Search for Virus in Human Malignancies. I. *In vitro* Studies.* (27648)

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The abundant and conclusive evidence relating viruses to neoplasia in animals has provided a basis for belief that at least part of the spectrum of malignancies in man may be of viral cause. On theoretical grounds, viruses of human malignancy may be unique and hitherto unrecognized, or they may be ordinary and well known in relation to other clinical illnesses in man. In the latter possibility, malignancy may be but a late manifestation of infection with an ordinary virus perhaps as a carrier of an oncogenic principle, as a primary oncogenic agent, or as a co-carcinogenic substance.

Studies in our laboratory have been directed toward detection of an infectious principle of viral or virus-like quality in controlled studies of tissues of human malignant origin. In these studies, animals or cell cultures of human or animal tissues were inoculated with extracts of human malignant tissues. Observations were made directly for viral presence as well as for the consequences of viral activity. The present report, the first in this series, is concerned with the *in vitro* studies in cell cultures. A second paper describes the findings in *in vivo* studies(1) in experimental animals.

Materials and methods. Clinical specimens. Tissues from human cases of malignancy were obtained from the Dept. of Hematology, Children's Hospital of Philadelphia; from Montgomery Hospital, Norristown, Pa.; and from Albert Einstein Medical Center, Philadelphia. Tables I and II summarize the kinds of tissues which were included in the studies. The human leukemic tissues were obtained, primarily, from the first 2 sources listed above while solid tumor materials were obtained from the last named source. Specimens such as bone marrow, tumors, organs and the like were obtained at surgery or at autopsy. Whenever possible, leukemic specimens were obtained prior to starting chemotherapy at the time of first diagnosis or at onset of remission. Heparinized blood was centrifuged shortly after collection and the plasma was stored at −70°C in sealed glass containers. Serum was stored at −20°C. Tissues intended for cell culture purpose were collected into Basal Medium Eagle (BME)(2) with 10% calf serum and antibiotics and were refrigerated at 4°C until used less than 24 hours later. All other tissues were stored frozen at −70°C or at −20°C until processed for testing. *Processing of specimens.* Saliva and bone marrow aspirates from leukemic patients were diluted sufficiently with Hanks' balanced salt solution (HBSS)(2) to permit pipetting and inoculation. Urine samples were dialyzed overnight at 4°C against 20 volumes of HBSS and were processed by several alternate cycles of low (2000 rpm) and high speed (40,000 rpm) centrifugation. The high speed sediment was resuspended in 1/10 the original volume of HBSS. Tumors and other autopsy tissues were homogenized for 10 minutes at 16,000 rpm in an Omnimix cup held in an ice bath, employing sufficient HBSS to give a 15% suspension. Extended blending periods were required for tissues which homogenized poorly. The crude supernate obtained following centrifugation for 20 minutes at 2000 rpm was used for inoculation purpose. In certain instances, crude urine or the 2200 rpm sediments of tissue extracts were resuspended in half of the supernate, homogenized with 0.5 volume of Freon 113 (fluorocarbon), and centrifuged at 2000 rpm for 10 minutes. Freon treatment was repeated once or twice on the clarified fluids. For filtration, the crude homogenate was clarified by passing through a Johns-Manville Celite 503 pad (prewashed with HBSS) followed by a Selas 02 filter which was used untreated or which had been primed with 0.05% gelatin in HBSS and washed with HBSS before use. The processed specimens were divided and either in-

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TABLE I. Specimens of Human Leukemia Studied in Cell Cultures of Primary Human Amnion and of Grivet Kidney.

Malignancy	Clinical materials: Specimens Kind	No.	Pre-inoc'n. treatment	No. of specimens on which observations were performed: Cytopathology–blind passage	Hemads.	Hemagglut.	Acridine orange	Fluor. antibody
Stem cell leukemia	Brain	1	Crude susp.	1	1	1	1	1
1 case	"		Freon 1X	1	1	1	1	1
(Aged 1 yr)	Lung	1	Crude susp.	1	1	1	1	1
	"		Freon 1X	1	1	1	1	1
	Liver	1	Crude susp.	1	1	1	1	1
	"		Freon 1X	1	1	1	1	1
	Spleen	1	Crude susp.	1	1	1	1	1
	"		Freon 1X	1	1	1	1	1
	Kidney	1	Crude susp.	1	1	1	1	1
	"		Freon 1X	1	1	1	1	1
	Thymus	1	Crude susp.	1	1	1	1	1
	"		Freon 1X	1	1	1	1	1
	Lymph nodes	1	Crude susp.	1	1	1	1	1
	" "		Freon 1X	1	1	1	1	1
	Bone marrow	1	Crude susp.	1	1	1	1	1
	" "		Freon 1X	1	1	1	1	1
Aleukemic lymphocytic leukemia	Serum	1	Crude susp.	1	1	1	1	1
1 case	Saliva	2	" "	2	2	2	2	2
(Aged 56 yr)	"		Freon 1X	1	1	1	1	1
	Urine	2	Dialyzed	2	2	2	2	2
	"		Freon 1X	1	1	1	1	1
	"		High speed sup't.	1	1	1	1	1
	"		" " sed.	1	1	1	1	1
Acute lymphocytic leukemia	Buffy coat	4	Crude susp.	4	4	4	0	0
16 cases	Plasma	16	" "	11	10	8	11	11
(Aged 10 mo., and 2, 3,	"		" , filtered	5	5	5	5	5
3, 4, 5, 6, 6, 6, 6, 6,	Bone marrow	18	" susp.	18	10	10	10	10
6, 7, 7, 8 & 14 yr)	" "		Freon 1X	3	3	3	0	0
	" "		" 2X	3	3	3	0	0
	" "		" 3X	3	3	3	0	0
	Lymph nodes	7	Crude susp.	4	4	3	2	2
	" "		" , filtered	3	3	2	1	1
	" "		Freon 1X	3	3	2	1	1
	" "		" 2X	3	3	2	1	1
	" "		" 3X	3	3	2	1	1
	Saliva	1	Crude susp.	1	1	1	0	0
	Urine	12	Dialyzed	12	8	4	0	0
	"		High speed sup't.	9	5	5	7	7
	"		" " sed.	9	5	5	7	7
			Freon 1X	1	1	1	0	0

TABLE I (continued)

Malignancy	Clinical material: Specimens Kind	No.	Pre-inoc'n. treatment	No. of specimens on which observations were performed: Cytopathology–blind passage	Hemads.	Hemagglut.	Acridine orange	Fluor. antibody
	Brain	3	Crude susp.	3	3	2	1	1
	"		" , filtered	2	2	1	2	2
	"		Freon 1X	2	2	0	0	0
	"		" 2X	1	1	1	0	0
	"		" 3X	1	1	1	0	0
	Lung	1	Crude susp.	1	1	1	1	1
	"		" , filtered	1	1	1	1	1
	"		Freon 1X	1	1	1	1	1
	"		" 2X	1	1	1	1	1
	"		" 3X	1	1	1	1	1
	Kidney	8	Crude susp.	5	4	2	1	1
	"		" , filtered	3	3	1	1	1
	"		Freon 1X	2	2	1	0	0
	"		" 2X	1	1	1	0	0
	"		" 3X	1	1	1	0	0
	Liver	7	Crude susp.	5	5	1	0	0
	"		" , filtered	2	2	1	0	0
	"		Freon 1X	3	3	1	0	0
	"		" 2X	1	1	0	0	0
	"		" 3X	1	1	0	0	0
	Spleen	8	Crude susp.	6	6	2	2	2
	"		" , filtered	2	2	2	2	2
	"		Freon 1X	3	1	1	0	0
	"		" 2X	1	1	0	0	0
	"		" 3X	1	1	0	1	1
	Thymus	4	Crude susp.	4	4	4	1	1
	"		" , filtered	1	1	1	1	1
	"		Freon 1X	2	2	0	0	0
	"		" 2X	1	1	1	0	0
	"		" 3X	1	1	1	0	0
Chronic lymphocytic leukemia	Plasma	2	Crude, filtered	2	2	2	1	1
2 cases	"		Freon 1X	1	1	1	0	0
(Aged 59, 71 yr)	Buffy coat	1	Crude susp.	1	1	1	0	0
	RBC	1	" "	1	1	1	0	0
	Saliva	2	" "	2	2	2	1	1
	"		Freon 1X	1	1	1	1	1
	Urine	2	Dialyzed	2	2	2	0	0
	"		High speed sup't.	2	2	2	2	2
	"		" " sed.	2	2	2	2	2
	"		Freon 1X	2	2	2	0	0

TABLE I (continued)

Malignancy	Clinical material: Specimens Kind	No.	Pre-inoc'n. treatment	No. of specimens on which observations were performed: Cytopathology–blind passage	Hemads.	Hemagglut.	Acridine orange	Fluor. antibody
Acute myelogenous leukemia	Liver	1	Crude, filtered	1	1	1	0	0
	Brain	1	" "	1	1	1	0	0
2 cases	Sternum	1	" "	1	1	1	0	0
(Aged 3, 10 yr)	Spleen	1	" "	1	1	1	0	0
	Kidney	1	" "	1	1	1	0	0
	Urine	1	High speed sup't.	1	1	1	0	0
	"		" sed.	1	1	1	0	0
	Bone marrow	1	Crude susp.	1	1	1	0	0
Chronic myelogenous leukemia	Plasma	1	" , filtered	1	1	1	1	1
1 case	"		Freon 1X	1	1	1	1	1
(Aged 44 yr)	Bone marrow	1	Crude susp.	1	1	1	1	1
	Saliva	1	"	1	1	1	1	1
	"		Freon 1X	1	1	1	1	1
	Urine	1	High speed sup't.	1	1	1	1	1
	"		" sed.	1	1	1	1	1
	Totals	121		211	187	150	102	102

oculated immediately or stored frozen at −70°C in sealed glass containers until used. *Cultures* of human normal or malignant tissues were prepared by the ordinary trypsin process or by fragmentation by forceps employing BME with 10% calf serum medium. After various periods of incubation, the cultures were frozen and thawed 3 times and the extracts were passed to tissue cultures without further processing or following clarification by centrifugation and/or filtration. Subcultures of the normal and malignant tissues were sometimes carried out for 1 or 2 passages. With certain tissues, the stainless steel grid technic of Jensen *et al.*(3) was carried out in Petri plates incubated in an atmosphere of 10% CO_2 in air.

Tissue cultures. Primary cell cultures of *human amnion* (PHA) were prepared by the trypsin process in BME with 10% human serum. The cells were maintained with BME containing 50% bovine amniotic fluid (BAF) following inoculation with the tissue suspensions. Trypsinized suspensions of *grivet monkey kidney* cells (GMK) were planted in HBSS containing 2% calf serum and Edamin yeast extract. For maintenance purpose, 50% BME, 50% BAF was employed. Cell strain WI-18 of *human fetal lung* (HFL)(4) was obtained from Dr. Leonard Hayflick, Wistar Inst. of Anatomy and Biology. The cultures were propagated in BME with 10% calf serum and were maintained in Eagle's minimum essential medium (ENEM)(5) without added serum.

Inoculation and observation of cell cultures. Fifteen cell cultures of each type were each inoculated with 0.2 ml of undiluted nonviscous or of 1:2 or 1:4 dilutions of viscous specimens. Change of fluid at 24 to 72 hr was sometimes necessary because of excessive debris but was usually performed at 7-9 days of incubation. The cultures were observed for 2 to 3 weeks for cytopathic change, for excess acid production, for cell hyperplasia, and for other abnormality. At the end of the observation period, about ⅓ of the cultures were tested for hemadsorptive properties (see below) and the remaining were subpassaged to the same cell culture type after a single freeze-thaw cycle. Unused fluids were stored frozen

TABLE II. Human Solid Tumors Studied in Cell Cultures of Primary Human Amnion and of Grivet Kidney.

Malignancy	Clinical materials: Specimens Kind	No.	Pre-inoc'n. treatment	No. of specimens on which observations were performed: Cytopathology–blind passage	Hemads.	Hemagglut.	Acridine orange	Fluor. antibody
Wilm's tumor	Tumor	3	Crude susp.	3	3	3	3	3
			" , filtered	3	3	3	3	3
			Freon 2N	3	3	3	3	3
Neuroblastoma	"	3	Crude susp.	3	3	3	3	3
Various:								
Breast cancer	Breast	5	" "	5	5	2	2	2
	Lymph nodes	2	" "	2	2	0	0	0
	Lung (metast.)	1	" "	1	1	0	0	0
Uterine "	Tumor	2	" "	2	2	1	1	1
Lung "	"	3	" "	3	3	1	2	2
" "	Lymph nodes	1	" "	1	1	0	0	0
Stomach "	Tumor	3	" "	3	3	1	1	1
" "	Liver (metast.)	2	" "	2	2	0	0	0
" "	Lymph nodes	1	" "	1	1	1	0	0
Bowel and rectal cancer	Tumor	13	" "	13	10	4	5	5
" " " "	Liver	1	" "	1	1	1	0	0
" " " "	Lymph nodes	1	" "	1	1	1	0	0
Other cancer	Tumor	8	" "	8	8	1	3	3
	Lymp nodes	2	" "	2	2	0	0	0
	Totals	51		57	54	25	26	26

at −20°C until tested for presence of hemagglutinin (see below). Blind passage of each was carried out in the manner described for 4 additional times at 2-3 week intervals. *Hemadsorption.* Cultures at passages 0, 2 and 4 were washed with medium 199 and examined for hemadsorption using a 0.1% suspension of fresh guinea pig erythrocytes in BME. A first reading was made following incubation for 20 minutes at room temperature and a second reading was made after shaking and further incubation at 4°C for 20 minutes. *Hemagglutination.* Fluids harvested from the frozen and thawed cultures at passages 1 and 3 or 5, were clarified by centrifugation at 2000 rpm for 20 minutes and were tested (a) without further treatment, (b) after heating at 56°C for 30 minutes, (c) after treatment with receptor destroying enzyme (RDE) in cholera filtrate, or (d) after extraction for 3 minutes in a chilled Omnimix microcup with ½ volume of Freon for GMK and HFL fluids or with 1/10 to ⅓ volume for PHA fluids. Fluids not tested immediately were stored frozen at −20°C and recentrifuged as necessary after thawing and prior to test. For the hemagglutination tests, equal volumes of fluid and red cell suspension were used. Guinea pig (0.8%) and chick erythrocyte (0.5%) suspensions were employed and both were incubated at 4°C and at room temperature. Two diluents, *i.e.,* phosphate buffered saline at pH 7.2 and citrate saline (0.05M citrate in 0.42% sodium chloride) at pH 7.4, were employed for each. The latter was employed to bind divalent cations which might suppress hemagglutination. Thus, each clinical specimen at each passage level in each cell line was tested for hemagglutination by 32 variables. The specimens which were untreated or were treated with RDE or heat, were tested at 1:2 and 1:10 dilutions while the Freon-treated samples were tested at a 1:3 dilution only. In later studies, 45 experimental and 5 control fluids treated as described above were tested with 0.5% type "O" human red cell suspension in citrate saline at 4°C.

Special studies. Acridine orange staining. Cell cultures of PHA, GMK and HFL on coverslips were prepared as described above, and were inoculated with the clinical specimens or with second or fifth tissue culture passage fluid from inoculated cell cultures or from direct cultures of human malignant tissues. Coverslips were collected after 5 to 14 days incubation and were fixed and stained according to Bertalanffy *et al.*(6). Examination was made using a Lietz fluorescent microscope equipped with an HBO 200 UV light source, a BG 12 exciting filter, an OG1 eyepiece filter, and a bright field condenser. *Fluorescence microscopy.* The inoculated coverslip cultures were washed once or twice with Earle's balanced salt solution (EBSS)(2) at pH 7.2 and once with absolute ethanol after which they were allowed to fix for 10 minutes with ethanol at room temperature. The air-dried preparations were treated for 30 minutes at 37°C with 0.05 to 0.1 ml of autologous patients' serum which had been inactivated at 56°C for 30 minutes and diluted 1:5 in veronal buffered saline solution containing 10% normal guinea pig serum. They were then washed twice with phosphate buffered saline solution at pH 7.2 and once with distilled water. Each was then treated for 30 minutes at 37°C with 0.05-0.1 ml rabbit antihuman globulin conjugated with fluorescein isothiocyanate. Prior to use, the fluorescein conjugate was absorbed 2 or 3 times with acetone extracted rhesus or grivet monkey liver powder. The preparations were washed 3 times in buffered saline, one time in water, air dried, mounted in semi-permanent mountant, and examined using the same optical system described above but employing a dark field condenser.

Control tests. Tissues obtained at autopsy from patients who showed no gross signs of malignancy were tested in PHA and in GMK cell cultures in the manner described for the specimens from cases of malignancy. Additionally, serial blind passages were made of each cell culture system and uninoculated cell cultures from each lot were examined in the manner described for the cultures which were inoculated. For positive control purpose in the fluorescent antibody studies, PHA cultures were infected wtih respiratory syncytial (RS) virus and examined by the indirect method employing acute and convalescent sera from a case of RS virus infection in man. The HFL and GMK cell cultures were simi-

TABLE III. Direct Cultivation of Human Malignant Tissues.

Malignancy	Tissues cultivated: Kind	No.	Observations: No. fluoresc. stained	Subpassage to "normal" cell cultures: PHA	GMK	HFL	No. harvests
Stem cell leukemia (1 case)	Brain, lung, kidney, spleen	1 each	0	x	x	ND	2 of each
Acute lymphocytic leukemia (2 cases)	Liver, spleen	2 "	0	x	x	"	4 " "
	Lung, thymus, brain, kidney	1 "	0	x	x	"	4 " "
	Bone marrow, lymph nodes	1 "	0	x	x	"	4 " "
Acute myelogenous leukemia	Bone marrow	1	1	ND	ND	x	2
Squamous cell carcinoma	Lymph node metastasis	1	1	"	"	x	4
Teratodermoid "	Tumor	1	1	"	"	x	2
Cystosarcoma phylloides	"	1	0	"	"	x	2 crude, 1 filt.
Cancer of:							
Breast (2 cases)	Tumor	2	1	"	"	x	2 of each
	Lymph node	1	0	"	"	x	2 crude, 1 filt.
Ovary	Tumor	2	1	"	"	x	1 of each
Uterus	"	1	0	"	"	x	4
Lung	"	1	0	"	"	x	1
Stomach	"	1	1	"	"	x	3
Intestine (14 cases)	"	12	4	"	"	x	1 or 2 of each
	Liver metastasis	1	0	"	"	x	1
	Lymph node metastasis	1	0	"	"	x	2 crude, 1 filt.
Pancreas	Tumor	1	0	"	"	x	1
Spleen	Metastasis, primary unknown	1	0	"	"	x	1
	Totals	42 specs.	10				

larly controlled in a measles virus system. Tests of inoculated cultures and autologous patients' sera were controlled, in the negative aspect, with sera from infants aged 6 to 9 months.

Results. Extracts of leukemic tissues. Table I summarizes results of attempts to recover in PHA or in GMK cell cultures of viruses or virus-like agents from cases of human leukemia. Altogether, 105 clinical specimens from 23 cases were examined. After a variety of treatments, 211 preparations were available for examination.

No significant cytopathic change, hyperplastic or other, was observed in any of the inoculated PHA cell cultures, even after 4 or 5 blind passages. Twelve strains of a virus which caused multinucleation were recovered from cultures of GMK which had received the clinical specimens. The agent, called MNA, was of indigenous monkey renal cell origin and was not related to the human malignant tissues. The agent is described below.

Cell cultures of PHA and GMK, both of which had been inoculated with 187 human malignant tissue preparations were tested for hemadsorption on primary passage and at the second and fourth subculture passages. No hemadsorption was observed with guinea pig erythrocytes when tested at 4°C or at room temperature.

One hundred fifty specimens of human malignant tissue were passed in PHA and GMK cell cultures and the cultures were tested on primary passage and in the third and fifth subculture passages for presence of hemagglutinins for guinea pig and chick erythrocytes. All cultures proved negative at all passage levels despite attempts to liberate or unmask hemagglutinins by various chemical and physical methods, incubation at different temperatures, or incorporation of citrate for binding divalent cation, as described in the section on materials and methods above. Additionally,

none of the specimens tested caused hemagglutination of human "O" erythrocytes.

None of the PHA, GMK, or HFL cultures which had been inoculated with any of the 102 human specimens and tested by acridine orange staining (6) at the second and fifth subpassage levels showed any "malignant shift" in the nucleic acid staining as compared with the uninoculated and subpassage controls. These same 102 preparations were examined by the indirect fluorescent antibody technic employing autologous patients' serum. No localization of patients' antibody on the cells was found.

Extracts of solid tumors. Fifty-one solid tumor specimens as shown in Table II were examined in a manner identical for the leukemic tissues described above. In all instances, the results were negative.

Extracts of tissues from non-malignant conditions also gave negative findings when examined in the manner of the tissues of malignant origin.

Cultivation of human malignant tissues and virus isolation attempts from the cell cultures. Forty-eight clinical specimens shown in Table III were planted in cell culture media. Ten of the cultures were observed directly by the fluorescent antibody technic. Fluids from all of the primary malignant cell cultures were passed to PHA, GMK and HFL as shown. Examinations were as described above. The findings were uniformly negative.

Multinucleating agent (MNA). As stated above, 12 strains of MNA were recovered from GMK cell cultures which had received human malignant tissues. Studies were carried out with 8 of these strains to ascertain whether this agent was present in the human malignant tissue or whether it was an indigenous contaminant of GMK. All evidence indicated its origin in the GMK itself.

MNA was isolated on a single occasion from an uninoculated GMK culture and was designated control MNA. Cultures of GMK which had been inoculated with human tissues of non-malignant source or with uninoculated control passage materials did not yield an MNA isolate, even though as many as 25% of the cultures were used for control purpose. Eight of the 12 MNA isolates and the control MNA were tested with sera from the autologous donor monkeys and were found to be neutralized. The control MNA was indistinguishable in neutralization tests with isolates derived from the cultures inoculated with human malignant material. The patients' sera did not neutralize their autologous MNA isolates and did not react in the fluorescent antibody system employing infected cultures. Further, attempts to reisolate the agent from the original clinical specimens gave negative results. These findings, collectively, showed that the MNA strains were not derived from the human malignant tissue but may have been evoked from a latent state by the malignant tissue extracts.

The MNA virus was found to be transmissible in primary cell cultures of rabbit kidney and of human foetal kidney but not in HeLa or in stable human amnion. The agent was not neutralized by antisera against measles, SV_5, SV_{40}, parainfluenza 2, or respiratory syncytial virus nor by pooled antisera against the various simian virus groups. The agent did not evoke tumors or leukemia within 2 years following subcutaneous inoculation into newborn hamsters.

Discussion. A variety of malignancies in several animal species have yielded viral oncogenic agents(7). The proportion of such malignancies shown to be of viral cause appears to be in proportion to the amount of effort which has been made to recover such agents. In certain of the animal tumors, however, no virus has been recovered while in others, the virus may be demonstrable initially but is no longer detectable after continued growth of the tumor or on serial transplant(8).

The concept of viral etiology in human malignancy is neither new nor unique, but has been held for several decades. Attempts to recover viruses from human malignant tissues by cell culture methodology have been carried out by a number of workers(7-14). While interesting leads were obtained, no virus of proven oncogenic quality or of reasonably established etiologic relationship to malignancy in man has been found to date.

The present study was carried out for the purpose of attempted recovery of viruses or of

virus-like agents from cases of leukemia and from other malignancies in man. Leukemia was of special interest because of the acute febrile onset of the illness which is suggestive of an infectious disease. A wide variety of approaches and variables in test procedures was employed in the studies and these are summarized in Table IV. Patients' secretions and excretions were tested as well as malignant tissues in the hope that the former might be less inclined to have present antibodies and other possible viral inhibitors. Crude and filtered patients' specimens were tested untreated and following treatment with Freon to remove hypothetical virus inhibitory substances. Three human or animal cell culture systems were employed and, in addition, primary cultures of the malignant tissues were made. These were observed for visible alterations in cell morphology or excessive acid production, for a shift in acridine orange staining, and for presence of antigen capable of detection by fluorescent antibody staining in tests with patients' autologous sera. Additionally, tests were made for hemadsorption and for hemagglutinins employing a number of different kinds of indicator cells and a variety of procedures to remove inhibitors or to provide for optimal conditions for demonstrating activity. Citrate saline, *e.g.*, was employed to remove divalent cation known to inhibit certain hemagglutinins(15).

TABLE IV. Brief Summary, Variables Employed in Attempts to Recover Viruses in Cell Cultures from Tissues of Human Cases of Malignancy.

- Clinical specimens
  - Leukemias and tumors
    - Secretions
    - Excretions
    - Solid and fluid tissues
- Specimen preparation
  - Crude suspensions of specimens or of their cell cultures
  - Filtrates
  - Freon treatment
- Cell cultures (primary inoculation and blind passage)
  - Primary human amnion
  - Primary grivet monkey kidney
  - Diploid fetal lung strain
  - Direct culture of malignant tissue
- Observation
  - Cytopathology
  - Excess acid production
  - Cell hyperplasia
  - Fluorescent antibody (autologous serum)
  - Acridine orange (malignant staining)
  - Hemadsorption (guinea pig red cells)
    - 4°C and room temperature
  - Hemagglutination (chick, guinea pig, human "O" red cells)
    - Treatment of specimens
      - Untreated
      - Freon treated
      - RDE treated
      - Heated 56°C
    - Conditions
      - 4°C and room temperature
      - Phosphate buffer and citrate saline

The present studies to recover viruses from human malignant tissues were remarkable for their failure to recover viruses and are in striking contrast to the frequent positive findings in tests of animal neoplasms. These exhaustive experiments, though negative, should be interpreted as examples of methods which failed to recover viruses and should not be construed as positive evidence for a non-viral etiology of malignancies of the human species.

The results indicate the difficulty likely to be encountered in the search for viruses of human malignancy and provide justification for large-scale attack on the problem.

The finding of a multinucleating virus, MNA, in cell cultures of grivet monkey kidney serves as a cogent example of the need for reliable controls and for caution in interpretation of apparent positive results.

Summary. Exhaustive investigations were made employing cell culture methods to recover virus from specimens from human cases of leukemia and other malignancies. Altogether, 316 clinical specimens were tested. Primary culture of the human malignant tissue and passage of extracts of clinical specimens to primary cell cultures and to a cell strain were done. The specimens were tested crude and after treatment to remove hypothetical inhibitors and the cultures were observed by a variety of procedures to detect virus by direct and indirect methods as well as before and after treatment to remove inhibitors. No detectable virus or virus-like agent of human malignant tissue origin was recovered. The finding of a simian virus multinucleating agent, MNA, emphasized the need for reliable controls and for caution in interpretation. The negative findings in these

experiments were interpreted as examples of methods which failed and were not construed as positive evidence for a non-viral etiology of malignancies in man.

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Chromosome Studies in Certain Subgenera of Spermophilus.* (27649)

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A considerable variation in chromosome number and morphology has been reported among ground squirrels belonging to the subgenera of Spermophilus(1). Furthermore, it was also noted that karyograms of all male specimens of Spermophilus and Ammospermophilus contained a medium size acrocentric Y chromosome(1).

Karyograms of *S. tridecemlineatus* (subgenus Ictidomys) and of *S. beldingi* (subgenus Spermophilus) have not been previously analyzed. It was of interest, therefore, to compare chromosomes of the above species with those described in *S. mexicanus* (Ictidomys) and *S. richardsonii* (*Spermophilus*)(1). Chromosomes of *S. tereticaudus* (subgenus Xerospermophilus) were also analyzed.

The purpose of this investigation was to: 1) evaluate chromosome analysis in comparative taxonomy of closely related species and 2) determine the validity of the medium size acrocentric Y chromosome as a specific characteristic of Spermophilus and Ammospermophilus.

Methods. The following animals were studied: 1) *Spermophilus beldingi beldingi,* 3 males and 2 females. 2) *Spermophilus tridecemlineatus tridecemlineatus,* 3 males and 1 female. 3) *Spermophilus tereticaudus neglectus,* 1 male and 3 females.

Animals were classified as previously described(1). Chromosomes from femoral marrow were analyzed by utilizing a colcemide, hypotonic citrate and acetic orcein squash method(1,2).

Results. Chromosome number. The data are listed in Table I. The modal diploid (2n) chromosome number in *S. beldingi* was 30, in *S. tridecemlineatus* 34 and in *S. tereticaudus* 36.

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