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DEVELOPMENT OF PLAQUE TECHNIQUES FOR TITRATION AND NEUTRALIZATION TESTS WITH AFRICAN HORSE-SICKNESS VIRUS (*)

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SUMMARY

Factors affecting plaque formation of African horse-sickness (AHS) virus in monkey kidney stable (MS) cells were studied, and the technique of plaque assay was standardized. Methods for neutralization tests and purification of virus using the plaque technique were also established. Using a standardized technique, sensitivity of plaque method was compared with tissue culture infective dose and titration by intracerebral inoculations in mice. The structures of plaques produced by several strains of AHS virus were compared. Differences were found in titer and size of plaque of the virus cultures in MS cell lines purified by cloning. The addition of protamine sulfate into the nutrient agar increased the size of plaques. Methyl cellulose was also used in place of agar.

Adaptation of neurotropic strains of African horse-sickness (AHS) virus to the monkey kidney stable (MS) cell line by Ozawa and Hazrati,[13] led to development of vaccine produced in MS cell cultures[14] instead of mouse brains. Although methods of titration and virus neutralization in tube cultures of MS cells have been established,[6] development of a plaque assay technique was needed in several aspects of virus studies.

Using trypsinized monkey kidney cells, Dulbecco and Vogt[2] developed a poliomyelitis virus assay by plaque technique, each virus particle producing one plaque. The technique was simplified by Hsiung and Melnick,[8] who used stoppered, flat prescription bottles instead of unsealed petri dishes. This report describes standardized techniques of plaque assay and plaque neutralization tests

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using 2-oz. prescription bottles. The techniques were used to compare plaque forming unit (PFU) with TCID50 and LD50 titers. Plaque structure of Asian strains and 9 types of AHS virus were also studied.

Materials and Methods

Virus.—The neurotropic attenuated type 9 Iranian strain (S2),[5] adapted to MS cell culture after 102 passages in mouse brains, was used in almost all of the experiments. However, viscerotropic Iranian strain (10/60),[16] adapted to MS cell culture after 4 passages in mouse brains, was used in 2 experiments, and virus purified by selecting single plaques for propagation was also used.

The structure of plaques of 7 viscerotropic strains of type 9 virus collected in Asia were compared: 4 Iranian strains (2/63, 4/63, 6/63, 7/63) from sporadic cases of the disease in Iran in 1963; 1 Turkish strain (Turkey) from infected horse blood; and 1 Indian strain (Indian 4) from the state of Madhya Pradesh, India. These were all adapted to MS cell culture after 5 passages in mouse brains and 1 strain from Pakistan (7/60), which was received from the Onderstepoort Veterinary Institute, South Africa, and was adapted to MS cell culture after 16 mouse brain passages.

Seven antigenically different neurotropic strains (types 1 to 7) were also obtained from Onderstepoort and designated A501, OD, L, Vryheid, VH, 114, and Karen. They were adapted to MS cell cultures after 102 passages in mouse brains.

Two other strains of type 9 virus isolated in India and Turkey, were also compared. The Indian strain (Indian 6) a neurotropic virus was received at the 105th mouse passage from the Indian Veterinary Institue, Mukteswar, and was passed through 2 more mouse brain passages before being adapted to MS cell culture. The Turkish strain from mouse brain (Elazig) was adapted to MS cell culture after 60 mouse brain passages.

Methods of adaptation of the virus to MS cells and passage of the virus in MS cell cultures have been described.[13] Fluid containing virus was centrifuged 10 minutes at 1,174 g, and the supernatant was dispensed in hemagglutination tubes fitted with rubber stoppers. All fluids were stored at 4 C.

Tissue Culture Cells.—The MS cell line was used. Cells were grown in bulk in 1-L. Roux flasks. The monolayers formed after 72 hours were washed once with phosphate buffered saline solution without calcium and magnesium salts (PBS^-) then trypsinized with 0.25% trypsin solution in PBS^-. The trypsinized cell suspension was diluted with an equal volume of growth medium and centrifuged 5 minutes at 150 g. The cellular sediment was diluted with growth medium to make the final concentration 1.5×10^5 cells per milliliter. Eight milliliters of this cell suspension was put into the plaque bottles (2-0z. prescription bottles). Monolayers were formed after 72 hours at 36 C., and the bottle cultures were used for titrations after 72 or 96 hours of incubation.

Growth Medium.—The growth medium was Earle's balanced salt solution containing 0.005% yeast extract, 0.5% lactalbumin hydrolysate, 0.0015% phenol red, 10.0% calf serum (heated 30 minutes at 56 C.), 100 units of penicillin/ml.,

100 microgramme of streptomycin/ml., and enough sodium bicarbonate to adjust the pH value to 7.2.

Maintenance Medium.—The maintenance medium was like the growth medium excepting calf serum, reduced to 2%.

Agar Overlay Medium.—The following formula was used:

1.6% special Agar-Noble *	50.0 m.
2 times concentrated maintenance medium (without phenol red and calf serum)	50.0 ml.
7.5% aqueous sodium bicarbonate solution	1.66 ml.
1.0 aqueous neutral red solution	0.15 ml.
Calf serum (heated)	10.0 ml.

Special agar washed 3 times in double distilled water (DDW), was dissolved in DDW to make a 1.6% solution, and was sterilized by autoclaving 20 minutes at 10 lb. of pressure per square inch. The melted agar was cooled to 43 C., and the other constituents, previously warmed to 43 C., were added just before the agar was used. Nine milliliters of this medium was pipetted onto the glass surface of each prescription bottle while holding the MS culture uppermost. When the agar had cooled to approximately 38 C. and floccules appeared the bottle was turned so that the agar set over the monolayer. The bottles were left for 30 minutes, at ambient temperature, before being placed with the agar overlay medium uppermost at 36 C., unless otherwise specified.

Inoculation.—Tenfold dilutions of virus were made in maintenance medium. Unless otherwise specified, the following technique was used to inoculate the plaque bottle cultures: After the MS cell growth medium was decanted, 0.1 ml. of virus dilution was placed on the cell sheet in each bottle. The virus was adsorbed 3 hours at 36 C. before 9.0 ml. of agar overlay medium was added. The cells were not washed before or after inoculation.

Titrations of virus in tube cultures were made in the manner previously described. [13]

Antiserums.—Three rabbit antiserums, used in the plaque neutralization technique, were prepared in the manner previously described. [6] Antigens were 6th passage of strain 10/60, 60th passage of strain Elazig, and 102nd passage of strain S2 viruses in mouse brains. Antiserums and control normal rabbit serums were heated 30 minutes at 56 C. before use.

Plaque Neutralization Test.—The standardized plaque technique was used in neutralization test of plaque-forming units. Equal volumes of tenfold virus dilutions and virus antiserums were mixed and incubated 1 hour at 36 C., then 0.1-ml. portions

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* Special Agar-Noble, Difco Laboratories, Detroit, Mich.

were inoculated in MS cell bottle cultures. They were adsorbed at 36 C., and then 9.0 ml. of overlay medium was added.

Methyl Cellulose Overlay.—Methyl cellulose * * was repeatedly washed with absolute ethyl alcohol and ether, and was air dried as described by Rapp *et al.* [17] Two grams of methyl cellulose was suspended in 50 ml. of DDW at 80 to 100 C., and then immediately autoclaved at 121 C. for 20 minutes. The suspension was cooled to 45 C. when an equal volume of double strength nutrient medium was added. Eight milliliters of this mixture was overlaid in each bottle culture. After incubation at 36 C. for 5 days, 8.0 ml. of standardized agar overlay medium was added.

Experiments and Results

Comparison of Different Agars.—Washed and unwashed Bacto agar, * Special agar-Noble, * and Bacto Nutrient agar * were compared.

Washing was done by suspending 20 Gm. of agar in 1 L. of DDW. The agar suspension was kept at 4 C. until the agar settled, then the DDW was siphoned. This was repeated 3 times. The agar was then dissolved in 1 L. of DDW, dispensed in 100-ml. volumes, and autoclaved 20 minutes at 10 lb. of pressure per square inch. Unwashed agar was prepared in the same manner, but the washing was omitted.

The 10th passage of strain S2 virus was used, 0.2 ml. of virus suspension being adsorbed 30 minutes before 10.0 ml. of agar overlay medium was added. The titer was read after incubating 12 days at 36 C.

At the end of the test, Special agar-Noble washed 3 times in DDW gave the highest titer and the largest plaques (Table 1), and this was used throughout the remainder of the experiments.

Concentration of Agar and Volume of Overlay.—Bottle cultures were overlaid with 6.0-, 8.0-, 10.0-, and 12.0-ml. volumes of 0.6, 0.8, 1.0, and 1.2% agar overlay medium. The 5th passage of strain S2 virus was used to inoculate bottle cultures; 0.2 ml. of virus dilution was adsorbed 3 hours at 36 C. before the agar overlay medium was added. Results were read after 10 days.

The titer of virus did not vary significantly in the tests. There was little variation in plaque size, although those developing in 1.2% agar were smaller than in the others. With 0.6% agar, difficulty was encountered, because the agar overlay medium occasionally collapsed when the bottle was inverted. With 6 and 12 ml. of overlay medium, it was sometimes difficult to read results.

Therefore, Special Agar-Noble washed 3 times in DDW was used at a final concentration of 0.8% in the overlay medium with 9 ml. for each 2-oz. bottle culture.

Concentration of Neutral Red.—Two-ounce bottle cultures inoculated with 14th

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* * Methyl Cellulose, Methocel 4,000 centipores, Dow Chemical Company, Midland, Mich.

* Bacto agar, Special Agar-Noble, and Bacto Nutrient agar, Difco Laboratories, Detroit, Mich.

passage of strain S2 virus in MS cell cultures were overlaid with 10 ml. of agar overlay medium containing varying concentrations of neutral red *—1:28,000, 1:37,300, 1:55,900, 1:74,500, and 1:111,800. Also 1 set of bottles was overlaid 1st with agar overlay medium without neutral red, a 2nd overlay containing neutral red 1:74,500 was added on the 5th day of incubation at 36 C. Plaque counts were made 10 days

TABLE 1—Comparison of Effect of Several Agars Used in Overlay Medium on Plaque Forming Units (PFU) and Plaque Sizes in Monkey Kidney Stable Cell Cultures

Agar and its washing		PFU			Plaque size (mm.)	
		10^{-4}	10^{-5}	10^{6}	Largest	Average
Bacto agar	Unwashed	45	3	1.9*	0.5	0.3
	Washed	107	10	5.3	1.0	0.5
Special Agar-Noble	Unwashed	55	6	2.9	0.7	0.3
	Washed	122	14	6.6	2.0	0.7
Bacto-nutrient agar	Unwashed	0	0	10^{4}**	..	..

* Average PFU per milliliter ** Less than 10^{4} PFU/ml.

after bottles were inoculated. The 2 highest concentrations of neutral red in the agar overlay medium destroyed the cell sheet within 3 days after overlaying. Plaque size and titer was not affected by the other neutral red concentrations or by time of adding neutral red overlay medium. Neutral red at a concentration of 1:74,500 incorporated in the first agar overlay medium was used for future work.

Number of Cells.—Two sets of bottle cultures were prepared in similar manner, 1 set, however, was prepared 4 days later. When the age of cultures became 7 days and 3 days, respectively, the cells were counted. The 7-day-old culture contained 4.5×10^6 live cells per bottle, and the 3-day-old culture had 3.2×10^6 cells per bottle.

Other bottle cultures were inoculated using 5th passage of strain S2 and Indian 4 viruses. After adsorbing 3 hours at 36 C., they were overlaid with agar. The results were read 10 days after incubation at 36 C.

In cultures inoculated 3 days after preparation, plaques 1.0 to 1.5 mm. were produced by both strains of virus, but plaques formed on cultures inoculated 7 days after preparation were too small to measure.

This indicated that plaque size was affected by density or age of the cells or both. Therefore, in subsequent work, cultures were used 3 to 4 days after preparation.

Effect of Diluent and Washing.—Ten-fold dilutions of 21st passage of plaque-purified 10/60 virus were prepared, using 2 diluting fluids: maintenance medium and PBS^{-}. Their pH values were 7.0.

One set of bottle cultures was left untreated after the growth medium was decanted, and the other set was washed twice with 5.0 ml. PBS^{-}. These cultures were inoculated with 0.2 ml. virus inoculation per bottle.

* Merck AG, Darmstadt, Germany.

The PFU titers of virus diluted in PBS⁻ were lower than those of the same virus diluted in maintenance medium (Table 2). From the results, it appeared that washing of cultures with PBS⁻ did not always increase PFU titers. Significant differences in plaque size were not observed.

Therefore, in subsequent work, unwashed 3-day-old cultures were inoculated with virus diluted in medium.

TABLE 2—Effects of Virus Diluting Fluid and Washing Cultures Prior to Infection on Plating Efficiency

Preparation of diluent		Av. PFU/ml.
Virus diluent	Cell washing	
Maintenance medium	Not washed	1.6×10^7
	Twice with PBS⁻	1.9×10^7
PBS⁻	Not washed	5.3×10^6
	Twice with PBS⁻	4.3×10^6

PBS⁻ = Phosphate buffered saline solution without calcium and magnesium salts, and PFU = plaque forming units

Adsorption Time.—Inoculated cultures were overlaid with agar overlay medium as indicated (Table 3). Each bottle culture was inoculated with 0.2 ml. of virus. In 2 experiments, the maximal PFU titer was obatined at 3 hours of adsorption at 36 C.

TABLE 3—Effect of Time of Adsorption at 36 C on Number of Plaque Forming Units (PFU)

Adsorption time (hr.)	Average PFU on 6 culture bottles	
	S2 virus plaque-purified 10/60 (passage 23)*	Type 6 virus, strain 114 (passage 6)**
½	12	
1	34	
1½	87	79
2	71	92
2½	89	120
3	139	137
3½	134	134
4	107	127

* 10^{-5} dilution of virus ** 10^{-4} dilution of virus.

Effect of Volume of Inoculum.—Measured amounts of 5th passage of strain S2 virus were used as inoculum (Table 4).

TABLE 4—Effect of Volume of Virus Dilutions on Plating Efficiency

Volume of inoculum (ml.)	Av. PFU/ml. × 10^5	
	Experiment 1	Experiment 2
0.1	6.7	4.1
0.2	5.3	2.8
0.3	4.2	3.0
0.4	3.4	2.1
0.5	3.4	2.1

PFU = Plaque forming units.

The plating efficiency was greatest when the smallest volume (0.1 ml.) of virus dilution was used as inoculum, and the titer per milliliter decreased as the volume of inoculum was increased.

Reproducibility and Precision of Plaque Assay.—Two strains of virus (23rd passage of strain 10/60 and 6th passage of strain 114 viruses) were stored at 4 C. and

TABLE 5—Reproducibility of Plaque Assay on Similar Suspensions of Virus

	No. of PFU/bottle culture				
	Strain 10/60 virus (dilutions)		Strain 114 virus (dilutions)		
Experiment	10^{-4}	10^{-5}	10^{-4}	$10^{-4.7}$	10^{-5}
1	126	12	118	30	18
2	140	14	126	25	11
3	152	17	154	22	20
4	152	12	126	26	11
5	172	16	142	22	16
6	120	10	115	16	12
7	111	10	110	27	16
8	143	13	110	26	17
Average	139	13	125	24	15
Log PFU/ml.	7.14	7.11	7.1	7.09	7.18

PFU = Plaque forming units.

used for plaque assays in different batches of MS bottle cultures.

Plaque assay by the standardized method was quite reproducible and accurate (Table 5; Fig. 1).

Plaque Neutralization Test.—In this experiment, 6th passage of type 6 virus (Strain 114) and 3 rabbit anti-type-9 virus serums (anti-S2, anti-10/60, and anti-Elazig strains) were used to investigate the serologic relationship between type 6 and type 9 viruses. For the control, heat inactivated normal rabbit serum was used.

It should be noticed that PFU of type 6 virus were neutralized by antiserums against type 9 virus, the titers being lowered approximately 10,000 times (Table 6). Similar partial cross neutralization between the 2 viruses types was repeated using MS cells in tubes. [6]

Comparison of Plaque-Forming Units, Tissue Culture Infective Dose, and Mouse Lethal Dose.—Serial passages of S2 virus strain in MS cell cultures were made, and the virus fluids of 5th, 14th, 50th, 75th, and 109th, passages were collected and then kept at 4 C. Dilutions of virus were made in maintenance medium. Three plaque bottle cultures were inoculated with each dilution of virus and were overlaid with agar overlay medium following the standardized technique. Four tube cultures were inoculated with each of the same virus dilutions, using 0.1 ml. of inoculum per tube culture. After adsorption 30 minutes at 36 C., maintenance medium was added to each tube. Also 4 mice were inoculated intracerebrally with each virus dilution, each being given 0.05 ml. of inoculum. The test results were read 10 days after inoculation.

TABLE 6—Neutralization of Type 6 Virus (Strain 114) by Rabbit Antiserums Against Type 9 Virus

Antiserums	No. of PFU by dilutions						
	10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Normal serum	TMTC	TMTC	TMTC	TMTC	280	30	3
Anti-S2 serum	250	20	2	0	0	0	0
Anti-10/60 serum	280	45	2	0	0	0	0
Anti-Elazig serum	TMTC	28	4	0	0	0	0

TMTC = Too many to count; PFU = plaque forming units.

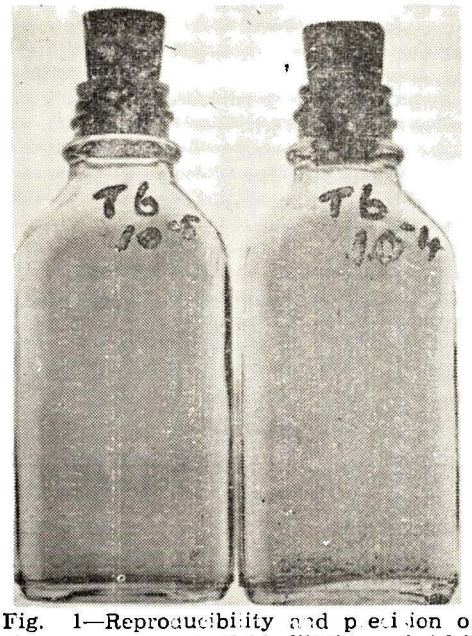

Fig. 1—Reproducibility and precision of plaque assay on tenfold dilutions of African horse-sickness type 6 virus (strain 114) inoculated on monkey kidney stable cell cultures at 14 days after inoculation. Ninety-five plaques at 10^{-4} dilution of virus (right), and 10 plaques at 10^{-5} dilution (left).

The PFU titers of 5th, 14th, and 24th passages of the virus were less than the titers in tubes, whereas titers of 50th, 75th, and 109th passages were greater than TCID50 titers (Table 7). The minimal LD50 of mice decreased as the virus passage level increased.

Plaque Structure for Virus Isolated in Asia.—Ten isolates of AHS virus collected in Iran, Turkey, India, and Pakistan were adapted to MS cell cultures after varying passage levels in mice. Using standardized plaque assay technique, plaque structure of these viruses was studied.

Although differences in size were found in plaques produced by different strains of the virus, all strains developed plaques of various sizes ranging from almost invisible by the unaided eye to 2.3 mm. maximal size (Table 8). However, it would be impossible to identify or distinguish one strain from another on the basis of plaque structure.

Plaque Structure for 9 Types of Virus. — Nine antigenically different types of AHS virus, types 1 to 8 and S2 (type 9), were compared in a test for characteristic differences in size or morphologic structure of plaques. These strains were used after 5 passages in MS cell culture.

The strains developed plaques of various sizes (Fig. 2). Types 1 and 9 viruses produced small plaques averaging 0.5 mm.; plaques of Types 2 and 6 averaged 1.0 mm.; whereas the remaining types had plaques averaging 1.5 mm. in diameter.

There was a variation in diameters of the largest plaques obtained with the

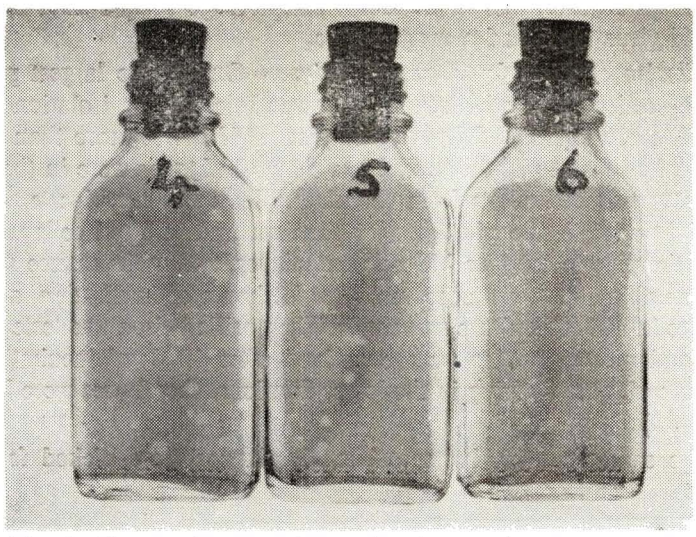

Fig. 2—Plaque structure for antigenically different types of African horse-sickness virus. Types 4, 5, and 6 (marked 4, 5, and 6) had various sizes of plaques.

strains. The largest plaques with types 3, 4, 8, 7, and 5 viruses measured 7.0, 5.0, 5.0, 4.5, and 3,5 mm., respectively. Types 2 and 6 viruses had plaques measuring 2.0 mm., whereas those of types 1 and 9 were 1.0 mm. (However, in previous work, plaques measuring 6.0 and 4.5 mm. were developed by types 6 and 9 viruses).

TABLE 7—Comparison of Sensitivity of Plaque Assay, Tube Culture and Intracerebral Titration in Mice

	Log_{10} virus titer/ml. for certain serial passages of strain S2 virus					
Method	5	14	21	50	75	109
Plaque assay	6.8	6.9	6.1	5.9	6.9	5.7
Tube culture titrations	7.2	7.2	6.7	5.5	6.3	5.5
Mouse intracerebral inoculation	7.5	6.7	3.2	2.2	1.8	1.4

TABLE 8—Plaque structure of African Horse-Sickness Viruses Isolated in Asia

Virus Strains			Plaque size (mm.)	
Adaption of passage levels in mice to MS cell culture		MS Cell Culture	Largest	Average
2/63	5	5	2.3	0.8
4/63	5	7	1.5	0.4
6/63	5	13	1.8	0.8
7/63	5	10	1.7	0.4
7/60	18	5	2.3	0.6
S2	103	5	1.5	0.5
Elazig	60	10	1.5	0.6
Turkey	10	14	2.3	0.9
Indian 4	5	5	1.5	0.5
Indian 6	107	6	1.5	0.5

MS = Monkey kidney stable.

There were differences in maximal and average plaque sizes in all strains. Plaques of all virus types had a ragged edge.

Purification of Virus by Plaque Technique.—One of the largest plaques produced in MS cell culture with strain 114, was selected for isolation of purified virus. The plaque was isolated from other plaques if it was more than 1 cm. from the margins of adjacent plaques and if less than 10 plaques were in the bottle culture. The virus was picked up from the plaque by a Pasteur pipette, its tip being bent at a right angle. The plug of agar medium and infected cells was then expelled from the pipette onto an MS cell monolayer, and maintenance medium was added. Cytopathic effect was observed after 72 hours of incubation, and an additional passage in MS cell culture was made. The purified virus was tested for plaque formation, and plaque bottles were examined after 12 days' incubation. Except for few minute plaques forming in the lower dilutions, the viruses were large plaque formers (Fig. 3).

In the same manner, large and small plaques were picked from cultures

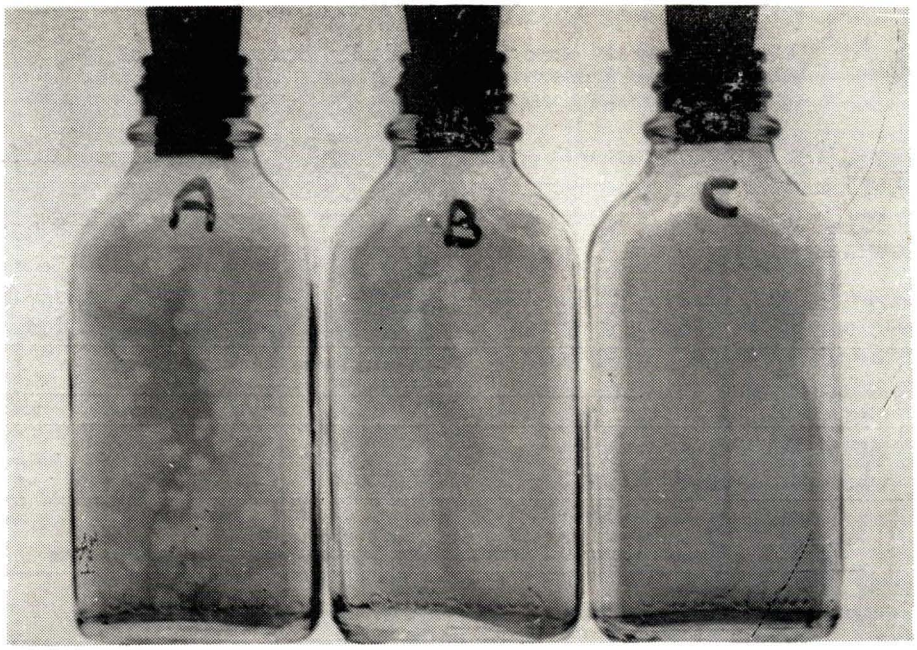

Fig. 3—Purification of African horse-sickness type 6 virus (strain 114) by plaque technique. Large plaques and a few microplaques (A), progeny of large plaques only in higher dilution (B), control bottle not infected (C). (Photograph was taken 12 days after inoculation.)

inoculated with 6th passage of strain 10/60 virus. Each pure virus was grown in MS cell culture and tested as previously described.

The size of plaques produced with the 2 purified viruses were as variable

as in the original mixed virus cultures.

Effect of Protamine Sulfate.—To investigate the possibility of increasing the size of plaques produced in MS cell cultures, protamine sulfate was added to agar overlay medium to have a final concentration of 0.5 mg./ml.

Nine serologically different types of AHS virus were tested simultaneously, each in 2 sets of bottle cultures. One set of bottle cultures was overlaid with the standardized agar overlay medium and the other with the medium containing protamine sulfate.

Plaque sizes were increased two- to six-fold in the cultures overlaid with medium containing protamine sulfate (Fig. 4).

Methyl Cellulose.—Bottle cultures were inoculated with 3 strains of virus: S2, 10/60, and 114. One set of bottles was overlaid with methyl cellulose and a 2nd set with the standardized agar overlay medium.

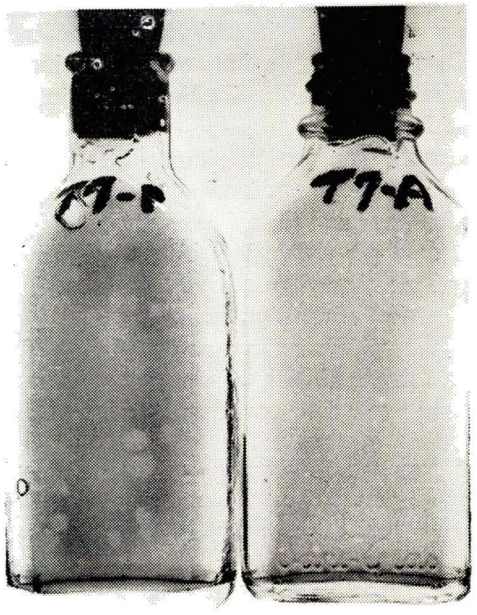

Fig. 4—Effect of protamine sulfate on plaque sizes of African horse-sickness type 7 virus (strain Karen). Overlaid with agar overlay (right, T7-A) and with the same medium with protamine sulphate added (left, T7-P).

Plaques formed in cultures overlaid with methyl cellulose medium were more uniform in size than those under agar overlay medium, but they were not larger.

Susceptibility of Different Monkey Kidney Stable Strains.—Four purified cell strains were made by the cloning method [15] from stock MS cell cultures, using 2 strains of type 9 virus (10/60 and S2). It was found that both virus strains produced larger plaques in 1 of 4 MS cell strains. Average sizes of plaques produced in this cell strain were 2 to 3 times larger than the average size of plaques produced in other MS cell strains.

Discussion

It is evident that Special Agar-Noble contains some inhibitors of plaque development with AHS virus. Washed Special Agar-Noble produces larger plaques and increases the number of PFU produced on MS cell cultures. Takemori and Nomura [19] found that minute plaque mutants of polio-virus are inhibited by an extract of agar. Liebhaber and Takemoto [10] reported that the addition of dextran or protamine sulfate to overlay mediums inactivates a polysaccharide sulfate inhibitor of plaque development. Significant increases in plaque diameters of AHS virus are demonstrated (Fig. 4). This may, therefore, be included in the overlay medium to increase the size of plaques of AHS virus.

Other methods may be used to inactivate or remove inhibitors from the overlay medium to increase the size of plaques, as described by Takemoto and Liebhaber.[20] The technique may not applicable to culture cells other than MS cells that are susceptible to AHS virus.[3, 4, 11, 12, 13] Attempts to produce plaques on baby hamster kidney cell cultures,[13] using standardized technique and varying the composition of the overlay medium, were not successful.

With MS cell cultures, plaques are not produced or only very minute plaques are produced in older cultures having an increased density of cells. The density of cells in bottle cultures are considered a factor affecting plaque size, because little difference is found in TCID50 titers of the same virus in cultures with different densities of cells.[14]

A significant difference is found in PFU titers when virus is diluted in PBS$^-$ and when it is diluted in maintenance medium: both containing calf serum and cations. This coincides with the report by Hazrati and Ozawa,[6] who compared TCID50 of virus using similar diluting fluids. Calf serum in maintenance medium may serve as a stabilizer, and the cations, Ca^{++} and Mg^{++}, that were not included in PBS$^-$ could promote adsorption of the virus to MS cells, like the adsorption of Japanese B encephalitis virus by hamster kidney cells.[18]

Another interesting observation is made on the maximum PFU titers, obtained after 3 hours of adsorption at 36 C. In previous experiments,[13, 14] it was found that AHS virus has a relatively long latent period (7 to 8 hours) when developing in MS cell cultures, and the titer decreases slowly during this period. Little difference is found, however, in TCID50 of the same virus titrated in tubes into which maintenance medium is added after varied adsorption periods.[14]

The minimum volume of inoculum (0.1 ml.) has the greatest plating efficiency. Although less inoculumn may give greater plating efficiency mathematically, 0.1 ml. is the most appropriate amount needed to cover the surface of cell culture prepared in a 2-oz. prescription bottle.

It is evident in comparisons of PFU, TCID50, and LD50 virus titers at different passage levels that passage of the virus in MS cells decreased the titer in mice. In the previous report,[14] passages in MS cells resulted in loss of antigenicity. Whether the loss of infectivity in mice has a direct relation to its loss in antigenicity was not studied in detail.

On the other hand, PFU titers were lower than TCID50 titers of early passages of S2 virus in MS cells, but these became higher after the 50th passage. This indicates that plaque forming virus dominates nonplaque forming virus, nonplaque formers become plaque formers by increasing the size of plaque, or plating efficiency of the virus is increased after passage. In any case, the sensitivity of plaque assay in MS cell cultures and the TCID may be related.

Summarizing the study on plaque structure, it is apparent that strains of AHS virus produce plaques of different sizes, and slight differences are found among average diameters of plaques produced with these strains without adding protamine sulfate. However, size of plaques produced by using the standardized plaque technique may have little value to distinguish virulent field isolates of virus

from its attenuated vaccinal strains.

The appearance of few minute plaques among larger plaques produced with plaque-purified type 6 virus (Fig. 3) may be due to (1) the presence of residual unadsorbed virus, (2) overlapping of minute plaques, or (3) back mutation caused by the influence of virus passage in MS cells using high concentration of virus. The last phenomenon was observed by Brown and Parker [1] who could not purify large plaque type by 3 serial clonings of plaques produced by western equine encephalomyelitis virus.

Purified MS cell strains appear to have different degrees of susceptibility to AHS virus and produce plaques of different size with similar virus inoculums.

There is no significant difference in the size of plaques formed when methyl cellulose was used instead of agar in the overlay medium.

Application of standardized plaque technique will play an important part in virus purification, neutralization, quantitative studies, and continued research on the properties of AHS virus.

REFERENCES

1. Brown, L. N., and Packer, R. A.: Some Factors Affecting Plaque Size of Western Equine Encephalomyelitis Virus. Am. J. Vet. Res., 25. (March, 1964): 487-493.

2. Dulbecco, R., and Vogt, M.: Plaque Formation and Isolation of Pure Lines with Poliomyelitis Viruses. J. Exptl. Med., 99. (1954): 167-182.

3. Erasmus, B. J.: Cultivation of Horse-Sickness Virus in Tissue Culture. Nature. 200. (1963): 716.

4. Erasmus, B. J.: Some Observations on the Propagation of Horse Sickness Virus in Tissue Culture. Bell. Off. int. Epizoot., 62. (1964): 55-60.

5. Hazrati, A., and Taslimi; H.: Studies on Horse-Sickness Virus Strains Isolated in Iran. Proc. XVIIth World Vet. Congress. 1. (1933): 535-543.

6. Hazrati, A., and Ozawa, Y.: Serological Studies of African Horse-Sickness Virus with Emphasis on Neutralization Test in Tissue Culture. Canad. J. Comp. Med. & Vet. Sci., 29, (1965): 173-178.

7. Howell, P. G.: The Isolation and Identification of Further Antigenic Types of African Horse-Sickness Virus. Onderstepoort J. Vet. Res., 29, (1962): 139-149.

8. Hsiung, G. D., and Melnick, J. L.: Plaque Formation with Poliomyelitis, Coxsackie, and Orphan (ECHO) Viruses in Bottle Cultures of Monkey Epithelial Cells. Virology, 1. 533-535.

9. Kanda, Y., and Melnick, J. L.: In Vitro Differentiation of Virulent and Attenuated Polioviruses by Their Growth Characteristics on MS Cells. J. Exptl. Med., 109. (1959): 9-24.

10. Liebhaber, H., and Takemoto, K. K.: Alteration of Plaque Morphology of EMC Virus with Polycations. Virology, 14. (1961): 502-504.

11. Mirchamsy, H., and Taslimi, H.: Adaptation of Horse Sickness Virus to Tissue Culture. Nature, 198, (1963): 704-706.

12. Mirchamsy, H., and Taslimi, H.: Visualisation of Horse-sickness Virus by the Fluorescent Antibody Technique. Immunology, 7, (1964): 213-216.

13. Ozawa, Y., and Hazrati, A.: Growth of African Horse-Sickness Virus in Monkey Kidney Cell Cultures. Am. J. Vet. Res., 25. (March, 1964): 505-511.

14. Ozawa, Y., Hazrati, A., and Erol, N.: African Horse-Sickness Live Virus Tissue Culture Vaccine. Am. J. Vet. Res., 26, (Jan., 1964): 154-163.

15. Paul, J.: Cell and Tissue Culture. 2nd ed. E. and S. Livingstone Ltd. Edinburgh, Scotland and London (1960): 220.

16. Rafyi, A.: La Peste Equine. Arch. Inst. Razi (1961): 60-106.

17. Rapp, F., Stevens, J. S., Taross, L., and Gordon, L. : Quantitative Determination of Infectious Units of Measles Virus by Counts of Immunofluorescent Foci. Proc. Soc. Exptl. Biol. & Med., 101, (1959) : 289-294.

18. Rhim, J. S., and Hammon, W. McD. : Quantitative Studies of Japanese B Encephalitis Virus in Hamster Kidney Cell Cultures. Proc. Soc. Exptl. Biol. & Med., 112, (1963) : 419-424.

19. Takemori, N., and Nomura, S. : Mutation of Polio-virus with Respect to Size of Plaque. II. Reverse Mutation of Minute Plaque Mutant. Virology, 12, (1960) : 171-184.

20. Takemoto, K. K., and Liebhaber, H. : Virus-Polysaccharide Interactions I. An Agar Polysaccharide Determining Plaque Morphology of EMC Virus. Virology, 14, (1961) : 456-462.