Review paper

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Ultrastructural findings in pigs experimentally infected with bovine spongiform encephalopathy agent

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Abstract

We report here an electron microscopic study of selected nervous system tissues from pigs infected experimentally with the agent of bovine spongiform encephalopathy (BSE). Generally, the ultrastructural neuropathology of BSE-affected pig brain resembled that of BSE-affected cattle brain. Spongiform change, in the form of membrane-bound vacuoles separated by septae into secondary chambers, dominated the pathology. Numerous astrocytic processes were visible in close conjunction with elongated microglial cells. Neuronal degeneration presented as either dystrophic neurites or by the formation of autophagic vacuoles. Altered subcellular organelles: mitochondria, electron-dense bodies, autophagic vacuoles, neurofilaments and "branching-cisterns" accumulated in abnormal neurites. Autophagic vacuoles appeared as neuronal cytoplasm of increased electron-density sequestrated by intracytoplasmic membranes. Tubulovesicular structures were numerous, particularly in the cerebellum. Unusual crystalloids were observed in the white matter. In conclusion, experimental BSE in pigs demonstrated ultrastructural pathology in keeping with that observed in other spongiform encephalopathies.

Key words: *prions, BSE, pigs, ultrastructure.*

Bovine spongiform encephalopathy (BSE) is a transmissible neurodegenerative disorder previously epidemic among cattle in the United Kingdom, with subsequent cases worldwide and which has now declined to a very low incidence due to rigorous controls [8,24,73,92]. Like other scrapie-like transmissible spongiform encephalopathies (TSE) [48], BSE is caused by an elusive pathogen which has historically been variously termed "a slow unconventional virus" [23], "virino" [33] and more recently, a "prion" [74]. Originally transmitted to cattle via meat-and-bone meal in commercial feedstuffs [91], BSE has been transmitted in primary inoculum to a wide range of food animal species [82] and in addition to mice [10,20,22], mink [75] and the common marmoset [2].

Neuropathologically, both natural and experimental BSE is characterized by spongiform change and astrocytosis in the neuropil and vacuolation of selected nuclei of the brain stem [81,84,85,88,90,91] and accumulation of the proteinase-resistant isoform of PrP (PrP^{Sc}) [83,84]. Scrapie-associated fibrils (SAF) or "prion rods" which are visualized by negative-staining electron microscopy in brain extracts [9,29,77] are also associated with disease,

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and there is a good correlation between degree of pathology and SAF yield [89].

To date, ultrastructural studies of spongiform encephalopathies have been conducted in primates, including humans [37,38,41,51,53,60]; ruminants (cattle, sheep, mule deer and elk) [4-6,16-18,26,27,46,66,67] and laboratory rodents [12,31,32,47,64,65,70; for review: 30,66,67]. The successful transmission of BSE to domestic pigs [14,86], which belong to the order *Artiodactyla* (even-toed hoofed animals), made it possible to study the pathology of this disease in another species and to test whether tubulovesicular structures, the only disease-specific structures observed *in situ* [49,53, 55,59,62,65] occur also in BSE-affected pig brain.

Material and methods

Experiment design and inoculation procedure

The pigs were infected at 1-2 weeks of age by multiple-route parenteral inoculation with a homogenate of bovine brain from natural BSE cases, as described in full previously [76,86,87]. All challenges were carried out in accordance with the Animals (Scientific Procedures) Act, 1986, under licence from the UK Home Office. Animals were sedated with azoperone (Stresnil; Janssen Animal Health) and killed by the intravenous injection of pentobarbitone sodium followed by exsanguination. When clinical disease developed, animals were killed and samples collected immediately postmortem.

Electron microscopy

Multiple samples, comprised 2-3 mm^3, of cerebral cortex, brain stem at the level of vestibular nuclei, ventral horns of the spinal cord, cerebellum and dorsal root ganglia, selected on the basis of the previously determined prevalence of light-microscopy changes [76,87], were fixed immediately after dissection in 2.5% glutaraldehyde, freshly prepared in phosphate buffer (pH 7.4), then postfixed in 1% osmium tetroxide and processed for routine electron microscopy. Comparable areas of brain from uninoculated pigs served as controls.

Results

In general, the ultrastructural features of BSE-affected pig brain were similar to those of BSE-affected cattle [46,66,67] and humans with TSEs [60,61]. Spongiform change in the form of membrane-bound vacuoles (Fig. 1) separated by membranes curled into secondary chambers dominated the pathology. A dense astrocytic reaction was accompanied by abundant elongated microglial cells. Of particular note was the finding of numerous astrocytic processes in close conjunction with microglial cells. Neuronal degeneration presented as either neuroaxonal dystrophy, as evidenced by dystrophic neurites, or autophagic vacuoles. Dystrophic neurites accumulated altered subcellular organelles: mitochondria (Fig. 1B), electron-dense bodies, neurofilaments and "branching-cisterns" (Fig. 2). Autophagic vacuoles appeared as a part or parts of the neuronal cytoplasm sequestrated by intracytoplasmic membranes (Fig. 3). Sequestrated cytoplasm was of higher electron density than the remaining cytosol. Discontinuity of plasma membranes was occasionally seen (Fig. 3B, arrow). Tubulovesicular structures (TVS) were numerous with the highest number of affected processes in the cerebellum (Fig. 4). Many large multivesicular bodies were seen (Fig. 5).

Discussion

Not unexpectedly, the overall neuropathology of experimental BSE in pigs resembled the main ultrastructural features of BSE in cattle and other TSEs [for review: 42,43,48,78,79]. However, the distribution of lesions in pigs was different from that in cattle. In particular, the cerebral and cerebellar cortices in pigs were more heavily involved. These quantitative differences in pathology are not unprecedented, as it is well established that the topography of lesions differs between species or between strains of the agent passaged in one species [3,15,21]. It remains unclear to what extent such differences are explicable in terms of targeting of the agent to different anatomical regions [11] or selective vulnerability of different neuronal populations, but both phenomena are considered to be important.

The observation of TVS in the pig established their occurrence in another animal model of experimental spongiform encephalopathy and in a mammalian species not previously used in TSE research. The molecular composition and the biological significance of TVS remains unknown [49,55]. TVS have been found in all naturally occurring and experimentally induced spongiform encephalopathies in which the appropriate examinations have been made [35]. Examples include natural Creutzfeldt-Jakob disease [1,53,55,60], Gerst-

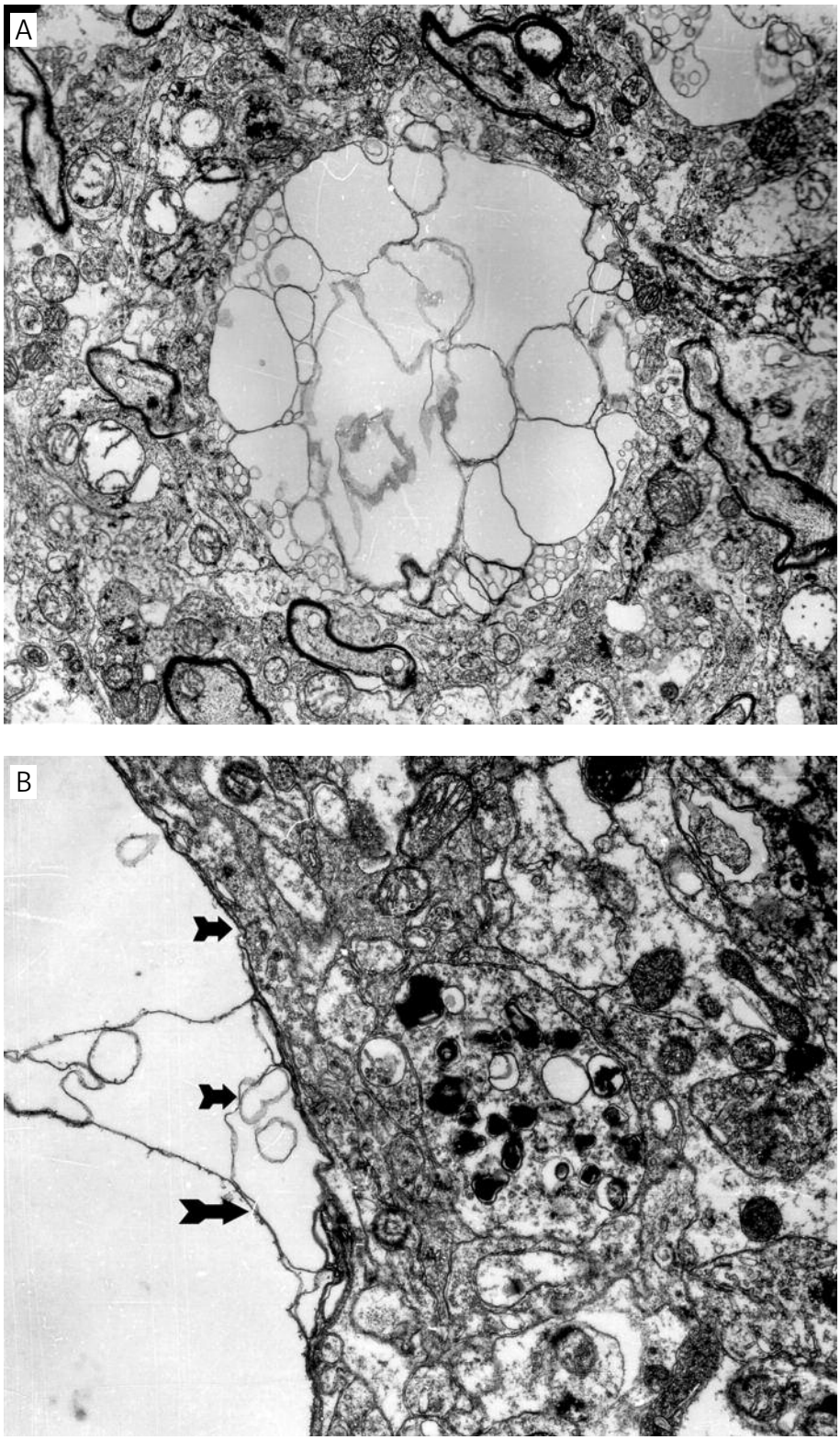

Fig. 1. A) A typical vacuole containing numerous secondary chambers. Original magnification, x 4400. **B**) A part of a vacuole, with curled membrane fragments within. A dystrophic neurite is seen in the vicinity. Original magnification, x 20 000.

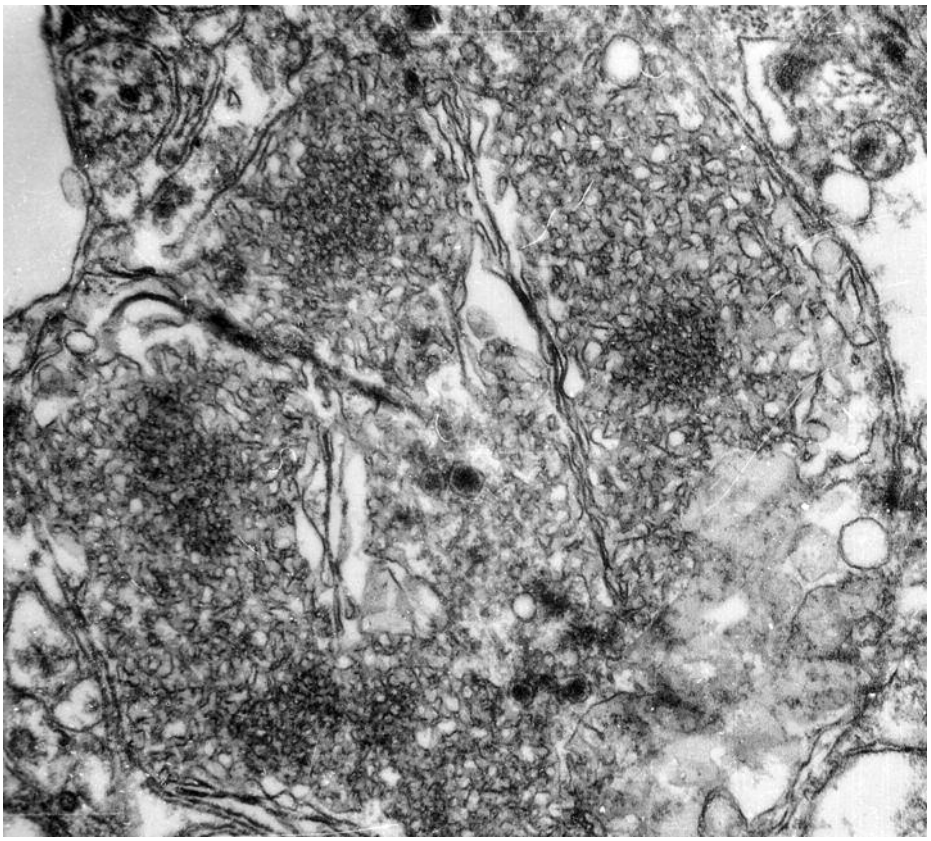

Fig. 2. A neurite containing branching cisterns. Original magnification, x 16 000.

mann-Sträussler-Scheinker disease (GSS) [51], BSE [46,66,67], and natural and experimental scrapie [4,62, 65,69,70]. This comprehensive association suggests that they represent a morphological component closely linked to the basic disease pathomechanism. This is further supported by the observation that TVS appear early in the incubation period. For example, in hamsters infected with the 263K strain of scrapie, in which the incubation period lasts approximately 8 weeks, TVS were observed 3 weeks postinoculation [62]. Additionally, the number of neuronal processes containing TVS generally correlates well with the infectivity titre [62]. Thus, the highest number of processes involved was seen in hamsters infected with the 263K strain of scrapie, followed by the next highest frequency in hamsters infected with the 22C strain of scrapie and in mice infected with the Fujisaki strain of CJD [62]. This corresponds to infectivity titres in brains of 10^4 LD_{50} for hamsters infected with the 263K strain of scrapie agent and 3.1 x 10^4 LD_{50} in CJD virus-infected mice, respectively. In contrast, the lowest number of neuronal processes containing TVS is reported for natural diseases like scrapie [4], BSE [46,66,67] or CJD and GSS [51,53,55,60,61]. The frequency of TVS-containing processes in BSE-affected pig brains was high but, as the titre there is unknown, it is impossible to judge whether this finding represents a true correlation or a coincidental finding. In conclusion, irrespective of what TVS represent, the necessity for further studies is obviously clear.

Autophagy is an important component of the ultrastructural picture of TSE [50,57,58,80], but its exact role and whether it is protective or destructive is not well established [39]. Autophagy was initially shown in scrapie-infected hamsters [7,63] and later in CWD-infected cervids [26], and the present authors consider it to be a deleterious process leading to neurodegeneration [56].

Different types of autophagy have been described: macroautophagy (here called just "autophagy"), microautophagy and chaperon-mediated autophagy [34]. One of these is macroautophagy, which is the intracellular bulk degradation of organelles. Its stages com-

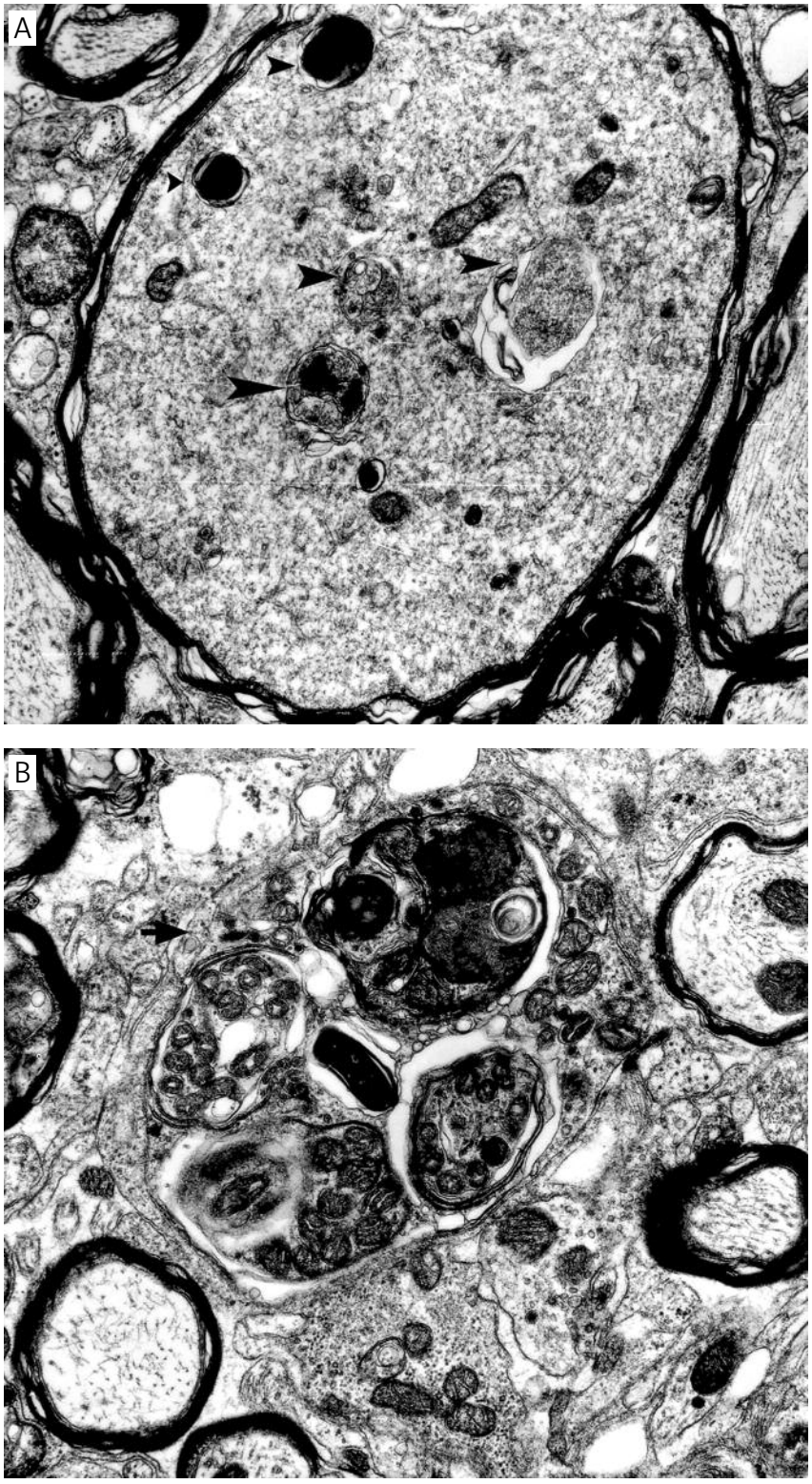

Fig. 3. A) A myelinated neurite containing numerous autophagic vacuoles (arrowheads). Original magnification, x 12 000. **B**) A neurite containing several autophagic vacuoles, the discontinuity of plasma membrane is labelled with an arrow. Original magnification, x 12 000.

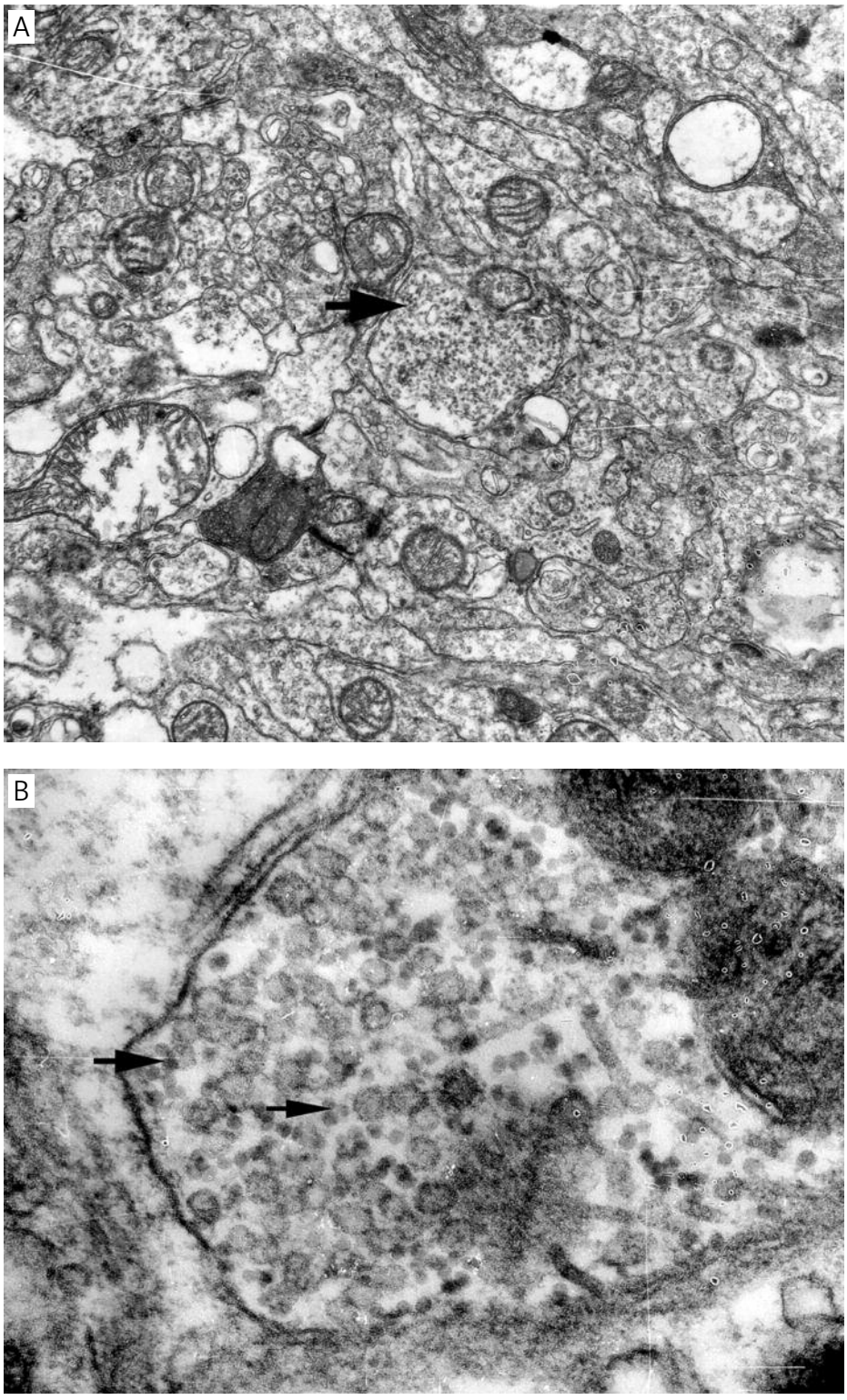

Fig. 4. Lower (**A**) and higher (**B**) magnification of TVS (arrows). Original magnification, (A) x 12 000, (B) 50 000.

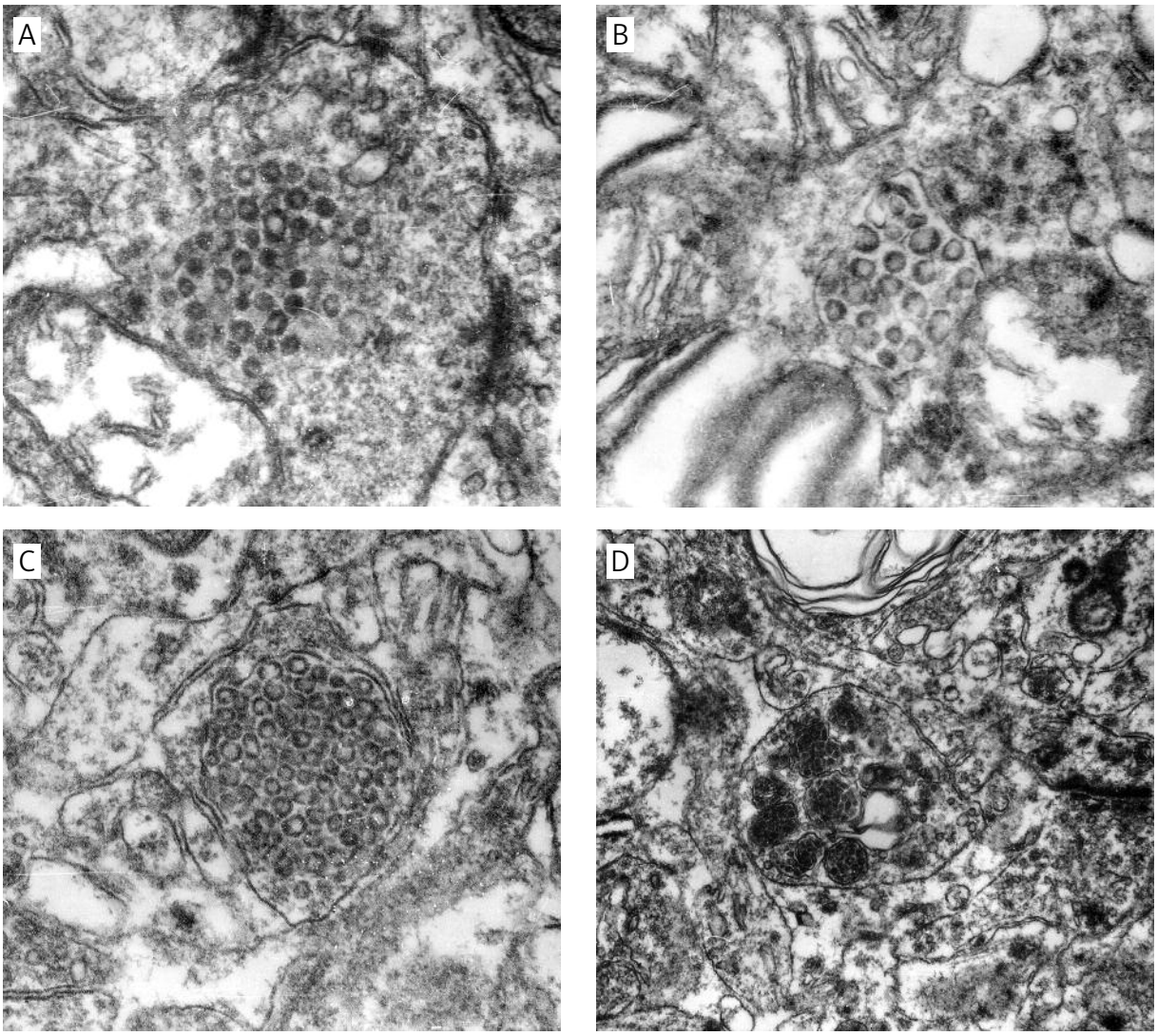

Fig. 5. Four examples of multivesicular bodies. Original magnifications (**A-C**), x 50 000; (**D**), x 30 000.

prise the formation of semi-circular membrane elongations which engulf a target portion of the cytoplasm (a cargo), forming a membrane sac which fuses with a lysosome to form an autophagosome. Such membranes are observed readily in scrapie-affected hamster brain [63] but not so readily in different models.

Several investigators have suggested that autophagy plays a beneficial role in prion infection [28]. For instance, imatinib, an autophagy inducer [93], not only delays the onset of clinical disease following peripheral inoculation but also clears PrP^{Sc} from scrapie-infected cultures [19]. Rapamycin, acting through the target of rapamycin (TOR) reduced the level of PrP^{Sc} and prolonged the incubation when given in the last third of the expected incubation period [28]. Recent observations suggest a "double-edged" role for autophagy [13]. For example, in Alzheimer's disease (AD), another protein-misfolding disorder, upregulation of autophagy contributes to beta-amyloid pathology [68]. This upregulation of autophagy may result in cell-death through distortion of neuronal metabolism or loss of synapses and dendrites. The abundance of dystrophic neurites containing abundant autophagic vacuoles and lysosomal dense bodies has been shown repeatedly in prion diseases and other protein-misfolding diseases [25,36,40, 44,45,52,54,71,72]. In Drosophila transfected with Ab42 (a major amyloidogenic peptide AD plaque forming), Ling *et al*. [68] found increased macroautophagy and dystrophic neurites typical of AD. Rapamycin increased the number of autophagic vacuoles and electron-lucent

areas in dystrophic neurites, probably reflecting enzyme leakage from post-lysosomal autophagic vacuoles, which may lead to neurodegeneration. Also, membrane erosion was seen in Aβ42-Drosophila and we also observed membrane discontinuity in dystrophic neurites in this study. Collectively, the very presence of dystrophic neurites may suggest that autophagy, over certain threshold tolerated by the brain may become deleterious.

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