Qualitative and Quantitative Differences in the Immune Response to Foot-and-Mouth Disease Virus Antigens and Synthetic Peptides

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SUMMARY

In cross-immunization studies using foot-and-mouth disease virus (FMDV) antigens and a synthetic peptide, from a region within virus coat protein VP1, it has been shown that intact virus will prime the immune system for intact virus, virus subunits and synthetic peptide but not for disrupted virus. In contrast, peptide will prime for a response to peptide and virus subunits but not to intact virus or disrupted virus. Furthermore, studies on antibody populations in anti-virus and anti-peptide antisera demonstrated clear differences in the nature of the antibody response to the two antigens. This result is reflected in protection studies carried out on animals immunized with virus particles or peptides where there is a clearer correlation between in vitro neutralization and protection in vivo following peptide immunization. Thus, it has been shown that there are major qualitative and quantitative differences in the immune response to the FMDV particle and synthetic peptide.

INTRODUCTION

Foot-and-mouth disease virus (FMDV) is a member of the family Picornaviridae. The infectious particle, which has a sedimentation coefficient of 146S, is composed of one molecule of ssRNA ($M_r$ $2.6 \times 10^6$) and 60 copies of each of four structural proteins (VP1 to 3 $M_r$ $24 \times 10^3$ and VP4 $M_r$ $10 \times 10^3$). Heating the virus to 56 °C for 30 min or lowering the pH to 6.5 disrupts the virus capsid to produce 12S particles (composed of five copies each of VP1, 2 and 3), aggregated VP4 and free RNA (Burroughs et al., 1971; Talbot et al., 1973). This 12S particle will elicit complement-fixing antibodies but it has very low immunizing activity (Brown & Newman, 1963). Of the four structural proteins VP1 appears to play a key role in the immunizing activity of the virion since cleavage of this protein with trypsin results in loss of immunogenicity (Wild et al., 1969). Furthermore, VP1 is the only protein which, in its isolated form, will evoke neutralizing antibodies (Laporte et al., 1973; Bachrach et al., 1975; Meloen et al., 1979). Detailed studies using enzymic and chemical cleavage fragments (Strohmaier et al., 1982) and synthetic peptides (Bittle et al., 1982; Pfaff et al., 1982) from VP1 of virus type O, Kaufbeuren have identified two areas (amino acids 141 to 160 and 200 to 213) which elicit neutralizing and protective antibodies. Of these two sequences the 141 to 160 peptide is immunodominant in its ability to raise antibodies that have a higher specific activity to neutralize virus infectivity and protect against experimental infection (Bittle et al., 1982).

The 141 to 160 peptide has been shown to prime the immune system so that a second sub-immunizing dose of the same peptide will elicit a serotype-specific neutralizing antibody response (Francis et al., 1985a, b). However, in a preliminary study the same peptide did not appear to prime for a subsequent response to virus particles (Francis et al., 1985a). This was in contrast to the results reported for poliovirus where synthetic peptides had been shown to prime the immune system to produce high titres of neutralizing antibody following secondary inoculation of a sub-immunizing dose of virus (Emini et al., 1983).

Although there have now been a number of reports on the properties of FMDV synthetic peptides, there has been very little information on the relationship between the immune
responses to synthetic peptide and native viral antigens. Indeed, this lack of information has led many to assume incorrectly that the immune response to the peptide is necessarily intended to mimic the immune response to the intact virion. In this report we have attempted to clarify the qualitative and quantitative differences by performing cross-immunization studies and by examining serological profiles and in vivo protection using virus and peptide antigens.

**METHODS**

_Virus antigens._ FMDV type O_1 Kaufbeuren was grown in baby hamster kidney monolayer cells and purified to give 146S particles using the method described by Brown & Cartwright (1963). This purified virus was used to produce 12S virus subunits by mild acid treatment. One volume of virus was mixed with two volumes of 0.05 M-NaH2PO4 to give a final pH of 6.5. This mixture was kept for 30 min at room temperature before being centrifuged overnight at 30000 g on a 15 to 25% sucrose gradient to isolate the 12S component. Purified virus was also disrupted to give isolated virus coat proteins by boiling for 2 min with 1% SDS and 0.1% mercaptethanol.

_Synthetic peptides._ O_1 Kaufbeuren peptides were synthesized by the solid-phase method using a Beckman model 990B peptide synthesizer at the Scripps Clinic (La Jolla, Ca., U.S.A.) (Bittle _et al._, 1982) based on the 213 amino acid sequence of VP1 published by Kurz _et al._ (1981). An additional non-natural cysteine residue was added to the carboxy terminus of each peptide. Coupling to keyhole limpet haemocyanin (KLH) was carried out using the cross-linking reagent m-male imidobenzoyl-N-hydroxysuccinimide ester (Liu _et al._, 1979).

_Anomals._ Female Dunkin–Hartley guinea-pigs, approximately 12 weeks old and weighing between 450 and 500 g, that had been maintained as a closed randomly bred colony at the Institute for Animal Disease Research, Pirbright, U.K., were used.

_Neutralization assay._ The neutralizing activity of serum samples against 100 TCID<sub>50</sub> of FMDV was determined using a microneutralization test (Francis & Black, 1983). Each test was performed in triplicate and the results were recorded as the mean log₁₀ reciprocal of the serum dilution that gave confluent cell sheets in 50% of the microplate wells (SN<sub>50</sub>).

_ELISA._ A modification of the indirect ELISA technique described by Voller & Bidwell (1976) was used to assay anti-virus particle and anti-peptide IgG responses. Briefly, microplates were coated overnight at room temperature with purified FMDV or uncoupled synthetic peptide at a concentration of 2 µg/ml. The plates were washed and test serum samples, at a range of twofold dilutions from 1 : 10, were added. After incubation for 1 h at 37 °C, plates were washed and anti-guinea-pig IgG-peroxidase conjugate was added. After a further 1 h at 37 °C the plates were washed and an enzyme substrate (0.04% o-phenylenediamine plus 0.04% hydrogen peroxide in phosphate/citrate buffer) was added. After 5 to 7 min, colour development was stopped with 12.5 µo sulphuric acid and the absorbance at 492 nm was measured in a Titertek Multiskan (Flow Laboratories).

The _A<sub>492</sub> values obtained from twofold dilutions of post-inoculation samples were plotted against the log₁₀ reciprocal antiseraum dilution and the antibody titre were calculated by reference to a negative standard (a 1:10 dilution of pre-inoculation serum). The results reported are the means of two tests, using duplicate wells for each serum dilution in each test.

_Challenge test._ O_1 FMDV suspension in a 0.02 ml dose containing 500 guinea-pig ID<sub>50</sub> was injected intradermally into the left hind footpad and the animals were examined daily for 7 days. Guinea-pigs with no lesions on any footpads or lesions only at the injection site were regarded as protected and those with more extensive lesions as unprotected.

**RESULTS**

**Peptide priming**

Four groups of 16 guinea-pigs were inoculated intramuscularly with 100, 10, 1 or 0 µg of 130-160–KLH peptide in incomplete Freund’s adjuvant (IFA). After 42 days they were further subdivided into 16 groups of four animals and reinoculated with either 10 µg 130-160–KLH peptide, 0.1 µg 146S virus particles, 10 µg 12S virus subunits or 10 µg disrupted virus in IFA. Serum samples, collected at regular intervals of 2 weeks, were analysed for neutralizing, anti-virus and anti-peptide activity.

The neutralizing antibody results (Fig. 1) showed that a 100, 10 or 1 µg dose of peptide primed the immune system for a secondary dose of 10 µg peptide, which was itself a sub-immunizing dose. This was best illustrated by the two doses of 10 µg 130-160–KLH regimen (Fig. 1b). In contrast a secondary inoculation of 146S virus particles, 12S virus subunits or disrupted virus produced little or no detectable increase in the neutralizing activity. The low level of activity seen after inoculation of peptide-primed animals with a 0.1 µg dose of virus particles was clearly due to their inherent immunogenetic activity as illustrated by the control group which had
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Fig. 1. Neutralizing antibody response of guinea-pigs primed with (a) 100 μg, (b) 10 μg, (c) 1 μg or (d) 0 μg of 130-160-KLH peptide and boosted (1) 42 days later with 130-160-KLH peptide (▲), 146S virus particles (●), 12S subunits (■) or disrupted virus (○).

received no primary peptide inoculation (Fig. 1d). It is possible that 100 μg peptide followed by 10 μg 12S virus subunits may have induced some neutralizing activity but this was low and was not seen in the group that received 10 μg peptide as a primary dose (Fig. 1b), despite the fact that this dose clearly primed for a second inoculation of peptide.

The anti-virus and anti-peptide ELISA results for the group of animals that received 10 μg peptide as the primary dose (Fig. 2) confirmed the neutralizing antibody data. Little or no enhanced activity was observed following a secondary inoculation of 146S virus particles, 12S virus subunits or disrupted virus. However, enhanced anti-peptide and anti-virus activity was observed after a secondary inoculation of peptide. It is interesting to note that the anti-peptide activity (as measured by this assay) was approximately 10-fold higher than the anti-virus activity.

Virus priming

Four groups of 16 guinea-pigs were inoculated intramuscularly with 1, 0.1, 0.01 or 0 μg of 146S virus particles in IFA. After 42 days they were further subdivided into 16 groups of four animals and reinoculated with 0.1 μg virus particles, 10 μg 12S virus subunits, 10 μg disrupted virus or 10 μg synthetic peptide (130-160-KLH) in IFA. Serum samples, collected at regular intervals of 2 weeks, were analysed for neutralizing, anti-virus and anti-peptide activity.

The results of neutralizing antibody assays (Fig. 3) showed that neutralizing activity could be boosted at 42 days using either 146S virus particles or 12S virus subunits. This secondary response was most noticeable in animals that received a 0.1 to 0.01 μg primary dose (Fig. 3b, c) but was less marked in those which received the 1 μg dose because it was masked by the higher ongoing response elicited (Fig. 3a). Secondary immunization of the 1 μg and 0.1 μg groups with synthetic peptide also resulted in some increased neutralizing activity. However, disrupted virus had no noticeable effect on the immune response. It should be noted that, as in the peptide
Fig. 2. Anti-peptide (▲) and anti-virus (●) antibody response of guinea-pigs primed with 10 μg 130-160-KLH peptide and boosted (↓) 42 days later with (a) 146S virus particles, (b) 12S subunits, (c) disrupted virus or (d) 130-160-KLH peptide.

Fig. 3. Neutralizing antibody response of guinea-pigs primed with (a) 1 μg, (b) 0.1 μg, (c) 0.01 μg or (d) 0 μg of 146S virus particles and boosted (↓) 42 days later with 130-160-KLH peptide (▲), 146S virus particles (●), 12S subunits (■) or disrupted virus (○).
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Fig. 4. Anti-peptide (▲) and anti-virus (●) antibody response of guinea-pigs primed with 0.1 μg 146S virus particles and boosted (●) 42 days later with (a) 146S virus particles, (b) 12S subunits, (c) disrupted virus or (d) 130-160-KLH peptide.

priming experiment, the 0.1 μg dose of 146S virus particles used for secondary immunization produced a low level of neutralizing antibody in unprimed animals; nevertheless secondary responses in the 0.1 μg and 0.01 μg primed groups could still be detected.

Anti-virus and anti-peptide ELISA results for the group that received a 0.1 μg primary dose of 146S virus particles (Fig. 4) confirm the boosting effect of 146S virus particles and 12S virus subunits although the antibody profile was qualitatively different. The 146S virus particle booster injection resulted in a 10-fold increase in anti-virus ELISA titre over anti-peptide ELISA titre (Fig. 4a) while a 12S virus subunit booster resulted in an equal increase in peptide and virus antibodies (Fig. 4b). Secondary immunization with peptide increased the peptide antibody titres but had little or no effect on the virus antibody titres (Fig. 4d). Disrupted virus had no detectable effect on the levels of peptide or virus antibodies (Fig. 4c).

Comparative serology of virus and peptide antisera

Results obtained in the peptide and virus priming experiments indicated that antibody profiles were qualitatively different according to the inoculation regimen adopted and the choice of immunogen. This led us to consider whether activity in two standardized pools of anti-virus or anti-peptide sera would reflect these differences. We therefore examined two pools that exhibited similar levels of virus neutralizing activity (1.65 to 1.85 log<sub>10</sub> SN<sub>50</sub>) for their anti-virus and anti-peptide titres in an indirect ELISA (Fig. 5). The virus antiserum (Fig. 5a) had 10-fold more anti-virus activity than anti-peptide activity while the peptide antiserum (Fig. 5b) had 10-fold more anti-peptide activity than anti-virus activity.

Protection afforded in vivo by anti-virus and anti-peptide antibodies

Results of neutralization tests on accumulated serum samples collected from a total of 738 guinea-pigs before virus challenge are presented in Fig. 6. They have been divided according to whether the animals had been previously inoculated with inactivated O<sub>1</sub> virus particles
Fig. 5. Neutralizing anti-peptide and anti-virus antibody activity in guinea-pig sera collected from animals immunized with (a) virus particles or (b) synthetic peptide.

Fig. 6. Results of virus challenge tests on guinea-pigs immunized with (a) virus particles or (b) synthetic peptide.

(n = 463; Fig. 6a) or synthetic peptides, based on the major immunogenic site around the 141 to 160 region of VP1 (n = 275; Fig. 6b). There was a clear correlation between the level of neutralizing antibodies in the serum before challenge and protection, as indicated by the increase in protected compared to unprotected animals as the titre increases. However, the profiles obtained with the two different immunogens differed markedly. Following immunization of the animals with virus particles we observed a wide range of neutralizing antibody titres that clearly resulted in varying degrees of protection. For example, guinea-pigs with titres of >2.0 log_{10} SN_{50} may be unprotected while others with titres of <1.0 log_{10} SN_{50} may be
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protected. An estimated 50% protection value for this group would be approximately 1.5 log$_{10}$ SN$_{50}$. In contrast the data obtained from guinea-pigs immunized with synthetic peptides indicated a more distinct cut-off between neutralizing antibody titres giving protection and those which fail to protect. Thus a titre of > 1 log$_{10}$ SN$_{50}$ would be expected to afford over 99% protection.

DISCUSSION

The results of the priming experiments have shown that while the peptide will prime well for a subsequent challenge of peptide it does not appear to prime for virus. This result supports the previously published observations on FMDV priming (Francis et al., 1985a) and suggests that peptide and virus immunogens elicit distinct immune repertoires. It is interesting that disrupted virus also failed to enhance a response in peptide-primed animals. This indicates that the amino acid sequence 130 to 160 within VP1 is not recognized by the peptide-primed immune system when it is present on intact but denatured VP1. This is in agreement with published data on the antigenicity and immunogenicity of this viral protein (Bachrach et al., 1975; Cartwright et al., 1982; Meloen & Barteling, 1986a, b). Low level priming by peptide for the 12S virus subunit suggests that the major epitope may adopt a more linear conformation on the sub-viral particles. However, it should be noted that owing to the reduced immunogenicity of this form of the virus we decided to use a considerably higher dose for the secondary inoculation than that of the intact 146S virus (i.e. 100-fold).

Further priming experiments demonstrated that virus primes for itself and 12S subunits but not for disrupted viral antigen. This again illustrates the difference in immunological recognition of the virus coat protein in various forms (i.e. as the intact virion, as 12S subunits or as isolated denatured proteins). The phenomenon of 146S virus-primed responses being boosted by 12S subunits has been described previously (Cartwright et al., 1980). However, a secondary immunization with synthetic peptide also enhances a neutralizing and anti-peptide response in virus-primed animals, a result which is in agreement with previously published data (Francis et al., 1985a).

These results appear to present a paradox since peptide does not prime for virus but virus primes for peptide. Nevertheless, it is not necessary to introduce complex arguments on priming at the B cell and T cell level because the result may simply be due to the lower amount of linear epitope that is present on the virus particle. Thus, let us assume that immunization with virus stimulates a wide range of B cell clones to proliferate and produce a broad spectrum of antibodies. It is likely that the majority (> 90 to 99%) of these antibodies will be to sites outside the 141 to 160 region and/or to those which incorporate this region as part of complex non-linear epitopes. A small number of B cell clones may be producing antibodies that recognize a more linear form of the 141 to 160 region similar to the antigenic structure present on the synthetic peptide. The memory B cells produced to these clones may therefore be selectively induced to proliferate following inoculation of 10 μg of a synthetic peptide covering much of the linear determinants. In contrast, immunization with peptide would stimulate B cell clones that produce antibodies to linear determinants only and secondary immunization with 0.1 μg of virus that contains only 0.003 μg of the 130 to 160 peptide determinant may have little stimulatory effect on these clones. Thus, using the FMDV system we are unable to confirm the dramatic priming effect of poliovirus peptides for poliovirus (Emini et al., 1983). However, it should be noted that in their experiments Emini et al. (1983) used high priming doses (1 mg) of poliovirus peptides which elicited a strong anti-peptide response but no neutralizing antibody. Had we chosen to use such doses of the FMDV peptide the primary neutralizing antibody response would have prevented us from observing any effect of a second inoculation. It may be that a high dose of a poor or non-immunogenic peptide from FMDV would produce the same effect as that seen with poliovirus. Alternatively since studies with monoclonal antibodies suggest that the A serotype of FMDV presents the 141 to 160 determinant in a more linear fashion (C. Bolwell, personal communication) it is possible that synthetic peptides may prime for this serotype.

Studies on virus and peptide antisera demonstrate the clear differences in the nature of the antibody response to the two antigens. These confirm our belief that in using a synthetic peptide
for immunization we are not necessarily attempting to mimic an anti-virus response but to enhance the response to a very restricted epitope and thus produce an immune status in the animal that could not be duplicated by traditional vaccination. Indeed, we have already demonstrated that this response is more broadly cross-reactive than an anti-virus response (Ouldridge et al., 1986) and in this study we show that these anti-peptide antibodies are highly protective \textit{in vivo}. The occurrence of high neutralizing antibody titres \textit{in vivo} that are not protective following virus immunization suggests that a range of antibodies, some to protective and others to non-protective epitopes, are competing to produce the \textit{in vitro} result. In contrast immunization with synthetic peptides stimulates a much more clonally restricted response which results in a population of antibodies directed against an important protective epitope on the virion and therefore is a clearer correlation between \textit{in vitro} neutralization data and \textit{in vivo} protection.

In conclusion this paper has demonstrated that there are major qualitative and quantitative differences in the immune response to the FMDV particle and the synthetic peptide. It is our belief that immunization with peptide results in the expansion of B cell clones secreting antibodies which are either unique or form a very small part of the immune repertoire elicited by virus. Furthermore, this anti-peptide antibody population appears to possess many desirable characteristics that could not be achieved by using conventional vaccines.

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**REFERENCES**


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