

CHRONIC INFECTION OF HeLa CELLS WITH JAPANESE ENCEPHALITIS VIRUS. GENERAL CHARACTERISTICS OF THE SYSTEM

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Summary. — Chronic infection of HeLa cells was induced by an attenuated variant of Japanese encephalitis (JE) virus (HeLa-K3 cell line). The chronic infection was characterized by alternating phases of degeneration and recovery of the cell monolayer. JE virus was regularly released into the medium of chronically infected cell cultures and virus-specific antigen was regularly demonstrated in the cytoplasm of 15—25% of cells. JE virus persisting in HeLa-K3 cells was sensitive to pancreatic ribonuclease and resistant to treatment with 4 M urea. HeLa-K3 cells did not undergo cytological or karyological transformation; they were susceptible to superinfection with heterologous viruses but resistant to reinfection with homologous virus.

Key words: Japanese encephalitis virus; HeLa cells; persistent infection

Introduction

Previously we induced persistent infection with Japanese encephalitis virus (JE; genus *Flavivirus*) (K₄₃ and K₃₃) in two cell systems of different origin: primary cultures of suckling mouse brain cells (GMS-K₃₃) and Vero cells (Vero-K₄₃). We found differences in the course of the infection and attempted to elucidate the basic mechanisms responsible for the persistence of virus in these systems. In the course of infection we isolated JE virus variants considerably differing from the original clones (further decrease in pathogenicity, prevailing ability to reproduce at suboptimal temperatures, appearance of the ability to cause a cytopathic effect in chick embryo cell cultures) (Gavrilov *et al.*, 1974a; Deryabin *et al.*, 1974).

The aim of the present work was to characterize infection of HeLa cells induced by a JE-K₃₃ virus variant. We studied the course of infection, the properties of cells in the course of virus persistence and some properties of the persisting virus.

Materials and Methods

Virus. JE virus clone 3, previously isolated from the chronically infected GMS-K₃₃ cells at their 108th passage level (Gavrilov *et al.*, 1974a), were used. JE virus clone 3 showed a lowered pathogenicity for suckling mice (causing infection in 35–40 % of intracerebrally inoculated animals), was apathogenic for adult mice by any route of inoculation and reproduced in SPEV cell cultures to titres of $10^{6.5}$ TCD₅₀/ml. A distinctive property of this clone was its ability to destroy chick embryo fibroblasts.

For superinfection of HeLa-K3 cells we used as homologous virus the K40 variant of JE virus obtained by cloning from the Peking-1 strain (possessing a high peripheral and cerebral activity for white mice). As heterologous viruses we used a flavivirus (tick-borne encephalitis virus strain Pan) and an alphavirus (Semliki-Zaisan strain 260). Standardized inocula (100 TCD₅₀/ml) were used in all experiments on superinfection. Resistance of HeLa-K3 cells to superinfection with isologous and homologous viruses was evaluated by resistance to the cytopathic effect (CPE) of these viruses.

Cell cultures. The HeLa cell line used was obtained in November 1976 from the tissue culture laboratory of our Institute (head A. S. Novokhatsky). According to this laboratory, the cell line was free of fungi, bacteria and mycoplasmas, but contained type A and D oncoviruses. For titration of JE virus we used the continuous pig embryo kidney cell line SPEV, susceptible to flavivirus reproduction, which had been derived in the Institute of Poliomyelitis and Viral Encephalitis, U.S.S.R. Academy of Medical Sciences. JE virus causes an intensive CPE in these cells on the 4th–5th day, reaching maximal titres of 6–7 log TCD₅₀/ml or PFU/ml.

Establishment of chronic infection. HeLa cells were infected with JE virus clone 3 in November, 1976. The cell monolayer was inoculated once at a multiplicity of infection of 1 TCD₅₀ per cell. After adsorption for 1 hr at room temperature the non-adsorbed virus was removed and the cultures were incubated further at 37 °C. The cells were grown in medium 199 supplemented with 10 % bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin. Subcultures were done after the monolayer became confluent. The medium was changed every other day or in the stage of marked degeneration every 2–3 days. Virus in the culture fluid and cell homogenate obtained by freezing and thawing was titrated periodically in SPEV cells. Specific antigen in HeLa-K3 cells was detected by indirect immunofluorescence with the use of immune ascitic fluid to JE virus obtained by immunization of white mice, anti-mouse rabbit serum labelled with fluorescein isothiocyanate and bovine albumin labelled with rhodamine sulphofluoride in a ratio of 1:1. Sera to tick-borne encephalitis and lymphocytic choriomeningitis virus served as controls.

Cytomorphological studies. Cell cultures grown on coverslips in penicillin vials were fixed in Bouin's solution after 1, 2, 3 and 4 days of growth and stained with haematoxylin and eosin. Mitotic activity was assayed by examination of 3000 cells in each sample and the forms of pathological mitoses were evaluated according to Blyumkin and Monastyreva (1971). The results were evaluated statistically by the methods of Fisher and Student. For cytogenetic investigations, cell suspensions were fixed according to Moorhead *et al.* (1960) and stained with azur-eosin according to Romanowski.

Isoenzyme analysis. To exclude the possibility of cell contamination of the HeLa-K3 line in the course of long-term passaging, we analysed the pattern of glucose-6-phosphate dehydrogenase (G6PDH) and lactate dehydrogenase (LDH) in uninfected and chronically infected HeLa cells by electrophoresis in vertical slabs of 7 % polyacrylamide gels and subsequent staining of the fractions by histochemical methods (Tsareva *et al.*, 1976).

Properties of the persisting virus. Media from chronically infected HeLa-K3 cell cultures were treated with 4 M urea (Sigma, U.S.A.) for 20 min at 37 °C (Nishimura *et al.*, 1968) or 100 µg/ml pancreatic ribonuclease (Reanal, Hungary; 40 Kunitz units/mg) for 20 min at room temperature. Untreated medium from HeLa-K3 cultures served as control (Gavrilov *et al.*, 1974b).

Results

Establishment of chronic infection

With the K3 variant of JE virus we established a chronic infection of HeLa cells, the HeLa-K3 cell line. In the course of the observation period of 205 days we carried out 38 subcultures (Fig. 1).

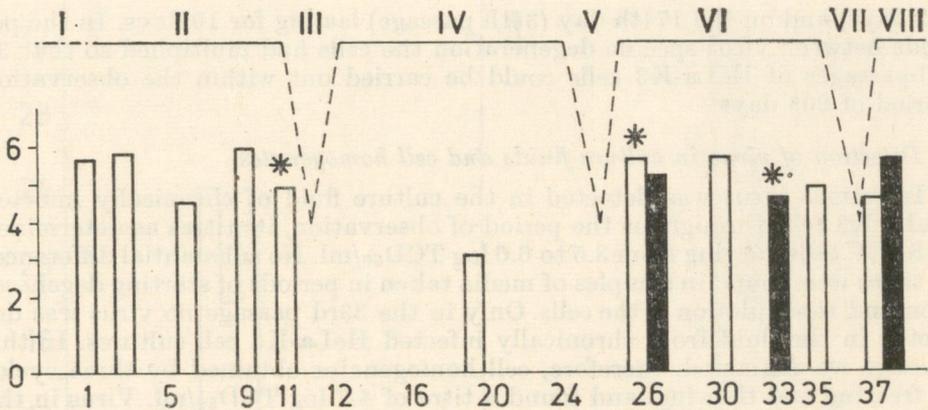


Fig. 1.

Characteristics of HeLa cell cultures chronically infected with JE virus

I—VIII: phases of cell growth stabilization; - - - periods with signs of specific cell degeneration
 Abscissa: passage number of HeLa-K3 cells in the course of 205 days (period of observation).
 Ordinate: virus titres in log TCD₅₀/ml values assayed in SPEV cells; empty columns: virus in cell homogenate; black columns: virus in culture fluid.
 Asterisks: specific fluorescence of viral antigen in HeLa-K3 cells at the indicated passage levels.

Within the first 20 days after onset of the experiment, the HeLa-K3 system showed an intensive cell degeneration. An equilibrium between virus and cell could only be achieved by frequent changes of medium which on the 20th day led to recovery of the cell monolayer making possible the first passage of HeLa-K3 cells. Since then marked cell degeneration was no more observed and HeLa-K3 cells were subcultured every 4—5 days up to the 6th passage in a 1:1 ratio and then for 50 days in a 1:2 ratio. In the 11th passage there appeared strong cell degeneration which persisted for 14 days. During this period the cultures could be maintained by regular changes of medium. On the 75th day, cell proliferation was renewed. It continued for 63 days, during which 13 subcultures were carried out. Cell degeneration reappeared in the system on the 122nd day of observation (24th passage) for a short time

Table 1. Susceptibility of HeLa-K3 cells to superinfection

Superinfecting virus	Intensity and time of appearance of a CPE HeLa-K3 cells	normal HeLa cells
JE virus, K3	0	++++, day 4
JE virus, K40	++, day 5	++++, day 4
Tick-borne encephalitis virus, Pan	++, day 4	++, day 4
Semliki-Zaisan, 260	++++, day 4	++++, day 4

++++: marked CPE; ++: partial degeneration of the monolayer; 0: absence of a CPE on day 6.

(12 days) and on the 174th day (35th passage) lasting for 10 days. In the periods between virus-specific degeneration the cells had multiplied so that 38 subpassages of HeLa-K3 cells could be carried out within the observation period of 205 days.

Detection of virus in culture fluids and cell homogenates

Infectious virus was detected in the culture fluid of chronically infected HeLa-K3 cells throughout the period of observation, its titres as determined in SPEV cells varying from 3.5 to 6.0 log TCD₅₀/ml. No substantial differences in titres were found in samples of media taken in periods of starting degeneration and repopulation of the cells. Only in the 33rd passage no virus was detected in the fluid from chronically infected HeLa-K3 cell cultures. In this passage we examined, therefore, cell homogenates obtained by three cycles of freezing and thawing, and found a titre of 4.7 log TCD₅₀/ml. Virus in the culture fluid was again detected on the 187th day (35th passage) in a titre of 5.0 log TCD₅₀/ml and on the 192nd day in a titre of 4.7 log TCD₅₀/ml. The virus titres in the cell homogenates at these intervals were higher, namely 5.5 log TCD₅₀/ml (Fig. 1).

Detection of virus-specific antigen in HeLa-K3 cells

Virus-specific antigen was demonstrated in 15–25% of HeLa-K3 cells at the levels of the 11th, 25th and 33rd subpassage (Fig. 1). Fluorescence was typical of JE virus and was localized in the perinuclear zone of the cytoplasm. The intensity of fluorescence in passages 11 and 33 was higher than in cells renewing their growth after the stage of degeneration (25th passage).

Susceptibility of the HeLa-K3 cell line to superinfection

HeLa-K3 cells proved to be highly resistant to superinfection with isologous virus (K3 of JE virus, used for establishing the chronic infection), relatively resistant to superinfection with homologous virus (K40 of JE virus) and susceptible to heterologous tick-borne encephalitis and Semliki forest viruses. In control HeLa cells, all these viruses produced a specific CPE after 4–5 days (Table 1). This CPE was somewhat less intensive in relation to uninfected and chronically infected cells after infection with the Pan strain of tick-borne encephalitis virus.

Cytomorphological and cytogenetic investigations on HeLa-K3 cells

The morphology of chronically infected HeLa-K3 cell cultures did not practically differ from that of control cultures. They consisted of epithelial-like cells with centrally located nuclei containing 1–5 large nucleoli (Fig. 2). The differences between the two cultures in mitotic activity and the number of pathological mitoses were insignificant (Fig. 3). The mitotic activity in chronically infected cultures was somewhat lower than in the controls. The highest numbers of dividing cells in either variant were observed in 72 hr old cultures. The following forms of pathological mitoses prevailed: staying behind of single chromosomes during the metaphase, 3-polar metaphases and

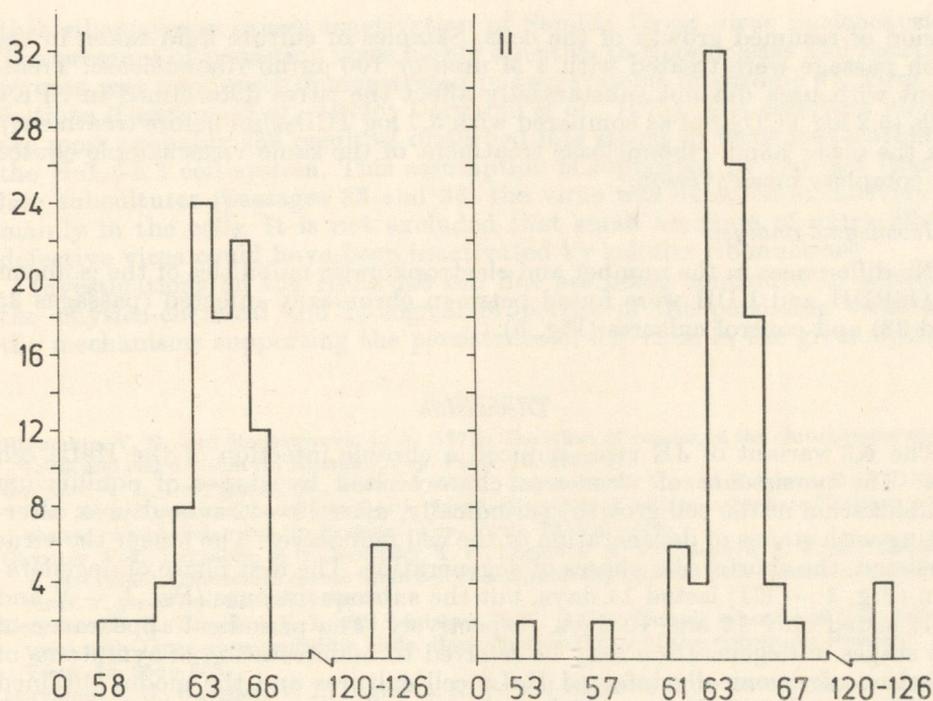


Fig. 4.

Karyological analysis of normal (I) and chronically infected (II) HeLa cells
Abscissa: No. of chromosomes; ordinate: % of cells

bridges in the anaphase. Colchicine-like metaphases, 4-polar anaphases and staying back of single chromosomes in the anaphase were infrequent.

Cytogenetic analysis also revealed no substantial differences between control and chronically infected cultures. The modal class in both cases represented cells with 63 chromosomes (Fig. 4) with variations from 53 to 67 chromosomes in the control and from 58 to 66 chromosomes in HeLa-K3 cells. The proportion of near-tetraploid cells (with 120–126 chromosomes) was 4% in control and 6% in HeLa-K3 cells. The same marker chromosome was found in both variants: a long submetacentric chromosome. But as distinct from control cultures, 2% of metaphases in HeLa-K3 cell cultures contained markedly changed chromosomes: shortened, distorted and partially broken into chromatids.

Sensitivity of the persisting virus to urea and ribonuclease

Because the titres of virus in culture fluids in the stages of degeneration and recovery of chronically infected cultures did not differ substantially from each other, we investigated some properties of the persisting virus in the

period of resumed growth of the cells. Samples of culture fluid taken in the 25th passage were treated with 4 M urea or 100 $\mu\text{g/ml}$ ribonuclease. Treatment with urea did not substantially affect the titres determined in SPEV cells (5.2 log TCD₅₀/ml as compared with 5.7 log TCD₅₀/ml before treatment). On the other hand, ribonuclease treatment of the same virus sample caused its complete inactivation.

Isoenzyme analysis

No differences in the number and electrophoretic mobilities of the isoforms of G6PDH and LDH were found between chronically infected (passages 35 and 38) and control cultures (Fig. 5).

Discussion

The K3 variant of JE virus induced a chronic infection of the HeLa cell line. The persistence of virus was characterized by stages of equilibrium (stabilization of the cell growth) periodically, after 11–12 subcultures, alternating with stages of degeneration of the cell monolayer. The longer the virus persisted, the shorter the phases of degeneration. The first phase of degeneration (Fig. 1 – III) lasted 14 days, but the subsequent ones (Fig. 1 – V and VII) lasted only 12 and 10 days, respectively. The periodical appearance of the stages of degeneration may be referred to manifestation of symptoms of infection of chronically infected HeLa cell cultures and the model obtained can thus be classified as a model of chronic infection of HeLa cells with JE virus. In previous model systems Vero-K₄₃ and GMS-K₃₃ (Gavrilov *et al.*, 1974a; Deryabin *et al.*, 1974) persistence of JE virus was manifested as a chronic (GMS-K₃₃) and persistent (Vero-K₄₃) infection. All three variants of JE virus used possessed characteristics of attenuation. JE virus variants with lowered pathogenicity may thus induce in different cell systems chronic or persistent types of infection. As distinct from GMS-K₃₃ cells, in which JE virus exerted a transforming effect (Gavrilov *et al.*, 1974a), we observed no cell transformation on persistence of the K3 variant of the same virus in HeLa cells. The absence of signs of transformation in HeLa cell cultures under conditions of chronic JE virus infection was most probably due to the resistance of cells of one of the oldest continuous lines.

According to the classification of Walker (1966), the present type of virus – cell interaction may be referred to autoregulatory infections (type IV), because for maintaining the equilibrium the addition of antisera was unnecessary and interferon production by the cells was not demonstrated.

Cells of the HeLa-K3 system produced infectious virus for the whole period of observation. As compared with the original virus, the persisting virus displayed different properties as manifested by its high sensitivity to pancreatic ribonuclease and resistance to the inactivating effect of 4 M urea. It can be assumed, therefore, that the population of persisting virus contained particles in the form of viral ribonucleoprotein or that the virions had a substantial defect in their outer envelope. Kaariainen and Soderlund (1971) found

that ribonuclease causes inactivation of Semliki forest virus nucleocapsids. The presence of persisting virus highly sensitive to ribonuclease and resistant to urea was previously demonstrated in two systems of chronic JE virus infections (Gavrilov *et al.*, 1974b; Deryabin *et al.*, 1975). The defective virions are most probably the result of disturbed late stages of virus maturation in the HeLa-K3 cell system. This assumption is supported by the fact that in late subcultures (passages 33 and 35) the virus was detected exclusively or mainly in the cells. It is not excluded that small amounts of extracellular defective virus could have been inactivated by cellular ribonuclease.

Investigations on the HeLa-K3 cell line are being continued to elucidate the physico-chemical and biological properties of the persisting virus and the mechanisms supporting the persistence of JE virus in the given system.

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Explanation of Figures (Plates XXXII-XXXIV):

Fig. 2. Morphology of normal (I) and chronically infected (II) HeLa cells.

Fig. 3. Mitoses in normal (I) and chronically infected (II) HeLa cells.

Fig. 5. Isoenzyme patterns of HeLa cells - I: uninfected, II: HeLa-K3

Left: glucose-6-phosphate dehydrogenase, right: lactate dehydrogenase.