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Autonomous nervous system innervation of lymphoid territories in spleen: A possible involvement of noradrenergic neurons for prion neuroinvasion in natural scrapie

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In experimental as well as in natural scrapie, transmissible spongiform encephalopathies (TSEs), the infectious agent, closely related to PrPsc, an abnormal isoform of the cellular prion protein, invades and replicates in lymphoid organs such as spleen before affecting the brain. To clarify the cellular requirements for the possible neuroinvasion of scrapie agent from the spleen to the central nervous system, we have studied the sympathetic innervation within the lymphoid territories of the spleen. These noradrenergic fibers originating from the coeliomesenteric ganglia were examined with regard to PrPsc-associated cells in spleen of adult sheep severely affected with natural scrapie. Using a double immunolabelling strategy, we demonstrated the proximity of noradrenergic endings with PrPsc-accumulating cells, strengthening its possible implication in the neuroinvasion process. *Journal of NeuroVirology* (2001) **7,** 447–453.

Keywords: noradrenaline; neuroinvasion; PrP; scrapie; tyrosine hydroxylase; immunohistochemistry

Introduction

The transmissible spongiform encephalopathies (TSEs) are neurodegenerative disorders without associated inflammatory syndrome, which include Creutzfeldt-Jakob disease (CJD) in humans, scrapie in sheep and goat, or bovine spongiform encephalopathy (BSE) in cattle. In scrapie, variant CJD and experimental murine TSEs, the immune system probably plays a critical role in the pathogenesis, not only as the initial peripheral site of replication of the TSE's agent, but also with regard to its implication in the neuroinvasion process (Beekes *et al*, 1996; Aguzzi, 1997; Race *et al*, 1998; Bruce *et al*, 2000; Glatzel and Aguzzi, 2000). How TSE's agent propagates from lymphatic tissues to the central nervous system (CNS) is unclear, but lymphoreticular system and peripheral nervous system are considered to be involved in scrapie neuroinvasion, in experimental (Kimberlin and Walker, 1988; Lasmezas *et al*, 1996; Baldauf *et al*, 1997; Klein *et al*, 1997; Groschup *et al*, 1999), as well as in natural disease (O'Rourke *et al*, 2000; van Keulen *et al*, 1996; van Keulen *et al*, 1999; van Keulen *et al*, 2000).

Besides the possible involvement of blood cells in the transport of TSE's agent (Klein *et al*, 1997; Houston *et al*, 2000), the autonomous nervous system that innervates the lymphoid organs may directly contribute to their dissemination from the spleen to the CNS (Fried *et al*, 1986; Groschup *et al*, 1999). Because PrPsc, an abnormal isoform of the cellular prion protein, copurifies with prion infectivity, it is considered as a component of the infectious agent (Bolton *et al*, 1982; Prusiner, 1997). Indeed, in sheep, PrPsc deposits are first detectable in the thoracic spinal cord or in the nucleus parasympatheticus nerve X, both connected to the

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coeliomesenteric ganglia (van Keulen *et al*, 1999; Andréoletti *et al*, 2000; van Keulen *et al*, 2000). In addition, PrPsc accumulation was also described in the enteric nervous system of the gastrointestinal tract, that is also originating from the coeliomesenteric ganglia (van Keulen *et al*, 1999). In this context, the purpose of our work was thus to investigate the distribution of noradrenergic fibers originating from coeliomesenteric ganglia, compared to the topography of PrPsc-associated cells in the spleen of sheep genetically susceptible and affected with natural scrapie. Sheep with severe symptoms of scrapie were used to have a maximum of PrPsc-associated cells in spleen together with PrPsc presence in the CNS and autonomous nervous system. Therefore, in this study using immunohistochemistry we show that in natural scrapie, PrPsc deposition found in spinal cord as well as in spleen is consistent with spread of TSE's agent from the spleen to the CNS (spinal cord and brain) via noradrenergic nerves of coeliomesenteric ganglia.

Results

PrPsc detection in brain, spinal cord, coeliomesenteric ganglia, and spleen of scrapie affected sheep

Besides clinical signs of scrapie, the sheep were diagnosed as infected with scrapie on the basis of cerebral detection of the pathological PrPsc by immunohistochemistry method using 4F2 monoclonal antibody. As an illustration, PrPsc was detected in the granular and molecular layers (Figure 1B) of the cerebellum of scrapie-infected sheep but never in the brain of healthy lamb (Figure 1A). In the spinal cord, PrPsc was detected in the grey matter of the dorsal horn (Figure 2B) as well as in the coeliomesenteric ganglia (Figure 2C). In the spleen, PrPsc was present within all secondary lymphoid follicles, mostly in the germinal centers and few cells labelled in the mantle zone (Figure 2D). Sections in which the primary antibody was omitted and replaced by a normal serum were used to evaluate the background staining that was nonexistent.

Autonomous innervation from coeliomesenteric ganglia to spleen

One anatomical link between spinal cord, coeliomesenteric ganglia, and spleen is represented by noradrenergic neurons of autonomous nervous system (Figure 2A). Using tyrosine hydroxylase (TH) immunohistochemistry, noradrenergic cell bodies of coeliomesenteric ganglia, were identifiable (Figure 3A). As shown after double-labelling experiment most of these neurons presented PrPsc deposits on their membrane (Figure 3B).

Noradrenergic processes from these cell bodies innervate the spleen; endings were located within the trabeculae (data not shown), around blood vessels (Figure 3C), within the white pulp, in the mantle zone near immune cells, in the outer zone, between germinal centers and mantle zone (Figure 3D). Using double-labelling experiments, noradrenergic fibers were seen adjacent and even close to PrPsc-accumulating immune cells detected with 4F2 mAb (Figure 3E, F) in almost all the germinal centers.

Discussion

In naturally occurring scrapie, it is assumed that the infection is initiated in the alimentary tract (Hadlow *et al*, 1982) and PrPsc—the TSE-specific abnormal prion protein (Bolton *et al*, 1982)—is first detectable in lymphoid tissues associated to intestine and also in tonsils and spleen (O'Rourke *et al*, 1998; Heggebo *et al*, 2000). In the next step of infectious agent spread, PrPsc is detected in peripheral nervous system in naturally affected sheep (van Keulen *et al*, 1999; Andréoletti *et al*, 2000; van Keulen *et al*, 2000), as

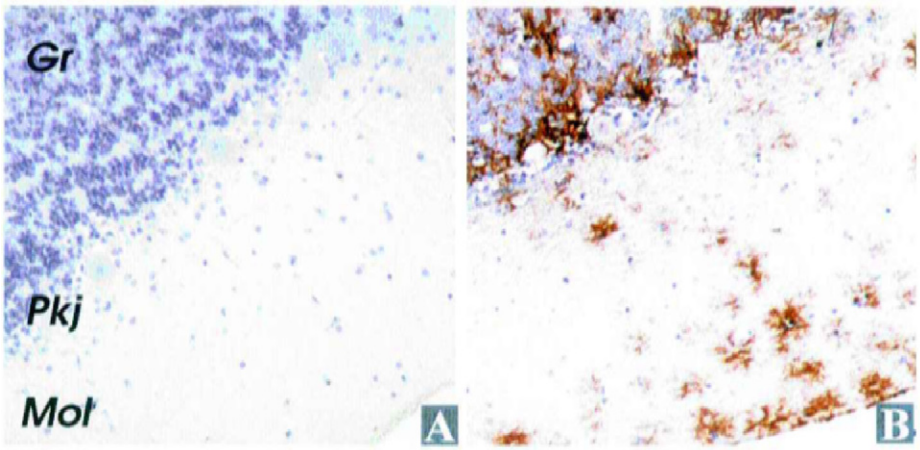

Figure 1 In contrast to the brain of a healthy lamb (A, ×100), in scrapie-infected sheep, PrPsc is detected using 4F2 antibody in the granular (Gr) and molecular (Mol) layers of the cerebellum, and not in Purkinje (Pkj) cells (B, ×100).

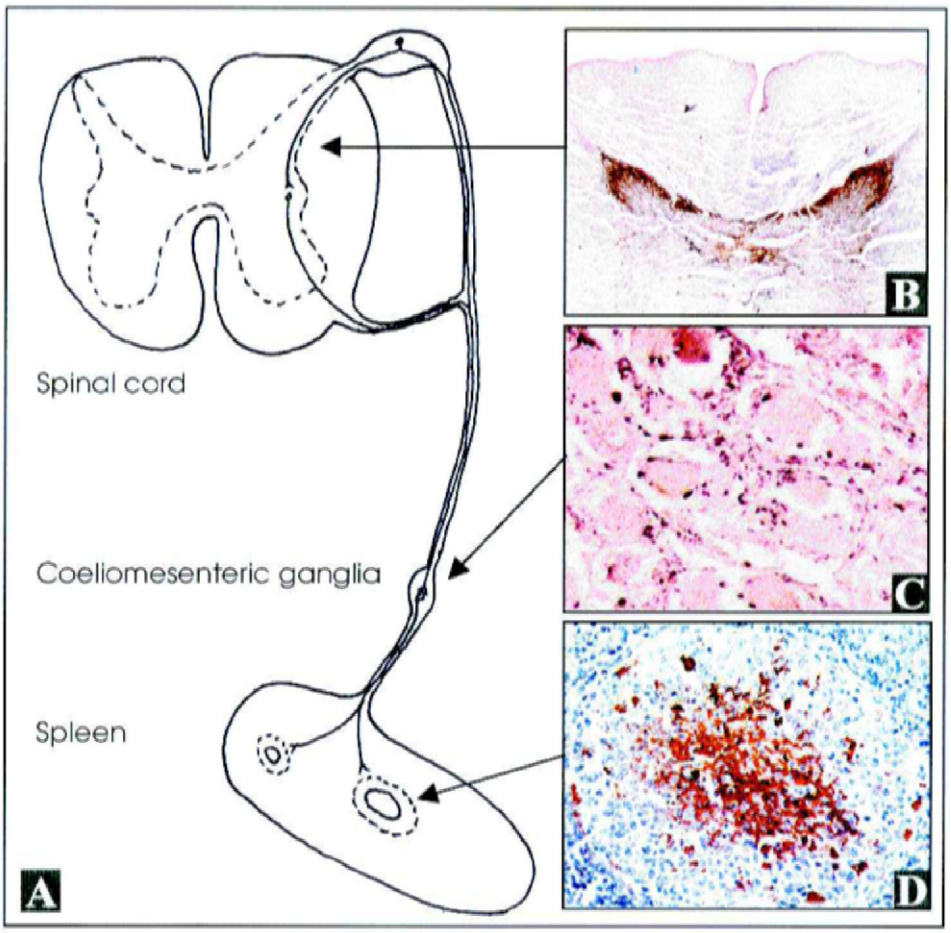

Figure 2 Schematic sympathetic innervation of spleen from spinal cord *via* coeliomesenteric ganglia (A). PrPsc accumulation sites detected using 4F2 antibody. In thoracic spinal cord, PrPsc was observed in the dorsal horns (B, ×20, brown deposits); in coeliomesenteric ganglia PrPsc was seen surrounding neural cell bodies (C, ×200, dark deposits); in spleen PrPsc was detected within germinal centers of secondary lymphoid follicules (D, ×100, red deposits).

in sheep inoculated intraperitoneally with scrapie (Groschup *et al*, 1999). Similarly, the distribution of PrPsc described in the present study supports the idea of an autonomous nervous system implication in neuroinvasion from the spleen in natural disease. The type of cells involved in the transport of prion infectivity from the lymphoid organs to the central nervous system (CNS) is actually unknown. Besides the conceivable transportation of TSE's agent by blood cells (Klein *et al*, 1997; Houston *et al*, 2000), another possibility is the involvement of peripheral nervous system. Indeed, in experimental models such as hamsters fed with scrapie, vagal nerve was identified as an alternative neuroinvasion pathway from the visceral organs to the medulla (Beekes *et al*, 1996; Baldauf *et al*, 1997; Beekes *et al*, 1998).

The present immunohistochemical study, using the tyrosine hydroxylase, a monoaminergic synthesising enzyme, allows to precise the nature of neuronal pathways potentially involved in prion spread. Our data revealed that PrPsc distribution, in the coeliomesenteric ganglia of naturally scrapie-affected sheep, was associated with noradrenergic neurons. PrPsc detected in coeliomesenteric ganglia might be due to TSE's agent transportation by blood cells, but because their localisation was restricted to cell bodies and along cell processes of the ganglia, it is more likely originating from the CNS or spleen, considering the neuroanatomical link between these organs. Indeed, PrPsc may be transported either in an anterograde or retrograde manner because PrPsc is detectable in the spleen of mice infected intracranially as well as in the brain after a peripheral inoculation (Fraser, 1970; Kimberlin *et al*, 1983a).

In addition, kinetic studies of PrPsc spread strongly suggested a very low transportation of TSE's agent that have been indirectly evaluated as being around 1 to 2 mm/day (Kimberlin *et al*, 1983b). This rate of spread is compatible with the slow axonal transport described in sympathetic neurons ranging from 0.2 to 8 mm/day (Hirokawa, 1997). This slow anterograde transportation is linked to different cytoskeleton components such as neurofilament or clathrine that might be coupled to PrPc, as recently shown for

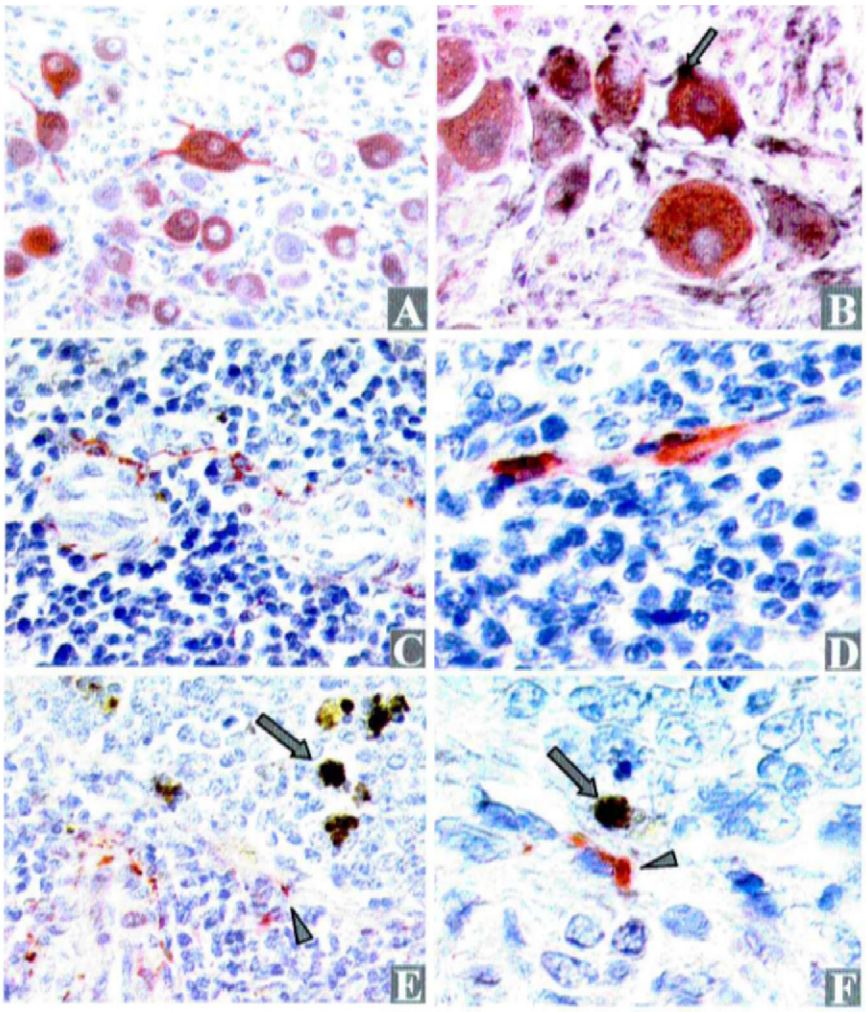

Figure 3 In the coeliomesenteric ganglia of scrapie-infected sheep, noradrenergic neurons were identifiable using anti-TH antibody (A, ×200, red deposit). A double-immunolabelling experiment showed that some noradrenergic cell bodies (B, ×400, red deposit) were associated with PrPsc on the cell membranes (B, black deposit, arrow). In the spleen, the noradrenergic fibers were identifiable using the same anti-TH antibody (red deposit) around blood vessels (C, ×200) and near several immune cells at the periphery of germinal centers (D, ×400). In double-labelling experiments, noradrenergic fibers (red deposit, arrowheads) are detected at proximity of PrPsc-expressing cells (black deposit, arrows), revealed using 4F2 antibody (E, ×400, and F, ×600).

caveolin in noradrenergic neurons (Mouillet-Richard *et al*, 2000). Our study in natural disease cannot clarify whether this possibility is most probable or if an ad-axonal transportation should be suggested. Nevertheless, we demonstrated that noradrenergic fibers, originating from these noradrenergic neurons of coeliomesenteric ganglia, entered deep inside the ovine spleen. The great majority of these noradrenergic fibers were detected around blood vessels, which is consistent with a classical role of blood pressure control or regulation of spleen contraction via the noradrenergic innervation detected in the capsule (Fried *et al*, 1986; Bencsik *et al*, 2000).

More interestingly, the close vicinity of noradrenergic fibers with PrPsc-associated cells within the follicular area together with the colocalisation of PrPsc within cell bodies of the coeliomesenteric ganglia comfort the idea of an implication of sympathetic nervous system in the transport of TSE's agent from the spleen to the CNS. Indeed, in sheep noradrenergic nerve terminals may make direct contacts with lymphoid cells, as demonstrated in rat spleen where, by electron microscopy studies, it was shown that they were separated by a gap junction of only 6 nm (Felten and Felten, 1988). If it is the case in sheep, it would be evocative of a very narrow relationship between immune and nervous system in prion spread within the body, as gap junctions are sites of cytoplasmic communication between adjacent cells, mediating diffusion of metabolites and signalling molecules (Saez *et al*, 2000). Besides, the intensity of PrPsc staining in follicular domains of spleen from natural scrapie cases vary not only with the stage of the disease but also with the breed and genotype of sheep (Hunter *et al*, 1996, 1997, 2000). Thus, it would be interesting to extend our study to other genotypes,

particularly $VV_{136}RR_{154}QQ_{171}$, considered as more susceptible to scrapie than $AV_{136}RR_{154}QQ_{171}$ and to $AV_{136}RR_{154}RQ_{171}$ that have been recently shown to accumulate PrPsc solely in the nervous system and not in the lymphoid tissue (Andréoletti *et al*, 2000). Nevertheless, in an older study, a $AV_{136}RR_{154}RQ_{171}$ sheep was found positive in the spleen, suggesting that the strain of scrapie may be also important to take into account (Somerville *et al*, 1997).

Finally, because the different strains of TSE's agent target different neuronal populations in the brain, we are currently attempting to analyse whether other types of neurons than noradrenergic in the coeliomesenteric ganglia may be used by the infectious agent as particular channels for its spread from the periphery to the CNS.

Materials and methods

Tissues

The brain, spinal cord, coeliomesenteric ganglia, and spleen of three sheep (3-year-old) with severe symptoms of scrapie, with a susceptible genotype to the disease ($AV_{136}RR_{154}QQ_{171}$) (Bossers *et al*, 1996; Hunter *et al*, 1996; Elsen *et al*, 1999) from the same flock of Texel × Charolais breed (Ardennes department) were fixed in 10% buffered formalin. As a control, spleen of a healthy sheep, with a genotype known as resistant to scrapie ($AA_{136}RR_{154}RR_{171}$) (Bossers *et al*, 1996; Hunter *et al*, 1996; Elsen *et al*, 1999) coming from another flock in which some clinical scrapie cases occurred in genetically susceptible animals (Prealpes breed, Drôme department) was immersed in buffered 10% formalin. Once fixed, samples were rinsed for a week in cold phosphate buffer (PBS 0.1 M, pH 7.4), then routinely dehydrated and embedded in paraffin. To ensure adhesion, sections (5 μm) were collected onto pretreated glass slides (Polylysin or StarFrost, Fischer Scientific, Pittsburgh, PA) and baked overnight at 57°C.

Antibodies

To detect the PrPsc in the scrapie-affected sheep, we used the 4F2 monoclonal antibody (courtesy of Pr G. Hunsmann, German Primate Center, Göttingen, Germany) (Krasemann *et al*, 1996). It was possible to distinguish the immunolabelling of PrPc from the pathological PrPsc by applying pretreatments described in the following staining procedure. Noradrenergic fibers of the autonomous nerve system were identified using a rabbit polyclonal antibody against the tyrosine hydroxylase (TH) (Institut Jacques Boy, France), the rate limiting enzyme in the synthesis of noradrenaline (Björklund and Hökfelt, 1985).

Immunostaining procedure

The slides were dewaxed and rehydrated in water, then used for immunohistochemical analysis. Every step was done at room temperature (24°C). Endogenous peroxidase activity was inhibited with 10-min incubation in 3% hydrogen peroxide in PBS, 0.1 M. Unspecific antigenic sites were blocked by a 30-min step in blocking reagent (Roche-Boehringer). Then the sections were incubated overnight with the primary antibody (TH, 4F2, Table 1). The sections were rinsed before the detection of the primary antibody using the ABC system (Vector), succinctly using first the biotinylated anti-rabbit (Vector) or anti-mouse antibody (Cliniscience) (30 min) and secondly a peroxidase-labelled avidin-biotin complex (ABC Vector) (30 min). These steps were followed by a 5-min wash and finally peroxidase was revealed by immersing the sections in aminoethylcarbazole solution (AEC, Dako) giving red deposits, in DAB (brown deposit), or DAB intensified with $NiCl_2$ (Biosys), thus giving black deposits.

To assess the specificity of pathological PrPsc immunostaining several pretreatments were necessary (Bell *et al*, 1997). Hydrated autoclaving (immersed in PBS, 0.1 M, pH 7.4, 15 min at 121°C in a pressure cooker), combined with an immersion for 15 min in 98% formic acid (Merck), proteinase K treatment (Roche-Boehringer, 20 μg/ml in PBS, 0.1 M, pH 7.4, 37°C for 15 min), or guanidium thiocyanate (Sigma, 4 M, for 60 min at room temperature) were applied to sections and led to the detection of the PrPsc form solely.

Double-staining procedure

We developed a double-immunostaining procedure, allowing the simultaneous detection of PrPsc and NA endings, by modulating the pretreatment sequences (5 min in formic acid and 10 min in PK instead of 15 min for each, but maintaining the hydrated autoclaving step, 15 min at 121°C), using both 4F2 antibody and anti-TH antibodies to visualise PrPsc-accumulating cells and noradrenergic cell bodies as well as fibers (Table 1). Following the first immunohistochemistry procedure, the

Table 1 Primary antibodies used for the detection of PrPsc, noradrenergic (NA) neurons and endings

Antibodies	*Species*	*Dilutions*	*Origin*
4F2 (PrPhu 79-92)	Mouse	1/200	GPC, Göttingen, Germany
TH (Tyrosine hydroxylase)	Rabbit	1/200	Jacques Boy Institute, France
Biotinylated anti-rabbit (secondary antiboby)	Goat	1/100	Vector, France
Biotinylated anti-mouse (secondary antiboby)	Rabbit	1/200	Zymed, Cliniscience, France

second one was performed, beginning with a new blocking step (Roche-Boehringer, 30 min). Then, the same procedure was applied. The specificity of the double-immunolabelling procedure was evaluated using healthy sheep samples, in which we checked that only TH immunolabelling was observable and neither PrPsc nor PrPc were detectable. In addition, on diseased samples we also controlled that there was no alteration of the first immunolabelling when in the second immunohistochemistry step the HRP complex was omitted. To reveal the histological organisation of the each sample of tissues, a very weak staining was obtained using aqueous haematoxylin. Finally, slides were mounted with Crystal Mount medium and observed under a microscope coupled to an image analysis workstation (ßiocom, Les Ulis, France).

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