JOURNAL OF VIROLOGY, July 1991, p. 3949–3953
0022-538X/91/073949-05$02.00/0

Vol. 65, No. 7

Antigenic Stability of Foot-and-Mouth Disease Virus Variants on Serial Passage in Cell Culture

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Received 31 January 1991/Accepted 9 April 1991

Two neutralizing monoclonal antibody (MAb)-resistant variants selected from an isolate of foot-and-mouth disease virus (FMDV) type A5 were repeatedly passaged in cell culture and monitored for susceptibility to neutralization by the selecting MAb. A variant isolated with a MAb to a conformational epitope (1-OG2) lost resistance in 20 passages, while a variant isolated with a MAb to a linear epitope (1-HA6) persisted for 30 passages. In both cases, the virus population emerging after passage was antigenically and genetically indistinguishable from the original wild-type parental virus (FMDV A5 Spain-86). Coinfection assays with the wild type and each variant, and between the variants, showed rapid conversion to a homogeneous population. Wild-type virus prevailed over the variants and for coinfection between the variants, the linear epitope variant 1-HA6. While both variants arose from a single nucleotide substitution and reversion to wild type occurred for each, it appears that the variant based on the continuous epitope (1-HA6) was more stable. We discuss the implications of these results for the antigenic diversity of FMDV and its relationship to virus evolution.

Foot-and-mouth disease virus (FMDV), a member of the family *Picornaviridae*, is the cause of an economically devastating disease of cloven-hoofed animals. The virus possesses a single-stranded positive RNA molecule greater than 8,000 nucleotides (2) encapsidated in an icosahedral capsid made of 60 copies each of four proteins: VP1, VP2, VP3, and VP4 (30).

The genomic heterogeneity of RNA viruses is now clearly established (for reviews, see references 16 and 18). The presence of mixtures of very similar genomes coexisting in a single population constitutes experimental support for the quasispecies model (21, 22). This model implies that molecules that replicate with limited fidelity will be organized as distributions of related but nonidentical genomes that include one (or more) master sequences. These may represent a small proportion of molecules in a dynamic equilibrium with a mutant spectrum consisting of a distribution of single- and multiple-residue mutants. The proportion of each mutant depends not only on the frequency with which it appears but also on its competitive behavior with all the variants present at that moment in the population (4, 16, 20).

The emergence of (genetic) variants during the replication of FMDV has been extensively characterized in cell culture (8, 11, 13, 15, 32, 33), even after extensive plaque purification (41), and in field isolates (27–29, 34, 35). Genetic and antigenic heterogeneity has also been described in virus populations recovered from infected swine (10) and in persistently infected cattle (24). This antigenic diversity of FMDV and emergence of new strains are significant impediments for the design of more broadly protective vaccines (for a review, see reference 9). A recent approach to study of the diversity of FMDV populations is to locate and characterize antigenic sites through the isolation of monoclonal antibody (MAb)-resistant variants (for a review, see reference 19). This has allowed the definition of multiple antigenic sites on FMDV, depending on the virus serotype analyzed. Most of these antigenic sites have been located on VP1, but recently neutralizing epitopes located outside (or at least partially outside) VP1 have been described for serotypes O (3, 26) and A (7, 8, 31, 38).

We have examined the behavior of two FMDV type A5 antigenic variants when passaged in tissue culture, both alone and in competition with the parental wild type or the other variant, but in the absence of neutralizing antibodies. The data presented show the appearance of antigenic strains similar or identical to the parental virus after serial passages in vitro of plaque-purified MAb-resistant variants. In addition, we found a quick domination of the parental type over the variants in coinfection experiments and the imposition of one variant virus strain over the other when the variants coinfected cell cultures. We discuss these results in relation to the antigenic diversity of RNA viruses and the implication in viral evolution.

Virus strains, MAbs, and cells. FMDV type A5 Spain-86 (A5 Sp-86) was obtained from an isolate of a recent outbreak in Spain (1986) and three times plaque purified. Production and characterization of the MAbs used have been described elsewhere (32). MAb-resistant variants 1-HA6 and 1-OG2 were obtained from the parental virus population (A5 Sp-86) as previously described (31). Serial passages of the variant strains in the absence of neutralizing MAbs were made on monolayers of a continuous bovine kidney cell line (LFBK; 37) in 25-cm^2 tissue culture flasks, using a multiplicity of infection of 1 to 3 PFU per cell. A 10-fold dilution of the recovered material was used to infect a new flask, and this procedure was repeated 20 to 30 times, depending on the virus studied. Cultures were also coinfected with equal mixtures of the parental A5 virus and each variant or with a mixture of the two variants, and serial passages were made in a similar fashion. The virus passage stocks are identified by strain designation and a postscript passage designation; i.e., 1-HA6P30 indicates the population recovered from virus variant 1-HA6 after 30 serial passages; A5:1-OG2C6 indicates the viral population recovered after coinfection and

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TABLE 1. Neutralization characteristics of different viruses passaged in tissue culture as monitored by plaque reduction neutralization assay

Passage no.	PRN-70[a] titer of viruses and virus mixtures					
	1-OG2[b]	1-HA6[c]	A5:1-OG2[b]	A5:1-HA6[c]	1-OG2:1-HA6	A5 Sp-86
0	<0.30	<0.30	<0.30	<0.30	0.30,[b] <0.30[c]	2.63,[b] 1.90[c]
2	<0.30	<0.30	1.43	<0.30	2.80, <0.30	—
4	<0.30	<0.30	2.27	<0.30	2.55, <0.30	—
6	<0.30	<0.30	1.95	<0.30	2.38, <0.30	—
8	<0.30	<0.30	2.14	0.60	2.42, <0.30	—
10	<0.30	<0.30	2.31	0.50	2.39, <0.30	—
12	<0.30	<0.30	2.46	1.09	2.93, <0.30	—
14	<0.30	<0.30	2.49	1.28	2.71, <0.30	—
16	<0.30	<0.30	2.30	1.93	2.54, <0.30	—
18	<0.30	<0.30	—	—	—	—
20	2.04	<0.30	—	—	—	—
22	2.69	<0.30	—	—	—	—
24	1.97	<0.30	—	—	—	—
26	2.04	<0.30	—	—	—	—
28	2.29	1.80	—	—	—	—
30	2.30	2.10	—	—	—	—

[a] PRN-70, 70% plaque reduction neutralization titer (log_{10} value of dilution of MAb needed to reduce the number of input plaques by 70%); see text. <0.30 indicates 70% endpoint not met at 1:1 virus/MAb mixture, MAb full strength; —, not determined. Except for controls using A5 Sp-86, initial passages of virus show resistance to neutralization characteristic of the selected variants under test.

[b] MAb 23OG2 was used to obtain PRN-70 titers.

[c] MAb 23HA6 was used to obtain PRN-70 titers.

six passages of the parental virus, A5, and variant 1-OG2. The in vitro neutralization tests were performed by plaque reduction assay as described by Stave et al. (36), using LFBK cells. The log_{10} neutralization titers were determined as 70% plaque reduction neutralization by the logit-log transformation method (39).

Sequencing of viral RNA. Viruses from different passages or coinfections were amplified in monolayers of BHK-21 cells and purified as previously described (5, 40). FMDV RNA was prepared from purified virus as described by Grubman and Baxt (25), and sequencing reactions were performed by the primer extension-dideoxynucleotide chain termination method, using oligonucleotide primers sited in the P1 region of the genome as previously described (7, 31). Sequence information was stored and analyzed by using software provided under license from the Genetics Computer Group, Madison, Wis. (14).

Stability of antigenic variants in cell culture. The two MAb-resistant variants, 1-HA6 and 1-OG2, used in this study were found to recognize two antigenic sites which differ in complexity (32). Variant 1-HA6 corresponds to a linear epitope located near the C terminus of VP1, and variant 1-OG2 corresponds to a conformationally dependent epitope located on VP2 (31). Each variant was independently passed in LFBK cells, and the progeny were monitored for antigenic characteristics in neutralization assays using MAbs 23HA6 and 23OG2. The results, expressed as 70% plaque reduction neutralization titers, are shown in Table 1. Variant 1-OG2, obtained under the pressure of MAb 23OG2 and therefore no longer neutralized by this MAb, was again fully neutralized by MAb 23OG2 after 20 passages. Variant 1-HA6, obtained with MAb 23HA6, lost its resistance after 28 passages and was now fully neutralized by 23HA6. When the average neutralization data of at least three different assays were plotted, there was a slow increase in neutralizable virus to about 30% of the total population and, after a period of stability, a rapid increase until all of the viral particles tested were neutralized again by the selecting MAb (Fig. 1). The behaviors of the two variants were similar except that the final loss of resistance for variant 1-HA6 occurred about 10 passages after 1-OG2. The possible contribution to selection of virus strains by antibodies in fetal calf serum used in the cell cultures had previously been dismissed (15) and was again checked here. No neutralizing activity was present in the fetal calf serum used in the current tests (data not shown).

Behavior of the variants on coinfection of cell culture. When cultures were infected with a mixture of variant 1-OG2 and the parental virus (A5 Sp-86), a neutralization titer similar to that of the wild type was obtained after four passages, suggesting loss of the variant strain (Table 1). In the case of mixed infection with variant 1-HA6 and the parental A5 virus, the full neutralization titer was not reached until 16 passages. Finally, in coinfection using the two variants (1-HA6 and 1-OG2) as inoculum, only virus showing a neutralization behavior of the 1-HA6 variant could be found after two passages, indicating a rapid loss of the 1-OG2 variant.

In the coinfection experiments, when the average data of three different neutralization assays were plotted, it was apparent that variant 1-OG2 disappeared from the viral population in a few passages when passaged either with parental virus or with variant 1-HA6 (Fig. 2A and C). In the case of variant 1-HA6 and parental A5 virus, the disappearance of the variant was slower, showing a gradual increase in neutralizable virus through 20 passages (Fig. 2B).

Nucleotide sequences of recovered viruses. To advance in the understanding of the molecular variations occurring in these two variants after serial passages in vitro, nucleotide sequences of both viruses were analyzed and compared with those of the original variant and the parental virus. Variant 1-OG2 had acquired resistance to MAb 23OG2 via a single nucleotide change in the VP2 coding region (Asp to Asn at amino acid 72), and 1-HA6 had acquired resistance to MAb 23HA6 via a single nucleotide change resulting in a C-terminal VP1 residue change (Asp to Gly at amino acid 198) (31). After serial passage, variants 1-OG2 and 1-HA6 lost resistance to neutralization by the selecting MAb. Viral RNA was

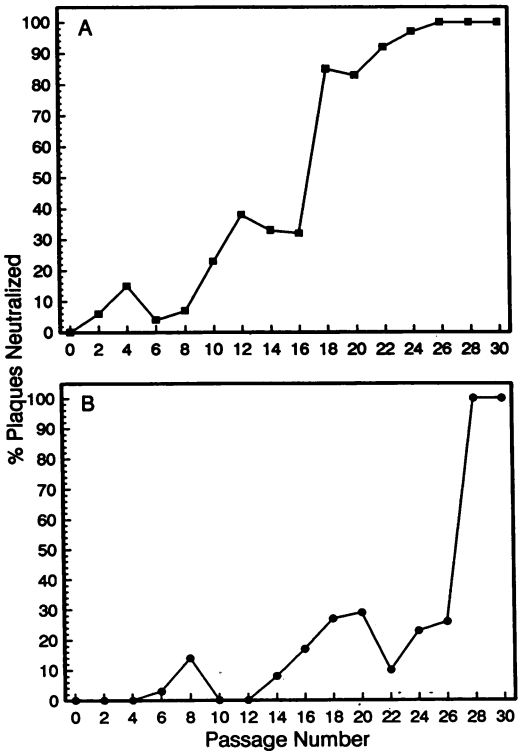

FIG. 1. Percentage of plaques neutralized for variants serially passed in cell culture. Data represent the averages of three assays showing the percentage of plaques able to be neutralized by the antiserum. Initial passages are represented by resistant variants which show characteristic resistance to neutralization; subsequent passages contain an increasing population of virus which can be neutralized. (A) Variant 1-OG2 tested with MAb 23OG2; (B) variant 1-HA6 tested with MAb 23HA6.

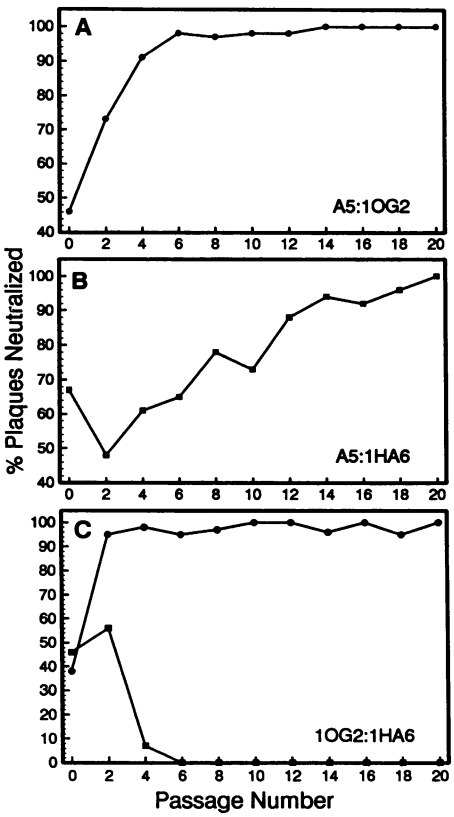

FIG. 2. Percentage of plaques neutralized in coinfection experiments following serial passage. The data are the averages of three assays of the percentage of neutralizable plaques as shown in Fig. 1. Values start at about 50% since the input fraction contains half wild-type virus (A5 Sp-86), which is fully neutralizable. (A) Variant 1-OG2 and parental FMDV A5 assayed with MAb 23OG2; (B) variant 1-HA6 and parental FMDV A5 assayed with MAb 23HA6; (C) variants 1-OG2 and 1-HA6 assayed with MAb 23OG2 (●) and MAb 23HA6 (■).

isolated after conversion (passage 20 for 1-OG2 and passage 30 for 1-HA6), and the nucleotide sequences of selected areas of the P1 region were determined. In each case, the parental A5 sequence was found (Table 2). In similar fashion, virus recovered from coinfection with parental type A5 was analyzed (A5:1-OG2, passage 6; A5:1-HA6, passage 20). In each case, the parental A5 sequence was found (Table 2). Finally, coinfection with the two variants resulted in the emergence after six passages of virus with the sequence of variant 1-HA6 (Table 2, 1-OG2:1-HA6 C6).

We have studied the behavior of two MAb-resistant variants of FMDV A5 in serial passages in tissue culture. The results show the emergence of a viral population antigenically and genetically indistinguishable from the wild type after serial passage of the MAb variants in the absence of external immunoselective pressure (Fig. 1). While both 1-OG2 and 1-HA6 displayed the sequence of the parental, wild-type FMDV A5 Sp-86 (31) on passage, it seems that the variant based on the continuous epitope (1-HA6) was more stable. Supporting this conclusion are the results of the coinfections between viruses: variant 1-OG2 disappeared soon after 2 passages when coinfected with the wild type; variant 1-HA6 persisted longer and gradually disappeared over 16 passes (Fig. 2). In addition, when the 1-HA6 and 1-OG2 variants were inoculated together, variant 1-HA6 emerged as the main viral population after just two passages.

When the variants were passaged, the emerging population of parental A5 virus quickly became dominant in the population. Both variants used here were selected by two plaque purifications under pressure of high concentrations of the selecting MAb and expanded in MAb-containing media before starting the serial passages. However, since antigenic variants for FMDV type O1 could be quickly isolated after extensive plaque purification (41), it is not possible to definitively exclude the persistence of a minute subpopulation of the parental virus in the original variant populations. Additionally, in the original isolation of MAb-resistant variants of type A5, MAb-resistant plaques emerged quickly (31) and sister isolates were regularly encountered not only with type A5 (31) but also with types A12 (7) and O1 (26), suggesting their presence in the original parental population of virus. In any case, whether a subpopulation was carried from the beginning or whether it appeared de novo during the serial replications in vitro, there seems to be a stable coexistence for a period of time before the apparent complete imposition of a virus different from the original average population. The imposition here was sharp, occurring in two passages (Fig. 1). Similar results have been obtained by others (27a): viruses genetically different from the original variants dominated upon replication in tissue culture.

The reasons for these population dynamics are not well

TABLE 2. Nucleotide and deduced amino acid changes determined for the indicated FMDV populations after serial passage and for coinfections in cell culture

Variant or mixture[a]	Sequence change[b]				Result[c]
	VP1		VP2		
	nt 2117	aa 198	nt 421	aa 72	
A5 Sp-86	A	Asp	G	Asp	wt
1-OG2	A	Asp	A	Asn	1-OG2
1-OG2 P20	A	Asp	G	Asp	wt
1-HA6	G	Gly	G	Asp	1-HA6
1-HA6 P30	A	Asp	G	Asp	wt
A5:1-OG2 C6	A	Asp	G	Asp	wt
A5:1-HA6 C20	A	Asp	G	Asp	wt
1-OG2:1-HA6 C6	G	Gly	G	Asp	1-HA6

[a] Virus neutralization-resistant variants (or wild type:variant mixtures) passaged in cell culture. P*n*, serial passage level; C*n*, passage level of coinfection.

[b] Nucleotide (nt) position (P1 region) and deduced amino acid (aa) residue at position number of VP1 or VP2 under observation.

[c] Genotype of VP1 and VP2 sites at passage level indicated. wt, wild type.

understood. A type A12 virus isolated from cattle and passaged only a single time in tissue culture was found to have three nearly equally represented populations of antigenic variants, suggesting the stable coexistence of these viruses in cattle (29). On the other hand, several variant strains of vesicular stomatitis virus could not dominate a growing population, despite their aggressive growth in pure culture, and thus were suppressed in the overall population (12). The reason for this competitive disadvantage (12; this report) is not always clear, but in the case of a number of A12 variants, some variants showed reduced amounts of virus-directed protein synthesis and reduced ability to inhibit cellular protein synthesis (7). The type A5 variants used in our study grow to high titer in cell culture and are infectious for suckling mice as well (31). The presence of nucleotide changes outside the P1 region between antigenically distinguishable viruses has not been deeply explored and, at this point, cannot be excluded as involved in the relative fitness of variant viruses. We are currently investigating several FMDV isolates with different host range capabilities, and initial results suggest that changes in the genome sequence outside of the capsid region contribute to the differences (25a).

Crystallographic data (1) indicate that most of the neutralizing epitopes described to date (for a review, see reference 19) are located on an elevated, disordered loop of VP1 or on areas on VP1, VP2, and VP3 that are in close proximity to the VP1 loop. Thus, these areas could be prone to accept substitutions which are neutral to virus integrity. However, these mutations could also interfere with the accessibility to the conserved sequence RGD of VP1, which has been identified as contributing to viral receptor site (6, 23), or with some other essential viral function, which could explain the selective disadvantage of virus variants versus parental virus.

As has been pointed out by Diez et al. (15), these mutations could appear before the exposure of the viral population to the immune system, with subsequent immunologic mediation of the population change. This capability resident in the population could provide a significant survival potential for the virus (21, 22). Our results show a reemergence of the dominant parental virus after multiple replication cycles in tissue culture in the absence of any known immunological pressure and support the suggestions of these authors. Moreover, our results indicate that these disadvantaged subpopulations continuously exist and can be competitive with wild-type virus under certain circumstances. It is possible that under external pressure, immunologic or otherwise, they could become fixed as the main population. We suggest that such a mechanism may play an important role in escape from the immune response and thus play an important role in viral evolution.

This work has been supported in part by U.S.-Spain Joint Commission S & T research grant CCA-8510/083 administered through USDA/OICD.

We thank M. Gonzalez-Juarrero for helpful discussions on neutralization assay methods and J. Card for technical information and assistance.

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