

ISOLATION OF HEPATITIS C VIRUS IN CULTURED LEUKOCYTES FROM HUMAN BLOOD

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ABSTRACT — The article presents the results of the author's long term research on use of human leukocyte cultures for the isolation of hepatitis C virus and studying its characteristics.

KEYWORDS — hepatitis C virus, chromosomal alterations, mitotic activity, electron microscopy (EM), buoyant density.

INTRODUCTION

Our previous data [1] established that human and animal hepatitis viruses actively replicate in the organs rich in reticular cells and lymphoid elements (liver, spleen, lymph nodes, bone marrow), and viremia especially in transmitted parenterally transmitted hepatitis is connected both with the blood plasma and leukocytes [2]. In our present study, a blood leukocyte culture was used for isolation of the viruses from patients with C hepatitis.

MATERIALS AND METHODS

The method of cultured leukocytes was based on the technique by Moorhead et al. [see in refs. [3, 4]. 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 4° 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 CO₂ at 37° C.

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) [3, 4]. 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. Contrasting was achieved by 3% phosphotungstic acid (pH 7.2). A 5EM-7A microscope was used.

An indirect immunofluorescence on blood smears was used. Patients were diagnosed based on the clinical survey data and the results of morphologic research of blood and bone marrow samples.

The buoyant density of the leukocytic hepatitis virus and its RNA were measured in cesium and sucrose gradients [4,5].

The amounts of RNA of hepatitis C and G viruses were measured by RT-PCR as previously described [1,4,5].

Phytohemagglutinin (PHA) was obtained from Sigma.

Leukocytes from the blood of healthy donors were stimulated with PHA in a final concentration of 0.02 mg/ml. PHA-stimulated leukocytes were infected with the virus and serially passaged. The support medium was RPMI-1640 with 20% of autologous donor serum and PHA (0.02 mg/ml).

The method is described in details in [4].

RESULTS

Previously, in patients diagnosed with hepatitis as well as in virus-bearing donors, a high percentage (up to 48%) of lymphocytes with chromosome alterations was observed (Fig. 1). In a special set of studies we tested the possibility of passage of the viral agents from those blood samples in leukocyte cultures from healthy donors.



Fig. 1. Chromosomal alterations (indicated with arrowheads) in leukocyte culture from a 'non A non B' hepatitis patient

We also marked a decrease of mitotic activity in leukocytes from patients with viral hepatitis, especially over the first days of the disease [3, 5]. This phenomenon was observed in leukocyte cultures also at later stages of infection. It is considered a virus-specific cytopathic activity, like alterations in chromosomes. In collaboration with A. Shubladze, I. Demytyev and J. Shahgildyan, we tested the infectious agents from blood sera by several features: chromosome altering activity in cultured leukocytes, decrease of mitotic activity in those cultures and delay of blast transformation in PHA-stimulated cultured lymphocytes.

Blood sera from patients with hepatitis obtained on days 3 to 5 of jaundice were added to cultured lymphocytes from healthy donors. 72 hrs later the cultures were studied cytologically and cytogenetically. Some cultures were used for serial passages. With the rise of the passage level, the initial sera were diluted 20 times. The sera from healthy donors and non-infected lymphocyte cultures were passaged in parallel as controls. The results are shown in Tables 1 and 2. They indicate a consistent decrease of the mitotic activity and blast-transformation capacity in the process of passage of the initial material (a 2- to 3-fold at passage 3). The number of lymphocytes with alterations in chromo-

somes (increased as early as at passage 1) rose 3 to 6 times at passage 3 of the infected material compared with the controls. It is important that no contaminating virus was detected in the sera samples over 3 serial passages in human embryo kidney cells as well as the material from passage 15 in lymphocytes.

Thus, the obtained results indicate to the possibility of in vitro passage of the agent contained in hepatitis patients blood sera. In addition to our previous data [1], the agent's cytopathic activity was manifested in inhibition of the mitotic activity and blast transformation of lymphocytes as well as chromosomal alterations. The viral nature of the agent was confirmed by the results of a cytogenetic study of leukocyte cultures from viral hepatitis patients and convalescents. Also, the cytoplasm of lymphocytes in infected cultures showed a specific fluorescence in the immunofluorescent assay using an immune serum against the virus. The electron microscopic (EM) study detected peculiar viral particles in leukocyte cultures from patients (Fig. 2, 3). Similar particles were detected in blood sera from patients at the early stage of hepatitis [1, 5].

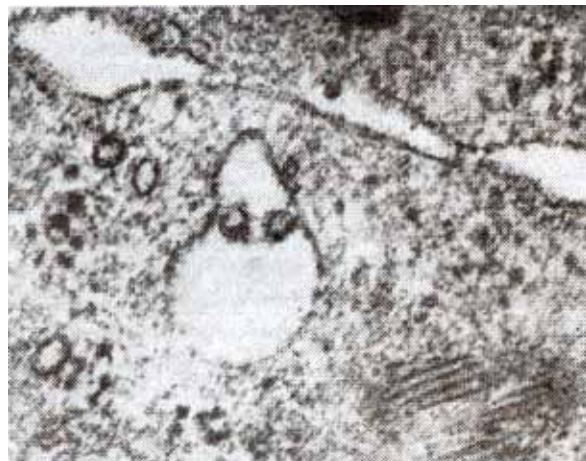
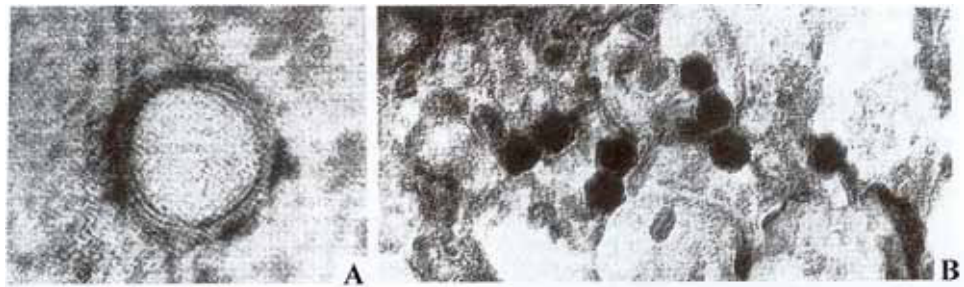


Fig. 2. Slice of a leukocyte culture from hepatitis C patient. Vacuoles contain virus-like particles. X 200,000

A series studies on the virus was carried out. Currently we have several isolates of the virus designated as 'leukocytic hepatitis virus (LHV)' obtained from the patients sera over the first days of the disease [1, 3, 4, 5].

Virus-like particles measuring ca. 50–55 nm were detected in slices of infected cultured leukocytes examined by EM, and in the peak fractions of 3H-uridine labeled cesium chloride gradient. The inner component of those particles represented electron

Fig. 3. Leukocytic hepatitis virus. Negative contrasting. A – Blood serum of a viral hepatitis patient, B – Multiple particles in a PHA-stimulated leukocyte culture, passage level 12. X 400,000.



dense nucleoid covered with a bilayer envelop 4 to 5 nm thick. Similar particles were found by negative contrast staining of sera from hepatitis patients purified from proteins on sefadex column [3]. LHV is non-pathogenic for small laboratory animals, green african monkeys and lacks hemagglutinating properties with erythrocytes from chickens, sheep, geese, mice, dogs, monkeys and humans. LHV has no shared antigenic determinants with HBVs antigen. The specificity of LHV was proved in a survey of 500 paired sera in complement fixation test, leukocytes migration delay and cutaneous assay. Cutaneous assay using formalin-inactivated LHV was clinically applied and proved positive in 15% of patients with hepatitis in the acute stage and in 75% of individuals with active chronic hepatitis [1, 5]. In the assays when the control antigen was applied (non-infected cultured leukocytes), negative results were obtained. The buoyant density of the two studied LHV strains is 1.26 g/ml (Fig. 4). Replication of LHV in human embryo kidney cell cultures in the presence of 3H-uridine resulted in accumulation of structures with the indicated density. Extraction of RNA from virions from the peak fractions of cesium chloride density gradient and subsequent research of it in sucrose gradient consistently isolated the higher-weight RNA with a sedimentation constant of 40-50S [2, 3, 5]. EM studies with negative contrasting of the virus and LHV-infected cultured leukocytes as described previously [4], revealed numerous spheric and oval particles 50 to 55 nm in size (Fig. 3). The inner component, electron dense nucleoid, is covered with a 4 to 5- nm-thick envelop. Non-infected leukocyte cultures did not contain such particles. Previously, we observed particles similar in size and structure in EM of slices of leukocytes from viral hepatitis patients (Fig. 4) [1].

DISCUSSION

The conducted research has shown that PHA-stimulated cultured leukocytes from healthy donors may successfully be applied for study of the etiology of parenteral viral hepatitis. EM study of negative

contrasted LHV samples as well as cultured leukocytes from hepatitis patients without hepatitis B antigenemia detected viral particles of a hexagonal shape 50 to 65 nm in size with a double envelop 5 nm thick. It allowed us to classify LHV as a Flaviviridae family member. In LHV, RNA of hepatitis C virus (1A subtype) was detected. The sedimentation constant LHV in cesium chloride density gradient (1.26 g/ml) was similar to that of hepatitis virus G particles (1.18–1.23 g/ml) [6]. However, no hepatitis virus G RNA was detected by RT-PCR in LHV [4, 7]. Instead, RNA of hepatitis C virus (1A subtype) was detected in one of the isolates of LHV after its passage in PHA-stimulated cultured leukocytes by RT-PCR [4,5]. In addition, RNA the NS non-structural protein of hepatitis C virus was regularly detected (66.7%) in leukocytes of patients with hepatitis [7, 8, 9].

Therefore, the conducted research showed the effectivity of using PHA-stimulated cultured leukocytes from healthy donors for studying etiology of parenterally transmitted viral hepatitis.

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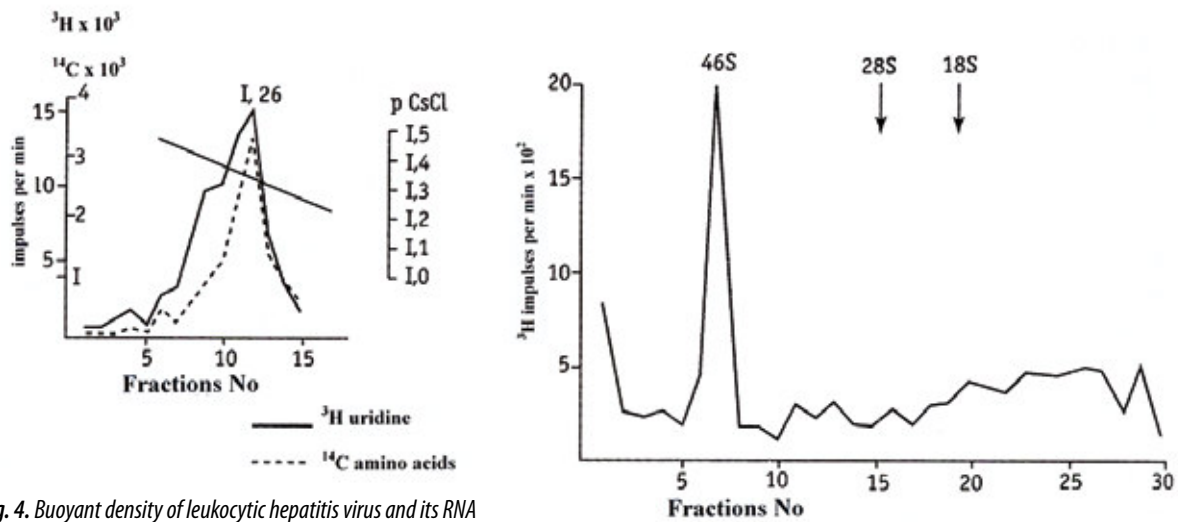


Fig. 4. Buoyant density of leukocytic hepatitis virus and its RNA

Table 1. Mitotic activity and chromosome alterations as a result of passageing of the agent from the blood sera of the viral hepatitis patients. (The serum agent was passageing in cultured leukocytes from healthy donors)

Dilution	Passage	Donor Sh.					Donor M.					
		1B		2B			1B		2B		H	
		1	2	1	2	1	1	2	1	2	1	2
Dilution	Initial stock	28	12/4	28	12/4		29		29	4.2	29	4/2
0.5·10 ⁻¹	1	16.7	25/10	13.3	—		18	12/4	20	16/8		—
0.25·10 ⁻²	2	10	24/12		20/8		20	20/20	24	14/12		—
0.125·10 ⁻³	3	15	20/8		21/12		24	32/32	13.4	28/24	28	8/0

1B and 2B: blood sera from viral hepatitis patients on days 3 and 5 of jaundice, H: blood serum from a healthy donor.

1 — mitotic activity, in per mille;

2 — Percentage of cells with chromosome alterations (the numerator is the total of cells with alterations, the denominator is the number of cells with coarse alterations)

Table 2. Delay of lymphocyte blasttransformation in leukocyte cultures infected with the material from patients with hepatitis

Dilution	Passage	Donor Sh.					Donor M.				
		1B		2B		H		1B		2B	
		48h	72h	48h	72h	48h	72h	48h	72h	48h	72h
	Initial stock	90.4	96.9	90.4	96.9	90.4	96.9	65.8	89.7	65.8	89.7
					78.3*	89.7	96.5	—	—	28.6*	68.3*
0.5·10 ⁻¹	1	55.8*	77.1	66.3*	78.3*	90.1	92.7	33.0	39.9*	40.3*	61.9*
0.25·10 ⁻²	2	63.0*	78.8*	66.0*	71.8*	97.7	93.2	30.1	49.8*	—	—
0.125·10 ⁻³	3	65.8*	78.6*	66.5*							

1B and 2B: blood sera from viral hepatitis patients on days 3 and 5 of jaundice, H: blood serum from a healthy donor.

Figures indicate the numbers of blastocells as percentage to the total cell numbers. Asterisk is a reliable difference from the control

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