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Production of Interferon by Human Mononuclear Leucocytes.* (29794)

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The production of interferon by suspensions of human leucocytes has been previously demonstrated(1). The present series of experiments was performed to determine whether (a) mononuclear cells or (b) polymorphonuclear leucocytes or (c) both cell types were responsible for the interferon produced under these conditions.

Materials and methods. Viruses. Sendai virus (F. Davenport) obtained from Dr. J. F. Enders was passed many times in allantois of embryonated egg. Stock Sendai was grown in 9-11-day-old embryonated eggs in the usual manner. To obtain high concentrations of virus to the order of 10^{8-9} EID_{50}, the harvested virus in allantoic fluid was ultracentrifuged at 40,000 rpm in the Spinco model L ultracentrifuge for 2½ hours. The virus was suspended in M199 with 50% skimmed milk and stored at −20°C. Infectivity titer was calculated by the method of Reed and Muench. The virus was titrated in embryonated eggs (EID_{50}) using the appearance of haemagglutinin as criterion of infection.

Sindbis virus (59G) obtained from Dr. R. Taylor was propagated in established human amnion cells (HA-FL) and titrated. For purposes of interferon assay, approximately 500 $TCID_{50}$ of the virus was used.

Antiserum. Sendai antiserum (HAI-titer = 1:1024) was prepared in rabbits inoculated 5 times intravenously at 3-day intervals with 0.5 ml of Sendai virus in allantoic fluid. The animal was bled 10 days after the last injection. The serum was inactivated at 56°C for 30 minutes and titrated by haemagglutination inhibition.

Tissue cultures. HA-FL cells (Dr. K. Rozee, Univ. of Toronto) were grown in either milk dilution bottles or screw-capped tubes in Hanks' Balanced Salt Solution containing 0.5% lactalbumin hydrolysate, 0.1% glucose, penicillin and streptomycin, and supplemented with 10% calf serum. Upon virus inoculation, no serum was included in the medium.

Iron. The iron powder used was obtained from Fisher Scientific Co. (Cat. # 1-62). Sterility was ensured by autoclaving.

Stains. Orcein stain was obtained from The British Drug House Ltd., Poole, England. A 2% solution was prepared(2) for use.

Dextran. 6% W/V dextran with 0.9% NaCl, Pharmachen Corp., Bethlehem, Pa.

Preparation of leucocyte suspensions. Fifty ml of heparinized blood was taken from healthy male or female donors in sterile flasks. To this sample, dextran (6%) was added to a final concentration of 1:6 in the blood sample. The blood was then incubated in 10 ml portions in sterile screw-capped tubes inclined at a 45° angle for 30 minutes or longer at 37°C.

After incubation, the plasma containing the suspension of leucocytes was removed and pooled. This sample was then divided into 2 portions.

From one portion, the plasma was removed by centrifugation at 2500 rpm for 15 minutes, and the sediment containing leucocytes was washed 3 times, and resuspended in 5 ml of medium 199 containing 10% calf se-

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rum. A total of 8 cell counts was performed on the suspension to determine the final concentration of cells. The cells were stained with acetic-orcein and counted in a haemocytometer (Neubauer), or by an electronic cell counter (Coulter).

From the second portion of plasma, for preparing a mononuclear leucocyte suspension, 3 ml of plasma were diluted with 10.0 ml of medium 199 in 125 ml Erlenmeyer flask, to which was added 0.5 g of iron powder. This suspension was placed in a shaker and agitated for 60 minutes or longer (up to 2 hours) at 37°C.

To remove the polymorphonuclear leucocytes containing phagocytosed iron particles, the wall of the test tube was placed in contact against a strong electromagnet for 10 minutes, at which time the supernate was carefully removed by pipetting and transferred to screw-capped tubes. Several samples of this suspension were stained by Wright's method for differential counting. For these studies a suspension consisting of at least 97% mononuclear cells was required. If adequate separation was not achieved initially, the procedure was repeated once or twice.

The cell suspension, now consisting of 97-100% mononuclear cells, was centrifuged at 2500 rpm for 15 minutes and washed 3 times with medium 199 containing 10% calf serum, and then resuspended in 5.0 ml of medium. Eight cell counts were then done to determine the cell concentration.

Interferon production. The 2 cell suspensions were then each divided into 2 aliquots. To one of these Sendai virus 10^{8-9} EID_{50} in 1.0 ml of medium was added, and to the other, a control, an equal volume of medium free of virus.

The 4 cell suspensions were then adjusted each to a final cell concentration of approximately 10^6 cells per ml in medium 199 containing 20% calf serum, and incubated in screw-capped tubes in 1.5 ml portions, at 37°C in a roller wheel for 24-48 hours.

At the end of incubation, the cultures were pooled into the corresponding 4 samples under test, and repeated differential and total cell counts were made. The specimens were centrifuged to remove the cells and the supernatant fluids were treated with hyperimmune Sendai-antiserum prepared in rabbits, or ultracentrifuged (Spinco model L) at 40,000 rpm for 2-4 hours to remove virus.

Interferon assay. The 4 supernatant specimens were assayed for interferon production in the following manner:—

Four-fold dilutions of interferon with 3 tubes per dilution were incubated with human amnion cells (HA-FL) for 18-24 hours, prior to inoculation of 500 $TCID_{50}$ of Sindbis virus.

For each test, in addition to virus-free cell and tissue culture controls, the following control cultures were included: (a) tissue culture plus iron 0.01% (b) tissue culture-Sendai antiserum toxicity control (c) each of these control cultures was incubated with and without the addition of Sindbis virus.

The cultures were examined on the 3rd and 4th days after incubation and readings were taken at the period in which control cultures showed complete cell destruction by the Sindbis virus.

The interferon titer was expressed as the highest dilution producing 50% protection of the cell sheet for a period of 2 days after complete destruction of the control cultures. The 50% protection titer (IND_{50}) was determined in the following manner:

4+—no protection—100% cell sheet destruction
3+—approximately 75% cell destruction
2+—approximately 50% cell destruction
1+—approximately 25% cell destruction
0—no visible cytopathic change.

The following scoring system was adapted to determine 50% end points, in order to obtain some quantitation of interferon titer (Rozee, K. R., personal communication).

Cytopathic effect (3 tubes)			Sum	Interferon "score"	% cell protection
					(%)
4+	4+	4+	12 (=100%)	0	0
3+	2+	2+	7	5	42
1+	2+	1+	4	8	67
0	0	0	0	12	100

The 50% end-point was determined by plotting the $\log_{10}$ dilution of interferon against the per cent cell protection as out-

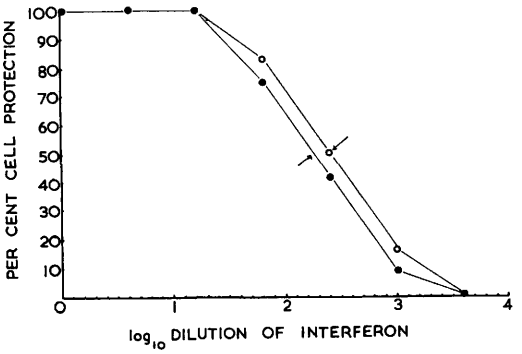

FIG. 1. (Exp. 4) Interferon titer (50% cell protection). ○, Mixed culture; ●, mononuclear cell culture; ↙, 50% end point.

lined above (Fig. 1).

Results. Preparation of leucocyte suspensions. Dextran (6%) sediments the red blood cells more efficiently than Phytohemagglutinin M. Thus, in a 10 ml sample of heparinized blood to which 2 ml of dextran are added, about 3-4 ml of plasma-leucocyte suspension can be obtained within 30 minutes of incubation at 37°C whereas in the same period only one-third to half the yield would be obtained by the use of Phytohemagglutinin. By the use of dextran, the average yield of white cells per 50 ml whole blood sample is in the range of 30-80 $\times$ 10^6 cells.

Differential leucocyte separation. The separation technique described by Hastings *et al* (3) and others(4,5,6) utilizing the phagocytic properties of polymorphonuclear cells by permitting them to ingest freely particulate iron was modified for our use (Fig. 2). During the course of separation it was observed that the efficiency of the technique depended largely on the density of the initial cell suspension to which the iron powder was added. In cases where large numbers of cells were present in small volumes, cells tended to clump, resulting in less efficient phagocytic activity and hence less efficient cell separation.

It was found by trial and error that optimal separation of cells with a final suspension consisting of 97-99% mononuclear cells was achieved by a 1:3 dilution of the initial plasma-leucocyte suspension with M199 to which 0.5 g of iron powder per 6 ml of suspension was added. Of the 29 blood samples tested, there were 8 suspensions in which there was inadequate separation, *i.e.*, less than 97% mononuclear cells in the final suspension. These were discarded from the experimental data. Thus, for purposes of comparison of the interferon production by cultures of mixed polymorphonuclear and mononuclear cells *vs* "pure" mononuclear cell cultures, at least a 97% mononuclear cell suspension as determined by repeated differential counts was deemed necessary.

Repeated differential cell counts on the test cell suspensions, 24 and 36 hours after incubation, showed that the polymorphonuclear leucocytes were reduced in numbers, but were still present in amounts ranging from 10-20% of the total counts. However, after 48 and 72 hours of incubation, the great majority of cells remaining in suspension were mononuclear in type.

Interferon production by leucocytes. A series of 17 experiments was performed in an attempt to determine whether polymorphonuclear cells, mononuclear cells, or both were responsible for the production of interferon under *in vitro* stimulation with Sendai virus.

Interferon production from cell cultures prepared from each individual donor was compared in equal cell suspensions of (a) "mixed" cultures and (b) cultures consisting largely of mononuclear cells. The results of 17 experiments are summarized in Table I. It may be seen that there is no significant difference in interferon production in either

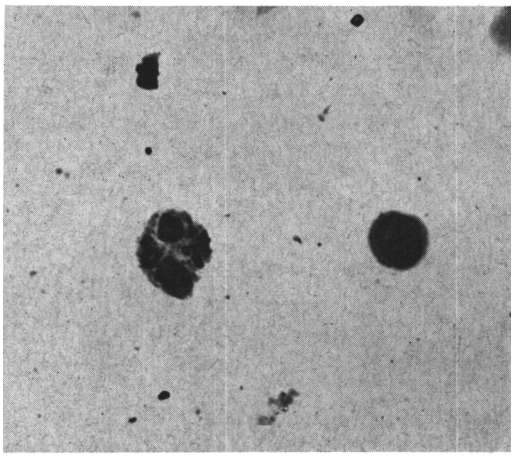

FIG. 2. Phagocytosis of iron particles by human polymorphonuclear leucocyte after 60 min incubation. Wright stain, × 600.

TABLE I. Comparison of Interferon Production by "Mixed" *vs* Mononuclear Human Leucocyte Suspensions.

Exp No.	Mononuclear cell (%) "mixed"	Mononuclear cell (%) "pure"	Interferon titer (IND_{50}) "mixed"	Interferon titer (IND_{50}) "pure"	IND_{50} ratio "mixed"/"pure"
1	55	97	74	62	1.19
2	44	99	177	125	1.42
3	30	97	256	256	1.0
4	50	98	251	178	1.41
5	44	97	256	190	1.35
6	66	98	104	64	1.62
7	26	99	250	145	1.72
8	45	98	205	94	2.18
9	48	99	112	99	1.13
10	48	100	115	205	.56
11	44	97	112	126	.89
12	35	99	132	115	1.15
13	44	98	82	82	1.0
14	35	98	120	100	1.20
15	34	99	132	85	1.55
16	35	99	151	112	1.35
17	30	99	127	95	1.34
Total					22.06
Mean					1.29
Standard deviation					.36

of the culture systems. The results indicate that normal mononuclear cells in these suspensions are capable of producing interferon.

Assay for intracellular and extracellular interferon. Since polymorphonuclear cells do not survive appreciably more than 48 hours *in vitro,* a series of 6 experiments was performed to demonstrate the possible effect of cell lysis on interferon yield in the following manner. After 24 hours' incubation, the control and infected tube cultures containing the respective "pure" mononuclear and "mixed" cell suspensions were pooled into the corresponding 4 samples under test. Each sample was divided into equal portions prior to centrifugation to remove the cells. One portion was disrupted ultrasonically to permit the release of any intracellular interferon. The results are summarized in Table II. It may be seen that the cell lysis has little or no effect on the yield of interferon as has been previously observed(7).

TABLE II. Assay for Intracellular and Extracellular Interferon.

Exp No.	Interferon titer: Supernatant fluid "Mixed"	Supernatant fluid "Pure"	Supernatant fluid + cell lysate "Mixed"	Supernatant fluid + cell lysate "Pure"
1	136	116	136	99
2	100	86	105	85
3	82	82	100	95
4	115	205	120	196
5	126	114	126	114
6	98	100	98	98

Relationship between leucocyte count in suspension and yield of interferon. From Table III it is apparent that the yield of interferon is approximately proportional to the number of cells stimulated *in vitro, i.e.,* the titer varies directly with the number of cells per ml of suspension.

TABLE III. Relationship Between Number of Leucocytes and Yield of Interferon.

Cell count per ml ($\times 10^6$)	Interferon titer: Exp 1	Exp 2	Exp 3	Exp 4
5.0	600	676	729	729
2.5	327	302	400	340
1.0	128	138	140	100
.5	70	63	70	56
.25	N.D.*	25	35	29

* Not done.

Production of interferon by polymorphonuclear leucocytes. In view of the evidence that mononuclear cells are capable of interferon production under appropriate *in vitro* stimulation with Sendai virus, an effort was made to determine whether polymorphonuclear leucocytes also share in this capacity. A series of 4 experiments was performed by adjusting both the "pure" mononuclear and "mixed" cell suspensions in such a way that both cultures would contain an approximate-

TABLE IV. Production of Interferon by Polymorphonuclear Leucocytes After 24 Hours Exposure to Virus.

Exp No.	"Pure" % mononuclear cell	"Pure" Total cell count per ml	"Mixed" % mononuclear cell	"Mixed" Total cell count per ml	IND_{50} "Pure"	IND_{50} "Mixed"
1	99	1.0×10^6	40	2.5×10^6	126	486
2	99	1.0×10^6	38	2.5×10^6	100	342
3	98	1.0×10^6	70	1.5×10^6	83	242
4	98	1.0×10^6	83	1.9×10^6	100	386

ly equal number of mononuclear cells (10^6 cells/ml). Under such circumstances the "mixed" cultures would contain an excess of cells consisting entirely of polymorphonuclear leucocytes. The interferon titers obtained in these cultures are shown in Table IV. A 3-fold or greater increase in interferon yield is demonstrated in each of the "mixed" cell suspensions. Since the yield of interferon is directly related to the total number of visible cells as previously demonstrated (Table III) it seems apparent that polymorphonuclear leucocytes are also responsible for the interferon obtained in these experiments.

Characterization of interferon. The interferon obtained in these experiments was characterized in the usual manner, namely (a) its insusceptibility to inactivation by Sendai antiserum, (b) inability to remove the inhibiting factor by ultracentrifugation, (c) its insusceptibility to heat inactivation at 56°C for 1 hour, (d) its inactivation by crystalline trypsin (1 mg/ml at 37°C for 3 hours).

Discussion. The production of an interferon by suspensions of human leucocytes has been demonstrated by Gresser(1) and confirmed in the present report. Heretofore, from experimental data it has been difficult to determine the cell type responsible for the production of this antiviral substance. Employing a differential separation technique, our results confirm the impression of others (1) that the mononuclear leucocytes are indeed capable of interferon production under appropriate stimulation *in vitro*.

Polymorphonuclear leucocytes do not survive in appreciable amounts for more than 48 hours' incubation *in vitro*. However, repeated cell counts in these experiments 24 and 36 hours after incubation still reveal large numbers of intact cells present in suspension. Since there is evidence that interferon production in leucocyte suspensions reaches peak levels within 24 hours after exposure to Sendai virus(1), it is reasonable therefore to attribute the interferon harvested 36 hours after incubation to the various cell types present in suspension during this period of time.

Within the limits of the technique employed there is a rough quantitation between interferon titer and the total number of cells in suspension(1) (Table III). Our experimental data would indicate that polymorphonuclear cells as well as mononuclear cells share in their ability to produce interferon under appropriate *in vitro* stimulation for the following reasons: (a) From Table I, it is apparent that the yield of interferon from the mixed and from the pure mononuclear cell suspension is similar in all experiments. (b) From Table IV an approximately 2-fold excess of cells in culture consisting almost entirely of polymorphonuclear leucocytes results consistently in 3-fold or greater yields of interferon when harvested 24 hours after exposure to virus.

The use of a separation technique to produce mononuclear cell suspensions such as that described here should be useful in studies of viremia in various phases of human viral illness(8,9,10). Berg(11), for example, has demonstrated the multiplication of Echo 9 virus in leucocyte suspensions. Similarly Berg and Rosenthal(12) have also shown the propagation of measles virus in human and monkey leucocyte cultures.

If the production of interferon by leucocytes as shown in these experiments also occurs *in vivo*, this probably plays an important role in limiting spread of virus during

illness, and also may help to explain some of the difficulty involved in demonstrating virus in blood during the course of viral illness.

Summary. A technique to produce suspensions of human mononuclear leucocytes has been employed to demonstrate the production of interferon by these cells *in vitro* using a Sendai-Sindbis virus system. There is also evidence from the experimental data that polymorphonuclear leucocytes participate in interferon production.

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Serum Lactic Acid Dehydrogenase Isoenzyme Patterns in Tropical Sprue.* (29795)

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Marked elevations in the levels of serum lactic acid dehydrogenase (S.L.D.) have been reported in acute myocardial infarction(1), renal diseases(2), carcinomatosis(3,4), leukemia(4,5), sickle cell disease(6,7), and megaloblastic anemias such as pernicious anemia(6,7,8) and tropical sprue(9,10). The elevation in the total S.L.D. is the result of an increase in one or more of the 5 normally occurring isoenzyme fractions of L.D.H. The fractions affected vary in different pathologic states. The elevation occurring in pernicious anemia and nutritional megaloblastic anemia has been shown by Wroblewski & Gregory to consist of an increase in the LD5 isoenzyme fraction(7). This seems to be unique among hematologic disorders in which isoenzymes LD3 and LD4 are the fractions most frequently affected(7). Data are presented in this report on the serum isoenzyme pattern in patients suffering from tropical sprue.

Materials and methods. The subjects under study consisted of 6 patients with tropical sprue in its acute phase, 4 partially but inadequately treated tropical sprue cases, and 7 normal controls. The clinical diagnosis of tropical sprue was based on the following criteria: glossitis, weight loss, macrocytic anemia, megaloblastic bone marrow and laboratory evidence of malabsorption, including abnormal vitamin A, xylose and butterfat absorption tests. In some instances, determination of increased fecal fat and abnormal pattern of tissue obtained by peroral jejunal biopsies were also available.

Disc electrophoresis in acrylamide gels was

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