JOURNAL OF VIROLOGY, Nov. 2008, p. 10465–10476 Vol. 82, No. 21
0022-538X/08/$08.00+0 doi:10.1128/JVI.00825-08
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Hidden Virulence Determinants in a Viral Quasispecies In Vivo▽

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Received 17 April 2008/Accepted 2 August 2008

The characterization of virulence determinants of pathogenic agents is of utmost relevance for the design of disease control strategies. So far, two classes of virulence determinants have been characterized for viral populations: those imprinted in the nucleotide sequence of some specific genomic regions and those that depend on the complexity of the viral population as such. Here we provide evidence of a virulence determinant that depends neither on a genomic sequence nor on detectable differences in population complexity. Foot-and-mouth disease virus is lethal for C57BL/6 mice showing the highest viral load in pancreas. Virus isolated from pancreas after one passage in mice showed an attenuated phenotype, with no lethality even at the highest dose tested. By contrast, virus from sera of the same mice displayed a virulence similar to that of the parental wild-type clone and virus isolated from spleen displayed an intermediate phenotype. However, viral populations from pancreas, spleen, and serum showed indistinguishable consensus genomic nucleotide sequences and mutant spectrum complexities, as quantified according to the mutation frequencies of both entire genomic nucleotide sequences of biological clones. The results show that the populations with differing virulences cannot be distinguished either by the consensus sequence or by the average complexity of the mutant spectrum. Differential harvesting of virus generated by cell transfection of RNA from serum and pancreas failed to reveal genetic differences between subpopulations endowed with differing virulences. In addition to providing evidence of hidden virulence determinants, this study underlines the capacity of a clone of an RNA virus to rapidly diversify phenotypically in vivo.

Genetic diversity is one of the most important features that allows a population to evolve in an ever-changing environment with shifting selecting pressures. RNA viruses display the highest replication error rates scored among components of our biosphere, with an average of approximately one misincorporation per 10^4 nucleotides copied, resulting in highly diverse replicating populations, termed viral quasispecies (19, 27). A viral quasispecies can act as a unit of selection, and its properties are influenced by the interactions among its individual components (19, 57). In a complex environment such as within an animal, the ability to generate a complex mutant spectrum may allow the viral populations to adapt and survive in the different intrahost environments, including the selective pressures generated by the host immune response (16, 39, 57). During replication and spread within an infected host, viral populations may also be subjected to frequent bottleneck events that may result in an accumulation of deleterious mutations and, consequently, in an average decrease in fitness (operation of Muller's ratchet) (3, 10, 18, 21, 22, 24, 30, 32, 38). Furthermore, the genetic diversity in a viral population, which has been termed the quasispecies cloud size, is an intrinsic property of the quasispecies that may influence the viral phenotype. This has been clearly evidenced by variations in viral diversity brought about by the copying fidelity of the viral polymerase, which may modulate viral virulence in vivo (39, 57). A role of the mutant spectrum in viral pathogenesis has been also evidenced for West Nile virus infections (9, 29). Knowledge of the evolutionary dynamics of viral populations in vivo is key to understanding viral pathogenesis.

In the present study, we use foot-and-mouth disease virus (FMDV), a picornavirus that causes a severe vesicular disease in livestock (5, 40), to study viral population dynamics and virulence in vivo. Most studies relating to the population dynamics of FMDV (reviewed in reference 16) have been carried out in cell culture, due in part to the difficulties of using natural hosts to characterize evolving FMDV mutant spectra. We have developed a mouse model for FMDV that mimics, at least partly, the course of infection in natural hosts and that allows several aspects of FMDV pathogenesis to be addressed (42). C57BL/6 mice are highly susceptible to FMDV infection when the virus is administered subcutaneously. Signs of disease begin at 24 h postinoculation (hpi), and within 2 or 3 days of the onset of disease, the animals die. An analysis of FMDV replication in different tissues indicated a systemic infection with virus present in all major organs (42). Mice developed microvesicles near the basal layer of the epithelium (42), an event that precedes the vesiculation which is characteristic of FMD.

In the present study we have analyzed the molecular evolution of FMDV in mice by serial passages of a biological clone of FMDV (C-S8c1 [described in reference 50]) in the mouse pancreas. We found that in one single passage, virus isolated from pancreas was attenuated for mice but displayed normal

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▽ Published ahead of print on 20 August 2008.

growth in cell culture. By contrast, viral populations isolated from serum showed a virulence similar to that of the parental virus. Furthermore, viral populations from spleen, which is a highly perfused organ (large blood input), presented a phenotype intermediate between that of the virus isolated from pancreas and that from serum. Intriguingly, the genetic diversities of these three viral populations were identical, and no differences were found among their corresponding consensus genomic sequences; nor could differences be unveiled in RNA from sequential viral harvests of BHK-21 cells transfected with viral RNA. The data support that the observed differences in virulence could be due to a dissimilar composition in genomes within the viral populations. Therefore, we propose that viral replication in mouse organs leads to an accumulation of attenuated genomes as a consequence of the random fixation of mutations and that the most virulent genomes from several mouse tissues would spread through serum in a higher proportion. According to this model, viral pathogenesis would be modulated by the proportion of attenuated and virulent genomes and by the interactions between them.

MATERIALS AND METHODS

Mice, viruses, infections, and preparation of samples. C57BL/6 mice maintained at Centro de Investigación en Sanidad Animal (CISA-INIA) were used for all the experiments. All mice were matched for sex and age (females, 8 to 10 weeks). Mice were inoculated subcutaneously in the left rear footpad (FP) (50 μl) or intravenously in the caudal vein (100 μl) with homogenized naïve tissues in phosphate-buffered saline (PBS) (negative controls) or with homogenized tissues from infected mice or FMDV from the supernatants of infected cells and examined for clinical symptoms every day. At 24 hpi, mice were bled, and some of them were sacrificed by perfusion with PBS or protease inhibitor cocktail (Sigma), as indicated by the manufacturer. Pancreas, heart, spleen, and lung from most of the euthanized mice were harvested, weighed, and homogenized using an Omni tissue homogenizer (Omni International); part of each tissue was homogenized in PBS, and part in Trizol (Invitrogen). Serum was isolated from whole blood collected from the caudal vein. A fraction of the pancreas from eight infected mice was fixed in 10% buffered formalin (pH 7.2) for histological staining. All the experiments with live animals were performed under the guidelines of the European Community (86/609) and were approved by the site ethical review committee.

Viruses and cells. Procedures for infection of BHK-21 cell monolayers with FMDV in liquid medium and for plaque assays in semisolid agar medium were carried out as previously described (15, 50). FMDV C-S8c1 is a plaque-purified derivative of natural isolate C1-Sta, Pau-Spain 70, a representative of the European subtype C1 FMDV (50). Biological clones from viral populations from serum and pancreas were obtained by isolating virus from randomly chosen, well-isolated, individual virus plaques, as previously described (4, 23).

Transfection of BHK-21 cell monolayers (70% confluent, about 7×10^5 cells) with FMDV RNAs (10^9 to 10^{11} RNA molecules) or RNA transcripts (about 10^{11} RNA molecules) was carried out using Lipofectin (Invitrogen) as previously described (44). Virus derived from RNA transcripts was collected from the supernatants of transfected cells at 72 h posttransfection (95% of cells displaying cytopathology [cpe]) and was passaged twice in BHK-21 cells before its use in biological studies. RNA extraction and nucleotide sequencing were performed to ensure that the virus maintained the mutations of the initial transcript. Viruses from transfections with FMDV RNA were harvested at short times (24 to 36 h) posttransfection (about 20% of cpe) and at late times (48 to 60 h) posttransfection (100% cpe), and no passage in BHK-21 cells was performed before using them in biological studies or for nucleotide sequencing. Intracellular virus was harvested by freeze-thawing of the transfected BHK-21 cells.

Histopathology. Samples from pancreas were fixed in 10% buffered formalin (pH 7.2) for histopathological studies. After fixation, samples were dehydrated through a graded series of alcohol to xylol and embedded in paraffin wax. Sections 4 μm thick were cut and stained with hematoxylin and eosin for histopathological analysis.

RNA extraction, cDNA synthesis, PCR amplification, and nucleotide sequencing. RNA was extracted from mouse tissues by treatment with Trizol (Invitrogen) according to the instructions of the manufacturer, from the supernatants of infected cells, or from biological clones, as previously described (48). Reverse transcription (RT) of FMDV RNA was performed using avian myeloblastosis virus reverse transcriptase (Promega) or Transcriptor reverse transcriptase (Roche), according to the manufacturer's instructions. For the determination of the consensus sequences (viral populations and biological clones), PCR amplification was carried out using the Expand high-fidelity polymerase system (Roche), as specified by the manufacturers. RT-PCR amplification of FMDV RNA from mouse tissues was performed using a 1:10 dilution of the extracted RNA. Viral RNA detection and semiquantification in samples from passage 2 mouse tissues were performed by RT-PCR amplification of two different genomic regions: residues 2767 to 4193 (spanning parts of VP3-, VP1-, 2A-, and 2B-coding regions) and residues 6308 to 7157 (spanning parts of 3C- and 3D-coding regions) (the numbering of residues of the FMDV genome is according to reference 53); amplifications were carried out in parallel with known amounts of standard C-S8c1 RNA, and the products were analyzed by electrophoresis in 1% agarose gels. For the molecular cloning of individual viral RNA molecules and the construction of FMDV infectious plasmids containing specific mutations, PCR amplification was carried out using *PfuUltra* DNA polymerase (Stratagene), due to its high copying fidelity (12), using procedures described previously (35). The FMDV-specific primers used for these amplifications and for sequencing have been previously described (21, 22). Nucleotide sequencing was performed using the BigDye Terminator cycle sequencing kit (ABI Prism; Perkin-Elmer) and an automated ABI 373 sequencer; all sequences were determined at least twice from independent sequencing reaction mixtures.

Viral RNA quantification. FMDV RNA quantification was performed by real-time RT-PCR using the LightCycler instrument (Roche) and the RNA master SYBR green I kit (Roche) as specified by the manufacturer. Quantification was relative to a standard curve obtained with known amounts of FMDV C-S8c1 RNA and was done using a procedure that has been described previously (25, 26).

Molecular cloning. Residues 2767 to 4192 (corresponding to parts of VP3-, VP1-, 2A-, and 2B-coding regions) and residues 4027 to 6009 (spanning parts of 2B-, 2C-, 3A-, and 3B-coding regions) of the FMDV genome were amplified by RT-PCR as described above. PCR amplification was carried out with a 1:10, 1:100, and 1:1,000 dilution of template RNA by use of *PfuUltra* DNA polymerase (Stratagene) to ensure both a basal error rate in the range of 7.0×10^{-5} to 1.4×10^{-4} substitutions per nucleotide and an adequate representation of the components of the mutant spectrum among the sequences determined (2, 34, 45). Cloning was carried out with the amplification products of the 1:10 dilutions sample when both the 1:100 and 1:1,000 dilutions of template yielded a visible band (controls and procedure detailed in reference 2). Molecular clones corresponding to the VP1 genomic region (residues 2767 to 4192) were obtained by ligation of cDNA into the pGEM-T Easy vector (Promega) and transformation into *Escherichia coli* DH5α cells. DNA from positive colonies was amplified with a TempliPhi amplification kit (Amersham) by following the manufacturer's protocol. Amplified cDNAs from the 2B-to-3B-coding region were digested with the restriction enzymes HindIII (position 4068) and EcoRI (position 5369) (New England Biolabs) and ligated to plasmid pGEM 4Z (Promega) previously digested with the same restriction enzymes and treated with shrimp alkaline phosphatase (New England Biolabs). Transformation of *E. coli* DH5α, colony screening by PCR amplification, and nucleotide sequencing of individual clones were carried out as previously described (2, 44). The genomic region sequenced corresponded to the entire VP1-coding region and residues 4072 to 5366 (spanning part of 2B, the complete 2C, and part of 3A). Seventy-four or 75 independently cloned viruses were sequenced from each compared FMDV population (79,000 to 80,000 nucleotides per viral population).

Characterization of mutant spectra. The complexity of mutant spectra was characterized by the analysis of the mutation frequency and normalized Shannon entropy, quantified as described previously (4, 36, 47).

Construction of FMDVs containing mutations in 2C. To obtain viruses containing substitutions I85V and I248T in 2C in the genetic background of C-S8c1, plasmid pMT28, which contains a cDNA copy of the entire FMDV genome, was used (25); residues 4027 to 6009 (spanning parts of 2B-, 2C-, 3A-, and 3B-coding regions) were amplified by RT-PCR, and the cDNAs were digested with the restriction enzymes BglII (position 4201) and RsrII (position 5839) (New England Biolabs) and ligated into pMT28 digested with the same enzymes and treated with shrimp alkaline phosphatase (New England Biolabs). Plasmids containing the complete FMDV genome with the mutations in 2C were linearized by digestion with NdeI (position 8140) (New England Biolabs) and transcribed with SP6 RNA polymerase (Promega) according to reference 7. RNA concentrations of infectious transcripts were estimated by agarose gel electrophoresis with known amounts of rRNA from yeast as the standard.

Statistical analyses. Data handling, analysis, and graphic representation was performed using Prism 2.01 (GraphPad Software, San Diego, CA). Statistical

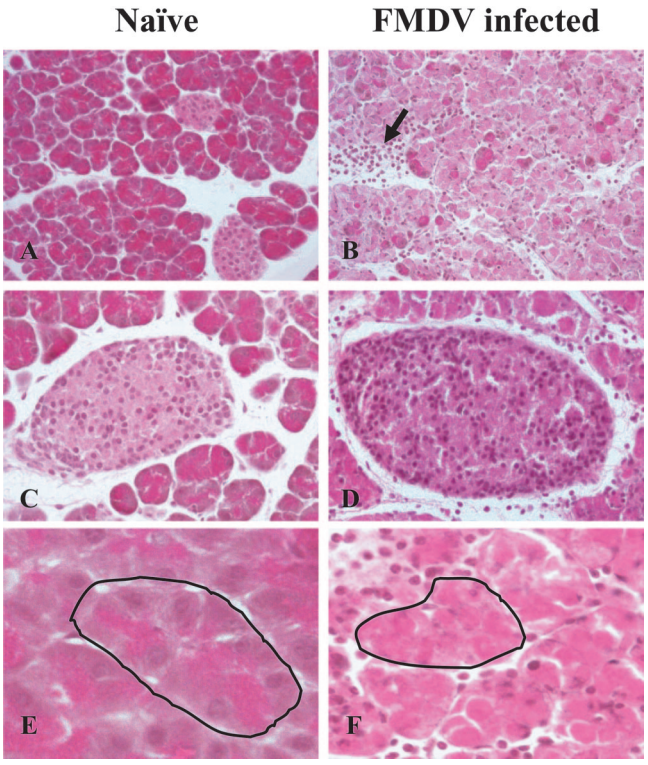


FIG. 1. Histopathology analysis of pancreatic tissue taken from representative C57BL/6 mice that were uninfected (A, C, and E) or that were infected with FMDV C-S8c1 (B, D, and F). Sections from formalin-fixed and paraffin-embedded pancreas samples were stained with hematoxylin and eosin. (A and B) Exocrine and endocrine pancreas. Lymphocytic infiltrates in the infected mice are indicated with an arrow (magnification, ×100). (C and D) The infected mice show a high lymphocytic infiltration in the islets of Langerhans (magnification, ×200). (E and F) Exocrine tissue at higher magnification. Loss of acinar tissue in infected mice; the acinus has been outlined (magnification, ×400).

differences were determined using a one-way analysis of variance (ANOVA) ($P < 0.05$) test or Fisher's exact test ($P < 0.05$).

RESULTS

FMDV causes acute pancreatitis in adult C57BL/6 mice. It has been previously described that FMDV replication in C57BL/6 mice causes a systemic infection resulting in mouse death at 36 to 48 hpi, with a 50% lethal dose of 50 PFU (42). Time course experiments showed virus replication in all major organs (heart, lung, brain, kidney, liver, spleen, pancreas, and thymus). The highest viral load at 24 hpi was observed for pancreas. To understand the effect of viral replication in pancreas, groups of mice were inoculated in parallel in the FP with 10^4 PFU of FMDV C-S8c1, and the consequences of the infection were evaluated. Groups of eight mice were sacrificed by perfusion with PBS at 24 hpi and their pancreases were collected and analyzed (Fig. 1). Macroscopic examination revealed enlarged and edematous pancreases. Histological examination showed acute inflammatory reaction (Fig. 1B) with proteolytic destruction of the pancreatic parenchyma (Fig. 1F), and interstitial hemorrhage, affecting more severely the acinar tissue than the endocrine pancreas. The damage to the acinar tissue was highly restricted, and the majority of the endocrine pancreas remained less affected than the exocrine pancreas. Nevertheless, mild inflammation in the islets of Langerhans was observed (Fig. 1D), with a high proportion of lymphocyte infiltration in both the endocrine and acinar tissues. These results indicate that the replication of FMDV in mouse pancreas causes a severe pancreatic injury.

Passage of FMDV C-S8c1 in mouse pancreas in vivo. To explore the molecular evolution of FMDV in vivo, and based on our previous observation that FMDV reaches higher levels in pancreas than in other tissues (42), FMDV was passaged in mouse pancreas in vivo. With this aim, four mice were inoculated in the FP with 10^4 PFU of FMDV C-S8c1. At 24 hpi mice were bled for viremia detection and sacrificed by perfusion with PBS (mice 1 to 4; Fig. 2) to obtain the pancreases for viral load and infectivity determinations. The amount of PFU per g of pancreas varied among individual mice (with differences of more than 2 $\log_{10}$), although all mice showed similar viremias at 24 hpi (mice 1 to 4; Table 1). However, the amounts of viral RNA in pancreases, as quantified by real-time RT-PCR, were similar for all mice tested (Table 1). This indicates a remarkable difference in the specific infectivities of viruses isolated from pancreases among FMDV C-S8c1-infected mice. To ensure that the low specific infectivity values did not reflect a loss of infectivity or the destruction of the viral particles due to the activation of pancreatic enzymes, six C57BL/6 mice were inoculated with FMDV C-S8c1 and sacrificed by perfusion at 24 hpi. Three mice were perfused with a protease inhibitor cocktail (mice 5 to 7, Fig. 2) and three mice with PBS (mice 8 to 10, Fig. 2). The amount of virus in pancreas determined by plaque assay was as variable among mice perfused with the protease inhibitor cocktail as among mice perfused with PBS (mice 5 to 10, Table 1). In addition, and in anticipation that homogenization (or any other mechanical disruption) of the pancreas might produce the activation of pancreatic enzymes, we evaluated the specific infectivity of FMDV C-S8c1 mixed with homogenized pancreases from naïve mice. FMDV C-S8c1 in the homogenized mixture showed a specific infectivity similar to that of FMDV C-S8c1 alone (Table 1). All these data indicate that the specific infectivity found in pancreases of FMDV C-S8c1-infected mice was not affected by pancreatic enzymes.

Viruses isolated from pancreas show an attenuated phenotype for mice. To continue the passage of virus from pancreases in mice, pancreases obtained from mice 1, 2, 8, and 10 (chosen based on the high specific infectivity of virus isolated from their pancreases) and perfused with PBS were homogenized in PBS and inoculated into naïve C57BL/6 mice (passage 2 in Fig. 2). The inoculations were according to the following experimental design: eight mice were inoculated with virus from the pancreas of mouse 1 (group 1 in Fig. 2), four with 10^3 PFU in the FP and four with 10^4 PFU intravenously; four mice were inoculated with 10^4 PFU of virus from the pancreas of mouse 2 in the FP (group 2); eight mice were inoculated with 10^4 PFU of virus from the pancreas of mouse 8 in the FP (group 8); and eight mice were inoculated with 10^4 PFU of virus from the pancreas of mouse 10 in the FP (group 10). The overall survival index of mice inoculated with virus from pancreas was 93.75%, only one mouse from group 2 died at 7 days postinoculation, and the rest of mice from all groups survived during a follow-up period of 30 days (Fig. 3A). By contrast, all

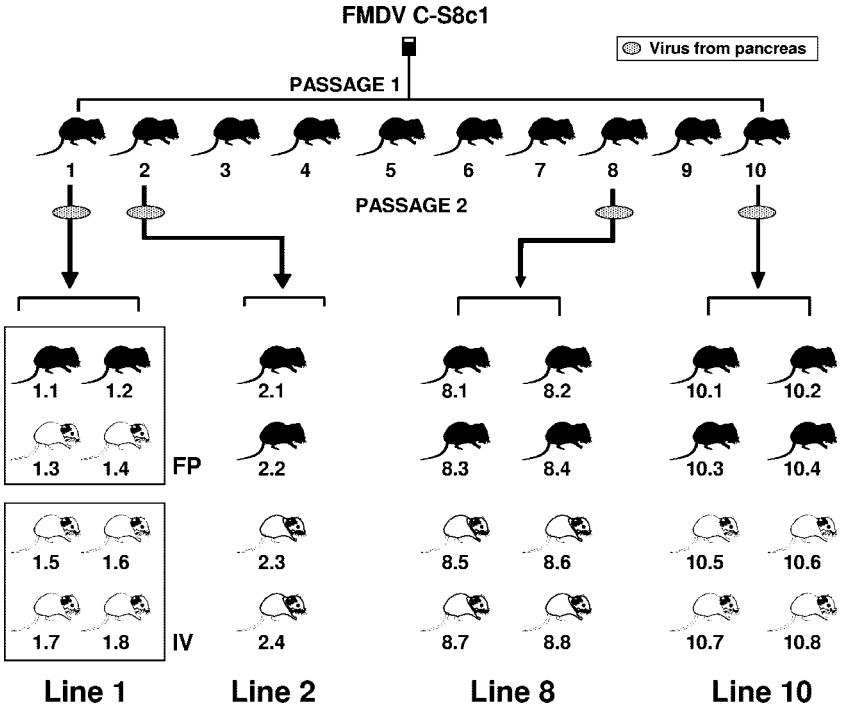


FIG. 2. Experimental design of FMDV C-S8c1 passages in mouse pancreas in vivo. Ten mice were inoculated with 10^4 PFU of FMDV C-S8c1 into the FP (passage 1). Virus isolated from pancreases of mice 1, 2, 8, and 10 were inoculated into naïve mice, as depicted for passage 2. Mice were inoculated with virus from the pancreas of mouse 1 as follows: four mice (1.1, 1.2, 1.3, and 1.4) were inoculated in the FP with 10^3 PFU, and four mice (1.5, 1.6, 1.7, and 1.8) were inoculated intravenously (IV) with 10^4 PFU. All other mice were inoculated with 10^4 PFU from pancreases of mice 2, 8, and 10 in the FP. Inoculated mice are divided in groups 1 to 4. Black mice indicate the animals that were sacrificed at 24 hpi in order to remove the pancreas and other tissues (described in Materials and Methods). White mice were maintained alive for clinical observations or until their sacrifice.

TABLE 1. Viral titers and viral RNA molecules in pancreases and sera from C-S8c1-infected mice

Mouse[a]	Serum viral titer (PFU/ml)	Pancreas		
		Viral titer (PFU/g)	Viral RNA (no. of molecules)[b]	Specific infectivity (PFU/molecule)[c]
1	4.2×10^6	1.3×10^6	8.4×10^{12}	1.5×10^{-7}
2	ND[e]	5.8×10^6	6.2×10^{13}	9.3×10^{-8}
3	5.6×10^7	3.9×10^3	4.3×10^{13}	9.2×10^{-11}
4	6.1×10^7	3.5×10^4	2.3×10^{13}	1.5×10^{-9}
5	1.2×10^8	$<1.0 \times 10^2$[d]	9.2×10^{13}	$<1.1 \times 10^{-12}$
6	ND[e]	5.5×10^4	1.3×10^{12}	4.3×10^{-8}
7	5.2×10^8	1.1×10^7	1.3×10^{14}	8.6×10^{-8}
8	4.3×10^8	2.5×10^6	8.5×10^{13}	2.9×10^{-8}
9	3.8×10^8	5.6×10^4	4.8×10^{13}	1.2×10^{-9}
10	3.1×10^8	1.3×10^8	4.3×10^{13}	3.1×10^{-6}
Control1[f]		1.1×10^6	3.6×10^{11}	3.2×10^{-6}
Control2[f]		1.4×10^6	6.7×10^{11}	2.1×10^{-6}
Control3[f]		6.5×10^6	2.4×10^{12}	2.7×10^{-6}

[a] Mouse number according to Fig. 2. Mice 1 to 4 and 8 to 10 were perfused with PBS, and mice 5 to 7 were perfused with a protease inhibitor cocktail.

[b] Viral RNA is expressed as the number of RNA molecules quantified by real-time RT-PCR (described in Materials and Methods) per g of tissue.

[c] Specific infectivity is expressed as the number of PFU per viral RNA molecule.

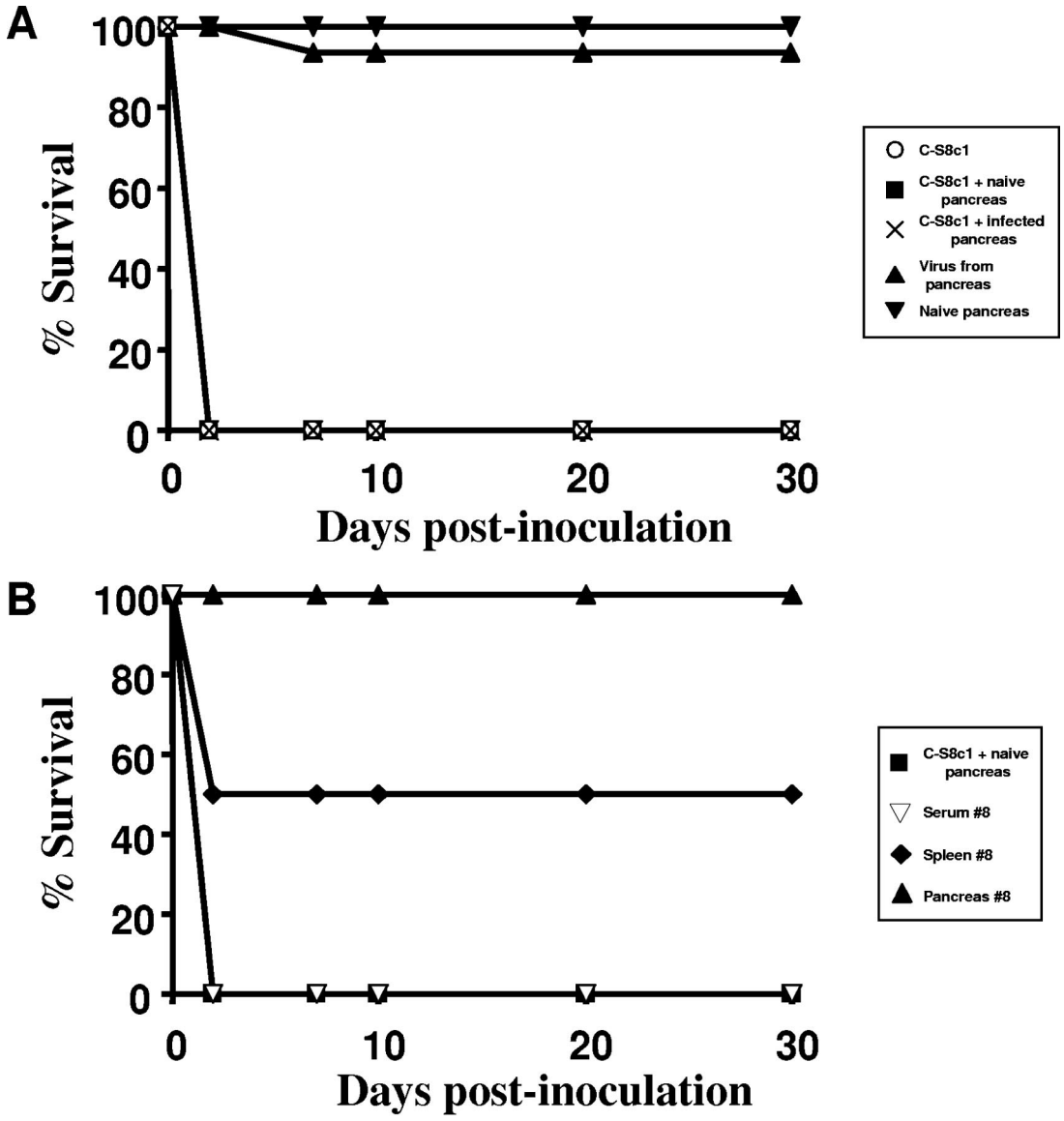
[d] No PFU were detected in this sample.

[e] ND, not determined.

[f] Controls 1 and 2 correspond to a mixture of pancreas from naïve mice and 5×10^6 PFU of FMDV C-S8c1. Control 3 is C-S8c1 from the supernatant of infected BHK-21 cells. In these three controls, viral titer is expressed as the number of PFU per ml and viral RNA as the number of RNA molecules per ml.

mice inoculated with 10^4 PFU of the mixture of FMDV C-S8c1 with naïve pancreas died at about 36 hpi (Fig. 3A). To rule out the possibility that pancreatic enzymes (or other virus-inactivating molecules) could be produced as a result of FMDV replicating in the pancreas, resulting in a decrease of infectivity, FMDV C-S8c1 was mixed with a pancreatic extract prepared from FMDV-infected mouse number 4 (Table 1). Mice inoculated with 10^4 PFU of FMDV C-S8c1 in a mixture with pancreatic extract died at about 36 hpi (Fig. 3A), indicating that virus in pancreas was not destroyed by any antiviral activity or pancreatic enzymes that might be activated by the viral infection.

To determine whether the reduced virulence displayed by virus from pancreas at passage 2 corresponded to low viral replication and expansion, samples from serum and pancreas at 24 hpi were titrated by plaque assay. Eleven out of 12 mice sacrificed by perfusion with PBS at 24 hpi showed no viremia (below detection limit [50 PFU/ml of serum]). Mouse 1.2 (Fig. 2) showed a viremia of 3.4×10^4 PFU/ml of serum. The viral infectivity in pancreas was negative by plaque assay for the 12 analyzed mice (below the detection limit of 10^2 PFU/g tissue). To ensure that not even minimal amounts of infectious virus were present in pancreas, infections in liquid medium in BHK-21 cells were carried out with extracts from pancreases 1.1 and 1.2. No infectivity was recovered up to 72 hpi. In addition, the level of viral RNA in pancreatic tissue was determined by RT-PCR. Five out of 12 mice showed viral RNA



C

Viral Population	Specific Infectivity (PFU/RNA molecule)
C-S8c1	2.7×10^{-6}
Pancreas	2.9×10^{-8}
Serum	1.5×10^{-4}
Spleen	1.8×10^{-5}

FIG. 3. Virulence for mice of viruses isolated from pancreases, spleens, and sera of FMDV C-S8c1-infected mice. (A) Percentages of survival of mice after inoculation into the FP of 10^4 PFU of FMDV C-S8c1 (4 mice), a mixture of FMDV C-S8c1 with naïve pancreas (4 mice), a mixture of FMDV C-S8c1 with infected pancreas of mouse 4 (4 mice), virus from pancreas (16 mice), or naïve pancreas (4 mice) during a follow-up period of 30 days. The reduced virulence for mice displayed by viruses from pancreas in relation to C-S8c1, C-S8c1 mixed with naïve pancreas, and C-S8c1 mixed with infected pancreas is statistically significant ($P < 0.0001$). (B) Percentages of survival of mice after inoculation into the FP of 10^4 PFU of a mixture of FMDV C-S8c1 with naïve pancreas (four mice), virus from the serum of mouse 8 (four mice), virus from the spleen of mouse 8 (four mice), or virus from the pancreas of mouse 8 (four mice) during a follow-up period of 30 days. The differences in virulence of viruses from pancreas, spleen, and serum are statistically significant ($P < 0.0071$). (C) Specific infectivity of C-S8c1 and viral populations from mouse 8 at passage 1, expressed as the number of PFU per viral RNA molecule, determined as described in Materials and Methods.

TABLE 2. Virus detection in pancreases, lungs, hearts, and spleens from mice inoculated with FMDV from mouse pancreas at passage 2

Mouse[a]	Tissue	Viral RNA[b]
1.1	Pancreas	−
	Lung	++
	Heart	−
	Spleen	−
1.2	Pancreas	+++
	Lung	++
	Heart	++
	Spleen	+++
2.1	Pancreas	++
	Lung	++
	Heart	+
	Spleen	++
2.2	Pancreas	+
8.1	Pancreas	−
8.2	Pancreas	+
8.3	Pancreas	−
8.4	Pancreas	+
10.1	Pancreas	−
10.2	Pancreas	−
10.3	Pancreas	−
	Lung	−
	Heart	+
	Spleen	+
10.4	Pancreas	−

[a] Mouse numbers are the same as in Fig. 2.
[b] Viral RNA was detected by RT-PCR amplification of two different genomic regions as described in Materials and Methods. The symbol − means $<10^7$ viral RNA molecules per g of tissue, while the symbols +, ++, and +++ correspond to 10^7 to 10^8, 10^8 to 10^9, and $>10^9$ viral RNA molecules per g of tissue, respectively. No virus was detected by plaque assay in any of the samples. The detection limit was 10^2 PFU per g of tissue.

TABLE 3. Substitutions found in the consensus sequence of FMDV isolated from pancreas at passage 1 in mice[a]

Pancreas of mouse[b]:	Nucleotide substitution[c]	Genomic region[d]	Amino acid substitution[e]	Conservation of residue[f]
1	U 879 C	IRES	—	Variable
	U 1358 C	L	I 107→T	Conserved
	C 2439 U	1B	—	
	C 3333 U	1D	—	
2	U 150 A	Fragment S	—	
	C 559 U	*cre*	—	Conserved
	A 1306 G	L	T 90→A	Conserved
	G 1410 A	L	—	
	A 4597 G	*2C*	*I 85→V*	*Conserved*
3	**U 5087 U/C**	**2C**	**I 248→T**	**Variable**
	U 7197 C/u	3D	—	
4	U 586 C/U	*cre*	—	Variable
	A 4597 G/a	*2C*	*I 85→V*	*Conserved*
	C 6570 C/A	3C	—	
5	A 1052 G	L	D 5→G	Variable
	U 5087 C	**2C**	**I 248→T**	**Variable**
	G 5295 A	2C	—	
6	C 3494 U	1D	S 96→F	Variable
	A 5537 G	3A	H 80→R	Variable
7	A 3075 A/U	1C	E 175→D	Variable
	A 4597 A/G	*2C*	*I 85→V*	*Conserved*
	A 5609 G/A	3A	Q 104→R	Variable
8	**U 5087 C**	**2C**	**I 248→T**	**Variable**
	U 6474 C	3C	—	
	U 6702 C	3D	—	
9	*A 4597 G*	*2C*	*I 85→V*	*Conserved*
	C 6096 U	3C	—	
10	C 1746 U/C	1A	—	
	C 3653 A/c	1D	T 149→K	Variable

[a] The complete genomes of FMDV populations from pancreas of passage 1 in mice were amplified by RT-PCR and sequenced as described in Materials and Methods. Repeated mutations are shown in the same typeface (boldface or italic): boldface indicates an amino acid change in a variable residue, while italic indicates an amino acid change in a conserved residue.
[b] Mouse numbers 1 to 10 correspond to the mouse numbers shown in Fig. 2.
[c] The numbering of residues was done according to reference 50. Lowercase letters indicate the presence of a minority nucleotide (<50 %) according to the sequencing peak pattern.
[d] The genomic region corresponding to each mutation is indicated.
[e] Dashes indicate mutations that do not give rise to amino acid substitutions.
[f] The conservation of the substituted nucleotide or amino acid in other FMDV isolates is according to reference 21.

levels of more than 10^7 viral RNA molecules per gram of tissue (Table 2), supporting the conclusion that FMDV had replicated in the pancreases of at least 5 mice, although no infectious virus was detected. Since the inoculated virus originated in pancreases of infected mice, the detection of viral RNA in pancreas could indicate a preferential tropism of the virus to replicate in pancreas. To test this possibility, other organs from mice of passage 2 were removed and analyzed for the presence of virus by plaque assay and by RT-PCR. No infectivity was detected in extracts from lungs, hearts, and spleens from mice 1.1, 1.2, 2.1, and 10.3 (Fig. 2), although most of the samples were positive for viral RNA by RT-PCR (Table 2). These data indicate that the virus replicated in all the organs tested and did not show any discernible preferential tropism for pancreas.

Genomic changes in *cre*, L, 2C, and 3A are associated with FMDV replication in mice. To identify genetic characteristics that might be relevant to the reduced pathogenicity displayed by FMDV isolated from pancreas, the entire genomic consensus nucleotide sequence of viruses isolated from the pancreases of 10 mice from passage 1 was determined as described in Materials and Methods. Each viral population analyzed showed nucleotide changes in the consensus nucleotide sequence relative to that for the parental virus, FMDV C-S8c1 (Table 3). Nineteen out of 29 mutations mapped in the region coding for nonstructural proteins, and 12 of these 19 mutations led to an amino acid substitution that affected protein L, 2C, or 3A (Table 3). Amino acid substitutions I85V and I248T in 2C were found repeatedly in virus from several animals (I85V in four mice, and I248T in three mice). However, these substitutions were never found together in the same viral population. These data suggest a role of the 2C protein in FMDV replication in mice.

To further investigate the molecular basis of FMDV adaptation to mice, virus was isolated from several organs of FMDV C-S8c1-infected mice. Spleens, lungs, and hearts were taken from mice 1, 2, 8, and 10 (Fig. 2) and homogenized. The consensus sequences from the viral population isolated from these organs were determined for the genomic regions *cre*, internal ribosome entry site (IRES), L, 2C, and 3A, as indicated in Materials and Methods. Most of the mutations found in these genomic regions correspond to the mutations found in the viral populations from the pancreas of the corresponding animal (Table 4). In addition, amino acid substitutions I85V and I248T found in 2C were present also in viral populations from organs other than the pancreas. Therefore, the 2C protein may play a role in FMDV replication generally in mice and not specifically in the pancreas.

TABLE 4. Mutations found in the consensus sequence of FMDV populations from lungs, hearts, and spleens of passage 1 mice[a]

Mouse[b]	Organ[c]	Nucleotide substitution[d]	Genomic region[e]	Amino acid substitution[f]	Conservation of residue[g]
1	Lung	U 5480 C	3A	I 61→T	Variable
	Spleen	A 641 A/g	IRES	—	Variable
		U 5480 C/U	3A	I 61→T	Variable
2	Lung	C 559 C/U	*cre*	—	Conserved
		A 1306 A/G	L	T 90→A	Conserved
		G 1410 G/A	L	—	
		G 1588 G/A	L	D 184→N	Conserved
		A 4597 A/G	*2C*	*I 85→V*	*Conserved*
	Heart	C 559 C/U	*cre*	—	Conserved
		A 1306 A/g	L	T 90→A	Conserved
		G 1410 G/a	L	—	
		G 1588 G/A	L	D 184→N	Conserved
		A 4597 A/G	*2C*	*I 85→V*	*Conserved*
	Spleen	C 559 C/U	*cre*	—	Conserved
		A 1306 A/G	L	T 90→A	Conserved
		G 1410 G/A	L	—	
		G 1588 G/A	L	D 184→N	Conserved
		A 4597 A/G	*2C*	*I 85→V*	*Conserved*
8	Lung	**U 5087 C**	**2C**	**I 248→T**	**Variable**
	Heart	**U 5087 C**	**2C**	**I 248→T**	**Variable**
	Spleen	**U 5087 C**	**2C**	**I 248→T**	**Variable**
10	Lung		—	—	
	Heart		—	—	
	Spleen		—	—	

[a] The consensus sequences of the genomic regions *cre*, IRES, L, 2C, and 3A of FMDV populations from lungs, hearts, and spleens from passage 1 mice are represented. Residues 389 to 1641 and 4345 to 5757 were analyzed for all mice except mouse number 1, for which only residues 389 to 1095 and 4345 to 5757 from lung and spleen could be amplified by RT-PCR. Repeated mutations are shown with the same typeface (boldface or italic): boldface indicates an amino acid change in a variable residue, while italic indicates an amino acid change in a conserved residue.

[b] Mouse numbers 1, 2, 8, and 10 correspond to the mouse numbers shown in Fig. 2.

[c] Organs from which viral RNA was amplified by RT-PCR and sequenced (described in Materials and Methods).

[d] See Table 3 footnote *c*.

[e] See Table 3 footnote *d*.

[f] See Table 3 footnote *e*.

[g] See Table 3 footnote *f*.

To study a possible relationship between substitutions I85V or I248T in 2C and FMDV virulence for mice, two infectious transcripts of FMDV C-S8c1 (termed C-S8c1 V-85 and C-S8c1 T-248, each carrying one of the two mutations in the 2C-coding region) were obtained from plasmid pMT28, as detailed in Materials and Methods. Infectious viruses harvested after two serial passages in BHK-21 cells of the supernatants from transfected BHK-21 cells with the infectious transcripts maintained each of the substitutions in 2C. The phenotypic properties of the resulting viruses were studied in vitro and in vivo. The viral titer of the two newly generated mutant viruses was similar to that of the parental virus infectious transcript, C-S8c1 pMT28. Their virulence in vivo was determined by inoculation of C57BL/6 mice in the FP with 10^4 PFU. No statistically significant differences in mouse survival at 48 hpi were observed for C-S8c1 V-85, C-S8c1 T-248, and C-S8c1 pMT28 ($P > 0.05$) (Table 5). Furthermore, the inoculated animals with each of the infectious transcripts carrying one of the mutations in 2C showed pathological signs similar to those observed for the infection with C-S8c1. This result excluded the association of point mutations in 2C with the attenuated phenotype found in virus isolated from pancreases of C-S8c1-infected mice.

TABLE 5. Similar phenotypic properties of infectious viruses recovered from FMDV C-S8c1 V-85 and C-S8c1 T-248 transcripts

FMDV population	Viral titer (PFU/ml)[a]	% Survival (no. of surviving mice/no. inoculated)[b]
C-S8c1 V-85	1.5×10^7	25 (1/4)
C-S8c1 T-248	1.6×10^7	0 (0/4)
C-S8c1 pMT28	2.3×10^7	0 (0/4)

[a] Viral titers of viruses obtained after two serial passages in BHK-21 cells for the supernatant of BHK-21 cells transfected with infectious clone transcripts.

[b] Virulence in mice is expressed as the percentage of mice that were alive at 48 hpi. No statistically significant differences between the survivals of mice at 48 hpi were noted among the three viral populations analyzed (one-way ANOVA; $P < 0.05$).

Viral populations from spleen and serum are pathogenic for mice. To investigate whether other viral populations from mice inoculated with FMDV C-S8c1 also showed an attenuated phenotype, the capacity of virus isolated from the spleen and sera of FMDV C-S8c1-infected mice to spread, cause disease, and kill mice was monitored (Fig. 3). The complete consensus nucleotide sequence of virus isolated from serum of mouse number 8 (Fig. 2) was identical to the consensus sequence of the genome from viruses from pancreas isolated from the same mouse (data not shown). Mice infected with virus from serum showed symptoms similar to those seen for mice infected with C-S8c1, and all mice died between 36 and 48 hpi. Mice inoculated with virus from pancreas showed 100% survival and mice inoculated with virus from spleen presented 50% survival at the same viral dose (Fig. 3B). To investigate the origin of these differences in virulence among viruses, the specific infectivity was determined. The three viruses displayed differences in specific infectivity, with viruses from pancreas having the lowest value (Fig. 3C). Thus, the low specific infectivity of viral populations isolated from mouse organs with respect to viruses isolated from serum might be associated with the attenuation of FMDV for mice. However, this possibility will require further study because C-S8c1, even when subjected to passages in BHK-21 cells, can vary its specific infectivity by orders of magnitude (23) (R. Agudo and E. Domingo, unpublished results). These results indicate that viruses isolated from different tissues of the same mouse, despite having the same consensus genomic nucleotide sequence, display remarkably different phenotypes in vivo.

Genomic diversity of viral populations from serum, spleen, and pancreas. To determine whether the quasispecies complexity of the viral populations isolated from spleen, serum, and pancreas of mouse number 8 (Fig. 2) could explain the differing virulences for mice displayed by these viruses, the mutation frequencies of their mutant spectra were compared. Seventy-four or 75 cDNA clones from the VP1- and 2C-coding regions of viruses from spleen, pancreas. and serum (approximately 80,000 nucleotides per viral population) were sequenced without virus adaptation to cell culture (see Materials and Methods). There were no statistically significant differences among the mutation frequencies of these three viral populations (Table 6). The normalized Shannon entropy val-

TABLE 6. Complexities of the mutant spectra of FMDV populations from serum, pancreas, and spleen of mouse 8 at passage 1

Virus population	Molecular cloning			Biological cloning		
	Substitutions/nucleotide (mutation frequency)[a]	Normalized Shannon entropy[b]	NS/S[c]	Substitutions/nucleotide (mutation frequency)[d]	Normalized Shannon entropy[e]	NS/S[c]
Serum	15/79,188 (1.9×10^{-4})	0.224	10/5 (2)	4/40,575 (9.9×10^{-5})	0.826	1/1 (1)
Pancreas	14/80,602 (1.7×10^{-4})	0.172	10/4 (2.5)	3/40,575 (7.4×10^{-5})	0.826	1/2 (0.5)
Spleen	24/80,602 (3×10^{-4})	0.259	19/5 (3.8)	ND[f]	ND[f]	ND[f]

[a] The mutation frequency is the number of different mutations divided by the total number of nucleotides sequenced. To determine the mutation frequency in each viral population, 74 or 75 cDNA clones were analyzed. There were no significant differences between the mutation frequencies for the three viral populations analyzed (one-way ANOVA; $P < 0.05$).

[b] Normalized Shannon entropy is a measure of the proportion of identical sequences in a distribution (57). To calculate the normalized Shannon entropy, residues 4364 to 5306 of each viral molecular clone were analyzed. Numbering of residues is as in reference 50.

[c] NS/S, ratio between nonsynonymous and synonymous mutations. The corresponding numerical value is shown in parentheses.

[d] Mutation frequency calculated with the entire genomic nucleotide sequence of five biological clones from serum and five biological clones from pancreas. There were no significant differences between the mutation frequencies of these two viral populations (one-way ANOVA; $P < 0.05$).

[e] Normalized Shannon entropy determined with the entire genomic nucleotide sequences of five biological clones from serum and of five biological clones from pancreas.

[f] ND, not determined.

ues were also similar for all viral populations analyzed. Moreover, the complete genomic nucleotide sequences from five biological clones from the virus isolated from pancreas and five from biological clones from the virus isolated from serum were determined, and mutation frequency and normalized Shannon entropy were calculated (Table 6). Again, the viral populations from serum and pancreas did not exhibit any significant difference in mutant spectrum complexity. These data suggest that the different phenotypes shown in mice by these viral populations cannot be attributed to a difference of genomic diversity in the viral populations.

Differential quasispecies genome composition in virus from pancreas and serum determines viral phenotype in vivo. We hypothesized that a different quasispecies genome composition, not reflected in mutant spectrum complexity, was the determinant of viral virulence in vivo. To test whether viral subpopulations within serum and pancreas could be distinguished by differences in their replications in BHK-21 cells, viral RNA from serum or pancreas from mice 8 and 10 was transfected into BHK-21 cells. RNA transfection was chosen to avoid any possible change in quasispecies composition due to selective viral entry into BHK-21 cells. A positive control transfected with C-S8c1 RNA was included in the assay. Extracellular virus was collected either at early times (24 to 36 h) posttransfection (when cytopathology affected 20% of BHK-21 cells) or late times (48 to 69 h) posttransfection (when cytopathology was complete). The viruses rescued at early and late times posttransfection were tested for their virulence in mice (Table 7). A significant increase in survival of mice was noted for RNA from pancreas harvested at late times posttransfection ($P < 0.0001$). To determine whether the intracellular virus population at early times posttransfection differed from the extracellular virus in terms of virulence, intracellular virus was harvested by freeze-thawing of BHK-21 cells transfected with RNA either from serum or pancreas or from C-S8c1. The data indicate (Table 7) that there is no significant difference between the virulences for mice of the extracellular and intracellular viral populations. Thus, the late subpopulation of virus derived from pancreas, but not the corresponding early subpopulations (intracellular as well as intracellular viral populations) derived from pancreases of the same animals, reproduced the attenuated phenotype for mice. Interestingly, no differences among the consensus nucleotide sequences of the entire viral genomes from the early and late viral subpopulations were observed (data not shown). Therefore, a differential rate of replication upon transfection of BHK-21 cells captured the difference in virulence for mice of the two subpopulations. Yet, no genomic signature could be associated with the attenuated phenotype.

DISCUSSION

Quasispecies dynamics of FMDV has been extensively studied in cell culture (reviewed in reference 17). However, very few studies have addressed quasispecies dynamics of FMDV in vivo. In an early evidence of quasispecies, two closely related variants of FMDV were identified in the same infected animal

TABLE 7. Virulence for mice of FMDV rescued at early or late times upon transfection of BHK-21 cells with viral RNA

Viral RNA[a]	Extent of CPE (%)[b]	Virulence for mice: % survival (no. surviving/no. inoculated) at 48 hpi[c]	
		Intracellular virus population	Extracellular virus population
C-S8c1	20	25 (1/4)	0 (0/6)
	100	0 (0/8)	
Serum 8	20	0 (0/4)	0 (0/6)
	100	0 (0/4)	
Serum 10	20	ND[d]	0 (0/6)
	100	0 (0/8)	
Pancreas 8	20	0 (0/4)	17 (1/6)
	100	75 (3/4)[e]	
Pancreas 10	20	0 (0/8)	17 (1/6)
	100	100 (8/8)[e]	

[a] Viral RNA was extracted either from C-S8c1 (control) or from sera or pancreases of mice 8 and 10 (as shown in Fig. 2) and used to transfect BHK-21 cells to rescue infectious particles, as described in Materials and Methods.

[b] Virus was collected from the supernatant of transfected cells when the cytopathic effect (CPE) affected 20% (24 to 36 h posttransfection) or 100% (48 to 69 h posttransfection) of BHK-21 cells.

[c] Mice were inoculated in the FP with 10^4 PFU of the virus harvested after transfection.

[d] ND, not determined.

[e] These viruses show significant decreased virulence in mice (Fisher's exact test; $P < 0.05$) in relation to the other viral populations shown in this table.

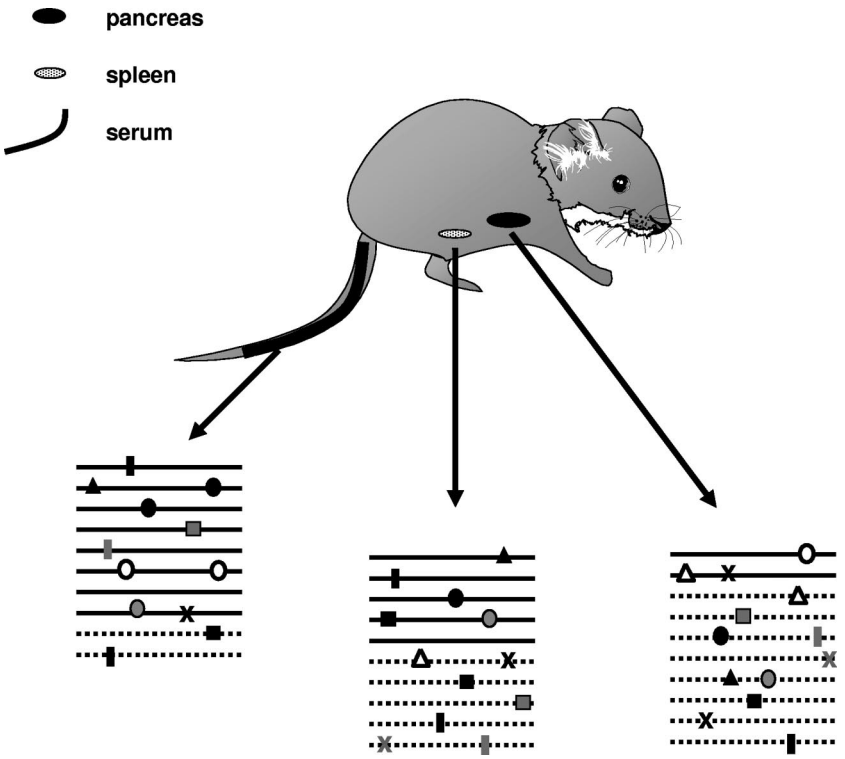


FIG. 4. Model of the interplay between different variants from the FMDV quasispecies as a determinant of an in vivo phenotype. Lines represent viral genomes, and symbols on the lines represent mutations. Viral populations isolated from the pancreas, spleen, or serum of a mouse are composed of "pathogenic" (black line) and "nonpathogenic" (dashed line) genomes in different proportions. A higher proportion of "pathogenic" than of "nonpathogenic" genomes (serum) would result in virulence for mice. An intermediate proportion of "pathogenic" and "nonpathogenic" genomes (spleen) would result in an intermediate virulence for mice. A quasispecies composed of a higher proportion of "nonpathogenic" than of "pathogenic" genomes would result in attenuation in mice (virus from pancreas).

in cattle (15). Adaptation of a swine FMDV isolate to guinea pigs occurred through heterogeneous viral populations, ending in the fixation of a critical substitution in nonstructural protein 3A (33). In a recent study, serial contact transmission of FMDV in pigs (equivalent to plaque-to-plaque transfer in cell culture [17]) led to reduction of virulence and interruption of transmission, despite the persistence of virus in the animals (8). In none of the above studies were FMDV subpopulations from different organs compared genetically or phenotypically. This report provides experimental evidence that viral quasispecies in vivo may comprise multiple FMDV subpopulations that evolve independently in different tissues within the same infected host. Tissues introduce distinct selective pressures and may represent population bottlenecks to viral spread, with a reduction of quasispecies complexity (24, 38). However, our data indicate that the quasispecies complexity of FMDV can be maintained upon viral replication in different tissues. In addition, biologically very significant differences in viral phenotype were observed among populations presenting identical consensus sequences and indistinguishable mutant spectrum complexities, as determined both by mutation frequency and by normalized Shannon entropy of specific genomic regions and the entire genome sequence of biological clones. These results are consistent with a model in which virus phenotype (virulence in mice) may be determined by the interplay between different variants within the quasispecies that may or may not facilitate expansion and replication in mice, without the requirement of a specific genetic signature associated with virulence (Fig. 4).

Anatomical sequestration of virus populations may provide a biological niche that could lead to the subdivision of the viral quasispecies into smaller, semi-isolated groups that differ in terms of phenotype and/or selective advantage. Our study was undertaken to investigate specific tissue and cell type factors that may contribute to the selection of such variants. FMDV C-S8c1 showed a propensity to invade the mouse pancreas at 24 hpi (42), resulting in acute pancreatic inflammation and the loss of pancreatic acinar tissue (Fig. 1A). Different mice had equivalent viral loads at 24 hpi but displayed differences of from 1 to more than 5 logarithmic units in specific infectivity. Specific infectivities for FMDV C-S8c1 with a history of passages in BHK-21 cells are in the range from 1×10^{-4} to 4×10^{-9} PFU/RNA molecule (23, 56). Therefore, it is not surprising that even lower specific infectivity values are scored for C-S8c1 after replication in mice, when measured in BHK-21 cells (Fig. 3 and Table 1). Thus, further research would be needed to attribute the phenotypic behavior to the specific infectivity of the virus. When virus from pancreas was inoculated into naïve mice, the virus showed an attenuated phenotype, with low mortality even at the highest viral dose used (10^4 PFU). Interestingly, the virus spread and replicated in all major organs, including the pancreas, suggesting that virus from the pancreas did not show discernible preferential tropism for

the pancreas. A similar phenomenon has been described for cytomegalovirus in which the cell type producing the most virus (hepatocytes) was not necessarily the one responsible for virus dissemination within the host (41). Thus, it appears that the difference between FMDV C-S8c1 and its progeny from pancreas with regard to virulence for mice lies at a level different from their mere abilities to replicate in certain mouse tissues.

The basis for the striking differences in virulence between C-S8c1 and its pancreatic derivatives was analyzed at the molecular level. The molecular characterization of pancreas viruses revealed several mutations, and I85V and I248T in 2C were found for several mice. These two substitutions were also found for other organs from the same mice (spleen, lung, and heart), suggesting a relevance of these two positions in 2C in the adaptation of FMDV to enable replication in mice. Mutations in 2C have been found upon adaptation of FMDV and other picornaviruses to different environments in cell culture and in vivo (6, 8, 20, 21, 28, 33, 38, 46, 59). Their possible biological significance is under study.

The changes found in the *cre* region and in the L and 3A proteins in FMDV populations isolated from pancreas, spleen, heart, and lung point out an influence of these genomic regions on FMDV replication in mice. It is interesting that in the *cre* region, a relatively small genomic region comprising only 54 nucleotides, two nucleotide changes (C559U and U586C) were found, located in the stem region of *cre*, which has been described as very important for replication (31). The application of the Mfold program (59) indicated that both substitutions caused an increase of Gibbs free energy in the predicted structure and that the change U586C generated a mispaired stem structure.

The various organs and cell types in an organism provide a rich milieu for the selection of viral variants, as evidenced, for example, by the presence of genetically distinct subpopulations of HIV-1 in different organs and even in different regions within the same organ (46, 54, 55, 58). It cannot be excluded, however, that some of the mutations scored in these studies may have resulted from genetic drift. Many examples have documented that a single point mutation may lead to important changes in the biology of diverse viruses (for examples, see references 1, 6, 14, 43, 49, and 51). Our study underscores, however, important phenotypic changes among viral populations in an individual mouse, with no detectable changes in the consensus sequences or in the complexity of the mutant spectra. Furthermore, the molecular and biological cloning analysis showed no differences among viruses from serum, pancreas, and spleen or in other parameters such as the ratio of nonsynonymous and synonymous mutations or the number of transitions and transversions in each viral population, pointing to factors in viral pathogenesis that may go unnoticed by our current methods to probe viral genome populations. One possibility is that attenuation was prompted by the limited capsid stability of the virus assembled in the pancreas. However, this is unlikely to be a major contributing factor because virus rescued at late times after transfection of BHK-21 cells maintains its attenuated phenotype.

Our current hypothesis is that the FMDV quasispecies from spleen, pancreas, and serum are composed of "nonpathogenic" genomes and "pathogenic" genomes in different proportions (Fig. 4). The molecular basis for how the interplay between different genomes may lead to a virulent or an attenuated population is unknown, but several possible mechanisms have been previously documented. A subset of "nonpathogenic" genomes can suppress the "pathogenic" genomes, as previously evidenced with attenuated and virulent poliovirus in vaccine preparations (11) or with the suppression of growth hormone deficiency syndrome associated with lymphocytic choriomeningitis virus infection by disease-negative virus variants (52). An alternative mechanism is that complementation among subsets of genomes is needed for pathology and that this requires minimum levels of some genome subsets. Complementation of a specific poliovirus mutant by a spectrum of mutants was required for the mutant to reach the brains of susceptible mice (57). In the case of FMDV, the so-called "nonpathogenic" genomes would reach the pancreas in a higher proportion than "pathogenic" genomes, although the latter might replicate faster at early stages of the infection and be poured into the bloodstream, enriching the circulating quasispecies with a higher proportion of "pathogenic" genomes. This hypothesis was supported by our transfection data, in which RNA from pancreas transfected in BHK-21 cells and harvested at short times posttransfection was pathogenic for mice, while the virus harvested at later times posttransfection was nonpathogenic for mice. The pathogenic versus nonpathogenic subpopulations distinguished by the time after transfection of BHK-21 cells did not correspond to a compartmentalization of intracellular versus extracellular virus. Indeed, intracellular virus (particles that were assembled at 24 to 36 h posttransfection) was as virulent for mice as virus shed from the cells at such times posttransfection. This suggests, again, that attenuation corresponds to subsets of genomes that replicate and assemble at later times. This is consistent with the hypothesis that the viral quasispecies at times shortly after posttransfection were enriched in subsets of genomes that we describe as "pathogenic," relative to their "nonpathogenic" counterparts, whereas at late times posttransfection the quasispecies was dominated by "nonpathogenic" genomes. Suppressive effects of mutant spectra have also been documented for FMDV and other viruses in cell culture and in vivo (13, 26, 37, 52). New techniques such as microarray analyses or a more exhaustive survey of individual genomes through pyrosequencing-based methodologies might help to discern the so-called "nonpathogenic" from "pathogenic" subpopulations of genomes in a quasispecies by some genetic signature that cannot be identified from the nucleotide sequences of limited numbers of genomes.

ACKNOWLEDGMENTS

This research was supported by grants AGL2004-0049 and BFU2005-00863 from Ministerio de Educación y Ciencia, Spain; by the EU, Network of Excellence, EPIZONE (contract no. FOOD-CT-2006-016236); and by Fundación Ramón Areces. CIBERehd is funded by the Instituto de Salud Carlos III. M.S.-R. was supported by an FPU fellowship from Ministerio de Educación y Ciencia. F.D.-S.S. was supported by a fellowship from INIA (Spain).

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