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THE FORMATION OF PLAQUES BY AFRICAN HORSE SICKNESS VIRUSES AND FACTORS AFFECTING PLAQUE SIZE (*)

by H. Mirchamsy and H. Taslimi

ABSTRACT

Under agar overlay, large and small plaques were observed for African Horse Sickness viruses.

The purification of large plaques by cloning was possible. By addition of protamine sulfate to the agar overlay only large plaques were produced. The plaque size variation can not be a suitable marker for determinatoin of variants of this virus since under methyl cellulose and starch gel overlays homogenous plaques were noticed.

Recent studies by Mirchamsy and Taslimi (1), demonstrated that strains of African Horse Sickness Virus (A H S V) may differ in antigenicity.

In order to improve the antigenic quality of vaccine strains of AHSV, studies were conducted with tissue culture adapted viruses to plaque purification.

The plaque technique developed by Dulbecco (2) was applied to A H S V.

During an evaluation of plaques number under agar overlay we noted variations in plaque size in all types of virus which were studied. It was then essential to study the causes of these variations. Several factors for plaque size variations, suggested by various investigators were studied, and data resulting from these observations are included in this report.

Materials and Methods

Virus strains — The African mouse adapted type 4, strain (V R Y) previously adapted to M S cells was used in all experiments. The plaque forming ability of

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the following African vaccine viruses type 1 (A 501), type 2 (O D), type 3 (L), type 5 (V H), type 6 (114), type 7 (Karen), African wild type 4 (S 205), Asian mouse adapted type 9 (S2-Shiraz) and Asian wild type 9 (S 10/60) were studied. The African types were kindly supplied by Onderstepoort laboratories of South Africa. All viruses had undergone several passages in M S cells prior to use in this study.

Stocks of viruses were prepared by inoculation of M S cells in 2-oz bottles. The bottles were incubated at 36°C and were harvested when 80 to 100% of the cells exhibited cytopathic changes. The cultures were quickly frozen, thawed and cell free medium was lyophilized in small ampoules sealed and kept at — 20°C. All plaque experiments were made with the same lot of lyophilized viruses. Different factors such as pH, period of virus absorption, composition and amount of overlay, temperature and duration of incubation, staining with neutral red and plaque counts were controlled as strictly as possible in all experiments. Techniques for determination of T.C.I.D.50/ml or virulence of viruses for mice are described in our previous reports.

Plaque assay. Line M S of monkey kidney were grown in 2-oz prescription bottles for 3-4 days. The growth medium consisted of 0.5% lactalbumine hydrolysate in Earle's balanced salt solution and 5 mg% yeast extract (Difco) plus 5% inactivated calf serum and antibiotics. The bottles were seeded with 1 x 10^6 cells in 6 ml medium and incubated 3-4 days at 36°C. Uniform monolayer was washed once with Dulbecco's phosphate buffer and inoculated with 0, 3 ml of virus diluted in the above medium. The bottles were then incubated for 3 hours at 36°C with occasional shaking after which they were overlaid with nutrient agar. The nutrient agar was prepared from 2 stock solutions. Solution 1 contained 2 per cent special Nobel Agar washed 6 times in bidistilled water, melted and cooled to 41°C in a water bath. Solution 2 was a 2 x medium used for growing cells but serum was omitted from overlay. This solution was also heated to 41°C. Equal parts of the two solutions were mixed before use and pH was adjusted to 7.4 with 7.5% NaHCO3. The excess seed fluid was first removed from the cultures and 6 ml of this overlay was then poured in each bottle. After solidification of the overlay the bottles were inverted and incubated in 36°C.

Enumeration of plaques was carried out after an incubation of 3 days. From a 0.1% aqueous neutral red solution, a 1 in 50,000 solution in the growth medium without serum was prepared. 2 ml was added to each agar surface and returned to the incubator for 1 hour to allow the dye penetration.

Bottles were then removed, reinverted and kept at 36°C. Plaques were counted after 12 and 36 hours.

Virus purification — Two successive passages of large and small plaques were made by using bottles containing a few plaques of type 4 (V R Y). In order to avoid possible contamination of plaques with viruses drained vertically into agar surface as observed by Mosley *et al* (3), before each trial the agar overlay of the area immediately surrounding a plaque was removed. The plaques were then picked out for subculture in M S cells and were transferred with a Pasteur pipette into 2 ml of nutrient medium as seed for further assays.

Protamine sulfate assay — The agar overlay was supplemented in some experiments with protamine sulfate (N.B.C.) to a final concentration of 0.4 mg/ml.

Neutral red trial — For study of the effect of neutral red on the plaque formation and size, the dye was incorporated in agar overlay to a final concentration of 1/50.000.

Methyl cellulose and Starch overlays —Methyl cellulose overlay consisted of 1.5% methyl cellulose (Fisher Scientific Co. 4000 centripoises, U.S.P.) with YLE medium and antibiotics without serum. The methyl cellulose was brought into solution by autoclaving the suspensions as described by Hotchin (4). 6 ml of this overlay was poured on to each 2-oz prescription bottle of M S cells infected with proper dilutions of A H S V, type 4 (V R Y). Starch gel overlay used by De Maeyer and Schonne (5) was prepared as suggested by the authors (6) as follows: ten grammes of Starch hydrolysed for gel electrophoresis (Connaught Med. Res. Lab. Toronto, Canada) was suspended in 20 ml of cold bidistilled water. This suspension was added with continuous stirring to 54 ml of boiling bidistilled water, was allowed to boil 10 minutes until it became transparent. The suspension was then cooled to 50°C. and 24 ml of 5 times concentrate (Y L E) previously warmed to 50°C was added. The pH of the overlay was then brought to 7.4 with a solution of 7.5% $NaHCO^3$. 6 ml of this overlay was poured on each 2-oz prescription bottle of M S cells previously infected with different dilutions of A H S V type 4 (V R Y).

Procedures for staining plaques under these two overlays with neutral red and the time for counting plaques were similar to that described for agar overlay. The methyl cellulose was however removed before staining by cooling bottles for one hour in a refrigerator, during which time the gel was liquefied.

Results

Plaque count — Inoculation of M S cells with either mouse adapted or wild types of A H S V resulted in the formation of plaques within 48 hours at 36°C. Microscopic examination of plaques at 48 hours showed necrotic areas without intact cells. Cultures stained after 72 hours of incubation showed a mixture of large and

small plaques. Under conditions of these experiments the number of plaques found in each bottle was directly proportional to the concentration of the original inoculum and a linear relationship was found for all viruses studied. In repeated experiments utilization of the same virus resulted in almost the same ratio of large to small plaques if the environmental factors were identical. The plaque types were referred to as small (less than 1 millimeter) and large (2 to 5 millimeters). The results of a representative series of experiments are presented in table 1. In all instances small and large plaques were observed 5 days after inoculation of the viruses (Fig. 1 to 9).

Observation on virus purification — Five single large and small plaques of type 4 (V R Y) were cultivated first in M S cells in Y L E medium, and then assayed for plaque formation under Nobel agar. Uniform large plaques were obtained in all cases (Fig. 11). Characteristics of these plaques are given in table 2. Attempts to isolate homogenous small plaques failed and in all 5 cases a mixture of large and small plaques were observed.

Effect of Protamine sulfate — In the presence of protamine sulfate in agar overlay medium, Strain V R Y, type 4 of AHSV which regularly showed large and small plaques, produced plaques not significantly different in Size. The smallest plaques had a diameter of about 1 mm (Fig 10).

Table 1. Plaque titration of A H S V on M S cells under agar overlay 5 days after infection

Virus	Origin	Passage level in M S cells	Titer		
			Neg. log. T.C.I.D./50 ml	P.F.U. ml	Neg. log. L.D.50/ml (mice)
Type 1	Mouse adapted vaccine strains	7	7.0	7.0×10^{6}*	7.90
Type 2		8	7.0	2.15×10^{8}	7.99
Type 3		9	6.5	1.2×10^{7}	7.72
Type 4		9	7.0	4.0×10^{7}	7.75
Type 5		8	6.5	5×10^{6}	8.21
Type 6		9	6.5	1.7×10^{7}	7.21
Type 7		8	6.5	6.2×10^{6}	7.36
Type 9		16	7.0	2.5×10^{7}	7.89
Type 4 (S. 205)	Virulent strains	4	5.0	2.6×10^{5}	3.51
Type 9 (S 10/60)		9	6.0	1.66×10^{7}	6.51

*Bottles in triplicate

Effect of neutral red — Neutral red when incorporated in agar overlay has been found toxic to many tissue culture systems. According to Waterson (7) this toxicity manifests itself by a reducion in number and clarity of the plaques in the case of fowplague.

Crowther and Melnick (8) have found that neutral red, at high dilutions which have no visible influence on the cells nor on the cytopathic response to virus, together with only small amounts of light, reduces the normal yield of infective poliovirus considerably.

Several batches of neutral red which we have assayed in overlay have been found slightly toxic for M S cells.

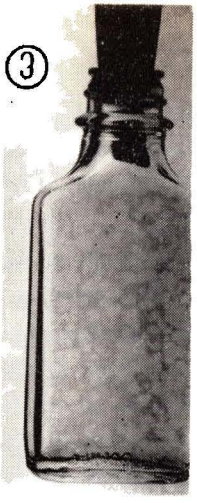
In some experiments incorporation of dye into agar overlay (Nobel or Difco Agar) delayed appearance of the first plaque until the 7th day. When first visible, the diameter of plaques was about 1 mm which increased in a week or so to a maximum of 3 millimeters. The distinctness of small and large plaques in this case was more difficult. A comparison between plaques of type 4 (V R Y) under Nobel agar with neutral red in overlay or added 72 hours after inoculation indicated that the latter method was more sensitive. Plaques stained three days after infection were distinct in 5 days and could be counted easily (3.65 x 10^7PFU/ml). In the former case plaques were observed 7 days after infection but the count was not possible until 12 days. The number of plaques was also significantly decreased (1.2 x 10^7PFU/ml).

Characteristics of plaques formed under methyl cellulose and starch — Comparative titrations of A H S V types 4 (V R Y) and 9 (S2-Shiraz) in M S cells were carried out as follows: The bottles of cells were incubated with 0.3 ml of 10^{-4}, 10^{-5} and 10^{-6} of virus (3 bottles for each dilution). After an adsorption time of 3 hours the excess

Table II. Characteristics of 5 large plaques isolated from A H S type 4 under agar overlay

Plaque No	Titer		
	P. F. U. ml	neg. log. L.D. 50/ml (mice)	neg. log. T.C.I.D./50 ml
1	3.6 x 10^7*	8.69	6.5
2	4.2 x 10^7	8.65	7.0
3	3.8 x 10^7	8.51	7.0
4	3.69 x 10^7	8.75	6.5
5	3.55 x 10^7	8.80	6.5
Original type 4 (V R Y)	4.0 x 10^7	7.75	7.0

*Bottles in triplicate



Figs. 1-9. Plaques 5 days post inoculation with 0.3 ml of 10^{-5} different types of mouse adapted vaccine Strains.

Fig. 10. Type 4 Strain VRY, AHSV, Agar overlay treated with Protamine Sulfate.
Fig. 11. Large plaques Pl, Type 4, Strain VRY of mouse adapted AHSV. 5 days post inoculation with 0.3 ml of 10^{-6}.

Fig. 12. Type 4 Strain VRY, AHSV. Normal agar overlay (Control).

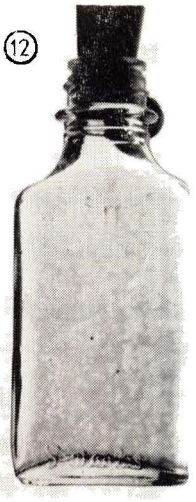


Table III. Comparative plaque titration of A H S V under different overlays (P F U/ml)

Virus	Overlays		
	Agar	Methly cellulose	Starch gel
Type 4 (Strain V R Y)	3.6×10^{7}*	5.2×10^{7}*	7.7×10^{7}
Type 9 (Strain S2 Shiraz)	2.8×10^{7}	4.4×10^{7}	6.2×10^{7}

*Bottles in triplicate

seed was removed and the bottles were divided into 3 groups. An agar overlay was added to one group, a methyl cellulose overlay to the second group and a Starch gel overlay to the third group. No infected controls were included in each experiment. This experiment was repeated 4 times. After 5 days a heterogenous population of small and large plaques were observed under agar overlay. At this time the plaques under methyl cellulose and starch gel were homogenous with an average size of 1 millimeter. The plaque counts for one experiment are given in table 3.

Discussion

Within the limits of these observations, it appeared that all types of A H S V studied, showed two distinct types of large and small plaques under agar overlay in M S cells. As the environmental and nutritional factors were carefully controlled and were identical in most of the experiments, these factors can not be the origin of plaque size variations.

Although the mechanism of the inhibitory effect of neutral red on A H S V plaque is obscure, it is evident that when the dye is added to the agar at the time of pouring bottles, the plaques will not be visible until seven days; variations in size will be less evident and the plaque count will be affected.

The fact that a significant increase in plaque size of the small type occurs by addition of Protamine sulfate to the Nobel agar, supports the observations of Takemoto and Liebhaber (9), Rapp (10) and Brown and Packer (11) who demonstrated the presence of a polysaccharide sulfate inhibitor in agar which can be eliminated by addition of Protamine sulfate.

The purification of large plaque variants was possible by cloning. This variant gave consistently large plaques and seems to be more virulent for mice, than the parent strain. Attempts to isolate small plaques failed, and in all instances a contamination with large plaques was observed.

In some preliminary experiments not reported here it was found that the number of large plaques may be increased considerably (50 to 100%) by allowing a certain interval of 30 to 60 minutes between removal of inoculum and addition of the agar overlay. This phenomenon indicates the interfering effect of agar overlay with an early interaction between some of virus particles and cells.

From data presented here one can assume that although all laboratory adapted or virulent types of A H S V studied consisted of two variants, different in size of plaques under agar overlay, this variation can not be used as a useful marker for isolation of A H S V variants since under methyl cellulose, starch or agar overlay supplemented with Protamine sulfate plaque size variation was not observed.

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