ACTA NEUROBIOLOGIAE EXPERIMENTALIS

Review Acta Neurobiol Exp 2008, 68: 113–121

The tubulovesicular structures – the ultrastructural hallmark for all prion diseases

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Tubulovesicular structures (particles – TVS) are the only ultrastructural marker for all prion diseases as seen by thin-section electron microscopy as opposed to "negative-staining" techniques. TVS are spheres or short rods of approximately 27 nm in diameter. That size of TVS is also the size of filter cut-off of infectivity as judged from the ultrafiltration studies and the size of the smallest infectious unit as recently estimated. TVS have been found in all naturally occurring and experimentally induced prion diseases, including variant Creutzfeldt-Jakob disease and human familial TSEs – fatal familial insomnia and Gerstmann-Sträussler-Scheinker disease. In longitudinal studies, the number of neuronal processes containing TVS correlates roughly with the incubation period and with infectivity. Hence, they are readily found in hamsters infected with the 263K strain of scrapie but it is very difficult to find them in human TSEs where titer is lower. The composition of TVS is unknown but they are not composed of PrP. Their consistent presence in all TSEs suggests the unexplained role at least of TSE pathogenesis.

Key words: prion diseases, scrapie, Creutzfeldt-Jakob disease, tubulovesicular structures

INTRODUCTION

Tubulovesicular structures (TVS) or "tubulovesicular bodies" are disease-specific structures encountered in transmissible spongiform encephalopathies (TSE) or prion diseases including Creutzfeldt-Jakob disease (CJD) and Gerstmann-Sträussler-Scheinker disease (GSS). Our earlier observations suggest that TVS appear early during the incubation period of experimental TSE and thus represent a primary pathologic event rather than a consequence of brain damage. The composition of TVS is unknown but immunogold electron microscopy studies show that TVS are not composed of prion protein (PrP). Thus, the significance of TVS is yet to be discovered (Liberski and Brown 2007).

Recently, structures similar to TVS were described in cell cultures infected with TSEs (Manuelidis 2007, Manuelidis et al. 2007). The latter finding made TVS even more interesting phenomenon.

Irrespective of whether PrP^{Sc} (or PrP^{TSE}) forms a part or the whole of the scrapie agent, the abnormal isoform of PrP (PrP^{TSE}) accumulates late in the brain in the course of disease either as amyloid plaques or as diverse extracellular deposits (Budka 1997, 2004, Ironside 1998, Kovacs et al. 2004). Some investigators have suggested that specific pathological phenomena – for example apoptosis, may be, in part at least, dissociated from PrP^{TSE} accumulation (Lasmezas et al. 1997). It is increasingly important to search for early pathological changes and to determine the association between such changes and PrP^{TSE} accumulations (Sikorska 2004).

In this short review we concentrate on the TVS or "scrapie-associated particles". These structures of unknown chemical composition and unresolved biological significance but they appear to be unique to the TSEs at the level of thin-section transmission electron microscope.

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Received 18 July 2007, accepted 9 October 2007

TVS – DESCRIPTION

TVS (also known as the scrapie-associated particles or tubulovesicular bodies) are unique to the TSEs, at the level of thin-section electron microscopy. TVS are described as spherical or elongated structures of approximately 27–30 nm in diameter (Fig. 1). TVS differ from other particulate structures of the CNS – i.e. spiroplasma-like inclusions (Fig. 2b); multivesicular bodies (Fig. 2c); branching cisterns (Fig. 2d) or accumulations of glycogen granules (Fig. 2e).

TVS have been identified in GSS, CJD, many rodent scrapie models, BSE, and FSE (feline spongiforme encephalopathy) (Jeffrey et al. 1995), and in natural scrapie of sheep. They were first described in NIH Swiss mice infected intracerebrally with the "Chandler" strain of scrapie (David-Ferreira et al. 1968). TVS were described as "particles and rods ranging in diameter from 320 to 360 A°" (32–36 nm). Interestingly, rods were covered with minute spikes. It is clear from these description and subsequent discussion that the diameter of TVS is variable when reported by different investigators – but these minute discrepancies are probably accounted for by the variable swelling or dehydration which may occur during preparation of tissues for electron microscopic examination. In natural scrapie in sheep TVS appeared as membrane-bound accumulations of round particles measuring 35 nm in diameter (Bignami and Parry 1971). The electron-dense core could be demonstrated in some of them. The membrane-bound accumulations of TVS also appear in cell-cultures infected with scrapie and CJD (Manuelidis et al. 2007). It seems that "cucumber-shaped bodies" measuring approximately 20 nm in diameter and 60 nm in length reported by Narang (1974a) may represent TVS despite differences in size.

TVS have been reported in the majority of models of scrapie in rodents studied so far, including the TG3 $PrP^{0/0}$ mice and are therefore capable of being generated in the absence of cytoplasmic PrP expression (Jeffrey et al. 2004). At low magnification, a process containing TVS is crowded with structures typically of higher electron density than other elements in this field.

TVS IN DIFFERENT FORMS OF TSES

Lamar and coauthors (1974) found TVS in dendrites of ICR mice inoculated intracerebrally or subcutaneously with the Klenck (not further characterised) isolate of scrapie isolated from a Suffolk ram. Spherical TVS reported by these investigators measured 30–35 nm in diameter; some of TVS, however, appeared as longer tiny rods. Interestingly, TVS could neither be detected in spleens taken from scrapie-affected mice nor in brain cell cultures. Narang and others (1980) reported TVS in several strains of mice infected intracerebrally with the Chandler strain of scrapie. In these models, TVS were seen only in postsynaptic terminals and measured 26 nm in diameter. When stained with ruthenium red (Narang 1974b), TVS appeared larger approaching 33 nm in diameter. In Swiss mice inoculated with the Chandler strain of scrapie, TVS were found in enlarged dendrites or unidentified neuronal processes (Barringer and Prusiner 1978, Barringer et al. 1979, 1981). Electron dense spherical TVS measured 23 nm in diameter and frequently formed "paracrystalline" tubular or vermicellar arrays (Barringer et al. 1981). Interestingly, when hamsters infected with the 263K strain of scrapie were studied, TVS could not initially be detected (Barringer and Prusiner 1978, Barringer et al. 1979, 1981). These negative data were originally used to suggest that TVS could not represent a significant ultrastructural finding as the 263K scrapie (Kimberlin and Walker 1977) model represents the highest titers of infectivity found in scrapie. Subsequently, however, three groups of investigators reported TVS in hamsters infected with the 263K strain of scrapie (Narang et al. 1987a,b, Liberski et al. 1988, 1989b, Gibson and Doughty 1989). Narang and coauthors (1987a,b) reported TVS measuring 22 to 24 nm in diameter in postsynaptic terminals accompanied by larger vesicular profiles measuring 100–110 nm in diameter and "smaller number of tubulofilamentous profiles" 200 nm long. Liberski and coworkers (1988, 1989b) identified TVS in the same hamster model not only in dendrites but also in presynaptic terminals. In the latter structures they were mixed with, but easily discriminated from synaptic vesicles. Affected processes also contained normal-appearing mitochondria, large vesicles (measuring 100–150 nm in diameter), multivesicular bodies and large electron lucent cisterns surrounded by "fuzzy" membranes. TVS measured 20 to 50 nm in diameter (mean, approximately 35 nm). Interestingly, when the archived electron micrographs were searched for the presence of TVS apparently not present at the time of publishing (Liberski 1987) they were readily identified. Thus, as is typical in many areas of pathology, once a new finding has been reported it

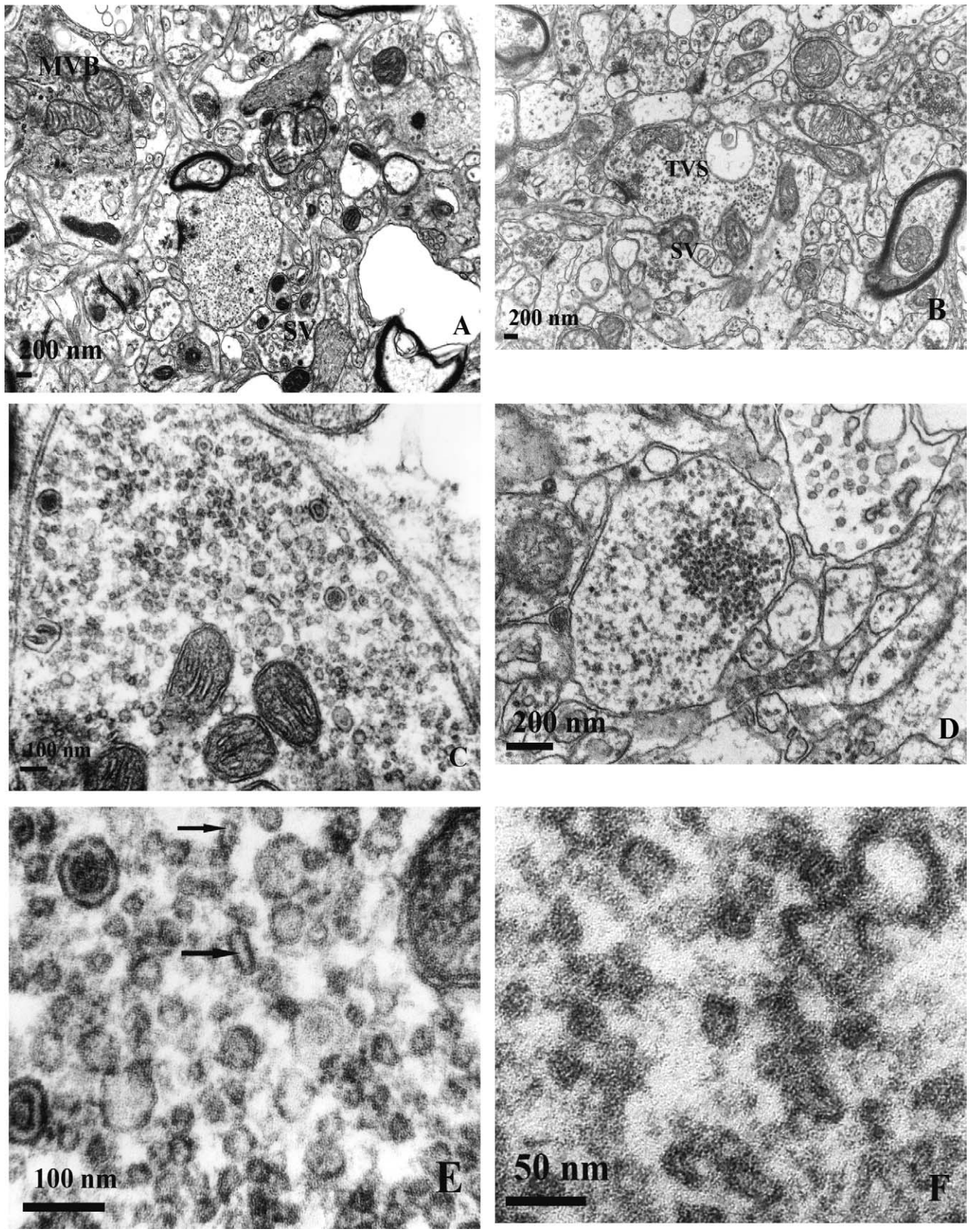

Fig. 1. Tubulovesicular structures (TVS) as seen in different magnifications indicated by bars. In (E) arrows point to the elongated form of TVS.

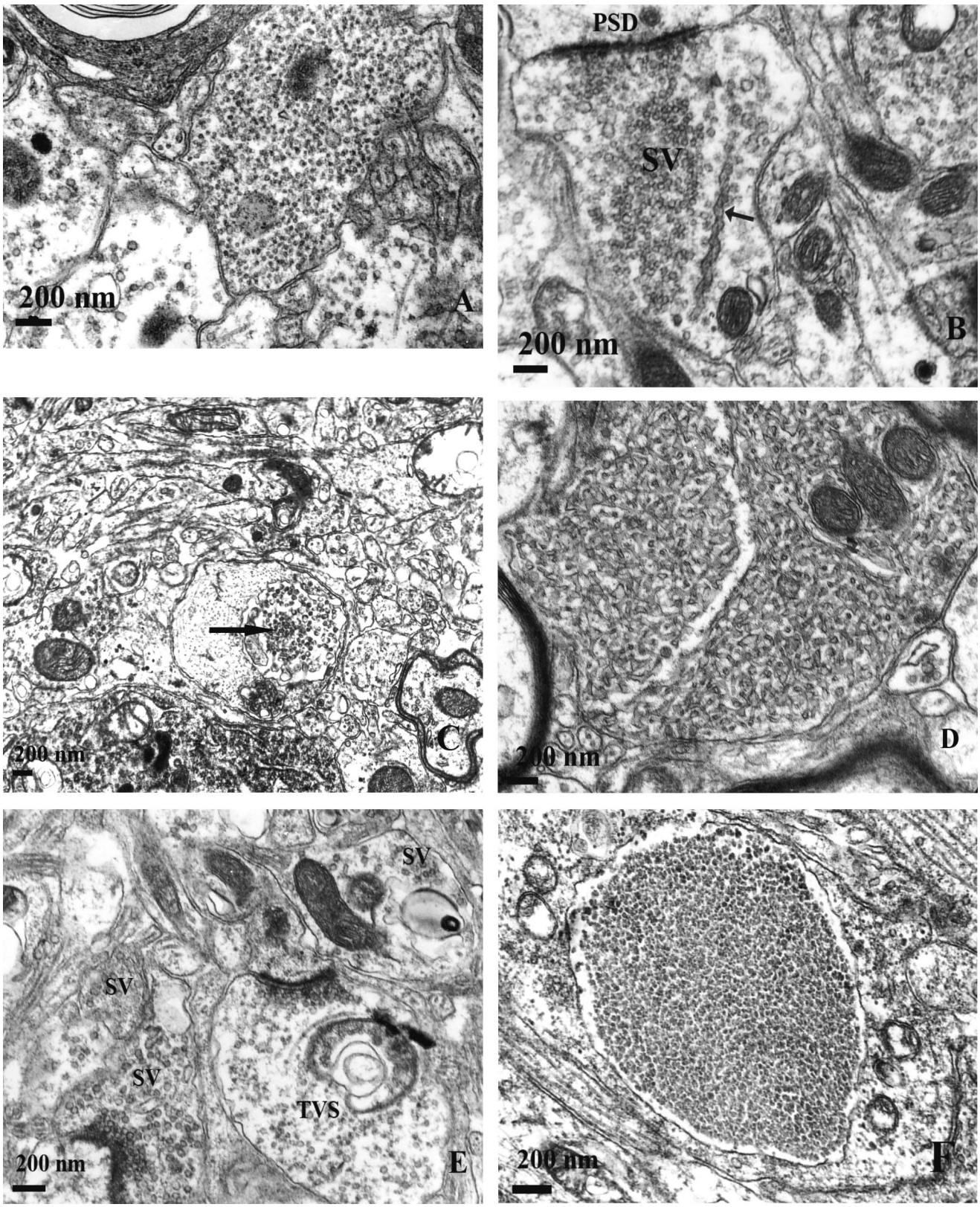

Fig. 2. Tubulovesicular structures (TVS; A, E) as seen in comparison with different particulate structures. (B) A spiroplasma-like particle or a "spiral"; (C) multivesicular body; (D) branching cisterns and accumulations of glycogen granules; (E) larger spheres in TVS-labeled process may represent SV; (F) accumulation of glycogen granules. (SV) Synaptic particles.

becomes clear that the phenomenon had been present all along but had not been recognized.

Gibson and Doughty (1989) reported the most extensive survey of TVS in different experimental scrapie models in rodents. In agreement with Liberski and others (1988, 1989a) these investigators found TVS in both presynaptic terminals and dendrites but the latter predominated (41 profiles of 109 in dendrites – as compared to 12 in presynaptic terminals). Gibson and Doughty (1989) reported the presence of dense "paracrystalline" arrays apparently absent in hamsters infected with the 263K strain of scrapie (Liberski et al. 1988, 1989a). TVS were most numerous in VL mice infected with the murine ME7 strain of scrapie followed by C3H mice infected with the 22C and 79A strains. Furthermore, when VM mice infected with the 87V strain of scrapie (a model producing a large quantity of amyloid deposits) were examined, numerous synaptic terminals containing TVS were found in the vicinity of amyloid plaques. TVS were also found in Cheviot sheep inoculated with the ME7 strain of scrapie but the number of affected processes was the lowest among reported models. In a further survey of 10 optimally perfused fixed sheep scrapie brains, TVS were also considerably harder to detect and less frequent than found in rodent models (Ersdal et al. 2003, 2004). Paracrystalline arrays of TVS are not seen in sheep scrapie.

In experimental CJD, TVS were reported infrequently or not at all (Kim and Manuelidis 1986). In chimpanzees inoculated either with human or chimpanzee CJD isolates, TVS were reported in one animal as spherical and elongated structures measuring 30–40 nm in diameter. TVS were reported in NIH Swiss mice infected intracerebrally or intraocularly with the Fujisaki strain of GSS (Liberski et al. 1988, 1989a). They were of the same diameter and localization as in hamsters infected with the 263K strain of scrapie and, in contrast to those reported from mice infected with scrapie, did not form "paracrystalline" arrays. In a companion paper, we detected TVS in hamsters infected with the Echigo-1 strain of CJD. In well-fixed brain biopsies infected with CJD and in two cases of GSS, TVS were readily identified but the number of affected processes was very low (Liberski et al. 1992, 1993, 2008, Liberski and Budka 1994). Thus, while more extensive studies of other neurodegenerative diseases are still required, TVS appear to be specific to the TSEs.

Only limited data are available concerning the number and density of TVS through the incubation time. In hamsters inoculated with the 263K strain of scrapie, TVS were initially observed as early as 3 weeks after intracerebral inoculation, but their number increased only with the onset of disease at 9 to 10 weeks after inoculation (Liberski et al. 1989b). Noteworthy, vacuolation and astrocytosis were detected at 8 weeks post-inoculation and, thus, followed the appearance of TVS. In Webster-Swiss mice, TVS were found 12 weeks and 16 weeks after intracerebral inoculation and intradermal (footpads) inoculation respectively (Narang 1988). In NIH Swiss mice infected with the Fujisaki (Fukuoka-1) strain of GSS, TVS were first seen 13 weeks after intracerebral inoculation and their numbers increased dramatically at 18 weeks after inoculation, when the first signs of clinical disease were noted (Liberski et al. 1989b). In contrast to the 263K, the Fujisaki strain of GSS showed vacuolation and astrocytosis at the same time as the appearance of TVS and the increase in the number of processes containing TVS paralleled the increasing intensity of vacuolation and astrocytosis. TVS were approximately twice as abundant in terminally ill mice following intraocular inoculation as in mice following intracerebral inoculation (Liberski et al. 1989b). By contrast, final intensity of vacuolation and astrocytosis was not dependent on the route of inoculation. Similar findings were described by Jeffrey and Fraser (2000) in the ME7 model which has an extended incubation period when compared with the 263K. TVS were first seen in the ME7 model at about 39% of incubation period considerably before any significant pathology could be detected and arguing that TVS are part of the primary disease process and not simply epiphenomena consequent upon widespread tissue degeneration. The numbers of TVS in this model increased in frequency with increasing incubation period and titer.

TVS therefore appear early in the incubation period, preceding the onset of clinical disease. Furthermore, in scrapie-infected hamsters, TVS preceded the appearance of other neuropathological changes. The approximately 1 000-fold lower infectivity titer of the Fujisaki strain of GSS, compared to the 263K strain, may have accounted for the delayed appearance of TVS in experimental CJD. The apparent correlation between the number of neuronal

processes containing TVS and infectivity titer may explain why in cell cultures infected with scrapie, TVS could not be found and why their number in experimental scrapie in sheep or CJD in humans was so low (Gibson and Doughty 1989).

ATTEMPTS TO ISOLATE TVS

The isolation and purification of TVS has been unsuccessful. However, when homogenates of scrapie-affected brains and spleens were sized by ultrafiltration with or without zonal centrifugation, spherical particles measuring 30–60 nm in diameter were detected in thin-section or negative-staining transmission electron microscopy (Siakotos et al. 1979). Occasionally, 30–60 nm spheres were accompanied by smaller spherical structures measuring 8–10 nm and 16–20 nm in diameter. Interestingly, the contrast of these particles was enhanced by use of ruthenium red (*vide supra*). Recently, Silveira and coauthors (2005), based on fractionation technique, reported that scrapie infectivity corresponded to particles size smaller than 50 nm. Corresponding size partices (25 nm) is reported by the newest study *in vitro* (Manuelidis 2007, Manuelidis et al. 2007). Collectively, these data suggest that the smallest infection unit of scrapie is located around the diameter of TVS, but the real connection between those two is, at the moment, conjectural.

IMMUNOGOLD-ELECTRON MICROSCOPY OF TVS

The chemical composition of TVS is unknown. Earlier studies reported that ruthenium red enhances contrast of TVS (Narang 1974b). These staining properties of TVS may be interpreted as an evidence for the presence of glycosyl residues within TVS. It was even tempting to speculate that TVS may be composed of PrP because this protein is a sialoglycoprotein (Bolton et al. 1985). To evaluate this problem we employed immunogold electron microscopy and anti-PrP antibodies.

In the 87V murine model, PrP-conjugated gold particles decorated typical stellate amyloid plaques and the cell surface of numerous dendrites (Jeffrey et al. 1994a,b, 1996). By light microscopy and semi-thin (1 μm) sections, discrete PrP^{TSE}-immunopositive plaques were observed in the 263K and 22C-H models in the subependymal region but not in the deep brain neuroparenchyma. These plaques were not discernible by routine H & E staining. Ultrastructurally, plaques were recognized as areas of low electron density containing haphazardly oriented fibrils heavily decorated with PrP-conjugated gold particles but not as stellate compact structures typical to mouse scrapie models. In all models, TVS-containing processes were readily detected and neither these processes nor TVS themselves were decorated with gold particles. Even if amyloid plaques were observed in close contact with TVS-containing neuronal processes, the plaques were decorated with gold particles while the processes remained unstained (Liberski et al. 1996, 1997, 1998). At higher magnification, amyloid fibrils were clearly visible with numerous round or short tubular particles attached to them. At such a magnification, these particles were membrane-bound and their diameter was approximately twice that of amyloid fibrils; they were virtually indistinguishable from TVS. No such particles were visible within amyloid plaques of six cases of Alzheimer's disease, which serve as a negative control. TVS located in areas adjacent to plaques in the 87V model and in areas of diffuse PrP immunolabeling in ME7 model were also unlabelled with anti PrP antisera. Absence of PrP immunoreactivity to TVS would indicate that these structures are not related to the putatively most infectious PrP particles of 20–27 nm identified by Silveira and others (2005) but the thorough examination of the latter particles by electron microscopy is also desired.

Using immunogold techniques we were unable to label TVS with anti-PrP antibodies. As these techniques proved to be sensitive enough to immunolabel not only amyloid plaques but also pre-amyloid accumulations of PrP (Jeffrey et al. 1992), we believe that the absence of staining reflects the real structure of TVS which are not composed of PrP. The absence of PrP from TVS would not support the suggestion put forward by Narang and coworkers that TVS are cross-sections of "thick tubules" or "tubulofilamentous particles" visualized by touch-preparations of scrapie-affected mouse and hamster brains (Narang 1994, Narang et al. 1987a,b, 1988) as these putatively contain PrP at their core. These "thick tubules" were claimed to represent an ultrastructural correlate of the whimsically invented "nemavirus" which in turn was proposed to be elusive scrapie agent. Indeed, one of us had previously suggested that "thick tubules" represent merely swollen microtubules as they

are observed in both scrapie-infected and sham-inoculated animals (Liberski 1995). Furthermore, Chasey (1994) in a comment to the Narang paper (Narang 1994) suggested that "thick tubules" may represent microtubular doublets, again these are entirely normal subcellular component.

THE SIZE OF INFECTIVITY

The size of TVS (27–35 nm, according to a technique used) correlates extremely well not only with the cut-off of the size of infectivity as measured by earlier ultrafiltration studies (Gibbs et al. 1965), but also with recently published 27 nm smallest infectivity size estimated on the basis of most advanced molecular biological techniques (Silveira et al. 2005). Such a numerical correlation should be explained as it may not be coincidental.

CONCLUSIONS

The number of neuritic processes containing TVS increases through the incubation period and have been shown to correlate with the incubation period and titer of infectivity in two longitudinal disease studies of scrapie and CJD. The latter notion was recently supported in a formal blinded study of the mouse brains infected with the ME7 strain of scrapie (Jeffrey and Fraser 2000). These studies therefore suggest that TVS may represent a primary pathogenetic event rather than a pathological product of disease, repudiating the hypothesis of Gibson and Doughty (1989) who interpreted TVS as breakdown products of microtubules.

The predominant theory of the scrapie agent is now the "prion hypothesis" (Prusiner 1998) and its derivatives which implies that a conformationally altered abnormal isoform (PrP^{Sc} or PrP^{TSE}) of a normal cellular membrane glycoprotein (PrP^{c}) is the agent and its accumulation merely mimicks replication. As already mentioned, recent *in vitro* studies elegantly showed that the nucleation process is responsible for generation of PrP^{TSE} but so far no infectivity has yet been demonstrated in this newly formed PrP^{TSE} (Lansbury and Caughey 1995, Hill et al. 1999) except in one experiment (Castilla et al. 2005). In the latter experiments, however, the titer of infectivity was disproportionably low in a comparison to the level of protein amplification. If an abnormal fraction of PrP is indeed the infectious agent (although it is no longer suggested in some quarters that protease resistant fraction of PrP^{TSE} is the agent) the absence of stainable PrP^{TSE} in TVS would indicate that they are not the ultrastructural correlate of the agent. However, TVS appear to be specific and unique to the TSEs, appearing before the earliest pathological changes and increasing in line with incubation period or titer. The very existence of TVS and their correlation with infectivity therefore urgently needs an explanation.

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