Suppression of Herpes Simplex Virus Infection by Antimitotic Substances in the Rabbit Cornea[1] (35505)

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(Introduced by Wladyslaw Manski)
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The antiviral spectrum of colchicine and its related compounds includes both RNA and DNA viruses. Thus in mice colchicine was effective in suppressing influenza and encephalomyocarditis viruses in the earlier stage of infection (1). Similarly, a prolongation of the survival time of mice infected with mengo virus was obtained with vinblastine (2). Vincristine was found to suppress Friend and Rauscher leukemia viruses in mice (3), but was ineffective against mengo (2), vaccinia, polyoma, Rous sarcoma, and encephalomyocarditis viruses (4). Demecolcine has been reported to suppress vaccinia, polio, ECHO, and Coxsackie B viruses in tissue culture and Newcastle disease virus in the developing chick embryo (5). Colchicine has been reported to be ineffective against polio (6) and REO (7) viruses in tissue culture and against influenza virus in the developing chick embryo (8).

We recently have obtained results indicating that these compounds have a suppressive effect against herpes simplex virus in the rabbit cornea and in tissue cultures of rabbit kidney cells. We have determined the effective dose and duration of action and have investigated the development of drug-resistant mutant virus strains, and we report the results of these studies below.

Materials and Methods. Virus and tissue culture. The F strain of herpes simplex virus was used in the present study. It was isolated in our laboratory in primary rabbit kidney (RK) tissue culture (9) from scrapings of a human cornea with herpetic keratitis, and it was then subjected to 3 passages in RK tissue cultures. The tissue culture medium consisted of 5% calf serum and 1% lactalbumin hydrolysate in Earle's balanced salt solution containing 100 units/ml of penicillin and 50 μg/ml of streptomycin. The pH was adjusted to 7.2 with sodium bicarbonate. The cultures used consisted of ca. 10^6 cells per a conventional tissue culture tube with 1 ml of the medium. The fourth passage of the virus was made in 50 culture tubes and the virus was harvested with the cells and medium at the time of the maximal cytopathic effects. The harvested material was pooled and redistributed into ampoules for storage at −70°. When thawed, this preparation had a titer of $10^{6.5}$ plaque-forming units/ml. Only this fourth passage virus was used for the animal experiments.

Animals. New Zealand white and chinchilla rabbits of both sexes, weighing 4–5 kg, were obtained from the Glouchester Rabbitory, Chepachet, Rhode Island and the Camm Research Institute, Inc., Wayne, New Jersey, respectively. They were maintained on laboratory chow.

Ocular inoculation. The rabbit eye was anesthetized with 0.5% Ophthaine solution (Squibb & Sons, Inc., New York, New York) and infected by producing a small abrasion at the center of the cornea by tapping it 5 times with cotton swabs soaked with the virus preparation (10).

Evaluation of corneal lesions. For evaluation of the lesions, 1% sodium fluorescein

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solution was applied and the lesion was examined with a blue pen light. The stained lesions were graded in terms of the relation of the size of the lesion to the size of the cornea (11) as follows, 1 = ¼, 2 = ½, 3 = ¾, and 4 = whole area of the cornea. Values of 1.5, 2.5, and 3.5 were used to express grades intermediate between the above grades.

Virus assay. For titration of virus infectivity, RK culture tubes were inoculated with 0.1-ml portions of various dilutions of the material being assayed and then 0.1 ml of an appropriate dilution of rabbit antiherpes antiserum was introduced 60 min after the original inoculation of the virus. Microplaques (12) thus formed were counted. The virus content of the cornea was assayed as follows: the rabbits were sacrificed with Nembutal and the corneas were excised by trepanation with a trephine 11 mm in diameter. Each cornea was ground in a mortar and sand with 5 ml of Earle's solution at pH 7.2. The ground material was centrifuged for 20 min at 2000 rpm and the supernatant fluid was titrated for infectivity. Serum virus neutralizing antibody titer was determined conventionally by 50% reduction of plaque counts using the stock virus.

Chemicals. The following chemicals were dissolved in Earle's solution containing phenol red, adjusted to pH 7.0 with 0.1 *N* NaOH or HCl and then sterilized by passing through a Swinnex-25 filter unit (Millipore Corporation, Bedford, Massachusetts). Unless otherwise specified, the compounds were made up in concentrations of 1 mg/ml and 2 drops (0.1 ml) were introduced with a medicine dropper into the lower fornix of the rabbit eye. Colchicine (Nutritional Biochemicals Corporation, Cleveland, Ohio); demecolcine (Colcemid: CIBA Pharmaceutial Company, Summit, New Jersey); vinblastine (Velban) and vincristine (Oncovin): Eli Lilly Company, Indianapolis, Indiana). Following antiviral substances were used: 5-iodo-2′-deoxyuridine (IDU) (Stoxil: Smith, Kline & French Laboratories, Philadelphia, Pennsylvania); 5-bromo-deoxyuridine (BDU) (Nutritional Biochemicals Corporation); 5-fluorodeoxyuridine (FU) (Hoffmann-La Roche, Nutley, New Jersey); 1-β-D-arabinofuranosyl cytosine hydrochloride (CA) (The Upjohn Company, Kalamazoo, Michigan).

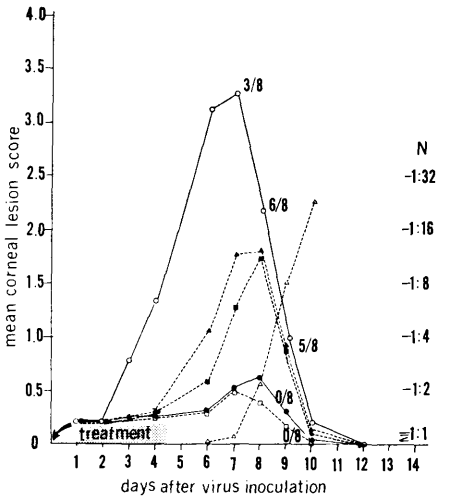

FIG. 1. Development of corneal lesions in rabbits during herpetic infection: (○) Earle's solution-treated controls (8 eyes); (▲) 1 mg/day of vincristine (8 eyes); (■) 250 μg/day of vinblastine (8 eyes); (●) 400 μg/day of colchicine (8 eyes); (□) 200 μg/day of demecolcine (8 eyes); and (△- -) *N* = virus neutralizing antibody titers in serum; (fractions) no. of opaque eyes/no. of total eyes.

Results. Development of corneal lesions. Twenty rabbits (New Zealand) were infected in both eyes with the virus. When punctate lesions had formed 24 hr later, 8 eyes were randomly selected for each of 5 groups, which were, respectively, treated with colchicine, demecolcine, vinblastine, vincristine and Earle's solution (control). The eyes were treated once daily for 1–4 days using the dose shown in Fig. 1. The grade of corneal lesions in the control group increased rapidly after day 3 and reached a maximum on day 7. The subsequent diminution of the lesions starting on day 8 was accompanied by an increase in the serum neutralizing antibodies against this virus. There was a corneal opacity on day 8 in 6 of 8 eyes in controls. A suppression of the lesions and the corneal opacity was noted in eyes trated with colchicine and demecolcine, while the eyes treated with vinblastine and vincristine showed partial protection against the lesions and the opacification.

In the next experiment, 6 rabbits (chinchilla) were bilaterally infected and the right eyes were treated with 100 μg/day of demecolcine for 1–8 days. The mean corneal lesion scores of treated and untreated eyes, respectively, were 0.2:0.2 on the 2nd day, 0.2:0.9 (3rd), 0.3:2.6 (4th), 0.6:3.2 (5th), 1.1:3.4 (6th), 1.5:3.8 (7th), 1.1:2.3 (8th), 0.3:2.0 (9th), and 0.1:1.5 (12th). The treated eyes showed no or significantly less conjunctival hyperemia, edema, and pus formation when compared with the untreated eyes. The ratio of the formation of corneal opacity on the 7th day was 0/6:2/6 and 0/6:5/6 on the 12th day. Four of the 6 untreated eyes developed further disciform keratitis and were persistently vascularized.

To determine the effect of a larger virus inoculum, 25 rabbits (chinchilla) were bilaterally infected by application of the virus with cotton swabs to the entire surface of the cornea instead of only to the center. The right eyes were treated with 200 μg/day of colchicine for 1–10 days. The development of corneal lesion scores for the treated and untreated eyes, respectively, were 0.2:0.2 (1st), 0.2:1.8 (2nd), 0.3:2.4 (3rd), 0.5:2.6 (4th), 0.8:2.7 (5th), 2.4:3.6 (6th), 2.9:3.3 (7th), 3.4:3.1 (8th), 3.3:2.6 (9th), and 2.6:2.2 (12th day), suggesting the breakthrough of protection between 6th and 9th days. On day 4, 7 rabbits were sacrificed for determination of the virus contents in the cornea (see below). Concurrent with the rapid development of the corneal lesions in this experiment there was a 50% mortality from encephalitis between days 6 and 14, which was not encountered in the experiments with smaller inocula.

Response to different doses of drugs. Thirty rabbits (chinchilla) were infected by application of the virus to the whole cornea of each eye. Twenty-four hr later, groups of 4 randomly selected eyes were each treated with 3 different concentrations of each of 4 compounds under study. The remaining 12 eyes were treated with Earle's solution (control). Treatment was given for 3 days and the corneal lesions were graded on day 4. After probit transformation of means of lesion scores (11), the dose–response patterns shown in Fig. 2 were obtained.

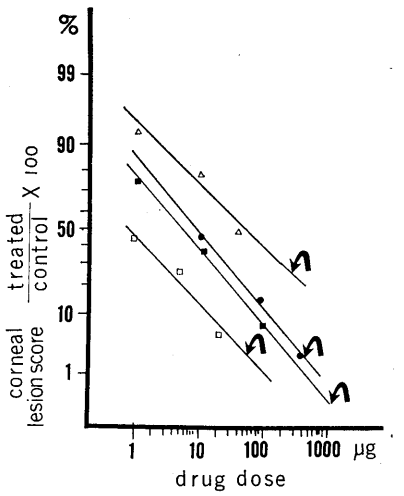

FIG. 2. Corneal responses to different doses of drugs: (△) vincristine; (●) colchicine; (□) vinblastine; (■) demecolcine. (arrows) indicate the points of formation of corneal opacity in the normal eye.

When drugs were applied to normal eyes for 4 days at concentrations shown by arrows in Fig. 2, there was the development of glittering corneal opacity (13) in 2 of 6 eyes beginning at the upper portion of the cornea, which returned to normal upon cessation of the drug applications. Since demecolcine was reported to be less toxic (14), we continued an application of demecolcine 100 μg/day to 12 normal eyes for 20 days. No development of corneal opacity was observed during this period.

Mode of suppression of the virus. Since there was an age-dependent sensitivity of the RK cells to the cytotoxicity of colchicine, with the younger cells being highly susceptible, in this experiment, we used 3-week-old cultures which had been refed twice weekly. Twenty min after the final refeeding with medium containing varying concentrations of cochicine, 1-ml amounts of fresh medium containing ca. 100 microplaque-forming units of the virus were added to the tube cultures. One group of RK culture tubes received the virus first, and later colchicine at various intervals. The effect of the two threshold concentrations of colchicine which suppressed the formation of microplaques after 24 hr are shown in Fig. 3. Since there was no significant difference in suppression between the

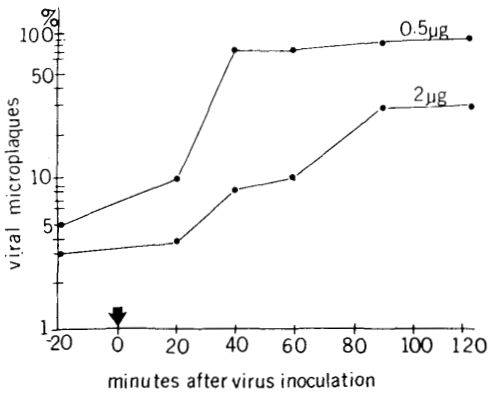

FIG. 3. Suppression of microplaque formation of herpes simplex virus by colchicine in rabbit kidney tissue culture cells.

addition of colchicine 20 min prior to or 20 min after the addition of the virus, this compound, under the present condition, did not prevent the adsorption of the virus onto cells. Rather, colchicine had interfered strongly with the earliest phase of viral synthetic processes, which may take place within 40 to 80 min following the virus challenge.

Development of drug-resistant mutant strains. In this experiment, RK tube cultures were refed with medium containing 10 μg/ml of colchicine and allowed to stand for 20 min at 37° after which 0.1 ml of stock virus was added to each tube and also to control tubes without colchicine. The tubes were then incubated for another 60 min. At the end of this period cultures were washed three times with 2 ml of fresh medium and then refed again with 1 ml of fresh medium without colchicine. Twenty-four hr after the inoculation of the tubes with the virus, the untreated cultures showed the maximal cytopathic effect (CPE), whereas the colchicine-treated cultures showed no changes. The average titer of the virus in the medium from the untreated cultures was $10^{6.2}$ plaque-forming units/ml, whereas it was $10^{3.7}$ in the colchicine-treated cultures.

We then divided the colchicine-treated cultures, one of which was left untreated and the other of which was again treated with colchicine as above on days 2 and 3. The former group of cultures showed CPE 48 hr after the original virus inoculation, but the latter showed suppression of the formation of microplaques until day 3, when a few microplaques were detected. A CPE developed in these cultures within the next 24 hr. The virus from these cultures was able to propagate (15) in both untreated and colchicine-treated cultures in new experiments, similar to the above. However, this virus was suppressed by 100 μg/ml of IDU and BDU and 10 μg/ml of CA, and like the parent virus it was not suppressed by 100 μg/ml of FU (16).

Comparable experiments were carried out similar to above colchicine with 100 μg of demecolcine, 5 μg of vinblastine, and 20 μg of vincristine. All of these compounds were capable of suppressing the virus during 1–3 days but there was the development of drug-resistant virus strains by the end of this period.

Virus strains form the colchicine-treated corneas. The results of the titration of the viral infectivity of the material from the infected corneas in 7 rabbits (see above) are summarized in Table I. As shown in Table I, the titer of the virus was suppressed in the colchicine-treated eyes to 8.7% of that in the untreated eyes. Moreover, 6 of 7 strains isolated in this manner from the colchicine-treated eyes were also not inhibited in colchicine-treated cultures used for the detection of drug-resistant mutants. In view of these *in vitro* results with respect to the development of resistance, it can be suggested that the second peak in the grades of the corneal lesions may have been produced by colchicine-resistant virus and/or the concurrent development of resistance (17) of the corneal tissue to colchicine in a later stage of the treatement.

Discussion. Depending on the intensity of the disturbances that colchicine produces in the cell, its effect can range from a simple inhibition of cellular migration (18), through arrest of mitosis at metaphase and profound interference with cellular activity which enables the cells to enter mitosis and finally to cell death (19). Virus multiplication may be affected by various stages of these colchicine-produced disturbances in physiology of host cells. Thus cells in mitosis are resistant to infection with herpes simplex virus (20) and

TABLE I. Determination of Virus Titers in the Infected Rabbit Corneas Treated with Colchicine.

Rabbit no.	Virus titers PFU[a] $\log_{10}$ Untreated (OS)	Treated (OD)	% OD/OS	Colchicine-resistance shown in cytopathogenicity in tissue culture: OS virus, Colchicine[b] With	OS virus, Colchicine[b] Without	OD virus, Colchicine[b] With	OD virus, Colchicine[b] Without
1	5.14	4.00		+[c]	4+	3+	3+
2	6.13	4.90		3+	4+	4+	4+
3	6.25	4.79		0	4+	4+	4+
4	6.13	5.14		0	4+	4+	4+
5	5.17	4.51		0	4+	4+	4+
6	6.12	5.10		+	4+	3+	4+
7	5.00	4.15		+	4+	2+	4+
Av	5.71	4.65	8.7				

[a] Cornea disc (11 mm in diameter) extracted in 5 ml. The values indicate plaque-forming units (PFU)/ml.

[b] Dose and method of application see text.

[c] Involvement of tissue culture by cytopathic effects: +, 25%; 2+, 50%; 3+, 75%; and 4+, 100%. Results from duplicate tube cultures.

vinblastine-produced metaphase cells were incapable of supporting the growth of Newcastle disease, vaccinia and polio viruses (21). The intracellular uncoating of rabbit pox virus was inhibited by colchicine, which was assumed to have blocked synthesis of messenger RNA (22). The latter type of inhibition was detectable even 15 hr after removal of the colchicine from the tissue culture medium. Furthermore, a loss of intercellular connections between the corneal cells due to the action of colchicine (18) may retard intercellular transfer of herpes simplex virus.

In perspective, the question arises of what metabolic pathways in the host cells that are affected by colchicine and its related compounds may be involved in the inhibition of the multiplication of a wide variety of viruses. The experimental utilization of properties of different drug-resistant mutants and a search for specific substances which can reverse the effect of these substances (19, 23) may provide useful information. In addition, a detailed analysis of cross resistance among drug-resistant mutants may be of value in suppressing the emergence of these mutants by the use of combinations of different drugs.

It first seemed possible that the healing of the rabbit cornea might be hindered by colchicine (18), due to its inhibitory activity of cellular migration and proliferation as shown *in vitro*. However, in contrast to the *in vitro* condition, colchicine and its related compounds did not appear to have inhibited the sliding capability of corneal epithelial cells to cover the superficial ulceration *in vivo*. Rather it appeared that the inhibition of cellular activity by these agents had not only suppressed the virus multiplication but also prevented rapid proliferation of highly susceptible cells. When treatment with the drugs was stopped, healing proceeded. Indeed, at later stages of the treatment regression of the corneal ulcer occurred in the presence of colchicine and demecolcine.

Summary. Colchicine, demecolcine, vinblastine, and vincristine were found to suppress infections of the cornea by a strain of herpes simplex virus in rabbits and of rabbit kidney tissue cultures. These agents were applied locally once every 24 hr for 1–4 or 1–10 days. The duration of treatment was limited by the necessity of avoiding the production of a glittering opacity of the cornea as a toxic manifestation of some of these agents. Because of its lower toxicity, demecolcine was found to be the agent of choice for studies of the antiviral activities of anti-

mitotic substances.

In the course of typical experiments, these agents, including demecolcine, aborted the infection at an early stage, reduced the local inflammatory reaction, and prevented the development of corneal opacity. When an overwhelming infection of the cornea or the kidney tissue cultures was produced, viral strains resistant to these agents developed.

1. Weinstein, L., and Chang, T.-W., Antibiot. Chemother. (Washington, D.C.) **10**, 180 (1960).

2. Johnson, I. S., Ann. N.Y. Acad. Sci. **130**, 52 (1965).

3. Chirigos, M. A., Ann. N.Y. Acad. Sci. **130**, 56 (1965).

4. Freeman, G., Kuehn, A., and Sultanian, I., Ann. N.Y. Acad. Sci. **130**, 330 (1965).

5. Katsilambros, L., Rev. Med. Moyen-Orient **19**, 318 (1962).

6. Kovács, E., Experientia **18**, 70 (1962).

7. Dales, S., Proc. Nat. Acad. Sci. U.S.A. **50**, 268 (1963).

8. Solovyov, V. D., and Mentkevich, L. M., Acta Virol. (Prague) Engl. Ed. **9**, 308 (1965).

9. Tokumaru, T., J. Immunol. **95**, 181 (1965).

10. Underwood, G. E., and Buthala, D. A., Ann. N.Y. Acad. Sci. **130**, 151 (1965).

11. Kaufman, H. E., Ann. N.Y. Acad. Sci. **130**, 168 (1965).

12. Farnham, A. W., Virology **6**, 317 (1958).

13. Kaufman, H. E., Capella, J. A., Maloney, E. D., Robbins, J. E., Cooper, G. M., and Uotila, M., H., Arch. Ophthalmol. **72**, 535 (1964).

14. Schär, B., Loustalot, P., and Gross, F., Klin. Wochenschr. **32**, 49 (1954).

15. Renis, H. E., and Buthala, D. A., Ann. N.Y. Acad. Sci. **130**, 343 (1965).

16. Herrmann, E. C., Proc. Soc. Exp. Biol. Med. **107**, 142 (1961).

17. Lettré, H., Ann. N.Y. Acad. Sci. **63**, 1022 (1956).

18. Weimar, V. L., Fellman, M. J., and Davis, M., Proc. Soc. Exp. Biol. Med. **131**, 1457 (1969).

19. Deysson, G., Int. Rev. Cytol. **24**, 99 (1968).

20. Roizman, B., Virology **13**, 387 (1961).

21. Marcus, P. I., and Robbins, E., Proc. Nat. Acad. Sci. U.S.A. **50**, 1156 (1963).

22. Joklik, W. K., J. Mol. Biol. **8**, 277 (1964).

23. Cutts, J. H., Biochem. Pharmacol. **13**, 421 (1964).

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