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Reverse Plaque Formation by Hog Cholera Virus of the GPE⁻ Strain Inducing Heterologous Interference

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A simple and rapid plaque procedure was developed for the assay of hog cholera virus (HCV) of a particular strain, GPE⁻, based on its intrinsic interference with vesicular stomatitis virus (VSV) on the primary swine testicle cells and on an established swine kidney cell line; the procedure is called the reverse plaque formation (RPF) method. The plaques were produced as colonies of HCV-infected cells which were VSV refractory, standing out in the background of a VSV-sensitive, disintegrated cell sheet. These plaques became visible after 15 to 20 h of superinfection with VSV done 2 days after an initial inoculation of the GPE⁻ strain. The plaque formation was inhibited by a specific antiserum against HCV. All cells within the plaque had HCV antigen detectable by fluorescent-antibody staining. The variations of reverse plaque count were low enough to permit virus titration. The relationship between virus concentration and the number of plaques was essentially linear. The titer measured by the RPF method was a little higher than that of the tube culture interference method.

Hog cholera virus (HCV) is noncytopathic and has been assayed by the cell culture fluorescent-antibody technique (10) or the END method (5, 6), the latter based on enhancement by HCV of growth of Newcastle disease virus (NDV) in primary swine testicle (ST) cell cultures. The GPE⁻ strain of HCV was a low-virulence mutant isolated during serial passage of the virus in guinea pig kidney cells; the mutant did not enhance NDV but interfered with NDV and Western equine encephalomyelitis virus (14). The GPE⁻ strain has been used for the preparation of live virus vaccine and assayed by its interference with Western equine encephalomyelitis virus or vesicular stomatitis virus (VSV) (2).

This paper describes the new plaque assay of the GPE⁻ strain. This method, named the reverse plaque formation (RPF) method, was based on the complete resistance of the GPE⁻-infected cells to the superinfection by VSV. The plaques were visible as intact cell colonies in the background of a disintegrated cell sheet.

MATERIALS AND METHODS

Viruses. The GPE⁻ strain of HCV was propagated in guinea pig kidney or ST cell cultures by incubation at 30°C for 5 to 6 days. The New Jersey serotype strain of VSV adapted to chicken embryos was grown in primary swine kidney cell cultures. Lapinized HCV of the LPC strain and virulent HCV of the ALD strain were used for the preparation of hyperimmune serum.

Cell cultures. The swine kidney cell line (SK-H) and ST cells were grown as monolayers in Eagle minimum essential medium containing 10% inactivated bovine serum without the antibody against bovine viral diarrhea virus, which shares partial antigen with HCV. The SK-H was established in our laboratory and was around the 420th passage level when used.

Reverse plaque procedure. The cells were grown to confluency in 60-mm petri dishes containing 5 ml of the growth medium. After removing the growth medium at the time of inoculation, the GPE⁻ strain in 0.5 ml of Hanks salt solution supplemented with 0.5% lactalbumin hydrolysate was absorbed for 60 min at 37°C in a 5% CO_2 incubator, and then shaken slightly every 15 min. The inoculum was aspirated, and the cultures were washed once with Hanks salt solution and then covered with 5 ml of methylcellulose overlay medium. To prepare the overlay medium, a 3% methylcellulose solution in water was mixed with an equal volume of a double-strength minimal essential medium supplemented with 5% bovine serum and 60 μg of kanamycin per ml, and the pH was adjusted to 7.2 with a 10% solution of sodium bicarbonate. The methylcellulose (4,000 centipoise) was repeatedly washed with absolute ethanol and ether and then air dried, according to the method of Rapp et al. (11). After incubation for designated periods, generally 2 days, the overlay medium was removed by washing with Hanks salt solution, and the cell monolayer was inoculated with VSV at a multiplicity of about 2 plaque-form-

ing units/cell in 0.5 ml and absorbed for 60 min at 37°C. Then the inoculum was removed, and 5 ml of a 1% agar overlay medium (pH 7.4) was added to the monolayer. After incubation for 15 to 20 h, the plates were stained with 2 ml of 0.01% neutral red in Hanks salt solution. After incubation for 2 h, the reverse plaques were visualized as red-colored colonies. For permanent record, the cultures were fixed with 5% formalin for 30 min and stained by Giemsa or phloxine solution.

Titration of the GPE⁻ strain by the tube culture interference method. This was carried out according to the method previously described (2). Tubes first received a serial decimal dilution of the GPE⁻ strain in 0.1-ml amount per tube, 10 tubes receiving each dilution. The tubes then received 0.5 ml of the ST cell suspension and were incubated at 37°C in a slant stationary position. After incubation for 4 days, the medium was discarded and the cultures were challenged with 10^3 mean tissue culture infective doses of VSV in 0.5 ml of culture medium supplemented with 5% bovine serum. The cultures were then incubated under the same conditions for 1 day, and the test results were read. The cultures without cytopathic effect were read as HCV positive, and the mean tissue culture infective dose titer was calculated by Kärber's method (4).

Staining of the plaque by fluorescent antibody. Hyperimmune serum obtained from swine immunized with HCV of the ALD strain was used for the preparation of fluorescent antibody. The reverse plaques were produced on SK-H on cover slips in the petri dish. The cover slip with plaques was washed once with phosphate-buffered saline (pH 7.2), air dried, fixed with acetone at room temperature for 10 min, and then stained with the fluorescent antibody at 37°C for 1 h.

Inhibition of reverse plaque formation. Each rabbit was given, in the lateral ear veins, a 2-ml injection of lapinized HCV of LPC strain. The serum was obtained from the blood of the rabbits collected 30 days after injection. The serum was inactivated at 56°C for 30 min. The neutralizing antibody titer of the antiserum measured by the END neutralization test (15) was 800 times the serum dilution. The undiluted antiserum was mixed with an equal amount of each serial log dilution of the GPE⁻ strain. In a control, the GPE⁻ strain samples were mixed with normal rabbit serum instead of HCV antiserum. The mixture was incubated at 37°C for 1 h and then subjected to reverse plaque assay using SK-H.

RESULTS

Development of reverse plaques. The incubation period after the initial inoculation with the GPE⁻ strain and before the challenge inoculation with VSV was varied from 1 to 4 days with a daily interval. Plaques produced after 1 day of incubation were clearly visible only through the microscope. Diameters of reverse plaques were estimated to be less than 0.3 mm. With 2-day incubations, all plaques became visible to the naked eye. Their diameter ranged from 0.5 to 2 mm (Fig. 1A). Incubation for 3 and 4 days gave larger plaques ranging, respectively, from 1 to 3 mm (Fig. 1B) and from 2 to 5

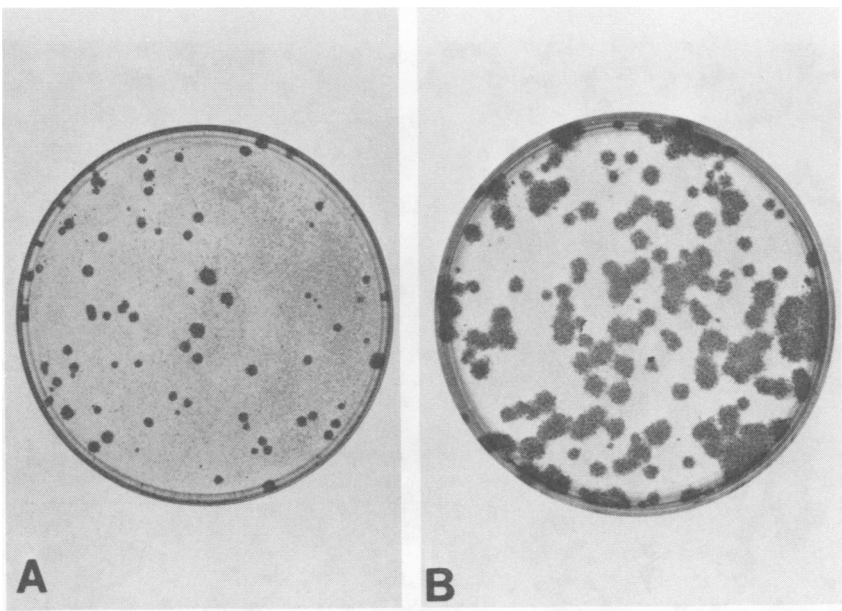

FIG. 1. *Reverse plaques produced by the GPE⁻ strain of HCV on monolayers of SK-H (A) and ST (B) cells. A cell monolayer grown in a 60-mm petri dish was inoculated with the GPE⁻ strain, incubated for 2 (A) and 3 days (B), challenged by VSV, incubated for additional 20 h, and stained by phloxine (A) and Giemsa (B).*

mm in diameter. The plaques were more readily recognized than those having a 2-day incubation. No significant increase of plaque count due to the extention of the incubation period was observed. Any difference in the type of cells, SK-H and ST, made no difference in the shape and number of plaques. Figure 2 illustrates a plaque produced on SK-H by a 2-day incubation and stained by Giemsa. Cells in a circular plaque were all intact and surrounded by disintegrated cell debris. Clear reverse plaques could be also developed on replicate dishes under a liquid medium without methylcellulose overlay medium; the secondary reverse plaques, however, were developed on the plates.

Specificity of reverse plaque formation. The reverse plaque formation was totally inhibited when the GPE^- strain was mixed with HCV antiserum before inoculation, whereas the control mixture without antiserum gave clear reverse plaques of expected numbers. Moreover, the fluorescent-antibody staining revealed the presence of HCV antigen in all cells composing the reverse plaque (Fig. 3). This indicates the HCV specificity of produced reverse plaques.

Dispersion of reverse plaque number in replicate plate. The number of reverse plaques counted on each of 20 replicate plates of SK-H and ST cell was recorded in Table 1. These reverse plaque numbers on SK-H and ST cell were in conformity with the Poisson distribution, and the 95% confidence limits were 72.7 ~ 66.1 and 29.3 ~ 25.7, respectively. These results indicate that RPF method using SK-H

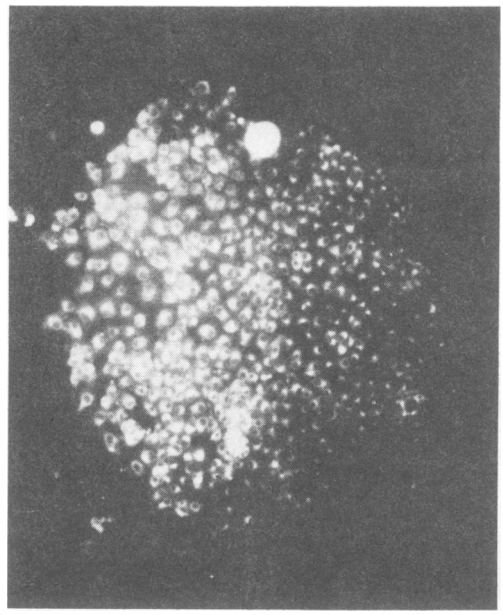

FIG. 3. *Specific fluorescence of a reverse plaque on SK-H cells. The plaque on SK-H was stained by fluorescent antibody specific to HCV. The virus antigen was detected in all cells composing plaque.*

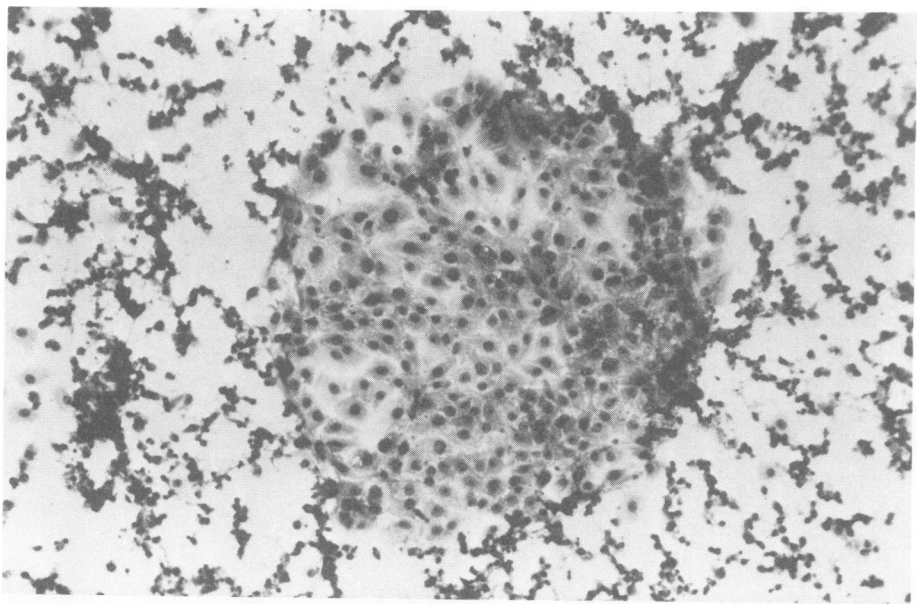

FIG. 2. *Reverse plaque induced by the GPE^- strain of HCV. Conditions were the same as Fig. 1A, except that cells were stained by Giemsa solution and observed microscopically.*

TABLE 1. *Reverse plaque number on SK-H and ST cells*

Cells used	Inoculum dose ($TCID_{50}$/ml)[a]	No. of reverse plaques in each of 20 plates (RPFU/0.5 ml)[b]	Mean	Variance	95% confidence limit
SK-H	$10^{2.0}$	83 81 76 76 75 73 73 71 70 70 70 69 69 68 63 62 62 62 58 57	69.4	47.6	72.7 ~ 66.1
ST	$10^{1.5}$	36 32 31 31 31 30 29 29 29 27 26 26 26 26 25 25 24 22 22 22	27.5	13.0	29.3 ~ 25.7

[a] The virus titers were measured by the tube culture interference method. $TCID_{50}$, Mean tissue culture infective dose.

[b] Reverse plaques were counted, challenging with VSV at 2 days after inoculation of GPE^- strain of HCV. RPFU, Reverse plaque-forming units.

and ST cells was low enough to permit the virus titration.

Dose response curve. The number of reverse plaques on SK-H and ST cells increased linearly with the concentration of the GPE^- strain (Fig. 4). These results indicate that each reverse plaque was initiated by a single infectious unit of the GPE^- strain.

Comparative titrations by RPF and tube culture interference methods. The GPE^- strain was titrated by means of the RPF method for comparison with the tube culture interference method. To compare the sensitivities of the RPF and tube culture interference methods, the same material was titrated by both methods, using SK-H and ST cells. Virus was serially diluted 10-fold. For each dilution, 5 plates for the RPF method and 10 tubes for the tube culture interference method were used. The results are shown in Table 2. Reverse plaque titers were a little higher than those of tube culture interference method.

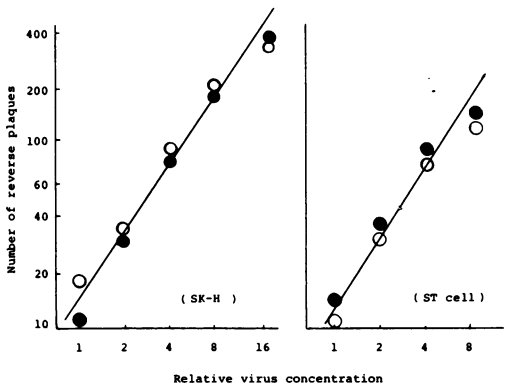

FIG. 4. *Proportionality between the relative concentration of the GPE^- strain of HCV and number of reverse plaques. Open and closed circles represent the results of two different experiments.*

TABLE 2. *Comparative titration of RPF and tube culture interference methods*

Expt. no.	Cells used	RPF method[a]	Tube culture interference method[b]
1	SK-H	2.9×10^5	$10^{5.2}$ (1.1×10^5)
2	ST	6.5×10^5	$10^{5.5}$ (2.19×10^5)

[a] Titers were expressed as RPF particles per milliliter.

[b] Titers were expressed as mean tissue culture infective dose ($TCID_{50}$/ml). Titers in parenthesis are number of interference inducing unit converted from $TCID_{50}$/ml by the Poisson formula, where 0.69 interference units equal 1 $TCID_{50}$.

DISCUSSION

The hemadsorption-negative plaque test (7) has been reported as a means of accurately assaying rubella virus as a noncytopathic virus. This test was based on the fact that cells infected with rubella virus were completely resistant to superinfection by NDV, and this state of resistance was termed "intrinsic interference" (7, 8). The capacity to induce intrinsic interference was demonstrated with some other viruses (3, 7-9, 12, 13, 16), and the plaque test of this type has been applied to some noncytopathic viruses (1, 13, 16). In addition to NDV, VSV was also added to a virus group blocked by intrinsic interference (3, 12). In this experiment, aimed at the development of a new plaque technique for the assay of HCV inducing heterologous interference, the RPF method represents a very simple, reliable, and sensitive procedure for determination of virus infectivity. The RPF method depended on a similar principle to the hemadsorption-negative plaque test except for using NDV as a challenge virus, and a GPE^--VSV interference relationship may

be included in a manner analogous to intrinsic interference. Hunt and Marcus (3) described that although the Sindbis virus was able to be detected by means of the hemadsorption-negative plaque test, a plaque assay using VSV as challenge virus for detection of this virus was not attempted. In our experiments, we have found that the RPF method was comparable to the hemadsorption-negative plaque test for viruses inducing intrinsic interference.

The reverse plaques were produced on both SK-H and ST cells under the same condition. When the cell monolayers were challenged with VSV within 24 h after initial inoculation of the GPE$^-$ strain, discrete foci containing a few VSV-resistant cells were microscopically observed on the plate. They were macroscopically apparent 2 days after inoculation of the GPE$^-$ strain. Further incubation after inoculation of the GPE$^-$ strain produced larger reverse plaques and eventually produced a monolayer in which whole cells were VSV resistant. These results indicate that incubation for 2 or 3 days after inoculation of the GPE$^-$ strain is the most effective method for counting reverse plaques on the plate as a standard procedure of the RPF method. The number of reverse plaques on plates increased linearly with the concentration of the GPE$^-$ strain and indicated that each plaque was initiated by a single infectious viral particle. The specificity of reverse plaque formation was confirmed by neutralization using antiserum against HCV and by fluorescent-antibody staining.

The RPF method offers a tool of assay for other viruses inducing intrinsic interference and also provides a convenient laboratory model for exploring the mechanism of the viral interference.

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