

COMPLEMENT FIXATION WITH HORSESICKNESS VIRUSES.

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For some years a neutralization test has been available for the immunological differentiation and identification of horsesickness viruses. This test involves the intracerebral inoculation of mice and has been used with antisera from horses, rabbits and ferrets (Alexander, 1935; McIntosh, 1955). With antisera prepared in rabbits it has been possible to group all horsesickness virus strains so far examined into seven immunological types (McIntosh, 1955). In these neutralization tests all strains grouped as homotypic were reciprocally cross-reactive while practically no immunological relationship was evident between the heterotypic strains. So far the neutralization test has been the only laboratory tool available for the antigenic study of these viruses.

Since complement fixation offered a possible means of studying further the antigenic differences between strains experiments were conducted to evolve a satisfactory test. Fixation of complement has been demonstrated with a large number of neurotropic viruses and as most horsesickness strains multiplied in suckling mouse brains to titres of 10^6 and 10^7 LD₅₀ it was hoped that fixation with these viruses would also be possible. This has been the case and this article deals with this work. From a publication of Polson and Madsen (1954) it is evident that these workers have used this test with horsesickness virus but no details of their methods were given.

METHODS AND MATERIALS.

Virus Strains.

Seven virus strains, viz., A501, OD, L., VRY, VH, 114 and PMB, each representing a different immunological type were selected to be used in this work. All of them had had at least 100 intracerebral mouse passages.

Antigens.

To prepare a batch of antigen a 10^{-1} dilution of stock virus suspension was inoculated intracerebrally into 60 to 80 suckling mice 3 to 6 days old. When the mice were *in extremis* on the second or third day after inoculation they were killed in ether vapour and their brains removed. These were weighed and from the number of mice injected usually 12 to 15 grams of tissue were obtained. After maceration of the brains with a pestle and mortar and alundum, they were extracted with acetone and ether according to the method of Casals (1949). The residual ether was removed under negative pressure from a water vacuum pump for one hour. Two volumes of saline, calculated on the weight of the original wet brain tissue, were added to the dried brains and the suspension held at 4° C. overnight. It was then centrifuged in the cold at 10,000 r.p.m. for one hour. The supernatant was the antigen which was pipetted off and merthiolate added. The antigens were stored at 4° C.

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Antisera.

Antisera against each of the above virus strains were obtained from guinea-pigs which had received repeated injections of virus. To avoid the production of complement fixing antibodies in these guinea-pigs against the heterologous mouse brain tissue the initial injections consisted of egg cultivated virus. For this purpose mouse-adapted virus was injected via the yolk-sac into hen eggs containing 8-day old embryos. The eggs were incubated at 32° C. and embryos which died on the 4th to 6th day after injection were harvested, macerated and stored at -20° C. One ml. of a 10⁻¹ dilution of these embryos was used to inject the guinea-pigs. Injections were given intraperitoneally and were administered at the following intervals: day 0, 30, 90, 94, and 120. With strains A501, OD and 114 the inocula on the 90th and 94th days consisted of infective suckling mouse brains at a dilution of 10⁻² instead of egg-cultivated virus. Infective suckling mouse brains at a dilution of 10⁻¹ were used with all strains on the 120th day inoculation. The guinea-pigs were bled for serum 10 days after this injection. The sera were heated at 56° C. for 50 minutes and stored at -20° C. in small volumes sufficient for one test.

Diluent.

All dilutions were done in a veronal-saline buffer solution containing Ca and Mg ions as recommended by Brooksby (1952).

Other Reagents.

The preparation of the complement, haemolysin and the erythrocyte suspension followed conventional methods.

Technique of the Test.

The antisera were diluted two-fold in volumes of 0.2 ml. beginning with a 1:2 dilution. After completion of the serum dilutions 0.2 ml. of complement containing 2 units was added followed by 0.2 ml. of antigen. Before it was used in the cross fixation tests each antigen was titrated to determine the dilution which gave the maximum complement fixing reactivity. The highest dilution of antigen which gave the highest serum titre was selected for use. This was usually a dilution of 1:4 or 1:8 of the antigen (1:3 of brain tissue) and represented about 8 antigen units. In a few batches of antigen used in the cross-fixation tests complement fixing activity ceased at dilutions higher than 1:4. Consequently the number of antigen units in these antigens was less than 8. For fixation the mixtures were incubated at 37° C. for 75 minutes. Four-tenths ml. of sensitised red cells (containing 2 units of haemolysin) was added and the tests were read after incubation at 37° C. for 30 minutes. The serum end-point was the highest dilution giving +3 or more fixation.

RESULTS.

The guinea-pig antisera employed in the experiments were capable of fixing complement in the presence of antigen and no anticomplementary activity from either reagent was evident. It appeared necessary to give the guinea-pigs injections of mouse brain propagated virus for satisfactory serum titres. The reason for this was not apparent although the slightly higher virus titres obtained when these strains are propagated in suckling mice in comparison with embryonated eggs may possibly be the reason. None of the guinea-pigs developed complement fixing antibodies against mouse brain tissue.

A series of reciprocal cross fixation tests with the seven heterotypic virus strains showed that all these strains are cross-reactive with each other and no difference between them was evident. The results of these tests are shown in Table 1. The four-fold difference in titre of some antisera against different antigens is not considered to be significant and is likely to be the result of using antigens of varying unit strength.

TABLE 1.

Showing Serum Titres Obtained in Cross-Fixation with Heterotypic Virus Strains.

Virus Strain.	Immunol. Type.	ANTISERA.							
		A. 501	O.D.	L.	VRV.	VM.	114.	PMB.	MP.
A501.....	1	128*	64	32	32	32	32	64	NT
OD.....	2	64	32	64	16	32	32	64	NT
L.....	3	64	32	64	16	16	64	64	NT
VRV.....	4	64	32	32	32	16	16	64	NT
VM.....	5	256	64	32	32	64	64	64	NT
114.....	6	128	32	64	32	16	128	64	NT
PMB.....	7	128	64	32	16	32	32	64	NT
MP.....	—	—	—	—	—	—	—	—	32

* = Serum titre expressed as reciprocal of dilution.

MP = Mimosa Park bluetongue strain propagated in brains of suckling mice and used here as a control antigen. The antiserum was obtained from adult mice injected intracerebrally (van den Ende, Linder and Kaschula, 1954).

NT = Not tested.

With a "box" titration in which dilutions of serum were tested against antigen dilutions it was observed that higher serum titres often occurred with higher dilutions of antigen rather than with the undiluted antigen. This phenomenon has been observed by Espana and Mammon (1947) and Casals (1949). Such a titration is shown in Table 2 with dilutions of L. antigen tested against L. antiserum. These results show that the antigen had a greater reactivity at a dilution of 1:8 and 1:16 than at lower dilutions.

TABLE 2.

Complement Fixation Test with L. Virus Antigen and Homologous Guinea-pig Hyperimmune serum.

Antigen Dilution.	SERUM DILUTIONS.							
	1:2.	1:4.	1:8.	1:16.	1:32.	1:64.	1:128.	1:256.
1:1.....	4	4	4	4	4	0	0	0
1:2.....	4	4	4	4	4	3	0	0
1:4.....	4	4	4	4	4	4	1	0
1:8.....	4	4	4	4	4	4	3	0
1:16.....	4	4	4	4	4	4	3	0
1:32.....	4	4	4	4	1	0	0	0
1:64.....	3	0	0	0	0	0	0	0

DISCUSSION.

From the results it is evident that with the antigens used in the present experiments no antigenic difference between the seven heterotypic strains can be shown and hence no confirmation of the viral grouping obtained with the neutralization test is possible. With influenza virus and the complement fixation test Lennette and Horsfall (1941) and Friedewald (1943) showed that soluble antigens freed of virus are immunologically specific for the serological type from which they are derived but fail to reflect the immunological difference between strains of one type. The antigen associated with the virus particle, on the other hand, shows a higher degree of strains specificity comparable with that obtained in neutralization or haemagglutination-inhibition tests. Polson and Madsen (1954) showed that neurotropic horsesickness virus suspensions contained infective particles of two different sizes, viz., 31.2 $\mu\mu$ and 50.8 $\mu\mu$ as well as a non-infective particle of 12 $\mu\mu$ diameter. They found that after complete removal of infective particles from virus suspensions by ultracentrifugation at 30,000 r.p.m. for 2 hours the supernatant fluid still contained about one half of the original complement fixing power. It remains to be seen whether the particulate complement fixing antigens of horsesickness virus would show a higher specificity than the antigens used in the present tests.

At the moment the value of the complement fixation test with horsesickness viruses lies in the facility with which it enables the identification of these viruses to be made. Recently this test was used successfully in the diagnosis of an outbreak of horsesickness in a pack of hounds. Fixation of complement occurred between antiserum from hyperimmune guinea-pigs and virus isolated in mice from one of the hounds. The antigen for the test was prepared from infective suckling mouse brains of the second passage—a passage level at which neutralization tests are somewhat difficult to interpret. While the immunological identity of viscerotropic horsesickness virus can be determined by neutralization tests on antisera from ferrets infected with viscerotropic virus, seven neutralization tests may be necessary for this purpose and a negative result is not conclusive as the unknown virus may belong to an additional and as yet unidentified immunological type. A negative biological test in horses may also be unsatisfactory as there is no certain method of determining the complete susceptibility of these animals. Horses can be immune to a horsesickness strain and still show no neutralizing antibodies in the serum.

Limited tests using horse antisera with mouse brain antigens and guinea-pig hyperimmune antisera with infective horse spleen containing virulent virus have not as yet resulted in fixation of complement.

SUMMARY.

Fixation of complement has been obtained using antigens prepared from suckling mouse brains and antisera from hyperimmune guinea-pigs.

With horsesickness viruses the complement fixation test is less type specific than the neutralization test and no antigenic differentiation between the seven heterotypic strains tested was possible.

The value of the complement fixation test in the diagnosis of the disease is discussed.

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