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Interferon Production by Human Leucocytes *in vitro*. Reduced Levels In Lymphatic Leukemia.* (31043)

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Humans with lymphatic leukemia, acute or chronic, have reduced resistance to infection by fungi and bacteria(1). They also seem to be peculiarly prone to develop serious infections by certain viruses which in normal subjects usually produce self-limited illnesses, *e.g.*, measles(2) chickenpox(3) and vaccinia (4). Other than these specific examples, however, there is no overall evidence of increased susceptibility to virus infection in leukemic patients.

The production of interferon by human leucocytes *in vitro* has been shown by several investigators(5,6,7). We have previously shown that both polymorphonuclear and mononuclear leucocytes share the capacity to produce interferon when stimulated by Sendai virus(6).

The present paper relates to similar studies of *in vitro* interferon production by suspension of leucocytes from patients with acute or chronic lymphatic leukemia.

Materials and methods. Viruses. Stock Sendai virus used to induce interferon formation in leucocyte cultures was grown in allantois of 9-11-day-old embryonated eggs and concentrated by high speed centrifugation as previously described(6). Sindbis virus was propagated and titrated in established human amnion cells (HA-FL)(6). For purposes of interferon assay, approximately 1,000 $TCID_{50}$ of the virus was used.

Tissue culture. Interferon assay was carried out using monolayer tube cultures of HA-FL cells as previously reported(6).

Preparation of leucocyte suspensions. The procedure for preparing leucocyte suspensions from normal donors and leukemic patients was as described previously(6). Dextran in a proportion of 1:6 was added to the heparinized blood sample to aid sedimentation of erythrocytes. The mixture was allowed to stand for 30 minutes or longer at 37°C. The leucocyte-rich plasma was removed and sedimented at 2,500 rpm for 15 minutes. The sedimented leucocytes were washed 3 times and resuspended in medium 199 containing 10% calf serum. A total cell count was then performed. Cytogenetic examination was also conducted in a number of normal control specimens and the karyogram shown to exhibit the normal number of 46 chromosomes.

Interferon production and assay. The production of interferon was carried out by the method already described(6). For comparative purposes, each blood sample obtained from leukemic patients was tested along with that of a normal control subject, during each experiment. Each cell suspension so prepared was divided into 2 aliquots. To one of these, stock Sendai virus at an input of 10-50 EID_{50}/cell was added, and to the other, the control, an equal volume of medium, free of

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TABLE I. Interferon Production by Leucocytes from Acute Lymphatic Leukemia as Compared with Non-Leukemic Controls.

Exp No.	Normal donor's No. initials age & sex	Patient's No. initials age & sex	Date of illness first diagnosed	Therapy* prior to blood sampling	Date of blood sampling	Hemogram on day of blood sampling: Hgb† (g %)	Platelets (per cu mm)	Total WBC (per cu mm)	Differential (%): Blast†	Myelo†	Juv†	Band	Mono†	PMN†	EOS†	Interferon titer (IND_{50})
1.		62. P.Z.M. 20 ♂	13- 2-64	a,b,c,d	21-4-65	12.5	20,000	1,900	13		1	26	25	34	1	5
		63. L.J.L. 16 ♂	2- 3-65	a,e,d,e	21-4-65	13.2	295,000	5,650	3		1	17	8	68	3	15
	66. W.M.M. 33 ♂				21-4-65								50	49	1	132
2.		63. L.J.L. 16 ♂	2- 3-65	a,e,d,e,f	1-6-65	12.1	128,000	1,000				2	66	32		3
	66. W.M.M. 33 ♂				1-6-65								46	54		132
3.		63. L.J.L. 16 ♂	2- 3-65	a,e,d,e,f	15-6-65	11.8	151,000	1,275			2	7	54	36	1	46
	70. R.H. 47 ♀				15-6-65								54	44	2	157
4.		63. L.J.L. 16 ♂	2- 3-65	a,e,d,e,f	28-7-65	7.9	44,000	1,000		1		2	57	35	5	13
	70 R.H. 47 ♀				28-7-65								50	49	1	182
5.		79. J.P.M. 4 ♂	2-12-65	a,e,r	8-7-65	10.3	394,000	5,000					45	52	3	65
		80. G.F. 13 ♂	14- 1-65	a,e,d,g	8-7-65	13.2	245,000	9,000					13	87		18
	78. F.J. 37 ♂				8-7-65								50	50		219
6.		85. P.J.K. 3 ♀	17- 7-65	None	17-7-65	10.5	18,000	6,200	50	1			42	7		20
	86. L.B. 18 ♀				17-7-65								44	54	2	160
7.		17. A.M. 6 ♂	19-12-63	c,d,e	3-8-65	13.0	95,000	2,000					61	39		16
	88. B.M.S. 6 ♀				3-8-65								46	52	2	132

(Table continued on following page.)

virus. The cell suspensions diluted to give a final concentration of approximately 1.5 × 10^6 cells/ml in medium 199 containing 20% calf serum, were incubated in screw-capped tubes in 1.5 ml portions at 37°C in a roller wheel for 24-48 hours. After incubation, the culture fluids were duly harvested, centrifuged at 3,000 rpm for 15 minutes to remove the cells, and Sendai virus was inactivated by treatment with hyperimmune Sendai antiserum(6), or by acidification to pH 2 with N HCl and allowed to act for 24 hours at 4°C (8).

Serial 3-fold dilutions of these preparations were tested in tube cultures of HA-FL cells for inhibitory activity against Sindbis virus using 3-4 tubes per dilution, and the interferon titer was expressed as a 50% protection titer (IND_{50}), as described previously (6).

In a portion of the experiments, mononuclear cells were separated from the polymorphonuclear cells by a technique using the phagocytic capacity of the latter to ingest fine iron particles, as reported previously(6). These relatively "pure" mononuclear cell suspensions were likewise tested for interferon production.

A total of 14 patients with lymphatic leukemia, both acute and chronic, and one with acute myelogenous leukemia comprised the study group. Diagnoses were established by routine examination of the bone marrow and peripheral blood. In each test, the interferon titer produced in leukemic cell suspensions was compared with those obtained in a control group of healthy subjects.

Results. Interferon production by cells of patients with lymphatic leukemia. The clinical history of the patients with regard to age, sex, duration of disease, duration of therapy, type of therapy, and hematological findings is shown in Tables I and II, along with the interferon assay results. It is apparent in almost all experiments, that the interferon yields in leucocyte cultures from the 14 leukemic patients, are consistently lower than those obtained from the control group.

It should be noted that all cases except one (P.J.K.) were under treatment with vari-

TABLE I. Interferon Production by Leucocytes from Acute Lymphatic Leukemia as Compared with Non-Leukemic Controls (continued).

Exp No.	Normal donor's No. initials age & sex	Patient's No. initials age & sex	Date of illness first diagnosed	Therapy* prior to blood sampling	Hemogram on day of blood sampling: Date of blood sampling	Hgb† (g %)	Platelets (per cu mm)	Total WBC (per cu mm)	Differential (%): Blast†	Myelo†	Juv†	Band	Mono†	PMN†	EOS†	Interferon titer (IND_{50})
8.		90. L.S. 6 ♂	12- 1-65	a,b,c,o,r,s	16-8-65	14.5	950,000	5,850		1		2	25	70	2	46
	89. C.G. 47 ♀				16-8-65								44	56		182
9.‡		82. S.S. 57 ♂	15- 4-65	c,d,e	9-7-65	8.5	33,000	23,500	93				4	3		55
	78. F.J. 37 ♂				9-7-65								50	50		132

* a, Prednisone; b, Purinethol; c, Methotrexate; d, Vincristine; e, 6-Mercaptopurine; f, Amethopterin; g, Riopan; h, Chlorambucil; i, Morphine; j, Digoxin; k, Warfarin; l, Trilafon; m, Phenobarbital; n, Melphalen; o, Blood transfusion; p, X-ray; q, Triethylene melamine; r, Tetracycline; s, Griseofulvin; t, Penicillin; u, Atropine.

† Hgb, hemoglobin; Blast, lymphoblast; Myelo, myelocyte; Juv, juvenile; Mono, mononuclear; PMN, polymorphonuclear neutrophil; EOS, eosinophil.

‡ Acute myelogenous leukemia.

TABLE II. Interferon Production by Leucocytes from Chronic Lymphatic Leukemia as Compared with Non-Leukemic Controls.

Exp No.	Normal donor's No. initials age & sex	Patient's No. initials age & sex	Date of illness first diagnosed	Therapy* prior to blood sampling	Date of blood sampling	Hemogram on day of blood sampling												Interferon titer (IND_{50})
						Hgb† (g %)	Platelets (per cu mm)	Total WBC (per cu mm)	Differential (%)									
									Smear cell	Blast†	Pro-lymph	Myelo†	Band	Mono†	PMN†	EOS†	BAS†	
1.		67. J.N.M. 57 ♂	10-10-64‡	a,h	1-6-65	9.5	94,000	284,000		1	2			96	1			81
	66. W.M.M. 33 ♂				1-6-65									50	49	1		132
2.		71. L.L.P. 50 ♂	2- 6-58	h	21-6-65	13.9	350,000	142,000			1			98	1			19
	72. P.O.M. 40 ♂				21-6-65									49	50	1		138
3.		76. E.P. 71 ♀	30- 6-65	i,j,k,l,m	5-7-65	12.5	152,000	316,000	34		5		1	58	2			13
	77. B.D.B. 26 ♂				5-7-65									33	67			135
4.		81. S.K. 51 ♂	28- 6-55	n,o	9-7-65	11.5	102,000	4,850				1	1	54	43	1		33
	78. F.J. 37 ♂				9-7-65									50	50			132
5.		83. G.B. 56 ♀	5- 9-52	l,p,q	12-7-65	13.4	200,000	24,000						90	10			45
		84. C.M.L. 62 ♂	17-12-62	i	12-7-65	12.0	400,000	10,200					1	51	48			45
	70. R.H. 47 ♀				12-7-65									46	54			166
6.		96. R.T.M. 59 ♂	9- 9-65	i,m,t,u	14-9-65	15.6	350,000	58,000	24		2			60	14			23
	97. R.MacP. 29 ♂				14-9-65									49	48		3	166

* As shown in Table I.
† Hgb, hemoglobin; Blast, lymphoblast; Myelo, myelocyte; Mono, mononuclear; PMN, polymorphonuclear neutrophil; EOS, eosinophil; BAS, basophil.
‡ Treatment commenced on readmission to hospital in 31-5-65.

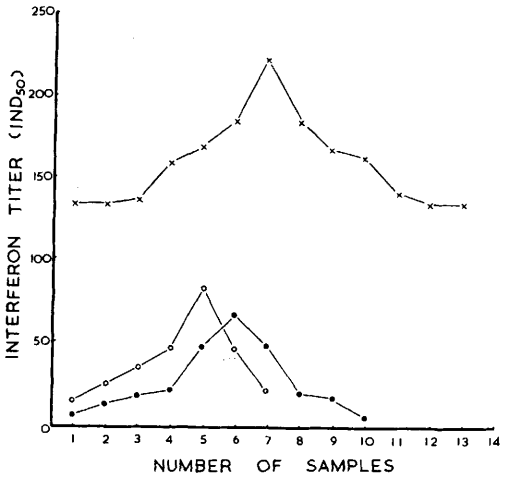

Fig. 1. Comparison of interferon titers from leucocytes cultures. x Control non-leukemic subjects; ● Acute lymphatic leukemia; ○ Chronic lymphatic leukemia.

ous antileukemic drugs at the time of testing, as indicated in Tables I and II. Duration of therapy in these patients varied from 24 hours to almost 10 years. Among the cases reported, 6 patients were on steroid therapy and/or other medications. Eight patients had not received steroids, but were receiving other forms of treatment. In the limited number of cases studied, there was no observed difference in interferon yields between the steroid-treated patients and those on other medications.

The difference in interferon yield between the normal controls and leukemic subjects can be seen in Fig. 1. The interferon yields from the control subjects which were assayed simultaneously with each leukemic blood sample, show a range varying from 132 to 219 IND_{50} with a mean value of 152.6. The leukemic cell suspensions, on the other hand, show yields varying from 3 to 65 IND_{50}, with a mean value of 24.7 for acute leukemia and from 13 to 81 IND_{50} with a mean value of 37 for chronic leukemia.

We have shown(6) that in normal subjects, both polymorphonuclear and mononuclear cells in suspensions can be induced by Sendai virus to produce interferon. An attempt was therefore made in the present series of experiments to study the production of interferon by polymorphonuclear cells, as opposed to that produced by the mononuclear cells from patients with lymphatic leukemia. In each individual case, interferon yields were obtained from the initial "mixed" cell suspensions as well as from suspensions containing largely mononuclear cells. The results are summarized in Table III. With one exception, (L.J.L.), it can be seen that there is no significant increase in interferon yield in those suspensions containing appreciable number of polymorphonuclear leucocytes, at the time the experiments were performed.

Characterization of interferon. All the preparations obtained from infected or uninfected leucocyte cultures to be assayed for interferon content were divided into two groups. One group was acidified to pH 2 and treated with a high titer rabbit anti-Sendai antiserum; in the other group, each preparation was divided into 2 portions, one portion acidified and the other portion treated with antiserum. The latter procedures were adopted in order to find out whether any other factors inhibitory to Sindbis virus were present in the leucocyte culture media. No inhibitory activity against Sindbis virus was detected in the uninfected culture media. On the other hand, interferon titers in both acidified and antiserum treated infected culture media were similar.

Representative preparations from infected leukemic and non-leukemic leucocyte cultures were subjected to various physical and chemical tests, designed to characterize interferon, as previously reported(6). The virus-inhibitory substance produced by leucocytes was found to be sensitive to trypsin, was not sedimentable by ultracentrifugation, was stable at 56°C for 1 hour, and was completely destroyed by boiling for 10 minutes. The specificity of this interferon was also demonstrated by its inhibitory activity in human fetal skin-muscle cells and by its non-protection in primary chick embryo cells against Sindbis virus.

Discussion. In the present series of experiments, a significantly lowered capacity for formation of viral interferon, using the

Sendai-Sindbis system, has been demonstrated in suspensions of leucocytes, obtained from leukemic donors.

Transformation of cells in tissue culture by a carcinogenic agent may lower the capacity of the cells to produce interferon. Rotem *et al* (9) have shown that 3 lines of transformed hamster cells derived from embryo cells, transformed *in vitro* with the carcinogenic hydrocarbons, produced less interferon and were less sensitive to interferon than normal hamster embryo cells. Comparative studies of interferon yields produced by various cell types under similar experimental conditions have shown that heteroploid cells, consisting of HA-FL, HeLa and Davis human embryonic lung cell, produced significantly less interferon than 3 human diploid cell strains, namely, fetal lung, embryonic kidney and lung respectively (Lee/Embil, unpublished data).

TABLE III. Comparison of Interferon Production by "Mixed" *versus* Mononuclear ("Pure") Leucocyte Suspensions from Lymphatic Leukemic Patients.

Type of leukemia	Patient's No., initials, age & sex	Type of culture	% Polymorphonuclear leucocyte	% Mononuclear leucocyte	Interferon titer (IND_{50})
Acute	63 L.J.L. 16 ♂	mixed	46	44	46
		pure	3	97	10
	79 J.P.M. 4 ♂	mixed	52	48	65
		pure	2	98	42
	80 G.F. 13 ♂	mixed	87	13	18
		pure	5	95	15
Chronic	67 J.N.M. 57 ♂	mixed	1	99	81
		pure	0	100	81
	71 L.L.P. 50 ♂	mixed	1	99	19
		pure	0	100	9
	81 S.K. 57 ♂	mixed	46	54	33
		pure	3	97	41
	83 G.B. 50 ♀	mixed	10	90	45
		pure	2	98	45
	84 C.M.L. 62 ♂	mixed	49	51	45
		pure	3	97	27
	96 R.T.M. 59 ♂	mixed	14	86	23
		pure	1	99	17

Leukemic cells cultured in suspension do not survive as long as normal cells(10). Gresser(5) has demonstrated that leucocyte interferon is produced shortly after virus inoculation. Similarly in the present data, an appreciable amount of interferon is detectable 24 hours after inoculation. It is unlikely, therefore, that the difference in growth characteristics of leukemic cells could account for the observed result.

The blood suspensions from 7 patients at the time of testing confirmed a substantial number of polymorphonuclear leucocytes. On the basis of morphology, these would be classified as non-leukemic cells; yet each of these cases showed low interferon levels when tested (Table III). This was further confirmed when the polymorphonuclear cells were removed from these suspensions and "pure" lymphocytic cultures were tested. In this latter instance, there was no significant lowering of the interferon titers. For these reasons, it is suggested that all leucocytes in these individuals have impaired capacity to produce interferon.

In the present series of cases, only one patient (P.J.K.) with acute lymphatic leukemia has received no treatment prior to testing. In this patient, a low level of interferon production was also noted. Chromosomal analysis of this patient's leucocytes by the technique of Moorhead *et al*(11) showed 34% of the cells with chromosomal breakages and 26% consisting of an extra autosome in the 21-22 groups as reported by Sandberg *et al*(12). Of the other patients in this series only 6 were being treated with prednisone, either alone or in combination with other medications, including 6-mercaptopurine, methotrexate, etc. The effect of the other antileukemic drugs on interferon production is not yet documented but in view of the fact that some of the medications interfere with normal RNA synthesis, *e.g.*, 6-mercaptopurine(13), the possibility of an indirect effect on the production of interferon should be considered.

Studies on the influence of cortisone on production of influenza viruses and interferon in chick embryos have been reported by Kilbourne *et al*(14) and also by Reinicke(15). They found a decreased yield of interferon in hydrocortisone treated eggs. Similarly, Isaacs has demonstrated a steroid-induced inhibition of interferon production in animals (16).

The inhibition of interferon production in these cells by interfering extraneous material incorporated within the cells is yet another possibility. Inhibition of viral interferon production by infection of carrier cell cultures with another virus has been demonstrated by Hermodsson(17). In his experiments the auto-interference of Newcastle disease virus in calf kidney cultures was inhibited by previous infection with Parainfluenza virus type 3. Recently, the isolation of pleuropneumonia-like-organism (PPLO) cultures from human leukemic bone marrow has been reported by several investigators(18,19), but to what extent the possible presence of PPLO organisms may account for reduced interferon titers is impossible to state.

No attempt has been made to recover any agent from the leucocytes of these leukemic patients in this study. However, it was observed during the experiments that these leucocytes upon incubation did not produce any substance that might display inhibitory activity against Sindbis virus.

Similar experiments on untreated patients with acute and chronic leukemia are essential. Further studies are in progress.

Summary. *In vitro* production of interferon by human normal and leukemic leucocytes, employing the Sendai-Sindbis system, has been compared. The interferon response of leucocytes derived from 13 treated cases of acute and chronic lymphatic leukemia, have been found to be markedly lower than that of normal controls. Likewise, one untreated case of acute lymphatic leukemia, also showed a depressed interferon level.

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Scleroderma: Dermal Amino Acid Composition with Particular Reference to Hydroxyproline.* (31044)

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Scleroderma is characterized by a severe rigidity, thickening, and pigmentation of the skin. Other than thickening and homogenization of collagen fibers seen with the light microscope, essentially no significant histologic(1,2) or chemical abnormalities have been detected. An apparent dependence of the stability of collagen on the hydroxyproline and proline residues in the protein raises the question of whether some abnormality related to hydroxyproline may be of etiologic significance. Therefore, this study was initiated to determine if an abnormality could be detected by gross amino acid analysis of dermis from individuals with scleroderma.

Since the conclusion of our work, Fleischmajer(3) has published data indicating that the hydroxyproline content of collagen isolated from 2 patients with scleroderma was normal. He also presented data which, with 2 exceptions, indicated a comparable concentration of hydroxyproline in dry dermis from an "affected area" and from an area "that was clinically normal in appearance" from 8 patients with the disease.

In our study, hydroxyproline was determined in samples of dermis from a series of patients with scleroderma and from controls; amino acid analysis by an ion exchange method was performed on a small number of the same specimens.

Since the data we obtained did not agree with that published(3), it was thought advisable to present it now even though adequate interpretation and discussion would not be possible.

Materials and methods. Material. Samples were obtained from 10 patients (2 men and 8 women) with scleroderma. All were classed as having typical acrosclerosis. The disease had varied in duration from 4 months to 3 years; it usually had begun with Raynaud's phenomenon and edema of the fingers and progressed to gradual sclerosis and decreased mobility of the dermal collagen of the hands,

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