

## STUDIES ON THE PATHOGENESIS OF AUJESZKY'S DISEASE. II. THE DISTRIBUTION OF VIRUS AFTER SUBCUTANEOUS INFECTION

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*Summary.* — The distribution of pseudorabies virus (PRV) after subcutaneous (sc) infection was followed in piglets by infectivity assay and immunofluorescence. The results of fluorescent antibody (FA) studies were compared with histological changes in the same organs and tissues. From the subcutaneous connective and adipose tissue and the adjacent muscles near to the site of inoculation where primary multiplication of the virus takes place, PRV either penetrates into the nerve bundles or is transported to the regional lymph nodes. The virus reaches the central nervous system (CNS) along the peripheral nerves. Although the neural spread appears to be the most important route by which PRV gains access to the susceptible neurons of the CNS, the possibility of haematogenic dissemination to some visceral organs should be also taken into account.

### Introduction

The role of the tonsillary and nasopharyngeal epithelial layer, as the site of primary virus multiplication, in piglets infected per os with PRV was emphasized in our previous work (Sabó *et al.*, 1968). The results of FA studies compared with the infectivity assay and histological examination supported the view that the neural spread was the most important route by which PRV reaches the CNS. In view of several experiments, in which sc inoculation was used to induce Aujeszky's disease (Bergman and Becker, 1967; Corner, 1965; Olander *et al.*, 1966), we decided to verify the role of peripheral nerves in virus spread after sc infection. Similarly to our previous work the same tissues and organs were subjected to infectivity assay, and FA and histological examinations.

### Materials and Methods

The procedures used for virus titration, FA tracing and histological examinations were described previously (Sabó *et al.*, 1968).

*Experimental animals.* Ten 7 days old piglets, weighing about 3000 g, were infected sc into the thigh of the left dorsal extremity with  $2 \times 10^6$  plaque forming units (PFU) of the virulent ČVOS strain of PRV (isolated from a case of natural Aujeszky's disease in swine) which had undergone 11 passages in chick embryo cells. The seronegativity of the animals was checked by neutralisation tests against 100 PFU of PRV in chick embryo cells. At 1-day intervals, samples were taken from the following organs and tissues: subcutaneous connective tissue and muscles

in the area of virus inoculation, homo- and contralateral inguinal and pelvic lymph nodes, femoral nerve, lumbosacral plexus, lumbal and sacral spinal ganglions, the lumbosacral, thoracic and cervical cord segments, several other parts of the CNS (hemispheres, cerebellum, basal ganglions and brain stem), liver, spleen, lungs, tonsils, kidney, and mediastinal and submandibular lymph nodes. Morphological studies were not performed on 2 animals which died 5 and 6 days after inoculation (p.i.) respectively.

### Results

#### Clinical observations

Starting from the 3rd day p.i., paresis of the dorsal extremities, followed by ataxia and paralysis, were observed. Tonicoclonic seizures often reported in animals with Aujeszky's disease were not seen, even in animals which died subsequently. Paralysis lasted for 2-3 days, leading to sudden death. In other animals, no further clinical symptoms developed in addition to the outlasting paralysis and a transitory loss of appetite.

#### Virological findings

PRV was detected in high titres in all animals examined at the site of inoculation and in the regional lymph nodes (Table 1). Starting from 48 hours p.i., PRV was regularly found in the femoral nerve, in the lumbosacral and thoracic cord and in the corresponding spinal ganglions. From the 3rd

Table 1. The results of infectivity assay in organs from 7-day old piglets after subcutaneous infection with  $10^6$  PFU of PRV

Piglet No.:	36	39	41	43	42	45	40	38	44	37
Day p.i.:	1	2	3	4	5*	5	6*	6	7	8
Clinical signs:	-	-	+	+	+	+	+	+	+	+
Site of inoculation	7.5	7.5	7	7.5	7	8	7	7	5.5	3.5
Inguinal lymph node	4	3	6	5.5	6	6	6	4.5	3	2.5
Pelvic lymph node	6	7.5	7	6	5.5	5.5	6	5.5	3	3
Popliteal lymph node	-	-	5.5	3.5	-	3.5	-	4.5	-	-
Lumbal cord	-	3.5	3.5	5	3	4.5	4	4	5.5	5
Thoracic cord	-	2	3	3	2	2	2	1	2	2
Cervical cord	-	-	2.5	3	3	3.5	3	3	3	-
Brain stem	-	-	2	2.5	2	2	2	2	3	-
Femoral nerve	-	3	5.5	6	2	5.5	2	2	3.5	3.5
Spinal ganglion	-	-	5	2.5	3	2.5	5	3	3.5	2.5
Tonsils and nasopharynx	-	-	4	-	-	4	-	2	-	3
Cervical lymph node	-	-	-	-	-	4	-	-	-	-
Lungs	-	-	3.5	4.5	-	5	-	5.5	-	-
Mediastinal lymph nodes	-	-	1	4	-	4	-	3	-	-
Spleen	-	3	3	5	-	3	-	3.5	-	-

\* The animals died.

- = Negative findings.

The titres are expressed in  $\log_{10}$  TCID<sub>50</sub> per g of tissue.

day p.i., PRV was isolated from the cervical cord, brain stem and popliteal lymph nodes. The isolation attempts were occasionally positive in lungs, spleen, mediastinal lymph nodes, tonsils and in the nasopharyngeal mucosa. PRV isolation from the lungs was always paralleled by such from the mediastinal lymph nodes, but the simultaneous presence of PRV in tonsils and submandibular lymph nodes was demonstrated only once. The successful isolation attempts in tonsils on the 3rd, 5th, 6th and 8th day p.i. are interesting also from the epidemiological point of view.

In no case was PRV detected in blood serum, livers, kidneys, brain hemispheres or the cerebellum.

#### *Fluorescent antibody studies*

*Tissues adjacent to the site of inoculation.* Specific fluorescence was found starting from 24 hours p.i. in all samples of subcutaneous tissue examined, namely in the fibroblasts, in adipose tissue cells (Fig. 1) and in mononuclears of the inflammatory infiltrate. The mononuclears harbouring the viral antigen were often met around the vessels, mainly venules. The same localisation of the fluorescence was seen in the interstitial connective tissue of striated muscles adjacent to the injection site. A bright fluorescence was also found in the sarcolemma of the muscle fibres (ring-shaped fluorescence around the transversally cut fibres) and in the endothelium of some venules (Fig. 2). In one case the antigen was also found in the endoneural cells of a little peripheral nerve branch.

*Nervous system.* In the peripheral nerves innervating the inoculated area (femoral nerve), in the homolateral lumbal plexus and in the lumbal spinal roots, a distinct specific fluorescence was found in Schwann's cells and endoneural fibroblasts starting from 24 hours p.i. (Fig. 3). In the transversally cut nerve bundles the fluorescence appeared as rings, leaving the axons free.

A bright fluorescence of viral antigen in the lumbosacral cord segments was found on the 4th and 5th day p.i. It was present in the cytoplasm and protoplasmic branches of astrocytes in anterior and lateral columns near to the entrance of anterior roots into the spinal cord (Fig. 4). Some fluorescing astrocytes were also found in higher cord segments. In the gray matter of anterior horns, the viral antigen was situated in neurons and glial cells. A bright fluorescence was seen in the nuclei or the cytoplasm, or both, of giant motor neurons (Fig. 5). Its specificity was verified in parallel sections stained with heterologous conjugates.

No virus was detected by immunofluorescence in the brain stem, basal ganglia, hemispherical cortex and cerebellum. In lumbosacral spinal ganglia, some pseudounipolar nerve cells and their satellites showed a distinct specific fluorescence.

*Regional lymph nodes.* The FA studies did not yield positive results as regularly as the infectivity assays. Specific fluorescence was seen more often in the pelvic than in the inguinal nodes. It never occurred in the contralateral nodes. It was confined to the endothelial cells of the lymphatics (vasa afferentia), marginal sinuses and to the cytoplasm of free macrophages in

their lumen. In one case was fluorescence seen in the cytoplasm of lymphocytes and reticular cells in the white pulp (Fig. 6).

We did not succeed in detecting viral antigen in the lungs, spleen, tonsils, and mediastinal and submandibular lymph nodes in spite of that PRV was isolated from these organs.

#### *Histological findings*

*The site of inoculation.* At 24 hours p.i., only slight mononuclear and polynuclear exudation, cedema and vasodilatation developed in the subcutaneous tissue near to the place of injection. Starting from the 2nd day p.i., a distinct inflammatory reaction was detectable at all time intervals examined. The cellular infiltrate consisted mainly of lymphocytes, macrophages and polynuclear leukocytes; later also plasmocytes occurred. Occasionally, the nerve branches were surrounded by mononuclears.

Changes similar to those in the subcutaneous connective and adipose tissues were found in the interstitium of the adjacent striated muscles. Some muscle fibres developed necrosis, their cross striation disappeared and their homogenized sarcoplasm was disintegrated. In such necrotic foci, an exudation of fibrin, erythrocytes and polynuclear leukocytes completed the histological picture (Fig. 7). Inclusion bodies or homogenisation of the nucleoplasm were occasionally met in the nuclei of some sarcolemma cells.

*Nervous system.* The early changes in the femoral nerve bundle and in the lumbosacral spinal roots consisted of focal lymphocytary infiltrates. Beginning from the 5th day p.i. demyelination was seen in the anterior and posterior nerve roots and in ventral and dorsal columns of the lumbosacral cord segments. In the homolateral spinal ganglia, homogenisation of the cytoplasm, tigrolysis and eosinophilic necrosis of some pseudounipolar neurons was found together with focal round cell infiltration and satellite cell proliferation (Fig. 8).

In the meninges of the lumbosacral cord, lymphocytary infiltration developed starting from the 2nd day p.i. On the 3rd day, some glial nodules and perivascular cuffings occurred in the anterior horn. Degenerative changes in the cytoplasm (tigrolysis, homogenisation) or total necrosis in some motoric neurons developed on the 4th day p.i. The nuclei of the degenerated cells exhibited changes suggestive of the virus presence: vacuolisation and swelling, aggregation of the chromatin and its attachment to the nuclear membrane and the appearance of small homogeneous eosinophilic droplets, but no typical inclusion bodies. The nuclei of the necrotic cells were pycnotic or had undergone karyorhexis. Focal necrosis of the whole gray substance surrounded by numerous mononuclears was regularly found in the L5-S1 segments of the spinal cord (Fig. 9). A reparative glial reaction developed in these foci between the 7th—8th day p.i.

Only scattered glial nodules and slight perivascular cuffings were detected in the gray and white matter of the higher cord segments. Similar changes were seen starting from the 5th day in the brain stem on the bottom of the IVth ventricle and in the central reticular substance. Some of the neurons showed slight degenerative changes, but no necrosis. The meninges of the

**Table 2. Comparison of histological and immunofluorescent findings in piglets infected subcutaneously with  $10^6$  PFU of PRV**

Piglet No.:		36	39	41	43	45	38	44	37
Day p.i.:		1	2	3	4	5	6	7	8
Clinical signs:		-	-	±	+	+	+	+	+
Inoculation site (subcutaneous connective tissue, muscles)	H	+	+	+	+	+	+	+	+
	I	+	+	+	+	+	+	+	-
Regional homolateral lymph nodes (inguinal, pelvic)	H	+	+	+	N	+	+	-	-
	I	+	-	+	-	+	-	-	-
Femoral nerve and spinal ganglion	H	N	+	+	+	+	+	+	+
	I	N	+	+	+	+	+	+	-
Lumbosacral cord	H	N	-	+	+	+	+	+	+
	I	N	-	-	+	+	-	-	-
Cervical cord	H	N	-	-	-	+	+	+	-
	I	N	-	-	-	+	+	+	-
Brain stem	H	N	-	-	-	+	+	±	-
	I	N	-	-	-	-	-	-	-

H = histological findings: + positive; - negative; N = not done.

I = immunofluorescent findings: + positive; - negative; N = not done.

hemispheres were infiltrated by lymphocytes but the cortex was found free of any pathological changes.

*Regional lymph nodes.* The marginal sinuses and vasa afferentia in the homolateral lymph nodes showed dilatation and endothelial swelling starting from the 1st day p.i., Their lumen was filled up with macrophages and polynuclear leucocytes. Occasionally, necrotic foci were found in the white pulp. The periglandulare adipose tissue was infiltrated with lymphocytes. No typical intranuclear inclusion bodies were seen in the activated reticular and mononuclear cells, although similar changes to those described above developed in their nuclei. The findings in the contralateral lymph nodes were negative.

No histological changes were detected in the other organs examined. As seen in Table 2, the occurrence of histological lesions was in good correlation with the results of the FA assay. An exception was the spinal cord on the 6th, 7th and 8th day p.i., when the neurons yielded no specific fluorescence despite of the overwhelming inflammatory reaction.

#### Discussion

The distribution of PRV in piglets infected subcutaneously differed considerably from that in animals infected per os. While in the latter maximal

virus levels were detected in tonsils, nasopharyngeal mucosa and cerebral cortex, after sc infection they were found at the site of injection, in the regional lymph nodes and spinal cord. The infectivity assay showed clearly that PRV spreads to the CNS along the neural routes. This was also confirmed by direct detection of the virus in Schwann's cells of the femoral nerve bundles using the FA method.

The immunofluorescent findings allow us to make several important conclusions about the spread of PRV in piglets after sc infection. At the injection site PRV penetrates either into the sessile elements of connective tissue (fibrocytes, adipose tissue cells) or into the mobile cells of the inflammatory infiltrate (macrophages). In the adjacent muscles it is present in sarcolemma cells of muscle fibres, in endothelial cells of venules and in endoneural cells of the nerve branches. In the peripheral nerves PRV is probably transported along the lymphatic spaces. Moreover, it penetrates into Schwann's cells and endoneural fibroblasts of nerve bundles, but the axons remains free. The presence of viral antigen in Schwann's cells was first demonstrated by Johnson (1964) by the FA method in mice infected experimentally with herpes simplex virus. PRV spreads into the spinal cord via both the ventral and dorsal nerve roots and multiplies in glial cell of anterior, lateral and posterior columns. Finally it enters the glial cells and neurons in the grey matter.

The results of virus assay and of the FA studies in infected piglets are in agreement with clinical symptomatology and histological picture. The typical signs of pseudorabies as observed after peroral infection did not develop after sc infection. Paralysis of the pelvic extremities, so that the animals took the position of a sitting dog, dominated in the clinical symptomatology. The histological changes in the CNS were in agreement with the clinical signs and were similar to those described by others (Bergmann and Becker, 1967; Corner, 1965; Olander *et al.*, 1966). Neuronal necrosis was confined to the lumbosacral cord and the inflammatory reaction ceased in cranial direction so that the cerebral and cerebellar cortex was free of any lesions. Quite opposite was the localisation of the inflammatory lesions after peroral infection. The changes were mostly found in the cortex, but not in the spinal cord (Sabó *et al.*, 1968). A similar relationship between the site of injection and the inflammatory-necrotic reaction in the CNS was found by Dow and McFerran not only in pig (1962), but also in sheep (1964) and cattle (1966).

From the inoculation site PRV is regularly transported to the lymph nodes. The values of infectious virus titres being higher at the site of inoculation, a passive transport of virus might be considered. The immunofluorescent findings, however, indicate not only that PRV is transported to lymph nodes but also that it multiplies in endothelial cells of sinusses and in the white pulp. When the amounts of virus transported through the lymph nodes are extraordinarily large, a part seems likely to enter the blood stream owing to the limited filtration capacity of the nodes (Málková, 1968). This should be regarded as the first source of virus in blood.

An another source of virus, as our immunofluorescent studies indicate,

is the endothelium of some venules. Certain amounts of PRV get into the blood already during the virus injection by the capillaries damaged by puncture. There are different opinions about the significance of viraemia in Aujeszky's disease. Mašič *et al.* (1965), on the one hand, consider viraemia a frequent phenomenon; McFerran and Dow (1965), on the other, found it only twice among 500 blood samples. We found no virus in the serum of our experimental animals. We suppose, therefore, as long as the virus gets in to the blood, it is engulfed by the mononuclear cells. The role of blood macrophages in the pathogenesis of some viral diseases was pointed out for experimental animals by Mims (1955, 1964) and by Gresser and Lang (1966) for man. The irregular occurrence of PRV in lungs, mediastinal lymph nodes, tonsils, and spleen might be explained by its dissemination by the blood leucocytes. If this were true, the negative isolation attempts from liver are rather surprising. Our negative histological findings in all visceral organs are unlike those of Corner (1965) who observed a number of inclusion bodies and conspicuous inflammatory changes in these organs.

Owing to the negative immunofluorescent findings in tonsils we were unable to determine whether the virus multiplies in the squamous epithelium as is the case after peroral infection. The presence of PRV in the nasopharyngeal area and tonsils after sc infection would have been important for the spread from swine to swine of the attenuated strain used for vaccination. Jamrichová and Škoda (1969), however, did not find the attenuated virus in nasopharynx and tonsils after sc infection. The spread and distribution of the attenuated strain is probably rather different and less extensive than that of the virulent strain.

We should like to conclude that, even after sc infection, the neural route is the main pathway of the spread of PRV in piglets.

A part of virus, however, is transported to the regional lymph nodes; another one passes into the blood and probably is delivered by means of blood leucocytes to certain visceral organs (lungs, spleen). Our FA studies did not confirm the assumption that PRV would gain access to the cells of the CNS by the haematogenic route.

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#### *Explanation of Photomicrographs:*

*Figs 1—6:* Positive specific fluorescence of PRV antigen in tissues and organs of sc infected piglets.

- 1 — Specific fluorescence of the subcutaneous adipose tissue near to the needle puncture. Piglet No. 39.
- 2 — Specific fluorescence in striated muscles adjacent to the injection site. The viral antigen in sarcolemma cells of muscle fibres, in the endothelium of a venule (in the center) and in the interstitial connective tissue. Piglet No. 33.
- 3 — Bright fluorescence in Schwann's cells and endoneural fibroblasts of a femoral nerve bundle. Piglet No. 41.
- 4 — The transition of the anterior root to anterior column of the lumbosacral cord. Note the ring-shaped fluorescence in Schwann's cells of the transversally cut nerve root and the fluorescence in astroglia cells of the spinal cord white matter. Piglet No. 43.
- 5 — Bright fluorescence of motoric neurons in the anterior horn of spinal cord. Piglet No. 43.
- 6 — Specific fluorescence in lymphocytes of the white pulp in a regional lymph node. Piglet No. 41.

*Figs 7—9:* Histological findings in piglets infected subcutaneously with PRV.

- 7 — Necrotic muscle fibres in the vicinity of the injection site. Piglet No. 41; phosphotungstic acid haematoxylin.  $\times 260$ .
- 8 — Focal degeneration of neurons and round cell infiltration in the homolateral spinal ganglion. Piglet No. 43; haematoxylin-erythrosin.  $\times 110$ .
- 9 — Focal necrosis in the anterior horn of the lumbosacral cord surrounded by a mononuclear infiltrate. Degenerated and necrotic neurons. Piglet No. 43; haematoxylin-erythrosin.  $\times 110$ .