seven times with guinea pig inclusion conjunctivitis. The clinical picture was comparable in both eyes, although only the left eye was infected, which again strongly suggests transfer of organisms. Finally, repeated conjunctival scraping had no appreciable effect on the infection, although it had an enhancing effect at 2 days. This last finding is consistent with our experience, in which weekly scraping in the rhesus monkeys had no appreciable effect and certainly did not produce scarring.

With this animal model it may be possible to fill in the important gaps in our understanding of the pathogenesis of trachoma. We do not yet have a clear understanding of the contribution of immunologic mechanisms to the development of the clinical disease nor of the role played by tarsal follicles in the development of tarsal scarring, the underlying cause of the binding sequelae of trachoma. A study of the animal model should allow clarification of the role played by both cellular and humoral immunity and should elucidate the role of locally produced immunoglobulins, including those of the secretory immune system.

We thank Dr. W. T. London of the National Institute of Neurological Diseases and Stroke, Bethesda, Md., for making available the isolation cages used in this study.

REFERENCES