ferences were found. TSR determined 2 weeks after ADRX while on 1% NaCl replacement therapy was significantly reduced by 17% in males and 32% in females. TSR determined after 2 weeks of hydrocortisone replacement (150 μg/day) in addition to 1% NaCl was found to return to normal level. Body weight was not affected by treatments. It was suggested that reduced TSR after ADRX may be due, in part, to reduced feed consumption rather than ADRX *per se*.

1. Fluckiger, E., Verzar, F., *J. Endocrinol.*, 1955, v13, 39.
2. Brown-Grant, K., *Endocrinology*, 1955, v56, 607.
3. Inbar, S. H., Freinkel, N., *Metabolism*, 1956, v5, 652.
4. Halmi, N. S., Barker, S. B., *Endocrinology*, 1952, v51, 127.
5. Verzar, F., Vidovic, V., *J. Endocrinol.*, 1952, v8, 321.
6. Perry, W. F., *Endocrinology*, 1951, v49, 284.
7. Shellabarger, C. J., *ibid.*, 1954, v55, 100.
8. Bastenie, P. A., Ermans, A. M., *ibid.*, 1958, v62, 245.
9. O'Neal, L. W., Heinbecker, P., *ibid.*, 1953, v53, 239.
10. Beck, R. N., *ibid.*, 1958, v62, 9.
11. Grosvenor, C. E., Turner, C. W., PROC. SOC. EXP. BIOL. AND MED., 1958, v99, 517.
12. Pipes, G. W., Turner, C. W., *Mo. Agr. Exp. Sta. Res. Bull. No. 617*, 1956.
13. Pipes, G. W., Grossie, J. A., Turner, C. W., PROC. SOC. EXP. BIOL. AND MED., 1960, v104, 491.
14. Cowie, A. T., Folley, S. J., *J. Endocrinol.*, 1946-48, v5, 282.
15. Benson, G. K., PROC. SOC. EXP. BIOL. AND MED., 1960, v103, 132.

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An Interferon Appearing in Cell Cultures Infected with Measles Virus.* (26692)

EDWARD DE MAEYER,† AND JOHN F. ENDERS
Research Division of Infectious Diseases, The Children's Hospital Medical Center, and Department of Bacteriology and Immunology, Harvard Medical School, Boston, Mass.

Several virus-cell systems have recently been shown to produce substances capable of inhibiting growth of related and unrelated viruses. The mode of action of these inhibitors has been clearly distinguished from interference due to viral components. Isaacs' interferon(1), Cooper and Bellett's Transmissible Interfering Component(2) and Ho and Enders' Viral Inhibitory Fluid(3) all seem to be similar. Ho and Enders(4) noticed that fluids of tissue culture, infected with the measles virus retarded growth of poliovirus and inhibited its cytopathic effect. We have been able to confirm these findings and to demonstrate that they depend upon the presence of an interferon-like agent which will be referred to subsequently as measles interferon. By this designation we do not imply that it is identical with influenza interferon as described by Isaacs and Lindemann, but only that, in our opinion, it represents one of a class of substances with similar properties. The evidence supporting this opinion will be presented.

Materials and methods. Cell cultures. Primary cultures of human amnion cells were prepared from trypsinized human amnion membranes. The culture medium consisted of equal parts of bovine amniotic fluid and Hanks' saline solution, enriched with 5% horse serum. This medium will be referred to as "regular BAF medium"(5).

Chick cell cultures were prepared from 9 to 10-day old chick embryos. Growth medium consisted of regular BAF medium with 2% chick embryo extract.

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† Fulbright Scholar—Supported by R. I. T. (Belgium) and Lederle International Fellowship Committee. Present address: The Rockefeller Inst., New York City.

A strain of HeLa cells, supplied by Dr. R. S. Chang,‡ and a line of human amnion origin, WS cells, supplied by Dr. R. L. Thompson,§ were also used. These cells were nourished with Eagle's basal medium, modified as described earlier(4), enriched with 10% calf serum. Concentration of serum was reduced to 5% when virus was added.

Viruses. Lines of the Edmonston strain of measles virus adapted to human amnion (5) and to chick embryo cells(7) were used. Of the former the 33rd and 34th human amnion passages were chiefly studied, and will be referred to as HA virus. Of the chick embryo adapted line, mainly the 14th and 15th chick embryo cell passages were used. This line will be referred to as CE virus.

Sindbis virus strain AR-339 was furnished by Dr. R. M. Taylor.

Type I (Brunhilde) and Type II (MEF) polioviruses were also used. Stocks of both polio and Sindbis viruses were grown in human amnion cell cultures. *Assay of virus.* Serial 10-fold dilutions of the virus suspension were prepared and 3 tube cultures were inoculated with 0.1 ml of each dilution tested. Infectivity titers are expressed as log 10 units of the $TCID_{50}$/0.1 ml.

Plaque assays of measles virus were performed in either HeLa or WS cell cultures as previously described(6). *Antisera.* Measles antiserum was obtained from a Cynomolgus monkey inoculated intracerebrally with the Edmonston strain virus(8). The complement fixing titer of this serum was 1/2048. *Complement fixation tests* were carried out by the drop method of Fulton and Dumbell as modified by Svedmyr and associates(9). *Ultracentrifugation* was performed in a Spinco L-preparative centrifuge. In all instances head #40 was used. *Assay of interferon.* Routine assays for interferon activity were done with Polio Type II or Sindbis as test agents and were carried out in human amnion cultures incubated in a roller wheel at 36.5°C. Only cultures at least 14 days old were used(10). After removal of the culture fluids, 0.5 ml of fresh regular BAF medium was added to each tube. Subsequently 0.5 ml of the fluid to be tested for interferon was added in the appropriate dilution, using 2 cultures per dilution. The challenge virus was added immediately afterwards. This challenge dose consisted of either 200 $TCID_{50}$ of poliovirus or 500 $TCID_{50}$ of Sindbis virus. At this concentration the cells of control cultures were completely destroyed 3 to 4 days after inoculation. The interferon titer was expressed as the highest dilution that at least in one culture clearly diminished the CPE. Usually the lower dilutions completely inhibited CPE, whereas at the endpoint partial CPE occurred.

Experiments and results. Production of interferon. By comparing the development of CPE and virus output in cultures inoculated with HA or CE measles virus, we found that, although the first cytopathic changes occurred at about the same time for both viruses, subsequently a significant difference appeared in spread of CPE as well as in amount of virus produced. These observations, to be described elsewhere in detail, led us to investigate the possibility of the emergence in the culture fluids of a substance inhibiting dissemination of infection and viral multiplication. The following is an example of several comparable experiments. Eight cultures of human amnion cells were inoculated with 100 $TCID_{50}$ of CE measles virus. Fluids were collected at several intervals, pooled, centrifuged at 30,000 rpm for 2 hours to remove infective virus and assayed for inhibitory activity against Sindbis virus in human amnion cells. The results are given in Table I. Inhibitory activity was also subsequently demonstrated in fluids of HeLa or WS cell cultures infected with the measles virus. The first signs of this activity were usually found at about the time the infective virus was released, and amount of inhibitor

TABLE I. Development of Interferon in Fluids of Cultures Infected with CE Virus.

No. of sample	Days after inoculation	CPE at time of collection	Interferon titer
1	6	0/±	0
2	11	++	1/16
3	15	+++/++++	1/32
4	18	++++	1/32

‡ Harvard School of Public Health.

§ Sterling Winthrop Research Inst., Rensselaer, N. Y.

TABLE II. Interferon Titers of Fluids from Cultures Infected with CE Virus.

Exp.	Type of culture	CPE at time of harvest	Measles titer at time of harvest, $TCID_{50}$/0.1 ml	Interferon titer	Challenge virus
117 E	HeLa	+++	2.5	1/4	MEF_1
157 E	"	++++	3.5	1/16	"
172 E	"	0	0	0	"
173 E		+	2.5	0	
		+++	3.25	1/8	
		+++/++++	3.25	1/16	
357 E	"	+	ND*	0	"
		++	ND	undil.	
		+++	ND	1/2	
		++++	ND	1/2	
618 E	"	±	ND	1/2	Sindbis
		+	ND	1/4	
		++++	ND	1/8	
674 E	"	+	ND	1/2	"
		++++	ND	1/32	
766 E	HA	±/+	2.5	1/2	"
		++	4	1/8	
		+++	4.5	1/16	
		++++	3.5	1/16, 1/32	

* ND = not done.

increased with advancing CPE. The results of several experiments summarized in Table II give interferon titers found in fluids of human amnion or HeLa cell cultures previously inoculated with the CE measles virus, and assayed in human amnion cell cultures.

That the inhibition of CPE also reflects a decrease in viral production is shown by the following experiment. Aliquots of HeLa cell prepared interferon were added to each of 4 human amnion cell tube cultures. As controls, 4 other cultures were supplemented with fluid derived from uninfected HeLa cell cultures. All cultures were then inoculated with 500 $TCID_{50}$ of Sindbis virus. Cytopathic effect was followed and the amount of Sindbis virus produced was determined by titrations in human amnion cell cultures. The results are given in Table III. The control cultures were completely destroyed after 3 days, and yielded virus up to $10^{5.5}$ $TCID_{50}$/0.1 ml. The cultures with interferon were followed over a 20-day period and did not show any sign of CPE. Some virus was reproduced in these cultures, although considerably (1000 ×) less than in control cultures. The medium was changed twice during this period, and the same amount of interferon as was present at onset of experiment was added to the fresh medium. *Mode of action.* It was demonstrated in the following 2 experiments that interferon did not inactivate the virus. A mixture of undiluted, HeLa cell prepared interferon and undiluted stock Sindbis virus was incubated at 37°C for 90 minutes. Infectious virus was then

TABLE III. Effect of Measles Interferon on Propagation of Sindbis Virus in HA Cell Cultures.*

Hr after inoc.	Amt of virus in culture fluid		CPE	
	Control	Interferon	Control	Interferon
0	2.5	2.5	0	No sign of CPE
6	.75	1.25	0	
12	3.5	2.75	0	
24	4.25	2.25	0	
36	5.25	3.25	+/++	
48	5.5	2.5	++/+++	
60	5.25	2.75	+++	
72	3.75	2.5	++++	
96		1.5		
120		1.5		
168		2		
216		0		
360		0		

* Cultures with and without interferon were inoculated with the same amount of Sindbis virus. At indicated intervals thereafter degree of CPE was recorded and infectivity titer of culture fluids was determined. Titers are expressed as $\log_{10}$ $TCID_{50}$/0.1 ml.

TABLE IV. Results of Varying Concentrations of Interferon and Challenge Virus.

Amt of virus added (ID_{50}/0.1 ml)	Concentration of interferon in culture				Control cultures
	1/4	1/8	1/16	1/32	
10^6	2	4	4	4	4
10^5	±	4	4	4	4
10^4	±	±	4	4	4
10^3	0	0	±	1	4
10^2	0	0	0	0	4

Cultures were previously incubated for 2 hr with indicated amount of interferon. Thereafter challenge virus (poliovirus Type I) was added, in 10-fold increasing concentrations. Readings of CPE taken every day. Table records reading on 3rd day after inoculation.
(4 = 100% CPE; 3 = 75% CPE, etc.).

measured by titration in human amnion cells. The endpoint was identical with that of a control mixture, indicating that no direct neutralization of the virus had occurred. In a second experiment, the virus-interferon mixture was left for one hour at 37°C and subsequently kept overnight at ± 4°C. Infectivity titrations were carried out in this experiment in chick embryo cell cultures. Again, no evidence was found for direct neutralization of the virus. It should be pointed out here that HeLa cell produced interferon does not protect chick cells against infection with Sindbis virus.

The influence of increasing amounts of test virus on degree of protection conferred by interferon was next investigated. Four different concentrations of HA cell produced interferon were tested each against 5 ten-fold increasing doses of poliovirus type I. Concentration of interferon required to inhibit the CPE varied directly with concentration of the challenge virus (Table IV). *Separation of interferon from infectious particles and complement fixing antigens.* That the inhibitory substance was obtained in a system where virus multiplication took place made it necessary to study the relationship of this inhibitor to the infectious and complement fixing properties of the virus.

Ultracentrifugation for 2 hours at 30,000 rpm was sufficient to sediment infective particles and complement fixing antigens, but did not significantly depress the inhibitory activity of the supernate. These results are compatible with the size of measles virus and CF particles, as determined by Benyesh and associates using other methods(11).

Filtration through Millipore filter membranes showed differences in filterability between infectious, complement fixing and interferon particles. Three 5 ml aliquots of HeLa cell grown CE measles were passed through Millipore filter membranes respectively of 100, 50 and 10 mμ average pore size.‖ The pH of the fluids varied between 7.1 and 7.2. The 3 filtrates were then assayed for infectious, complement fixing and inhibitory activity. All infectivity was removed after passage through the 100 mμ membrane (Table V). The amount of complement fixing antigen passing through decreased with pore size, but some activity was left even in the filtrate of the 10 mμ membrane. In contrast the concentration of interferon remained undiminished even after passage through the membrane of smallest pore size.

To obtain further information concerning the relationship of the interferon to the virus itself, we determined the effect of measles antiserum. Equal amounts of interferon and the antiserum diluted 1/10 were mixed. In one experiment the mixtures were incubated for 2 hours at 37°C, then assayed against Sindbis virus. In a second experiment the mixtures were first incubated at 37°C and kept overnight at 4°C before assay. No effect of the antiserum on inhibitory activity was found. *Some chemical properties of measles interferon.* In view of results obtained by others with similar substances(4, 12), the possibility that measles interferon is a protein was examined by submitting it to the action of trypsin. A 30 minute exposure of interferon fluid to an equal volume of 0.25% crystalline trypsin at pH 7.4 and 37°C completely destroyed inhibitory activity. Activity of samples treated with trypsinogen remained unchanged.

Incubation with either ribonuclease or deoxyribonuclease at a concentration of 100γ/

‖ Millipore Filter Corp., Bedford, Mass. Dishes of the VF (10 mμ) VM(50 mμ) and VC(100 mμ) filters in 47 mm diam. size were used.

TABLE V. Influence of Pore Size on Infectivity, Complement-Fixing Activity and Interferon Titer.*

		Infectivity (titer in PFV/0.5 ml)	Complement fixing activity		Interferon titer
			Undiluted	Diluted ½	
Before filtration		3140	+	+	1/8
After filtration	100 mμ pore size	0	+	±	1/8
	50 " " "	0	+	0	1/8
	10 " " "	0	±	0	1/8

* Fluid used in this experiment was obtained from a HeLa cell bottle culture, previously inoculated with CE measles agent. Fluid was harvested at time of maximum CPE. After low speed centrifugation an equal amount of phosphate buffer was added. Filtration through Millipore membranes of indicated pore size.

ml for 30 min at 37°C had no effect.

A one hour exposure to ether (20% by volume) at room temperature did not destroy the interferon.

The interferon also proved wholly or partially resistant to treatment with fluorocarbon or chloroformoctanol. A mixture of equal parts of genetron¶ (trifluorotrichloroethane) and interferon was vigorously shaken for 5 min at room temperature, then centrifuged at 1000 rpm for 5 minutes. Assay of the aqueous phase revealed only a very slight loss of activity. After one chloroform-octanol extraction, however, only about 50% of the original activity remained in the fluid.

Heating for 2 hours at 56° had no effect on interferon titer. Stability at this temperature is in contrast to that of measles virus infectivity which is rapidly destroyed under these conditions(13). The resistance of measles interferon to various physical and chemical treatments studied is summarized in Table VI along with the behaviour of the infectious and complement fixing components of the virus under the same conditions.

Discussion. The data provide information on the nature and properties of an inhibitor of viral activity appearing in measles infected tissue cultures. Production and release of this inhibitor is broadly coincident with that of infective virus. Degree of inhibition varied directly with amount of virus used as challenge. The inhibitor can be distinguished from the infective unit. The data also suggest that it is smaller than the complement fixing antigen. It also differs from both these viral components by its failure to be affected by specific measles antibody.

Its mode of action remains largely unknown. Since we have shown that it does not combine directly with the test virus, it probably acts like other interferons at the cellular level.

The influence of the inhibitor on spread of infection in the system in which it is produced has not been investigated in this study. Some chemical properties of the inhibitor, however, have been defined. Digestion by trypsin shows that it is a protein, or that at least part of the activity is due to a protein. Treatment with ether or with genetron did not reduce the activity. Some loss occurred after one chloroform octanol extraction. Heating for 2 hours at 56° was without effect. These

TABLE VI. Some Physical and Chemical Properties of Measles Interferon as Compared with Those of Infectious and Complement Fixing Particles.

	Interferon	Infectivity	Complement fixing antigen
Inact. by trypsin	+	+	0
" " RNase or DNase	0	0	0
" " ether	0	+	0
" " Genetron	0	+*	0
Inact. after 2 hr at 56°	0	+†	0
Sedimented after 2 hr at 100,000 g	0	+	+
Retained by pore size of 100 mμ (Millipore filter discs)	0	+	0

* Only 10% of original activity left after one extraction.

† Inactivation not complete.

¶ Genetron 226. Division of Allied Dye and Chemical Corp., General Chemicals, Inc. (New York) Medford, Mass.

properties may be of value in testing for its presence in materials such as blood or crude tissue extracts.

The physical and chemical properties of this agent, as well as the way it is obtained, exhibit certain similarities and differences as compared with influenza interferon(12), VIF (4) and transmissible interfering component (2,14). For example, all these factors are inactivated by trypsin and are not neutralized by specific antibody. As with measles interferon, a direct correlation between amount of inhibitor produced and extent of infection of the cell system has been shown for interferons produced with influenza(15) and vesicular stomatitis virus(2). Ho and Enders, however, found with chick-adapted Type II poliovirus that maximal yields of VIF were obtained at a stage earlier than that of maximal virus production. On the other hand, in some instances influenza interferon was produced only after virus production had passed its peak(16). Another difference lies in the ease with which influenza interferon is destroyed by ether treatment as contrasted with the resistance of measles interferon to this reagent. It is of interest in this connection that the infective component of both measles and influenza virus is ether sensitive.

In spite of these differences it is reasonable to assume that measles interferon, influenza interferon, T.I.C. and VIF may be related though not identical substances. Whatever their ultimate relationship may be, it is clear that these factors represent part of the mechanism of cellular resistance to viral infection *in vitro*. It is probable that *in vivo* as well, they prove to play a role in limitation of viral infections.

Summary. An inhibitor of viral activity appears in cell cultures inoculated with live measles virus. This inhibitor has been separated from the infectious particles and complement fixing antigens produced in the same cultures. It is not neutralized by measles antiserum. It does not inactivate the virus when mixed with it, but reduces the extent of cytopathic changes as well as quantity of virus produced.

1. Isaacs, A., Lindemann, J., *Proc. Roy. Soc. B.*, 1957, v147, 258.

2. Cooper, P. D., Bellett, A. J. D., *J. Gen. Microbiol.*, 1959, v21, 485.

3. Ho, M., Enders, J. F., *Proc. Nat. Acad. Sci.*, 1959, v45, 385.

4. ———, *Virology*, 1959, v9, 446.

5. Milovanovic, M. V., Enders, J. F., Mitus, A., PROC. SOC. EXP. BIOL. AND MED., 1957, v95, 120.

6. De Maeyer, E., *Virology*, 1960, v11, 634.

7. Katz, S. L., Milovanovic, M. V., Enders, J. F., PROC. SOC. EXP. BIOL. AND MED., 1958, v97, 23.

8. Enders, J. F., Katz, S. L., Medearis, D. N., *Perspectives in Virology*, 1959, 103.

9. Svedmyr, A., Enders, J. F., Holloway, A., PROC. SOC. EXP. BIOL. AND MED., 1952, v79, 296.

10. Frothingham, T. E., *ibid.*, 1959, v100, 505.

11. Benyesh, M., Pollard, E. C., Opton, E. M., Black, F. L., Bellamy, W. D., Melnick, J. L., *Virology*, 1958, v5, 256.

12. Isaacs, A., Burke, D. C., *British Med. Bull.*, 1959, v15, 185.

13. Black, F. L., Reissing, M., Melnick, J. L., *Advances in Virus Research*, 1959, v6, 205.

14. Bellett, A. J. D., Cooper, P. D., *J. Gen. Microb.*, 1959, v21, 498.

15. Wagner, R., *Bact. Rev.*, 1960, v24, 151.

16. Burke, D. C., Isaacs, A., *Brit. J. Exp. Path.*, 1958, v39, 452.

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