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The Effect of an Interferon Inducer on Influenza Virus*

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The double-stranded complex of polyriboinosinic acid and polyribocytidilic acid (poly I·poly C) was shown to inhibit effectively the infection of human cells with 4 common human respiratory pathogens—influenza A2, rhinovirus 13, respiratory syncytial and parainfluenza-1 viruses. This protection was observed with levels of poly I·poly C which did not induce the release of detectable amounts of interferon. Mice could be protected from pulmonary influenza A2 infections by intranasal instillation of poly I·poly C before challenge with virus. Administration of poly I·poly C directly to the respiratory tract was found superior to systemic administration in the case of the pulmonary infection studied.

The control of viral respiratory diseases remains one of the major problems facing virologists and public health officials. Vaccines have greatly changed the character of many other serious viral diseases, but among the respiratory diseases they have not been strikingly successful. Influenza virus, because of its changing antigenic character, has been a major cause of illness and has managed to spread through some populations before adequate vaccines could be developed or administered. This problem is particularly serious with influenza because of its propensity to cause severe illness and mortality in the aged and infirm. While the vaccine approach has had some definite successes and will have undoubted future gains, 2 other approaches to controlling influenza, and other viral respiratory diseases, are being explored with increasing promise of success. These are (1) the use of drugs which have direct antiviral properties, and (2) the enhancement of host defence mechanisms, such as the stimulation of endogenous interferon (Isaacs, 1963; Baron, 1963; Finter, 1966). Both of these approaches are of particular significance in influenza, since they can be used without concern for antigenic variation of the influenza virus, and will provide protection for persons with high risk of complications while adequate vaccines are being developed and administered.

Since the original description of interferon 12 years ago by Isaacs, it has been hoped that this substance could be exploited to combat viral illnesses. The use of interferon in many animal and some human experiments has given evidence that this is a useful approach, but the technical difficulties of producing adequate amounts of sufficiently purified human interferon will probably prevent extensive investigation of its clinical usefulness in the immediate future. However, the discovery that many substances, other than viruses, can stimulate endogenous interferon production and produce resistance to virus infections has led to an extensive search for such agents which might be clinically useful. Poly I·poly C is a synthetic, double-stranded RNA of polyriboinosinic acid and polyribocytidylic acid which has been shown to be a very effective inducer of interferon (Field et al., 1967).

The present study was carried out as an initial step in evaluating the potential role of poly I·poly C in the control of human respiratory diseases. *In vitro* experiments, using human cells, were performed to determine if poly I·poly C would protect these cells from infection with common human respiratory virus pathogens. It has been reported by Field et al. (1968) that poly I·poly C induces resistance to rhinoviruses *in vitro*. We have previously reported that influenza, parainfluenza, respiratory syncytial and rhinoviruses are sensitive to poly I·poly C in cultures of human cells (Hill, Baron & Chanock, 1969). In order to test the effect of poly I·poly C on a common human respiratory virus *in vivo*, the compound was studied in experimental mouse influenza using a strain of influenza A2 adapted to be pathogenic for mice.

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MATERIALS

Poly I·poly C

Single-stranded polyriboinosinic acid and polyribocytidylic acid were obtained in lyopholized form (P. L. Laboratories). Each was dissolved in saline and mixed in equimolar amounts to form the double-stranded complex in a final concentration of 1.0 mg/ml. The pH was adjusted to 7.6 with phosphate buffer.

Tissue culture

Primary embryonic kidney cell cultures and AH-1 and AH-2 cell cultures (NIH Media Section) were grown in stoppered tubes. The AH-1 and AH-2 cells are 2 continuous lines of diploid fibroblasts derived from the same adult human vaginal specimen; the AH-1 and AH-2 are indistinguishable morphologically and in their response to human interferon. All cells were maintained with Eagle's basal medium with 2% foetal bovine serum.

Mice

General purpose NIH strain mice (14 g–16 g) were used in all experiments.

Viruses

Vesicular stomatitis virus (Indiana strain) (VSV) with a titre of $10^{8.5}$ PFU/ml was prepared in chick embryonic fibroblasts. Respiratory syncytial (RS) virus (Long strain) with a titre of $10^{7.5}$ PFU/ml was prepared in HEp-2 cells. Parainfluenza-1 (strain 20993) with titre of $10^{4.2}$ $TCID_{50}$/ml was prepared in primary African green monkey kidney cells. Rhinovirus 13 with a titre of $10^{4.3}$ $TCID_{50}$/ml was prepared in WI-38 cells. Influenza A2/Ann Arbor/60 with a titre of $10^{6.7}$ $TCID_{50}$/ml was prepared in primary Rhesus monkey kidney cells. Influenza A2/Hong Kong/68 with titre of $10^{4.9}$ $TCID_{50}$/ml was prepared in primary human embryonic kidney cell cultures. Influenza A2/Taiwan/64, which had been adapted to produce lesions in mouse lung, was prepared in 12-day-old chick embryos; allantoic fluid gave a titre of $10^{4.3}$ MPD_{50}/ml (mouse pneumonia $dose_{50}$) by intranasal inoculation.

METHODS

In vitro *experiments*

Monolayer cell cultures in stoppered tubes were incubated overnight (14–18 hours) at 37°C with a maintenance medium containing concentrations of poly I·poly C from 0.01 μg/ml to 100 μg/ml. The cultures were rinsed 3 times with 2.0 ml of Hanks' BSS to remove poly I·poly C from the cells, and then the challenge virus was added. After virus adsorption for 1–2 hours at 37°C the cultures were again washed 3 times with 2.0 ml of Hanks' BSS. The cultures were re-fed with maintenance medium free of poly I·poly C and observed for development of cytopathic effect (CPE) or haemadsorption (HAd) and the observations recorded when the entire cell sheet showed evidence of virus infection. In some experiments culture fluid and cells were harvested and assayed for virus yield.

In vivo *experiments*

A total of 0.05 ml of poly I·poly C solution (50 μg poly I·poly C) was administered to mice lightly anaesthetized with ether by instilling drops into the nose. Control mice received saline in the same manner. Intraperitoneal administration was carried out under similar anaesthesia. Mice were challenged by intranasal inoculation of the allantoic fluid pool of influenza A2/Taiwan/64 diluted in Hanks' BSS. The schedules of poly I·poly C administration and virus challenge doses are indicated in Tables 2 and 3. Ten days after inoculation, the mice were autopsied and the lungs scored for consolidated lesions as follows:

4+ = completely consolidated
3+ = 75% consolidation
2+ = 50% consolidation
1+ = 25% consolidation
± = < 25% consolidation

Interferon assays

Supernatant tissue-culture fluid for interferon studies was collected 24 hours after rinsing away poly I·poly C from cell cultures not challenged with virus. Interferon was assayed by yield reduction of Sindbis virus in AH-1 or AH-2 cells, the titre being expressed as the highest dilution of test material that induced a 0.5 $\log_{10}$ reduction in virus yield during a single growth cycle. For assay of *in vivo* interferon induction, groups of 6 mice, which were not challenged with virus, were sacrificed at 2 h, 6 h, 24 h and 48 h after administration of poly I·poly C and the serum and lungs pooled from each group. The lungs were homogenized to a 20% suspension in phosphate-buffered saline. Mouse interferon levels were determined by yield reduction of GD-7 virus haemagglutination in CCL-1 cells during a single growth cycle.

RESULTS

In vitro *experiments*

All of the viruses tested were inhibited by the pretreatment of cells with poly I·poly C as shown in

TABLE 1
PROTECTION OF HUMAN CELL CULTURES AGAINST VIRUSES BY PRETREATMENT WITH POLY I·POLY C

Virus	$TCID_{50}$/tube	Tissue culture [a]	Concentration to give 100% inhibition of CPE or HAd (μg/ml) [b]	Concentration to give 50% inhibition of CPE or HAd (μg/ml) [b]	Maximum concentration without clear decrease in CPE or HAd (μg/ml) [b]	Minimum concentration to give 0.5 $\log_{10}$ reduction in virus yield (μg/ml) [b]
VSV	$10^{6.8}$	HEK	10	1.0	0.1	0.01
VSV	$10^{6.8}$	AH-2	10	1.0	0.1	ND
Influenza A2 (Ann Arbor/66)	$10^{4.8}$	HEK	10	1.0	0.1	ND
Influenza A2 (Hong Kong/68)	$10^{2.2}$	HEK	10	1.0	0.1	ND
RS	$10^{6.2}$ (PFU)	HEK	10	0.1	0.01	0.01
Parainfluenza-1	$10^{4.2}$	HEK	1.0	<1.0>0.1	0.1	ND
Rhinovirus 13	$10^{2.5}$	AH-2	10	1.0	0.1	ND

[a] HEK = Human embryonic kidney cell cultures; AH-2 = diploid fibroblast cell line derived from adult human vaginal biopsy by NIH Media Section.
[b] Concentration in media for 16 hours before inoculation.

Table 1. Complete protection of these human cells against all the viruses tested was observed when they were pretreated with 10 μg/ml of poly I·poly C and observed for CPE or HAd. Significant protection was afforded by 0.1 μg poly I·poly C per ml. The respiratory virus pathogens, which included respiratory syncytial virus, parainfluenza-1 virus, rhinovirus 13 and influenza A2 virus, were at least as sensitive to the effect of poly I·poly C as was VSV which is known to be sensitive to interferon. When virus yields were measured for VSV and respiratory syncytial virus it was found that the minimum concentration of poly I·poly C required to produce a 0.5 $\log_{10}$ reduction in yield was 0.01 μg/ml. No reduction in CPE was noted with this minimum concentration. It was found that with 10 μg/ml–50 μg/ml of poly I·poly C, which completely protected the cells against CPE or HAd, some virus was recovered. It was found that if cells were observed for 2–5 days after the controls showed complete CPE or HAd some cytological evidence of infection did develop in the protected cultures; this could be prevented or further delayed by maintaining the protective concentrations of poly I·poly C in the media after virus challenge. When the supernatant fluid was assayed for interferon, none was detected in those cultures treated with less than 50 μg poly I·poly C per ml. In HEK treated with 50 μg poly I·poly C per ml, culture fluid harvested 24 hours after washing away the poly I·poly C had an interferon titre of 10 units/ml. Thus, significant resistance was induced by 1/500 the level of poly I·poly C required to induce production of detectable amounts of interferon.

In vivo *experiments*

Mice treated with 50 μg of poly I·poly C intranasally before intranasal challenge with influenza virus had a significant decrease in incidence and extent of pneumonia compared to saline-treated controls. When single or multiple 50-μg doses of poly I·poly C were administered intranasally between 7 hours and 30 hours before virus inoculation with 2 MPD_{50}, significant protection was observed in all treated groups, as shown in Table 2. The number of mice with pneumonic lesions was 6 out of 37 (16%) in the treated groups and 6 out of 10 (60%) in the control group. A single dose of poly I·poly C either 7 hours or 30 hours before inoculation was as effective as 2 or 4 doses given in the 30 hours before inoculation. In another experiment shown in Table 3, the number of mice developing pneumonic lesions after intranasal challenge with 10 MPD_{50} per mouse was reduced from 10 out of 10 in control animals to 1 out of 10 with intranasal treatment with 50 μg of poly I·poly C 6 hours before virus challenge, whereas

TABLE 2

INTRANASAL TREATMENT OF MICE WITH POLY I·POLY C AT DIFFERENT TIMES BEFORE INTRANASAL CHALLENGE WITH INFLUENZA A2 [a]

Number of poly I·poly C doses administered [b]	No. of hours before virus challenge	Total no. of mice with pneumonia/ total no. of mice	Frequency of pneumonic lesions
1	30	2/9	22%
1	7	0/10	0
2	30, 7	2/10	20%
4	30, 7, 3, 1	2/8	25%
Total of mice treated with poly I·poly C		6/37 [c]	16%
Control mice—no poly I·poly C		6/10 [c]	60%

[a] Challenge dose 2 MPD_{50}.
[b] Each dose 50 μg of poly I·poly C.
[c] P = <0.05 with Yates correction.

intraperitoneal treatment with 100 μg of poly I·poly C 6 hours before virus challenge did not significantly alter either the frequency or extent of the pneumonic lesions. In experiments where poly I·poly C was administered intranasally 24 hours or more after challenge with virus, the mice developed lesions as severe as, or more severe than, those in control mice.

Interferon levels in mice

Titres of interferon after intranasal administration of 50 μg of poly I·poly C rose to a peak of 1250 units/ml in the serum and 17 500 units/g in lung tissue at 6 hours and remained elevated at 125 units and 6250 units respectively 48 hours later. After intraperitoneal injection of 50 μg of poly I·poly C a peak serum titre of 16 000 units/ml in serum and 6250 units/g in lung were reached, but interferon could not be detected after 48 hours (Table 4).

TABLE 3

INTRANASAL AND INTRAPERITONEAL TREATMENT OF MICE WITH POLY I·POLY C 6 HOURS BEFORE INTRANASAL CHALLENGE WITH INFLUENZA A2 [a]

Route of poly I·poly C administration	Dose of poly I·poly C	No. with pneumonic lesions/total no. in group	Mean lesion score of those with lesions
Intranasal	50 μg	1/10	0.5
Intranasal	Saline	10/10	1.5
Intraperitoneal	100 μg	8/9	2.0

[a] Challenge dose 10 MPD_{50}.

TABLE 4

INTERFERON LEVELS IN SERUM AND LUNG AFTER INTRAPERITONEAL AND INTRANASAL ADMINISTRATION OF 50 μg POLY I·POLY C TO MICE

Route of poly I·poly C administration	Specimen assayed	No. of hours after poly I·poly C administration			
		2	6	24	48
Intranasal	Serum	<10	1 250	350	125
	Lungs	625	17 500	8 000	6 250
Intraperitoneal	Serum	10 000	16 000	2 000	<10
	Lungs	1 750	6 250	625	<10

DISCUSSION

The *in vitro* and *in vivo* findings suggest that inducers of interferon are capable of inhibiting human respiratory virus pathogens. The results of the *in vitro* experiments demonstrated that poly I·poly C is capable of inducing human cells to become resistant to infection by common human respiratory virus pathogens. Further, it was shown that all those viruses tested, influenza A2 (Ann Arbor/60 and Hong Kong/68), rhinovirus 13, respiratory syncytial virus and parainfluenza-1 virus, are approximately as sensitive to this drug as is VSV which is known to be highly sensitive to interferon. These viruses were all inhibited at levels of poly I·poly C below those required to liberate detectable amounts of interferon into the medium of the human cell cultures used in these experiments. This is consistent with previous studies which indicated that subdetectable levels of induced interferon are capable of inducing protection at the cellular level (Buckler, Wong & Baron, 1968). We have found, as others have reported (Field et al., 1968), that strains of the same virus differ in their sensitivity to poly I·poly C. Cell lines also differ in their response to interferon and to poly I·poly C. When comparing the sensitivity of different viruses, both the cell line and strain of virus must be considered. Furthermore, considerable differences in the interferon-inducing activity are noted when poly I·poly C preparations from different sources are compared (Buckler & Baron, unpublished observation).

The studies of mouse influenza pulmonary infections indicate that experimental virus infections can be prevented or diminished by prior treatment with poly I·poly C. The inability to demonstrate amelioration of the infection when treatment is delayed until 24 hours after inoculation when virus replication has begun may be due to the effect described by Taylor (1941), i.e., fluid instilled intranasally during influenza infection causes a worsening of the pulmonary lesions by spreading the infection throughout the lung. Since poly I·poly C does not have a direct virucidal action, this effect of fluid spreading the pulmonary infection may overshadow any protective effect afforded by the treatment. We are currently testing the use of aerosol delivery of poly I·poly C to mice after infection to see if treatment after inoculation with influenza virus can be effective in this model system. It was found that the use of a low challenge dose of virus (2–10 MPD_{50}) was essential if a protective effect of poly I·poly C was to be demonstrated. With doses of virus of 100 MPD_{50} or greater, no protective effect of poly I·poly C was observed. This is in agreement with work of others who showed that interferon was most effective in protecting mice against low challenge doses of virus (Finter, 1966). The superiority of intranasal administration over intraperitoneal administration may be due to the higher relative pulmonary concentration and longer duration of interferon after administration by the former route. These findings are similar to those of Cathala & Baron (unpublished results) who found different blood–cerebrospinal fluid distribution of interferon after local or systemic administration of poly I·poly C, and Weissenbacher et al. (unpublished results) who showed a similar finding for interferon in blood and aqueous of the eye. Kleinschmidt [1] has shown that statolon is more effective in protecting mice against influenza PR/8 when it is administered intranasally than when administered systemically.

Taken together the findings suggest that an interferon inducer which is effective in the cells of the human respiratory tract will probably provide protection against many types of viral respiratory pathogens, including influenza.

[1] Kleinschmidt, W. J. (1969). Paper presented at the Second Conference on Antiviral Substances of the New York Academy of Sciences, 1969.

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