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Animal-Derived Antigenic Variants of Foot-and-Mouth Disease Virus Type A_{12} Have Low Affinity for Cells in Culture

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We recently have shown that binding of foot-and-mouth disease virus (FMDV) to cells in culture requires an arginine-glycine-aspartic acid (RGD) sequence in the G-H loop of the capsid protein VP1 (P. W. Mason, E. Rieder, and B. Baxt, Proc. Natl. Acad. Sci. USA 91:1932–1936, 1994). In this report, we show that FMDV type A_{12} viruses found in infected bovine tongue tissue (BTT) differ from their tissue culture-grown derivatives at amino acid residues near the RGD. Viruses genetically engineered to contain VP1 sequences found in animal tissue (BTT viruses) were antigenically different from their tissue culture derivatives and bound to BHK cells more poorly than did the tissue culture-adapted viruses. Passage of the genetically engineered BTT viruses in BHK cells resulted in the rapid selection of variants with cell-binding properties, antigenic characteristics, and sequences typical of tissue culture-adapted viruses. These data indicate that residues near the RGD are critical for cell binding and that interpretations of antigenic variation of FMDV can be affected by virus cultivation in vitro.

Studies of the three-dimensional structure of foot-and-mouth disease virus (FMDV) have revealed that the prominent surface feature formed by the loop between the G and H β strands (G-H loop) of VP1 is partially disordered under some conditions (1, 20, 25). The G-H loop also has exceptional genetic variability, as shown by the existence of a large number of antigenic variants containing mutations in the loop sequence (3, 8, 13, 24, 26, 28, 30). Despite its physical and genetic flexibility, the loop contains a conserved three-amino-acid sequence, arginine-glycine-aspartic acid (RGD). On the basis of this finding, and of the fact that the RGD sequence is important for binding several extracellular matrix proteins to a class of cell surface proteins known as integrins (15), Pfaff et al. proposed that the receptor for FMDV could be an integrin (26). This possibility was supported by studies showing that synthetic peptides containing the RGD sequence specifically inhibit FMDV binding to cells (2, 12) and confirmed by a study demonstrating that genetically engineered viruses with mutations in the RGD sequence are unable to bind to or infect cells in culture (21). This latter study also showed that several alterations in the sequence surrounding the RGD did not have a major influence on virus binding to cells in culture (21).

Differences in growth, antigenic, and immunogenic properties have been noted between field strains and tissue culture-adapted FMDVs (5); however, the biochemical basis for these differences is unknown. Diez et al. (6) have shown that serial passage of FMDV C-S8 in BHK cells resulted in selection of antigenic variants containing changes in the G-H loop of VP1, and monolayer culture-propagated FMDV type A_{22} and its suspension culture-adapted derivative differ in VP2 sequences and binding to BHK cells (4). The FMDV genome is highly mutable, and virus populations which contain mixtures of genomes are referred to as quasispecies (22). The quasispecies nature of FMDV could permit the rapid selection of variants upon introduction into tissue culture, consistent with findings of Meyer et al. (23) showing that genomes of viruses derived from infected bovine tissues differ from the genomes detected following growth in tissue culture. Proliferation of virus variants with altered biological characteristics has been described for many different RNA viruses, notably human immunodeficiency virus and FMDV (7), but many of these observations have been based on characteristics of tissue culture-propagated viruses.

To investigate the relationship between tissue culture-derived serotype A_{12} variants and viruses growing in infected animals, we sequenced the G-H loop of viruses present in bovine tongue tissue (BTT) from cattle infected with the A_{12} Vallee strain 119 (from a 1932 outbreak in England). One sample, BTT-1, was derived from an infected-tongue homogenate prepared in 1958. A second sample, BTT-2, was obtained from an animal infected by placing it in contact with a virus-inoculated steer. Culture-adapted derivatives of BTT viruses, BTT-2/BHKp1 and BTT-1/BHKp2, were isolated from BHK cells following either one or two passages, respectively. cDNA fragments spanning the VP1 coding region were amplified from all four samples by PCR (31), and sequences of the G-H loop were determined from the PCR products (14), revealing differences from the prototype A_{12} virus (29) and other variants of A_{12} (24, 30) at positions 147, 149, and 152 of VP1 (Table 1).

Selection of tissue culture variants with mutations in their G-H loops indicated that a subpopulation in the original tongue tissue was more fit in culture, preventing study of the viruses present in the animals. To compare BTT viruses and their culture derivatives, full-length cDNA molecules containing the four different G-H loop sequences shown in Table 1 were generated and used to produce infectious RNAs by using T7 RNA polymerase (27). The four synthetic RNA genomes were transfected into BHK cells by electroporation to produce first-cycle viruses with sequences identical to those of their parent plasmids (21). Sucrose density gradient analyses showed that similar amounts of [^{35}S]methionine-labeled virus particles

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TABLE 1. Properties of viruses recovered from BHK cells transfected with infectious-clone RNAs carrying VP1 sequences from animal-derived or cell culture-grown viruses

RNA source[a]	Sequence[b]	cDNA clone[c]	Yield (PFU/ml)[d]	% Reactivity with MAb[e]:				% Binding to BHK cells[f]
				2PD11	6EE2	2FF11	7SF3	
A_{12} 119ab	RGDFGSLAP	pRM-FSP	4.0×10^6	53	48	62	45	47 ± 8
BTT-1	---S-S--P	pRM-SSP	1.0×10^6[g]	54	45	41	51	12 ± 4
BTT-1/BHKp2	---S-S--L	pRM-SSL	2.5×10^6	64	59	77	24[h]	46 ± 3
BTT-2	---S-P--P	pRM-SPP	5.0×10^2[g]	62	51	53	53	4 ± 0.2
BTT-2/BHKp1	---S-P--L	pRM-SPL	1.5×10^6	56	46	56	16[h]	44 ± 2

[a] Source of VP1 sequence. A_{12} 119ab is the prototype sequence, BTT-1 and BTT-2 are sequences obtained directly from BTT, and BTT-1/BHKp2 and BTT-2/BHKp1 are sequences from tissue culture-grown viruses.
[b] Sequence of amino acids 144 to 152 of VP1. Dashes indicate identity to the A_{12} 119ab sequence in pRM-FSP ($pRMC_{35}$ [25]); sequences were determined by direct sequencing (14) of PCR products (31) derived from viral cDNA (see text).
[c] Clones are designated by amino acid residues at positions 147, 149, and 152 of VP1.
[d] PFU recovered from BHK cells transfected with the indicated RNAs by electroporation (2).
[e] Percent labeled virus bound to the indicated MAb at an immunoglobulin G concentration of 0.8 μg/ml.
[f] Percent labeled virus, recovered from electroporated cells, that bound to cells in 2 h at room temperature (2). Results are averages ± standard deviations for three experiments.
[g] Plaques were turbid.
[h] Similar to reactivity observed with MAb escape mutants altered at position 152 (3).

were produced by BHK cells transfected with the various transcripts (data not shown), indicating that their G-H loop sequences had no effect on RNA replication or virion assembly. However, these virions showed differences in their ability to bind to BHK cells (Table 1). Specifically, viruses recovered from cells transfected with BTT/BHK RNAs attached to cells as well as did the prototype virus, whereas virus particles recovered from BTT RNAs bound to BHK cells very poorly, and virus with the BTT-2 sequence was very inefficient at producing plaques on BHK cells (Table 1). These differences in binding were also observed with primary bovine kidney cells: 30% binding was achieved for both viruses with BTT/BHK sequences, and <10% binding was achieved for the viruses with BTT sequences.

Monoclonal antibody (MAb) reactivities revealed that the tissue culture-adapted viruses were antigenically different from viruses present in bovine tongue. These studies employed four well-characterized virus-neutralizing MAbs (3), including one (7SF3) which was generated by using a synthetic peptide corresponding to the G-H loop of VP1 of the A_{12} prototype virus (3). Both viruses containing BTT sequences reacted well with MAb 7SF3 (Table 1), whereas the two viruses with tissue culture-adapted sequences reacted poorly with this MAb (Table 1), whose epitope has been mapped to position 152 of VP1 in neutralization escape studies (3). The poor reactivity with this G-H loop-specific MAb did not reflect an overall change in the antigenic structure of the virion, since the three other MAbs, which recognize sites distant from the G-H loop (3), reacted well with all four viruses (Table 1).

The molecularly defined BTT viruses harvested from electroporated BHK cells did not readily cause cytopathic effect in this cell type, consistent with their turbid-plaque phenotype (Table 1). However, after four blind passages in BHK cells, virus populations that were as cytopathic as BTT/BHK viruses were obtained. Sequence analyses of fourth-passage BTT populations revealed a mixture of genomes, so several plaques were picked from each sample and further characterized. Several different variants were detected in these analyses (Table 2), and representatives of each of the genetic types were checked for binding to BHK cells; all of these viruses bound well to BHK cells (Table 2).

Selection of variants with enhanced binding to BHK cells indicates that amino acids 147 and 152 of VP1 influence cell binding. Among the viruses we detected, sequence variation in the G-H loop occurred only at these two positions and all nucleotide substitutions resulted in amino acid changes. Interestingly, all variant genomes differed from their parents by a single nucleotide, and in all cases the substitutions were transitions, consistent with the prevalence of transitions versus transversions among picornavirus mutants (9, 13, 17). These

TABLE 2. Properties of viruses obtained from fourth passage of electroporation-derived viruses

cDNA clone	Original sequence[a]	Sequence detected after passage[b]	Frequency[c]	PRN_{50}[d] for:			% Binding to BHK cells[e]
				2PD11	2FF11	7SF3	
pRM-SSP	---S-S--P	---S-S--L	1/5	1.7	3.0	>3.3[f]	63 ± 4
		---L-S--P	4/5	1.7	3.3	2.1	69 ± 11
pRM-SSL	---S-S--L	---S-S--L	2/2	1.7	3.0	>3.3[f]	60 ± 8
pRM-SPP	---S-P--P	---F-P--P	3/6	1.4	2.7	2.4	35 ± 4
		---S-P--S	3/6	1.4	3.0	>3.3[f]	59 ± 21
pRM-SPL	---S-P--L	---S-P--L	2/2	1.4	3.3	>3.3[f]	42 ± 16

[a] Sequence of amino acids 144 to 152 of VP1. Dashes indicate identity to the A_{12} 119ab sequence in pRM-FSP ($pRMC_{35}$ [25]), RGDFGSLAP.
[b] Sequence found in individual plaques examined, determined as described in footnote *b* to Table 1.
[c] Frequency with which sequence was found.
[d] Log of MAb concentration (nanograms per milliliter) that reduced plaque number by 50%.
[e] Percent labeled virus bound in 2 h at room temperature (2). Results are averages ± standard deviations for two experiments.
[f] No neutralization at the highest MAb concentration tested (2 μg/ml).

viruses comprise at least two antigenically distinguishable groups, based on their ability to be neutralized by three MAbs. Two of these MAbs, 2PD11 and 2FF11, which react with residues outside the G-H loop (3), displayed similar neutralization titers with all six viruses tested (Table 2). However, only those viruses which had a P at position 152 were neutralized by 7SF3 (Table 2), which recognizes an epitope within the G-H loop (3) (see above). Several of the amino acid substitutions detected in these six viruses were observed in earlier descriptions of antigenic variants of this virus (24, 30), indicating that tissue culture propagation has influenced previous interpretations of antigenic structure of the A_{12} virus. Rapid selection of variant populations from homogeneous molecularly defined genomes demonstrates that enhanced binding to cells is a powerful selective pressure. The fact that a single variant was detected following inoculation of each of the infected animal tissues in BHK cells (Table 1) was probably due to a biased repertoire of sequences in the infected tissue, favoring growth of a single isolate upon introduction into tissue culture.

Although the mechanism by which substitutions at residues 147 and 152 alter binding of FMDV to cells is unclear, changes at these positions could alter the ability of RGD to be recognized by its receptor, or residues 147 and 152 could be included in the binding pocket of the cellular receptor. The relationship of residues 147 and 152 to improved cell binding must be complex, since alteration of either S at residue 147 or P at residue 152 can produce a virus with a high-binding phenotype (Tables 1 and 2). Furthermore, the additional P found at position 149 of the BTT-2 virus appears to have only a modest effect on binding, since it was retained in all three tissue culture-adapted derivatives of BTT-2. The three-dimensional structure of type A_{12} virus is not known, but the structure of serotype O_1 has been solved under conditions which stabilize the otherwise-flexible G-H loop of VP1. In this structure, amino acids 147 to 152 are present in a 3_{10} helix prominently displayed on the surface of the virion (20). On the basis of this structure, several hypotheses can be made about the effect of individual amino acid substitutions if a similar helix were formed by the G-H loop of the A_{12} virus: (i) the presence of a hydrophilic residue at position 147 (S) would disrupt potential hydrophobic interactions of the helix with the surface of VP2, (ii) the presence of a P at position 149 would shorten or disrupt the helix, and (iii) the presence of a P at position 152 would destabilize the helix.

Adaptation of viruses to cell culture systems can result from changes at several different genetic loci, including changes in the nonstructural protein genes, as for the picornavirus hepatitis A (10, 11). We have identified specific amino acid sequences of FMDV that are rapidly replaced by more fit sequences upon passage in culture. These sequences probably function in cell binding in conjunction with the RGD sequence, which is absolutely required for virus replication in tissue culture (21). These results indicate that sequences required for efficient propagation of FMDV in tissue culture can be different from those prevalent in infected animals, and suggest that viruses with different cell-binding characteristics could evolve during an outbreak or during the infection of a single individual. Correlation between virus adaptation to different culture conditions and selection of antigenic variants of FMDV agrees with observations of other viruses, including swine influenza (16) and hepatitis A (18, 19).

In addition to being important in the understanding of virus-host interactions, the finding that the cell-binding structures and antigenic determinants of FMDV overlap could have practical implications. Specifically, this fact could complicate disease surveillance, evaluation of antigenic variation, and the production of vaccines from field strains, since these procedures typically rely on cultivation of virus in tissue culture.

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REFERENCES

1. **Acharya, R., E. Fry, D. Stuart, G. Fox, D. Rowlands, and F. Brown.** 1989. The three-dimensional structure of foot-and-mouth disease virus at 2.9 Å. Nature (London) **337:**709–716.
2. **Baxt, B., and Y. Becker.** 1990. The effect of peptides containing the arginine-glycine-aspartic acid sequence on the adsorption of foot-and-mouth disease virus to tissue culture cells. Virus Genes **4:**73–83.
3. **Baxt, B., V. Vakharia, D. M. Moore, A. J. Franke, and D. O. Morgan.** 1989. Analysis of neutralizing antigenic sites on the surface of type A_{12} foot-and-mouth disease virus. J. Virol. **63:** 2143–2151.
4. **Bolwell, C., A. L. Brown, P. V. Barnett, R. O. Campbell, B. E. Clarke, N. R. Parry, E. J. Ouldridge, F. Brown, and D. J. Rowlands.** 1989. Host cell selection of antigenic variants of foot-and-mouth disease virus. J. Gen. Virol. **70:**45–57.
5. **Cowan, K. M., N. Erol, and A. P. Whiteland.** 1974. Heterogeneity of type Asia 1 foot-and-mouth disease virus and BHK-21 cells and the relationship to vaccine production. Bull. Off. Int. Epizoot. **81:**1271–1298.
6. **Diez, J., M. G. Mateu, and E. Domingo.** 1989. Selection of antigenic variants of foot-and-mouth disease virus in the absence of antibodies, as revealed by an in situ assay. J. Gen. Virol. **70:**3281–3289.
7. **Domingo, E., J. Díez, M. A. Martínez, J. Hernández, A. Holguín, B. Borrego, and M. G. Mateu.** 1993. New observations on antigenic diversification of RNA viruses. Antigenic variation is not dependent on immune selection. J. Gen. Virol. **74:**2039–2045.
8. **Domingo, E., M. G. Mateu, M. A. Martínez, J. Dopazo, A. Moya, and F. Sobrino.** 1990. Genetic variability and antigenic diversity of foot-and-mouth disease virus, p. 233–266. *In* E. Kurstak, R. G. Marusyk, F. A. Murphy, and M. V. H. Van Regenmortel (ed.), Applied virology research, vol. 2. Plenum Publishing Corp., New York.
9. **Drake, J. W.** 1993. Rates of spontaneous mutation among RNA viruses. Proc. Natl. Acad. Sci. USA **90:**4171–4175.
10. **Emerson, S. U., Y. K. Huang, C. McRill, M. Lewis, and R. H. Purcell.** 1992. Mutations in both the 2B and 2C genes of hepatitis A virus are involved in adaptation to growth in cell culture. J. Virol. **66:**650–654.
11. **Emerson, S. U., Y. K. Huang, and R. H. Purcell.** 1993. 2B and 2C mutations are essential but mutations throughout the genome of HAV contribute to adaptation to cell culture. Virology **194:**475–480.
12. **Fox, G., N. R. Parry, P. V. Barnett, B. McGinn, D. J. Rowlands, and F. Brown.** 1989. The cell attachment site on foot-and-mouth disease virus includes the amino acid sequence RGD (arginine-glycine-aspartic acid). J. Gen. Virol. **70:**625–637.
13. **Gebauer, F., J. C. de la Torre, I. Gomes, M. G. Mateu, H. Barahona, B. Tiraboschi, I. Bergmann, P. Augé de Mello, and E. Domingo.** 1988. Rapid selection of genetic and antigenic variants of foot-and-mouth disease virus during persistence in cattle. J. Virol. **62:**2041–2049.
14. **Higuchi, R. G., and H. Ochman.** 1989. Production of single-stranded DNA templates by exonuclease digestion following the polymerase chain reaction. Nucleic Acids Res. **17:**5865.
15. **Hynes, R. O.** 1992. Integrins: versatility, modulation, and signaling in cell adhesion. Cell **69:**11–25.
16. **Kilbourne, E. D., A. H. Taylor, C. W. Whitaker, R. Sahai, and A. J. Caton.** 1988. Hemagglutinin polymorphism as the basis for low- and high-yield phenotypes of swine influenza virus. Proc. Natl. Acad. Sci. USA **85:**7782–7785.
17. **Kuge, S., N. Kawamura, and A. Nomoto.** 1989. Strong inclination toward transition mutation in nucleotide substitutions by poliovi-

rus replicase. J. Mol. Biol. **207**:175–182.
18. **Lemon, S. M., L. N. Binn, R. Marchwicki, P. C. Murphy, L.-H. Ping, R. W. Jansen, L. V. S. Asher, J. T. Stapleton, D. G. Taylor, and J. W. LeDuc.** 1990. In vivo replication and reversion to wild type of a neutralization-resistant antigenic variant of hepatitis A virus. J. Infect. Dis. **161**:7–13.
19. **Lemon, S. M., P. C. Murphy, P. A. Schields, L.-H. Ping, S. M. Fienstone, T. Cromeans, and R. W. Jansen.** 1991. Antigenic and genetic variation in cytopathic hepatitis A virus variants arising during persistent infection: evidence for genetic recombination. J. Virol. **65**:2056–2065.
20. **Logan, D., R. Abu-Ghazaleh, W. Blakemore, S. Curry, T. Jackson, A. King, S. Lea, R. Lewis, J. Newman, N. Parry, D. Rowlands, D. Stuart, and E. Fry.** 1993. Structure of the major immunogenic site on foot-and-mouth disease virus. Nature (London) **362**:566–568.
21. **Mason, P. W., E. Rieder, and B. Baxt.** 1994. RGD sequence of foot-and-mouth disease virus is essential for infecting cells via the natural receptor but can be bypassed by an antibody-dependent enhancement pathway. Proc. Natl. Acad. Sci. USA **91**:1932–1936.
22. **Mateu, M. G., M. A. Martínez, E. Rocha, D. Andreu, J. Parejo, E. Giralt, F. Sobrino, and E. Domingo.** 1989. Implications of a quasispecies genome structure: effect of frequent, naturally occurring amino acid substitutions on the antigenicity of foot-and-mouth disease virus. Proc. Natl. Acad. Sci. USA **86**:5883–5887.
23. **Meyer, R. F., M. Pacciarini, E. J. Hilyard, S. Ferrari, V. N. Vakharia, G. Donini, E. Brocchi, and T. W. Molitor.** Genetic variation of foot-and-mouth disease from field outbreaks to laboratory isolation. Virus Res., in press.
24. **Moore, D. M., V. N. Vakharia, and D. O. Morgan.** 1989. Identification of virus neutralizing epitopes on naturally occurring variants of type A_{12} foot-and-mouth disease virus. Virus Res. **14**:281–296.
25. **Parry, N., G. Fox, D. Rowlands, F. Brown, E. Fry, R. Acharya, D. Logan, and D. Stuart.** 1990. Structural and serological evidence for a novel mechanism of antigenic variation in foot-and-mouth disease virus. Nature (London) **347**:569–572.
26. **Pfaff, E., H.-J. Thiel, E. Beck, K. Strohmaier, and H. Schaller.** 1988. Analysis of neutralizing epitopes on foot-and-mouth disease virus. J. Virol. **62**:2033–2040.
27. **Rieder, E., T. Bunch, F. Brown, and P. W. Mason.** 1993. Genetically engineered foot-and-mouth disease viruses with poly(C) tracts of two nucleotides are virulent in mice. J. Virol. **67**:5139–5145.
28. **Rieder Rojas, E., E. Carrillo, M. Schiappacassi, and R. Campos.** 1992. Modification of foot-and-mouth disease virus O1 Caseros after serial passages in the presence of antiviral polyclonal sera. J. Virol. **66**:3368–3372.
29. **Robertson, B. H., M. J. Grubman, G. N. Weddell, D. M. Moore, J. D. Welsh, T. Fischer, D. J. Dowbenko, D. G. Yansura, B. Small, and D. G. Kleid.** 1985. Nucleotide and amino acid sequence coding for polypeptides of foot-and-mouth disease virus type A12. J. Virol. **54**:651–660.
30. **Rowlands, D. J., B. E. Clarke, A. R. Carroll, F. Brown, B. H. Nicholson, J. L. Bittle, R. A. Houghton, and R. A. Lerner.** 1983. Chemical basis of antigenic variation in foot-and-mouth disease virus. Nature (London) **306**:694–697.
31. **Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich.** 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239**:487–491.