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THE ISOLATION AND IDENTIFICATION OF FURTHER ANTIGENIC TYPES OF AFRICAN HORSESICKNESS VIRUS

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The significance of the antigenic plurality of strains of African horsesickness virus was first realised by Theiler (1908, 1915, 1921). In numerous experiments over a number of years, he showed that the immunity in horses and mules after challenge by homologous strains was solid, but when heterologous strains were used a percentage of animals contracted the disease in varying degrees of severity. The extension of this work was seriously handicapped by the economic implications of using large numbers of experimental animals, with the result that for many years only two strains of virus were used for prophylactic immunisation.

No further progress was made in the evaluation of naturally occurring strains until the virus of African horsesickness was adapted to propagation in adult white mice (Nieschulz, 1932, 1933; Alexander, 1933). Alexander (1935) developed an intracerebral protection test in this host which proved to be an accurate, economical and practical means of differentiating the antigenic types of the virus. The earlier studies made use of convalescent or hyperimmune horse sera, but the frequent presence of heterologous antibody made the interpretation of experimental results difficult. McIntosh (1958) overcame this problem by using hyperimmune sera produced in the insusceptible rabbit and in a study of 84 strains of virus, established the existence of seven distinct immunological types.

Materials and Methods

Virus strains

During the course of successive horsesickness seasons, numerous specimens are received by the Onderstepoort laboratory from equines, showing symptoms of horsesickness. In addition, countries in the Middle East and south-western Asia affected by the recent epizootic of horsesickness, forwarded specimens for confirmation of the diagnosis and typing of the virus. These specimens consisted of blood collected at the height of the febrile reaction into a preservative of either equal parts of Edington's oxalate-carbolic-glycerine diluent (Theiler, 1930) or heparin in a concentration of 10 units per ml. Alternatively spleen was collected from fatal cases post-mortem and submitted in 50 per cent buffered-glycerine.

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Antisera

Type specific hyperimmune sera were prepared by a technique essentially similar to that used by McIntosh (1958). The strains of virus used as antigens were, with one exception, those in current use for the production of polyvalent vaccine. The exception was virus strain 47 58 of Group 4 which replaced the poorly antigenic strain VRY but which nevertheless, as far as can be determined by current methods, is immunologically identical with the orignal type strain.

Families of 3 to 4 day old suckling mice were infected intracerebrally with a 1 : 1000 dilution of antigen. After three days incubation, when the majority were *in extremis*, they were killed with ether and the brain tissue harvested. Five brains were pooled in a screw-capped bottle, sealed and stored at 20 C. Sufficient aliquots were prepared for the immunisation of not less than two rabbits. Suspensions of 10 per cent mouse brain were prepared in M_{50} phosphate buffer from the frozen material. After centrifugation at 2,000 rpm for 30 minutes in an angle centrifuge, each rabbit received an initial injection of 2·5 ml of antigen intraperitoneally, followed by 10 intravenous injections at 4 to 5 day intervals. Rabbits immunised by this method were bled 10 days after the last injection. The sera without preservative were stored at 20 C.

Specific antisera to the recently isolated virus strains were prepared in young adult guinea-pigs. The antigens were prepared in suckling mice as described above. The inoculation schedule differed, however, in that each guinea-pig received 1·0 ml of brain emulsion intraperitoneally, followed by a second injection two weeks later. Serum from not less than five guinea-pigs was collected 10 days after the second injection, pooled and stored at 20 C.

A positive control serum for the complement fixation test was collected from a horse artificially infected and bled 30 days later. This convalescent serum was inactivated at 56 C for 30 minutes before use.

Isolation of virus

Blood specimens were diluted with equal parts of distilled water. The preserved tissue specimens were washed free from glycerine in three changes of sterile saline. Three to four grams of spleen were ground in a mortar with sterile alundum until the tissue had been reduced to a pulp, a 10 per cent tissue suspension was then prepared in phosphate buffered peptone diluent. Each preparation was then centrifuged at 2,500 rpm for 30 minutes, the supernatant fluid pipetted off and penicillin (100 units ml) and streptomycin (2 mgm ml) added. These preparations were held at 4 C before further use.

Two families of suckling mice were inoculated intracerebrally with 0·03 ml of each preparation and examined twice daily thereafter. Deaths recorded among mice previously showing prostration and nervous symptoms were considered specific and as far as possible only mice *in extremis* were selected for further passage.

Passage of each virus was continued with a 1 : 1000 dilution of brain tissue. Infective brain tissue suspensions of the third serial passage were mixed in equal parts with an M_{50} phosphate buffer containing 10 per cent lactose and 2 per cent peptone, dispensed into ampoules, freeze dried and stored at 20 C for future reference.

Complement fixation

For the purpose of rapid identification the brains of moribund suckling mice were harvested, pooled and macerated in saline to give a 10 per cent suspension. This preparation was allowed to stand overnight at 4 C and on the following

morning was centrifuged in an angle head M.S.E. centrifuge at an estimated speed of 10,000 rpm for 30 minutes. The clear supernatant fluid was withdrawn and used at a 1:2 dilution as antigen. A test was set up by mixing unit volumes of two-fold dilutions of known positive and negative horse sera, two units of complement and unit volume antigen. After incubation for $1\frac{1}{2}$ hours at 37°C the haemolytic system containing optimal amboceptor dilution and 2 per cent sheep red cells was added. The degree of haemolysis was determined after a further half-an-hour incubation at 37°C. Controls on the sera, antigens as well as all the reagents used in these tests were included.

Neutralisation tests

Sterile stock five-fold dilutions of the prepared rabbit and guinea-pig antisera were made in buffered peptone diluent and stored at −20°C. Whenever required the sera were rapidly thawed and 0·3 ml of each dilution placed into a block of pyrex test tubes to cover the entire series of sera at each dilution. A suitable dilution of antigen previously determined, to contain approximately 100 Ld_{50} doses of virus per 0·03ml in mice was then prepared and added in equal volume to each tube. The serum virus mixtures were incubated overnight at 4°C followed by one hour at 37°C immediately before inoculation. Each mixture was injected intracerebrally into a group of six adult mice in a volume of 0·03 ml. Mortality was recorded daily and the end points determined after 15 days observation.

Experimental Results

(1) *Isolation of new type strains*

(*a*) *Virus Strain* 18/60

The registered specimen 18/60, from which this antigenic type was isolated, was obtained from a horse 2762 which was born at Onderstepoort on 5 November, 1953. This horse had been immunised annually with the polyvalent vaccine prepared from the established antigenic types, but died on 10 April, 1960 from a natural infection of horsesickness. On the morning before death the animal showed a mild febrile reaction with slight filling of the temporal fossae. Inadvertently it was worked under the saddle and shortly after returning to the stable, collapsed and died. A post-morten examination performed within two hours of death showed lesions associated with the cardiac form of the disease. A portion of spleen was excised and stored at −20°C. From this material the presence of a virus was indicated by the mortality in suckling mice after intracerebral injection as shown in Table 1.

Table 1.—*Fate of suckling mice injected intracerebrally with spleen suspension—specimen* 18/60

Passage level	Days after inoculation									
	1	2	3	4	5	6	7	8	9	10
Gen. 1.....	0/6*	0/6	0/6	0/6	0/6	0/6	0/6	3/3	3/0	—
2.....	0/8	0/8	1/7	2/5	5/0	—	—	—	—	—
3.....	0/5	0/5	4/1	1/0	—	—	—	—	—	—

* 0/6 indicates number of mice found dead/number of mice surviving

(*b*) *Virus Strain* 7/60

A portion of spleen preserved in 50 per cent glycerine-saline was received from the Pakistan Animal Husbandry Institute, Peshawar, and registered as specimen 7/60. This material was collected after death, from a pony which died 10 days after the subinoculation of 5 ml of blood, taken from two reacting donkeys. These donors represented the third serial passage of the virus in this host. On post-mortem examination lesions characteristic of the cardiac form of the disease were evident. The mortality in suckling mice is shown in Table 2.

TABLE 2.—*Fate of suckling mice injected intracerebrally with spleen suspension—specimen* 7/60

Passage level	Days after inoculation									
	1	2	3	4	5	6	7	8	9	10
Gen. 1.....	0/9	0/9	0/9	0/9	0/9	0/9	8/1	1/0	—	—
2.....	0/7	0/7	4/3	3/0	—	—	—	—	—	—
3.....	0/9	0/9	7/2	2/0	—	—	—	—	—	—

(2) *Identification of virus strains*

(*a*) *Action of sodium desoxycholate*

The inactivation of the arbor viruses by sodium desoxycholate under controlled conditions was used by Theiler (1957) as a preliminary test in the classification of viruses pathogenic for suckling mice.

The technique used by Theiler was followed closely. Infected suckling mouse brain of each virus strain in the form of a 10 per cent suspension in 0·75 per cent bovalbumin in 0·85 per cent saline was clarified by centrifugation at 10,000 rpm for an hour in a refrigerated M.S.E. centrifuge. Duplicate aliquots of each supernatant fluid were set up. To the one was added an equal volume of the diluting fluid in which was dissolved sodium desoxycholate in a concentration of 1: 500, to the other an equal volume of the diluting fluid alone. Both tubes were then incubated at 37°C for one hour after which the virus titres were determined by decimal dilution in adult mice, by the intracerebral route.

The results are shown in Table 3.

(*b*) *Action of diethyl ether*

Antigens prepared as previously described were incubated for 18 hours at 4°C with diethyl ether added to give a final concentration of 20 per cent (Andrewes & Horstmann, 1949). The mixtures were held in screw-capped bottles firmly closed to prevent evaporation of the ether. Control specimens without ether were similarly prepared. Virus infectivity was determined in suckling mice after removal of the ether by evaporation under negative pressure. The results are given in Table 3.

TABLE 3.—*Action of sodium desoxycholate and diethyl ether on African horsesickness virus*

Virus Group and Strain	Passage Level	Virus Titre		Difference	Virus Titre		Difference
		D.C.A.	Control		Ether	Control	
1 A 501	Over 100	3·50*	3·50	0·00	7·75	7·5	−0·25
2 OD	Over 100	3·30	3·50	0·20	6·1	5·9	−0·2
3 L	Over 100	3·50	3·50	0·00	6·4	6·9	0·5
4 VRY	Over 100	3·60	4·30	0·70	6·1	6·2	0·1
5 VH	Over 100	3·40	3·25	0·15	5·5	6·0	0·5
6 114	Over 100	5·50	5·80	0·30	6·0	6·3	0·3
7 Karen	103	4·50	4·10	−0·40	5·5	6·1	0·6
18/60	4	4·70	4·82	0·12	4·5	5·0	0·5
7/60	4	5·00	5·10	0·10	5·1	5·4	0·3
Rift Valley fever Smithburn	M 102† E 56 M 16	2·80	7·00	4·20	5·25	7·1	1·85

* Figures indicate logarithmic index end points calculated by the method of Reed and Muench.
† Neurotropic R.V.F. virus: 102 passages in mice followed by 56 passages in fertile hens' eggs and then a further 16 passages in suckling mice.

Result

The results of these two experiments show that representative strains of the recognised seven antigenic groups of horsesickness virus, as well as the two freshly isolated strains, are not inactivated by 1: 1000 sodium desoxycholate nor by 20 per cent diethyl ether whereas Rift Valley fever virus selected as a control to represent an accepted arbor virus (Theiler, 1957; Casals, 1959) is inactivated by both.

(*c*) *Complement fixation*

Infective suckling mouse brain tissue of the second serial passage was harvested and treated as above. The protocols of complement fixation tests carried out with these antigens in the presence of prebleed and convalescent horse serum are shown in Table 4.

TABLE 4.—*Complement fixation with newly isolated virus strains*

Antigen	Convalescent horse serum						Prebleed horse serum					
	1: 4	1: 8	1: 16	1: 32	1: 64	1: 128	1: 4	1: 8	1: 16	1: 32	1: 64	1: 128
18/60 Gen. 2	4*	4	4	4	3	0	0	0	0	0	0	0
Pos. Control HS Antigen Type 7	4	4	4	4	3	0	0	0	0	0	0	0
Normal Mouse Brain Control	4	2	0	0	0	0	0	0	0	0	0	0
Serum anticomplementary control	4	1	0	0	0	0	0	0	0	0	0	0
7/60 Gen. 2	4	4	4	4	3	1	0	0	0	0	0	0
Pos. Control HS Antigen Type 7	4	4	4	4	3	0	0	0	0	0	0	0
Normal Mouse Brain Control	1	0	0	0	0	0	0	0	0	0	0	0
Serum anticomplementary control	1	0	0	0	0	0	0	0	0	0	0	0

* Values of 4, 3, 2, 1 and 0 indicate degrees of fixation of complement

Result

Fixation of complement by both antigens is clear. The fact that the fixation end point happens to be at the same serum dilution level is quite fortuitous, as the antigens had not been standardised previously.

(*d*) *Serum virus neutralisation tests*

The results of the preliminary screen tests with the two new virus strains are shown in Table 5 from which it will be seen that there was no detectable neutralisation by undiluted antisera of the known antigenic types in the case of strain 18/60 and to low titre by type 6 only in the case of strain 7/60.

TABLE 5.—*Screen neutralisation tests—strains* 18/60 *and* 7/60

Antigen		Type Antisera						
Virus	Ld_{50}	1	2	3	4	5	6	7
18/60.....	100	0	0	0	0	0	0	0
7/60.....	60	0	0	0	0	0	35*	0

* Reciprocal of serum dilution giving 50 per cent protection

A complete series of cross neutralisation tests was then set up to determine the antigenic relationship of the new strains to the existing seven antigenic types. Antisera to types 1 to 7 were produced in rabbits, those to the new strains, in guinea-pigs as described above.

TABLE 6.—*Cross neutralisation of virus strains by type antisera*

Antigen			Type Antisera								
Type	Strain	Ld_{50}	1	2	3	4	5	6	7	8	9
1	A 501	177	280*	5	0	0	0	0	0	0	0
2	OD	50	7	386	5	0	0	5	0	0	0
3	L	20	11	0	3125	0	0	0	13	0	0
4	47/58	30	0	7	0	625	0	0	0	0	0
5	VH	30	0	0	11	0	280	25	11	0	0
6	114	30	0	0	0	0	0	125	0	0	0
7	Karen	56	0	0	0	0	0	0	328	0	0
	18/60	30	8	5	0	5	5	5	0	125	0
	7/60	63	7	6	5	5	8	48	5	5	125

* Reciprocal of the end point dilution giving 50 per cent protection

Results

Reference to Table 6 shows that there is no complete cross neutralisation either between the two new virus strains themselves or between them and any of the recognised antigenic types, though the slight overlap between 7/60 and Group 6 as indicated in the preliminary screen test, again is apparent. Whether any significance can be attached to the neutralisation of strains 18/60 and 7/60 to low titre by heterologous type antisera produced in guinea-pigs is not clear.

The results obtained in this series of experiments confirm the validity of the classification of antigenic types by previous workers (McIntosh, 1958).

Therefore, it is proposed to designate virus strains 18/60 and 7/60 as the prototypes of Groups 8 and 9.

Isolation of additional strains of Groups 8 and 9 viruses

While this work was in progress numerous other strains of horsesickness virus were isolated from material either collected or submitted for routine investigation. In this report no mention will be made of those which have been shown to fall into Groups 1–7 inclusive, but the origin and date of collection of those which fall into Groups 8 and 9 are detailed in Table 7.

TABLE 7.—*Origin and date of collection of additional Group 8 and 9 virus strains*

Antigenic Group	Strain	Origin	Date of Collection
8	18/60	Onderstepoort—Republic of South Africa	10. 5.60
	55/58	Vryburg—Republic of South Africa	10. 4.58
	53/61	Elizabethville—Katanga	29. 4.61
9	7/60	Peshawar—W. Pakistan	10.12.59
	10/60	Bushir—Iran	1. 4.60
	13/60	— —Iran	15. 4.60
	14/60	— —Iran	15. 4.60
	15/60	— —Iran	15. 4.60
	24/60	Jaipur—India	27. 5.60
	25/60	Jaipur—India	27. 5.60
	26/60	Jaipur—India	27. 5.60
	27/60	Jaipur—India	27. 5.60
	28/60	Jaipur—India	27. 5.60
	29/60	Jaipur—India	28. 5.60
	30/60	Jaipur—India	28. 5.60
	31/60	Jaipur—India	28. 5.60
	34/60	Nowshera—W. Pakistan	3. 9.60
	36/60	Kamary—Afghanistan	—
	40/60	Baghdad—Iraq	20. 9.60
	42/60	Baghdad—Iraq	20. 9.60
	45/60	Peshawar—W. Pakistan	15. 9.60
	46/60	Peshawar—W. Pakistan	20. 9.60
	49/60	Peshawar—W. Pakistan	20. 9.60
	52/61	Peshawar—W. Pakistan	5. 4.61
	58/61	Peshawar—W. Pakistan	1. 5.61
	60/61	Elazig—Turkey	—
	98/61	Peshawar—W. Pakistan	18.11.61

Result

An additional two strains of Group 8 virus have been identified, the one collected two years previously in Vryburg situated about 250 miles from Onderstepoort, the origin of the prototype, and the other a year later from Katanga, Central Africa.

During the period December, 1959, to November, 1961, a total of 24 strains of Group 9 viruses have been identified from areas as widely separated as West Pakistan, India, Iran, Iraq and Turkey. In fact no strain of virus belonging to another antigenic group has been identified from that area, the scene of the recent epizootic.

It is of interest to note that no Group 9 virus has been isolated or identified in South Africa, although there is considerable evidence to show that this virus type is present, from the high titre antibodies found in immunised horses exposed season after season to natural infection. There remains in the Onderstepoort collection a large number of specimens awaiting investigation.

Discussion

The study of the immunological properties of the antigenically complex virus of horsesickness will remain a subject of the greatest importance where prophylactic immunisation with a polyvalent vaccine, based upon the use of live attenuated strains of virus, is accepted as the rational approach to the control of the disease. In recent years the distribution of the disease and the boundaries of enzootic infection, have been extended considerably, so that the way has been opened for an approach to the many problems from a number of different angles.

In Africa the 18/60 strain of virus, the prototype of the new Group 8 of antigenic types, was only one of many strains belonging to other groups that were isolated during the normal seasonal recrudescence of the disease, in a notoriously bad enzootic area, amongst a predominantly immunised population. It was shown therefore, that the anticipated plurality of virus types did in fact appear simultaneously. In the Middle East from a wide-spread epizootic of horsesickness in a predominantly susceptible population there were isolated 24 strains of virus all of which belonged to a new antigenic Group 9 of which strain 7/60 is the prototype. At this stage it would be rash to conclude dogmatically that only one antigenic type of virus was involved in the epizootic, but at least only one was isolated and it, appears to have been the dominant, if not exclusive, type over a very wide area. It would seem, therefore, that it is a matter of prime importance for research workers in this field to watch the position closely and to determine, on a basis of international co-operation, when and where a multiplicity of virus types appears and the conditions under which they do emerge, such as the immune status of the equine population.

It is generally accepted today that never has a change in antigenic structure of any isolated horsesickness virus occurred on artificial, or needle passage in any host system, no matter how prolonged the passage series. What then is the origin of the different antigenic types? Does a change take place during a period of quiescence in a hypothetical reservoir of infection? The answer to this question must await the discovery of that reservoir. Does a mutation take place during a cyclical development in the insect vector? This question probably will only be answered when transmitting species of culicoides have been colonized and the

disease transmitted, under control, by means other than parenteral injection. In the light of the experience in the Middle East where the continued spread and persistence of infection must have been due to biological transmission, the insect vector would appear not to be the site of antigenic variation when a susceptible population is involved.

In this connection the experience in South Africa over a period of almost half a century merits attention. Initially a bivalent vaccine was used with a certain measure of success in the field. In due course as a result of improvements of methods and technique, a quadrivalent vaccine was developed and used for a period of some years with a considerable measure of success. Slowly but surely the percentage of immunised animals that broke down to natural infection increased. From these, antigenically aberrant strains of virus were isolated to be attenuated laboriously for inclusion in vaccines characterised by wider polyvalency. Today a total of nine such distinct antigenic groups have been identified and the natural question springs to mind—is this the end? At present this question cannot be answered.

Without entering into any discussion as to the limitations of the techniques used for the antigenic classification of virus strains, it behoves us to profit by the experience of the past and direct attention to the advisability or otherwise of monovalent immunisation as opposed to polyvalent immunisation as a means of control of the Middle East epizootic, at present and in the future. Theoretically there appears to be no doubt that that outbreak could be controlled by the widest possible use of a monovalent vaccine of high potency based upon a selected strain of Group 9. Mass production of the necessary vaccine would be simplified and expedited though its administration would not be affected. However, would such action not favour the development of antigenic variants or mutants? Possibly the wisest course, with an eye to the future, would be the immediate use of a vaccine characterised by the widest possible antigenic polyvalency, incorporating strains of proved potency. Alternatively monovalent immunisation to reduce the immediate economic loss could be practised but followed without delay by polyvalent immunisation.

In addition to the plurality of antigenic types of horsesickness virus it is well known that the virulence of different strains, even within the same antigenic group, may vary within wide limits. Until such time as a Group 9 virus has been isolated in South Africa no experiments on horses involving a strain of that group will be carried out. Published reports of research in the Middle East are scanty but from personal communications (Shah & Pavri, 1962) it would appear that at least some of the strains isolated are characterised by relative avirulence. This conclusion is drawn from the reported number of recoveries amongst naturally infected susceptible horses and the detection in the laboratory of neutralising antibody in the serum of animals, that must have undergone an inapparent infection. Relative avirulence of the predominant strains of virus would appear to be an explanation for the protection afforded by a polyvalent vaccine (O.I.E., 1961) which did not contain a Group 9 antigen though it may in part be due to the demonstrated overlap with Group 6, or a common antigenic factor between virus groups.

In identifying strains of virus isolated in mice from equines beyond the borders of the Republic of South Africa, in fact from outside the continent of Africa it is understandable that workers at Onderstepoort, located in a notoriously heavily infected enzootic area, were loth to use susceptible horses for confirmatory clinical

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diagnostic purposes. Therefore, the group specific complement fixation test (McIntosh, 1956) was used as the preliminary step. The finding that representatives of all nine antigenic types were not inactivated by 1: 1000 desoxycholate nor by 20 per cent diethyl ether is of importance in that these tests may be used to differentiate a virus strain from other accepted arbor viruses.

Finally it is expedient to point out that the spread of horsesickness to an extended area outside the previously accepted limits of enzootic infection, has proved to be a quite remarkable stimulus to further research into this disease and it is hoped that active collaboration with workers in other institutes will be fruitful in throwing additional light on methods of control with a view to ultimate eradication of infection.

Summary

Two new strains of horsesickness virus were isolated in suckling mice by the intracerebral route.

These strains were identified as horsesickness by:—

(*a*) Demonstration of fixation of complement by the group specific complement fixation test.

(*b*) Non-inactivation by sodium desoxycholate and diethyl ether under standard conditions.

(*c*) Preliminary screen serum virus neutralisation tests.

Detailed serum virus cross neutralisation tests showed that these virus strains belong to two new antigenic types, which have been designated Groups 8 and 9.

The two strains were isolated from widely separated geographical regions and are regarded as the prototypes of the two new groups which have been shown to include other strains of virus.

The significance of the finding is discussed in relation of control of horsesickness by mass immunisation.

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