ABSTRACT. There are no antigenic differences between known strains of Aujeszky’s Disease virus or the Pseudorabies virus (PV). The characterization of these viruses has been based on pathological and/or pathogenic aspects of the host. In this study, PV (RC/79 strain) virulence markers have been characterized based on their capacity to produce changes in monolayer cell cultures and by effects generated in experimentally infected rabbits. By analyzing the cytopathic effect induced on Vero cells by the virus, it was possible to visualize the formation of rounded-up cells at the beginning of the infection and later on the appearance of multinuclear or syncytial cells. Lysis plaques (4-5 mm) under agar had rounded, well defined borders. Studies carried out in cell cultures and experimental infections in rabbits showed the virulent nature of the RC/79 strain, for all cases studied. In this animal model intense pruritus was seen at the site of inoculation, with the appearance of a lesion exuding serum and blood from 6-24 h before death. For all cases, the mortality rate was 100%, even when smaller viral doses (10^2 PFU/ml) were used, which indicates that there was an absence of dose response. The rabbit marker was used to characterize the RC/79 strain of the PV as strongly virulent. This model could also be used in epidemiological studies to diagnose AD, differentiating between virulent and attenuated strains. It represents a useful tool that can be used to assess naturally attenuated virulent isolates that are potential vaccine candidates. Furthermore, the rabbit model may be useful to determine the degree of attenuation in viral strains that have been submitted to laboratory modifications.

Key words: Aujeszky’s Disease Virus, Virulence Markers, Rabbit Inoculation.

INTRODUCTION

The Pseudorabies virus (PV) is the etiological agent of Aujeszky’s disease (AD). AD is characterized by producing marked respiratory and/or nervous changes, particularly in the natural host, the pig. Since PV was first isolated in the Republic of Argentina,\(^1\) the incidence of the disease in the main pig producing areas of Argentina has increased.\(^{5,12,20}\) In response to this data and in the absence of an official plan of campaign against AD, it is necessary to carry out epidemiological investigations.

Such epidemiological studies are an integral part of future control programs that will depend on the effective identification of isolates of Pseudorabies virus strains.
PV is a member of the Herpesviridae family, Alphaherpesvirinae sub-family. Unfortunately there are no antigenic differences between the known strains of the virus, hence their classification and characterization has been based on their physio-chemical properties and on pathological and pathogenic aspects in the host.\textsuperscript{7}

From a practical point of view it is important to identify genetic markers that are related to virulence. These markers will then be used to distinguish between virulent and attenuated viral strains. For PV such markers have still not been defined. For example, a relationship between virulence and s (size of lysis plaques) or rct (ability to grow at high temperatures) has not been found.\textsuperscript{21}

On the other hand, two models have been identified which distinguish between virulent and attenuated strains of PV. The first of these is known as the “rabbit marker”, in which attenuated strains do not cause pruritus at the inoculation sites of rabbits\textsuperscript{19} or in rats.\textsuperscript{19} The second model involves the assessment of CE in cell cultures, such that attenuated strains cause rounding-up of cells, whereas virulent strains cause the formation of syncytial cells. These differences have been demonstrated in pig kidney cells,\textsuperscript{11} calf kidney cells\textsuperscript{22} and monkey kidney cells.\textsuperscript{19}

It has also been established by immuno-chemical techniques that in attenuated PV strains, the viral Ag can mainly be seen in the cytoplasm of infected cells, while the virulent strains show nuclear fluorescence.\textsuperscript{16,22}

The aim of this study is to characterize the virulence markers of the Pseudorabies virus, RC/79 strain. This strain was first isolated in Argentina in our laboratory by the experimental inoculation of rabbits and by in vitro infection of Vero cell monolayers.

**MATERIALS AND METHODS**

**Viral Strain.** Herpes suis type 1, RC/79 strain, was used. It was isolated in Río Cuarto in 1979 in chicken embryo fibroblast cultures. Isolates were taken from the central nervous system of pigs that were infected during an epidemic outbreak, and a titer of 1 x 103.5 DICC50/ml was detected.\textsuperscript{1} The virus was passaged ten successive times in Vero cell cultures until a titer of 1 x 108 DICC50/ml was obtained. This viral stock was kept at 20°C and was maintained in the laboratory for successive passages.

**Cellular System.** Vero cells from a line originally derived from kidney cells of the African green monkey (Cercopithecus aethiops) were used in all experiments. The cells were grown as monolayers on glass or plastic surfaces in Eagle-Earle’s minimal essential medium (MEM), supplemented with 8% fetal bovine serum and 30 µg/ml of glutamine and antibiotics. Cells from two sources were used indiscriminately. One source, which had undergone between 105 and 171 passages, originated from Micrology department in the Exact Sciences Faculty, (Facultad de Ciencias Exactas) in the University of Buenos Aires (UBA). The other source, strain 76, with 32-40 passages, was obtained from the ABAC (Asociación Banco Argentino de Células, The Argentine Cell Bank Association) in the city of Pergamino, province of Buenos Aires.

**Rabbits.** To study viral strain pathogenicity, 8 female New Zealand white rabbits (Oryctolagus cuniculus) were used. The animals weighed from 1.4 to 2.3 kg and were 1.5 to 2.5 months old. The animals were provided by the Central Animal House at the National University of Río Cuarto.

**Determination of virulence markers by in vitro assays.** To characterize the type of cytopathic effect (CE) produced by the RC/79 viral strain of AD, Vero cell monolayers were grown in 24 well plates or in 15 ml glass bottles. They were infected with a viral dilution, which produced an approximate rate of infection of 1:1. The cells were then incubated at 37 °C. Viral damage was assessed by microscopic examination over a period of 3 days.

**Characterization of the lysis plaques.** In parallel, the type of lysis plaque generated by the virus strain was characterized. This was carried out by infecting four bottles as described above, to achieve a 1:1 infection ratio. To these bottles plaque medium (double concentration of MEM plus 2% methylcellulose in double distilled water) was added and the cultures were incubated for 4 days at 37 °C. At the end of this time the lysis plaques were revealed following the method described by Dulbecco.\textsuperscript{5} As a control, four bottles were left with non-infected cellular monolayers. These controls were subjected to the same procedure as the infected monolayers.

**In vivo assays. Studies on pathogenicity in rabbits.** Virulence of the RC/79 strain was determined in rabbits. 8 animals were inoculated with 1 ml of viral suspension in MEM supplemented with 2% inactivated fetal bovine serum. The suspension was inoculated subcutaneously, in the inferior dorsal flank.

To analyze the influence of viral dose on the clinical response, 3 animals were inoculated with a viral dose of 102 PFU/ml and the remaining 5 animals received 105 PFU/ml. Observation of the symptoms was carried out for as long as the test allowed, which was on average for no more than 120 h post infection (p.i.). All the animals were kept in the animal house, at ambient temperature and in individual cages.

**RESULTS**

**Characterization of CE.** Vero cell monolayers infected with PV-RC/79 were checked daily. The formation of groups of large, refractive rounded-up cells were observed at the beginning of the infection (16-24 h p.i.). Later on during the infection, these cells made up the focus of lysis with detachment of cells from the glass walls, revealing holes in the cell culture (Fig. 1-arrow a).

In parallel, between 24 and 36 h p.i., it was possible to
see the appearance of multinuclear or syncytial cells produced as a consequence of cellular fusion (Fig. 1, arrow b).

A certain number of cells that did not undergo lysis remained united to others by cytoplasmic connections and had the appearance of asteroid formations (Fig. 2).

In Fig. 3 the control non-infected Vero cell monolayers can be seen.

Characterization of lysis plaques. Fig. 4 shows the lysis plaques caused by PV-RC/79 under agar. These were seen after 24 h p.i., and reached their greatest number and size at day three p.i.

It can be observed that the plaques are bright, with regular borders and have a diameter of 4-5 mm.

Tests of pathogenicity in rabbits. Table 1 shows the results obtained from inoculation tests carried out in rabbits. Animals were infected with different concentrations of virus, which varied from 102 to 105 PFU/ml. Changes in the clinical response to these different viral concentrations were not found, since all the animals died.

In each of the infected rabbits, periods of nervous excitement were observed during which they moved violently within their cages, followed by periods of tranquillity. Also, pruritus was observed on each animal at the inoculation site, which occurred between 6 and 24 h before death. As a consequence of the pruritus the animals savagely bit the area around the injection site, and this auto-mutilation resulted in the area around the site being left completely hairless, with cutaneous abrasions that exuded blood and serum (Fig. 5a). In the same figure it is possible to see differences in the texture of the animal’s fur. Before viral in-
occlusion (Fig 5b), the fur was flat and silky while after infection the fur became rough and bristly.

The rabbits that received smaller viral doses (102 PFU) took longer to die, on average 120 h p.i.. The time of death was 1.9 times greater than for the 5 animals that received 105 PFU that died between 54 and 66 h p.i.

DISCUSSION

Studies carried out by A. Sabin in 1934, on the Herpes simplex virus (HSV) and the Pseudorabies virus in animals, have revealed that these two viral types are related. The reasoning behind this conclusion was based on the fact that both viruses are pantotrophic, neuro-invasive, produce similar intracellular inclusions and have a similar range of hosts. Furthermore, the epidemiological patterns of HSV and PV are also similar.

Later a more detailed study was carried out that made comparisons between both viruses. In this article cell cultures infected with PV were described. There was initially the appearance of few areas of focal necrosis and rounded-up cells, and a few h later (8-10 h) it was possible to see the formation of syncytial cells in the monolayer, where the cell walls disappeared and consisted of a large multinucleated syncytial mass. Based on studies carried out by T. Tokumaru in 1957, these two different classes of CE were attributed to genetic heterogeneity within the viral population. This author demonstrated that the difference in the characteristics of CE was due to strain virulence. It was concluded that in general the attenuated strains caused rounding-up of cells while the virulent strains initially caused cellular rounding-up but also caused the formation of syncisius.

In this study, characterization of the PV-RC/79 strain was based on cytopathogenic effects on Vero cell monolayers, and it was possible to observe a progression in CE. This progression started with the rounding-up of cells and lead to the formation of syncisius and cellular detachment as a consequence of cellular lysis (Fig. 1a, b and 2).

With the aim of demonstrating differences between lysis plaques induced by virulent and attenuated strains, Tokumaru carried out single step growth curves for each viral type, using immune sera. It was shown that the viral strains that generate clinical symptoms of Pseudorabies in rabbits and rats, and that induced formation of syncisius in cellular monolayers, have a tendency to generate large or “L” lysis plaques, however no specific values were given. Sabin on the other hand, described plaques produced by virulent strains that had diameters of between 5-10 mm.

In the characterization of the type of lysis plaques generated by PV-RC/79, it was observed that the sizes of these plaques (4-5 mm) correspond to those described for virulent strains (Fig. 4). This was reaffirmed by the results from the type of CE induced by the virulent viral strain, as well as by its progression toward the formation of syncisius.

From another point of view, the virulent character of PV-RC/79 was revealed by results obtained from the rabbit
Table 1: Determination of virulence of the Pseudorabies virus, RC/79 strain, by clinical response and mean time of death, in white rabbits inoculated subcutaneously.

<table>
<thead>
<tr>
<th>RC/79 strain Dilution:</th>
<th>Nº of inoculated rabbits</th>
<th>Nº of deaths</th>
<th>% mortality</th>
<th>Mean time of death @</th>
<th>Range</th>
<th>Pruritus</th>
</tr>
</thead>
<tbody>
<tr>
<td>105 PFU</td>
<td>5</td>
<td>5</td>
<td>100</td>
<td>62</td>
<td>54 - 66</td>
<td>Yes</td>
</tr>
<tr>
<td>102 PFU</td>
<td>3</td>
<td>3</td>
<td>100</td>
<td>120</td>
<td>114 - 126</td>
<td>Yes</td>
</tr>
</tbody>
</table>

@ Hours post inoculation (p.i.)

infection tests. Subcutaneous injection of rabbits with a suspension of animal tissue suspected of containing the AD virus, was described in an empirical way by the first researchers into this disease. Its virulent character was confirmed by the authors as strongly virulent, generating symptoms of pruritus and death in rabbits. The virulent character of the RC/79 strain, as suspected since it was first isolated following its history of generating 80% mortality in piglets. The virulent character of the RC/79 strain is also supported by the absence of a dose response. Animals were given viral inoculation of between 102 and 105 PFU, with lower viral doses only the average time of death was modified, and in all cases the fatality in rabbits was 100% (Table 1).

In a previous work our group has demonstrated that the PV-RC/79 strain is sensitive to heat and trypsin. By comparing this data with those obtained in this rabbit infection test, it is possible to see that the RC/79 strain is strongly virulent, which is in agreement with the results obtained by Platt et al.

The "rabbit marker" has an important role in epidemiological studies. Its use would help in the diagnosis of Pseudorabies by distinguishing between virulent and attenuated strains. Furthermore this test represents a useful tool when screening for naturally attenuated viral isolates that may be used as vaccines and/or it may be used to control the degree of attenuation of viral strains that are modified in the laboratory.

It must be pointed out that some authors have described strains of the AD virus that cause high mortality in the field but do not produce pruritus following intracerebral or subcutaneous inoculation, at least following 2 or 3 passages. This observation reaffirms the fact that the RC/79 strain, by generating the symptoms of pruritus and death with only one subcutaneous passage in rabbits and with lower viral doses (102 PFU), is strongly virulent.

With this experience it has been possible to characterize the PV-RC/79 strain with virulence markers and in parallel we have adjusted a valuable diagnostic tool consisting of the experimental inoculation of rabbits with clinical samples. This technique is available in our laboratory and it is possible to apply it to AD control or eradication programs.

ACKNOWLEDGEMENTS

This work was financed with assistance from the Secretaría de Ciencia y Técnica (SEC y T) of the Universidad Nacional de Río Cuarto, item 477 and by CONICOR.

We would like to thank Sr. Victor Saldaño who is the
general coordinator of the Central Animal House (Bioterio Central) in the Universidad Nacional de Río Cuarto, for his generous collaboration and for caring for the animals.

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