A NON-SPECIFIC COMPLEMENT-FIXING SYSTEM IN FOOT-AND-MOUTH DISEASE AND EXPERIMENTS ON THE ABSORPTION OF HETEROLOGOUS ANTIBODY FROM SERUM

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The lack of specificity which is often found in association with guinea-pig antisera of high titre decreases the precision of the complement-fixation test in foot-and-mouth disease. Tests for immunological type of some field strains have shown marked overlapping fixation. An obvious matter for investigation, therefore, is the use of sera absorbed with virus of the heterologous types. The present series of experiments was begun to determine if a method of absorption similar to that used by Henle, Henle, Groupi & Chambers (1944) for antisera to the influenza virus would be practicable in the case of foot-and-mouth disease. This method depends on ultracentrifugation for removal of the absorbing virus together with the antibody with which it has combined. Successful absorption of antibody from foot-and-mouth disease antisera has been made in some instances with this technique, but the results were not always reproducible. A more definite finding emerged from the control observations, since it was found that, before the ultracentrifugation which followed absorption, the serum-virus mixture fixed complement to a considerable degree, even after storage overnight at 4° C. and after heating at 56° C. for 30 min. The present paper deals with the investigation of this effect and with the results of some of the absorption experiments.

METHODS

Both viruses and antisera used were of guinea-pig origin throughout. The stock guinea-pig strains of the three types, Strain 1 (Vallée O type), Strain GB (Vallée A type) and Strain GC (Waldmann C type) were maintained by serial passage intradermally on the tarsal pads. The pads were collected after 24 hr. when the primary lesions were well-developed. In some cases pads were stored for several weeks at −20° C. For the preparation of antigen or for absorption of sera, suspensions were made by grinding minced pads with sand in 0·9 % NaCl, or in this solution buffered with veronal, at the rate of one pad per ml. of suspending fluid. The suspensions were centrifuged at about 2000 r.p.m. in a small laboratory centrifuge for 5 min. before use.

The antisera were prepared as described earlier (Brooksby, 1952) by hyperimmunizing guinea-pigs from 8 to 12 weeks after their initial infection with the virus. The guinea-pigs were killed and bled out ten days after the hyperimmunization.

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The complement-fixation technique has also been described (Brooksby, 1952). Each tube contained 1 ml. of complement dilution, 0·4 ml. of serum dilution, 0·2 ml. of antigen and 1 ml. of the sensitized cell suspension. The complement dilutions were made in veronal-buffered saline and a series of sub-dilutions of a 1/25 stock solution prepared so that the 1 ml. doses pipetted into each successive tube in the set of five contained 0·09, 0·13, 0·2, 0·3 and 0·45 ml. of the 1/25 stock solution. Fixation was carried out in a water-bath at 37° C. for 30 min., after which the sensitized cells were added without removing the tubes from the bath. A further 30 min. were allowed for haemolysis, and the tubes were then removed from the water-bath and centrifuged as soon as possible for 3 min. at 2000 r.p.m. The percentage haemolysis in each tube was then estimated, using a photoelectric absorptiometer. The probit of the percentage haemolysis for each tube in the set of five was then plotted against the logarithm of the dose of 1/25 complement used. The line so obtained indicated the amount of complement necessary for 50 % haemolysis. The difference between the control and fixing mixtures was then calculated and is referred to as the log. dose of complement fixed. By plotting these results against the dilutions of serum used, a serum titre figure is obtained, that is, a dilution of serum estimated to fix a selected log. dose of complement, usually log. 0·3 or twice the 50 % unit of complement.

RESULTS

A typical experiment for the demonstration of the non-specific stable complement-fixing material which will be termed, for convenience, the non-specific complex, was made as follows. A centrifuged virus suspension was mixed with an equal volume of a 1/4 dilution of hyperimmune antiserum, which had been inactivated at 56° C. for 30 min. The mixture was incubated at 37° C. for one hour, stored overnight at 4° C. and then heated at 56° C. for 30 min. This material was then tested for its ability to fix complement, 0·4 ml. being added to tubes containing 1·0 ml. of the serial complement dilutions. 0·2 ml. of veronal buffer was added to bring the volume up to the standard 1·6 ml. Fixation was for 30 min. at 37° C. The dilutions of mixtures of virus of types O, A and C with type A and C antisera fixing log. 0·3 of 1/25 complement are shown in Table 1. The variation in the fixation produced by overnight mixtures of any of the three viruses with a given serum can be seen to be insignificant, and the virus-serum complex involved in the reaction would therefore seem to be quite definitely non-specific. The titres of the two sera obtained in orthodox tests, in which the reagents were added in the order complement—antiserum—virus, are also shown in Table 1. Comparison of the results in columns 3 and 4 of Table 1 indicates that the amount of complex formed would appear to be independent of the usual specific : non-specific ratio of the serum.

Several possible factors which might influence the result have been investigated: (*a*) change in the medium used for dilution of the serum and suspension of the virus from veronal buffered saline to unbuffered saline was without effect; (*b*) serum stored frozen at − 20° C. gave the same result as lyophilized reconstituted serum;

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(*c*) incubation of the serum-virus mixtures at 37° C. for 60 min. was omitted in some experiments without affecting the result. Similarly the final heating at 56° C. for 30 min. could be omitted or prolonged to 60 min. The formation of the virus-serum complex seems to take place during the overnight storage of the mixture. At what time the specific fixing activity of the virus with the antiserum decreases and allows the demonstration of the non-specific effect has not yet been determined. Once the non-specific complex is formed it is heat stable (56° C. for 30 min.) and resists storage (several days). (*d*) Normal serum failed to produce the effect with similar virus suspensions.

Table 1. *Comparison of serum titre in overnight 'complex formation' and routine complement-fixation*

Serum type and batch no.	Virus type	Serum titre estimated by dilution of overnight mixture	Serum titre estimated in routine 30 min. fixation test
C_{73}	O	1/32	1/20
	A	1/42	1/13
	C	1/42	1/168
A_{78}	O	1/109	1/13
	A	1/118	1/256
	C	1/102	1/7

In each case, the titre of antiserum is the dilution of the serum in the mixture estimated to fix log. 0·3 of 1/25 complement.

Removal of the non-specific complex by ultracentrifugation

In all experiments in which a non-specific complex was demonstrated it was further shown that ultracentrifugation in the L40 rotor of the Spinco ultracentrifuge at 35,000 r.p.m. for 50 min. would remove the complement-fixing activity. Such a procedure would remove 99 % of the infectivity of the virus suspension (Bradish, Brooksby, Dillon & Norambuena, 1952) and it would therefore be presumed to remove virus with any antibody which had combined with it. The smaller complement-fixing component of the foot-and-mouth disease virus system would not be removed to any great extent by such centrifugation unless aggregation had taken place in the presence of antibody. The role of the small component in this phenomenon therefore remains doubtful.

Attempts to demonstrate the effect with antigens other than the virus of foot-and-mouth disease

The possiblity that the effect might be due to a species-specific antigen or a Forssman-type antigen was tested by using pad suspensions from normal guinea-pigs and from guinea-pigs infected with the virus of vesicular stomatitis. In preparing the pad 'antigen' from normal guinea-pigs, blisters were induced on the pads of anaesthetised guinea-pigs with pledgets of hot, wet, cotton wool. The guinea-pigs were killed while still anaesthetised and the detached epithelium was collected. The pad antigens from both normal and vesicular stomatitis-infected

guinea-pigs failed to produce the effect with antisera to the virus of foot-and-mouth disease.

Absorption of heterologous antibody

When removal of the non-specific complement-fixing complex by ultracentrifugation had been demonstrated, the specificity and titre of the antibody remaining in the serum assumed new interest. The results of absorption have not always been the same, in contrast to the regularity with which the non-specific complex itself is formed. In some cases non-specific absorption was so great that it reduced the homologous titre of a serum by eightfold. Absorption of this order is illustrated in Table 2 in which the effect of absorbing O serum with A virus and A serum with O virus is illustrated. When loss of specific antibody is so great it is impossible to draw conclusions about the existence of a non-specific antibody.

Table 2. *Absorption of antibody*

	Type O serum				Type A serum			
	Before absorption. Complement fixation with virus of type		After absorption with A virus. Complement fixation with virus of type		Before absorption. Complement fixation with virus of type		After absorption with O virus. Complement fixation with virus of type	
Dilution of serum	O	A	O	A	O	A	O	A
1/8	0·77	0·23	0·46	0	0·1	—	0	0·61
1/16	0·76	0·19	0·18	0	0·06	—	0·01	0·38
1/32	0·75	0·07	0	0	0·04	—	0	0·03
1/64	0·58	0	0	0	0·02	0·68	0	0

The figures indicate the log. dose of 1/25 complement fixed by the serum with excess antigen of the type indicated.

In other cases the technique was successful to the point of decreasing to zero the heterologous titre of a serum while leaving the homologous titre unchanged. This is best illustrated in the results of two experiments in which two successive absorptions were carried out on the same serum. The plan of these experiments is shown in the key to Tables 3–6 and further evidence for formation of the non-specific complex is shown in the body of these tables. It is confirmed that an equal amount of complex formation takes place in homologous and heterologous mixtures. It is surprising that complex formation was no less at the second absorption cycle than at the first, and may even have been slightly greater. The fixation with the absorbed serum and homologous and heterologous virus is shown in Tables 4 and 6. Absorption in this instance has not reduced significantly the specific or homologous titre while the non-specific or heterologous fixation has disappeared. Further, the same result was obtained after absorption by either homologous or heterologous virus. These findings may be due to a combination of factors of which the most probable are that the available virus was, in this experiment, insufficient to absorb more than a small fraction of the homologous antibody and that this specific antibody was present in much greater excess than the non-specific. The influence of the

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amount of virus used on the degree of absorption has not been investigated, since the variation in titres of pad suspensions prepared under the conditions described is not usually outside the range of experimental error in infectivity titration.

From the results of the absorption experiments, it is not possible to indicate the relationship between formation of the non-specific complex and the removal of heterologous antibody from the serum. The amount of fixation by the complex is

Key to the experiments of which the results are given in Tables 3–6.

C serum	Absorbed with O virus = C/O	Absorbed with O virus = C/O/O Absorbed with C virus = C/O/C
C serum	Absorbed with C virus = C/C	Absorbed with O virus = C/C/O Absorbed with C virus = C/C/C

In Tables 3–6 the figures indicate the log. dose of 1/25 complement fixed by the mixture concerned.

Table 3. *Demonstration of formation of non-specific complex in two cycles of absorption*

	First cycle C serum + O virus (C/O)		Second cycle C/O serum + O virus (C/O/O)		Second cycle C/O serum + C virus (C/O/C)	
Dilution of serum	Before centri-fugation	After centri-fugation	Before centri-fugation	After centri-fugation	Before centri-fugation	After centri-fugation
1/16	0·66	0·07	0·64	0·08	0·73	0·19
1/32	0·37	0	0·57	0·06	0·60	0·10
1/64	0·26	0	0·38	0·02	0·43	0·03
1/128	0·09	0	0·21	0·02	0·29	0

Table 4. *Fixation with doubly absorbed sera* (*Table* 3 '*after centrifugation*')

	Untreated serum + virus		First cycle C/O serum + virus		Second cycle C/O/O serum + virus		Second cycle C/O/C serum + virus	
Dilution of serum	C	O	C	O	C	O	C	O
1/16	—	0·33	—	0·16	—	0	—	0
1/32	—	0·28	—	0·08	—	0	—	0
1/64	—	0·14	—	0	—	0	—	0
1/128	0·50	0·01	0·56	0	0·54	0	0·46	0
1/256	0·33	—	0·42	—	0·33	—	0·28	—

Table 5. *Formation of non-specific complex in two cycles of absorption, first with homologous virus*

	First cycle C serum + C virus (C/C)		Second cycle C/C serum + O virus (C/C/O)		Second cycle C/C serum + C virus (C/C/C)	
Dilution of serum	Before centri-fugation	After centri-fugation	Before centri-fugation	After centri-fugation	Before centri-fugation	After centri-fugation
1/16	0·61	0·32	0·66	0·16	0·78	0·18
1/32	0·45	0·19	0·52	0·10	0·65	0·10
1/64	0·26	0·08	0·34	0·04	0·47	0·08
1/128	0·12	0	0·20	0	0·29	0·05

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surprisingly large, the more so when, as in the experiments of Tables 4 and 6, there is no decrease in the homologous antibody after removal of the complex. Further evidence on this point is provided by an experiment in which the neutralizing titre of a serum was measured in unweaned mice before and after complex formation and removal by ultracentrifugation. There was no decrease in the homologous serum titre in the two tests. Unfortunately the heterologous neutralizing power was below the level of significance of the test in both cases.

Table 6. *Fixation with doubly absorbed sera* (*Table* 5 '*after centrifugation*')

Dilution of serum	Untreated serum + virus		First cycle C/C serum + virus		Second cycle C/C/O serum + virus		Second cycle C/C/C serum + virus	
	C	O	C	O	C	O	C	O
1/16	—	0·33	—	0·17	—	0	—	0·06
1/32	—	0·28	—	0·14	—	0	—	0
1/64	—	0·14	—	0	—	0	—	0·02
1/128	0·50	0·01	0·7	—	0·50	—	0·50	—
1/256	0·33	—	0·45	—	0·38	—	0·31	—
1/512	0·15	—	0·15	—	0·16	—	0·08	—

DISCUSSION

It has been shown that a mixture of antiserum and the virus of foot-and-mouth disease, stored overnight at 4° C., has the property of fixing complement. The effect is considered as 'fixation' rather than as an anticomplementary effect since it involves an antigen-antibody mixture. Ultracentrifugation sufficient to remove virus particles also removes the fixing power of the mixture and it seems likely therefore that an antigen-antibody complex is formed. This complex is of somewhat unusual character since it retains its fixing power after heating at 56° C. for 30 or 60 min., and since its formation is independent of the usual type specificity of serological reactions in foot-and-mouth disease. The lack of specificity may be explained on the hypothesis that the antibody concerned is a group antibody, specific for foot-and-mouth disease virus irrespective of type. In this event it would be difficult to explain the fact that the amount of fixation produced by the complex is considerably greater than the usual heterologous or overlapping fixation in orthodox tests.

In the double absorption tests, also, complex formation appeared to be unaffected by the removal of much of the heterologous fixing power from the serum in the first cycle of absorption. Further quantitative studies are necessary to elucidate this problem but the present findings demonstrate in a new way the 'disease specificity' of foot-and-mouth disease antisera.

SUMMARY

Stored mixtures of the virus and corresponding antisera in foot-and-mouth disease have equal complement-fixing activity whether they are prepared from virus and serum of homologous or heterologous types. On ultracentrifugation the complement-fixing activity is removed from the mixture and the remaining antibody is

more sharply type-specific in routine complement-fixation tests than the original serum.

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