AN ELECTRON MICROSCOPE ANALYSIS OF NERVES INFECTED WITH THE B VIRUS*

By E. De ROBERTIS, M.D.;

(From the Department of Biology, Massachusetts Institute of Technology, Cambridge)

Plates 29 to 31

(Received for publication, June 10, 1949)

The demonstration with the electron microscope that the nerve contains a fibrous component, probably axonic, the so called neurotubules (De Robertis and Schmitt (1)) has led to the supposition that these structures might be involved in the transfer of virus particles through the axon. This hypothesis was first tested with the poliomyelitis virus (2). In experimentally infected monkeys, neurotubules were found which contained dense particles of the order of size generally assumed for poliomyelitis virus. The rate of progression of the particles inside the nerves is also within the range determined by indirect studies of the poliomyelitis virus transfer (Bodian and Howe (3)). It was concluded that although the identification of these dense particles could not be definitely stated they appeared to be related to the advancing virus infection (4).

In the present investigation the pantropic B virus was used. This virus follows a regular and constant pattern of progression when injected into the gastrocnemius muscle of the rabbit. As Sabin (5) has shown, up to 48 hours after injection the virus grows in the muscle but is not detectable in the nerve or in the cord. Between 72 and 96 hours the virus is detectable in the sciatic nerve, but in small concentration. Later, multiplication of the virus particles occurs in the sciatic and cord and reaches a maximum at the onset of paralysis on the 6th day. At this moment the virus can be detected also in the left sciatic as a result of centrifugal spread from the spinal cord (Sabin). The results which will be described here show a clear correlation with the above-mentioned findings of Sabin inasmuch as dense particles can be found in the neurotubules of nerves known to contain virus and their concentration with time follows a pattern similar to the one demonstrated by the virus assay.

Material and Technique

The virus inoculations were made by Professor A. Sabin of the Children's Research Hospital, University of Cincinnati. The author is most grateful for his generous collaboration and valuable criticism.

---

*This work was supported in part by a grant from the trustees under the wills of Charles A. King and Marjorie King.
†Fellow of the United States Public Health Service.
‡Present address, Instituto de Investigacion de Ciencias Biologicas, Montevideo, Uruguay.
NERVES INFECTED WITH B VIRUS

A constant amount of B virus suspension (0.5 cc. of 10 per cent suspension of rabbit spinal cord) was injected into the right gastrocnemius muscle of six rabbits. (5). 26, 48, 72, 96, 120, and 144, hours later the animals were sacrificed and the sciatic branches and trunk supplying the gastrocnemius were fixed in 10 per cent formaldehyde. The 144 hour rabbit showed paralysis of both posterior extremities when killed, while the others had no sign of paralysis.

Pieces of the fixed nerves were dissected under a binocular wide-field microscope until the nerve bundles were freed of all visible collagenous tissue (epi- and perineurium). The nerve bundles were then sectioned at 4 μ with the freezing microtome and fragmented with sonic vibration of 9 kc. After light centrifugation, the supernatant was put on a grid for observation. Several techniques were used to wash the neurotubules clear of most of the amorphous material found in the background. Observations were carried out on untreated material and also on material stained with 0.1 per cent phosphotungstic acid at pH 5 or shadowed with chromium at 11°. For most of the observations an RCA Type EMU microscope was used.

RESULTS

During the first 48 hours after the injection of B virus, essentially normal neurotubules similar to those described before (1) were found in both the right and left sciatic nerves of the 24 and 48 hour rabbits (Fig. 1). In the right sciatic of the 72 hour rabbit most of the neurotubules also have a normal structure but a small proportion of them show the presence of spherical particles ranging in size between 300 and 600 Å and showing characteristics which will be described below. At 96 hours the number of neurotubules containing particles seems slightly increased, particularly in the middle and proximal part of the right sciatic, while no particles are found in the left contralateral nerve.

In the right sciatic nerve of the 120 hour rabbit, a considerable increase of neurotubules with particles is observed, of which Fig. 2, treated the same as the normal neurotubule (Fig. 1), is a representative example. In Fig. 2 a group of neurotubules containing particles varying in size between 300 and 600 Å and with different degrees of electron densities are seen. In addition to these which are considered as small and medium sized particles in other electron micrographs a few clumps of larger particles were observed.

The 144 hour right sciatic nerve showed the maximum number of changes. A thorough study of several parts of the nerve was made by dividing it into three segments of about equal length. It was found that the proximal segment, near to the spinal cord, had the largest number of neurotubules containing particles. Although in this segment normal neurotubules or bundles of neurotubules were found, most of them contained particles of varying sizes. From this observation it seems probable that at the time of paralysis a large number of the nerve fibers were affected by the B virus.

Figs. 3 and 4 show representative bundles of neurotubules of this nerve, in which the normal structure has been considerably distorted by the presence of numerous dense particles which range in size between 400 and 800 Å. In Fig. 3 the edges are scalloped, presumably owing to the underlying particles. In
Fig. 4 the largest and densest particles tend to form large clumps which bulge on the surface of the bundle. In Figs. 3 and 4 the preparation had been previously stained on the grid with phosphotungstic acid to demonstrate the banded structure of the neurotubules (De Robertis and Schmitt (1)). However, in this case no sign of banding is observed. A study of the fine structure, both in stained or shadowed preparations, shows that the normal banding is usually more difficult to detect in neurotubules containing particles (Figs. 5 and 5 a). This fact is evident in Fig. 5, which shows many tubules containing particles in which the cross-banding is more or less effaced in contrast with the normal neurotubules, also present, which show typical banding. In this figure all the gradations in particle diameter between 300 and 1000 Å can be observed in a single bundle of tubules.

The relation between the particles and the neurotubule can be best studied in preparations shadowed with chromium. Figs. 6 to 9 show single neurotubules or bundles of them in which the particles are all arranged linearly within the limits of the neurotubules and produce a definite bulge on the surface. This fact is particularly striking when one observes the scalloped shadows of these neurotubules (Fig. 8) which is in contrast with the almost straight shadows of normal neurotubules. In most of the cases of small and medium sized particles, they appear to be located within the edges of the neurotubules; however, in the case of larger particles the topographical interpretation is more difficult (Figs. 8 and 9). The clumps of large dense particles are generally associated with the bundles of tubules and in some cases seem to be directly embedded in them (Fig. 9). However, in other cases clumps of large (600 to 1000 Å) particles can be seen isolated on the supporting film or separating the neurotubules of a normal bundle.

In the 144 hour experiment a proximal segment, 20 mm. in length, of the left sciatic nerve was also examined. A number of neurotubules containing particles were found but in much lower concentration than in the similar segment of the right sciatic nerve.

**DISCUSSION**

Studies of the human B virus infection (Sabin and Wright (6)) and particularly the analysis of the disease produced experimentally in rabbits and monkeys (6–9) suggested that the B virus, although pantropic, invades the central nervous system by way of the nerve axons. This fact was further confirmed by Sabin (5) who showed that cutting of the sciatic nerve prevented migration of virus from the muscle into the attached peripheral portion of the nerve.

Direct knowledge of the size and morphology of the virus particles involved in such neurotropic infection is still lacking. The virus isolated by Sabin and Wright from a human infection is known to be readily filterable through the Berkefeld V and N, Chamberland L3, and single disc Seitz filters and to
NERVES INFECTED WITH B VIRUS

sediment at 14,000 x. p. m. in about 3 hours (8). These properties would indicate a particle diameter of about 1250 Å or less. Filtration through gradocal membranes yielded data indicating its size as 1000 to 1500 Å (10).

In our study, particles generally ranging between 300 and 1000 Å have been observed in nerve material from experimentally infected animals. It is interesting to note, however, that at the beginning of the virus transfer through the nerve (72 to 96 hours) the particles found range mainly between 300 to 600 Å. In the later stages (120 to 144 hours), coinciding with a considerable virus growth (5), some of the now numerous particles reach diameters of 600 to 1000 Å. It is also interesting to note that in individual electron micrographs (Figs. 5 and 9) great variation in the particle size can be observed.

Owing to the lack of direct morphological information about the elementary bodies it is difficult to be certain of the exact nature of the dense particles here observed.

The supposition that they may represent "products" of the inflammatory reaction of the nerve, due to the passage of the virus, gains some support in the final stages, when an interstitial neuritis develops (8), but it is less justified for the early stages of virus transfer when the nerve fiber appears intact on histological study by ordinary methods. Neither do they represent the type of degenerative change previously described in neurotubules under in vitro or in vivo degeneration (11). This of course does not exclude the possibility of other types of degenerative processes not yet investigated.

The fact that the dense particles are only found at times when, to judge from the work of others, the virus assay is positive and that they increase in number in later stages when there is a considerable virus multiplication (5) may support the hypothesis that they actually represent the elementary bodies of the virus. If this is the case, the different sized particles might be interpreted as representing stages in the transfer and development of the virus inside the nerve axon.

As in the case of nerves infected with the poliomyelitis virus (4), it is suggested that, though identification of the particulate material is not yet warranted, the morphological changes described in the neurotubules appear to be associated directly with the virus infection.

These results, together with those of the preceding paper (4), indicate the possibility of using the electron microscope analysis of the axon for the study of the pathogenesis of diseases caused by neurotropic agents, as well as for the study of the problem of virus-host cell relationships.

CONCLUSIONS

Rabbits were infected with B virus in the right gastrocnemius muscle and both sciatic nerves were fixed and analyzed under the electron microscope, after periods varying between 26 and 144 hours.
Starting at 72 hours, a few neurotubules of the right sciatic show the presence of spherical particles, ranging between 300 and 600 Å. The number and size of the particles increase with time and appear to reach a maximum at 144 hours when the paralysis starts. At this moment also the proximal part of the left sciatic nerve shows the presence of dense particles. The relation of the particles with the periodic structure of the neurotubules was studied both in preparations stained in phosphotungstic acid and those shadowed with chromium. The small and medium sized particles are located within the edges of the neurotubules, and the large particles appear to be attached to the neurotubules.

The possible significance of the dense particles is discussed.

BIBLIOGRAPHY

10. Personal communication of Dr. Elford to Dr. Sabin.
EXPLANATION OF PLATES

PLATE 29

Fig. 1. Normal neurotubule. × 43,000.

Fig. 2. Neurotubules from a rabbit sciatic nerve, 120 hours after inoculation with B virus. Small and medium size particles, some indicated with arrows. × 54,000.

Fig. 3. Bundle of neurotubules filled with dense particles, 144 hours after the infection with B virus. Edges are scalloped by the presence of particles. Stained with phosphotungstic acid. Particles indicated by arrows. × 48,000.

Fig. 4. Same as in Fig. 3. Large clumps of dense particles are attached to the sides of the bundle. × 36,000.
(De Robertis: Nerves infected with B virus)
FIG. 5. Bundle of neurotubules 144 hours after infection with the B virus. Stained with phosphotungstic acid. The periodic banded structure of the neurotubules is more or less obscured by the presence of particles of varying size. For better localization of the particulate material see the above diagram (Fig. 5 a). \( \times 62,000 \).

Fig. 5 a. Diagram representing the upper portion of Fig. 5.
(De Robertis: Nerves infected with B virus)
PLATE 31

Neurotubules of the rabbit sciatic nerve infected with B virus, 144 hours after inoculation. Chromium shadowing at an angle of 11°.

Fig. 6. Neurotubule showing the presence of small particles. $\times$ 31,000.

Fig. 7. Bundle of neurotubules filled with small particles. $\times$ 31,000.

Fig. 8. Neurotubule with medium sized particles. $\times$ 38,000.

Fig. 9. Bundle of neurotubules with medium sized and large particles forming a big clump at the right end. $\times$ 38,000.
(De Robertis: Nerves infected with B virus)