STUDIES ON PERSISTENT INFECTIONS OF TISSUE CULTURES*

IV. Evidence for the Production of an Interferon in MCN Cells by Myxoviruses

By WERNER HENLE, M.D., GERTRUDE HENLE, M.D., FRIEDRICH DEINHARDT, M.D., and VICTOR V. BERGS,‡ Ph.D.

(From the Division of Virology, The Department of Public Health and Preventive Medicine, School of Medicine, The University of Pennsylvania, and The Children's Hospital of Philadelphia, Philadelphia)

As a result of persistent latent infections with Newcastle disease (NDV), mumps, and hemadsorption Type 1 (HA-1) viruses (1) cell cultures of the MCN and Lung-To strains[1] acquire resistance to certain cytopathogenic viruses, especially the virus of vesicular stomatitis (VSV). The resistance so induced was shown to fulfill all the criteria of viral interference (2). However, in quantitative analyses of these systems marked discrepancies became apparent between the cells of persistently infected cultures, which could be proven to contain and release infectious NDV, mumps or HA-1 viruses, and the number of cells resistant to VSV. At most only 10, and generally less than 2 per cent of the cells could be shown to harbor infectious virus, and not more than one infectious unit, as a rule, at any given time. On transfer of a few virus-producing cells to fresh MCN cultures, the increase of infectious virus was insufficient in quantity to account for the rapid establishment of interference in the total cell population (3). No evidence was obtained to indicate the production of non-infectious, interfering virus particles, as assayed by hemagglutination and complement fixation tests.

A new approach to this problem was afforded by the recent discovery (4–6) that following adsorption of heat- or ultraviolet-inactivated myxoviruses onto the allantoic membrane of the chick embryo a substance is released which is void of hemagglutinating and virus-specific complement-fixing activity, but is capable of inducing resistance in susceptible cells to viral infections.

---

[1] Isolated respectively by McCulloch from a leukemic patient and in this laboratory from human embryonic lung.

This substance has been called *interferon*. Some success has been reported also with production of interferon-like components in cell cultures (7). Initial attempts to detect interferon in persistently infected MCN cultures failed. It was found, however, that a substance, resembling interferon in every aspect, was readily produced in uninfected MCN cultures upon addition of ultraviolet-inactivated myxoviruses. Subsequent restudy of persistently infected cell lines, employing increased concentration of cells per milliliter of medium yielded evidence of the presence of an interferon-like substance in this system. These findings form the subject of this report.

Materials and Methods

Cell Cultures.—The techniques employed for the growth and maintenance of MCN and HeLa cells have been described (8). Primary cell cultures of human and rabbit kidney and of mouse embryos at the 15th to 16th day of gestation were prepared by trypsinization using essentially the method described by Youngner (9). The cells were grown in medium 199 containing 2 per cent calf serum.

Viruses.—For the preparation of interferon the following myxoviruses were employed: The Victoria strain of Newcastle disease virus (NDV); the Barnes strain of mumps virus; the WS strain of influenza Type A virus; and the F strain of Sendai virus, kindly supplied by Dr. Morihide Yamada. All these agents were adapted to the allantois of the chick embryo. Following allantoic inoculation of appropriately diluted seed viruses the allantoic fluids were collected after 24 to 48 hours of further incubation of the eggs at 37°C., except for the mumps virus preparations, which were harvested after 5 days. The viral concentrations per milliliter ranged from $10^{2.4}$ to $10^{3.4}$ hemagglutinin units and from $10^{8.0}$ to $10^{9.5}$ chick embryo-infectious doses.

The allantoic fluids were dialyzed overnight in the cold room against 20 volumes of $M/100$ phosphate-buffered saline solution at pH 7.0. Thereafter, 15 to 20 ml. aliquots of the dialyzed preparations were exposed in open Petri plates to a General Electric germicidal lamp at a distance of 7 inches under continuous mechanical rocking (10). After irradiation for 90 seconds the materials were transferred to fresh Petri plates and exposed for another 90 seconds. This procedure was generally sufficient to inactivate all infectious virus as determined by 2 serial allantoic passages. These preparations were employed for production of interferon as described in the experimental section.

For challenge of the resistance of cells exposed to interferon the following viruses were used: the Indiana strain of vesicular stomatitis virus, propagated in MCN cells; and the PR8 strain of influenza Type A and Sendai viruses, maintained as described above.

Further technical details are described in the text.

EXPERIMENTAL

The Production and Assay of Interferon.—The first experiment to be presented serves as a typical example for the production of interferon in MCN cells and assay of its activity.

Bottle cultures, 8 days after seeding, were washed twice with Hanks' solution and the cell sheets were then covered with 25 ml. of a mixture of 10 × concentrated medium 199 (2.5 ml.) and ultraviolet-inactivated mumps virus (22.5 ml. undiluted dialyzed allantoic fluid). The virus preparation used had an infectivity titer in chick embryos of 10^8 $EID_{50}/ml.$

prior to inactivation and was non-infectious thereafter as attested by 2 blind passages. It contained 2560 HA units/ml. After 2½ hours of incubation at 37°C. the cultures were washed 6 times and then refed with medium consisting of 33 per cent No. 199 and 67 per cent Scherer's maintenance solution. The bottles were incubated at 37°C. and the media were collected 24 hours later to serve as interferon (I) preparation. Tests for infectious virus and hemagglutinins were negative. Control (C) preparations were obtained in the same manner by substituting irradiated normal allantoic fluid for the ultraviolet-inactivated virus. The cells in the two sets of bottle cultures were refed and then challenged with 10^4 TCD_{50} of VSV. The cultures, which had been treated with inactivated virus showed extensive interference in that no lesions were discernible after 5 days of further incubation. The cell sheets in the control bottles were completely destroyed in 2 days.

The interferon (I) and the control (C) preparations were adjusted to a pH of 7.6 (2) and then added in 0.8 ml. volumes to adequate numbers of MCN culture tubes from which the media had been removed. The tubes contained approximately 3×10^5 cells. After an incubation period of 24 hours at 37°C. VSV was titrated in the two sets of cultures by inoculation of 0.2 ml. of diluted virus per tube, using 4 tubes per dilution. Several cultures remained unchallenged as a check on the interferon and control preparations. VSV was titrated also in fresh MCN cultures, but, since the titers obtained always matched those recorded in the C series, these data are omitted from all further discussion. The degrees of cellular destruction were estimated microscopically at daily intervals after challenge and the titration endpoints were determined on the basis of a minimum of 2+ readings or a 50 per cent or greater loss of cells. The difference between the VSV titers in the I and C sets at given days was taken as the protective index, a value used for simplification in the presentation of data to be described below.

As can be seen in Table I, the interferon preparation afforded definite but transitory protection. In the controls VSV lesions developed rapidly and the cultures were destroyed in 2 to 3 days. In the interferon set lesions developed more slowly so that a protective index of 100,000 was noted on the 2nd day. This value decreased rapidly, however, in the ensuing days and by the 7th day it had been reduced to 10.

In other experiments interferon was prepared as described but in addition to the media removed at 24 hours the cells were collected, and after disintegration by sonic oscillation, tested for interferon activity. In accordance with the results recorded by Isaacs and Lindenmann (4) for chorioallantoic membranes, less activity was found in the cells than in the media. Only the media were employed thereafter.

In further tests, interferon-containing media were removed at 24 hours. The cell sheets were then refed and these media were harvested after an additional 24 hours of incubation. The 2nd harvest still had significant interferon activity but distinctly less than the first. All further experiments were carried out, therefore, with material obtained at 24 hours.

No significant differences were noted in the protection obtained whether or not the interferon was removed from the test cultures prior to challenge with VSV. According to Lindenmann et al. (6) the establishment of resistance by interferon in chorio-allantoic membranes requires considerable time.

A 24 hour interval was chosen therefore between addition of interferon and challenge. It was noted that with an increase of the incubation period to 48 hours less protection became apparent. Intervals shorter than 24 hours have not as yet been studied.

Table II summarizes the results of experiments conducted with interferon preparations derived from MCN cultures with ultraviolet-inactivated mumps, NDV, Sendai, and the WS strain of influenza A viruses It can be seen that

TABLE I

Titration of VSV in MCN Cultures 24 Hours after Addition of Interferon (I) or Control Medium (C)

Dilution of VSV	Average degree of cellular destruction*									
	Days after VSV challenge									
	1		2		3		5		7	
	C	I	C	I	C	I	C	I	C	I
10–2	2	±	4	*3–4*	4	4	4	4	4	4
10–3	2	0	3–4	1	4	3–4	4	4	4	4
10–4	*2*	0	3–4	(±)	4	*3*	4	4	4	4
10–5	1	0	3	0	4	1–2	4	*4*	4	4
10–6	±	0	3	0	4	0	4	1	4	4
10–7	0	0	*2–3*	0	4	0	4	±	4	*4*
10–8	0	0	1–2	0	*4*	0	*4*	0	*4*	0
Titer‡..........	10^{-4}	$<10^{-2}$	10^{-7}	10^{-2}	10^{-8}	10^{-4}	10^{-8}	10^{-5}	10^{-8}	10^{-7}
Protective index, *log*......	>2.0		5.0		4.0		3.0		1.0	

The interferon was prepared with ultraviolet-inactivated mumps virus in MCN cultures.
* 0, no cellular destruction; ±–4, increasing degrees of cellular destruction.
‡ The titers are based on 2 + or greater destruction.

all the irradiated viruses yielded active interferon. Failures were noted, however, as with the WS preparation in Experiment 5. The occasional failures have not been explained but would seem to depend largely on the concentration of virus in the irradiated preparations used for interferon production. Indeed, as will be shown below, the inactivated virus preparations could not be diluted very far and still yield interferon. Presumably for the same reasons the amount of interferon produced, and with it the extent of protection afforded varied considerably. The protective indices were generally highest on the 2nd day, ranging from more than 1 million to as low as 1000. The higher the values were on the 2nd day the longer some degree of protec-

tion was found to persist. In some instances, as in Experiment 2 of the table, considerable resistance was noted even on the 7th day after VSV challenge. In such cases the suspicion must be entertained that possibly some virus escaped inactivation by ultraviolet irradiation and initiated persistent latent

TABLE II

Summary of Results Obtained with Interferon Prepared in MCN Cultures with NDV, Mumps, Influenza (WS), and Sendai Viruses and Challenge with VSV

Exp. No.	Interferon prepared with	Protective index, *log*							
		Days after VSV challenge							
		1	2	3	4	5	6	7	8
1	NDV	>4.0	4.0	2.0		0.5			
2	NDV		>3.0			>3.0		>4.0	
3	Mumps	>2.0	5.0		4.0	3.0		1.0	
4	NDV	>3.0	3.0	2.0		1.0			
	Mumps	>5.0	>5.0	>5.0		>5.0*		3.0	2.0
	WS	>4.0	3.0	1.0		0			
	Sendai	>4.0	4.0	2.0		1.0			
5	NDV	>4.0	>6.0	6.0			3.0		0
	Mumps	>4.0	6.0	6.0			3.0		1.0
	WS	1.0	0	0			0		0
	Sendai	>4.0	>6.0	6.0			3.0		0
6	NDV undil.	>3.0		>5.0		>5.0			
	1:3	3.0		2.0		2.0			
	1:9	0		0		0			
	Mumps undil.	>3.0		>5.0		>5.0			
	1:3	>3.0		>5.0		>5.0			
	1:9	>3.0		>5.0		5.0			
	1:27	0		0		0			

All VSV titrations were carried out 24 hours after addition of interferon or control material to MCN cultures.

* Medium renewal at this date.

infection in the test MCN cultures, converting the temporary protection by interferon into the prolonged resistance previously described (1). The results of Experiment 6 indicate that upon dilution of interferon preparations the protective effect is rapidly lost; *i.e.*, 3- to 9-fold dilutions still revealed interferon activity but with a further 3-fold step in dilution no protection was observed.

The interferon preparations induced resistance also to viruses other than VSV. The PR8 strain of influenza A and the F strain of Sendai viruses undergo incomplete cycles of propagation in MCN cultures with the emergence of non-infectious hemagglutinins (11, 8). In order to observe this phenomenon inoculation of large doses of virus is required; *i.e.*, 10^8 or more infectious units for chick embryos.

TABLE III

Effect of Interferon on Production of Non-Infectious Hemagglutinins in MCN Cultures Challenged with Influenza (PR8) or Sendai Viruses

Exp. No.	Interferon prepared with	Challenge virus	Hemagglutinin titers*	
			Hrs. after challenge	
			2	72
1	NDV	PR8	1:2‡	1:8
	Control		1:2	1:192
2	NDV	Sendai	1:2	1:128
	Mumps		1:2	1:128
	WS		1:2	1:512§
	Sendai		1:2	1:128
	Control		1:2	1:1024
3	NDV (24)‖	Sendai	—	1:12
	NDV (48)		—	1:12
	Mumps (24)		—	1:2
	Mumps (48)		—	1:3
	Control		1:6	1:256

* The cells were sonically disintegrated in the medium prior to test.

‡ Disintegrated cells often cause atypical patterns of sedimented red cells due to cellular debris in low dilutions.

§ The WS interferon was shown to be ineffective also against VSV, see Table II, Experiment 5.

‖ The interferon preparations were harvested after 24 and 48 hours, respectively.

Small bottle cultures containing 2×10^6 cells were washed and then overlayed with 10 ml. of either interferon or control preparations. After 24 hours of further incubation the cultures were washed again and 10 ml. of a 1:5 dilution of allantoic fluids infected with PR8 or Sendai viruses were added. The inocula were removed after an adsorption period of 2 hours. The cultures were washed 4 to 6 times and then refed with standard medium. Some of the cultures were harvested at this time for base line titrations, the others after 72 hours of further incubation at 37°C. The cells were suspended in the culture media and then disintegrated in the treatment vessel of a Raytheon sonic oscillator. The final materials were titrated for hemagglutinating activity.

The results are shown in Table III. It is evident that the interferon-treated MCN cultures yield from 8 to 100 times less incomplete virus progeny than

the cells exposed to the control materials. Again, variations in the potency of the interferon preparations are evident.

Comparison of Interference and Interferon

In an effort to correlate the extent of interference by inactivated virus to the production of interferon the following type of experiment was carried out.

Sets of adequate numbers of MCN cultures were inoculated with 1 ml. amounts of increasing 3-fold dilutions of (a) irradiated mumps virus; (b) irradiated NDV; and (c) irradiated normal allantoic fluid, using 8 cultures per dilution. These preparations contained 1280, 640, and 0 HA units/ml. After 3 hours of incubation the inocula were removed, the cultures washed and refed in the usual manner. Following further incubation for 24 hours the media were collected and pooled according to groups. The cells were refed and challenged with 10^1 to 10^4 TCD$_{50}$ of VSV using 2 tubes of each group for each dose. The media were transferred in 0.8 ml. volumes to 8 fresh MCN tube cultures each per group and challenged 24 hours later with VSV using the same dosages and 2 cultures per dose per group.

The essential results of this experiment are shown in Table IV, giving the extent of cellular destruction by VSV on the 5th day after challenge. It can be seen that interference induced in the cells by the irradiated virus at the endpoint dilution of effectiveness is slightly more solid than the protective action of the interferon preparation derived from the respective cells. It is evident that when interference is obtained in the cells by inactive virus the media removed from these cells contain interferon activity.

Some Properties of Interferon Derived from MCN Cells

It has been shown by Isaacs et al. (4) and Lindenmann et al. (6) that interferon derived from the chorioallantoic membrane of chick embryos differed in several of its properties from interfering, ultraviolet- or heat-inactivated influenza virus. Table V summarizes the results of corresponding experiments carried out with interferon produced in MCN cultures.

In the first experiment preparations of (a) interferon derived from MCN cells after adsorption of inactivated mumps virus in the usual manner and (b) the original irradiated virus were subjected to centrifugation at 20,000 R.P.M. for 30 minutes. The supernates were separated and the sedimented virus of (b) was resuspended in Hanks' solution to the original volume. Other aliquots of the preparations were twice absorbed with 10 per cent chicken red cells in an ice bath and the absorbed fluids were saved for analysis. In the second experiment, interferon derived from MCN cells by exposure to inactivated NDV and the irradiated virus employed for interferon production were used. Aliquots were mixed with specific anti-NDV serum and incubated at 4°C. for 2 hours prior to test. Other aliquots were exposed to 0.001 per cent trypsin (Difco 250) for 2 hours at 37°C. The various preparations were then added to groups of MCN culture tubes and these were challenged with various doses of VSV 24 hours later. The degree of cellular lesions on the 3rd day after challenge with 10^2, 10^3, and 10^4 TCD$_{50}$ of VSV are shown in the table.

It is seen that interferon was not sedimentable at 20,000 R.P.M. nor adsorbed onto red cells, nor neutralized by specific immune serum in contrast to the

TABLE IV

Relation of Interference by Irradiated Virus to Interferon Production in MCN Cultures

U.V. virus	Challenge TCD$_{50}$ of VSV	Test	Average VSV lesions on 5th day after VSV challenge				
			Dilution of U.V. virus				
			Undiluted	1:3	1:9	1:27	1:81
Mumps	10^4	U.V. virus*	0	0	0	4	4
		Interferon‡	0	±	1	4	4
	10^3	U.V. virus	0	0	0	4	4
		Interferon	0	0	1	4	4
	10^2	U.V. virus	0	0	0	1	4
		Interferon	0	0	±	4	4
	10^1	U.V. virus	0	0	0	±	4
		Interferon	0	0	±	4	4
NDV	10^4	U.V. virus	0	2	4	4	
		Interferon	0	4	4	4	
	10^3	U.V. virus	0	±	4	4	
		Interferon	0	4	4	4	
	10^2	U.V. virus	0	0	4	4	
		Interferon	0	3	4	4	
	10^1	U.V. virus	0	0	±	4	
		Interferon	0	2	4	4	
None§	10^2	U.V. control	4	4			
		Interferon control	4	4			
	10^1	U.V. control	4	4			
		Interferon control	4	4			

* 24 hours after addition of irradiated virus, the media were removed, the cell sheets were washed and refed, and the cultures were then challenged with VSV.

‡ The media removed at 24 hours were pooled according to groups and transferred to fresh MCN cultures. These were challenged 24 hours later with VSV.

§ Normal allantoic fluid.

results obtained with the interfering activity of the U.V. (ultraviolet-treated) viruses. Trypsin affected interferon but not the irradiated virus. Thus, the interferon derived from MCN cells behaved like the interferon produced in chorio-allantoic membranes as reported by Isaacs and his associates (4–6).

Influence of Source of Test Cells on Interferon Activity and Production

It was of interest to determine to what extent interferon produced in one type of cell would protect other cells and whether cells other than MCN cultures would yield interferon. While no extensive surveys were carried out regarding these points, some information has been gathered to show that the source of the cells plays a role with respect to both questions.

Table VI summarizes data of experiments in which interferon preparations derived from MCN cells by addition of irradiated NDV, mumps, Sendai, and WS viruses were tested for protection against various challenge viruses in cultures of HeLa, human and rabbit kidney, and mouse embryo fibroblastic cells. It can be seen that protection was obtained in all the cells except HeLa

TABLE V

Properties of Interferon as Compared to Irradiated Virus

Virus used	Treatment	Average VSV lesions on 3rd day after challenge					
		Interferon			U.V. virus		
		Challenge dose of VSV (TCD$_{50}$)					
		10^2	10^3	10^4	10^2	10^3	10^4
Mumps	None	0	0	0	0	0	0
	20,000 r.p.m. supern.	0	0	0	4	4	4
	20,000 r.p.m. sediment	n.d.	n.d.	n.d.	0	0	0
	Absorbed RBC	0	0	0	4	4	4
NDV	None	0	0	0	0	0	2
	+ immune serum	0	0	1	4	4	4
	+ trypsin	4	4	4	0	0	2

cultures when challenged with VSV (Experiments 1 to 4). HeLa cells remained susceptible also to the various myxoviruses (Experiment 5). The effectiveness of the various interferon preparations used in this last test is attested by the results obtained in MCN cells on challenge with VSV (Table II, Experiment 4). The lack of protection against cytopathic effects of the challenge virus in this experiment was confirmed by hemagglutination tests with sonically disintegrated cells derived from parallel cultures. The HA titers observed in the interferon series did not differ significantly from those obtained with the corresponding control preparations.

Representative examples of attempts to produce interferon with various irradiated myxoviruses in cells other than MCN cultures are summarized in Table VII. In the first experiment shown, mouse embryo fibroblasts yielded an interferon which protected homologous and MCN cells to similar degrees

but failed to induce resistance in HeLa cultures. These results were indis-
guishable from those obtained with interferon derived from MCN cells. No
detectable amounts of interferon were elicited in HeLa cultures under identical
experimental conditions. Additional efforts to derive interferon from HeLa
cells, likewise, failed (Experiment 2). None of the preparations studied pro-

TABLE VI

Effectiveness of Interferon Derived from MCN Cultures in Various Types of Cultured Cells

Exp. No.	Interferon prepared with	Cells used in test	Challenge virus	Protective index, *log*		
				Days after VSV challenge		
				2	3	5
1	Mumps	HeLa	VSV	0	0	0
		MCN		5.0	4.0	3.0
2	Mumps	HeLa	VSV		0	0
		MCN			6.0	5.0
	NDV	HeLa			0.5	0
		MCN			4.0	3.0
3	Mumps	Mouse fibroblasts	VSV		>2.0	2.5
		HeLa			0	0
		Rabbit kidney			>1.5	>2.5
		MCN			>3.0	3.5
4	NDV	Mouse fibroblasts	VSV		>1.5	>2.5
		Human kidney			>2.5	>3.5
		MCN			>2.5	>3.5
5*	NDV	HeLa	NDV	0	0	0
	Mumps		Mumps	0	0	0
	Sendai		Sendai	0	0	0
	WS		WS	0	0	0

* For the results in MCN cultures with VSV challenge see Table II, Experiment 4.

tected MCN cultures against VSV or HeLa cells against the three myxoviruses
used. In the third experiment interferon preparations produced in MCN and
human kidney cultures were compared. While the two preparations induced
some resistance to VSV in both types of cells, the protection in the homologous
interferon-cell combinations was somewhat more pronounced than in the
heterologous set-ups.

These as yet limited tests revealed that HeLa cells differ from the others
studied in that they were neither protected by the interferons employed nor

TABLE VII

Production of Interferons in Various Types of Cultured Cells and the Degree of Mutual Protection Induced

Exp. No.	Interferon		Test		Criterion	Results					
						Interferon			Control*		
	Cells	U.V. virus	Cells	Virus		Days after challenge					
						1	2	3	1	2	3
1	MCN	Mumps	MCN	VSV 10^3 TCD$_{50}$	CPE‡	0	0	0	1	4	4
			Mouse fibroblasts			0	0	0	±	4	4
			HeLa			1	4	4	1	4	4
	Mouse fibroblasts	Mumps	MCN	VSV 10^3 TCD$_{50}$	CPE	0	0	1	1	4	4
			Mouse fibroblasts			0	0	1	1	4	4
			HeLa			1	4	4	1	4	4
	HeLa	Mumps	MCN	VSV 10^3 TCD$_{50}$	CPE	1	4	4	2	4	4
			Mouse fibroblasts			±	4	4	±	4	4
			HeLa			1	4	4	1	4	4
2	HeLa	NDV Mumps WS Sendai	MCN	VSV 10^0 to 10^6 TCD$_{50}$	Protective index	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0
		NDV WS Sendai	HeLa	NDV WS Sendai	HA	1:2 1:2 1:2	— — —	1:256 1:192 1:512	1:2 1:2 1:2	— — —	1:256 1:192 1:512
3	MCN	NDV	MCN	VSV 10^3 TCD$_{50}$	CPE	0	0	±	1	4	4
			Human kidney			0	1	2-3	1	3	4
	Human kidney	NDV	MCN	VSV 10^3 TCD$_{50}$	CPE	0	3	4	1	4	4
			Human kidney			0	0	±	1	3	4

* Media from cultures inoculated with normal allantoic fluid were added to the test cultures instead of interferon.

‡ Average VSV lesions.

were they capable of yielding interferon upon exposure to inactivated myxo-
viruses. It was of obvious interest to determine whether the irradiated viruses
per se would induce interference in HeLa cells. In general, little or no protec-
tion was obtained with inactivated mumps, Newcastle disease, WS, and
Sendai viruses when VSV was used for challenge. However, transitory degrees
of resistance were noted with challenge by a myxovirus such as NDV. It
was thought possible that this temporary resistance in this case was not
caused by interference in the sense of an intracellular blockade of viral repro-
duction (12) but by destruction of cell receptors by the irradiated virus followed
by receptor regeneration and a correspondingly delayed infection by the
challenge agent. Indeed, addition of receptor-destroying enzyme of *Vibrio
cholerae* (RDE, obtained from the Behringwerke, Marburg, Germany) pro-
tected HeLa cultures to some extent against NDV but not VSV.

Interferon-Like Substances in Persistently Infected Cultures

As pointed out in the introduction, tests for interferon in MCN_{NDV},
or MCN_{mumps} cultures initially failed. In these tests the media from cultures
mained as routine were collected and then subjected to high speed centrifuga-
tion or absorbed with chicken red cells in order to remove virus particles. The
media thus treated were then mixed with potent specific anti-viral sera and
transferred to MCN cultures. Challenge with VSV 24 hours later revealed
no evidence of resistance. In the light of the data described in the preceding
sections, it appeared to be possible that these experiments failed for quanti-
tative reasons. Efforts were made, therefore, to increase the number of cells
in relation to the volume of medium employed by a technique developed by
Hummeler and his associates (13).

MCN and MCN_{mumps} cells were seeded (5×10^6 cells in 50 ml. of medium) into 500 ml·
round prescription bottles which were rotated on a slow roller apparatus. The medium con-
sisted of 55 per cent Scherer's maintenance solution, 30 per cent medium 199, 10 per cent
horse serum, and 5 per cent unheated calf serum. The last named ingredient was required
to grow the cells in even sheets under these conditions (13). The cultures were refed at 2
to 3 day intervals and after about 2 weeks of rotation heavy layers of cells had formed. At
this time the media were renewed again and collected 3 to 4 days later for interferon assays
(A preparations). The cells were fed once more, but now with only 10 ml. of 30 per cent
medium 199 in Scherer's maintenance solution. These media were harvested 2 to 4 days
later. (B preparations). At the time of the last medium collection the MCN_{mumps} cultures
contained a total of 5.8 to 9.4×10^7 cells; the control cultures 1.7 to 1.8×10^8 cells. Simi-
lar differences in cellular multiplication have been observed previously (1). The various
media obtained from the infected and control cultures were clarified at 2000 R.P.M. for 20
minutes and then centrifuged at 25,000 R.P.M. for 1 hour. The supernates were carefully
withdrawn and tested for hemagglutinating activity with negative results. They were mixed
with human mumps hyperimmune globulin (Hyland Laboratories, Los Angeles) in a final
concentration of 1:20 and incubated at 37°C. for 60 minutes. Tests for active virus were nega-
tive. The final preparations were then inoculated in 0.9 ml. amounts into adequate numbers of
MCN tube cultures from which the media had been removed. After incubation periods

of 24 hours at 37°C. the cultures were challenged with VSV (10^1 to 10^5 TCD_{50}). In Experiment 1 the cultures were washed prior to challenge but not in Experiment 2.

The results are summarized in Table VIII. It is seen that the preparations derived from the persistently infected cultures were capable of inducing

TABLE VIII

Evidence for Interferon Production in Persistently Infected MCN Cultures

| Exp. No. | Preparation | | | VSV lesion on 3rd day after challenge | | | | |
| | Code | Cells | Dilution | Dose of VSV (TCD_{50}) | | | | |
				10^1	10^2	10^3	10^4	10^5
1	A* 4th day	MCN	Undiluted	4	4	4	4	—
		MCN_mumps	"	0	0	3	4	—
	B* 2nd day	MCN	Undiluted	—	4	4	—	—
		MCN_mumps	"	—	0	2	—	—
2	A 3rd day	MCN	Undiluted	—	4	4	4	4
		MCN_mumps	Undiluted	0	0–2	1–3	3	3–4
			1:3	—	0–±	2–3	2–3	3
			1:27	—	2–3	2–3	3	4
		MCN_mumps, 60 min. 60°C.	Undiluted	—	3–4	4	4	4
	B 4th day	MCN	Undiluted	—	4	4	4	4
		MCN_mumps	1:3	—	0	0	0	1–2
			1:9	—	0	0	0	1–2
			1:27	—	0	0	0	2–4
		MCN_mumps + trypsin	Undiluted	—	3	4	4	4

* A, serum-containing medium (50 ml. per culture); (4th day) = collected after 4 days of contact with cell culture.
  B, serum-free medium (10 ml. per culture).

some degrees of resistance to VSV in MCN cells but not those obtained from control cultures. The extent of protection observed was not as striking as that noted with the best interferon preparations produced by ultraviolet-inactivated viruses (see Table II). In the first experiment both the 10 ml. of serum-free medium, collected after 2 days of incubation with persistently infected cells (preparation B) and the 50 ml. of serum-containing medium harvested after 4 days of contact with the cells (preparation A) gave about

equal protection per 0.9 ml. used but only against 100 TCD_{50} of VSV when readings were made on the 2nd day after challenge. In the second experiment the B preparation, collected on the 4th day of contact with infected cultures, protected MCN cultures to a significantly better extent (protective index of 10,000 on the 2nd day after challenge), even in dilution 1:27, than preparation A, harvested after 3 days of incubation with MCN_{mumps} cells. An aliquot of A was heated at 60°C. for 1 hour and a sample of B was exposed to 0.001 per cent trypsin at 37°C. for 2 hours prior to the addition of the hyperimmune globulin. Both procedures destroyed the protective effects in accordance with the properties of the interferon described by Isaacs and his associates (4–6).

DISCUSSION

The data recorded have shown that transitory exposure of MCN cultures to ultraviolet-inactivated myxoviruses leads to the subsequent release into the media of a substance which on transfer to fresh MCN cultures protects the cells against infection with vesicular stomatitis (VSV), influenza A (PR8), and Sendai viruses. The media so obtained were free of detectable viral hemagglutinating and complement-fixing activity. The first named challenge virus is readily propagated and maintained by serial passages in MCN cultures, whereas the other two agents undergo only incomplete reproductive cycles with the emergence of non-infectious hemagglutinating particles (NIHA) so that they cannot be passed in series (11, 8). The protection afforded by this substance against VSV is temporary in nature and the cultures ultimately succumb to the infection. With the influenza and Sendai viruses no late breakthrough is detectable. This difference in response to the challenge viruses is explainable on the basis that any small amounts of VSV remaining in the cultures are capable of initiating spreading infections as the cells gradually lose their resistance. In contrast, any cells that may become infected with remaining influenza or Sendai virus particles yield only NIHA and thus further cycles of reproduction are prevented even though the cells may no longer be protected by interferon action. Only when a large proportion of cells is infected at one time by these agents enough NIHA is produced to become detectable by the available techniques.

As far as tested, the protective substance has similar properties as the interferon described by Isaacs and his associates (4–7) and thus it appears to be justifiable to call it an interferon. It is not neutralized by specific antibodies to the virus used for its production; it is not sedimented by centrifugal speeds sufficient to precipitate the virus; it is not adsorbed onto red cells; but it is largely inactivated by trypsin which has no effect upon the virus.

Interferons were obtained following exposure to inactivated myxoviruses also from mouse embryo fibroblasts and human kidney cells but not from HeLa

cultures. The active preparations induced some degrees of resistance to VSV or myxoviruses in the types of cultures studied except in HeLa cells. Only a small number of cell types were employed for interferon production as well as interferon assay. Yet, the limited cross-protection tests carried out under these conditions suggest some degree of host-specificity in that interferon preparations derived from one type of cell may be somewhat more protective for homologous than heterologous cultures. These interrelationships and the range of viruses affected by interferon activity require further study.

The role of interferon in establishing interference, likewise, requires further analysis. Data presented above denote that there is a close relationship between interference by ultraviolet-inactivated virus and the presence of interferon in the system. Cultures receiving minimal interfering doses of inactivated virus regularly yielded media just prior to viral challenge which exhibited some interferon activity. On the other hand, interferon protects for a relatively short period of time when compared to viral interference and, as discussed above, shows some degree of host specificity. Both points do not necessarily imply that interference and interferon action are based on different principles. The interference induced by inactivated virus may persist for somewhat longer periods because the affected cells may continue to yield interferon, whereas interferon acting on its own may rapidly be utilized or inactivated. The problem of host-specificity of interferon obviously does not enter into interference tests since under these conditions any interferon produced can act only upon cells within the same culture.

While the MCN cells were shown to be capable of yielding interferon on exposure to inactivated myxoviruses the question remains whether fully active virus, likewise, will produce this substance. It has been shown by Burke and Isaacs (7) that upon infection of pieces of chorio-allantoic membranes with active influenza viruses interferon is released in detectable quantities only after 24 hours of incubation. More recently it was observed (R. R. Wagner, personal communication) that allantoic fluids collected 72 hours after inoculation of chick embryos with active influenza viruses contain an interferon in high concentration which protects chick embryo fibroblast cultures against large doses of equine encephalomyelitis virus. In the present studies, MCN cultures persistently infected with mumps or Newcastle disease viruses, and in consequence resistant to superinfection with VSV, failed to yield interferon, when maintained under routine conditions. However, when the cell population was substantially increased in relation to the volume of medium employed, some interferon activity could readily be demonstrated in the cultures.

The finding of interferon in the persistently infected MCN cultures, although in only relatively small concentrations, suggests that this substance may play a decisive role in the establishment of resistance to VSV and other viruses and with it in the maintenance of persistent infection. Further experimentation

will have to answer the question whether interferon is derived only from virus-yielding cells, that is, from the 2 to 10 per cent of the cells which can be proven to contain and produce virus (1, 3), or whether the remaining cells are also to some extent engaged in its production. If the latter were the case the mechanism by which they enter into production, likewise, will have to be determined. Because of the transitory nature of the protective effect of interferon, one may expect that a proportion of the cells are regaining susceptibility at given times and that then infectious virus and interferon compete for these cells. The former may win in some instances to insure persistence of the viral infection of the cultures.

SUMMARY

In previous reports of this series, it was shown that persistent infection of MCN cultures with certain myxoviruses rendered the cells insusceptible to superinfection by several cytopathogenic viruses. It was thought that production of an interferon might be the cause of this resistance and efforts to confirm this suggestion have been presented.

Addition of ultraviolet-inactivated myxoviruses (mumps, Newcastle disease, influenza A, and Sendai) to MCN cultures for periods of 2 to 3 hours, followed by washing and refeeding of the cells, led to the subsequent release into the media of a substance which induced in fresh MCN cells a transitory resistance to infection by vesicular stomatitis virus, and prevented incomplete reproductive cycles of influenza A and Sendai viruses. Media containing this substance were free of detectable hemagglutinating activity and viral complement-fixing antigens. The substance was not neutralized by specific antiviral sera; it was not sedimentable by high speed centrifugation; it was not adsorbed onto red cells; but it was inactivated by trypsin. Thus, its properties matched those of the interferon described by Isaacs and his associates.

A comparison of the extent of resistance induced in MCN cells by decreasing doses of ultraviolet-inactivated myxoviruses (interference test) and the protection afforded by the media removed from the cultures prior to challenge and transferred to fresh MCN tubes (interferon test) revealed that wherever interference became detectable in the cells, the media of the corresponding cultures contained some interferon.

Interferon was obtained by inactivated myxoviruses also from primary cell cultures by the same techniques, but not from HeLa cells. Interferons derived from one type of culture may protect others equally well or show a certain degree of host specificity in that resistance in homologous cells may be somewhat more pronounced than in heterologous cultures. No resistance could be induced in HeLa cells by the interferon preparations employed.

Interferon was detected also in MCN cultures, persistently infected with mumps virus. Its concentration was apparently too small in carrier cultures

maintained as routine to be measurable. However, when the cells were grown in heavy sheets in roller bottles, and especially when the volume of medium was reduced for several days prior to harvest, interferon became readily detectable.

These results strengthen the suggestion that interferon may play a decisive role in the establishment and maintenance of persistent infections in the system under study. Its nature, source, mode of action, and exact role in persistent infection remains to be elucidated.

BIBLIOGRAPHY

1. Henle, G., Deinhardt, F., Bergs, V. V., and Henle, W., Studies on persistent infections of tissue cultures. I. General aspects of the system, *J. Exp. Med.* 1958, **108**, 537.
2. Bergs, V. V., Henle, G., Deinhardt, F., and Henle, W., Studies on persistent infections in tissue cultures. II. Nature of the resistance to vesicular stomatitis virus, *J. Exp. Med.*, 1958, **108**, 561.
3. Deinhardt, F., Bergs, V. V., Henle, G., and Henle, W., Studies on persistent infections in tissue cultures. III. Some quantitative aspects of host cell-virus interactions, *J. Exp. Med.*, 1958, **108**, 573.
4. Isaacs, A., and Lindenmann, J., Virus interference. I. The interferon, *Proc. Royal Soc. London, Series B*, 1957, **147**, 268.
5. Isaacs, A., Lindenmann, J., and Valentine, R. C., Virus interference. II. Some properties of interferon, *Proc. Royal Soc. London, Series B*, 1957, **147**, 268.
6. Lindenmann, J., Burke, D. C., and Isaacs, A., Studies on the production, mode of action and properties of interferon, *Brit. J. Exp. Path.*, 1957, **38**, 551.
7. Burke, D. C., and Isaacs, A., Some factors affecting the production of interferon, *Brit. J. Exp. Path.*, 1958, **39**, 452.
8. Deinhardt, F., and Henle, G., Studies on the viral spectra of tissue culture lines of human cells, *J. Immunol.*, 1957, **79**, 60.
9. Youngner, J. S., Monolayer tissue cultures. I. Preparation and standardization of trypsin-dispersed monkey kidney cells, *Proc. Soc. Exp. Biol. and Med.*, 1954, **85**, 202.
10. Henle, W., and Paucker, K., Interference between inactivated and active influenza viruses in the chick embryo. I. A re-evaluation of factors of dosage and timing using infectivity titrations for assay, *Virology*, 1958, **6**, 181.
11. Henle, G., Girardi, A. J., and Henle, W., A non-transmissible cytopathogenic effect of influenza virus in tissue culture accompanied by fornua of non-infectious hemagglutinins, *J. Exp. Med.*, 1955, **101**, 25.
12. Henle, W., Interference phenomena between animal viruses: A review, *J. Immunol.*, 1950, **64**, 203.
13. Hummeler, K., Ketler, A., and Hinuma, Y., data to be published.