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Production of Hantaan Virus from Human Immortalized Retina Cell and Its Immunogenicity

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Abstract Hantaan virus production, using human immortalized retina cell (PER.C6), was investigated to develop an inactivated virus vaccine. To infect Hantaan virus into PER.C6, two infection methods (medium-to-cell and cell-to-cell) were tried, and IFA results showed that the cell-to-cell infection method was very useful for producing Hantaan virus-infected PER.C6. Hantaan virus production was significantly affected by the growth rate of PER.C6 and the content of FBS in medium. Higher specific growth rate of infected PER.C6 and lower FBS content induced higher production of Hantaan virus. The inactivated human cell-culture vaccines with various EIA titers were prepared, their antibody responses were compared with those of inactivated suckling mouse brain vaccines (Hantavax®), and the result showed their immunogenicities were slightly higher than those of inactivated suckling mouse vaccines. Therefore, this study shows the possibility of the development of Hantaan virus vaccine from a human cell culture.

Key words: Hantavirus, infection method, immunogenicity

Hantaan virus (HTV), the etiological agent of Korean hemorrhagic fever (KHF), was isolated from the lungs of the Korean striped mouse by Lee *et al.* in 1976 [12]. Since then, various HTV-related viruses which cause clinically similar symptoms to those of Korean hemorrhagic fever have been found around the world, and in 1983, the disease caused by these viruses was named HFRS (hemorrhagic fever with renal syndrome) by WHO. In 1985, HFRS-related viruses were proven to form a new genus in Bunyaviridae through the study on antigenic relations of HFRS-related viruses and named Hantavirus [18]. Subsequently, many species, such as HTN, SEO, PUU, PH, Thai, DOB, and TPM, were established and 23 species have been classified to date.

The main causative virus of HFRS in Korea is Hantaan virus belonging to HTN and it is related to the severe type of HFRS, with an around 10- 15% mortality rate. Since the outbreak during the Korean War, HFRS has been recognized as a dangerous health-threatening disease in Korea and hundreds of people have been hospitalized annually. In 1984, WHO recommended the rapid development of an effective inactivated vaccine, and HTV vaccine was for the first time developed using suckling mouse brain in Korea and has been marketed. There was also a report on the development of inactivated cell-culture vaccine using golden hamster kidney cell (GHKC) in China [22]. However, up to now, no study on vaccine developed from a human diploid cell strain (HDCS) has been reported, except for some publications on HTV infection of human endothelial cells [17, 23], human lung cancer cell line [9], and human adherent mononuclear cells [15]. In this study, HTV vaccine was prepared from the human retina endothelial diploid cell (PER.C6).

Although the large-scale production and downstream processing of a virus are well known procedures, cultivation conditions for efficient virus production are sometimes highly specific to an employed virus-animal cell line system and hence should be carefully investigated. There are a variety of cultivation factors that influence the production of any particular virus: cell growth rate, medium composition, and culture temperature. To enhance the production of HTV in the presently used virus-cell line system, the effects of various important factors, such as infection methods, cell growth rate, and FBS content on HTV production, were investigated in T25 flasks. In addition, the inactivated human cell-culture vaccines with various HTV content were prepared by mass production using T175

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fl: sks, and their immunogenicities were examined in guinea pi ; and compared to those of inactivated suckling mouse br ain vaccines.

M ATERIALS AND METHODS

C lls, Seed Virus, Media, and Antiserum

T e ROK 84-105 strain of HTV, originally isolated from th : blood of a Korean HFRS patient in 1984, was used in th s study. All experiments handling live virus in this study w re conducted under biosafety level-3 containment.

Seed Hantaan virus was prepared from the culture o strain ROK 84-105 in the brain of suckling mouse a d the subsequent purification was carried out using th combination protocol of homogenization, high-speed c ntrifugation and microfiltration. This seed virus was p opagated in the PER.C6 cells between 43 and 50 of p ssage numbers, which are the immortalized diploid cell st ain derived from human retina endothelial cells.

Except when specially mentioned, DMEM (catalog no. 1 995-065, Gibco BRL) with 10% FBS (catalog no. 2 140-079, Gibco BRL) was used as a growth medium for P R.C6, DMEM with 5% FBS as a growth medium for V roE6 as well as a HTV propagation medium in PER.C6, a d DMEM with 2% FBS as a propagation medium for V roE6. Throughout this study, the CO_2 level was maintained a 10% and temperature at 37°C (MCO 175, Sanyo).

The GP antiserum to HTV was prepared in two SPF-grade 3 0-350 g guinea pigs by immunization with two doses of th virus. At the beginning of immunization, the well-mixed s lution of seed virus and Freund's complete adjuvant (c atalog no. F5881, Sigma) was injected intramuscularly, o Day 15 a second dose of seed virus and Freund's in complete adjuvant (catalog no. F5506, Sigma) was injected, a d on Day 30 the GP antisera to HTV was collected and p oled [21]. FITC (fluorescein-isothiocyanate) conjugated a ti-guinea pig IgG was purchased from Sigma (catalog n . F6212). The IFA (indirect immunofluorescence assay) ti er of the above antisera to HTV-infected VeroE6 cells v as about 1:4,096.

I A, PFU, EIA, and Cell Number Count

I A was performed to determine the infection rate of in fected cells on a multispot slide [4, 5, 6]. The cells to be e amined were delivered to the appropriate number of s ots, immediately allowed to air-dry and fixed in acetone f r 10 min at 4°C. Thereafter, slides were reacted with a propriately diluted HTV antiserum and stained with F TC conjugated anti-guinea pig IgG. All the reactions were c nducted in a moist box for 30 min at 37°C and the slides v ere thoroughly washed with PBS after each reaction. Stained s ides were mounted (catalog no. 1000-4, Sigma) and the in fection rate was visually examined under UV light.

The plaque forming unit (PFU) of HTV was quantified as follows; a 6-well plate (catalog no. 152795, Nunc) with confluent VeroE6 was prepared and the culture media were removed. Three-tenth ml of appropriately diluted HTV was gently added into each well of a 6-well plate, incubated for 90 min at 37°C while shaking every 20 min, and finally removed by suction. Three ml of overlay medium which was prewarmed to 42°C was added to each well, followed by 7 days incubation period at 37°C. Two ml of staining medium per well was layered, and continued for another 1-3 days incubation at 37°C, and finally, HTV plaques were counted visually. The compositions of the overlay medium and the staining medium were EMEM (catalog no. 12-668E, Biowhittaker) with 5% FBS and 1% agarose (catalog no. A6138, Sigma), and EMEM with 1% agarose and 4% neutral red (catalog no. 15330-079, Gibco BRL), respectively.

Viral antigen titer (EIA titer, EU/ml) was determined by Hantaan virus antigen ELISA kit (enzyme-linked immunosorbent assay kit; GCLS, Korea) [9, 10], which binds the monoclonal antibody of nucleocapsid protein (N) of ROK 84-105 strain, and assayed by the method recommended by the kit manufacturer.

After trypsinization and neutralization, cells were appropriately diluted and counted on a hemacytometer (catalog no. Z35,962-9, Sigma). Cell concentration was calculated by the manufacturer's method.

Vaccine Preparation

The culture medium of HTV-infected PER.C6 was clarified by high-speed centrifugation (6,000 rpm, 30 min, 4°C), and then ultracentrifuged at 35,000 rpm at 4°C for 4 h (50.2Ti, Beckman). The virus pellet was resuspended by overnight-soaking in a small volume of TNE buffer (Tris-NaCl-EDTA buffer, pH 7.5) and dialyzed through an MWCO 100,000 Da-dialysis membrane (catalog no. 131420, Spectrum) against PBS (pH 7.4). This concentrated HTV solution was inactivated by incubation for 14 days at 4°C in 0.025% formalin and then dialyzed. Inactivation was verified by IFA examination of a 7 day-infected VeroE6, known as a sensitive cell line, to HTV [4], and no infected VeroE6 was found. To enhance the vaccine immunogenicity, an aluminium hydroxide gel was used as an adjuvant [3]. Aluminium content and pH of prepared vaccines were 500 μg Al/ml and 7.4, respectively. These aluminium hydroxide-adjuvanted vaccines were used for immunogenicity tests.

Immunogenicity in Guinea Pigs

The immunogenicities of inactivated human cell-culture HTV vaccines were measured by IFA titer and PRNT (plaque reduction neutralizing test) titer (*the minimum requirements for biological products of Korea, K-FDA*), and they were compared with those of the conventional vaccines (7,000 EU/ml and 10,240 EU/ml (Hantavax®)) prepared from suckling mice brains. One group comprised of five SPF-

grade guinea pigs. Each group was intramuscularly immunized three times every 10 days with each test sample (Vaccines and Phosphate-buffered saline (pH 7.4) as a negative control), and bled by a heart puncture 10 days after the final immunization. Antiserum was pooled, inactivated at 56°C for 30 min, and stored at -20°C for the neutralizing antibody titer assay (PRNT titer). And, also, a small amount of blood was collected every 10 days and pooled for IFA titer assay.

For PRNT titer assay, monolayers of VeroE6 cells were prepared in 6-well plates and washed twice with sterile PBS (pH 7.4). One ml each of challenge HTV, whose titer was about 333 PFU/ml, was neutralized for 90 min at 37°C after mixing with 1 ml each of serially 4-fold-diluted antisera (4^1, 4^2, 4^3, 4^4). Here, diluent was EMEM with 2% FBS. Three-tenth ml of the neutralized virus was added to each well of the 6-well plates, and challenge HTV producing about 100 plaques per well was inoculated into another plate as a virus control. After adsorbing the virus for 90 min at 37°C and removing the supernatant, 3 ml of overlay medium (EMEM with 1% agarose and 5% FBS) per well was added, solidified at room temperature, and cultivated for 7 days at 37°C. Thereafter, each well was stained during the 2-3 day incubation with 2 ml of staining medium (EMEM with 1% agarose, 5% FBS, and 0.013% neutral red) per well, plaques were visually counted, and the vaccine potency (PRNT titer), which is the reciprocal of the highest dilution that results in 50% reduction in the number of plaques compared to the virus control, was calculated by the *Reed-Muench* formula [13, 14].

RESULTS

Infection of HTV into PER.C6

Seed Hantaan virus prepared as mentioned in *Materials and Methods* was added to the T25 flask with the PER.C6 monolayer. In all cases, DMEM with 5% FBS was used as an HTV propagation medium.

To obtain the PER.C6-adapted HTV, 0.5 ml of seed virus was inoculated onto the normal PER.C6 monolayer in the T25 flask. After the first passage culture of seed virus using a PER.C6 monolayer for 10 days, IFA was tried in order to examine the HTV infectivity on the PER.C6 cells, and it was found that about 10% of the cells were infected. But the infection rate of HTV was not further increased, even after being cultured for a longer period. In order to achieve more rapid infection, 0.5 ml of 10 day-culture medium of the first passage was consecutively adsorbed onto a fresh PER.C6 monolayer. However, strangely, no infected cells were found. Further attempts with blind passages by the same medium-to-cell infection method were unsuccessful.

The other infection method, which is the cell-to-cell infection mode, was examined. The first passage culture, the PER.C6 monolayer infected with seed virus for 10 days, was trypsinized and neutralized, and 0.5 ml out of 5 ml cell suspension was added to the T25 flask with a normal PER.C6 monolayer and further passages were tried by the same cell-to-cell method. On Day 10 after subsequent infection, the infection rate was 20-30% for the second passage and nearly 100% for the third passage culture, respectively. From the fourth passage, the culture medium of the previous passage was used for further passages (medium-to-cell infection mode) and the medium of the sixth passage culture, which completely infected the PER.C6 monolayer in 10 days after infection, was used as PER.C6-adapted HTV for further study. In addition, the analysis of HTV-infected PER.C6 by microscopy did not show any cytopathic effect, as known already on HTV [8, 11].

The VeroE6 cell, known as a susceptible cell line for HTV [11, 14], was well infected with the medium-to-cell infection method. 7 days after being cultured with the seed virus, about 70% of VeroE6 was infected. Accordingly, PER.C6 seems to be less sensitive to HTV than VeroE6. In the case of PER.C6, probably a very low HTV titer in the medium of the first passage culture, having a very small number of infected cells, is thought to be one of the reasons why the infection failed in further passages.

From these results, it can be concluded that, for a less HTV-sensitive cell line like PER.C6, the cell-to-cell infection method used in this study is a useful method to obtain higher infection rate.

Growth Characteristics of PER.C6-Adapted HTV in PER.C6

One-half ml of PER.C6-adapted HTV was added to the T25 flask with a fresh PER.C6 monolayer and its MOI (multiplicity of infection) was 0.017. DMEM with 5%

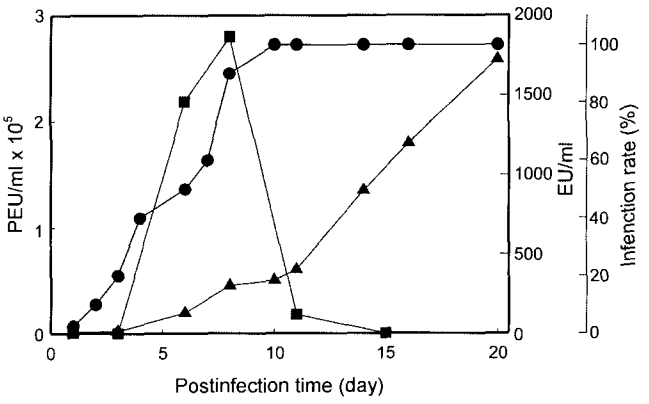

Fig. 1. Virus profile during postinfection time.
—▲—, EIA titer, EU/ml; —■—, Active virus titer, PFU×10^5; —●—, Infection rate, %.

FI S was used as an HTV propagation medium, and cu ture was continued for 20 days after infection (Fig. 1). Fr)m Day 10 of postinfection, some cells began to be de ached due to deterioration of the culture environment, ar l at the end of culture, most of the cells were suspended in the culture medium. IFA confirmed that nearly all of the ce ls at around Day 8 were infected. The PFU titer of HTV in culture medium had the peak value of 2.8×10^5 PFU/ml or Day 8 and decreased rapidly thereafter. This trend was qu ite similar to that reported on HTV infection into human en dothelial umbilical vein cells (HUVEC) and human en dothelial saphenous vein cells (HSVEC), both of which ha d PFU peak titer on Day 3 of postinfection [17]. On the ot ıer hand, the EIA titer of HTV, which detects both active an d inactive HTV, kept increasing until the end of culture al er a short plateau around the highest PFU value. It is li ely that the virus excreted by the lysis of detached cells ca uses this continuous increase of EIA titer in the late phase.

To elucidate the trend of EIA titer after Day 20 of p stinfection, its behavior was investigated for a longer p riod. Initial MOI was 0.0017, 0.017, and 0.17. As shown ir Fig. 2, the production rate of HTV in the medium was h gher at the higher initial MOI, and in all cases, EU titer h d the highest value at around Day 18 after infection and b gan to rapidly decrease thereafter. The decrease of EU ti er appeared to be caused by the virus degradation.

IFA data on Day 3 after infection showed a significant d fference in the infection rate, which was that the higher M OI induced a higher infection rate (Fig. 3). The time r quired for the complete infection of PER.C6 monolayer v as longer at the lower MOI, which was about 18, 10, and 5 days for 0.0017, 0.017, 0.17 of MOI, respectively.

From these results, it can be concluded that the infection a id production rate of HTV for PER.C6 is strongly d pendent on the initial MOI, and, in batch culture using a T flask, the maximum PFU time (infectious virus titer) and the maximum EU time (total titer of infectious and noninfectious viruses) are different.

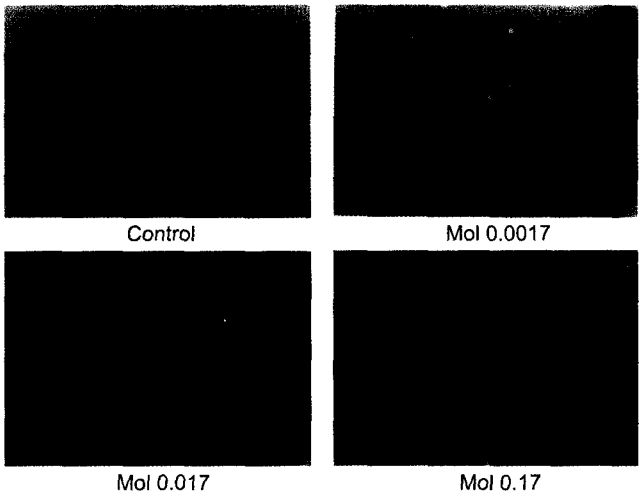

Fig. 3. Infection rates on Day 3 of postinfection.

Virus Propagation Characteristics According to Successive Passage of PER.C6-Adapted HTV and Dependency on Altered Cell-to-Cell Infection Mode

The propagation characteristics of PER.C6-adapted HTV through longer passages were investigated. The culture period of all passages was 8 days when the infectious virus concentration in medium nearly reached a peak. In all cases, DMEM with 5% FBS was used as a propagation medium. From the sixth passage to the eighth passage, the EIA titer of HTV in medium was dramatically reduced from 1,000 EU/ml at the sixth passage to 55 EU/ml at the eighth passage, as shown in Fig. 4. This dropping of the EIA titer by HTV passage was very revealing, since it suggests that the use of HTV in medium as an inoculum

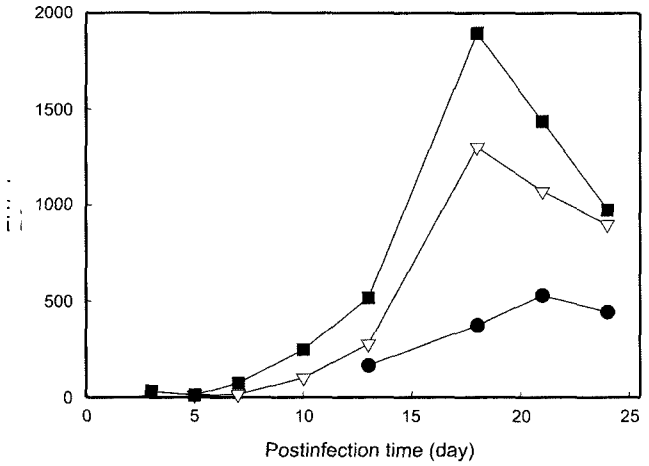

Fig. 2. Profile of EIA titer according to different MOI. -●-, 0.0017 of MOI; -▽-, 0.017 of MOI; -■-, 0.17of MOI.

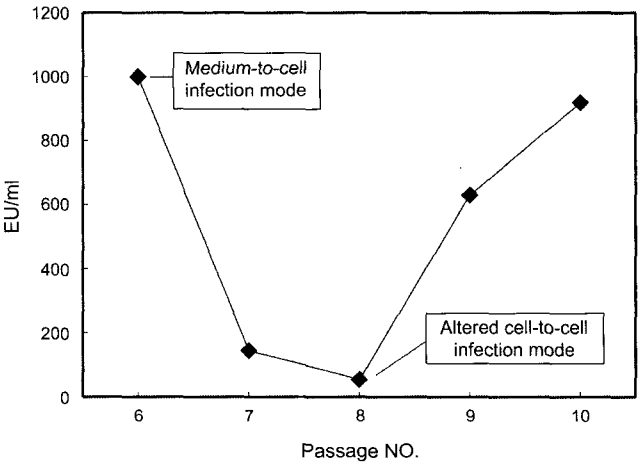

Fig. 4. Effect of passage and infection mode on virus production.

(medium-to-cell infection mode) is not suitable for HTV production from PER.C6.

To alleviate this undesirable situation, the altered cell-to-cell infection mode, which allows both infected and normal cells to grow together in an empty T25 flask, was tried. Thus, a 1/10 (0.5 ml) volume of infected cells at the eighth passage and a 1/10 (0.5 ml) volume of normal PER.C6 monolayer were mixed, and the mixture was placed in a T25 flask having 4 ml of DMEM with 5% FBS and cultured. This altered cell-to-cell infection mode was repeated until the tenth passage, and the culture period of all passages was 8 days. Surprisingly, the EIA titer in the medium increased sharply, from 55 EU/ml at the eighth passage to 920 EU/ml at the tenth passage and recovered the EIA titer level of the sixth passage. This altered cell-to-cell infection mode was quite different from the medium-to-cell infection mode in that the former provided PER.C6 a continuous growth during the infection period. Therefore, the virus production in altered cell-to-cell infection mode seemed to be somewhat related to the host growth rate.

Correlation Between HTV Production and PER.C6 Growth Rate

To investigate if the host growth rate had an effect on HTV production, PER.C6-adapted HTV was propagated by the PER.C6 with the different growth rates during the infection period. One-half ml each of PER.C6-adapted HTV was adsorbed onto the PER.C6 cells in the T25 flasks with the different initial confluencies (Low: ~20%, Middle: ~50%, High: ~70%), which were prepared to induce the different growth rates. As a propagation medium, DMEM with 5% FBS was used. Figure 5A shows the relationship among cell growth, virus production, and postinfection time. Cells were cultured for 9 days after infection and entered the resting phase at around Day 8 after infection in all cases. As expected, growth curves with different slopes were obtained, and average specific growth rates ($\Delta C/(\Delta t \times C_{AVE})$) before the resting phase were 0.380, 0.312, and 0.196 day^{-1} for 20%, 50%, and 70% initial confluency, respectively. Here, ΔC is the difference in cell concentration, Δt the difference in time, and C_{AVE} the average cell concentration for the designated period.

In all cases, the EIA titer of HTV particle secreted into medium increased during the culture period and its rate of increase was strongly dependent on the specific growth rate of PER.C6; the higher the specific growth rate, the higher HTV production rate was induced. Figure 5B also reveals that the higher growth rate of PER.C6 played a very important role in producing HTV particles secreted into medium. In this figure, a direct correlation between the specific growth rate of PER.C6 and the HTV yield per unit cell ($Y_{V/C}$) can be seen. Here, $Y_{V/C}$ was calculated by the equation of $(V_{day9} - V_{day2})/(C_{day9} - C_{day2})$. V_{day9} and V_{day2} represent the EU/ml of Day 9 and Day 2 after infection, and C_{day9} and C_{day2} stand for the cell concentration of Day 9 and Day 2 after infection. These results suggest that the production of HTV particle by PER.C6 is closely growth-associated.

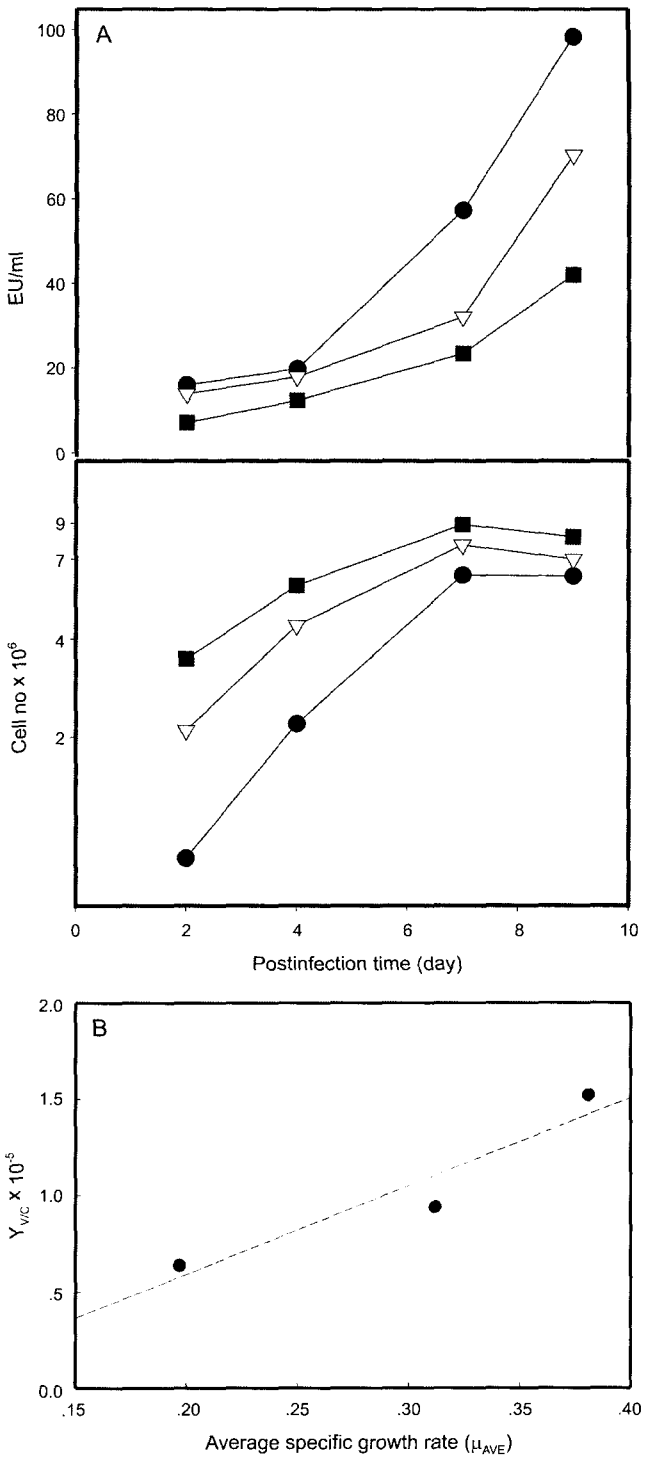

Fig. 5. A, Effect of PER.C6 growth rate on virus production. —●—, Low confluency; —▽—, Middle confluency; —■—, High confluency: B, Correlation between specific growth rate and virus yield.

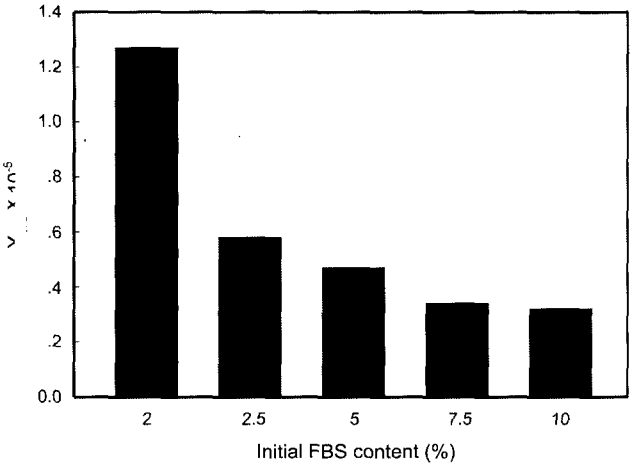

Fig. 6. Effect of initial FBS content on virus production.

Effect of FBS Content on HTV Production

Effect of FBS content on HTV production was investigated. DMEM with each 0.5, 1.0, 1.5, 2.0, 2.5, 5.0, 7.5 and 10% FBS was used as propagation media. In all cases, initial confluency was about 50%, and 4.5 ml each of infection medium was added into T25 flasks after 0.5 ml of PER.C6-adapted HTV was adsorbed. Cells were cultured for 9 days after infection. Ranging from 2.0% to 10% of FBS, cell growth curves were nearly the same for 9 days, entering resting phase at Day 8 and having average specific growth rates between 0.307 and 0.347 day^{-1}. On the other hand, the media with lower than 2% FBS allowed only one to three days of growth. Virus yield per unit cell ($Y_{V/C}$) was much higher at the lower FBS content, with between 2.0% and 10% having similar growth rates (Fig. 6). These results imply that the FBS content in medium is a very important factor to support the growth of PER.C6, and that excess FBS content has an adverse effect on the HTV production in PER.C6.

Immunogenicity of Cell-Culture HTV Vaccine

Human cell-culture HTV vaccines with 2,500, 5,000, 10,240, and 16,000 EU/ml were prepared as explained in *Materials and Methods* to investigate the immunogenicity in guinea-pig, and their antibody titers (which are IFA titer and PRNT titers) were compared with those of conventional vaccines (7,000 EU/ml and 10,240 EU/ml (Hantavax®)) prepared from suckling mouse brain. The medium from the tenth passage culture was purified, inactivated, and finally mixed with aluminium hydroxide gel. Western blot showed that the band pattern of HTV purified from HTV-infected PER.C6 culture was exactly the same as that of HTV from suckling mouse brain (data not shown). In both cases, one major band at around 50 kDa was found and was presumed to be N protein.

To determine the IFA titer, all sera were serially diluted 2-fold. All cases, except human cell-culture HTV vaccine of 2,500 EU/ml, showed the same IFA trend during the immunization period (Table 1). The first booster dose dramatically increased the IFA titer, but a further boost no longer elicited the immune response of guinea pig. The final IFA titers, 10 days after the second booster dose, were 2,560, 5,120, 5,120, 5,120 for 2,500, 5,000, 10,240, 16,000 EU/ml of inactivated human cell-culture vaccine, respectively, and 5,120 for both titers of inactivated sucking mouse brain vaccine. Human cell-culture vaccines between 5,000 and 16,000 EU/ml showed the same final IFA titer as in inactivated sucking mouse brain vaccines between 7,000 and 10,240 EU/ml (Hantavax®).

Figure 7 shows the results of the PRNT titer representing the HTV-neutralizing ability. Unlike the results of IFA titer, vaccines with higher EIA titer showed higher neutralizing antibody titer, irrespectively of vaccine source. Furthermore, human cell-culture vaccine with 10,240 EU/ml had a slightly higher PRNT titer than the inactivated suckling mouse brain vaccine with the equal EIA titer (Hantavax®).

In summary, when the sucking mouse brain vaccines and the human cell-culture vaccines with different EIA titers were compared, their antibody responses in sera showing the immune system-eliciting ability of vaccine were nearly identical. Accordingly, it was concluded that the human cell-culture vaccine prepared in this study induced similar or higher immune response in guinea pig compared to the conventional vaccine prepared from suckling mouse brain.

Table 1. Antibody response (IFA titer, reciprocal of highest dilution) trends of human cell-culture vaccines and suckling mouse brain vaccines in guinea pig.

Immunization day	PBS	Suckling mouse brain vaccines		Human cell-culture vaccines			
		7,000	10,240	2,500	5,000	10,240	16,000
0 (Initial dose)	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
10 (First booster dose)	Negative	160	160	40	40	40	160
20 (Second booster dose)	Negative	5,120	5,120	2,560	5,120	5,120	5,120
30	Negative	5,120	5,120	2,560	5,120	5,120	5,120

N.A.: Not assayed.
PBS: Phosphate-buffered saline (pH 7.4) as a negative control.

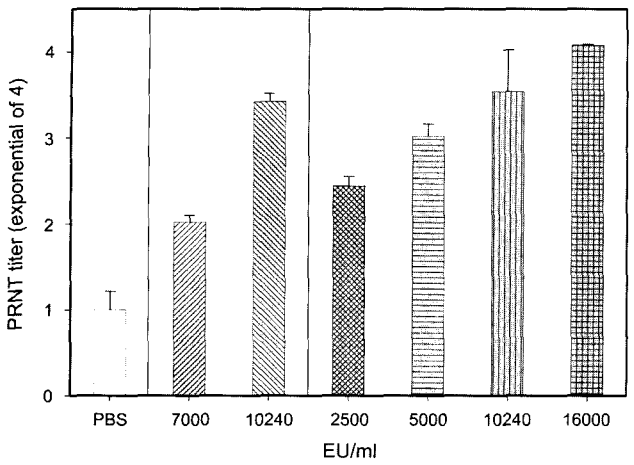

Fig. 7. Potency (PRNT titer) comparison of human cell-culture vaccines and suckling mouse brain vaccines in guinea pig.
Negative control: ☐ PBS; Suckling mouse brain vacciness: ▨ 7,000 EU/ml, ▧ 10,240 EU/ml; Cell-culture vacciness: ▩ 2,000 EU/ml, ▤ 5,000 EU/ml, ▥ 10, 240 EU/ml, ▦ 16,000 EU/ml.

DISCUSSION

Human immortalized retina endothelial cell, PER.C6, was hardly infected by Hantaan virus from sucking mouse brain, due to its low susceptibility even in repeated blind passage. However, the repeated cell-to-cell infection method made it possible to completely infect HTV into PER.C6. It was likely that the repeated cell-to-cell infection method was useful in getting the cell-adapted virus from a cell line with low sensitivity to target virus. During the culture of HTV-infected PER.C6, no cytopathic effect was induced by HTV infection. The difficulty of direct infection and the absence of CPE implied that human eye retina might be free of the viral damage by HTV infection. In fact, there has been no report on eyesight loss or permanent eye tissue damage due to HTV infection, except mild temporary clinical symptoms such as mild photophobia, eyeball pain, and blurred vision in the febrile phase of HFRS [14].

As evidenced by the data of the specific growth rate and the virus EIA titer level in medium, the growth rate of infected PER.C6 was of great importance in high-level production of Hantaan virus. However, the actual mechanism of growth rate on the stimulation of HTV production was not investigated in the present study. Ogura *et al.* [16] reported that the degree of Sendai virus release in the growing phase is much higher than in the resting phase of infected mouse 3T3 cell, and the decrease of viral replication and transcription rates in the resting phase is related to reduced virus production. The decreased rate of viral RNA synthesis is likely to cause decreased virus production in HTV-infected PER.C6 with lower specific growth rate. Another plausible mechanism is that cell growth rate is connected with the virus secretion rate from Golgi, where the assembly and budding of Hantavirus particles occur [19], to medium through cell membrane. Bae *et al.* [1, 2] showed that the secretion of hGCSF from recombinant *Saccharomyces cerevisiae* is enhanced at the higher specific growth rate and the secretion efficiency is directly proportional to cell growth rate. It is plausible to deduce the correlation between cell growth rate and virus secretion efficiency, because the secretion pathway of Hantavirus from infected cells is similar to that of *Saccharomyces cerevisiae*, although this is the case of a microorganism. Therefore, it is suggested that both mechanisms are probably closely related to Hantavirus production in PER.C6, and further investigation is in need.

There have been some reports that porcine reproductive and respiratory syndrome virus (PRRSV) in CRL11171 cell culture [20] and rubella virus in primary rabbit kidney cell culture [7] propagated significantly better when the cell culture medium was supplemented with FBS or FCS [20]. These studies show that serum components play an important role in cell growth as well as virus propagation. However, we found a suppression effect of excess FBS on Hantavirus production in PER.C6. This is consistent with the result that FCS and CS had the suppressive activity on infectious bursal disease virus (IBDV) growth [24]. In the range of FBS content to support enough growth rate of infected PER.C6, it was confirmed that higher FBS content had a negative effect on virus production. This implies that FBS should be supplemented with the lowest level possible for HTV production in PER.C6.

The vaccine from human diploid cell strain (HDCS) is needed for safer vaccination of people in areas endemic of HFRS. This is the first report that PER.C6 (HDCS) has been used for the development of an inactivated cell-culture vaccine against HFRS. Its immunogenicity was assessed in guinea pig and compared to that of suckling mouse brain vaccine. The sera of suckling mouse brain vaccine groups and human cell-culture vaccine groups were positive for IFA and PRNT titers. The groups which received three doses of the human cell-culture vaccines with different EIA titers gave slightly higher neutralizing antibody response compared to the suckling mouse brain vaccine groups in the similar EIA titer range. These results suggest that the human cell-culture vaccine against HFRS is very effective, even in humans.

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