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# Dose-response relationships in a microneutralization test for foot-and-mouth disease viruses

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## SUMMARY

Two-dimensional quantal microneutralization tests on foot-and-mouth disease viruses, in which neutralizing antibody activity was titrated against a serial range of virus doses, demonstrated a variety of dose-response curves some of which were rectilinear, others clearly curvilinear. Moreover, in the case of the non-linear responses obtained with some antisera, the shape of the curve was such that antibody titres recorded with doses of virus ranging from $10^3$–$10^5$ TCD 50 were closely similar. Studies were carried out on the effect of varying the conditions of the test on the shape of the dose-response curve: significant differences were obtained after treatment of the antiserum–virus mixtures with anti-species globulin, and when the test was assayed in cells of differing susceptibility to infection.

## INTRODUCTION

Studies of foot-and-mouth disease (FMD) viruses using quantal neutralization tests have indicated that the relation between $\log_{10}$ neutralizing antibody titre and $\log_{10}$ number of median infective doses (TCD 50) of virus used in the test is rectilinear (Martin & Chapman, 1961). However, similar investigations of other viruses have demonstrated that the dose-response relation in such tests is not always in the form of a straight line but may describe a smooth or sigmoid curve (Fazekas de St. Groth, Withell & Lafferty, 1958; Fazekas de St. Groth, 1961). During the development of a microneutralization test for FMD viruses we have frequently observed that the relation departs from strict linearity. Evidence will be presented herewith, together with the results of studies of the effect of various factors on the outcome of the test.

## MATERIALS AND METHODS

### *Virus*

Virus antigen for use in the neutralization test was prepared by passaging of cloned, stock virus in baby hamster kidney (BHK21) or pig kidney (IB-RS-2) cells as required; infective tissue culture harvests were clarified of cell debris by centrifuging at 600 *g* for 10 min and samples were stored at −70 °C.

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*Antiserum*

Guinea-pig antisera were prepared against purified, 146S, inactivated virus antigen as described previously (Rweyemamu, Booth & Pay, 1977).

*Cells*

BHK21 and IB-RS-2 (de Castro, 1964) monolayer cells were routinely propagated in Roux flasks, the growth medium consisting of Eagle's medium with 10% tryptose phosphate broth, 10% bovine serum, 0·025% sodium bicarbonate and 100 units/ml of penicillin and streptomycin.

*Microneutralization test*

Antiserum was inactivated at 56 °C for 30 min and then diluted 1/4 in growth medium, containing 0·025% (w/v) sodium bicarbonate and 0·025 M tris(hydroxymethyl)aminomethane, the pH having been adjusted to 7·4 by addition of N-HCl. Twofold serial dilutions of the antiserum in the tris-buffered growth medium were made in flat-bottomed, tissue culture grade microtitre plates, using 0·05 ml diluting loops. Two identical series of dilutions were prepared for each virus dose to be tested.

Serial 0·5 $\log_{10}$ dilutions of virus were made in tris-buffered growth medium and 0·05 ml of each of these was added to a duplicate series of antiserum dilutions. The resulting mixtures were incubated at 20 °C for 1 h, after which they received 0·05 ml of cell suspension, and were then incubated at 37 °C for 48 h before being simultaneously fixed and stained with 0·1% crystal violet in 10% formol saline. The infectivity titre of the original virus suspension was determined by incubation of each 0·5 $\log_{10}$ dilution together with 0·05 ml of tris-buffered growth medium, in quadruplicate, before addition of the cell suspension. Furthermore, dilutions of antiserum were tested, in the absence of virus, for cytotoxic activity. Infectivity titres and titres of neutralizing antibody activity with each dose of virus tested were calculated using the method of Karber (1931), and were used to construct a dose-response curve. The concentrations of BHK21 and IB-RS-2 cells used in the procedure were $1{\cdot}5 \times 10^6$ and $5 \times 10^5$/ml respectively.

*Inactivation and purification of virus*

Infective FMD virus, in BHK21 tissue culture harvests, was inactivated at 4 °C for 48 h with 0·05% (v/v) acetylethyleneimine (AEI), after which the reaction was stopped by addition of excess sodium thiosulphate (Brown, Hyslop, Crick & Morrow, 1963). The non-infective virus particles were then purified according to Brown & Cartwright (1963); the crude fluids were concentrated by precipitation with saturated ammonium sulphate and clarified and pelleted in the ultracentrifuge; the resuspended pellets, treated with 1% sodium lauryl sulphate, were finally purified by zonal centrifugation in 15–45% sucrose gradients for 2·5 h at 30000 rev./min in the MSE 3 × 23 swing-out rotor. Treatment with trypsin, where required, was done by incubating the concentrated, pelleted virus with 1 mg/ml of the enzyme before treatment with detergent and zonal centrifugation.

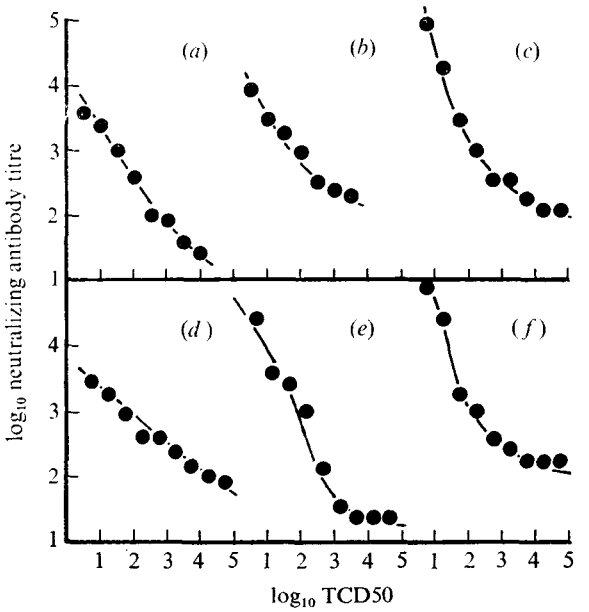


Fig. 1. Relation between antibody titre and virus dose (TCD 50) in quantal microneutralization tests on foot-and-mouth disease viruses and homologous antisera: (*a*) Asia 1 Ankara, virus titre $10^{4\cdot5}$ TCD 50/0·05 ml; (*b*) SAT2 Ken 3/57, virus titre $10^{4\cdot1}$ TCD 50/0·05 ml; (*c*) A(K 18/66), virus titre $10^{5\cdot2}$ TCD 50/0·05 ml; (*d*) SAT2 Uga 6/70, virus titre $10^{5\cdot2}$ TCD 50/0·05 ml; (*e*) C Noville, virus titre $10^{5\cdot1}$ TCD 50/0·05 ml; (*f*) $O_1$BFS 1860, virus titre $10^{5\cdot2}$ TCD 50/0·05 ml.

*Absorption of antiserum*

Antiserum, diluted 1/4, was mixed with $10^7$ normal BHK21 cells per ml, then incubated for 1 h at 20 °C followed by overnight at 4 °C, before being subjected to two successive cycles of centrifuging at 40000 rev./min for 2 h in the MSE 10 × 10 angle rotor.

*Antiglobulin serum*

Rabbit anti-guinea-pig IgG serum (Miles Laboratories Ltd) was absorbed with normal BHK21 cells as described above and was then diluted 1/10 with growth medium; 0·025 ml of this was added to neutralization tests as required.

## RESULTS

The relation between $\log_{10}$ neutralizing antibody titre and $\log_{10}$ virus neutralized in two-dimensional, quantal microneutralization tests assayed in BHK21 cells, was determined for several different FMD viruses, grown in BHK21 cells, and their respective homologous antisera. A variety of dose-response curves was obtained, some of which were evidently rectilinear whereas others were clearly and reproducibly curvilinear (Fig. 1). Moreover, with some of the higher-titred antisera there was a tendency for non-linear response curves to level off gradually,

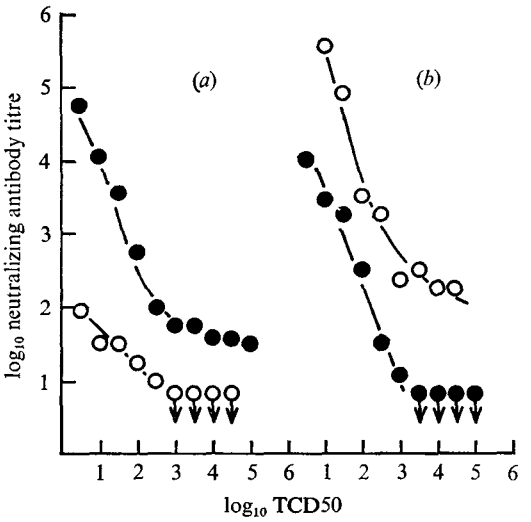


Fig. 2. Relation between virus dose (TCD 50) and antibody titre in cross-neutralization tests with homotypic virus strains A Pando (●—●, titre $10^{5\cdot5}$ TCD 50/0·05 ml) and A(K 18/66) (○—○, titre $10^{5}$ TCD 50/0·05 ml): (*a*) A Pando antiserum; (*b*) A(K 18/66) antiserum.

so that eventually there was little difference in the antibody titres detected with doses of virus in excess of $10^{3}$ TCD 50 (Fig. 1*e*, *f*).

One possible explanation for this apparent stabilization of antibody titre with respect to virus dose was that a non-specific inhibitor of virus infectivity was present in the antiserum. However, this seemed unlikely, since neutralization tests carried out on serologically distinct virus strains of the same immunological type frequently failed to demonstrate significant titres of antibody activity with doses of virus greater than $10^{3}$ TCD 50 (Fig. 2).

To determine if the non-linear response curves were due to delayed expression of virus cytopathic effects in the presence of high concentrations of antibody, the two-dimensional tests were read after four days' incubation instead of the usual two. This extension resulted in no significant change in the results.

With poliovirus, neutralization of infectivity is considerably enhanced during prolonged incubation of antiserum–virus mixtures (Gard, 1957). In the FMD virus microneutralization test, identical dose-response curves were obtained whether the antiserum–virus mixtures were incubated for 1 h at 20 °C, before addition of the BHK21 cells, or for 1 h at 20 °C followed by overnight at 4 °C.

Wallis & Melnick (1967) have shown that aggregates of virus particles are more refractory to neutralization by specific antibody than are virus monomers, and furthermore, that such aggregates can be removed from virus suspensions by filtration through membranes whose pore size is up to four times the diameter of the virus particle. However, dose-response curves with unfiltered FMD virus and virus that had been passed through Millipore membranes of average pore diameter 100 nm were very closely similar.


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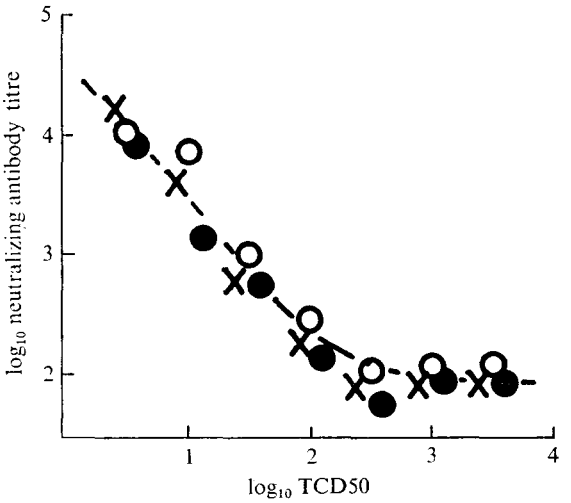


Fig. 3. Effect of addition of non-infective homologous virus antigen, on the relation between virus dose (TCD 50) and antibody titre in microneutralization tests on $O_1$BFS 1860 virus (titre $10^{4.5}$ TCD 50/0·05 ml); infective virus mixed 1:1 with: ●—●, 64-fold excess of non-infective, purified virus; ○—○, 64-fold excess of non-infective, trypsin-treated purified virus; × – ×, growth medium.

*With most viruses, assays of infectivity account for only a small fraction of the* total number of particles detectable by physical means, so that differences in the proportion of infective to non-infective virus from one virus suspension to another might be expected to influence the sensitivity of tests for detecting neutralizing antibody activity. Fazekas de St. Groth & Webster (1963) have shown that addition of inactivated influenza virus particles to preparations of the infective virus, before dilution as antigen in two-dimensional quantal neutralization tests, may depress the antibody titres recorded with higher doses of the virus, the effect being dependent upon the final concentration of the non-infective antigen. To determine if the sensitivity of the FMD virus microneutralization test was significantly affected by increasing the concentration of non-infective virus particles in the antigen preparation, the following experiment was carried out.

$O_1$BFS 1860 virus preparation with a titre of $10^{4.0}$ TCD 50/0·05 ml and 4 units of complement-fixing antigen/0·025 ml was mixed with an equal volume (0·5 ml) of *either* purified, 146S homologous virus particles which had been inactivated with 0·05% AEI before purification *or* purified, inactivated, homologous virus particles which in addition had been treated with trypsin to destroy surface-neutralizing antigens (Wild & Brown, 1967). The two inactivated virus preparations each contained 256 units of complement-fixing antigen/0·025 ml. Two-dimensional microneutralization tests carried out with the supplemented virus preparations gave results which were indistinguishable from those performed in parallel with virus that had been diluted twofold in medium (Fig. 3). These results indicated that variation by as much as 64-fold in the proportion of non-infective to infective particles in FMD virus preparations did not significantly

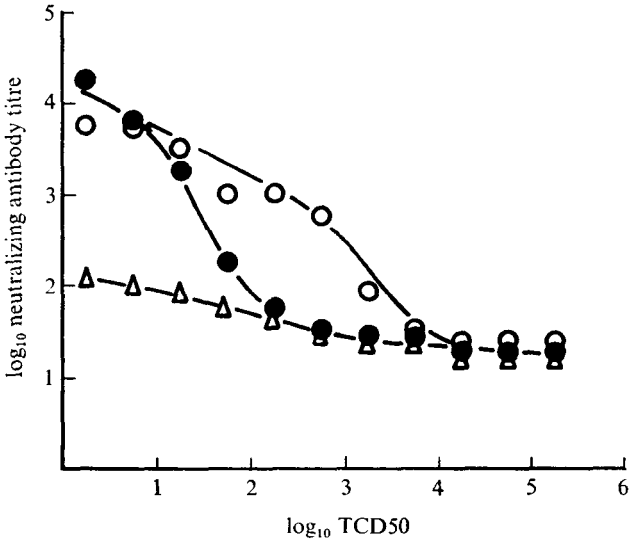


Fig. 4. Effect of anti-species globulin on the relation between virus dose (TCD 50) and antibody titre in microneutralization tests on $O_1$BFS 1860 virus (titre $10^{5\cdot7}$ TCD 50/0·05 ml) and homologous guinea-pig antiserum: ●—●, control, no anti-species globulin; ○—○, anti-guinea-pig IgG added to pre-incubated virus–antiserum mixtures; △—△, anti-guinea-pig IgG added to serial dilutions of the antiserum before addition of the virus.

affect the sensitivity of the quantal neutralization test with doses of up to $10^{3\cdot5}$ TCD 50.

It has been shown that antibodies specific for the tissue culture cells employed as indicators in neutralization tests may manifest non-specific virus-inhibiting activity (Timbury, 1963; Axler & Crowell, 1968). Although the antisera employed in the present study had all been prepared by immunization of guinea-pigs with purified virus antigen, the possibility that anti-cell antibodies might be present, at concentrations that could influence the results of the microneutralization test, was examined by determining the effect of absorbing the antisera with normal BHK21 cells as described in Materials and Methods. In all cases the dose-response curves with absorbed and unabsorbed sera were identical.

Mixtures of virus and immune serum may contain infective antibody complexes whose presence could be expected to influence the results of neutralization tests. To find out if such complexes affected the shape of the dose-response curve in the FMD virus microneutralization test, advantage was taken of the fact that infective virus-antibody complexes are neutralized by treatment with antibodies specific for immunoglobulins of the animal species that was used for preparing the virus antiserum (Ashe & Notkins, 1966; Cihak, 1973; Huggett, Rodriguez & McKee, 1972; Rweyemamu, Booth & Pay, 1977). Mixtures of virus and antiserum were prepared and incubated in the usual way for 1 h at 20 °C, and were then incubated for a further hour with 0·025 ml of rabbit anti-guinea-pig IgG serum before adding the BHK21 cells. Controls consisted of an identical series of virus–antiserum mixtures incubated with growth medium in place of the anti-globulin serum.


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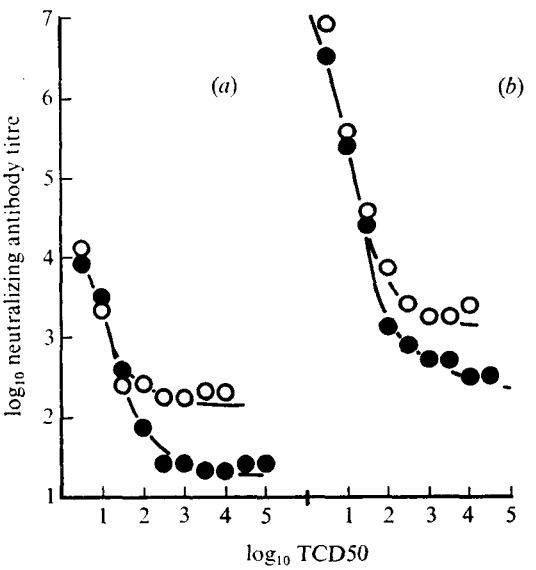


Fig. 5. Effect of indicator cell type on the relationship between virus dose (TCD 50) and antibody titre in microneutralization tests on virus strains: (*a*) $O_1$BFS 1860 and (*b*) A(K 18/66), and their respective antisera; ●—● BHK21 cells; ○—○ IB-RS-2 cells.

Infectivity titre in BHK21 and IB-RS-2 cells: $O_1$BFS 1860 virus, $10^{5\cdot5}$ and $10^{4\cdot5}$ TCD 50/0·05 ml, respectively; A(K 18/66) virus, $10^5$ and $10^{4\cdot5}$ TCD 50/0·05 ml, respectively.

The results (Fig. 4) showed that the anti-globulin preparation did not affect the titre of the virus antigen used in the test but produced marked enhancement of neutralizing antibody titres, with doses of virus of less than $10^3$ TCD 50, and alteration in the shape of the dose-response curve. To demonstrate the ability of the anti-globulin serum to combine with virus-neutralizing antibody, in some tests the anti-globulin serum was incubated with dilutions of the virus antiserum before addition of the infective virus antigen; this treatment resulted in lower antibody titres being detected, particularly with doses of virus of up to $10^2$ TCD 50. The observed enchancement of neutralizing titres by treatment of pre-incubated virus–antiserum mixtures with the anti-globulin serum indicated that infective virus–antibody complexes played an important role in determining the outcome of the two-dimensional, quantal neutralization test with FMD viruses and antisera raised in guinea-pigs.

The degree of neutralization detected in virus–antiserum mixtures may be influenced by the kind of cell system in which the assays for residual infectivity are carried out (Kjellén & Schlesinger, 1959; Tyrrell & Horsfall, 1953). In the present study, different results were recorded when microneutralization tests were assayed in parallel in BHK21 and IB-RS-2 cells (Fig. 5); higher titres of antibody were demonstrated in the IB-RS-2 cells, especially with doses of virus in excess of $10^2$ TCD 50. Moreover, the dose-response curves in both cell systems were not parallel, but tended to converge with doses of virus of less than $10^2$

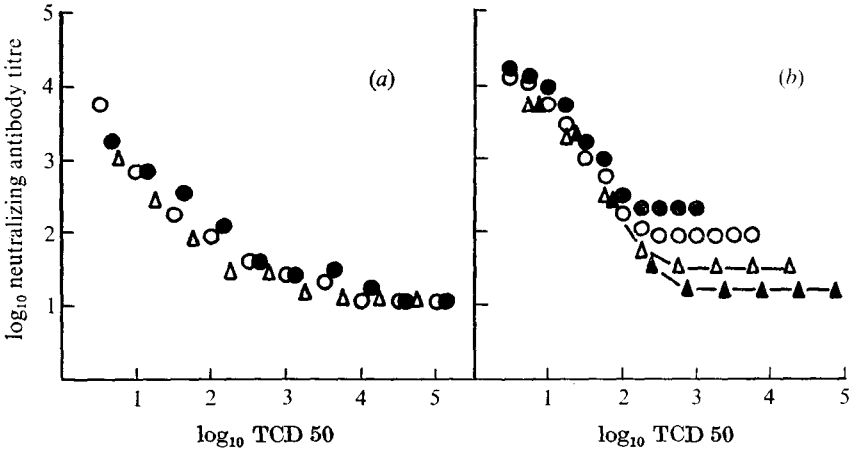


Fig. 6. Relation between virus dose (TCD 50) and antibody titre in microneutralization tests assayed in BHK21 cells, on different occasions, with the same batch of virus antigen and antiserum: (*a*) C Noville virus, infectivity titre ●—● $10^{5\cdot7}$ TCD 50/0·05 ml, ○—○ $10^{5\cdot5}$ TCD 50/0·05 ml, △—△ $10^{5\cdot2}$ TCD 50/0·05 ml; (*b*) $O_1$ BFS 1860 virus, infectivity titre ▲—▲ $10^{5\cdot4}$ TCD 50/0·05 ml, △—△ $10^{4\cdot75}$ TCD 50/0·05 ml, ○—○ $10^{4\cdot25}$ TCD 50/0·05 ml, ●—● $10^{3\cdot5}$ TCD 50/0·05 ml.

TCD 50. Closely similar results were obtained whether the virus antigen had been grown in BHK21 cells or given three additional passages at low input multiplicity in IB-RS-2 cells. A further finding in these studies was that the enhanced sensitivity of the IB-RS-2 cells for detecting neutralizing antibody was coincident with reduced sensitivity for detecting virus infectivity, the difference amounting to $10^{0\cdot8}$ TCD 50 on average. The possibility was considered that such discrepancies occurred because the seeding concentration of the IB-RS-2 cells was threefold lower than that of the BHK21 cells. However, reducing the concentration of the BHK21 cells proportionately was found to have no significant effect on the final results, although the tests had to be incubated for two days longer than with the usual cell concentration.

Differences in sensitivity for detecting virus and antibody were occasionally demonstrated in microneutralization tests carried out, on separate occasions, with different batches of BHK21 cells. With the majority of viruses included in this study (Fig. 1), repeated testing of homologous antiserum showed that antibody titres seldom varied by more than $10^{0\cdot3}$ either side of the mean value calculated with each virus dose used in the test (Fig. 6*a*). However, in the course of a prolonged series of tests on a standard batch of the $O_1$BFS 1860 virus and homologous antiserum, evidence was obtained that wide variation in sensitivity occasionally did occur. Thus, just as in the comparison between BHK21 and IB-RS-2 cells, tests with different batches of BHK21 cells displayed variation in sensitivity for detecting neutralizing antibody which appeared to be related inversely to sensitivity for detecting infectivity (Fig. 6*b*). Furthermore, the between-test differences in antibody titre became evident only with virus doses greater than $10^2$ TCD 50, and were, at maximum, about 10-fold.

## DISCUSSION

For reasons of economy and ease of performance, quantal tests have been long preferred to other more precise methods for quantifying virus-neutralizing antibody activity, based on counting of virus plaques, particularly in laboratories engaged in routine diagnosis and large-scale serological surveillance. In recent years the application of quantal tests has been considerably extended by the development of so-called microneutralization techniques performed in disposable plastic trays and requiring only small volumes of reagents. Despite these advances, factors affecting the sensitivity and accuracy of this kind of test are poorly understood. Horsfall (1939) and Horsfall & Lennette (1941), in studies of influenza virus neutralization in mice, found that the relation between virus dose and antibody titre was strictly rectilinear. On the other hand, Fazekas de St. Groth, Withell & Lafferty (1958) also working with influenza virus, but in eggs, demonstrated that the dose-response relation was curvilinear or sigmoid. With FMD viruses, only rectilinear responses have been reported hitherto (Martin & Chapman, 1961). However, our evidence shows that whereas this pattern is true for some virus–antiserum combinations, for others the relation is clearly curvilinear.

These differences in response are not easily explained. Prolonged incubation of two-dimensional FMD virus microneutralization tests failed to alter the shape of the curve, indicating that the non-linear form was not due to retardation of development of virus cytopathic effects in the presence of high concentrations of antibody. Also ineffectual was filtration of virus antigen preparations, to remove possible aggregates of virus particles, such as have been found to be refractory to neutralization in studies of several viruses including picornaviruses (Wallis & Melnick, 1967). A further possibility considered was that the various dose-response curves were due to differences in the amount of non-infective virus in antigen preparations used in the microneutralization test; Fazekas de St. Groth & Webster (1963) have shown that addition of non-infective antigen to neutralization test antigens, in sufficient quantity, resulted in depression of antibody titres. However, in the present study, addition of 64-fold excess of inactivated, purified FMD virus particles to the undiluted, infective homologous antigen, produced no significant change in the dynamics of the neutralization response. Another possible explanation for the different dose-response curves was that the antisera employed in the test contained anti-cell antibodies, although this seemed unlikely since absorption with tissue culture cells failed to modify the results.

Several workers have demonstrated the presence, in virus–antiserum mixtures, of infective immune complexes whose activity can be neutralized by treatment with anti-species globulin (Ashe & Notkins, 1966; Cihak, 1973; Huggett, Rodriguez & McKee, 1972; Rweyemamu, Booth & Pay, 1977). Addition of this to microneutralization tests on FMD viruses resulted in an enhancement of antibody titre and alteration in the shape of the dose-response curve. This finding suggests, not unreasonably, that the proportion of the input virus dose which becomes


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manifest as infective complexes is important in determining the sensitivity of quantal neutralization tests on FMD viruses. Nevertheless, the formation of infective immune complexes, or their expression as recognizable cytopathic activity, must be completely suppressed in the presence of high concentrations of antibody, otherwise it is difficult to imagine how neutralizing antibody activity can be detected at all in quantal neutralization tests, especially with high doses of virus. This question is especially pertinent to the present study in which, with some antisera, the antibody titres recorded with virus doses ranging from $10^3$ to $10^5$ TCD 50 were closely similar. This phenomenon has been put to practical use in our laboratory since it has been found possible to carry out serological type identification of FMD virus isolates, simply and rapidly, by titrating selected high-titred representative type-specific antisera against undiluted tissue culture harvests of unknown viruses, in the microneutralization test (Rweyemamu, Booth & Pay, in preparation).

It is well known that the host cell plays an important role in the outcome of neutralization tests (Kjellén & Schlesinger, 1959; Tyrrell & Horsfall, 1953). This was also found to be true in the present study, in which the two-dimensional microneutralization test displayed higher antibody titres when assayed in IB-RS-2 cells compared with BHK21 cells. The difference in sensitivity was more pronounced with the higher doses of virus tested and the dose-response curves obtained in each cell system were not parallel. A further finding was that the increased sensitivity of the IB-RS-2 cells for demonstrating antibody appeared to correlate with reduced sensitivity for detecting infectivity, as in the study of Tyrrell & Horsfall (1953). A similar phenomenon was observed in tests assayed in batches of BHK21 cells of differing virus sensitivity and, again, the dose-response curves obtained on different occasions were not exactly parallel, indicating that error in titration of the virus dose was not the reason for the disparities. These findings show that the sensitivity of microneutralization tests, assayed in a standard cell system, can be expected to vary with the quality of the host cells and, more importantly, that tests carried out on different occasions, with manifestly the same dose of infective virus, cannot be expected always to demonstrate identical titres of neutralizing antibody activity.

The variety of linear and curvilinear responses obtained in two-dimensional microneutralization tests poses a problem in deciding on what criterion to adopt for comparison of results. Fazekas de St. Groth (1961) derived a method for overcoming this dilemma, in which the data obtained in the quantal test were used to calculate the so-called neutralizing potency (pN) of the antiserum with a particular virus antigen. However, this mathematical treatment does not appear to be suitable for all virus systems. Stalder, Oxman & Herrman (1975) have shown, with herpes simplex virus, that the pN value varies considerably, depending on the dose of virus employed in the test. We have encountered similar difficulties in the present study (unpublished data), and for purposes of comparison prefer to adhere to the conventional practice of determining the end-point of the two-dimensional microneutralization test as that dilution of the antiserum which completely neutralizes exactly 100 TCD 50 of virus. Two-dimensional tests allow


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this reading to be made with greater accuracy than do simpler antibody titrations that employ a single virus dilution containing an estimated 100 TCD50, a factor important for detecting the slight serological differences that are prevalent among intratypic variants of FMD virus, and frequently of considerable significance for selection of suitable candidate strains for vaccine manufacture.

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