Protective Role of Foot-and-Mouth Disease Virus Antibody

*in vitro and in vivo* in Guinea-pigs

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SUMMARY

Plaque reduction neutralization assays, using foot-and-mouth disease virus (FMDV) type A, strain 119 and immune serum from convalescent guinea-pigs infected with this strain of virus and performed with monolayers of a swine kidney cell line, resulted in biphasic neutralization curves because of the presence of as many as 30 to 50% of non-neutralized virus particles at peak activity. These results were found using gum tragacanth, agar, agar containing DEAE-dextran, and methylcellulose overlays and were also found using monolayers of guinea-pig embryo tongue and guinea-pig embryo heelpad cells. Non-neutralized virus in immune serum–FMDV mixtures was neutralized after the addition of anti-species antibody, demonstrating that the non-neutralized virus fraction consisted of virus in the form of infectious immune complexes. These complexes were not infectious when inoculated intraperitoneally into suckling mice or intracardially into guinea-pigs. They were infectious, however, if inoculated intradermally into the tongue or rear heelpads of guinea-pigs. Low doses of passively transferred immune serum did not protect guinea-pigs against the formation of primary vesicles after intradermal tongue or heelpad challenge with virus but did protect against systemic spread of virus to the remaining uninoculated feet. Higher doses of passively transferred immune serum protected against tongue challenge but even higher doses were required to protect against heelpad challenge. The role of antibody in protection against the systemic spread of FMDV may be due to infectious immune complexes being removed from the blood by the reticuloendothelial system. In the dermis of the tongue and heelpad, the immune complexes remain infectious, resulting in the formation of local vesicles except when these tissues contain very high concentrations of antibody.

INTRODUCTION

Although guinea-pigs have been used for many years in potency tests for foot-and-mouth disease (FMDV) vaccines (Terpstra et al., 1976; Mackowiak et al., 1966), relatively little is known of the immune response that protects these animals against virus challenge. Antibody of the IgM class develops 2 to 3 days after infection followed by IgG class antibody (Cowan & Trautman, 1965), and these antibodies can protect suckling mice against fatal infection with the virus (Cowan & Trautman, 1965; Hardy & Moore, 1981; Knudsen et al., 1979). However, studies *in vitro* using plaque reduction neutralization (PRN) assays performed on swine kidney cell lines indicate that guinea-pig immune serum incompletely neutralizes FMDV; this results in the formation of infectious immune complexes (Hardy & Moore, 1981). In the current study, our purpose was to examine and characterize the protective effects of FMDV antibody obtained

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from guinea-pigs that are completely protected against FMDV challenge. Our results show that virus-antibody reactions result in the formation of immune complexes which are infectious both in vivo and in vitro and we characterize the role of antibody and immune complexes in antibody-mediated protection of guinea-pigs against FMDV.

**METHODS**

**Virus.** The virus used was FMDV type A12, strain 119 that had been passaged 30 times in guinea-pigs by inoculation of 24 h vesicular fluids (Knudsen et al., 1979, 1982) and then passaged twice in Mengeling–Vaughn porcine kidney (MVPK) cells (Dinka et al., 1977). This virus preparation contained, per ml, $10^{9} + 50\%$ mouse lethal doses (MLD$_{50}$), $10^{9} + 1$ p.f.u. when assayed on MVPK cells, $10^{6} + 50\%$ guinea-pig infectious doses (GPID$_{50}$) when titrated by epidermal inoculation of guinea-pig tongue, $10^{7} + 50\%$ GPID$_{50}$ when titrated by epidermal inoculation of heelpads, and $10^{3} \text{GPID}_{50}$ when titrated by intracardial inoculation of guinea-pigs. In titrations in guinea-pigs, five animals per dilution were used and infectious endpoints were determined as described under "Mouse and guinea-pig serum neutralization test" below. Unless specified otherwise, all GPID$_{50}$ referred to in the text are based on titrations in heelpads.

**Cell cultures.** The medium used for cell cultures was minimal essential medium (MEM) with Earle’s salts supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), gentamicin (500 μg/ml) and 5% foetal calf serum.

Late-term pregnant guinea-pigs were killed by CO$_{2}$ anaesthesia and embryos were aseptically removed. Guinea-pig embryo heelpad (GPEH) cells were obtained by surgically excising the pads and cutting them into small pieces with a scalpel and forceps, and then treating with a 0.05% solution of 1:250 trypsin in 0.02% EDTA for 2 h with stirring at 37°C. Large debris was removed by sedimentation under gravity and small debris was removed by centrifugation at 20 $g$ for 5 min. Cells were removed from the supernatant by centrifugation at 200 $g$ for 10 min then the cell pellet was resuspended in MEM and the last centrifugation step was repeated. Five ml of cell suspension were placed into 25 cm$^2$ sterile disposable plastic tissue culture flasks and incubated at 37 °C, after which an overlay was added. Overlays consisted of MEM containing 0.6% gum tragacanth (McVicar et al., 1974), 0.5% Noble agar, 0.5% Noble agar containing DEAE-dextran (100 μg/ml) (Booth, 1977) or 0.6% methylcellulose (Hardy & Moore, 1981). In some experiments, an equal volume of 1:20 goat anti-guinea-pig immunoglobulin (Miles Laboratories) was added to each virus-serum mixture. Control tubes received equal volumes of normal goat serum. These mixtures were then incubated for an additional 1 h at 5 °C, after which 0-1 ml was inoculated into each well of a six-well 35 mm plastic disposable multidish containing a confluent monolayer of MVPK, GPET or GPEH cells. Virus controls were diluted 1:5 in MEM before inoculation. The cultures were incubated at 37 °C for 1 h, after which an overlay was added. Overlays consisted of MEM containing 0-6% gum tragacanth (McVicar et al., 1974), 0-5% Noble agar, 0-5% Noble agar containing DEAE-dextran (100 μg/ml) (Booth, 1977) or 0.6% methylcellulose (Hardy & Moore, 1981). In some experiments, an equal volume of 1:20 goat anti-guinea-pig immunoglobulin (Miles Laboratories) was added to each virus-serum mixture. Control tubes received equal volumes of normal goat serum. These mixtures were then incubated for an additional 1 h at 5 °C, after which 0-1 ml was inoculated into cell cultures in multidishes. The plates containing MVPK cells were incubated for 18 h and those containing GPET and GPEH cells were incubated for 40 h and then stained with a crystal violet-formalin solution (McVicar et al., 1974). Results are expressed as percent p.f.u., i.e. the mean number of p.f.u. per 0-1 ml in test wells divided by the mean number of p.f.u. in control wells, $\times 100$.

**Preparation of sera.** Convalescent serum was prepared from guinea-pigs that had been inoculated subcutaneously in the right heelpad with 100 GPID$_{50}$ of virus. The animals developed prominent lesions on the non-hairy surfaces of the four feet and usually the dorsal surface of the tongue at 3 to 5 DPI. At 40 DPI, five of the animals were challenged with $10^{3} \text{GPID}_{50}$ of FMDV in the tongue and right heelpad. They showed no signs of vesicles. The remaining 35 animals were anaesthetized with CO$_{2}$ and exsanguinated by cardiac puncture and the serum was collected and pooled. All convalescent serum used in these studies was from this pool. Pooled serum was heated at 56 °C for 30 min and filtered through a 0-22 μm filter.

Hyperimmune serum was prepared by inoculating guinea-pigs subcutaneously in the neck with 0-5 ml of emulsified complete Freund’s adjuvant (CFA) containing 0-25 ml of CFA (Difco) and 0-25 ml of 0-05 μM phosphate-buffered saline (PBS) (pH 7-2) containing 10 μg of purified inactivated FMDV (Knudsen et al., 1979, 1982). The immunity was boosted at 30 days post-vaccination (DPV) by inoculating 10 μg of inactivated FMDV intradermally into the flank. These animals were exsanguinated at 40 DPV.
Protective role of FMDV antibody

Animal inoculations. Outbred female Hartley strain guinea-pigs (Dutchland Farms, Denver, Pa., U.S.A.), weighing 300 to 400 g were used. Guinea-pigs were inoculated with 10 μl of FMDV preparation intradermally in the anterior tip of the tongue and the metatarsal region of the right heelpad as described previously (Knudsen et al., 1982), and 0.2 ml were inoculated intracardially into guinea-pigs anaesthetized with CO₂.

For experiments involving the passive transfer of serum, hyperimmune serum, normal guinea-pig serum or various amounts of convalescent serum were inoculated subcutaneously in the neck. Twenty-four h later, 1 ml of blood was collected for determination of antibody levels and the protective immunity of the animals was challenged by intradermal inoculations of tongue and the right heelpad with 10³ GPID₅₀ of FMDV. The animals were observed for 7 days for the development of vesicles at the inoculation site (primary vesicles) and for the development of vesicles on the three un inoculated feet (secondary sites).

Mouse and guinea-pig serum neutralization test. Serum neutralization was assayed in mice (Cunliffe & Graves, 1963) by intraperitoneally inoculating 0.03 ml of serum–virus mixture containing 100 MLD₅₀ of FMDV into each of 10 suckling mice (Knudsen et al., 1979). Serum neutralization was assayed in guinea-pigs by inoculating 10 μl of serum–virus mixture containing 100 GPID₅₀ of FMDV intradermally into the right heelpad. For intracardial inoculations, 10 μl of the mixture was diluted to 0.2 ml with PBS just before inoculation. Endpoints were determined by the development of primary vesicles (tongue and right heelpad inoculations) or the development of a vesicle on the tongue or any foot (intracardial inoculations) within 7 days of inoculation. The neutralizing activity of each serum was calculated by the method of Spearman-Karber (Finney, 1964) and expressed as -log₁₀ of the 50% protective dose per ml (PD₅₀).

RESULTS

Plaque reduction neutralization studies on heterologous MVPK cells

To characterize the ability of specific antibody to neutralize FMDV, convalescent serum was diluted and mixed with constant amounts of virus and then assayed by PRN on MVPK cells. Fig. 1 shows that, as antibody was diluted, a biphasic neutralization curve resulted because of the presence of variable amounts of non-neutralized virus. Maximum neutralization occurred at a 1:4 dilution or less (81% neutralization) and 1:256 (82% neutralization), and the least amount of neutralization occurred at 1:16 (64% neutralization). Neutralizing antibody declined rapidly at 1:1024 and 1:4096 dilutions. Plaque size averaged 1.2 mm diam. at 1:4 dilution, 1.5 mm at 1:16 and 2.5 mm at 1:256. Plaque sizes in control wells averaged 2.5 mm.

Fig. 1 also shows that the addition of goat anti-guinea-pig immunoglobulin to the virus–serum reaction mixtures neutralized the non-neutralized fraction of FMDV, demonstrating that non-neutralized virus fractions were composed of immune complexes.

Effect of overlay on plaque reduction neutralization

Because it has been shown that the overlay can affect the neutralization of FMDV on swine kidney cells (Booth, 1977) we performed the PRN with agar, agar containing DEAE-dextran and gum tragacanth overlays. Fig. 2 shows biphasic neutralization curves containing large fractions (>20%) of non-neutralized virus with all three overlays, although agar overlays containing DEAE-dextran resulted in the largest amounts of non-neutralized virus. In separate experiments (data not shown), it was found that results similar to those obtained with gum tragacanth overlay were obtained with methylcellulose overlay.

Other parameters of the plaque reduction neutralization assay

Additional studies using MVPK cells and gum tragacanth overlays (data not shown) demonstrated that the infectious immune complexes could not be washed off the cell monolayers after a 2 h adsorption period, that similar biphasic neutralization curves were obtained with six randomly chosen plaque-purified variants and that the addition of guinea-pig complement did not alter the size of the non-neutralized fraction. Similar results have been described by Hardy & Moore (1981).

Plaque reduction neutralization using primary guinea-pig embryo cells

Because serum neutralization may vary with the cell culture in PRN assays, we investigated the neutralization of FMDV by guinea-pig convalescent serum on cells derived from the
epithelium of the tongue (GPET cells) and heelpads (GPEH cells) of guinea-pig embryos. Fig. 3 shows four separately performed but representative PRN curves from experiments using monolayers of GPET cells. Although the size of the non-neutralized fraction varied from one experiment to the other, it is clear that there was a sizeable non-neutralized fraction and the biphasic shapes of the curves are similar to those obtained with MVPK cells. Fig. 3(d) also shows that the non-neutralized FMDV fraction was neutralized by the addition of goat anti-guinea-pig immunoglobulin at a dilution of 1:10³. Results similar to those of Fig. 3 were obtained with GPEH cells (data not shown). In these studies, the fibroblastic GPET and GPEH cells readily grew into monolayers, and plaques were clear, sharp and ranged in size from pinpoints at low serum dilution to several mm at higher serum dilutions.

**Serum–virus neutralization in mice**

The inoculation of serum–FMDV mixtures into suckling mice forms the basis of a widely used assay for the detection of serum neutralizing antibody to FMDV. We were interested in determining whether the immune complexes that were infectious *in vitro* were capable of killing suckling mice. Table 1 shows that the presence of infectious immune complexes in the serum–virus mixtures was not apparent in suckling mice because the mice were protected against FMDV at serum dilutions greater than 1:2048 which results in a mean PD₅₀ of 4.77 for the three assays.
Table 1. Neutralization of FMDV by guinea-pig convalescent sera in suckling mice

<table>
<thead>
<tr>
<th>Experiment number</th>
<th>Reciprocal of serum dilution</th>
<th>Number of dead suckling mice*</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>16</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>32</td>
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<td></td>
<td>64</td>
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<td></td>
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<td></td>
<td>1024</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>2048</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>4096</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>NGPS† (1:50)</td>
<td>10</td>
</tr>
</tbody>
</table>

* Ten mice used per dilution.
† Normal guinea-pig serum.

Table 2. Infectivity of immune complexes for guinea-pigs after intradermal inoculation into the tongue and right heelpad and intracardial inoculation

<table>
<thead>
<tr>
<th>Inoculation route†</th>
<th>Reciprocal of serum dilution</th>
<th>Intracardial</th>
<th>Tongue</th>
<th>Right heelpad</th>
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<tbody>
<tr>
<td></td>
<td>2</td>
<td>ND‡</td>
<td>6</td>
<td>0</td>
</tr>
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<td></td>
<td>10¹</td>
<td>10</td>
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<td>0</td>
</tr>
<tr>
<td></td>
<td>10²</td>
<td>9</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10³</td>
<td>5</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10⁴</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10² (NGPS)§</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>PD₅₀</td>
<td>4·9</td>
<td>2·6</td>
<td>&lt;2·3</td>
</tr>
</tbody>
</table>

* 10 animals per group.
† Each inoculum contained 10² intracardial, tongue or heelpad GPID₅₀ of FMDV mixed with the appropriate dilution of serum.
‡ ND, Not done.
§ Normal guinea-pig serum.

Intracardial and intradermal inoculation of immune complexes into guinea-pigs

The previous studies demonstrated that immune complexes were infectious in vitro for GPET and GPEH cells and it was considered important to determine whether the immune complexes were infectious for guinea-pigs. Consequently, serum–virus mixtures were inoculated directly into two of the tissue sites of FMDV replication, the tongue and right heelpad, and into the blood by means of intracardial inoculation. To ensure that the animals inoculated by each route received the same infectious dose of FMDV, the serum–virus mixtures were adjusted to contain 100 GPID₅₀ per inoculation vol. for each route. Table 2 shows that the immune complexes formed at all serum dilutions were infectious when inoculated intradermally into the right heelpad (PD₅₀ less than 2·3). Immune complexes formed with low dilutions of serum were less infectious when intradermally inoculated (PD₅₀ = 2·6) and immune complexes were infective after intracardial inoculation into the vascular system only when formed with very high dilutions of serum (PD₅₀ = 4·9).

To ensure that vesicles of the tongue and heelpad resulted from infectious immune complexes rather than free virions, 8 µl of undiluted goat anti-guinea-pig immunoglobulin were added to 2 µl of a serum–virus mixture containing 10³ GPID₅₀ of FMDV and a 1 : 100 final dilution of convalescent serum. The animals (five per group) inoculated intradermally in the tongue and heelpad with 10 µl of this mixture did not develop tongue or heelpad vesicles at 3 DPI, although
Fig. 3. Four examples of FMDV plaque reduction neutralization by convalescent serum on GPET cells. Panel (d) shows convalescent serum (●) and convalescent serum plus anti-species globulin (○).

Table 3. Protective effects of passively transferred immune serum in normal guinea-pigs against intradermal tongue and heelpad challenge

<table>
<thead>
<tr>
<th>Serum source (ml)</th>
<th>Antibody titre* (PD₅₀)</th>
<th>Primary sites</th>
<th>Secondary sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyperimmune (10)</td>
<td>5.3</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Convalescent (10)</td>
<td>4.0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Convalescent (1-0)</td>
<td>2.8</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Convalescent (0-1)</td>
<td>&lt;2.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NGPS (5-0)</td>
<td>&lt;2.1</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Antibody titres of pooled sera from recipient animals obtained after passive serum transfer and just before intradermal FMDV challenge.

† Each animal from groups of five guinea-pigs was inoculated with 10³ GPID₅₀ of FMDV intradermally into the tongue and right heelpad 24 h after passive serum transfer.

two of five animals in each group did develop primary vesicles between 4 and 7 DPI. All animals that were inoculated with this serum–virus mixture but to which normal goat serum was added developed prominent primary vesicles of the tongue and feet within 2 DPI. This shows that it was virions in the form of immune complexes, formed with serum dilutions of at least 1 : 10² or less, that caused the primary vesicles of the tongue and heelpads.
Protective role of FMDV antibody

Protective role of passively transferred serum

To evaluate the protective role of FMDV antibody *in vivo*, hyperimmune serum and decreasing concentrations of convalescent serum were passively transferred to normal guinea-pigs. Each animal was then doubly challenged by intradermally inoculating the tongue and the right heelpad. The animals were observed for the development of vesicles at the inoculation sites and for the spread of virus to the remaining uninoculated three feet. Table 3 shows that the passive transfer of hyperimmune serum resulted in complete protection against either tongue or heelpad FMDV challenge. A 10 ml volume of convalescent serum protected the guinea-pigs against FMDV administered in the tongue, but not in the heelpad; 1 ml of convalescent serum protected them against generalized FMDV but not against primary tongue or heelpad lesions. Thus, protection against challenge by passively transferred immune serum was dependent on the concentration of immune serum and the virus inoculation site.

**DISCUSSION**

The serum used in this study was obtained from convalescent guinea-pigs that were completely protected against intradermal tongue or heelpad challenge with FMDV. Studies of this serum should therefore provide insight into the protective role of antibody to FMDV in guinea-pigs. *In vitro* studies of virus-antibody reactions showed both neutralized and non-neutralized virus. The non-neutralized virus was in the form of immune complexes as shown by secondary neutralization with anti-species immunoglobulin (Booth, 1977; Hahon, 1970). The size of the non-neutralized fraction, which consisted of 10 to 50% of infectious virions, varied with the serum concentration in the form of a biphasic neutralization curve. The formation of infectious immune complexes was not dependent on *in vitro* culture conditions (Booth, 1977) because similar biphasic neutralization curves were found with guinea-pig and swine cell cultures and with four different cell culture overlays.

Hardy & Moore (1981) have described affinity-purified 12S subunit-specific FMDV antibody from hyperimmune guinea-pig sera which binds to but is otherwise highly inefficient in neutralizing FMDV. Other workers using monoclonal antibodies to mouse mammary tumour virus have shown that the non-neutralized fraction may be due to non-neutralizing antibodies binding to virus and thereby sterically blocking the binding of neutralizing antibodies (Massey & Schochetman, 1981). These latter two studies suggest that the non-neutralized fraction of virus in our study is dependent on the relative concentration of 12S subunit-specific antibodies which prevent neutralization by 140S virion-specific antibody.

These *in vitro* results suggested that FMDV immune complexes would be infectious *in vivo* and raised questions about the efficacy of antibody-mediated protection in guinea-pigs. Studies using either passive serum transfer or the inoculation of immune complexes into the vascular system and tissues of guinea-pigs showed that protection was dependent on antibody concentration as well as the site of FMDV challenge. Low concentrations of passively transferred serum (1-0 ml) protected guinea-pigs against systemic spread of FMDV from tongue or heelpad vesicles. Any virus entering the vascular system from vesicles should be bound by passively transferred antibody and form an immune complex. The intracardial inoculation of immune complexes (Table 2) showed they were not able to reach susceptible sites from the vascular system. Thus, it is likely that FMDV antibody protects against the systemic spread of FMDV by forming immune complexes that are removed from the vascular system by Fc-dependent (Phillips-Quagliata *et al.*, 1971), or complement-dependent (Munthe-Kaas *et al.*, 1976) phagocytosis by macrophages and cells of the reticuloendothelial system (Benacerraf *et al.*, 1959). This mechanism may also function in the peritoneal cavity of suckling mice, resulting in the Fc- or complement-mediated phagocytosis of the intraperitoneally inoculated infectious immune complexes before they can reach lethal viral replication sites in muscle (Skinner, 1951).

In contrast to results from intracardial inoculation, immune complexes were infectious after intradermal inoculation of the tongue and feet. In animals receiving passively transferred immune serum, 5 to 10 ml of convalescent serum was required to protect against a tongue challenge and 10 ml of hyperimmune sera to protect against a heelpad challenge, indicating that
high concentrations of antibody in intracellular fluids of the dermis of the tongue and feet can protect against intradermal challenge. Tongue tissues may require lower antibody levels for protection than heelpad because antibody and other immune components can infiltrate faster into the warmer, highly vascularized tongue epithelium than the more environmentally exposed, non-hairy heelpad epithelium (Knudsen et al., 1982). At very high concentrations of antibody in the PRN (Fig. 1, 2, 3), the lowest number of plaques are usually found, and these plaques are reduced in size. This suggests that, in vivo, the high concentration of antibody in intracellular spaces greatly reduces the number of infectious virions and probably slows the rate of cell-to-cell spread of non-neutralized FMDV in the form of immune complexes sufficiently to allow non-specific inflammatory mechanisms (Knudsen et al., 1982) to eliminate residual virus.

We have shown that FMDV antibodies in unfractionated guinea-pig sera, despite the formation of infectious immune complexes, can protect guinea-pigs against intracardial or intradermal challenge. However, this protection is dependent on antibody concentration and the route of virus inoculation. To gain deeper insight into the protective role of antibody in foot-and-mouth disease, it would be of considerable interest to test by passive transfer the protective capacities of IgG subclasses and antibodies having different viral component specificities.

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REFERENCES


