J. Comp. Path. 2014, Vol. 151, 384–393

Available online at www.sciencedirect.com

ScienceDirect

ELSEVIER
www.elsevier.com/locate/jcpa

EXPERIMENTALLY INDUCED DISEASE

Protective Effects of Intranasal Immunization with Recombinant Glycoprotein D in Pregnant BALB/c Mice Challenged with Different Strains of Equine Herpesvirus 1

N. A. Fuentealba[*,‖], C. N. Zanuzzi[†,§,‖], M. R. Scrochi[*,†,‖], G. H. Sguazza[*], M. E. Bravi[*,#], V. Cid de la Paz[*,¶], S. G. Corva[‡], E. L. Portiansky[§,‖], E. J. Gimeno[§,‖], C. G. Barbeito[†,§,‖] and C. M. Galosi[*,#]

** Department of Virology, † Department of Histology and Embryology, ‡ Department of Epidemiology, § Department of General Pathology, School of Veterinary Sciences, National University of La Plata, Buenos Aires, ‖ National Scientific and Technical Research Council (CONICET-CCT La Plata), ¶ Scientific Research Commission of Buenos Aires Province (CIC-PBA) and # Argentinean Agency for the Promotion of Science and Technology (ANPCyT), Argentina*

Summary

Equine herpesvirus (EHV)-1 induces respiratory infection, neurological disorders and abortion in horses. Most of the currently available attenuated or inactivated vaccines against this infection are administered intramuscularly and only provide partial protection against the respiratory disease. The present study examines the effect of intranasal immunization with purified EHV-1 recombinant glycoprotein D (gD) in BALB/c mice followed by challenge with three different EHV-1 strains during early to mid-pregnancy. The induced viral infection was evaluated by virus isolation, DNA detection by polymerase chain reaction, histopathology and immunohistochemical localization of antigen in the lung, placenta and uterus. Non-immunized mice showed clinical signs of infection, positive virus isolation from lungs and uteri, and abortion induced by one of the virus strains. Endometrial lesions developed in some of these animals that have been described previously only in horses. Immunized mice and their offspring had no viral infection or typical lesions. Intranasally administered gD therefore induced partial or complete protection against three different EHV-1 strains in BALB/c mice.

Keywords: equine herpesvirus 1; glycoprotein D; intranasal inoculation; mouse abortion model

Introduction

Equine herpesvirus (EHV)-1 induces respiratory infection, neurological disorders and abortion in horses throughout the world (Jackson *et al*., 1977; Smith, 1997); however, the pathogenesis of abortion is poorly understood. Primary infection induces a humoral immune response and production of neutralizing antibodies, but naturally infected animals do not develop long-lasting protection and remain susceptible to new infections throughout their lives (Allen and Bryans, 1986; Crabb and Studdert, 1995). EHV-1 and other alphaherpesviruses have developed many strategies to evade the host immune system (Ambagala *et al*., 2004; Koppers-Lalic *et al*., 2005; Van de Walle *et al*., 2008). Up to now, no vaccine is able to prevent the infection and most of the attenuated or inactivated vaccines currently available are administered intramuscularly and only provide partial protection against the respiratory disease. These vaccines do not prevent cell-associated viraemia nor do they protect fully against abortion (Walker *et al*., 1999; Minke *et al*., 2004).

Correspondence to: N. A. Fuentealba (e-mails: nadiafuentealba@hotmail.com, nadiafuentealba@fcv.unlp.edu.ar).

0021-9975/$ - see front matter
http://dx.doi.org/10.1016/j.jcpa.2014.06.003

Envelope glycoproteins play an important role in the infectivity and pathogenicity of EHV-1 and constitute major targets for the host immune system (Packiarajah *et al*., 1998). These glycoproteins have been incorporated into recombinant vaccines (Love *et al*., 1993; Osterrieder *et al*., 1994; Munro *et al*., 1999). Experimental studies using individual or combined glycoproteins B, C, D and H have been performed in mice (Guo *et al*., 1990; Tewari *et al*., 1994, 1995; Osterrieder *et al*., 1995; Kukreja *et al*., 1998; Zhang *et al*., 1998; Ruitenberg *et al*., 1999) and in the natural host (Foote *et al*., 2005, 2006). These studies have involved different vaccination strategies and they mainly used non-purified glycoproteins and parenteral routes of immunization. Most of the vaccines have been evaluated using the murine respiratory model of the disease (Awan *et al*., 1991, 1995; Iwai *et al*., 1998; Walker *et al*., 1999), but for the abortion model only DNA encoding glycoprotein D (gD) of was used (Walker *et al*., 2000). Glycoprotein D is a component of the viral envelope involved in viral entry into host cells (Whittaker *et al*., 1992).

The aim of the present study was to determine whether intranasal administration of gD was protective against respiratory disease and abortion in BALB/c mice challenged with different strains of EHV-1 during early to mid-gestation.

Materials and Methods

Animals

Specific pathogen-free 5-week-old BALB/c mice were provided by the Department of Laboratory Animals (School of Veterinary Sciences, National University of La Plata, Buenos Aires, Argentina) and kept in conventional animal rooms. Temperature, light and ventilation were controlled and animals received food and water *ad libitum*. All experimental procedures were carried out in compliance with the Guide for the Care and Use of Laboratory Animals of the National Research Council (ILAR, 2010) and supervised by the Institutional Committee for Care and Use of Laboratory Animals (School of Veterinary Sciences, National University of La Plata).

Immunization and Antibody Detection

Glycoprotein D was expressed in insect cell lines derived from *Trichoplusia ni* (High Five™ cells, Invitrogen, Carlsbad, California, USA) and purified as described by Fuentealba *et al*. (2014).

Mice were immunized intranasally with 50 µl (300 µg/ml) of gD in phosphate buffered saline (PBS), under light anaesthesia with isoflurane (Baxter Co., Deerfield, Illinois, USA). The same dose was administered for a second time on day 20. Non-immunized mice received intranasal PBS.

Blood was taken from the maxillary vein 10 days after each immunization and pooled serum samples (six mice/pool) were tested for antigen-specific immunoglobulin (Ig) G by indirect enzyme-linked immunosorbent assay (ELISA) using a soluble antigen produced with AR8-infected Madin–Darby bovine kidney (MDBK) cells. Control antigen was prepared following the same procedure with uninfected MDBK cells. An optimum dilution of 1 in 40 was determined for the serum samples. Anti-mouse IgG peroxidase (Sigma–Aldrich, St Louis, Missouri, USA) was used as secondary antibody and ABTS (2,2′-azino-di-[3-ethyl-benzothiazoline-6-sulphonic acid]) solution containing H_2O_2 was used as substrate–indicator solution. Absorbance was determined by use of an ELISA reader (Thermo Scientific Multiskan FC, Vantaa, Finland) at 405 nm and the cut-off value was determined as two average optical density (OD) values of negative serum (IAEA, 1989).

Virus Strains and Cell Cultures

Three virus strains (AR11, AR52 and HH1) were used in this study. The Argentinean AR11 and AR52 strains were isolated from mares after an

Table 1
Experimental design

Experimental group	*Number of mice per group*	*Inoculation time*	*Treatment of animals*
I+/P+/V I−/P+/V I−/P+/S	6	Day 7 of pregnancy	Four killed at 96 hpi and two continued until day of parturition
I+/P−/V I−/P−/V I−/P−/S	6	In accordance with the respective pregnant group	Two killed at 48 hpi, two killed at 72 hpi and two observed until 40 dpi

I, immunized; P, pregnant; V, virus inoculation; S, sham-inoculated; +, positive; −, negative; hpi, hours post infection; dpi, days post infection.

abortion storm and from a case of neonatal disease in 2004 and 2005 (data not published), respectively. The HH1 Japanese reference strain was isolated from a case of abortion and was provided by Dr. T. Mikami (University of Tokyo, Japan). Rabbit kidney (RK13) cells were used to grow virus. Each strain was propagated and after three cycles of freezing and thawing, the cell suspensions were centrifuged at 10,000 g for 30 min to remove cell debris and the virus suspensions were fractionated, quantified by the standard Reed and Muench method (1938) and stored at −70°C until used.

Experimental Design

Twenty days after the second immunization, a group of female mice were caged with males at a ratio of 3:1 and pregnancies were confirmed by the presence of vaginal plugs. Then, the pregnant mice were caged individually. The assays were developed sequentially according to the availability of pregnant mice.

The following experimental groups were established for each virus strain (Table 1):

Group I+/P+/V: immunized, pregnant, virus-inoculated mice ($n = 6$);
Group I−/P+/V: non-immunized, pregnant, virus-inoculated mice ($n = 6$);
Group I−/P+/S: non-immunized, pregnant, sham-inoculated mice ($n = 6$);
Group I+/P−/V: immunized, non-pregnant, virus-inoculated mice ($n = 6$);
Group I−/P−/V: non-immunized, non-pregnant, virus-inoculated mice ($n = 6$);
Group I−/P−/S: non-immunized, non-pregnant, sham-inoculated mice ($n = 6$).

All mice from each experimentally-infected group (I+/P+/V, I+/P−/V, I−/P+/V and I−/P−/V) were lightly anaesthetized (Eöry *et al.*, 2013) and inoculated intranasally with $\sim 10^{6.3}$ $TCID_{50}$/50 µl of each virus strain at day 7 of pregnancy (early to mid-gestation). Non-pregnant mice were inoculated on the same day. Mice from each sham group (I−/P+/S and I−/P−/S) were inoculated intranasally with 50 µl of supernatant of RK13 cells kept in Eagle's Minimum Essential Medium (EMEM) with 2% fetal calf serum (M-EMEM). Four mice from groups I+/P+/V, I−/P+/V and I−/P+/S were killed by intraperitoneal injection of 0.1 mg/g body weight of ketamine (Laboratorios Holiday, Buenos Aires, Argentina) and 0.01 mg/g body weight of xylazine (Laboratories Richmond, Buenos Aires, Argentina) at 96 hours post infection (hpi), and two mice in these groups were left alive until natural parturition. The number, size and development of embryos were recorded and lesions in uteri and placentae were described. Two mice from groups I+/P−/V, I−/P−/V and I−/P−/S were killed at 48 hpi or 72 hpi, or left alive until ∼40 days post infection (dpi).

Clinical Assessment, Collection and Processing of Samples

Animals were weighed before mating and then daily from day 7 of pregnancy (or on the respective day for non-pregnant mice). The body weights of the mice were checked to verify their normal distribution by the Shapiro−Wilk test. The analysis was performed only on day 7 (initial weight) regardless of strain or treatment. The mean body weight values among groups of mice were compared by one-way ANOVA. In addition, after virus inoculation, the general condition of the mice was determined twice daily by assessing the presence of clinical signs, premature births and/or parturition. Premature loss of pregnancy was identified when there was a sharp fall in the body weight of the pregnant mice. Heparinized (20 IU/ml) and non-heparinized blood samples were collected from all experimental groups before killing and were processed immediately for virus isolation (Galosi *et al.*, 2004) and IgG detection. The left lung of each animal was selected for histological studies, while the right lung was used for titration of viral infectivity and processed for DNA detection by polymerase chain reaction (PCR). The placentae and uteri were processed for histological studies, virus isolation or PCR. Fifty percent of the offspring of females that remained alive were killed by deep anaesthesia and processed for virus isolation and PCR. The remaining offspring and their mothers were monitored daily and 30 days after birth they were bled for IgG detection.

Virus Isolation

Samples were processed by preparing a 10% (weight/volume) homogenized suspension in M-EMEM and clarified by centrifugation at 6,000 g for 20 min. The supernatants were inoculated into RK13 cells and these were observed for cytopathic effect for 1 week. Supernatants from cell cultures without cytopathic effect were re-passaged twice before being considered as definitively negative.

Titration of Viral Infectivity

Tenfold serial dilutions of lung homogenates in M-EMEM were inoculated onto preformed cell monolayers and after 3 h of incubation at 37°C the cells were overlaid with M-EMEM and checked for

Table 2
Antibody detection by ELISA (absorbance values at 405 nm)

Group	*Primary immunization*	*Secondary immunization*	*Hours post infection*	*30 days post partum or 40 days post infection*
Infection with AR11 strain				
I+/P+/V	136	131	418 (96 hpi)	410 (mother) 339 (offspring)
I−/P+/V	113	120	141 (96 hpi)	423 (mother) 386 (offspring)
I−/P+/S	125	139	141 (96 hpi)	158 (mother) 147 (offspring)
I+/P−/V	132	137	150 (48 hpi) 408 (72 hpi)	400
I−/P−/V	138	145	139 (48 hpi) 147 (72 hpi)	362
I−/P−/S	146	132	130 (48 hpi) 143 (72 hpi)	141
Infection with AR52 strain				
I+/P+/V	143	139	406 (96 hpi)	381 (mother) 370 (offspring)
I−/P+/V	155	152	138 (96 hpi)	394 (mother) 385 (offspring)
I−/P+/S	138	141	137 (96 hpi)	142 (mother) 140.5 (offspring)
I+/P−/V	137	149	139 (48 hpi) 390 (72 hpi)	379
I−/P−/V	128	131	134 (48 hpi) 147 (72 hpi)	400
I−/P−/S	121	134	138 (48 hpi) 137 (72 hpi)	137
Infection with HH1 strain				
I+/P+/V	152	147	446 (96 hpi)	413 (mother) 373 (offspring)
I−/P+/V	121	139	137 (96 hpi)	394 (mother) Offspring not determined
I−/P+/S	141	153	135 (96 hpi)	136 (mother) 139.5 (offspring)
I+/P−/V	147	138	145 (48 hpi) 429 (72 hpi)	409
I−/P−/V	128	131	139 (48 hpi) 153 (72 hpi)	407
I−/P−/S	121	134	143 (48 hpi) 146 (72 hpi)	147

Table 3
Virus isolation from lungs of mice infected with EHV-1

Group	*Virus titre*					
	HH1		**AR11**		**AR52**	
	96 hpi		96 hpi		96 hpi	
I+/P+/V	10^{2} $TCID_{50}$†		Negative		Negative	
	$10^{0.35}$ $TCID_{50}$*					
I−/P+/V	$10^{3.87}$ $TCID_{50}$‡		$10^{2.62}$ $TCID_{50}$‡		$10^{2.87}$ $TCID_{50}$‡	
	$10^{0.48}$ $TCID_{50}$*		$10^{0.62}$ $TCID_{50}$*		$10^{0.28}$ $TCID_{50}$*	
	48 hpi	72 hpi	48 hpi	72 hpi	48 hpi	72 hpi
I+/P−/V	Negative	Negative	Negative	Negative	Negative	Negative
I−/P−/V	$10^{3.75}$ $TCID_{50}$‡	$10^{4.25}$ $TCID_{50}$‡	$10^{2.75}$ $TCID_{50}$‡	$10^{3.25}$ $TCID_{50}$‡	$10^{2.75}$ $TCID_{50}$‡	$10^{3.25}$ $TCID_{50}$‡
	$10^{0.35}$ $TCID_{50}$*	$10^{0.35}$ $TCID_{50}$*	$10^{0.35}$ $TCID_{50}$*	$10^{0.35}$ $TCID_{50}$*	$10^{0.35}$ $TCID_{50}$*	$10^{0.35}$ $TCID_{50}$*

*Average standard deviation.
†All mice of the group were positive. Average at third passage over cells.
‡All mice of the group were positive. Average at first passage over cells.

cytopathic effects over the next week. Supernatants of cells were re-passaged twice before being considered as negative. Viral titres (log $TCID_{50}$/lung) were calculated by the Reed and Muench method (1938) at 96 hpi.

Polymerase Chain Reaction

Samples were processed using a commercial kit (Wizard Genomic DNA Purification Kit, Promega, Madison, Wisconsin, USA) for DNA extraction. In addition, cells inoculated with a third passage of samples and negative for virus isolation were processed for DNA extraction. PCR was performed as described by Galosi *et al.* (2001).

Histopathology and Immunohistochemistry

Samples of lungs, placentae and uteri were fixed in 10% neutral buffered formalin for 24 h, processed routinely and embedded in paraffin wax. Sections (3 μm) were stained with haematoxylin and eosin (HE). Immunohistochemistry (IHC) was used to detect EHV-1 antigens (Eöry *et al.*, 2013; Zanuzzi *et al.*, 2014) and IgA. Briefly, EHV-1 antigens were detected using a primary rabbit polyclonal anti-EHV-1 antibody produced in our laboratory, diluted 1 in 1,500 in PBS with bovine serum albumin 0.1%. Labelling was 'visualized' with anti-rabbit EnVision® detection system + HRP (Dako, Carpinteria, California, USA). To detect IgA protein direct IHC using primary goat anti-mouse IgA (Southern Biotech, Birmingham, Alabama, USA) labelled with peroxidase was used.

Results

Antibody Detection and Clinical Signs

Values of OD ≥300 were considered positive in the ELISA. Antigen-specific serum IgG was detected in immunized mice of groups I+/P+/V and I+/P−/V after two immunizations, at 96 hpi and 72 hpi, respectively. IgG was also detected in mice and offspring of group I+/P+/V and I−/P+/V on day 30 after parturition and in surviving mice of groups I+/P−/V and I−/P−/V at ∼40 dpi (Table 2).

According to the Shapiro−Wilk test the initial body weights were normally distributed ($P > 0.05$). After challenge, neither body weight loss nor clinical signs of infection were observed in mice of immunized groups (I+/P+/V and I+/P−/V). Premature births were not recorded and the number, size and development of the embryos and offspring of mice of group I+/P+/V challenged with each virus strain was similar to those of the control group (I−/P+/S).

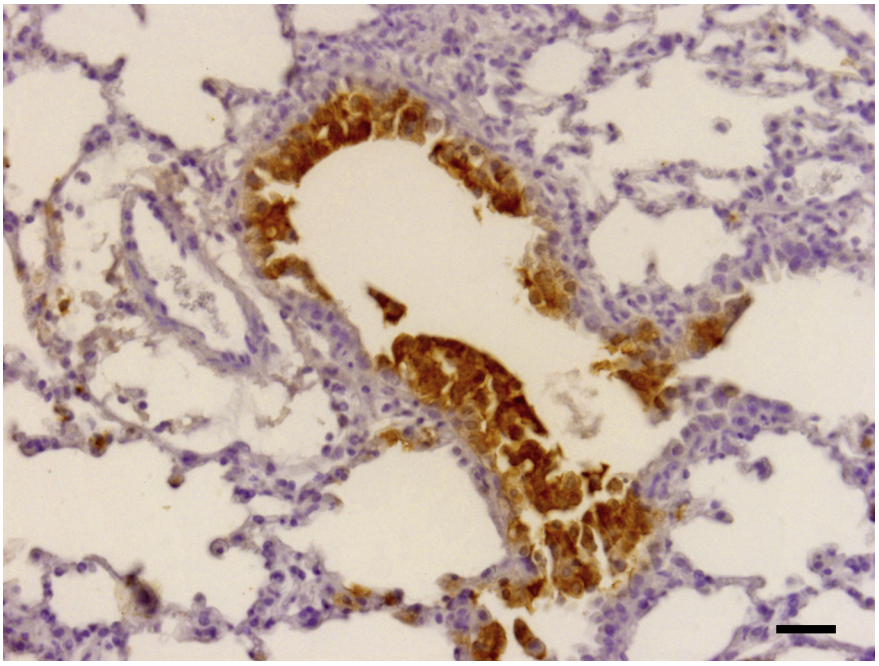

Fig. 1. Lung of a mouse from group I−/P+/V challenged with strain AR11. There is immunohistochemical labelling of EHV-1 antigen in the bronchiolar epithelium. IHC. Bar, 30 μm.

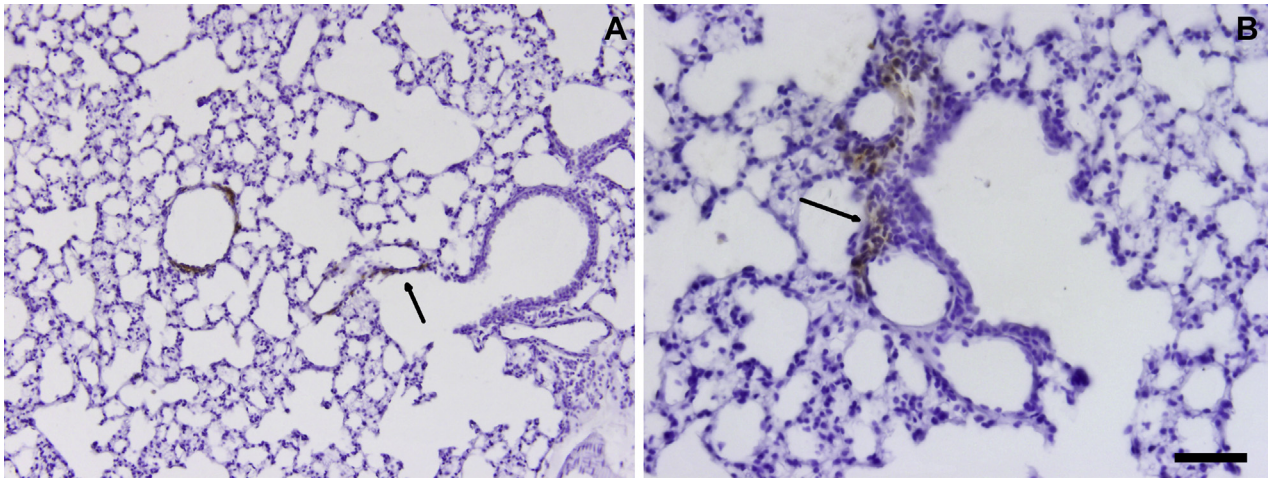

Fig. 2. Lung of a mouse from group I+/P+/V challenged with strain AR11. There is immunohistochemical labelling of IgA in peribronchiolar tissue. IHC. Bar, 100 μm in (A), 50 μm in (B).

Mice of groups I−/P+/V and I−/P−/V showed ruffled fur, hunched posture, depression and dyspnoea at 24 hpi with all of the virus strains studied, although these signs were more severe with strain HH1 at 72 hpi. Slight loss of body weight was observed in mice of group I−/P+/V from 48 to 72 hpi with all of the virus strains. Mice of group I−/P+/V challenged with AR11 or AR52 strains and killed at 96 hpi had few embryos (2 ± 1) and some small haemorrhagic spots indicative of presumptive abortion, whereas those challenged with HH1 strain only showed small haemorrhagic spots and no embryos. Mice of group I−/P+/V challenged with AR11 or AR52 that were left alive until parturition delivered an average of three offspring at term. However, HH1-inoculated mice of this group showed an abrupt decrease in body weight at 5 dpi and no offspring, which may have been associated with abortion. Mice of group I−/P−/V showed a decrease in body weight from 24 to 72 hpi for all of the virus strains tested, although the differences between this group and mice in groups I+/P−/V and I−/P−/S were not significant. None of the sham-inoculated mice (groups I−/P−/S and I−/P+/S) developed clinical signs. The mean number of embryos and neonates ($n = 6$) from mice of group I−/P+/S was in accordance with the mean number expected for this mouse strain.

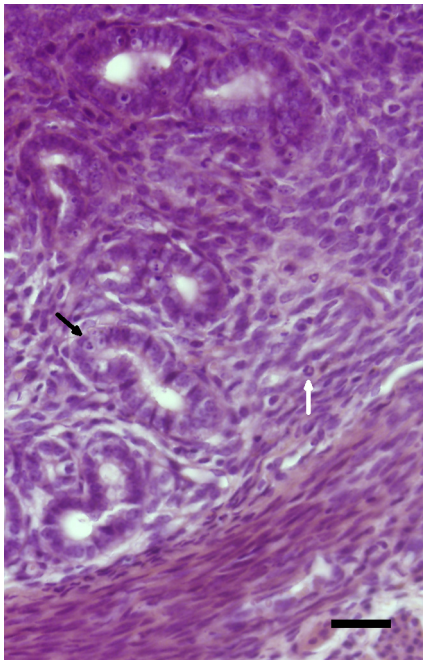

Fig. 3. Uterine horn of a mouse from group I−/P+/V challenged with strain HH1 and showing apoptosis in the glandular epithelium (black arrow) and neutrophils (white arrow) in the lamina propria. HE. Bar, 30 μm.

Virus Isolation and DNA Detection

Virus isolation from the lungs of group I+/P+/V challenged with strain HH1 was positive only after the third passage in RK13 cells (10^2 $TCID_{50}$), whereas it was negative in mice inoculated with strains AR11 or AR52. Virus isolation was also negative in the lungs of mice of group I+/P−/V challenged with each strain.

Viral titres from the lungs of mice in challenged groups are shown in Table 3. The virus was not recovered from plasma rich in leucocytes from immunized mice challenged with any strain, but it was isolated from two females of group I−/P+/V and one of group I−/P−/V challenged with strain HH1. Virus isolation from uteri of mice of group I−/P+/V was positive in four mice challenged with strain HH1, in

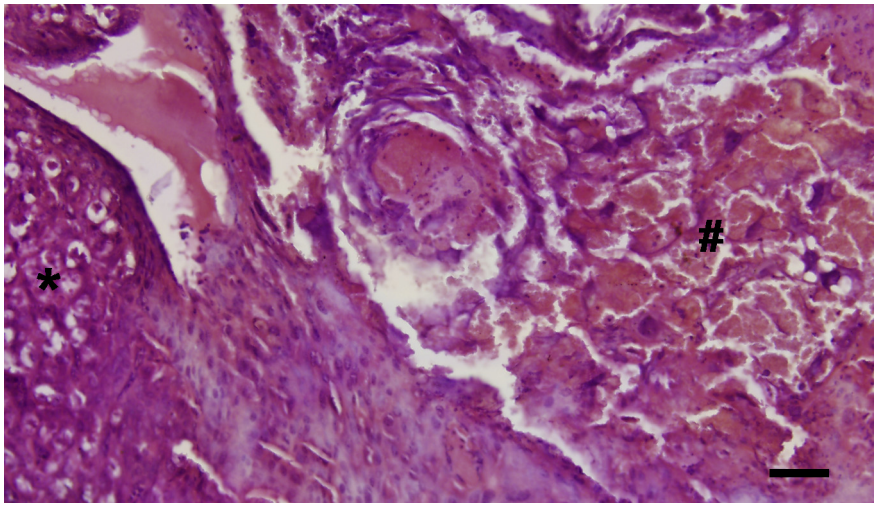

Fig. 4. Placenta of a mouse from group I−/P+/V challenged with strain AR52 showing normal spongiotrophoblast (*) and a necrotic area (#). HE. Bar, 30 μm.

three mice inoculated with AR11 and in one mouse inoculated with strain AR52. In addition, lungs and uteri that were positive for virus isolation were also positive for DNA. Virus isolation and PCR from placentae, embryos and neonates of mice of groups I+/P+/V and I−/P+/V challenged with strains AR11 or AR52 were negative. Fetal resorptions were found in the groups challenged with each strain, but virus isolation was negative. Cells of third passage of each analyzed sample were also negative by PCR.

Histopathology and Immunohistochemistry

Lesions such as inflammatory infiltration, loss of normal alveolar architecture, desquamation and necrosis of bronchial, bronchiolar and alveolar epithelia were found in the lungs of non-immunized animals (groups I−/P+/V, I−/P−/V) challenged with the three virus strains. Intranuclear inclusion bodies were also present in some epithelial cells. Viral antigens were detected by IHC in bronchial and bronchiolar epithelia (Fig. 1). Lungs of mice from groups I+/P+/V and I+/P−/V challenged with strains HH1, AR11 or AR52 showed no lesions and the detection of viral antigens was negative. IgA-positive cells were detected, some at the bronchial epithelium and others in peribronchial tissue (Fig. 2).

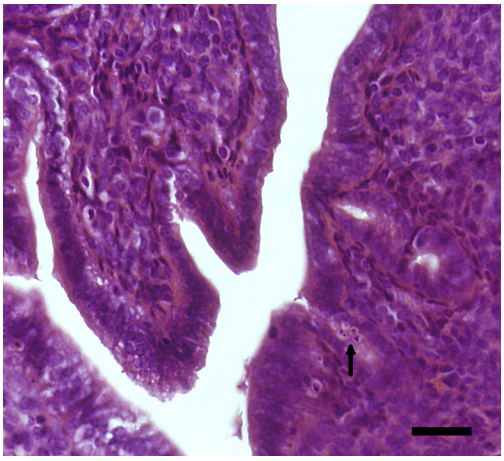

Fig. 5. Uterine horn of a mouse from group I+/P+/V challenged with strain HH1 showing remodelling in the luminal epithelium (arrow). HE. Bar, 30 μm.

Mice of group I−/P+/V challenged with strain HH1 showed a large number of dead cells in connective tissue, epithelium and glandular lumina of uteri. Inflammatory cells, including macrophages and neutrophils, were also observed (Fig. 3). Mice of this group challenged with strain AR11 showed no typical herpesvirus lesions (e.g. syncytial formation or inclusion bodies) in their placentae or deciduae, although a large number of apoptotic epithelial cells and inflammatory cells were observed in the uterine connective tissue, probably as a result of a non-specific inflammatory response. Mice challenged with strain AR52 showed neutrophilic infiltration and necrotic foci in uteri and placentae (Fig. 4). Mice of groups I−/P+/S and I+/P+/V challenged with the three virus strains showed few extensive epithelial remodelling areas and apoptotic cells in the uteri (Fig. 5).

Discussion

The present study evaluated the effect of intranasal immunization with purified recombinant gD in mice challenged with three different strains of EHV-1 during early to mid pregnancy, and compared the lesions found in uteri and placentae of these animals. Immunized pregnant mice did not produce antigen-specific IgG in serum before challenge; however, virus

isolation from their lungs was negative for two virus strains. It is known that secretory IgA blocks the interaction between cellular receptors and pathogens and that intranasal immunization is more effective in stimulating mucosal immunity (Watanabe *et al.*, 2002; Ito *et al.*, 2003). Therefore, gD may partially protect against the respiratory and abortigenic forms of the infection through inducing a secretory immune response in the upper respiratory tract. The development of mucosal immunity in immunized pregnant mice may prevent or reduce the viraemia and its consequences, as occurs in mares (Smith *et al.*, 1992). The detection of IgG on day 30 after parturition in the offspring could be due to the transfer of maternal immunity via colostrum.

The duration of pregnancy in mice from groups I+/P+/V and I−/P+/V was not significantly different. Similar findings were reported by Kukreja *et al.* (1998) in mice immunized with glycoprotein B and challenged during late pregnancy. The use of similar doses and titres for these three strains showed that strain HH1 induced a higher abortion rate than AR11 or AR52, thus indicating a possible higher pathogenicity.

Virus isolation was positive from uteri, but not from placentae and embryos, which were also negative by PCR. These results agree with those of Awan *et al.* (1995). Smith (1997) also reported abortion in infected mares, positive virus isolation from placentae and negative isolation from fetal tissues, findings that may be explained by the rapid expulsion of fetuses induced by specific viral-endothelial tropism.

The most common lesions described in placentae of infected mares (Smith, 1997; Smith *et al.*, 2000) and mice (Awan *et al.*, 1995; Iwai *et al.*, 1998; Walker *et al.*, 1998, 1999) include ischaemia, necrosis and atrophy of trophoblasts. Vascular lesions and inflammatory changes have also been reported in the endometrium of naturally or experimentally infected mares (Smith *et al.*, 1992; Carlton *et al.*, 1995). To our knowledge, there are no previous descriptions of changes in the endometrium of EHV-1-inoculated mice. In the current study, some of the lesions described above were found in mice from group I−/P+/V, and they varied with the infecting virus strain. Although equine and murine placentae are histologically different, there are similarities in the local immune response (Croy *et al.*, 2009).

The variability in the histopathological changes reported in infected mares may also occur in the murine model, as reported here. In this regard, in the present study we reported uterine lesions that have been previously described in horses, but not in mice. The embryonic losses reported here after the inoculation of strain HH1 agree with those described by Awan *et al.* (1995) at the same stage of pregnancy using a more virulent strain of virus. However, the effects on the conceptuses are highly variable and depend on the virus strain.

Since immunized pregnant challenged mice (group I+/P+/V) showed few lesions in placentae and uteri, the local immune response in the upper respiratory tract could have prevented or reduced the access of virus to the bloodstream, thereby inhibiting viraemia and infection of the reproductive tract.

It remains as an open question whether the abortions were caused primarily by maternal factors or by the viral infection. Further studies are required in order to determine the significance of IgA in the upper respiratory tract of mice immunized intranasally with gD and challenged with EHV-1 strains. This information will inform strategies for testing gD immunization in horses with a view to increasing the immune protection at weaning, when a decline in maternal antibodies makes animals more susceptible to infection.

Conflict of Interest Statement

There are no conflicts of interest with respect to authorship or the publication of this article.

Acknowledgements

We thank Professor M. Ayala (Department of Laboratory Animals, School of Veterinary Sciences, National University of La Plata) for professional assistance. We also thank our technicians R. Villegas, R. Mario, C. A. Leguizamón and G. Guidi for their support. This study was supported by grants from the Scientific Research Commission of Buenos Aires Province (1376/12), the Department for Science and Technology of the National University of La Plata and by the Argentinean Agency for the Promotion of Science and Technology (PICT 2011-1123), Argentina.

References

Allen GP, Bryans JT (1986) Molecular epizootiology, pathogenesis and prophylaxis of equine herpesvirus-1 infections. In: *Progress in Veterinary Microbiology and Immunology*, R Pandey, Ed., Karger, Basel, pp. 78–144.

Ambagala APN, Gopinath RS, Srikumaran S (2004) Peptide transport activity of the transporter associated with antigen processing (TAP) is inhibited by an early protein of equine herpesvirus-1. *Journal of General Virology*, **85**, 349–353.

Awan AR, Baxi M, Field HJ (1995) EHV-1 induced abortion in mice and its relationship to stage of gestation. *Research in Veterinary Science*, **59**, 139–145.

Awan AR, Gibson JS, Field HJ (1991) A murine model for studying EVH-1-induced abortion. *Research in Veterinary Science*, **51**, 94–99.

Carlton WW, McGavin MD, Acland HM (1995) Reproductive system: female. In: *Special Veterinary Pathology*, WW Carlton, MD McGavin, Eds., Thomson Mosby, St Louis, pp. 529–530.

Crabb BS, Studdert MJ (1995) Equine herpesviruses 4 (equine rhinopneumonitis virus) and 1 (equine abortion virus). *Advances in Virus Research*, **45**, 153–190.

Croy BA, Wessels J, Linton N, Tayade C (2009) Comparison of immune cell recruitment and function in endometrium during development of epitheliochorial (pig) and hemochorial (mouse and human) placentas. *Placenta*, **30**, 26–31.

Eöry ML, Zanuzzi CN, Fuentealba NA, Sguazza GH, Gimeno EJ *et al.* (2013) Effects of different anesthetics in the murine model of EHV-1 infection. *Veterinary Pathology*, **50**, 849–856.

Foote CE, Love DN, Gilkerson JR, Rota J, Trevor-Jones P *et al.* (2005) Serum antibody responses to equine herpesvirus 1 glycoprotein D in horses, pregnant mares and young foals. *Veterinary Immunology and Immunopathology*, **105**, 47–57.

Foote CE, Raidal SL, Pecenpetelovska G, Wellington JE, Whalley JM (2006) Inoculation of mares and very young foals with EHV-1 glycoproteins D and B reduces virus shedding following respiratory challenge with EHV-1. *Veterinary Immunology and Immunopathology*, **111**, 97–108.

Fuentealba N, Sguazza G, Scrochi M, Bravi M, Zanuzzi C *et al.* (2014) Production of equine herpesvirus 1 recombinant glycoprotein D and development of an agar gel immunodiffusion test for serological diagnosis. *Journal of Virological Methods*, **202**, 15–18.

Galosi CM, Barbeito CG, Vila Roza MV, Cid de la Paz V, Ayala MA *et al.* (2004) Argentine strain of equine herpesvirus 1 isolated from an aborted foetus shows low virulence in mouse respiratory and abortion models. *Veterinary Microbiology*, **103**, 1–12.

Galosi CM, Vila Roza MV, Oliva GA, Pecoraro MR, Echeverría MG *et al.* (2001) A polymerase chain reaction for detection of equine herpesvirus-1 in routine diagnostic submissions of tissues from aborted foetuses. *Journal of Veterinary Medicine Series B*, **48**, 341–346.

Guo PX, Goebel S, Perkus ME, Taylor J, Norton E *et al.* (1990) Coexpression by vaccinia virus recombinants of equine herpesvirus 1 glycoproteins gp13 and gp14 results in potentiated immunity. *Journal of Virology*, **64**, 2399–2406.

Institute for Laboratory Animal Research (ILAR) (2010) *Guide for the Care and Use of Laboratory Animals (2010)*. http://dels.nas.edu/Report/Guide-Care/12910?bname=ilar.

International Atomic Energy Agency (IAEA) (1989) *Manual of ELISA*.

Ito R, Ozaki YA, Yoshikawa T, Hasegawa H, Sato Y *et al.* (2003) Roles of anti-hemagglutinin A and IgG antibodies in different sites of the respiratory tract of vaccinated mice in preventing lethal influenza pneumonia. *Vaccine*, **21**, 2362–2371.

Iwai H, Kodera A, Sekiya N, Nakumura A, Kirisawa R (1998) Effect of equid herpesvirus 1 infection on parturition of mice. *Journal of Equine Veterinary Science*, **9**, 25–27.

Jackson TA, Osburn BI, Kendrick JW (1977) Equine herpesvirus 1 infection in horses: studies on the experimentally induced neurologic disease. *American Journal of Veterinary Research*, **38**, 709–719.

Koppers-Lalic D, Reits EA, Ressing ME, Lipinska AD, Abele R *et al.* (2005) Varicelloviruses avoid T-cell recognition by UL49.5-mediated inactivation of the transporter associated with antigen processing. *Proceedings of the National Academy of Sciences USA*, **102**, 5144–5149.

Kukreja A, Walker C, Fitzmaurice T, Awan A, Love DN *et al.* (1998) Protective effects of equine herpesvirus 1 (EHV-1) glycoprotein B in a murine model of EHV-1 induced abortion. *Veterinary Microbiology*, **62**, 303–311.

Love DN, Bell CW, Pye D, Edwards S, Hayden M *et al.* (1993) Expression of equine herpesvirus 1 glycoprotein D by using a recombinant baculovirus. *Journal of Virology*, **67**, 6820–6823.

Minke JM, Audonnet JC, Fischer L (2004) Equine viral vaccines: the past, present and future. *Veterinary Research*, **35**, 424–443.

Munro KI, Wellington JE, Love DN, Whalley JM (1999) Characteristics of glycoprotein B of equine herpesvirus 1 expressed by a recombinant baculovirus. *Veterinary Microbiology*, **68**, 49–57.

Osterrieder N, Wagner R, Brandmüller C, Schmidt P, Wolf H *et al.* (1995) Protection against EHV-1 challenge infection in the murine model after vaccination with various formulations of recombinant glycoprotein gp14 (gB). *Virology*, **208**, 500–510.

Osterrieder N, Wagner R, Pfeffer M, Kaaden OR (1994) Expression of equine herpesvirus type 1 glycoprotein gp14 in *Escherichia coli* and in insect cells: a comparative study on protein processing and humoral immune responses. *Journal of General Virology*, **75**, 2041–2046.

Packiarajah P, Walker C, Gilkerson J, Whalley JM, Love DN (1998) Immune responses and protective efficacy of recombinant baculovirus-expressed glycoproteins of equine herpesvirus 1 (EHV-1) gB, gC and gD alone or in combinations in BALB/c mice. *Veterinary Microbiology*, **61**, 261–278.

Reed LJ, Muench H (1938) A simple method of estimating fifty per cent end points. *American Journal of Hygiene*, **27**, 493–497.

Ruitenberg KM, Walker C, Wellington JE, Love DN, Whalley JM (1999) ADN-mediated immunization with glycoprotein D of equine herpesvirus 1 (EHV-1) in a murine model of EHV-1 respiratory infection. *Vaccine*, **17**, 237–244.

Smith KC (1997) Herpesviral abortion in domestic animals. *Veterinary Journal*, **153**, 253–268.

Smith KC, Whitwell KE, Binns MM, Dolby CA, Hannant D *et al.* (1992) Abortion of virologically negative foetuses following experimental challenge of

pregnant pony mares with equid herpesvirus 1. *Equine Veterinary Journal*, **25**, 36–40.

Smith KC, Whitwell KE, Mumford JA, Hannant D, Blunden AS *et al*. (2000) Virulence of the V592 isolate of equid herpesvirus-1 in ponies. *Journal of Comparative Pathology*, **122**, 288–297.

Tewari D, Nair SV, De Ungria MC, Lawrence GL, Hayden M *et al*. (1995) Immunization with glycoprotein C of equine herpesvirus-1 is associated with accelerated virus clearance in a murine model. *Archives of Virology*, **140**, 789–797.

Tewari D, Whalley JM, Love DN, Field HJ (1994) Characterization of immune responses to baculovirus-expressed equine herpesvirus type 1 glycoproteins D and H in a murine model. *Journal of General Virology*, **75**, 1735–1741.

Van de Walle GR, Peters ST, VanderVen BC, O'Callaghan DJ, Osterrieder N (2008) Equine herpesvirus 1 entry via endocytosis is facilitated by alphaV integrins and an RSD motif in glycoprotein D. *Journal of Virology*, **82**, 11859–11868.

Walker C, Packiarajah P, Gilkerson JR, Love DN, Whalley JM (1998) Primary and challenge infection of mice with equine herpesvirus 1, strain HSV25A. *Virus Research*, **57**, 151–162.

Walker C, Perotti VM, Love DN, Whalley JM (1999) Infection with equine herpesvirus 1 (EHV-1) strain HVS25A in pregnant mice. *Journal of Comparative Pathology*, **120**, 15–27.

Walker C, Ruitenberg KM, Love DN, Millar Whalley J (2000) Immunization of BALB/c mice with DNA encoding equine herpesvirus 1 (EHV-1) glycoprotein D affords partial protection in a model of EHV-1-induced abortion. *Veterinary Microbiology*, **76**, 211–220.

Watanabe I, Hagiwara Y, Kadowaki S, Yoshikawa T, Komase K *et al*. (2002) Characterization of protective immune responses induced by nasal influenza vaccine containing mutan cholera toxin as a safe adjuvant (CT112K). *Vaccine*, **20**, 3443–3455.

Whittaker GR, Taylor LA, Elton DM, Giles LE, Bonass WA *et al*. (1992) Glycoprotein 60 of equine herpesvirus type 1 is a homologue of herpes simplex virus glycoprotein D and plays a major role in penetration of cells. *Journal of General Virology*, **73**, 801–809.

Zanuzzi C, Scrochi M, Fuentealba N, Nishida F, Portiansky E *et al*. (2014) Effects of equid herpesvirus 1 (EHV-1) AR8 and HH1 strains on BALB-c mice. *Archives of Virology*, **159**, 141–145.

Zhang Y, Smith PM, Tarbet EB, Osterrieder N, Jennings SR *et al*. (1998) Protective immunity against equine herpesvirus type-1 (EHV-1) infection in mice induced by recombinant EHV-1 gD. *Virus Research*, **56**, 11–24.

[*Received, May 6th, 2014*
Accepted, June 21st, 2014]