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Research paper

Features of follicular dendritic cells in ovine pharyngeal tonsil: An in vivo and in vitro study in the context of scrapie pathogenesis

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ABSTRACT

Although the alimentary tract has been suggested as the most likely portal of entry in natural scrapie, a growing amount of data indicates that the respiratory system and more specifically the pharyngeal tonsils serve as a natural portal of entry for scrapie.

This study describes for the first time the broad cell populations in the lymphoid compartment of pharyngeal tonsils and more specifically inside the lymphoid follicles where the scrapie agent accumulates during the period of latency.

Follicular dendritic cells (FDCs), stromal cells located in the light zone of the germinal centre of lymphoid follicles, seem to be the principal causal factor in the accumulation of the infectious agent in transmissible spongiform encephalopathy (TSE) diseases. Knowing that efficient lymphoreticular prion propagation requires PrPc expression, we analysed the expression of PrPc with the mouse monoclonal antibody Pri 909 both *in situ* and on FDC-cluster-enriched cell suspensions.

In situ, a positive staining was observed in the germinal centre of pharyngeal lymph follicles. The germinal centre labelling was due to the presence of a follicular dendritic network as revealed after immunogold staining of isolated FDC clusters. Our results suggest that the pharyngeal lymphoreticular system and more specifically PrPc expressing follicular dendritic cells could serve as a prion "reservoir" during the latency phase, thus playing a key role during the scrapie lymphoinvasion.

1. Introduction

In humans and in many animal species, the tonsils are a part of the pharyngeal mucosal immune system (Ogra, 2000) and form a ring of lymphoid tissue in the pharyngeal wall, known as the Waldeyer's ring (Perry and Whyte, 1998).

In sheep, the palatine (*tonsilla palatina*), the lingual (*tonsilla lingualis*) and the tonsil of the soft palate (*tonsilla veli palatini*) are located in the oropharynx; the pharyngeal (*tonsilla pharyngea*) and tubal tonsils (*tonsilla tubaria*) in the nasopharynx, and the paraepiglottic tonsil (*tonsilla paraepiglottica*) in the laryngopharynx (Barone, 1997). Defined as tonsils with or without crypts based upon their relationship with the surface epithelium, they consist mainly of secondary lymph follicles separated by T-dependent zones, areas through which antigens can pass and where effector cells accumulate.

In sheep, the palatine and the pharyngeal tonsils contain the largest volume of lymphoid tissues and the greatest epithelial surface area of the oropharyngeal and the

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nasopharyngeal parts of the Waldeyer's ring, respectively (Casteleyn et al., 2007, 2008). Palatine tonsils are bilateral, are the size of a hazelnut and present one to three crypts covered by the buccal epidermoid epithelium (Gabriel and van den Broeck, 2003; Cocquyt et al., 2005). The non-cryptic pharyngeal tonsil is a unique and circumvoluated elevation located on the caudal part of the pharyngeal septum. The lymphoid compartment consists of secondary follicles lying underneath the pseudostratified columnar ciliary epithelium.

Massive intraepithelial lymphocyte infiltration suggests that these tonsils are perfectly adapted to sample foreign antigens.

Relatively easily accessible, palatine and pharyngeal tonsils thus appear to be of strong interest in the diagnosis of several diseases. It is well known that animal tonsils are the sites of entry and replication of several pathogens. Studies on the foot and mouth disease virus (FMDV) in sheep confirm that the tonsils are the main predilection sites for the persistence of this aphtovirus (Kitching and Hughes, 2002; Horsington and Zhang, 2007). High levels of PrPd have been detected in palatine tonsil follicles of sheep naturally infected with the scrapie agent (Jeffrey et al., 2001). Tonsillar biopsies can thus be useful in guaranteeing food safety (Thuring et al., 2002). Infection via the nasal route may also be possible in scrapie infected flocks. Recent experiments postulate that the upper respiratory tract, and more specifically the pharyngeal tonsil, serves as a natural portal of entry for the scrapie agent (Hamir et al., 2008). This hypothesis is sustained by the presence of microfold M cells in the ovine nasopharyngeal mucosal epithelium (Stanley et al., 2001; Casteleyn et al., 2010), since these cells have been shown to efficiently transcytose prion infectivity in an in vitro co-culture system (Heppner et al., 2001).

The mucosa-associated lymphoid tissues play an important role in scrapie pathogenesis. PrPd accumulates inside the germinal centres (McBride et al., 1992) before spreading to the sites of neuroinvasion. The cells implicated in prion pathogenesis are follicular dendritic cells (FDCs) which reside in the germinal centre (Jeffrey et al., 2000; Mabbott and Bruce, 2001). Numerous studies using chimaeric mice indicate that mature follicular dendritic cells are necessary for prion propagation within the lymphoreticular system (Brown et al., 1999). In the absence of mature FDCs, neuroinvasion following peripheral challenge is significantly impaired in scrapie pathogenesis (Mabbott et al., 2000; Montrasio et al., 2000). The ability to recognise ovine FDCs is limited. In fact, among the various anti-FDC antibodies commercially available, DRC1 (Naiem et al., 1983; Johnson et al., 1986), FDC-M1 (Kosco et al., 1992) and CNA.42 (Raymond et al., 1997; Lezmi et al., 2001) all fail to recognise specifically FDCs from all the secondary ruminants lymph organs. FDC-B1, a new mouse monoclonal antibody, has recently been produced and characterised by Melot et al. (2004). The antigen detected by FDC-B1 is expressed exclusively on the surface of FDCs in all ruminant lymphoid organs.

The aims of our study were (i) the identification of the FDCs inside the pharyngeal tonsil; (ii) the study of their morphological characteristics and (iii) the analysis, in vivo and in vitro, of the PrPc expression on their cell membrane. This approach will be useful for future comparative studies on the implication of the FDC network in the replication of the scrapie agent in the nasopharyngeal mucosa of sheep with different polymorphisms in the host PrP gene.

2. Materials and methods

2.1. Sample collection

Pharyngeal and palatine tonsils were obtained at a local abattoir from clinically healthy sheep aged from 4 to 6 months. These animals showed the ARR/ARR genotype resistant toward scrapie.

2.2. Immunohistochemistry

Some specimens were immersed in Tissue-Tek OCT embedding medium (SAKURA, Zouterwoude, the Netherlands), snap-frozen and stored at −20 °C until processing for immunohistochemistry. Sections were cut at −15 °C with a microtome (MICRON HM 500 OM) and deposited on glass slides coated with poly-L-lysine (Sigma, St. Louis, USA), air-dried, fixed in acetone for 10 min at 4 °C, and stored at −80 °C until use.

After rehydration, the cryosections were incubated for 1 h at room temperature with antibodies directed against the selected lymphoid cell populations or with those which react with ovine PrPc. Immune cells were stained with the following antibodies: *follicular dendritic cells* (FDCs) identified with two mouse monoclonal antibodies: FDC-B1 (undiluted hybridoma supernatant) kindly provided by Melot et al. (2004) and CNA.42 (1/100) (Dako, Glostrup, Denmark), *B cells* revealed with a mouse anti-human CD79 αcy mAb (clone HM 57) (1/1000) (Dako, Glostrup, Denmark) and *T cells* with a rabbit anti-CD3 polyclonal antibody (1/600) (Dako, Glostrup, Denmark). Cellular prion protein expression was analysed on tissue sections and on FDC clusters with a mouse anti-human monoclonal antibody Pri 909 (SPIbio, Montigny le Bretonneux, France) raised against an epitope located at amino acid sequence 214–230 in the protein and diluted at 1/100 in PBS. The samples were rinsed three times in the same buffer. They were then incubated with a conjugated goat anti-mouse (for FDC-B1, CNA.42, CD79 and anti-PrPc mAb) or goat anti-rabbit (for CD3) immunoglobulin peroxidase labelled polymer (Amplification EnVision® System-HRP, Dako, Glostrup, Denmark) for 30 min at room temperature. Peroxidase activity was revealed with 9ethyl-3-aminocarbazole (AEC, Zymed, San Francisco, USA) combined to H_2O_2 as substrate for 12 min. The specificity of the antibodies was tested using an isotype control antibody and negative controls were obtained by omitting the primary antibody. The specimens were counterstained with Mayer's haematoxylin.

2.3. Isolation of FDC clusters

Three protocols were tested. The first two compared the efficiency of two enzymatic cocktails in terms of FDC cluster numbers and of PrPc expression by freshly isolated FDCs. Pharyngeal tonsils were cut into 1 mm thick slices. The