Bovine Fibroblasts Response to Foot-and-Mouth Disease Virus: Influence of Integrins and Soluble Factors in Resistance

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Abstract

Foot and mouth disease may be the most economically devastating animal disease nowadays. Blanco Orejinegro (BON) cattle are one of the native creole breeds from Colombia; this breed carries important genetic traits for survival and reproduction in the tropics showing *in vitro* and *in vivo* resistance to pathogens. Here we evaluate primary fibroblast cultures of BON cattle for their phenotypic resistence/susceptibility (R/S) polymorphism toward FMDV, and to determine whether a correlation exists between the R/S polymorphism and expression of integrin αV-β3, and/or the antiviral activity in the supernatants of those cultures. Our data show that different R/S patterns to FMDV *in vitro*. Between 2% and 85% of the fibroblasts in the PCF-BON samples expressed integrin aV-b3. Interestingly, a correlation between R/S and expression of this receptor was observed for the A24 FMDV subtype. Highly resistant cultures showed a low level of integrin expression. Furthermore a correlation was observed between resistance and antiviral activity (AVA) of supernatants from infected samples. Supernatants with high AVA were always from very resistant samples for subtype A24, or from very resistant or resistant samples for subtype O1; supernatants with low AVA were from susceptible samples. In most samples, the factor in the supernatants responsible for AVA was not only resistant to heating, but AVA increases after heating. To our knowledge, this is the first report showing resistance to FMDV infection *in vitro* using primary cultures from bovines.

Keywords: Cattle; Infection; Resistance; Virus receptors

Introduction

Foot and mouth disease virus (FMDV) is a ssRNA+ member of the *Picornaviridae* family; it has seven serotypes and a large number of subtypes. It affects domestic livestock cloven-hoofed animals, causing substantial loss of milk, reduction in the growth rate, among others [1]. Foot and mouth disease (FMD) may be the most economically devastating animal disease nowadays [2]. In some countries of the Americas, FMDV is eradicated and some of them are FMD free countries on which vaccination is practiced [3].

Blanco Orejinegro (BON) cattle are one of the native creole breeds from Colombia; this breed carries important genetic traits for survival and reproduction in the tropics [4]. It has been show that BON cattle is resistant to illnesses, and is clearly resistant to the larva of *Dermatobia hominis* [5], *Brucella abortus* [6] and it has been show that BON cattle exhibit a polymorphism for resistance/susceptibility (R/S) to Vesicular Stomatitis Virus infection *in vitro* [7] and to FMDV [8]. It has been shown that FMDV resistance could be due to mutations on integrin receptors which are responsible for virus adhesion [9].

Two types of integral membrane components of host cells are implicated as FMDV receptors, heparan sulfate used by the O serotype *in vitro* [10], and several types of integrins that bind the capsid protein of all FMDV serotypes [11]. The α_V-β_3 integrin was the first integrin reported to be a natural receptor for FMDV [12].

Interferon (IFN)-α/β is one of the most important factors involved in natural resistance to viral diseases [13]. Viral induction of IFN is mediated by double-stranded RNAs (dsRNAs), that are uncommon constituents of cells but are produced as intermediates or final products during viral RNA replication [14]. Other factors can play an important role in individual resistance; they include lack of cell receptors required for virus adhesion and penetration into cells [15], and apoptosis that suppresses viral replication and dissemination [16]. Also, soluble antiviral factors have been detected in the supernatants of cell cultures that can inhibit different viruses [17].

The aim of this study was to examine primary fibroblast cultures of BON (PCF-BON) cattle for their phenotypic R/S polymorphism toward FMDV subtypes A24 and O1, and to determine whether a correlation exists between the R/S polymorphism and expression of integrin α_V-β_3, and/or the antiviral activity (AVA) in the supernatants of those cultures.

Materials and Methods

Collection of frozen BON fibroblast and primary cell cultures

Ear biopsies from 60 animals were taken from five herds of pure BON cattle located in five farms in Colombia. Handling of animals and all sampling procedures were performed in compliance with the recommendations of the "Guide for the Care and Use of Laboratory Animals of the National Research Council (Academy Press, 1996, Washington, USA) and previous approval for the Committee for ethics in animal experimentation.

All reagents were from Sigma-Aldrich® (St. Louis, MO) unless indicated otherwise. To obtain primary cell cultures the ear was washed, shaved and disinfected. The biopsy (diameter 0.8 mm) was removed with a sterile metallic punch and kept at 4°C in transport medium containing MEM, 2% penicillin-streptomycin-amphotericine B (PSA) and 2% fetal bovine serum (FBS; Gibco°-Invitrogen, UK) pH 7.2-7.4. Less than 8 h after sampling, the biopsy was washed three times with phosphate-buffered saline (PBS) pH 7.2-7.4 containing 2% PSA, and the skin discarded. The cartilage and subcutaneous tissues were minced finely, placed in 25 cm² cell culture flasks covered with growth medium (MEM with 2 mM L-glutamine, 1% MEM vitamins, 1% MEM non-essential amino acids, 1% PSA and 10% FBS pH 7.2-7.4), and incubated at 37°C in 5% CO_2. Every third day 50% of the medium was replaced with fresh growth medium. When the fibroblasts reached 30-40% confluence, the remaining pieces of tissue were discarded by gently shaking with PBS and again the cells were fed with fresh grown medium. When 80% confluence was reached, the cells were trypsinized and cultured in 150 cm² flasks. Once they reached 80% of confluence they were cryopreserved.

Cell lines

Cells of baby hamster kidney clone 21 (ATCC #CCL10), were used to replicate the FMDV subtypes. They served to determine the virus titer, and as control of susceptibility to FMDV in experiments of phenotypification of BON cells. Moreover these cells are insensitive to antiviral treatments such as IFN, due to a genetic defect in the expression of the IFN receptor. Cells of African green monkey kidney clone 76 (Vero - ATCC CRL-1587) served to titrate Vesicular stomatitis virus (VSV) serotype New Jersey (VSV-NJ) by plaque forming units (PFU), and as a substrate for biological quantification of AVA due to Vero cells are sensitive to IFN, but are incapable of producing IFN.

Viruses

The reference A24-Cruzeiro (A24) and O1-Campos (O1) FMDV subtypes were from the Laboratory of Animal Biotechnology (Corpoica-CEISA, Bogotá). To prepare viral stocks, BHK monolayers were infected separately with both subtype and the virus was harvested 18-24 h post-infection (hpi), when the cytopathic effect reached 80-90% of the culture. Supernatants were centrifuged at 800×g for 10 min at 4°C, and cell debris was discarded. Aliquots (100 μl) were stored at -70°C. The virus was titrated in BHK cells by the tissue culture infective dose 50 per ml ($TCID_{(50)}$/ml) method. VSV-NJ, was used to quantify AVA in supernatants of primary BON fibroblasts infected with FMDV.

$TCID_{(50)}$/ml to FMDV in PCF-BON

The $TCID_{(50)}$/ml of PCF-BON for the FMDV subtypes A24 and O1 was determined with each subtype for the 60 samples. Briefly, all the wells of a 96-well plate were seeded in 50 μl of growth medium with 5 × 10⁴ cells of a given PCF-BON sample (one plate per sample and per subtype), and immediately infected with 50 μl of serial ten-fold viral dilution (from 1:1 × 10⁻² to 1:1 × 10⁻⁷; 14 wells/dilution) of each virus subtype; 12 wells of each plate contained uninfected PCF-BON as cell controls. The plates were incubated for 72 h at 37°C in 5% CO_2, and stained with crystal violet. The titer was determined by the method of Spearman-Karber. Two 96-well plates seeded with 3 × 10⁴ BHK cells and submitted to the same virus treatment as above were consistently included for each subtype as virus controls.

R/S index (RSI)

The RSI results from the comparison of the $TCID_{(50)}$/ml in control

BHK cells infected with FMDV subtype A24 or O1 over the $TCID_{(50)}$/ml obtained for each PCF-BON sample infected with FMDV, and is expressed as the logarithm of the titer in control BHK cells over the titer in BON cells, such that:

$$R/S\,index = \log_{10}\left[\frac{TCID_{(50)}\,/\,ml\,BHK}{TCID_{(50)}\,/\,ml\,PCF-BON}\right]$$

All PCF-BON samples whose RSI was <1 were considered susceptible (S) because they needed similar or at least 10 fold higher concentrations of virus than did BHK cells to cause cytopathic effects in 50% of the cells; samples whose RSI was >1 but <3 were considered resistant (R) because they needed between 11 and 1000 fold more virus to reach the same cytopathic effect as did BHK cells; samples whose RSI was >3 were considered very resistant (VR) because they needed >1000 fold more virus to destroy 50% of the PCF-BON cells as compared to BHK cells.

Quantification of the expression of integrin α_V-β_3 in BON fibroblast cultures

The cells from 30 PCF-BON samples tested above were harvested using a scraper, taken up in 1 ml PBS, centrifuged, and resuspended so as to have 1 × 10⁷ cells/ml in buffer A (cold PBS pH 7.5, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1% FBS and 0.1% sodium azide). All subsequent steps were performed on ice. Approximately 10⁶ cells in 100 μl of buffer A were incubated with 2 μl of MAB1976Z (Chemicon®) anti-integrin α_V-β_3 monoclonal antibody (stock, 1 mg/ml) for 30 min, washed with buffer A and incubated in the dark with goat anti-mouse immunoglobulin G conjugated with fluorescein isothiocyanate during 30 min, washed twice with buffer A and resuspended in 1% paraformaldehyde in PBS. Fluorescent staining was analyzed by flow cytometry using a Facs scan (Coulter EPICS XL). Background fluorescence was determined using isotype control antibodies.

Quantification of AVA in supernatants

A biological test was applied to the supernatants of the 30 PCF-BON samples to quantify AVA activity, a 24-well plate was seeded with 2.5 × 10⁵ fibroblasts of each sample per well; each well was infected with 10 $TCID_{(50)}$/ml of of one or the other FMDV subtype (12 wells per subtype) in a final volume of 1 ml/well. Supernatants were collected 12, 24, 36 and 48 hpi (3 wells/time point), and the supernatants of each time point of each fibroblast culture were mixed to obtain the total amount of factor with AVA produced in 48 hpi. To inactivate the virus, the supernatants were treated with 50 μl of 0.1 N HCl/ml and stored at -70°C.

Quantification of AVA was performed on Vero cell monolayers in two 24-well plates per sample. The monolayers were treated with 1 ml of two-fold dilutions (1:2 to 1:128) of the supernatants, three wells for each dilution of each supernatant, one plate per subtype; three wells were used as controls (one contained uninfected cells [control cells], and two contained cells infected with VSV-NJ without treatment with supernatant [virus control]). After 24 h at 37°C in 5% CO_2, the monolayers were infected with 20 PFU/well of VSV-NJ, covered with growth medium containing 0.4% agarose and incubated for 24 h at 37°C in 5% CO_2. One international unit (IU) of AVA corresponds to the highest dilution of supernatant that prevents the formation of 50% of plaques in comparison with virus control (infected monolayer without addition of supernatant). The supernatants were classified as having low, intermediate or hight AVA if the AVA was 2-4, 8-16 or $\geq$32 IU/ml, respectively.

Effect of heating on the AVA

To determine whether the AVA in the supernatants is heat resistant, the supernatants pertaining from the 30 PCF-BON samples tested for AVA were heated before addition to the Vero cell monolayers. Briefly, Vero cells (2.5×10^4) were seeded in each well of 96-well plates; 24 h after seeding, the monolayers were treated with 100 μl/well of a 1:2 dilution of the supernatants (three wells per supernatant) obtained from PCF-BON samples infected with FMDV and previously heated at 95°C for 3 min or unheated. Twenty-four hours later, each well was infected with 10 $TCID_{(50)}$/ml of VSV-NJ; each plate contained 6 wells with uninfected cells (control cells) and 6 wells with cells infected with 10 $TCID_{(50)}$/ml but without supernatant (virus control). After 24 h the virus was inactivated with 10% formaldehyde in PBS and the monolayers stained with crystal violet. Protection of the monolayers was determined using an inverted contrat phase microscope by comparing the integrity of the monolayer in the wells containing treated cells with the wells containing control virus.

Statistical design

The data were analyzed by descriptive statistics, and the student-T test was applied to determine the level of significance. Correlation analysis was performed by means of the Spearman correlation coefficient. All the analyses were made with the program GraphPad Prism® (GraphPad Software, Inc.) version 4.00 and Excel®; the significance level was set at $p < 0.05$.

Results

R/S polymorphism in primary BON fibroblast cultures

Sixty PCF-BON samples were evaluated for R/S for FMDV subtypes A24 and O1. The $TCID_{(50)}$/ml varied considerably, between 1.9 and 8.7 $\log_{(10)}$ $TCID_{(50)}$/ml for A24, and between 3.0 and 8.8 $\log_{(10)}$ $TCID_{(50)}$/ml for O1 (not shown). Subtype O1 tended to present higher $TCID_{(50)}$/ml in almost all fibroblast cultures than subtype A24. Both subtypes led to total destruction of the BHK monolayer (susceptibility control). The cytopathic effect of A24 was limited to small plaque formation in all PCF-BON except one whose $TCID_{(50)}$/ml was higher (7.5 $\log_{(10)}$ $TCID_{(50)}$/ml) than that of the BHK control (8.7 $\log_{(10)}$ $TCID_{(50)}$/ml). Subtype O1 in PCF-BON led to total destruction of the monolayer, and four samples presented $TCID_{(50)}$/ml higher than the BHK control (data not shown).

The RSI of subtypes A24 and O1 was between -1.2 and 5.6, and -0.6 and 5.1 respectively (Figure 1); hence, the phenotypic polymorphism in BON fibroblasts is larger for A24, because the range of variation is higher for A24 than for O1. Although the distribution of the RSI was a continuum, three categories S, R, VR were distinguished (Methods). With this classification, of the 60 samples infected with A24, only 7% were classified as S, 30% as R, and 63% as VR (Figure 1); with O1, 38% of the cultures were classified as S, 42% as R and only 20% as VR. These results suggest that there is a high degree of polymorphism for R/S to FMDV infection, but the PCF-BON are more susceptible to subtype O1 and polymorphism is greater for subtype A24.

Thus the response of PCF-BON to viral infection is clearly different for each subtype: whereas for A24 the VR category is the most and the S category the least frequent, for O1 the VR is the least frequent and there is no difference between the S and R categories. Only 33% (20/60) of the samples showed a coincidence between the three categories of R/S after infection with either subtype (Table 1). Interestingly 66% (40/60) of the samples neither coincided in R/S after infection with either of the two
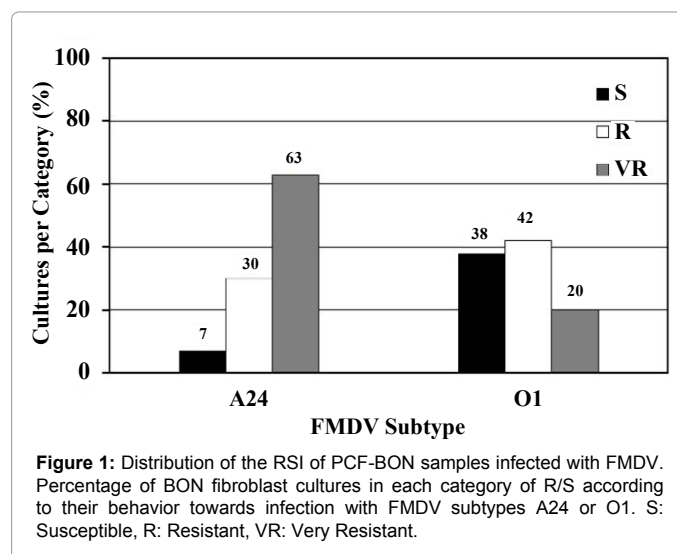
subtypes because these samples belonged to different R/S category for the two subtypes (Table 1), nor did they follow a similar pattern.

No correlation was noted between the R/S to viral infection and the farm from which the sample was collected, suggesting that the phenomenon of R/S is inherent to the BON breed. All further experiments were performed with the same 30 randomly selected samples, out of the 60 samples.

Polymorphism for R/S to FMDV correlated with integrin α_V-β_3 expression level

Thirty samples were evaluated for expression of integrin α_V-β_3. There was a wide polymorphism in the expression of this integrin among the fibroblast cultures, ranging from 2% and 85% of the cells in the PCF-BON samples evaluated. Surprisingly, the PCF-BON sample with the lowest level of integrin expression (2%), presented a very different $TCID_{50}$/ml for the two FMDV subtypes (3.2 $\log_{(10)}$ $TCID_{(50)}$/ml for A24 and 8.8 $\log_{(10)}$ $TCID_{(50)}$/ml for O1; not shown) and was classified as VR and S to infection with subtype A24 and O1 respectively. Conversely, the PCF-BON sample with the highest level of integrin expression (85%), showed a similar $TCID_{50}$/ml (4.9 and 4.5 $\log_{(10)}$ $TCID_{(50)}$/ml for A24 and O1 respectively; not shown) for both subtypes and was classified in the R category.

The average integrin α_V-β_3 expression was calculated for each category of R/S in the PCF-BON infected with FMDV subtype A24 or O1. VR fibroblasts to A24 expressed a lower level of integrin compared

Figure 1: Distribution of the RSI of PCF-BON samples infected with FMDV. Percentage of BON fibroblast cultures in each category of R/S according to their behavior towards infection with FMDV subtypes A24 or O1. S: Susceptible, R: Resistant, VR: Very Resistant.

	Total	A24	O1
Coincidence	20	10 VR	10 VR
		7 R	7 R
		3 S	3 S
No coincidence	40	28 VR	19 R
			9 S
		11 R	1 VR
			10 S
		1 S	1 R
Total	60	60	60

Table 1: Polymorphism in R/S after infection of PCF-BON. PCF-BON showed a high polymorphism in R/S after FMDV infection. Not all the phenotyped PCF-BON coincide by category of R/S to the two FMDV subtypes used (A24 and O1). VR: very resistant, R: resistant, S: susceptible.

to the R or S categories (Figure 2) with a statistically significant difference in the expression of integrin between the three categories (p=0.03); when the comparison was done between two categories, a statistically significant difference in the average percentage of integrin expression was observed between the S and VR categories and between the R and VR categories (p<0.05). However for subtype O1 the average integrin expression was similar for all the three categories; and no statistical difference (p=0.69) in expression of the integrin was observed. Thus, when the average of the percentages of integrin α_V-β_3 expression was compared, the VR samples to subtype A24 expressed on average lower levels of integrin than the average in the other categories, in which the level of integrin expression was similar.

Finally the correlation between the RSI to FMDV subtype A24 or O1 and the expression of integrin α_V-β_3 was analyzed. Both correlation coefficients (r) were negative (-0.55 and -0.02 for subtypes A24 and O1 respectively; not shown), indicating that when expression of the integrin receptor is high, the PCF-BON are more sensitive to FMDV infection; the correlation was better for A24 than for O1.

Polymorphism for R/S to FMDV is correlated with AVA of supernatants from PCF-BON infected with FMDV

The level of AVA in the supernatants of the 30 PCF-BON samples infected with FMDV was determined, i.e. the capacity of serial two-fold dilutions of the supernatants of the samples to inhibit VSV-NJ replication in Vero cells. As positive control of AVA induced by FMDV infection, the supernatants of BHK cells infected with 10 $TCID_{50}$/ml of either A24 or O1 were used; these supernatants could inhibit VSV replication up to a dilution of 1:64, indicating that the supernatants of BHK cells infected with either subtype have 64 IU/ml AVA. Serial two fold dilutions of FBS in medium without serum were used as negative control for AVA: these serial dilutions did not protect Vero cells from VSV-NJ infection, indicating that the AVA is not due to factors present in the FBS.

The supernatants from the 30 samples yielded AVA that ranged between 2 and 64 IU/ml (not shown). The AVA was classified as low, intermediate or high (see Methods). Figure 3 shows that for supernatants from samples infected with subtype A24, 23.3% had low, 33.3% intermediate, and 43.3% high AVA. In contrast for subtype O1, 44.8% had low, 41.4% intermediate and only 13.8% had high AVA. It is remarkable that for subtype A24 most of the cultures are VR o R, and most of the CPF-BON infected with this subtype produce high levels of AVA.

Five supernatants from fibroblast cultures infected with subtype A24 presented 64 IU/ml but only one from a culture infected with subtype O1; notably, all these fibroblast cultures were VR to FMDV infection (not shown). The lowest level of AVA was 2 IU/ml and was present in two supernatants from samples infected with A24, both S, and in three supernatants from samples infected with O1, but here each sample belonged to a different category. Thus, except for one sample, PCF-BON samples that were VR to FMDV infection secrete factors into the supernatant capable of blocking viral replication to both subtypes; on the other hand, for samples that were R, the AVA was very diverse between the two subtypes, and all the supernatants from samples classified as S for both subtypes had low AVA (data not shown).

The correlation coefficient (r) between RSI and AVA was 0.57 for A24 and 0.53 for O1. Hence after FMDV infection, when the PCF-BON was VR, it had the capacity to produce and secrete into the supernatant,

factor(s) with AVA; when the PCF-BON sample was S, it did not produce factor(s) with the capacity of affecting VSV-NJ replication. It is remarkable that for subtype A24, all the supernatants from samples classified as S presented low AVA, and those with high AVA were from samples classified as VR (Table 2). No clear correlation could be established between the AVA and R/S in supernatants from samples infected with subtype O1 (29 supernatant tested), except that all the
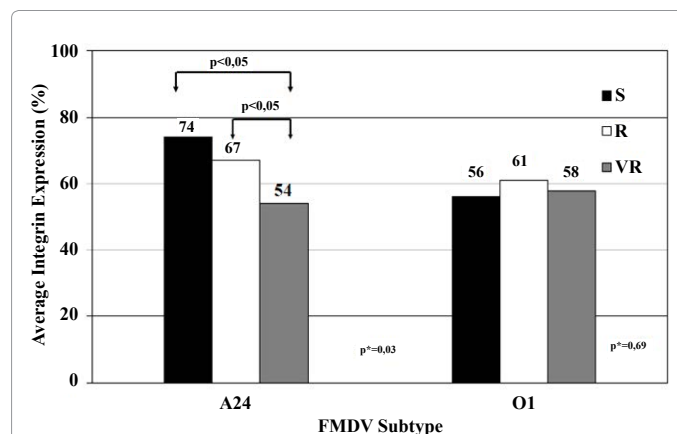

Figure 2: Level of expression of integrin α_V-β_3 in PCF-BON samples. Average integrin α_V-β_3 expression in PCF-BON classified in different categories of R/S in response to FMDV subtype A24 or O1; above each rectangle is the percentage of PCF-BON in each R/S category. p: statistically significant differences between two categories, p*: statistically significant difference between the three categories. S: Susceptible, R: Resistant, VR: Very Resistant.
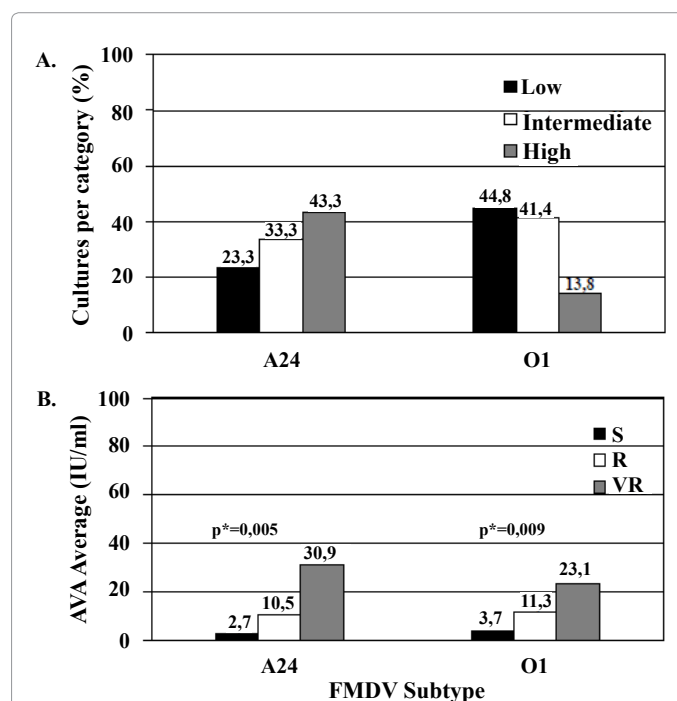

Figure 3: AVA of supernatants from PCF-BON samples infected with FMDV. A. Distribution of samples according to the capacity of AVA induced by infection with FMDV subtype A24 or O1; above each rectangle is the percentage CPF-BON in each category of AVA. B. Average of AVA in each R/S category to FMDV infection with subtype A24 or O1, above each rectangle is the average AVA. p*: statistically significant difference between the three categories. S: Susceptible, R: Resistant, VR: Very Resistant.

FMDV Subtype	R/S Category	Number of CPF-BON in each AVA level		
		High	Intermediate	Low
A24	VR	10	8	1
	R	0	5	3
	S	0	0	3
O1	VR	2	4	1
	R	2	8	5
	S	0	0	7

Table 2: AVA of supernatants from PCF-BON pertaining to several categories of R/S to FMDV. AVA of supernatants from PCF-BON that are very resistant (VR), resistant (R) or susceptible (S) to FMDV A24 or O1 infection. The PCF-BON samples are classified as high, intermediate or low producers of AVA.

supernatants from S PCF-BON samples to infection subtype O1 had low AVA.

When the average of the AVA in supernatants of PCF-BON infected with FMDV was calculated for each category of R/S to FMDV infection, the supernatants obtained from cultures infected with A24 and classified S contained 2.7 IU/ml AVA, as compared to 10.5 and 30.9 IU/ml for supernatants from R and VR samples (Figure 3), with a statistically significant difference between the three categories (p=0.005). The AVA in the supernatants obtained from cultures infected with subtype O1, was 3.7, 11.3 and 23.1 IU/ml for samples S, R and VR respectively (Figure 3), with a statistically significant difference between the three categories (p=0.009). Therefore, the PCF-BON classified in the S category for both FMDV subtypes secreted significantly less anti-VSV-NJ factor(s) than the R or VR samples.

The AVA factor(s) present in supernatants from PCF-BON infected with FMDV is (are) heat resistant

To determine the nature of the factor with AVA present in the supernatants of FMDV infected PCF-BON samples, possibly IFN or an RNase, an aliquot of each supernatant was heated or not at 95°C for 5 min; if the AVA is due to IFN, it should be heat-sensitive, and if it is due to an RNase, it is most likely heat-resistant.

The level of AVA was increased after heating in most of the supernatants (67% and 48% for subtypes A24 and O1 respectively, not shown); in some supernatants the AVA was not affected by heating (13% and 21% for subtypes A24 or O1 respectively), and in some cases the AVA decreased in supernatants from PCF-BON after heating (20% and 31% for subtypes A24 and O1 respectively). These results suggest that a heat-resistant product (possibly RNases) cloud be active in the supernatants in which the AVA is maintained or increased after heating, while in supernatants in which the AVA decreases, IFN is probably the active element.

Discussion

FMDV is one of the most important viral diseases in bovine animals. The economic importance of this illness is due to the highly infectious nature of FMDV and to its international impact on the trade of animals. It is therefore highly important to characterize bovine animal species capable of being infected with FMDV.

Here we demonstrate that a correlation exists between R/S to FMDV infection of PCF-BON samples and expression of integrin α_V-β_3, and between R/S to FMDV infection and the AVA of the supernatants of these infected cells. As a first scientific study to relate R/S of PCF-BON to FMDV with either AVA in the infected cell supernatants or integrin expression, these results address, locally prevalent ideas of resistance of BON livestock to the certain viral diseases.

To our knowledge, this is the first study searching for natural resistance to FMDV in primary cell cultures from bovines, which are natural hosts of this virus. A similar study was reported by Chinsangaram et al. [18], using secondary cells from pig kidney and embryonic bovine kidney, transfected with plasmids coding for porcine and bovine IFN- or -β, and evaluating the susceptibility of these cells to FMDV. Other studies using primary cell cultures to study R/S to viruses different from FMDV have been reported [19,20] but this is the first one that report natural resistance to FMDV in natural host.

The results reported here show that the behavior of PCF-BON to infection depends on the subtype. A higher percentage of primary fibroblasts were R and VR to subtype A24 than to O1 (Figure 1). The cytophatic effect produced in BHK (susceptible control cells) was characterized by total destruction of the monolayer with either subtype, but the two subtypes produced different effects in PCF-BON, O1 leading to total destruction of the monolayer, and A24 producing a cytophatic effect characterized by small foci. This could reflect the difference in behavior of FMDV subtypes in cultures *in vitro*, corresponding to their affinity for cell receptors [10,21-23].

Interestingly, PCF-BON samples classified as VR to A24 express lower levels of integrin α_V-β_3 that those classified S (Figure 2). Since binding between integrin receptor and virus is mediated by a highly conserved RGD motif located on an exposed loop of the viral VP1, resistance to A24 could be associated with low expression of integrin α_V-β_3, the natural receptor for FMDV [21,23]. A correlation between resistance to A24 infection and receptor expression had not previously been reported in primary bovine cells. In contrast, expression of integrin α_V-β_3 in PCF-BON infected with O1 was similar for the three categories of resistance (Figure 2). *In vitro*, subtype O1 is known to use not only integrin α_V-β_3, but also heparan sulfate as receptor [10] a receptor expressed to high levels in different cell types and $_V$-β_6 integrin [24] which appears to be constitutively expressed in the normal airways of cattle [25]. This could explain why no correlation exists between R/S and integrin α_V-β_3 expression for subtype O1(r=-0.02), while a correlation exists for subtype A24 (r=-0.55). It would be pertinent to prevent heparan sulfate-mediated or α_V-β6 Integrin adhesion/penetration of FMDV into PCF-BON and then determining the true susceptibility of PCF-BON to subtype O1. If the only explanation for the high susceptibility of BON fibroblasts to subtype O1 is the choice of receptor used *in vitro*, the phenomena reported here would probably be of little use *in vivo* for this FMDV subtype.

It is also important to note that R/S phenotype of PCF-BON to FMDV its been evaluated in correlation with α_V-β6 Integrin, which is present at the basal layers of the stratified squamous epithelium of the oral mucosa and coronary bands in cattle [25]. This evaluation could be very important for a most accurate approach of the BON resistance to FMDV.

The correlation observed between the R/S phenotype and AVA of the supernatants was similar for A24 and O1 (r=0.57 and r=0.53 respectively), suggesting that other factors in addition to factors with AVA secreted into the supernatants could be associated with the R/S described here. The AVA could be explained by soluble factors in the supernatants of the fibroblast cultures such as IFN or RNases. Using porcine and bovine IFN expressed in *Escherichia coli*, Chinsangaram et al. [18] demonstrated that α and β IFNs were equally effective in inhibiting replication of FMDV subtype A12 in cells such as primary fibroblasts from bovine embryo kidneys. These observations corroborate the results reported here, and the AVA in the supernatants of BON fibroblast cultures could be mediated at least in part by IFN.

A cell infected by a Picornavirus such FMDV can activate IFN production, and IFN activates the 2-5A/RNase L or PKR antiviral pathway [18]. In some cases RNase L activated by IFN could be released into the supernatants from lysis of infected cells; this could imply that the AVA of the supernatants would be heat resistant, because RNases are usually heat resistant [17,26,27]. Our results show that in most supernatants the AVA increases or is maintained after heating. Hence an RNase could be one of the factors with AVA in the supernatants, and because not all supernatants retained their AVA after heating, the AVA could in some cases be associated with RNases, and in others with IFN activity.

In conclusion, PCF-BON show a high polymorphism *in vitro* in response to FMDV infection and this polymorphism is different for the FMDV subtype A24 than for O1. In A24 the R/S of PCF-BON is correlated with expression of integrin α_V-β_3, and the capacity of supernatants from these infected cells to inhibit VSV replication. Thus, PCF-BON with low RSI to A24 express high levels of integrin α_V-β_3, and PCF-BON with high RSI to FMDV A24 produce high amounts of factor with AVA. Conversely for subtype O1, there is no correlation between R/S of PCF-BON and integrin α_V-β_3 *in vitro*, but a correlation exists between RSI and AVA from supernatants of infected PCF-BON. It is remarkable that stimuli triggered by viruses as closely related as FMDV A24 and O1 can affect PCF-BON in such very different ways.

This study is the first to show natural resistance *in vitro* of BON cattle to FMDV infection, a re-emerging viral agent world-wide. These cattle could serve as excellent model breed to control FMDV infection if resistance is confirmed *in vivo*.

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