Neuralulation of Foot-and-Mouth Disease Virus. II. Further Parameters Related to the Sensitization of the 140S Virion by Antibody

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SUMMARY

The reaction of foot-and-mouth disease virus (FMDV) with 12S subunit/140S virion cross-reactive (sensitizing) antibody was studied in order to elucidate the requirements for neutralization versus sensitization. The presence of sensitizing antibody in immune serum caused an atypical in vitro neutralization response curve and a non-neutralized fraction. Cell-associated (cytophilic) antibody was not present in the system. Dissociation of the immune complex was not a factor and sensitized virus adsorbed to host cells via the regular virus receptor site(s). This finding led to the conclusion that sensitizing antibody is specific for non-critical sites. Dosing of the neutralization reaction mixtures with fractionated antibody of alternative antigenic specificities had an antagonistic effect on the neutralization response, suggesting steric hindrance. Cell receptor sites were a factor in sensitization since different host systems had different susceptibilities for sensitized antigen. The results suggest that in vitro neutralization of FMDV requires the attachment of multiple antibody molecules as proposed by the multi-hit theory of neutralization. The in vitro measurement of serum neutralizing activity as an indication of the in vivo immune response is discussed.

INTRODUCTION

The in vitro interaction of A12 FMDV and homologous antibody was previously reported (Hardy & Moore, 1981) to be atypical, consisting of a non-neutralized virus peak in antibody excess which often displayed an enhancement of infectivity above the virus control values. We demonstrated that the infectious virus was complexed with antibody (sensitized) and could be neutralized with anti-globulin. Furthermore, it was shown that neutralization versus sensitization was dependent on the antigenic specificity of the antibody. Protein subunit (12S) immunoadsorbent column fractionation of the serum produced three antibody fractions, one which was 140S virion-specific and two which were 12S/140S cross-reactive. The antibody fraction specific for the 140S intact virion neutralized virus effectively, while the antibody fraction reactive with both the 140S virion and the 12S protein subunit mostly sensitized or neutralized ineffectively. The enhancement of virus infectivity was associated only with the 12S/140S cross-reactive antibody. Although the atypical neutralization response was absent from the reaction of virus with the 140S virion-specific antibody, a small amount of virus was found to be sensitized. The following study was conducted to provide further information on the mechanisms involved in the sensitization and neutralization of virus.

Two theories which attempt to define virus neutralization have emerged in recent years: the...
single-hit and multi-hit models of neutralization. The single-hit model contends that there is just one critical site on the virion. Neutralization is interpreted as the result of the binding of a single antibody molecule to that site (critical site hypothesis; Dulbecco et al., 1956; Mandel, 1960). The one-hit theory was reiterated but modified by Mandel (1976) in his study of the neutralization of poliovirus. His critical reaction hypothesis allows for neutralization to occur by a one-hit mechanism at any capsid site provided the reaction is efficient, i.e. critical. The opposing multi-hit model proposes that more than one immunoglobulin molecule must bind to the surface of the virion to render it non-infectious (Daniels, 1975) and suggests that neutralization requires special dispositions of antibody attached to certain critical areas on the virus surface (Della-Porta & Westaway, 1978).

The extent to which the FMDV neutralization reaction conformed to these models is discussed.

**METHODS**

**Virus.** The large plaque antigenic variant 'ab' (Cowan, 1969) of FMDV type A₁₂ strain 119 (A₁₂) was used in this study. The virus stocks for neutralization assay and virus for purification were prepared in bovine kidney (BK) and baby hamster kidney (BHK) cells respectively as described previously (Hardy & Moore, 1981).

**Inactivation and purification of virus.** BHK-grown A₁₂ virus was inactivated with 3 mM-ethyleneimine (BEI), and concentrated and purified by density-gradient centrifugation as described previously (Hardy & Moore, 1981).

**Antiserum.** Immune serum to A₁₂ was produced in guinea-pigs by subcutaneous inoculation (Cowan, 1968) of a 10 μg dose of BEI-inactivated purified 140S virus antigen. The inoculum was prepared and the antisera were collected as described by Hardy & Moore (1981).

**Fractionation of antiserum by immunoadsorbent chromatography.** Preparation of the 12S protein subunit antigen, the cyanogen bromide-activated Sepharose 4B immunoadsorbent (Pharmacia), and fractionation of the serum on the column was performed as described previously (Hardy & Moore, 1981). The fractions were screened by Ouchterlony-type immunodiffusion analysis for 140S or 12S antigenic cross-reactivity (Hardy & Moore, 1981) and pooled into 140S virion-specific (140S), 1 M-sodium thiocyanate (NaSCN)-eluted 12S subunit cross-reactive (1 M-NaSCN 12S/140S) and 2 M-NaSCN-eluted 12S subunit cross-reactive (2 M-NaSCN 12S/140S) serum fractions.

**Cell culture.** The Mengeling and Vaughn foetal porcine kidney cell line (MVPK-1 clone 7; Dinka et al., 1977) was grown and maintained in Eagle’s minimum essential medium (MEM F-15; Grand Island Biological, Grand Island, N.Y., U.S.A.) containing 1 mM-sodium pyruvate, 5% (v/v) foetal bovine serum, 100 units/ml penicillin and 50 μg/ml streptomycin. Secondary (2°) and tertiary (3°) BK cells were prepared by the method of Bachrach et al. (1962) and grown and maintained in MEM with 10% (v/v) foetal bovine serum and antibiotics as described for MVPK cells. Monolayer cultures of both cell types were prepared for assay as described for MVPK by Hardy & Moore (1981).

**Neutralization assays.** Neutralizing activities of the antisera were measured in a plaque reduction neutralization test (PRNT) (Hardy & Moore, 1981). Further details concerning controls and modifications of the above method are described in Results. The number of p.f.u. was plotted versus antibody dilution based on original serum volume before fractionation. Neutralizing titres (70% reduction) of the sera were calculated by computer using the logit-log transformation method (Trautman & Harris, 1977).

**Secondary neutralization with anti-globulin serum.** Secondary neutralization of the virus was accomplished by the addition of anti-globulin (Majer, 1972). Rabbit anti-guinea-pig
immunoglobulin G was prepared and secondary neutralization of virus was performed as described previously (Hardy & Moore, 1981).

RESULTS

Study of antiserum–cell interaction and effect of blocking cell receptors on virus infectivity in the non-neutralized peak

Cytophilic antibody (Hawkes & Lafferty, 1967), if present, might be implicated as the cause of the non-neutralized fraction and the enhancement of virus infectivity. To test this, antiserum was adsorbed on cell cultures to determine whether the adsorption could remove the sensitizing antibody from the serum. The unfractionated and fractionated sera were each diluted to 1:10, applied in 0.1 ml vol. to eight MVPK cell cultures and allowed to adsorb at 37 °C for 1 h. The sera were then aspirated. When the cell-adsorbed unfractionated serum was assayed for neutralizing ability in a PRNT, no difference in response was seen when compared to unadsorbed serum (Fig. 1). To test for cytophilic enhancement of infectivity, the cell cultures on which the four serum fractions had been adsorbed were compared to untreated cultures for plaquing efficiency. The cultures were gently washed three times with Hanks’ balanced salt solution containing 10% lactalbumin hydrolysate (HLH) to remove free antibody, and inoculated with the standard concentration of virus (60 p.f.u./0.1 ml). The mean p.f.u. count for all five cell groups (eight replicates each) was not significantly different (data not shown).

To ascertain if sensitized virus might adsorb via a site other than the virus capsid receptor site(s), BEI-inactivated virus was adsorbed to the cells to block cell receptors for virus before the addition of the virus–serum mixtures of a standard PRNT. BEI-inactivated virus (100 μg/0.1 ml) was preadsorbed in 0.1 ml vol. to MVPK cell cultures for 1 h at 4 °C to prevent penetration, eclipse of virus and regeneration of new or formerly blocked cell receptors. Previous titrations of the BEI-inactivated virus established that the 100 μg dose achieved inhibition of virus infectivity (data not shown). The cultures were gently washed with HLH to remove unbound inactivated virus and inoculated with reaction mixture dilutions which represented the non-neutralized peak of the serum fractions showing poor neutralization responses. Controls consisted of the standard virus adsorbed to both inactivated virus-treated and untreated cultures. The entire dilution schemes for the three serum fractions were assayed in parallel on normal, untreated cells. The inoculated cultures were incubated at 4 °C for 30 min and at 37 °C for an additional 30 min. The results (Fig. 2) demonstrated a 90 to 100% drop in infectivity in the peak region of the neutralization response when assayed on the preadsorbed cultures. The dilution sets assayed on untreated cells, however, showed the non-neutralized fraction to be still present. The control virus mixture assayed on BEI-inactivated virus-adsorbed cells also showed a 100% drop in infectivity when compared to the untreated parallel.

Effect of dosing the neutralization reaction mixtures with antibody of alternative specificity

The presence of sensitizing antibody might sterically hinder the attachment of neutralizing antibody and prevent neutralization. To test this hypothesis, a group of neutralization-competition experiments were performed. A standard neutralization test with virus controls was conducted with the three fractionated serum pools and incubated in the normal manner. Each dilution mixture of the respective antibody titrations was then divided into equal volumes and given a second dose of an equal volume of antibody fraction or medium as outlined in Table 1. These reaction mixtures were again incubated at 37 °C for 1 h and assayed for survivors. The 1:83 (final) serum fraction concentration was chosen since it corresponded to the non-neutralized peak region of the 1 M-NaSCN 12S/140S fraction and
Table 1. Experimental format to test the effect of dosing prereacted \( A_{12} \) FMDV neutralization mixtures with immunoadsorbent-fractionated antibody of alternative specificity

<table>
<thead>
<tr>
<th>Second serum dose</th>
<th>1:83 140S virion-specific†</th>
<th>1 M-NaSCN fraction†</th>
<th>2 M-NaSCN fraction†</th>
<th>1:25 140S virion-specific</th>
<th>Fig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial serum dose + virus</td>
<td>HLH only</td>
<td>1 M-NaSCN 12S subunit cross-reactive fraction</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 M-NaSCN 12S subunit cross-reactive fraction</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>3 (a)</td>
</tr>
<tr>
<td>2 M-NaSCN 12S subunit cross-reactive fraction</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td>3 (b)</td>
</tr>
<tr>
<td>140S Virion-specific</td>
<td>X</td>
<td></td>
<td>X</td>
<td></td>
<td>3 (c)</td>
</tr>
</tbody>
</table>

* Virus–antibody dilution mixtures from a standard neutralization test were incubated for 1 h at 37 °C, divided into equal parts and dosed (X) with a second equal volume of medium or antibody fraction. The mixtures were again incubated for 1 h at 37 °C, and then assayed for surviving virus infectivity. HLH, Hanks' balanced salt solution containing 10% lactalbumin hydrolysate.
† Final concentration 1:83 after addition to neutralization test.
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Fig. 3. Effect of dosing prereacted $A_{12}$ FMDV neutralization mixtures with fractionated antibody of alternative specificity. (a) 1 M-NaSCN eluate + virus mixture dosed with the following: HLH (☐); 1:83 1 M-NaSCN eluate antibody (△); 1:83 140S virion-specific antibody (○); 1:25 140S virion-specific antibody (●). (b) 2 M-NaSCN eluate fraction + virus mixture dosed with the following: HLH (●); 1:83 140S virion-specific antibody (○). (c) 140S virion-specific antibody + virus mixture dosed with the following: HLH (●); 1:83 1 M-NaSCN eluate antibody (□); 1:83 2 M-NaSCN eluate antibody (△). (1) indicates mean virus control; (2) indicates 1:83 1 M mean serum-reacted virus control; (3) indicates 1:83 140S mean serum-reacted virus control; (4) indicates 1:25 140S mean serum-reacted virus control; (5) indicates 1:83 2 M mean serum-reacted virus control.

should, therefore, effect the largest change in infectivity. Controls containing 1:83 dilutions of all three fractions and standard virus amounts were performed in addition to the regular virus control and treated in the same manner as the test mixtures.

The results of this experiment are presented in Fig. 3. Fig. 3 (a) shows that the addition of homologous antibody to the 1 M-NaSCN 12S/140S neutralization response shifts the curve to
Table 2. In vitro neutralization plaque reduction titres of anti-A<sub>12</sub> FMDV serum fractions

<table>
<thead>
<tr>
<th>Serum fraction</th>
<th>Primary neutralization</th>
<th>Secondary neutralization</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MVPK</td>
<td>BK</td>
</tr>
<tr>
<td>Unfractionated</td>
<td>1.45</td>
<td>3.76</td>
</tr>
<tr>
<td>140S Virion-specific</td>
<td>2.33</td>
<td>2.50</td>
</tr>
<tr>
<td>1 M-NaSCN 12S subunit cross-reactive eluate</td>
<td>0.13</td>
<td>3.24</td>
</tr>
<tr>
<td>2 M-NaSCN 12S subunit cross-reactive eluate</td>
<td>0.68</td>
<td>NT†</td>
</tr>
</tbody>
</table>

* Serum fractions were assayed on MVPK and 2° BK cell cultures. Primary neutralization reaction mixtures were secondarily neutralized with rabbit anti-guinea-pig immunoglobulin G for 1 h at 37 °C. Titre values represent the 70% plaque reduction log neutralizing activities of the serum fractions.
† NT, Not tested.

Influence of host cell on the occurrence of the non-neutralized fraction

When the neutralization tests were assayed in parallel on 2° or 3° BK cell cultures, the results were somewhat different from those found with MVPK cells (Hardy & Moore, 1981). Susceptibility for the sensitized virus was readily observed with the 2° BK cells but not with the 3° BK cells. A non-neutralized peak as seen on MVPK cell cultures could not be demonstrated on either 2° or 3° BK cells. However, when the immune complexes in the primary neutralization mixture were subjected to antiglobulin precipitation (secondary neutralization) and assayed in parallel on 2° BK cells (Table 2), the enhancement of titres showed that the virus was sensitized. Furthermore, the degree of sensitization and the secondary neutralization titres closely paralleled those obtained on MVPK cells (Hardy & Moore, 1981).

DISCUSSION

As was shown in the previous report (Hardy & Moore, 1981), 12S subunit–140S virion cross-reactive antibody was responsible for the non-neutralized fraction on MVPK cells and was mostly, if not entirely, sensitizing antibody. Sensitization could occur by two possible
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mechanisms: (i) either as a stage in the process of neutralization which does not proceed to completion due to dissociation of the reactants in the mixture or on attachment to the host cell, or (ii) as a distinct process of antibody attachment to non-critical sites on the virus surface (Majer, 1972). The ability of anti-globulin to secondarily neutralize is explained for the first hypothesis as a stabilization of the virus-antibody complexes through aggregation (Majer, 1972). The stability of the primary FMDV neutralization response over time (Hardy & Moore, 1981), and the failure of BK cells to show a non-neutralized peak in this study, indicates that dissociation does not occur, as do the reports of other investigators (Mandel, 1958; Ashe et al., 1969; Hahon, 1970; Lewenton-Kriss & Mandel, 1972). The inactivating effect of anti-globulin in the second hypothesis may be explained as the result of blocking neighbouring critical sites after attachment to antiviral antibodies bound to non-critical sites (Majer, 1972). The second hypothesis which infers that sensitizing antibody is specific for non-critical site(s) appears to be most appropriate to the results of this study.

Other explanations for the sensitizing phenomenon were also ruled out. Antibody that sensitizes only because it is cytophilic was found not to be a factor in the FMDV non-neutralized peak. Booth et al. (1978) reported the same findings for FMDV, as well as Kjellen & Schlesinger (1959) and Hawkes & Lafferty (1967) for other virus systems. Thermolabile accessory factors were not a requirement for sensitization or 140S virion-specific antibody-mediated neutralization because the serum was heat-inactivated. Furthermore, the addition of complement did not affect the neutralization response of the sensitized A12 virus used in this study (M. M. Hardy & D. M. Moore, unpublished results). Similar results were found by Radwan & Burger (1973) and Rweyemamu et al. (1977).

Brown & Smale (1970) demonstrated by electron microscopy studies that the anti-FMDV 12S/140S cross-reactive antibody attachment site is different from the anti-140S virion-specific site(s). Cartwright et al. (1980) suggested that the 12S antigen site is a part of, or a different conformation of, the intact 140S site. The successful blocking of sensitized virus infection in this study by the preadsorption of inactivated free virus suggests that the attachment site of sensitized virus to the cell was not significantly different from unbound virus. This finding supports the theory that 12S subunit cross-reacting antibody is specific for non-critical sites. A similar segregation of neutralizing versus sensitizing ability based on antibody specificity was reported by Wadell (1972) for adenovirus.

Steric hindrance of neutralizing antibody by sensitizing antibody was strongly suggested by the results of the neutralization-competition experiments. The 140S virion-specific antibody was less efficient in neutralizing virus when 12S/140S cross-reactive antibody was present. The ability of 12S/140S cross-reactive antibody to reverse 140S virion-specific neutralization, which occurred regardless of the order of addition of the components, indicated that the phenomenon was independent of respective antibody affinities. The competition for limited sites or space on the virion is further supported by the finding that the sum of the neutralizing activities of the fractions exceeds that of the unfractionated serum.

The sensitization of FMDV by the 140S (critical) antibody can be explained best in terms of the Della-Porta & Westaway (1978) multi-hit model: sensitization occurs since a proportionately increasing number of virion critical sites remain unoccupied at low antibody concentration. If neutralization occurred through the attachment of one antibody molecule, an increase of neutralization upon reaction with anti-globulin would not have been observed. In a mathematical model for FMDV neutralization, Trautman & Harris (1977) postulated that, of the estimated 5 to 10 critical sites on FMDV for IgG, all but one or two must react with antibody to effect neutralization.

Svehag (1968) and other investigators (Kjellen & Schlesinger, 1959; Booth et al., 1978) deduced that the fate of the virus–antibody complex, as well as the efficacy of the antibody as a virus-neutralizing factor, depends upon how the complex is handled by the host cell. It has
been suggested that the difference in response of two cell lines can be related to reduced detection of virus infectivity, not increased detection of neutralization (Booth et al., 1978). We suggest that attachment of 12S/140S antibody to the capsid is sufficient in the BK cell system to block binding sites. The MVPK cell system may have additional virus receptor sites not present in the BK system. A similar explanation for the variations in neutralization responses with the host system was suggested by the multi-hit model (Della-Porta & Westaway, 1978). In our study, the requirements for neutralization were obviously more stringent for the MVPK system than the BK system. These differences in recognition of neutralization indicate that a one-hit-induced physical transition of the virion as suggested by Mandel (1976) did not occur.

The FMDV neutralization system exhibited most of the phenomena cited by Della-Porta & Westaway (1978) as evidence of a multi-hit mechanism of neutralization (i.e. a non-neutralized fraction, neutralization of sensitized virus by anti-immunoglobulin, enhancement of virus infectivity by antibody, influence of host cell on amount of neutralization). In addition, the atypical shape of the non-neutralized peak observed in this study appears related to the number of non-critical antibody molecules attached to the virion. We suggest that sensitizing 12S/140S antibody is only able to neutralize in areas of large antibody excess and in the zone of equivalence. Non-neutralized virus within the peak would represent particles with insufficient bound antibody to completely coat the surface, but too much for good lattice formation. Cowan (1968) demonstrated that the 140S virion reacted with a maximum of 50 to 60 molecules of IgG in antibody excess and approx. 20 molecules at the equivalence zone of the precipitin curve.

In conclusion, although it is generally felt that the virus neutralization test provides a reliable index, it is evident that neutralization of infectivity of animal viruses by antibody, using in vitro tests, cannot provide a complete understanding of the in vivo neutralizing response in animals. The findings of this report show that the sensitivity and accuracy of in vitro neutralization tests can be expected to vary with the virus or antigen, the antiserum specificity to that antigen, and the host cell. These variations can be eliminated through the use of anti-globulin which standardizes the results without regard to those variables while enhancing the sensitivity. In vitro tests are helpful in the attempt to elucidate the mechanism of primary or direct neutralization of viruses if that reaction is not interfered with by sensitizing antibody. However, since in vivo neutralization may be affected differently by the presence of sensitizing antibody, investigators will have to make independent judgements as to whether the expediency and ease of in vitro testing versus the measurement of total significant antibody (i.e. true in vivo protective antibody) will best meet their needs.

REFERENCES


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