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Homologous Interference by a Foot-and-Mouth Disease Virus Strain Attenuated for Cattle

By

PATRICIA POLACINO, G. KAPLAN and E. L. PALMA

Departamento de Virología, Centro de Investigaciones en Ciencias Veterinarias, Instituto Nacional de Tecnología Agropecuaria, Buenos Aires, República Argentina

With 3 Figures

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Summary

An attenuated strain of foot-and-mouth disease virus (FMDV) of the A_{24} Cruzeiro subtype grew less well than wild-type virus in primary bovine fetal kidney (PBK) cells resulting in a 4-log lowered efficiency of plaque formation. Both wild-type and attenuated virus grew equally well in baby hamster kidney (BHK) cells and in suckling mice. Using PBK cells, virus-specific RNA of the wild-type accumulated up to 6 hours after infection. In contrast, PBK cells infected with the attenuated strain made less than 20 per cent of the RNA synthesized by wild-type virus.

Infection of the cell with wild-type virus followed by superinfection with attenuated virus led to almost complete inhibition of viral RNA synthesis, an effect which is dependent on the concentration of input attenuated virus. Three subsequent undiluted successive passages of the attenuated virus resulted in a preparation which no longer interfered with wild-type RNA synthesis and which induced more of its own viral RNA synthesis in PBK cells.

The basis of this interference was considered. Interference occurred intracellularly and was directed only against viruses within the genus FMDV. A role for interferon was ruled out. Attempts to demonstrate the physical presence of defective interfering (DI) particles of FMDV in the attenuated strain failed; but, cyclic patterns of infectivity were produced during successive undiluted passages.

Introduction

Passaging of viruses in unnatural hosts is one of the most common methods for attenuating viral strains during vaccine development. Serial

passages through hosts, such as embryonated eggs (1, 22), mice (22), rabbits (2), one-day-old chicken (15), and tissue cultures (14), have succeeded in attaining attenuation of FMDV for cattle, as shown by a loss pathogenicity without a reduction in immunogenicity. The criteria for selection of such strains are highly empirical, since little is known about the attenuation mechanism. Inhibition of FMDV multiplication is generally attributed to modified viral strains or to interferon. Although some interferon is produced by bovine fetal kidney (PBK) cells infected with a wild-type FMDV strain (4), a much higher interferon titer is measured when the infecting strain is a modified one (21). Interference of FMDV by temperature-sensitive mutants (*ts*) also occurs when PBK cells are incubated at the nonpermissive temperature; in this case, synthesis of interferon is detected, also (18).

Another kind of interference, widely reported in different animal virus systems, is that produced by defective interfering (DI) particles. This is characterized by being intracellular and homotypic. Within the picornaviruses, poliovirus DI particles have been the most thoroughly studied (9). In the case of FMDV, although DI particles have not been described, their role during interference remains a possibility.

Here we report interference studies with an attenuated strain of FMDV. Attenuation was correlated with its interfering capacity against the homologous wild-type strain. In addition, attempts were made to identify the possible role of interferon or DI particles during interference.

Materials and Methods

Cells and Viruses

BHK_{21} Clone 13 cells and primary cultures of PBK cells were used throughout this work. Both kinds of cells either in monolayers or in suspended cell cultures were propagated in Eagle's MEM, supplemented with 10 per cent calf serum (GIBCO) (6). Wild-type FMDV is of the serotype A, subtype 24, strain Cruzeiro, isolated from bovine tongue epithelium and passaged less than 6 times through BHK_{21} cells. The same virus was attenuated for cattle by passaging in chick embryos (attenuated strain) (1). Wild-type and attenuated strains of FMDV subtypes O_1 and C_3 were also studied. Both the wild-type and the attenuated strains were kindly provided by the Foot-and-Mouth Disease Pan American Center (Río de Janeiro, Brazil). Bovine enterovirus strain (BEV) came from the strain collection of our Institute's (INTA) Virology Department (11). Virus stocks, grown in BHK_{21} cells, were stored at $-70°$ C.

Cloning of FMDV

BHK_{21} cells grown in 20 cm^2 monolayers plates were infected with either the wild-type or the attenuated strains of A_{24} Cruzeiro virus, and incubated at 37° C with an agar overlay. Isolated plaques from each strain were picked at 48 hours post-infection (p.i.) and grown up by inoculation into tubes with BHK_{21} cells. Three successive cloning steps were carried out. Plaques were developed with 0.02 per cent neutral red solution.

Virus Titration

Viral titers were determined by two different procedures: a) plaque assay on BHK_{21} cell monolayers (5) and b) tissue culture infectious doses ($TCID_{50}$) in PBK microplate cultures. Also *in vivo* pathogenicity in 6 day-old suckling mice was carried out by intraperitoneal inoculation; the lethal dose (LD_{50}) was calculated 7 days later, by using the Reed-Muench method (19).

Purification of Radioactive Labeled Virus

BHK_{21} cells grown in roller bottles were infected with A_{24} Cruzeiro wild-type virus or the attenuated strain in MEM without serum and exposed to 5 μg/ml of Actinomycin D (grade II, Sigma Chemical Co.) and 0.025 M Hepes buffer (N-2 hydroxyethylpiperazine-N'2'-ethanesulfonic acid) pH 7.5. For labeling the nucleic acid of the virus 10 μCi/ml of ^{3}H-uridine (NEN Co., 37.9 Ci/mmol) was utilized. Virus was harvested at the time of total cytopathic effect (CPE). The supernatant was clarified at $10{,}000 \times g$ for 10 minutes and the virus was concentrated at $100{,}000 \times g$ for 120 minutes, at 4° C. After the pellet was resuspended in NET buffer (0.05 M Tris-hydrochloride pH 7.4; 0.1 M NaCl; 0.01 M EDTA), and treated with 0.5 per cent sodium deoxycholate (DOC), virus was purified by ultracentrifugation in a 10—30 per cent sucrose-NET (w/v) gradient at $100{,}000 \times g$ for 180 minutes at 4° C. After fractionation and determination of TCA-insoluble radioactivity (6), fractions containing FMDV were collected.

Cesium Chloride Density Gradients

The purified virus was diluted with an equal volume of NET buffer (pH 7.4) and pelleted at $200{,}000 \times g$ for 50 minutes at 4° C. The pellet was resuspended in NET buffer and CsCl was added to a final concentration of 1.42 g/cm^3. Centrifugation was carried out at $150{,}000 \times g$ for 16 hours at 4° C. The gradient was then fractionated and acid-insoluble radioactivity was used to determine virus peak location.

Viral Interference Assays: Inhibition of Nucleic Acid Synthesis

PBK cells, suspended at a concentration of 5×10^6 cells/ml, were infected with the A_{24} wild-type strain. After adsorption for 45 minutes at 37° C the cells were centrifuged at $800 \times g$ for 2 minutes, and the supernatant was discarded.

The cellular pellet was resuspended in MEM and superinfected with the attenuated virus strain under the same conditions of adsorption and elimination of excess virus described for the wild-type. Details on multiplicity of infection (MOI) are given under "results" for each experiment. The infected cells were then resuspended in MEM containing Actinomycin D (5 μg/ml) and Hepes buffer (0.025 M). To measure virus specific RNA synthesis, 0.5 μCi/ml of ^{14}C-uridine (NEN Co., 53.2 mCi/mmol) were added, and the TCA-insoluble radioactivity was measured at different times.

Results

Cytopathic Differences Between Wild-Type and Attenuated Strains in PBK Cells

With the purpose of establishing differential markers for the wild-type and the attenuated strains of A_{24} Cruzeiro virus, infectivity assays were done on both PBK and BHK_{21} cells, in addition to pathogenicity assays in suckling mice. Table 1 shows that both strains behaved similarly in

Table 1. *Characterization of wild-type and attenuated strains of FMDV A_{24} Cruzeiro*

Virus strain	Infectivity		Pathogenicity
	PBK cells log $TCID_{50}$/ml	BHK_{21} cells log PFU/ml	Suckling mice log LD_{50}/ml
Wild-type	6.5	7.3	6.8
Attenuated	2.5	6.6	7.0

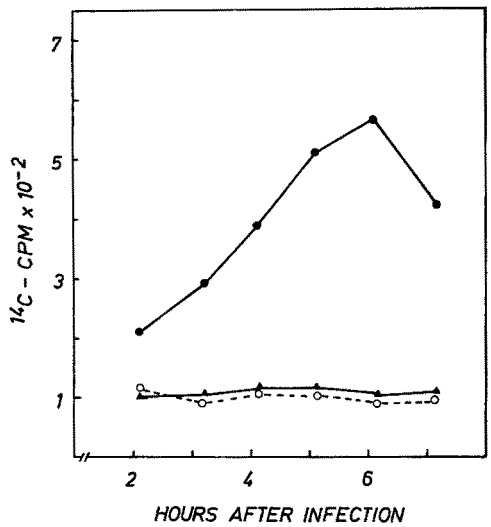

Fig. 1. Inhibition of wild-type virus RNA synthesis by superinfection with an attenuated strain ◉ PBK cells (5×10^6), were infected either with the wild-type strain of A_{24} Cruzeiro (MOI = 1) (•), with the attenuated A_{24} Cruzeiro strain (MOI = 1) (○) or successively with both the wild-type and the attenuated strains at the same multiplicity (▲). After adsorption, cells were centrifuged at low speed and the supernatant discarded. The cells were then resuspended in MEM containing both Actinomycin D (5 μg/ml) and Hepes buffer pH 7.4 (0.025 M), and labeled with 0.5 μCi/ml of ^{14}C-uridine at 100 minutes post-infection. Aliquots were removed at different time intervals and their TCA-insoluble radioactivity was determined

BHK_{21} cells and in suckling mice, while the attenuated strain showed a much lower titer in PBK cultures. This defined both BKH_{21} cells and suckling mice as permissive and PBK cells as semipermissive for the attenuated strain. Correlation between attenuation for cattle and conditional growth in PBK cells was shown to be a general rule for the attenuated strains of the O_1 and C_3 FMDV subtypes as well as with the attenuated A_{24} strain (data not shown).

Interference of the Attenuated Strain with Replication of the Wild-Type Virus in PBK Cells

To determine whether the attenuated strain was able to interfere with wild-type replication, PBK cells were infected with the wild-strain (MOI=

Table 2. *Interference by attenuated virus at different multiplicities on wild-type virus RNA synthesis*

Attenuated strain MOI[a] of the superinfectant virus	Viral RNA cpm	^{14}C-Uridine incorporation %
0	1800	100
0.05	1900	105
0.5	850	47
5	350	19

[a] PBK cells, infected with wild-type A_{24} Cruzeiro virus (MOI = 10), were superinfected 45 minutes later with the attenuated homologous strain. Radioactive viral RNA production by the infected cells was determined 7 hours after superinfection (see Materials and Methods)

10), and 45 minutes later, superinfected with the attenuated strain (MOI= 10). Inhibition of viral mucleic acid synthesis was used as an indicator of interference (Fig. 1). Nucleic acid synthesis remained at a low background level when cells were infected with the attenuated strain. Cells infected with wild-type virus synthesized considerable viral RNA up 6 hours. Cells previously infected with wild-type virus and then superinfected with attenuated virus were inhibited to the background level. This means that the attenuated strain completely inhibited replication of the wild-type one. If the MOI of the superinfecting attenuated virus were reduced, an equivalent reduction in the degree of interference took place (Table 2). These results indicate that interference depended on the concentration of attenuated virus. Also, it was not due to competition for cellular receptors, since the challenge virus was added to cells prior to the interfering virus.

Table 3. *Decreased interference by passaged attenuated virus*

Virus	^{14}C-Uridine incorporation cpm	%
Wild-type	1950	100
Attenuated P 1	360	18
Attenuated P 2	270	14
Attenuated P 3	690	35
Wild-type + attenuated P 1	420	21
Wild-type + attenuated P 2	860	44
Wild-type + attenuated P 3	1480	76

PBK cells were infected with wild-type strain (MOI = 10) and then superinfected with the attenuated strain (MOI = 5), that had been passaged in BHK_{21} cells, the infected PBK cells were incubated in MEM containing 5 μg/ml of Actinomycin D and labeled at 110 minutes p.i. with ^{14}C-uridine (0.5 μCi/ml). Incorporated radioactivity was measured 7 hours p.i. Control cells were infected with only one of the virus strains. Pi = passage number in BHK_{21} cells

Table 4. *Characterization of viral clones derived from the attenuated strain after 2 passages in BHK_{21} cells*

Virus clone	Original strain	Infectivity		Pathogenicity
		PBK cells log $TCID_{50}$/ml	BHK_{21} cells log PFU/ml	Suckling mice log LD_{50}/ml
34	Wild-type	6.2	5.5	6.8
835	Attenuated	1.5	6.0	5.7
104	Attenuated	5.5	6.1	6.8

Relationship Between Degree of Interference and the Number of Passages of the Attenuated Virus in BHK_{21} Cells

The interfering capacity of the attenuated strain was determined during 3 successive passages of the virus in BHK_{21} cells. Using inhibition of viral RNA synthesis the interfering capacity of the virus decreased after each successive passage with a concommitant increase in viral RNA synthesis induced by the 3rd passage virus (Table 3). This suggests a change in the virus population possibly due to the selection of viral particles that have lost their capacity for interference and/or gained their ability to induce viral RNA synthesis. To test this possibility attenuated virus from the second successive passage in BHK_{21} cells was cloned three times and the behavior of several isolated clones was examined by titration on PBK cells, on BHK_{21} cells or in mice. Table 4 shows two representative clones derived from the attenuated strain. One of them (clone 835) retained its attenuation features (low titer on PBK cells) while the other clone (numbered 104)

Table 5. *Homologous interference by attenuated A_{24} virus*

Virus	Infectivity PBK cells log $TCID_{50}$/ml
A_{24} wild-type	6.5
A_{24} attenuated + A_{24} wild-type	1.5
C_3 wild-type	7.5
A_{24} attenuated + C_3 wild-type	2.5
BEV	6.0
A_{24} attenuated + BEV	6.0

PBK-cell microplates were infected with attenuated A_{24} virus (MOI = 1). Later at 24 hours the cells were washed and superinfected with dilutions of wild-type strains of FMDV A_{24} Cruzeiro or C_3 Resende and with bovine enterovirus (BEV). Control plates were infected with only the corresponding wild-type strains. Infectious titers were measured after further incubation for 48 hours

behaved more like the wild-type virus. Interference assays with these two clones showed that clone 835 interfered with wild-type strain RNA synthesis while clone 104 did not.

Role of Interferon During Interference

Although interferon has been described in FMDV infections (4), in the experiments described here interferon appeared not to play a role during interference because of the following observations:

a) interference assays were done in the presence of 5 μg/ml of Actinomycin D, a drug which prevents interferon-mediated events (7).

b) cells superinfected with virus-freed medium from cells infected with the attenuated virus (100,000 × *g* supernatant) did not interfere with wild-type virus multiplication, while the pelleted virus did so.

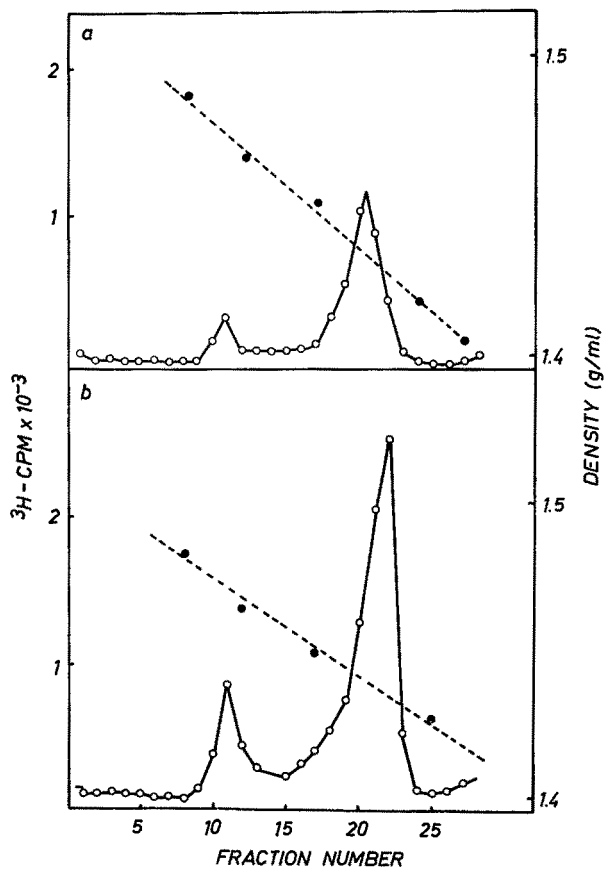

Fig. 2. CsCl density gradients of the wild-type and the attenuated strains of A_{24} Cruzeiro virus. ^{3}H-uridine-labeled virus was purified in sucrose gradients and then analyzed by CsCl density gradients, as described. *a* Attenuated strain; *b* wild-type strain

c) interference was not-cell specific (7) because attenuated virus suspensions made in BHK_{21} cells were able to interfere in PBK cells (Fig. 1, Table 3).

d) interference was homologous for FMDV strains and not for the unrelated picornavirus BEV. Table 5 shows the results of interference assays in subcultures of PBK cells infected with the attenuated virus and superinfected 24 hours later with wild-type FMDV of the subtypes A_{24} or C_3, or with BEV.

Interference occurred with both FMDV strains indicating it to be genus-specific. The unrelated BEV in comparison, was unaffected by attenuated FMDV.

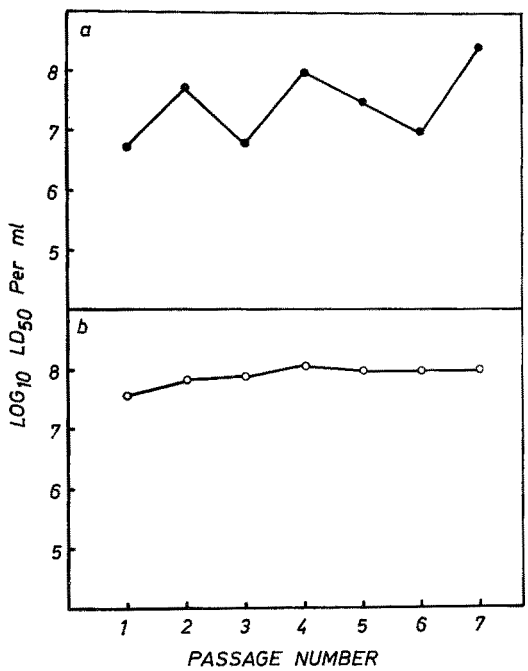

Fig. 3. Infectious titers of attenuated A_{24} Cruzeiro virus in serial diluted- and undiluted-passages. Monolayers of BHK-21 cultures (3×10^6 cells) were infected with attenuated virus, either *a* undiluted (MOI = 10—20) or *b* diluted (MOI = 0.1). After adsorption for 60 minutes, both cultures were incubated with MEM (5 ml) at 37° C, until total cytopathic effect was obtained. The virus harvests were then used as inocula for a subsequent passage in BHK-21 cells. The virus thus produced was titrated in suckling mice for pathogenicity determination (LD_{50})

Role of Defective Interfering (DI) Particles in the Production of Interference

DI particles have been detected for different picornaviruses, although not yet for FMDV. Their indentification has been based on: a) a lower density in CsCl gradients compared to standard virus [e.g. polio virus (9)];

b) slower migration of their viral RNA in agarose gels [e.g. some other picornaviruses (13)]; and c) cyclic variation of their infectivity during successive passages of undiluted virus (10, 16). All the three methods were tried for the attenuated virus stocks in attempts to demonstrate DI particles. The results obtained by ultracentrifugation in CsCl density gradients are shown in Fig. 2. Two bands of radioactivity at 1.43 gm/ml and 1.47 gm/ml were seen. Particles at lower than 1.43 gm/ml were detected. Both bands present in about the same proportion; if one normalized the radioactivity to infectious FMDV, known to band at 1.43 gm/ml, then the equivalent percentage of the material at 1.47 gm/ml would indicate a lack of difference between the wild-type and attenuated strains.

Therefore the 1.47 gm/ml material was unlikely to represent DI particles. Sizing of viral RNAs from the attenuated and wild-type strains showed that they coelectrophoresed in agarose gels. These studies were compromised, however, by partial degradation of the RNAs possibly due to virus-associated ribonucleases (3, data not shown). Finally, in undiluted passages of the attenuated strain, the cyclic model typical of DI particles was clearly seen, although the maximal variations were only about 1.5 logs; diluted passages, in comparison, gave constant high titers with less than half a log of variability (Fig. 3).

Discussion

A correlation between attenuation for cattle and low titers in PBK cells for a strain of FMDV has permitted the characterization of a viral interference phenomenon produced by modified strains of FMDV against the wild-type virus.

The attenuated A_{24} Cruzeiro strain interfered with the homologous wild-type strain multiplication as well with the heterologous O_1 and C_3 wild type strains. Such interference was demonstrated by infectivity assays (Fig. 1 and Table 5) and by viral nucleic acid studies (Table 2).

Different factors that might be responsible for the observed interference were examined in the present paper. The possibility of interferon production was first considered because previous studies with FMDV had established that temperature-sensitive mutants or modified viruses, which multiplied slowly, induced the synthesis of interferon leading to inhibition with standard virus replication (18, 20). However, in this paper the data don't support interferon as the basis for interference (Fig. 1) (Table 2, 3, 5).

Another possible mechanism for explaining interference by the attenuated strain is defective interfering (DI) particles which have been shown to play a role in viral interference for most animal virus systems (8, 9). The features of the interference phenomenon in the FMDV system described here support, only in part, the role of DI particles: i) the interference mechanism occurs at the intracellular level; ii) the degree of interference was dependent on

the concentration of the pelleted attenuated virus; iii) interference is specific for the FMDV genus (Table 5); and iv) a cyclic infectivity pattern was seen when serial undiluted passages of the attenuated strain were made (Fig. 3a). However DI particles could not be identified, either by CsCl gradient centrifugation or by agarose gel electrophoresis.

This study has demonstrated that the viral interference observed in PBK cultures was homologous and due to an intracellular effect of the attenuated virus. Previous studies have correlated reduced viral pathogenicity in cows to attenuated FMDV vaccines (12, 14, 17). Therefore, the ability of attenuated strains to interfere may play a major role in its own reduced pathogenicity as well as in its ability to prevent virulent wild-type or revertant strains from causing disease.

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Authors' address: Dr. E. L. Palma, Dpto. de Virología—INTA, c.c. 77, 1708—Morón, República Argentina.

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