Intranasal Immunization of Guinea Pigs with an Immunodominant Foot-and-Mouth Disease Virus Peptide Conjugate Induces Mucosal and Humoral Antibodies and Protection against Challenge†

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Vaccination against foot-and-mouth disease (FMD) is currently achieved by parenteral administration of products prepared from tissue culture-grown virus inactivated by an aziridine (15). These vaccines were shown to be effective by their successful application in western Europe and some countries in South America, notably Argentina and Uruguay, following their introduction in the 1950s (12, 15). However, parenteral vaccination rarely elicits sufficient secretory immunity to prevent initial infection, so vaccinated animals can become persistently infected virus carriers (17, 38). This has led to the implementation of trade restrictions against countries that vaccinate against the disease, particularly because the currently validated serologic test cannot distinguish between vaccinated and infected animals.

Synthetic peptides corresponding to the immunodominant region of FMD virus (FMDV) have been shown to elicit protective levels of neutralizing antibodies in serum when inoculated parenterally into mice, guinea pigs, and pigs (10, 29, 40); however, the induction of mucosal immunity to FMD has not been well studied. The guinea pig is a useful small animal model for studies of FMDV due to its capacity to support viral infection and exhibit unambiguous clinical signs and its low cost (compared to cattle and swine) and modest housing requirements. Since mucosally administered peptides have been shown to elicit both systemic and mucosal immunity to several antigens (4, 5, 41), we investigated whether this approach was efficacious for an FMDV-derived peptide. Guinea pigs were immunized intranasally with a peptide corresponding to the major immunogenic region of serotype A virus coupled to the keyhole limpet hemocyanin (KLH) carrier protein, with and without the mucosal adjuvant cholera toxin (CT). Systemic and mucosal antibody titers were measured in conjunction with an assessment of serum virus neutralization titers and protection upon homotypic virus challenge.

MATERIALS AND METHODS

Synthetic peptides. A chemically synthesized peptide corresponding to the region including amino acids 141 to 159 of VP1 of the serotype A, subtype 12, virus (VP1141-159), with the sequence CH3CO-C-G-G-V-V-L-P-R-P-V-C-F-E-K-G-M-N-Y-T-V-R; Sigma), was used as a negative control for enzyme-linked immunosorbent assays (ELISAs). A second, unrelated peptide, laminin-B1 chain (amino acids 641 to 660, with sequence R-Y-V-V-L-P-R-P-V-C-F-E-K-G-M-N-Y-T-V-R; Sigma), was used as a negative control for assays of secretory immunoglobulin A (SIgA).

Virus and neutralization assays. The American variant of FMDV (serotype A, subtype 12) was grown in monolayers of BHK 21 cells (37). Neutralization assays were performed as previously described (9). Briefly, mixtures of equal volumes of 10-fold virus dilutions and sera (either undiluted or diluted 1/10 or 1/50) were incubated at 25°C for 15 min. The mixtures were then added to monolayers of BHK cells grown in 96-well microtiter plates. After a 3-day incubation at 37°C in a humidified atmosphere containing 5% CO2, the cells were stained with crystal violet/Histochoice (Amresco, Inc., Solon, Ohio). The neutralization titers of the sera were determined by the difference between the titer of the virus alone and the titers of the mixtures.

Vaccine preparation. The vaccines were prepared by conjugation of the synthetic peptide VP1141-159 to maleimide-activated KLH. For coupling, 12 mg of Invert maleimide-activated mKLH (Pierce, Rockford, Ill.) was reconstituted with 1.2 ml of distilled water and conjugated to 10 mg of the peptide according to the manufacturer’s instructions. The conjugate was then exhaustively dialyzed overnight at 4°C against phosphate-buffered saline. The total protein in each sample was quantified by using the Bio-Rad (Hercules, Calif.) protein assay kit according to the manufacturer’s instructions.
For parenteral vaccination, MPL + TDM + CWS adjuvant (catalog no. M-6661; Sigma Chemical Co.) was reconstituted with 2 ml of the diluted peptide solution (in sterile saline) and maintained at 37°C until administration to the animals. All animal procedures were approved by the University of Connecticut and Plum Island Animal Disease Center Institutional Animal Care and Use Committees in advance of the work and complied with all pertinent U.S. Department of Agriculture and National Institutes of Health guidelines.

Immunization. Thirty-two female Duncan-Hartley guinea pigs, weighing approximately 250 g each, were divided into four groups of eight animals each. Animals were immunized as follows. Group 1 received 100 μg of FMDV peptide conjugate plus 10 μg of CT in a total volume of 50 μl (25 μl per nostril) on week 0, with identical booster immunizations given at weeks 1, 2, and 4. Group 2 received 100 μg of FMDV peptide conjugate without CT at the same time points. Group 3 received 100 μg of the FMDV conjugate administered intramuscularly in Sigma adjuvant (MPL + TDM + CWS; Ribi) at week 0, followed by an identical booster at week 4. Group 4 received unconjugated KLH (100 μg) plus CT (10 μg) intranasally at the same times as described for groups 1 and 2. All intranasally immunized animals received 25 μl/nostril, administered nontraumatically by using a 200-μl plastic pipette tip. Parenterally immunized animals received approximately 0.3 ml of the vaccine in two subcutaneous locations (one on each side) plus a third injection into the peritoneal cavity, as described in the manufacturer’s instructions. Sera and nasal secretions were collected on a weekly basis from all the animals, which were shipped to Plum Island Animal Disease Center (U.S. Department of Agriculture, Agricultural Research Service) for challenge. These samples were assayed by epitope- and iso-type-specific ELISA and for virus-neutralizing activity.

Peptide-specific IgG responses. Evaluation of peptide-specific antibody production was performed by indirect ELISA using 96-well polystyrene Immunolon-4 HBX microliter plates (Dynex Technologies, Chantilly, Va., and Fisher Scientific, Agawam, Mass.). Wells were coated with 50 μl of a 10-μg/ml solution of unconjugated VP1141–159 peptide in a 0.05 M Na2CO3 buffer, pH 9.6, overnight at room temperature and stored at 4°C until needed. Prior to the assay, plates were washed five times with 125 μl of phosphate-buffered saline containing 0.05% Tween 20 (PTA) and 0.1% bovine serum albumin (BSA)/well, using an EL-403 plate washer (with shaking; Bio-Tek, Winoski, Vt.). The plates were then incubated with 50 μl of blocking solution (PTA–5% BSA)/well for 1 h at 37°C and then washed as described above. Serum samples from weeks 0 to 4 were diluted 1:100 (prechallenge) or 1:500 after virus challenge (week 8 samples). Primary antibody samples were diluted in PTA–0.1% BSA, and 50 μl of each sample was applied in duplicate to the microtiter plate. After a 3-h incubation at room temperature. The OD of each sample was measured at 405 nm with an EL-311 Bio-Tek plate reader after a 100-min incubation at room temperature.

Challenge and protection. Five weeks after the initial inoculation, guinea pigs were challenged by footpad injection of 107 50% tissue culture infective doses (TCID50) of homotypic FMDV. All animals were monitored for characteristic FMD lesions on the footpads for 8 consecutive days after challenge. The severity of lesions was scored as follows. Animals showing primary lesions at the site of virus injection, without spreading to other feet, were categorized as partially protected. Secondary lesions were considered to be a sign of systemic viremia, and animals displaying such lesions were considered unprotected. Guinea pigs were considered completely protected if no lesions were observed on any feet throughout the period of observation.

Statistical analysis. Data were analyzed by using one-way analysis of variance (ANOVA) and the Tukey-Kramer multiple comparison test (as indicated in the figure legends) with the GraphPad Inc. (San Diego, Calif.) InStat software (version 3.0). In cases where unequal variances were observed (using the Bartlett’s statistic), all values were log10 transformed prior to analysis. Zero values were assigned a value of 0.001 to allow this transformation to have meaning. For instance, when values fell outside the limits of detection, nonparametric Kruskal-Wallis and Dunn multiple comparison tests were used. Values of P of <0.05 were considered to be statistically significant.

RESULTS

Antipeptide antibody response. Guinea pigs immunized with peptide conjugated to KLH produced strong serum antibody responses, regardless of the route of immunization. Weekly intranasal immunizations with peptide conjugates induced the highest level of response over the first 4 weeks of study, with the majority of animals (73%) seroconverting within 2 weeks and 93% (15 of 16) by week 4. Supplemeting the intranasally administered peptide conjugate with CT did not result in any augmentation of the serum IgG anti-FMDV response (P > 0.05) (Fig. 1). As anticipated, the parenterally immunized animals mounted a vigorous serum IgG anti-FMDV response, especially following a booster immunization at week 4 followed by virus challenge.

S-IgA responses in nasal secretions. To determine whether intranasally vaccinated guinea pigs produced a peptide-specific S-IgA response within the respiratory mucosa, ELISAs were performed on nasal wash samples collected on weeks 0 through 4. Within 2 weeks of immunization, a considerable S-IgA anti-FMDV response was detected in the nasal secretions of 75% (6 of 8) of the animals immunized intranasally with the peptide-KLH conjugate alone (Fig. 2). Surprisingly, only 14% (1 of 7) of similarly vaccinated guinea pigs responded when CT was mixed with the conjugate (P < 0.05). This latter group mounted a significant response in comparison to sham-immunized animals at weeks 3 and 4 (P < 0.05), but the response
was only about half of that achieved by animals not receiving the CT. Correcting the data by dividing the responses by the total IgA present in each sample did not appreciably affect the conclusions but increased the error terms (data not shown). As anticipated, none of the animals in either the sham- or parenterally immunized groups mounted a S-IgA anti-FMDV response at any time point.

**Serum virus neutralization assay.** In vitro virus neutralization activity in serum was determined with samples taken at weeks 0 (preimmunization), 4 (postimmunization), and 8.
It is well established that mucosal administration of vaccines is much more effective than parenteral immunization for inducing secretory immunity, especially when antigen is applied directly to the mucosal surface at which immunity is desired. Coadministration of potent mucosal enterotoxin adjuvants such as CT is often required to induce a strong mucosal response and is sometimes needed to prevent the induction of tolerance (19). In addition, mucosal vaccination frequently induces immunity at distant mucosal sites and often results in a concomitant serum IgG response, especially when coadministered with CT (42). Moreover, coadministration of CT with peptides can help to circumvent the inherently low immunogenicity of peptides when administered by the mucosal route due to the peptides’ high degradability (9, 20, 30) and inefficient uptake and processing in mucosa-associated lymphoid tissues (23).

Among the benefits of mucosal immunization are the ease of administration, the low risk of adventitious infection or anaphylactic reaction, and the low stress associated with the vaccination process. Mucosal vaccines against several viruses that infect via mucosal surfaces, such as poliovirus (32), rotavirus (2), and influenza virus (7), have been developed; however, little information is available on mucosal immunity to FMDV. Peptide-based vaccines represent a promising alternative to the currently used chemically inactivated viral vaccine. Synthetic vaccines have been shown to induce a rapid, safe, and protective immunity to a wide variety of diseases, including influenza (31), group A streptococcus infection (6, 33), measles (34), canine parvovirus infection (13), and FMD (3, 8–10, 14, 16, 29, 30, 35, 37, 40). Moreover, the ease with which peptide vaccines can be redesigned is critically important for combating the spread of rapidly mutating RNA viruses such as FMDV. The availability of an intranasally administered peptide vaccine could facilitate the rapid vaccination of entire herds by minimally trained farm personnel, with little risk to the animals or their handlers.

The immunodominant epitope of FMDV is expressed within the animals or their handlers. It is well established that mucosal administration of vaccines is much more effective than parenteral immunization for inducing secretory immunity, especially when antigen is applied directly to the mucosal surface at which immunity is desired. Coadministration of potent mucosal enterotoxin adjuvants such as CT is often required to induce a strong mucosal response and is sometimes needed to prevent the induction of tolerance (19). In addition, mucosal vaccination frequently induces immunity at distant mucosal sites and often results in a concomitant serum IgG response, especially when coadministered with CT (42). Moreover, coadministration of CT with peptides can help to circumvent the inherently low immunogenicity of peptides when administered by the mucosal route due to the peptides’ high degradability (9, 20, 30) and inefficient uptake and processing in mucosa-associated lymphoid tissues (23).

DISCUSSION

FMD is a highly contagious disease that is capable of spreading rapidly among animals within a herd, often resulting in pandemic outbreaks that cover thousands of square miles. Aerosol transmission is believed to be an important route of infection, with the initial site of viral replication often occurring in pharyngeal epithelial cells of the upper respiratory tract. For this reason, a vaccine that induces mucosal immunity in the upper respiratory tract may prevent the initial infection and thereby decrease the likelihood of establishing carrier animals and the subsequent emergence of viral escape variants.

<table>
<thead>
<tr>
<th>Immunogen/route</th>
<th>Titer&lt;sup&gt;a&lt;/sup&gt;</th>
<th>% with the following levels of protection upon challenge</th>
<th>Total</th>
<th>Partial</th>
<th>None</th>
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<tbody>
<tr>
<td>Peptide-KLH plus CT/i.n.</td>
<td>1.78 ± 0.29</td>
<td>14&lt;sup&gt;e&lt;/sup&gt;</td>
<td>86</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Peptide-KLH (no CT)/i.n.</td>
<td>1.81 ± 0.28</td>
<td>86&lt;sup&gt;e&lt;/sup&gt;</td>
<td>14</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Peptide-KLH/parenteral</td>
<td>1.50 ± 0.33&lt;sup&gt;e&lt;/sup&gt;</td>
<td>100&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Sham (KLH) plus CT/i.n.</td>
<td>0.00 ± 0.00&lt;sup&gt;f&lt;/sup&gt;</td>
<td>13</td>
<td>26</td>
<td>62</td>
<td></td>
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</table>

<sup>a</sup> i.n., intranasal.

<sup>b</sup> Titer is expressed as log<sub>10</sub> TCID<sub>50</sub>s/ml.

<sup>c</sup> Group did not differ from sham-immunized group (P > 0.05).

<sup>d</sup> P < 0.001 by one-way ANOVA.

<sup>e</sup> P < 0.05 by one-way ANOVA.

<sup>f</sup> Groups differed from sham-immunized group (P < 0.01) according to Kruskal-Wallis and Dunn multiple comparison tests (nonparametric).
apparent antigenic variants were isolated (39). A mucosal vaccine may reduce the likelihood of this occurrence by blocking the initial infection, reducing viral load, limiting viral replication, or preventing the transmission of virus in mucous secretions.

In the present study, guinea pigs were immunized via the intranasal route to determine whether mucosal immunity could be induced in the presence or absence of CT. The intranasal route of immunization proved to be quite effective for inducing both mucosal and systemic antibody responses, especially in animals immunized with the KLH-peptide conjugate alone. Curiously, CT did not appear to act as an adjuvant with respect to the FMDV peptide-KLH conjugate, and if anything, it appeared to blunt the response. This lack of adjuvant property was manifested in lower serum and mucosal antibody responses, lower virus neutralization titers, and only a moderate level of protection upon virus challenge. These observations are somewhat perplexing in light of the work of Hartman et al. (21), in which native and mutant forms of CT and the heat-labile enterotoxin of Escherichia coli were found to be effective adjuvants in guinea pigs when administered intranasally with either attenuated or heat-killed Shigella vaccines. However, most of the data presented in that report were for heat-labile enterotoxin and its mutants, and the one group given an intranasal immunization of heat-killed Shigella flexneri plus CT actually demonstrated less protection upon challenge than those given killed S. flexneri alone.

Despite a large body of work spanning over 100 years, the mechanisms of immune protection against FMDV are not yet clear. The protection observed in the present study was probably mediated by the humoral arm of the immune system, in concert with CD4+ T helper 2 cells, a mechanism well documented for antiviral protection (22, 36). Neutralizing antibodies in serum also seem to play an important role in protecting some domestic species against FMD (24–26, 28), presumably due to phagocytosis by macrophages following virus opsonization (11, 27).

In the present study, all animals vaccinated with the FMDV peptide conjugate raised comparable levels of neutralizing antibodies; however, in keeping with the findings of several previous reports, a high degree of variability was observed on an animal-to-animal basis both within and between groups. It should be noted that animals completely devoid of neutralizing antibody activity were rarely protected. Curiously, several animals in the group given FMDV peptide-KLH conjugate plus CT had significant serum and mucosal anti-peptide immune responses in addition to substantial virus neutralization titers yet exhibited only limited protection upon virus challenge. It remains unclear why in vitro assessments of neutralizing antibody titers were imperfectly correlated with protection upon virus challenge; however, this observation has been reported previously (18).

Taken together, the results presented in this report indicate that intranasal immunization with the FMDV peptide-KLH conjugate was an extremely effective inducer of serum and mucosal anti-FMDV antibody responses, neutralizing antibody activity, and protection upon challenge. Three weekly immunizations via the intranasal route induced a more rapid and robust response than a single immunization via the parenteral route, with serum and mucosal antibodies evident within 2 weeks of vaccination. Complete protection was observed in nearly all of the intranasally immunized animals (7 of 8); however, coadministration of the FMDV peptide-KLH conjugate with the mucosal adjuvant CT was not as effective.

Unfortunately, no intranasal challenge model has been developed for the guinea pig, and attempts to induce disease via intranasal inoculation with FMDV were unsuccessful (data not shown). Thus, the importance of ongoing studies to examine the efficacy of the intranasal route of vaccination in target species such as swine cannot be overestimated.

REFERENCES


