It has been demonstrated that a double-stranded ribonucleic acid (RNA), such as the polyinosinic-polycytidylic acid complex (In • Cn), induces antiviral resistance against a large number of viruses in a wide variety of tissues grown in culture because of interferon production. In addition, the interferon-like resistance and interferon production induced by In • Cn has been enhanced by the use of polybasic substances such as neomycin, streptomycin, and protamine sulfate. When In • Cn is applied topically to the rabbit eye, it is mildly therapeutic and significantly prophylactic against herpes simplex keratoconjunctivitis. Though herpes simplex virus (HSV) has been noted to be relatively resistant to the antiviral action of interferon, In • Cn has already been used to treat early stages of human herpes simplex keratoconjunctivitis.

Because of the theoretically possible beneficial effects of In • Cn on human ocular HSV, a study was undertaken to evaluate the in vitro effects of In • Cn on ocular tissues in culture. This report considers the antiviral effects of In • Cn on conjunctiva, cornea, and iris-ciliary body against
Fig. 1. Effect of In-Cn and neomycin on the yield of herpes simplex virus in various rabbit eye cultures infected with 5 TCID<sub>50</sub>. Assays were performed on supernatant fluid taken from cultures on the third day after infection.

HSV, as well as the efficacy of neomycin as an enhancer of the interferon-inducing effect of In·Cn in such tissues.

Materials and methods

Three-week-old New Zealand albino rabbits of both sexes were used for all tissue-culture source material. Primary rabbit kidney (PRK) tissue cultures were prepared by trypsinization. Minimal essential medium (MEM) plus 10 percent fetal bovine serum (FBS) were used as growth medium for the rabbit kidney tissue. The cultures were maintained with MEM, 2 percent FBS, and 1 percent glutamine at 37° C. in a 5 percent CO<sub>2</sub> atmosphere.

Growth medium for rabbit ocular tissue was Basal Media Eagle (BME) with 20 percent FBS and 1 percent glutamine supplemented with the addition of streptomycin, penicillin, tetracycline, and mycostatin (SPTM). Maintenance medium was BME and 2 percent FBS. All rabbit tissue cultures were grown and matured in screw-top tubes at 37° C.

Primary rabbit ocular tissue cultures were prepared from cornea, iris-ciliary body, and conjunctiva. Corneal explants were made by shaving the surface of the cornea with a scalpel blade and placing the tissue in 2 ml. of growth medium. Every effort was made to remove just epithelium, but all tissues contained a small number of stromal cells.

Iris-ciliary body cultures were prepared by dissecting this structure from the eye, mincing the tissue, and placing the segments in 2 ml. of growth medium. Conjunctiva was dissected from the limbus to the cul-de-sacs, minced, and placed in 2 ml. of growth medium.

Rabbit kidney monolayers were complete in about five days, while ocular monolayers took seven to ten days to grow. In·Cn was obtained in a concentration of 5 mg per milliliter* and was diluted to a concentration of 100 ng per milliliter with the appropriate maintenance medium.

Viral resistance was initiated by incubating cultures of all four tissue types with 100 μg of In·Cn at 37° C. The influence of neomycin on viral resistance was determined by adding 300 ng of neomycin sulfate to cultures containing 100 ng of In·Cn. Control tubes were incubated without In·Cn, and additional controls were incubated with neomycin alone.

After 24 hours of incubation, the media were decanted and three tubes of each culture were challenged with 5 or 50 tissue culture infective doses (TCID<sub>50</sub>) of HSV.† After an adsorption period of 2 hours at 37° C., the cultures were washed three times to remove excess virus and re-fed with maintenance medium. Cultures were then observed for seven days for cytopathic effect (CPE). After three days, the media were harvested, the cultures were re-fed, and virus titers were determined as TCID<sub>50</sub> in PRK cells by a

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*Obtained from Grand Islands Biological Company.
†Herpes simplex virus, 11183 strain, obtained from Dr. W. Ashe of the National Institutes of Health, Bethesda, Md.
Results

The results are shown in Figs. 1 and 2. The various eye tissue cultures incubated with In-Cn and challenged with 5 TCID₅₀ of HSV, yielded 1/10 to 1/200 as much virus as did the virus controls (Fig. 1). This effect was clearly enhanced by neomycin treatment, which further decreased virus yield by a factor of 5 to 2,000. The difference in virus yield between virus control cultures and those treated with In-Cn plus neomycin was greater than 2 log₁₀ in all tissues. In addition, iris-ciliary body and PRK cultures yielded no virus at all after In-Cn plus neomycin treatment. Controls had significant virus yields, identical to those obtained with neomycin alone.

Cultures, pretreated with In-Cn and infected with 50 TCID₅₀ HSV, yielded less virus than controls, but the degree of reduction was smaller and more variable than when the cultures were challenged with the lesser viral inoculum (Fig. 2). In fact, no diminution in virus yield was noted in conjunctival cultures. Neomycin enhancement at this viral challenge dose was slight in most tissues and absent in iris-ciliary body cultures.

Discussion

Though HSV is known to be relatively resistant to the inhibitory action of interferon, this study demonstrates that In-Cn, an interferon inducer, confers antinherpetic resistance to various ocular tissue cultures. This protection is enhanced when In-Cn is combined with a polybasic substance such as neomycin. In addition, the degree of protection in tissue culture is clearly related to the intensity of virus challenge in that viral resistance was noted with inoculums of 5 TCID₅₀, but the protection cytotoxic endpoint of seven days. A 0.7 log reduction in virus yield is statistically significant (P < 0.05) in a single assay. All results were confirmed in at least one or two additional experiments, thereby further increasing the reliability of the differences.
was apparently overwhelmed by inoculums of 50 TCID₅₀. Additional experiments have been carried out to show that it is interferon that is the protective factor after In-Cn treatment of iris and corneal tissue cultures.

Polybasic substances, such as neomycin, have been shown to enhance the interferon stimulating effect of polynucleotides in nonocular tissue cultures. The present findings show similar enhancement in several types of ocular tissue cultures. The available evidence indicates that the polybasic substances may act by increasing cellular uptake and ribonuclease resistances of ribopolynucleotides.

The ability of large viral inoculums to overcome interferon protection has been noted consistently in vivo but has not been generally observed in cell culture systems. For example, in rabbit eye studies, if systemic interferon protection is in-
duced with intravenous typhoid vaccine, anterior chamber growth of Newcastle disease virus is inhibited if the intracameral inoculums of this virus are low. Challenges with high virus inoculums produce an incidence of infection equal to controls, although the onset of disease may be delayed. On the other hand, in mouse embryo cultures, low doses of vesicular stomatitis virus are actually less sensitive to the antiviral action of interferon than are high doses of virus. Recent in vitro studies with interferon in other viruses of the herpes group, mouse and human cytomegaloviruses, concur with the observations of this study in that they are highly sensitive to the antiviral action of interferon when small virus challenge doses are used. However, when virus doses in excess of 100 TCID₅₀ are employed, the interferon effect is diminished by about 100-fold. These findings with the herpes
viruses, but not generally with other viruses in culture, suggest that the herpes virus group may be unusual in its interaction with the interferon system in vitro. Study of the controlling mechanisms in tissue culture may lead to an understanding of the systems which govern the resistance to interferon of large inocula of most viruses in vivo. The possibility that the size of the fraction of the virus population, which is not inhibited by interferon in a given system, is related to the virus dose-dependent resistance, is being studied.20

The observations of this investigation may be pertinent to the therapeutic potential of In·Cn, with the obvious limitation that cells in vitro may be quite different from cells in situ. The prophylactic response to In·Cn treatment of experimental herpetic keratoconjunctivitis in rabbits is greater than is the therapeutic re-
Fig. 6. (A) Normal rabbit conjunctiva tissue culture. (B) CPE in rabbit conjunctiva tissue culture 96 hours after infection with 50 ID₅₀ per milliliter of HSV.

This may be due to the relatively small virus dose which is applied after induction of interferon in a prophylactic study. In contrast, the same virus dose may replicate to much higher levels before interferon is induced in a therapeutic study and, thereby, lead to a resistant condition.

Herpes keratitis in man is typically a more indolent disorder. The number of virus particles and infected cells may be significantly lower in the human case than in the experimental counterpart. In Cn may, therefore, prove more effective against the milder human infection than it does in rabbits. Furthermore, the ability to enhance activity with polyanions, such as neomycin, may help. In any event, because of the demonstrated prophylactic ability of the interferon system and the potential therapeutic efficacy available by local therapy, well-controlled clinical stud-
ies seem in order if In·Cn proves to be nontoxic topically.

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REFERENCES