Isolation of Viruses from Leukaemic Patients

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[WITH SPECIAL PLATE]

Brit. med. J., 1964, 1, 927-929

The discovery that viruses cause chicken and mouse leukaemias has stimulated a world-wide search for a similar cause of human leukaemia. Morphological evidence of "virus-like" particles in human leukaemic cells has been produced by many electron-microscopists, but these findings cannot be taken as proof that the virus particles are the cause of the tumours when there is no other evidence (Dmochowski, 1963). The biological evidence thus far has been either equivocal or negative, and is summarized in two recent reviews by Harris (1963) and Southam (1963). I shall quote only two papers: Grace *et al.* (1960) claimed an increased incidence of tumours in Swiss mice inoculated with extracts prepared from human tumours; the other, by Girardi *et al.* (1962), reports a "surprising" failure to isolate any virus from human tumours by tissue-culture methods.

The experiments described in this paper started in 1960 and were designed to isolate, in tissue cultures, viruses from the bone-marrow of leukaemic patients. A virus was isolated from each of 10 cases of human leukaemia and was maintained by serial passage in tissue cultures. A preliminary comparative study of their biological properties is now completed and the results are summarized here.

Materials and Methods

Inoculum.—Through the courtesy of various pathologists in London and Bristol, samples of bone-marrow were obtained by sternal, tibial, or iliac-crest biopsy, and inoculated direct into culture tubes of human kidney tissue. The inoculation was usually carried out at the patient's bedside. If this was not possible the bone-marrow samples were kept in sterile bottles at 0° C. for one to four hours before the inoculation. Twenty-five samples of bone-marrow from leukaemic patients and 13 from patients with diseases other than leukaemia were studied.

Tissue Cultures.—For the preparation of tissue cultures whole human embryos and human foetal kidneys were obtained (the majority through the courtesy of Dr. H. E. M. Kay, of the Royal Marsden Hospital, London) from pregnancies terminated between the third and sixth months. "Single" cell suspensions were prepared by trypsinization either according to the technique of Melnick (1955), or at 4° C. overnight. The cell suspensions were used immediately or after storage at -70° C., according to the method described by Dougherty (1962). Tube monolayer cell cultures were grown in a medium containing 10-20% calf serum and 0.5% lactalbumin hydrolysate in Hanks's salt solution and antibiotics. The cultures were kept static for five days at 37° C. and then rolled at the same temperature. The maintenance medium had the same components as the growth

medium, except that the concentration of serum never exceeded 10%. It is important to note that some calf sera were found to be highly inhibitory for the growth of the viruses isolated from the leukaemic patients, and it was necessary, therefore, to select appropriate sera.

Virus Preparations.—These consisted of culture media from infected tissue cultures, diluted in buffered salt solution. No purification was attempted and the preparations were stored undiluted at -70° C.

Infectivity.—Growth of virus was estimated by following cytopathic changes in the infected cultures. The infectivity titrations were carried out by the tenfold serial dilution method, groups of cultures being inoculated at each dilution level. The end-point was determined from the proportion of positive and negative tubes at each dilution level.

Preparation of Immune Sera and Neutralization Tests.—Three virus preparations, each comprising 10 ml. of infectious tissue-culture medium derived from a different leukaemic patient, were inoculated individually into three rabbits. The inoculation was repeated after one week. The serum from the rabbits was collected one further week after the second inoculation. Cross-neutralization tests were made with each immune serum against all nine "strains" of virus isolated from the leukaemic patients. Pre-immunization sera from the same rabbits were used as controls. Equal quantities of virus, at increasing dilution, and sera (1:10) were mixed and incubated at room temperature for 30 minutes. Then 0.2 ml. of the mixture was inoculated into each tissue-culture tube. The infectivity titres were compared with those obtained with virus mixed with control sera. The neutralization tests with sera from leukaemic patients were carried out in a similar way. Sera from non-leukaemic patients, or apparently normal volunteers, were used as controls.

Results

Virus Isolation and Serial Passages

The isolation of viruses from the bone-marrow was successful in 10 out of 25 cases of human leukaemia. From the other 15, no virus was isolated in seven cases, and no conclusion was possible in eight cases, as the experiments were stopped in the early passages by fungal or bacterial contamination.

One "strain" of virus was lost by bacterial contamination in the course of serial passage in tissue culture. Table I summarizes the clinical histories of the nine patients giving a successful virus isolation. Eight were suffering from acute leukaemia, and one from chronic myelogenous leukaemia in the acute phase: only three were children. These viruses have all been maintained by serial passage in tissue culture.

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TABLE I.—Summary of Clinical Histories of Leukaemic Patients

Hospital	Initials	Sex	Age	Diagnosis
Edgware General ..	P.T.	F	11 years	Acute leukaemia
" " ..	I.G.	F	61	" "
" " ..	R.D.	M	Adult	Acute lymphoblastic leukaemia
" " ..	K.H.	M	"	Unspecified leukaemia
" " ..	C.W.	M	"	Chronic myeloid leukaemia
Great Ormond Street	C.C.	F	Child	Acute leukaemia
Middlesex ..	—	?	"	" "
Central Middlesex	-L.	M	Adult	" "
West Middlesex ..	R.D.	F	32 years	Naegeli type leukaemia

As controls, bone-marrow samples from 12 patients with diseases other than leukaemia were inoculated into tissue cultures. No virus was isolated from any of these as judged by the absence of cytopathic change in human cell cultures after 6–17 successive blind passages.

Table II gives the clinical histories of these cases. At least six had non-leukaemic changes in the bone-marrow, indicating either a primary disease of the marrow or secondary changes as a result of disease in another organ or system.

TABLE II.—Summary of Clinical Histories of Control Patients

Hospital	Initials	Sex	Age	Diagnosis
Royal Hospital for Sick Children, Bristol ..	S.J.	M	1 year	Cerebral palsy
" " ..	J.B.	M	11 years	Idiopathic thrombocytopenic purpura
" " ..	S.T.	F	4 "	Congenital (familial) benign neutropenia with hypergammaglobulinaemia. Circulating leuco-agglutinins
" " ..	C.K.	M	7 mths	Hydrocephalus-haemangioma
" " ..	S.D.	F	11 years	Rheumatoid arthritis
" " ..	S.W.	F	6 "	Idiopathic thrombocytopenic purpura
Royal Marsden ..	J.M.	M	1 year	Renal tubular disorder
" " ..	G.B.	M	Adult	Polycythaemia
" " ..	-S.	F	"	? Anaemia
" " ..	-H.	F	"	Cricoid carcinoma
" " ..	-H.	M	"	" "
" " ..	-C.	M	"	" "

Cytopathic Change and Infectivity

The tissue cultures inoculated with bone-marrow from the leukaemic patients showed cytopathic changes after some three to five blind passages. In subsequent early passages cytopathic changes were detectable four to five days after the inoculation, but later these occurred after only two days. The cells in infected monolayer cultures at first lost contact with each other, making it impossible to detect their outer border. Later they became rounded up, clumping together in groups until finally they left the glass (Special Plate, Figs. 1 and 2). Only a few cells remained on the glass surface, and these formed a network with cytoplasmic bridges joining one cell to another. Concomitantly, the pH of the culture medium dropped below 6.8.

The medium from cultures with pronounced cytopathic changes was infectious for other cultures to a dilution of 10^5 – 10^6 . One to ten infectious doses produced cytopathic changes in five days, while 10^5 – 10^6 infectious doses produced cytopathic changes in only two days.

The infectivity in undiluted medium from cultures showing cytopathic changes was retained after storage at -70°C ., but was lost after storage at -20°C . It was also lost after ether treatment and after heating at 56°C . for half an hour, but retained after heating at 37°C . for five hours. Preparations of viruses were infectious after filtration through a bacteria-tight sintered-glass filter and through a collodion membrane of a.p.d. 85 μ .

Neutralization Tests

The sera obtained from three rabbits immunized with medium from infected cultures neutralized 100 tissue-culture infectious doses (TCID) of the "homologous" strain.

In cross-neutralization tests these sera also neutralized the same number of TCID of any of the other eight "strains." Pre-immunization sera from these rabbits gave no neutralization.

The results of neutralization tests with sera from leukaemic patients are still in a preliminary stage. Only eight sera from leukaemic patients have been studied. None of these was from a patient whose bone-marrow was used for the isolation of virus. All cases were of chronic lymphatic leukaemia, with the exception of one of acute myelogenous leukaemia. In seven out of eight cases 1:10 dilutions of sera neutralized 10–100 TCID of virus. The result was negative with one serum. The sera from nine normal volunteers or patients with diseases other than leukaemia showed no neutralization; two sera from patients with "anaemia" gave a doubtful result.

Discussion

A virus, cytopathic for human embryo cells in culture, has been isolated from 10 cases of human leukaemia. Uninoculated tissue cultures or tissue cultures inoculated with "control" bone-marrow samples showed no cytopathic changes even after several blind passages of the culture medium. The presence of a virus in these infected cultures has been confirmed by electron microscopy (Inman *et al.*, 1964).

The virus could be maintained by serial passage in tissue culture and killed infected cells. This cytotoxic action increased in the course of the passages.

Attempts are currently being made to classify and identify the virus. Herpes simplex has often been found in association with leukaemia. The failure of an anti-herpes antiserum (obtained by courtesy of Dr. H. G. Pereira) to neutralize the virus excludes herpes simplex from the present series.

Three different virus isolates produced antibodies in rabbits, and cross-neutralization experiments showed that all nine strains of virus were antigenically related. Thus one virus, or group of viruses, is frequently associated with leukaemia. The number of controls is, unfortunately, too small, as yet, to prove an *exclusive* association of these viruses with human leukaemia. No firm conclusion is therefore possible, as yet, regarding the relation of these viruses to the disease itself. It has *not* been proved that they are the cause of the leukaemia, and the possibility that they were simply passenger viruses cannot be ruled out. The frequency of the association is, however, remarkable.

The next steps in the investigation include: first, an extensive study of virus-neutralization by sera from leukaemic patients and controls; second, renewed attempts to reproduce the disease in small animals—to date, virus-inoculated mice, hamsters, and ferrets remain in perfect health (experiments with the collaboration of Drs. F. C. Chesterman and June East); and third, the effect of the virus on primates.

Summary

Bone-marrow samples from patients with leukaemia were inoculated into human embryo tissue cultures. A virus was isolated from each of ten cases, and became cytopathic for the cells in the course of serial passage. Comparable cultures inoculated with bone-marrow from control cases showed no changes even after many "blind" passages.

The viruses, which are thermolabile and ether-sensitive, have, so far, produced no pathological changes after inoculation into newborn mice, hamsters, and ferrets.

The viruses are antigenic for the rabbit, are antigenically related to each other, and are weakly neutralized by sera from patients with leukaemia. It is therefore reasonable to assume that one virus, or a group of antigenically related viruses, is frequently associated with human leukaemia.

The results, so far, do not justify any conclusion regarding the relationship of these viruses to leukaemia itself. The frequency of the association is, however, remarkable.

My especial thanks are due to Dr. H. E. M. Kay, of the Royal Marsden Hospital, who, in co-operation with many surgeons, has supplied the foetal kidneys and also some bone-marrow samples. In addition, foetal material has been provided by Mr. M. W. Hemans, St. Albans City Hospital; Mr. E. R. Rees, Gynaecological Metropolitan Hospital, London; Mr. D. N. S. Robertson, Stoke Mandeville and Amersham Hospitals.

I should also like to thank Dr. V. J. Marrian, of the Royal Hospital for Sick Children, Bristol, for bone-marrow samples; and Dr. F. C. Chesterman for organizing the supply of leukaemic material in co-operation with Dr. H. F. Brewer, St. Bartholomew's Hospital, London; Dr. G. Discombe and Dr. Joan Clarke, Central Middlesex Hospital; Dr. R. M. Hardisty and Dr. K. M. Dormandy, Hospital for Sick Children, Great Ormond Street; Dr. J. G. A. McSorley, Whittington Hospital, London; Dr. J. G. Selwyn, West Middlesex Hospital; and Dr. J. W. Stewart, Middlesex Hospital.

My thanks are also due to Dr. A. Pomerance, Central Middlesex Hospital, and to Dr. E. K. Blackburn, Royal Infirmary, Sheffield, for the supply of sera from normal and leukaemic patients respectively; and also to Hilary Morgan for technical assistance, and A. J. Pringle for photographic assistance.

I have appreciated the advice and encouragement of Dr. G. F. Marrian and Dr. R. J. C. Harris.

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Electron Microscopy of Virus Particles in Cell Cultures Inoculated with Passage Fluid from Human Leukaemic Bone-marrow

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[WITH SPECIAL PLATE]

Brit. med. J., 1964, **1**, 929-931

The presence of virus particles in murine and avian leukaemias has been demonstrated in the electron microscope by numerous workers. There seems little doubt that such particles are a causative agent in these conditions. Among the many relevant reviews are those of Bernhard (1960), Bernhard and Granboulan (1962), and Dmochowski (1961). Table I summarizes some of the reports of the electron microscopy of known leukaemogenic murine and avian viruses. Virus-like particles have also been reported in the lymph nodes (Dmochowski *et al.*, 1959), bone-marrow (Braunsteiner *et al.*, 1960; Murphy and Furtado, 1963), and blood (Almeida *et al.*, 1963; Burger *et al.*, 1964) of human leukaemic patients. Reports of viruses associated with leukaemia in man are summarized in Table II.

In the preceding paper (Negroni, 1964) the presence of a cytopathic virus, derived from the bone-marrow of leukaemic patients, has been demonstrated by serial passage in cultured human kidney cells. These infected cultures have been examined in the electron microscope in an attempt to support the virological evidence by direct demonstration of virus particles in infected cells and media. The present paper is a preliminary report on this work.

Methods

Tissue Cultures.—The methods of preparing and inoculating cell cultures are described in the preceding paper (Negroni, 1964).

Electron Microscopy

Cell cultures were prepared for examination in the electron microscope in one of three ways.

1. **Thin Sections of Whole Cells.**—Cells were detached from the walls of the culture-tube, centrifuged for less than five minutes at $1,000\times g$, and the supernatant culture fluid removed. The cells were then fixed in cold buffered osmic acid for half to one hour, dehydrated in ethanol and embedded at 60°C . in methyl/butyl methacrylate (25:75) containing 1% uranyl nitrate. Thin sections were cut on an A. F. Huxley pattern ultramicrotome (Cambridge Instrument Co. Ltd.) and mounted on carbon-coated grids. Some sections were treated on the grid with lead stain (Karnovsky, 1961). The sections were examined in a Siemens Elmiskop I electron microscope.

2. **Thin Sections of Pellets.**—After separating out the cells as in No. 1 above, the culture fluid was clarified by centrifuging for 20 minutes at $2,000\times g$ in an M.S.E. clinical centrifuge. The supernatant was then centrifuged for 30 minutes at $78,000\times g$ in a "Spinco" model L ultracentrifuge and the resulting pellet fixed, dehydrated, embedded, and sectioned as in No. 1 above. In some cases immune or control rabbit antiserum was added to the clarified culture fluid (1:5) half an hour before ultracentrifugation at either $30,000\times g$ or $78,000\times g$.

3. **Negative Contrast.**—Inoculated tissue cultures were prepared for negative contrast electron microscopy by the method of Almeida and Howatson (1963).

Measurement of Virus Particles.—In some sections the virus has an oval appearance (Special Plate, Fig. 5), apparently due to a sectioning artifact. Therefore the long and short axes of all particles have been measured and their mean calculated. Only particles that were believed to have been sectioned medially were measured; these represent approximately 30% of the total

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G. NEGRONI: ISOLATION OF VIRUSES FROM LEUKAEMIC PATIENTS

FIG. 1.—Uninoculated human kidney tissue culture. 16 mm. cruciform phase-contrast objective (Trevan, 1961). ($\times 100$.)

FIG. 2.—One hundred and fifth passage of virus. Comparable tissue cultures from a titration experiment, five days after the inoculation of: (a) 100 infectious doses of virus in 0.1 ml. inoculum (medium diluted $10+^3$). (b) 0.1 infectious doses in 0.1 ml. inoculum (medium diluted $10+^6$). 16 mm. cruciform phase-contrast objective (Trevan, 1961). ($\times 100$.)