Inhibition of Human Cytomegalovirus *in Vitro* by Double-Stranded Polyribocytidylic-inosinic Acid (Poly $I_r \cdot C_r$) (33909)

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Glasgow and his associates (1) have shown that human cytomegalovirus is relatively insensitive to the antiviral action of interferon *in vitro*. With the availability of potent inducers of interferon such as double-stranded synthetic RNA (2), we thought that it would be of interest to examine the effect of one of these inducers, double-stranded polyribocytidylic-inosinic acid (poly $I_r \cdot C_r$) on human cytomegalovirus infection *in vitro*.

Materials and Methods. Cells. The WI-38 cells and AH-1 cells were obtained from the media unit of the NIH. AH-1 is a line of human fibroblasts derived from vaginal tissue of an adult woman and has been in continuous culture for more than 3 years. All cells were grown and maintained in RPMI no. 1640 with 20% fetal bovine serum (FBS).

Viruses. The Davis strain of human cytomegalovirus has been carried in this laboratory for 4 years by serial passage in WI-38 cells. Virus pools were grown in these cells in RPMI no. 1640 with 20% fetal bovine serum and when cytopathic effects involved more than ¾ of the monolayers, additional FBS was added to bring the serum concentration to 50% and the cultures were frozen at −70°. They were then frozen and thawed 3 times, pooled, centrifuged at 500*g* for 10 min to remove debris and 1 ml samples were frozen at −70° for subsequent use. The virus was titered by a plaque assay similar to that described by Plummer and Benyesh-Melnick (3), except that the assay was done in closed 25 cm^2 Falcon plastic flasks rather than petri dishes. Vesicular stomatis virus (VSV), Indiana Strain was obtained from Dr. Samuel Baron and grown an titered in primary chick embryo fibroblasts (VSV titer = 2.4×10^8 pfu/ml).

Poly $I_r \cdot C_r$. Polyriboinosinic acid and polyriboncytidylic acid were purchased from P-L Laboratories, Milwaukee, Wisconsin. They were dissolved in 0.15 *M* NaCl, buffered at pH 7.2 with 0.01 *M* phosphate. Heating to 40° facilitated solution as did the use of a Dounce homogenizer. The single stranded homopolymers were then mixed in equimolar proportion to yield a viscous opalescent product. A $MgCl_2$ solution was added to give a final concentration of $5 \times 10^{-3} M$. There was a hypochromic shift of about 35% indicating the formation of a double-stranded complex. The final product remained acid insoluble after treatment with 2 μg/ml of pancreatic ribonuclease (Worthington) at 37° for 2 hr in the presence of 0.5 *M* NaCl.

Interferon. Human interferon was prepared in human lymphocyte cultures by infecting them with Sendai virus. After dialysis, acid treatment, and centrifugation, the interferon titer was 500 units/ml when assayed with VSV on human amnion cells (1 unit produces a 50% inhibition of virus yield).

Plaque reduction experiments with poly $I_r \cdot C_r$ and interferon. All experiments were done with monolayer cultures of WI-38 and AH-1 cells in closed 25 cm^2 plastic flasks. The dilutions of poly $I_r \cdot C_r$ and interferon were made in growth medium (RPMI 1640 with 20% FBS) and permitted to remain in contact with the cells for 24 hr at 37°. The materials were then removed and the cultures were washed 3 times with phosphate buffered saline (PBS). Appropriate virus dilutions of human CMV were then added (0.2ml per flask) and absorption for 1 hr at 37° was permitted. Inocula were then removed, the cultures were washed 2 times with PBS and overlayed with 6 ml of the methylcellulose medium (4). After 1 week, an additional 6 ml of overlay was added. After 14 days the cultures were chilled to 4°, the overlays, were removed, the monolayers were fixed for 1 hr in buffered 10% formalin, stained with 0.03% methylene blue and mi-

TABLE I. Effect of Poly $I_r \cdot C_r$ on Human Cytomegalovirus Plaque Formation in WI-38 and AH-1 Cells.

Treatment (μg/ml)	WI-38		AH-1	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2
Control	83[a]	72	151	98
Poly $I_r \cdot C_r$, 1	76	67	86	39
10	42	61	3	0

[a] pfu/flask (mean of 3 replicate cultures).

croplaques were counted with a dissecting microscope at 13–30× magnification.

Results. Table I shows representative experiments in which poly $I_r \cdot C_r$ was tested against human cytomegalovirus in WI-38 and AH-1 cell cultures and Table II shows similar experiments with human interferon. Initially we only used WI-38 cells and we found no significant decrease in plaques with 25 and 50 units of human interferon. With poly $I_r \cdot C_r$ at 1μg/ml we also found no decrease in plaque formation, however, with 10 μg/ml in occasional experiments there was 50% decrease in plaques. Since WI-38 cells are known to be relatively resistant to interferon and to respond poorly to poly $I_r \cdot C_r$ (5), we repeated all of the experiments with AH-1 cells which are more sensivive to the antiviral action of interferon when tested with VSV and Sindbis viruses (personal communication, Dr. Samuel Baron, NIAID, NIH). With our preparation of human interferon, 25 units/ml produced approximately a 50% reduction in plaques while 50 units/ml produced almost a 90% plaque reduction. With poly $I_r \cdot C_r$ at 10μg/ml, the 2 experiments shown in Table I had 98–100% plaque reduction and in repeated experiments 90–100% plaque reductions were consistently obtained with no morphologic evidence of cell injury or toxicity produced by the poly $I_r \cdot C_r$.

Discussion. Although herpes simplex virus has been reported to be relatively resistant to the action of interferon both *in vitro* and *in vivo* (6), recently it was shown to be inhibited by poly $I_r \cdot C_r$ and that herpes keratitis in rabbits can be effectively treated with poly $I_r \cdot C_r$ (7). These findings suggest the possibility that human infections with members of the herpesvirus family may be susceptible to treatment with potent interferon inducers.

In addition to producing severe and generalized cytomegalic inclusion disease in neonates and infants, human cytomegalovirus has been associated with pulmonary and generalized disease in patients with malignant lymphomas and in patients with transplanted organs who are receiving immunosuppressive drug regimens. If interferon inducers are active against human cytomegalovirus *in vivo*, they might have considerable therapeutic value in these situations. The finding that poly $I_r \cdot C_r$ is active against human cytomegalovirus *in vitro* makes the possibility of *in vivo* action plausible and if problems of drug toxicity and delivery of therapeutic concentrations to involved tissues can be resolved, clinical trials in patients with cytomegalic inclusion disease might be indicated.

It is difficult to obtain good direct evidence

TABLE II. Effect of Human Interferon on Human Cytomegalovirus Plaque Formation in WI-38 and AH-1 Cells.

Treatment (units/ml)	WI-38		AH-1	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2
Control	95[a]	21	401	197
H-IF, 25	100	12	191	73
50	107	14	43	20

[a] pfu/flask (mean of 3 replicate cultures).

that the antiviral effects observed with the poly $I_r \cdot C_r$ are actually mediated through the interferon mechanism. Since interferon can be blocked by treatment of cells with actinomycin or inhibitors of protein synthesis, it would be desirable to show that such compounds would block the effects of poly $I_r \cdot C_r$ on cytomegalovirus. Unfortunately, the toxicity of metabolic inhibitors prevents such tests in the plaque assay system used in the present study. Nevertheless, since the antiviral effects of poly $I_r \cdot C_r$ in other virus host cell systems seem to be mediated through interferon, it is reasonable to assume that similar mechanisms prevail with cytomegalovirus.

In support of this is the fact that poly $I_r \cdot C_r$ is much more active against cytomegalovirus in AH-1 cells than in WI-38 cells and that AH-1 cells are more susceptible to the antiviral effects of interferon than WI-38 cells.

Summary. Poly $I_r \cdot C_r$, a synthetic double-stranded RNA which is a potent interferon inducer, produces marked plaque reduction of human cytomegalovirus in human fibroblasts. The material is active in human fibroblasts which are sensitive to the antiviral action of interferon (AH-1 cells) while it is not active in human fibroblasts which are relatively resistant to interferon (WI-38).

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