Sensitivity of Human Conjunctival Tissue Cultures to Adenovirus 8, Herpes Simplex and Vaccinia Viruses. Induction of Resistance by an Interferon Inducer[1] (37161)

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Adenovirus type 8 is the principal cause of epidemic keratoconjunctivitis in man in natural and experimental infections (1). Though the clinical recognition of this syndrome can be confirmed by suitable laboratory studies, the extremely slow growth of this virus in many tissue culture systems has hampered the development of more efficient isolation methods.

The conjunctiva is both the portal of entry and of final localization of adenovirus type 8 in man. Keeping this in mind it seemed interesting to find out whether and to what extent human conjunctival cells propagated in tissue culture would support the replication of this virus as well as of other common agents causing viral conjunctivitis, like herpes simplex and vaccinia viruses. The sensitivity of the conjunctival cells, infected with these viruses, to interferon and an interferon inducer, polyinosinic-polycytidylic acid (poly I·poly C) was also determined.

Materials and Methods. Human Conjunctival Tissue Culture (HCTC). Human conjunctiva was obtained at the time of routine ocular surgery from patients without conjunctival disease. The tissue, washed 3 times with antibiotics containing Hank's Balanced Salt Solution (BSS), was cut into 1-mm pieces, placed into 30 ml Falcon flasks containing 5 ml of Minimal Essential medium (MEM) with 10% fetal calf serum (FCS), 1% glutamine and antibiotics, kept in a humidified 5% CO_2 incubator at 37°. The cells reached confluency in about 3 weeks. Secondary cultures (1:2 split) prepared by scraping off the cells reached confluency in about 5 days. Subcultures could be maintained for five to six weeks without visible changes in cell morphology, in maintenance medium which was MEM, 2% FCS, 1% glutamine and antibiotics. This medium was changed twice a week.

The 6th to 12th subcultures of HCTC were used for all experiments. For virus inoculations, tube tissue cultures were prepared.

Viruses. Adenovirus type 8, Trim strain, was obtained from the Research Resources Branch of the National Institutes of Health. Its titer in HeLa cells was $10^{2.5}$ tissue culture infectious doses ($TCID_{50}$)/0.1 ml according to the National Institute of Allergy and Infectious Diseases Catalog of Research Reagents 1968. Herpes simplex virus (HSV), strain 11123 was obtained from Dr. Ashe of the National Institutes of Health. Its titer in primary rabbit kidney cells was $10^{6.5}$ $TCID_{50}$/0.2 ml. Vaccinia virus (VV), the Mill-Hill strain, obtained from Dr. S. Baron of the National Institutes of Health had a titer of $10^{6.0}$ $TCID_{50}$/0.2 ml in primary rabbit kidney cells. Sindbis virus obtained from Dr. S. Baron of N.I.H., was propagated in chick embryo tissue culture. Its titer was $10^{9.0}$ plaque-forming units/ml. Polyinosinic-polycytidylic acid (poly I·poly C) 1000 μg/ml served as the interferon inducer. Human interferon was prepared by infecting human diploid BUD 8 cells with MOI of 1 of Chikungunya virus and harvesting the fluid after 24 hr. Interferon was treated at pH 2 at 4° for 4 hr to inactivate residual virus.

1. Sensitivity of HCTC to HSV, VV and adenovirus 8. Serial 10-fold dilutions of herpes

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simplex virus, vaccinia virus or adenovirus 8, were prepared in maintenance medium and 0.2 ml of each dilution was inoculated into tubes containing human conjunctiva cell monolayers. After 3 hr adsorption for adenovirus 8 and 2 hr for VV and HSV, supernatant fluids containing unadsorbed virus were removed. Each culture was washed 3 times with 2 ml of Hank's Balanced Salt Solution, maintenance medium was added (1 ml) and the tubes were incubated at 37° in 5% CO_2. All tubes were inspected daily for 6 weeks for specific cytopathic effects.

The infectivity titer was calculated as the highest dilution of virus which infected 50% of the tubes. At selected intervals, the supernatant fluids and cells from some tubes were frozen for subsequent virus titration.

2. Interferon induction and sensitvity of HCTC. Tubes with monolayers of HCTC were incubated overnight with dilutions of human interferon or poly I·poly C and challenged with Sindbis virus (MOI: 20 to 100). After 24 hr, virus replication was checked by hemagglutination techniques using gander cells (2). The minimal effective dose is defined as the smallest amount of poly I·poly C which inhibited the yield of Sindbis virus HA by 0.5 $\log_{10}$.

Interferon was assayed in the tissue culture fluids 24 hr after polyI·polyC treatment by transferring serial dilutions to "U" cells (continuous line of human amnion cells insensitive to poly I·poly C) and using Sindbis virus HA reduction assay. One unit of interferon activity is contained in the highest dilution of the sample which inhibits the yield of Sindbis virus HA by 0.5 $\log_{10}$.

3. Induction of resistance to HSV, VV and adenovirus 8 by poly I·poly C in HCTC. The induction of resistance to viral infection was measured after overnight treatment of HCTC cells with differing amounts of poly I·poly C. Tubes with HCTC were incubated at 37° in 5% CO_2 with 1 ml of maintenance medium containing 100 μg, 10 μg, 1 μg and 0.1 μg of poly I·poly C. Controls were incubated in the same manner with 1 ml of maintenance medium alone. After 20 hr incubation, the media were decanted and the cells were challenged with 10, 100, 1000, or 10,000 $TCID_{50}$ of HSV or VV using 3 tubes per each viral dilution. Because of the low titer of the initial stock of adenovirus 8, we used only 10, 100, or 300 $TCID_{50}$ as a challenge dose for that virus.

After adsorption, the cells were washed three times with Hank's Balanced Salt Solution, fed with 1 ml of maintenance medium and incubated at 37°. After 48 hr incubation, the media from each dilution of HSV or VV were harvested, pooled and titrated in primary rabbit kidney cells. Cells and media from tubes containing adenovirus 8, were harvested after 72 hr and 7-day incubation periods. The pooled materials from each set of cultures were frozen and thawed four times, and the infectivity titer of each pool was determined in HCTC.

Results. The cells which grew out from the explanted fragments of conjunctiva tissue were spindleform, fibroblast-like. All explants were successful except for a few which were contaminated with fungus. Eight lines of HCTC were established and 3 of them were subcultured 18 times. The cells could be maintained for up to 6 weeks without visible changes in cell morphology. No good cell growth could be obtained after the 18th passage.

1. Sensitivity of HCTC to HSV, VV and adenovirus 8. When 100,000 conjunctival cells were exposed to 300 $TCID_{50}$ of adenovirus 8, new infectious virus appeared at 48 hr and reached the maximum titer of $10^{4.5}$ $TCID_{50}$/0.2 ml at 5 days. Typical cytopathic changes usually started appearing 48 hr after inoculation in well defined foci at the margin of the cell sheet. When the inoculum was about 50 $TCID_{50}$, the CPE appeared at 3–4 days (Figs. 1 and 2). The CPE progressed to complete destruction of the monolayer after 2 weeks.

When HCTC were inoculated with HSV or VV, there was rapid development of cytopathic changes and good viral growth similar to that obtained in primary rabbit kidney cells.

2. Interferon induction and sensitivity of HCTC. When pretreated with human interferon or poly I·poly C, the conjunctival cells developed resistance to Sindbis virus. This was measured by the Sindbis viral HA yield

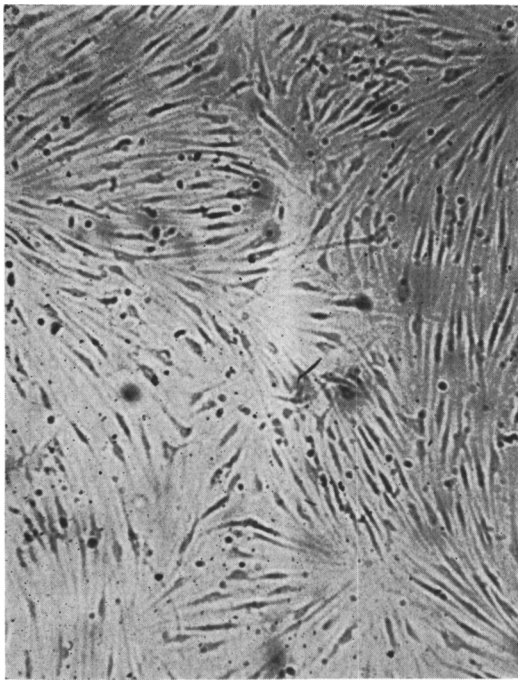

FIG. 1. Normal human conjunctival tissue culture.

reduction assay and by inhibition of the cytopathic effect. (Table I). The interferon preparation titered 20,000 units and the minimal effective dose of poly I·poly C was 0.05 μg/ml. Since this preparation of interferon contains 1000 IU of interferon, the HCTC is highly sensitive to human interferon, and to the interferon inducer.

Interferon induction was assayed in the supernatant of the tissue cultures, 24 hr after poly I·poly C treatment. The amount of interferon induced varied between 30 and 80 units in several tests as measured by HA reduction of Sindbis virus in "U" cells. Since "U" cells are 10 times less sensitive to the international reference interferon than are many strains of diploid human fibroblasts, the interferon yield in international units may be considered to be 300 to 800 units.

3. Induction of resistance to HSV, VV and adenovirus 8 by poly I·poly C in HCTC. The results are shown in Figs. 3 and 4. When human conjunctival tissue cultures were pretreated with 100 μg of poly I·poly C, HSV growth was completely inhibited (Fig. 3). At lower doses of poly I·poly C there was proportional inhibition of HSV with the greater inhibition occurring at the lower viral challenge doses.

As shown in Fig. 4, different doses of poly I·poly C applied to human conjunctival tissue cultures inhibited vaccinia virus growth in a manner similar to herpes simplex virus inhibition. The degree of protection was directly proportional to the dose of poly I·poly C applied and inversely proportional to virus challenge dose.

Conjunctival cells pretreated with 100, 10, 1 or 0.1 μg of poly I·poly C were not protected against 10, 100, or 300 $TCID_{50}$ of adenovirus type 8. The cytopathic effect was not reduced and there were no differences between the adenovirus yield in treated cells and untreated controls, harvested 72 hr and 7 days after poly I·poly C treatment.

Discussion. Chang (3) cultivated epithelial-like cells from normal human conjunctiva which could be passaged several times and were susceptible to poliomyelitis virus. We report here on human conjunctival fibroblasts,

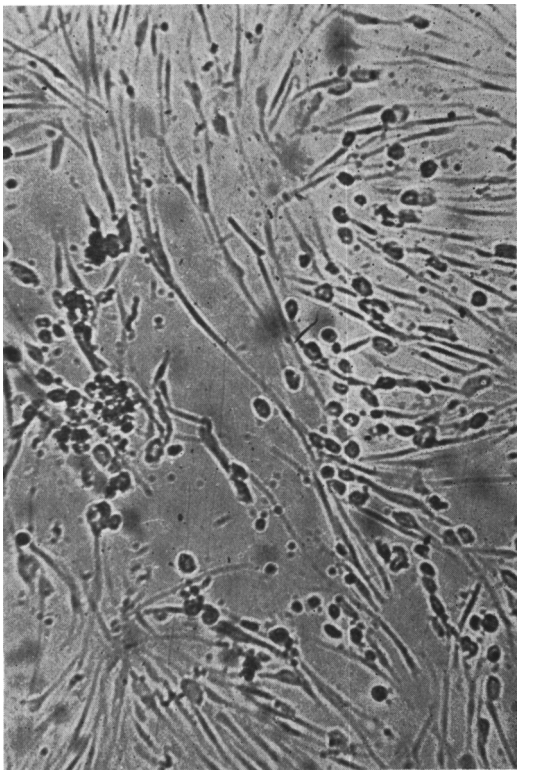

FIG. 2. Human conjunctival tissue culture 72 hr after infection with 50 $TCID_{50}$ of adenovirus 8.

TABLE I. Sensitivity of HCTC to Human Interferon and Poly I·Poly C.

Treatment		CPE	Yield of sindbis virus HA
Human interferon dilutions	1/10	0 0 0	0
	1/100	0 0 0	0
	1/1000	0 0 0	0
	1/10000	4 4 4	16
	Virus control	4 4 4	64
poly I·poly C μg	100	0 0 0	0
	10	0 0 0	0
	1	0 0 0	0
	0.1	4 4 4	16
	0.01	4 4 4	64
	Virus control	4 4 4	64

which could be maintained for prolonged periods without changes in cell morphology and which could be passaged 18 times. We also demonstrated the high susceptibility of HCTC to adenovirus type 8. A clear cytopathic effect was produced in short time. Although adenovirus 8 has been propagated in several tissue cultures, it is one of the most difficult adenoviruses to isolate, because cytopathic changes develop very slowly and cell cultures must be incubated for prolonged times (4). Attempts were made by Hanna and Jawetz to enhance the infectivity of adenovirus 8 but in no case was it possible to increase the titer above $10^{2.5}$ when the prototype strain TRIM was used in Maben cells (5). Clyde and Denny grew adenovirus 8 in human amnion cells (6). When these were incubated for prolonged periods after inoculation, or the cultures had been aged, they recovered 10^3–10^5 $TCID_{50}$ of virus per ml. Golden and McKee increased the infectivity titer of adenovirus 8 from 10^1 to 10^5 by using a washing technique (7). It was also shown that dual infection with adenovirus 8 and mycoplasma results in adenovirus type CPE up to a 10^{-9} dilution of the tested material (7). We obtained a maximum adenovirus 8 yield of $10^{4.5}$ $TCID_{50}$/ml, five days after infection of HCTC. This higher susceptibility was not due to contamination with mycoplasma, as immunofluorescence tests for mycoplasma in HCTC were negative.

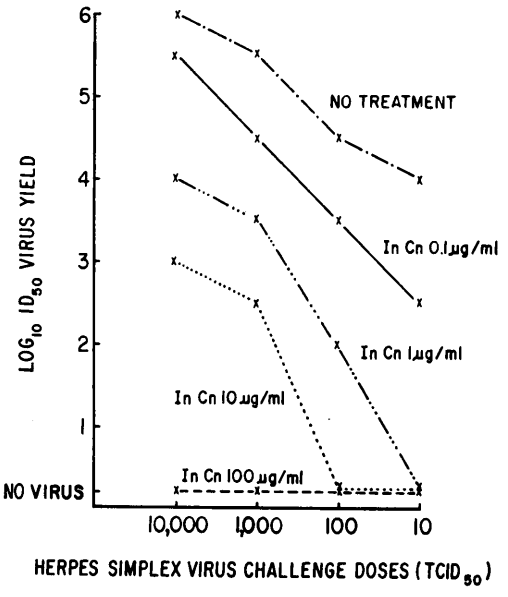

FIG. 3. Effect of different doses of polyI · polyC on the yield of simplex virus on HCTC. Cells were challenged with 10,000, 1,000, 100, or 10 $TCID_{50}$ of herpes simplex, and virus yields were measured 48 hr after infection.

The HCTC grown in this laboratory was highly sensitive to a standard adenovirus 8 strain, as suggested by the early appearance of CPE after inoculation of 300 $TCID_{50}$ and the high yield of virus. Experiments should be performed to compare adenovirus 8 growth in other human fibroblasts, *i.e.*, skin fibroblasts, WI38 and also in human embryo kidney cells. Virus isolation attempts also should be done in an outbreak of epidemic keratoconjunctivitis (EKC) using HCTC as

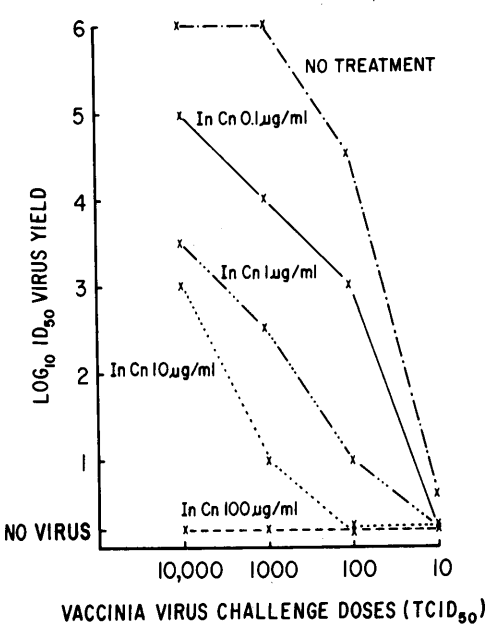

FIG. 4. Effect of different doses of polyI • polyC on the yield of vaccinia virus on HCTC. Cells were challenged with 10,000, 1,000, 100, or 10 $TCID_{50}$ of vaccinia virus and virus yields were measured 48 hr after infection.

compared with other human cells. The percentage of successful isolations and time required for producing typical CPE should be established and compared with other cell systems in use.

The limited sensitivity of adenovirus type 2, 7, and 12 and also of types 1, 3, 4, 5, 8, 11, and 18 to interferon, was demonstrated by Gallagher *et al.* (8) in plaque-reduction assays using human embryo kidney cell cultures. We examined the sensitivity of adenovirus 8 to the interferon inducer poly I·poly C in human conjunctiva tissue cultures and within the limits of the techniques applied, the adenovirus 8 was not sensitive to doses of poly I·poly C which completely blocked the replication of HSV. Although this may suggest that poly I·poly C may not be useful in EKC, the variation of virus susceptibility to interferon in different cell systems, indicate that a direct trial in man still should be performed.

In a previous paper (9) we demonstrated that poly I·poly C conferred antiherpetic resistance to various ocular and rabbit kidney tissue cultures and that the protection was clearly related to the virus challenge dose. The viral resistance was noted with an inoculum of 5 $TCID_{50}$ but the protection was markedly diminished with an inoculum of 50 $TCID_{50}$ of HSV. Different cell cultures vary in their sensitivity to poly I·poly C and interferon. Poly I·poly C is capable of inducing resistance to many viral infections in a wide variety of tissue cultures, but the amount required is different for different tissues (10). This report describes protection in human conjunctiva tissue cultures even when they were pretreated with a minute amount of poly I·poly C (0.05 µg), using a high virus challenge dose (10,000 $TCID_{50}$). With lower challenge doses of vaccinia or herpes simplex virus and with higher concentration of poly I·poly C the inhibition of virus yield was increased. The observed high sensitivity to interferon of HCTC may be a factor which allowed the strong protection by poly I·poly C.

Summary. Fibroblast like cells were grown from explanted fragments of human conjunctiva. The cultures could be maintained for up to six weeks and passaged several times. This tissue supported the replication of adenovirus 8, herpes simplex virus and vaccinia virus. The cytopathic changes caused by these viruses were typical. The human conjunctival tissue culture was sensitive to adenovirus 8 which yielded a titer of $10^{4.5}$ $TCID_{50}$/ml. This tissue was also highly sensitive to human interferon and produced interferon after polyinosinic-polycytidylic acid (poly I·poly C) treatment. Poly I·poly C protected the tissue against challenge with herpes simplex and vaccinia viruses, but not against adenovirus 8 challenge.

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