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COMPARATIVE STUDIES ON THE SEROLOGICAL RESPONSES OF HORSES TO AFRICAN HORSESICKNESSS VIRUS (*)

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Introduction

Serological responses of animals to African horsesickness (AHS) virus was first studied quantitatively by Alexander, who introduced the serum neutralization (SN) test for immunologic studies of AHS virus (1).

Application of SN test based on the original technique or with further modification (4, 12) in the study of varying problems associated with immunity, proved that equines recovered from a natural or experimental infection as well as those exposed to attenated vaccine strains of AHS virus, developed a demonstrable neutralizing antibody (NA) which was proved to be related to the immunity of the animals.

The formation of complement fixing (CP), precipitating (P), and hemagglutination inhibiting (HI) antibodies in animals exposed to AHSvirus, have also been demonstrated by several workers (6, 9–11, 16–18, 21) but the first appearance and persistence of the antibodies have not yet been throughly investigated.

The present communication consists of a quantitative study of antibodies directed against several strains of AHS virus with varying levels of pathogenicity. It attempts to compare the appearance and persistence of P and CF antibodies with onset and duration of homologous NA in horses exposed to AHS virus.

Materials and Methods

*H*ORSES. Susceptible horses, 1.5–2.5 years old, were used for all experiments. The animals were maintained in an insect-proof stable, approximately 7 days before and for the following 14 days after inoculation. The susceptibility of each individual horse to AHS was confirmed by showing the absence of hor-

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sesichness antibodies in its serum before being used in the experiment.

*V*IRUS STRAINS. The following strains of type 9 AHS virus, at varying levels of pathogenicity, were used as virulent virus:

1 . Fifth intracerebral passages of strain 1/63 and 2/63 (5).

2 . First passage of strain 10/60–6 (6th passage of the strain in mouse brain) in BHK-21, cell line (5).

3 . First or 2nd passages of 10/60–6 or Alg 16/66–5 in a mosquito (*Aedse albopictus*) cell line (14).

As the attenuated strains of the virus, strains OD and S2 of types 2 and 9 AHS virus, respectively, were selected to be used in the experiment, the 102nd passage of these virus strains with or without 6 or 7 further passages in monkey kidney stable (MS) cell cultures were employed (15).

Recording of clinical responses. The animals were closely observed for appearance of any response to the virus inoculation and the body temperature was recorded twice daily.

Virus isolation. Determination of virus from the blood of inoculated horses was carried out by injecting defibrinated blood samples, diluted with an equal volume of sterile distilled water into suckling mice. Each sample was inoculated into 10 mice, each of which was given 0.025 ml of inoculum intracerebrally.

Further passages and identification of the isolates were performed whenever it was necessary, as has been described before (3, 5).

Collection and assay of sera. Serum samples were collected immediately before inoculation and thereafter at varying intervals during the course of the experiments. Sera were stored frozen at –20 to –30 C before being assayed for AHS antibodies as follows.

1 . *NA.* Virus NA titers of the sera were determined against 7th passage of AHS virus strains, homologous to the respective virus strains which were used for animal inoculation. MS cell culture tubes were used as the host system in all tests. The technique of cell preparation, and the procedure of neutralization test were as previously described (4, 13).

To minimize the possible errors resulting from factors affecting the test, all available serum samples obtained from one animal were titrated simultaneously.

The sera were inactivated at 56°C for 30 min immediately before use. Four-fold dilutions of the serum samples were mixed with equal volume of virus suspension containing 40-100 TCID 50 virus/0.1 ml. The virus serum mixtures were incubated at 37°.C for 60 min and then each mixture was tested for infective

virus by inoculating 4 cell culture tubes using 0.2 ml of the mixture as inoculum. The cultures were examined for characteristic cytopathic effect (CPE) every other day for 7 days, and serum titers expressed as the logarithm of reciprocal; the highest dilution of serum that completely prevented CPE in 50% of the cell culture tubes was calculated by the method of REED and MUENCH (19).

2. *Precipitating antibodies* (PA). PA titrations were performed according to the technique previously described (6). 0.2 log10-fold dilutions of the serum were prepared in veronal buffer solution. Then each serum dilution was allowed to react against an optima dilution of P horsesickness antigen in agar diffusion medium, using two separate antigen antibody systems for each dilution. The highest dilution producing a visible line of precipitation, in at least one antigen-antibody system, was taken as the P titer of the serum.

P horsesickness viral antigens were produced from ultrasonically disrupted cells of suckling mouse brain or monkey kidney tissue culture system infected with the virus. The sera obtained from horses inoculated with the virus propagated in suckling mouse brain were tested against antigen prepared from infected cell cultures, and *vice versa*. In such a case no nonviral precipitin line, due to the mouse brain or tissue culture materials, was observed in the agar diffusion to confuse the results.

3. CFA. Series of tubes containing 0.1 ml of 0.3 log10-fold dilutions of inactivated serum samples were prepared. After completion of the serum dilution, 0.1 ml of a freshly prepared dilution of antigen was added followed by 0.1 ml of complement containing 2U. the fixaotion was allowed to occunr at 4°C for 18h followed by 30 min encubation in a 37°C water-bath. 0.2 ml of sensitized sheep red cells, containing 2U of himolysin was then added and the results were read after a further incubation in the water-bath for 30 min. To facilitate the reading the tubes were read in comparison with a set of hemoglobin color stan- dard, after being centrifuged at 1,000 rpm for 5 min.

The serum titers were the highest dilution, giving 50% or more fixation.

In each test, necessary controls such as serum control, normal and positive antigen controls, hemolysin and veronal controls were included and the complement dilution was titrated simultaneously.

Acetone ether extracted antigen prepared from suckling mouse brain infected with AHS virus (2), was used in testing sera of horses inoculated with tissue culture adapted virus, while the antigen used in testing sera of horses inoculated with virus propagated in mouse brain was produced from ultrasonically disrupted cells of infected MS cell cultures (6). Two to 4 U antigen were used in the tests.

The antigens, complement and hemolysin were freeze-dried and stored at –20°C until use. All dilutions were done in a cold veronal-buffered dilution

containing 0.1% gelatin.

Results

Responses of Horse to Virulent Strains of AHS Virus

1. *Virus propagated in mouse brain.* The 5th mouse brain passage of strains 1/63 and 2/63 was employed. The strains were isolated from sporadic cases of African horsesickness in Iran during the year 1963, and were identified as type 9 of the virus.

Susceptible horses inoculated with these strains at their 5th passage level in mouse brain developed a typical case of AHS which, under the condition of the experiment, terminated in the death of at least 50% of inoculated animals.

During two separate experiments from inoculated horses, two animals, i.e. horse No. 62 (inoculated with $10^{7.5}$ mouse ID 5° of strain 1/63), and horse No. 69 (inoculated with $10^{8.0}$ mouse ID 5° of strain 2/63), after developing a severe case of AHS and showing clinical reactions of the disease from 5th to 14th day postexposure, eventually recovered. The body temperature, the results of virus isolation and the serological responses of these horses are shown in figure 1,2 and 3.

Virus was isolated during the febrile reaction and identified as the strains used for inoculation of the animals. NA was first detectable in the serum as early as the 4th day postinfection. The antibody reached its highest titer on the 10th to 12th day and maintained the same or a somewhat lower titer throughout the period of observation.

CF and P antibodies, on the other hand, were demonstrated in circulation a few days after detection of NA. It is worth mentioning that although

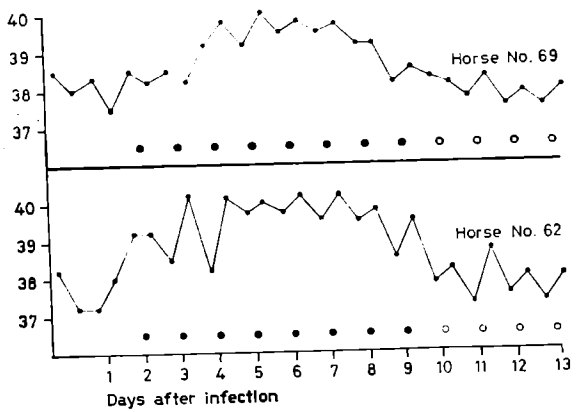

Fig. 1. Postexposure temperature (°C) of horses inoculated with 5th intracerebral mouse passages of strain 1/63 or strain 2/63 AHS virus. ● = virus was isolated. o = virus was not isolated.

P antibody was detectable 2–3 days after the appearance of CF antibody, it seemed that the development of both antibodies was similar. Both reached highest titer later than NA and they declined more rapidly.

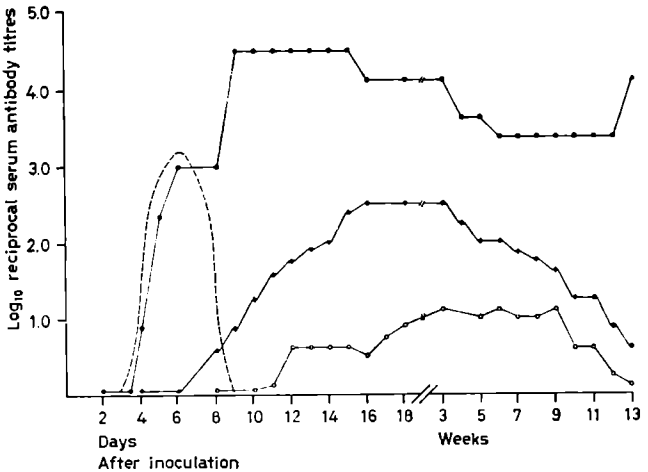

Fig. 2. Neutralizing (●——●), precipitating (o——o) and complement fixing (◆——◆) antibody responses in horse No. 62 following intravenous inoculation of 5th mouse passage of strain 1/63, type 9 AHS virus. ---- = febrile or viremia period.

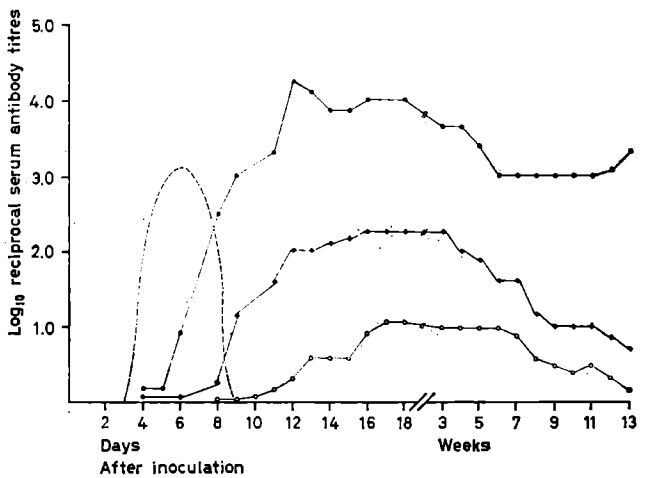

Fig. 3. Neutralizing (●——●), precipitating (o——o) and complement fixing (◆——◆) antibody responses in horse No. 69 following intravenous inoculation of 5th mouse passage of strain 2/63, type 9 AHS virus. ---- = febrile or viremia period.

2. *Virus propagated in cell culture.* Strains Alg 16/66 and 10/60 of type 9 AHS virus, isolated from naturally infected horses, were adapted to mosquito (*A. albopictus*) or BHK cell lines after being passaged in suckling mouse brain for 5 and 6 times, respectively. First or 2nd passage of the cell culture adapted strains were used to infect several horses. Clinical reactions of AHS with varying severity including rises in body temperature were observed in the inoculated animals, and AHS virus was isolated from blood samples collected during the febrile period.

The results of serological tests in these animals are presented in table I. The early appearance and persitence of antibodies in one of the horses is shown in comparison with the viremia period in figure 4.

The data presented here indicate that inoculated horses showed serological responses very similar to those explained in previous experiment. The virus strains stimulated the production of the 3 antibodies which were detectable in serum from the end of the febrile period for at least 3 months. The P and CF antibodies appeared to decline considerably while the high NA levels were maintained until the end of the experiment.

Table I. Serological responses of horses to African horsesickness virus propagated in cell culture

Number of horses	Virus	Febrile period, days	Antibody	Logarithmic group mean antibody titer at indicated days postexposure										
				4	7	10	14	21	28	35	42	63	84	120
	S 10/60[1]		NA[3]	0	0	0	2.1	3.8	3.5	3.8	3.5	3.5	3.4	3.0
4	SM-6	7–14[2]	CF	0	0	0	0.8	2.1	2.1	2.1	1.9	1.8	1.2	0.9
	Aa-1		P	0	0	0	0	0.8	0.8	0.6	0.6	0.6	0.6	0.4
	S 10/60		NA	0	0	0.6	3.6	4.0	4.0	4.0	3.6	3.6	3.6	3.6
2	SM-6	6–13	CF	0	0	0	1.5	1.8	2.4	2.1	1.8	1.5	1.2	0.8
	Aa-2		P	0	0	0	0	0.6	1.2	0.8	0.6	0.4	0.4	0.2
	Alg 16/66		NA	0	0	0	1.8	3.6	3.2	3.0	3.3	3.0	3.3	3.3
2	SM-5	6–12	CF	0	0	0	1.6	1.8	2.0	2.0	1.8	1.6	1.2	0.8
	Aa-2		P	0	0	0	0.2	0.6	0.8	0.6	0.6	0.6	0.4	0.2
	S 10/60		NA	0	–	1.6	3.8	–	4.0	–	–	4.0	–	–
4	SM-6	6–12	CF	0	–	0.2	1.5	–	2.1	–	–	1.5	–	–
	BHK-1		P	0	–	0	TR	–	0.8	–	–	0.8	–	–

1 Strain (S) 10/60, 6th passage in suckling mice, 1 passage in *Aëdes albopictus* cell culture.
2 From 7 to 14 days postexposure.
3 Neutralizing (NA), complement fixing (CF), and precipitating (P) antibodies.

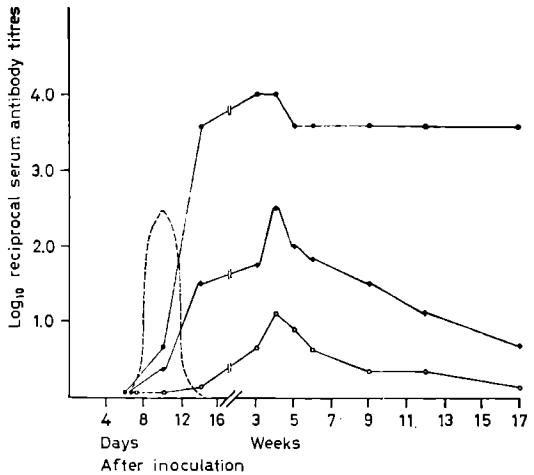

Fig. 4. Neutralizing (●——●), precipitating (○——○) and complement fixing (◆——◆) antibody responses in a horse following inoculation of 2nd passage of neurotropic AHS virus, strain 10/60, in a mosquito cell line. ---- = febrile period.

Responses of Horse to Attenuated Strains of AHS Virus

1. *The neurotropic mouse-attenuated virus.* A 10-percent saline suspension of brain material of suckling mice inoculated with the OD (type 2) and S2 (type 9) AHS virus strains were used for inoculation of horses. The animals received two inoculations at approximately 4-week intervals. The serological responses of the animals are summarized in table II.

Both viruses stimulated the formation of neutralizing and CF antibodies. P antibody response simulated that of CF antibody in horse inoculated with strain OD, while the first inoculation of the strain S2 failed to initiate development of detectable P antibody in inoculated animals; a second inoculation, however, produced a rapid serological response for all 3 antibody types.

2. *The neurotropic tissue-culture adapted virus.* Neurotropic virus strains OD and S2 used in the previous experiment were adapted to MS cell cultures and then serological responses of several horses to these strains were investigated. The 7th passage of the virus strains in MS cell cultures was harvested and the culture fluid containing virus, with or without infected cells, was used for animal inoculation, as indicated in tables III and IV.

From the results summarized in table III, it can be concluded that the first administration of these viruses, although it stimulated the formation of NA in horses, did not always produce detectable CF and P antibodies. In fact, under the conditions of the experiment, if CF and P antibodies were detectable, the concentration was low and persisted only for a short time and a relatively

Table II. Serological responses of horse to neurotropic mouse-attenuated African horsesickness virus

Number of horses	Virus strain	Antibody	Log. antibody titers at indicated days postexposure									
			7	14	21	28	35	42	49	56	7[1]	14
		NA[2]	0	0	1.8	1.8	2.4	3.2	3.6	2.4	3.6	3.6
1	OD-102[3]	CF	0	0	1.2	1.5	1.8	1.8	1.8	1.8	1.8	2.1
		P	0	0	0	0.4	0.6	0.8	0.6	0.8	0.8	1.2
		NA	0	0.6	2.4	2.4	2.4	2.0	2.0	1.8	3.2	3.2
1	S2-102	CF	0	0	1.5	1.5	1.2	1.2	0.8	0.2	1.6	1.6
		P	0	0	0	0	0	0	0	0	0.6	0.6

1 7 days after 2nd inoculation.
2 Neutralizing (NA), complement fixing (CF), and precipitating (P) antibodies.
3 102nd passage of strain OD in mouse brain.

Table III. Serological responses of horse to neurotropic tissue-culture adapted African horsesickness virus

Number of horses	Virus	Antibody	Log. antibody titers at indicated days postexposure										
			7	14	21	28	35	42	48	56	7[1]	14	21
		NA[2]	0	0	1.2	2.6	2.4	2.6	3.0	2.6	3.0	3.0	3.0
1	OD-102	CF	0	0	0	0	0	0	0	0	0.8	0.6	0.6
	MS-7	P	0	0	0	0	0	0	0	0	0	0	0
	OD-102[3]	NA	0	0	1.4	2.6	3.0	2.6	2.8	2.8	3.6	3.6	3.6
1	MS-7	CF	0	0	1.5	1.8	1.8	1.8	1.8	1.5	2.1	2.1	2.1
	with inf. cells	P	0	0	0.2	0.6	0.6	0.4	0.4	0.2	0.6	0.6	0.6
		NA	0	0.6	1.8	1.8	–	–	–	–	2.4	2.4	1.8
1	S2-102	CF	0	0	0	0	–	–	–	–	0.6	0	0
	MS-7	P	0	0	0	0	–	–	–	–	0.2	0	0
	S2-102	NA	0	0.8	1.8	1.8	–	–	–	–	3.0	2.4	2.4
1	MS-7	CF	0	0.2	0.4	0.4	–	–	–	–	0.4	0.4	0.4
	with inf. cells	P	0	0	0	0.4	–	–	–	–	0.6	0.4	0.2

1 7 days after 2nd inoculation.
2 Neutralizing (NA), complement fixing (CF), and precipitating (P) antibodies.
3 102nd passage of the strain in mouse brain, 7th passage in MS cells with infected (inf.) cells.
0 = no antibody was demonstrated.

Table IV. Serological responses of horses to various amounts of neurotropic tissue culture adapted, strain S2, African horsesickness virus

Number of horses	Amount of virus	Antibody	Logarithmic group mean antibody titer at indicated days postexposure										
			14	21	28	35	42	49	70	100	120	15[1]	30
		NA[2]	0	0.3	1.0	1.5	1.5	1.8	1.8	1.8	1.8	2.4	2.4
2	3.8[3]	CF	0	0	0	0.3	0.4	0.6	0.6	0.4	0	0.4	0
		P	0	0	0	0	0	0	0	0	0	0	0
		NA	0	0.3	1.2	1.2	1.4	1.4	1.2	1.2	1.2	2.4	2.4
2	4.4	CF	0	0	0	0	0	0	0	0	0	0.3	0.3
		P	0	0	0	0	0	0	0	0	0	0	0
		NA	0	0.8	1.2	1.6	1.8	1.8	1.8	1.6	1.2	2.6	2.6
2	5.0	CF	0	0	0.3	0.6	0.6	0.6	0.3	0	0	0 3	0
		P	0	0	0	0	0	0	0	0	0	0	0
		NA	1.0	1.5	1.8	1.8	1.8	1.6	1.2	1.2	1.2	2.6	3.0
2	5.6	CF	0	0	0.3	0.8	0.4	0.3	0.3	0	0	0.6	0
		P	0	0	0	+	+	0	0	0	0	+	0

1 Days after 2nd inoculation.
2 Neutralizing (NA), complement fixing (CF), and precipitating (P) antibodies.
3 $10^{3.8}$ $TCID_{50}$.
0 = no antibody was demonstrated.
+ = Trace of antibody

good response was produced when virus-containing cells were included in the inoculum used for animal inoculation.

The second inoculation resulted to a rise in NA titers in all horses; while the rise in CF and P antibodies titers, if any, was of no significance.

Discussion

The early observation of the fact that animals recovered from AHS infection possessed a solid and durable immunity to the infective virus strain, led several investigators to administer the serum of recovered or hyperimmunized horses in AHS immunization (22, 23). The NA in the serum of recovered animals was clearly demonstrated, in 1935, by applying a serum neutralization test (1).

The extensive works on immunization of horses with the neurotropic attenuated strains of AHS virus clearly indicated the formation of NA and showed that although there was a tendency of horses to develop NA to heterologous strains, the immunological responses of horses to AHS virus was mostly specific to the type of virus strain and could be used as a reliable indicator of the immune status of the exposed or vaccinated animals.

CF antibody to AHS virus was first obtained from guinea pigs which had received repeated injection of neurotropic attenuated strains of AHS virus. The guinea pig antiserum prepared against each of 7 heterotypic virus strains

was capable of fixing complement in the presence of all types of viral antigens and, hence, no identification of different types of the virus was possible (11).

In the horse, on the other hand, it was reported that recovery from infection resulted in the development of CF antibody. In a horse injected with 2 ml of blood containing type 7 horsesickness virus, the CF antibody was detectable in the blood as early as 5 days after febrile reaction, this persisted for a short time and then disappeared (8). In another report, CF antibody was reported to be detectable in infected horses 9 days after thermal reactions (20).

Formation and persistence of AHS P and HI antibodies in the horse, on the other hand, have not been throughly investigated, although the P and HI tests have been applied successfully in the study of AHS virus (6, 9, 10, 16, 17).

In the present experiments, the formation and persistence of neutralizing, P and CF antibodies in horses exposed to AHS viruses were compared. Horses surviving infection with several strains of AHS virus at varying levels of pathogenicity developed neutralizing, P and CF antibodies.

NA, being detectable in serum approximately 2–3 days after the onset of febrile reaction appeared sooner than the other antibody types, and persited at a higher titer level for a much longer period. The CF and P antibodies were observed after appearance of the NA, but P antibody was always detected a few days after the first detection of CF antibody. These two antibody types reached their peaks later than NA and declined more rapidly.

CF antibody and NA were detected in sera obtained from blood samples collected during viremia. The P antibody was not detected in these samples. collected during viremia. The P antibody was not detected in these samples.

The provisional diagnosis of AHS is usually confirmed by isolation and subsequent serological identification and typing of the infecting virus. If the detection of antibodies in convalescent animals is to be used on a particular occasion as a confirmatory diagnosis of the disease, the detection of all 3 main antibody types could be used, providing the serum samples were obtained within the first 2–4 weeks after recovery.

The serological responses of the horse to attenuated strains of AHS virus seem to be dependent on the degree of attenuation. The virus strains which have lost their pathogenicity for horses through more than 100 serial mouse intracerebral passages were shown to have retained their antigenic properties producing AHS antibbodies when used for horse inoculation.

The capability of the same strains after several passages in MS cell cultures in producing CF and P antibodies, decreased considerably, on the other hand, while their capacity to stimulate the formation of NA was retained to a high degree.

Administration of various amounts of neurotropic tissue-adapted AHS virus, showed that the NA, following the use of larger amounts of virus, formed earlier than after the use of smaller amounts (table IV). It was also shown that MS cell-propagated virus stimulated a better serological response when it was associated with infected cells. This could be attributed to the administration of larger amounts of virus particles, or to higher concentration of viral antigens which existed in the infected cells (7).

Based on the present data, which confirm early appearance and longer persistence of NA in recovered or immunized animals, we may conclude that the NA is the more reliable indicator of the immune status of equines following artificial immunization or natural infection.

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