Effect of IUDR and amethopterin on experimental herpes simplex keratitis

Martin E. Corwin,* Virginia Coleman, Sidney Riegelman, Masao Okumoto, Ernest Jawetz, and Phillips Thygeson

The antimetabolite, S-iodo-2'-deoxyiuridine (IUDR), altered significantly the normal course of herpes simplex keratitis in rabbits when given every 2 hours during the day (8:00 A.M. to 4:00 P.M.) for 2 days only.

In qualitative experiments, virus was recovered as regularly from herpes simplex virus-infected rabbits receiving IUDR as from control rabbits. In a quantitative experiment performed on a few rabbits, virus was recovered in the same titer from IUDR-treated rabbits as from control rabbits for the first 2 days of therapy. Not until the third day was a lower titer observed in the IUDR-treated rabbits.

The addition of amethopterin did not alter the clinical picture, course of the experimental disease, or the virus recovery rate from herpes-infected rabbits.

Kaufman introduced the use of the antimetabolite, 5-iodo-2'-deoxyuridine (IUDR), in experimental and human herpes simplex keratitis. The drug's favorable effect on experimental herpes keratitis in rabbits has been confirmed in our laboratory and by the work of others. Its contribution to the management of the disease in humans is presently under investigation at the University of California San Francisco Medical Center and at various other centers of ophthalmology throughout the world.

This paper describes our recent experience with IUDR in experimental herpes simplex keratitis. We were able to modify the therapeutic regimen previously followed without changing the favorable effect of the IUDR, and to make qualitative and quantitative studies of the virus yield from IUDR-treated animals.

Materials and methods

Rabbits. The experimental animals were New Zealand white rabbits weighing 4 to 5 pounds each. They were obtained from a local breeder.

Virus. The strain of herpes simplex virus was the PH strain, also known as the O strain, and obtained originally from Dr. W. McD. Hammon. Infected mouse brain from a twenty-fourth passage...
IUDR and amethopterin in herpes simplex keratitis

### Table I. Scale of weighted scores for grading the severity of ocular lesions

#### I. Cornea

<table>
<thead>
<tr>
<th>A. Opacity, degree of density (area which is most dense is taken for reading)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scattered or diffuse area, details of iris clearly visible</td>
</tr>
<tr>
<td>Easily discernible translucent areas, details of iris slightly obscured</td>
</tr>
<tr>
<td>Opalescent areas, no details of iris visible, size of pupil barely discernible</td>
</tr>
<tr>
<td>Opaque, iris invisible</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>B. Area of cornea involved</th>
</tr>
</thead>
<tbody>
<tr>
<td>One quarter (or less) but not zero</td>
</tr>
<tr>
<td>Greater than one quarter, less than one half</td>
</tr>
<tr>
<td>Greater than one half, less than three quarters</td>
</tr>
<tr>
<td>Greater than three quarters up to whole area</td>
</tr>
</tbody>
</table>

Score equals \((A + B) \times 10\) total maximum = 80

#### II. Conjunctiva

<table>
<thead>
<tr>
<th>A. Redness (refers to palpebral conjunctivas only)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vessels definitely injected above normal</td>
</tr>
<tr>
<td>More diffuse, deeper crimson red, individual vessels not easily discernible</td>
</tr>
<tr>
<td>Diffuse beefy red</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>B. Chemosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Any swelling above normal (includes nictitating membrane)</td>
</tr>
<tr>
<td>Obvious swelling with partial eversion of the lids</td>
</tr>
<tr>
<td>Swelling with lids about half closed</td>
</tr>
<tr>
<td>Swelling with lids about half closed to completely closed</td>
</tr>
</tbody>
</table>

C. Discharge

| Any amount different from normal (does not include small amount observed in inner canthus of normal animals) | 1 |
| Discharge with moistening of the lids and hairs just adjacent to the lids | 2 |
| Discharge with moistening of the lids and considerable area around the eye | 3 |

Score \((A + B + C) \times 2\) total maximum = 20

---

was suspended (20 per cent) in skimmed milk and stored in a dry ice cabinet for several weeks. The LD₅₀ of the stock virus was 10⁻⁶ when titrated in mouse brain. The PH strain, which has been used in our laboratory for many years, produces in rabbits a severe keratitis with low mortality from encephalitis. The first signs of keratitis appear regularly within 48 hours.

**Drugs.** The compound 5-iodo-2'-deoxyuridme* is poorly soluble in water and is therefore commonly used as an 0.1 per cent aqueous solution. To enhance its solubility and thus its potential activity, the drug was dissolved in a sterile, semiaqueous solution containing polyethylene glycol. In this nonirritating, water-miscible, neutral vehicle—sterilized by autoclave and while still hot—it was possible to dissolve 0.9 per cent IUDR. The solution was then cooled at once by immersion in ice.

A fifty milligram per cent solution of amethopterin was prepared by dissolving sterile amethopterin powder in some of the same vehicle used to make the IUDR solution.

**Tissue culture.** Maben cells, derived from adenocarcinoma of the lung and carried in continuous culture in our laboratory, were used for all virus isolation attempts. The growth medium was composed of 10 per cent calf serum, 45 per cent Hanks balanced salt solution, and 45 per cent mixture 199 containing penicillin, 300 meg. per milliliter and streptomycin, 500 meg. per milliliter. The maintenance medium was 2 per cent horse serum, 40 per cent mixture 199, 57 per cent Hanks balanced salt solution, 0.5 per cent peptone, and 0.5 per cent dextrose, and also contained penicillin, 300 meg. per milliliter and streptomycin, 500 meg. per milliliter.

Cell sheets were prepared in test tubes as follows: 80,000 to 80,000 cells were seeded into tubes to which 0.5 ml. growth medium was added. The tubes were incubated at 37°C for 3 to 5 days. Confluent monolayers were then ready for use. All of the growth medium was decanted from the tubes before inoculation, and after inoculation 0.5 ml. maintenance medium was added. The tubes were held for 12 days and read daily for signs of the cytopathogenic effect (C. P. E.) typical of herpes simplex infection in this cell line—a degeneration consisting of areas of rounded refractile cells in plaques, limited at first, then spreading, multiplying, and gradually enlarging, with the center cells dropping off the glass. With this strain of virus in Maben cells, the sequence

---

*Supplied by Dr. Guisachi of Smith Kline & French Laboratories.
of events always progresses to complete infection of the cell sheet, all cells eventually rounding, clumping, and dropping into the medium.

Corneal inoculation and preparation of specimens. The corneal epithelium of both eyes of New Zealand rabbits received five vertical and five horizontal scratches with the edge of a Lindner platinum spatula. Each scratch was approximately half the diameter of the rabbit's cornea and down to the level of superficial stroma. A drop of mouse brain suspension of PH virus was then instilled in the fornix. The lids were held closed for 1 minute. The right eye was examined at frequent intervals for evidence of corneal staining, corneal opacities, chemosis, injection, and discharge. The cornea of the left eye was scraped with the edge of a Lindner spatula at intervals of 24 to 72 hours to obtain material for virus isolation. Half of the corneal epithelium was removed at each scraping, the superior and inferior halves being scraped alternately so as to allow a period of at least 48 hours for epithelial regeneration between scrapings of the same area.

Each epithelial specimen to be tested for virus was placed in 0.5 ml of mixture 199. The entire sample was then pipetted onto one cell sheet. Appearance of the typical cytopathogenic effect established the presence of herpes simplex virus infection.

Specimens destined for virus titration were placed in 0.5 ml of maintenance medium in a 2 ml glass tube. These tubes were placed in the refrigerator for up to 2 hours and the specimens ground as soon as possible with a chilled, fitted, Teflon pestle. Tenfold dilutions from $10^{-1}$ to $10^{-6}$ were then prepared in mixture 199. From each dilution, 0.3 ml was inoculated onto one cell sheet. The cell sheet was observed until C.P.E. was unequivocal or until the cells were discarded as uninfected.

Scoring system. In order to simplify the task of comparing the rabbits, a modification of the scoring system described by Draize and co-workers was devised (Table I).

Results

In a previous paper we reported the results of using IUDR (0.1 per cent) on experimental herpes keratitis in rabbits every 2 hours around the clock for 5 days. Subsequently, as described above, we developed for experimental use an 0.9 per cent solution of the compound. This concentration was used in all three of the following experiments. Clinically the course of the disease was the same whether treated with 0.1 per cent IUDR or 0.9 per cent IUDR.

Experiment 1. Fifty rabbits in 5 groups of 10 rabbits each were inoculated with a drop of mouse brain suspension of PH virus. Forty-eight hours after inoculation, Groups A, B, C, and D received IUDR (0.9 per cent) as follows:

- Group A. IUDR every 2 hours around the clock for 5 days.
- Group B. IUDR every 2 hours around the clock for 10 days.

Fig. 1. Comparison of epithelial ulceration as measured by fluorescein stain in IUDR-treated animals and control animals. Refer to section I, B (Table I) for scoring system.

Fig. 2. Comparison of epithelial ulceration as measured by fluorescein stain in IUDR treated animals (2 and 5 days) and control animals at various intervals after infection. Refer to section I, B (Table I) for scoring system.
IUDR and amethopterin in herpes simplex keratitis

Group C. IUDR every 2 hours during the day (8:00 A.M. to 4:00 P.M.) for 5 days.

Group D. IUDR every 2 hours during the day (8:00 A.M. to 4:00 P.M.) for 10 days.

The rabbits in Group E were untreated controls.

The right corneas of all rabbits were examined at frequent intervals. The usual clinical course of herpes simplex keratitis was modified characteristically by IUDR whether it was given for 10 days or for only 5 (Fig. 1), and treatment during the day only was as effective as treatment around the clock. In a variation of the experiment, the effect of 5 days of daytime IUDR therapy was then compared with the effect of only 2 days of daytime therapy (Fig. 2). No clinical difference between the results of 5 days' and 2 days' treatment could be demonstrated, nor was there any significant difference in virus recoverability whether the animals were treated for 5 days, for 2 days only, or not at all (Fig. 3).

Experiment 2. Forty rabbits, inoculated as in Experiment 1, were divided into four groups of 10 rabbits each. Forty-eight hours after inoculation, Groups A, B, and C received IUDR (0.9 per cent) as follows:

Group A. IUDR every 2 hours during the day for 5 days.

Group B. IUDR and amethopterin every 2 hours during the day for 5 days.

Group C. Amethopterin alone every 2 hours during the day for 5 days.

The rabbits in Group D were untreated controls.

All animals were examined at frequent intervals. Within 24 hours the rabbits in Groups A and B showed the improvement characteristic of IUDR-treated rabbits (Fig. 4). Group C, which received amethopterin alone, pursued a clinical course indistinguishable from that of the untreated animals in the control Group D. Virus could be recovered with equal ease from all four groups.

Experiment 3. Sixteen rabbits, inoculated as in Experiment 1, were divided into two groups of 8 rabbits each. The animals in Group A received IUDR every 2 hours during the day for 5 days; those in Group B were untreated controls. The right eyes were examined at frequent intervals. The corneas of the left eyes were scraped to obtain material for qualitative virus isolation. The corneas of 3 rabbits in each group were also scraped to obtain specimens for quantitative virus determinations. The IUDR-treated animals showed the usual clinical improvement within 24 hours, and herpes virus was recovered with equal ease and regularity from both groups (Fig. 5). In the titration experiments, moreover,
Discussion

Our initial experience in Experiment 1 showed clearly that only minimal amounts of IUDR were necessary to alter the usual clinical course of herpes simplex keratitis in rabbits. We were unable to detect any greater improvement by using higher concentrations of IUDR (0.9 per cent) or by extending the therapy to one drop every 2 hours for 10 days. Clinical improvement and virus recoverability proved to be dis-associated, however, since we were able to recover virus from rabbits treated with IUDR for 2 days and 5 days with the same ease as from control rabbits.

The reason for this dissociation between virus recovery and clinical appearance is difficult to determine. Perhaps with our method of inoculation, virus was deposited in the superficial layers of the stroma where it multiplied without interference from the IUDR. Other possibilities are (1) that the virus strain we used was peculiar in some respect, or (2) that the IUDR exerted a suppressive effect on the inflammation.

This problem was examined by Holmes and associates, who treated herpes-infected rabbits with IUDR 36 to 48 hours after infection. These animals received one drop of IUDR every hour during the day and every 2 hours at night. In the treated eyes a rapid drop in virus titer occurred during the first 12 hours of treatment. The titer remained low for 4 days and then increased. Meanwhile, the clinical disease worsened after 3 days of therapy, and at the end of 6 days all animals had severe disease. In contrast, our animals usually responded rapidly to treatment but continued to yield virus.

Holmes states that IUDR inhibits corneal epithelial cell DNA as well as viral DNA. Clinically this did not seem to be the case with rabbits. Uninfected rabbits, in which a standard corneal abrasion was made within the area of a 7 mm. corneal trephine, were given 0.9 per cent IUDR for 5 days during the day. These abrasions healed in the same time as the abrasions in a control group which received no therapy.

In an attempt further to suppress herpes virus production in infected rabbits, we combined amethopterin with IUDR in Experiment 2. Amethopterin prevents the synthesis of thymidine, which is essential for the formation of DNA. Since IUDR appears to act at a terminal stage of DNA synthesis, the combined therapy could theoretically be of value. In fact it ap-
IUDR and amethopterin in herpes simplex keratitis

appeared to be no better than IUDR alone. One possible explanation is that the amethopterin in the dosage used in our experiment was not absorbed by the corneal epithelium.

In a final attempt to determine why clinical improvement should occur despite the continued presence of virus, virus titer were determined in 3 rabbits in each group of Experiment 3. Only after 2 days of IUDR therapy were lower titers observed in the three IUDR-treated rabbits than those in the 3 controls.

It is evident that in our hands IUDR dramatically alters the clinical appearance of herpetic keratitis in rabbits but suppresses virus production only slightly. Perhaps a small decrease in virus concentration (titer diminished by less than one log) reflects a sufficient reduction in virus production to account for the clinical difference between control and treated rabbits.

REFERENCES

4. Leopold, I.: Personal communication.