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Citation	Biken journal : journal of Research Institute for Microbial Diseases. 1974, 17(2), p. 51-58
Version Type	VoR
URL	https://doi.org/10.18910/82673
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BIKEN JOURNAL Vol. 17, 51-58, 1974

A NEW METHOD FOR PROPAGATION OF HVJ (SENDAI VIRUS) *IN VITRO*: HIGH SENSITIVITY AND PRODUCTIVITY IN SPREADING CULTURES OF FRAGMENTED CHORIOALLANTOIC MEMBRANE OF CHICK EMBRYOS

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(Received January 31, 1974)

SUMMARY A high titer of HVJ was obtained *in vitro* by infecting HVJ at low multiplicity to minced fragments of chorioallantoic membrane (CAM). The HVJ produced on incubation of this system manifested all the known biological activities of HVJ from *in ovo* systems, i.e., hemagglutinating, hemolytic, fusion and neuraminidase activities and infectivity.

The system was characterized and standardized with a view to its potential use for various experimental purposes.

INTRODUCTION

HVJ is routinely prepared by infection and propagation of stock virus in the chorioallantoic membrane(CAM) of chick embryos. The sensitivity of this system to HVJ is very high, so that much HVJ can be produced on infection at low multiplicity. Moreover, the HVJ produced in this system manifests all of the known characteristics of HVJ, i.e., hemagglutinating, hemolytic, neuraminidase and fusion activities and infectivity to various permissive cells.

However, this system has several disadvantages. Namely: (1) Production varies in different eggs. (2) The conditions for HVJ growth cannot be altered because the virus is grown in quite limited conditions, in chorioallantoic fluid of living embryos. (3) Handling of the infected eggs is troublesome and requires much space.

Thus, establishment of a more suitable *in vitro* system for propagation of HVJ would be very useful. However, *in vitro* systems have seldomly been employed for growing HVJ, because the virus does not grow well in primary cultures of chick embryo fibroblasts or many types of cultured mammalian cell lines (Darlington et al., 1970), and further the HVJ particles produced in such less-productive systems frequently manifest reduced or defective nature in the characteristic biological activities of HVJ, such as hemolytic and fusion activities and infectivity (Ishida and Homma, 1960, 1961; Matsumoto and Maeno, 1962; Hosaka, 1962).

HVJ is usually propagated on CAM, but no *in vitro* system using CAM has yet been developed, although this has been achieved with

influenza virus (Barry, 1961, 1965) and Newcastle disease virus (Tamm, 1973).

This paper describes an *in vitro* system for production of a high titer of virus with all the known biological activities of HVJ. This is achieved by infecting minced fragments of isolated CAM with HVJ at low moi (multiplicity of infection). Since this system seems potentially useful for various experimental purposes, its characterization and standardization are also described.

MATERIALS AND METHODS

1. *Virus*

The Z-strain of HVJ adapted to eggs was used. A dose of 0.2 ml of 10^{-3} dilution of stock virus (infected chorioallantoic fluid) was injected into the chorioallantoic cavity of 10 day old embryonated chicken eggs, stage 36, (Hamburger and Hamilton, 1951). After incubation for 72 hr at 35 C, the chorioallantoic fluids (CAF) were harvested, pooled and used as a fresh sample of HVJ.

2. *Hemagglutination(HA) titration*

Salk's pattern method (Salk, 1944) was employed. Aliquots of 0.5 ml of two-fold serial dilutions of samples in SSC (0.15 M NaCl, 0.015 M sodium citrate) were mixed with 0.5 ml of 0.5%(v/v) of fowl red blood cells in SSC. The HA titer (HAU/0.5 ml) was expressed as the reciprocal of the lowest dilution which agglutinated the red cells in 60 min in room temperature.

3. *Assay of hemolytic activity*

One ml of HVJ, partially purified by differential centrifugation (20,000 rev/min), was mixed with 2 ml of 2%(v/v) of fowl red blood cells in SSC. The mixtures were incubated at 37 C for 60 min and then centrifuged at 1,500 rev/min for 5 min. The hemoglobin liberated into the supernatant was measured from the absorbance at 490 nm.

4. *Assay of fusion activity*

Ehrlich's ascites tumor cells were washed twice with SSC and once with BSS (balanced salt solution), and suspended at a concentration of 10%(v/v) in BSS with 2 mM of $CaCl_2$ (Okada and Murayama, 1966). Then 0.5 ml of the cell suspension was mixed with 0.5 ml of HVJ sample at 0 C for 10 min and then shaken at 37 C for 30 min (Okada and Murayama, 1966). Cell fusion was estimated microscopically.

5. *Assay of neuraminidase activity*

A mixture of 0.25 ml of HVJ sample and 0.25 ml of fetuin (10 mg/ml) as substrate was incubated at 30 C for 60 min. The *N*-acetyl-neuraminic acid liberated was assayed with 2-thiobarbituric acid (Aminoff, 1959).

6. *Infectivity assay*

Aliquots of 0.2 ml of 10-fold serial dilutions of samples in PBS (Dulbecco's phosphate buffered saline) were injected into the chorioallantoic cavity of five 10-day old embryonated eggs. After 3 days of incubation, the chorioallantoic fluids were harvested and assayed for hemagglutinin production. The infectivity of HVJ was expressed as 50% egg infective dose (EID_{50}) and the ID_{50}: HA ratio (I/A ratio) was calculated.

7. *Test of neutralization with anti-HVJ antiserum*

Aliquots of 0.5 ml of 4-fold dilutions of rabbit anti-HVJ antiserum (supplied by Dr. J. Kim in our laboratory) with PBS were mixed with 0.5 ml of HVJ sample (10^5 EID_{50}/ml). After incubation for 2 hr at room temperature, 0.1 ml of each mixture was put into a 30 mm culture dish immediately after inoculation of minced fragments (see PROCEDURES). HA titration was carried out 4 days later.

PROCEDURES

1. *Preparation of minced fragments of CAM*

CAM of 8–10 days embryos (stage 34–36) was harvested. After washing with PBS, the CAMs from 5 eggs were very thoroughly minced with sharp scissors (at least 500 times) in the corner of a petri dish. The minced fragments were washed repeatedly with PBS to remove liberated red blood cells and centrifuged at 1,000 rev/min for 5 min. The packed fregments were suspended in 30 volumes of culture medium, consisting of Eagle's MEM supplemented with 10% fetal bovine serum (Microbiological Associates Inc., U.S.A.), and 5 ml volumes of the suspension were put into 50 mm Falcon plastic culture

dishes (or 2 ml into 30 mm dishes) and incubated in a CO_2-incubator at 37 C.

2. *HVJ-infection of the system*

The following three ways of infection were found suitables.

1) *In ovo* infection

Samples of 0.2 ml of HVJ appropriately diluted with PBS were injected into the chorioallantoic cavity of 10-day chick embryos. After incubation for 2 hr, CAM was harvested from the eggs and minced as described above.

2) Infection of minced fragments immediately before cultivation

Appropriately diluted HVJ was added to a suspension of minced fragments of CAM to give the desired multiplicity of infection of virus. After incubation for one hr at 37 C, the infected fragments were washed by centrifugation, resuspended in fresh culture medium and inoculated into culture dishes.

3) Infection immediately after inoculation of minced fragments

Samples of 0.1 ml of HVJ were dropped into 30 ml culture dishes (or 0.2 ml samples into 50 mm dishes), just before cultivation.

After incubation of the infected cultures for desired periods, the culture fluids were harvested for use in various experiments.

RESULTS

1. *In vitro production of HVJ from spreading cultures of minced fragments of CAM, infected with HVJ before and after cultivation*

When minced fragments of CAM were cultured, the cells spread out from the fragments over the plastic dishes 8 to 24 hr after inoculation. Subsequently marked radial spreading of epithelial components from the free edges of the fragments was observed (Fig. 1a-c).

Production of HVJ from the cultured fragments after infection at various times, is shown in Table 1. On infection with HVJ, either before inoculation or immediately after inoculation, the culture fluid contained 2,048 HAU/0.5 ml after incubation for 3 days *in vitro*. This high yield of HVJ is similar to the maximal yield in the *in ovo* system.

Cytopathic effects(CPE) were observed in the spreading epithelial sheet (Fig. 1d), but it was noted that some epithelial sheets did not exhibit noticable CPE(Fig. 1e), even at the time of maximal production of HVJ in the culture fluid. The fibroblastic cells, occasionally spread out together with the epithelial

TABLE 1. *In vitro production of HVJ from the fragmented CAM system*

Dilution of CAF[a]	Infection[b]			Titer of HVJ in culture fluids (HAU/0.5 ml)	
				24 hr	72 hr
10^{-1}	Before cultivation	*in ovo*	1)[b]	128	2,048
		in vitro	2)	128	2,048
		in vitro	3)	128	2,048
	After 3 days cultivation[c]			16	256
10^{-5}	Before cultivation	*in ovo*	1)	(−)	2,048
		in vitro	2)	(−)	2,048
		in vitro	3)	(−)	2,048
	After 3 days cultivation[c]			(−)	32

[a] CAF (infected chorioallantoic fluids), containing ca. 10^{10} EID_{50}/ml of HVJ.

[b] For details of methods for infection and meanings of 1), 2) and 3), see PROCEDURES.

[c] After 3 days cultivation, 0.2 ml of HVJ sample was dropped into 50 mm dish.

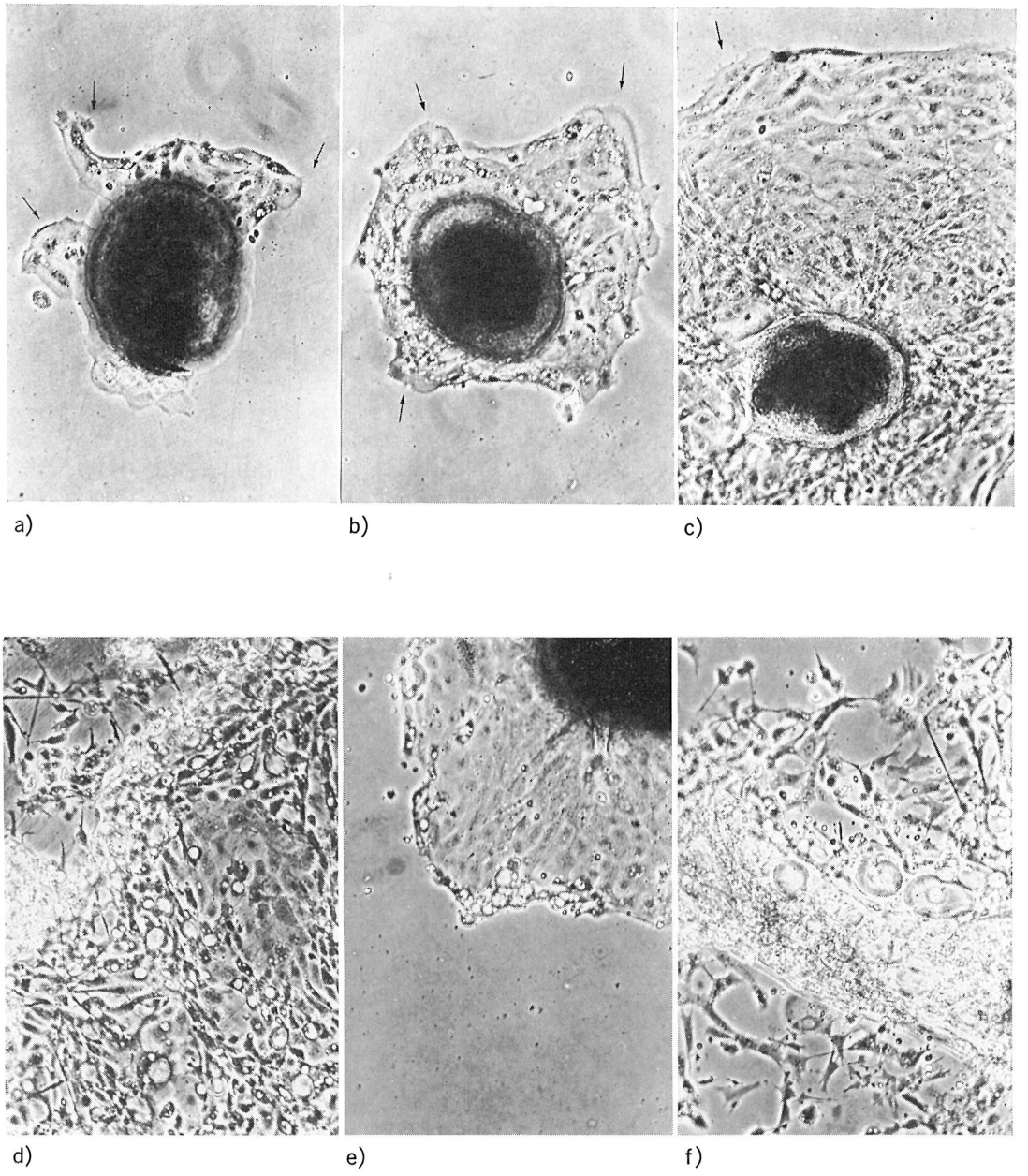


FIGURE 1. *Spreading of constituent cells from plated CAM fragments and their cytopathic effects (CPE) due to HVJ infection. a-c, Spreading of epithelial components after plating. Free edge activities of the epithelial sheets are observed (arrow). a, 12 hr cultivation. b, 24 hr cultivation. c, 72 hr cultivation. d, CPE in epithelial components, cultivated after infection with 10^5 EID_{50}/ml of HVJ. 72 hr cultivation. e, Epithelial components showing scarecely any CPE. The same culture dish as d. f, Mesenchymal components immediately beneath the epithelial sheet, destroyed by CPE, show no CPE. The same culture dish as d. Phase contrast micrographs: magnification* $\times 200$.

components, did not show noticable CPE (Fig. 1f).

2. *Characteristics of HVJ produced in the in vitro system*

The EID_{50}: HA ratio (I/A ratio) of the hemagglutinating (HA) particles obtained in the *in vitro* system was $10^{6.0}$. Therefore, the particles exhibiting the HA activity shown in Table 1 are actually infectious virion particles of HVJ.

Maximal production of HVJ was attained even on inoculation of 10^4 EID_{50}/ml of HVJ (Table 1). This implicates that the virion particles produced in the *in vitro* system possess the capacity to infect neibouring cells in the culture progressively.

Besides this completeness of infectivity, i.e. infectivity both *in ovo* and *in vitro*, the HVJ particles also well-manifested all the biological activities (hemolytic, fusion and neuraminidase activities) of HVJ produced in the *in ovo* system.

3. *Dependence of the HVJ yield on the dose of inoculated fragments*

Fig. 2 clearly indicates that the HVJ yield

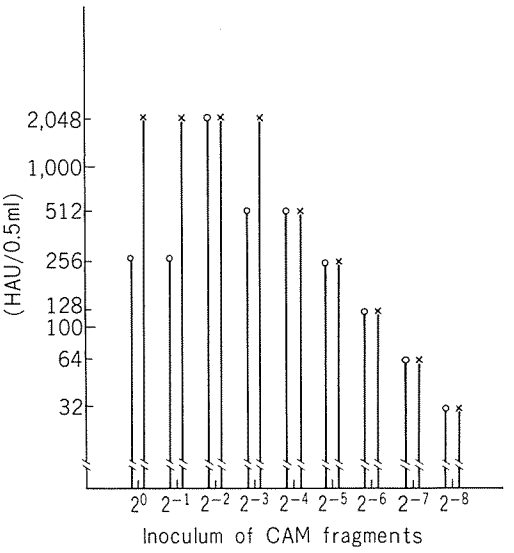


FIGURE 2. *Dependence of HVJ yield on dose of inoculated fragments. The HVJ titer in culture fluids was assayed 3 days* (○) *and 5 days* (×) *after infection. For infection, 10^{-4} dilution of CAF was added to suspensions of each dilution of CAM fragments to attain a final dilution of CAF of 10^{-5} (ca. 10^5 EID_{50}/ml). Abscissa (Inoculum of CAM fragments); Packed volume(ml) of CAM fragments inoculated into dishes (diameter: 50 mm) containing 5 ml of culture medium. For details, see PROCEDURES 2.2).*

TABLE 2. *Sensitivity of the system to HVJ, inoculated at various moi*

Dilution of CAF[a]	No. of positive dishes / total dishes		Titer of HVJ in cuture fluids (HAU/0.5 ml)		
	72 hr ($10^{7.5}$ per 0.1 ml)	120 hr (10^8 per 0.1 ml[c])	24 hr	72 hr	120 hr
10^{-1}	3/3	3/3	128	2,048	512
10^{-2}	3/3	3/3	32	1,024	1,024
10^{-3}	3/3	3/3	8	1,024	2,048
10^{-4}	3/3	3/3	(−)	512	2,048
10^{-5}	3/3	3/3	(−)	256	2,048
10^{-6}	3/3	3/3	(−)	128	2,048
10^{-7}	3/3	3/3	(−)	32	2,048
10^{-8}	0/3	1/3	(−)	(−)	(64)[b]
10^{-9}	0/3	1/3	(−)	(−)	(64)[b]
10^{-10}	0/3	0/3	(−)	(−)	(−)

[a] CAF (infected chorioallantoic fluids), containing ca. 10^{10} EID_{50}/ml of HVJ. Samples of 0.1 ml were inoculated into 30 mm Falcon plastic dishes containing 2 ml of culture fluid.
[b] HA titer in the positive case.
[c] $TCID_{50}$ of CAF measured in the present system.

in culture fluids is directly proportional to the dose of inoculated fragments, at least, on inoculation of less than 2^{-3} ml of precipitated fragments. Thus, production of HVJ per cell seems to be constant, and infection proceeds quantitatively, so that the maximal yield of HVJ is determined by the quantity of host cells in the system.

4. *Sensitivity of the system to infection at various moi*

The data shown in Table 2 indicate that the system is very sensitive to infection. Maximal production of HVJ (2,048 HAU/0.5 ml) was attained on inoculation of HVJ at the relatively low moi, 10^2–10^6 EID_{50}/dish. Even on inoculation of 10^0–10^1 EID_{50}/dish, *in vitro* infection was sometimes established and detectable amounts of HVJ were produced. This indicates that 10^3 EID_{50} corresponds to ca. 10^1–10^2 $TCID_{50}$ in the present system. Since the system shows this high sensitivity, it will be very useful in screening tests for detection of HVJ.

Neutralization of infectivity by anti-HVJ antiserum was tested. Complete inhibition of infection at about 10^4 EID_{50} of HVJ was attained by 4^{-3}-fold dilution of anti-HVJ antiserum, with a hemagglutination-inhibition (HAI) titer of 1,024 HAI/0.5 ml (Table 3). Thus, this system will also be useful for either detection or titration of anti-viral antibodies.

TABLE 3. *Neutralization test with anti-HVJ-antiserum in the fragmented CAM system*[a]

Serial dilution of antiserum	Titer of HVJ in culture fluids[b] (HAU/0.5 ml)
4^{-1}	(−)
4^{-2}	(−)
4^{-3}	(−)
4^{-4}	32
4^{-5}	64
4^{-6}	128
4^{-7}	128
4^{-8}	128

[a] HVJ samples used for infection were 10^{-5} dilutions of CAF, and the titer of antiserum was 1,024 HAI/0.5 ml. See MATERIALS AND METHODS for details.

[b] Assayed 4 days after inoculation of virus-antibody complex.

DISCUSSION

HVJ was produced in high titer by culturing minced fragments of CAM, infected with a low moi of HVJ. The HVJ produced in this *in vitro* system showed the same biological activities as HVJ produced in the *in ovo* system. Namely, it showed hemagglutinating, hemolytic, neuraminidase and fusion activities and infectivity to both *in ovo* and *in vitro* systems.

Monolayer cultures of some types of epithelial cells, such as primary cultures of chicken lung (Portner and Kingsbury, 1971), chick, mouse, calf and monkey kidney (Shibuta et al., 1971; Watanabe and Okada, unpublished data) and a cell line (MDBK) from bovine kidney (Mountcastle et al., 1971), exhibit fairly high sensitivities and productivities of HVJ. However, the fragmented CAM system described in this paper has definite advantages over these other systems. In particular, it should be noted that it is a very convenient system, and that the CAM cells, used as hosts for HVJ, are the same as in the *in ovo* system, which has been widely used for many years.

In many *in vitro* systems HVJ losses its hemolytic and fusion activities and infectivity (Ishida and Homma, 1960, 1961; Matsumoto and Maeno, 1962; Hosaka, 1962). This has been a major obstacle in applying these systems to various experimental purposes. However, the HVJ particles produced in the present *in vitro* system manifest all of the known biological activities of HVJ produced in the *in ovo* system.

Because the fragmented CAM system has these many advantages, it should be useful for various experimental purposes. For ex-

ample: (1) It provides a cheap and easy way to label HVJ. In labeling HVJ with ^{3}H-uridine (Okada et al., 1974), the labeling efficiency was about 10-fold more than that using the *in ovo* system. Moreover, it was shown that HVJ labeled in this way reacted with cell membranes just in the same way as HVJ prepared *in ovo*. (2) The sentitivity of the system is very high (Table 2), so that it can be used for screening many samples for detection of infective viruses under controlled and constant conditions. (3) As shown in Table 3, the system can be used for detection and titration of anti-viral antibody by measuring neutralization of infectivity. (4) It can be used in various analytical experiments on cell-virus interactions in a high productive system under controlled experimental conditions.

Other experiments (Watanabe and Okada, 1974) suggested that the main cells producing virus in the fragmented CAM system are the allantoic epithelial cells, and that the mesenchymal cells, which are also supposed to be exposed to infection, scarecely participate in virus production. This situation is similar to that of *in ovo* propagation of HVJ in the chorioallantoic cavity.

No data were obtained on the participation of chorionic epithelial cells and cells of the vascular systems in HVJ production in the present system. The spreading epithelial cells which show no apparent CPE (Fig. 1e) might be representative of these cells.

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