Studies on Human Leukemia.* (25062)

L. Dmochowski, C. E. Grey, J. A. Sykes, C. C. Shullenberger and C. D. Howe
Section of Virology and Electron Microscopy, Section of Hematology, and Dept. of Medicine, University of Texas M. D. Anderson Hospital and Tumor Institute; and Dept. of Microbiology, Baylor University College of Medicine, Houston

Studies on relationship of viruses to origin of tumors have led to important discoveries through employment of methods such as use of newborn animals, electron microscopy of ultrathin sections of different tumors, and tissue culture(1-13). Results of our studies on mouse leukemia and chicken leukosis, based on electron microscopy alone or combined with biological and biophysical methods, have been reported(14-19). In view of results obtained by others(20-23) and ourselves, a study of the relationship of viruses to origin of human leukemia was undertaken.

Material and methods. Lymph nodes obtained by surgical biopsy from patients with different types of leukemia, lymphoma and from patients with non-neoplastic diseases were used. Part of material was immediately fixed for electron microscopy(14); part used for tissue culture; part for histology; and the rest, finely minced and resuspended in 5.3% glucose for storage at −70°C. Material for tissue culture was minced, trypsinized in 0.2% trypsin ("Difco" in Ca- and Mg-free Hanks B.S.S.) for 20 minutes at room temperature, centrifuged for 10 minutes at 1,200 × g at 0°C and resuspended in complete medium. Eagle's basal medium with 20% inactivated human serum and 100 units/ml penicillin and 100 μg/ml streptomycin was used. Resuspended cells were grown in T-30 culture flasks. Medium changes were made not less than once a week. Recently, somewhat better results have been obtained using M199 with amino acid mixture, vitamins and glutamine (as used in Eagle's medium), 0.5% lactalbumin hydrolysate (General Biochemicals) and 15-20% calf serum (Colorado Serum Co., Denver). For phase-contrast and electron microscope studies, part of successfully grown material was maintained in Sykes-Moore tissue culture chambers(24). For electron microscopy, cells were fixed 20 minutes in buffered 1% osmium tetroxide, dehydrated and embedded in n-butyl methacrylate using similar time intervals. An RCA EMU 3A electron microscope during earlier and an EMU 3E model during later part of studies were used. Cell-free extracts of biopsy material were tested on monkey kidney cell cultures. Both original and tissue culture material were inoculated into newborn Swiss (Holtzman), C_3HZb/Bi, and C_3H/Bi mice.

Results. Electron microscopy of ultrathin sections of biopsy material revealed changes in cytoplasmic constituents of cells of leukemic and non-leukemic lymph nodes, similar to those observed in cells of mouse leukemia and chicken leukosis(14-19). In addition, characteristic virus particles have been found in cells from lymph nodes of certain cases of leukemia (Table I). No virus particles could be found in several hundred sections of lymph nodes from 5 non-leukemic patients. The particles are present in inclusion bodies (Fig. 1 and 1a) and scattered outside the cytoplasm (Fig. 2). They have an average diameter of 900 Å, an internal dense core, surrounded by one or 2 limiting membranes. There appears

TABLE I. Electron Microscope Study of Lymph Nodes from Biopsy Cases of Human Leukemia.

Diagnosis	Age	Sex	Virus-like particles observed
Acute lymphatic	15	♀	Yes
	28	♂	"
	18	♀	No
	45	♂	"
	22	♀	"
	19	♂	Yes
Chronic monocytic	71	♂	No
Acute monocytic	20	♀	"
Acute myeloid	22	♀	Yes
Lymphosarcoma	48	♂	"
	62	♂	No
Hodgkin's disease	23	♂	"

* Presented at April 1959 meeting of Am. Assn. for Cancer Research, Atlantic City. This work supported in part by Research Grants from Nat. Cancer Inst., N.I.H., U.S.P.H.S., and Am. Cancer Soc.

to be no difference in size or internal structure of particles in lymph nodes of different types of leukemia and lymphosarcoma.

In cases of acute lymphatic, myeloid leukemia, and lymphosarcoma, in which virus particles were observed, an occasional isolated area in the enlarged lymph node showed presence of these particles in approximately one out of 30 sections. In other areas of this same tissue, no particles were found in several hundred sections examined.

Cells grown *in vitro* from lymph nodes of a number of patients with different types of leukemia and lymphosarcoma revealed changes suggestive of a cytopathogenic agent. Increased granularity of cytoplasm, vacuolization, formation of inclusions, leading occasionally to scattered breakdown of cells, were frequently observed (Fig. 3, 4, and 5). The number of cases examined and results following varying number of passages are shown in Table II. Similar studies of 4 non-leukemic lymph nodes failed to reveal any changes.

Electron microscopy of cells grown in tissue culture from a case of malignant lymphoma (lymphosarcoma) which showed the described changes *in vitro* revealed virus particles in inclusion bodies similar to those seen in ultrathin sections of biopsy specimens (Fig. 6 and 6a).

Cell-free extracts from lymph nodes of 8 patients (2 acute lymphatic, 1 chronic lymphatic, 2 acute myeloid leukemia and 3 malignant lymphoma) have been tested on monkey kidney cell cultures up to 10 sequential passages. Changes, suggestive of a cytopathogenic effect, occurred in all cases of tested leukemic lymph nodes from passages 2 through 8, but were not observed in 9th or 10th passage. Material from tissue culture passages has been tested in mice and part

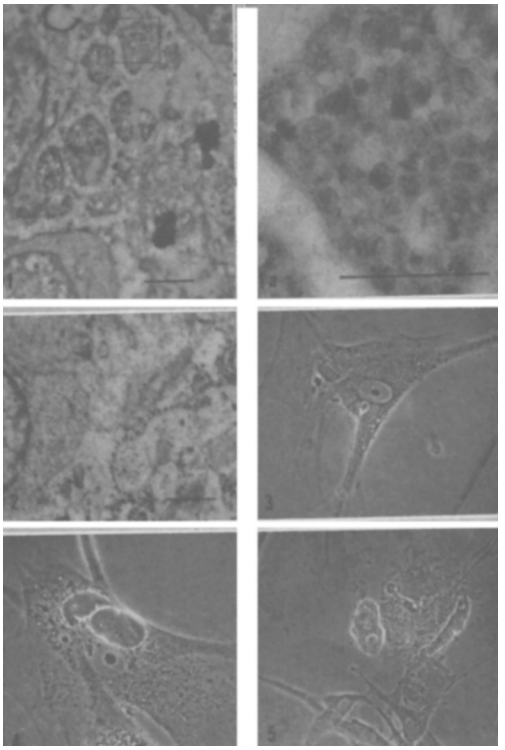

FIG. 1. Electron micrograph of section of biopsy specimen from lymph node of case of lymphosarcoma. Inclusion bodies with virus particles present in cytoplasm of cell. Magnification 6480 ×.

FIG. 1a. Inclusion body with virus particles at higher magnification. 38,880 ×.

FIG. 2. Extracellular virus particles in specimen shown in Fig. 1. Magnification 6480 ×.

FIG. 3. Phase photomicrograph of cell from case of acute monocytic leukemia, 7th passage, 22-day S-M chamber culture, showing perinuclear inclusion. Magnification 108 ×.

FIG. 4. Phase photomicrograph of cells from case of acute myeloid leukemia, 7th passage, 20-day S-M chamber culture, showing cytoplasmic inclusions. Magnification 135 ×.

FIG. 5. Breakdown cells from same case as Fig. 4, 7th passage, 7-day S-M chamber culture. Magnification 108 ×.

TABLE II. Behavior of Human Leukemic and Lymphomatous Tissues *In Vitro*.

Type of tumor	No. grown *in vitro*	Cytopathogenic changes: No. with changes	Cytopathogenic changes: No. of passage	Cytopathogenic changes: Appearance in weeks
Acute lymphocytic leukemia	7	4	2 : 10 : 4 : 1	2– 4
" granulocytic "	4	3	4 : 4 : 6	4– 6
" monocytic "	1	—	—	—
Chronic lymphatic "	2	1	8	12
Hodgkin's disease	2	2	1 : 1	2– 4
Lymphoma excluding Hodgkin's	8	5	1 : 1 : 1 : 2 : 4	2–12
Normal lymph nodes	4	—	—	—

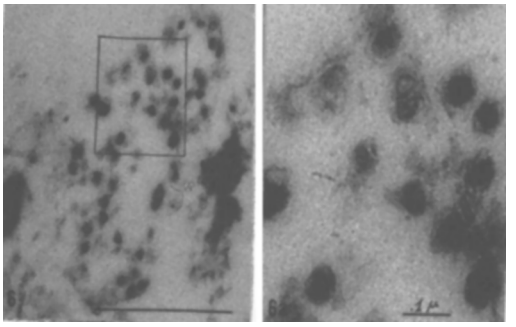

FIG. 6. Electron micrograph of section of cells grown *in vitro*, from lymph node of same case as Fig. 1, 7th passage, showing virus particles. Magnification 18,240 ×.

FIG. 6a. Virus particles at higher magnification in area shown in Fig. 6. Magnification 59,280 ×.

stored at −70°C for future use. Cell-free extract from a non-leukemic lymph node induced no changes during 5 sequential passages on monkey kidney cell cultures.

Results of bioassays of cell-free extracts of biopsy specimens and of fluids from monkey kidney cultures treated with these extracts in newborn Swiss (Holtzman), C_3HZb/Bi and C_3H/Bi mice are shown in Tables III, IV, and V. The incidence of spontaneous leukemia in Swiss (Holtzman) mice is approximately 1% at 12 months of age, and less than 0.5% at much later age in C_3HZb/Bi and C_3H/Bi mice. The induction up to 18% of leukemia at an early age by material from one case of acute lymphatic leukemia cannot be considered significant, in view of the development of leukemia in 10% of uninoculated litter-mate control animals at a similar age. In addition, newborn Swiss (Holtzman) mice injected with untreated monkey kidney culture fluids developed increased incidence of leukemia (Table VI). The incidence of mammary tumors (MT) and hemorrhagic disease (H) in inoculated animals was comparable to that in uninoculated litter-mate controls.

Discussion. The study of ultrathin sections of lymph nodes from 12 patients with leukemia or malignant lymphoma revealed in cells of all cases, vacuolization of cytoplasm, breakdown of mitochondria and endoplasmic reticulum, which were also found in cells of lymph nodes from 5 patients with non-neoplastic disease. In addition to these changes, inclusion bodies with spheroidal particles of a characteristic structure were found in the cytoplasm of cells from lymph nodes of 3 patients with acute lymphocytic, 1 with acute myeloid leukemia, and 1 with lymphosarcoma. The virus particles are in all cases of similar size and appearance. They have not been observed in cells of non-leukemic lymph nodes. The particles may represent a virus unconnected with leukemia.

Changes suggestive of presence of a virus have been observed in cells of lymph nodes grown *in vitro* from 15 out of 24 cases with different types of leukemia and lymphosarcoma. Such changes have not been seen in cells of lymph nodes during a similar number of passages in tissue culture from 5 patients with non-neoplastic disease. They have been

TABLE III. Newborn Mice Injected with Extracts of Human Leukemic Organs.

Exp.	Diagnosis	Duration of exp. (mo)	Mice used (litter-mates)	No. with leukemia/No. inj.	Avg age of leukemia development (mo)
HL #1	Acute lymphocytic leukemia	15–17	Swiss —Inj.	44/244 (+2H)	9
			—Controls	6/65	10
			C_3HZb—Inj.	3/50 (+1MT) (+1H)	10
			—Controls	0/6	—
HL #2	Acute myeloid leukemia	15	Swiss —Inj.	3/15	8
			—Controls	1/7	14
			C_3HZb—Inj.	1/14 (+1H)	5
			—Controls	0/11	—
HL #3	Acute myeloid leukemia	12	Swiss —Inj.	1/23 (+2H) (+2MT)	5
			—Controls	1/9	8
			C_3HZb—Inj.	0/6	
			—Controls	0/5	

TABLE IV. Newborn Mice Injected with Extracts of Human Leukemic Organs.

Exp.	Diagnosis	Duration of exp. (mo)	Mice used (litter-mates)	No. with leukemia/No. inj.	Avg age of leukemia development (mo)
HL #5	Lymphosarcoma (follicular lymphoma)*	11	Swiss —Inj.	5/72 (+3MT) (+1H)	5
			—Controls	2/34	7
			C_3HZb—Inj.	1/25	6
			—Controls	0/13	—
HL #6	Lymphosarcoma (lymphocytic type)	10	Swiss —Inj.	5/54	5
			—Controls	2/32	8
			C_3HZb—Inj.	0/28	—
			—Controls	1/16	8
HL #8	Chronic lymphocytic leukemia	8	Swiss —Inj.	1/30	6
			—Controls	1/20	5
			C_3HZb—Inj.	1/28	6
			—Controls	0/14	—

* And chronic lymphocytic leukemia.

TABLE V. Newborn Mice Injected with Fluids from Tissue Cultures Treated with Extracts of Human Leukemic Organs.

Exp.	Diagnosis	Duration of exp. (mo)	Mice used (litter-mates)	No. with leukemia/No. inj.	Avg age of leukemia development (mo)
HL #5	Lymphosarcoma (follicular lymphoma)	11	Swiss —Inj.	3/60	7
			—Controls	3/40	5
			C_3H —Inj.	1/40	4
			—Controls	0/18	—
HL #6	Lymphosarcoma (lymphocytic type)	10	Swiss —Inj.	12/177 (+3MT)	6
			—Controls	5/92 (+3MT)	8
HL #7	Acute lymphocytic leukemia	9	Swiss —Inj.	12/176 (+2MT)	6
			—Controls	7/96 (+3MT)	8

found in cells of leukemic lymph nodes, both positive and negative for presence of virus particles in electron microscope studies. In one case of lymphosarcoma in which virus particles were seen in the biopsy specimen, similar virus particles were also demonstrated in cells of this material grown *in vitro* which had shown cytopathogenic changes.

Monkey kidney cell cultures, when used for testing extracts of leukemic lymph nodes, present a drawback because of presence of simian viruses. However, control cultures treated with fluids from uninoculated monkey kidney cultures failed to show any changes. Extracts of lymph nodes have also been tested on human embryo cultures. In tests, so far carried out, changes have been observed during first passage which could not be transmitted serially. Experiments to isolate an infective agent in human embryo cultures are being continued using different technics.

In bioassays of cell-free extracts of biopsy material from leukemic lymph nodes and of fluids of monkey kidney cell cultures treated with extracts of these lymph nodes, no significant difference in incidence of leukemia was observed between inoculated and uninoculated litter-mate control animals. The increase in incidence of leukemia in uninoculated littermate controls and in mice, following inoculation of untreated culture material, is of interest. Inhibition of hemagglutination tests (25), using sera of Swiss (Holtzman) mice from our colony against polyoma virus (ob-

TABLE VI. Newborn Mice Injected with Untreated Monkey Kidney Fluids.

Duration of exp. (mo)	Mice used (litter-mates)	No. with leukemia/No. inj.	Avg age of leukemia developm't (mo)
7 to 10	Swiss–inj.	6/104	6
	–controls	5/41	5

tained through the courtesy of Dr. Sarah Stewart), have shown high titers indicating infection with the virus. It should be noted, however, that fluids from cultures of lymph nodes showing cytopathogenic changes failed to give hemagglutination of guinea-pig red cells(26).

Summary. 1) Electron microscope studies have been carried out on ultrathin sections of lymph nodes from 6 patients with acute lymphatic, 1 with acute and 1 with chronic monocytic, 1 with acute myeloid leukemia, 2 with lymphosarcoma and 1 with Hodgkin's disease. Virus particles of approximately 900 Å in diameter have been observed in cells of lymph nodes from 3 cases of acute lymphatic, 1 of acute myeloid leukemia and 1 of lymphosarcoma. Similar studies of sections of lymph nodes from 5 non-leukemic patients have failed to reveal presence of virus particles. 2) Cytopathogenic changes have been observed in cells of lymph nodes grown *in vitro* from 15 out of 24 patients with leukemia and lymphosarcoma. Cells of lymph nodes grown *in vitro* from 4 non-leukemic patients have not shown changes observed in lymph nodes from leukemic patients during similar number of passages. Cell-free extracts of lymph nodes from 8 patients tested have induced changes in monkey kidney cell cultures which could be serially transmitted. These changes have not been seen in similar cultures treated with extract of a non-leukemic lymph node. Extracts of leukemic lymph nodes which induced changes in monkey kidney cell cultures did not have a similar effect in human embryo cultures. 3) Cell-free extracts of biopsy material from leukemic and lymphomatous lymph nodes and fluids from monkey kidney cell cultures treated with these extracts have not shown specific leukemia-inducing activity in Swiss (Holtzman), C_3HZb/Bi and C_3H/Bi strain mice. 4) It is of particular interest that in one patient with lymphosarcoma, not only were virus particles observed in ultrathin sections of the biopsy specimens, but also similar virus particles have been found in cells from the same specimen, grown *in vitro* for 6 sequential passages during which cytopathogenic changes had been observed.

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Received June 1, 1959. P.S.E.B.M., 1959, v101.