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A complement fixation technique for the quantitative measurement of antigenic differences between strains of the virus of foot-and-mouth disease

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The early literature on the specificity of the immunological types of foot-and-mouth disease virus included references to minor antigenic differences which occurred between strains of virus of the same type. Such strains were called variants. The value of these observations, however, was limited by the techniques then available for differentiation. Bedson, Maitland & Burbury (1927) applied an *in vitro* serum neutralization test in their comparison of two strains of virus of Type A. They recorded results which indicated that higher serum neutralization titres were obtained with homologous serum–virus mixtures than with heterologous mixtures.

Progress in this field of investigation resulted from the development of the complement fixation test. Traub & Möhlmann (1946) used this test to demonstrate antigenic differences between three strains of virus of Type A and also drew attention to the possible significance of these observations in relation to the efficiency of protection by vaccines. Subsequent work at this Institute was directed to the correlation of the results obtained by cross-vaccination, cross-serum neutralization and cross-complement fixation tests. In the first of these experiments Galloway, Henderson & Brooksby (1948) compared three strains of virus of Type A, A119 (A_{12} Pirbright) with MP and M1 from the Mexican outbreak of 1947–49. The comparison of a further ten pairs of strains of Type A, Type O, Type SAT1 and Type SAT2 (Rice & Brooksby, 1953; Brooksby, 1952; Davie, 1962; Martin, Davies & Smith, 1962; Hyslop, Davie & Carter, 1963; and unpublished work) has confirmed that there are strains of virus of the same immunological type which can be classified as antigenic variants or subtypes within the types and that in general there is good correlation between the results of the three tests. The results of the two serological tests and the cross-vaccination tests showed that three of the ten pairs were of the same subtype and that seven of them were dissimilar.

It has been found that the reproducibility of the results obtained in the complement fixation test and its convenience are of advantage when a large number of strains of virus of one type have to be examined. The complement fixation test was applied by Traub & Möhlmann (1946) to demonstrate differences in the titre of immune sera reacting with homologous and heterologous antigens. Brooksby, Galloway & Henderson (1948) used the amount of complement fixed as a measure of such reactions, while Graves (1960) combined the titration of complement with the titration of antisera in order to compare homologous and heterologous antigen–

antibody combination. Bradish, Brooksby & Tsubahara (1960) and Bradish & Brooksby (1960) made a detailed examination of the reactions of foot-and-mouth disease antigens and antisera in the complement fixation test and established certain principles for the comparison of virus strains. They demonstrated that the amount of complement fixed by the reaction of an antigen with an antiserum was in proportion to the concentration of antibody when the antigen was present in limited excess in the reacting mixture; that a comparison of the amounts of complement fixed by two or more strain-specific antisera in an excess of one antigen allowed a more sensitive detection of differences between strains of virus than when two antigens and one antiserum were used; and that, if two or more strain-specific antisera were titrated with one preparation of antigen, the reactions of each mixture of antigen and antibody throughout the dilution series could be measured as the amount of complement fixed at optimum proportions of antibody with limited excess of antigen and compared as the cross-fixation ratio of each serum to that of the homologous mixture. When the cross-fixation ratios of a range of strain-specific antisera were compared in their reactions with the virus strains used to prepare the sera, the relationship of one virus strain to the other could be expressed as the product of their cross-fixation ratios one with the other. Two virus strains were distinct if the cross-fixation product was significantly different from unity.

MATERIALS AND METHODS

A series of virus strains of type O was examined, including the established subtypes O_1, O_2 and O_3. The other virus strains included nine strains isolated from field outbreaks of disease in Britain from 1957 to 1961 and several other strains of virus maintained at Pirbright for experimental purposes. It was not known whether any of these strains had a common origin.

The virus antigens and antisera used in the tests were prepared in guinea-pigs. Original virus samples recovered from cattle were adapted to growth in guinea-pigs by intradermal inoculation of the metatarsal pad and serial passage of infected pad material. A virus was considered to be adapted to the guinea-pig when secondary lesions developed on the metacarpal pads or tongue following metatarsal pad inoculation, usually at the third to sixth passage. The guinea-pigs used for passage of the virus strains and the preparation of sera were kept in strict isolation, one room being set aside for each strain examined.

Antigens

The antigens were prepared from infected guinea-pig pad material. The pads and vesicle fluid were collected 24 hr. after intradermal inoculation of the metatarsal pads. Antigen suspensions were prepared by grinding one pad in 1 ml. of 0·04M phosphate buffer, pH 7·6, to give a 1/10 suspension and clarified by centrifugation with a laboratory centrifuge at 3000 r.p.m. for 10 min. The suspensions were prepared immediately before the tests and the infected pads were used within 48 hr. of collection. The infectivity of these antigens was in the region of 10^8 mouse ID50 per ml.

Antisera

Guinea-pig antisera were used in the tests. 600–800 g. guinea-pigs were inoculated intradermally with infected pad material of high titre and, if secondary lesions developed, the guinea-pigs were held for 4 weeks. They were then given two intramuscular injections of 1 ml. of a 1/5 suspension of fresh infected pads at an interval of 4 days. Ten days after the last inoculation, the guinea-pigs were bled and the serum collected and pooled. The pooled serum was filtered through a Seitz E.K. pad, inactivated at 56° C. for 30 min. and stored in convenient quantities at −20° C. The stored serum was diluted as described below before the tests and inactivated for a further 30 min. at 56° C.

The complement fixation test

This followed the basic procedure developed and described by Brooksby (1952) for the 'short' or 'routine' test. The complement used was normal guinea-pig serum freeze-dried and stored under vacuum. Immediately before each test the dried complement was reconstituted and diluted in veronal buffer with added calcium and magnesium. The dilution chosen, usually 1/20 or 1/25, was that at which a 0·1 ml. dose would give approximately 50 % haemolysis in the complement control titration. This standard dilution was found to be constant within each batch of dried complement. Subdilutions were prepared in which seven doses of complement in a logarithmic series of 0·09, 0·13, 0·2, 0·3, 0·45, 0·67 and 1·0 ml. were obtained when pipetting a standard 1·0 ml. amount of complement dilution into each tube. Five doses of complement within this series were chosen to cover the expected 50 % haemolytic end-point in each set of five tubes containing the reacting mixtures of antigen and antiserum or control titrations of the reactants alone.

The haemolytic indicator system consisted of a 1·5 % suspension of washed sheep cells sensitized and incubated with 4 minimum haemolytic doses of glycerinated haemolytic horse serum for 30 min. before use.

The appropriate complement dilutions in 1 ml. volumes were put into sets of five tubes. To these were added 0·4 ml. of diluted serum and 0·2 ml. of antigen suspension. Control titrations of antigen and serum alone were included in the tests and the total volume of each tube was made up, where necessary, to 1·6 ml. with veronal buffer.

After incubation for 30 min. in a water bath at 37° C., 1 ml. of the haemolytic indicator system was added to each tube and incubation was continued for another 30 min. The red cells which had not been haemolysed in the tubes were then sedimented by centrifugation and the degree of haemolysis in each tube determined with a simple colorimeter.

Titration of antisera

Each serum was titrated within a 1·25-fold dilution series from 1/32 to 1/298 against a 1/10 suspension of each antigen. The volume of complement required for 50 % haemolysis after absorption by the antigen–antibody complex was determined graphically by plotting the percentage haemolysis between 20 and 80 %

against the log. dose of complement in the tubes. The log. dose of complement required for 50 % haemolysis was indicated by a line drawn between two adjacent points above and below 50 %. Control titrations of the antigen, serum and complement were made in each test.

Assay of complement-fixing activity

The complement-fixing activity of the antisera with the antigens was calculated by the method described by Bradish *et al.* (1960) using the formula

$$\alpha B = (V_i - V_0)/v_i.$$

V_i is the volume of complement required for 50 % haemolysis in the test, V_0 is that volume of complement required for 50 % haemolysis by the most anticomplementary reagent, usually the antigen, in the control tests and v_i is the volume of whole serum used in the test. The complement-fixing activity αB is thus expressed as the volume of complement fixed per unit volume of serum.

When the complement-fixing activity of an antiserum at all the points in a dilution series is calculated in this way, it is found that there is depression of fixation when antibody on the one hand and antigen on the other is in excess in the reacting mixtures. There are, however, two or three points within the dilution series which exhibit close proportional relationship to one another. The amounts of complement fixed at these points can be used to calculate a mean value for the reaction slope which expresses the specific reaction of the antibody with the antigen in the test when these are both present in optimum proportions for maximum fixation of complement.

The reaction slope can be plotted as in Fig. 1 by using the equation above and choosing an appropriate value for v_i. Thus,

when $$\alpha B = (V_i - V_0)/v_i,$$

then $$V_i = (\alpha B \times v_i) + V_0.$$

Cross-fixation ratios

In practice, a number of strain-specific antisera are titrated with one preparation of antigen and the complement-fixing activity of each serum is calculated by the method described. When a strain-homologous antiserum prepared from the antigen is included in the range of sera tested, the complement-fixing activity of each antiserum can be expressed as the cross-fixation ratio of each serum to that of the homologous strain-specific serum.

Thus, when two antisera, A (homologous) and B (heterologous), react with one preparation of antigen, the cross-fixation ratio of heterologous serum B to homologous serum A equals

$$\frac{\text{complement-fixing activity of serum } B}{\text{complement-fixing activity of serum } A}.$$

The values obtained are found to be highly specific to each serum and this specificity is not affected by variations in the concentration of antigen or the components of the haemolytic system.

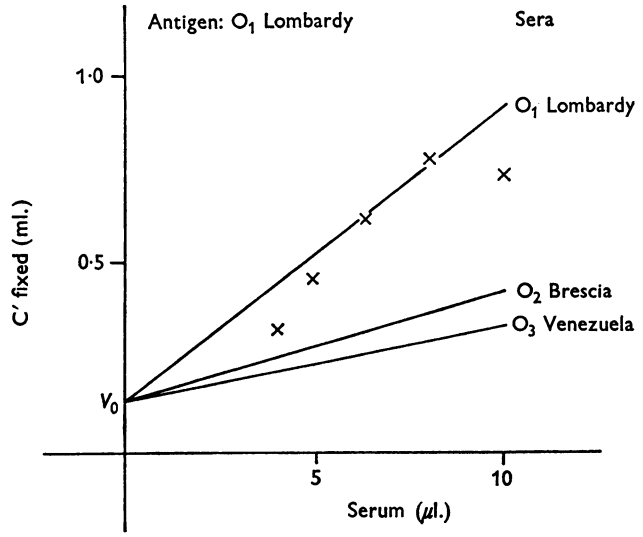

Fig. 1. The titration of three subtype specific sera in the presence of one antigen.

Cross-fixation products

When a series of strain-specific sera is tested with the virus antigens used to produce the sera, and the amounts of complement fixed by homologous and heterologous mixtures of antigen and antibody reacting in optimum proportion are compared as cross-fixation ratios, the relationship of the antigens to each other can be expressed as the product of the cross-fixation ratios of their strain-specific sera. Identical strains of virus have cross-fixation products of 1·0 while antigenically different strains have cross-fixation products of less than 1·0, depending upon the degree of difference.

RESULTS

Serum titrations

Table 1 shows the results of a complement fixation test in which a serum prepared from O_1 Lombardy virus is titrated in the presence of its homologous antigen. The volumes of complement fixed at the second and third dilutions of the series are proportionately greater per unit volume of serum. The inhibition of fixation in the first reaction is caused by antibody excess and that in the last two reactions by antigen excess. The second and third readings are therefore used to calculate the mean complement fixation by the serum reacting with this antigen when the two are present in optimum proportions.

When a range of strain-specific sera had been prepared, a series of complement fixation tests was set up in which all the sera were titrated in parallel with each of the antigens used to prepare the sera. Figure 1 illustrates a test in which three sera were titrated with O_1 Lombardy antigen.

Cross-fixation ratios

In each test the homologous serum–antigen reaction was included and the amounts of complement fixed by the heterologous reactions were expressed as cross-fixation ratios of the homologous reaction. The expression of the results of the tests

Table 1. *The assay of the complement-fixing activity of O_1 Lombardy antiserum with O_1 Lombardy virus (Fig. 1)*

	Reacting mixtures. Constant antigen + serum dilutions	μl. serum in test (v_i)	ml. C′ fixed in test (V_i)	Actual ml. C′ fixed ($V_i - V_0$)	ml. C′ fixed/ml. serum $\frac{V_i - V_0}{v_i}$	
1	1/40	10·0	0·741	0·623	0·062	
2	1/50	8·0	0·776	0·658	0·082*	
3	1/62·5	6·4	0·624	0·506	0·079*	
4	1/78	5·1	0·442	0·324	0·064	
5	1/98	4·1	0·320	0·202	0·049	
6	Antigen control (no serum)	0·0	0·118	—	—	V_0
7	Serum control 1/40 (no antigen)	10·0	0·084	—	—	
8	C′ control (no antigen, no serum)	0·0	0·080	—	—	
				Sum 2 and 3	0·161	
				Mean 2 and 3	0·081	

$\therefore$ Complement-fixing activity, αB, of O_1 Lombardy serum with O_1 Lombardy virus $= 0{\cdot}081$ ml. C′/μl. antiserum,

and V_i at serum dilution 1/40 (10μl.) $= (\alpha B \times v_i) + V_0$,

$= (0{\cdot}081 \times 10) + 0{\cdot}118$.

$= 0{\cdot}928$ ml. C′ dilution.

(1) v_i is the volume of undiluted serum used in the test, V_i is the volume of complement required for 50 % haemolysis, and V_0 is the volume of complement for 50 % haemolysis in the most anticomplementary control, the antigen.

(2) Reaction No. 1 exhibits antibody-excess inhibition of complement fixation and Nos. 4 and 5 antigen-excess inhibition. These results are not, therefore, included in the calculation of complement-fixing activity at optimum proportions of antiserum with antigen.

* Region of maximal proportional fixation.

as ratios has the advantage that repeated tests with different preparations of the same antigen and different preparations of the haemolytic system are standardized as a proportion of the homologous reaction in each test. Table 2 gives the cross-fixation ratios of six antigens reacting in a series of tests with the six antisera prepared from these antigens.

Cross-fixation products

When the cross-fixation ratios of the six sera reacting with the six antigens had been determined, the antigenic relationship between pairs of antigens was expressed as the product of their cross-fixation ratios, one with the other (Table 3). Antigens O_1, O_2, O_3 and O_6 have cross-fixation products of less than 0·5 with one another and are therefore widely different. Antigen number 1411, however, has a cross-fixation product of 0·96 with antigen O_2 and the two virus strains have a close antigenic relationship. This relationship is reflected in the cross-fixation products of this antigen with the other virus strains. Antigen number 1698 is more difficult to classify as it exhibits some relationship with the two strains O_1 and O_2.

The use of the cross-fixation product to assess the antigenic relationship between strains of virus is of value when using strain-specific sera which may have a wide

Table 2. *The cross-fixation ratios of six strain-specific antisera reacting with the six virus strains used to produce the sera*

Virus antigens	Antisera					
	O_1	O_2	O_3	O_6	1411	1698
O_1	1·0	0·37	0·265	0·9	0·57	1·4
O_2	0·83	1·0	0·24	1·23	1·85	1·49
O_3	0·76	0·24	1·0	1·04	0·24	0·96
O_6	0·37	0·08	0·2	1·0	0·2	0·47
1411	0·47	0·52	0·15	0·81	1·0	0·86
1698	0·44	0·36	0·16	0·54	0·59	1·0

Table 3. *Cross-fixation products showing the antigenic relationship between virus strains*

O_1	1·0					
O_2	0·31	1·0				
O_3	0·2	0·06	1·0			
O_6	0·33	0·10	0·21	1·0		
1411	0·27	0·96	0·04	0·16	1·0	
1698	0·61	0·54	0·15	0·25	0·51	1·0
	O_1	O_2	O_3	O_6	1411	1698

range of homologous antibody titre. Virus strain O_6, which is used to produce stock type-specific serum, has had many passages in guinea-pigs and does therefore produce a serum of very high antibody titre. This is reflected in the high levels of fixation produced with heterologous antigens of the same type. When, however, O_6 antigen reacts with the heterologous sera the levels of fixation are low and the antigenic variation between the strains of virus becomes apparent.

Over twenty strains of virus of type O have been examined by this technique. Antigenic differences such as those exhibited by subtype strains O_1, O_2 and O_3 have been demonstrated in several of the strains examined and indicate the existence of at least eleven subtype groups within immunological type O. There were several strains such as BFS 1411 which could be classified as identical or very closely related to the subtype reference strains but there were other strains such as BFS 1698 which could not be classified with such certainty. A classified list of virus strains of Type O is given in Table 4. These virus strains have been recovered from epizootics in the field and some have been used for vaccine production in different parts of the world. The strains were examined at the World Reference Laboratory for Foot-and-Mouth Disease at Pirbright, using the complement-fixation technique described.

DISCUSSION

These experiments describe the development of a technique for the definition of subtype differences between strains of virus of one immunological type. The complement fixation test has been found preferable to other laboratory techniques such as mouse serum neutralization tests, agar gel diffusion or the colour test, which do not provide reproducible results of the same precision. The best results were

Table 4. *The classification of strains of foot-and-mouth disease virus of Type O*

Subtype no.	Designation of strain	Origin of material examined by W.R.L.	Other references to subtype differentiation
O_1	Lombardy	Cattle epithelium ex Tübingen, Germany,* 1958	Traub & Möhlmann (1946); Schneider (1950)
	M 11	Field sample ex Mexico, 1952	Graves (1960)
	BFS 1698	British Field Sample, 1961	—
O_2	Brescia	Cattle epithelium ex Tübingen, Germany*	Ubertini (1951, 1954); Schneider (1950); Girard, Mackowiak & Robin (1952)
	Italian O '1950'	Cattle epithelium ex Brescia, Italy†	—
	Channel Islands 1/57	W.R.L. sample	Graves (1960)
	BFS 1312, BFS 1411, BFS 1426, BFS 1428, BFS 1429	British Field Samples: 5 in 1958, 1 in 1959, 1 in 1960	—
O_3	Venezuela	Cattle epithelium ex Tübingen, Germany*	Schneider & Kosch (1951); Michelsen & Thiesen (1951)
	Ven 1	Field Sample ex Venezuela, 1950	Graves (1960)
O_5	India	Mukteswar‡ Goat 'O', 1961	—
	India 1/62	W.R.L. sample	—
O_6	Pirbright	O_{Y_1} British Field Sample, 1924. Reference guinea-pig strain used for production of type-specific antiserum for use by W.R.L.	—
O_7	Italy 1/58	Cattle epithelium ex Brescia, Italy†	Nobili (1962)
	Poland 1/59	W.R.L. sample	—
O_8	Brazil 1/60	Cattle epithelium ex Bahia, Brazil,§ 1960	New subtype reported by Pan-American Foot-and-Mouth Disease Centre and confirmed by W.R.L.
O_9	Kenya 102/60	W.R.L. sample	—
O_{10}	Philippines 3/58	W.R.L. sample	—
O_{11}	Indonesia 1/62	W.R.L. sample	—

Samples sent for identification to the World Reference Laboratory for Foot-and-Mouth Disease (W.R.L.) are given a reference number which indicates the country of origin, the serial number of the sample and the year of collection.

Reference strains supplied by other Institutes

* Bundesforschungsanstalt für Viruskrankheiten der Tiere, Waldhäuser Höhe, Tübingen, Germany.

† Istituto Zooprofilattico Sperimentale delle Provincie Lombarde, Brescia, Italy.

‡ Indian Veterinary Research Institute, Mukteswar-Kumaon, Uttar Pradesh, India.

§ Pan American Foot-and-Mouth Disease Centre, Rio de Janeiro, Brazil.

obtained with fresh antigen of high infectivity and antiserum of high antibody titre. While the antibody level of serum was found to be stable under normal conditions of storage, antigens prepared on different occasions produced different levels of fixation. Minor variations in the concentration of antigen or in the reagents used in the haemolytic system would not, however, upset the specificity of the reactions if the complement-fixing activity of the reacting mixtures of antigen and antibody were expressed as cross-fixation ratios.

If the antigenic relationship of two virus strains has to be determined, it is necessary to prepare strain-homologous antisera and to compare the cross-fixation ratios of both sera tested with both antigens. A study of the results tabulated in Table 3 demonstrates that the complement-fixing activity of an antiserum is a reflexion of two properties, its specificity in relation to the test antigen and also its antibody titre. Thus O_6 antiserum, because of its high antibody titre, exhibits a high cross-fixation ratio with all the virus antigens. The other sera, on the other hand, have low cross-fixation ratios with O_6 antigen and the subtype differences, expressed as cross-fixation products, become evident. O_6 serum is therefore very useful in a routine typing test because it gives a high level of fixation with a wide range of Type O antigens, but it would be unwise to assume that strong fixation with this serum would necessarily indicate close antigenic relationship.

When a collection of subtype virus strains and strain-specific reference sera has been prepared and their homologous and heterologous reactions one with the other are known, an unknown virus strain can be classified fairly rapidly. A strain-specific antiserum is prepared from the unknown virus in 6–8 weeks and the virus is then tested with its homologous and the known reference subtype sera. A comparison of the results with those of the stock subtype strains will show whether the virus under investigation is likely to be placed within a known subtype group or not. If it can be so placed, the stock virus will react with the new serum as if it were homologous and the cross-fixation product will not be significantly different from unity.

Virus strains have therefore been classified into subtype groups in which the cross-fixation product of each strain within the group is 0·5 or more with the reference subtype strain. While this level of cross-fixation product has been arbitrarily fixed, it is considered that a vaccine produced from one strain within the group will give good protection against challenge by a field strain of virus of the same group classification. This technique for the classification of virus strains has been applied to strains of other types by Davie (1962) and has confirmed the results of other workers who, using a variety of techniques, have reported subtype differences.

SUMMARY

1. A complement fixation test has been developed to measure the minor antigenic differences which occur between strains of the virus of foot-and-mouth disease of the same immunological type.

2. Strain-specific sera were prepared from each of the virus strains examined and then titrated with each of the antigens.

3. The amounts of complement fixed in each reaction were measured in the region of maximal fixation where the antigen and antibody were present in optimal proportions.

4. Heterologous antigen–antibody reactions were compared with the homologous reaction in each test and expressed as cross-fixation ratios.

5. Pairs of antigens could then be compared by taking the product of their cross-fixation ratios one with the other. The values of cross-fixation product so obtained ranged from 1·0 between identical strains to 0·01 between strains of different immunological type.

6. Strains of virus which exhibit cross-fixation products of 0·5 or more with one another have been classified within subtype groups.

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