"Treatment-Resistance" to Idoxuridine in Herpetic Keratitis* (33419)

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Whenever a new antimicrobial drug has been used on a large scale in medical treatment, the selection pressure exerted by that drug has favored the emergence of drug-resistant microbial variants. Failure of clinical treatment has often been the first indication of the emergence of drug resistance. Idoxuridine (5-iodo-2-deoxyuridine, IUDR) was the first drug employed on a significant scale in the United States for the treatment of a human viral infection, herpes simplex keratitis (1). During the past 4 years ophthalmologists, observing patients with herpetic keratitis who failed to respond to IUDR treatment, have speculated as to the nature of this "resistance" and its possible increasing frequency.

Buthala (2), Smith (3) and others (4–6) have studied the emergence of IUDR-resistant herpes simplex viruses (HSV) in cell culture. Repeated passage of HSV in medium containing IUDR promptly yielded strains that could replicate in the presence of IUDR up to 500 μg/ml. About 1 in 1000 or 1 in 10,000 particles appeared to be highly IUDR-resistant, and this resistance was stable on passage. On the other hand it has been reported (7) that some HSV strains are fully susceptible to IUDR *in vitro* yet are treatment-resistant *in vivo*, because they escape the action of the drug by rapidly progressing into the depths of the infected cornea. Therefore, it was of interest to determine whether HSV strains of increased resistance to IUDR could be isolated from patients whose herpetic keratitis had failed to respond to IUDR treatment. As a corollary we determined the influence of IUDR treatment on the frequency of HSV isolation from herpetic keratitis in patients, and made observations on IUDR resistance induced in HSV isolates *in vitro* and in herpetic keratitis of rabbits.

Materials and Methods. Patients. Seventy-seven patients with entirely typical dendritic keratitis were referred for virus isolation by the eye clinic or by individual ophthalmologists. The duration of the eye lesions varied from a few days to 4 months. All patients were subjected to a complete ophthalmological examination, including biomicroscopy, before specimens for virus isolation were obtained.

Virus isolation. Material for attempted HSV isolation was obtained by swabbing corneal epithelium with a sterile cotton swab that was immediately placed into 1 ml of fluid cell culture medium (2% calf serum in Eagle's minimum essential medium containing penicillin 100 μg/ml and streptomycin 100 μg/ml). The swab was agitated, fluid expressed, and inoculated into cell cultures immediately. An occasional specimen had to be held at −60° for a few days. Only 1 specimen per patient was examined. Monolayers of Maben cells (a line derived from human adenocarcinoma) were used, incubated in stationary tubes at 37° and inspected for cytopathic effects daily. Isolates were identified by neutralization with specific anti-herpes rabbit serum (7).

Susceptibility of isolates to IUDR in vitro. A plaque assay in strain L cells was performed as described previously (7). Measured amounts of IUDR were incorporated in a methocel overlay. A standard laboratory strain (PH) was included in each test for comparison, and as a control for the potency of the IUDR.

HSV keratitis of rabbits. Keratitis was induced in 18 rabbit eyes as described in detail elsewhere (7). Within 48 hr all eyes ex-

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TABLE I. Patients with Typical Dendritic Lesions of Herpetic Keratitis.

	No. of patients	Herpes virus isolated	
		Number/total	% Positive
I. Patients received no IUDR prior to collection of specimen	27	21/27	77.8
II. Patients who had received IUDR during 3 weeks prior to collection of specimen but not during 48 hr preceding specimen	10	6/10	60.0
III. Patients who had received IUDR during the 48 hr preceding collection of specimen	40	5/40	12.5
TOTAL with typical dendritic lesions	77	32/77	41.6

hibited typical epithelial lesions. Pretreatment specimens were obtained by scraping the epithelium, and then 1 drop containing IUDR 0.1% was placed in each infected eye every 4 hr day and night for 5 days. Epithelial scrapings were obtained again 1 or 2 days after the end of IUDR administration. The isolates obtained before treatment were compared with those obtained after treatment by plaque assay for IUDR susceptibility.

Results. Influence of IUDR on isolation rate of HSV from herpetic keratitis. In herpetic keratitis involving the corneal epithelium, HSV is usually isolated with ease. Single specimens tend to give an isolation rate of 75% or more. If most HSV were susceptible to IUDR, attempted isolation should frequently fail in patients receiving the drug and should succeed in those not receiving the drug. Conversely, if most HSV became resistant to IUDR during treatment, the isolation rates should be similar in patients with active epithelial lesions regardless of recent IUDR administration. During 1964–1967, 77 patients with typical dendritic lesions of herpetic keratitis were studied (Table I). In all of them, epithelial involvement was manifest at the time of securing single specimens of corneal epithelium for virus culture. Twenty-seven of these patients had not received IUDR prior to the examination. The isolation rate in these individuals was 77.8%, comparable to some earlier series. In the remaining 50 patients IUDR had been administered, but the clinical activity of herpetic keratitis had continued unabated. These 50 patients thus were "treatment-resistant" to IUDR on clinical grounds. Ten of these individuals had stopped using IUDR 48 hr or more before the specimen was obtained. From 6 of them, virus was cultured, a rate of 60%. By contrast, virus was isolated from only 5 of 40 patients (12.5%) who continued using IUDR during the 48 hr preceding specimen collection. This suggests strongly that in the "clinically IUDR-resistant" patients the infecting HSV was markedly inhibited by IUDR, even after weeks of drug use, and that IUDR-resistant HSV does not prevail in IUDR-treated herpetic keratitis.

Measurement of IUDR-susceptibility of HSV isolates from "IUDR-resistant keratitis." Isolates were obtained from 12 patients with herpetic keratitis that persisted for days or weeks during the application of IUDR. The drug had been given in 0.1% concentration in the form of eye drops instilled every 2 hr day and night, and occasionally as ointment placed into the conjunctival sac every 6–8 hr. The isolates were passed once or twice in cell culture soon after the appearance of the initial cytopathic effects, in order to make infective pools. The pools were stored at —60° and assayed repeatedly against twofold concentrations of IUDR incorporated into the cell culture overlay in a plaque reduction test. Every test included a stable laboratory strain of HSV (PH) for comparison; it regularly showed a reduction in plaque count of 95–98% with 2 μg/ml IUDR, and of 99.5% or greater with 8 μg/ml IUDR.

Ten of the 12 isolates from "IUDR-resistant keratitis" were indistinguishable in this IUDR susceptibility assay from the la-

TABLE II. Number of Plaques Expressed as Percentage of Number in Drug-free Control.

IUDR (μg/ml) in overlay	PH[a] IUDR-sens.	PH[b] IUDR-resist.	Reg[c]	McGil[c]	Rabbit eye[d] before IUDR	Rabbit eye[e] after IUDR
0	100	100	100	100	100	100
0.5	18	76	92	61	51	42
1.0	12	69	45	47	22	19
2.0	3	36	31	30	7	5
4.0	0.4	12	31	29	0.9	0.8
8.0	0.2	6	14	18	<0.1	<0.1
16.0	<0.1	1	10	12	<0.1	<0.1

[a] Standard laboratory strain PH included in every assay.

[b] Variant derived from *a* after 6 passages in concentrations of IUDR increasing from 0.5 μg/ml to 8 μg/ml in cell culture.

[c] Isolate from a patient with herpetic keratitis that failed to respond promptly to IUDR by clinical observation.

[d] Strain WAL isolated from 2 rabbit eyes 48 hr after inoculation of cornea, and prior to treatment.

[e] Strain WAL isolated from 4 rabbit eyes (pooled specimens) on day 8 after inoculation, one day after end of 5 days' treatment with IUDR 0.1% as described in text.

boratory strain PH. These 10 isolates exhibited a plaque reduction of 95–98% with IUDR 2 μg/ml incorporated in the methocel overlay. Two isolates (McGil, Reg) appeared to be significantly more resistant. IUDR 16 μg/ml or more was required to reduce the plaque count by 85–90% and IUDR 2 μg/ml reduced the count by only 70% (Table II). From repeated plaque-reduction tests, it was judged that early passages of isolates McGil and Reg were 10–30 times more resistant to IUDR than the other strains tested. However, beginning with the fifth passage of these two strains in cell culture, IUDR resistance was no longer demonstrable. Some possible explanations are discussed below.

Emergence of IUDR-resistance during exposure of HSV in vitro. In view of the rarity of IUDR-resistant HSV isolates and their apparent lack of stability, it was desirable to examine the emergence of IUDR-resistant HSV in cell culture for comparison with the results reported by others (2–6). The HSV laboratory strain PH was grown in L cells in increasing concentrations of IUDR. When cytopathic effects appeared, usually in 3–5 days, a portion of culture supernate containing also detached cells was passed to a fresh cell monolayer with a higher concentration of IUDR. The IUDR level permitting some viral replication and cytopathic effects was thus increased from 1 to 8 μg/ml. Repeated passage at the latter concentration resulted in delayed and very slowly progressing cytopathic effects. The titer of infective virus was 10^5 PFU/ml as compared to 10^8 PFU/ml in drug-free controls. Assay of these IUDR-passed strains indicated a significantly greater IUDR-resistance than the parent strain (Table II). The stability of these derived strains in the absence of IUDR was not investigated.

Attempts to demonstrate IUDR-resistant HSV in herpetic keratitis of rabbits treated with IUDR. We may have failed to isolate some IUDR-resistant HSV from human keratitis because the original inoculum was not propagated in the presence of IUDR. If HSV were IUDR-dependent, or IUDR-resistance were very unstable; or if IUDR-resistant HSV were present as a very small proportion of the viral population and replicated more slowly than the IUDR-susceptible population, we may have failed to detect them in first passage. Therefore we produced herpetic keratitis in rabbits, removed specimens of corneal epithelium before and after IUDR treatment of these animals, and isolated HSV in the presence and in the absence of IUDR in cell culture. We have shown earlier that

HSV can usually be recovered from the rabbit cornea after 5 days of treatment, in spite of apparent "clinical cure" of lesions (7).

HSV strains PH or Wal were inoculated into the eyes of 12 rabbits. Specimens from their corneas were taken on day 2 after infection (prior to the initiation of IUDR treatment) and on day 8 after infection (1 day after the end of IUDR treatment). Parallel specimens were pooled and inoculated into duplicate cell cultures, with and without IUDR 1 μg/ml. Virus was recovered in all 8 instances with no noticeable difference in the time of onset or progression of cytopathic changes in the cell cultures with or without IUDR. Assays for IUDR resistance were done on the first passage of these isolates. There was no difference in IUDR-susceptibility of the isolates obtained before treatment and those obtained after 5 days of IUDR treatment (Table II). The plaque count of all 8 isolates was reduced 95–98% by IUDR 2 μg/ml with an input of from 80 to 7000 plaque-forming units per plate. Thus no evidence was obtained for the presence of significant numbers of IUDR-resistant particles in the virus population of IUDR-treated corneas.

Discussion. The results presented in this paper cannot be compared without reservations with those published earlier (2–6) because of differences in the techniques employed. Furthermore, we attempted purposely to evaluate the impact of IUDR drug use on the eye in large numbers of patients with herpetic keratitis and set experimental conditions to imitate natural ones. Buthala (2) concluded that a single passage of HSV in the presence of IUDR resulted in a virus culture resistant to IUDR. Smith (3) also recovered IUDR-resistant HSV fairly readily from infected and IUDR-treated cell cultures. In our hands, repeated passage of HSV in cell culture yielded an IUDR-resistant strain, but this strain replicated less well in the presence of IUDR than the IUDR-sensitive parent strain did in drug-free medium. This could be explained by the observation that the cell line (L) used in this experimental system grew more slowly in the presence of IUDR, and—at a concentration of 8–16 μg/ml IUDR in the medium—had a shortened span of viability, as evidenced by staining with neutral red. One might speculate that for this reason IUDR-resistant particles were not released from cells in significant numbers, thus accounting for our uniformly lower titers in IUDR-grown HSV pools. This could be analogous to the situation in the human cornea, where proliferation of cells proceeds slowly, even in the absence of IUDR. Under natural conditions the application of IUDR to HSV-infected eyes might yield resistant mutants relatively frequently, but if these mutants replicated more slowly than the IUDR-sensitive members of the virus population they might soon be lost. In fact, IUDR application to herpetic keratitis continued in 1967 to suppress the viral population of most HSV-infected eyes (Table I), and only rare isolates exhibit IUDR-resistance by direct assay. We have shown earlier (7) that many isolates from patients who fail to respond to IUDR treatment are fully IUDR-susceptible by assay, but rapidly progress into the depths of the cornea and thus escape IUDR action *in vivo*. The survey of isolation frequency in relation to IUDR administration does not support the belief that widespread biochemical IUDR-resistance has yet emerged.

The marked suppression of HSV population in most infected eyes treated with IUDR does not seem compatible with the postulated emergence of IUDR-resistance on the same scale as resistance to benzimidazoles or guanidine (6). Thus we conclude that IUDR-resistant HSV do not readily replace the IUDR-susceptible HSV population during IUDR treatment *in vivo*, although there can be no doubt that IUDR-resistance can be observed readily *in vitro*.

Among 12 isolates from the eyes of patients who failed to respond to IUDR treatments, only 2 isolates were unequivocally resistant to the drug by plaque-reduction assay. This biochemical resistance was not stable and could be detected only during the first 5 passages in cell culture, whereas some plaque-purified resistant mutants were quite stable (2, 5, 8). The reason for this difference is not clear unless population dynamics,

as described above, could be responsible. It is of interest that these 2 strains, both isolated from children, are highly encephalitogenic when inoculated into rabbit cornea.

Underwood *et al.* (4, 5) observed the emergence of IUDR-resistant HSV during treatment of experimental keratitis in rabbits. After 6 or 7 days of treatment with 0.1% IUDR, new lesions appeared and 3 of 4 isolates from such lesions were resistant to IUDR *in vitro*. In the rabbit HSV keratitis model employed in our laboratory, treatment was administered for only 5 days, and we failed to induce IUDR-resistance in any of the HSV isolates obtained 1 and 2 days after the end of IUDR treatment. Again we are at a loss to explain the differences in findings.

From the results presented here we conclude that the use of IUDR in a large majority of patients with HSV keratitis has had little impact to date on the emergence of IUDR-resistant HSV strains. The rabbit model suggests that IUDR-resistance may not be a frequent outcome of short IUDR treatment (5–7 days). While IUDR-resistant HSV can be produced by laboratory manipulations, the dynamics of virus populations may not favor the emergence of IUDR-resistant strains in human disease. The problem should be reinvestigated after several additional years of IUDR use.

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Correlation of Infectivity and Hemagglutinins of Reoviruses* (33420)

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Reovirus type 3 usually produces much less hemagglutinins (HA) than types 1 or 2 (1), whereas the infectivity titer of all three types reaches about the same level. Lerner *et al.* (2) suggested that HA units and infectivity units are correlated, since both were inactivated at about the same rate when the reoviruses were incubated over a prolonged period at 37°. Zalan and Labzoffsky (3) on the other hand showed that the HA of type 3 reached maximal titer only after a 2-week incubation time at 37° whereas maximal infectivity titer was reached 4–5 days after inoculation. They also reported that after incubation at 37° for 3–4 months the infectivity declined and disappeared, but the HA titer remained constant for as long as 8 months; hence there was no parallel increase or decrease of HA and infectivity. Also Usmankhodzhayev and Zakstelskaya (4) reported that the stability of infectivity and HA of these viruses was not the same at 37°. The infectivity decreased steadily over a period of 40 days, whereas the HA of reovirus types 1 and 2 remained constant, or showed a slight increase over the first 30 days and then declined rapidly; reovirus type 3 HA however was rapidly inactivated. From previous investigations we also demonstrated an infectious and noninfectious HA

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