J. Hyg., Camb. (1974), **72**, 397
Printed in Great Britain

A study of foot-and-mouth disease virus strains by complement fixation

I. A model for the fixation of complement by antigen/antibody mixtures

By A. J. FORMAN
Animal Virus Research Institute, Pirbright, Woking, Surrey, GU24 0NF

(*Received* 28 *September* 1973)

SUMMARY

An examination was made of the relations between antigen, antibody and fixation of complement with foot-and-mouth disease virus (FMDV). It was found that complement fixation in this system follows the same principles as models developed in other antigen/antibody systems. The assumption that there is a relation of direct proportionality between the amount of complement fixed and the amount of antiserum reacting with constant antigen was found to be incorrect. An alternative method was proposed for the quantitative differentiation of FMDV strains by comparing the titres of an antiserum when reacting with optimum amounts of homologous or heterologous antigens.

INTRODUCTION

The use of complement fixation for the quantitative study of reactions between antigen and antibody was pioneered by Wadsworth and his co-workers (Wadsworth Maltaner & Maltaner, 1938*a*, *b*, *c*) and further developed by Mayer and others (Mayer, Osler, Bier & Heidelberger, 1948; Osler, Mayer & Heidelberger, 1948; Osler & Heidelberger, 1948*a*, *b*).

Fulton & Dumbell (1949) developed a method to study serological relations between strains of influenza virus. From the early work of Traub & Möhlmann (1946) and Brooksby, Galloway & Henderson (1948), which showed that foot-and-mouth disease virus (FMDV) strains could be differentiated by complement fixation, a method was developed for the measurement of serological differences between strains (Brooksby, 1952). The method was based on the comparison of the amount of complement fixed by homologous and heterologous mixtures using fixed antigen doses and varying the amount of antiserum and of complement in the mixtures.

In this paper, data are presented from experiments carried out to investigate the relations between antigen, antibody and fixation of complement in an FMDV system and, from the results obtained, a different approach is suggested for the differentiation of FMDV strains by complement fixation.

MATERIALS AND METHODS

Reagents

Diluent. Veronal-buffered saline (VBS) was prepared using complement-fixation buffer tablets (Oxoid Ltd, London)* with the addition of gelatin to 0·1 % w/v and was used for the dilution of all reagents used in the test.

Antigens. The virus strain used was FMDV type O, strain O_6 (OVI) except where a heterologous reaction was involved, when the strain was O_1 Lombardy (O_1 Lom). Virus was grown in baby hamster kidney (BHK 21) cell monolayers and the whole virus particles (140S antigen) were purified from the harvests by the method described by Brown & Cartwright (1963). The antigen was diluted in VBS and stored at 4° C. for a maximum of 1 week before use.

Antiserum. Guinea-pig antiserum was prepared against the OVI virus by the method described by Brooksby (1952). Before use the serum was inactivated for 30 min at 56° C.

Complement. The source of complement was pooled guinea-pig serum, adsorbed with washed sheep erythrocytes as described by Kabat & Mayer (1961), and stored in small volumes at −70° C. A fixed dilution of complement was used in the test to produce 75 to 80 % haemolysis in control mixtures.

Haemolytic system. Sheep erythrocytes (Wellcome Research Laboratories, Beckenham, England), stored in Alsever's solution, were washed according to the method of Kabat & Mayer (1961) and diluted in VBS.

The concentration of the erythrocyte suspension was estimated by lysing 1·0 ml. of the suspension with 9·0 ml. of water and measuring the optical density (O.D.) of the lysate at 541 nm. in a Pye Unicam SP500 Series II Spectrophotometer. The suspension was then appropriately diluted to give a lysate with an O.D. of $0{\cdot}300 \pm 0{\cdot}005$.

Rabbit anti-sheep haemolytic serum (Wellcome) was diluted 1/10 and stored in small volumes at −20° C. Erythrocytes were sensitized by slowly adding an equal volume of a 1/800 dilution of haemolytic serum and incubating in a water bath for 30 min. at 37° C. with occasional mixing. The sensitized erythrocyte suspension was then stored at 4° C. before use.

Protocol of the test

The reaction mixtures were prepared in disposable spectrophotometer cuvettes (Walter Sarstedt (U.K.) Ltd, Leicester) of 1 cm. path length and 3·5 ml. capacity. Reagents were added, using fixed volume micro-pipettes (Eppendorf Gerätebau Netheler u. Hinz GmbH, Hamburg, Germany) in the following order and volume: (i) antigen, 500 μl.; (ii) complement, 500 μl.; (iii) antiserum, 500 μl.; (iv) haemolytic system, 1000 μl.

In control mixtures, reagents were replaced as appropriate by the addition of the same volume of VBS in the same order.

The reaction mixtures were prepared in a water-bath at 0° C. They were then

* The constituents of the diluent are as follows: barbitone, 0·575 g./l.; calcium chloride, 0·028 g./l.; sodium chloride, 8·5 g./l.; barbitone soluble, 0·185 g./l.; magnesium chloride, 0·168 g./l.; pH approximately, 7·2.

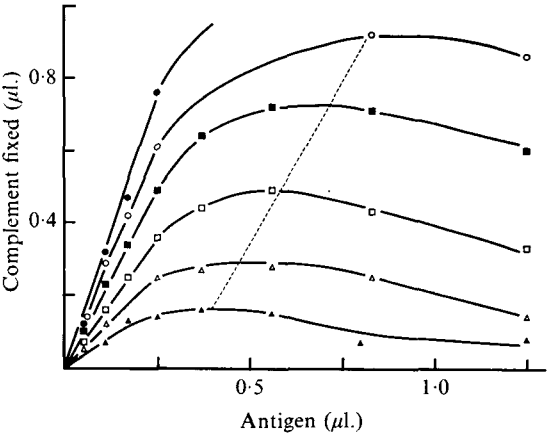

Fig. 1. The effect of varying the amount of antigen on the amount of complement fixed. Each curve represents a constant amount of antiserum. Serum (μl.): ●, 0·200; ○, 0·166; ■, 0·138; □, 0·115; △, 0·096; ▲, 0·080.

incubated in a water-bath for 30 min. at 37° C. before the addition of sensitized erythrocytes, which was followed by a further incubation for 45 min. at 37° C. The tubes were then chilled and centrifuged at 600 *g* for 10 min. to sediment unlysed cells. The O.D. of each mixture was then measured at 541 nm. and the proportion of lysis calculated relative to that in control tubes showing 100 % lysis (O.D. 0·600 ± 0·010).

The amount of complement fixed in each reaction mixture showing lysis between 10 % and 90 % was calculated, using the alternation formula of von Krogh (1916), viz.

$$\log x = \log k + (1/n) \log y/1-y,$$

where x is the amount of complement remaining after primary fixation, k is the amount of complement required for 50 % lysis (1 C'H50), $1/n$ is a constant and y is the proportion of erythrocytes lysed.

The value of $1/n$ was established for the system as being 0·20 ± 0·02. The value of k was calculated using the same formula, from complement control mixtures where x was taken as the amount of complement added.

RESULTS

Variation of amount of antigen with constant antiserum

Fig. 1 shows the effects of varying the amount of antigen in a series of mixtures, each with the amount of antiserum constant. For any fixed amount of antiserum, the response to increasing antigen is at first a linear increase in the amount of complement fixed, followed by a region of maximum fixation where the proportion of antigen to antibody is optimal and then a range of relative antigen excess with a progressive decrease in fixation. Smaller amounts of antibody resulted in lower maximum fixation and a shallower slope in the zone of linear response.

The linear response is in the region of antiserum excess and under these circumstances the relation of amount of antigen to amount of complement fixed is directly

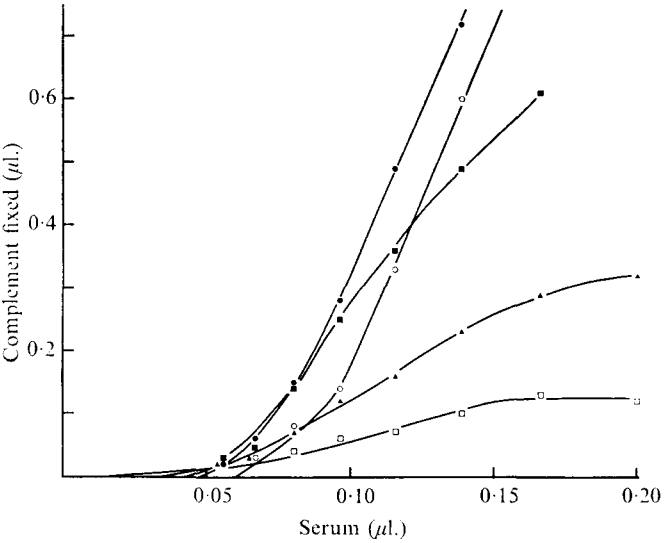

Fig. 2. The effect of varying the amount of antiserum on the amount of complement fixed. Each curve represents a constant amount of antigen. Antigen (μl.): ○—○, 1·25; ●, 0·56; ■, 0·25; ▲, 0·11; □, 0·05.

proportional. In the region of maximum fixation the slope is small, so that the optimum amount of antigen for a particular amount of serum is not well defined. However, the broken line in Fig. 1, which passes approximately through points of maximum fixation for each amount of serum, shows that the amount of antigen which is optimal increases with an increase in the amount of serum in the mixture.

Variation of amount of antiserum with constant antigen

Figs. 2 and 3 represent the effect of varying the amount of antiserum in a series of mixtures, each containing constant antigen. Fig. 2 is derived from the same experiment as Fig. 1. Comparison of these figures will reveal that the lines of constant antigen amounts of 0·25 μl., 0·11 μl. and 0·05 μl. in Fig. 2 represent antigen below the optimum over the range of amounts of serum used. While these curves are sigmoid, the lines representing 1·25 μl. and 0·56 μl. of antigen are linear over a large part of their range and are almost parallel.

Fig. 3, from a different experiment, shows lines of constant antigen amounts from 3·1 μl., which was close to optimal over the serum range used, to 15·6 μl., which represents a greater than fourfold antigen excess. The linear response to increasing serum with constant antigen is apparent, as is the parallelism of all but one of the lines.

It is evident from these figures that the relation of complement fixed to amount of serum with antigen constant, although linear, is not one of direct proportionality since the lines do not pass through the origin.

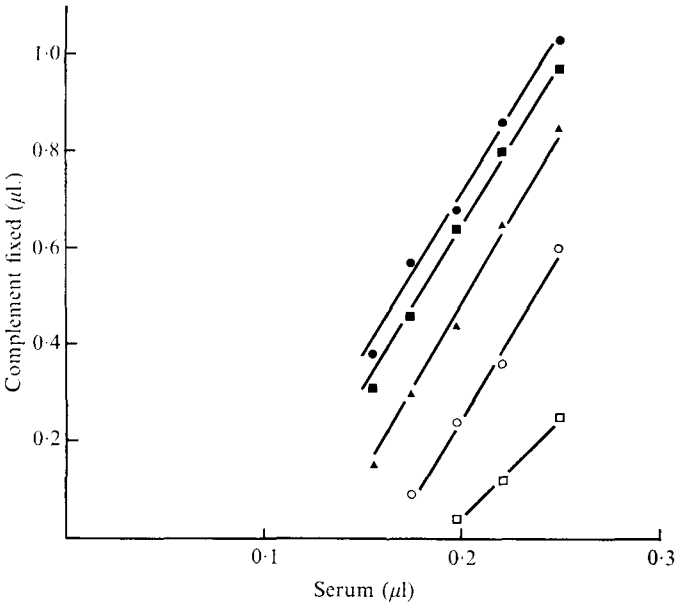

Fig. 3. The relation of the amount of antiserum to the amount of complement fixed. The lines represent constant antigen amounts over a range from close to optimal to a > fourfold antigen excess. Antigen (μl.): ●, 3·1; ■, 4·6; ▲, 6·9; ○, 10·4; □, 15·6.

Table 1. *Amounts of antigen and antiserum in optimum proportion mixtures, derived from Fig.* 1

Amount of antigen (μl.)	Amount of antiserum (μl.)	Ratio of antigen:antiserum
0·40	0·080	5·0
0·47	0·096	4·9
0·58	0·115	5·0
0·72	0·138	5·0
0·83	0·160	5·0

Variation of amounts of antigen and antiserum while maintaining them in optimal proportions

The broken line in Fig. 1, although inexact in position, is drawn to suggest the likelihood of a linear response between the amount of complement fixed and the amount of antigen which is optimum in each case. The ratio of antigen to antiserum in each optimum proportion mixture can be calculated from this (Table 1) and is apparently constant (the range of error in the ratios is approximately $\pm 0{\cdot}4$).

Fig. 4 is derived from Fig. 1 by plotting the points of maximum fixation of complement for each amount of antiserum. The graph shows a linear response between the amount of complement fixed and the amount of serum in each mixture. It demonstrates that, at least within this range of fixation, linear interpolation or extrapolation from two or more points could be used to ascertain the amount of serum required to fix a certain defined amount of complement. If this is taken as 0·5 C′H50 (0·69 μl. in Fig. 4), then 0·138 μl. of serum in a volume of 500 μl. is

https://doi.org/10.1017/S0022172400023639 Published online by Cambridge University Press

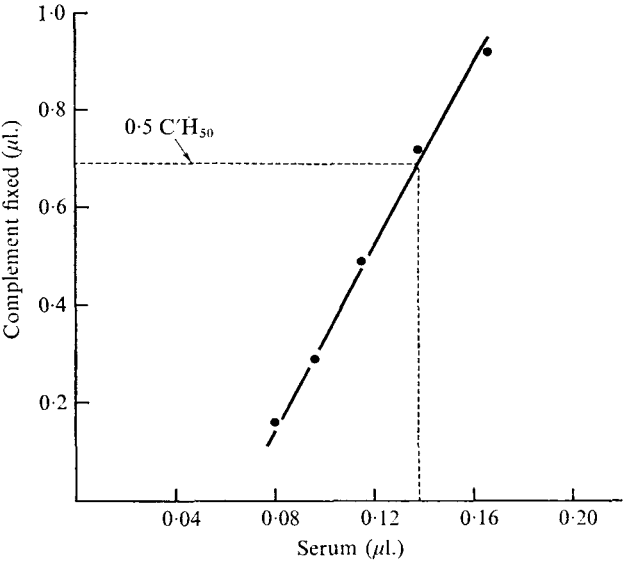

Fig. 4. The relation of the amount of antiserum to the amount of complement fixed in the presence of an optimal amount of antigen.

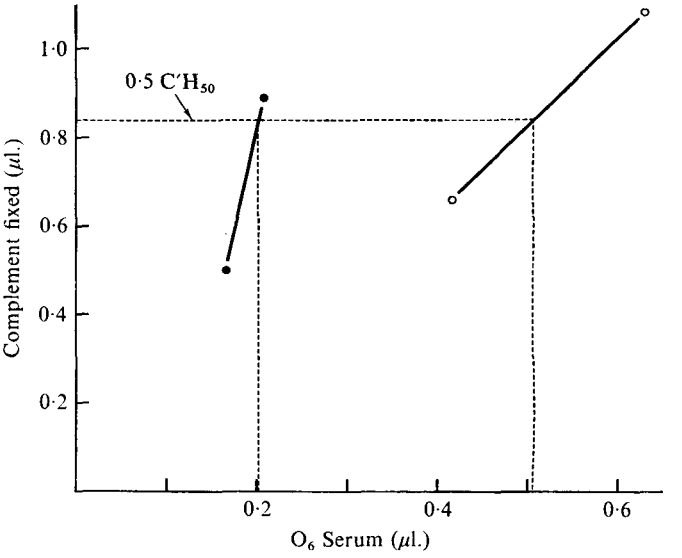

Fig. 5. The graphical estimation of titres of O_6 (OVI) antiserum with homologous and heterologous (O_1 Lom) antigens. Antigen: ●, O_6; ○, O_1.

required to fix this amount of complement with an optimal amount of antigen, i.e. the serum titre can be defined as 0·138/500 or 1/3623.

Reactions with homologous and heterologous antigens

Experiments carried out using a strain-heterologous antigen (O_1 Lom antigen with OVI antiserum) demonstrated that a similar pattern of fixation occurred but with a greater amount of antiserum required in the heterologous system, compared with the homologous system, for the same amount of complement

https://doi.org/10.1017/S0022172400023639 Published online by Cambridge University Press

fixed. Homologous and heterologous antigens were tested over a suitable range of serial twofold dilutions against two levels of antiserum each, pretitrated to give maximum complement fixation in the desired range. The amount of complement fixed in each mixture was calculated and curves analogous to those of Fig. 1 were drawn for both antigens at each level of antiserum. From these curves the amounts of complement fixed with optimum antigen were determined and plotted (Fig. 5). The serum titres for the homologous and heterologous reactions were then found by linear interpolation as described above. Thus, the serum titre with homologous antigen (1/2475) was greater than with the heterologous antigen (1/990). It can also be seen from Fig. 5 that a greater proportional increase in the amount of antiserum is required in the heterologous reaction than in the homologous reaction for a similar increase in the amount of complement fixed.

DISCUSSION

The results presented in this paper suggest a model for complement fixation in this system which is consistent with those obtained by other workers, using more precise methods in more closely defined systems. Osler *et al.* (1948), using a single antigen/antibody system (pneumococcal capsular polysaccharide), presented data which bear a close resemblance to these results; and Shulman (1958) gave experimental and theoretical evidence for a similar model of complement fixation by complexes of antibody, quinidine as a haptene and platelets.

In each of these models there was evidence for lines of constant antigen concentration having regions of approximately parallel linear response, indicating that the relation between complement fixed and amount of antibody is not one of direct proportionality.

Bradish, Brooksby & Tsubahara (1960), studying the specificity of FMDV antigens, made the assumption that in constant antigen excess there was a direct relation between the amount of antibody and the amount of complement fixed. This was the basis for their calculation of complement-fixing activity and has been used by many workers since for subtype differentiation studies (Graves, 1960; Davie, 1964; Darbyshire, Hedger & Arrowsmith, 1972). However, the results from this paper suggest that the initial assumption was incorrect, so that this method of calculation of complement-fixing activity is invalid.

The estimation of a serum titre, as described above in relation to Fig. 4, is the basis of conventional chequerboard titrations. The linear response shown between the amount of complement fixed and the amount of immune complex is probably part of a sigmoid curve (Wallace, Osler & Mayer, 1950; de Almeida, Silverstein & Maltaner, 1952; Shulman, 1958) but the data in Fig. 4 suggest that in this system it is reasonable to assume this linearity over the range of fixation used in the test. The use of only two points on this line (as in Fig. 5) to determine a serum titre introduces the possibility of error in the estimate. However, if the error in position of the points is small and large extrapolation is avoided, the error in the estimate of the serum titre should in practice be acceptable. The reproducibility of results will be considered in a later paper (Forman, 1974).

As shown in Fig. 5, an antiserum will have a lower titre when reacting with a heterologous antigen than with its homologous antigen. It would appear that the expression of a difference in titres as a proportion (i.e. heterologous titre/homologous titre) is a valid method of measuring the relation between the two antigens. This expression was the basis of a technique for differentiation of FMDV strains by Roumiantzeff, Stellman & Dubouclard (1965) and Roumiantzeff, Dubouclard, Fontaine & Gilbert (1966) in a system using 5 C'H50 and a chequerboard titration of antigen and antiserum. The method described in this paper differs from that of Roumiantzeff *et al.* in the method of calculating serum titres and in using a lower dose of complement, enabling a more accurate measurement of residual complement in antigen/antibody mixtures compared with that in antigen, antiserum and complement control mixtures.

REFERENCES

DE ALMEIDA, J. O., SILVERSTEIN, A. M. & MALTANER, F. (1952). Principles governing the practical application of complement fixation tests. *Journal of Immunology* **68**, 567–76.

BRADISH, C. J., BROOKSBY, J. B. & TSUBAHARA, H. (1960). The complement-fixation test in studies of components of the virus system of foot-and-mouth disease and its antibodies. *Journal of General Microbiology* **22**, 392–404.

BROOKSBY, J. B. (1952). The technique of complement-fixation in foot-and-mouth disease research. *Agricultural Research Council Report Series* No. 12. London: H.M. Stationery Office.

BROOKSBY, J. B., GALLOWAY, I. A. & HENDERSON, W. M. (1948). Strains of virus of foot-and-mouth disease recovered from outbreaks in Mexico. Complement fixation tests. *Proceedings of the Society for Experimental Biology and Medicine* **69**, 70–7.

BROWN, F. & CARTWRIGHT, B. (1963). Purification of radioactive foot-and-mouth disease virus. *Nature, London* **199**, 1168–70.

DARBYSHIRE, J. H., HEDGER, R. S. & ARROWSMITH, A. E. M. (1972). Comparative complement-fixation studies with sub-type strains of foot-and-mouth disease virus. *Journal of Hygiene* **70**, 171–80.

DAVIE, J. (1964). A complement-fixation technique for the quantitative measurement of antigenic differences between strains of the virus of foot-and-mouth disease. *Journal of Hygiene* **62**, 401–11.

FORMAN, A. J. (1974). A study of foot-and-mouth disease virus strains by complement fixation. II. A comparison of tube and microplate tests for the differentiation of strains. *Journal of Hygiene* **72**, 407.

FULTON, F. & DUMBELL, K. R. (1949). The serological comparison of strains of influenza virus. *Journal of General Microbiology* **3**, 97–111.

GRAVES, J. H. (1960). The differentiation of sub-type (variants) of foot-and-mouth disease virus by serological methods. I. Complement fixation test. *American Journal of Veterinary Research* **21**, 687–90.

KABAT, E. A. & MAYER, M. M. (1961). *Experimental Immunochemistry.* 2nd edit. Illinois: Charles C. Thomas.

VON KROGH, M. (1916). Colloidal chemistry and immunology. *Journal of Infectious Diseases* **19**, 452–77.

MAYER, M. M., OSLER, A. G., BIER, O. G. & HEIDELBERGER, M. (1948). Quantitative studies of complement fixation. I. A method. *Journal of Immunology* **59**, 195–206.

OSLER, A. G. & HEIDELBERGER, M. (1948*a*). Quantitative studies of complement fixation. III. Homologous and cross-reactions in Pneumococcal type III and type VIII systems. *Journal of Immunology* **60**, 317–25.

OSLER, A. G. & HEIDELBERGER, M. (1948*b*). Quantitative studies of complement fixation. IV. Homologous and cross-reactions in chicken and duck egg albumin systems. *Journal of Immunology* **60**, 327–37.

OSLER, A. G., MAYER, M. M. & HEIDELBERGER, M. (1948). Quantitative studies of complement fixation. II. Fixation of complement in the reaction between type III Pneumococcus specific polysaccharide and homologous antibody. *Journal of Immunology* **60**, 205–12.

ROUMIANTZEFF, M., DUBOUCLARD, C., FONTAINE, J. & GILBERT, H. (1966). Méthodes sérologiques utilisées pour l'étude des variantes du virus aphteux. *Bulletin de la Société des Sciences Vétérinaires et de Médecine comparée Lyon* **68**, 41–54.

ROUMIANTZEFF, M., STELLMAN, C. & DUBOUCLARD, C. (1965). Technique de fixation quantitative du complément appliquée à l'étude du virus de la fièvre aphteuse. *Bulletin de la Société des Sciences Vétérinaires et de Médecine comparée de Lyon* **67**, 1–23.

SHULMAN, N. R. (1958). Immunoreactions involving platelets. *Journal of Experimental Medicine* **107**, 665–90.

TRAUB, E. & MÖHLMANN, H. (1946). Untersuchungen über immunologische Varianten der Typen A und B des Maul- und Klauenseuchevirus. *Berliner und Münchener tierärztliche Wochenschrift* **1**, 1–5.

WADSWORTH, A., MALTANER, F. & MALTANER, E. (1938*a*). Quantitative studies of the reaction of complement fixation with tuberculous immune serum and antigen. *Journal of Immunology* **35**, 93–103.

WADSWORTH, A., MALTANER, F. & MALTANER, E. (1938*b*). Quantitative studies of the reaction of complement fixation with syphilitic serum and tissue extract. *Journal of Immunology* **35**, 105–15.

WADSWORTH, A., MALTANER, F. & MALTANER, E. (1938*c*). Quantitative studies of the complement fixation reaction with syphilitic serum and tissue extract: technique of the practical quantitative test. *Journal of Immunology* **35**, 217–34.

WALLACE, A. L., OSLER, A. G. & MAYER, M. M. (1950). Quantitative studies of complement fixation. V. Estimation of complement fixing potency of immune sera and its relation to antibody nitrogen content. *Journal of Immunology* **65**, 661–73.