Production of Interferon by Suspensions of Human Leucocytes.†‡ (27072)

ION GRESSER* (Introduced by J. F. Enders)
Research Division of Infectious Diseases, Children's Hospital Medical Center and Department of Medicine, Harvard Medical School

Multiplication of several animal viruses in suspensions of leucocytes has been previously demonstrated(1). Since it is now well established that cultures of human cells produce interferon-like substances we considered it of interest to determine whether human leucocytes of the circulating blood exposed to virus might respond in the same manner. The results of such experiments form the basis of this preliminary communication.

Methods and materials. Suspensions of Leucocytes. Procedures for obtaining venous blood and preparation of suspensions of leucocytes utilizing Bacto-Phytohemagglutinin (Difco) have been previously described(1). After cell counts, washed leucocytes (500,000-5 million/ml) were resuspended in 5-10 ml of medium consisting of 5% heat inactivated horse serum, 5% beef embryo extract, 45% bovine amniotic fluid and 45% Hanks' balanced salt solution. Leucocyte preparations were incubated in rubber stoppered§ prescription bottles 10 cm × 4 cm kept on stationary racks at 37°C. Virus was in most instances added one hour after preparation of cells.

Viruses. Relevant data on viruses employed are as follows: Sendai virus (F. Davenport) allantois of embryonated egg, 27 passages; Sindbis virus (R. Taylor AR-339) human amnion (HA) cells, 10 passages; Polio virus II (MEF_1) chick embryo cell cultures 143, HA cells, 2 passages; Polio virus I (Brunhilde) human uterus cell cultures, 3 passages; Measles virus, Edmonston strain, human kidney cells, 29 passages.

Preparation of Stock Sendai Virus. Sendai virus was grown in 9-11 day embryonated eggs. The inoculum, consisting of a 10^{-3} dilution of stock virus was introduced into the allantoic cavity. After 72 hours incubation at 35°-37°C, allantoic fluid was harvested, centrifuged for 10 minutes at 500 rpm and the supernate centrifuged at 4°C for 2 hours at 110,000 g (Spinco L preparative centrifuge #40 rotor). The sediment was resuspended in a mixture of 50% bovine amniotic fluid medium and 50% skimmed milk and stored at −70°C.

Virus Assay. Infectivity titers were calculated by the formula of Reed and Muench. Sendai virus was titrated in embryonated eggs (EID_{50}) taking as criterion of infection appearance of hemagglutinin in allantoic fluids. Sindbis, poliovirus and measles viruses were prepared and titrated in HA cells. In this system more than 25% cell destruction was considered evidence of viral multiplication. Preparation and maintenance of HA cells has been previously described(2).

Antiserum. Anti-Sendai rabbit serum (HAI-titer = 1:1280) was obtained through the courtesy of Dr. J. Lewis of the Sterling Winthrop Co., Rensselaer, N. Y.

Assay of Interferon. All preparations to be tested for interferon were centrifuged at 110,000 g at 4°C for 2 hours. The supernate (interferon) was shown to be free of infectious virus and hemagglutinin and as an additional precaution to eliminate infectious virus, anti-Sendai rabbit serum in excess was added in certain instances. Interferon was assayed in primary tube cultures of HA cells previously incubated in stationary racks at 35°-37°C for 4 or more weeks. Two fold dilutions of interferon (2-3 tubes/dilution) were incubated with cells for 18-24 hours prior to inoculation of 100 $TCID_{50}$ of Sindbis virus. Complete destruction of cells in control cultures regularly occurred in 3 days. Interferon titer was expressed as the highest dilution

† The author gratefully acknowledges the constant help and guidance of Dr. J. F. Enders. We also wish to express our gratitude to Miss Alison Downie for technical assistance, Dr. E. Engel for technical advice, Dr. S. V. Kevy and Dr. H. McDevitt for arranging to supply us with blood specimens.

‡ Supported in part by grant from U.S. Public Health Service, Department of HEW.

* Postdoctoral Research Fellow of National Foundation.

§ The West Co., Phoenixville, Pa.

which protected more than 75% of the cell sheet in at least one of 2 cultures for at least 3 days after complete destruction of cells had occurred in control cultures.

Experimental. Production of Interferon by Leucocytes. Previous experiments suggested that after inoculation of high concentrations of chick-adapted Sendai virus into primary cultures of human amnion cells, an incomplete cycle of viral replication ensued. This was associated with rapid production of high titers of an interferon-like substance(3). Sendai virus was therfore chosen as the principal interferon-inducing virus and was inoculated in high concentrations (10^7-10^8 EID_{50}/ml) into suspensions of leucocytes ranging from 500,000-5 million cells/ml. On serial passage of this chick adapted virus in human leucocytes no multiplication of infecious virus was demonstrated. Thus after inoculation of $10^{7.7}$ EID_{50}/ml of virus, only $10^{4.7}$ EID_{50}/ml was recovered after 72 hours. No infectious virus was detected in the medium after 2 further passages of this material utilizing fresh preparations of washed leucocytes. On two occasions measles virus (Edmonston strain) was employed as the test agent for interferon production since it had been previously demonstrated that this strain multipled in suspensions of human leucocytes(1).

Examination of stained preparations of leucocytes 3 and 7 days after addition of virus revealed that the great majority of remaining cells were mononuclear. Only a few polymorphonuclear cells were observed. No morphologic cellular abnormalities were noted though tests for viability were not performed.

Leucocyte suspensions prepared from the peripheral blood of 20 healthy and ill donors were tested for interferon production (Table I). After inoculation of the suspensions with either Sendai or measles virus, interferon in varying titers was detected in 21 of 22 preparations (15/16 patients). In one case (pt. #16) no interferon was demonstrated. The leucocytes from this person were inadvertently maintained in a medium subsequently shown to be markedly toxic for primary cultures of human amnion cells. It is probable therefore that this medium was toxic for leucocytes as well, though this was not established.

In no instance was interferon found in uninoculated suspensions: (10 suspensions representing 10 subjects). In 2 patients (pts. #18 and #19) with typical measles attempts to isolate the virus from whole blood were unsuccessful, as well as an attempt to isolate this virus from a suspension of one patient's leucocytes which was incubated for 3 days at 37°C.

In considering the results presented in Table I, it should be emphasized that because of a number of variables, interferon titers cannot be accurately compared. The number of leucocytes in different suspensions varied between ½ and 5 million cells/ml and different lots of cultures of human amnion cells were employed in assaying interferon. It is possible that the sensitivity of primary cultures of amnion cells varies in respect to both interferon and challenge virus.

Identification of Inhibitor. Though all interferon preparations were ultracentrifuged to remove infectious Sendai virus, only representative preparations were subjected to various physical and chemical tests. It was found that neither ultracentrifugation nor antiserum to the interferon-inducing virus diminished the viral inhibitory effect of these preparations. Dialysis for 24 hours at a pH of 4, and heating at 56°C did not affect the interferon titer; but incubation with crystalline trypsin (1 mg/1 ml 37°C 1 hour) reduced the titer from 1:384 (pt. #2) to <1:8.

Quantitative Relationship between Number of Leucocytes and Yield of Interferon. From Table II it is apparent that after inoculation of 10^8 EID_{50}/ml of chick adapted Sendai virus into suspensions of leucocytes decreasing by a factor of 10 the concentration of interferon found 72 hours later was roughly proportional to the number of cells. Though erythrocytes were present in all leucocyte preparations they played no role in interferon production since most of them were lysed shortly after addition of Sendai virus. This agent has been shown to cause hemolysis(4). Moreover, no interferon was detected after exposure to the virus in suspensions of erythrocytes which contained very few leucocytes (Table II).

Rapid Production of Interferon. After infection of primary cultures of human amnion cells with Sendai virus interferon was demonstrated in high titer within 12 hours after

TABLE I. Production of Interferon by Human Leucocytes after Exposure to Virus.

	Patient	Age	Sex	Disease	Interferon-Inducing Virus	Interferon Titer[1] (hr of harvest)[2]
1.	EE	35	M	Normal	Sendai	24 (48 hr)
2.	IG	32	M	"	"	384[3]
				"	Measles	>96
				"	Polio I	<4
	IG	32	M	"	None	<4
				"	Sendai	<4 (2 hr)
				"	"	256
	IG	32	M	"	"	>384
3.	BJ	45	F	Acute Barbiturate Ingestion	"	24
4.	CF	80	F	Myocardial Infarct Convalescent	"	>192[4]
5.	PT	78	F	Congestive Heart Failure	"	>3072
6.	AP	80	F	Cirrhosis	"	1024
7.	CG	28	F	Obesity	"	>768
	CG	28	F	"	"	64[5]
8.	JY	3	F	Acute Myelomonocytoid Leukemia[6]	None	<4
					Sendai	>192
9.	JJ	82	M	Chronic Lymphatic Leukemia[7]	None	<4
					Sendai	24
10.	PT	2	M	Acute Myelomonocytoid Leukemia with Acute Varicella (1 day)[8]	None	<4
					Sendai	96
11.	ML	2	F	Acute Lymphoblastic Leukemia[9]	"	64
12.	FE	16	M	Congenital Agammaglobulinemia	"	32
					Measles	12
					Sendai	>1024
13.	JW	18	M	idem.	"	32
14.	EA	11	M	"	"	>256
15.	DW	14	M	Aseptic Meningitis	None	<4
					Sendai	>384
16.	DG	3	M	" "	None	<4
					Sendai	<4
17.	NM	14	M	Mumps Sx. 60 hr duration	None	<4 (3 & 7 days)
18.	IN	4	M	Measles 24 hr rash	"	<4 " " " "
19.	JH	2	F	? Measles 24 hr rash	"	<4 " " " "
20.	HB	2	M	? Infectious Mononucleosis-5 days duration	"	<4 " " " "

[1] Expressed as reciprocal of dilution of medium that inhibited 100 $TCID_{50}$ of Sindbis virus.
[2] All media assayed for interferon were collected 72 hours after viral inoculation unless otherwise specified.
[3] Interferon titer of this preparation was >192 against Poliovirus II (MEF_1).
[4] Interferon titer <4 when leucocytes were subjected to 2 cycles of freezing and thawing prior to inoculation of Sendai virus.
[5] Leucocytes were incubated for 3 days prior to inoculation of challenge virus, to decrease number of polymorphonuclear leucocytes in suspension.
[6] WBC count in peripheral blood 245,000/mm³. On examination of stained preparations of peripheral blood all cells were believed to be "blasts."
[7] WBC count in peripheral blood 10,000/mm³ 75% lymphocytes.
[8] WBC count in peripheral blood 6,500/mm³ 60% "blasts."
[9] WBC count in peripheral blood 550/mm³. All cells seen on examination of stained preparation either immature "blasts" or lymphocytes.

TABLE II. Relationship Between Number of Leucocytes and Production of Interferon.

No. of Leucocytes/ml Suspension	Interferon Titer*
5,200,000	>3072
520,000	384
52,000	32
5,200	12
520	<2
52	<2
5	<2
Erythrocytes Suspension†	<2

* Reciprocal of dilution of medium, 72 hr after addition of virus.
† Containing <4000 WBC/ml.

infection and maximal titers were attained at 24 hours(3). Though medium from leucocyte suspensions was routinely harvested for interferon assay 72 hours after inoculation, it can be seen from Table III that interferon was liberated in high titer in one experiment as soon as 6 hours after inoculation.

Production of Interferon by Cells of Patients with Agammaglobulinemia. Interferon was formed as readily by leucocytes taken from 3 patients with congenital agammaglobulinemia as it was by cells from normal sub-

jects (Table 1). The clinical course of patient #12 F.E. has been previously described(5,6). He had not received gamma globulin for over one year and his gamma globulin level was usually 25 mg% (normal 600-800 mg%). Patient #13 J. W. is the uncle of Patient #14 E. A.(7). Gamma globulin was administered to uncle and nephew one month prior to testing of their leucocytes and gamma globulin levels on the day leucocytes were withdrawn were 40 mg% and 250 mg% respectively.

Attempts to Detect the Presence of Virus by Assaying Media for Interferon. It is well known that viremia occurs in certain viral diseases. It seemed possible therefore that after incubation of leucocytes taken during the viremic phase, interferon might be produced by leucocytes infected *in vivo*. Emergence of interferon in the medium would provide a criterion for the presence of virus. Attempts were therefore made to detect interferon in the media of uninoculated leucocytes from a normal subject (#2), 3 leukemic patients (Nos. 8,9,10) and 6 patients suffering from acute viral diseases (Nos. 15,16,17,18,19,20) (Table 1). No interferon was demonstrated.

Despite these negative results, an attempt was made to demonstrate presence of the virus of rubella by adding various materials from patients with this infection to suspensions of leucocytes and subsequently assaying the medium for interferon. To preparations of washed leucocytes were added aliquots of throat washings and blood from 3 patients with typical rubella. Materials from 2 of these patients had been collected one day before the onset of rash as well as on the day of rash and had been stored at −70°C for one year prior to testing. Material from the 3rd patient was collected on the day prior to onset of rash and had been stored at −20°C for 7 weeks. Two passages were made in leucocyte suspensions (4 million/ml). No interferon was detected in the medium after assay on the 3rd and 7th day of either the 1st or 2nd passages.

Discussion. A consideration of the various properties of the viral inhibitory factor produced by human leucocytes after infection with Sendai and measles virus would seem to justify employing the term interferon(8). That this interferon was formed and liberated by the leucocytes themselves was supported by the following facts: 1) no interferon was demonstrated in the original viral inoculum (Sendai) which had been prepared by ultracentrifugation of infected allantoic fluid and resuspension of the pellet in medium free of interferon. 2) no interferon was demonstrated in medium nourishing uninoculated leucocytes. 3) no interferon was present in the medium nourishing leucocytes ½-2 hours after inoculation of virus (Table 1, pt. #2 and Table III). 4) amount of interferon released appeared roughly proportional to number of leucocytes. 5) no interferon was detected when leucocytes were lysed by rapid freezing and thawing prior to introduction of virus (patient #4, footnote 4). 6) no interferon was detected in a suspension of erythrocytes exposed to virus.

Since interferon was produced shortly after inoculation there can be little doubt that surviving cells originally present in the donor's blood were responsible for production of interferon rather than descendants of these cells. Evidence was obtained that mononuclear cells were capable of producing interferon. Thus leucocytes incubated for 3 days at 37°C prior to inoculation (case #7, footnote 5) produced interferon. Very few polymorphonuclear cells were seen on examination of stained preparations of these cells at time of inoculation. Moreover, only lymphoblasts or lymphocytes were observed in a differential count of leucocytes of patient #11, yet interferon was

TABLE III. Production of Interferon by Leucocytes at Various Times After Exposure to Virus.

Time in hr	½ hr	1 hr	6 hr	12 hr	16 hr	24 hr	40 hr
Pt. #5 2,600,000 cells/ml	—	<4	—	—	1024	—	1024
Pt. #6 3,700,000 cells/ml	<4	—	>768	768	—	1024	—

— indicates not tested.

elaborated by these cells. Whether or not polymorphonuclear cells also produce interferon cannot be determined from the data now available.

The number of variables in the experimental system precluded quantitative comparison of interferon titers. It should be entirely feasible, however, to control these variables and thus derive significant information on rate and degree of interferon production by leucocytes from patients with a variety of diseases.

Whether interferon-like substances are actually produced by leucocytes *in vivo* remains uncertain though there appears to be no reason to consider this unlikely. In this regard it is of interest that leucocytes from patients with classical congenital agammaglobulinemia produce interferon *in vitro*. It is well known that this group encounters relatively little difficulty with naturally acquired viral diseases and it has been suspected that their recovery may depend on factors other than conventional antibody.

Though we have been unable to demonstrate production of interferon by uninoculated leucocytes taken from patients with acute viral diseases, it should be emphasized that in these cases no virus was isolated from peripheral blood. If leucocytes were taken during the viremic phase of a disease such as measles it is entirely possible that interferon might be produced by these cells when subsequently maintained *in vitro*. This supposition would seem to find support from the following considerations: 1) measles virus has been isolated from the buffy coat of leucocytes from patients with measles(9). 2) measles virus has been propagated in suspensions of human and monkey leucocytes(1). 3) measles virus, as we have shown on 2 occasions, does induce production of interferon by leucocytes *in vitro*.

We were unable to detect interferon in the medium of normal leucocytes inoculated with material from patients with rubella. However, this approach to the detection of certain non-cytopathogenic agents might prove to be of considerable value, since it is possible that such agents adapted to the human host might multiply in human leucocytes maintained *in vitro* and reveal their presence by the production of interferon.

Summary. Suspensions of leucocytes from 2 normal donors and 18 patients with various diseases, including 3 patients with congenital agammaglobulinemia were exposed to Sendai or measles virus and maintained *in vitro* for several days. Production of interferon occurred in 21 of 22 preparations. It is suggested that interferon-like substances may be liberated by leucocytes *in vivo*, after infection with some viruses and that production of interferon by leucocytes may provide a criterion for presence of non-cytopathic viruses.

1. Berg, R. B., Rosenthal, M. S., PROC. SOC. EXP. BIOL. & MED., 1961, v106, 581.

2. Milovanovic, M. V., Enders, J. F., Mitus, A., *ibid.*, 1957, v95, 120.

3. Unpublished data.

4. Fukai, K., Suzuki, T., *Med. J. Osaka Univ.*, 1955, v6, 1.

5. Rosen, F. S., Kevy, S. V., Merler, E., Janeway, C. A., Gitlin, D., *The Lancet*, 1961, v1, 700.

6. ———, ———, ———, ———, ———, *Pediatrics*, 1961, v28, 182.

7. Gitlin, D., *Agammaglobulinemia in Cellular and Humoral Aspects of the Hypersensitive States.* Hoeber & Harper, 1959, p. 375.

8. Isaacs, A., Burke, D. C., *Brit. Med. Bull.*, 1959, v15, 185.

9. Peebles, T. C., Unpublished data.

Received September 25, 1961 P.S.E.B.M., 1961, v108

Fetal Plasma Potassium.* (27073)

J. N. BARKER (Introduced by M. H. F. Friedman)
Department of Physiology, Jefferson Medical College, Philadelphia, Pa.

Christianson and Jones(1) have summarized evidence that fetal plasma potassium concentration is far higher than the normal adult range. Reported values are as high as 17 meq/l(2), while others are around 9 meq/l.

* Aided by U. S. Public Health Service Grant.