Effect of cyclophosphamide on primary herpes simplex uveitis in rabbits

Jang O. Oh

Intracameral injection of the normal rabbit eye with $10^3$ 50% tissue culture infectious doses of type 1 herpes simplex virus (HSV) caused uveal inflammation, biphasic elevation of intraocular pressure (IOP), and virus growth in the ocular tissues. A series of intramuscular injections of an immunosuppressive agent, cyclophosphamide (Cy), did not affect the ocular changes or the multiplication of virus in the uveal tissues in the early stage of the disease (days 1 to 6). During this period neither circulating antibody nor macrophage migration inhibition factor (MIF) against HSV appeared in either control or Cy-treated animals, suggesting that the disease is initiated by infectious processes. After day 7, however, virus growth in the uveal tissues of the Cy-treated animals was much more abundant and more prolonged than in the tissues of the control eyes, but uveal inflammation in the Cy-treated animals was much milder and subsided earlier than in the control animals. The Cy treatment suppressed completely the second rise of IOP which normally appears on day 7. These effects of Cy in the late period coincided with a significant suppression of both circulating antibody and MIF, indicating that the late stage of the uveitis was mediated by immune mechanisms. We conclude therefore that primary herpes simplex uveitis in the rabbit is dually mediated, first by the mechanisms of infection and later by the mechanisms of immunity.

Key words: primary herpes simplex uveitis, intraocular pressure, virus replication, immune reactions, immunosuppression, cyclophosphamide, rabbit

Herpes simplex uveitis is often a severe, chronic disease with a high incidence of secondary glaucoma. Although the precise role of herpes simplex virus (HSV) in the pathogenesis of the uveitis is not clear, two mechanisms have been postulated: (1) that the inflammation is the direct result of invasion of the iris and other uveal tissues by the infectious virus and (2) that it is an immune-mediated reaction resulting from hypersensitivity to viral antigens.

The results of our previous study in experimental animals indicated that primary and secondary uveitis are induced by different pathogenetic mechanisms: primary uveitis largely by HSV infection of uveal tissue and secondary uveitis largely by immunological mechanisms. To define the precise role of infectious mechanisms in the primary disease, further studies have been carried out in rabbits treated with an immunosuppressive agent, cyclophosphamide (Cy). This paper describes the results of the study, which indicated that both infectious and im-

From the Francis I. Proctor Foundation for Research in Ophthalmology, University of California, San Francisco.

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Reprint requests: Dr. Jang O. Oh, Francis I. Proctor Foundation, 5315, University of California San Francisco, San Francisco, Calif. 94143.
munological mechanisms participate in the pathogenesis of this disease.

Materials and methods

Rabbits. We used healthy New Zealand White (NZW) male rabbits, weighing about 2 kg each. None of them showed neutralizing antibody against HSV before we used them.

Virus. We used the Shealey strain of type 1 HSV. As in our previous study, the virus was partially purified to avoid inducing uveitis by nonviral antigens from a contaminated preparation. We grew the virus in a primary culture of NZW rabbit kidney cells and maintained it in a medium consisting of 95% medium 199, 5% NZW rabbit serum, and antibiotics (100 U of penicillin and 100 μg of streptomycin per milliliter). The harvested culture of HSV was then centrifuged at 800 × g for 10 min at 4°C. The supernatant fluid was collected and recentrifuged at 140,000 × g for 1 hr at 4°C. This time we discarded the supernatant fluid, resuspended the pellet in the same amount of phosphate-buffered saline solution (PBS) at pH 7.3, and centrifuged the new suspension at 140,000 × g for 1 hr. This procedure was repeated twice.

The final pellet was suspended in a volume of PBS that was one-tenth the volume of the original suspension. This final preparation was then divided into small volumes and stored at −70°C until needed. Its infectivity was 3.5 × 10^6 50% tissue culture infectious doses (TCD_{50}) per milliliter.

Cyclophosphamide. Cytoxan (Mead Johnson Laboratories, Evansville, Ind.) was dissolved in sterile distilled water in a concentration of 20 mg/ml just before use.

Virus titration. The infectivity of HSV in the uveal tissues was titrated by inoculating each of two tubes of Vero cells with 1.0 ml of a serial 10-fold dilution of iris tissue homogenate. The infectivity titer was determined by the Reed and Muench method. The technique we used to prepare the iris tissue homogenate was described previously.

Titration of HSV-neutralizing antibody. We mixed 0.5 ml of serial twofold dilutions of unheated serum with 0.5 ml of our partially purified HSV containing about 100 TCD_{50}. We kept this mixture at room temperature for 1 hr and then inoculated 1.0 ml of it into Vero cell tubes, which were incubated at 37°C for 7 days. The titer was the reciprocal of the highest dilution of the specimen that completely inhibited HSV-induced cytopathic effects.

Measurement of macrophage migration inhibition. Rabbits were inoculated intraperitoneally with 40 ml of sterile paraffin oil; 72 hr later the peritoneal exudate was harvested in Eagle's minimum essential medium (MEM) containing 0.3 U of heparin, 100 U of penicillin, and 100 μg of streptomycin per milliliter. We sedimented the cells by centrifugation at 170 × g for 10 min, washed the packed cells twice with 100 ml of MEM, and resuspended the packed cells in MEM after the second washing. The cell concentration was 10^8 cells/ml.

We then drew the cell suspension into capillary tubes, sealed one end of each tube with "Seal-ease" (Clay-Adams, Inc., New York, N. Y.), and pelleted the cells by centrifugation at 170 × g for 5 min. The capillary tubes were broken at the cell-fluid interface and attached to sterile Mackness-type migration chambers with silicone grease. The chambers were then filled with a special medium (90% MEM + 10% unheated rabbit
serum), with or without heat-inactivated HSV of predetermined optimum concentration for migration-inhibition factor (MIF).

With an inverted microscope, we examined the capillary tubes 2 hr after they had been planted (to check for any spilling of cells) and again at 24 hr to measure the macrophage migration. After incubating the tubes for 24 hr at 37° C, we photographed the migration area with a Polaroid camera and measured it with a compensating polar planimeter (Keuffel & Esser Co., Morristown, N. J.). The mean area of migration was calculated by measuring duplicate tubes. The results were expressed by the following equation:

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\text{% inhibition of migration} = \left(1 - \frac{\text{mean area of migration with HSV}}{\text{mean area of migration without HSV}}\right) \times 100
\]

Evaluation of ocular lesions. Persons without prior knowledge of the Cy treatment schedule examined the eyes with a biomicroscope before each intraocular inoculation and daily thereafter. The uveal inflammation was scored according to a modification of the criteria established by Hogan et al.\textsuperscript{5} Five clinical signs—flare, cells, and fibrin clots in the anterior chambers, iritis, and lens precipitates—were each graded from 0 (no reaction) to 3 (maximum reaction). The scores of each of these signs of uveal inflammation were averaged separately for each group of similarly treated rabbits.

Measurement of intraocular pressure. As in our previous study,\textsuperscript{9} intraocular pressure (IOP) was measured daily for 11 days with the Mackay-Marg electronic tonometer. At least 10 tonograms were recorded for each eye, and the lowest consistent value was taken as an IOP of the eye. We then calculated the mean IOP and the standard deviation for each group. To prevent cross-infection, we used a new sterile TonoTip (Biotronics, Inc., Redding, Calif.) for each measurement of each rabbit eye.

Experimental design and results

Exp. A. Effect of Cy on clinical manifestations. Primary uveitis was produced in both eyes of 24 rabbits by the intracameral injection of 0.1 ml of HSV suspension containing about 10\textsuperscript{2} TCD\textsubscript{50}. The inoculated rabbits were then divided into two groups of 12 rabbits (24 eyes) each. On postinoculation day 2 and every second day thereafter, the rabbits in group I received a series of intramuscular injections of 80 mg of Cy and intramuscular injections of penicillin (100,000 U) and streptomycin (100,000 mg). The rabbits in group II served as controls and were not treated. We examined the eyes daily with the slit lamp and measured the IOP.

As shown in Fig. 1, cells and flare in the anterior chambers of the control rabbits began to appear on day 2, reached their peak on day 4 or 5, and persisted more than 11 days after the virus inoculation. Cells and flare in the Cy-treated rabbits were of the same severity as in the controls up to day 8 but had almost completely subsided by day 11. There was much less fibrin in the eyes of the treated rabbits than in the controls throughout the experiment.

Iritis and lens precipitates were also milder in the Cy-treated group on day 8 and had greatly diminished or disappeared by day 11. In the control eyes these changes were more severe and lasted longer.

As was the case in our previously reported study of rabbits with primary HSV uveitis,\textsuperscript{9} there was a biphasic rise of IOP. In the control rabbits the initial rise occurred on day 2, with return to the preinjection level on day 5, and a second rise on day 7 that lasted for over a week (Fig. 2). In the Cy-treated rabbits, however, the second rise was completely suppressed.

Exp. B. Virological and immunological studies. As in Exp. A, primary uveitis was
produced in both eyes of 32 rabbits by the intracameral injection of 0.1 ml of HSV suspension containing approximately $10^3$ TCD$_{50}$. The inoculated rabbits were then divided into two groups of 16 rabbits (32 eyes) each. Every second day from postinjection day 2, the rabbits in group I received a series of intramuscular injections of 80 mg of Cy as well as antibiotics in the same manner as in exp. A. Again the rabbits in group II served as controls. On days 3, 7, and 11, from four to six rabbits were randomly chosen from each of the two groups and were sacrificed. From these animals, blood was taken for neutralizing antibody titration, peritoneal exudates were used to measure macrophage MIF, and the eyes were removed for virological study.

As shown in Fig. 3, on days 3 and 7 the amounts of HSV recovered from the eyes of both groups (Cy-treated and untreated) were about the same. On day 11, however, the eyes of the Cy-treated rabbits contained far more virus than the control eyes. The range was from $10^3$ to $10^6$ TCD$_{50}$ in the Cy-treated group and from less than 10 to $10^2$ TCD$_{50}$ in the control group.

As shown in Fig. 4, no neutralizing antibody could be detected in the blood of the control animals on day 3. But it could be detectable on day 7 and was present in moderately high titers (1:8 to 1:32) on day 11. Throughout the study, however, almost no antibodies could be detected in the blood of the Cy-treated rabbits. MIF was also markedly inhibited in the Cy-treated group compared with the controls (Fig. 5). The average percentage of migration inhibition on day 11 was 48% in the control rabbits and 22% in the treated rabbits (p < 0.01).

Discussion

In this study, Cy treatment did not affect uveal inflammation or virus multiplication in
the uveal tissues in the early stage of the disease (prior to day 7). During this period, neither circulating antibody nor MIF against HSV could be demonstrated in control or Cy-treated animals. This suggested that viral infection of the uveal tissues, rather than immune-mediated reactions, was responsible for the early phase of the uveitis. In the late phase of the disease (after day 7), however, the Cy-treated animals showed far milder uveitis of much shorter duration than did the control animals. This clinical improvement in the Cy-treated rabbits coincided with significant inhibition of both circulating antibody and MIF and with sharp enhancement of viral growth in the uveal tissues. It seemed clear that the late phase of the uveitis was mediated mainly by immune mechanisms rather than by the infectious process.

We showed in an earlier study\(^9\) that there was a biphasic elevation of IOP in primary herpes simplex uveitis in rabbits. In this study an initial rise was first observed on day 2 and lasted through day 5, and a second rise began on day 7 and lasted for over 10 days. The initial rise could be suppressed by aspirin (indicating a prostaglandin-mediated change), but the second rise could be suppressed, not by aspirin but by corticosteroid, a drug which is known to possess immunosuppressive activity.\(^9\) In our present study, Cy treatment also abolished the second rise, suggesting that the second rise was associated with an immune reaction in the uveal tissues.

The specific immunological process responsible for the late phase of primary herpes simplex uveitis is not yet clear. Cy treatment suppressed both circulating antibody and MIF, and therefore still unanswered is the question of which immunological component was responsible for the late phase of the uveitis. Several immunological mechanisms have been thought to be responsible for the inflammation in HSV infection. (1) Viral antigens on the host-cell surface may interact with sensitized lymphocytes, or with antiviral antibody and complement, leading to the destruction of infected cells long before they could break down as a direct reaction to viral replication.\(^10\)\(^,\)\(^11\) (2) Stimulation of sensitized lymphocytes by viral antigens may release various lymphokines that could mediate inflammation.\(^12\) (3) Virus-antibody complex may produce immune-complex disease.\(^13\)\(^,\)\(^14\) Future studies may identify which of these mechanisms is responsible for the late phase of primary herpes simplex uveitis.

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**REFERENCES**