

# LEUKOCYTIC CULTURES IN ISOLATION AND STUDY OF VIRUSES AT ACUTE LEUCOSIS AND CHRONIC MYELOLEUCOSIS IN HUMANS

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## INTRODUCTION

Previously, human leucocyte cultures derived from the blood of healthy donors stimulated with PHA were successfully used for isolation of human hepatitis C virus from the blood of patients [1]. In the present study, the same cultures were used for isolation and cultivation of oncoviruses at acute leucosis and chronic myeloleukosis. This work was carried out in collaboration with A.K. Shubladze, E.P. Ugriumov, A.F. Bocharov.

## MATERIALS AND METHODS

The method of *cultured leukocytes* was based on the technique by Moorhead et al. [see in refs. [1, 2]. Venous blood was collected into sterile tubes containing 1 or 2 drops of concentrated heparin. Blood samples were centrifuged at 1000 rpm 10 min or left for 18 hrs at 40 C. The plasma was removed, and the leukocyte film was collected off the surface of erythrocytes. Leukocytes were then suspended in cultural medium 199 containing 25–30% of the autologous plasma to a final concentration of 2–3 or 6–7 million cells per 1 ml. An equal amount of leukocytes from healthy donors was added followed by PHA to a final concentration of 0.1–0.2 mg per 10 ml of cell suspension. The cells were incubated in an atmosphere with 2.5–5.0 CO<sub>2</sub> at 37° C. For a long-term culturing of leukocytes from patients with leucosis, leukocytes from healthy donors and PHA were not added.

For EM studies, cells were taken off the glass mechanically or with versene solution and washed off by centrifuging in medium 199 (1500 rpm). After that cells were fixed either with 1.6% glutaraldehyde for 1 hr followed by 1% osmium at 40 C for 45 min, or with 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2). (Osmium fixator was prepared in acetate-veronal buffer, pH 7.2) [1, 2]. Slices were prepared using LKB-8800 A microtome and contrasted with 1% uranyl acetate in 70% methanol for 15 min at room temperature followed by 1.5 % lead citrate for 10 min at room temperature. For negative contrasting, concentrated virus was placed on a net with a formvar underlayer dusted with coal and exposed to electric field. Con-



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trasting was achieved by 3% phosphone-tungsten acid (pH 7.2). A 5EM-7A microscope was used. The density of the oncovirus was defined in a linear density gradient sucrose solutions [2, 3, 4].

The virus activity was measured in the reverse transcriptase (RT) assay by Spiegelman et al. [2, 4]. The proteins of the leucosis-like virus were fingerprinted [1, 4]. In serological studies, a rabbit serum against the virus isolated from leukocytes of a chronic myeloleukosis donor was used [2, 3, 4, 5].

An indirect immunofluorescence on blood or bone marrow smears was used. A total of 101 samples of bone marrow from patients were studied [1, 2, 3, 4]. Patients were diagnosed based on the clinical clinical survey data and the results of morphologic research of blood and bone marrow samples.

## RESULTS

The oncovirus was isolated through cocultivation of short-living PHA-stimulated leukocytes from patients with acute leucosis and from healthy donors. The virus was passaged in PHA-stimulated cultured leukocytes from healthy donors. For passageing of the isolated virus, healthy leukocytes were inoculated before adding PHA. For the inoculate, destroyed infected leukocytes suspension free from cell debris, was used. For passageing of the oncovirus isolated from long-living cultures from patients with chronic myeloleukosis, primary human embryo fibroblasts and human embryo kidney cells were used. The clarified cultural suspension from the previous passage was used inoculate for the next passage.

The comparative study of the two types of leukocyte cultures from leucosis patients (short-living

PHA-stimulated and long-living) showed an extreme difficulty of isolation and culturing of the human leucosis agent. In our opinion, use of primary leukocytes is a helpful approach to solve this problem. Leukocytes upon stimulation by PHA transform into blastocytes, which produce morphologically formed biologically active virus passaged on in cultured PHA-stimulated leukocytes.

Another approach is obtaining passageable lymphoblastoid cultures from white elements of the peripheral blood from leucosis patients. From patients with acute leucosis, we isolated three strains of the virus named 'leukocytic human leucosis virus'. In EM studies of ultrathin slices, C and A types particles typical for oncoviruses in size and structure were observed over 15 passages. Particles of the same types were also detected in the cultural fluid and infected leukocytes homogenates (Fig. 1). In control PHA-stimulated leukocyte cultures, no viral or virus-like particles were detected. The isolated leukocyte leucosis virus strains proved apathogenic for laboratory animals and chicken embryos and did not show cytopathic activity after inoculation of various primary and pasaged cultures. They did not cause chromosome alterations in infected leukocytes, but in a number of cases they stimulated the mitotic activity of the leukocytes, which was the direct indication of the presence of the virus. Leukocytic leucosis virus is instable to high temperature and is completely inactivated with ether. Its buoyant density is  $1.16 \text{ g/cm}^3$  [2, 4, 5]. The reverse transcriptase activity of the virus was shown by using exo- and endogenous templates, the latter being the virion RNA with a sedimentation constant of 70 S. Similar viral particles were detected also in the cultural fluid.

The obtained results were used in our further studies on etiopathogenesis of human leucosis. We attempted to derive passageable cell lines from the blood of leucosis patients whose cells would contain morphologically formed oncovirus. Out of 74 assays conducted, only 5 cases proved successful. Those cells were from a patient with chronic myeloleucosis [3]. The observation showed that only few cells or groups of cells preserved vitality and attached to the glass (or plastic) as transparent cells with protrusions. Besides, some cells in the cultures had rounded shape. In the process of culturing they might enlarge transforming into giant cells with perinuclear graining. In some cases, giant and polynuclear cells formed clusters, from which cells with protrusions stemmed and that event was a good prognostic feature. The cultures began growing as cells with protrusions forming first foci (islets), then colonies followed by a monolayer. The histological studies showed that cultures were made up of large cells with big oval nuclei containing a few visible

nucleoli with a thin cytoplasm, sometimes vacuolized. The cultures metabolized actively, acidifying the medium rapidly. Cells of some patients were passaged for as long as 7 months [3]. Ultrathin slices of the cultured cells in EM studies revealed viral particles of type C with characteristic morphology. Particles were spheric or ellipsoid in shape, 87 to 130 nm in diameter, with a 65 to 70 nm nucleoid. Type C particles were observed in EPR channels and intracellular space (Fig. 2). We did not observe big clusters of particles. The characteristic budding of virions occurred on the plasma membrane or inside vacuoles formed by enlarges sites of the EPR. The cells looked viable and possessed all the typical organoids. The cytoplasm was partially vacuolized.

Thus using long-lived leukocyte cultures we showed that cultured cells contained oncovirus-like particles even without PHA stimulation.

We paid a special attention to the leukocyte leucosis virus as its isolation and passageing were based on use of human leukocytes and serum only, so contamination with any bovine oncoviruses from bovine serum was excluded. As it was shown, the oncovirus

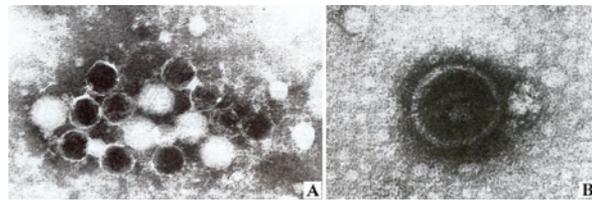


Fig. 1. Leukocytic leucosis virus (oncovirus type C) isolated in human leukocyte culture. Negative contrasting. A – multiple particles in a PHA-stimulated culture. B – single particle.  $\times 100,000$



Fig. 2. Oncovirus type C in intracellular space and budding off cells. Leukocytic culture from a chronic myeloleucosis patient.  $\times 250,000$

from leucosis patients was propagated in the leukocytes from healthy donors.

Human embryo fibroblasts and human embryo kidney cells were used in further passaging of the oncovirus. The virus' capability to replicate in primary human embryo cell subcultures was studied by radioisotopes and EM. In 6 studied cultural fluid samples an RNAase-resistant fraction labeled with <sup>3</sup>H-uridine was detected in the density range of 1.15–1.18 g/cm<sup>3</sup>, which corresponds to the density of the known oncoviruses. Such particles did not replicate in the presence of actinomycin D and were absent from non-inoculated control leukocyte cultures (Fig. 3).

In the cytoplasm of inoculated cells studied by EM, osmophilic clusters of fibrillar and membrane structures contained viral particles of type A with a diameter of 87 nm (nucleoids 45 nm). Virions of type C were also detected budding from the cell membrane. Some type C particles were found outside cells. Inside cells, condensed mitochondrial matrix and enlarged lamellar complex were observed [1, 2, 3]. RNA extracted from particles of 1.16 g/cm<sup>3</sup> was analysed by centrifuging in sucrose gradient [1, 2, 4, 5]. It is a 70 S molecule characteristic for oncoviruses. A reverse transcriptase assay detected 70 S RNA molecules marked with small fragments of newly synthesized DNA. In non-inoculated cultures, such structures were never found.

Long-living cells from 5 chronic myeloleucosis patients also supported replication of the oncovirus. Cells that began growing into a culture were the most likely producers of the virus. It is remarkable that the progeny of those cells preserved virus-producing capacity. Type C virions formed on the outer cytoplasm membrane, occurred outside the cells and had centered optically dense nucleoids. Type A particles were found inside the cells in fibrillar osmiophilic clusters. Viral particles synthesized after inoculation of embryonic cells also had a buoyant density of 1.16 g/cm<sup>3</sup> [1]. The active reproduction of this virus in human embryonic cells did not cause either transformation or cytoplasmic changes in the clusters.

In the next series of experiments, we studied the structure of the oncovirus and compared it with other mammalian oncoviruses. The previously described technique Gautsch et al., 1978 [4, 6] allows to study individual protein structures, e.g. the major inner virion protein product of the gag gene. This protein accounts for roughly 25 % of the total virion proteins of oncoviruses, so it can be easily obtained by PAAGE. The peptide mapping of it is very informative for typing of novel isolates as has been shown in a number of cases [2, 3, 4, 6].

On Figure 4, the main proteins of the virus isolated from leucosis patients is presented. (The virus was concentrated from 400 ml of cultural fluid by cen-

trifuging followed by purification in sucrose density gradient). After PAAGE the proteins p15, p24 and gp70 characteristic for mammalian oncoviruses can be clearly seen. The major inner p24 protein was studied in collaboration with J. Elder, J. Gautsch and R. Lerner from the Oncovirus Lab, Scripps Institute, San Diego, CA. The p24-containing band was cut out of the slab, iodated with NaI125 and treated with either trypsin or chymotrypsin. The obtained polypeptides were separated by two dimensional electrophoresis on cellulose sheets [2, 4]. The migration pattern of the obtained peptides differed from those obtained previously for p24 of the all known oncoviruses (Fig. 5).

The further concentration and purification of the virus allowed to use it in experiments. The specificity of the isolated virus was studied using immunomorphological and serological approaches. Ouhterloni gel precipitation was applied initially. Hyperimmune sera against (i) mouse leucosis virus, (ii) feline leukemia virus and (iii) type D virus from HE-p2 cells did not give any precipitation. Out of 12 sera from rabbits immunized with purified virus, only two contained specific antibodies. This fact indicated to a low antigenicity of the virus. In further studies by IF these sera were used in a 1:4 dilution. The immune rabbit sera preliminary were adsorbed with human (healthy donors) and bovine sera in order to get rid of non-specific rabbit antibodies against serum components.

Human embryo fibroblasts infected with leukocytic leucosis virus, showed a cytoplasmic fluorescence for 24 hrs p.i. onwards. First, the fluorescence granular, later it grew confluent. The dynamics and morphology of the fluorescence corresponded to the data obtained for the other vertebrate oncoviruses [2, 3, 4, 5].

After these experiments, the immune rabbit sera were used to survey of smears of bone marrow and blood autopsies from leucosis patients. 73 individuals were surveyed. A specific fluorescence of the bone marrow cells cytoplasm analogous to that of the inoculated fibroblast cultures was detected in 19 out of 31 patients with various forms of leucosis and in 9 of 42 control group individuals. The difference was statistically highly reliable ( $p < 0.01$ ).

The cytoplasmic fluorescence was observed in 15 out of 25 cases of acute leucosis and in 4 out of 6 cases of chronic myeloleucosis. The control group consisted of 42 patients with so called 'border conditions': plasmocytosis, reticuloscleroderma, myelomic disease, Werlhof disease and others. Only 1 person was healthy donor of the bone marrow sample.

The blood samples studied from 70 of the total of the 73. Fluorescence of the leukocyte cytoplasm in the smears was detected in 10 out of 12 patients with acute and chronic forms of leucosis and in 5 out

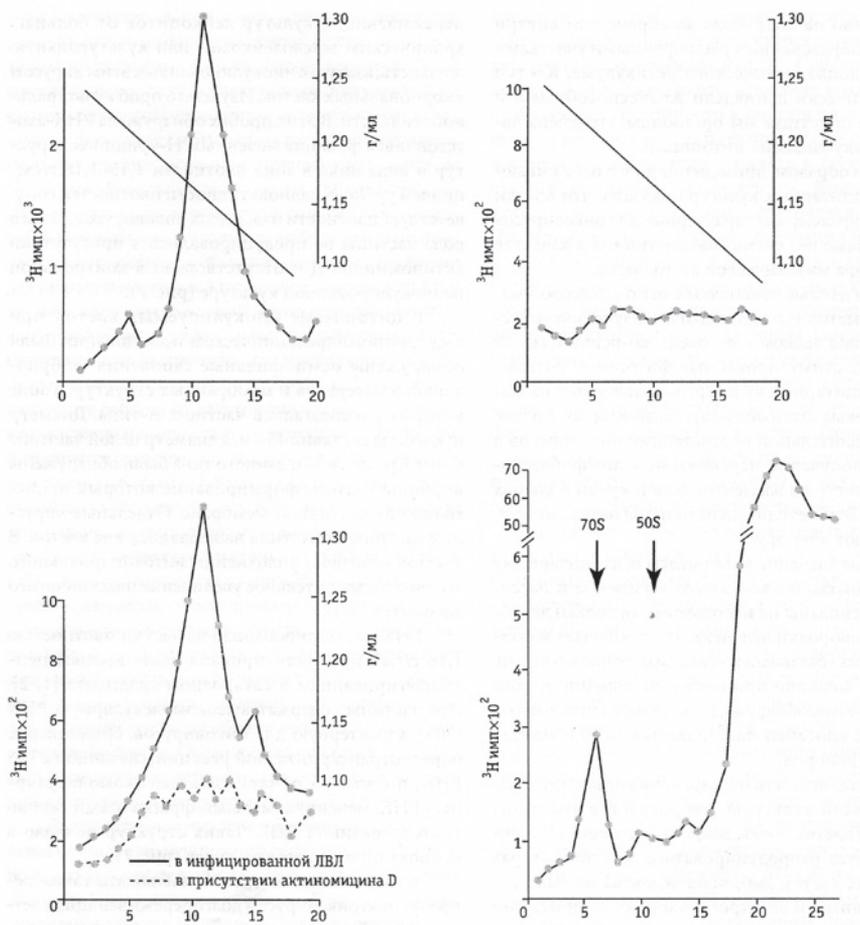
of 58 control group individuals: 2 blood donors and 3 patients with rheumatoid arthritis. The difference between the two groups was statistically reliable ( $p < 0.01$ ). The results of the conducted survey are presented in Table 1.

The next step of our study was examination of the virus in complement fixation test (CFT) for detection of antibodies against this virus in human blood sera.

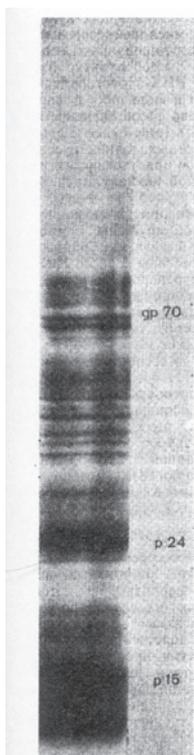
In total, 128 blood sera from healthy donors and 74 sera from patients with acute and chronic myeloleucosis were surveyed by CFT with leukocytic leucosis viral antigen. The results are shown in Table 2. These results indicate that antibodies against the studied virus widely occur in sera of leucosis patients. The specific fluorescence of the cell cytoplasm and the nature of the fluorescence agree with the data on the other oncoviruses. This similarity was observed in both bone marrow and blood smears from patients with acute leucosis and chronic myeloleucosis as well as from some patients with reticulo-scleroderma, myelomic disease, rheumatoid arthritis and even some clinically healthy persons. The observed similarity appears fairly explicable given the wide spread of oncoviruses in humans and animals.

## DISCUSSION

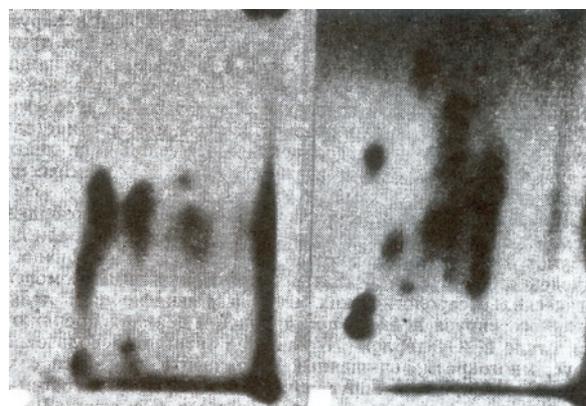
The conducted research has shown a successful application of cultured leukocytes from human blood for isolation of oncoviruses from patients with acute leucosis and myeloleucosis alike. Our data suggest that the two approaches in studying the viral nature of human leucosis, (i) primary PHA-stimulated leukocyte cultures for isolation of new viral strains and (ii) passaged lymphoblastoid cultures from white blood of patients for studies of virus persistence in the cells in the long run. These two approached supplement rather than exclude each other.



**Fig. 3.** Buoyant density of leukocytic leucosis virus and its RNA in sucrose gradient A – Density of the oncovirus under study in the sucrose gradient; B – Same, uninfected culture; C – Oncovirus under study, grown in the presence of actinomycin D in the cultural medium: broken line; Same virus, grown in the absence of actinomycin D in the cultural medium: solid line; D – Density of the viral RNA in the sucrose gradient



**Fig. 4.** Proteins of the oncovirus isolated from a leucosis patient, studied in PAAGE



**Fig. 5.** Tryptic (A) and chemotryptic (B) peptides of the oncovirus p24 protein

**Table 1.** Detection of the oncoviral antigen in the bone marrow and blood smears using the immunofluorescence technique

Source	Patients with leucosis			Control group		Reliability of the difference
	Acute leucosis	Chronic myeloleucosis	Chronic lympholeucosis	Healthy donors	Patients with other conditions	
Bone marrow smears	15/25	4/6	0/1	0/1	9/41	$\chi^2=11.119$ ( $p<0.01$ )
Total	19/32			9/42		
Blood smears	2/2	5/6	3/4	2/28	3/30	$\chi^2=24.140$ ( $p<0.01$ )
Total	10/12			5/58		

Fractions: the numerator is the number of positive samples, the denominator is the number of samples studied.

**Table 2.** Antibodies against human embryo fibroblasts infected with the virus under study in blood sera from leucosis patients and healthy donors

Serum samples	Studied	Antibodies titers in CFT with the studied oncovirus antigen				Total and % of the positive samples	Reliability of the difference
		Negative	1:10	1:20	1:40		
Leucosis patients	74	29	12	24	9	45(68.81%)	$p<0.01$
Healthy donors	128	104	10	4	10	24(18.75%)	$p<0.01$

CFT: Complement fixation test.

The leukocytic cultures technique proved efficient in isolation of oncoviruses. Passageing of the isolated viruses in leukocytic cultures as well as in primary fibroblasts and embryo kidney cells allows to grow the virus in amounts sufficient for further studies.

In our study, both immunologic techniques, immunofluorescence and complement fixation, detected the examined viral antigen or antibodies against it reliably more frequently in samples from leucosis cases compared with samples from control groups. This fact is a strong indication to the etiologic role of the studied virus in human leucosis [2, 4, 5, 7].

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The article is devoted to the investigation of human leukocyte cultures derived from the blood of healthy donors stimulated with PHA were successfully used for isolation of human hepatitis C virus from the blood of patients. The same cultures were used for isolation and cultivation of oncoviruses at acute leucosis and chronic myeloleukosis. The research was conducted in a *careful* and *objective* way and can be recommended for publishing in the medical Journal *Archiv Euromedica*.

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