Arch. Inst. Razi, 1966, 18, 43 - 52

SEROLOGIC STUDIES OF AFRICAN HORSE–SICKNESS VIRUS WITH EMPHASIS ON NEUTRALIZATION TEST IN TISSUE CULTURE (*)

by

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In place of mice, monkey kidney stable (MS) cell cultures were used successfully in serologic studies of African horse-sickness virus.

The maintenance medium containing 2% serum was chosen as the virus diluent. Maximum neutralization occurred after 1-hour incubation at 37 C., and maintained the same titer during an additional 4-hour incubation period. No significant difference was observed between neutralization titers titrated using the same antiserum mixed with two different passage levels of virus.

Rabbit and guinea pig antiserums prepared using virus grown in MS cell cultures had antibody titer as high as those prepared in the same manner using mouse brain suspension.

African horse-sickness virus strains isolated in Asia were serologically identified using a standard neutralization technique in tissue culture. All the strains were closely related to each other and had antigenic similarity to Type 6 virus (strain 114).

Introduction

As early as 1935, Alexander[1] described the technique of neutralization tests using African horse-sickness virus and the anti-serum obtained from either recovered or immunized horses.

The technique was based on intracerebral injection of virus and serum mixture in mice, and was proved to be useful for immunologic studies of the virus.

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* Reprinted from Canadian Journal of Comparative Medicine and Veterinary Science, Gardenvale, Que., Vol. 29, No. 7, July, 1965.

Using the same method and antiserums prepared in rabbits, McIntosh[7] classified 42 strains of the virus isolated in Africa into seven serologic groups.

Neutralization tests in mice have been accepted as the best means for studies of the virus, and have been used for identification and serologic classification of African horse-sickness virus by other workers. [2,3]

Recently, however, African horse-sickness virus has been successfully adapted to tissue culture cells by the authors and other workers. [10,8,9] The primary purpose of the present investigation was to explore the usefulness of the new host cell systems in serologic studies of the virus.

Materials and Methods

Tissue Culture.—Monkey kidney stable (MS) cells were used throughout the experiment. The method of cultivation, the source of the cell line, and its susceptibility to African horse-sickness virus have been described in a previous paper.[9]

The growth medium (YLE, 10% calf serum) consisted of Earls' buffered salt solution containing 0.5% lactalbumin hydrolysate, 0.005% yeast extract, 10% calf serum heated at 56 C. for 30 minutes, 100 units of penicillin and 100 gamma of streptomycin per ml., and 0.0015% phenol red.

The maintenance medium (YLE, 2% calf serum) contained the same constituents the only difference being the reduced concentration (2%) of inactivated calf serum.

Virus Strains.—Seven antigenically different neurotropic vaccine strains A501, OD, L Vryheid, VH, 114, and Karen were received from the Onderstepoort Veterinary Institute at the 100 mouse passage level.

Neurotropic S2 strain was originally isolated in Iran, and 100 passages in mouse brain were carried out at Razi Institute.[3]

Another Iranian strain, 10/60, was isolated at the Onderstepoort Veterinary Institute[2] and was received at the 4th mouse passage. A few additional passages of these strains were made in mouse brain before adapting them to MS cells.

Two strains from Turkey were received from Elazig Institute. One of them was designated "Turkey strain" and it was isolated at the Razi Institute from infected horse blood received from Turkey. Another strain, designated "Elazig strain", was isolated in Elazig and 50 mouse brain passages were made at this institute. Ten additional passages in mouse brain were made in Iran on both strains.

Pakistan strain, 7/60, was isolated in Onderstepoort and received at the 16th passage. [2]

Indian strain, Ind.6 (isolated from Rajasthan), was received at the 105 adult mouse passage level. This strain was isolated during the recent outbreaks in India. It was selected from 7 strains kindly supplied by Dr. Sharma from the Indian Veterinary Institute, Mukteswar. Two additional passages of this strain were made before adapting it to MS cells.

Strains 2/63, 3/63, 4/63, 6/63 and 7/63 were isolated from sporadic cases of the diseases in Iran during the year 1963 and the 5th passage of each strain was used for adapting to MS cells.

The method of adaptation to tissue culture cells and subcultivation techniques have been described in the previous paper.[9] Virus fluids were harvested when the infected cells produced wide spread cytopathic effect. After shaking well, the medium was harvested and centrifuged at 2,000 r.p.m. for 15 minutes. The supernatant was used as virus suspension.

All infected mouse brains were stored at —25C. and viruses grown in tissue cultures stored at 4 C. throughout the experiment.

Titration of Virus in MS cell Cultures.—Serial ten-fold dilutions of virus were prepared using the maintenance medium, YLE + 2% calf serum, except during the experiment to choose the best diluent.

Titrations of virus were made in ordinary roller tube cultures prepared by seeding 1.5 ml. of cell suspension which contained approximately 160,000 cells/ml. After 3 to 4 days complete cell sheets were formed, and the growth medium was discarded just before infection. Four tube cultures were infected with each dilution of virus. For more precise titration five tube cultures were used for each dilution. Each tube culture was inoculated with 0.1 ml. of virus dilution, and icubated at 37 C. for 30 minutes. Fresh maintenance medium, 1.5 ml. per tube, was added and all the tube cultures were incubated at 37 C. for 6 to 7 days and were observed under the microscope every other day.

Fifty per cent or more cytopathic effect (CPE) was considered as the indication of infection. Fifty per cent end points were calculated by the Reed and Muench method[12], and expressed as the reciprocal of the log. TCID50/ml.

Antiserums.—Antiserums against each of the neurotropic vaccine strains including the S2 strain, and against strain 10/60 were obtained from hyperimmunized rabbits. For each strain five healthy rabbits were intravenously injected, each receiving 1 ml. of freshly prepared suckling mouse brain emulsion. To prepare the emulsion, approximately 1 part of infected brains was macerated in 9 parts of sterile saline. The mixture was centrifuged at 3,000 r.p.m. for 30 minutes and the supernatant fluid was used as the virus suspension.

Each rabbit received 10 consecutive injections at weekly interval. Antiserum was collected from the five rabbits 10 days after the last injection, and pooled.

Another anti-S2 serum was prepared in sheep. The animal received 5 consecutive intravenous injections, weekly. After 10 days' rest 5 additional intravenous injections were given at weekly intervals. The serum was collected 10 days after the last injection.

Anti-10/60 serum was also obtained from hyperimmunized guinea pigs. Five young adult guinea pigs were subcutaneously injected, each receiving 1 ml. of infected suckling mouse brain emulsion prepared in the same manner as the above. After 2 weeks the second injection and another week later the third injection were given. Antiserum was collected from them 10 days after the last injection and pooled.

Another anti-10/60 serum was prepared both in rabbits and guinea pigs by using virus grown in MS cell cultures. The method of preparation was exactly the same as the above except for the antigen. The 55th passage of the virus in MS cell cultures was used as the antigen, after 15 minutes centrifugation at 2,000 r.p.m.

Anti-Elazig serum was prepared both in rabbits and guinea pigs by using MS or mouse adapted virus as antigens. The animals were immunized in the same manner as described for the anti-10/60 serum preparation. The 60th passage of the strain in mouse brain and the 10th passage in MS cells were used as antigens.

Anti-7/60 serum was prepared in sheep at the Onderstepoort Veterinary Institute, South Africa.

All antiserums were inactivated at 56 C. for 30 minutes before use.

Neutralization Tests.—Serial ten-fold dilutions of the virus were prepared using the maintenance medium. Three tenth ml. of each dilution was mixed with an equal volume of diluted or undiluted antiserum. For the control, normal serum of the same species of animal was used. Each virus-serum mixture was incubated at the desired temperature for the required time.

Four or five tube cultures were inoculated with each dilution, each receiving 0.1 ml. of virus serum mixture. Infected cultures were incubated for approximately 30 minutes at 37 C. and then fresh maintenance medium was added to them.

The difference between the virus titer and the titer of the virus-serum mixture was taken as the Neutralization Index (NI) of the antiserum.

Experiments and Results

1) *Effect of Calf Serum in the Diluent on the Titer of Virus Incubated at* 37 C.—Ten-fold dilutions of S2-10 (10th passage of S2 strain in MS cell cultures) were

prepared using three different diluents, YLE, YLE+1% calf serum, and YLE+5% calf serum.

The virus was titrated in MS tube cultures after the following incubation periods: 0, 1, 2, 5, 15, and 25 hours' incubation at 37 C.; 1 hour incubation at 3 C. after 20 hours storage at 4 C.; 1 hour at 37 C. and 20 hours' storage at 4 C.; 2 hours at 37 C. and 20 hours' storage at 4 C.

The results are summarized in Table 1. It is evident from the results that the titer of virus diluted in YLE without calf serum decreased very rapidly during incubation at 37 C. and that there is no significant difference in the titer of virus diluted either in YLE+1% calf serum or YLE+5% calf serum.

Table 1. Effect of Calf Serum in the Diluent on the Titer of Virus Incubated at 37C.

Incubation and time	YLE	YLE +1% C.S.	YLE +5% C.S.
No Incubation	7.0+	7.5	7.5
37 C., 1 hour	6.0	7.0	7.0
37 C., 2 hours	6.0	7.0	7.0
37 C., 5 hours	5.0	6.8	6.8
37 C., 15 hours	4.5	6.8	6.8
37 C., 25 hours	4.5	6.8	6.8
4 C., 20 hrs.; 37 C., 1 hour	6.0	7.0	7.5
37 C., 1 hr.; 4 C., 20 hours	5.5	7.5	7.0
37 C., 2 hrs.; 4 C., 20 hours	5.5	7.5	7.5

+ = logarithm $TCID_{50}$/ml.

Therefore, the maintenance medium, YLE+2% calf serum was used as the diluent in subsequent work.

2) *Effect of Incubation period of Virus-serum Mixtures on the Neutralization. Tter of Horsesickness Antiserum.*—The neutralization titer of rabbit-S2 antiserum was titrated against S2-10 (10th passage of strain S2 in MS cell cultures). The virus-serum mixtures were incubated at different temperatures for various periods of time. The results are summarized (Table 2).

Table 2. Neutralization Index of Rabbit-S2 Antiserum Against S2 strain of Horse-sickness Virus Measured after Iucabiton of Serum Virus Mixture at Different Temperatures for Various Periods of Time.

Incubation and time	Log of Neutralization Index
No incubation	5.0
37 C., 30 minutes	5.3
37 C., 1 hour	6.0
37 C., 2 hours	6.0
37 C., 4 hours	6.0
4 C., 20 hrs,; 37 C., 1 hour	6.0
37 C., 30 min.; 4 C., 1.5 hours	5.8
37 C., 1 hours; 4 C., 20 hours	6.0
37 C., 2 hours; 4 C., 20 hours	6.0

It appears from the results that the maximum neutralization occurred after 1 hour incubation at 37 C. and maintained the same during additional incubation period up to 4 hours. Twenty hours incubation at 4 C. before or after 1- or 2- hours incubation period at 37 C. did not make any difference in neutralization titer.

3) *Effect of Virus Passages in MS Cell Cultures on Neutralization Titers.*—The object was to determine if passage levels of virus in MS cultures made any difference on the neutralization titers.

In Tables 3 and 4 are listed the results

of two experiments showing the neutralizing titers of Horsesickness antiserums against high and low passage levels of virus.

Table 3. Neutralization Titers of Several Horsesickness Antiserums Against S2 Virus of Two Different Passage Levels.

Antiserum	Exp. No.	S2−10+	S2−75
Rabbit-S2 antiserum...	1	5.0‡	5.4
	2	5.5	5.6
Rabbit-T.6 antiserum..	1	3.0	3.0
	2	3.5	3.6
Rabbit-T.3 antiserum..	1	0.5	0.3
Rabbit-T.4 antiserum..	1	0	0
Rabbit-T.5 antiserum..	1	0	0

+10th passage of strain S2 in MS cell cultures.
‡Logarithm of Neutralization Index of the serum

In the first experiment, the 10th and 75th passages of strain S2 were used. Hyperimmune serums used were anti-S2, T.6, T.5, T.4, and T.3 serums prepared in rabbits using infected mouse brain suspensions.

In the second experiment, the 10th and 76th passages of strain 10/10 in MS cell cultures were used as antigens. Four different anti-10/60 serums were prepared using two different animals, rabbits and guinea pigs, and two different antigens, infected mouse brain suspension (MO) and infected MS culture fluid (MS).

Table 4. Neutralizing Titers of 10/60 Antiserums Against 10/60 Virus of Two Different Passage Levels.

Antiserum	10/60-10+	10/80-67
Rabbit-10/60 antiserum (MO)‡.....	5.1++	5.2
Rabbit-10/60 antiserum (MS).....	5.6	5.8
G. pig-10/60 antiserum (MO).....	5.6	6.0
G. pig-10/60 antiserum (MS).....	5.6	6.0

+10th passage of strain 10/60 in MS cell cultures.
‡MO = Infected mouse brain suspension was used for antiserum preparation.
MS = MS tissue culture virus was used for antiserum preparation.
++Logarithm of Neutralization Index of the serum.

The diluent used was YLE+ 2% calf serum and virus-serum mixtures were incubated at 37 C. for 1 hour.

No significant difference was observed between the neutralization titers using the same antiserum mixed with high and low passage levels of virus.

4) *Comparison of Antiserums Prepared Using Two Different Antigens.*— The object was to compare antibody titers in the antiserums prepared by using two different antigens, i.e. infected mouse brain suspension and infected MS culture fluids. Antiserums were prepared in rabbits and guinea pigs in the manner described previously.

The same amount of neutralizing antibody in the antiserums was titrated

using different strain of virus.

In Table 5 and 6 are listed the results of two experiments showing the neutralization titers of anti-Elazig and anti-10/60 serums prepared as above.

High titer neutralizing antibodies were present in the serum of rabbits and guinea pigs immunized with injections of either infected MS tissus culture fluid or infected mouse brain suspension.

Table 6. Neutralizing Antibody in Anti-Elazig serum Obtained from Animals Immunized with Antigens of Two Different Sources.

Antiserum	Virus strain		
	Elazig	Turkey	6/63
Rabbit-Elazig antis. (MO)	6.0‡:	6.0	5.8
Rabbit-Elazig antis. (MS)+	6.2	6.0	6.0
G. pig-Elazig antis. (MO)	6.4	6.2	5.8
G. pig-Elazig antis. (MS)	6.5	6.0	6.0

+(MO) = Infected mouse brain suspension was used for antiserum preparation.
(MS) = MS tissue culture virus was used for antiserum preparation.
‡: Logarithm of Neutralization Index of the serum.

Table 5. Neutralizing Antibody in Anti-10/60 serum Obtained from Animals immunized with Antigens of Two Different Sources.

Antiserum	Virus strain				
	10/60	Turkey	Elazig	S2	T.6
Rabbit-10/60 antis. (MO)+	5.1‡	4.1	4.8	5.3	3.0
Rabbit-10/60 antis. (MS)	5.6	5.3	5.2	5.0	—
G. pig-10/60 antis. (MO)	6.0	5.8	5.8	—	4.0
G. pig-10/60 antis. (MS)	6.2	6.0	6.0	—	—

+(MO) = Infected mouse brain suspension was used for antiserum preparation.
(MS) = MS tissue culture virus was used for antiserum preparation
‡= Logarithm of Neutralization Index of the serum.

5) *Cross Neutralization Tests and Serological Identification of Horsesickness Virus Strains Isolated in Asia.*—Cross neutralization tests were carried out in MS cell tube cultures using a standard technique. Tenfold dilutions of virus were prepared using YLE+2% calf serum; each dilution of virus was mixed with an equal volume of antiserum heated at 56, C. for 30 minutes; virus-serum mixtures were incubated at 37 C. for 1 hour; 0.1 ml. of the mixture was inoculated onto 3 to 4 days old MS tube cultures (4 tubes per dilution); infected tube culture were incubated at 37 C. for 30 minutes; 1.5 ml. of fresh YLE+2% calf serum was added to each tube; and the results were read 7 days after infection.

The strains of virus used for cross neutralization tests were either 9th, 10th or 11th passages in MS cell cultures.

The results shown in Table 7 indicate that both anti-S2 and anti-10/60 serums contain relatively high neutralizing antibodies against Type 6 but not against the other types of vaccine strains.

Table 7. Cross Neutralization Tests of Horsesickness Virus in MS Cell Cultures.

Virus strain . . .	A501	OD	L	Vryheid VII		114	Karen	S2	10/60
	T.1	T.2	T.3	T.4	T.5	T.6	T.7		
Rabbit-T.1 antiserum	4.2	—	—	—		—	—	0‡	0
Rabbit-T.2 antiserum	—	4.5	—	—	—	—	—	0	0
Rabbit-T.3 antiserum	—	—	3.8	—	—	—	—	0	0
Rabbit-T.4 antiserum	—	—	—	4.8	—	—	—	0	0
Rabbit-T.5 antiserum	—	—	—	—	4.0	—	—	0	0
Rabbit-T.6 antiserum	—	—	—	—	—	4.5	—	3.0	3.8
Rabbit-T.7 antiserum	—	—	—	—	—	—	3.5	0	0
Rabbit-S2 antiserum	0	0	0	0	0	3.6	0	5.8	5.5
Rabbit-10/60 antiserum	0	0	0	0	0	3.0	0	5.3	5.8

*Logarithm of Neutralization Index of the Serum.
‡ 0 or negligible

It was also observed that among the seven different types of antiserums only anti-T.6 serum neutralized both Asian strains, S2 and 10/60, in some degree. The neutralization indices of the others were of no significance.

Further investigations have been made to demonstrate the antigenic similarity of several other Asian isolates and their relations with type 6 virus. The results summarized in Table 8 indicate clearly that all the Asian isolates are closelv related to each other and all have an antigenic similarity with Type 6 virus.

Table 8. Antigenic Similarity of Several Asian Isolates of Horsesickness Virus and Their relation to Type 6 virus.

Virus strain	S2	10/60	2/63	3/63	4/63	6/63	7/63	Turkey	Elazig	Ind. 6	7/60	T.6
Rabbit-S2 antiserum	5.8	5.5	5.5	5.5	5.0	5.7	5.2	4.6	4.7	5.7	5.3	3.8
Sheep-S2 antiserum	6.0	6.0	6.0	5.8	5.5	6.1	5.7	5.0	5.2	6.1	5.8	2.8
Rabbit-10/60 antiserum (MO)*	5.3	5.8	5.5	5.8	5.0	5.6	5.0	4.1	5.0	5.8	5.5	3.0
Rabbit-10/60 antiserum (MS)	5.3	5.5	5.8	6.0	5.5	6.1	5.2	5.3	5.2	6.1	5.6	3.5
Rabbit-Elazig antiserum (MO)	—	—	5.5	5.5	5.5	5.8	5.7	6.0	6.0	5.7	5.5	—
Rabbit-Elazig antiserum (MS)	—	—	5.5	5.8	5.5	6.0	5.7	6.0	6.2	6.1	5.8	—
Sheep-7/60 antiserum	5.5	6.0	5.8	6.0	5.8	6.1	6.0	5.7	5.8	6.1	6.2	—
Rabbit-T.6 antiserum	3.5‡	3.8	—	—	—	—	—	3.6	3.0	—	—	4.5

*(MO) = Infected mouse brain suspension was used for immunization of animal.
(MS) = Ms tissue culture virus was used for antiserum preparation.
‡: Logarithm of Neutralization Index of the antiserum.

Discussion

In recent years, several workers have developed various serologic methods to characterize African horse-sickness virus such as complement fixation[6], serum

neutralization in mice[1], heamagglutination[5], and agar diffusion[4]. Among these methods, neutralization tests in mice have been found to be the most type specific, while the others have not been entirely satisfactory.

Recent developments in new host cell systems[8,9] made way not only for studies of the properties of African horse-sickness virus and its vaccine production,[8,10] but also for diagnostic and serologic studies on the virus.

The host cell system appeared to be most suitable for neutralization tests. The use of MS tube cultures instead of mice in neutralization tests is far more economical also saving labor and space.

Presence of 1% or more calf serum in the diluent of virus appears to stabilize the infectivity of virus during incubation at 37 C. Therefore, maintenance medium which contains 2% calf serum was used as the diluent. This was also found to be opptimum for maintaining MS cell cultures[10].

The incubation of virus-serum mixtures was made in the incubator at 37 C. with maximum neutralization titers occuring after 1 hour incubation. If these tubes containing virus-serum mixtures were placed in the water bath 37 C. the temperature of the contents must have reached the desired temperature quicker than in the incubator, and the maximum neutralization titers would be obtained before 1-hour incubation period.

High passage level virus (10/60 and S2) in MS cell cultures was neutralized as much as or slightly more than low passage level virus when mixed with the same antiserum. The difference in titer was, however, of no significance.

It was demonstrated that tissue culture virus could stimulate antibody response in rabbits and guinea pigs as well as the antigens prepared from infected mouse brains. This is of significance because repeated injections of antigens prepared from mouse brains sometimes result in death of animals due to anaphylactic shock.

All the examined strains of African horse-sickness virus isolated in Asia were found to be serologically identical. According to Howell[2], Asian strains belong to the serological Type 9. However, as shown in Tables 7 and 8, there is clear indication that all the isolates in Asia are also antigenically similar to Type 6. Therefore, further investigations are under way on the relationship between Howell's Types 6 and 9 using more advanced plaque technique.

The finding that all the examined strains of African horse-sickness virus isolated in the Middle East and the other countries in Asia are serologically identical is of particular interest when we observe the numerous variants that have been identified from African outbreaks of the disease.[11]

ACKNOWLEDGMENT

The authors thank Dr. A. Rafyi and Dr. H. Mirchamsy of Razi Institute, Dr. W. M. Moulton, and Dr. E. Traub of Near East Animal Health Institute for their helpful advice, M. I. Hopkins and Dr. S. Bahrami for their technical assistance.

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