

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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Received 4 October 2002/Accepted 18 March 2003

Guinea pigs immunized intranasally with a keyhole limpet hemocyanin-linked peptide, corresponding to the prominent G-H loop of the VP1 protein of foot-and-mouth disease virus, raised substantial levels of antipeptide and virus-neutralizing antibodies in sera and of peptide-specific secretory immunoglobulin A in nasal secretions. In groups of animals immunized intranasally without adjuvant, 86 percent were fully protected upon challenge with homotypic virus. Surprisingly, animals given the peptide conjugates plus the mucosal adjuvant cholera toxin were afforded only partial protection in that primary lesions were observed in most animals, although spread to other feet was prevented. These results indicate that intranasal inoculation with the peptide offers a potential route of vaccination against foot-and-mouth disease and may be useful for eliciting protection in the upper respiratory tracts of susceptible animals.

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-S-G-V-R-G-D-F-G-S-L-A-P-R-V-A-R-Q-L-OH, was synthesized by Genemed Synthesis Inc. (South San Francisco, Calif.). An additional cysteine residue (represented by the double-underlined letter) was added at the N terminus of the peptide to facilitate its conjugation to KLH. A structurally unrelated 14-mer peptide (amino acid sequence C-G-Y-G-P-P-K-K-K-A-K-V-G-G [catalog no. C-4547; Sigma, St. Louis, Mo.]) 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 (S-IgA).

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 defined as the differences 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 Inject 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.

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† Agricultural Experiment Station publication 2126, Storrs Agricultural Experiment Station, Storrs, Conn. Center of Excellence for Vaccine Research publication 082.

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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 until the animals 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 isotype-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 microtiter 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, Winooski, 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, another wash was performed under the same conditions described above, followed by the addition of goat anti-guinea pig IgG conjugated to alkaline phosphatase (50 µl; Chemicon Inc., Temecula, Calif.) at a dilution of 1:2,000 (adsorbed with 1 µg of guinea pig IgM/ml and 0.1 µg of guinea pig IgA/ml; Inter-Cell Technologies, Inc., Hopewell, N.J.) and an overnight incubation at 4°C. A final wash was performed, followed by the addition of the *p*-nitrophenyl phosphate substrate (1 mg/ml in 0.05 M Na₂CO₃ and 1 mM MgCl₂ buffer, pH 9.8). Product formation was determined by measuring the optical density (OD) of each well at 405 nm with an EL-311 Bio-Tek plate reader after a 100-min incubation at room temperature. The net anti-FMDV peptide response for each sample was calculated by subtracting the mean OD of the control peptide from the mean OD of each specific peptide. Variation between plates was corrected by normalizing all values to a positive control included on each plate.

Peptide-specific IgA response. Indirect ELISAs were performed on nasal wash samples collected at weeks 0 through 4 from each of the animals in the study. Polystyrene 96-well Immunolon-4 HBX microtiter plates were coated with 10 µg of the peptide VP1₁₄₁₋₁₅₉ or of laminin-B1/ml in PTA-0.1% BSA. Wells were blocked with 50 µl of PTA-5% BSA/well for 1 h at 37°C and then washed (as described above) immediately prior to sample application. Each sample was diluted 1:20 with PTA-0.1% BSA to a volume of 50 µl and applied in duplicate to microtiter plates, after which the plates were incubated at room temperature for 4 h. The plates were then washed as described above and incubated for 4 h at room temperature with a 1:400 dilution of rabbit anti-guinea pig IgA (Bethyl Labs, Montgomery, Tex.). After the plates were washed, a 1:4,000 dilution of secondary antibody (alkaline phosphatase-conjugated goat anti-rabbit IgG [ICN, Aurora, Ohio]) was applied to the wells and the plates were incubated overnight at 4°C. After a final wash, the *p*-nitrophenyl phosphate substrate was added (as above) and incubated for 100 min at room temperature. The OD of each sample was then measured at 405 nm as described above. Specific IgA response was defined as the difference between the average of peptide-specific response and nonspecific laminin-B1 response.

Quantitation of total IgA. Nasal wash samples were assayed for total IgA concentration in an effort to control for unavoidable variations in wash efficien-

cies and sample recovery. Total IgA concentration was determined by capture ELISA. Plates were coated with 50 µl of capture antibodies (rabbit anti-guinea pig IgA; Bethyl Laboratories)/well at a concentration of 10 µg/ml and incubated overnight at room temperature, followed by storage at 4°C until use. Prior to the application of the samples, plates were warmed to room temperature and blocked with PTA-5% BSA for 3 h.

An IgA standard curve (0, 250, 500, 1,000, 2,000, and 4,000 ng/ml) was included on each plate, and total IgA values were calculated by interpolation from the standard curve. All samples were diluted 1:200 and incubated at room temperature for 4 h.

After the plates were washed (as described above), sheep anti-guinea pig IgA, diluted at 1:200, was used as a secondary antibody to detect IgA specifically bound by the capture antibody and was incubated for 3 h at room temperature, followed by washing (as described above). Detection of sheep anti-guinea pig IgA was performed by incubation of the plates with an alkaline phosphatase-labeled donkey anti-sheep IgG (Sigma) diluted at 1:200 for 4 h at room temperature. A final wash was performed, followed by the addition of *p*-nitrophenyl phosphate substrate (as above). Product formation was determined by measuring the OD of each well at 405 nm with an EL-311 Bio-Tek plate reader after a 50-min incubation at room temperature.

Challenge and protection. Five weeks after the initial inoculation, guinea pigs were challenged by footpad injection of 10⁵ 50% tissue culture infective doses (TCID₅₀s) 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 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 instances 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. Supplementing 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

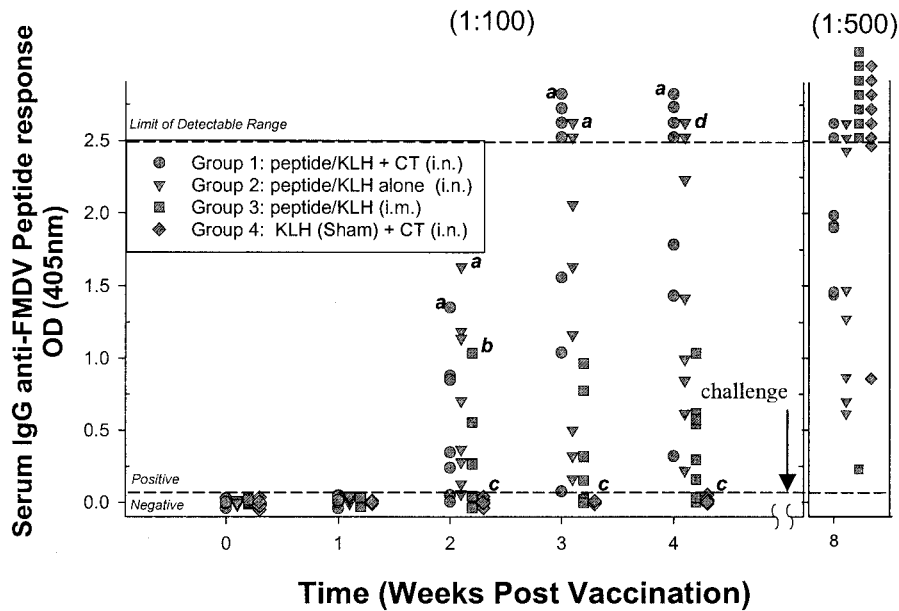


FIG. 1. Serum IgG anti-FMDV response. Pre- and postvaccination sera were assayed for anti-peptide immune response by using ELISA as described in Materials and Methods. Sera collected on weeks 0 through 4 were assayed at a 1:100 dilution, while convalescent-phase sera (week 8) were assayed at a 1:500 dilution. OD values exceeding 2.5 were considered to be outside the limits of detection. Statistically significant differences between immunized and sham-immunized groups (c) at each time point are denoted by lowercase letters (a, $P < 0.001$; b, $P < 0.05$; d, $P < 0.01$). Each group consisted of seven or eight animals. i.n., intranasal; i.m., intramuscular in RIBI.

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 paren-

terally 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

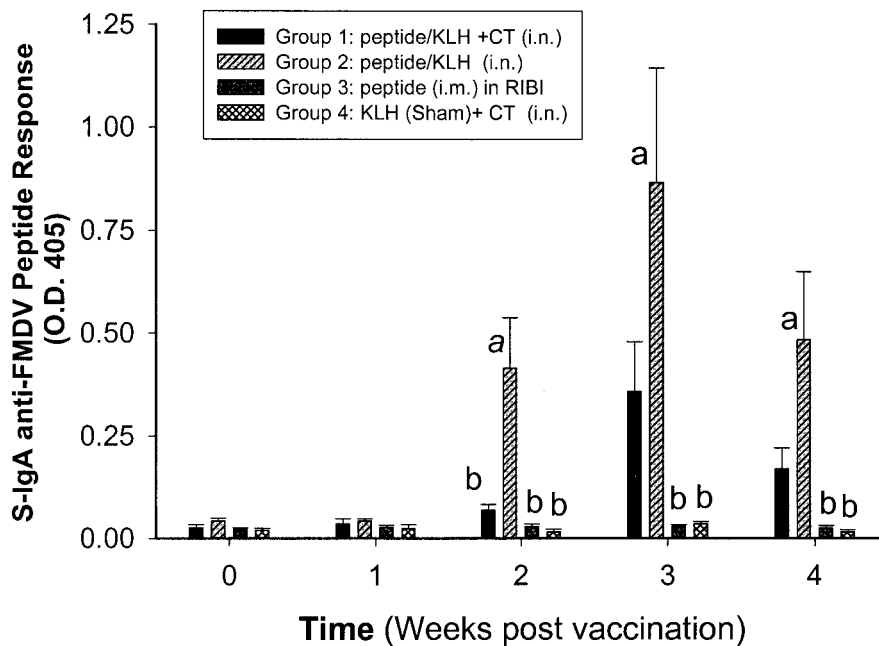


FIG. 2. Nasal S-IgA anti-FMDV response. Pre- and postvaccination nasal wash samples were assayed for anti-peptide immune response by using ELISA as described in Materials and Methods. All nasal wash samples were diluted 1:50 prior to their application to microtiter plates. Statistically significant differences between groups at each time point are denoted by lowercase letters ($P < 0.01$). Each group consisted of seven or eight animals. i.n., intranasal; i.m., intramuscular.

TABLE 1. Serum virus neutralization titers and protection upon virus challenge

Immunogen/route ^a	Titer ^b		% with the following levels of protection upon challenge		
	Week 4	Week 8	Total	Partial	None
Peptide-KLH plus CT/i.n.	1.78 ± 0.29	2.57 ± 0.25	14 ^e	86	0
Peptide-KLH (no CT)/i.n.	1.81 ± 0.28 ^c	3.06 ± 0.15 ^c	86 ^f	14	0
Peptide-KLH/parenteral	1.50 ± 0.33 ^d	3.38 ± 0.28 ^d	100 ^f	0	0
Sham (KLH) plus CT/i.n.	0.00 ± 0.00 ^d	4.44 ± 0.35 ^d	13	26	62

^a i.n., intranasal.

^b Titers are expressed as log₁₀ TCID₅₀s per milliliter.

^c *P* < 0.05 by one-way ANOVA.

^d *P* < 0.001 by one-way ANOVA.

^e Group did not differ from sham-immunized group (*P* > 0.05).

^f Groups differed from sham-immunized group (*P* < 0.01) according to Kruskal-Wallis and Dunn multiple comparison tests (nonparametric).

(postchallenge). Titers are expressed as log₁₀ TCID₅₀s/ml. As shown in Table 1, virus neutralization activity was induced in all three groups immunized with FMDV peptide-KLH conjugates. In each case, the neutralization titers were significantly higher following virus challenge, with the exception of those of the intranasally immunized group given CT as an adjuvant. Although the prechallenge neutralization titers appeared to correlate with protection, this correlation was imperfect both on an animal-by-animal basis (data not shown) and with respect to group 1 (peptide-KLH plus CT). The neutralization titers for the sera collected from sham-immunized animals following challenge were considerably higher than those of all three immunized groups, providing further evidence that these groups had significantly limited virus replication.

Protection against virus challenge. Guinea pigs were challenged with 10⁵ TCID₅₀s of homotypic virus injected into a hind footpad. In groups of animals immunized intranasally without adjuvant, 86 percent were fully protected upon challenge with homotypic virus, compared to 100% protection observed with the parenterally immunized animals (Table 1). Curiously, most of the animals receiving the FMDV peptide-KLH conjugates plus CT via the intranasal route developed large lesions at the site of injection but none displayed the secondary lesions that are characteristic of systemic viremia. Approximately two-thirds of the sham-immunized animals exhibited severe primary lesions at the site of injection and secondary lesions on other footpads. The remaining animals in this group were either partially protected (2 of 8) or had no lesions at all (1 of 8). The protection observed with the intranasally or parenterally FMDV peptide-KLH-immunized groups showed a highly statistically significant difference from that of the sham-immunized group (*P* < 0.01).

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.

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 a highly flexible loop spanning amino acids 134 to 158 of the viral structural protein VP1 (1). Peptide vaccines corresponding to this site have been shown to induce significant levels of anti-FMDV neutralizing antibodies and have conferred protection in guinea pigs, swine, and cattle upon challenge (3, 8–10, 14, 16, 29, 30, 35, 37, 40). One study found that peptide vaccines conferred only partial protection to cattle, and several

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 antipeptide 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.

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