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ACCUMULATION DYNAMICS OF THE AGENT OF AMYOTROPHIC LEUKOSPONGIOSIS AND THE DEVELOPMENT OF DEGENERATIVE CHANGES IN THE CENTRAL NERVOUS SYSTEM OF GUINEA PIGS

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Summary. — After retrobulbar inoculation into guinea pigs, the agent of amyotrophic leukospongiosis was detected in the central nervous system (CNS) as early as 7 days post-infection (p.i.), whereas specific morphological changes appeared since days 14 to 21 p.i. Virological and morphological findings suggested that motoneurons of the spinal cord were first damaged in the course of the pathologic process which then proceeded upwards.

Key words: slow CNS infection; pathogenesis of amyotrophic leukospongiosis; motoneurone; spongiosis

Introduction

Based on clinico-morphological, epidemiological and virological studies carried out in 1971—1975 we described a specific progressive amyotrophy named "amyotrophic" leukospongiosis" (AL) (Votyakov *et al.*, 1971; 1975*a,b;* Votyakov *et al.*, 1985). Two squirrel monkeys inoculated with the brain suspension of a patient who died of AL have attracted the disease within 16 and 23 months, respectively. Comparison of the course of the disease and morphological changes of the CNS in humans and experimentally infected squirrel monkeys, as well as the isolation of the infectious agent that appeared significantly different from conventional viruses, suggested that AL was a slow virus infection (Votyakov *et al.*, 1983; 1984; 1985*a*, *b*; Poleshchuk *et al.*, 1985). AL was further transferred to other species of laboratory animals. Guinea pigs appeared to be the most sensitive (Kolomiets *et al.*, 1986); their use enabled a detailed investigation of the pathogenesis of experimental AL. The present paper is devoted to some peculiarities and time course of development of degenerative changes in the CNS of guinea pigs with experimental AL.

Materials and Methods

Clinico-morphological observations. Patient D., 44 years old, a veterinarian, in autumn of 1979 felt an awkward sensation in his hands. In January 1980 he felt weakness in his arms: fibrillation of body muscles and atrophy of hands developed. He also felt a slight weakness in

his legs. His condition aggravated. During 1981 the atrophy of body muscles became well marked. In March 1982 a deep atrophic tetraparesis was registered. The functions of cranial nerves were preserved. After a short remission, the patient fell asleep in a semi-sedentary posture. In November 1982 respiratory disorders aggravated so that the patient died.

At autopsy a marked atrophy of anterior horns was observed in the spinal cord. Death of motoneurons, mostly cervical and lumbar ones was seen, only single neurons were preserved. In the lateral horns the number of neurons was reduced to 5—7. A moderate proliferation of astroglia was found in the anterior horns. The axons of anterior and lateral collumns were vacuolized, some of them were wrinkled. Myelin membranes enveloping the axons were well stained. In posterior columns single spongiform cavities were detected. The number of neurons in the nuclear groups of medulla oblongata, pons and piriform cerebellar neurons was unchanged. In cortical area IV the death of pyramidal neurons of layers VI and V was registered, the remaining neurons had apically convoluted projections. Spongiform cavities were formed in the neuropil of layer III. Subcortical white matter showed spongiform changes and a moderate oligodendroglial proliferation. The case was diagnosed as amyotrophic leukospongiosis (Votyakov *et al.*, 1987).

Experimental animals. Sixty guinea pigs weighing 250—300 g were used. The animals of the infected group (42 animals) were inoculated retrobulbarly (behind both eyes) with 0.25 ml of 10% brain suspension taken from the anterior central gyrus of the left hemisphere. Control animals were given a suspension prepared in an analogous fashion from a brain of an individual who died in a car accident.

Electron microscopy and histological examination. At 7 day intervals p.i. intravascular perfusion with a mixture consisting of 1% paraformaldehyde and 2% glutaraldehyde in 0.15% mol/l phosphate buffer (pH 7.3) was performed in 6 guinea pigs for 1—2 min. This was followed by perfusion with 4% glutaraldehyde in the same buffer for 15 min. The brain was then removed and pieces of about 1 mm^3 were isolated from different CNS areas. They were postfixed for 1 hr in 4% glutaraldehyde and then for 1 hr in 4% OsO_4. The preparations were dehydrated and embedded into Araldit. Ultrathin sections were examined in electron microscope EM-100-CX-II. Histological preparations were processed as described (Votyakov *et al.*, 1971), the following staining techniques were used: haematoxylin-eosin, Nissl stain and Cluver-Barrer stain modified according to Viktorov (Votyakov *et al.*, 1983; 1985*b*; Poleshchuk *et al.*, 1985).

For virological assays at 7 days intervals p.i. the whole blood was collected from the heart of 3 experimental guinea pigs under ether anesthesia; hemispheres, cerebellum, medulla oblongata, as well as cervical, thoracic and lumbar portions of the spinal cord were removed using separate instruments. A 10% suspension was prepared as described from each brain area and another 3 guinea pigs were inoculated retrobulbarly behind both eyes in 0.25 ml vol. The animals were clinically examined and after the appearance of the signs, in 2 animals out of 3 histological examination was carried out in order to confirm the diagnosis. The titre of AL agent was determined according to Kimberlin *et al.* (1983).

Results

No clinical signs of the disease were observed within 20 to 23 days after retrobulbar inoculation. Guinea pigs gained weight, were active and mobile. On days 28—30 the hair of the infected animals lost its natural luster, and a certain loss of weight, as compared to control animals, was registered. On days 35—38 the guinea pigs lost considerable weight (up to 50 g), their hair was unkempt and falling, the animals became immobile and weakly responded to external stimulation. On days 40—42 the survivors developed characteristic signs of experimental AL: they lost their hair on most part of the body, they showed a marked atrophy of body muscles and posterior limbs, and their breathing grew hurried and shallow due to involvement of intercostal muscles. About 40% of the animals had pareses and paralyses of the limbs; they were sacrificed by bleeding in the agonal state. The rest

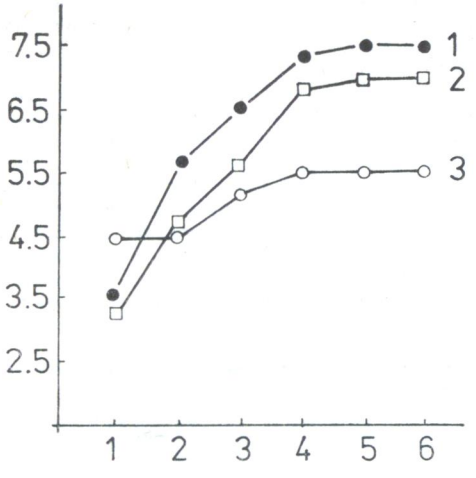

Fig. 1
Concentration of AL agent in different zones of central and peripheral nervous systems 1 — thoracic portion of the spinal cord, 2 — hemispheres, 3 — optic nerve Abscissa: weeks post-infection; ordinate: log LD_{50}'

of the experimental animals died and were subjected to virological and histological examinations.

Guinea pigs of the mock-infected group remained normal throughout the observation period. After death of the last animal of the infected group, they were subjected to virological and morphological examinations.

As soon as 1 week p.i., a transmissive infectious agent was detected in all areas of CNS. Its concentration was measured in the tissues during the next 2 weeks when it was found to reproduce intensively. Thus, for example, the titre in the cerebellum, as well as in the cervical and thoracic portions of the spinal cord reached 6.0 log LD_{50} and in large hemispheres and in the lumbar portion of the spinal cord 5.0 and 5.5 log LD_{50}, respectively.

After the appearance of marked clinical signs and until the death of infected animals (4 to 6 weeks post-infection), the AL agent reached a plateau titre and showed essentially no changes, its variations in different CNS areas being within 0.5 log LD_{50} (Fig. 1). Titration of the AL agent has shown that on week 1 p.i. the titre was by 1.5—2.0 log LD_{50} higher in the optic nerve than in the CNS. However, a further increase was recorded within 2 weeks only. During this period the titre of the agent in the optic nerve reached a "plateau" and remained essentially unchanged thereafter. It shoul be noted that its concentration in the optic nerve was still by 2.0 to 2.5 log LD_{50} lower than in the CNS.

Histological examination on day 7 p.i. failed to detect any pathologic changes in the CNS of infected animals as compared to the control ones. Ultrastructural examination showed that some astrocytes of the anterior spinal horns (thoracic portion) and of the hemispheres (frontal part) were swollen and enlarged with translucent cytoplasm. In some neurons, often in those located near altered astrocytes, the lamellar part of Golgi system was elongated, the number of layers was increased and vesicular component

was hypertrophic. The cisterns of granular endoplasmic reticulum were slightly dilated. By the end of week 2 (day 14) the first degenerative changes were detected. In the cytoplasm of single spinal neurons focal chromatolysis and vacuolization were registered. More often were affected the neurons in the anterior and lateral horns of the cervical and thoracic portions of the spinal cord. The neurons of layers III and V of the motor cerebral cortex, hippocampal neurons and Purkinje cerebellar cells were occasionally hyperchromatic. Electron microscopy revealed a noteworthy reaction of astrocytes with mitochondrial destruction, as well as the appearance of osmiophilic inclusion bodies, dilatation of projections and formation of vacuoles. The cytoplasm of the spinal neurons showed a dramatic hypertrophy of the cisterns of granular endoplasmic reticulum and appearance of vacuoles (50 to 500 nm diameter) which had an oval or irregular shape. Some spinal and cerebral neurons looked darker owing to an increased quantity of ribosomes and mitochondria and increased osmiophilicity of cytomembranes. In the Purkinje cells lamellar bodies were found.

Presynaptic terminals in the neuropil of anterior spinal horns and in the cerebral cortex were swollen; they contained fewer synaptic vesicles which formed small groups. In single projections small granular bodies appeared which failed to change the overall light background. Mitochondria had an irregular shape, they looked swollen with translucent, rarely with more dense matrix.

The most marked changes in the anterior and lateral horns of the spinal cord as detected by light microscopy were found 3 weeks p.i. The nuclei of neurons were hypertrophic with focal lysis of chromatin and formation of limited electron translucent areas. In the cytoplasm lysis of Nissl substance was noticed: the larger and smaller vacuoles were occasionally confluent and formed "package" -type structures. Signs of satellitosis were also registered. In addition to well preserved neurons, other were hyperchromatic and wrinkled. Low marked spongiform changes were found in the neuropil. In the cerebral cortex (layers III and V), hippocampus and cerebellum (Purkinje cells), besides hyperchromatic cells, shrinkage of single neurons with focal chromatolysis and small cytoplasmic vacuoles also occurred. A moderate proliferation and hypertrophy of macroglia was observed in all regions of CNS. No signs of demyelinization were detected.

On week 4 the degree of pathologic changes in the CNS increased. Signs of severe dystrophy were registered essentially in all neurons of the anterior and lateral horns of spinal cord. Posterior horn neurons were also damaged but dystrophic changes in them were less expressed showing formation of small vacuoles in the cytoplasm and focal chromatolysis. Spongiform changes were observed not only in the grey matter but also in the anterior and lateral columns and the brain stem.

Cytoplasmic vacuoles were found in some neurons of layers III and V of the cerebral cortex predominantly in precentral gyrus; in some neurons nucleolysis with ghost cell formation was also seen. Hippocampal neurons, as well as cerebellar Purkinje cells (mostly deep inside the gyri) were characte-

rized by analogous changes. The nuclei of small neurons in the granular layer of the cerebellum showed signs of nucleolysis. The neuropil and white matter were occasionally spongiform in limited areas of the hemispheres. In addition, slight macroglial proliferation was detected in the white matter.

Ultrastructural investigations 3 to 4 weeks p.i. demonstrated dilatation of the cisterns of granular endoplasmic reticulum in the cytoplasm of motoneurons resulting in the formation of giant cavities with breaks of membrane structures, destruction of some organelles, vacuolization, formation of osmiophilic inclusions, disappearance of microtubules and focal translucent hyaloplasm (Fig. 2-I).

Similar changes, though less marked, were registered in some neurons of layers III-V of the cerebral cortex and cerebellar Purkinje cells. Some vacuoles in the neuronal cytoplasm contained membrane-like structures which resembled accumulations of "soap-bubbles" or "daughter vacuoles" (Fig. 3-I).

Vacuolization of the nuclei of some neurons and astrocytes was noteworthy. Intranuclear vacuoles were more often detected in motoneurons and astrocytes of spinal anterior horns, in Purkinje cells and neurons of the cerebellum granular layers and less often in pyramidal neurons and astrocytes of cerebral cortex. Intranuclear vacuoles (1 to 3 per section plane) had an oval or irregular shape and were not filled with any structures (Fig. 3-III). The enveloping membrane was occasionally destroyed and the nucleoplasm around vacuoles was translucent; the chromatin was dispersed or concentrated in clumps and the nucleoli were mostly preserved. In the projections of the neuropil in spinal cord and cerebral cortex vacuoles were seen; alike the cytoplasm of neurons, some of them contained daughter membrane formations (Figs. 2-II; 3-I and IV.)

Examination of the white matter showed structural changes resulting in spongiform state of the anterior and lateral columns of the spinal cord (Fig. 4-II). In myelinated fibres the neurofilaments, microtubules and mitochondria were destroyed. The axons were wrinkled and periaxonal cavities between axolemma and myelinic membrane were formed (Fig. 2-III). This finally lead to complete death of the axon, the myelin membrane being relatively undamaged. Within some of these cavities budding myelin lamellae were found forming protrusions and "myelin balls". The latter were located in periaxonal cavities or completely occupied the gap in the place of dead axon (Fig. 2—IV). Rarely the death of the axon was followed by separation of fibers in the myelin membrane. Vacuolization of oligodendrocyte cytoplasm occasionally occurred and cavities were formed owing to exfoliation of external lamellae of the myelin layer. No primary demyelinization with appearance of bare axons was observed in any of the sections examined. Endothelial cells and vascular pericytes were without ultrastructural changes. No perivascular infiltration with blood-borne cells was observed. Around vessels the astrocyte projections were empty and looked like "void vacuoles" (Fig. 3-II).

At the end of incubation period by week 5—6 p.i. the animals with signs of AL revealed severe degenerative changes of the spinal cord neurons

(Fig. 4-I); in the anterior horns, mostly in the cervical and thoracic portions, many neurons were lacking. In cortical layers III and V slight satellitosis and neuronophagia were seen with signs of focal disappearance of neural cells (Fig. 4-III), and also starshaped accumulations formed by 5 to 7 astrocytes. Focal absence of Purkinje cells and thinning of the granular layer were found in the cerebellum. Some of the preserved piriform neurons were hyperchromic with marked proliferation of Bergman glia. Focal spongiform changes and proliferation of macroglial cells were registered in the white matter (Fig. 4-IV).

Ultrastructural examination of CNS at the terminal stage of disease showed increasing dystrophic changes associated with destruction of plasma membranes of some neurons. As a result, their cytoplasm occupied the intracellular space. The nuclei of neurons were diminished, chromatin was condensed to form large clumps. Because of the destruction of nuclear membranes the boundary between the nucleus and the cytoplasm often disappeared. In the area of dead neurons a large number of astrocyte projections filled with glial filaments was observed. In the pyramidal neurons preserved in the cerebral cortex lipid-containing inclusions accumulated which occasionally occupied nearly all the cell cytoplasm.

Morphological findings indicate that by week 6 of the disease severe pathologic changes developed in the CNS of the animals of experimental group. Thoracic portion of the spinal cord was most severely damaged. Primary signs of degenerative changes in the CNS were registered one week before the first clinical signs of the disease. The above-described changes were not registered in control animals throughout the observation period.

Discussion

The results obtained indicate that upon retrobulbar inoculation of guinea pigs the incubation period was significantly shortened and the pathologic process developed more rapidly than after other administration routes. Shortening of the incubation period seems to be related to the administration of a high dose of the infectious material into close vicinity to CNS and also to the spread of the agent along the optic nerve. Moreover, good blood supply of the eye also promoted rapid dissemination of the pathogen in the body (Votyakov *et al.*, 1987*a*).

The analysis of light and electron microscopic data has shown that signs characteristic of AL can only be detected in the nerve tissue. The basic signs are the death of the spinal motoneurons, proliferation of macroglial cells, spongiform changes of the white matter and the absence of marked demyelinization. First signs of neuronal damage were detected in the spinal cord and by the end of the incubation period they were also found in the brain. Most neurons died owing to vacuolar or hydropic degeneration. At the same time, some neurons were shrunken and hyperchromic. In the course of the infectious process not only destruction but also regeneration appeared as expressed by hyperplasia and hypertrophy of astrocytes and increased number of their projections.

Formation of vacuolar-membranous structures observed in the cell vacuoles and their projections seem to be a specific pathomorphological sign of AL. The presence in the CNS of vacuoles and cavities containing membrane fragments, and smaller vesicles is one of characteristic features of Creutzfeldt-Jacob disease (CJD) and scrapie (Erman *et al.*, 1984; Roikhel *et al.*, 1983). It should be also noted that neuronal vacuolization occurs after accumulation of the AL agent at high concentrations in the CNS which has been earlier reported by other investigators who studied the pathogenesis of scrapie (Kimberlin *et al.*, 1983; Lampert *et al.*, 1971). In addition, degenerative changes in the axon, with myelin membrane relatively unaffected, can be considered as another specific pathomorphological sign of AL. These changes can be identified as periaxonal-cavito-vacuolar spongiosis with low marked myelinosis. Primary development of spongiform change of the white matter can to some extent distinguish AL from other subacute transmissive spongiform encephalopathies. They are characterized by the development of status spongiosus in the neuropil, although Mizutani *et al.* (1981) described a form of CJD associated with primary damage of the white matter. At the same time, the appearance of lamellar bodies in the altered Purkinje cells seems to result from a general neuron pathology because such changes were observed in other diseases of CNS (Erman *et al.*, 1984).

The specific histological changes in experimental AL indicated primary damage of motoneurons and of the white matter in the spinal cord. This process had an ascending course in good accord with the histological findings obtained in the CNS of humans who died of AL (Votyakov *et al.*, 1975; 1987*b*).

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Legend to Figures (Plates LXXI–LXXIII):

Fig. 2. Ultrastructural changes in the spinal cord (Th_{1-3}) of guinea pigs with experimental AL.
I – Osmiophilic inclusion and destruction of Golgi system membrane in the cytoplasm of motoneuron. The projections adjacent to the motoneurons look empty, magn. 53 000×
II – Neuropil of spinal anterior horn. Membrane structures of different configuration and electron density are located in the centre of the projections (arrows). Mitochondrion (m) is preserved. magn. 40 000×
III – Anterior column of the spinal cord. Shrinkage of axons (a) and formation of periaxonal cavities. magn. 20 000×
IV – Anterior column of spinal cord. Necrosis of the axon and formation of a cavity (ca) into which distorted myelin sheaths (ms) are protruding, magn. 40 000×

Fig. 3. Ultrastructural changes in the CNS of guinea pig with experimental AL.
I – Formation of vacuoles containing daughter membrane structures in the cytoplasm of pyramidal neuron (Vn) and in the adjacent projections of the neuropil (Vo); magn. 53 000×
II – Cerebral cortex. Vacuole (v) formation in the perivascular space results from swollen and empty astrocytic projections. BM – basement membrane. In the centre of one of degenerating projections a mitochondrion (m) with dense mitochondrial matrix can be seen; magn. 40 000×
III – Nucleus (n) of a spinal anterior horn motoneuron; in its transclucent nucleoplasm vacuoles (v) are located; magn. 40 000×
IV – Myelinated area of the spinal anterior horn neuropil. A "void" vacuole (v) in the axoplasm (a). Neurofilaments, microtubules and mitochondria are preserved. Magn. 40 000×

Fig. 4. Morphological changes in the CNS of a guinea pig with experimental AL.
I – Thoracic portion of the spinal cord: degeneration of motoneurons in the anterior horn; magn. 120×
II – Thoracic portion of the spinal cord: spongiosis of the white matter in the anterior columns; magn. 60×

III — Fragment of the cerebral cortex taken from the anterior gyrus: hyperchromia and disappearance of pyramidal neurons; magn. 30×
IV — cerebellum: focal disappearance of Purkinje cells, thinning of the granular layer, proliferation of Bergman glia; magn. 30× . Stained with cresylviolet according to Nissl (I, II, III) and according to Olson (IV).

Book Review

Current Topics in Vector Research, Volume 3

K. F. Harris (Ed.): Current Topics in Vector Research, vol. 3, 25 Figs., pp. Springer Verl., Nwe York—Berlin—Heidelberg—London—Paris—Tokyo, 1987, price DM 136.—

In the Volume 3 of the series contributions are collected concerning arboviruses and insect-borne plant viruses and their vectors. In Chapters 1 B. H. Kay and H. A. Standfast give the well selected but comprehensive informations on ecology of arboviruses and their vectors in Australia. The next chapter written by D. J. Gubler concerns the current research on dengue, which is the most important arbovirus disease of humans, in terms of both morbidity and mortality. Epidemiology, with special attention to risk factors associated with dengue haemorrhagic fever, clinical studies, laboratory diagnosis, prevention and control are discussed in the light of the most recent findings.

The authors of Chapter 3 K. Kiritani, F. Nakasuji, and S. Miyai review mathematical theories in epidemiology of plant diseases, with special reference to insect-borne virus diseases. Recent contributions of system approaches in rice dwarf virus epidemiology are also discussed. The purpose of the Chapter 4 of Y. Robert is to give information on the present situation of aphid vector monitoring in Europe, concentrating on aphids as virus vectors.

Chapter 5 is called "Nepoviruses of the Americas". The intention of the authors R. Stace-Smith and D. C. Ramssdell is to fill a niche in the literature and to stimulate an awakening interest in this important group of plant viruses. Also the next chapter is concerning nepoviruses. D. C. Ramsdell explains to the reader viral replication, translation, and assembly of nepoviruses. In the Chapter 7 "Soil-Borne Viruses of Plants" authors C. Hiruki and D. S. Teakle are concerned mainly with those properties of the viruses that are relevant to their association with the soil. Such properties as particle stability, the possession of a divided genome, and the ability to be aequired, retained, and transmitted by a vector are discussed.

The last chapter of Y. C. Paliwal is on immunoelectron microscopy of plant viruses and mycoplasma, describing this group of techniques and their applications for rapid diagnosis of virus/mycoplasma diseases, detection of viruses in seeds, determination of serological relationships, localization of antigens on virus particles, etc.

Today, as never before, researchers from different areas of human, animal, and plant health cooperate for a common goal: the control and eventual elimination of vector-transmitted diseases causing so much human suffer. The hope of the editors of Current Topics in Vector Research is that the book series will both reflect and stimulate this newly emerging cooperation.

M. Labuda, Bratislava