Growth of Equine Arteritis Virus in Cells Derived from Infectious Canine Hepatitis Virus-Induced Hamster Tumor and Transformed Cells

Morikazu SHINAGAWA, Ryo YANAGAWA and Tsuneki INOUE

Department of Hygiene and Microbiology, Faculty of Veterinary Medicine, Hokkaido University, Sapporo-shi, Hokkaido 060

Yutaka AKIYAMA

Equine Health Laboratory, Japan Racing Association, Setagaya-ku, Tokyo 154

(Received for publication September 8, 1975)

Abstract. The growth of equine arteritis virus (EAV) was found in HT-7 and HS cells derived from hamster tumor and hamster transformed cells. EAV produced plaques of countable size in both HT-7 and HS cell cultures 2 days after infection. Infective progeny virus appeared in HS cells from 10 to 12 hr after infection. Viral yield in HT-7 and HS cells reached about $10^7$ PFU/ml 24 hr after infection. The appearance of viral antigen detected by the fluorescent antibody technique correlated to that of infective progeny virus.

Equine arteritis virus (EAV) is an enveloped RNA virus 60 nm in average size [14] and regarded as a member of nonarbovirus [4]. Although only the genus Equus is known as the natural host and animal susceptible to EAV, various kinds of cultured cells have been reported to support the growth of EAV. They are cells of horse kidney [12], horse testicles [5], rabbit kidney [11], hamster kidney [17], cat kidney and swine kidney [9], as primary or secondary cultured cells, and such cell lines as Equine Derm NBL-6 [1], BHK 21 [5], HmLu [9], RK13 [10], LLC-RK1 [16], LLC-MK2 [13], BSC1 [2], Vero [9] and JINET [9].

The present report concerns the growth of EAV in HT-7 cells derived from a hamster tumor induced by infectious canine hepatitis virus (ICHV) and HS cells originated from an ICHV-transformed focus.

Materials and Methods

Virus and cells: Strain Bucyrus of EAV which had passed 5 times in horse kidney cells was received from Dr. M. Ogata, of the Department of Veterinary Microbiology, Faculty of Agriculture, University of Tokyo, Tokyo. The virus was allowed to propagate once in primary horse kidney cells at the senior author's laboratory and stored at $-80^\circ$C before use. EAV which had serially passed 14 times through HS cells (to be described below) was also used.

HT-7 cells were established from a hamster tumor which had been induced by ICHV [8]. They were cultured in Earle's balanced salt solution containing 0.5% lactalbumin hydrolysate (LE medium) and supplemented with 10% calf serum. They contained no demonstrable amounts of ICHV-specific T antigen, which could be detected by the fluorescent antibody (FA) technique or complement fixation test, during the period of this study. HS cells were established from a hamster-embryo culture containing a few foci of transformed cells induced by strain Woc-4 of ICHV. FA technique failed to demonstrate the presence of ICHV-specific
Table 1. Growth of two preparations of EAV in HS–7, HS and BHK 21 cells

<table>
<thead>
<tr>
<th>EAV preparation</th>
<th>Host cell</th>
<th>Virus yield (PFU/ml) assayed using the cells of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HT–7</td>
</tr>
<tr>
<td>EAV propagated in horse kidney cells</td>
<td>HT–7</td>
<td>$3 \times 10^5$</td>
</tr>
<tr>
<td></td>
<td>HS</td>
<td>$3.5 \times 10^6$</td>
</tr>
<tr>
<td></td>
<td>BHK 21</td>
<td>$2.3 \times 10^6$</td>
</tr>
<tr>
<td>EAV propagated in HS cells</td>
<td>HT–7</td>
<td>NT$^{(3)}$</td>
</tr>
<tr>
<td></td>
<td>HS</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td>BHK 21</td>
<td>NT</td>
</tr>
</tbody>
</table>

Remarks.

1) Cells were infected with EAV at a multiplicity of infection of about 2 PFU/cell, and harvested 24 hr after infection.

2) No countable plaques were produced.

3) Not tested.

T antigen in HS cells on and after the 6th passage. HS cells, after 35 to 50 passages, were cultured in LE medium supplemented with 10% calf serum and used for the present experiments. BHK 21 cells were supplied by Dr. T. Uchida, of the Sapporo Medical College, Sapporo, and cultured in Eagle’s minimum essential medium containing 10% calf serum.

Plaque assay: Cells grown in multi-dish Disposo trays (24 holes, φ16 mm; Limbro Chemical, Vineyard, New Jersey) were washed once with LE medium containing 50 μg/ml of DEAE-dextran (Pharmacia, Uppsala) and were inoculated with 0.1 ml of virus suspension diluted with LE medium containing 50 μg/ml of DEAE-dextran. After virus adsorption at 37°C, 1.5 ml of Eagle’s minimum essential medium containing 0.5% lactalbumin hydrolysate, 2% calf serum and 2% methylcellulose (Shin-etsu Chemical Co., Tokyo) was added. The culture was incubated at 37°C for 2 to 3 days. After removing the medium, the culture was fixed and stained with 4% formalin containing 0.1% gentian violet for 30 min, followed by washing and plaque counting. Virus titer was expressed as plaque forming units (PFU)/ml.

FA study: Cells grown on the coverglass were infected with EAV at a multiplicity of infection of about 5 PFU/cell. After 1.5 hr of adsorption, they were fed LE medium containing 2% calf serum. At the scheduled time after infection, the coverglass was withdrawn, washed 3 times with phosphate-buffered saline (PBS), dried, and fixed with acetone at −20°C for 5 min. The fixed cells were allowed to react with anti-EAV rabbit serum [7] at room temperature for 1 hr and washed 5 times with PBS. They were allowed to react again with anti-rabbit IgG goat γ-globulin labeled with fluorescein isothiocyanate at 37°C for 30 min and washed again 5 times with PBS. Examination was done and photography taken by a Nikon fluorescent microscope, type ETC (Nippon Kogaku Co., Tokyo).

Results

HT–7 and HS cells in plastic trays produced clear plaques at 2 days of incubation (Fig. 3). The number of plaques produced was directly related to virus dilution. Plaques formed 2 or 3 days after virus infection were found suitable for counting (Fig. 3, A, B, D and E), because a prolonged incubation period would cause fusion of plaques and nonspecific degeneration of cells (Fig. 3, C). On the other hand, BHK 21 cells did not produce plaques (Fig. 3, F). Numerous degenerated cells, however, were found microscopically in the cultures of BHK 21 cells inoculated with low dilutions of EAV (10- and 102- fold dilutions of stock virus). HT–7 and HS cells were thus able to be used for rapid plaque assay of EAV.

The effect of the adsorption period on plaque production was then examined. Fig. 1 shows that plaques increased in number with the advance in adsorption time up to 120 min. After 120 min of adsorption some cells became rounded and others were detached from the wall. Therefore, 90 min
was adopted as the period of virus adsorption for further experiments.

Whether EAV was required to be adapted to HT-7, HS and BHK 21 cells before it could grow in these cells was examined. When Vero cells were used, the virus could grow sufficiently after it was adapted to the cells [6]. HT-7, HS and BHK 21 cells were infected with 2 preparations of EAV. After isolation one of the preparations was passed 6 times in horse kidney cells only, and the other 6 times in horse kidney cells and then 14 times in HS cells. Viral yields were titrated with HT-7, HS or BHK 21 cells. As shown in Table 1, HT-7 and HS cells supported good growth of both EAV preparations. BHK 21 cells, however, did not support good growth of either EAV preparation. The virus yield in BHK 21 cells was almost one-tenth of that in HT-7 or HS cells. It was thus clarified that EAV could grow in HT-7 and HS cells, but not in BHK 21 cells, without preliminary adaptation.

The growth curve of EAV in HS cells is shown in Fig. 2. EAV progeny production began 10 to 12 hr after infection. Thereafter, infective virus increased progressively and reached a level of $8 \times 10^6$ PPU/ml 24 hr after infection.

Viral growth in HT-7, HS and BHK 21 cells was also examined by the indirect FA technique. The findings obtained with the infected HS cells are as follows (Fig. 4, A-D). A faint fluorescence was found around the perinuclear region of the cytoplasm in a few cells 10 hr after infection. It became clearer and was expressed as irregular granules fusing with one another 12 hr after infection (Fig. 4, A). Occasionally, the faint fluorescence was diffused all over the cytoplasm of such cells. Thereafter, cells with fluorescence increased in number and cytoplasm
was filled with fluorescence in infected cells (Fig. 4, B). Round cells which had a cytoplasmic fluorescence began to be found 18 hr after infection. Most of the cells with fluorescence became round by 6 hr later (Fig. 4, C). At this time after infection a cytopathic effect was found in Giemsa-stained preparations, which showed the heavily stained cytoplasm, the round shape of cells, pycnosis and occasional karyolysis (Fig. 4, D).

Almost the same results were obtained from EAV-infected HT-7 cells (Fig. 4, E). In infected BHK 21 cells, however, a cytoplasmic fluorescence became apparent 14 hr after infection. The cells with fluorescence were fewer than infected HT-7 and HS cells. Their fluorescence was weaker than that of these two types of cells (Fig. 4, F) under the same conditions of infection. No fluorescence in nuclei was found in any cell at any time after infection. The time of appearance of cytoplasmic fluorescence agreed with the time of production of progeny virus in HS cells (Fig. 2).

Discussion

HT-7 and HS cells supported growth of EAV. Plaques of countable size were formed in cultures of both cells 2 days after infection. These two types of cells may, therefore, be useful for the studies of EAV.

HT-7 and HS cells were derived from the tumor and the transformed cells induced by ICHV. Although T antigen was no more detectable in these cells and ICHV genome might have been lost from them, it seemed better to use these cells within the limit of laboratory work of EAV.

Progeny EAV was produced in HS cells around 12 hr after infection in the present investigation. This result agreed with that obtained by Maess et al. [14] in BHK 21 cells. The granular fluorescence found around the perinuclear region may correspond to virions accumulated in cytoplasmic vacuoles [15]. Crawford and Henson also observed cytoplasmic viral antigen in endothelial cells and macrophages in an infected horse [3].

EAV was reported to produce plaques in BHK 21 cells cultured in Petri dishes [5, 14]. In the present investigation, however, it failed to produce plaques in BHK 21 cells cultured in plastic trays. It seems that these cells may be less sensitive to EAV than HT-7 and HS cells and may not allow EAV to produce plaques. Hyllseth [5] succeeded in making EAV grow enough in BHK 21 cells, although he used the virus which had passed 2 or 3 times through BHK 21 cells before use. It is possible that the difference in results obtained between Hyllseth and the present authors may have been caused by the difference in the culture history of BHK 21 cells used.

Acknowledgments: The authors wish to express their thanks to the Japan Racing Association. This work was supported in part by a grant-in-aid from this association.

References

 tion of the equine arteritis virus using the fluorescent antibody technique. Thesis for Master's degree submitted to the Faculty of Veterinary Medicine, Hokkaido University, Sapporo.


Explanation of Figures

Fig. 3. Plaques of EAV produced in HT-7, HS and BHK 21 cell cultures. The cultures were fixed and stained at the indicated time after infection. The number shown at the bottom indicates the dilution (log 10) of inoculated virus. Control, C, was inoculated with LE medium. A, B and C: HS cells. D and E: HT-7 cells. F: BHK 21 cells.

Fig. 4. Immunofluorescent micrographs and a light micrograph of EAV-infected cells. A: HS cells, 12 hr after infection. B: HS cells, 16 hr after infection. C: HS cells, 24 hr after infection. D: HS cells, 24 hr after infection; Giemsa stain. E: HT-7 cells, 18 hr after infection. F: BHK 21 cells, 18 hr after infection.