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Apoptosis as pathogenic mechanism of infection with vesicular stomatitis virus. Evidence in primary bovine fibroblast cultures

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ABSTRACT: To determine whether fibroblasts from Blanco Orejinegro cattle, exhibit any level of resistance to infection against vesicular stomatitis virus (VSV) serotypes Indiana (VSV-I) or New Jersey (VSV-NJ), 30 fibroblast cultures were phenotyped to evaluate their resistance/susceptibility. Thirty three % of Blanco Orejinegro fibroblast cultures were classified as very resistant, 50% as resistant, and 17% as susceptible to VSV-I infection, whereas 20% were classified as very resistant, 50% as resistant and 30% as susceptible to VSV-NJ infection. Therefore, there appears to be a large variation in phenotypic polymorphism among the fibroblasts to infection by VSV. To elucidate the mechanisms responsible for this diversity, we searched for a possible relationship between resistance/susceptibility and production of factors with antiviral activity; however fibroblasts did not secrete factors with antiviral activity. We examined also whether apoptosis where induced by infection and its correlation with the polymorphism of resistance/susceptibility to VSV. Using morphological analyses, hypoploidy measurements, and level of phosphatidyl serine expression, high levels of apoptosis were measured in VSV infected fibroblasts. However, no correlation exists between apoptosis and the category of resistance/susceptibility to infection, indicating that apoptosis is a pathogenic mechanism of VSV.

Introduction

In Colombia, there are seven local cattle breeds that are part of the country's natural resources. Among them, the "Blanco Orejinegro" cattle occupies a very important place within the coffee-producing regions. This breed has not been submitted to selection and harbours large genetic diversity; in addition it is slowly becoming extinct (Arboleda, 1980; Bedoya *et al*., 2001). There are reports indicating that *in vivo,* Blanco Orejinegro cattle are resistant to infestation by the larva of *Dermatobia hominis* (Buitrago and Gutierrez, 1999). We have recently shown that primary fibroblast cultures of Blanco Orejinegro infected with foot-and-mouth disease virus (FMDV) subtypes A24 or 01 present broad phenotypic polymorphism (López-Herrera *et al*., 2005). A statistically significant correlation exists between the antiviral activity of the media from fibroblast infected with FMDV and the phenotype of resistance/susceptibility. That is, the supernatants of fibroblast with high antiviral activity are resistant to both FMDV subtypes, whereas those with low antiviral activity are susceptible to infection.

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In Colombia both serotypes of vesicular stomatitis virus (VSV), Indiana (VSV-I) and New Jersey (VSV-NJ) are endemic. VSV is an enveloped virus, member of the *Rhabdoviridae* family, genus Vesiculovirus. In its natural hosts (horses, cattle and pigs) VSV causes clinical sings that are indistinguishable from those produced by FMDV, producing vesicular lesions in the oral cavity, extending to the teats and to the coronary band of the hoofs. In humans, a few sporadic cases of VSV have been described (de Mattos *et al*., 2001). The viral genome is composed of a single strand RNA negative of about 11 Kb that codes for 5 proteins (reviewed in Lichty *et al*., 2004): nucleocapsid, phosphoprotein, matrix, envelope glycoprotein, and RNA-dependent RNA polymerase.

It has been shown that for a few members of the *Picornaviridae* and *Rhabdoviridae* families to which FMDV and VSV belong respectively, resistance mechanisms are induced by interferon (IFN) type-I that activates the 2´-5´oligoadenylate synthetase (2-5)-RNAse L, and the double stranded RNA-dependent protein kinase. Both pathways lead to inhibition of viral as well as cell protein synthesis, and to apoptosis of the infected cells (Biron and Sen, 2001; Chinsangaram *et al*., 2001).

Apoptosis is a genetically regulated and coordinated cell death mechanism that is essential for the control of embryonic development and cell homeostasis. It is activated via a variety of stimuli and is characterized by several morphological changes and biochemical processes, including chromatin condensation, intranucleosomal cleavages by activation of endonucleases and endogenous proteases, and finally cell fragmentation into apoptotic bodies, thereby avoiding inflammatory responses (O'Brien, 1998).

Apoptosis has been also described as a means of counteracting certain infections by inhibiting replication of pathogenic microorganisms. Velilla *et al*. (2005) recently demonstrated that monocytes of the peripheral blood of a group of people repeatedly exposed to human immunodeficiency virus type-1 (HIV-1) but uninfected, have a higher propensity to apoptosis induced by infection with HIV-1 *in vitro* compared to apoptosis induced by HIV-1 infection in monocytes of a population at low risk of infection. The authors suggest that such monocytes from exposed non infected subjects, when infected *in vitro* with HIV-1 initiate apoptosis to avoid replication and dissemination of the virus. In the case of Influenza A virus, it was demonstrated that apoptosis of monocytes and macrophages is a mechanism used to limit replication of the virus (Fesq *et al*., 1994; Ohyama *et al*., 2003); the authors proposed that during infection, apoptosis is induced to block early events of the replicative influenza A virus cycle. Nevertheless, Nunoi *et al*. (2005) suggest that in influenza A virus infection, apoptosis in organs such as the brain and liver corresponds to a mechanism of viral pathogenesis. Similarly, it has been described that infection of mice by cytomegalovirus can damage the brain by induction of apoptosis in cells of the central nervous system (Reuter, 2005). On the other hand, apoptosis induced by reovirus in several tissues of 2-day old mice is associated with the expression of the viral protein sigma 1S (Hoyt *et al*., 2005), that determines the extent of apoptosis in the heart and in the central nervous system, converting apoptosis into a pathogenic mechanism and a virulence factor.

In the present study, we demonstrate that apoptosis is induced in fibroblast of Blanco Orejinegro cattle infected by VSV-I or VSV-NJ. Our results also show that there is a phenotypic polymorphism in resistance/susceptibility of such fibroblast infected with VSV that is not associated either with the expression of antiviral activity factors or with apoptosis induced by the infection. Apoptosis as observed in Blanco Orejinegro fibroblast is a pathogenic mechanism of infection triggered by VSV, and not a resistance mechanism.

Materials and Methods

Cell Cultures

All cells used in this study were grown or incubated at 37ºC in 5% CO_2 for the times indicated.

Primary Fibroblast Cultures

A group of 30 Blanco Orejinegro fibroblast samples from the cell bank of the Immunovirology-Biogenesis Group were cultivated in RPMI-1640 (Sigma®) growth medium supplemented with 1% penicillin-streptomycin, 1% vitamins, 1% L-glutamine (Sigma®) and 10% fetal calf serum (Gibco®). Phenotypification of resistance/susceptibility and apoptosis after infection with VSV-I or -NJ was carried out *in vitro* using these cells, and the culture media were recovered to quantify antiviral activity.

Baby hamster kidney (BHK) and African green monkey kidney (Vero) cells

Kidney cells from hamsters (BHK) and from African green monkeys (Vero) were from the bank of the

Immunovirology-Biogenesis Group and were cultivated in minimum essential medium (MEM) (Sigma®) supplemented as above for the RPMI medium. BHK cells served to titer the virus and as susceptibility control in determining the index of resistance/susceptibility of the fibroblast to infection with either VSV serotype. The supernatants of the infected BHK and fibroblast cell cultures served to assay the antiviral activity. Vero cells were used to quantify the antiviral activity, since these cells are sensitive to antiviral activity (including to IFN), but are incapable of producing the last one (Emery and Morgan, 1979).

VSV stocks

The VSV-I and -NJ serotypes were from the Immunovirology-Biogenesis Group. Virus stocks were produced by infecting BHK monolayers in 75 cm^2 culture flasks with 2 ml of VSV-I or -NJ containing titers of 8.8 or 8.5 $\log_{(10)}$ of 50% tissue culture infectious dose/ml ($TCID_{(50)}$/ml) respectively, incubating the cells for 1 h to allow adhesion and penetration of the virus, and then adding medium to 15 ml. The culture media from the infected cells were collected 24 h post infection when cytopathic effects were detected in 80% of the cells using an inverted microscope, the media was collected, centrifuged at 220 xG for 5 min at 4ºC, and the supernatants aliquoted (300 µl) and kept at -70ºC. They were used for phenotypification of resistance/susceptibility of fibroblasts, to quantify the antiviral activity and to determine induction of apoptosis.

Virus titer -$TCID_{(50)}$/ml

The titer of the VSV stocks was determined by the $TCID_{(50)}$/ml method on BHK cells cultivated in 96-well plates (50.000 cells per well). Briefly, using MEM supplemented with 5% fetal calf serum, serial ten-fold dilutions from 10^{-1} to 10^{-12} of VSV-I or -NJ were prepared. 100 µl of the virus dilution were added to each well containing a monolayer of BHK cells (7 wells per dilution, and 12 wells with uninfected cells as cell controls). The plates were incubated for 24 h. The virus was then inactivated by addition of 100 µl phosphate buffer-saline (PBS) containing 10% formaldehyde and exposed to UV light for 1 h. Finally, the number of wells presenting cytopathic effects for each dilution was quantified, and compared to the wells with control cells. The virus titer ($TCID_{(50)}$/ml) was determined by calculating the virus dilution that produced a cytopathic effect in 50% of the infected wells (Leennette, 1995).

Plaque forming units (PFU)

To quantify antiviral activity, the PFU produced by the VSV-NJ stock were determined (Leennette, 1995). Briefly, starting from virus stock, ten-fold dilutions from 10^{-1} to 10^{-7} of the virus were prepared in MEM supplemented with 5% fetal calf serum, and used to infect Vero monolayers in 24-well plates, 3 wells per dilution and 200 µl/well; 3 wells also contained uninfected cells. After 1 h, the virus inoculum was removed, 1 ml/well of a semisolid medium (MEM with 5% fetal calf serum and 0.4% agarose) was added and the plates incubated for 24 h. The virus was inactivated with 500 µl PBS containing 10% formaldehyde and exposed to UV light for 1 h. The monolayers were coloured with crystal violet and the cytopathic effect determined by the number of plaques formed per well.

Phenotypification of resistance/susceptibility in Blanco Oreginegro Fibroblast

The resistance/susceptibility of each fibroblast culture to infection by VSV-I or -NJ was determined by comparing the $TCID_{(50)}$/ml of each one of the 30 samples with the $TCID_{(50)}$/ml of the control susceptible BHK cells. Plates of 96 wells were seeded with 5 x 10^4 cells/well of each fibroblast culture (two wells per sample) in 100 µl of RPMI growth medium. After 24 h, each well was infected with 100 µl of ten-fold dilutions of the virus from 10^{-2} to 10^{-7}, 14 wells per dilution, and one plate for each VSV serotype. Each plate included 12 wells with uninfected cells as control. The cells were incubated 24 h and after that the virus was inactivated by the addition of 100 µl of PBS containing 10% formaldehyde, exposed to UV light for 1 h, and stained with crystal violet. Destruction of the monolayer was evaluated considering each well with cytopathic effects as positive. The $TCID_{(50)}$/ml titer was determined by the method of Spearman-Karber (Leennette, 1995). In parallel, the titer was also evaluated in the BHK cells serving as controls, using the same viral dilutions as for the fibroblast samples.

The resistance/susceptibility index was calculated comparing the $TCID_{(50)}$/ml value obtained with BHK cells with the $TCID_{(50)}$/ml value of each fibroblast sample infected with either VSV serotype, and is expressed as the log of the $TCID_{(50)}$/ml in BHK over the $TCID_{(50)}$/ml of the fibroblast, such that:

$$R/S\ index = \log_{10}\left[\frac{TCID_{(50)}/\ ml\ BHK}{TCID_{(50)}/\ ml\ \text{Fibroblast}}\right]$$

Based on the results thus obtained, the infected fibroblast samples were grouped in three categories: (1) all the samples with an resistance/susceptibility index ≤1 that require an equal concentration or a 10-fold excess of virus over BHK cells to reach a 50% level of cytopathic effect are classified as susceptible, (2) all samples in which the resistance/susceptibility index lies between 1.01 and 3 that require an 11- to a 1000-fold virus excess over BHK cells to reach the 50% level of cytopathic effect are classified as resistant, and (3) and all samples with an resistance/susceptibility index ≥ 3.01 that require more than a 1000-fold excess virus over BHK cells to reach the 50% level of cytopathic effect are classified as very resistant.

Induction and quantification of antiviral activity

To determine whether synthesis and secretion into the supernatant of factors with antiviral activity are induced in Blanco Orejinegro fibroblast infected with VSV-I or -NJ, 2 x 10^5 cells/well were seeded in 24-well plates containing 1 ml of RPMI medium. After 24 h, the medium was removed and the cells infected with 10 $TCID_{(50)}$/ml of VSV-I or -NJ in 100 µl of RPMI medium containing 5% of fetal calf serum (12 wells for each virus sample). After 1 h, to allow adhesion and penetration of the virus into the cells, 900 µl of growth medium were added to each well. The supernatants of each well were recovered 12, 24, 36 or 48 h post infection (the medium from 3 wells for each point time) and placed at –70ºC. The supernatants collected at various time points were combined to obtain a pool of supernatants for each fibroblast sample containing the total antiviral activity produced up to 48 h post infection. The virus contained in the supernatants was inactivated either by decreasing the pH to 2 for 12 h at 4ºC which does not destroy the acid-resistant IFN, or by heating to 65ºC for 30 min which does not destroy thermo-resistant RNases. Supernatants from BHK cells infected with VSV-I or -NJ and treated in conditions identical to the fibroblast samples and served as positive controls. Such BHK-infected cells produce large amounts of factors with antiviral activity, as for instance IFN, but are insensitive to IFN because they contain a genetic defect in the expression of the IFN receptor (Kramer *et al*., 1983).

To quantify the antiviral activity, a biological standard as established by Ahmed *et al*. (2003) was used. The wells of 24-well plates were seeded with 1.5 x 10^5 Vero cells per well. After 24 h, the monolayers were treated with 2-fold dilutions (1:2 to 1:128) of the pools of supernatants obtained from the VSV infected fibroblast and incubated for 24 h (3 wells per dilution; 3 wells without supernatant served as controls). Each well was then infected with 15 PFU of VSV-NJ in 100 µl of growth medium for 1 h; the medium was replaced by 1 ml of MEM supplemented with 5% fetal calf serum and 0.4% of agarose. After 24 h, the virus was inactivated with PBS containing 10% formaldehyde, the mixtures exposed to ultraviolet light for 1 h and the cells stained with crystal violet. The antiviral activity is expressed in International Units (IU) where 1 IU of antiviral activity is the maximum dilution of supernatant capable of decreasing the PFU by 50% compared to control infected cells not treated with the supernatants.

Apoptosis in Blanco Orejinegro fibroblast induced by infection with VSV-I or –NJ

Apoptosis induced by infection of each of the 30 fibroblats samples with VSV-I or -NJ (1 molecule of virus/cell; MOI = 1) was evaluated based on morphological analyses, hypoploidy and the expression of phosphatidyl serine induced by infection. Spontaneous apoptosis (control) was estimated in uninfected fibroblast treated as the infected samples.

Morphological analyses

To evaluate the morphology and the viability of the cells 24 or 48 h post infection with VSV by epifluorescence microscopy, 10^5 cells in 25 µl of PBS were stained with 1 µl of ethidium bromide (100 µg/ml; Sigma®) and 1 µl of acridine orange (100 µg/ml; Sigma®). The parameters of apoptotic morphology were characterized by loss of nuclear lobules, condensation of chromatin and loss of cell volume. Moreover, based on the difference of ethidium bromide and acridine orange penetration into living or dead cells, it was possible to distinguish between normal cells in which chromatin is bright green, early apoptotic cells with highly condensed or bright green fragmented chromatin, late apoptotic cells with highly condensed or orange-colored fragmented chromatin, and necrotic cells with orange-coloured chromatin (Vélez-Pardo *et al*., 2002). A total of 200 cells per fibroblast sample were counted per well 24 and 48 h post infection, and for each serotype.

Hypoploidy

For each fibroblast culture, 10^6 cells were infected with VSV-I or -NJ at a MOI = 1 and incubated for 24 or 48 h. The cells were mechanically detached from the

plate and then fixed with 70% ethanol for 30 min at 4ºC, washed twice with PBS containing 1 mM KCl, resuspended in 500 µl of 100 µg/ml of propidium iodide, and incubated for 30 min in the dark. The number of hypoploidal cells was determined by flow cytometry (Coulter EPICS, XL) using acquisition of 10.000 cells, the data was analyzed by CellQuest program. Cells that contained a low level of DNA compared to the normal DNA content of uninfected fibroblast were considered hypoploid (Mitchels *et al*., 2003).

Expression of phosphatidyl serine

Phosphatidyl serine is expressed on the outer cell membranes during the initial phases of apoptosis. To quantify these residues, the commercial TACS™ Annexin V-FitC kit (TREVIGEN®) was used. In 16-well plates, 5 x 10^5 fibroblast were seeded, infected with VSV-I or -NJ at a MOI = 1, and incubated for 24 or 48 h. They were then mechanically detached, washed with 1 ml PBS, centrifuged at 1500 rpm for 5 min at 4ºC, resuspended in 100 µl of a solution containing 89 µl of binding buffer (solution included in the kit), 10 µl of propidium iodide at 50 µg/ml and 1 µl of Annexin, and incubated for 30 min at room temperature in the dark. Finally, 400 µl of PBS were added and measurements performed by flow cytometry using acquisition of 10.000 cells, the data were analyzed by CellQuest program. Uninfected fibroblasts were used to evaluate spontaneous apoptosis.

Statistical analyses

The showed data for resistance/susceptibility phenotypes, antiviral activity and apoptosis quantification are the result of the average of three repetitions of each experiment. To evaluate the statistics normal distribution of the data, the statistics test of Kollmogorov-Smirnov, Shapiro Wilks and Bartlett's were performed. Since the data did not present a normal distribution, non parametric analyses such as the Mann-Whitney method were performed with a confidence level of 95%. The statistical analyses were carried out using the GraphPad Prism® version 3.02.

TABLE I.

Phenotypification of the resistance/susceptibility of 30 primary fibroblast cultures to VSV-I or VSV-NJ infection. To classified each fibroblast culture in a category of resistance/susceptibility, the challenge was repeated thrice with each virus serotype.

Phenotype	VSV-I (%)	VSV-NJ (%)
Very resistant	33	20
Resistant	50	50
Susceptible	17	30
Total	100	100

Results

Fibroblast samples infected with VSV-I or -NJ present different resistance/susceptibility phenotypes

The titers of the VSV cultivated in BHK cells were 7.3 $\log_{(10)}TCID_{(50)}$/ml for both serotypes. In the fibroblast samples it varied from 1.36 to 6.66 $\log_{(10)}TCID_{(50)}$/ml for VSV-I, and between 2.15 and 6.93 $\log_{(10)}TCID_{(50)}$/ml for VSV-NJ. The resistance/susceptibility index that resulted from the comparison between the $TCID_{(50)}$/ml in fibroblasts and BHK cells (see Materials and Methods) varied between 0.7 and 6 for VSV-I, and 0.43 and 5.21 for VSV-NJ. These results indicate that there exists polymorphism in the fibroblast samples with respect to resistance infection with VSV-I that it slightly greater than with VSV-NJ.

Based on the results of the resistance/susceptibility index, the fibroblasts were grouped in three categories of resistance/susceptibility: very resistant, resistant and susceptible (see Materials and Methods). As demonstrated in Table 1, the fibroblasts samples present a considerable polymorphism with respect to infection by either VSV serotype, but with different patterns of very resistant, resistant and susceptible to the two serotypes: Of the samples infected with VSV-I, 33% were very resistant, 50% were resistant, and only 17% were susceptible, indicating that 83% of the samples show resistance to infection. In comparison, 70% showed resistance to VSV-NJ (20% were very resistant and 50% resistant) and there were 30% susceptible samples. Consequently, the fibroblasts behave differently to infection by the two VSV serotypes.

Quantification of the antiviral activity present in the supernatants of Blanco Orejinegro fibroblast infected with VSV

We have previously shown that the supernatants of Blanco Orejinegro fibroblast infected with FMDV pro-

duce factors with antiviral activity that protect Vero monolayers from infection with VSV (López-Herrera *et al*., 2005). Consequently, we investigated whether the fibroblast samples infected with VSV also secrete factors with antiviral activity capable of protecting Vero cells from infection with VSV-NJ. To quantify the antiviral activity present in the supernatants fibroblast or BHK cells, the virus remaining in the medium was inactivated by lowering the pH or by a heat treatment (see Materials and Methods). Quantification was carried out

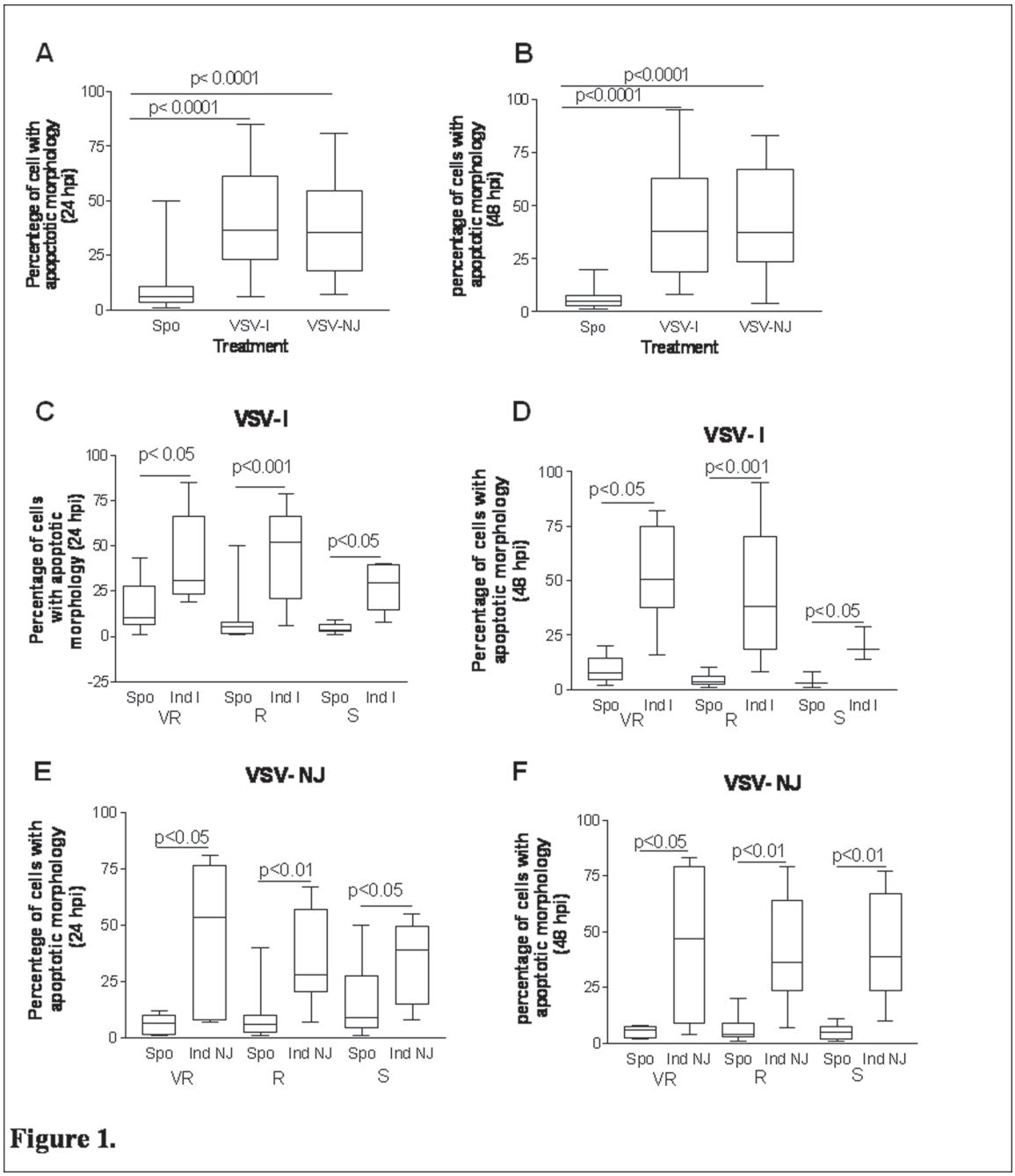

FIGURE 1. Morphological assay of spontaneous versus induced apoptosis measured after 24 and 48 h in VSV-I or VSV-NJ infected Blanco Orejinegro Fibroblasts. Apoptotic morphology spontaneous (Spo) or induced by infection (Ind-I: induced by VSV-I; Ind-NJ: induced by VSV-NJ) was visualized by epifluorescence microscopy. **A** and **B**: Average of spontaneous and induced morphological apoptosis after 24 and 48 h respectively. **C** and **D**: spontaneous versus induced morphological apoptosis induced by VSV-I in very resistant (VR), resistant (R) and susceptible (S) fibroblasts 24 and 48 h post infection respectively. **E** and **F**: Same situation that C an D, but infected with VSV-NJ. The horizontal lines in each box represent average, the lower and upper borders of the boxes are the 25 and 75 percentiles respectively, and the short horizontal bars correspond to the minimum and maximum values. P: level of statistical significance.

in Vero cells treated with the supernatants recovered from the fibroblast or the BHK cells infected with VSV-I or VSV-NJ. Vero cells were not protected from infection with VSV-NJ by the supernatants from VSV-infected fibroblasts, although they were protected from infection by up to a 1:64 fold dilution of the supernatants from VSV-infected BHK cells. Hence, after infection, BHK cells produce 64 IU/ml of antiviral activity, a phenomenon not observed with infected fibroblast (data not shown).

VSV Infection of fibroblast induces apoptosis

Some degree of spontaneous apoptosis was observed in uninfected fibroblast samples maintained *in vitro* and, except for one case, was always much lower than the VSV induced apoptosis in fibroblast infected for 24 or 48 h.

Effect of VSV infection on the morphology of fibroblast samples

The level of apoptotic morphology of fibroblasts infected with each VSV serotype was higher than in uninfected samples. After 24 h, the average percentage of apoptosis in uninfected cells was 6%, compared to 37% and 36% in cells infected with VSV-I and -NJ respectively (Fig. 1A). After 48 h, the values were 5% in uninfected cells, and 38% in cells infected with each VSV serotype (Fig. 1B). The difference between spontaneous apoptosis and apoptosis induced by VSV is statistically highly significant ($p < 0.0001$) after 24 and 48 h. These results show that VSV can induce cell death in fibroblasts irrespective of the virus serotype or the duration of infection.

The effect of infection on the morphological modifications of the fibroblast of the three categories very resistant, resistant and susceptible were evaluated. At both time points considered, there was a large difference in the percentage of induced apoptotic cells between the categories compared to uninfected cells in which spontaneous apoptosis was very low (Fig. 1C, D). Nevertheless, the major difference between spontaneous and induced apoptosis was observed in the resistant category with $p = 0.001$. These results show that there is a statistically significant difference between spontaneous apoptosis and apoptosis induced by infection for all three categories of infected fibroblasts.

When spontaneous apoptosis was analyzed and compared between the three categories, a higher incidence of apoptosis was visible with the very resistant fibroblast. However, this difference was statistically significant at 48 but not at 24 h post infection ($p = 0.0163$ versus $p = 0.0751$ respectively; results not shown).

Similar results were obtained when comparing the categories of resistance/susceptibility of fibroblasts infected with VSV-NJ, except that at 24 h post infection, cells classified as resistant presented a large decrease in average apoptosis compared to cells infected with VSV-I (28% and 52% for serotype VSV-NJ and serotype VSV-I; Fig. 1E, C).

When the spontaneous apoptotic morphology between the three categories of resistance/susceptibility to infection by VSV-NJ was compared, the fibroblasts with a susceptible phenotype tended to present a higher level of spontaneous apoptosis at 24 h post infection, whereas at 48 h post infection the behavior of the cells was very similar between the three categories. Induced apoptotic morphology was compared between the different categories of resistance/susceptibility to VSV-NJ infection in fibroblasts belonging to the three categories. Although a slight tendency to show a higher level of apoptosis post-infection was noted in the very resistant category, there were no statistically significant differences in induced apoptosis between the three categories, neither at 24 nor at 48 h post infection (data not shown).

Induction of hypoploidy in VSV infected fibroblasts

An evaluation of late apoptotic events based on the content of double-stranded DNA in uninfected fibroblasts (spontaneous) and in infected fibroblasts (induced) with each VSV serotype, showed that the percentage of apoptotic cells is larger in infected than in uninfected fibroblasts at both time points (Fig. 2A, B). After 24 h, VSV-I infected fibroblasts presented a higher level of hypoploidy than samples infected with VSV-NJ (55% and 42% of apoptotic cells, respectively). However, at 48 h post infection there was no difference between the two serotypes (81.2 and 81.7% respectively). Therefore, VSV infection of Blanco Orejinegro fibroblasts induced apoptosis, whereas in the uninfected control cells, cell death was very low, at 24 and at 48 h post infection (1.9 and 4.0%, respectively). The results of hypoploidy observed in infected VSV is statistically highly significant with each serotype ($p < 0.0001$) compared to spontaneous apoptosis in the same sample, where it is not significant.

It was also noted that hypoploidy in very resistant, resistant and susceptible classified fibroblasts and infected with VSV-I is higher than in uninfected cells (Fig. 2C, D) at 24 and at 48 h post infection. As can be seen

in Figure 2C there is a strong induction of apoptosis in cell cultures infected with VSV-I in the three categories. The percentage of apoptotic cells was about 50%, although the level of spontaneous apoptosis was insignificant in these cultures. Surprisingly, after 48 h of infection the evolution of cell death was very different between the three categories (Fig. 2D). In susceptible cell cultures infected with VSV-I, apoptosis reached 92%, whereas in resistant cell cultures cell death was 87%. Intriguingly, in the case of the very resistant cell cultures, there was little variation between 24 and 48 h post infection (from 55% to 62% respectively). These

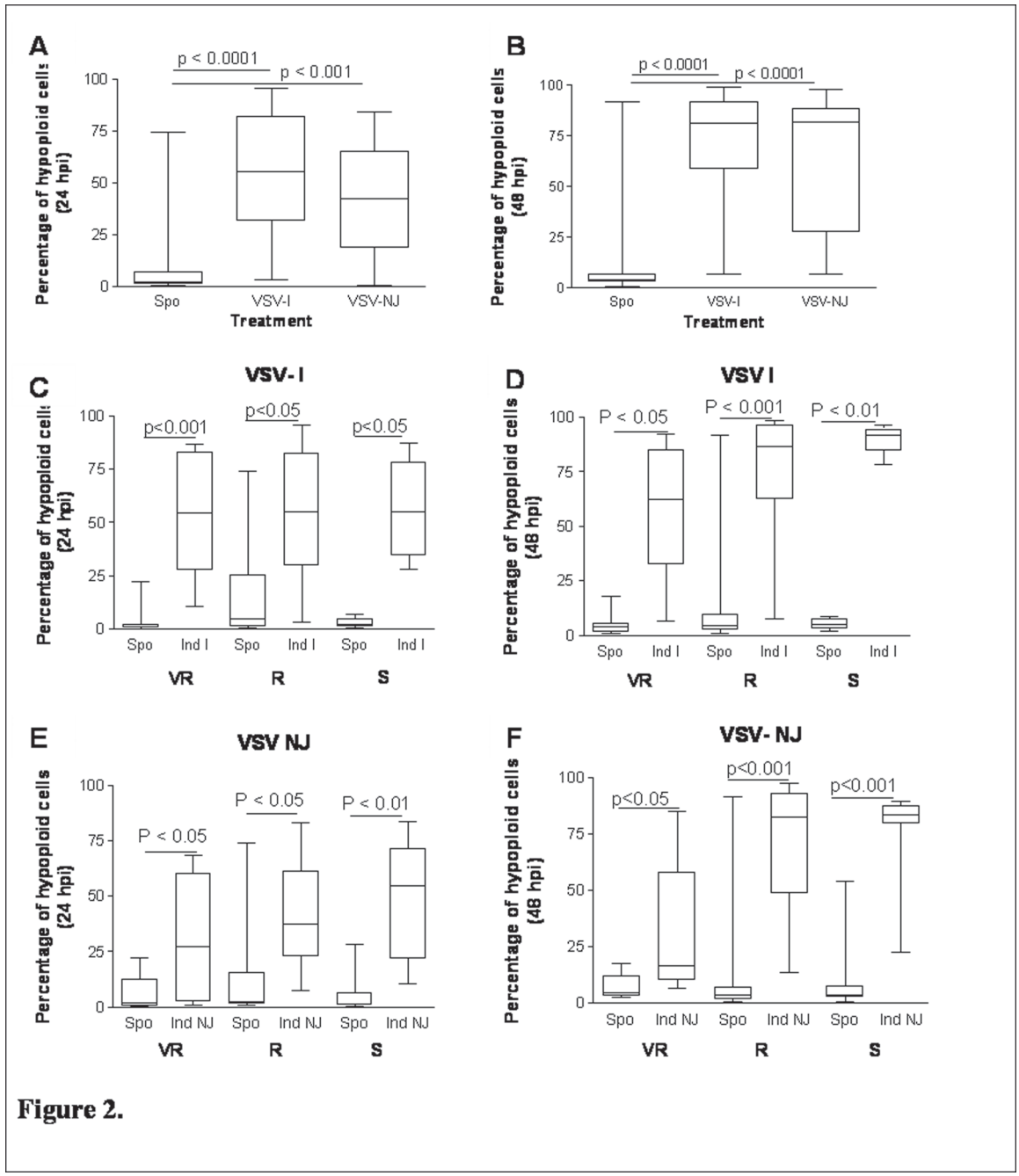

Figure 2.

FIGURE 2. Percentage of hypoploid cells with features of apoptosis measured in Blanco Orejinegro Fibroblasts infected with VSV-I or VSV-NJ for 24 and 48 h. Number of hypoploid cells determined by flow cytometry. **A** and **B**: general average of spontaneous hypoploidy versus hypoploidy induced by infection with VSV-I or VSV-NJ in the 30 fibroblasts samples for 24 and 48 h. **C** and **D**: spontaneous hypoploidy versus induced hypoploidy produced by infection with VSV-I in very resistant (VR), resistant (R) and susceptible (S) fibroblasts, 24 and 48 h post infection respectively. **E** and **F**: Same situation that C an D, but infected with VSV-NJ. Other indications like in Figure 1.

results demonstrate that infection with VSV-I induce high levels of apoptosis in Blanco Orejinegro fibroblasts.

As opposed to the results of cell death induced by VSV-I, in VSV-NJ infected fibroblasts each of the three resistance categories presents a large variation in the response to infection. After 24 h, the percentage of apoptotic cells in the very resistant cultures was 27%, in the resistant cultures it was 37%, and in the susceptible cultures it was 55%. It should be noted that a slight increase in spontaneous apoptosis was observed in uninfected cultures; nevertheless, when comparing the spontaneous to the induced apoptosis in each of the three

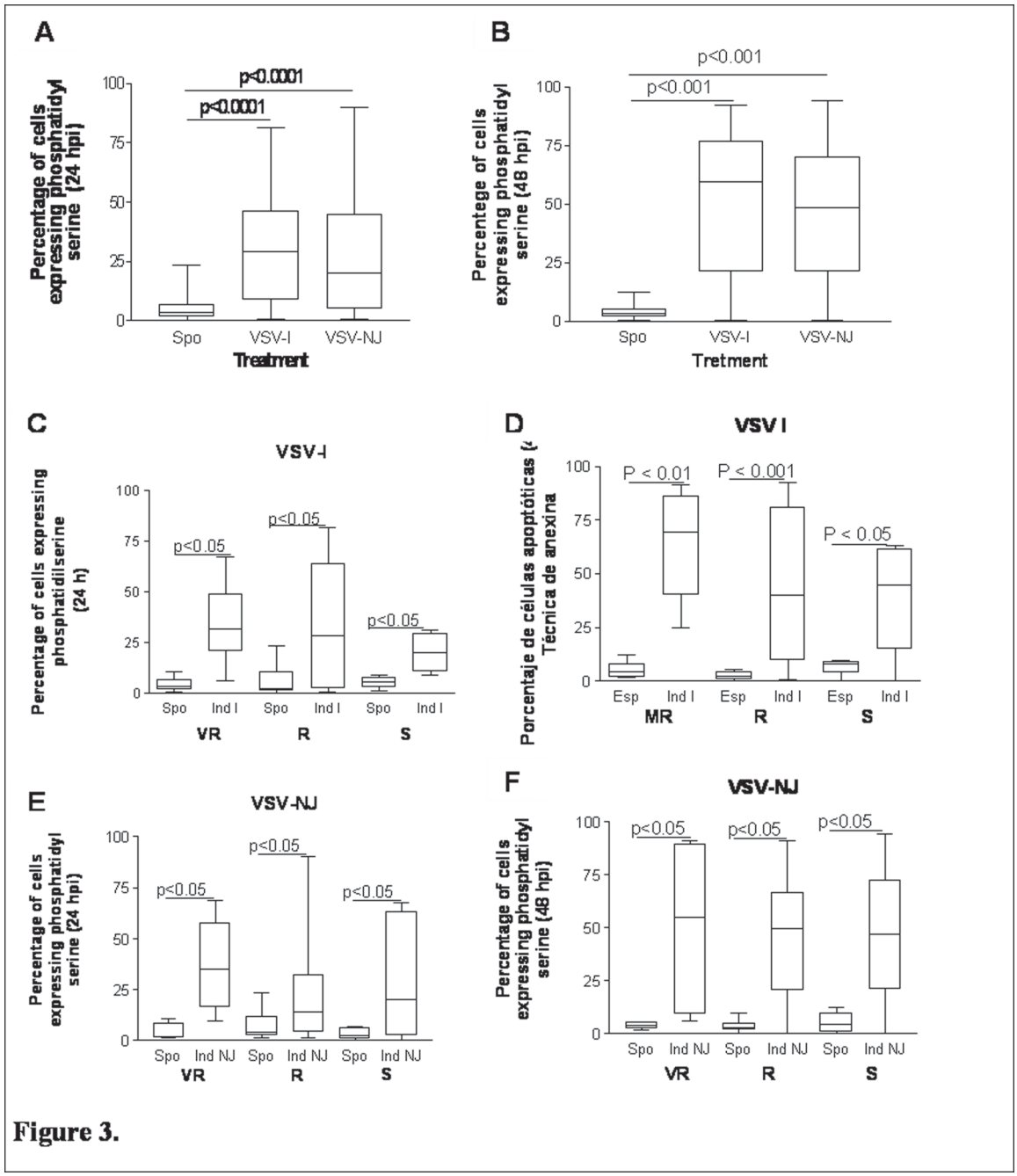

Figure 3.

FIGURE 3. Expression of phosphatidyl serine on the outer surface of cell membranes with characteristics of early apoptosis in VSV-I or VSV-NJ infected Blanco Orejinegro Fibroblasts for 24 and 48 h. Phosphatidyl serine residues expressed on the outer cell membrane quantified by flow cytometry. **A** and **B**: Comparison of the general average of spontaneous and induced expression of phosphatidyl serine by infection of the 30 fibroblasts samples with the two VSV serotypes for 24 or 48 h. **C** and **D**: spontaneous versus induced expression of phosphatidyl serine by VSV-I in very resistant (VR), resistant (R) and susceptible (S) fibroblasts, after 24 and 48 h respectively. **E** and **F**: Same situation that C an D, but infected with VSV-NJ. Other indications like in Figure 1.

categories, the difference was statistically significant (Fig. 2E). After 48 h, there was an increase in induced apoptosis in the cell cultures of categories resistant and susceptible as also observed with VSV-I; yet, in the very resistant cultures there was a slight decrease in cell death (Fig. 2F). Taken together, these results suggest that VSV-NJ infected cells are poorer inducers of apoptosis than VSV-I infected fibroblasts cultures.

Expression of phosphatidyl serine in VSV infected fibroblasts

First, the average level of expression of phosphatidyl serine in the 30 uninfected fibroblasts cultures or infected with either VSV serotype was evaluated, but without taking into consideration the category to which the samples belong. In uninfected cells, a low level of phosphatidyl serine was detected, but upon infection with either serotype, this level increased dramatically 24 and 48 h post infection (Fig. 3A, B). After 24 h, both serotypes induced a similar level of apoptosis, and at 48 h post infection there was an important increase in cell death. The percentage of cells expressing phosphatidyl serine after infection with either serotype was significantly higher that spontaneous expression ($p = 0.0001$) at both time points considered.

Upon evaluating the percentage of cells expressing phosphatidyl serine in each category of resistance/susceptibility and infected with VSV-I, there was an increase in apoptotic cells as compared to spontaneous apoptosis in uninfected cells. After 24 h, the frequency of induced apoptosis varied between 20 and 32%, but the difference between spontaneous and induced apoptosis was statistically significant (Fig. 3C). After 48 h, there was a large increase in induced apoptosis in the case of the very resistant cultures (70%), whereas the increase in the two other categories was less important (40% and 45% for the resistant and susceptible categories respectively; Fig. 3D). Similar results were observed in fibroblasts samples infected with VSV-NJ for 24 or 48 h (Fig. 3E, F). Thus, the annexin assay confirms the results obtained by the morphological and hypoploid assays, i.e.: VSV is a potent inducer of apoptosis in Blanco Orejinegro fibroblasts.

Taken together, the results obtained using the three methods to evaluate induction of apoptosis in Blanco Orejinegro fibroblasts samples infected with the two VSV serotypes suggest that VSV is a strong inducer of apoptosis, independent of the resistance/susceptibility level of the fibroblasts. This indicates that the apoptosis observed in infected fibroblasts is an integral part of a pathogenic effect of virus infection, since spontaneous apoptosis in these cultures is low and apoptosis induced by either VSV serotype is independent of the resistance/susceptibility category of the Blanco Orejinegro fibroblasts.

Discussion

VSV is an endemic virus in Colombia that produces a disease whose clinical symptoms are similar to those produced by infection with FMDV. The presence of VSV is one of the main barriers to exportation of bovines and pigs and their by-products to those countries which are either free of the disease or in which the disease is under a tight sanitary control.

The Blanco Orejinegro cattle breed is a natural resource of Colombia that is on its way to extinction, and popular ideas exist about its resistance to VSV. Our results show that Blanco Orejinegro fibroblasts is polymorphic for resistance/susceptibility to infection by VSV-I and -NJ, and support the results reported by Lopez-Herrera *et al*. (2002) about resistance/susceptibility to VSV, and polymorphism in resistance/susceptibility to VFA (Lopez-Herrera, 2005).

VSV serotypes I and NJ are recognized VSV serotypes that produce a disease with similar clinical characteristics in sick animals. Even though no significant difference has been described for these two serotypes except to antibody response, the resistance/susceptibility polymorphism observed in fibroblasts due to infection is greater with VSV-I than VSV-NJ. Likewise, for VSV-I, a larger percentage of fibroblasts are in the very resistant or resistant category (83.3%) than for VSV-NJ (70%).

FMDV infected fibroblasts secrete factors with antiviral activity into the medium capable of inhibiting replication of VSV-NJ in Vero cells (López-Herrera *et al*., 2005). Nevertheless, neither of the two VSV serotypes induced antiviral activity in Blanco Orejinegro fibroblasts. Surprisingly however, in the supernatants recovered from BHK cells infected with VSV, antiviral activity was detected up to a 1:64 dilution.

The matrix protein of VSV is reportedly (Ahmed *et al*., 2003) implicated in inhibition of host gene expression triggered by an antiviral response such as IFN type 1. It is also believed to inhibit the function of the three cell RNA polymerases, preventing protein synthesis. The absence of factors with antiviral activity in the supernatants of the examined fibroblast could be associated with inhibition of gene expression in VSV infected fibroblast via matrix protein. It should be re-

called that bovine fibroblasts are natural target cells of VSV, whereas BHK cells serve as model for research purposes.

Our results also indicate that both VSV serotypes induce apoptosis in fibroblasts, a phenomenon that has been associated with pathogenesis in cells infected by the virus (Nunoi *et al*., 2005; Reuter, 2005). The change in the nuclear morphology of the infected cells observed by epifluoresence microscopy is an indication of apoptotic cells. This phenomenon can also be associated with necrosis of the cell. However, our results also indicate that infected fibroblast present a low level of DNA as compared to uninfected ones. In addition, a large difference in phosphatidyl serine expression is observed in infected versus uninfected. These observations confirm the link that exists between the presence of VSV and apoptosis, and could form a basis to explain the formation of vesicular lesions *in vivo*. Apoptosis induced by VSV is a mechanism that leads to the destruction of tissues and death of infected mice, as described by Sur *et al*. (2003). Nevertheless the damage caused could result from a combination of mechanisms directly or indirectly associated with viral infection. Recent studies have shown that the matrix protein and other viral components are responsible for the induction of apoptosis in infected cells (Kopecky *et al*., 2001). It was also shown that viral matrix protein can inhibit synthesis of cell proteins by blocking the three cell RNA polymerases, also leading to apoptosis (Ahmed and Lyles, 1998; Ahmed *et al*., 2003; Kopecky *et al*., 2003).

Apoptosis has also been reported to be a resistance mechanism against virus infection (Fesq *et al*., 1994; Velilla *et al*., 2005). However, our results show no significant differences in induction of apoptosis by VSV between the three categories very resistant, resistant and susceptible of Blanco Orejinegro fibroblasts. For this reason, the polymorphism in resistance/susceptibility described here suggests the presence of another mechanism implicated in resistance of Blanco Orejinegro fibroblasts to infection by VSV. In a previous study (López-Herrera *et al*., 2005), we proposed that resistance of Blanco Orejinegro fibroblasts to FMDV infection is associated with a low level of expression of integrin α_v-β_3, the natural receptor for this virus. Likewise, the samples classified as very resistant or resistant could present low levels of the receptor as opposed to those classified as susceptible. Low expression of the receptor for VSV could explain the resistance to this virus described here, but this receptor has not been characterized yet.

In conclusion, our results demonstrate that there exists a considerable polymorphism in the resistance/susceptibility of Blanco Orejinegro fibroblasts to infection by VSV and confirm that a native Colombian breed, which is on its way to extinction, is resistant, at least *in vitro*, to infection by viral agents that produce vesicular diseases. In addition, we demonstrated that natural resistance to infection of Blanco Orejinegro fibroblasts by VSV-I and VSV-NJ serotypes is not due to the production of factors with antiviral activity, and we also showed that apoptosis induced by VSV infection in fibroblasts is a mechanism of viral pathogenesis.

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