In vivo analysis of the stability and fitness of variants recovered from foot-and-mouth disease virus quasispecies

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We have analysed the ability to infect pigs of two foot-and-mouth disease virus (FMDV) variants isolated at low frequencies from virus populations (quasispecies) generated in pigs on infection with a parental virus, C-S8c1. A monoclonal antibody-resistant mutant (MARM21), and a variant isolated at early times post-infection (S-3T1), each exhibiting a unique amino acid substitution in VP1, were able to cause disease in pigs, both by direct inoculation or by contact transmission. The symptoms developed were similar to those produced by C-S8c1 or the related virus C-S15c1. The VP1 sequence of viral RNA directly recovered from lesions of infected animals confirmed the stability of the variant genotypes. Pigs infected with S-3T1, consistently showed an advance of 12 to 24 h in the emergence of fever and lesions when compared to animals infected with C-S8c1 or the remaining variants, an observation consistent with its early isolation. The ability of FMDV variants to compete in vivo with C-S8c1 was investigated in coinfection experiments. Analysis of the proportion of each of the competitors in lesions of co-infected pigs revealed that none of the variants was completely overgrown by the parent. However, coinfection with C-S8c1 and MARM21 resulted in lesions in which C-S8c1 was predominant, indicating a selective disadvantage of this variant in swine. In contrast, lesions from swine co-infected with C-S8c1 and S-3T1 contained similar proportions of the two viruses. These results document fitness variations in vivo among components of the mutant spectrum of FMDV quasispecies.

Introduction

Populations of RNA viruses consist of multiple variants collectively termed virus quasispecies (Eigen & Schuster, 1979; Domingo et al., 1985). A complex equilibrium between a high rate of mutation and the competitive fitness of each of the arising variants determines, in a given environment and time, the composition of the quasispecies, which includes one or several master sequences, and a multitude of variants present in different proportions (Eigen & Biebricher, 1988; Domingo et al., 1985; Holland et al., 1992). This property endows RNA viruses with a high potential for virus variation and adaptation (Holland et al., 1982; Domingo & Holland, 1994). This is the case for foot-and-mouth disease virus (FMDV), an aphthovirus of the family Picornaviridae, which causes one of the most economically important animal diseases (Bachrach, 1968). A number of studies have been done that illustrate the potential for genetic and antigenic FMDV variation, as well as the complex dynamics of virus populations (Domingo et al., 1980; King et al., 1981; Rowlands et al., 1983; Sobrino et al., 1983; Escarmís et al., 1996; reviewed in Domingo et al., 1990, 1992). The heterogeneity of FMDV populations allows the isolation of MAb-resistant (MAR) mutants at frequencies close to 10⁻⁵ (Martínez et al., 1991). Analysis of the repertoire of MAR mutants selected from virus populations passaged in cell culture using MAb SD6, directed to an important antigenic site on the G–H loop of VP1 (Acharya et al., 1989; reviewed in

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‡ This paper is dedicated to the memory of our colleague Dr Donald Morgan.
Brown, 1995; Mateu, 1995), revealed a limited spectrum of amino acid replacements in this antigenic site. Two of the MAR mutants isolated showed a reduced fitness in BHK-21 cells relative to the parental virus (Martinez et al., 1991). Attempts to analyse the virus populations recovered from pigs experimentally infected with FMDV resulted in the isolation of a spectrum of mutants resistant to neutralization with MAb SD6. In addition, a virus variant was isolated from a pig at early times post-infection (p.i.) which did not become dominant in the virus populations recovered later in the infection (Carrillo et al., 1990). These results were consistent with the complexity and dynamics of FMDV quasispecies in vivo, but the ability of these virus variants to grow in susceptible animals, their fitness relative to the parental population, and their potential to contribute to the antigenic diversity of the virus in vivo remained to be studied. In an attempt to explore the potential of virus variants present in low proportions in the virus quasispecies in vivo to contribute to FMDV diversification we have studied the ability of different FMDV variants recovered from infected pigs to produce the disease, as well as the extent to which they compete with the parental population in co-infection experiments in this animal model.

Methods

**Viruses.** The FMDV isolates and the passage history in BHK-21 cells of the virus stocks used in this study were as follows. (i) C-S8c1 is a derivative of a type C1 field variant isolated from pig in Catalonia (Spain) in 1970 (Domingo et al., 1980); after three amplifications the virus population was plaque-purified four times (Carrillo et al., 1990). (ii) MARM21, a variant resistant to neutralization with MAb SD6, was isolated from a pig infected with C-S8c1, and exhibits a unique amino acid substitution, S(139) → R, within VP1 with respect to the parental virus (Carrillo et al., 1990). (iii) S-3T, isolated from a blood sample collected day 1 p.i. from a pig infected with C-S8c1, and exhibits a unique amino acid substitution, T(135) → A, when compared to the parental virus (Carrillo et al., 1990). (iv) C-S15c1 is a derivative of a type C1 field variant isolated from a pig in Catalonia (Spain); after two amplifications the virus population was plaque-purified three times. The VP1 protein of this virus has eight amino acid substitutions with respect to that of C-S8c1 (Sobrino et al., 1986). For animal inoculation, each of the viruses were amplified, by two tissue culture passages, from about 7 × 10^5 to 8 × 10^6 p.f.u. The average VP1 gene sequence of the virus stocks obtained was described below and confirmed that no nucleotide substitutions arose during amplification.

**Infection of animals.** Pigs (Landrace × Large White, 2 months old) were inoculated with 10^6 p.f.u. of the selected viruses, by injection at the coronary matrix of the foot. For this purpose, virus stocks were titrated in BHK-21 cells. The titres obtained in this cell line were comparable to those determined in two different pig-derived cell lines: IBSR-2 and PK-15. In all cases, animals were free of previous FMD contact, and this was confirmed by the absence of detectable anti-FMDV antibodies by serum neutralization tests. Rectal temperature and emergence of lesions was scored daily until day 4 p.i. and material from lesions was collected and kept frozen.

**Reactivity with MAbs.** A liquid phase radioimmunoassay (Robertson et al., 1984) was used to compare the binding of FMDV C-S8c1 and S-3T, to a panel of anti-FMDV neutralizing MAbs (Table 1). Briefly, purified 140S virions (Sáz et al., 1989) were radio-iodinated by the Iodo-gen method (Fraker & Speck, 1978) and equal amounts of viral radioactivity were incubated with culture supernatants from the different MAbs. The radioactivity recovered after precipitation and washing was measured in a γ-counter. Titres were expressed as the ratio of c.p.m. measured for each of the viruses compared (Sáz et al., 1989).

**In situ immunoenzymoassay.** The proportion of each of the FMDV variants present in samples from co-infected animals was determined by means of the differential reactivity of plaques of the competing viruses to anti-FMDV MAbs. Under the reaction conditions used, MAb SD6 (Mateu et al., 1987), which reacts with FMDV C-S8c1, was used to differentiate this virus from C-S15c1 or MARM21, which have 100-fold lower reactivity. Likewise MAb 15AC311 (D. M. Moore and others, unpublished results), which has 10-fold higher reactivity with S-3T, allowed distinction between this virus and C-S8c1, whose p.f.u. were not detectable in the assay. The procedure followed to assess virus reactivity was as described by Díez et al. (1989). Briefly, to 100 p.f.u. from lesion samples were directly plaqued in BHK-21 cells in the presence of 0.7 % agar. Virus plaques were transferred onto a nitrocellulose filter, which was then incubated with a 1:100 dilution of the selected MAb, and developed using rabbit anti-mouse peroxidase (Mateu et al., 1987). The total number of plaques present on the filter was estimated by a second incubation with a 1:100 dilution of swine anti-FMDV polyclonal serum, with binding revealed using a rabbit anti-swine peroxidase.

**Nucleotide sequencing.** Samples of vesicular fluid from lesions on infected animals were extracted with phenol and chloroform and ethanol precipitated. The resuspended RNA was used for direct sequencing of the FMDV VP1 gene by primer extension, as described (Sobrino et al., 1986; Martinez et al., 1988).

Results

**In vivo infections with the parental FMDV C-S8c1 and related variants.**

We have analysed the ability of two FMDV variants, MARM21 and S-3T, isolated from pigs experimentally infected with C-S8c1 (Carrillo et al., 1990), to replicate in swine, relative to the parental virus. In addition C-S15c1, a derivative of a field isolate closely related to C-S8c1, was also included in the comparisons. We first tested the ability of the parental C-S8c1 virus and the three related variants to produce clinical infection in pigs. For each virus, three animals were inoculated with 10^8 p.f.u. and two additional animals were kept in the same room to allow contact transmission. All the animals developed clinical symptoms, including vesicular lesions. In general, they also developed fever (temperature above 40 °C) from day 2 p.i. (Fig. 1). Pigs infected with variant S-3T displayed fever and presented secondary lesions at locations different from the site of inoculation on the day 1 p.i., which represents emergence of lesions 12 to 24 h before the animals infected with the other viruses. These observations are consistent with the isolation of S-3T at an early time after infection.

Direct nucleotide sequencing of total RNA extracted from two independent lesions on each of the infected animals confirmed that the average VP1 gene sequence was, in all cases, indistinguishable from that of the virus preparation used for infection. These results indicate that the different FMDV
Table 1. Reactivity of FMDV C-S8c1 and S-3T, with different MAbs

<table>
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<tr>
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* All the MAbs used neutralized the virus against which they were raised. Unless indicated the MAbs were of IgG isotype and are described in Methods.
† Subtype of the FMDV isolate used for production of the MAb.
‡ A radioimmunoassay was used to estimated the reactivity of the 140S virions with the different MAbs (see Methods). Values are expressed as the ratio between the c.p.m. obtained for each MAb with variant S-3T and those obtained with C-S8c1.
§ Unpublished results, presented here.
|| The isotype of this MAb was IgM.

variants are infectious for pigs, both by direct inoculation and by contact transmission. They also document the conservation of the average nucleotide sequence of the VP1 gene in the virus populations recovered from infected animals, including those infected by contact exposure.

**Antigenic analysis of the S-3T, FMDV variant**

The S-3T variant showed a single nucleotide substitution, A(403) → G, responsible for the amino acid replacement T(135) → A in VP1 (Carrillo et al., 1990). To analyse the effect of this replacement on the antigenicity of S-3T, a radioimmunoassay was used to compare the reactivity of S-3T and C-S8c1 with a panel of 58 different MAbs. In general, no significant differences were found between the two viruses. Only MAb 15AC311 showed a 10-fold increase in binding to S-3T relative to C-S8c1 (Table 1). This result implied the presence of an antigenic difference between the S-3T variant and its parental virus. As expected from the VP1 sequence conservation, this antigenic difference was also observed among the viruses recovered after infection of pigs with S-3T, and provided us with a tool for quantification of the proportion of each of the two viruses in mixed populations.

**Experimental co-infection of pigs with different FMDV variants**

The ability of FMDV variants to compete in vivo with the parental virus C-S8c1 was investigated in co-infection exper-
Fig. 1. Change of rectal temperatures in pigs experimentally infected with different FMDV variants. Groups of three animals were inoculated with each of the FMDV variants C-S8c1, S-3T1, MARM21 or C-S15c1 (see Methods). The virus used for inoculation is indicated at the top of each panel. An animal inoculated with C-S8c1, indicated with a stippled triangle, died at day 4 p.i. The remaining animals were slaughtered at day 5 p.i.

Discussion

In recent years, an increasing amount of information has been obtained on the structure and complex dynamics of RNA virus quasispecies, including the high potential for virus evolution conferred by the mutant spectrum of virus populations (Kilbourne, 1991; Domingo & Holland, 1994). In addition, there is evidence for in vivo involvement of variants present at low frequencies in the quasispecies in important biological properties such as virus pathogenesis and its implications in the control of the disease (Domingo & Holland, 1992; Coffin, 1995). In this report we have assessed the ability of FMDV variants from the quasispecies of FMDV C-S8c1 replicating in swine to infect this natural host. Variant MARM21, an escape mutant resistant to neutralization with MAb SD6, was present at a frequency close to $10^{-5}$ (Carrillo et al., 1990) in the vesicular fluid from a pig infected with C-S8c1.
Variant S-3T₁ was isolated from blood of a C-S8c1-infected pig at early times p.i. Each of the viruses showed a unique amino acid substitution in the VP1 protein that has allowed their identification in mixed infections by nucleotide sequencing or by differential reactivity with a MAb. These low frequency variants, as well as C-S8c1 and C-S15c1 variants, produced disease in vivo (Fig. 1). In all cases, contact transmission from inoculated animals was observed. Average VP1 sequences in RNA directly extracted from lesions confirmed that the genotype of the viruses recovered from inoculated and contact infected animals was, for each of the viruses analysed, indistinguishable from those used to establish infection. Thus, at least in the RNA region analysed, no mutations were selected during experimental infection and transmission. Pigs inoculated with variant S-3T₁ showed an advance in the emergence of clinical symptoms – fever and vesicular lesions were observed during day 1 p.i – while pigs infected with MARM21, C-S8c1 or C-S15c1 developed symptoms at day 2 p.i. (Fig. 1). The advance in the emergence of the symptoms produced by S-3T₁ was consistent with its early isolation, and shows that S-3T₁ is a stable variant with an altered pathogenicity in swine. The molecular basis of this phenotype is under study.

MAbs 15AC311 and SD6 were used to quantify the phenotype of the viruses recovered from co-infection experiments in pigs with C-S8c1 and each of the related variants.
selected from lesions of animals infected with C-S8c1 and were amplified in cell culture to the minimal extent that allowed animal inoculation, and therefore modifications of the original ability of the variants to compete with the parental virus cannot be excluded. However, the results obtained support a reduced relative fitness of MARM21 relative to its parental virus, C-S8c1. Selective disadvantage has also been noticed among other FMDV MARM in competition experiments with their parental quasispecies in cell culture (González et al., 1991; Martínez et al., 1991). The C-S8c1 phenotype was also predominant among the viruses recovered from the four lesions of animal 59, co-infected with C-S15c1. Dominance of C-S8c1 was not so evident in animal 60, also co-infected with C-S15c1. In this case, lesions in which each of the competitors was predominant were found. Further work is required to understand the basis of the differential representation of the competing viruses in independent lesions, an observation also made for competition of variants S-3T1 and C-S8c1.

Variant S-3T1 was present at a low proportion at early times of infection and never became dominant in the virus populations of the pig from which it was recovered (Carrillo et al., 1990). However, the analysis of lesions from animals 64 and 65 indicated that viruses with the S-3T1 phenotype are not significantly overgrown by those with the C-S8c1 phenotype. This result indicates the lack of a selective disadvantage of S-3T1 relative to its parental quasispecies during replication in pigs. It has been reported by De La Torre et al. (1990) that variants present in minority proportions can be competed by the average quasispecies even if they exhibit higher relative fitness. Assuming that the fitness of the S-3T1 variant was not modified during its isolation and amplification in cell culture, this threshold effect could be a possible explanation for the low proportion of S-3T1 in the animal from which it was isolated. A number of examples of a threshold for phenotypic expression have been described for RNA viruses (reviewed in Domingo & Holland, 1997).

Experiments aimed at fitness determination in vivo, such as those reported here, are subjected to a number of indeterminants, much more severe than those encountered in cell culture. Indeterminants include multiple biological environments that may affect the stability of the virus and its ability to infect different types of susceptible cells, the possibility of bottleneck events when the virus invade a new tissue or organ within the animal and the difficulty in determining comparable infectious doses among competitors, among others. In spite of these limitations, an increasing number of studies are addressing fitness determination in vivo (reviewed in Domingo & Holland, 1997), due to the relevance of the population complexity relative to the outcome of virus pathogenesis. The results reported here provide evidence of the potential of low frequency FMDV quasispecies variants with relevant phenotypic changes to produce and transmit the disease in a natural host. Also, differential fitness of these variants relative to the parental quasispecies from which they were selected is shown.

Fig. 2. In situ immunoassay of p.f.u. recovered from lesions of FMDV-infected pigs. P.f.u. obtained on BHK-21 cells from lesions of animals inoculated with (a) C-S8c1, (b) MARM21 or (c) a mixture of C-S8c1 and MARM21 (corresponding to lesion 63T, in Table 2), were transferred onto nitrocellulose filters and incubated with MAb SD6 as described in Methods. The p.f.u. with a C-S8c1 phenotype were revealed by incubation with MAb SD6. The total number of p.f.u. was estimated by incubation with an anti-FMDV polyclonal serum.

In all cases, p.f.u. with phenotypes corresponding to each of the competitor viruses were found in the lesions analysed, which indicates that vesicle formation is likely to be originated by more than one infectious virus; a clonal origin of FMD vesicles would have introduced a potential source of virus diversification during natural transmission (Chao, 1990; Duarte et al., 1992; Clarke et al., 1993). In those lesions in which the average VP1 RNA sequence was directly obtained, the results were consistent with the phenotypic quantification of the viruses recovered (Table 2).

Lesions from animals 62 and 63 co-infected with C-S8c1 and MARM21 predominantly produced p.f.u. with the phenotype of the parental virus. A precise interpretation of competition experiments has to take into account the potential of virus quasispecies to alter their relative fitness during individual propagation (Bolwell et al., 1989; Martínez et al., 1991; Clarke et al., 1993). In our case, virus variants were
The sustained presence of related FMDV variants in infected hosts is likely to contribute to the adaptability and persistence of this important pathogen.

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