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Isolation of Hemorrhagic Nephroso-nephritis Virus in Cell Cultures

By

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With 1 Figure

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Summary

Viruses in blood specimens from patients with hemorrhagic nephroso-nephritis were isolated in monolayer cultures of human embryo kidney cells and passaged in these cultures and in primary and continuous swine kidney cell cultures. The presence of viruses was demonstrated by interference with the growth of heterologous cytopathogenic viruses (poliovirus and Sindbis virus) and by direct and indirect immunofluorescence using human convalescent sera and sera from rabbits hyperimmunized with infected cell cultures as sources of antibody.

The specificity of viruses isolated was confirmed by the regular appearance in convalescent sera from patients with HNN of antibodies which inhibited the multiplication of viruses in cell culture and blocked staining by the immunofluorescence method. The viruses isolated were also neutralized and FA staining bloked by homologous immune rabbit sera.

Virus was present in greatest quantity on the 5th day after inoculation of cell cultures. When virus prepared in tissue culture was inoculated into various types of laboratory animals, including African green monkeys, no illness developed except occasionally among newborn white mice.

1. Introduction

Individual attempts to isolate and cultivate under laboratory conditions the virus of hemorrhagic nephroso-nephritis (HNN, hemorrhagic fever with renal syndrome) have not yet given reproducible results. The first indications that the agent of HNN was able to reproduce in cell cultures was given by the occasional appearance of cytopathic changes, which were not found with any regulraity (1, 2). The present report describes the cultivation and identification of the virus of HNN in tissue cultures using the techniques of interference and immunofluorescence.

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2. Materials and Methods

2.1. Materials from Patients

For isolation of viruses blood specimens were collected during the first 5 days of illness from patients with HNN living in the Primorye Territory of the Far Eastern part of the USSR. Sera and blood clots were studied separately. Thirty per cent suspensions were prepared from the clots by grinding with quartz sand in medium 199 containing 20 per cent calf serum. The suspensions were centrifuged and the supernatant fluids used for the inoculation of cell cultures. Viral reproduction in cell cultures and the susceptibility of laboratory animals, were studied.

For preparation of hyperimmune sera we used strains of virus which had been isolated from the blood of patients with HNN and had been passaged 5 to 7 times in cell cultures and identified with sera from convalscent patients and hyperimmune rabbit sera using interference and immunofluorescence methods as described below. For the initial identification of the virus sera from convalescent patients served as the source of specific gamma globulin. Later sera of hyperimmunized rabbits were also used.

Sera for serological investigations were collected one to three months after the onset of illness from convalescent patients living in foci in the Primorye Territory and also from the Bashkir Autonomous Soviet Socialist Republic in the European part of the USSR. As controls we used sera from permanent residents of Leningrad where HNN is not found.

The gamma globulin fractions of human convalescent and hyperimmune rabbit sera were prepared by salting out with ammonium sulfate and by the alcohol method of Cohn.

2.2. Cell Cultures

The following trypsinized monolayer cultures were studied:

a) Primary fibroblasts: chick embryo (CEF), white mouse (MF), human embryo (HEF), Japanese quail (QF).

b) Primary kidney cells: chick embryo (CEK), swine embryo (SEK), human embryo (HEK).

c) Continuous cell lines: swine embryo kidney (PS), HEp-2, Detroit-6, guinea pig kidney (GPK), monkey kidney (MK), rabbit kidney (RK), human kidney (HK), and Syrian hamster kidney (BHK-21).

Trypsinization of the original tissues for preparation of primary cultures was performed at room temperature for 4 to 6 hours or at 4° C for 18 to 20 hours. Cell cultures were grown on medium 199 containing 10 per cent calf serum and 0.5 per cent lactalbumin hydrolysate or 5 per cent beef blood hydrolysate. Chick embryo and Japanese quail fibroblasts were grown on Gey's medium with 5 per cent calf serum. Cells were grown for three to 5 days on 5×10 mm glass cover slips in test tubes. After the appearance of complete monolayers of cells, cultures were infected and the growth medium replaced with maintenance medium 199 containing 5 per cent calf serum.

The presence of HNN virus in the infected cell cultures was detected by the presence of a cytopathic effect (CPE), by interference with the growth of heterologous cytopathic viruses, and by direct and indirect immunofluorescence.

2.3. Interference Reaction

As indicator viruses we used poliovirus type 1 (vaccine strain LSC) and Sindbis virus in doses of 10 to 100 infectious doses per culture tube, three to seven days after inoculation with HNN virus. Results of the interference reaction were recorded one to five days after challenge.

2.4. Fluorescent Antibody (FA) Methods

2.4.1. Direct FA

An 1 per cent solution of gamma globulin prepared from HNN human convalescent serum or hyperimmune rabbit serum was conjugated at 4° C for 18 hours with fluores-

cein-isothiocyanate (FITC) in a ratio of 10 to 15 mg fluorochrome to 1 gram of protein. Conjugates were freed of unconjugated FITC by dialysis and fractionation on anionic Dowex-1 (Dow Chemical Corp.).

The protein concentration in specific purified conjugates was 1 per cent with fluorochrome to protein ratio of 8 to 9×10^{-3}. Nonspecific fluorescence was inhibited by counterstaining with equine albumin tagged with rhodamine sulfofluoride at a dilution of 1:8 to 1:16 (3). Fixed preparations were stained with conjugate mixtures at 37°C in a moist chamber, washed twice for 5 minutes in buffered physiologic saline (pH 7.2), rinsed with distilled water, dried and examined under fluorescence microscope ML-2 using lamp DRSh-250, exciter filters GS-8-2, FS-1-2, SS-15-2, and interfering filter 1 or 2.

2.4.2. Indirect FA

Fixed preparations were covered with drops of HNN convalescent serum in dilutions 1:2—1:10 and placed in a moist chamber for 30 minutes following which they were washed twice for 10 minutes in buffered physiologic saline (pH 7.2—7.4) and air dried. They were then stained with a mixture of FITC-globulin from rabbits immunized with human globulin and equine serum albumin tagged with rhodamine sulfofluoride in final concentrations of 1:6—1:8 and 1:8—1:16, respectively.

2.5. Susceptibility of Laboratory Animals to Viruses Isolated

Virus was suspended in medium 199 at an approximate concentration of 10^5 tissue-culture (HEK) infectious doses per ml. Rabbits were injected intravenously and intraperitoneally, guinea pigs intraperitoneally, newborn and adult white mice intra-

Table 1. *Fluorescent Antibody Titer of Sera Obtained from Different Geographical Areas*

HNN patient No.	Geographical focus of HNN source of sera	Serum antibody Titer by FA method			
		Direct FA		Indirect FA	
		Acute serum	Convalescent serum	Acute serum	Convalescent serum
1	Primarye Territory	0	1:4	0	1:8
2	Primarye Territory	0	1:4	0	1:8
3	Primarye Territory	0	1:3	0	1:4
4	Bashkir ASSR	0	1:4	0	1:6
5	Bashkir ASSR	0	1:2	0	1:3
Control No.	Source of control sera				
1	Leningrad residents (10 specimens)		0		0
2	Rabbits hyperimmunized with HNN virus		1:16		1:64
3	Rabbits hyperimmunized with tickborne encephalitis virus		0		0

peritoneally or intracerebrally, and newborn white mice intracerebrally or by combined intracerebral, intraperitoneal, intramuscular, intranasal and peroral routes. African green monkeys were inoculated intravenously and intracerebrally. Observations of the inoculated animals were carried out for four weeks after inoculation.

Table 2. *Demonstration of HNN Virus Reproduction in Tissue Culture of Various Methods*

Experiment No.	Type of cell culture	Designation	Virus detected by:			
			CPE	FA-method		Interference[1]
				Direct	Indirect	
	Primary cell cultures:					
1	Human Embryo Kidney	HEK	±	++	++	++
2	Human Embryo Kidney (Subculture)	HEK-Sub	±	+	+	+
3	Human Embryo Fibroblast	HEF	—	—	—	—
4	Chick Embryo Fibroblast	CEE	—	—	—	—
5	Japanese Quail Fibroblast	QF	—	—	—	—
6	Chick Embryo Kidney	CEK	—	—	—	—
7	Mouse Fibroblast	MF	—	—	—	—
8	Swine Embryo Kidney	SEK	—	++	++	+
	Continuous Cell Lines:					
9	Swine Kidney	PS	—	+	+	+
10	Human Amnion	AM	—	—	—	—
11	Human Kidney	RH	—	—	—	—
12	HEp-2	HEP-2	—	—	—	—
13	Monkey Kidney	MK	—	+	+	+
14	Detroit-6	D-6	—	—	—	—
15	Guinea Pig Kidney	GAK	—	—	—	—
16	Rabbit Kidney	RK	±	—	—	—
17	Mouse Fibroblast	L	—	—	—	—
18	Syrian Hamster Kidney	BHK-21	—	+	+	+

[1] with Sindbis or polioviruses.
— = Negative. ± = Weakly Positive. +, ++ = Positive.

Table 3. *Detection of HNN Virus (Strain FE-1) in HEK*

Virus dilutions	CPE[1] after challenge with poliovirus type 1/LSC 5 days post infection						
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Cultures infected with HNN virus	+++	+++	++	+	—	—	—
Non-infected cultures	++++	++++	+++	+++	+++	++	—

[1] Criteria for +, ++, +++, ++++ (+ to ++++ = % cell sheet destroyed from 25% to 100%).

3. Results

Virus from patients with HNN was first isolated in trypsinized HEK cell monolayer cultures. It was isolated more frequently from blood serum than from clots. In a typical study of 15 patients from an outbreak of HNN in the Primorye Territory virus was isolated in six cases from blood serum and in only two cases from clot.

Sera obtained from patients during the acute phase of HNN (before the 5th day) and during the period of convalescence were studied by both indirect and direct FA methods. Antibodies were consistently demonstrated in convalescent but not in acute sera suggesting that the viruses isolated in tissue culture are the specific agents of HNN. When sera of convalescent HNN patients from different geographic foci were examined they were found to give distinct immunofluorescence with the same virus, further suggesting that there is a common agent or group of related agents of HNN in both Far East and Europe. A comparative study of sera from the Primorye Territory and Bashkir ASSR is presented in Table 1. Additional evidence that the viruses isolated are identical or closely related is given by the fact that sera from rabbits hyperimmunized with one virus gave specific immunofluorescence with all others tested.

In trials of 20 different tissue cultures (Table 2) it was found that the growth of HNN virus was most regularly maintained by passages in trypsinized monolayer cultures of primary human embryo kidney and swine embryo kidney cells and in a continuous line of swine embrye kidney cells (PS). Several of the cultures investigated showed weak cytopathic effects which increased after multiple serial passages of virus-containing material.

The presence of virus in the cultures was demonstrated and dynamics of viral multiplication studied by the FA and interference reactions (Table 3). With the FA technique specific fluorescence was first detected in cell cultures 12 hours after infection, and maximum fluorescence developed in four to five days. By the eight to ninth day of cultivation the immunofluorescence had either disappeared or was weakly expressed. Direct and indirect FA methods gave similar results, and all control preparations were negative. The viral antigens were noted to be localized in the cytoplasm (Fig. 1).

Cell Cultures by Interference or Immunofluorescence

Immunofluorescence[2] with HNN convalescent serum dilutions										
Direct FA						Indirect FA				
	1:2	1:4	1:6	1:8	1:10	1:2	1:4	1:8	1:16	1:32
Cultures infected with HNN virus	+	+	±	−	−	+	+	+	−	−
Non-infected cultures	−	−	−	−	−	−	−	−	−	−

[2] Performed 5th day post infection.

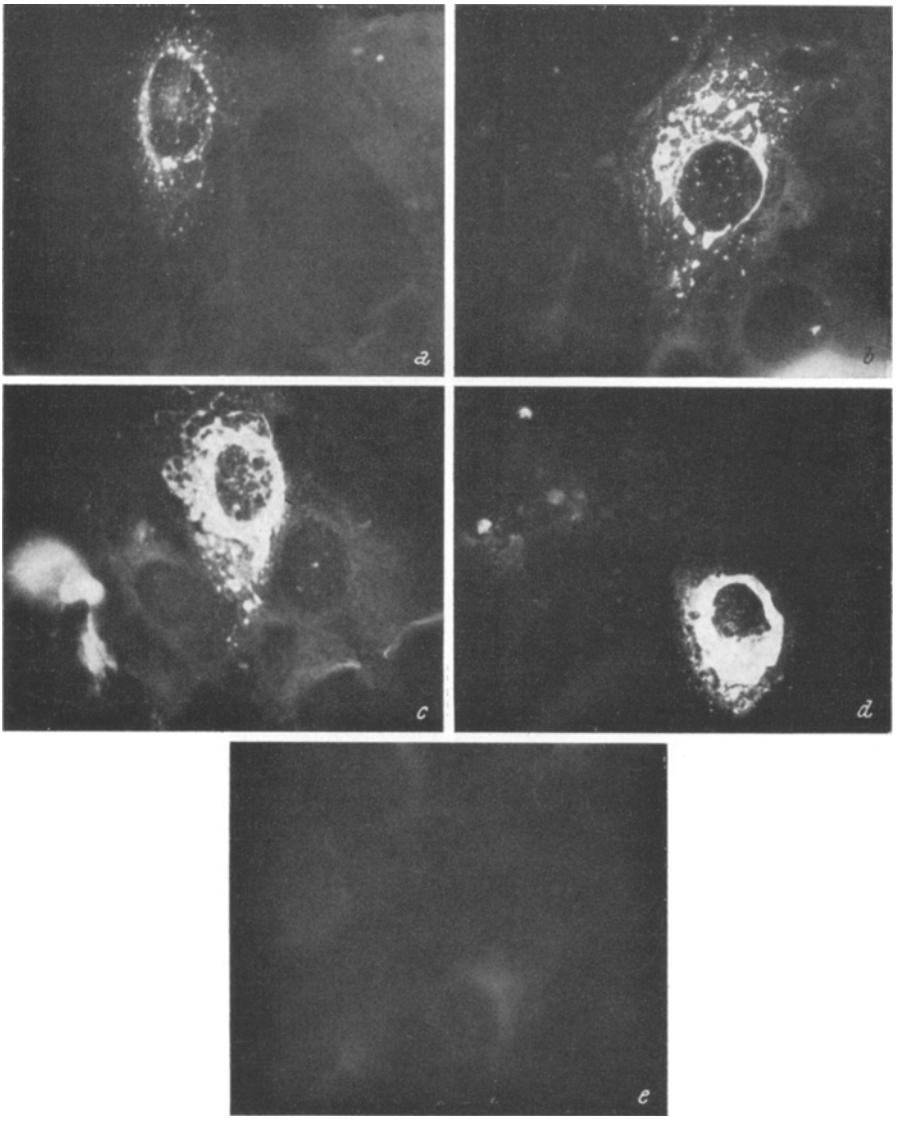

Fig. 1. Monolayers from embryonic human kidney infected by the virus of hemorrhagic nephrose-nephritis and stained by indirect fluorescent antibody method: a) Infected 12 hours before; b) Infected 24 hours before; c) Infected 72 hours before; d) Infected 120 hours before; e) Control — non infected tissue culture

The interference phenomenon was only weakly expressed until 3 days post infection, becoming quite distinct on the 5th to 7th day (Table 5). Optimal dose for the indicator cytopathogenic viruses was 10—1000 $TCID_{50}$ per culture.

When virus-containing material was incubated with convalescent human serum prior to inoculation of cell cultures, viral multiplication, using both inter-

ference phenomenon and FA techniques as indicators, failed to take place, demonstrating specific neutralization of virus.

As seen from Table 6, with the occasional exception of newborn white mice the laboratory animals tested did not show signs of clinical illness following inoculation

Table 4. *Dynamics of Multiplication of HNN Virus in HEK Cultures as Demonstrated by Immunofluorescence*

Time *p.i.* (days)	Presence of specific fluorescence (indirect method) in cultures infected with indicated dilutions of virus-containing material							
	1:1	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
1	+	+	−	−	−	−	−	−
2	+	+	+	±	−	−	−	−
3	+	+	+	+	±	−	−	−
4	+	+	+	+	±	−	−	−
5	+	+	+	+	+	−	−	−
6	+	+	+	+	+	−	−	−
7	+	+	+	+	+	−	−	−
8	±	±	+	+	+	−	−	−
10[1]	−	−	±	±	±	−	−	−
12[1]	−	−	−	−	−	−	−	−

[1] Nonspecific Degeneration of Cells.

Table 5. *Dynamics of Multiplication of HNN Virus in HEK Cultures as Demonstrated by Interference with Polio 1/LSC Virus*

Interval (days) between initial HNN infection and challenge with poliovirus 1/LSC (10 $TCID_{50}$)	CPE 72 hours after challenge No. cultures showing CPE/ No. cultures challenged
2	3/4
3	2/4
4	1/4
5	0/4
6	0/4
7	0/4
8	0/4
9	1/4

Each day 4 HNN-infected cultures and 4 uninfected control cultures were challenged with poliovirus.

with HNN virus. However among some of the animals inoculated the appearance of specific antibody was observed, titers rising after repeated inoculations.

The viruses isolated were stable when stored at −20°C for at least 6 months and at +4°C for at least 2 months.

Table 6. *Susceptibility of Laboratory Animals to HNN Virus (Adapted to HEK Cultures)*

Species of animal		Inoculation		Clinical signs[1]	Antibody formation[3]
		Route	Virus dose (10^5 $TCID_{50}$/ml) No. ml injected		
Rabbit		*i.v.*	5.0	—	+
		i.p.	5.0	—	+
Guinea pig		*i.p.*	2.0	—	n.d.
White Rat		*i.p.*	3.0	—	n.d.
White	Adult	*i.p.*	1.0	—	n.d.
Mouse	Newborn	*i.c.*	0.02	+[2]	n.d.
		Combined	0.1	+[2]	n.d.
African Green		*i.v.*	5.0	—	+
Monkey		*i.c.*	1.0	—	+
Cat		*i.p.*	5.0	—	n.d.

[1] Animals observed 4 weeks.
[2] Death of individual animals 10—12 days post infection, following a 24—48 hours period of lethargy and weakness.
[3] After repeated inoculations.
+ = Positive. — = Negative. n.d. = Not done.

References

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