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CHARACTERIZATION OF SHEEP (*OVIS ARIES*) PALATINE TONSIL INNERVATION

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Abstract—Palatine tonsils (PTs), together with ileal Peyer's patches, rank among the first colonization sites for infectious prions. After replicating in these lymphoid tissues, prions undertake the process of "neuroinvasion," which is likely mediated by the peripheral nerves connecting lymphoid tissues to the central nervous system (CNS). To study the connections between the tonsils and the CNS, we injected fluorescent tracers into the PTs of lambs; the highest number of Fast Blue (FB)–labeled neurons was found in cranial cervical ganglia (CCG), whereas a progressively decreasing number of cells were detected in proximal glossopharyngeal, proximal vagal, trigeminal, pterygopalatine, and cervicothoracic ganglia. Immunohistochemistry was carried out on tonsil and ganglia cryosections. Immunoreactivity (IR) for tyrosine hydroxylase (TH), dopamine β-hydroxylase (DBH), neuronal nitric oxide synthase (nNOS), calcitonin gene-related peptide (CGRP), substance P (SP), and calcium-binding protein S100 (S100), was observed in the fibers around and within PT lymphoid nodules. In the trigeminal, proximal glossopharyngeal and vagal ganglia the retrogradely-labeled neurons showed nNOS-, SP- and CGRP-IR. In all ganglia some retrogradely-labeled neurons showed nNOS-, SP- and CGRP-IR colocalization. It is worth noting that only 66±19% and 75±13% of retrogradely-labeled neurons in CCG showed TH- and DBH-IR, respectively. The present results allow us to attribute PT innervation mainly to the sympathetic component and to the glossopharyngeal, vagal and trigeminal cranial nerves. Furthermore, these data also provide a plausible anatomic route through which infectious agents, such as prions, may access the CNS, i.e. by traveling along several cranial and sympathetic nerves, as well as by migration via glial cells.

Key words: fluorescent tracers, immunohistochemistry, sympathetic innervation, prion diseases.

The palatine tonsils (PTs) are one of the components of Waldeyer's ring (vonWaldeyer-Hartz, 1884), along with the lingual, pharyngeal, tubal, paraepiglottic, and soft palate tonsils. The components of Waldeyer's ring, together with gut-associated lymphoid tissue (GALT), play a key role in protecting the body against ingested microorganisms. GALT is composed of several types of lymphoid nodules, especially in the small and large intestine, including Peyer's patches (PPs), the isolated lymphoid nodules and cryptopatches found in the small intestine, and the lymphoglandular complexes in the large intestine (Cesta, 2006). PTs and ileal PPs seem to be the first colonization and replication sites of prions, the infectious agents causing human and animal transmissible spongiform encephalopathies (TSEs), following oral infection with sheep scrapie (the TSE "prototype" disease) and bovine spongiform encephalopathy (BSE) agents (Andreoletti et al., 2000; Jeffrey et al., 2001; van Keulen et al., 2002). After replication in PTs and ileal PPs, infectious prions take part in a process called "neuroinvasion," a long journey from the host's peripheral tissues to the CNS (Aguzzi and Polymenidou, 2004; Mabbott and MacPhearson, 2006; Aguzzi and Heikenwalder, 2006; Beekes and McBride, 2007). Replication of TSE agents in lymphoid tissues appears to be essential for an efficient neuroinvasion (Press et al., 2004; Mabbott and MacPherson, 2006). Following lymphoid tissue colonization, prions presumably spread to the CNS along sympathetic and parasympathetic nerve fibers. These fibers belong to the autonomic nervous system (ANS) and are of the general visceral efferent (GVE) type (Brodal, 1981; Jenkins, 1983); however, general visceral afferent (GVA) fibers could also be a pathway for prion neuroinvasion (Press et al., 2004; Beekes and McBride, 2007).

During sheep scrapie, the first CNS structures affected by the typical spongiform lesions are the parasympathetic nucleus of the vagus nerve (PNV) and the intermediolateral cell column (IML) of the spinal cord segments T8–T10 (Andreoletti et al., 2000; van Keulen et al., 2000, 2002). From these structures, prions then spread bi-directionally (caudo-cranial and cranio-caudal, respectively) to the ad-

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Abbreviations: ANS, autonomic nervous system; CCG, cranial cervical ganglia; CGRP, calcitonin gene-related peptide; CNS, central nervous system; CT-FITC, cholera toxin subunit B; CTG, cervicothoracic ganglia; DBH, dopamine β-hydroxylase; DGG, distal glossopharyngeal ganglia; DiI, carbocyanine dye, 1,10, di-octadecyl-3,3,30,30,-tetramethylindo-carbocyanine perchlorate; DY, Diamidino Yellow dihydrochloride; FB, Fast Blue; FITC, fluorescein isothiocyanate; GALT, gut-associated lymphoid tissue; GG, geniculate ganglia; GVA, general visceral afferent; GVE, general visceral efferent; IHC, immunohistochemistry; IML, intermediolateral cell column; IR, immunoreactivity; MG, mandibular ganglia; NOS, nitric oxide synthase; OG, otic ganglia; PBS, phosphate-buffered saline; PGG, proximal glossopharyngeal ganglia; PNV, parasympathetic nucleus of the vagus nerve; PPG, pterygopalatine ganglia; PPs, Peyer's patches; PrP^{Sc}, infectious prions–scrapie; PTs, palatine tonsils; PVDF, polyvinylidene fluoride; PVG, proximal vagal ganglia; RT, room temperature; SG, spinal ganglia; SP, substance P; S100, calcium binding protein S100; TBST, 10 mM Tris–HCl, 133 mM NaCl, and 0.2% Tween 20; TG, trigeminal ganglia; TH, tyrosine hydroxylase; TSEs, transmissible spongiform encephalopathies; WB, Western blot.

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Table 1. Injection sites of fluorescent tracers in experimental animals

	Lamb 1	Lamb 2	Lamb 3	Lamb 4	Lamb 5	Lamb 6	Lamb 7
Right PT	FB	—	—	FB	FB	DY	FB
Left PT	FB	FB	FB	FB	FB	DY	FB
Right vagosympathetic trunk	—	—	—	—	DY	FB	DiI
Tongue	—	—	DY	—	—	—	—

Dashes indicate no injected sites.

jacent CNS segments (Brandner, 2003). The simultaneous involvement of PNV and IML is difficult to explain if we assume that prions reach these grey matter districts from the same portal of entry. PPs are a plausible starting point from which prions could reach and colonize the IML, although the PNV, which is located at a greater distance from ileal PPs than the IML, is unlikely to be simultaneously involved. Since PTs are considered a putative prion entry site in sheep scrapie (Andreoletti et al., 2000; van Keulen et al., 2002), they could perhaps play a role in early PNV involvement. This theoretical "neuroinvasion model" could provide a more plausible explanation for the simultaneous prion colonization of both the PNV and the IML through neural dissemination. This could also be explained by presuming a hematogenous route of prion transfer to the CNS. Nevertheless, some experimental data, such as those demonstrating inhibition or delay of scrapie progression after chemical and immunological sympathectomy, would argue in favor of prion dissemination through peripheral nerves (Glatzel et al., 2001; Press et al., 2004).

In veterinary anatomy treatises, PT innervation is ascribed to the tonsillar branch of the glossopharyngeal nerve (Habel, 1975; Barone, 1981). Nevertheless, since some nerve fibers that reach the pharynx could also be involved in PT innervation and, since there is no agreement among the various authors on the latter (see Discussion), an in-depth investigation of PT innervation would be worthwhile.

In the present paper, we studied the innervation of sheep (*Ovis aries*) PTs using both retrograde tracer and immunohistochemistry (IHC) techniques. Our main objective was to evaluate the entity of PT innervation by specific peripheral nerves. A further aim was to evaluate the phenotype of the fibers and neurons innervating ovine PTs. Our data should provide a better definition of PT innervation in sheep and, consequently, identify some additional pathways putatively utilized by sheep scrapie prions and, possibly, also by other TSE agents during neuroinvasion.

EXPERIMENTAL PROCEDURES

All the procedures described below were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and with Italian legislation regarding experimental animals, after approval by the Scientific Ethics Committee for Experiments on Animals of the University of Bologna (Prot Rif. BQ/af PROT. 50932-X/10–All. 98). All possible efforts were made to minimize the number of animals used and their suffering.

Injection of retrograde fluorescent tracers (Table 1)

In preliminary experiments, four different types of fluorescent tracers were tested: the retrograde tracers Fast Blue (FB; 2% aqueous solution; Sigma-Aldrich Chemie, Steinheim, Germany, code F5756) and Diamidino Yellow dihydrochloride (DY; 2% aqueous solution; Sigma-Aldrich, code D0281), along with the anterograde and retrograde tracers cholera toxin subunit B (CT-FITC; 10 mg/ml in PBS 0.01 M; Sigma-Aldrich; code C1655), and the carbocyanine dye, 1,10,di-octadecyl-3,3,30,30,-tetramethylindo-carbocyanine perchlorate (DiI; 2% in dimethyl sulfoxide; Molecular Probes, Eugene, OR, USA, code D-282). At the end of the preliminary tests, we chose to inject only FB and DY tracers into the tonsils, although DY did not retrogradely label a large number of neurons. The DY, FB and DiI tracers were also injected in the vagosympathetic trunk (see below). The CT-FITC and DiI tracers failed to label any neuronal cells and fibers after injection into the PTs.

Surgical procedures. Six young (2–4 months old) and one older (9 months old) female sarda breed lamb were maintained on a diet based on hay and water *ad libitum* for 1 week before the experiment. For 24 h prior to surgery, the animals were not given any food. They were tranquilized using atropine sulfate (0.04 mg/kg i.m., ATI s.r.l., Ozzano Emilia, Bologna, Italy), xylazine hydrochloride (0.22 mg/kg i.m., Rompun®; Bayer S.p.A., Milano, Italy) and acepromazine maleate (0.07 mg/kg i.m., Prequillan®; ATI s.r.l., Ozzano Emilia, Bologna, Italy). After recumbency and venous catheterization, induction and deepening of anesthesia were achieved by a rapid injection of thiopental sodium (10 mg/kg i.v., Pentothal sodium®; Intervet Productions s.r.l., Aprilia, Latina, Italy).

The fluorescent tracer was injected through the soft palate, after opening the mouth manually. To better visualize the PTs, endotracheal intubation was carried out only after PT injection in the animals scheduled to undergo injection of the vagosympathetic trunk (lambs 5, 6 and 7). In these subjects, intubation allowed the use of gaseous anesthesia (Isoflurane Vet; Merial Italia SpA, Milano, Italy) until the end of the surgical procedure.

After opening the mouth and illuminating the PTs with a straight blade laryngoscope, the fluorescent retrograde tracer FB was injected with a 10 μl Hamilton microsyringe. The needle was introduced into two sites at the level of the opening of the tonsillar crypts; a total amount of 10 μl FB was slowly injected into each PT. The syringe was gently withdrawn and any tracer leakage was removed from the palatine mucosa. It should be noted that this operation, in older ovine, is very difficult due to the increased distance between the mouth opening and PT location.

In five lambs (lambs 1, 4, 5, 6, and 7), the PTs were bilaterally injected with tracer, whereas lambs 2 and 3 received the tracer only in the left tonsil.

To verify whether both the glossopharyngeal and vagus nerves are involved in PT innervation, two additional experiments were carried out. The first one aimed at defining the involvement of the sensory component of the glossopharyngeal nerve in PT innervation; we therefore injected one animal (lamb 3) with 10 μl FB in the left PT and 100 μl DY between the middle and the caudal third of the left side of the tongue, since this area should be innervated by the glossopharyngeal nerve but not the vagus (Barone, 1981). In the second experiment, in order to demonstrate the role of the vagus nerve in PT innervation (and, hence, try to distinguish the proximal glossopharyngeal ganglia—PGG—from the proximal vagal ganglia—PVG), we injected three additional lambs with 10 μl DY (lamb 5), or FB (lamb 6), or DiI (lamb 7) in the