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Deposition of disease-associated prion protein involves the peripheral nervous system in experimental scrapie

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Abstract There is some evidence that the peripheral nervous system (PNS) is involved in the pathogenesis of transmissible spongiform encephalopathies (TSEs). The TSE-specific abnormal prion protein (PrP^{sc}) is considered as surrogate marker for infectivity. We traced the deposition of PrP^{sc} by immunocytochemistry in sheep and hamsters inoculated intraperitoneally with scrapie. The trigeminal, dorsal root, celiac, thoracic, and nodose ganglia contained ganglion cells and fewer satellite cells with prominent granular PrP^{sc} deposition. As a novel deposition pattern, punctate deposits in adaxonal location were seen along nerve fibers of peripheral nerve adjacent to ganglia. Such prominent involvement of the PNS in two different experimental scrapie models emphasizes the need to consider the PNS in natural scrapie and other TSEs including bovine spongiform encephalopathy as potential source of infectivity.

Key words Transmissible spongiform encephalopathy · Scrapie · Prion protein · Peripheral nervous system · Immunocytochemistry

Introduction

It is unknown by which route(s) the agent of transmissible spongiform encephalopathies (TSEs) spreads to the central nervous system (CNS) after peripheral infection. Since substantial evidence indicative for a link between bovine spongiform encephalopathy (BSE) and a new variant of Creutzfeldt-Jakob disease (vCJD) has emerged [8, 9], understanding of the pathogenesis of TSEs has become a scientific and public health priority. Elegant disease modeling established the requirement for the normal isoform of protease-resistant or prion protein (PrP) in peripheral tissues for TSE pathogenesis, representing a bottleneck for the spread of the infectious agent from peripheral sites to the CNS [5]. A neuro-immune connection in TSE pathogenesis was recently proposed [1]. Indeed, data on the peripheral nervous system (PNS) in natural [11, 13, 26] and experimental scrapie [4, 18, 19], BSE-infected nonhuman primates [6], experimental CJD [21] and BSE [29] suggest a neural pathway to the CNS. In experimental BSE after oral inoculation, infectivity was detected in dorsal root and trigeminal ganglia during preclinical stages of disease [29]. As a consequence, the UK government banned the use of beef-on-the-bone in December 1997, and the European Union struggles to implement a ban on specified risk materials from cattle and small ruminants including sheep. Sheep and goats can also be orally infected with BSE which produces clinical and histopathological symptoms as well as distribution of infectivity currently indistinguishable from scrapie [10]. According to current knowledge, an exposure of small ruminants to BSE infective meat and bone meal (MBM) may have occurred during the massive BSE epidemic in the United Kingdom as well as in countries where British MBMs were fed.

In the context of the ongoing debate, it is mandatory to exactly localize and characterize infected tissues and cells in affected animals. In this study we have, therefore, systematically investigated by immunohistochemistry the distribution of the TSE-specific abnormal protease-resistant or prion protein (PrP^{sc}) deposition in spinal cord, peripheral nerve ganglia and along adjacent nerves of four sheep and four hamsters experimentally infected with scrapie.

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Materials and methods

Experimental scrapie in sheep

Four female Merinoland sheep, aged 4–6 years, came down with disease 41, 44, 50 and 56 months, respectively, after intraperitoneal (0.1 ml of 10% brain homogenate) inoculation with scrapie. These sheep carried the scrapie susceptibility mediating prion gene allele PrP^{ARQ} (i.e., PrP with alanine, arginine and glutamine at the positions 136, 154 and 171) in homozygous form. The PrP genotype of all sheep providing samples was sequenced as described before [16]. Inocula were derived from German field scrapie cases. Tissue samples used as negative controls were obtained from four healthy, non-infected, age-matched sheep of the same PrP genotype.

Tissue samples from brain, spinal cord (cervical, thoracic, lumbar, sacral and coccygeal levels) with respective dorsal root ganglia (DRG), trigeminal, thoracic and celiac ganglia were formalin-fixed for 3 days, immersed in 98–100% formic acid for 1 h and embedded in paraffin. For immunohistochemistry, sections were immersed a second time in 98–100% formic acid for 15 min, then equilibrated in digestion buffer (50 mM TRIS-HCl, 1 mM ethylenediamine tetraacetic acid, 0.1% Tween 20, pH 7.8) for 5 min and treated with proteinase K solution (1 µg/ml in digestion buffer) for 15 min at 37 °C, followed by autoclaving in distilled water for 15 min (121 °C) in a pressure cooker. Antibodies used included monoclonal antibody (mAb) L42 diluted 1 : 500 or polyclonal rabbit serum Ra 38/16 diluted 1 : 2580. The avidin-biotin-complex (ABC) technique was performed using the VECTASTAIN Elite ABC-Kit, using diaminobenzidine or VIP as chromogen.

Experimental scrapie in hamsters

Syrian hamsters were taken from a series of outbred animals intraperitoneally infected with 100 µl of a 10% 263K scrapie brain homogenate [2] and from a series of control animals similarly mock-infected with normal brain homogenate. For the preparation of tissue samples, four male scrapie-hamsters and two male control animals were killed at the end-point of disease [118 ± 12 (SE) days post infection; age of animals: 140–170 days] or at 138 and 144 days post mock infection (age of animals: 170–190 days), respectively. Brain, cervical and thoracic spinal cord with attached DRG and left and right nodose ganglia (NG) with attached vagus nerve were removed. Brains and spinal cords were sliced coronally, all with corresponding left and right DRG. Immunocytochemistry was performed on semi-serial sections from formalin-fixed (fixation time: 4–6 days) and paraffin-embedded tissues, using the 3F4 mouse anti-PrP mAb [17] after section pretreatment with a three-tiered protocol of hydrated autoclaving, concentrated formic acid, and guanidine isothiocyanate [12], using the ABC technique with diaminobenzidine as chromogen. On selected blocks, double staining for PrP (3F4) and neurofilament proteins (Dako, clone 2F11, 1:50) was performed. 3F4 binding was visualized by the ABC technique and anti-neurofilament binding was detected with the alkaline phosphatase-anti-alkaline phosphatase (APAAP) technique with fast Blue BB salt as blue chromogen.

Results

Experimental scrapie in sheep

Spinal cord

A consistent immunolabeling pattern was found throughout the whole length of the spinal cord (cervical, thoracic, lumbar, sacral and coccygeal levels). Fine granular staining was found throughout the gray matter. Intense diffuse immunolabeling was seen in the substantia gelatinosa dorsalis. Granular intracellular deposits were demonstrated in some neurons.

Trigeminal, dorsal root, celiac, and thoracic ganglia

In all diseased animals, some ganglion cells showed dotty intracellular immunolabeling (Fig. 1); PrP^{sc} deposition was occasionally seen also in satellite cells (Figs. 1, 2 A) and, most prominently in the celiac ganglia, along axons of ganglion cells (Fig. 2 A). Such immunoreactivity was similar in both spinal and vegetative ganglia and was seen in all blocks of trigeminal nerve, celiac and thoracic ganglia (the latter two examined in two sheep), all 18 blocks with cervical DRG, all 24 blocks with thoracic DRG, and 16 out of 17 blocks with lumbar DRG (three sheep examined).

Adjacent peripheral nerves

Fine granular immunostaining could be seen occasionally in peripheral nerves adjacent to ganglia, suggesting a pattern of peri-/adaxonally PrP^{Sc} deposition (Fig. 2 B).

Experimental scrapie in hamsters

Spinal cord

As previously described [4], PrP^{sc} was heavily deposited in a mostly granular pattern in most brain areas and the spinal cord. At all levels, the ‘butterfly’ area of spinal gray matter was strongly immunolabeled, whereas mock-inoculated hamsters did not show any signal (Fig. 3).

NG and DRG

The NG and all cervical and thoracic DRG from inoculated animals showed prominent PrP^{sc} deposition as intensely stained cytoplasmic granules. Such immunoreactivity occurred within the majority of ganglion cells and some satellite cells (Fig. 4).

Adjacent peripheral nerves

In hamster spinal root or spinal nerve parts adjacent to DRG of all blocks, and in nerve parts adjacent to the NG and attached vagus nerves, scant punctate deposition was seen along nerve fibers (Fig. 4). This labeling appeared to be located between myelin sheath and axon (Fig. 4 insets).

The distribution pattern was consistent for all brains, spinal cords and ganglia within this scrapie model. Negative staining controls, with normal serum or irrelevant antibody replacing the anti-PrP antibody, did not show any labeling.

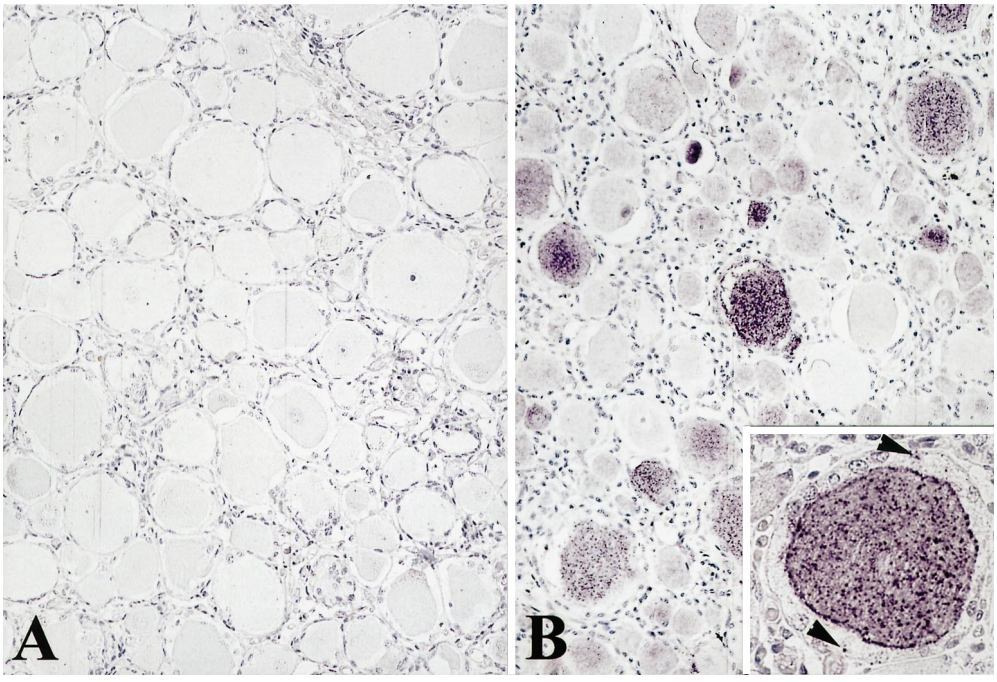

Fig. 1 Spinal ganglia of a healthy sheep (**A**) and of a scrapie-inoculated sheep (**B**) with finely granular PrP^{sc} deposition in many neurons and some satellite cells (*arrowheads* in the *inset* of **B** show tiny deposits in satellite cells) only in scrapie-inoculated animals. **A**, **B** × 110, *inset* × 310

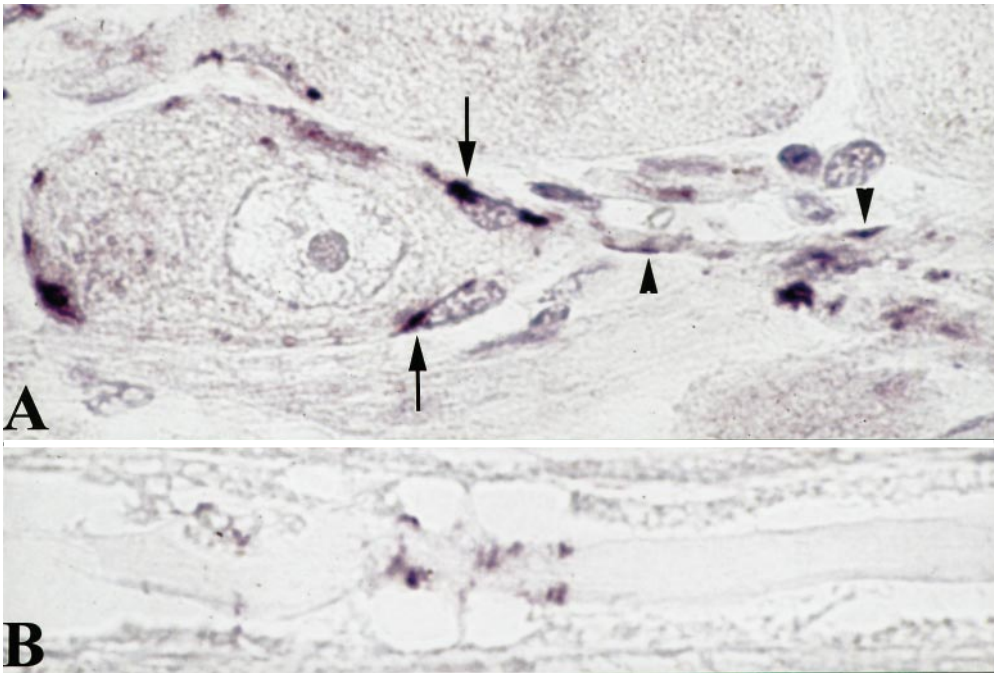

Fig. 2 **A** Celiac ganglion with PrP^{sc} deposition in satellite cells *(arrows)* and along the adjacent axon *(arrowheads)*; exceptionally, this neuron does not show granular PrP^{sc} deposition. **B** Nerve fiber of the trigeminal root; there is finely granular PrP^{sc} deposition on the surface of an axon. **A** × 1500, **B** × 1400

Discussion

In natural scrapie, high amounts of infectivity were detected in brain and spinal cord and, albeit at considerably lower levels, ileum, colon, spleen, several lymph nodes, tonsils, proximal sciatic nerve, thymus, bone marrow, liver, lung and pancreas (in order of decreasing concentrations [13]). In a scrapie-diseased sheep, infectivity was also demonstrated in peripheral nerves such as Nervus (N.) axillaris, N. ulnaris, N. medianus, N. ischiadicus, N. tibialis, N. fibularis and N. saphenus [11]. Deposition of PrP^{sc} has been shown to correlate well with infectivity in scrapie [2, 3, 15, 24]. Moreover, PrP^{sc} is analogous to the infectious agent itself according to the prion theory [23]. PrP^{sc} has been demonstrated, by immunohistochemical methodology, in brain [20, 26], lymphoid tissues [22, 25, 27], trigeminal nerve ganglia and retina (Hardt M, Baron T, Groschup MH, submitted) of scrapie-diseased sheep.

This study of experimental scrapie in two species consistently documents PrP^{sc} in sensory ganglion cells and associated peripheral nerve fibers, and thus further sup-

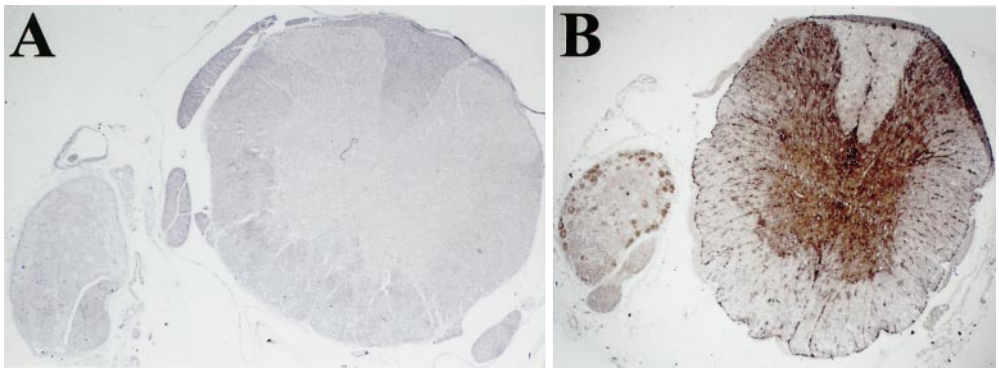

Fig. 3 Low-power view of spinal cord with DRG, in mock inoculated (**A**) and intraperitoneally inoculated (**B**) hamsters. There is abundant immunoreactivity for PrP^{sc} in spinal gray matter and DRG neurons only in scrapie-inoculated animals (*DRG* dorsal root ganglion). **A**, **B** × 24

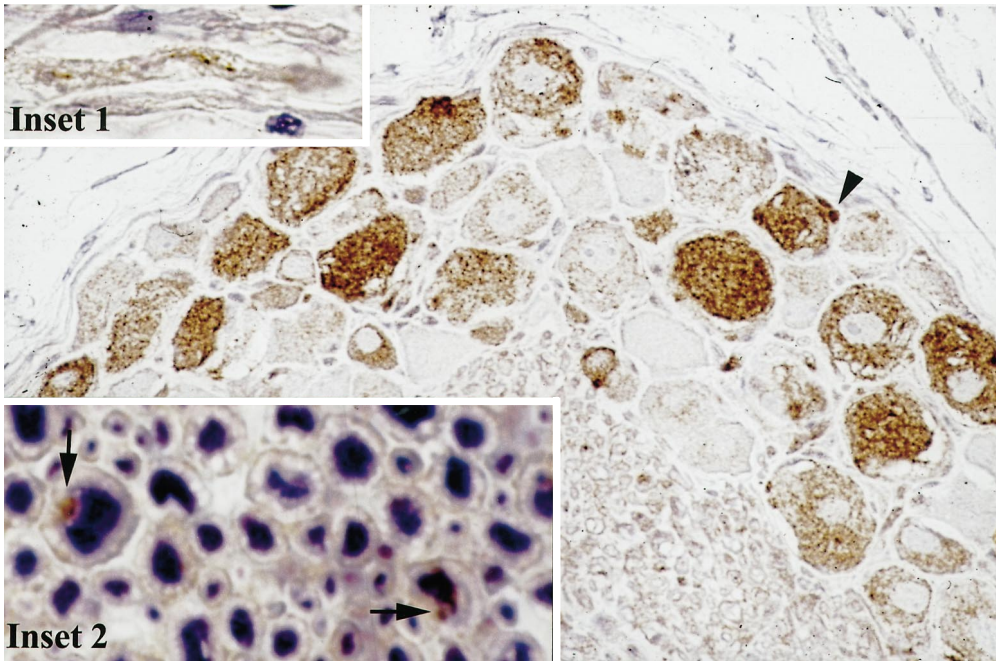

Fig. 4 Higher-power view of DRG in inoculated hamster. There is abundant granular immunoreactivity for PrP^{sc} in the cytoplasm of most ganglion cells and of a few satellite cells *(arrowhead)*. In addition, scant punctate deposition is seen along nerve fibers *(inset 1)*; *inset 2* the PrP^{sc} deposits (*brown label*, indicated by *arrows*) appear to be located between myelin sheath and axon (*blue label* anti-neurofilament protein binding). × 310, *inset 1* × 760, *inset 2* × 1900

ports a role for the PNS in TSE pathogenesis. In the spinal cord of rodents experimentally infected with scrapie, infectivity was previously found to target the mid-thoracic cord (T4–T9) [18]. This region showed also the earliest and most prominent spinal deposits of PrP^{sc} [2, 3]. In the brain, PrP^{sc} appears first in the dorsal motor nucleus of the vagus nerve (DMVN) [4]. Infection of DRG and NG can best be explained by retrograde spread of the TSE agent along splanchnic and vagal nerve fibers to neuronal cell bodies in the spinal intermediolateral nuclei and the DMNV, respectively, and then via dorsal root interconnections and the solitary tract nucleus or by direct anterograde spread along sensory fibers of the splanchnic and vagus nerves [19]. However, this report describes the detection of PrP^{sc} at late-stage disease; thus, it cannot establish the direction of spread of infection, either to the CNS via abdominal nerves, or alternatively by centrifugal spread. Further work is in progress to identify the time course of PNS involvement.

While neuronal PrP^{sc} was deposited as intracytoplasmic granular inclusions in ganglion cells, possibly in lysosomes which may harbor PrP^{sc} [14, 28], the exact site of deposition or transport of PrP^{sc} in peripheral nerve fibers remains to be established. The normal cellular isoform of PrP was shown to traffic by rapid anterograde axonal transport [7], whereas our preliminary light microscopical results indicate periaxonal rather than intra-axonal deposition of PrP^{sc}. The pattern of the adaxonal deposits suggests accumulation in Schwann cell nodal processes (Fig. 2 B) and in the inner loop of Schwann cells (Fig. 4). While we found these deposits consistently in experimental scrapie, similar deposits along peripheral nerve fibers could be found also in one human case each of Gerstmann-Sträussler-Scheinker disease and sporadic CJD (J. A. H. and H. B., Acta Neuropathol, this issue). This suggests that this pattern of nerve fiber involvement as a more generalized phenomenon of both human and animal TSEs.

There is recent concern, although speculative at present, that BSE might have infected UK sheep herds. The pathogenesis of BSE in sheep resembles more closely that of natural and experimental scrapie in sheep than of BSE in cattle [10]. Together with earlier reports [11, 13], our study of experimental scrapie emphasizes the need to consider the PNS as potential source of infectivity, especially

in the event of BSE transmission to sheep. Such involvement of the PNS might develop already before clinical disease manifestation, with obvious implications for potential TSE transmission by oral ingestion.

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