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REGULAR PAPER

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Tubulovesicular particles occur early in the incubation period of murine scrapie

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Abstract Tubulovesicular bodies are structures, apparently specific to the transmissible spongiform encephalopathies, which are of unknown composition and significance. Prion protein (PrP) is absent from tubulovesicular bodies when tissues are examined by immunogold electron microscopy. In the F1 cross of C57 and VM mice (CVF1) infected with ME7 scrapie there is a marked degeneration of hippocampal CA1 neurons. In this model the earliest changes seen, at about 100 days post inoculation (dpi) are a degeneration of axon terminals and synaptic loss. Terminal disease is around 250 dpi. In blind coded trials we counted the number of tubulovesicular particles and estimated their density in 56–76 electron micrographs taken from the stratum radiatum of each of one or two CVF1 ME7-infected mice at 84, 100, 126, 154 and 181 dpi and from four normal brain inoculated control mice. Tubulovesicular particles were present from 98 dpi and the density of particles increased with increasing incubation period. The very early occurrence of tubulovesicular particles, before the presence of significant pathology, argues that tubulovesicular particles are a part of the primary disease and are not epiphenomena.

Key words Tubulovesicular particles · Scrapie · Electron microscopy · Prion protein · Transmissible spongiform encephalopathy

Introduction

Scrapie is the archetype of a group of slowly progressive transmissible spongiform encephalopathies (TSE), which includes bovine spongiform encephalopathy (BSE) and Creutzfeldt-Jakob disease (CJD). So called tubulovesicular particles or tubulovesicular bodies (TVB), perhaps better named as scrapie-associated particles, were first described in the brains of scrapie infected mice by David-Ferreira et al. [2] and ever since have been regularly, if inconsistently, described in electron microscopic studies of the brains of various experimental and natural transmissible spongiform encephalopathies. TVB have been described in most rodent models of scrapie [3, 9], in natural sheep scrapie [1], CJD [7] and in BSE [8]. We have also seen such particles in feline spongiform encephalopathies (M.J., personal observation). The particles have not been reported in spleens or in infected brain cell cultures [9].

The molecular structure of TVB is unknown but staining of thin sections by ruthenium red enhances their appearance, suggesting that they contain glycosyl residues [11]. TVB identified in ME7, 87V and 22CH models of murine scrapie and stained by immunogold methods were negative for PrP epitopes using the 1A8 and 1B3 antibodies [4, 10].

Descriptions of the size and shape of the particles differs, probably because of differences in post-mortem delay and different fixation methods. Most reports describe the particles as spherical with a diameter of between 30–35 nm. The particles are pleomorphic with some appearing as short rods or ellipsoids and they can be found in axon terminals but are mainly seen in dendrites.

In previous investigations of the cause of neuronal loss in the hippocampus of a murine scrapie model and the toxicity of disease specific accumulations of PrP, we have shown that a very selective axon terminal degeneration occurs at some neuroanatomical sites and in some disease models in association with PrP accumulation. In the F1 cross of C57 and VM mice infected with ME7 scrapie we have also determined the number and type of synapses in the stratum radiatum of the hippocampus of ME7-infected mice [5]. Using this well-characterised model of scrapie we determined the number and density of TVB present at selected stages of the incubation period and related their occurrence to the sequence of pathological changes found.

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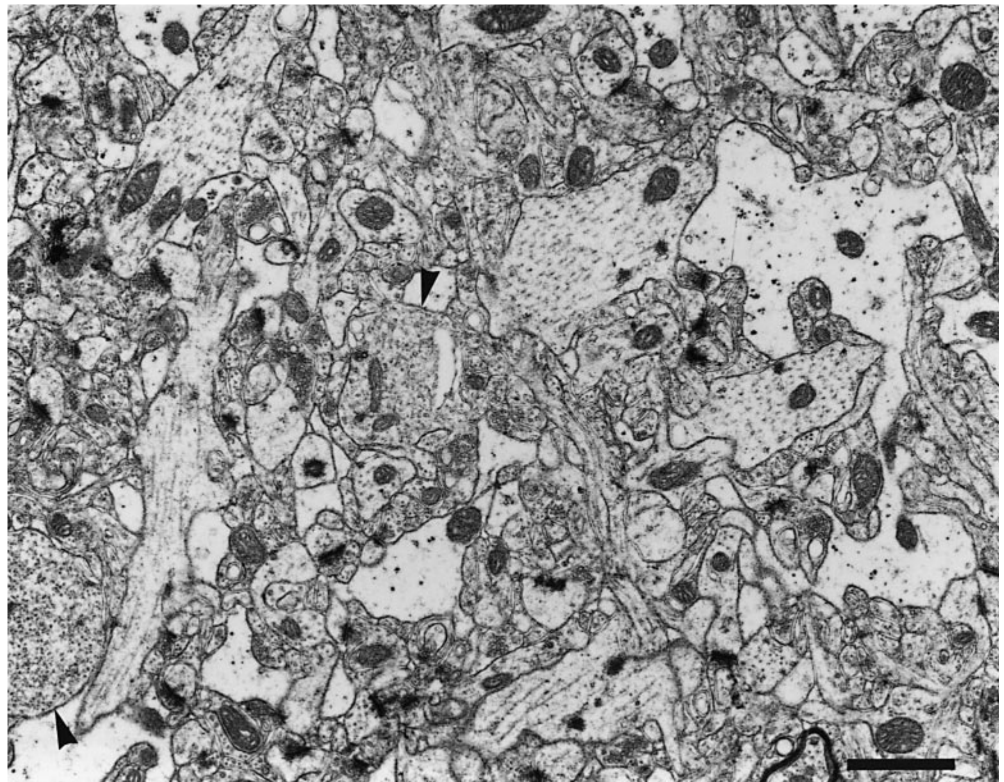

Fig. 1 CA1 hippocampus from a scrapie-infected mouse killed at 98 dpi and showing part of a counting field used for morphometric studies. The field has two process which contain TVB (*arrowheads*) one of which is only partly represented within the counting frame. In this sample taken at 98 dpi there are no other degenerative changes present in the field or in the adjacent fields (*dpi* days post infection, *TVB* tubulovesicular bodies). *Bar* 1 μm

Materials and methods

The methods have previously been published in detail [5]. In summary, the F1 cross of C57 BL and VM mice were infected by intracerebral inoculation into the left cerebral hemisphere with 0.02 ml of a 5×10^{-2} dose of unspun ME7 scrapie brain. Normal brain inoculated controls were also prepared.

Four normal brain inoculated controls and two scrapie-infected mice each at 80, 98,126, 154 and 181 dpi were perfused with calcium-free Tyrodes solution at 37 °C for 30 s followed by 500 ml of warm 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer pH 7.4 for 5 min and 700 ml of cold fixative at 4 °C for 20 min. Three 1-mm^2 regions were taken from the hippocampus of each animal and embedded in Araldite. From each of two blocks taken from a single hippocampal slice, tissue sections were cut at 1 μm and stained with toluidine blue. Mesas were then selected from each section which showed representation of CA1 and where pyramidal neuron primary dendrites were perpendicular to the plane of section. Strings of silver sections were cut from these mesas and placed onto formvar-coated slot grids and stained with uranyl acetate and lead citrate.

To perform TVB counts 16–18 pairs of micrographs were taken from two adjacent pairs of sections at a magnification of 7,000. The initial photograph was taken 200 μm from the neuron cell body layer in the stratum radiatum. Photographs were then taken of alternate fields sampled in a line parallel to the pyramidal neuron cell bodies but were positioned to avoid cell bodies, blood capillaries and bundles of myelinated fibres, and from 126 dpi, to avoid vacuoles. Two blocks were photographed from each mouse. At the end of each photographic session a calibration grid was also photographed to provide an accurate measure of the exact magnification used.

The photographs were coded and a rectangular unbiased counting frame of known area was placed over the photographs and the TVB within the frame were counted. A TVB containing process was recognised in photographs as a cluster of spherical or ellipsoidal particles approximately 30–35 nm in diameter with an electron-lucent core.

One mouse at 154 dpi and one mouse at 181 dpi did not show any significant vacuolation and only traces of PrP accumulation when H&E- or PrP-stained sections from the ipsilateral hemisphere were examined. These mice were not, therefore, at the expected stages of disease relative to their incubation period but are nevertheless included in this study.

Results

TVB were infrequently identified in micrographs taken from 98 dpi (Fig. 1) but were not found in controls or in blocks taken from either of the two infected mice examined at 84 dpi. The frequency of tubulovesicular particles showed an increasing trend with increasing incubation period (Table 1). The density of TVB was zero at 84 dpi and values of 0.09, 0.18, 0.16 and 0.27 TVB were obtained per 100 mm^2 at 98, 126, 154, and 181 dpi, respectively (Table 1). TVB were identified in dendrites and in axon terminals but the cellular location of some TVB could not be determined. Sometimes, within an individual process, very large numbers of particles accumulated, leading to distension of the process containing them (Fig. 2). In such processes the particles were sometimes arranged in regular paracrystalline-like arrays.

We have previously described the sequence of pathological changes in this disease model in some detail [5]. In summary, extremely sparse granular PrP accumulation, sparse vacuoles, degenerate axon terminals and synaptic loss may be detected in the stratum radiatum at 98 dpi. However, these features are all rare or extremely subtle, for example at 98 dpi only two degenerate axon terminals were seen in 160 micrographs (each representing an area

Table 1 The number and density of TVB found per tissue block at selected incubation periods and in controls. All micrographs represented approximately the same areas of 100 μm². Mean densities are based on total number of TBVs identified divided by the total area examined. The values for mean density are means of time points (*dpi* days post infection, *TVB* tubulovesicular bodies)

Animal ID	dpi	No. of micrographs	No. of TVB	Mean density (100 μm²)
R3744	84	36	0	
R3744	84	36	0	
R6175	84	36	0	
R6175	84	36	0	0
R2626	98	36	2	
R2626	98	30	0	
R1282	98	36	9	
R1282	98	34	1	0.09
R2727	126	36	11	
R2727	126	32	12	
R2985	126	36	0	
R2985	126	36	2	0.18
R3355	154	36	0	
R3355	154	34	0	
R3164	154	36	12	
R3164	154	36	10	0.16
R2804	181	36	2	
R2804	181	36	8	
R1905	181	38	14	
R1905	181	36	16	0.27
R1867	Control	36	0	
R1867	Control	36	0	0
R3524	Control	36	0	
R3524	Control	36	0	0
R3815	Control	36	0	
R3815	Control	36	0	0
R2001	Control	36	0	
R2001	Control	36	0	0

of approximately 100 μm²). Loss of dendritic spines occurs at 126 dpi and astrocytic glial fibrillary acidic protein labelling is increased from 154 dpi. Mice showed neuronal loss from about 160 dpi and initial, clinical disease at 201 dpi and were terminally ill at 250 dpi. TVB in later stages of disease were found in the same micrographic fields as degenerate axon terminals and glial processes, but at 98 dpi and 126 dpi they were not found adjacent to other pathological features associated with TSE, which were subtle at this stage of the disease process.

Discussion

Although tubulovesicular bodies have been recognised in the brains of TSE affected animals for more than three decades, they have attracted little attention, partly because they are relatively inconspicuous and partly because of a number of false claims for similar structures and for structures subsequently recognised as artefacts in electron microscopy preparations (for review see [9]). However, we have previously carefully characterised the sequence and severity of lesions at light and electron microscopical levels in the hippocampus of ME7-infected CVF1 mice [5] and can, therefore, be confident that TVB are present well before the onset of typical pathological changes. Thus, TVB are probably not a sequela to other degenerative changes. Although the precise nature of these structures is still unknown, they are likely to be of significance in the pathogenesis and may possibly be of value in the early diagnosis of scrapie, BSE and CJD.

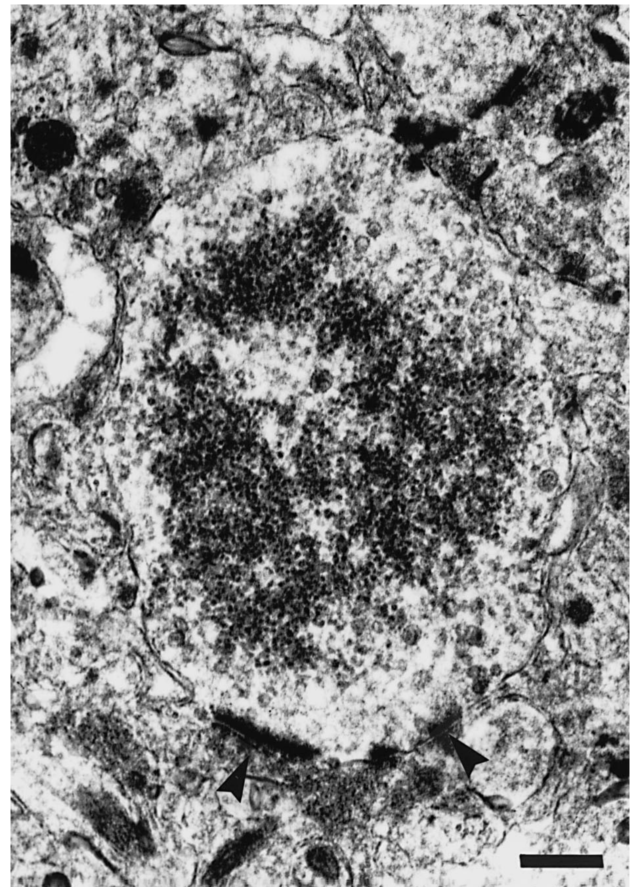

Fig. 2 Detail of TVB-containing dendrite (*arrowheads* show postsynaptic densities) showing pleomorphic size and shape of particles. *Bar* 0.3 μm

TVB have also been found before the onset of clinical disease in 263K hamster scrapie and in experimental CJD [6]. In this study subjective estimations of the number of TVB suggested that their density in the early stages of the incubation period was low but increased dramatically at later stages of disease. These authors have suggested, therefore, that the numbers of TVB are related to titre. The present formal blinded study of the frequency of TVB would support these observations, i.e. there is a gradual increase in the frequency of TVB which corresponds with increasing titre. However, brain infectivity titres are already at substantial levels when TVB were first identified (at 100 dpi the titre in the hippocampus is 4.4 log IC/ID 50/20 μl.; data not shown).

The counting method we employed was subject to a number of variables and it is probably unwise to try and

correlate infectivity and TVB density too closely. Because the method of ultrastructural morphometrics is time consuming it was possible to examine only one or two animals at each time point. One each of the mice sampled at 154 and 181 dpi did not show vacuolar or other degenerative changes expected at this stage of infection. These mice had fewer TVB than age-matched infected mice, i.e. the two mice with incubation periods of 154 dpi show quite different TVB numbers. It is likely that the calculated TVB average densities determined for the 154 and 181 time points should have been higher had animals with more representative lesions been sampled. It is inappropriate, therefore, to compare too closely the frequency of TVB obtained for small numbers of mice with infectivity titres established from much larger groups of mice. Nevertheless, the results suggest that the numbers of tissue TVB increase with increasing incubation period.

Tubulovesicular particles are apparently unique to the TSE as they have not been reported in other chronic degenerative conditions, either within the veterinary or medical literature. They are, however, apparently found in the brains of animals and man with TSE, although their existence in the inherited forms of CJD and Gerstmann-Sträussler-Scheinker disease has yet to be established. They do not contain PrP [4, 10] but are morphologically pleomorphic and on occasion they may assume paracrystalline arrays; they occur very early in the incubation period at a time when pathological changes are few. It is clear that whatever the nature of TVB (whether these structures are the elusive virino or virus or whether they are simply morphological indicator of early infection) they are worthy of further study. A method more reliable and sensitive than electron microscopy is necessary to assay tissues for the frequency of TVB to take forward further investigation of these structures.

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References

1. Bignami A, Parry HB (1971) Aggregations of 35-nanometer particles associated with neuronal cytopathic changes in natural scrapie. Science 171: 389–391
2. David-Ferreira JF, David-Ferreira KL, Gibbs CJ (1968) Scrapie in mice: ultrastructural observations in the cerebral cortex. Proc Soc Exp Med 127: 313–320
3. Gibson PH, Doughty LA. (1989) An electron microscopic study of inclusion bodies in synaptic terminals of scrapie-infected animals. Acta Neuropathol 77: 420–425
4. Jeffrey M, Goodsir CM, Bruce ME, McBride PA, Scott JR, Halliday WG (1992) Infection specific prion protein (PrP) accumulates on neuronal plasmalemma in scrapie infected Mice. Neurosci Lett 147: 106–109
5. Jeffrey M, Halliday WG, Bell J, Johnston AR, MacLeod NK, Ingham C, Sayer R, Brown DA, Fraser JR (2000) Synapse loss associated with abnormal PrP precedes neuronal degeneration in the scrapie-infected murine hippocampus. Neuropathol Appl Neurobiol (in press)
6. Liberski PP, Yanagihara R, Gibbs CJ, Gajdusek DC (1990) Appearance of tubulovesicular structures in experimental Creutzfeldt-Jakob disease and scrapie preceeds the onset of clinical disease. Acta Neuropathol 78: 349–354
7. Liberski PP, Budka H, Sluga E, Barcikowska M, Kwiecinski H (1992) Tubulovesicular structures in Creutzfeldt-Jakob disease. Acta Neuropathol 84: 238–243
8. Liberski PP, Yanagihara R, Wells GAH, Gibbs CJ Jr, Gajdusek DC (1992) Comparative ultrastructural neuropathology of naturally occurring bovine spongiform encephalopathy and experimentally induced scrapie and Creutzfeldt-Jakob disease. J Comp Pathol 106: 361–381
9. Liberski PP, Budka H, Yanagihara R, Gibbs CJ, Gajdusek DC (1993) Tubulovesicular structures. In: Liberski PP (ed) Light and electron microscopic neuropatholgy of slow virus disorders. CRC Press, Boca Raton, pp 373–392
10. Liberski PP, Jeffrey M, Goodsir C (1997) Tubulovesicular structures are not labeled using antibodies to prion protein (PrP) with the immunogold electron microscopy techniques. Acta Neuropathol 93: 260–264
11. Narang HK (1974) Ruthenium red and lanthanum nitrate a possible tracer and negative stain for scrapie particles. Acta Neuropathol 29: 37–43