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Plasmid DNA Encoding Replicating Foot-and-Mouth Disease Virus Genomes Induces Antiviral Immune Responses in Swine

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DNA vaccine candidates for foot-and-mouth disease (FMD) were engineered to produce FMD virus (FMDV) particles that were noninfectious in cell culture or animals. The prototype plasmid, pWRM, contains a cytomegalovirus immediate-early promoter-driven genome-length type A12 cDNA followed by the bovine growth hormone polyadenylation site. BHK cells transfected with this plasmid produced virus, but the specific infectivity of pWRM was much lower than that achieved with in vitro-generated RNA genomes. To improve the infectivity of the plasmid, a cDNA encoding the hepatitis delta virus ribozyme was added to the 3′ end of the FMDV cDNA. The resulting plasmid, pWRMH, exhibited slightly increased infectivity in cell culture and produced virus when inoculated into suckling mice. A third plasmid, pWRMHX, was created by removal of the sequences encoding the cell binding site found in capsid protein VP1 of pWRMH. Although cells transfected with pWRMHX produced viral capsids, this plasmid was not lethal in suckling mice, indicating that particles lacking the cell binding site were not able to initiate secondary infectious cycles. Swine inoculated with pWRMHX did not show any signs of disease and produced neutralizing antibodies to FMDV, and 20% of the vaccinated animals were protected from challenge. A derivative of pWRMHX, pWRMHX-pol$^-$, harboring a mutation designed to inactivate the viral polymerase was much less immunogenic, indicating that immunogenicity of pWRMHX resulted, in part, from amplification of the viral genome in the animal.

Foot-and-mouth disease (FMD), an economically important viral disease of livestock, is a problem in many developing countries and poses a continuous threat to FMD-free nations of North America and Europe. The impact of an outbreak in an FMD-free country could be severe, since it could result in international trade embargoes. Available responses to disease incursion include slaughter, disinfection, and vaccination. Vaccination around an outbreak is an attractive control strategy, but currently available FMD vaccines are not produced or utilized in the United States due to safety concerns based, in part, on findings that have linked outbreaks to release of virus from vaccine production plants or the presence of residual live virus in chemically inactivated vaccines (2).

FMD virus (FMDV) infection results in a severe disease that develops rapidly, with symptoms that are often apparent within 24 h following exposure to the virus. Thus, effective vaccines need to stimulate a strong immune response prior to infection since postexposure boosting of vaccine-induced responses cannot compete with the rapid onset of disease. Currently used FMD vaccines are whole virus inactivated with an imine and emulsified with aluminum hydroxide-saponin or an oil adjuvant. These vaccines elicit antibodies that neutralize the virus in vitro and efficiently protect livestock from the disease. Inactivated whole virus vaccines can be effective as part of disease control programs, and despite the occurrence of outbreaks due to the problems of safety mentioned above, they have been used to eradicate FMD from Western Europe, Uruguay, and Argentina. Attempts to produce safer vaccines for FMD have included synthetic peptides that contain highly immunogenic sequences from VP1 (5). Although these peptides induce antibodies that efficiently neutralize the virus in vitro, protection of livestock from virus challenge has been disappointing and has not correlated with prechallenge neutralization titers detected in the sera from these animals (7, 22–24, 35). Although it is unclear where this disparity arises, the importance of antibodies to epitopes present on other portions of the capsid in conferring protection to livestock (20, 30) and the intrinsic variability of the virus (8) could explain why peptide vaccines do not protect as well as whole capsid-based vaccines (35). To produce a new generation of safer FMD vaccines, we have used genetic engineering to develop attenuated FMD virions (21, 26). In one case, attenuation has been achieved by removing the coding region for a viral proteinase, L, that is responsible for inhibition of translation of host-cell mRNAs during infection (4, 18, 26). In another case, the virus has been attenuated by deletion of the sequences encoding three amino acid residues, RGD, that comprise the cell binding site (21). Viruses attenuated by the latter method are unable to spread between normal cells but can be propagated in cells containing a novel, genetically engineered receptor (31).

Immunization with naked DNA has been shown to be effective in inducing protective immunity against several diseases. These DNA vaccines rely on in situ production of antigens from inoculated nucleic acid molecules (9). First described by Wolff et al. in 1990 (40), direct DNA inoculation of viral genes has elicited immune responses against several viruses. Early work demonstrated that naked DNA inoculation could provide immune responses to influenza virus (33, 36), rabies virus (41), human immunodeficiency virus (38), and bovine herpesvirus (6). Over the last few years, DNA vaccines have been demonstrated to induce both humoral and cellular immunity against many different viral disease agents (17, 37). Delivery of DNA by a bioballistic approach, the propelling of DNA-coated gold or tungsten particles into the epithelial layer of the skin, has also been tested and, in some cases, has produced an immune

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TABLE 1. Infectivity of FMDV-encoding nucleic acids in BHK cell monolayers

Nucleic acid	PFU/μg[a]
pRMC35 RNA	>100,000
pRMC35 DNA	1
pWRM DNA	43
pWRMH DNA	66

[a] Determined by using Lipofectin (see Materials and Methods).

response superior to that obtained with injection of naked DNA molecules (11).

DNA vaccination has many of the benefits of live attenuated vaccines or live-vectored vaccines plus some additional advantages, including (i) ease of construction of recombinant DNA molecules expressing foreign genes, (ii) ability to elicit cell-mediated immune responses, (iii) elimination of risks associated with use of live agents, (iv) stability, (v) no problems associated with preexisting immunity to the agent or vector, and (vi) cost (9, 17, 37). Despite these promising aspects, the amounts of antigen produced in inoculated animals can be small, the levels of humoral immune responses are lower than those elicited by traditional antigen-containing vaccines (17, 33, 41) and protection against disease may be dependent upon boosting of the immune response following challenge.

Here we show that a DNA vaccine based on a genome-length FMDV nucleic acid that undergoes genomic amplification in inoculated animals can immunize swine against FMD.

MATERIALS AND METHODS

Cells, viruses, and plasmids. BHK (clone 21) cells were propagated as described by Rieder et al. (32); the CHO 11.1 cell line, expressing an immunoglobulin-based receptor for FMDV type A12 (scAb-ICAM1), was propagated as described by Rieder et al. (31). The eukaryotic expression plasmid pcDNA3 was obtained from Invitrogen (San Diego, Calif.). Wild-type FMDV type A12 and the genetically engineered chimera expressing the serotype O1 VP1 G-H loop substituted for the equivalent portion of the A12 capsid were derived from infectious cDNAs pRMC35 (32) and pRM-A/O (30), respectively. All pcDNA3-based plasmids were prepared by using viral cDNA from pRMC35 (32) and pRM-RGD$^-$ (21). The virus used to challenge swine was a bovine passage 78 preparation of a type A12 Vallee strain 119 isolate (21).

Plasmid engineering. The PCR (34) was used to construct a DNA fragment containing an *Sst*I site 14 bases upstream of the RNA start site of the cytomegalovirus (CMV) immediate-early gene promoter, followed by the remainder of the promoter (3) fused to the 5′ end of the FMDV A12 genome. This fragment of DNA was inserted into the *Sst*I site in the CMV promoter found in pcDNA3, and the cDNA corresponding to the remainder of the FMDV genome was added from plasmid pRMC35 by standard techniques. The resulting plasmid, designated pWRM, contained a complete immediate-early CMV promoter, the full-length A12 FMDV genome with a 3′ 15-base poly(A) tail followed by a *Not*I site, and the bovine growth hormone (BGH) polyadenylation site (see Fig. 1). A cDNA encoding bases 686 to 769 of the hepatitis delta virus (HDV) genome (39) encoding a minimal self-cleaving ribozyme (25) was inserted at the *Not*I site of pWRM to produce a plasmid designated pWRMH (see Fig. 1).

Plasmid pWRMHX, harboring a deletion of the sequences encoding the cell binding motif (the tripeptide RGD) found in VP1, was produced from pWRMH by substitution of the sequences encoding this region from plasmid pRM-RGD$^-$ (see Fig. 1) (21).

Plasmid pWRMHX-pol$^-$ was created from pWRMHX by mutation (13) of the codons specifying the highly conserved Gly-Asp-Asp motif required for polymerase activity of the picornavirus 3D protein (15). The specific mutation selected (GGA GAC GAC → GGC CGG GAC) produced a Gly-Arg-Asp sequence that was expected to block polymerase activity. All plasmid maps were confirmed with restriction endonucleases, and all fragments arising from PCR amplification were sequenced by using Sequenase (Amersham, Arlington Heights, Ill.) to confirm the sequence and to ensure that no fortuitous changes were introduced.

In vitro RNA synthesis and cell transfection. T7 transcripts were generated from restriction endonuclease-linearized plasmid DNAs with use of the MegaScript T7 kit (Ambion, Austin, Tex.) as described by the manufacturer. Dilutions of RNA and DNA preparations were introduced into BHK cell monolayers by using Lipofectin (Life Technologies, Gaithersburg, Md.), and plaques that formed in the monolayers were detected by staining 72 h later (32).

Animal inoculation and challenge. Plasmid DNAs were prepared by two cycles of CsCl centrifugation by standard methods. Seven- to 10-day-old suckling mice (outbred Swiss) were inoculated with nucleic acids diluted in phosphate-buffered saline (PBS) (Life Technologies) by intramuscular inoculation of 50 μl into each of the rear legs and monitored for 14 days to determine the lethal dose. For vaccination studies, DNA was suspended in PBS at a concentration of 1 mg/ml. Yorkshire-cross swine (weighing 20 to 40 kg) were inoculated with 200 μg of DNA; intradermal (i.d.) inoculations were administered at two sites on the right ear; intramuscular (i.m.) inoculations were administered as a single inoculation into the triceps muscle of the right fore leg. Some swine were inoculated in the same muscle with 2 μg of binary ethylenimine (BEI)-inactivated (1) type A12 virus emulsified in mineral oil (18). Swine were challenged by the same protocol utilized by Rieder et al. (30), except that the challenge virus consisted of approximately 10^5 PFU (equivalent to 10^5 bovine i.d. lingual infectious doses) of the challenge virus described above.

Serological tests. Neutralization assays were performed as described by Rieder et al. (30).

RESULTS

Construction of an infectious plasmid DNA for FMDV. Infectious RNA molecules can be transcribed from plasmid pRMC35, which contains a full-length FMDV cDNA with 35 cytosine residues in the position of the long, heterogeneous length poly(C) tract that characterizes natural FMDV genomes (32). However, pRMC35 DNA itself is very poorly infectious (Table 1). To produce an infectious DNA molecule, we inserted the genome-length cDNA containing 35 C's into pcDNA3, between the CMV promoter and the cDNA encoding the BGH polyadenylation site (see Materials and Methods for construction details). The resulting plasmid, pWRM, was expected to initiate its CMV promoter-driven transcript (3) with the first base of the FMDV genome (Fig. 1).

pWRM DNA was more infectious than the T7-driven plasmid DNA pRMC35 (32) when transfected into cells with Lipofectin (Table 1). Although these data indicated that the CMV promoter was able to produce viable transcripts, the fact that pWRM was significantly less infectious than RNA transcripts prepared from pRMC35 (Table 1) suggested a problem with translocation of the plasmid to the nucleus of the cell, with transcription, with termination, or with export of the plasmid-derived transcript from the nucleus.

Ribozyme sequences improve the infectivity of plasmid-encoded genomes. Since pWRM was unlikely to produce a transcript with a correct 3′ end, due to the presence of the BGH polyadenylation signal following the viral cDNA-encoded poly(A) tract, a second plasmid was constructed with the cDNA encoding an 85-nucleotide HDV ribozyme (see Materials and Methods) following the viral poly(A) tract in pWRM. This plasmid, pWRMH, was constructed so that the ribozyme would cleave the transcript such that the FMDV cDNA-encoded 15-nucleotide poly(A) tract and an additional GC would remain (Fig. 1). pWRMH, containing this ribozyme, was only slightly more infectious than pWRM in cell culture (Table 1). This twofold-increased infectivity was observed in multiple experiments (results not shown). The surprisingly modest increase in infectivity suggested that the ribozyme might not be active. However, functional activity of the ribozyme was confirmed by testing the cleavage activity in in vitro-generated transcripts (results not shown).

Suckling mice inoculated with pWRMH succumb to FMDV infection, but those inoculated with a plasmid encoding an attenuated viral genome did not die. The ability of FMDV nucleic acids to produce virus particles in vivo was evaluated by inoculating suckling mice with pWRMH DNA and RNA transcripts from pRMC35. Both RNA and DNA were lethal when administered by the i.m. route (Table 2). Furthermore, the animals inoculated with nucleic acids died with the same time course and clinical signs as those observed for live virus-injected mice, and virus recovered from moribund mice con-

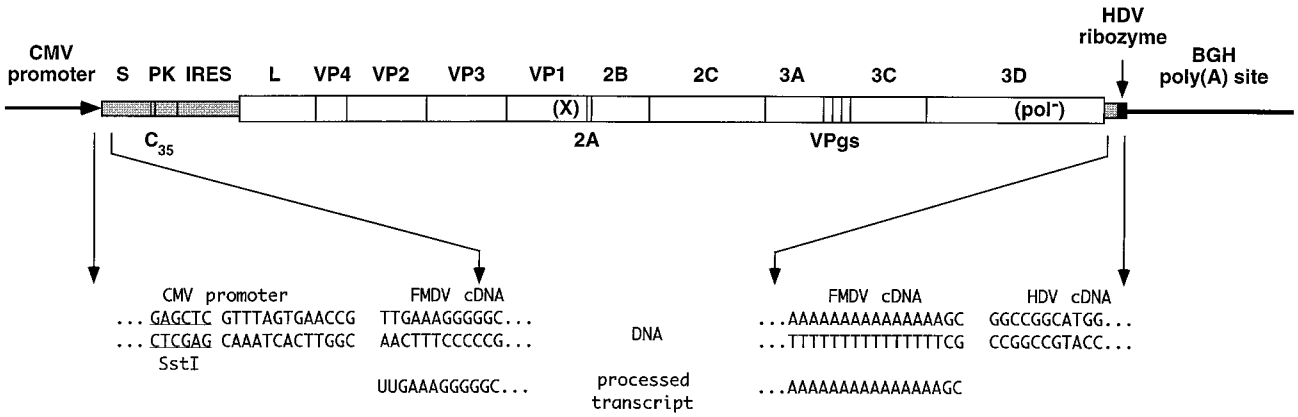

FIG. 1. Schematic diagram of CMV-driven derivatives of pRMC35. Open boxes show protein-coding regions of FMDV cDNA. Shaded boxes show noncoding regions of FMDV cDNA, including the S fragment (S), poly(C) tract of 35 nucleotides (C_{35}), pseudoknots (PK), the internal ribosome entry site (IRES), and the 3′ untranslated region (3′UTR). The closed box shows the position of the HDV ribozyme in pWRMH, and the thick lines show the CMV promoter and BGH terminator from pcDNA3. The nucleotide sequences at the junction of the CMV promoter and the S fragment of the FMDV genome found in all CMV-driven plasmids (pWRM, pWRMH, pWRMHX, and pWRMHX-pol$^-$) are shown at the bottom left, and the nucleotide sequences at the poly(A)-ribozyme junction present in all ribozyme-containing plasmids (pWRMH, pWRMHX, and pWRMHX-pol$^-$) are shown at the bottom right. The position of the RGD deletion present in pWRMHX and pWRMHX-pol$^-$ is indicated by "(X)" in VP1 (see the text). The position of the polymerase mutation in pWRMHX-pol$^-$ is indicated by "(pol$^-$)" in 3D (see the text).

firmed that the cause of death in these animals was FMDV infection (results not shown). Introduction of a cellular receptor binding site mutation which prevents virus spread between normal cells but not infectivity of RNA (21) produced a plasmid DNA that was not lethal in mice (Table 2). This plasmid, pWRMHX, contains the codons for NP substituted for GVRGDF in the sequences encoding the G-H loop of VP1. Thus, the inability of pWRMHX to kill suckling mice is in agreement with our studies showing that viruses harboring this mutation are innocuous in mice (21).

Recently, a cell line, CHO 11.1, has been developed to propagate receptor binding site-deleted type A12 viruses (31). Using this cell line, we confirmed that receptor binding site-deleted virus was produced by cells transfected with pWRMHX. Specifically, wild-type CHO cells (which cannot support second cycle replication by type A12 virus since they lack a functional receptor [19]), were transfected with pWRMHX and pWRMH. Viruses produced by cells transfected with either plasmid were able to form plaques on the CHO 11.1 cells, but only the lysates from pWRMH-transfected cells were able to form plaques on BHK cells. This confirmed that cells transfected with pWRMHX produced virions that were unable to infect normally susceptible cells.

pWRMHX induces FMDV-neutralizing immune responses in swine, and swine immunized with pWRMHX are partially protected from challenge. Preliminary studies in adult BALB/c mice demonstrated that some animals inoculated three times with 100 μg of pWRMHX by the i.d. or i.m. route produced low levels (1:20) of neutralizing antibodies to FMDV and antibodies to structural proteins detectable by radioimmunoassay (results not shown). However, an experiment with a larger sampling of 8-week-old outbred mice failed to produce any antibody responses. In addition, adult outbred mice inoculated i.m. with pWRMH, which caused death in newborn mice, did not show any signs of infection and failed to produce detectable antibodies to FMDV (results not shown). Although it is unclear why these DNAs did not efficiently immunize mice, it is possible that there was a host or tissue block to replication or translation of viral genomes in adult mice.

Swine were selected for evaluation of the pWRMHX vaccine candidate since they are economically important hosts known to be highly susceptible to FMDV infection. The first swine experiment contained two groups of five animals each that were inoculated four times with 200 μg of pWRMHX by the i.d. or i.m. route. This experiment also included three animals that were not inoculated and two animals that were vaccinated with 2 μg of a BEI-inactivated virus. None of the animals in this study showed any signs of illness following inoculations (no lesions and no more than a single day with a temperature over 40°C in the 10 days following each inoculation). In contrast to the murine studies, many of the swine inoculated with pWRMHX produced detectable levels of neutralizing antibodies following a single injection (one of five in the i.d. inoculation group and four of five in the i.m. inoculation group [Fig. 2]). Animals given a single inoculation of the BEI-inactivated virus produced superior immune responses, and antibody levels continued to climb for 10 weeks following inoculation. Radioimmunoprecipitation assays confirmed that sera from swine inoculated with DNA or BEI-inactivated virus immunoprecipitated virus capsid proteins (results not shown). In addition, the sera collected at week 10 were also able to neutralize a genetically engineered virus (A/O chimera [30]) that contains the major immunogenic site of serotype O1 substituted for the equivalent site of type A (Table 3). Comparison of the abilities of the sera from the BEI virion-vaccinated and DNA-vaccinated swine showed that the ratios of neutralization titers for the A12 and A/O viruses were similar (Table 3). Thus, the DNA vaccine, like the BEI-inactivated virus, was able to

TABLE 2. Infectivity of FMDV-encoding nucleic acids in newborn mice

Nucleic acid	No. of mice surviving/total no. tested at dose (μg/mouse) of:						LD_{50}[a]
	0.001	0.01	0.1	1.0	10	100	
pRMC35 RNA	10/10	3/9	2/10	0/10			9 ng
pWRMH DNA	9/10	3/10	4/10	2/10			42 ng
pWRMHX DNA					12/12	13/13	>100 μg

[a] LD_{50}, 50% lethal dose determined from the data given by the method of Reed and Muench (29).

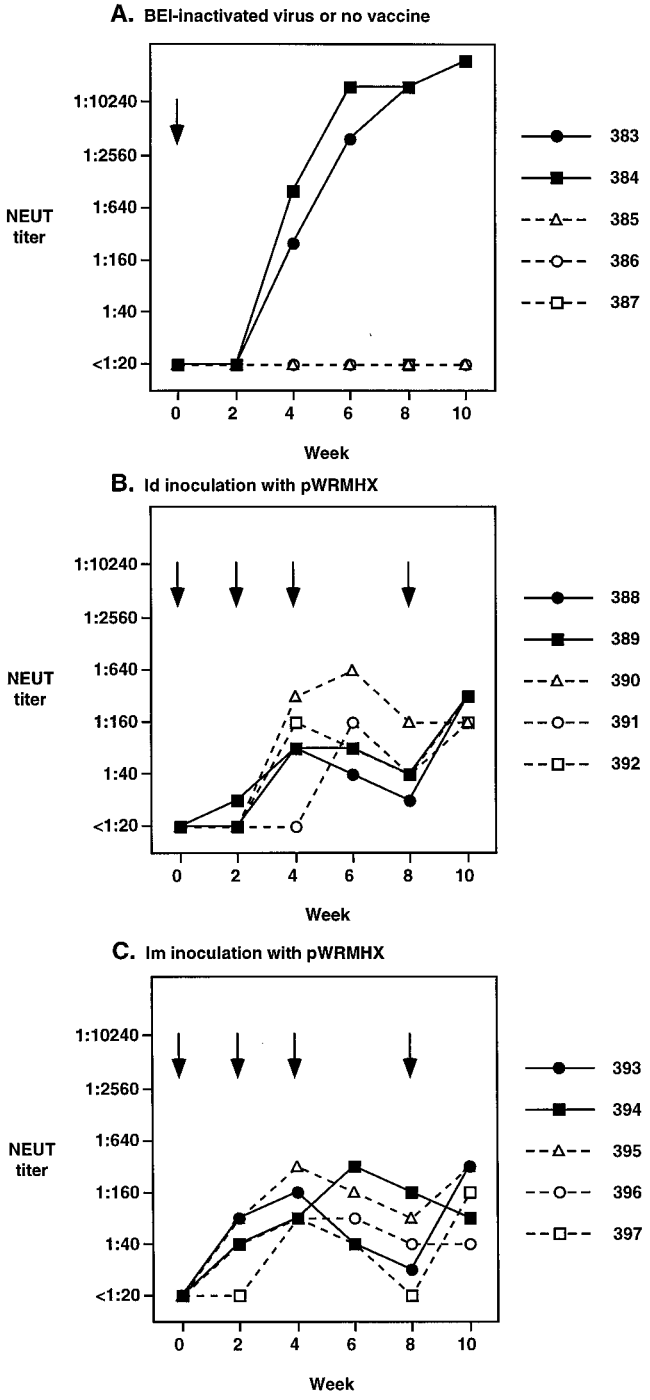

FIG. 2. Plaque reduction neutralization (NEUT) of type A12 virus (serum dilution that reduces the number of plaques by 50%) determined for sera collected at the indicated times. (A) Titers detected in sera from animals given either one inoculation of 2 μg of BEI-inactivated virions (no. 383 and no. 384) or no vaccine (no. 385 to 387); (B) titers detected in sera from animals inoculated i.d. with DNA (200 μg/inoculation); (C) titers detected in sera from animals inoculated i.m. with DNA (200 μg/inoculation). Arrows indicate the times of inoculations.

induce antibodies to epitopes residing outside of the immunodominant G-H loop of VP1.

Following the fourth DNA immunization, these animals were challenged by inoculating one of the nonimmunized animals (no. 385) with a bovine-passaged virus and allowing the infection to spread to all susceptible animals. The outcome of this contact challenge is shown in the last column of Table 3. As expected, the two animals inoculated with the BEI vaccine did not show any signs of infection, whereas the animals given no vaccine (including the inoculated animal and both remaining unvaccinated swine) developed severe FMD, with lameness and fevers. Postchallenge, DNA-vaccinated animals no. 392 and 393 showed no sign of disease (Table 3) and animals no. 390 and 395 exhibited mild signs of disease (based on the criteria shown in footnote *b* of Table 3). Although there was not a complete correlation between prechallenge neutralizing antibody titer and protection from disease (Table 3), the antibody titers detected in all DNA-vaccinated animals were in the range that produced only partial protection in our earlier studies (18, 30).

TABLE 3. Summary of immune responses and postchallenge disease data for swine

Vaccine and route	Animal no.	Neutralization titer[a]		Degree of disease postchallenge[b]
		A12	A/O chimera	
BEI virus (i.m. in oil)	383	1:32,000	1:10,240	None
	384	1:32,000	ND	None
None	385	<1:20	<1:5	Severe
	386	<1:20	<1:5	Severe
	387	<1:20	<1:5	Severe
pWRMHX DNA				
i.d.	388	1:320	1:40	Severe
	389	1:320	1:40	Severe
	390	1:160	1:20	Mild
	391	1:320	1:40	Severe
	392	1:160	1:40	None
i.m.	393	1:320	1:80	None
	394	1:80	1:20	Severe
	395	1:320	1:40	Mild
	396	1:40	1:10	Severe
	397	1:160	1:40	Severe

[a] Prechallenge titer (serum dilution that reduces the number of PFU by 50%) versus type A12 virus (A12) or type A12 virus containing the serotype O1 G-H loop of VP1 (A/O chimera) from week 10 (Fig. 2); one inoculation of 2 μg of BEI-inactivated virions or four inoculations of 200 μg of DNA.

[b] Based on daily observations and necropsy completed on day 11 postchallenge, the degree of disease was defined as follows: none, none or 1 day with a temperature of >40°C and no lesions on any feet; mild, 1 or 2 days with a temperature of >40°C and lesions on only one foot; and severe, 2 or more days with a temperature of >40°C and lesions on all four feet.

Immunogenicity of pWRMHX in swine is dependent on genome replication. To demonstrate the importance of genome replication in inducing an immune response, a vaccine candidate that was unable to produce a genome capable of replicating in the cells of the inoculated animal was generated. This plasmid, pWRMHX-pol$^-$, contained a mutation in a conserved sequence in the polymerase (see Materials and Methods) that prevents genome replication (results not shown). An evaluation of pWRMHX-pol$^-$ in swine showed that, in contrast to pWRMHX, the plasmid encoding the defective polymerase did not induce an immune response to FMDV (Table 4).

DISCUSSION

We have described a method for delivering attenuated viruses to animals in a DNA form that induces an immune response without disease. The basis for this delivery system is the production of replicating RNA genomes from inoculated

TABLE 4. Swine immune responses to pWRMHX are dependent on the ability of the encoded genome to replicate

Vaccine[a]	Animal no.	Neutralization titer
BEI virus	7	1:1,280
pWRMHX-pol$^-$DNA	8	<1:20
	9	<1:20
	10	<1:20
pWRMHX DNA	11	1:160
	12	1:80
	13	1:80

[a] Serum samples were collected at week 9. Animal no. 7 received one inoculation of 2 μg of BEI-inactivated virions at week zero, and animals no. 8 to 13 received three i.m. inoculations of 200 μg of DNA at weeks 0, 3, and 6.

naked DNA molecules. These naked DNA vaccines differ from traditional naked DNA vaccines (see Introduction) in that they encode RNA molecules that undergo replication in the cells of the inoculated animals. Similar types of replicating RNA molecules have been introduced into animals as plasmid DNAs for HDV (27) and Sindbis virus (10, 12) and as RNAs for Semliki Forest virus (42) and Sindbis virus (16).

The production of infectious RNA genomes from a transfected cDNA-containing plasmid DNA was first described over 15 years ago for poliovirus (28). However, this DNA, which did not include an RNA polymerase type II promoter, was poorly infectious. For the FMDV cDNA-containing plasmids described here, addition of a strong type II promoter from the CMV immediate-early gene and the polyadenylation signal from BGH improved infectivity of plasmid DNAs by about 50-fold, but DNA from this plasmid, pWRM, remained 1,000-fold-less infectious than RNAs generated in vitro. This profound difference in infectivity suggests that pWRM DNA encountered problems at the level of nuclear entry, transcription, or RNA exit from the nuclear compartment. Alternatively, the transcribed products could have initiated or terminated at incorrect positions, errors that would be expected to decrease infectivity by interfering with genome replication. We were unable to directly determine if the viral genomes generated from the CMV promoter initiated at the predicted first base of the viral genome, and it remains possible that initiation of transcription based on the mapping of the immediate-early gene transcript (3) could have been altered by fusion to the 5′ end of the FMDV cDNA at the predicted +1 position of the promoter. However, the product from pWRM was unlikely to have terminated at the end of the FMDV cDNA-encoded poly(A) tract, due to the presence of the sequence encoding the BGH polyadenylation signal. Since a transcript that terminated at, or near, the site found on the natural viral genome was expected to be more infectious than the one carrying a portion of the BGH mRNA, we were surprised that addition of a ribozyme cDNA, following the poly(A) tract at the 3′ end of the viral cDNA, only slightly improved the infectivity of the plasmid DNA. However, it is possible that rapid removal of the polyadenylation signal could interfere with nuclear export (14), thus preventing cytoplasmic replication of the genomic transcript. Furthermore, the transcript terminating with the poly(A) tract added at the BGH polyadenylation site might be able to serve as an efficient template for second-strand RNA synthesis, so addition of the ribozyme might not be expected to substantially improve the ability of the genome to be replicated. Finally, the presence of fortuitous splice sites within the viral cDNA could result in catastrophic deletions, which would be expected to severely reduce replicative potential of transcripts produced in cells in culture or in inoculated animals.

Work with DNA-encoded RNA genomes (10, 12) and directly injected RNA genomes (16) has shown that genomic amplification can improve expression of encoded genes. In our system, replication of the genomes clearly enhanced immunogenicity of the encoded capsids, since mutation of the active site of the viral polymerase gene dramatically reduced the immunogenicity of the plasmid DNA in swine. In addition, genome replication was confirmed by the presence of antibodies to nonstructural proteins in sera of swine immunized with pWRMHX but not in the sera from animals inoculated with the pol$^-$ derivative (results not shown). This requirement for genome replication and the fact that swine responded to epitopes outside the immunodominant loop of VP1 suggest that viral capsids were produced in the nucleic acid-inoculated swine.

Although our DNA vaccine candidate was not as effective as the inactivated whole virus vaccine, the neutralizing antibody responses that it elicited were consistent with observed protection. Moreover, pWRMHX-vaccinated swine produced antibodies to multiple epitopes on the viral capsid, and neutralizing antibody responses (≥1:20) were detected in seven of eight swine following a single i.m. injection. However, some of these responses did not significantly increase following boosting by the same route, but preliminary studies have indicated that boosting is possible if the plasmid DNA is delivered by a gene gun (results not shown). Finally, the low in vitro infectivity of the plasmid DNA relative to that of RNA genomes suggests that improvements in plasmid design could increase infectivity in vitro, which are likely to result in improved immunogenicity in animals. Current studies are aimed at further characterizing the immune response to this DNA, developing plasmid DNAs with improved infectivity, and investigating alternative delivery systems.

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