# Pathogenetic Studies of Herpes Simplex Virus Infection of the Rabbit Eye[1] (37666)

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The pathogenesis of ocular infection by herpes simplex virus (HSV) is far from being well understood. We lack the precise knowledge of the relative importance, to the disease process, of virus replication, interferon production and immune response. Each of these parameters has been investigated, in separate experimental studies, by various authors using different techniques and approaches (1–9).

The present study was undertaken to quantitate and compare the kinetics of the 3 major parameters of the infectious process simultaneously in the same animals, at the same intervals after infection and in those eye tissues which show typical clinical signs.

*Materials and Methods. Infection of rabbits.* Eighty New Zealand albino rabbits weighing 1.5–2.0 kg were used. Both corneas were scarified by 2 superficial 5 mm crisscross scratches and infected with $2.10^4$ $TCID_{50}$ of herpes simplex virus (HSV). The Mayo strain of HSV obtained from the Research Resources Branch, NIH was used. Its titer in secondary rabbit kidney TC was $10^{-7}$ per 0.05 ml.

Clinical signs were recorded every second day for a 2-wk period.

*Collection of samples.* At 6 hr, 1 day, 2, 4, 6, 8, 11, 12 and 14 days postinfection, 3–4 animals were exsanguinated by cardiac puncture, the serum was separated and stored at −20°. These animals were picked at random and marked accordingly on the day of infection. Entire corneas, bulbar conjunctivas and irides were removed *in situ* and profusely rinsed with a jet of Hanks' balanced salt solution (BSS) which, like the tissue culture medium, contained 100 units of penicillin, 100 μg of streptomycin, 10 μg of Achromycin and 25 units of Mycostatin/ml. Homologous eye tissues were pooled and 10% w/v extracts were made up in Eagle's minimum essential medium (MEM) with 2% fetal calf serum (FCS) by trituration with glass powder. The extracts were clarified by centrifugation (4500 rpm) and stored at −60° until used for titrations.

*Assays.* Secondary rabbit kidney cells grown in Linbro TC microplates (supplied by Bellco Co.), 48 flat bottom cups/plate, were used for all titrations throughout the experiments. The plates were kept in a humidified, 5% $CO_2$ incubator at 37°. For virus titrations, 10-fold serial dilutions of all tissue extracts were prepared, each dilution was tested in 4 cups. The highest dilutions causing CPE in 50% of cups at 7 days, were considered as virus titers. Interferon was prepared from all the extracts in small cellophane bags dialyzed for 24 hr against a HCl–KCl buffer, (pH 2.0) and for the next 24 hr against Hanks' BBS at 4°. The interferon preparations were tested in 4-fold dilution steps from 1/3 to 1/192, each dilution in 4 cups. After overnight incubation, the cultures were washed 3 times and challenged with 100 $TCID_{50}$ of vesicular stomatitis virus (VSV). The highest interferon dilutions causing protection in 50% of cultures were considered as interferon titers. A laboratory reference preparation of interferon was included in each test. This was a rabbit serum

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withdrawn 2.5 hr after iv injection of 1 mg of polyinosinic–polycytidylic acid (In·Cn). Its titer was $10^4$ units/ml as measured by the VSV-CPE protection assay. Extracts exhibiting interferon activity were retested with addition of 5 $\mu$g of RNase and $10^{-3}$ *M* EDTA/ml to ensure the removal of residual In·Cn present in the sample. For antibody titration, a standard neutralization test was applied. Serial 2-fold dilutions of the tested material, heated for 30 min at 56°, were mixed with equal volumes of HSV, 100 $TCID_{50}$ and incubated at 37° for 1 hr. Each serum or extract dilution was tested in 4 cups. The highest dilutions showing inhibition of HSV-CPE in 50% of cups at 7 days were recorded as antibody titers. Eye tissue extracts which contained virus were, before being used in neutralization tests, freed of virus by pressure filtration through Millipore filters, pore size 50 nm.

The In·Cn preparation contained 1000 $\mu$g/ml in 0.01 *M* PBS, pH 7.6. Its biological activity was tested before, during and after completion of the experiments by the VSV-CPE protection assay in secondary rabbit kidney tissue culture (RKTC), where 0.0005 $\mu$g of the preparation protected 50% of the cultures against 100 $TCID_{50}$ of VSV. In·Cn treatment was started 1 hr after infection in 40 rabbits. On the day of infection, the rabbits received 10 drops (500 $\mu$g) into each cul-de-sac. On consecutive days until Day 13 postinfection (pi), the treatment consisted of 4 drops (200 $\mu$g) delivered into each eye. Rabbits of the control group (40 rabbits) received sterile saline.

*Results. Cornea.* As shown in Fig. 1 virus titers in corneal extracts peaked on Day 2 and thus coincided with the onset of corneal epithelial lesions. In comparison, corneal stromal disease began on Day 8, coinciding with the appearance of serum antibody (Fig. 3). All the lesions persisted past the time of virus "elimination." The disease manifestations in the rabbits have been considered in detail elsewhere (10). The decline of corneal virus titers coincided with the appearance and rise of serum antibody which occurred between Days 8 and 11 (Fig. 3, control). However, antibody in the corneal extracts did not appear until Day 14 (Table I), a time at which corneal virus had been undetectable for 2 days (Fig. 1). No interferon was found in the 10% corneal extracts at any time.

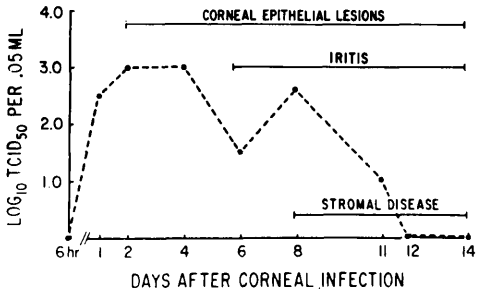


FIG. 1. Time course of HSV multiplication in rabbit corneas.

*Conjunctiva.* Figure 2 (control line) shows that although virus titers in conjunctival extracts peaked on Day 1, conjunctivitis began on Day 6. Conjunctivitis persisted until Day 12, 1 day after "elimination" of virus. Serum antibody appeared on Day 8 (Fig. 3), but tissue antibody in the richly vascularized conjunctiva was first detected on Day 14 (Table I) a time when conjunctival virus had been undetectable for 3 days (Fig. 2). No interferon was found in the conjunctival extracts at any time.

*Iris.* Although no virus was detected in the iris or the blood, iritis began on Day 6 and persisted until Day 14. Both iris (Table I) and serum antibody (Fig. 3) appeared on Day 8. Since iris antibody was determined in 10% extracts, its actual titer on Day 8 is in fact 10 times higher than in the serum. No interferon was detected in the iris at any time.

*Treatment with In·Cn beginning 1 hr postinfection.* It has been reported that posttreatment of this infection with In·Cn ameliorated the disease (11). The strong prophylactic effect has been confirmed (12). Other authors (14) pointed out that posttreatment was substantially less effective. To study this point further, we initiated topical treatment 1 hr after infection and measured the same parameters of infection and host defenses. This therapy significantly decreased virus titers (av 10-fold decrease) in the conjunctiva (Fig. 2). This was associated with the appearance of 12 units/ml of interferon

TABLE I. Virus Neutralizing Antibody in Extracts of Pooled Eye Tissues.

| Time after infection | Conjunctiva | | Cornea | | Iris | |
|---|---|---|---|---|---|---|
| | Treated | Control | Treated | Control | Treated | Control |
| 6 hr | <5 | <5 | <5 | <5 | <5 | <5 |
| 1 day | <5 | <5 | <5 | <5 | <5 | <5 |
| 2 days | <5 | <5 | <5 | <5 | <5 | <5 |
| 4 days | <5 | <5 | <5 | <5 | <5 | <5 |
| 6 days | <5 | <5 | <5 | <5 | <5 | <5 |
| 8 days | <5 | <5 | <5 | <5 | 5 | 10 |
| 11 days | <5 | <5 | <5 | <5 | <5 | 10 |
| 12 days | <5 | <5 | <5 | <5 | <5 | 5 |
| 14 days | <5 | 10 | <5 | 10 | 5 | 15 |

in the 10% conjunctival extract at 5 hr after start of treatment, but not thereafter. No interferon could be detected in the serum. Virus titers in the treated corneas were not significantly different from those in control corneas and no interferon was detected in the corneas of treated rabbits. Lesions of the treated conjunctivas and corneas were slightly but insignificantly milder (10).

Serum antibody titers in the treated rabbits were slightly, but consistently lower as illustrated in Fig. 3. Similarly, no antibody was detectable in 14 day conjunctival and corneal extracts from treated rabbits, whereas controls had titers of 10 units/ml. Finally, iris antibody in treated animals was reduced to at least one half on Days 8, 11, 12 and 14 (Table I).

*Discussion.* The present study was concerned primarily with the time relationships between herpetic eye disease and virus replication, interferon and antibody production. An important finding was that the kinetics of virus replication was not always correlated with the onset of clinical signs in a given eye tissue. Although corneal epithelial lesions coincided with peak virus titers, conjunctivitis began 5 days after peak virus titers. Since the rate and extent of virus replication in the cornea and conjunctiva were practically the same, the delayed appearance of conjunctivitis might be due to the difference in vascularity between the 2 tissues. Speculatively, toxic products from lysed cells are less likely to be removed from the avascular cornea, thus leading to an earlier appearance of corneal lesions.

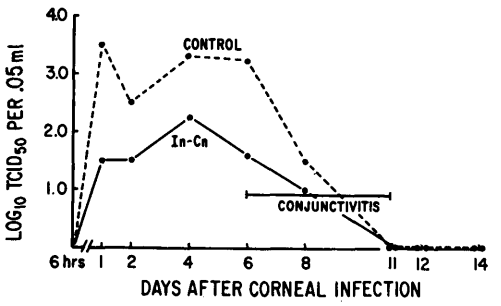


FIG. 2. Time course of HSV multiplication in rabbit conjunctivas.

Iritis developed in the absence of detectable HSV in that tissue. Lack of correlation between virus levels and lesions have been observed (4) but not in all studies (3) employing iododeoxyuridine (IDU).

The appearance of local antibody did not correlate with elimination of HSV from the cornea or conjunctiva. Iris antibody exceeded serum antibody by a factor of 10 on Day 8. It is likely, therefore, that it was produced by immunocompetent cells invading the iris. These immune cells or the local antibody may have contributed to iritis but such possibilities must be tested.

Local antibody (Table I) did not correlate in time with the corneal or conjunctival lesions. Consistent with this finding is the observation that incidence of stromal disease was increased by immunosuppression but unaltered by actively induced antibody (5). Although subdetectable levels of antibody cannot be excluded with the present methodology, such levels are generally considered ineffective in altering the course of

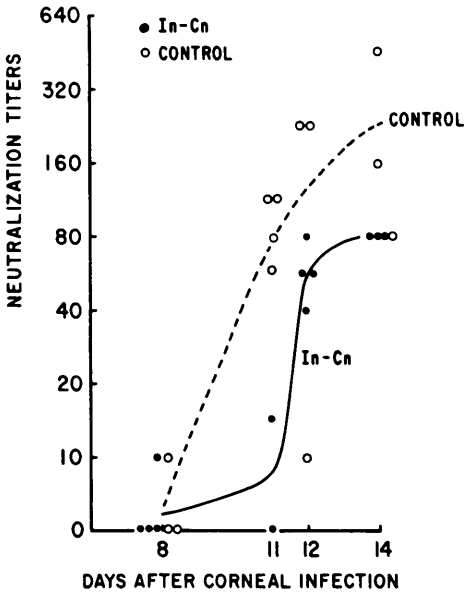


FIG. 3. Serum antibody production in HSV-infected rabbits.

established viral infections (15).

The present findings indicate that the interferon system may not play an important role during recovery from this HSV infection of rabbit eyes since no virus induced interferon was detected in eye tisues during the entire period of observation. Others, using more concentrated extracts, have detected 4–8 units of corneal interferon during HSV infections (9). It is doubtful that these levels of interferon could retard an already established HSV infection which becomes relatively resistant to interferon after initiation of infection (18).

Topical treatment with In·Cn beginning 1 hr after HSV infection induced low levels of conjunctival interferon and a 10-fold decrease in conjunctival virus. A slight protection against conjunctivitis was also observed (10). No decrease of corneal virus was detected. Thus, although pretreatment with this dose of In·Cn is strongly protective (12, 13), posttreatment is much less effective (14). With larger dose of In·Cn stronger therapeutic effects were reported (8).

In our experiments In·Cn also had a slight but consistent depressing effect on antibody production. This may reflect the decreased antigenic stimulation (due to suppressed virus replication in the treated conjunctivas) which apparently overrides the immune enhancing effect reported for In·Cn (16).

Considering that the available information argues against antibody and interferon as mediators of recovery in the present system, future studies might concentrate on the possible role of other mechanisms (17). It also seems desirable to investigate whether the clinical effectiveness of pretreatment with In·Cn is correlated with more distinct alterations of virus replication, interferon and antibody production.

*Summary.* The present study of experimental herpes simplex virus keratoconjunctivitis of the rabbit was undertaken to compare virus production, interferon and antibody production in those eye tissues which show typical clinical signs. Although corneal epithelial lesions coincided with peak virus titers in the cornea, conjunctival lesions began 5 days after peak virus titers in the conjunctiva. Iritis developed in the absence of detectable HSV in that tissue. Thus, virus titers were not always correlated with onset of clinical signs. Simarily, appearance of local antibody did not correlate with elimination of HSV from the cornea or conjunctiva. Stromal disease coincided with the appearance of serum antibody and iritis ensued 2 days earlier than local antibody in this tissue. Since iris antibody exceeded serum antibody by a factor of 10 on Day 8, it is likely that it was produced by immunocompetent cells invading the iris. The inability of the HSV infection to induce detectable concentrations of interferon and the relative insensitivity of HSV to the action of interferon indicates that the interferon system may not play an important role during natural recovery from HSV infection of rabbit eyes. Topical treatment with In·Cn beginning 1 hr after HSV infection induced low levels of conjunctival interferon, a 10-fold decrease in conjunctival virus as well as a slight suppression of circulating antibody, but only a slight protective effect.

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