Serum and tear antibodies to *Chlamydia* after reinfection with guinea pig inclusion conjunctivitis agent

**Raga Malaty, Chandler R. Dawson, Ira Wong, Catherine Lyon, and Julius Schachter**

Repeated inoculation of the eyes of guinea pigs with the naturally occurring *Chlamydia psittaci* agent, guinea pig inclusion conjunctivitis (GPIC), showed that animals gradually become susceptible to reinfection with the passage of time after primary infection. Higher levels of serum IgG antibody had a significant association with resistance to challenge inoculation only with a high dose (250 ELD$_{50}$) but not with a low dose (25 ELD$_{50}$) inoculum. With each inoculum, however, some animals with high serum antibody were susceptible. The presence of antibodies in tears did not correlate with resistance to the first low-dose challenge inoculation, but both tear IgG and secretory antibody did have a significant association with resistance on the second rechallenge with a high-dose inoculum. Topical treatment of the eye with immune serum or tears during primary infection reduced the amount of agent in the conjunctiva only during the period of application. Local treatment of the eye with heat-killed vaccine prior to primary infection did not produce detectable antibody or protect animals against challenge inoculation; this local immunization did "prime" the animals, however, so that they had an accelerated antibody response after infection. Although there is abundant evidence that local immunity has an important role in resistance to challenge inoculation with GPIC, serum and tear antibody levels correlate equally well with resistance to repeated ocular challenge inoculation. Effective immunization procedures for this chlamydial infection then would involve stimulation of both local and systemic immune responses. (INVEST OPHTHALMOL VIS SCI 21:833-841, 1981.)

**Key words:** *Chlamydia*, secretory antibody, conjunctivitis

Guinea pig inclusion conjunctivitis (GPIC) is a naturally occurring infection caused by a member of the *Chlamydia psittaci* group. The disease can involve the genital tract as well. The natural initial disease episode is mild, self-limited, and leaves the animal relatively resistant to reinfection. Experimental infection, however, results in a severe conjunctivitis within 3 to 6 days that is also self-limited and resolves in 3 to 4 weeks. Previously infected animals require a high dose of inoculum to produce disease, and even then the infection is shortlived and replication of the organism is severely limited.

The mechanisms that control this infection in the natural host are not yet clearly defined. After inoculation, susceptible animals develop serum and secretory antibodies against GPIC agent, and cell-mediated immunity...
can be demonstrated by skin tests and migration inhibition tests.\textsuperscript{2, 5} The disease resolves and agent disappears simultaneously with the appearance of local antibody and cellular immunity. Watson et al.\textsuperscript{8} claimed to show that passively transferred serum antibody did not confer immunity against infection. In cyclophosphamide (Cytoxan)-treated guinea pigs inoculated with GPIC, the agent persists and serum and tear secretory antibody responses are retarded, although the skin tests are positive by 10 days after infection.\textsuperscript{7}

Local immunity appears to be important in GPIC. For example, guinea pigs with primary genital inoculation are susceptible to challenge inoculation in the eye but are resistant to reinoculation of the urogenital tract, primary eye infection, however, confers immunity to challenge inoculation of both eye and vagina.\textsuperscript{8, 9} Topical administration of killed chlamydial agent has not successfully immunized primates to trachoma agent or guinea pigs to GPIC.\textsuperscript{10, 11} "Enteric immunization" with live GPIC resulted in partial protection to eye and vaginal challenge.\textsuperscript{12}

The guinea pig disease serves as a useful model for investigating the complex problems of immunity presented by trachoma and other human chlamydial infections because GPIC, like \textit{C. trachomatis} in humans, infects the epithelial surfaces of eye and genital tract and can cause recurrent disease. Although GPIC is a \textit{C. psittaci} strain, there is no evidence that the host immune response differs in any substantial fashion from that caused by \textit{C. trachomatis} strains that also infect mainly mucous epithelium. Indeed, eye infections of guinea pigs with GPIC and of owl monkeys with \textit{C. trachomatis} from endemic trachoma have a number of similarities, including exuberant growth of the agent initially in the conjunctival epithelium, rapid resolution of the infection in 2 to 4 weeks, and relatively solid resistance to infectious challenge immediately after the initial disease episode.\textsuperscript{1, 2, 3, 13, 14} In endemic trachoma, affected children are exposed to frequent reinfection with chlamydial agent. To evaluate the host response to repeated chlamydial infections, we did repeated experimental inoculations of the eyes of guinea pigs with GPIC. This study delineated the relationship between serum and tear antibody levels and susceptibility to reinfection. We also studied the susceptibility to ocular challenge after ocular applications of immune sera or tears and after immunization by the application of killed agent to the external eye.

### Materials and methods

**Chlamydial agent and inoculum.** A single pool of the twelfth egg passage of GPIC agent (GP86) isolated here was used for all inoculations.\textsuperscript{13} Aliquots of 50% yolk sac grown agent were stored at $-70^\circ\text{C}$ until used. The titer of this pool was about $10^7$ egg lethal doses (ELD) per milliliter, and material was titrated before every inoculation. The inoculum consisted of either 25 ELD\textsubscript{50} the "low dose," or 250 ELD\textsubscript{50} the "high dose," delivered in 0.025 ml from a lambda pipette.

**Animals.** For each of these experiments Hartley-strain guinea pigs were obtained from a herd known to be free of GPIC (Charles River Laboratories, Boston, Mass.).

**Smears.** Conjunctival scrapings were taken with a Kimura spatula, and the cells were spread thinly on a glass slide. Slides were stained with Giemsa and examined microscopically for the number of chlamydial inclusions. A total of 300 epithelial cells from each eye were counted to determine the number of inclusions per 100 epithelial cells.

### Table I. Ocular challenge inoculations with GPIC

<table>
<thead>
<tr>
<th>Group</th>
<th>Primary inoculation (dose)</th>
<th>Rechallenge inoculations (dose)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>60 days</td>
</tr>
<tr>
<td>Group I</td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>Group II</td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>Group III</td>
<td>High</td>
<td>Low</td>
</tr>
</tbody>
</table>

High dose = $250$ ELD\textsubscript{50} GPIC; low dose = $25$ ELD\textsubscript{50} GPIC.
Fig. 1. Serum and tear antibody responses to GPIC measured by microtiter immuno-fluorescence in animals challenged 60 days after primary infection. Tear antibody levels declined rapidly after initial infection, although serum antibody levels then reached a peak.

**Tear and serum antibody.** Tears were collected from each eye by placement of Weck-Cel sponges that absorbed about 0.05 ml from the lower conjunctival fornix. Each sponge was placed in 0.45 ml of saline in a screw-capped vial to give a dilution of 1:10. Thus the lowest titer measured in tears was 1:10, and levels less than this were not detected. Blood was collected by clipping the animals’ toe nails close to the nail bed. Serum and tears were stored at —70°C.

Both serum and tears were tested for the presence of IgG antibody against GPIC agent by the microimmunofluorescent technique with a fluorescein conjugated goat anti-guinea pig globulin. In addition, tears were tested for secretory antibody by a four-layer test with rabbit anti-guinea pig secretory component* and fluorescein conjugated goat anti-rabbit globulin.

**Vaccine preparation.** Material for topical immunization was made by harvesting eggs infected with the same strain and passage level of GPIC. Infected yolk sacs were homogenized with an equal volume of phosphate-buffered saline. After three cycles of slow (500 x g) and fast (17,000 x g) centrifugation, 0.1 ml of 1% formalin was added for each 100 ml of yolk-sac suspension to inactivate the agent, and the mixture was kept at 4°C for 48 hr. After another centrifugation at 17,000 x g for 20 min the solids were suspended in normal saline. The vaccine corresponded to 10⁴ ELD₅₀/ml of the starting material. A placebo vaccine was prepared by the same method, starting with uninfected yolk sacs. Both vaccine and placebo were stored in 1 ml aliquots at —20°C until use.

**Experimental design, reinoculation experiment.** In the first experiment all animals received the same primary inoculum of 250 ELD₅₀ (high dose) of GPIC in each eye (Table I). Conjunctival smears, tears, and blood were collected prior to inoculation and then on days 2, 4, 6, 8, 21, 28, 45, and 60 after inoculation. After primary infection animals were divided into three groups (I, II, and III) and challenged at different intervals.

At 60 days after primary infection, guinea pigs in group I received a challenge inoculum of 25 ELD₅₀ (low dose) in the right eye (Table I). Conjunctival smears were taken 2, 4, 6, 8, 14, and 20 days after inoculation. Blood and tears were collected just before infection challenge and then 7 and 14 days after inoculation. After primary infection animals were divided into three groups (I, II, and III) and challenged at different intervals.
oculum, and group I animals received a second, high-dose challenge. At 120 days group III received the first challenge inoculation with a low dose, and the other two groups were rechallenged with the low dose. At 150 days all animals were rechallenged with high dose. At 210 days all surviving animals were divided into two groups; one group received a low dose and the other a high dose.

An infectious take was defined by the presence of typical chlamydial inclusions in conjunctival smears 48 hr after challenge inoculation. About 45 days after primary inoculation a respiratory infection with Bordetella bronchiseptica became epizootic in the guinea pig colony, and three to four animals died in each group.

**Experimental design, topical application of antibody.** In a second experiment three groups of five guinea pigs free of previous GPIC infection received topical applications of immune serum, immune tears, or normal serum. One drop of serum (IgG titer 1:128) or tears (secretory antibody titer 1:3) was placed in one eye of each animal five times daily for 2 days before and 5 days after a high-dose inoculum (250 ELD₉₀) of GPIC was given to both eyes. The tears were from a pool that titered 1:16 and was diluted 1:5 for use. Tears and serum were collected before inoculation and 7, 14, 21, and 28 days after challenge. Conjunctival smears were taken before inoculation and 3, 4, 5, 6, 12, 16, 21, and 28 days after inoculation.

**Experimental design, topical immunization.** In a third experiment immunization was done by administering the vaccine preparation to one eye of nine guinea pigs and placebo to the other eye. Placebo was given to both eyes of four other guinea pigs. Vaccine and placebo were administered on alternate days for three days, then two more doses were given at intervals of 7 days. Vaccines were presented to the conjunctiva by placing a gel-foam sponge soaked with 0.05 ml of vaccine under the lower lid for 10 min. Challenge inoculation was done with a low dose (25 ELD₉₀) of GPIC 3 weeks after the last immunization. Smears, sera, and tears were taken before immunization, after immunization but before infection, and then at 3, 6, 16, 31, 35, and 49 days after inoculation.

**Results**

**Primary infection.** In the reinoculation experiment all animals became infected and had chlamydial inclusions in their conjunctival smears after primary inoculation with the high dose of GPIC. The serum IgG antibody levels rose rapidly to day 21 and by 60 days reached a geometric mean titer for all animals of 1:159 (Figs. 1 to 3). After 60 days there was a steady decrease in serum antibody titers in the absence of reinfection. Tear antibody reached peak levels 28 days after inoculation and then declined sharply. The highest geometric mean tear IgG antibody levels before reinoculation were 1:11, and the highest levels of secretory antibody were 1:37. Tear
antibody levels then decreased to minimal levels by 60 days. Thus at 60 days tear IgG and secretory antibody levels were at their lowest as serum IgG antibody reached a peak.

First challenge inoculation. Groups of animals received their first challenge inoculation with low dose of GPIC at 60, 90, or 120 days after primary infection (Table II). More animals became susceptible with longer intervals before the first challenge inoculation, and there was significant difference in susceptibility over the three times (p < 0.025).

After the first challenge inoculation in group I (only one of nine infected) the mean serum antibody titers continued to decline (Fig. 1), but in groups II and III (11 and 17 infectious takes) there was an increase in the mean serum and tear antibody levels after infection (Figs. 2 and 3).

Second or subsequent challenge inoculations. On subsequent challenge inoculations there were significantly more takes with the high dose (19 takes of 34 challenged) than with the low dose (one take of 22 challenged) (Table II). Animals that were infected on the first challenge inoculation became more resistant to subsequent infectious challenges even with the high-dose inoculum.

Serum antibody levels and resistance to challenge inoculation. When the titers of all animals at the time of first challenge with low dose were compared, the mean titer of susceptible animals was 1:76 and that of resistant animals was 1:128 (Table III), a difference that was not statistically significant. The one animal without antibody in the resistant group had polymorphonuclear leukocytes in the smear but no inclusions and developed a significant rise in antibody titer. Even if this animal is regarded as infected, the difference between the two groups is still not significant.

Among animals challenged with the high doses for the first time (i.e., after the primary

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**Fig. 3.** Antibody to GPIC in animals challenged 120 days after primary infection.

**Table II.** Infectious takes after first and subsequent inoculation of the eye of guinea pigs with GPIC

<table>
<thead>
<tr>
<th>Challenge on day:</th>
<th>60</th>
<th>90</th>
<th>120</th>
<th>150</th>
<th>210</th>
</tr>
</thead>
<tbody>
<tr>
<td>First challenge:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low dose</td>
<td>1/9*</td>
<td>5/9</td>
<td>6/8t</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Subsequent challenge:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low dose</td>
<td>—</td>
<td>—</td>
<td>1/12</td>
<td>0/10</td>
<td></td>
</tr>
<tr>
<td>High dose</td>
<td>5/6</td>
<td>—</td>
<td>9/19</td>
<td>5/9</td>
<td></td>
</tr>
</tbody>
</table>

*Number of animals infected/number inoculated.

† Distribution between infected and not infected at three times differs significantly (chi square = 7.44, p < 0.025).
Table III. Serum antibody levels and susceptibility to challenge infection with GPIC—first challenge with low dose (25 ELD₅₀)

<table>
<thead>
<tr>
<th>Serum IgG micro-IF titer</th>
<th>Take</th>
<th>No take</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:8</td>
<td>—</td>
<td>1*</td>
</tr>
<tr>
<td>1:16</td>
<td>2</td>
<td>—</td>
</tr>
<tr>
<td>1:32</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>1:64</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>1:128</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>1:256</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>1:512</td>
<td>—</td>
<td>1</td>
</tr>
<tr>
<td>1:1024</td>
<td>—</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>12</td>
<td>14</td>
</tr>
<tr>
<td>Geometric mean titer</td>
<td>1:76</td>
<td>1:128</td>
</tr>
</tbody>
</table>

Micro-IF = microimmunofluorescent.

*This animal developed a significant rise in antibody titer and had polymorphonuclear leukocytes in the smears but was classified as "no take" because no inclusions were found. If it is regarded as a "take," the mean titer of the "take" group is 1:64 and of the "no take" group is 1:159 (0.1 > p > 0.05).

infection and one low-dose challenge) the mean titer of the 11 susceptible animals was 1:106 and of the nine resistant ones 1:553 (Table IV), a significant difference (p < 0.01) by Student’s t test. Thus there is good correlation between serum antibody titer and resistance to challenge inoculation with this higher dose. It should be noted, however, that some animals became infected despite very high serum antibody levels.

Tear antibody levels and resistance to challenge inoculation. With the first challenge inoculation with the low dose, neither IgG nor secretory antibody in tears had a significant association with resistance. On subsequent challenge with the high dose inoculum, however, there was a high degree of association of the presence in tears of both IgG and secretory antibodies with resistance to reinfection (Table V). Between inoculations tear antibody levels appeared to decline more rapidly than serum antibody (Figs. 1 to 3).

In group III there was an apparent rise in tear antibody at day 90, although the animals had not been challenged (Fig. 3). It is unlikely that this resulted from crossover infection (from animals in group I inoculated at 60 days) because the serum antibodies in group III continued to decline throughout this period. Moreover, it should be noted that the lowest detectable level of tear antibodies was 1:10, so that the apparent rise of tear antibody in group III (Fig. 3) is exaggerated in the graph because a titer of "zero" for tears is really "less than 1:10." The standard deviations on day 60 and day 90 also overlap considerably.

Effect of pretreatment with tear or serum antibody. To compare the effect of pretreatment of the external eye with immune serum, immune tears, or normal serum on susceptibility to challenge primary infection, animals were treated in the right eye with one of the three preparations five times daily for 2 days before and 5 days after a high-dose challenge inoculum of GPIC. In the group treated with immune serum the onset was delayed by at least 1 day on the treated side. The number of inclusions was significantly reduced on days 4 and 5 while the animals were under treatment with immune serum regardless of the eye treated. During treatment, then, immune sera definitely retarded growth of the agent even with this relatively large inoculum. At cessation of treatment, however, the mean number of inclusions increased in animals receiving serum antibody. There was a similar but less marked reduction of inclusions in animals during treatment...
with immune tears that was significant only on day 4.

**Topical vaccination.** Both the vaccinated and unvaccinated control guinea pigs acquired infection, and there was no difference in severity of the infection. Although there was a 30% reduction in the maximum number of inclusions in both eyes of the vaccinated animals, this difference was not statistically significant. Although none of the animals had detectable antibody before challenge inoculation, IgG antibodies were higher in the serum and tears of vaccinated animals on the tenth day after infection. On day 14 those differences were significant for serum and tear IgG (sera, \( p < 0.05 \); tears, \( p < 0.01 \)).

The titer of tear secretory antibody was also higher early in the infection in both eyes of the vaccinated guinea pigs, but this difference was not statistically significant. Even though one eye was vaccinated, the tear antibody response was the same in both eyes.

### Discussion

GPIC may be considered a model to study immunity and immunization in trachoma. Like the human *C. trachomatis* infections, trachoma and inclusion conjunctivitis, GPIC (a *C. psittaci* agent) infects mainly the mucus epithelium of the eye, genital tract, and probably the intestinal tract. In both naturally occurring and experimental infections, GPIC agent first presents to epithelial surfaces. There are relatively few inflammatory cells in uninflamed guinea pig conjunctiva, although infiltration of polymorphonuclear leukocytes and lymphocytes occurs within hours after inoculation. Thus the relationship of the organism to its host epithelial cell is of prime importance. Attachment of antibody, exposure to ultraviolet light, or heat treatment of chlamydial elementary bodies modifies the host cell response so that the infectious particles are eliminated.16, 17

There is abundant evidence that local immunity plays an important role in resistance to challenge inoculation with GPIC. With the repeated infections of the same site done in this study, however, specific serum IgG antibody was as good as indicator of immunity as the presence of antibody in tears. A number of other studies have shown a good correlation between serum antibody and recovery from or resistance to infection. Rank et al.18 found that in immunosuppressed guinea pigs inoculated vaginally with GPIC, recovery from infection was associated with antibody formation and return of B-cell function, even though T-cell responses remained suppressed; animals with intact T-cell formation but with no B-cell activity, however, did not recover from the infection. Thus adequate systemic antibody formation appears to be an essential step in the development of immunity to GPIC. Nevertheless, local exposure of the site also appears to be critical, since parenteral immunization or infection at another site (e.g., vagina or urethra) does not protect the eye against infectious challenge to the same degree as does previous eye infection.3, 9

Previous experiments have shown that sufficiently high challenge doses will overcome resistance even at 45 days after primary infection.4 The challenge doses in the present experiments (25 and 250 ELD<sub>50</sub>) were selected to ensure an adequate test of immunity resulting in some breakthrough infections. It is not surprising that resistance to challenge infection can be overcome because repeated or recrudescent episodes of infection are characteristic of the whole chlamydial group.

In this study, serum and tear antibody levels correlated with resistance to the high-dose inoculation. This contradicts much of the previous work by Murray et al.4 and Wat-

### Table V. Tear antibody level and susceptibility to first high-dose challenge infection with GPIC

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Tear IgG</th>
<th>Tear IgA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Present</td>
<td>2*</td>
<td>3</td>
</tr>
<tr>
<td>Absent</td>
<td>7 2</td>
<td>9 0</td>
</tr>
</tbody>
</table>

\( p = 0.012 \) \( p = 0.0013 \)

*Number of animals.

† Probability that association of resistance to infection and detectable tear antibody occurs by chance (Fisher’s exact test).
son et al., 5, 6 who stated that secretory antibody and cell-mediated immunity appeared to be more critical than serum antibody. In those studies, however, the immunofluorescent test measured complement-fixing antibodies to Chlamydia, not IgG antibody against elementary body particles. Nichols et al., 12 have noted also that secretory antibody on mucous surfaces do not correlate well with the degree of immunity to later challenge. With the use of immunosuppression others have shown that B-cell activity and serum antibody correlates with resistance to infectious challenges. 5, 18 Nevertheless, topical application of immune serum or immune tears did have a significant effect in the temporary suppression of chlamydial replication in the conjunctiva. This suggests that both IgG and secretory antibody can be effective at the conjunctival surface.

Although there is a clear association of serum antibody and resistance to challenge infection, the mechanism of immunity to GPIC has not yet been elucidated. Attachment of antibody to infective particles appears to allow lysosomal enzymes to enter the inclusion vacuole. 17 It is not evident why such high titers of antibody are necessary for this in the intact animal. It is also possible that the level of serum antibodies simply indicates the state of all other immune mechanisms.

It seems that both local and systemic immune responses are necessary to achieve resistance to infectious challenge with GPIC. Moreover, effective immunization should produce adequate levels of circulating antibody. Although the natural infection appears to be limited to mucosal epithelial surfaces, enough antigen must enter the body to stimulate B cells to produce IgG antibody. It is probable that the GPIC agent stimulates lymphoid tissue in the nasopharynx and gut after eye infection. Since local immunity clearly has an important role, however, an effective vaccine would have to stimulate local immunity. In this study, the local immunization with relatively low-titer material did not produce detectable levels of antibody but did appear to prime the animals so that they developed accelerated antibody responses on infectious challenge inoculation. Thus it would be worthwhile to evaluate the combined application of parenteral and topical immunization with material of sufficient antigenicity as a means of producing protection to frequent small infectious challenge doses such as those that occur in endemic trachoma.

REFERENCES