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ULTRASTRUCTURE OF CRANIAL NERVES OF RATS INOCULATED WITH RABIES VIRUS

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ABSTRACT - The V and VII cranial nerves of rats inoculated with rabies virus were studied by electron microscopy. The results were compared with the same cranial nerves of rats inoculated with rabies virus but vaccinated against the disease. The findings are those of axonal degeneration and intense demyelination of the nerves of the group of rats not vaccinated. The vaccinated rats showed some ultrastructural irrelevant alterations when compared with the other group. The degree of ultrastructural alterations found in the group of rats not vaccinated suggests that in rabies severe damage of the cranial nerves occurs and that this may be closely related to the clinical picture of the disease (hydrophobia). Furthermore, as far as the authors know, this has not been considered in the classic descriptions of rabies and it is possible that an immunologic process may take part in the demyelination observed in the present study.

KEY WORDS: rabies, cranial nerves, electron microscopy.

Ultraestrutura de nervos cranianos de ratos inoculados com o vírus da raiva

RESUMO - Os autores estudaram o quinto e o sétimo nervos cranianos de ratos inoculados com o vírus da raiva. Os resultados foram comparados com os mesmos nervos cranianos de ratos inoculados com o vírus da raiva, porém vacinados contra a doença. Os achados no grupo não vacinado foram de degeneração axonal e intensa desmielinização dos nervos examinados. No grupo vacinado foram encontrados apenas discretas alterações da mielina, sem relevância do ponto de vista patológico. As grandes alterações ultraestruturais encontradas no grupo de ratos não vacinados sugerem que na raiva ocorram acentuadas alterações nos nervos cranianos e que tais alterações devem estar intimamente relacionadas ao quadro clínico da doença (hidrofobia). Além disso, é possível que tais alterações estejam associadas a um processo imunológico responsável também por acometimento sistêmico dos nervos periféricos.

PALAVRAS-CHAVE: raiva, nervos cranianos, microscopia eletrônica.

Rabies is an acute infectious disease caused by a virus included in the rhabdovirus group which has an internal capsule containing RNA[8]. The virus has an elongated form ("bullet-shaped") and its size has been estimated to be about 75 nm in diameter by 180 nm in length. The disease occurs all over the world, with exception of few countries. In man the lethality rate is nearly 100 per cent. Its main characteristic is an encephalomyelitis preceded by a biting attack of a sick animal. The incubation period varies from less than three weeks in the case of head injuries up to six months or more for wounds of the extremities[10].

Rabies in man starts with 2 or 4 days of prodromal symptoms characterized by slight fever, occipital headache, malaise, anorexia, sore throat and tinglings about the site of biting. Mental and

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neuromuscular overactivity develop rapidly. The pulse is rapid, there is dilatation of the pupils, lacrimation, increased salivation and excessive perspiration. Paralytic symptoms may be predominant from the beginning or may appear at any stage of the disease. Sometimes the course of the paralysis follows the pattern of the ascending type[8]. The outstanding clinical symptom is related to the act of swallowing and the sight, smell or sound of liquids may precipitate spasms of the muscles of the throat, hence the term of "hydrophobia" to designate the disease. Periods of intense excitement and convulsions occur which are responsible for the death of the patients.

The purpose of the present study was to establish, by electron microscopy, the cranial nerves involvement in experimental rabies in order to evaluate if this could help in explaining part of the symptomatology of the disease, particularly the so called "hydrophobia".

MATERIAL AND METHODS

The authors used in this investigation two groups (Group I and Group II) of ten adult white male rats (Wistar) weighing aproximately 330 g each.

Group I was inoculated with 0.20 ml of a street rabies virus preparation having a titer of $10^{5.23} LD_{50}$/0.03 ml titered in mice weighing 14 g. The virus was inoculated by intramuscular route in the left hind leg of the rats. Based in a previous study[11] in which alterations in the sciatic nerves of rats inoculated with rabies virus were described after the 7th day of inoculation, we sacrificed one rat every second day starting on the 6th day of inoculation.

In Group I, 7 rats were sacrificed, the others succumbed to the disease. From each sacrificed rat the left V and VII cranial nerves were removed. From them, specimens of 5 to 8 mm in lenght were obtained. The specimens were immediately immersed in a cold (4°C) solution of glutaraldehyde in cacodilate buffer 0.15M. After two hours of fixation the specimens were cut in small pieces of about 1 mm thick and washed in two changes of cacodilate buffer 0.15M + 0.2M NaCl for 15 minutes each. Post fixation was carried out for 30 minutes at room temperature in cold 2% osmium tetroxide in cacodilate buffer 0.30M. After being washed in distilled water they were stained by uranil acetate 2% for 24 hours. The specimens were again washed in distilled water during 5 minutes and after being dehydrated in ascending grades of alcohols and acetone 100% they were embedded in a mixture of Polylite. Thin sections were obtained from a Sorval MT2, collected on copper grids, stained by uranil acetate and lead citrate[16] and examined in cross section in a "Philips EM 300".

By means of immunofluorescent techniques rabies virus was detected in the brain of the rats from the day 12 onwards.

Group II was submitted to the same procedure as Group I. Group II, however, was vaccinated against rabies starting in the first day of inoculation. The vaccine was prepared from brains of suckling mice inoculated with the CVS strain of rabies virus and then inactivated with betapropiolactone 1:4,000. Vaccine concentration was 2% and the titre was superior to 10,000 LD_{50} calculated by the Habel's method[5]. The vaccine was used in a dose of 0.2 ml daily by IM route.

As in Group I, the rats of Group II also presented evidences of rabies virus in their brains as shown by immunofluorescent techniques.

RESULTS

In Group I, from the first day - the day in which rabies virus was inoculated - to the 7th day the rats did not show any clinical signs. From the 8th day onwards, however, a left hind leg paresis was observed in almost all the rats. The paresis rapidly progressed to a paralysis and in the 11th day the same happened with the right hind leg. After a period of 24 to 48 hours of involvement of the right leg almost all rats showed paralysis of the four limbs.

In Group II, the same evolution was observed in the majotiry of the rats, but in this group the paresis did not reach the degree of paralysis observed in the rats of Group I. In Group II, also, the general state of the rats was much better than that observed in Group I: in Group II at least five rats were normal in the 15th day of post-inoculation.

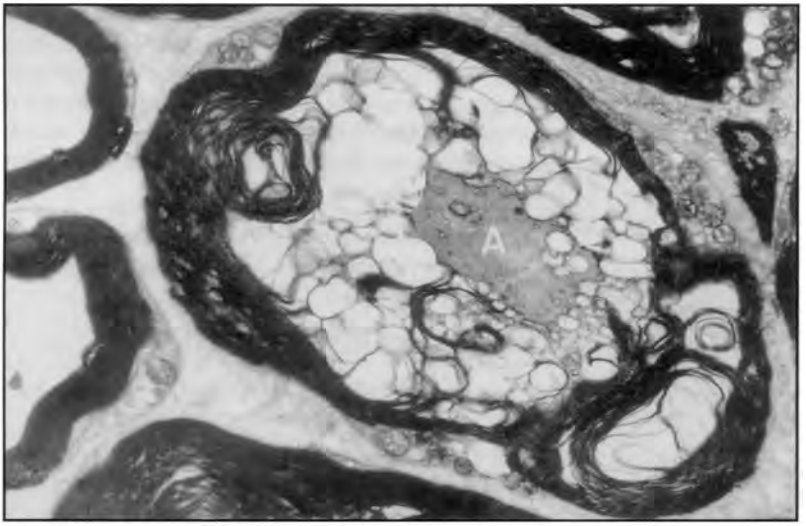

Fig 1. Electron micrograph of an axon in cross section showing intense dissolution of the myelin sheath: the myelin lamellae are in disaray and a great number of vacuoli are seen around the axoplasm which shows alteration on its shape and size (A). Left are 2 normal axons. X 8,500. V cranial nerve. Group I.

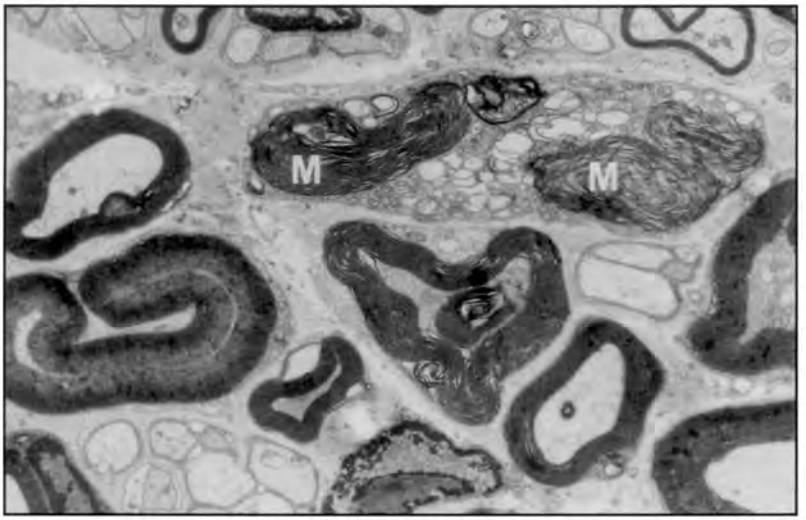

Fig 2. Upper right is an axon with 2 balls of myelin (M) disposed independently in each pole of a Schwann cell. The cytoplasm of the Schwann cell shows a great number of vacuoli. Normal myelinated and unmyelinated fibers are seen around. X 4,500. VII cranial nerve. Group I.

In Group I, the analysis of the V and VII cranial nerves showed ultrastructural alterations from the 9th day post-inoculation onwards. At that time, the alterations were those of mild degree of demyelination. But from this period onwards the alterations got progressively worse and reached their maximum state in the 17th and 19th day post-inoculation: in electron microscopy great disarrengement of the lamellae of the myelin sheath was present (Figs 1,5). These alterations of the myelin sheath were so important that sometimes large masses of myelin ovoid and great amounts of electron dense material which represents masses of myelin in disorganization were observed dispersed

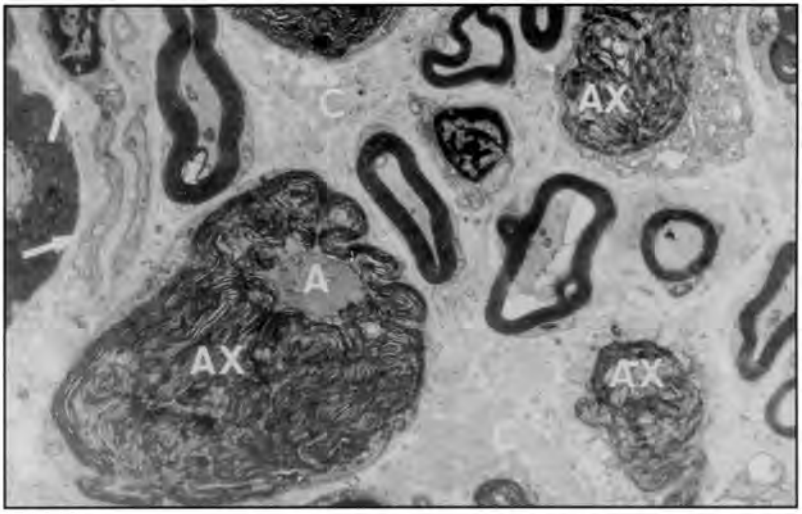

Fig 3. This picture shows at least 3 axons (AX) whose myelin sheath are under dissolution: that on the left shows a great disproportion between the amount of myelin and the axoplasm (A) which is not located in the center of the fiber. No axoplasm is identified in the other two axons which show alterations in the myelin sheath. A great amount of collagen (C) is seen among the structures. Upper left is a fibroblast (arrows). Few small myelinated normal fibers are seen around. X 6,500. VII cranial nerve. Group I.

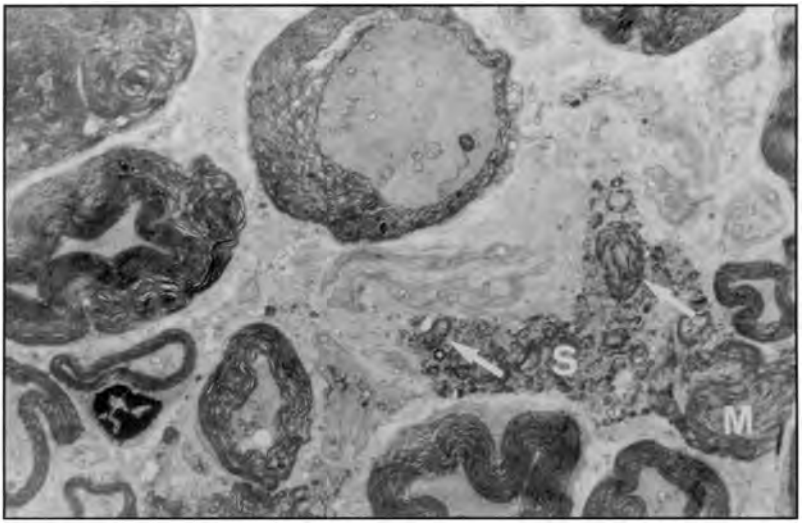

Fig 4. At right are seen debris of a Schwann cell (S) surrounded by numerous collagen fibrils. In the cytoplasm of the Schwann cell there are myelin bodies (arrows) and a large mass of myelin in dissolution in one of its pole (M). Upper, left and down fibers in the process of demyelination are seen. X 4,500. VII cranial nerve. Group I.

among the structures (Figs 2,3,4,6). The diameter of the axons of the cells with alterations of the myelin sheath become smaller than the normal pattern (Figs 1,3) and sometimes the axons were hardly identified within the electron dense masses of disorganized myelin. In the axoplasm it was difficult to identify the inner structures namely the neurotubules, neurofilaments and mitochondria (Figs 4,6). In the Schwann cells the cytoplasm was scanty, nuclei hardly seen and mitochondria

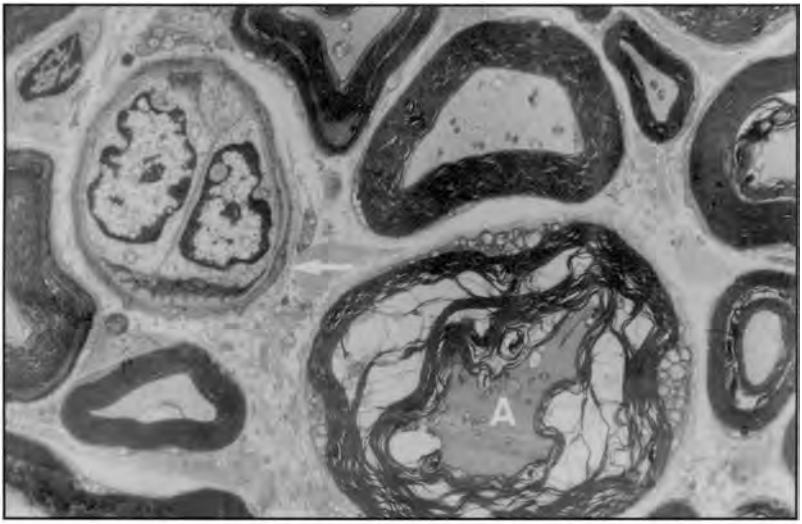

Fig 5. Botton is an axon in which the myelin lamellae are in disaray. The axoplasm (A) shows great alteration on its size and shape. Upper left is a vasa nervorum containing 2 white blood cells in its lumen. The capillary wall (arrows) does not show any ultrastructural abnormality. X 4,500. V cranial nerve. Group I.

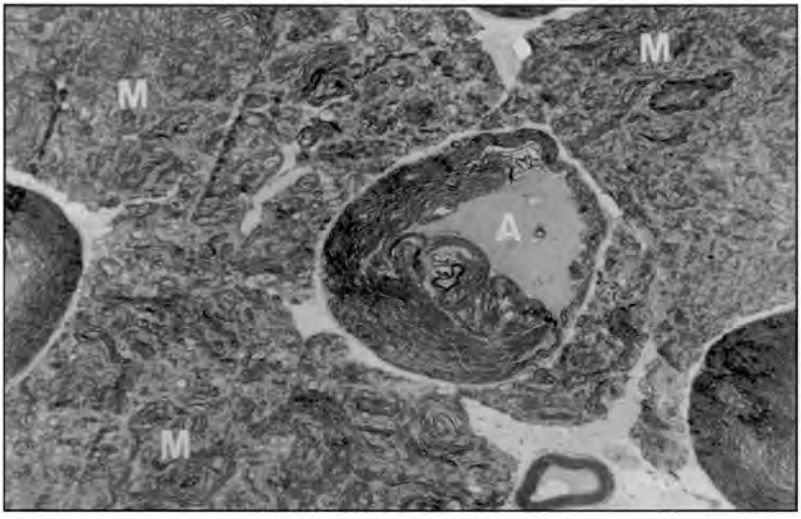

Fig 6. A great amount of myelin in dissolution is seen among the structures. The aspect is that of a "lake" of myelin (M) which does not exhibit any visible axoplasm. In the center, is an axon which shows alteration of the size, shape and location of its axoplasm (A). X 4,500. V cranial nerve. Group I.

appeared grouped in one region of the cytoplasm. The other cytoplasmic organelles of the Schwann cells were also hardly seen but vacuoli and cellular debris were frequently observed.

In all specimens examined no ultrastructural alterations were observed in the vasa-nervorum or even in the endoneural cells. Although intensively examined, no particles resembling rabies virus were seen in the material studied.

DISCUSSION

After death, rabies virus is commonly detected in the CNS of man and animals affected by the disease. Medulla, temporal lobe and cerebellum are the sites of the CNS where the virus reaches its highest concentration[8]. The virus may also be detected in tissues other than the nervous system, particularly in the submaxillary glands, lacrimal glands, parotid glands and kidneys. The virus is not detected in blood, spleen, liver, lymph nodes, intestine, gonads or bone marrow of dogs[7,8].

In brain, rabies-infected neurones seem to remain structurally intact[7,14]. Although perineuronal and perivascular mononuclear cell infiltration occur, neuronolysis or neuronophagia are mild and it is difficult to understand the nature of the damage produced on a nerve cell by rabies virus. Cytoplasmic inclusion bodies can be demonstrated in the neurons of human rabies. These structures were first described by Negri in 1903[7] and when present are pathognomonic of rabies. By means of fluorescent antibodies and electron microscopy it was demonstrated that Negri bodies contain rabies virus[7,12,13].

In rabies no much emphasis has been given to the involvement of the peripheral nerves, although in the majority of the cases paralysis, often of ascending type, is the initial symptom of the disease. The authors in a previous paper[11] showed by means of electron microscopy severe demyelination and axonal degeneration in the sciatic nerve of rats inoculated with rabies virus. Based on this, it was thought that the same ultrastructural changes could be found in the cranial nerves. In fact, the findings of the present study have shown the occurrence of demyelination and axonal degeneration of the V and VII cranial nerves of rats inoculated with rabies virus. In view of this, it may be speculated that in rabies systemic involvement of the peripheral nerves occurs and if these alterations also occur in the lowest cranial nerves they would help to explain the spasmodic contractions of the muscles of deglutition leading the patient to chok when attempting to swallow. From these observations, it may be speculated that in rabies systemic involvement of the peripheral nerves occurs including those from dorsal region and those from the sympathetic nervous system. This would help to explain a great number of signs and symptoms presented by patients with rabies and which are not directly related to the involvement of the CNS. In the literature there are reports of ascending paralysis and defects in immune response in cases of human and experimental rabies[6,17,19].

According to the WHO[15] very little is known about the peripheral nerve involvement in cases of rabies and histopathological study of the peripheral nerves in humans has not been adequately documented. The only good reports which refer histopathological changes in peripheral nerves of human rabies are those from Tangchai and Vejjajiva[18], who found axonal degeneration, leucocyte infiltration and nonspecific vascular congestion in 9 cases of human rabies, and Chopra et al.[2] who published a paper presenting 11 cases of human rabies starting with ascending paralysis indistinguishable from Landry's paralysis. These last authors studied different nerves taken at autopsy by means of light microscopy and using appropriated staining for myelin sheats. The most striking pathological features were mild to moderate myelin fibre loss, segmental demyelination, remyelination and axonal loss. Using also single teasing fibres technique they found Wallerian degeneration in ten cases.

It is generally agreed that rabies virus is carried from the site of exposure to the CNS by the peripheral nerve. Dean et al.[3] have postulated that the rate of propagation of the rabies virus along nerve pathways is approximately 3 mm per hour. Kaplan et al.[9] and Dean et al.[4] have also suggested that local anesthetics injected intramuscularly proximal to the site of exposure have a sparing effect due to the anesthetic action on the metabolism of the inoculated tissue. Baer et al.[1] investigating the spread of fixed rabies virus after its inoculation into the rear foot-pads of rats found that the removal of the corresponding sciatic nerve reduced the mortality of the rats.

Although all these theories of centripetal spreading via neural routes have been accepted for many years, the presence of rabies virus in the peripheral nerves has not been shown and how it moves within nerves has remained a perplexing problem.

Unfortunately, no evidence of the rabies virus was found by the authors neither in the present work or in the previous one which dealt with sciatic nerves, although hundreds of grids had been examined. If the theories of centripetal spreading are correct, it may be possible that the undetection of rabies virus in the peripheral nerves by electron microscopy is due to an alteration of its shape: by loosing of its capsule the virus would not keep the characteristic "bullet-shaped" aspect which would be misleading the examiners. If this happened, by electron microscopy the viral RNA molecules would become undistinguishable from the rest of the axoplasm content. This would explain the sparing effect of neurectomy and anesthetics injected proximal to the site of exposure.

The striking axonal degeneration along with demyelination of the cranial nerves studied in the present work suggest that both processes may take part in the pathogenesis of the disease and that they may be of immunoallergic in nature. It is difficult, however, to establish by electron microscopy which of these two processes, demyelination and axonal degeneration, is primary or secondary. A good method which could help in clarifying this point would be teasing fiber technique and this is a matter for future studies.

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