JOURNAL OF VIROLOGY, Jan. 2003, p. 1219–1226 Vol. 77, No. 2
0022-538X/03/$08.00+0 DOI: 10.1128/JVI.77.2.1219–1226.2003

Evidence of the Coevolution of Antigenicity and Host Cell Tropism of Foot-and-Mouth Disease Virus In Vivo

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Received 23 May 2002/Accepted 8 October 2002

In this work we analyze the antigenic properties and the stability in cell culture of virus mutants recovered upon challenge of peptide-vaccinated cattle with foot-and-mouth disease virus (FMDV) C3 Arg85. Previously, we showed that a significant proportion of 29 lesions analyzed (41%) contained viruses with single amino acid replacements (R141G, L144P, or L147P) within a major antigenic site located at the G-H loop of VP1, known to participate also in interactions with integrin receptors. Here we document that no replacements at this site were found in viruses from 12 lesions developed in six control animals upon challenge with FMDV C3 Arg85. Sera from unprotected, vaccinated animals exhibited poor neutralization titers against mutants recovered from them. Sequence analyses of the viruses recovered upon 10 serial passages in BHK-21 and FBK-2 cells in the presence of preimmune (nonneutralizing) sera revealed that mutants reverted to the parental sequence, suggesting an effect of the amino acid replacements in the interaction of the viruses with cells. Parallel passages in the presence of subneutralizing concentrations of immune homologous sera resulted in the maintenance of mutations R141G and L147P, while mutation L144P reverted to the C3 Arg85 sequence. Reactivity with a panel of FMDV type C-specific monoclonal antibodies indicated that mutant viruses showed altered antigenicity. These results suggest that the selective pressure exerted by host humoral immune response can play a role in both the selection and stability of antigenic FMDV variants and that such variants can manifest alterations in cell tropism.

The first evidence that antigenic changes could be associated with alterations in cell tropism was obtained with human influenza virus, by documenting that monoclonal antibody (MAb) escape mutants displayed changes in recognition of specific types of sialic acid molecules (38). More recently, this concept has acquired new impetus in view of structural evidence of some overlapping between antigenic sites and receptor recognition motifs in a number of viruses (4, 40), including the important animal picornavirus foot-and-mouth disease virus (FMDV) (3, 12, 20, 31, 39, 42). An Arg-Gly-Asp (RGD) triplet located within the G-H loop of capsid protein VP1 (1) is directly involved in the interaction with integrin receptors (7, 16, 18, 19, 33) and with neutralizing antibodies (15, 24, 30, 42). Correlation between variations in FMDV antigenic sites and cell interaction was suggested by the impaired binding to BHK-21 cells of FMDV variants derived from infectious cDNA, which included replacements downstream of the RGD motif (34).

Serial passage of FMDV in cell culture selects for FMDV mutants capable of using heparan sulfate as a receptor (6, 17, 36). Passage of FMDV C-S8 c1 100 times in BHK-21 cells resulted in dominance of virus mutants which could enter cells via a pathway which is independent of integrins and heparan sulfate and whose nature remains unknown (5). Because RGD was no longer a requirement to enter BHK-21 cells, a number of antigenic variants resistant to neutralizing antibodies were isolated from the multiply passaged FMDV C-S8 c1 populations (35). These variants contained one or several amino acid replacements within the G-H loop of VP1, including modifications within the RGD triplet with sequences such as RED, RGG, or even GGG (35). Furthermore, mutants in which the RGD and surrounding residues were replaced by unrelated amino acids were infectious for BHK-21 cells (4). However, current evidence is that integrins are the receptors used by FMDV in cattle (27), and no evidence of the use of alternative receptors for FMDV in vivo has been reported.

In a large-scale vaccination experiment involving a total of 138 bovines (41), four types of peptides containing sequences of FMDV C3 Arg85 were used as immunogens: peptide A, which included the G-H loop of capsid protein VP1 (antigenic site A [24]); peptide AT, in which an FMDV T-cell epitope comprising residues 21 to 40 of VP1 (10) was linked to site A; peptide AC, composed of site A and the carboxy-terminal region of VP1 (antigenic site C); and peptide ACT, in which the three peptides were collinearly represented. None of the tested peptides afforded protection to more than 40% of the cattle challenged with virulent homologous FMDV. Viruses

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from lesions developed in nonprotected animals were analyzed by direct nucleotide sequencing of the VP1-coding region, without passage of the viruses in cell culture. A significant proportion (41%) of the lesions analyzed contained viruses with a single amino acid replacement within antigenic site A (41). The replacements found were R141G, L144P, and L147P, within the VP1 G-H loop sequence AGAARR(141)GDL(144)AHL(147)AAAHARHLP. These replacements modify the antigenic properties of type C FMDVs (24, 26), suggesting that isolation of these mutants in vivo might be the result of selection of antigenic variants that escaped neutralization by anti FMDV antibodies in peptide-vaccinated cattle.

In this report we provide evidence in favor of this hypothesis. Propagation in cell culture of virus variants allowed characterization of the antigenic changes produced by VP1 amino acid replacements R141G, L144P, and L147P. Interestingly, these experiments also revealed the instability of mutants L144P and L147P, which rapidly reverted to the wild type sequences when grown in BHK-21 cells, suggesting that the antigenic changes produced by these replacements were associated with alterations in the cell receptor usage.

MATERIALS AND METHODS

Cells, viruses, and infections. Fetal bovine kidney cells were obtained as primary cell cultures and used at the second passage (termed FBK-2) throughout this study. BHK-21 and FBK-2 cell monolayers were grown in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum, at 37°C in an atmosphere of 5% CO_2. Cells were subcultured every 2 to 3 days. FMDV mutants L9(R141G), 40(L147P), 25(L144P), L5(L144P), and L12(L144P) were isolated from peptide-vaccinated cattle upon challenge with a virulent type C_3 FMDV isolate (C3 Arg85) as previously described (41). Mutants are identified by the animal from which they were isolated followed by the amino acid replacement (given in parentheses) found in capsid protein VP1. Each mutant exhibited a single substitution within antigenic site A. FMDV mutants and the parental virus C3 Arg85 were amplified by serial cytolytic passages in BHK-21 or FBK-2 cells. Cell monolayers were initially infected with material from independent lesions, and 16 to 20 h postinfection, when cytopathic effect was evident, supernatants were collected and used to infect fresh cell monolayers as well as to determine site A nucleotide sequence as describe below. The third passage of viruses in FBK-2 cells were used for neutralization assays, viral growth curves, starting of serial passages with or without specific immune sera, and determination of reactivity against MAbs in enzyme-linked immunosorbent assay (ELISA).

Plaque assays were performed on BHK-21 cells as follows. Virus dilutions, in a volume of 0.3 ml per 60-mm-diameter culture dish, were added onto the confluent cell monolayer (about 10^6 cells), and the plates were incubated for 1 h at 37°C to allow virus internalization. Then, the virus inoculum was removed and the cells were overlaid with 2.5 ml of semisolid Eagle's medium (0.7% Noble agar; 1% fetal calf serum; 25 mM HEPES, and gentamicin D [50 mg/ml] in Eagle's medium). At 48 h postinfection, the cells were fixed with 4% formaldehyde and stained with crystal violet. For visualization of very small plaques, the semisolid medium contained 0.6% agar and the plaques were allowed to develop for 96 h at 37°C.

Nucleotide sequencing of the capsid-coding RNA region. Total RNA was extracted from lesions of animals using guanidine thiocyanate (9). Viral RNA was phenol extracted from supernatants of infected cells as described previously (14). FMDV RNA was copied into cDNA, and the capsid-coding region was amplified using *Taq* polymerase (reverse transcription [RT]-PCR) either with the sets of primers previously described (14, 41) or with primers designed for efficient RT-PCR amplification of the capsid-coding region of FMDV C3 Arg85. The new primer sets used were as follows (sequences are given in 5′-3′ polarity, and numbering is from 5′ end of the genome RNA of FMDV C3 Arg85 [13]): P1Low (CAGCAGTTGTTTCGCCGGTGCGA) and SacLow (AGTTTGTGTTTCTCCTCCGT) (complementary to positions 3858 to 3880 and positions 3303 to 3322, respectively) and P1Up (GGAGCCGGGCAATCCAGCCCGCG) and SacUp (ACCGCGTCCAGTGAGGCTGA) (spanning positions 1685 to 1707 and positions 3098 to 3117). Nucleotide sequencing was performed from 3′ overlapping DNA fragments with the fmol DNA sequencing kit (Promega), as described previously (14). For this purpose, the following internal primers were used: 4+fin (CAGCGGTCTTTTCGGTGCTC) (spanning positions 1912 to 1931), 2+up (CGGGTACGCAACAGTTGAGG) (spanning positions 2041 to 2060), 2 half up (CCCCCACCAATTCATCAACC) (spanning positions 2368 to 2387), and VP4Low (CCGCTGAAGGCAGAGCTGGC) and VP2Low (ATGACGACAAGCGTCCAGGG) (complementary to positions 1898 to 1917 and positions 2465 to 2484, respectively).

MAbs and polyclonal sera. All MAbs used in this study were raised against whole FMDV serotype C particles and have been described previously (2, 21, 22, 24, 25). Neutralizing MAbs 7AB5, 7JD1, 7FC12, 7AH1, 7CA8, 7CA11, 7EE6, and 7CH1 recognize some of the multiple continuous epitopes within site A in capsid protein VP1. MAbs 2E5, 3G11, 2A12, 3E9, 5C4, and 5H10 interact with discontinuous epitopes within antigenic site D (21). MAb 1BH8 recognizes a discontinuous epitope, and its binding is inhibited after trypsin treatment of FMDV C3 particles (2). MAb 7DF10 recognizes a conformation-dependent epitope (25) that has not been mapped yet.

Bovine polyclonal sera from animals that had been vaccinated and given boosters with synthetic peptides (41) were obtained just before challenge with FMDV C3 Arg85. They include (i) sera from unprotected peptide-vaccinated animals L9 and 40 (inoculated with peptides AT and ACT, respectively), L12 (vaccinated with AT), and L5 and 25 (both vaccinated with ACT); (ii) sera from protected peptide-vaccinated animals L1 and 12 (vaccinated with peptides ACT and A, respectively); and (iii) sera from control animals 1.1 (inoculated with a chemically inactivated vaccine formulated with FMDV C3 Arg85; positive control) and 1.3 (inoculated with phosphate-buffered saline [PBS]-oil adjuvant; negative control).

Immunochemical assays and neutralization of infectivity. Neutralization of infectivity was performed by a plaque-reduction neutralization assay. Briefly, 150 to 200 PFU of C3 Arg85 or mutant viruses was incubated with serial dilutions of each bovine polyclonal serum or of MAb for 1 h at room temperature, followed by 30 min at 4°C. The mixtures were then plated onto BHK-21 cell monolayers and viral plaques were visualized by crystal violet staining. Serum neutralization (SN) titers are expressed as the reciprocal of the logarithm of the bovine serum dilution that caused a 50% plaque reduction.

Capture ELISA experiments were carried out to quantify the reactivity of MAbs with FMDV mutants (32). To this end, an equal amount of each FMDV mutant and C3 Arg85 was used. Virus was quantitated by reactivity in an ELISA using the nonneutralizing MAb 7BH11, which recognized a conserved epitope. ELISA plates (Maxisorp; Dynatech) were coated overnight at 4°C with an appropriate dilution of an anti-FMDV C3 Arg85 hyperimmune rabbit serum and saturated with 5% skim milk in PBS. After washing with PBS–0.05% Tween 20, an appropriate dilution of C3 Arg85 or of FMDV mutants was added and incubated for 1 h at room temperature. Then, 10-fold serial dilutions of each MAb were added and incubated for 1 h at room temperature. After addition of a 1:500 dilution of rabbit anti-mouse immunoglobulin G horseradish peroxidase conjugate, plates were developed with 2,2-azinobis-3-ethylbenthiazoline-6-sulfonic acid (ABTS) and hydrogen peroxide substrate. Absorbance was determined at 405 nm, and titers were calculated as the dilution of MAb giving 50% absorbance, relative to 100% absorbance values obtained with MAb 7BH11, which recognizes a conserved epitope in FMDV of serotype C (25). For each MAb, a relative reactivity index (Ri) was calculated as the ratio between the MAb titer for the mutant virus and that for the parental virus. Relative reactivity was expressed as positive ($Ri > 0.1$), intermediate ($0.01 < Ri < 0.1$), weak ($0.001 < Ri < 0.01$), or negative ($Ri < 0.001$).

Virus growth curves. Individual BHK-21 cell monolayers were infected at a multiplicity of infection (MOI) of 10 PFU per cell with either C3 Arg85 or a third passage in FBK-2 cells of the mutant viruses. After 1 h of absorption at 37°C, monolayers were washed with 0.1 M phosphate buffer at pH 5.5, and after adjusting to neutral pH, fresh medium containing 2% calf fetal serum was added to the culture and infection was allowed to proceed at 37°C. At different times postinfection, samples from the supernatants were withdrawn and titrated on BHK-21 cell monolayers.

Serial passages of FMDV mutants. Serial infection of BHK-21 and FBK-2 cell monolayers with FMDV mutants L9(R141G), 40(L147P), or 25(L144P) was carried out in the presence, or not, of neutralizing concentrations of sera from animal L9, 40, or 25, respectively. In order to determine the subneutralizing concentration of each bovine serum, monolayers were infected, at an MOI of 0.001 PFU per cell, in the presence of different serum dilutions: 1/20, 1/60, 1/200, 1/400, and 1/800. The minimal antiserum dilution that showed a nearly complete cytopathic effect was used for the serial passages. The progeny virus of each infection was diluted 1/1,000 in Dulbecco's modified Eagle's medium and was used to infect fresh cell monolayers up to a total of 10 passages. As a control,

mutant FMDVs and C3 Arg85 were serially passaged 10 times in the presence of nonimmune cattle serum.

Statistical analysis. In order to statistically test the hypothesis that the selection of mutations in the VP1 gene observed in the peptide-vaccinated animals was mediated by immune pressures, we analyzed the proportion of synonymous to nonsynonymous substitutions within the G-H loop coding region (8, 28) by means of a test implemented in MEGA (Molecular Evolutionary Genetics Analysis Software, Arizona State University, Tempe). This test estimates the number of nonsynonymous substitutions per nonsynonymous site and the number of synonymous substitutions per synonymous site (29) plus the respective variances, and uses them in a z test.

RESULTS

Antibody-mediated selection in the generation of antigenic variants of FMDV in peptide-vaccinated cattle. Lack of protection of peptide-vaccinated cattle could partly be due to selection of antigenic variants of FMDV during challenge with virulent virus, as judged by the high proportion of lesions that contained virus with an amino acid replacement within site A at the G-H loop of capsid protein VP1 (41). To further explore this possibility, we analyzed viruses from 12 independent lesions developed in six control animals included in that study, which were inoculated with PBS-oil adjuvant and then challenged with virulent virus C3 Arg85 in parallel with peptide-vaccinated animals. No mutations were observed in the VP1-coding region of any of the viruses found in the 12 lesions analyzed (results not shown). The significance of the difference in the selection of mutants between peptide-vaccinated and control animals was sustained by a statistical analysis based on the proportion of synonymous to nonsynonymous substitutions to estimate positive selection within the G-H VP1 loop coding region (see Materials and Methods). The results of this analysis revealed significant evidence ($P < 0.1$) of positive selection in the mutations found in peptide-vaccinated cattle. These results suggest the requirement of a specific anti-FMDV immune response elicited by the peptide antigens in the selection of virus variants in cattle.

To test whether the replacements found within site A in viruses from peptide-vaccinated cattle conferred FMDV C3 with some resistance to neutralization, sera from vaccinated and control cattle were tested for their ability to neutralize mutants L9(R141G), 40(L147P), and C3 Arg85 (Table 1). All sera tested, except those from animals immunized with the inactivated virus vaccine (positive control serum) or inoculated with PBS-oil adjuvant (negative control serum), exhibited neutralization titers for C3 Arg85 that were higher than those for mutants L9(R141G) and 40(L147P). Remarkably, no detectable neutralization of mutant 40(L147P) by sera from animals 40 (from which it was isolated), 12, and 25 was observed (SN titer < 0.7). This mutant also showed different extents of neutralization by sera from other peptide-vaccinated animals (Table 1). Mutant L9(R141G) showed partial neutralization by serum from animal L9, from which it was isolated, and various degrees of neutralization by the other sera tested. All mutants were neutralized (maximum SN titer of >4) by serum from cattle immunized with the whole-virus, inactivated vaccine (Table 1). Neutralization of mutants 25(L144P), L12(L144P), and L5(L144P) could not be tested due to the poor plating efficiency of these viruses in cell culture (see below).

The results indicate that during challenge of cattle with virulent FMDV, antigenic variants with amino acid replacements at or near the RGD triplet were produced in animals immunized with synthetic peptide antigens but not in control animals. The mutant FMDVs showed different degrees of resistance to neutralization by peptide-vaccinated animal sera, suggesting that they were generated by positive selection during replication in partially immune cattle.

TABLE 1. Neutralization of viral mutants by sera from partially immunized cattle

Animal serum[a]	Peptide vaccine[b]	Protection to challenge[c]	SN titers (mean ± SD) against[d]:		
			L9(R141G)	40(L147P)	C3 Arg85
L9	AT	−	1.4 ± 0.1	1.2 ± 0.2	2.5 ± 0.3
L12	AT	−	2.0 ± 0.2	1.3 ± 0.1	>2.8
L5	ACT	−	1.3 ± 0.2	1.1 ± 0.1	2.3 ± 0.1
25	ACT	−	1.6 ± 0.1	<0.7	2.3 ± 0.1
40	ACT	−	2.4 ± 0.4	<0.7	2.5 ± 0.4
12	A	+	1.9 ± 0.1	<0.7	2.2 ± 0.5
L1	ACT	+	2.3 ± 0.2	0.8 ± 0.1	2.6 ± 0.2
1.1[e]	Inactivated FMDV	+	>4	>4	>4
1.3[f]	PBS	−	<0.7	<0.7	<0.7

[a] Sera from peptide-vaccinated cattle are those described by Taboga et al. (41).
[b] The peptides employed to formulate the vaccines used for cattle immunization (41) are described in Materials and Methods.
[c] Vaccinated animals were challenged by tongue-intradermal inoculation with 10^4 50% mouse lethal doses of FMDV C3 Arg85 and periodically inspected for vesicular lesions up to 7 days after challenge; the animals were scored as protected (+) and unprotected (−) (37).
[d] The amino acid replacement in the VP1-coding region found in viruses recovered from unprotected animals, relative to the C3 Arg85 sequence, is indicated in parentheses in the subheads. Results are given as the logarithm of the serum dilution causing a 50% reduction in the number of PFU in BHK-21 cells (see Materials and Methods). Each value is the average of at least three determinations.
[e] This serum was obtained from an animal immunized with a whole-FMDV C3 Arg85 inactivated vaccine.
[f] This serum was obtained from an animal inoculated with PBS-oil adjuvant.

Unequal stability of mutant FMDVs in cell culture. To test the capacity of the FMDV mutants from cattle to replicate in cell culture, samples of FMDV mutants L9(R141G), 25(L144P), L5(L144P), L12(L144P), and 40(L147P), taken directly from vesicular lesions, and of C3 Arg85 were employed to infect BHK-21 cells and FBK-2 cells, as detailed in Materials and Methods. Each virus was subjected to serial passages in both cellular types, and cytopathic effect was observed in all cases. Virus titers produced in FBK-2 cells ranged from 8×10^5 to 7×10^6 PFU/ml at 48 h postinfection. The exception was L12(L144P), which produced a titer of 6×10^2 PFU/ml in both cell lines, with the plaque assays conditions used. The stability of these mutants was assessed by comparing the amino acid sequences of antigenic site A of the viruses recovered after two and three passages in BHK-21 and BFK-2 cells (Table 2). After two passages in BHK-21 cells, reversion to the C3 Arg85 sequence was observed in those virus populations derived from mutants L144P, while only a partial reversion was found in the progeny of mutant 40(L147P). All mutants were more stable upon passage in FBK-2 cells than in BHK-21 cells. The viral progeny recovered after three passages of 25(L144P) and L5(L144P) in FBK-2 cells showed heterogeneity at position 144 of VP1 (mixture of P and L), while substitutions in mutants L12(L144P) and 40(L147P) were maintained (Table 2). In contrast, the replacement of variant L9(R141G) was maintained after at least three passages in FBK-2 and BHK-21 cells.

TABLE 2. Stability of FMDV mutants in cell culture

FMDV mutant[a]	Replacement observed during cell passage[b]			
	BHK-21		FBK-2	
	2	3	2	3
L9(R141G)	G-141	G-141	G-141	G-141
L5(L144P)	L-144	ND	P-144	P/L[c]-144
L12(L144P)	L-144	ND	P-144	P-144
25(L144P)	L-144	ND	P-144	P/L[c]-144
40(L147P)	P/L[c]-147	ND	P-147	P-147
C3 Arg85	NR	ND	NR	ND

[a] The FMDV mutants analyzed are described in Materials and Methods. The amino acid replacements found in each mutant relative to the C3 Arg85 sequence are indicated.

[b] The amino acid found at positions 141, 144, and 147 of VP1-coding region in FMDV mutants after passages in BHK-21 or FBK-2 cells. No replacements were found after two passages of C3 Arg85. Abbreviations: ND, not done; NR, no replacements found.

[c] A band pattern indicating similar proportions of P and L was observed in the sequencing gels.

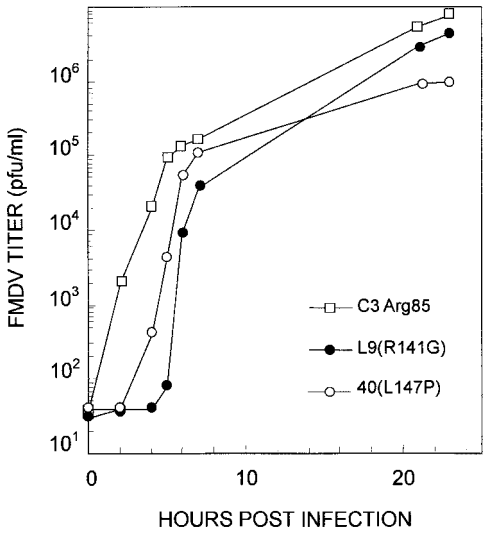

FIG. 1. One-step growth curve of C3 Arg85, L9(R141G), and 40(L147P). BHK-21 cell monolayers were infected for 1 h at 37°C at an MOI of 10 PFU per cell, rinsed with phosphate buffer at pH 5.5, and then incubated in Eagle's minimal essential medium containing 2% fetal calf serum. At the times indicated, the amount of virus released from the infected cells was determined by plaque assay as described in Materials and Methods. Each value represents the average of duplicate assays.

No other amino acid replacements were detected, neither in the complete capsid-coding region of any of the mutant viruses recovered after 2 or 3 passages in BHK-21 or FBK-2 cells, respectively, nor within antigenic site A of C3 Arg85, passaged under the same conditions (Table 2).

These results indicate that viruses harboring replacements L147P and L144P showed an unequal capacity to maintain these mutations upon replication in BHK-21 or FBK-2 cells, while viruses containing replacement R141G maintained the substitution in both cell cultures. Given the higher stability of the G-H loop of VP1 when the FMDVs from cattle were passaged in FBK-2 cells, working stocks to further study the behavior of the mutant FMDVs were prepared in FBK-2 cells.

Growth characteristics of viral mutants. Previous results showed that amino acid replacements R141G, L144P, and L147P in VP1 affected binding of FMDV C1 to BHK-21 cells (26) and also the binding of several MAbs to antigenic site A (42, 43), suggesting that these residues may play critical roles in cell tropism and antigenicity (4). To assess whether the replacements found at these residues in the FMDV mutants isolated from cattle affected their growth capacity in cell culture, single-step growth curves were carried out in BHK-21 cells with mutants L9(R141G) and 40(L147P). Variants carrying replacement L144P were not included in this analysis, since it was not possible to obtain viral preparations with appropriate titers in which this mutation was dominant. Viral titers recovered at different times postinfection were compared with those obtained with the parental C3 Arg85 (Fig. 1). Mutant L9(R141G) showed a significant delay in the onset of virus production, since at 4 to 5 h postinfection titers in the culture medium were more than 1,000 times lower than those produced by C3 Arg85 (Fig. 1). A similar behavior, though less pronounced, was observed with mutant 40(L147P). A complete cytopathic effect was observed 8 h postinfection with C3 Arg85 and 24 h postinfection with L9(R141G) and 40(L147P).

FMDV mutants L9(R141G) and 40(L147P) produced small plaques with diameters of 2 to 3 mm and 4 mm in BHK-21 cells, respectively, while C3 Arg85 produced plaques with diameters of 6 mm, under the plating conditions detailed in Materials and Methods. Mutants L12(L144P), L5(L144P), and 25(L144P) exhibited a heterogeneous plaque phenotype consisting of large plaques, similar to those produced by C3 Arg85, and very small plaques (1 mm) that were detectable only after 96 h postinfection.

Antibody pressure contributes to the stability of FMDV mutants. To examine a possible role of immune pressure in the stability of the mutants isolated from partially immunized cattle, FMDV mutants L9(R141G), 25(L144P), and 40(L147P) were subjected to serial passages in both FBK-2 and BHK-21 monolayers in the presence of a subneutralizing concentration of the serum from the animal from which each FMDV mutant was isolated, as described in Materials and Methods. Parallel passages of the FMDV mutants and C3 Arg85 were carried out in the presence of the same concentrations of nonimmune cattle serum. After 10 serial passages, the nucleotide sequence of the site A-coding region was determined (Table 3). Passage of L9(R141G) and 40(L147P) in FBK-2 or BHK-21 cells in the presence of preimmune serum resulted in reversion to the C3 Arg85 sequence. In contrast, mutations G141 in L9(R141G) and P147 in 40(L147P) remained dominant upon passage of the viruses in the presence of homologous serum (Table 3). Therefore, sera from partially immune cattle generally contributed to the stability of amino acid replacements at site A. An exception was FMDV 25(L144P), since P-144 was not maintained under any passage conditions tested. Interestingly, the virus recovered after 10 passages in FBK-2 in the presence of nonimmune serum included the replacement P144S, which represents a new mutation at this position. This replacement also had a dramatic effect on binding of FMDV C1 to cells and antibodies (24, 26, 42). Other passage conditions resulted in reversion to C3 Arg85 sequence (Table 3). These results suggest that direct selective pressure exerted by host humoral

TABLE 3. Serial passages of viral mutants in the presence of homologous sera from partially immunized cattle

FMDV mutant	Passage[b] (cell line)	Amino acid mutation at[a]: A G **V** **R** R G D **L** A H **L** A A H A R H L **P**
C3 Arg85	Initial	R L L
	10 (FBK-2), with nonimmune serum	R L L
	10 (BHK-21), with nonimmune serum	R L L
L9(R141G)	Initial	G
	10 (FBK-2), with nonimmune serum	R[c]
	10 (FBK-2), with serum from animal L9	G
	10 (BHK21), with nonimmune serum	R[c]
	10 (BHK21), with serum from animal L9	G
40(L147P)	Initial	P
	10 (FBK-2), with nonimmune serum	L[c]
	10 (FBK-2), with serum from animal 40	P
	10 (BHK21), with nonimmune serum	L[c]
	10 (BHK21), with serum from animal 40	P
25(L144P)	Initial	P
	10 (FBK-2), with nonimmune serum	S[d]
	10 (FBK-2), with serum from animal 25	L[c]
	10 (BHK21), with nonimmune serum	L[c]
	10 (BHK21), with serum from animal 25	L[c]

[a] Site A VP1 amino acid sequence of FMDV C3 Arg85, according to Taboga et al. (41). Amino acids in boldface type are V140, R140a, L144, L147, and P155, respectively. Only mutated amino acids are shown below.
[b] Passages were initiated with the virus from the second [25(L144P)] or third passage [viruses C3 Arg85, L9(R141G), and 40(L147P)] in FBK-2 cells. Passages in the presence of homologous immune serum or nonimmune serum were carried out as described in Materials and Methods.
[c] Reversion.
[d] Mutation.

immune response played an important role in both the selection and stability of some antigenic variants of FMDV.

Antigenic characterization of FMDV mutants with MAbs. Antigenic site A of FMDV serotype C consists of multiple, overlapping epitopes (24). Amino acid substitutions at this site may affect one, a few, or all the epitopes defined by site A-specific MAbs. To evaluate whether the amino acid replacements at site A of FMDV mutants isolated from partially immune cattle led to alterations in FMDV antigenicity, the reactivity of the mutants and of C3 Arg85 with MAbs 7CA11, 7JD1, 7AB5, 7EE6, 7FC12, 7CA8, 7AH1, and 7CH1 was measured by ELISA and SN assays. Replacement L147P abolished the binding of MAb 7JD1 and diminished the reactivity with MAbs 7FC12, 7CA8, 7CA11, and 7EE6 (Fig. 2A). Likewise, replacement L144P abolished the binding of MAbs 7FC12 and 7CH1 and diminished reactivity with MAbs 7AB5, 7JD1, and 1BH8. Substitution R141G did not alter the ELISA reactivity with any of the anti-site A MAbs tested. Eight additional MAbs (1BH8, 2E5, 3G11, 2A12, 3E9, 5C4, 5H10, and 7DF10) directed to the discontinuous antigenic site D in type C FMDV were also included in these experiments. The recognition by anti-site D MAbs was not affected by any of the three replacements, except for a diminution in reactivity of MAb 1BH8 with L144P mutants in ELISA (Fig. 2A). Replacement L147P had a drastic effect in preventing neutralization by five out of the six MAbs directed to continuous site A epitopes (Fig. 2B). For L9(R141G), only a slight decrease in neutralization was noticed with MAb 7AB5. Replacements R141G and L147P did not affect neutralization by discontinuous MAbs (Fig. 2B). No SN assays could be performed with mutant L12(L144P), due to its poor plating efficiency on BHK-21 cells.

These results indicate that the three replacements found in mutant viruses have remarkable effects on the antigenic properties of FMDV. In particular, substitutions L144P and L147P had a profound effect on the antigenicity of FMDV. Therefore, amino acid substitutions in the G-H loop of capsid protein VP1 of FMDV selected during replication in partially immunized cattle manifest alterations in both host cell tropism and antigenicity.

DISCUSSION

In the present study we have analyzed the growth capacity and stability in cell culture, as well as the antigenic properties, of serotype C FMDV mutants with single amino acid replacements R141G, L144P, and L147P, in antigenic site A, a major antigenic site of FMDV. These variants were generated during viral replication in cattle in the presence of high titers of neutralizing antibodies elicited against peptide constructions which included the amino acid sequence of antigenic site A. The variants were isolated from lesions of cattle, and RNA was subjected to RT-PCR amplification and nucleotide sequencing without virus amplification in cell culture (41).

In each of the mutants analyzed, the amino acid replacement found in antigenic site A was the only one found in the capsid-coding region. The three amino acids that were replaced in the variants are thought to be essential for integrin recognition of FMDV (17, 33). The isolation of mutant L9(R141G) from partially immunized cattle, with no other replacements in the consensus sequence of the viral capsid, is in contrast with results on isolation of mutants of FMDV C1 (clone C-S8c1) in cell culture. In the latter case, dispensability of the RGD triplet

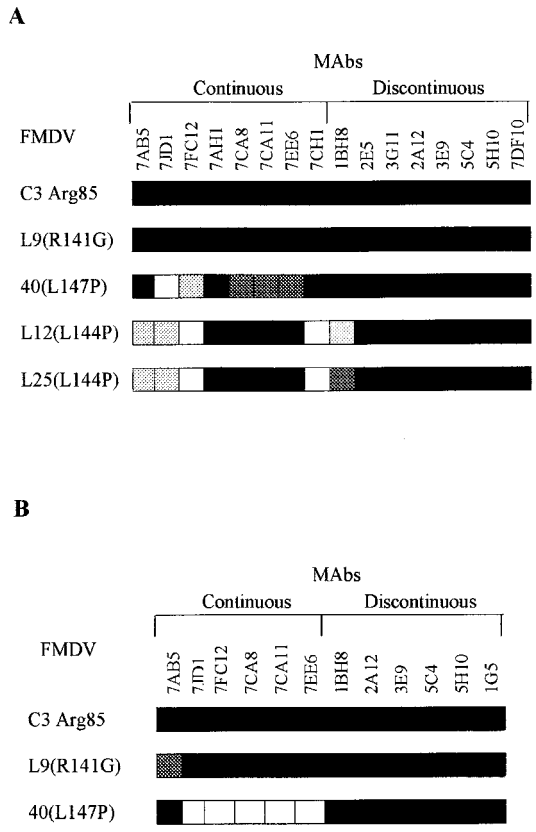

FIG. 2. Reactivity of MAbs with FMDV C3 Arg85 and mutants. (A) MAbs that recognize distinct continuous epitopes within site A and MAbs that define discontinuous epitopes outside site A (2, 20, 23) were used. ELISA was performed as described in Materials and Methods. For each MAb a relative Ri was calculated as the ratio between the MAb titer for any mutant virus and the reactivity for C3 Arg85. (B) Neutralization of infectivity was performed using a 50% plaque reduction neutralization assay (see Materials and Methods). SN titers are expressed as the reciprocal of the logarithm of the bovine serum dilution that caused 50% plaque reduction. The relative Ri was calculated as the ratio between the neutralizing titer for any mutant and the neutralizing titer for C3 Arg85 for each MAb. In both panels, Ri values are shown as follows: solid box, positive ($Ri > 1$); dark gray box, intermediate ($0.01 < Ri < 0.1$); light gray box, weak ($0.001 < Ri < 0.01$); open box, negative ($Ri < 0.001$).

was conditioned to additional replacements at exposed capsid sites (4, 6, 22). Such replacements allowed the use of an alternative receptor for entry of FMDV in BHK-21 cells (5).

The triplet RGD is highly conserved among natural FMDV isolates (11, 12), and this is probably due to the use of integrin receptors in vivo (7, 16, 23, 27). It is therefore very intriguing that the nonconservative amino acid replacements R141G, L147P, and L144P have been found among viruses isolated from lesions in cattle (Table 2). Evidence that these variant viruses had an altered interaction with cellular integrins was provided by the experiments in which the viruses directly isolated from lesions were grown in cell cultures. Sequence analysis of the capsid-coding region of the viral populations recovered after infection of BHK-21 cells revealed an unequal stability of each of the viruses in this cell line (Table 2). After two passages, mutants with the replacements at VP1 position L144P and L147P resulted in viral populations that reverted totally or partially to the parental sequence. After three passages in FBK-2 cells, two of the three variants bearing replacement L144P showed a partial reversion to the parental sequence, while mutant 40(L147P) remained dominant (Table 2). Sequence analyses of heterogeneous L144P populations are required to understand the molecular basis of viral growth phenotype. The reason for the relatively higher stability of these mutations in FBK-2 cell was not investigated, but it could be due to the fact that these cells derive from a primary culture of bovine cells, and therefore, they might maintain selective constraints present in cells from vaccinated animals. The selective disadvantage associated with these replacements in cell culture was confirmed by their reversion after 10 passages in BHK-21 or FBK-2 cells in the absence of specific neutralizing antibodies (Table 3). Interestingly, while P-147 reverted to the parental sequence, P-144 was replaced by S-144, a residue expected also to impair cell recognition and antibody binding (26, 43).

Mutant L9(R141G) was stable upon three passages in BHK-21 and FBK-2 cells (Table 2). The results denote that, in the context of the capsid proteins of C3 Arg85, an unaltered RGD motif is not essential for replication in both cattle and cell culture, suggesting functional flexibility of FMDV to enter cells in vivo. It may be significant, however, that mutant L9(R141G) shows a 4-h delay in the initiation of virus release in cell culture (Fig. 1) and that 10 serial passages in the absence of specific antibodies resulted in reversion of the amino acid replacement (Table 3). We cannot exclude the possibility that differences in the replication kinetics between the mutants and C3Arg85 could be mediated by virus-cell interactions affecting steps in the virus cycle other than receptor binding. However, our results suggest that despite its selection in partially immunized cattle, mutant L9(R141G) is affected in cell recognition and initiation of the productive infectious cycle. A decrease in plaque size in BHK-21 cell monolayers of L144P mutants and to a lesser extent of L9(R141G) and 40(L147P) is also consistent with an impairment of viral multiplication compared with their parental FMDV C3 Arg85. The results suggest that the three mutations found in partially immunized cattle influence the efficiency of integrin recognition. Further work is required to analyze the interaction of these mutants with the major FMDV integrin receptors $\alpha_v\beta_3$ and $\alpha_v\beta_6$ identified to date (7, 17) and to understand their interaction with bovine FMDV receptors (18).

The likely involvement of the host antibody response in the selection of variants L9(R141G) and 40(L147P) was indicated by neutralization experiments (Table 1) and also by dominance of these mutations during serial passage, conditioned to the presence of anti-FMDV antibodies (Table 3). Homologous serum (from animal 40) and sera obtained from protected (animals L1 and 12) and unprotected cattle (animals L9, L12, L5, and 25) were not efficient in neutralization of mutant 40(L147P). The reduction observed in neutralization titers of L9(R141G) by homologous serum (animal L9) was of lower magnitude, but it also evidenced a decrease in the neutralization efficiency by host cattle antibodies compared with neutralization of C3 Arg85 (SN titer of 1.4 against L9, versus 2.5 against C3 Arg85, Table 1). The observed differences in the neutralization of mutants L9(R141G) and 40(L147P) by heterologous sera may reflect a different proportion of antibodies

directed to the epitopes that compose antigenic site A (24, 41). No differences in neutralizing titers were detected between 40(L147P) or L9(R141G) and C3 Arg85 with a serum obtained from conventionally vaccinated FMDV cattle (animal 1.1). In addition to antibodies directed to antigenic site A, the inactivated FMDV vaccine induces a broad spectrum of antibodies against site C (the carboxy-terminal region of VP1) and the complex discontinuous antigenic site D (21, 24). These results emphasize the importance of antigenic sites other than site A in FMDV neutralization and suggest once more that animal immunization with an antigen representing a single antigenic site may promote selection of virus variants bearing amino acid substitutions at critical positions of the antigenic site (39, 41).

The ELISA results against a panel of neutralizing MAbs confirmed that replacements L144P and L147P resulted in a significant alteration of the antigenic properties of the virus (Fig. 2A). Likewise, replacement L147P abolished neutralization by five out of the six MAbs against site A that were tested (Fig. 2B). In contrast with the reduction in the neutralization titers observed with cattle sera (Table 1), replacement R141G did not significantly affect either the binding in ELISA or the neutralization exerted by the MAbs tested (Fig. 2). Replacement R141G alone, in the context of the C-S8c1 capsid, also had a minimal effect in the interaction of peptide antigens with MAbs (43). These results suggest differences in the epitopes contributed by residue 141 that are recognized by sera from peptide-vaccinated cattle and by the murine MAbs used in this study.

The relevance of antibody selection in maintenance of replacements L147P and R141G is supported by the requirement of subneutralizing concentrations of homologous sera for mutant stability upon serial passages in BHK-21 and FBK-2 (Table 3). The immune selective pressure exerted by a limited repertoire of antibodies directed against site A may force low-fitness, rare mutants from the mutant spectrum to become dominant in partially immune animals. Consequently, in cell culture a similar mechanism could be involved in maintaining the equilibrium in the viral quasispecies. On the contrary, mutant 25(L144P) reverted to the parental leucine in FBK-2 and BHK-21 cells under the same passage conditions, suggesting that other selective pressures influence the stability of the virus in these cell lines.

Evidence from several viral systems, following the pioneer studies of Skehel and colleagues with influenza virus (38), supports the concept of coevolution of antigenicity and host cell tropism both in cell culture and in vivo (4, 34). This coevolutionary potential is likely to contribute to expansions in cell tropism and host range, as suggested by the use of alternative receptors, often belonging to disparate cell surface macromolecules by the same virus (6, 37, 40). For FMDV, the first demonstration of the potential for coevolution of antigenicity and host tropism was obtained through structural studies (42) that showed that the RGD involved in integrin recognition was also critically involved in the interaction with a neutralizing antibody. The results have been extended to other antibodies as well as to variant forms of peptide antigens (30, 43). The analysis of FMDV escape viruses isolated from partially immunized cattle that differed in a single amino acid replacement within antigenic site A has revealed an amazing combination of alterations in antigenicity and capacity to infect and be maintained in cultured cells. These results with FMDV mutants generated in vivo provide further evidence of the high potential for virus adaptation in the face of an immune response, with consequences for virus pathogenesis.

ACKNOWLEDGMENTS

We thank O. Zabal, Culture Cell Laboratory of INTA, Buenos Aires, Argentina, for supplying the FBK-2 cells; Pan American Foot-and-Mouth Disease Center (PAHO/WHO) for supplying monoclonal antibodies; L. Jones for help in statistical analyses; and J. Lorenzo for technical assistance.

Work at the Instituto de Biotecnología was supported by INTA. Work at CBMSO-INIA was supported by grants PM97-0060-C02-01, BMC 2001-1823-C02-01, BIO 99-0833-02-01, and FAIR CT97-3665 and by Fundación Ramón Areces.

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