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Electrophysiological and Morphological Alterations in Peripheral Nerves by the Pig Paramyxovirus of Blue Eye Disease in Neonatal Pigs

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Summary

The pig paramyxovirus of blue eye disease (PPBED) produces central nervous system (CNS) damage leading to death in piglets. However, when PPBED was injected into the muscle and came into contact with hind limb peripheral nerves and was transported to the CNS, it did not cause death and could be a mechanism by which to induce protection. This study analyses whether PPBED causes electrophysiological and morphological alterations in infected hind limb peripheral nerves. It also studies, whether PPBED induces the onset of haemagglutination inhibitory antibodies (HIA) when it is transported to the spinal cord after medial gastrocnemius (MG) intramuscular injection. PPBED was detected by an immunohistochemical method and nerve morphology was studied using electron microscopy. The physiological status of the nerve was evaluated with electrophysiological techniques. The electrical threshold of the infected MG nerve increased four- or five-fold compared to that in the ipsilateral lateral gastrocnemius or in the MG nerve on the control side. The infected nerve fibres underwent myelin sheet disarrangement and their internal fibre diameter decreased. PPBED induced the onset of HIA.

Introduction

The pig paramyxovirus of blue eye disease (PPBED) is a novel member of the paramyxoviridae family (Moreno-López et al., 1986). It has two envelope glycoproteins: the 66-kDa haemagglutinin/neuraminidase protein and the 59-kDa fusion protein. As with many other paramyxoviruses, the first glycoprotein may recognize cellular receptors and the second may elicit the fusion of the viral envelope with the cell membrane (Horikami and Moyer, 1995). There is also a matrix protein of 40 kDa, associated with the inner face of the viral envelope involved in virus assembly and budding. Two other proteins, the 52-kDa phosphoprotein and the 200-kDa large protein participate in viral RNA transcription and replication. A nucleoprotein (68 kDa) covers the negative single strand of viral RNA, giving it a helicoidal symmetry (Sundqvist et al., 1990).
In neonatal pigs, PPBED infects the nervous system and causes death. In adult pigs, it does not produce neurological alterations but is a cause of reproductive failure (Stephano-Hornedo and Guy, 1985). During outbreaks occurring in neonatal pigs, morbidity and mortality percentages varied from 20 to 90 % and from 40 to 100 %, respectively. Economic loss is not only due to neonatal deaths. The virus also affects the farm reproductive records because infected boars undergo epididymitis, orchitis and testicular atrophy (Stephano-Hornedo and Guy, 1986). Furthermore, infected pregnant sows suffer embryonic and fetal death with subsequent return to oestrus. A birth decrease of approximately 20 % was recorded in affected farms. In 1989–90, the epidemiological data obtained from swine farms in 12 of 31 Mexican states showed that 21 % of the piglets had increased titres of PPBED antibodies (Carreón-Nápoles, 1994).

PPBED infects the central nervous system (CNS) using the antegrade and retrograde nerve transport systems (Ramírez-Herrera et al., 1997). It induces neuropathological disorders such as: cutaneous hyperexcitability, motor incoordination, hind limb paralysis, convulsions and death (Ramírez-Tabche and Stephano-Hornedo, 1982; Stephano et al., 1988). These signs suggest damage to both peripheral nerves and CNS. Histopathological lesions in the CNS consist of focal and diffuse gliosis, perivascular cuffing, glial and neuronal necrosis and meningitis (Kennedy et al., 1994; Moreno-López et al., 1986). The lesions in the peripheral nervous system have not been characterized. Therefore, it would be of interest to study: to what extent the peripheral nerve structure and function are damaged; whether nerve fibre damage occurs only in those fibres transporting the virus; and whether immune system stimulation occurs by exposure of suckling pigs to active PPBED using parenteral routes.

In this study we analysed the ultrastructural and the electrophysiological properties of peripheral nerves infected with PPBED. We also studied the seroconversion induced by PPBED after the experimental infection.

Materials and Methods

Virus

The virus was obtained from a natural outbreak in 1987, which occurred on a swine farm in the county of La Piedad, Michoacan, México. Infected brain samples were negative to pseudorabies virus and hog cholera virus. Brain homogenates had haemagglutinating activity (1/128–1/1024) upon chick, rabbit, mouse, rat, goat and human erythrocytes indicative of ongoing viral activity. The virus was purified by ultracentrifugation, thereafter isolated in PK-15 and in Vero cells. A cytopathic effect was characterized by syncytia formation, intracytoplasmic inclusions and haemadsorption activity. The isolated virus showed a similar protein migration pattern to the porcine paramyxovirus La Piedad, Michoacan (Sundqvist et al., 1990). The isolated PPBED was stored for experimental purposes. For the present study, PPBED was obtained from the brain of an experimentally infected pig by preparing a 10 % homogenate in 0.1 M phosphate-buffered saline solution (PBS). The homogenate was centrifuged at 1600 \text{ g}, the supernatant was filtered through nitrocellulose membranes (0.22 μm) and centrifuged at 40 000 \text{ g} for 2 h. The pellet was resuspended and adjusted to contain 256 haemagglutinating units (HU) per ml.

Virus inoculation

Thirteen pigs, 5–8 days old and weighing 1.5 kg, received a 1-ml dose of virus suspension injected into the left medial gastrocnemius (MG) muscle and 1 ml of PBS into the right MG muscle.

Electrophysiological study

This study was performed on nine suckling pigs aged 5–8 days, 11 days after PPBED MG intramuscular injection. Six pigs served to establish the optimal conditions for surgery, adequate
dosage of anaesthetic drugs and muscle relaxants in this animal species. Three pigs were used to obtain the electrophysiological data. All animals received atropine (0.08 mg/kg) and were anaesthetized with pentobarbital (32 mg/kg). Each animal was catheterized in the accessory cephalic vein for the administration of pentobarbital and/or vecuronium bromide (0.35 mg/kg). Body temperature was maintained by a thermal cushion. A tracheotomy was performed to keep the animal under artificial ventilation using a respiratory pump. MG and lateral gastrocnemius (LG) nerves were dissected in both the control and the virus-injected hind limbs and the spinal cord and dorsal roots were exposed by laminectomy. The animal was placed in a stereotaxic apparatus, the head was fastened with two screws placed inside the auditive meatus and two screws in the nasal cavities. Both hind legs were firmly attached to separate bars of the stereotaxic apparatus. Two pools were made using the skin of each hind limb and a third spinal cord pool was formed using the skin of the back. All pools were filled with mineral oil (at 37°C). The cut ends of the MG and LG of both legs were placed on bipolar silver electrodes to apply electrical stimuli or record electrical responses. An electrode was also placed on the L7 and S1 dorsal roots to record the afferent signal arrival as cord dorsal potential (CDP). To obtain the conduction velocity, we first established the value of the electrical stimulus threshold capable of producing the CDP afferent response. We calculated the conduction velocity knowing the time after stimulus to the recorded CDP and the distance between the stimulation and the recording sites.

The heteronymous monosynaptic reflex (HMR) elicited by electrical stimulation of the LG nerve was recorded in the MG nerve, or vice versa. The stimulus intensity varied from one to eight times the threshold. The responses observed in the spinal cord were classified as voltage waves appearing at different stimulus intensities. The CDPs were recorded at the L7 lumbar segment or the S1 sacral segment. Once the experiment was over the pig was killed with an overdose of sodium pentobarbital and disconnected from the respiratory pump.

Tissue processing and antibody staining

Eleven and 40 days after the intramuscular injection (four pigs at each time), the animals were anaesthetized with sodium pentobarbital (30 mg/kg intraperitoneally) and fixed with paraformaldehyde (6 %) and glutaraldehyde (3 %) using intracardial perfusion. Three segments of 5 mm from the left (infected) and right (control) MG, the sciatic nerve, the dorsal and ventral roots and transverse slices of lumbar spinal cord segments (L4–L7) were processed for paraffin embedding in paraplast plus. The presence of the virus was detected by an immunohistochemical method (Ramírez-Herrera et al. 1997). Briefly, paraffin-embedded tissue sections of 6 μm were adhered to microslides and treated with 1 % trypsin for 30 min, rinsed and incubated overnight in anti-PPBED mouse polyclonal antibody at 4°C (1/100). Later, sections were incubated overnight with 10 % normal rabbit serum in PBS. Thereafter, sections were rinsed and incubated at room temperature for 1 h in peroxidase-labelled anti-mouse immunoglobulin G (IgG) polyclonal rabbit IgG (1/1000). The reaction was revealed with diaminobenzidine tetrahydrochloride. Sections were counterstained with haematoxylin.

Electron microscopy study

The nerve lesions caused by the PPBED were detected by electron microscopy in nerve tissue samples from two pigs injected in the MG muscle; they were killed 11 days after injection. The tissue samples were fixed with 3 % glutaraldehyde for 30 min, rinsed with PBS and postfixed with 2 % osmium tetroxide in 0.1 m sodium cacodylate buffer for 15 min at 4°C. Samples were rinsed exhaustively with PBS at 4°C to remove excess osmium. Thereafter, tissue samples were dehydrated with ethanol, infiltrated with toluene and toluene and Epon 812 mixtures and embedded in Epon 812 (Dawes 1971). Thin sections (90 nm) were obtained with an ultramicrotome and placed on copper grids. They were counterstained with lead citrate and uranyl acetate. Sections were observed under a Zeiss electron microscope.

Anti-PPBED antibody production in pigs

As pigs survived for 40 days after PPBED intramuscular injection, we detected anti-PPBED antibodies which could help, in part, to explain their survival. Haemagglutination inhibition tests
(Carbrey et al. 1974) were performed to determine the anti-PPBED levels. Seven pigs, 5–7 days old, were injected with the virus into the left MG muscle and PBS was injected into the right MG muscle. Blood samples were taken at 0, 15, 30 (six pigs), 60 (five pigs) and 180 (three pigs) days after inoculation. On the 30th day the pigs received a challenge dose (256 HU/ml) of PPBED by the intranasal route. We used 8 HU of PPBED and a 0.5 % mouse red blood cell suspension. Serum titres were expressed as the highest dilution having complete haemagglutination inhibition.

**Results**

**Electrophysiological study**

Three pigs were properly maintained to complete the following recordings: conduction velocity to evaluate the functional integrity of afferent fibres and HMR to evaluate the monosynaptic transmission.

The electrical stimulus applied to the right LG at one time point produced a CDP appearing with a delay of 2.6 ms, giving a conduction velocity of 57.7 m/s to the stimulated afferent fibres (Fig. 1a, panel 1). When stimulus intensity was increased to twice the threshold value, the CDP became triphasic (Fig. 1a, panel 2) showing faster fibres of 60 m/s and slower fibres of 56.04 m/s, although all these fibres may correspond to group I afferent fibres. A second and a third CDP were clearly distinguished, with a four-time threshold stimulus intensity (Fig. 1a, panel 3). The fibres generating such CDPs had conduction velocities of 45.26 and 34.15 m/s, respectively.

The electrical stimulus needed to activate infected group I MG nerve fibres was 525 % more intense than the stimulus in control (i.e. non-infected) nerves. However, the conduction velocity of these fibres was 68.9 m/s. A second CDP of 46 m/s conduction velocity fibres also appeared with this stimulus intensity as seen in Fig. 1b (panel 1). The increase of stimulus intensity apparently recruited a low percentage of fibres of low conduction velocity. We assume this because the third CDP was not clearly observed (Fig. 1b, panels 2 and 3). The electrical stimulation of the LG of the infected side, which does not innervate the infected MG muscle, generates CDPs with a threshold level similar to that applied on the MG or LG of the control side (compare Fig. 1a with Fig. 1c). When the stimulus intensity was increased to twice the threshold value, the second CDP clearly appeared (Fig. 1c, panel 2), and with four times the threshold value, a third CDP also appeared (Fig. 1c, panel 3).

To establish whether low velocity conduction afferent fibres entering a different spinal cord segment were affected, we recorded CDPs in the S1 spinal cord segment. The CDPs recorded in the S1 segment produced by an electrical stimulus of the control MG nerve were similar to those obtained in the L7 segment. Figure 2a (panels 1, 2 and 3, bottom traces) shows the CDPs produced by electrical stimulation of MG at one, two and four times the threshold value, respectively. When the infected MG nerve was stimulated, the threshold value for most excitable fibres was 428 % higher, compared to the stimulus applied on the control side MG nerve. In the infected side, low conduction velocity fibres were not activated even at two and four times the threshold value, respectively (Fig. 2b, panels 2 and 3). In order to obtain the second CDP by stimulating the infected nerve, it was necessary to increase the stimulus to eight times the threshold value, resulting in a 1700 % higher intensity stimulus compared to the stimulus level needed for the second CDP on the control side. This is shown in Fig. 2b (panel 4), the trace displays the first and second CDP, the low amplitude of CDP II is remarkable (Fig. 2b, panels 1, 2, 3 and 4).

The conduction velocities and the threshold values were analysed by ANOVA test and compared to the infected and the control nerves. The mean value for conduction velocities for low threshold afferent fibres in the control nerves was 62.32 ± 4.41 m/s \((n = 3)\), versus 68.27 ± 2.82 m/s \((n = 3)\) in infected nerves with \(P < 0.120\) and no
The mean threshold value to activate the first cord dorsal potential in control nerves was 0.66 ± 0.152 V (n = 3) versus 3.33 ± 0.757 V (n = 3) in infected nerves, which has a significant difference (P < 0.004).

The HMR was not detected in any of the pigs analysed (Fig. 2a,b; upper traces in all panels). Only a polysynaptic reflex was observed in two assays evoked with an electrical stimulus more than twice the threshold value (not illustrated).

The experiments performed by the electrophysiological method showed a clear increase in the stimulus threshold value of afferent fibres in the infected nerve. However, the damage occurring in neurones and/or efferent fibres remains to be established.

Fig. 1. (a) Cord dorsal potential (CDP) recorded in the L7 spinal cord root entry from the control side; the electrical stimulus was applied to the right lateral gastrocnemius (RLG) nerve. Panels 1, 2 and 3 indicate one, two and four times the threshold value stimulus, respectively. (b) CDP recorded in the L7 spinal cord root entry from the infected side; the electrical stimulus was applied to the infected medial gastrocnemius nerve. (c) CDP recorded in the L7 spinal cord dorsal root entry and the electrical stimulus was applied to the ipsilateral (uninfected) LG nerve.
Attempting to clear this up, we analysed a motor function involving the activation of motor units. Thus, we recorded the activity of fictive locomotion in pigs when they came up from deep anaesthesia.

Fictive locomotion was recorded in the MG nerves of control and infected sides. Figure 3 shows the trace of a locomotion episode in three consecutive panels. The upper trace in each panel corresponds to the control side MG nerve record, and the bottom trace, to the infected side MG nerve record. In each panel, the traces corresponding to the control side exhibit a locomotion pattern with rhythm and periodic neurone discharges, with an average duration of 3233.3 ± 903 ms (n = 10 steps). In contrast, on the infected side, no motor neurone discharges were observed. In these pigs, to clarify whether PPBED was infecting motoneurones, PPBED was injected intramuscularly and revealed by immunostaining. Its detection in intramuscular nerve fibres was shown by the development of a brownish colour typical of peroxidase reaction with diaminobenzidine (Fig. 4a). The reaction was undetectable in sections of control MG muscle (not illustrated). The neurones of the spinal cord ventral horn were clearly labelled by the presence of the virus (Fig. 4b), but not all neurones developed the immunoreaction. This occurred in all sections analysed (n = 20). The four animals killed at day 40 post-inoculation exhibited results similar to those found in pigs analysed at day 11 post-inoculation.

**Electron microscopy study**

This study was performed to detect the ultrastructural alterations produced by PPBED in peripheral infected nerves.
PPBED produced an estimated 40% decrease in inner fibre diameter in the infected MG nerve (Fig. 5b, arrow double sense). Also a myelin sheet disaggregation occurred and was observed as the formation of concentric myelin loops (Fig. 5b, arrow). Meanwhile, in the MG nerve of the control side, the fibres showed a normal appearance, where nerve fibres are covered with a compacted myelin sheet establishing close contact (Fig. 5a). In the infected sciatic nerve there was an appearance similar to that described for the infected MG nerve fibres, although less severe (Fig. 5c). In infected ventral root sections, the nerve fibres were severely damaged, resembling the damage observed in the infected MG fibres, where some fibres had an inner diameter decrease of approximately 40% (double sense arrow), and the myelin sheet also underwent disaggregation (Fig. 5d, arrow).

**Seroconversion in pigs**

This study was performed to detect changes in anti-PPBED titres induced as a response to the inoculation with active PPBED. This may help to explain the pigs’ prolonged survival after exposure and challenge. All the pigs studied seroconverted (Fig. 6) remaining healthy during the study.

**Discussion**

The damage produced by PPBED in peripheral nerves was reflected in the intensity of the electrical stimuli required to reach the threshold level, and consequently, the CDP I response. In order to obtain the CDP II, the stimulus required was 17 times larger than under normal conditions. Thus, large and small diameter afferent fibre electrical
properties were damaged. In the remaining 'healthy' fibres, possibly large diameter afferent fibres, the conduction velocity was apparently not affected, as the afferent fibre arrival time was not modified by the presence of PPBED. This suggests that some afferent fibres remained in normal condition. In contrast, low conduction velocity afferent fibres (possibly small diameter fibres) were drastically damaged, because CDP II and CDP III were abolished. Fictive locomotion in the infected MG nerve was affected by the virus. This suggests that the system may be damaged at the motoneurone level, because efferent fibres were structurally disturbed.

As previously reported (Ramírez-Herrera et al. 1997), the tissue sections of infected sciatic nerve showed nerve fibres having internal and peripheral immunoreactivity, suggesting that such fibres had innervated the infected MG muscle and transported the PPBED in a retrograde manner. Most of the fibres displayed immunoreactivity in the periphery of the fibre, therefore such fibres may not innervate the infected MG muscle and may not transport the virus.

The arrival of PPBED at ventral horn neurones was detected as they developed an immunoreaction. The reaction was specific for neurones whose fibres transported the virus. This is assumed because not all neurones developed the reaction, although they were close together. These cells were located in L7 spinal cord segment.

The virus inoculated in the MG muscle has to travel a long distance, which increases as the pig grows. This may help the pig to overcome the infection because it delays the arrival of the virus at the CNS. This allows the generation of the immune response, which is partially shown by the increase of haemagglutination inhibition antibodies, as well as by the presence of viral proteins in popliteal lymphatic ganglion cells (Ramírez-Herrera et al. 1997). These observations support the idea that PPBED, once transported retrogradely by peripheral nerves, could be used to produce a humoral immune response.
The structural alterations caused by PPBED clearly observed by electron microscope, correlate to electrical alterations in afferent and efferent fibres. However, it is not clear whether nerve damage is caused by demyelination or by a viral mechanism inside the nerve fibres, or an inflammatory response. Infected nerves showed damage in the myelin sheet, as well as within infected nerve fibres.

Under natural conditions, the neonatal pigs infected by the PPBED might suffer virus invasion of the nervous system through the antegrade or retrograde nerve transport systems (Ramírez-Herrera et al. 1997). Independently of how the PPBED invades the CNS, when pigs are in late stages of the disease, severe damage of peripheral nerves governing respiratory motor function could occur. It may block the conduction of their

Fig. 5. Photographs obtained using electron microscope showing (a) control and (b) infected medial gastrocnemius nerve fibres. (c) Sciatic and (d) ventral root nerve fibres from the infected side. Bar calibration 1.3 μm.
respective motoneurones, finally leading to respiratory failure and eventual death of the pigs. Neural motor damage is shown in our findings, particularly in the generation of neuronal discharges structuring the fictive locomotion pattern.

Mild lesions have been previously been reported as caused by other viruses (Ugolini 1995). PPBED lesions could be due to the synthesis of protective antibodies, as RNA viruses do not affect cellular metabolism as DNA viruses do (Roizman and Sears 1990; Ugolini 1995). Lesions observed in these experiments were also similar to those produced by neurotoxins causing wallerian degeneration (Delgado-Lezama and Muñoz-Martinez 1990).

In experiments performed on neonatal pigs, we could not record HMR in infected nerves nor in the control hind limb nerves. This absence of response may be due to scarce heterosynaptic connections, or perhaps because electrophysiological conditions have been well established in cats but not in pigs (Dueñas et al. 1990). It would be very helpful if pigs were used in electrophysiological studies, because the use of cats is limited by their role as pets. Thus, the pig, as a farm animal, may have less restrictions in these types of studies.

In conclusion, PPBED can be transported retrogradely to the pig CNS. The infected peripheral nerves show a great increase in their threshold electrical stimulation. The infected peripheral nerves displayed ultrastructural alterations in fibres invaded by the virus. The pigs receiving the virus injection intramuscularly developed antibodies against PPBED, but it did not cause the pigs death.

References


