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NOTES

Receptor Binding Site-Deleted Foot-and-Mouth Disease (FMD) Virus Protects Cattle from FMD

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Binding of foot-and-mouth disease virus (FMDV) to cells requires an arginine-glycine-aspartic acid (RGD) sequence in the capsid protein VP1. We have genetically engineered an FMDV in which these three amino acids have been deleted, producing a virus particle which is unable to bind to cells. Cattle vaccinated with these receptor binding site-deleted virions were protected from disease when challenged with a virulent virus, demonstrating that these RGD-deleted viruses could serve as the basis for foot-and-mouth disease vaccines safer than those currently in use. This strategy may prove useful in the development of vaccines for other viral diseases.

Foot-and-mouth disease virus (FMDV), which comprises the *Aphthovirus* genus of the family *Picornaviridae*, causes a devastating disease of livestock (2, 18). The virion consists of a single-strand, positive-sense RNA genome packaged in an icosahedrally symmetric shell composed of 60 copies each of four structural proteins, VP1 to VP4. Chemically inactivated vaccines have been widely used to control foot-and-mouth disease (FMD), but frequent revaccination is required to maintain protective immunity (2, 18). Furthermore, the presence of residual live viruses in these inactivated vaccines and the escape of viruses from vaccine production plants have been implicated in FMD outbreaks (8). Here, we describe the use of a genetically inactivated FMDV as an effective vaccine.

The three-dimensional structure of FMDV has revealed a prominent surface feature formed by the loop between the G and H β strands of VP1 (referred to herein as the G-H loop) that is partially disordered under some conditions (1, 14, 17). Despite its flexibility, the G-H loop contains a highly conserved arginine-glycine-aspartic acid (RGD) sequence that has been implicated in receptor binding on the basis of synthetic peptide inhibition studies (5, 13). Using a genetic engineering approach, we confirmed that the RGD sequence is required for binding viruses to host cells by demonstrating that viruses with amino acid substitutions within the RGD sequence were unable to bind to or infect cells (16). However, viruses created with a single point mutation in the sequences encoding the RGD segment reverted to virulence by regaining the RGD sequence (16), consistent with the well-known variability of the FMDV genome (11, 12).

To create a stable, genetically inactivated FMDV for use as a vaccine, we constructed a virus in which the entire RGD sequence was deleted to prevent reversion to virulence. Specifically, a genome-length cDNA of FMDV type A_{12} (20) in which the codons specifying the wild-type amino acid sequence SGS**GVRGDF**GSL were replaced with codons for SGS**NP**GSL was engineered, and synthetic RNA transcripts derived from this cDNA were transfected into baby hamster kidney (BHK) cells by electroporation (16). (The deleted or inserted residues are underlined and in boldface type.) Cells transfected under these conditions produced levels of virus particles similar to those produced by cells transfected with wild-type RNA. Preliminary experiments showed that these particles did not bind to cells, were noninfectious, and were recognized by monoclonal antibodies representing four different epitopes of FMDV type A_{12} (6, 7). Interestingly, one of these epitopes includes portions of the G-H loop (7), indicating that this deletion had little, if any, effect on the antigenic structure of the RGD-deleted virus.

By expanding our previously published electroporation methods (16), we produced large numbers of cells transfected with the RGD-deleted RNA. The virus particles present in the culture fluid harvested from these cells were concentrated by polyethylene glycol precipitation and further purified by sucrose density gradient centrifugation (6, 16). The amount of virus present in these partially purified preparations was estimated from VP2 protein content on Western blots (immunoblots) (21). Western blots were generated with four twofold dilutions of wild-type virus quantitated by UV absorbance measurements (3) and developed by using polyclonal guinea pig serum specific for FMDV and ^{125}I-labeled protein A (New England Nuclear, Boston, Mass.). The density of the band for VP2, which was not altered in the receptor binding site-deleted virus, was quantitated in each sample by using Molecular Dynamics ImageQuant version 3.15 software. The VP2 normogram generated from the wild-type virus dilutions was used to establish the amount of virus present in the RGD-deleted virus preparation analyzed in the same Western blot. These experiments revealed that under conditions in which saturating amounts of RNA were used (16) approximately 1 μg of par-

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TABLE 1. Comparison of cattle immune responses and protection from FMD elicited by vaccination with RGD-deleted virus and BEI-inactivated wild-type virus

Vaccine	Animal	Neut titer[a]	Protection[b]
Mock	57	<0.7	No
	109	<0.7	No
	148	<0.7	No
RGD deleted	15	3.4	Yes
	103	2.5	Yes
	143	2.5	Yes
BEI inactivated	21	2.5	Yes
	50	2.8	Yes
	101	2.2	Yes

[a] Neutralizing (neut) antibody titers (log of serum dilution yielding a 70% reduction in PFU) obtained 28 days postvaccination.

[b] The cattle were challenged at day 29 postvaccination by exposure to an infected pig and observed for clinical signs. No, temperature over 39°C for more than 3 days (Fig. 1) and vesicles in the interdigital area and oral cavity; Yes, no clinical signs.

tially purified RGD-deleted virions could be obtained from 6 $\times$ 10^6 transfected cells.

BHK cells and baby mice were inoculated with the RGD-deleted virus preparations to demonstrate that this virus could not regain its infectivity or virulence. Five hundred nanograms of RGD-deleted virus was diluted in tissue culture medium, inoculated into cultures of BHK cells, and incubated for 72 h at 37°C. Although no cytopathic effect was visible, the culture was lysed by freeze-thaw cycling and passaged onto fresh cells. A plaque assay of the second-passage material on BHK cells did not reveal any infectious agent. One hundred nanograms of RGD-deleted virus was inoculated intraperitoneally into 20 7- to 10-day-old outbred Swiss mice. None of the mice died or showed any signs of infection. Side-by-side lethal dose titrations of virus recovered from cells transfected with the wild-type genome-length RNA from $pRMC_{35}$ (20) yielded 50% lethal dose values of 1.2 $\times$ 10^6/100 ng. Therefore, these studies with mice demonstrate that the RGD-deleted virus is attenuated by more than 10^6-fold relative to the wild type. All work with animals was performed in accordance with U.S.D.A. Agricultural Research Service and institutional guidelines.

Either wild-type or RGD-deleted virus (2 μg) was inoculated into the coronary band and the dermis of the snout of one of two adult Yorkshire swine. The animals were observed for signs of FMD for 2 weeks. The animal that received 2 μg of wild-type virus developed classical FMD (fever and lameness with vesicles on all four feet and the snout) within 5 days of inoculation, whereas the animal inoculated with 2 μg of the RGD-deleted virus did not show any signs of disease. As expected, radioimmunoprecipitation analyses of serum collected 28 days postinfection from the animal inoculated with the

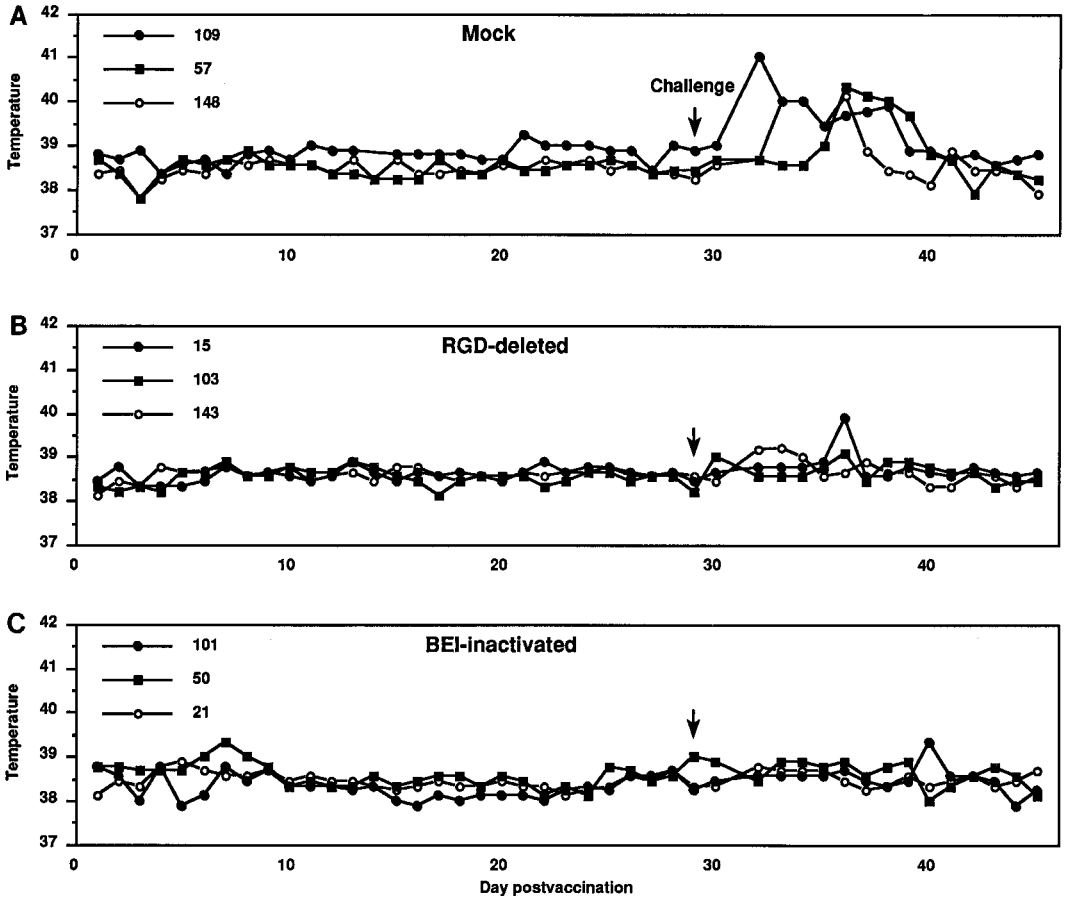

FIG. 1. Temperatures postvaccination. Steers were mock vaccinated (A) or vaccinated at day 0 with the RGD-deleted (B) or BEI-inactivated (C) vaccine and challenged at day 29. Temperatures are indicated in degrees Celsius, and the numbered symbols represent individual animals.

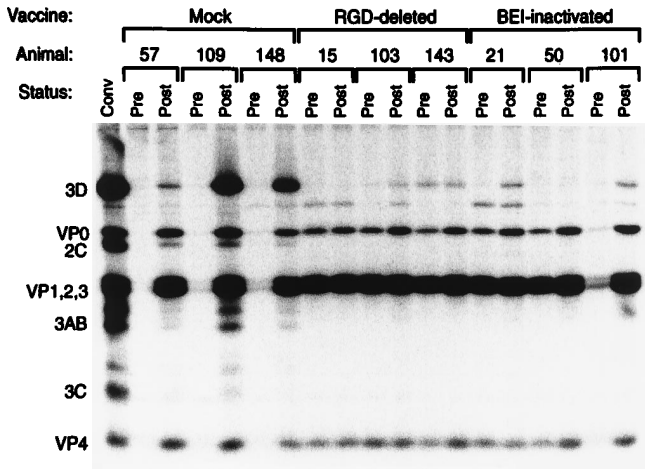

FIG. 2. Comparison of the abilities of prechallenge and postchallenge sera to immunoprecipitate proteins from FMDV-infected cells. Serum samples were collected from the indicated animals at 28 days following vaccination (Pre); the animals were challenged the next day, and a second serum sample was collected from each animal 28 days later (Post). Lane 1, convalescent serum (Conv). Serum samples were reacted with radiolabeled lysates of virus-infected cells, and immune complexes were precipitated and analyzed as described previously (19). The positions of the viral proteins are listed on the left-hand side of the autoradiogram.

wild-type virus revealed strong reactivity to structural proteins and the nonstructural proteins 3D, 3AB, and 2C, indicating that the wild-type virus had replicated in the animal (9, 15). In contrast, the 28-day postinoculation serum obtained from the pig inoculated by this route with the RGD-deleted virus showed very low levels of reactivity with structural proteins and no detectable reactivity with nonstructural proteins (results not shown), indicating that the RGD-deleted virus did not replicate in this animal (9, 15).

To test the usefulness of the RGD-deleted virus as a vaccine, we conducted a vaccination challenge study with nine 18- to 20-month-old Hereford steers. Three steers were vaccinated intramuscularly with a tissue culture medium-mineral oil (9:1 Marcol 52/Montanide 888) emulsion (mock vaccine), three animals were vaccinated intramuscularly with 2 μg of sucrose gradient-purified, binary ethylenimine (BEI)-inactivated (4) wild-type virus emulsified in oil, and the remaining three animals were vaccinated intramuscularly with an oil emulsion containing 2 μg of the RGD-deleted virus. Animals were observed for signs of FMD for 4 weeks. None of the animals developed fever or vesicles, classic signs of FMD. Four weeks postvaccination, serum samples were collected from all nine animals and tested for their ability to neutralize the virus in vitro. Table 1 shows that the RGD-deleted and the BEI-inactivated vaccines generated similar serum neutralization titers in steers, whereas the mock-vaccinated steers did not show any response to FMDV. These results indicate that the two vaccines performed equally well in their ability to elicit neutralizing antibodies in cattle at the dose tested.

The nine animals listed in Table 1 were combined in a single large room and exposed to a pig which had developed severe clinical manifestations of FMD after infection with a virulent cattle-passaged strain of FMDV type A_{12} (Vallee strain 119, cattle passage 78 [10], kindly provided by J. House). The cattle were examined daily for the onset of clinical signs (lameness, vesicle formation on the tongue, or fever). If temperatures over 39°C were noted, the animals were sedated and examined closely for vesicular lesions on their feet and in their oral cavities. All six vaccinated animals were protected from clinical disease, whereas all three mock-vaccinated animals demonstrated clinical FMD within 7 days of exposure to the infected pig, with temperatures over 40°C for 3 days (Fig. 1) and lesions on the tongue and all four feet.

The effectiveness of the vaccine was further evaluated by determining if viral challenge had produced immune responses to viral antigens by comparison of the abilities of pre- and postchallenge sera to precipitate viral proteins from radiolabeled infected cell lysates (Fig. 2). For one animal (animal 143), a weak reaction to 3D was observed in prechallenge serum, consistent with the fact that antibodies to 3D are often observed in sera from vaccinated animals (9, 15). Figure 2 also shows that antibodies to nonstructural proteins 2C, 3AB, and 3C were present in postchallenge sera of mock-vaccinated animals. On the basis of previously established criteria (9, 15), the presence of antibodies to two or more of these antigens demonstrates extensive viral replication, consistent with the observed clinical signs in these animals (Table 1; Fig. 1). Several minor differences between pre- and postchallenge sera were also noted among the six vaccinated animals, and in several cases antibodies to 3D were detected in postchallenge serum samples. However, following challenge none of the vaccinated animals developed antibodies to 2C, 3AB, or 3C (Fig. 2), indicating that vaccination had prevented, or severely limited, viral replication (9, 15). Interestingly, one of the BEI-inactivated vaccine-vaccinated animals (animal 101) showed an increase in antibodies to structural proteins and the appearance of reactivity with 3D after challenge (Fig. 2), suggesting that limited, but clearly detectable, viral replication had taken place in the face of challenge in this animal. The possibility that virus replication occurred in this animal is consistent with the finding that this animal showed the lowest prechallenge titer of neutralizing antibodies (Table 1).

This study demonstrates that the RGD-deleted vaccine performed as well as, or exceeded, the accepted BEI-inactivated vaccine with respect to protection from challenge, generation of serum-neutralizing antibodies, and generation of an immune response which restricts replication of the virus upon challenge. This is the first demonstration that a safe and effective vaccine can be prepared by genetically removing the cell binding site from a virus.

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