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"Tubulofilamentous particles" are not scrapie-specific and are unrelated to tubulovesicular structures

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Abstract. "Tubulofilamentous particles" has been defined as "thick" tubules, apparently distinguishable from other cytoskeletal elements, in touch-preparations from scrapie- and Creutzfeldt-Jakob disease (CJD)-infected brains. I report here that "tubulofilamentous particles" are nonspecific findings unrelated to the subacute spongiform virus encephalopathies (SSVE) as they are observed in brains of control animals. I further suggest that these "tubulofilamentous particles" are unrelated to "tubulovesicular structures" (TVS) which are the only particles consistently observed in brains of naturally occurring and experimentally induced SSVE.

Key words: scrapie, tubulovesicular structures, prion diseases

INTRODUCTION

The subacute spongiform "virus" encephalopathies (SSVE) are a group of neurodegenerative disorders which include kuru, Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker (GSS) syndrome, and familial fatal insomnia in man, natural scrapie in sheep, goats and moufflons, transmissible mink encephalopathy, chronic wasting disease of captive mule deer and elk in the USA, bovine spongiform encephalopathy (BSE) and its analogues in several exotic species of antelopes, cheetah and puma in zoological gardens and feline spongiform encephalopathy in domestic cats (for review, Liberski 1993). These disorders are caused by still not completely understood pathogen(s) variously referred to as a virus (usually with the adjectives unconventional or atypical), agent, prion or virino. These names reflect, in part, different views on the molecular structure of the elusive pathogen. Those who view this pathogen as composed only or predominantly of one protein, PrP, use the term "prion" (Prusiner 1993) (hence the term "prion disorders") to stress the unusual characteristics of the pathogen which is, in many aspects, different from "conventional" viruses and viroids. The "virino" hypothesis suggests that the pathogen is a molecular chimera composed of a still to be discovered nucleic acid and a shell protein which is host-encoded (Dickinson and Outram 1979, Kimberlin 1990). The virus hypothesis simply suggests that the pathogen is yet to be isolated and there is no evidence to date to prove that it is outside the spectrum of conventional viruses (Diringer 1993). To complicate the problem further, Narang put forward recently another hypothesis - that the agent is ssDNA virus of unusual structure, called "nemavirus", which is visualized as "tubulofilamentous particles" by thin-section electron microscopy or as "thick tubules" when the touch preparation method was applied (Narang et al. 1987, 1988, Narang 1992, 1993). Moreover, Narang claims that "tubulofilamentous particles" are longitudinal profiles of "tubulovesicular structures" (TVS). TVS were discovered more than a quarter of century ago by David-Ferreira and colleagues (1968) and are regarded as the only disease-specific structures for all SSVE observed by thin-section electron microscopy (Liberski et al. 1993), in contrast to scrapie-associated fibrils (SAF; Merz et al. 1981) or prion rods (Prusiner et al. 1983) which are visualized by negative staining. I report here that these "tubulofilamentous particles" are merely nonspecific findings related neither to the subacute spongiform virus encephalopathies (SSVE) nor to the TVS as they were observed in brains of control animals and represent swollen microtubules.

METHODS

Weanling, 4- to 6-week-old NIH Swiss mice (Animal Production Area, Frederick Cancer Research and Development Center, Frederick, MD), inoculated intracerebrally with 0.03 ml of a 10% suspension of brains from mice affected with the Fujisaki strain of Creutzfeldt-Jakob diseases (CJD) virus, were sacrificed at the terminal stage of disease by intracardiac perfusion using 180 ml of 1.5% glutaraldehyde and 1% paraformaldehyde prepared in phosphate buffer (pH 7.4). Outbred, 6-week-old golden Syrian hamsters were inoculated intracerebrally with 0.05 ml of a 10% brain suspension of the 263K and 22C strains of scrapie, and perfused with 100 ml of 1.25% glutaraldehyde and 1% paraformaldehyde prepared in cacodylate buffer (pH 7.4) followed by 50 ml of 5% glutaraldehyde and 4% paraformaldehyde. To obtain additional control, rats were injected intracisternally with $HgCl_2$ to develop mercury encephalopathy and processed analogously to the hamsters. It is noteworthy, experiments with rats were performed in a "scrapie-free" laboratory. Brain tissues from animals sham-inoculated with saline served as controls. Perfused animals were kept at $4^{o}C$ for 2-4 h, after which the brains were removed, and several approximately 1 mm^3 samples, dissected from different anatomical regions, were postfixed in 1% osmium tetroxide for 1-2 h, dehydrated through a graded series of ethanols, and embedded in Embed or Epon 812. Specimens were examined with Hitachi 11A,

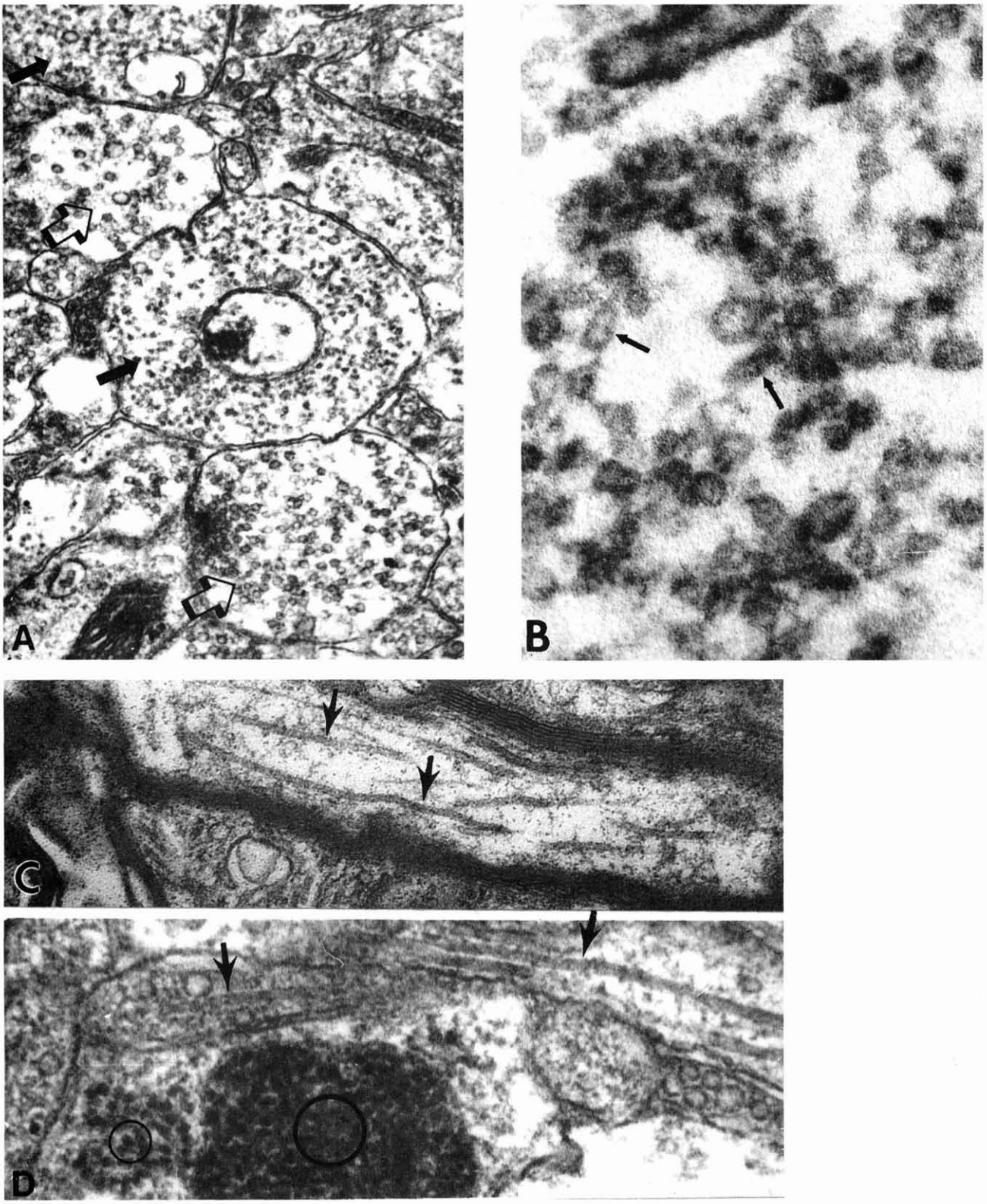

Fig. 1. Low (A) and high (B) power electron micrographs of tubulovesicular structures, TVS (arrows) in comparison to synaptic vesicles (open arrows). Note short tubular forms of TVS (arrows) at "B". Swollen microtubules or "tubulofilamentous particles" (arrows) in longitudinally cut axons (C) and dendrites (D) of sham-inoculated hamster (D) and $MgCl_2$-intoxicated rats. Glycogen granules are seen in (D) (circles). Bars = 100 nm.

Philips 300 and Zeiss 109 transmission electron microscopes.

RESULTS

To search for "tubulofilamentous particles" as defined and illustrated by Narang (1992, 1993), the files of more than 15,000 electron micrographs were re-analysed. In both mice, hamsters and rats, whether inoculated with CJD or scrapie virus, sham-inoculated or $HgCl_2$-intoxicated, thick "tubulofilamentous particles" were easily found (Fig. 1), particularly in longitudinally cut myelinated axons (Fig. 1C and D). These "tubulofilamentous particles" were indistinguishable from swollen microtubules (Peters et al. 1991) and, as reported by Narang (1992, 1993) occasionally showed cross striations. "Tubulofilamentous particles" were particularly easily found in those brain areas which were suboptimally fixed. In contrast, TVS were easily found only in all 3 models of SSVE but not in control animals and appeared as spheres approximately 35 nm in diameter or short tubules never longer than 50 nm (Fig. 1A and B). They were also never observed as long structures cut in longitudinal plane. It is thus completely untenable that they may be represented also by tubules longer than 1 μm.

DISCUSSION

The demonstrated presence of "tubulofilamentous particles" in normal mouse and hamster brains suggests that they are not scrapie-specific and represent merely swollen microtubules probably resulted from less than optimal fixation. Indeed, Narang's figure 2 (1992) clearly shows numerous areas of myelin sheath dissolution which are regarded as stigmata of bad fixation. Thus, the hypothesis of Narang (1992, 1993) that these structures represent the yet-to-be discovered scrapie virus, for which the name "nemavirus" has already been ascribed (1992, 1993), is untenable. In the following discussion I shall re-analyse the spurious relationship between "tubulofilamentous particles", tubulvesicular structures (TVS) (Liberski et al. 1988, 1989, 1990, 1993) and scrapie-associated fibrils (SAF) (Merz et al. 1981) or prion rods (Prusiner et al. 1983).

TVS also known as "scrapie-associated particles", were discovered by David-Ferreira and colleagues (1968) in experimental scrapie and subsequently observed in natural and experimental scrapie (Bignami and Parry 1971, Field and Narang 1972, Narang 1973, 1974a,b, Baringer and Prusiner 1978, Narang et al.1980, 1987, Baringer et al. 1981, Gibson and Doughty 1989), CJD (Liberski et al. 1991, 1992), and bovine spongiform encephalopathy (BSE) (Liberski et al. 1992). Ultrastructurally, TVS appear as spheres or short rods measuring approximately 35 nm in diameter (Liberski et al. 1993). Their chemical composition is unknown. In both experimental scrapie and CJD, TVS appear early during the incubation period and their appearance precedes the development of clinical signs and pathological lesions in the brain (Narang et al. 1988, Liberski et al. 1990). Narang has claimed (1992, 1993) that "tubulofilamentous particles" represent TVS cut longitudinally. However, this claim has never been substantiated and it is rather difficult to reconcile the length of "tubulofilamentous particles" which approaches more than 1 mm in longitudinal section with that of TVS which measure approximately 35 nm in diameter, no more than 50 nm in length and which have never appeared as long structures cut in longitudinal sections. As demonstrated in our micrographs, "tubulofilamentous particles" represent normal subcellular organelles, possibly swollen microtubules.

The same arguments apply to the spurious relationship between "tubulofilamentous particles", TVS and SAF. Narang has claimed that "tubulofilamentous particles", visualized as "thick tubules" (Narang et al. 1987, 1988, Narang 1992, 1993) in touch-preparations, undergo transformation into SAF following treatment with sodium dodecyl sulfate (SDS). Grids which were not treated with SDS did not show SAF. Grids treated with proteinase K (PK) alone did not show SAF while the addition of DNase or mung bean nuclease produced SAF (Narang 1993). This sequence of enzymatic diges-

tion and SDS treatment corresponds to that already reported by McKinley et al. (1991) who demonstrated that SDS treatment and proteolytic digestion are mandatory for the formation of prion rods which, like SAF, are composed of prion protein (PrP) (Barry et al. 1985, Merz et al. 1987). As inhibitors of proteolytic enzymes were not used by Narang prior to SDS treatment, SAF were formed even in the apparent absence of PK; similar data have already been reported by Beekes et al. (1993). Such an interpretation is further substantiated by Narang's immunogold localization of PrP along SAF (1993) which recapitulates only already published data (Barry et al. 1985, Merz et al . 1987).

In conclusion, it seems that "tubulofilamentous particles" are swollen microtubules unrelated to TVS and that the formation of SAF follows SDS treatment and proteolytic digestion. Finally, the Narang hypothesis that scrapie virus belongs to a new virus group, designated "nemavirus", should be rejected.

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