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STUDIES ON THE PATHOGENESIS OF AUJESZKY'S DISEASE. III. THE DISTRIBUTION OF VIRULENT VIRUS IN PIGLETS AFTER INTRANASAL INFECTION

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Summary. — In 7 days old piglets infected intranasally (*in*) with 5×10^3 plaque forming units (PFU) of a virulent strain of pseudorabies virus (PRV) the course of disease was followed and the distribution of virus was studied by infectivity assay and immunofluorescence. The tissues and organs subjected to fluorescent antibody (FA) tracing were examined also histologically. High titres of virus reaching 10^8 $TCID_{50}$ per gram of tissue, positive immunofluorescent localization of the antigen and inflammatory changes were observed in nasal and tonsillary mucosa as early as 24 and 48 hours post infection (p.i.) respectively. First clinical symptoms of a general character which rapidly converted to typical symptoms of Aujeszky's disease were seen 48 hours p.i. The detection of virus in the olfactory bulb by titration and immunofluorescent technique preceded the detection of virus in the brain stem by 24 hours. The development of the *in* experimental infection in piglets with PRV was similar to the peroral infection, except that the former was faster because the virus reached the central nervous system (CNS) sooner.

Introduction

The study of the peroral infection of 7 days old piglets revealed direct evidence on the sites of the primary multiplication of PRV, the mechanism of its spread into the CNS along the trigeminal nerve and the distribution of virus in individual stages of infection (Sabó *et al.*, 1968). There are also reports on the successful *in* infection of pigs (Akkermans, 1963; Corner, 1965; McFerran and Dow, 1965; Olander *et al.*, 1966). Some data (Kojnok, 1965) even showed that except of rather drastic intracerebral inoculation only the *in* route enables a successful infection of pigs.

The aim of the present paper was to supplement the so far known facts (Sabó *et al.*, 1968; Rajčáni *et al.*, 1969) by data on the clinical picture of the development of *in* infection of piglets and the spread of virus from the site of its entrance to the individual organs by use of various techniques: virus titration and FA and histological examination.

Materials and Methods

The virus, animals, method of virus titration in organs from infected animals and techniques of histological and immunofluorescent examination were the same as those used previously (Sabó *et al.*, 1968).

Histological examination. Both the sections stained by FA technique and restained with haematoxylin and erythrosin and the paraffin-embedded material prepared by standard histological technique were examined.

Infection of animals. Five 7 days old piglets (lacking specific serum antibodies against the infecting virus as revealed by a preliminary neutralization test) were *in* infected with 5×10^3 PFU of the virulent strain ČVOŠ, by instillating with a hypodermic syringe one half of the virus dose into each nostril. The intranasal instillation was carried out very carefully to avoid a possible injury of the nasal mucosa and without anaesthesia. Specimens were taken as follows: every 24 hours p.i. one animal was dissected (except the 3rd day p.i., when animals Nos 3 and 4 had to be dissected at the same time because of their clinical state) and the following organs were taken for virological, immunofluorescent and histological examination: nasal mucosa, tonsils, throat mucosa, bronchi, lungs, oesophagus, submandibular and parotis salivary glands, cervical, mediastinal, mesenteric and inguinal lymph nodes, liver, spleen, kidney, myocard, muscles from various parts of the body, stomach, small and large intestine, and urinary bladder. Besides that the following parts of the nervous system were examined: olfactory bulb, various parts of cerebral cortex and the adjacent parts of white matter, striatum, thalamus, cerebellum, brain stem, spinal cord and trigeminal nerve.

Results

Clinical observations

First clinical symptoms in *in* infected piglets were observed 48 hours p.i. They were of a rather general character, e.g. loss of appetite, torpidity,somnolence and mild increase of temperature. Several hours later the general condition of animals rapidly worsened and first symptoms characteristic of Aujeszky's disease appeared, viz. the uncoordinated twitching of muscle groups in different parts of the body developed further into expressed tonicoclonic seizures and muscle tremor. The animals remained voiceless, breathed with difficulties and during acute spasmic strokes seemed to suffocate. Before the appearance of spasmic strokes the animals vomited and pressed their heads onto the bottom and sides of the cage. In the developed stage of the clinical disease, the animals showed high fever; in the agony, however, their temperature dropped below normal.

Virological findings

From the 24th hour p.i. virus was isolated in a high titre (10^8 $TCID_{50}/g$) from nasal, tonsillary and throat mucosa. From these sites virus was isolated in a high titre also during the next days (Table 1) in all animals. The virus presence was demonstrated by isolation experiments also in the trachea ($10^{2.5}$ $TCID_{50}/g$) and lungs ($10^{2.0}$ $TCID_{50}/g$) at 24 hours p.i.

In the CNS, virus was first found in the olfactory bulb already 48 hours p.i. in a surprisingly high titre ($10^{4.5}$ $TCID_{50}/g$), so that it was possible to demonstrate it also by immunofluorescence. At the same time virus was detected in the trigeminal nerve in a titre identical with that found in the cervical lymph node ($10^{4.0}$ $TCID_{50}/g$). At this time interval virus was not found in other parts of the body.

Table 1. Comparison of virological, immunofluorescent and histological findings in organs and tissues of 7 days old piglets infected intranasally with a virulent strain of pseudorabies virus

Animal No.:		1	2	3	4	5
Days p.i.:		1	2	3	3	4
Clinical signs:		—	+	+	+	+
Nasal mucosa	V	8	7	8	7	6.5
	F	+	++	+	+	+
	H	+	+	+	++	+
Tonsils	V	8	6.5	6.5	6	6.5
	F	+	+	+	++	+
	H	+	+	+	+	+
Trigeminal nerve	V	0	4	5.5	4.5	5
	F	N	N	+	++	N
	H	N	N	N	N	+
Olphactory bulb	V	0	4.5	5.5	7	6
	F	N	+	+	++	++
	H	N	N	+	+	+
Cerebral cortex	V	0	0	0	5.5	5
	F	N	N	—	+	++
	H	N	N	—	+	+
Brain stem	V	0	0	3	4.5	4.5
	F	N	N	—	+	+
	H	N	N	—	+	+
Cervical lymph nodes	V	0	4	5	3.5	5
	F	—	—	—	—	—
	H	—	—	—	—	—
Inguinal lymph nodes	V	0	0	0	2.5	2.5
Cervical cord	V	0	0	0	3.5	3.5
Throat	V	8	7.5	7	6.5	6.5
Lungs	V	2	3	3.5	3.5	3
Trachea	V	2.5	3.5	2.5	4	2.5
Oesophagus	V	0	0	2	2.5	3
Kidneys	V	0	0	0	0	2.5

V = Virus titres in log $_{10}$ $TCID_{50}$ values per gram of tissue; 0 means < 10 $TCID_{50}$ per 0.1 g.
F = Immunofluorescent findings: + = distinct, ++ = bright, — = no fluorescence of viral antigen, N = not done.
H = Histological findings: + = inflammatory changes with inclusion bodies, ++ = widespread inflammatory changes with inclusion bodies, — = no histological changes.
N = not done.

Three days p.i. virus was isolated in animal No. 3 also from the brain stem ($10^{3.0}$ $TCID_{50}$/g) and the oesophagus ($10^{2.0}$ $TCID_{50}$/g). In animal No. 4, which was the only one dissected immediately after death (i.e. 80 hours p.i. and 8 hours after the dissection of animal No. 3) virus was present in various parts of the cerebral cortex ($10^{5.5}$ $TCID_{50}$/g) and in the cervical spinal cord. This animal displayed the highest virus titres so far observed by us in the brain tissue, namely in the olfactory bulb ($10^{7.0}$ $TCID_{50}$/g).

A rare finding of virus in the kidneys was obtained in animal No. 5, dissected 4 days p.i.

Those organs and tissues which were negative for virus in isolation experiments are not indicated in Table 1.

Virus isolation from blood samples taken close before the dissection of animals was always negative. In these experiments the whole blood was inoculated into tissue cultures.

Immunofluorescence findings

A positive fluorescence of viral antigen was observed in nasal and tonsillary mucosa as early as 24 hours p.i. In the nasal mucosa the antigen was located in cells of the ciliated epithelium; 48 hours p.i. positive fluorescence was detected also in cells of the inflammatory infiltrate of the connective tissue under the basal membrane (Fig. 1). In this phase of the infection the number of fluorescent cells was higher than that in later phases when a massive desquamation of the epithelium occurred. A focal fluorescence was observed in some cases in the throat epithelium and squamous epithelium of tonsils and throat archs. In tonsils, the most intensive specific fluorescence was located in the depth of the tonsillary crypts. The most developed fluorescence in tonsils was seen on the 4th day p.i., when the fluorescence in nasal mucosa was less pronounced due to massive desquamation of its epithelium. In this phase we observed sporadic positive fluorescent lymphocytes around the crypts and in the lymphatic follicles.

The bundle of the trigeminal nerve and the ganglion semilunare Gasseri showed positive fluorescence 3 and 4 days p.i. In the trigeminal nerve, the viral antigen was present in the cytoplasm of endoneural fibroblasts and Schwann's cells. In the ganglion mentioned the fluorescence was seen both in the cytoplasm and in the nuclei of some pseudounipolar neurons and in their satellites (Fig. 2).

At the site of the transition of the trigeminal nerve root into the brain stem we found the site where the structure of the peripheral nerve passed into the structure of the CNS. In this area positive fluorescence was observed in Schwann's cells and in astroglial cells (Figs 3 and 4).

The CNS displayed fluorescence of viral antigen first in animal No. 2, dissected 48 hours p.i., in neurons and glial cells of the olfactory bulb (Fig. 5). The other parts of the cerebral cortex were negative at this time. Animal No. 3 (72 hours p.i.) showed an extensive fluorescence in the olfactory bulb and some glial cells of the white matter were also positive. Animal No. 4 (dissected immediately after death, 80 hours p.i.) showed positive fluorescence also in the cortex of the frontal lobe of the hemispheres, striatum and thalamus (Fig. 6). An extensive fluorescence was also seen on the 4th day p.i. in most neurons of cerebral cortex as well as of subcortical gray matter (thalamus, nc. caudatus, putamen).

The brain stem displayed positive fluorescence in animals Nos 4 and 5 3 and 4 days p.i., respectively. The viral antigen was present in glial cells and neurons of the bottom of the IVth ventricle and of the reticular substance.

A negative finding was obtained in the cervical lymph node in spite of the proved virus titre of $10^{5.0}$ $TCID_{50}$/g in this tissue.

Histological findings

At 24—48 hours p.i. the nasal mucosa appeared hyperaemic and infiltrated by polynuclear leucocytes, lymphocytes and macrophages. The epithelium was partially sloughed. With regard to the immediate fixation of materials after their withdrawal, the desquamation may be considered as an intravital reaction. Three and 4 days p.i. we observed focal necroses of the epithelium of mucosa in the nose and throat. The connective tissue under the basal membrane was markedly infiltrated by polynuclear leucocytes and macrophages (Fig. 7). Nuclei of mononuclears vacuolized and the nuclear chromatin showed either a loss of affinity for stains or an aggregation on the surface of the nucleus. Some nuclei contained a homogeneous basophilic substance hardly distinguishable from swollen nucleoli. Typical eosinophilic intranuclear inclusions occurred only rarely. They were occasionally in cells lining the ducts of mucous glands.

Inflammatory alterations in the lumen of tonsillary crypts consisted of cell debris infiltrated by polynuclear and mononuclear leucocytes. Foci of vacuolar disintegration and even necrosis of squamous epithelium of crypts were also detected; intranuclear inclusions appeared only rarely. The infiltration of the mucous connective tissue by inflammatory cells was observed in the pharynx and around the tonsils.

The ganglion Gasseri showed 4 days p.i. focal mononuclear infiltrations containing predominantly lymphocytes (other ganglia were examined only by immunofluorescence). An increase of perinuclear satellites around some degenerated or necrotic neurons was also observed. Nuclei of these neurons showed karyorhexis, their cytoplasm was homogeneous, free of tigroid, weakly eosinophilic and vacuolized.

Histological alterations in the CNS were observed in animals Nos 4 and 5 4 and 5 days p.i., respectively. The meninges of the cerebral cortex were infiltrated predominantly by lymphocytes, mainly at the basal part of the frontal lobe of hemispheres. The cortex and white matter contained numerous perivascular round cell infiltrations. Neurons in frontal part of the cortex and in the olfactory bulb were necrotic, nuclei of neurons were vacuolized and looked empty, the cytoplasm was strongly eosinophilic, homogeneous and free of tigroid. The vicinity of damaged neurons displayed a strong oedema, numerous mononuclear cells and an activation of the microglia. Nuclei of some neurons contained typical intranuclear inclusions of type A surrounded by a halo. However, nuclei were more often filled up by homogeneous and weakly basophilic substances. Necrotic ganglion cells were not seen in thalamus, striatum and brain stem in such extent as in the described parts of the cortex. Neurons of the cerebellar cortex showed an intact appearance. The inflammatory alterations in the brain stem of piglets Nos 4 and 5 were followed in semiserial sections at a distance of 300 μ. The following centres of the gray matter were mainly inolved: nc. vestibularis, nc. tractus spinalis nervi trigemini, nc. tractus solitarii, nc. gigantocellularis, nc. gracilis and nc. cuneatus. Individual segments of the spinal cord showed no changes. A similarly negative histological finding was obtained with cervical lymph nodes, lungs, kidneys, liver and spleen.

Discussion

On comparing the distribution of PRV in the organism of 7 days old piglets after *in* and peroral infection (Sabó *et al.*, 1968) we do not find any substantial differences except that the virus multiplication at the site of its entrance into the organism after peroral infection (tonsils) is more pronounced than that after *in* infection, the latter case being characterized by a simultaneous virus multiplication in nasal and tonsillary mucosa. However, the appearance and the development of the clinical symptoms of the disease are much faster after the *in* infection (already on the 2nd day p.i.), whereas in the case of the peroral infection the first clinical symptoms are observed only on the 6th day p.i. Virus was detectable after *in* infection already 48 hours p.i., whereas in perorally infected animals only after 96 hours p.i.

We are convinced that under natural conditions pigs can be infected by two routes, namely (1) perorally, when virus first multiplies in tonsils and then penetrates into lymphatic tissue, mainly into fibres of the corresponding nerves (trigeminal, glosso-pharyngeal), along which it reaches the brain stem; and (2) by aerogenous infection, when (but partially also in receiving the food) the virus first multiplies in the nasal, eventually also simultaneously in the oropharyngeal mucosa and penetrates into the CNS along either the fila olphactoria or the trigeminal nerve. With regard to the local anatomical situation, the virus reaches the CNS via the fila olphactoria sooner than via another cranial nerve, as confirmed by our titration and immunofluorescent findings.

The present results confirmed the previous assumptions concerning the possibility of dissemination of some viruses via the neural pathways (Goodpasture, 1925; Hurst, 1933, 1934, 1936; Sabin, 1937). A similar route of spread of herpes simplex virus in mice was observed by Johnson (1964) by the FA method.

In analyzing the mechanism of spread of viruses along a nerve, Wright (1953) admitted three possible ways: via the axon, the neural lymphatics and the neural extracellular spaces. Wright considers the spread via interspaces as the most probable.

Our assumption on the neural spread of PRV from the nasal mucosa into the olfactory bulb via fila olphactoria is supported by electron microscopic findings of de Lorenzo (1960) showing that the olfactory receptors reach up to the surface of the olfactory mucosa where a close communication between neural endings and the external environment exists. The mechanism of spread of the infection along neural fibres in this particular case seems to be similar to that where other peripheral nerves are involved. Moreover, in our case the penetration of the virus into a neural fibre would have been facilitated by the possible penetration and spread of the virus via the nerve fibre without a necessary previous multiplication of the virus in the extraneural tissue. This view is also supported by the observation of Roberts (1962) who proved the spread of ectromelia virus from the nasal mucosa into the olfactory bulb via the neural route regardless of the fact that encephalitis did not appear.

We should like to mention our negative fluorescent and histological results concerning the presence of virus in lungs, especially with regard to the fact that many authors consider lungs as an important organ for the development of the infection with PRV and as a suitable tissue for the isolation of virus from field cases of Aujeszky's disease. The histological alterations in lungs were found neither after peroral (Sabó *et al.*, 1968) nor subcutaneous (Rajčáni *et al.*, 1969) infection with the mentioned strain of PRV, in spite of positive detection of the virus in lungs by titration. However, the examination of dissected piglets of the same age group from cases of field outbreakes of Aujeszky's disease regularly revealed an expressed pneumonia and a heavy oedema of lungs. These discrepancies may be apparently accounted for by a different affinity of individual virus strains to the lung tissue.

In considering all three routes of infection (peroral, intranasal and subcutaneous) studied in our laboratory, we can draw a conclusion important for the field practice, namely that for the isolation of PRV from pigs the following tissues are the most suitable for examination: tonsils, cervical lymph nodes and nasal mucosa; and the trigeminal nerve, brain stem and olfactory bulb of the neural tissues.

A mere isolation of PRV from an extraneural tissue is not sufficient for the diagnosis of the disease with regard to the observations of Kojnok (1965) and ourselves that virulent PRV can survive in tonsils of older (6 weeks) animals for a rather long period (18—20 days) without causing a clinical form of Aujeszky's disease (Sabó, 1969).

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Explanation of Photomicrographs:

Immunofluorescent and histological finding in piglets *in* infected with PRV.

Fig. 1. Specific fluorescence in some cells of the respiratory epithelium and in the subepithelial connective tissue, 3 days p.i. ×100.

Fig. 2. Fluorescence of viral antigen in pseudounipolar neurons in the ganglion semilunare nervi trigemini and in some satellite ganglion cells, 3 days p.i. ×100.

Fig. 3. The transition of the peripheral nerve structure into the structure of CNS in the root of trigeminal nerve. The cryostat section treated by specific conjugate was restained by haematoxylin and erythrosin; about ×60. The area marked by arrows corresponds to Fig. 4.

Fig. 4. Specific fluorescence in Schwann's cells of the peripheral nerve and in astrocytes (upper part), 3 days p.i. ×120.

Fig. 5. Viral antigen in neurons of the olfactory bulb, 3 days p.i. ×100.

Fig. 6. Specific fluorescence in neurons and glial cells of the frontal lobe of the hemispheres, 4 days p.i. ×100.

Fig. 7. Histological changes in the nasal mucosa 3 days p.i. Desquamated epithelium, an intensive infiltration of the mucous connective tissue with mononuclear cells and polynuclear leucocytes. Haematoxylin and erythrosin, ×120.