Arch. Inst. Razi, 1967, 19, 121 - 130

PLAQUE ASSAY OF AFRICAN HORSE-SICKNESS VIRUS IN AFRICAN GREEN MONKEY KIDNEY CELL LINE

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Plaque production by African horse-sickness (AHS) Virus in Rhesus monkey kidney stable (MS) cell line has been described by Ozawa and Hazrati (1964), Hopkins, Ozawa and Hazrati (1966), and Mirshamsy and Taslimi (1966). Attempts were made by these workers to use plaque morphology to distinguish between the 9 different types or between viscerotropic and neurotropic strains, but the results were not conclusive.

Susceptibility of African green monkey kidney cells to AHS Virus has been briefly reported by Ozawa and Hazrati (1964) who also tested various other cell lines such as MS, BHK, Hep-2, and FL for their susceptibility.

This report established a plaque technique, using a green monkey kidney cell line designated VERO, which was used to study various characteristics of AHS virus.

MATERIALS AND METHODS

Virus.

Seven antigenically different neurotropic strains of AHS virus (types 1 - 7) designated A501, OD, L, Vryheid, VH, 114, and Karen were obtained from the Onderstepoort Veterinary Laboratory. They were passed 102 times in mouse brains, adapted to MS cell line by 5 successive passages, then adapted to VERO cells and passed 1 to 3 times before being used in plaque experiments. Type 8 virus designated 18/60 was also obtained from Onderstepoort and adapted to cell lines after 25 passages in mouse brains. Type 9 virus, strain S2, (Hazrati and Taslimi 1963) was passed 102 times in mouse brains and adapted to tissue culture cell lines in the same manner as above. Large plaque-forming (LP) viruses of types 7 and 9 were serially purified twice by propagating selected LP virus in cell cultures then plating and selecting the largest plaques.

Cell line cultures.

The African green monkey kidney cell line (VERO) established by Yasumura and Kawakita (1963) was maintained at the National Institute of Health of Japan, and the 152nd passage was brought to Iran. MS cells established by Kanda and Melnick (1959) were also obtained from the same Institute.

Both MS and VERO cells were grown in nutrient medium (YLE) with 10% heated calf serum. Medium YLE was prepared by adding to Earle's balanced salt solution 0.5% lactalbumin hydrolysate, 0.005% yeast extract, and 0.0015% phenol red. The maintenance medium contained all these constituents except for the reduced concentration (1.0 to 2.0%) of calf serum. All these media contained 100 units of penicillin and 100 ug of streptomycin per millilitre. The final pH was approximately 7.3.

These cell lines were subcultured approximately every 4 days. Cells dispersed with 0.25% trypsin were suspended in nutrient medium to make the final concentration approximately 7-8 x 10^4 cells/ml. To each 2-ounce prescription bottle, 8 ml. of this cell suspension was dispensed. Monolayers were usually formed within 3 days' incubation at 37°C.

Agar overlay medium.

The following formula was used:

Double concentration of YLE without phenol red	100 ml.
Sodium bicarbonate solution (7.5%)	3.32 ml.
Neutral red solution (1%)	0.3 ml.
Calf serum heated at 56°C for 30 minutes	20 ml.

To the mixture warmed to 43°C in a water bath, an equal volume of 1.6% agar cooled to 43°C was added. Unless specified, Difco Noble agar washed 3 times in double distilled water was used after autoclaving 20 minutes at 10 lb/sq. in. In some experiments protamine sulphate at the concentration 0.5 mg/ml. was added before mixing with the above nutrient solutions.

Infected cultures in 2-ounce bottles were overlaid with 9 ml. of nutrient agar.

In place of agar, methyl cellulose (BDH) was used in one experiment according to the method described by Schulze and Schlesinger (1963). Infected cultures were overlaid with 9 ml. of this medium at 4°C. After 8 days incubation at 37° C, a second overlay containing agar and neutral red was applied to stiffen the methyl cellulose gel so that bottles could be inverted to examine the plaque formation.

Virus inoculation and titration.

Unless otherwise specified the following technique was used. Virus dilutions were made in maintenance medium. The growth medium was decanted from

cell cultures in 2-ounce bottles. They were infected with 0.1 ml. of diluted virus. After an adsorption period of 5 hours at 37°C. during which time the bottles were rocked every 30 minutes, 9 ml. of agar overlay medium was added. The results were calculated from a minimum average of 3 bottles to a maximum of 8 bottles per dilution.

Titration of virus in tube cultures and mice were made in the manner previously described by Ozawa et al. (1965).

Neutralization tests.

Hyperimmune sheep and rabbit sera used in the plaque neutralization tests were prepared in the manner previously described (Hazrati and Ozawa 1965). A hyperimmune horse serum was prepared by administering a total of 10 intravenous injections of S2 virus grown in mouse brains. Six months after the 1st injection 9 consecutive weekly injections followed. Serum was collected 2 weeks after the last injection. All sera were heated for 30 minutes at 56°C. before use.

For plaque neutralization tests, equal volumes of virus diluted in maintenance medium and sera were mixed and incubated for 60 minutes at 37°C. before infecting cell monolayers with 0.1 ml. of the mixture.

EXPERIMENTS AND RESULTS

Effect of diluent and washing.

The PFU titers of type 7 virus diluted either in phosphate buffered saline (PBS) or PBS without calcium and magnesium salts (PBS^-) were approximately 0.4 log lower than those diluted in maintenance medium containing 2% calf serum. Washing of VERO cell sheets with PBS^- resulted in further decreases in titers (maximum 0.8 log lower).

In subsequent work, therefore, virus was diluted in maintenance medium and cell monolayers were not washed except to remove residual unadsorbed virus when selecting large plaque forming virus.

Adsorption time.

Plaque bottles infected with type 7 virus were incubated at 37°C. for periods up to 8 hours. At 30 minute intervals bottles were removed and overlaid with nutrient agar. During the first 5 hours incubation, PFU titres increased almost linealy, and maximum titers were obtained after 6 hours' adsorption (Fig. 1).

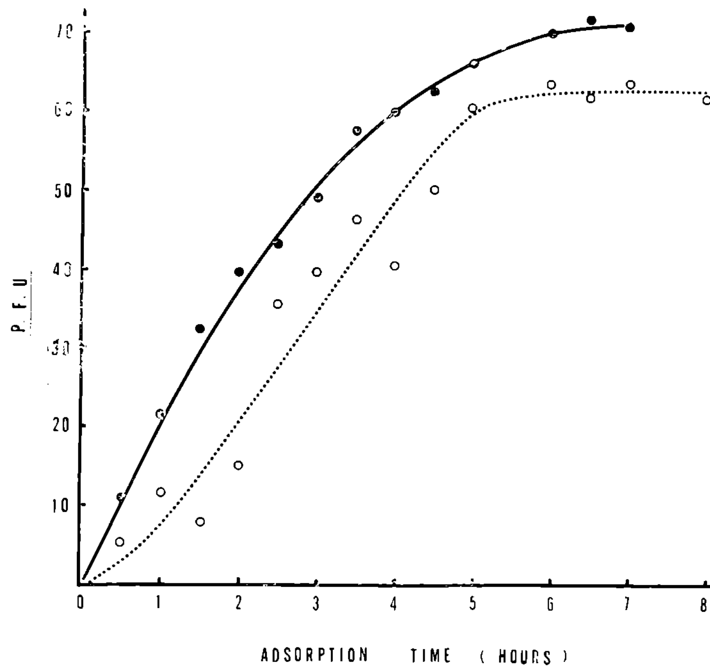

Fig. 1. Effect of time of adsorption at 37°C on number of plaque forming units (PFU) of type 7 horse-sickness virus.
The dotted line: Experiment No. 1; the solid line: Experiment No. 2 with a higher input multiplicity.

Effects of volume of inoculum, concentration of neutral red, density of cells.

When cultures in 2-ounce bottles were infected with aliquots of 0.1 to 0.5 ml. of 10^{-4} dilution of type 7 virus, the plating efficiency was greatest when 0.1 ml. was used. However, there were only small differences in titre using inoculums up to 0.5 ml. per bottle.

Infected cultures were overlaid with 9 ml. of nutrient agar containing various concentrations of neutral red - 1:41,200; 1:82,400, 1:164,800. Another set of bottles were overlaid with nutrient agar not containing neutral red and 8 days later a second overlay containing 1:41,200 neutral red was added. Neutral red at a concentration of 1:41,200 showed slight inhibitory effect, but PFU titers and the size of plaque in the others were practically the same.

The effect of the density of VERO cells at the time of infection on the plaque titer and size was studied by triplicate experiments. Cell sheets at concentrations between $7x10^5$ to $30x10^5$ cells per bottle were infected either with type 7 or type 9 virus. Neither plaque titres not size were affected by these densitites of cells.

Plaque neutralization test.

Neutralization indices of specific hyperimmune sera prepared in horse, sheep, and rabbit were tested. Using type 9 virus, neutralization indices in plaque assay of 2.66, 3.14, and 3.62 were obtained with the type specific antisera prepared in horse, sheep and rabbit, respectively. The PFU titer of type 7 virus was reduced by $10^{2.26}$ PFU with the anti-type 7 rabbit serum.

Comparison of PFU, TCID50, and MLD50 in mice.

PFU titres were estimated by using 5 to 6 bottles for each dilution. The TCID50 were titrated by using 8 tube cultures for each tenfold dilution. The MLD50 was estimated using 5 adult swiss albino mice per dilution inoculating each intracerebrally with 0.02 ml. of virus dilution. The results read on the 14th day after infection are shown in Table 1. The TCID50 titres were $10^{0.2}$ to $10^{0.6}$ higher than PFU titres and MLD50 titres were even lower than PFU titres.

TABLE I

COMPARISON OF PLAQUE FORMING UNITS, TISSUE CULTURE INFECTIVE DOSE AND MINIMAL LETHAL DOSE IN MICE.

Strain	Passes in cell cultures	Titers per millilitre		
		PFU	TCID50	MLD50
S2	8th	6.3	6.8	6.2
Karen	7th	5.8	6.4	5.5
S2	8th	6.2	6.7	—*
Karen	8th	6.2	6.4	—
S2	plaque purified	6.8	7.4	—
Karen	plaque purified	6.4	6.4	—

* not titrated.

Plaque structure for 9 serologically different types.

The nine different types of AHS virus were simultaneously examined for morphological differences. Results were read after 11 days. Those plaques measuring less than 1 mm after 11 days were considered small plaques (SP). Type 3 could be easily distinguished from the other types by its uniform large plaques (LP) averaging 4-5 mm with only 1-2% SP. Types 4, 5, 6, and 7 viruses were not easily differentiated although type 7 virus produced more uniform size plaques with only about 7% SP (Fig. 2), whereas the others had 20% SP. Types 1, 2, and 8 were

similar each giving a wide variation in plaque size from pin point to 3 mm. Type 9 virus produced smallest plaques, most of which were pin point plaques that did not develop more than 0.2 mm in size by 22 days. Largest plaques of type 9 virus were about 1.5 mm in diameter on the 11th day increasing to 3 mm on the 22nd day.

Stability of large plaque forming virus was studied by using plaque-purified viruses of types 4, 7, and 9. The non-purified original virus gave 20, 7, and 80% SP respectively with types 4, 7, and 9. After purification type 7 gave less than 1%, and type 4 three % SP observed in low dilutions of virus whereas type 9 gave 26% SP even after two successive purifications.

Effect of different agars and protamine sulphate.

Type 9 virus overlaid with Washed Noble agar formed larger plaques and gave a higher titre than with unwashed Noble agar which sometimes prevented plaque formation. The addition of protamine sulphate 0.5 mg/ml to either washed or unwashed agar overlay further increased both plaque size and titre. A large plaque forming strain of type 7 virus was unaffected by any of these overlay media. Comparing the 9 virus types overlaid with washed Noble agar and with washed Noble agar + protamine sulphate, the plaque size and titre of virus types 5, 8 and 9 was increased when protamine sulphate was present while types 2, 4 and 6 showed a decrease in plaque size and titre which was not observed when the protamine sulphate concentration was reduced to 0.25 mg/ml. The plaque size of types 1, 3 and 7 being similar with either overlay.

A new Difco product disignated "Purified agar" was employed for comparison. The following combinations were used: (A) washed Noble agar, (B) washed Noble agar + protamine sulphate, (C) unwashed Purified agar, and (D) unwashed Purified agar + protamine sulphate. Sets of cultures infected with virus types 1, 5, 7 and 9 were overlayed with these agar overlay. It was noticed with types 1, 5, 7, and 9 virus that unwashed "Purified agar" was not as good as washed Noble agar showing a slight decrease in plaque size. The addition of protamine sulphate to Purified agar increased the size of plaques of types 1, 5, and 9 virus, when compared with those formed under unwashed Purified agar. However, these plaques were still lightly smaller than those formed under washed Noble agar with protamine sulphate (Fig. 2, 3, and 5). Type 7 virus gave the same titres with A, B, C, D overlav media, the average sizes of plaques being more or less the same when read more than 2 weeks after infection (Fig. 4). Type 9 virus produced plaques only with B and D overlays. The number of plaque being less with D but the average size being almost double those formed under B.

Bottle cultures infected with types 1, 5, 7, and 9 viruses were overlaid with methyl cellulose medium. The results read on the 10th day after infection were not as clear as those under usual nutrient agar. A disadvantage was a tendency for plaques to be elongated due to the movement of methyl cellulose medium in infected bottles. No advantage was observed in the use of methyl cellulose instead of agar.

Reproducibility of plaque assay.

Type 7 virus was titrated 6 times in plaque bottles at various intervals during 28 days' storage at 4°C. Six bottles were used on each occasion each receiving 0.1 ml of 10^{-4} dilution of virus. Titres of virus after 0, 7, 11, 15, 21, and 28 days' storage were $10^{6.39}$, $10^{6.34}$, $10^{6.35}$, $10^{6.47}$ and $10^{6.37}$ PFU/ml, respectively.

DISCUSSION

The VERO cell line was found suitable for studying AHS Virus by plaque technique, although plaques were initially smaller and took longer to develop than those reported by Hopkins et al. (1966) in monkey kidney stable (MS) cell line.

Some factor present in maintenance medium but not in PBS or PBS⁻, either facilitates virus adsorption or preserves infectivity of virus during the adsorption period. Rhim and Hamon (1963) also found a medium containing glucose and bovine albumen superior to PBS and PBS⁻, and by using this medium that washing with PBS before or after adsorption had no effect on the plaque counts.

The time required for maximum virus adsorption, 6 hours, is double that required in MS cells reported previously (1966). However, as a routine 5 hours adsorption is adequate and better suited for our work programme.

The fact that cell concentrations of 7 to 30 x 10^5 cells per 2-ounce bottle had no effect on plaque size and titre eliminates the necessity for accurate cell counting and for selecting accurate timing of infection.

Comparing PFU, TCID50, and Mouse LD50 titres in African green monkey cells, results were similar to those obtained using MS cells (Hopkins et al. 1966) where the PFU titre was lower than the TCID50 titre up to the 24th passage of virus in MS cells and the mouse LD50 titre decreased as the passage in cell culture increased.

The morphology of plaques produced by AHS virus strains could not be used with accuracy to differentiate different types of virus, because of the large variation in plaque sizes and even in the variation of SP and LP on different occasions. It was however, noticed that type 3 virus (strain L) produced uniform large plaques both in VERO and MS cell cultures. The average plaque size was larger than any other strains used in the present experiment. The addition of 0.5 mg protamine sulphate per ml of agar overlay medium did not always increase the size of SP and under some conditions decreased the plaque size. This is at variance with the results previously reported when using MS cells. However the addition of protamine sulphate always increased the size of type 9 virus and was beneficial whenever using unwashed agar.

Plaques developing using unwashed Difco Purified agar overlay were not as large as those obtained with washed Noble agar. The authors found no advantage in the use of methyl cellulose overlay instead of agar.

The PFU titre determined by the standardized plaque assay method was reproducible with high accuracy. It was also confirmed that the virus grown in

MS and GM tissue cultures is stable at 4°C without any loss of plaque titre after 4 weeks.

It has been established that VERO cells may be used for plaque assay with AHS virus in place of MS cells with the advantages that the density of VERO cells at the time of infection is not a critical factor for plaque size, the edges of plaques are clear and smooth. The VERO cells used are more resistant to chemical and physical treatment than MS cells, and the overlaid cultures may be kept at 37°C up to 4 weeks after infection.

SUMMARY

Factors affecting plaque formation of AHS virus in African green monkey kidney (VERO) cell line cultures were studied, and the technique of plaque assay was standardized. The technique was employed to estimate plaque reduction by various antisera and to compare the structures of plaques produced by the 9 antigenically different types of AHS virus. Protamine sulphate at 0.5 mg/ml. did not always increase the size of plaques, and even reversal effects were seen with certain large-plaque forming viruses. The sensitivity of plaque assay technique was compared with tissue culture infective dose and titration by intracerebral inoculation in mice. Although PFU titres were not always as high as TCID50, plaque assay was reproducible and accurate.

ACKNOWLEDGMENTS

This work was undertaken at the Near East Animal Health Institute, a project established by the United Nations Development Programme/Special Fund through the Food and Agriculture Organization in cooperation with the Ministry of Agriculture in Iran.

The authors thank Dr. M. Kaveh of the Razi Institute, Dr. W. M. Moulton and Dr. E. Traub of the Near East Animal Health Institute for their advice and support, and Dr. S. Bahrami for his technical support.

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Fig. 2 — 5. Effect of protamine sulphate in two different agars. (A) washed Noble agar, (B) washed Noble agar + protamine sulphate, (C) unwashed Purified agar, and (D) unwashed Purified agar + protamine sulphate.

Fig. 2 Plaques of type 1 virus 15 days after infection. (x ½).

Fig. 3. Plaques of type 5 virus 15 days after infection. (x ½).

Fig. 4. Plaques of type 7 virus 19 days after infection. (x ½).

Fig. 5. Plaques of type 9 (S2) virus 19 days after infection. (x ½).