
Resistance to reinfection with a chlamydial agent (guinea pig inclusion conjunctivitis agent). AHMAD AHMAD, CHANDLER R. DAWSON, CHIEKO YONEDA, BRIGITTA TOGNI, AND JULIUS SCHACHTER.

Although most chlamydial infections are chronic or recurrent, infection of the guinea pig’s eye with guinea pig inclusion conjunctivitis (GPIC) agent induces a marked resistance to reinfection. To characterize this resistance to GPIC agent, we compared the disease and infection in previously infected guinea pigs with that in animals infected for the first time. In animals experiencing primary infection, even the lowest dose (10 egg-lethal doses [ELD50]) produced the disease and chlamydial inclusions in conjunctival smears, but the incubation period became progressively shorter with the highest inocula (104 and 105 ELD50). In animals with previous infection only these two highest inocula produced disease and infection, but the disease was short-lived, and replication of the agent was severely limited. The mechanism of this resistance may be due to secretory antibody in the tears, cellular immunity, or other local factors.

Natural infections with chlamydial agents do not ordinarily induce solid immunity. As a result, chlamydial diseases such as trachoma and inclusion conjunctivitis are usually chronic and recurrent. In two host-parasite systems, however, a marked degree of resistance to chlamydial eye infection can be induced. (1) The ocular inoculation of owl monkeys (Aotus trivirgatus) with trachoma agent produces an acute conjunctivitis that subsides and leaves the animal resistant to challenge inoculation. (2) In the same way, the naturally occurring chlamydial disease, guinea pig inclusion conjunctivitis (GPIC), is followed by resistance to further infectious challenge with GPIC agent. Since neither systemic immunization nor passive transfer of antibody prevents eye disease, it has been suggested that secretory immunoglobulins are largely responsible for such local immunity. In order to characterize further this resistance to GPIC agent, we have determined the infectious dose necessary to infect and produce disease in the eyes of previously infected animals.

Materials and methods
Agent. A single pool of the ninth egg passage of a GPIC agent (gp 86) originally isolated in this laboratory was used in all these experiments. Individual aliquots of 50% yolk sac–grown agent were stored at -70° C. until used. The titer of this pool was about 105 egg-lethal doses (ELD50) per milliliter.

Animals and inoculation. We carried out the study with two groups of Hartley strain guinea pigs (19 animals in each group) that were known to be free of GPIC. To inoculate the eyes, 0.1 ml. of various concentrations of the agent was dropped to the anesthetized cornea through a plastic well, and the agent was kept in contact with the cornea for a period of 5 min. We examined the animals’ eyes with a modified slit lamp before inoculation and at intervals after inoculation.

Smears. To quantitate the growth of the agent in the eye, we collected conjunctival smears for microscopic study at each examination. After Giemsa staining of the slides, we counted the number of chlamydial inclusions per 100 epithelial cells on each slide.

Light and electron microscope. A portion of the cornea was fixed for electron microscopy in 2% glutaraldehyde with a cacodylate buffer, postfixed with osmium, and embedded in araldite plastic for transmission electron microscopy. Another portion was fixed in formalin for paraffin embedding, and sections were stained with Giemsa stain and with hematoxylin and eosin for light microscopy.

Experimental design. The first group of 19 guinea pigs was inoculated with 104 ELD50 of GPIC agent, and all developed a typical keratoconjunctivitis that lasted for an average of 3 weeks, with inclusions in the conjunctival smears of all 19 animals. One month after inoculation, when the disease had totally subsided and the smears were negative, the same animals were rechallenged with serial 10-fold increasing doses of agent (10, 102, 104, and 105 ELD50), and two guinea pigs received 10% yolk sac only. At the same time the other 19 guinea pigs that had not yet been infected (primary infection group) were also inoculated with the same serial 10-fold increasing doses of agent and with 10% yolk sac (Table I).

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June 1977

Fig. 1. Clinical disease following primary challenge and rechallenge with 10-fold dilutions of GPIC. In primary inoculations, even the lowest dilution produced disease and the intensity of disease was the same at all dilutions, but the incubation period was shortened with the higher inocula. In rechallenged animals, disease developed only with the two highest inocula (10^4 and 10^5 ELD_{50}), and it was short-lived.

Table I. Number of nonimmune and previously infected guinea pigs inoculated with 10-fold dilutions of GPIC

<table>
<thead>
<tr>
<th>Inoculum (ELD_{50} of GPIC agent)</th>
<th>Number of guinea pigs inoculated</th>
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<tbody>
<tr>
<td></td>
<td>Primary challenge</td>
</tr>
<tr>
<td>10% Yolk sac</td>
<td>2</td>
</tr>
<tr>
<td>10^6 ELD_{50}</td>
<td>3</td>
</tr>
<tr>
<td>10^9 ELD_{50}</td>
<td>3</td>
</tr>
<tr>
<td>10^10 ELD_{50}</td>
<td>3</td>
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<tr>
<td>10^15 ELD_{50}</td>
<td>5</td>
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At 48 hr, six animals in each group were sacrificed for microscopy. From both the primary inoculation and rechallenge groups, one animal was selected from those receiving an inocula of 10 to 10^6 ELD_{50}, and two animals from those receiving 10^7 ELD_{50}. Thus a total of 11 GPIC-infected and two yolk sac control animals remained in each group. All 26 surviving animals were then examined twice weekly for clinical disease, as shown by conjunctival hyperemia, conjunctival chemosis, epithelial keratitis, and pannus. Conjunctival smears to detect chlamydial inclusions were taken at each examination.

Results

Clinical disease. The duration of the keratoconjunctivitis in the animals with primary infection and in those rechallenged is compared in Fig. 1. Since guinea pig conjunctivas usually have lymphoid follicles, only hyperemia and chemosis were considered as signs of active conjunctival inflammation. The most frequent corneal lesion was diffuse punctate epithelial keratitis that stained with fluorescein. Prior to inoculation, all these guinea pigs had very fine superficial vessels extending not more than 1 mm. beyond the limbus. After inoculation, the vessels became engorged, but there was only minimal increase in the length of the pannus.

All the guinea pigs experiencing primary infection developed similar disease regardless of the dose of agent, although the incubation period was progressively shorter as the dose was increased. In animals with primary infection, the disease had subsided by postinoculation day 25. One eye of one of the two control animals (inoculated with yolk sac) developed transient hyperemia on day 11 but was inclusion-negative.

In the rechallenged group, keratoconjunctivitis developed only in animals inoculated with 10^4 or 10^5 ELD_{50} of agent, and even in the five surviving animals the disease subsided by postinoculation day 8. In the rechallenged group the two animals that had been inoculated with 10% yolk sac developed a conjunctivitis in both eyes that subsided by day 4, probably due to hypersensitivity to yolk sac. One animal that had received 10^5 ELD_{50} of GPIC agent also had conjunctivitis in both eyes on day 2 only, but no agent was recovered.

GPIC agent in conjunctival smears. The prevalence and duration of inclusion-positive smears had a close correlation with clinical disease (Fig.
Fig. 2. Percentage of animals with inclusion-positive conjunctival smears in primary challenge and rechallenge. The presence of inclusions correlated closely with clinical disease. The single inclusion at day 15 in one animal rechallenged with 10 ELD<sub>50</sub> may be random shedding of agent in an animal with latent infection.

2). All the animals with primary infection had inclusions, even those that had received the lowest dose of agent (10 ELD<sub>50</sub>). At higher doses (10<sup>4</sup> and 10<sup>5</sup> ELD<sub>50</sub>), the onset of infection was progressively earlier, and the smears became positive on the second postinoculation day (Fig. 2). In most of the primarily infected animals the smears remained positive through day 22, but by day 25 all were negative.

In the rechallenged group, the conjunctival smears were inclusion-positive until day 8 in animals that received 10<sup>2</sup> and 10<sup>3</sup> ELD<sub>50</sub> (Fig. 2). They were first noted on day 2 in the group receiving 10<sup>2</sup> and on day 4 in the group receiving 10<sup>3</sup> ELD<sub>50</sub>. In one eye of an animal reinoculated with 10 ELD<sub>50</sub>, a single inclusion was noted on day 15, but this was not associated with clinical disease and so may have been due to spontaneous shedding of the agent in a persistently infected animal.

There was a marked difference between the number of inclusions in the conjunctival smears from animals with primary infections and in those from rechallenged animals (Fig. 3). In primary infection, 19% of epithelial cells had inclusions on days 4 and 8, but this declined to a mean of 1.2% by day 18. In reinfected animals the mean inclusion rates were 0.2% to 0.3% on days 2, 4, and 8, and the maximum in any one animal was 4%. The difference between primary and rechallenged animals was significant from day 2 to day 18 after inoculation.

**Histology of the cornea.** The cornea sections stained with Giemsa stain or with hematoxylin and eosin from normal guinea pigs had some mononuclear cellular infiltration noted at the limbus. Specimens from animals 48 hours after primary or rechallenge inoculation with 10 to 10<sup>4</sup> ELD<sub>50</sub> had no more limbal reaction than normal eyes. In animals rechallenged with 10<sup>5</sup> ELD<sub>50</sub> there was a moderate mononuclear cell (probably lymphocyte) infiltration at the limbus, and a few polymorphonuclear cells were present in the limbus and the overlying epithelium. These eyes also had round cells, presumably lymphocytes, in the central corneal stroma, but no inflammatory cells were in the corneal epithelium.

Electron microscopy revealed GPIC agent in the corneal epithelium of one eye of an animal inoculated with 10<sup>3</sup> ELD<sub>50</sub>. The GPIC inclusions were present in the flattened, superficial cells of the corneal epithelium, an indication that these cells were metabolically active enough to support the replication of this agent. Whenever the agent was seen in a superficial epithelial cell, usually several neighboring deeply seated cells were infected at the same time.

**Discussion.** In this study, primary infection increased resistance to challenge inoculation by at least 10<sup>4</sup> ELD<sub>50</sub>. Clinical disease was usually, but not always, associated with growth of the agent in the conjunctiva. Even in the animals rechallenged with 10<sup>4</sup> and 10<sup>5</sup> ELD<sub>50</sub>, the duration of replication of the agent (2 to 8 days) and the number of inclusions (1 to 4 per 100 epithelial cells in positive smears) was dramatically less than in animals undergoing primary infection (Fig. 3). Murray and Radcliffe reported "solid
Fig. 3. Relative amounts of GPIC agent in smears from eyes with infection. At day 2, 34 eyes in each group were tested, and from days 4 to 25, 20 to 24 were included in each group. In the re-inoculated animals the greatest number of inclusions noted was 4 per 100 epithelial cells following a challenge dose of $10^4$ ELD$_{50}$, but no inclusions were present after day 8. The difference between the two groups is significant from day 2 to day 18 by Student's $t$ test.

homologous immunity" to challenge eye infection in previously infected animals challenged with $3 \times 10^8$ ID$_{50}$ of GPIC. In our experiments, a solid resistance to challenge was manifested only with challenge doses of $10^8$ ELD$_{50}$ or less, but there was limited replication with higher challenge doses ($10^9$ or $10^{10}$ ELD$_{50}$).

Although the growth of the agent was significantly reduced in reinfected animals, the cellular response to reinfection was much more vigorous with $10^6$ ELD$_{50}$, suggesting an enhanced reactivity to the agent. Kazdan, Schachter, and Okumoto$^8$ may have observed a similar kind of enhanced response in animals rechallenged with GPIC 4 months after initial disease. In those experiments corneal vascularization was "significant" although inclusions were present in the eye for only 9 days.

Since systemic infection or immunization to GPIC does not confer immunity to eye infection, local factors must account for this substantial increase in resistance to infection of the eye.$^5$ The causes of this local immunity might include (1) local (secretory) antibody production; (2) local cell-mediated immunity (CMI); (3) enhanced mobilization of leukocytes; (4) a change in the response of the conjunctival epithelial cells, perhaps associated with local antibody or CMI; and (5) local interferon production. Antibody appears in tears with ocular but not systemic GPIC infection,$^4$ and passive transfer of serum antibody does not confer resistance to eye disease,$^5$ so that local antibody may be an important mechanism in resisting infection. Nevertheless, tear antibody does not neutralize the organism completely.$^4$ Cellular immunity to *Chlamydia* is found after eye or systemic infection,$^9$ but enhanced CMI against *Chlamydia* in eye tissues has not yet been demonstrated. Since the human chlamydial eye infections, trachoma and inclusion conjunctivitis, are chronic and do not lead to a solid immunity, it would be of interest to characterize in detail those factors in the local response to GPIC which confer such a high degree of resistance.

We thank Mrs. Catherine Lyon, Ms. Joan Williamson, and Mrs. Ruth Lee Thygeson for help in the preparation of this manuscript.


Key words: *Chlamydia*, conjunctivitis, immunity.

REFERENCES


It has been suggested that corticosteroids plus the effective antibiotics are more beneficial than antibiotics alone in reducing inflammation in infectious keratitis. Topical and subconjunctival corticosteroids as adjuncts to gentamicin therapy were evaluated by microbiological assay and clinical parameters in experimentally induced *Pseudomonas keratitis* in rabbits. Various strengths of dexamethasone given concurrently or delayed in conjunction with gentamicin therapy were studied. There was no statistically significant difference between combinations and the antibiotics alone in this experimental model.

Over the past few years, ocular infections by *Pseudomonas aeruginosa* have been increasing, especially in patients hospitalized with underlying debilitating ocular and systemic diseases. Uncontrolled, these infections can lead to a rapid loss of vision and destruction of the eye. Although new antibiotics are bactericidal, frequently it is the secondary inflammatory process which causes destruction of the cornea. The use of corticosteroids in corneal infections has been controversial. It has been suggested that corticosteroids plus the effective antibiotics are more beneficial than the antibiotics alone in reducing the disease process. Aronson and Moore have recommended combined high-dosage topical corticosteroid therapy in active *Pseudomonas* keratitis. Some have questioned whether high-dose steroids would enhance or prolong *Pseudomonas* viability.

The rationale for the addition of steroids to the appropriate antibiotic in the treatment of infectious keratitis is to reduce the severity of the inflammatory response, thus reducing corneal scarring. Newmark and Ellison have suggested that weaker steroid concentrations are better because they promote anti-inflammatory effects without suppressing local host resistance. The purpose of this study was to evaluate the effects of gentamicin and corticosteroids in combination, in the treatment of *Pseudomonas* keratitis in rabbits. Similar studies have been done in the past but have not been approached microbiologically. The parameters used as end points in this study were (1) the microbiological assay and (2) the clinical assay of keratitis.

**Materials and methods**

**Animals.** Female New Zealand white rabbits (weighing 2 kg.), free from ocular lesions and disease, were used in the study.

*Pseudomonas* strain. *P. aeruginosa* strain, pyocine type 6, from a lyophilized pool was prepared with sterile saline on a blood agar plate for each experiment. This strain was sensitive to 10 ag gentamicin sulfate disks. A 24 hr. growth was used for inoculation. This species was originally isolated from a clinical case of *Pseudomonas keratitis*.1

**Drugs.** Commercially available gentamicin sulfate (Garamycin; Schering Corp., Bloomfield, N. J.) and dexamethasone disodium phosphate (Decadron; Merck, Sharp & Dohme, West Point, Pa.) were used topically and subconjunctivally. Two drops (0.12 cc.) of gentamicin sulfate, 3.0 mg./cc., were given four times a day; a subconjunctival injection of 10 mg. of gentamicin in 0.25 cc. was also administered in the evening. Dexamethasone was given in a similar manner, 2 drops (0.12 cc.) four times a day. In certain rabbits, subconjunctival injections of 1 mg. of dexamethasone in 0.25 cc. were given in addition to the topical steroid. In eyes receiving more than one type of agent, subconjunctival injections were given in different quadrants, and there was a delay of 5 min. between different drops so as to minimize dilution factors. The control solution for dexamethasone was the vehicle supplied by Merck, Sharp & Dohme. The control for antibiotic was normal saline.

**Experimental design.** Each cornea (in both eyes) was inoculated centrally with a 30-gauge microsyringe (Hamilton Co., Reno, Nev.) containing 0.03 ml. of a suspension of approximately 100 viable *P. aeruginosa* organisms intrastromally. This suspension was prepared from a 24 hr. growth of the above strain of *Pseudomonas* at 37°C on blood agar plates. Dilutions were done with physiological saline to produce a standardized suspension to 95% transmission at a wavelength of 540 nm. against a clear blank in a Coleman spectrophotometer (Coleman Instruments, Oak Brook, Ill.). Injections were made under a topical proparacaine hydrochloride (Ophthalm, Alcaine) anesthetic in the central corneas of all rabbit eyes with the use of an operating microscope. This dosage, based on preliminary study, was found to


