PLAQUE FORMATION BY HERPES SIMPLEX VIRUSES IN GMK (ESTABLISHED GRIVET MONKEY KIDNEY) CELLS

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INTRODUCTION

Plaque assays of herpes simplex virus have been performed by many investigators using different virus strains and different cells. Since the primary rabbit kidney cells were used for plaque titration of herpes virus by Kaplan¹⁾, a variety of primary^{3) 6) 13) 21) 25)} and established^{4) 5) 8) 10) 11) 13) 14) 16) 20) 22) 23) 33)} cells have been employed.

Although the established cell line which is uniform in shape and in metabolism has been desired for various experiments, almost of the plaque assays of herpes simplex virus by the cell lines were carried out under fluid overlay in which in many cases added antiserum to prevent the secondary plaque formation, and furthermore the plaques formed were often microscopic. Yazaki et al.³³⁾ reported that HF strain, a standard herpes virus strain, could form the plaques in HeLa cell monolayers only after high adaptation to HeLa cells, and investigated on a simple agar overlay medium by which a high titration efficiency could be obtained.

Another problem is the different behaviour against cells of virus strains and this probably results in the difference of plaque characteristics. Munk and Donner²⁰⁾ isolated the herpes viruses from various symptoms and described that the viruses could be divided into two types by plaque size, which were in close agreement with the appearance of the cytopathogenic effect elicited by these strains.

The purpose of the present study is to find out the recommendable procedures and an established cell line for plaque assay applicable to two strains of herpes simplex virus which differ in their cytopathogenic effects.

MATERIALS AND METHODS

Cells GMK cells (established African grivet monkey kidney cells) were used throughout this experiment. The growth medium consisted of Hank's saline

plus 0.5% lactalbumin hydrolysate and 10% bovine serum. In one experiment, HeLa, FL, MS, Hep-2, KB, BSC-1, PS and CE (chick embryo) cells were used. The growth medium was the same as above except for PS and CE cells of which medium was Earle's in place of Hank's saline. CE cells were prepared according to procedures of Taniguchi and Yoshino²⁵⁾.

Viruses HF and M strains of herpes simplex virus were used. The latter was isolated in GMK cells from a patient with Herpes labialis by Dr. Maruta of the Department of Dermatology, Kurume University, and kindly supplied from him. The viruses were grown in GMK cells maintained with the medium of Earle's saline plus 0.125% lactalbumin hydrolysate and 0.5% skim milk. The stocks of the viruses were produced in the following manner: One day old cell monolayers, after washing twice with the medium, were inoculated with 0.5ml virus, adsorbed for two hours and added 10ml of the medium after washing three times. The whole culture was harvested after 24 hours of incubation at 37°C by sonication at 20 kc/sec for three minutes in ice water after freezing and thawing. The stocks were distributed in small vials and preserved in -80°C freezer until used. HF and M strains used in this experiment were the stocks which were serially passed ten and nine times in GMK cells, respectively.

Plaque technique GMK cell cultures were prepared in 2 oz bottles with 1,000,000 cells in 5ml of the growth medium. The cell monolayers, which were formed after two days of incubation at 37°C, were washed twice and inoculated with 0.2ml of an appropriate dilution of virus. The virus was allowed to adsorb for two hours at 37°C with intermittent agitation to prevent drying of the cells before adding 4ml of the agar overlay medium without neutral red. Three ml of the second overlay medium with neutral red was added after three days of incubation at 37°C. The reading of plaques was done from the following day. The agar overlay medium consisted of 1.5% agar in Earle's salt solution supplemented with skim milk, lactalbumin hydrolysate and sodium bicarbonate of such concentrations as will be described in the experiments.

Pock formation Eleven days embryonated eggs were inoculated with 0.1 ml of virus dilution, and incubated at 36°C. After three days of incubation the pocks formed on the chorioallantoic membrane (CAM) were observed by dipping it in saline.

Additives to the overlay medium Protamin sulfate (Salmine) was obtained from NBCo; deae dextran and sodium dextran sulfate 2000 (from dextran with M. W. 2×10^6) from Pharmacia, Uppsala.

RESULTS

Cytopathogenic effect

Both strains initiate to cause the distinct cell necrosis 12 hours after inoculation, but the feature of cytopathogenicity is different in two strains. HF strain causes the formation of polynucleate giant cells which develop rapidly by

Fig. 1. Cytopathogenic effect of GMK cells infected with herpes simplex virus.
Left, HF strain; Right, M strain

recruiting adjoining cells into themselves. On the other hand, M strain causes the formation of rounded and clumped cells, and occasionally the formation of small giant cells³⁶⁾.

Effects of skim milk, lactalbumin hydrolysate and sodium bicarbonate on the plaque formation

The formation of plaques was carried out in the presence of 0.5% skim milk and 0.125% lactalbumin hydrolysate or either of them or none in the agar overlay medium containing 0.225% sodium bicarbonate. Table 1 shows that the

TABLE 1
Requirement of skim milk and lactalbumin hydrolysate for plaque formation

Skim milk (0.5%)	—*	+	—	+
Lactalbumin hydrolysate (0.125%)	—	—	+	+
HF	0.2** (P-1)	1.4 (0.5-2.5)	3.2 (P-3)	5.8 (0.5-4.5)
M	<0.01	<0.01	0.03 (P-1)	2.0 (P-1.5)

* presence (+) or absence (-) in the agar overlay medium

** $\times 10^7$ PFU (plaque forming unit) / ml

Numbers in parentheses represent the plaque size in mm; p, plaque size smaller than 0.5 mm.

both were essential to the formation of plaques by herpes viruses. The plaques could not sufficiently develop in the presence of either of them or none, especially in the case of M strain.

The effect of different concentrations of skim milk in the overlay medium with 0.125 % lactalbumin hydrolysate on the formation of plaques was therefore investigated. The concentration of 0.1 % of skim milk decreased in some extent the number of plaques formed, while the concentrations of 0.2 to 1.0 % resulted in the formation of the same number of plaques as shown in Table 2.

TABLE 2
Plaque formation at the different concentrations of skim milk in agar overlay medium

Concentration of skim milk	0.1 %	0.2 %	0.5 %	1.0 %
HF	3.9*	4.9	5.8	4.9
M	1.4	1.9	2.0	1.8

* $\times 10^7$ PFU / ml

TABLE 3
Plaque formation at the different concentrations of lactalbumin hydrolysate in agar overlay medium

Concentration of lactalbumin hydrolysate	0.06%	0.125%	0.25%	0.5%	1.0%
HF	5.3*	6.3	5.3	4.7	6.1
M	2.1**	2.2	2.2	1.5**	1.4**

* $\times 10^7$ PFU / ml

** obscure plaques

Table 3 shows the effects of different concentrations of lactalbumin hydrolysate in the overlay medium with 0.5 % skim milk. The concentrations tested did not affect the feature of plaques formed by HF strain, while M strain decreased the number of plaques in the concentration of 0.5 and 1.0 %, and gave the plaques of which margin was obscure. In addition, the obscure plaques appeared at the lowest concentration tested though the number of plaques did not decrease. The most favourable concentration of lactalbumin hydrolysate for plaque formation by both strains of herpes virus was therefore 0.125 or 0.25 %.

Calf serum and bovine serum albumin fraction V have been introduced in the agar overlay medium for plaque titrations of many viruses. As shown in Table 4, both strains of herpes viruses, especially M strain decreased the number of plaques in the presence of bovine albumin in every concentration examined instead of skim milk. In the other side, calf serum could be replaced for skim milk

TABLE 4
Plaque formation in the presence of calf serum or bovine albumin V fraction instead of skim milk

	HF	M
Skim milk, 0.5%	4.6*	2.1
Calf serum, 2 %	4.6	1.4
4 %	5.5	1.5
10 %	4.6	1.7 ^d
Bovine Albumin, 0.11 %	3.4	0.3
0.22 %	2.0	0.1
0.55 %	3.3	<0.01

* $\times 10^7$ PFU / ml

although the latter was advantageous in the point that the fluctuation of the number of plaques by lots was not observed and the plaques formed a greater contrast with the white background.

The different concentrations of sodium bicarbonate were tested for plaque formation. Table 5 shows that 0.225 % sodium bicarbonate was adequate for plaque formation by both virus strains. In 0.113 %, the plaques formed by HF strain had no clear margin though the number of plaques was as same as in 0.225 %, and those formed by M strain decreased significantly as compared with those in the case of 0.225 %. The highest concentration tested, 0.68 %, resulted in no plaque formation. Thus, 0.225 % of sodium bicarbonate could be adopted as an adequate concentraion for plaque formation by both virus strains.

TABLE 5
The effect of the concentration of sodium bicarbonate in agar overlay medium on the plaque formation.

Concentration of NaHCO ₃	0.113 %	0.225 %	0.45 %	0.68 %
HF	5.0*	4.6	3.7	—
M	0.3	2.1	1.4	—

* $\times 10^7$ PFU / ml

From the experiments above mentioned, the agar overlay medium consisted of 1.5 % agar in Earle's balanced salt solution supplemented with 0.5 % skim milk, 0.125 % lactalbumin hydrolysate and 0.225 % sodium bicarbonate was adopted in the following experiments. The second overlay medium was the same as the first one except containing 0.004 % neutral red. Fig. 2 shows the plaques formed by HF and M strains according to this procedure.

Washing fluid and virus diluent

The following kinds of fluid were used for washing the monolayer cultures

Fig. 2. Plaques formed by herpes simplex virus in GMK cell monolayers.
Left, HF strain; Right, M strain

and diluting the virus; 1) Puck's saline A, 2) Earle's balanced solution with 0.225% sodium bicarbonate plus 0.125% lactalbumin hydrolysate, and 3) Earle's balanced solution with 0.225% sodium bicarbonate plus 0.125% lactalbumin hydrolysate and 0.5% skim milk which is the same as the constituent of the agar overlay medium deprived of agar. The results, as shown in Table 6, indicated that there was no significant difference among them for the plaque formation.

TABLE 6
*The effect of virus diluting and cell washing fluid
on the plaque formation*

Diluting and washing fluid	Puck's saline A	Earle's saline	
		+ LH	+ LH, SM**
HF	5.8*	5.8	6.2
M	2.0	1.6	2.1

* $\times 10^7$ PFU / ml

** LH : 0.125% lactalbumin hydrolysate

SM : 0.5% skim milk

Appearance of plaques after staining

The plaques were counted every day after staining with second overlay medium to ascertain whether the number of plaques could increase or not day

after day and when the number reached the maximum. Table 7 shows that the number of plaques of HF strain reached maximum on the following day of the second overlay, while in the experiment on M strain, two days were necessary to reach the maximum number of plaques. Thus, for titration of M strain, the plaque count must be done at least two days after second overlay.

TABLE 7
Appearance of plaque after second overlay

	days after 2nd overlay		
	1	2	3
HF	4.6*	4.5	
	5.2	5.1	
M	2.0	2.1	2.0
	1.6	1.9	2.0

* $\times 10^7$ PFU / ml

Virus adsorption

After inoculating the virus on cell monolayer, the cultures were overlaid at different intervals of time as indicated in Table 8. It was found that about 50 % of both HF and M strains were adsorbed by cells within 30 minutes at 37°C, and practically all the viruses within an hour and a half. Washing of inoculated monolayers before adding overlay medium did not affect the number of plaques formed.

TABLE 8
Adsorption time and number of plaques formed

Adsorption time (hrs)	HF	M
0	0.3*	<0.01
0.5	2.9	0.8
1.0	5.3	0.8
1.5	6.6	1.1
2.0	6.9	1.3
2.5	6.6	1.1

* $\times 10^7$ PFU / ml

Virus concentration and number of plaques

To determine if one plaque is produced by one infective virus particle, titrations were carried out using a series of virus concentrations. Two tenth ml of virus dilution were inoculated and the cultures were overlaid as described above. Fig. 3 shows that there is a linear relationship between the virus concentration and the number of plaques.

Fig. 3. Relationship between virus concentration and number of plaques.

Fig. 4. Distribution of plaque size of HF strain. Numbers in parentheses represent the total number of plaques in each experiment.

Fluctuation of plaque size

HF and M strains developed the plaques of 1 to 4.5 mm and 0.5 to 1.5 mm diameter in 4 and 5 days, respectively. It was difficult to examine the distribution of plaque size of M strain as the plaques were too small to measure the size correctly. On the other hand, the distribution could be easily examined by counting the number of plaques of which size was included in a given range. Fig. 4 shows the distributions of four separate experiments, indicating the normal distributions with the mean value of 2.0 to 2.3 mm in every case. This means possibly that there is no invisible plaque on the cell monolayer. In addition, it may be also the case in M strain because the size of the plaques is fairly uniform in the range of 0.5 to 1.5 mm.

The effect on plaque formation of some additives

Plaque formation was carried out by adding some reagents in the first overlay medium. The additives were omitted from the second overlay medium.

TABLE 9
*The effect of various additives to overlay medium
on plaque formation*

	HF	M
Control	6.9* (1.5-5.5)	1.3 (0.5-1.5)
MgCl ₂ , 40mM	7.1 (P-4.5)	1.0 (0.5-1.0)
Deae dextran, 100 µg/ml	7.7 (P-4.5)	1.3 (0.5-1.5)
Yeast extract, 0.14%	6.7 (0.5-5.0)	1.1 (0.5-1.5)
Dextran sulfate, 500 µg/ml	5.8 (P-1.0)	<0.01
Protamin sulfate, 500 µg/ml	6.9 (1.0-5.5)	1.3 (1.0-1.5)
Agarose, 1%**	6.6 (0.5-4.5)	1.3 (0.5-2.0)

* $\times 10^7$ PFU/ml

Numbers in parentheses represent the plaque size in mm :

p, plaque size smaller than 0.5 mm.

** Overlay medium with agarose instead of agar in "Control".

The results were shown in Table 9. Three reagents (40 mM MgCl₂, 100 µg/ml deae dextran and 0.14% yeast extract) among five tested had no effect on the number and the size of plaques formed by HF and M strains. Dextran sulfate in the concentration of 500 µg/ml decreased the number and the size of plaques of HF strain and formed no plaques of M strain. Protamin sulfate in the concentration of 500 µg/ml had no effect on plaque titer, but the plaques formed were clearer than others including the control. Especially, the plaques formed by M strain in the presence of protamin sulfate were very clear and in small extent but significantly larger in size, resulting in more uniform size. Using of 1% agarose in place of agar did not affect on plaque formation by both strains.

Plaque formation in a variety of cells

Nine different cells were tested on the formation of plaques by HF and M

strains according to the procedures described above except CE cells. Plaque formation in CE cells was performed according strictly to the procedures by Taniguchi and Yoshino²⁵⁾. At the same time, eleven days embryonated eggs were inoculated with 0.1 ml of the virus dilutions to compare the pock formation on the CAM and the plaque formation in the cell monolayers. The results were shown in Table 10. HeLa, MS, KB, PS and CE cells showed the decrease of the

TABLE 10
Plaque formation in a variety of cells

	HF		M	
GMK	5.2	(0.5-4.5)*	1.9	(0.5-1.5)
HeLa	2.4	(P -0.5)	0.2	(P)
FL	6.7	(P -1.5)	1.4	(P)
MS	4.3	(P -1.5)**	<0.001	
Hep-2	7.7	(P -1.5)	<0.001	
KB	3.4	(P -1.0)**	<0.001	
BSC-1	5.3	(P -3.0)	1.8	(P -0.5)**
PS	0.1	(P -0.5)	0.6	(P -1.0)**
CE	2.3	(0.5-2.0)	0.2	(1.0)
CAM	5.6	(1.0-3.0)***	1.9	(1.5-2.0)***

* $\times 10^7$ PFU / ml

Numbers in parentheses represent the plaque size in mm.

** Read 3 days after second overlay.

*** $\times 10^7$ pock forming units / ml

Numbers in parentheses represent the pock size in mm.

number and the size of plaques compared with GMK cells. The same titers of viruses were obtained by FL and BSC-1 cells. Hep-2 cell indicated slightly higher titer in HF strain although no plaque formation by M strain. M strain formed no plaque in Ms, Hep-2 and KB cells despite that the virus strain caused the distinct cytopathogenic change under the maintenance medium without agar. It was found that only BSC-1 cells could be used for titrations of both strains of herpes simplex virus in place of GMK cells, though the counting of the plaques of M strain must be done 3 or 4 days after the second overlay because of their delayed maximum appearance. The equal titers were obtained in both virus strains by pock formation on CAM and by plaque formation in GMK cells.

DISCUSSION

From the experiments described above, the procedures for plaque assay of herpes simplex viruses may be recommended as follows: The GMK cell monolayers are overlaid after adsorption of virus for two hours at 37°C. The overlay medium consists of 1.5% agar in Earle's salt solution plus 0.5% skim milk,

0.125 % lactalbumin hydrolysate and 0.225 % sodium bicarbonate. Introducing 500 μ g/ml of protamin sulfate in agar overlay medium makes the plaques clearer, probably by protecting cells from degeneration under agar¹⁸⁾, though the reagent is essentially not necessary for the plaque formation. On the third day, the second overlay medium is added of which constituent is the same as the first one except with neutral red. According to these procedures, HF and M strains develop the clear plaques with the size of 1 to 4.5 mm and 0.5 to 1.5 mm, respectively.

Farnham⁴⁾ reported the formation of plaque in HeLa cells under fluid overlay and the plaques formed were too small to be visible. The similar microscopic plaques under fluid overlay were reported by Wheeler in HeLa cells⁵⁾ and by Nii and Kamahora in FL cells¹⁴⁾. On the other hand, several investigators^{10) 16)} reported the visible plaques although they used the same virus-cell system as that of Farnham. The plaques were developed in Chang cells¹¹⁾ and in primary and stable grivet monkey kidney cells²¹⁾ by using methylcellulose in place of agar to rule out the possibility of inhibitory effect of agar components⁷⁾. By the same reason, carboxymethyl cellulose¹³⁾ or soft agar³⁵⁾ was also employed. In spite of these reports, the presence of inhibitor in agar against herpes simplex virus is doubtful, because many instances were reported that the viruses are possible to form the visible plaques under agar overlay in stable cells^{20) 23)}, and in primary cells^{2) 6) 12) 25) 34)}. In our present study, both HF and M strains could develop the clear plaques in GMK cell monolayer under agar and obtained the same titer under agarose. Addition of deae dextran or protamin sulfate into agar overlay medium, which is thought to combine with agar inhibitor and to neutralize its inhibitory effect²⁸⁾, did not affect the number and the size of plaques formed. Thus, the possibility of inhibitory effect of agar component could be ruled out.

Skim milk and lactalbumin hydrolysate were essential for the development of plaques. The former could be replaced by serum, and the latter might be a source of amino acids which were required by herpes viruses for their proliferation^{29) 34)}. $MgCl_2$ with which the proliferation of enteroviruses was enhanced¹⁵⁾¹⁹⁾ did not affect the plaque formation by herpes viruses. Dextran sulfate which was known to affect the proliferation of various viruses inhibited the development of plaques by both strains and it resulted in no plaque formation in the case of M strain.

There was a linear relationship between the number of plaques and the virus concentrations in both cases of two strains studied. This indicates that one plaque is produced by one infective virus particle¹⁾. It is therefore confirmed that this system can be used for titration of herpes simplex viruses.

By the plaque technique recommended here, the normal distribution was shown on the size of plaques formed by HF strain. The distribution pattern of plaque size of M strain was not possible to examine because of their small size,

but they were relatively uniform in size and became more uniform in size of 1.0 to 1.5 mm without affecting the number of plaques when added protamin sulfate in agar overlay. Thus, it is thought that there is no invisible plaque in GMK cell monolayer in both cases.

The titer obtained by the present technique using GMK cells was the same as the pock forming titer and higher than in CE cells. BSC-1 cells showed the same titer as GMK cells, but the plaques developed more slowly than those in GMK cells. It is not clear why MS, Hep-2 and KB cells did not permit M strain to develop any plaques despite they produced the typical cytopathogenic change following infection with the virus strain.

Munk and Donner²⁰⁾ isolated 40 strains of herpes simplex virus from a variety of sites representing various clinical manifestations and investigated the characteristics of their plaques in HeLa and Hep-2 cells. They divided the isolated strains into two groups by plaque size, that is, one of 0.7 mm and the other of 2 mm in size, and they indicated that the former group caused the cytopathogenic effect such as round cells and small syncytia, and the latter caused large syncytia. M and HF strains used in the present study showed the similar relationship, that is, M strain, which caused rounding and clumping of cells and rarely small giant cells, produced a small plaque of 0.5 to 1.5 mm in size, while HF strain, which caused the multinucleate giant cells (large syncytia), produced a large plaque of 1.0 to 4.5 mm in size. Takemori and Nomura⁷⁾ reported that the growth of the small plaque former of poliovirus (m mutant) was inhibited by extracts from agar, and the same phenomenon was observed by Colter et al.²⁸⁾ on the variants of encephalomyocarditis virus which formed small and medium plaques. The small plaque formers, M strain, could not develop the large plaque even if the agar was replaced by agarose as reported by Thiry on s mutants of Newcastle disease virus²⁷⁾. It was found that the plaque size of some viruses reversed by polycation^{9) 24) 26) 28)}, but the plaques of M strain remained almost unchanged in the presence of protamin sulfate or deae dextran in agar overlay medium. Thus, the presence of inhibitor in agar specifically affecting on M strain might be denied.

SUMMARY

The improved overlay medium for plaque titrations was investigated on two strains of herpes simplex virus using GMK cells. The most favourable constituents of the overlay medium was as follows: 1.5% agar, 0.5% skim milk, 0.125% lactalbumin hydrolysate and 0.225% sodium bicarbonate in Earle's saline. The second overlay with neutral red was done after 3 days. In this system, HF and M strain developed the clear plaques with the size of 1 to 4.5 mm and 0.5 to 1.5 mm, respectively. There was a linear relationship between the virus concentration and the number of plaques. The plaque forming titer in GMK cells was as same as the pock forming titer on CAM, and higher than that in

chick embryo cells. The possibility of inhibitory effect on the plaque formation of agar component could be ruled out by the experiment of adding protamin sulfate into overlay medium or of replacing the agar by the agarose.

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