Relationship Between Virus Neutralization and Serum Protection Bioassays for IgG and IgM Antibodies to Foot-and-Mouth Disease Virus

By R. TRAUTMAN AND C. E. BENNETT

Plum Island Animal Disease Center, Science and Education Administration, U.S. Department of Agriculture, Greenport, NY 11944, U.S.A.

(Accepted 30 August 1978)

SUMMARY

The time interval between administering the serum and the virus was found to influence the results of the in vivo mouse protection test for foot-and-mouth disease antibodies. In particular, for both IgG and IgM antibodies to strain A12 virus, the mouse protection index increased from zero to a maximum at about 6 h and remained high for at least five days.

Variations in the antiserum concentration, on a log scale, had a proportional effect on the mouse protection index, if between 1 and 3. The constant of proportionality was unity for IgM and 2 for IgG antibody. Comparison with in vitro neutralization tests revealed essentially parallel neutralization curves. The lower serum titre in the protection test, if computed for less than $10^3$ LD$_{50}$/dose, was accounted for by the simple dilution of the inoculated serum into the volume of the mouse. Consequently, in the low titre range, the same virus-antibody reaction and its effect are operable in each of the two tests. Analysis of literature data in which both the in vivo protection test and the in vitro neutralization test results were available on the same sera showed consistency with the above conclusions for both cattle and swine sera.

The protection test had a highly atypical survival pattern occurring at antibody concentrations expected to neutralize more than $10^3$ LD$_{50}$/dose. The resulting in vivo dampening effect on virus titre is postulated to be caused by the excess antibody of the passive immunity test interfering with the spread of infection. The effect is analogous to an anomaly caused by not removing the inoculum in quantal tissue culture assays and it prevents quantification of antibody levels in strong sera.

INTRODUCTION

Many routine serum bioassays for antibodies to foot-and-mouth disease virus (FMDV) are conducted at either constant virus and variable serum or vice versa. The serum endpoint and the virus endpoint, respectively, of such assays, are evidently selected points on an underlying neutralization curve obtained from a 'block' or 'chequerboard' design for which both the virus and the serum are varied. The underlying neutralization curve was found to be different for IgM and IgG antibodies for the in vitro neutralization reaction assayed in suckling mice (Trautman & Harris, 1977). In this paper, the underlying neutralization curve for the in vivo mouse protection test is presented for the first time for the FMDV system.
The protection test in suckling mice commonly calls for undiluted serum inoculated subcutaneously 1 h before inoculating varying dilutions of virus (Cunha et al. 1957). The variable serum and constant virus protocol has also been used to a lesser extent (Cunha, 1960). Extensive data from a block design have never been reported and the test has not previously been applied to IgM antibodies. The protection test in mice is an artificially acquired passive immunity test and is purported to be a better measure of the efficacy of vaccines in cattle than the \textit{in vitro} neutralization reaction that is also tested in suckling mice (Cunha & Honigman, 1963; Gomes & Astudillo, 1975). Here, the detailed relationship between the two tests is used to explain the anomalous and highly variable additional protection afforded by very strong antisera.

\section*{METHODS}

\textbf{Virus and antisera.} Foot-and-mouth disease virus, strain A_{12}, and the antiserum were supplied by Dr P. D. McKercher of this Center. The virus was passaged in baby hamster kidney cell cultures. The two bovine antisera against homologous virus were: 4 to 5 day post inoculation, representing the IgM antibody class, and 69 day post inoculation, representing the IgG antibody class. Original data were also supplied by Dr McKercher from vaccine studies on strain O_{1}-Brugge virus in swine (McKercher & Bachrach, 1976) and strain A_{24} virus in cattle (McKercher et al. 1975). Fresh O_{1}-Brugge virus from cattle tongue tissue and convalescent bovine sera were supplied by Dr J. W. McVicar of this Center. The tongue tissue virus was passaged once in secondary bovine kidney cell cultures to obtain stock for the extensive mouse protection tests.

\textbf{Mice.} Suckling mice of the Rockefeller H strain were used at 4 to 7 days of age with at least 16 mice (two litters of 8) at each dilution. With pooled test results, some experiments had up to 192 mice at each dilution.

\textbf{Mouse protection test.} The standard procedure of Cunha \textit{et al.} (1957) was extended to block tests as follows: 0.5 log dilutions (except where noted) of serum were administered subcutaneously (s.c.) as 0.1 ml; then 0.5 log dilutions of virus were inoculated intraperitoneally (i.p.) as 0.05 ml, 2 to 3 h later, for the standard test, and 18 to 24 h later, for the special test. Single dilutions of the IgG serum and the IgM serum were used for the time course study from 0 to 120 h for the period between serum and virus inoculations.

\textbf{Serum neutralization tests.} A block design of 0.5 log dilutions of serum and 0.5 log dilutions of virus was used. The reaction mixtures were incubated at 4 °C for 18 h (except where noted) and then further diluted with buffer solution, when required, before inoculation of 0.03 ml into suckling mice (Cunliffe & Graves, 1963) to make an extended block design. The method of plotting chosen here permits data from such extended block designs for the \textit{in vitro} neutralization reaction to be displayed on the same graph as the conventionally computed dose-response quantal assay data. The further dilution before inoculation is needed to obtain data for reaction mixtures that have so much remaining virus that all the mice are killed. Curiously, the additional dilution technique is used with plaque assays but rarely, if ever, with quantal assays. A requisite of the procedure is that further dilution of the reaction mixture does not cause dissociation of the immune complexes.

\textbf{Computations.} The Spearman-K\ddot{a}rber method for computing quantal end points was used (Finney, 1964). Standard conventions were used as follows: for constant virus and variable serum, the endpoint is termed a ‘serum dose’ and denoted by PD_{50} (50% protective dose) for the neutralization test and SP_{50} (50% serum protection dose) for the protection test. For constant serum and variable virus, the endpoint compared to the virus control is
Neutralization and protection bioassays for FMDV

Neutralization and protection bioassays for FMDV

called an ‘index’ and is denoted by VNI (virus neutralization index) for the neutralization test and MPI (mouse protection index) for the protection test. All results are reported in log₁₀ units.

Quantal virus endpoints can also be expressed as the fraction of virus remaining on a log scale, as is done here, which is equivalent to terms used variously by other authors such as: \( \log \frac{v}{v_0} \), where \( v \) is the final and \( v_0 \) the initial concentration of virus; the log of the surviving virus fraction; \(-\log \) (virus neutralized); \(-\)MPI; or \(-\)VNI. All notations reflect a measurement of residual infectivity. Serum concentrations are plotted as the independent variable with higher dilutions on the right and the axis labelled as \(-\log\) (serum dilution), which is equivalent to \( \log \frac{s_0}{s} \), where \( s \) is the final and \( s_0 \) the undiluted concentration of serum. All notations reflect a measurement of antibody activity.

The theoretical mass-action curves are drawn on the figures using reasonable parameters (Trautman, 1976; Trautman & Harris, 1977). The best fitting straight lines are inappropriate since the main underlying effect is curvilinear.

RESULTS

Time course

Fig. 1 presents the effect of time between the s.c. serum and i.p. virus inoculations in the mouse protection test. The graph shows for both IgM and IgG antibodies that the mouse protection index (MPI) increases for about 6 h and then remains essentially constant for five days, the longest period tested. Note that the 1 h period of the standard protocol for this test is in a region of high variability and low sensitivity. The curves were drawn from zero because there was no protection found when the virus was given before the serum.

Comparison of protection and neutralization block assays

Fig. 2 presents the data obtained with the IgM antiserum using the two assay methods. The curve for the in vivo protection test, with 2 to 3 h between serum and virus inoculations, shows a definite relationship between protection and antibody level. The control curve on the right for the in vitro neutralization test is similar to that previously reported for IgM (Trautman & Harris, 1977). When the errors of the assays are considered, there appears to be no significant difference in the shapes of the two patterns of Fig. 2 and both are adequately described as having a unit slope for the limiting straight parts. However, the mouse protection curve is displaced towards higher serum concentrations by about 2–3 logs, for reasons that will be explained after presenting the neutralization curves for the IgG antibody.

Fig. 3 presents a much more extensive study of the two assay methods for the IgG class of antibody. The curve on the far left of Fig. 3 is for the mouse protection test with 2 to 3 h interval between serum and virus inoculations. The curve in the centre is for a special protection test in which the period was extended to 18 to 24 h. Observe that the special test results are displaced to higher serum dilutions. Both protection tests show a sharp down-turn of the curve at about \(-3\) on the ordinate scale. Such a ‘dampening’ effect on the titre of virus did not occur with the in vitro neutralization reaction. Additional experiments on the dampening effect are presented in the last section.

When we consider the errors in the assays as shown by a large scatter in the points, there appears to be no significant difference in the shapes of the three patterns above \(-3\) on the ordinate scale. The mouse protection curves have a straight section with a slope of 2 and so does the neutralization curve. Whether these slopes are actually the same will be discussed
Fig. 1. Time course of mouse protection test. Bovine IgG and IgM antibodies to FMDV-A₁₂ virus were used at a constant dilution; the time after serum inoculation varied from 0 to 120 h; 16 mice/dilution; ○–○, IgG at -1.5 log dilution; □–□, IgM at -1.0 log dilution.

Fig. 2. Block assays for bovine IgM antibody to FMDV-A₁₂ virus. Data on the left (○–○) are for the mouse protection test and data on the right (●–●) are for the neutralization test; 16 mice/dilution were used in both tests. The solid lines have a unit slope in the linear part.
Neutralization and protection bioassays for FMDV

below. There is a significant lateral displacement between the curves for which measurements on the size of the mice are pertinent. The mean weight of 800 mice (4 to 5 days) was measured as 3.3 g with a standard deviation of 0.3 g. If the serum is diluted at 24 h by the vol. of the mouse, then 0.1 ml into 3.3 ml would have a log dilution factor of 1.52. The displacement between the centre and right-hand curves is 1.6 log units. Thus, the displacement is mainly accounted for by the dilution of the serum by the mouse in the protection test.

**Correlation between assay titres**

As part of another study, McKercher & Bachrach (1976) measured both the PD₉₀ and the SP₉₀ values on numerous swine and guinea pig antisera to strain O₁-Brugge vaccine. The published graphs for the two assays under a variety of conditions were essentially parallel lines. For one set of individual data on 72 swine sera obtained 90 days post vaccination, the mean difference between the serum titres was 1.44 ± 0.05 log units. The standard error of 0.05 represents a standard deviation of the difference population of 0.38, a value entirely accounted for by the internal error of 0.2 on each measurement because there are four operations involved in the computation. The mean difference of 1.44 corresponds to a dilution of the 0.1 ml serum dose into a vol. of 2.75 ml, which could have been the average vol. of the mice used.

McKercher et al. (1975) also measured the MPI and the PD₉₀ values on many cattle sera after vaccination with strain A₂₄ virus. One bleeding, involving 32 animals six months after the first vaccination and one month after revaccination, gave the results presented in Fig. 4, where the MPI is plotted against the PD₉₀. The two different assay results are undoubtedly highly correlated. Because both variables are subject to error, both regression
Fig. 4. High correlation between protection and neutralization tests for 32 bovine sera after vaccination with FMDV-A4 virus. Undiluted serum was used 1 h before virus for mouse protection index (MPI) and 100 LD_{50}/dose virus was reacted with serum for 1 h at 25°C for protective dose (PD_{50}) measurements. Both regression lines are shown because both variables are subject to error and have slopes of 1.7 and 2.5. (Data from McKercher et al. 1975.)

The infectivity titration at 1/316 serum dilution is quite similar to the virus control titration in terms of the shape and spread of the distribution of survival values, although the
Neutralization and protection bioassays for FMDV

Fig. 5. Protection map for passive immunity to FMDV-O1-Brugge virus in suckling mice. Paired figures are the 95% confidence limits for the percentage survival observed at each combination of serum and virus challenge. The curve is the interpolated 50% survival. Results represent ten experiments with 16 to 160 mice/dilution used.

50% endpoint is about $-1$ log unit on the left-hand scale. At the left-hand side of the graph at 1/31.6 serum dilution, a virus endpoint cannot be computed in the vertical column because the survival is essentially constant. The serum titration for constant virus is represented by the survival values along any horizontal row. At low challenge levels, in the upper right, the distribution is quite broad and asymmetrical, but at high levels, in the lower left, the distribution is very narrow and symmetrical.

The interrupted line in Fig. 5 represents the locus of the 50% endpoint values selected by inspection of confidence limits. The straight part in the centre is of slope 2, the same as that found for the mouse protection test in Fig. 3. At the lower left of the figure, the 50% line demonstrates a 'knee' and falls off precipitously from the extension of the linear function. The most striking effect of the more concentrated antibody is to protect all the mice from all the virus administered. As will be amplified in the Discussion, this protection means neutralization of all the virus administered as well as virus resulting from replication.
DISCUSSION

The experiments reported here show that in the range from none to 3 'logs' of virus titre reduction, the mouse protection test and the virus neutralization tests are statistically equivalent measures of antibody concentration, with their numerical difference accounted for by the dilution of the test serum by the vol. of the suckling mouse host used. The reason for such a close similarity is that this virus-antibody reaction proceeds rapidly at body temperature. The mouse thus experiences essentially the same distribution of infectious complexes whether the reaction took place in vitro or in vivo at the site of inoculation (when the serum level at the site is the same). Some differences in details can now be discussed.

Firstly, the seemingly different values reported here of 2·3, 1·6, and 1·44 for the log difference in serum titres can be considered estimates of the dilution phenomenon under different conditions. The neutralization test mixture is frequently incubated at 37 °C for 30 to 60 min; here, overnight at 4 °C was chosen for convenience and to avoid temperature inactivation. In previous studies with strain A24 virus, the 37 °C curve was displaced from the 4 °C curve a few tenths of a log unit toward lower serum dilutions (Trautman & Harris, 1977); thus, an increase in the temperature of the neutralization reaction would decrease the serum difference between the in vivo and in vitro tests. The effect of increasing the time interval between serum and virus inoculations also decreases the difference; about 0·3 log units, by changing from the 2 to 3 h interval to the 18 to 24 h one (from Fig. 3). The variation in all the tests reported is so high that determination of the precise effect of all these factors was unwarranted.

Secondly, a significant difference in slopes of the underlying curves might be expected. The mouse can respond to infectious complexes at the same time they are being formed in the in vivo test. Thus, more infectivity might be observed than in the equilibrium reaction mixture. The data presented do not show such a trend to a smaller slope for the straight part of the curves in the in vivo test compared with the in vitro one. It should be noted that precise values of the slopes are difficult to obtain. Previous analysis led to the conclusion that the slope of the in vitro test is between 2 and 3, but probably 3 (Trautman & Harris, 1977).

Thirdly, the dampening effect was described fully by Dulbecco et al. (1956) for tissue culture assays, but applicability to in vivo assays seems to have been overlooked. The explanation involves reduction and replication cycles of the virus with possible abortion of the initial infection. We point out that the observed nature of antibody-antigen reactions, expressed by the percentage law (Andrewes & Elford, 1933), is sufficient to explain the dampening effect without invoking mechanisms of cell-mediated immunity. The quantification of the phenomenon was attempted by Dulbecco et al. (1956) but needs much more analysis. The qualitative notions can be illustrated by assuming that each infected mouse cell produces 100 infectious virions and considering that the cells are continuously bathed with circulating antibody. Here we describe two extreme examples using only the 50 % survival curve of Fig. 5.

(i) Select an individual mouse which has a low level of circulating antibody at the 1/316 dilution of administered serum. From the solid line representing the percentage law in antibody excess, we would expect the antibody to reduce the virus concentration 1 log unit from the inner left hand scale on the ordinate axis. If a 10⁷ dose of virus is given to that mouse, the antibody would reduce this to 10⁶, which on replication would increase to 10⁸ infectious particles. The antibody would then reduce these released virions again by 1 log unit. The alternating cycles of reduction and replication might be thus: 10⁷ -> 10⁶ ->
Neutralization and protection bioassays for FMDV

$10^8 \rightarrow 10^7 \rightarrow 10^6 \rightarrow 10^5 \rightarrow 10^3 \rightarrow 10^2 \ldots$ This upward cascade would probably lead to death of that mouse, as indicated by the 0% survival in the lower right of Fig. 5.

(2) Select an individual mouse which has a high level of circulating antibody at the left in Fig. 5. The expected percentage law reduction is given by the straight line and is 3 log units at the 1/31.6 dilution. If the same $10^7$ dose is given, as in the first example, the alternating cycles of reduction and replication might now be: $10^7 \rightarrow 10^4 \rightarrow 10^6 \rightarrow 10^3 \rightarrow 10^2 \ldots$. This decreasing sequence of infectious particles would probably permit the mouse to survive, as indicated by the near 100% survival figures in the lower left of Fig. 5.

It can be seen that survival in the dampening region depends on the antibody not being depleted and on the reduction by antibody being greater than the augmentation of replication. The dampening effect is independent of the challenge dose, to a first approximation.

Fourthly, the mouse protection test and the virus neutralization test are used to establish the antibody level in the bovine host from which the serum was obtained. For a measure of low antibody concentration, especially for the presence of antibody in screening tests, both tests are equivalent but have different standard errors. Some of the variability of the standard 1 h protection test is due to the fact that the antisera takes several hours to become uniformly distributed after the subcutaneous inoculation.

The analysis here is proposed to explain why high antibody concentrations cannot be measured quantitatively. More work is needed to establish the precise location of the 'knee' on the curve that identifies the dampening effect. The location of the knee on the serum dilution axis depends on the serum tested and the time interval of the test, as is shown in Fig. 3. The location of the knee on the virus dose axis depends on the replication factor of infectious virions produced per host cell. The results presented are only for homologous reactions; however, an analysis of the data of Cunha & Honigman (1963) and Gomes et al. (1972) reveals no conflict with heterogeneous reactions. For such an analysis all values of the MPI above 3 were discarded and the effective dilution by the mouse on the serum protection test was considered. Block analysis of cross-reacting systems would be worthwhile in the future.

The protection map, given in detail for the first time in Fig. 5, displays the survival expected at various serum levels and various challenge doses of virus for the passive immunity test in suckling mice. This map is proposed for analysing the interaction of infection and replication processes together with antibody-antigen reactions that produce infectious complexes.

REFERENCES


(Received 23 February 1978)