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PrP^{CWD} lymphoid cell targets in early and advanced chronic wasting disease of mule deer

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Up to 15% of free-ranging mule deer in northeastern Colorado and southeastern Wyoming, USA, are afflicted with a prion disease, or transmissible spongiform encephalopathy (TSE), known as chronic wasting disease (CWD). CWD is similar to a subset of TSEs including scrapie and variant Creutzfeldt–Jakob disease in which the abnormal prion protein isoform, PrP^{CWD}, accumulates in lymphoid tissue. Experimental scrapie studies have indicated that this early lymphoid phase is an important constituent of prion replication interposed between mucosal entry and central nervous system accumulation. To identify the lymphoid target cells associated with PrP^{CWD}, we used triple-label immunofluorescence and high-resolution confocal microscopy on tonsils from naturally infected deer in advanced disease. We detected PrP^{CWD} primarily extracellularly in association with follicular dendritic and B cell membranes as determined by frequent co-localization with antibodies against membrane bound immunoglobulin and CD21. There was minimal co-localization with cytoplasmic labels for follicular dendritic cells (FDC). This finding could indicate FDC capture of PrP^{CWD}, potentially in association with immunoglobulin or complement, or PrP^{C} conversion on FDC. In addition, scattered tingible body macrophages in the germinal centre contained coarse intracytoplasmic aggregates of PrP^{CWD}, reflecting either phagocytosis of PrP^{CWD} on FDC processes, apoptotic FDC or B cells, or actual PrP^{CWD} replication within tingible body macrophages. To compare lymphoid cell targets in early and advanced disease, we also examined: (i) PrP^{CWD} distribution in lymphoid cells of fawns within 3 months of oral CWD exposure and (ii) tonsil biopsies from preclinical deer with naturally acquired CWD. These studies revealed that the early lymphoid cellular distribution of PrP^{CWD} was similar to that in advanced disease, i.e. in a pattern suggesting FDC association. We conclude that in deer, PrP^{CWD} accumulates primarily extracellularly and associated with FDCs and possibly B cells – a finding which raises questions as to the cells responsible for pathological prion production.

Introduction

Chronic wasting disease (CWD) is the only prion disease, or transmissible spongiform encephalopathy (TSE), known to affect free-ranging wildlife (Spraker *et al.*, 1997; Williams & Young, 1992, 1993). In endemic areas of Colorado and Wyoming, USA, up to 15% of free-ranging mule deer are infected (Miller *et al.*, 2000) and the potential of CWD transmission to livestock or humans is unknown. Even transmission routes among deer remain obscure, although epidemiological evidence suggests lateral transmission (Miller *et al.*, 2000). The pathogenesis of CWD is beginning to unfold. Recent studies have revealed the abnormal isoform of the prion protein (PrP^{res}) in lymphoid tissue (Sigurdson *et al.*, 1999) in a pattern very similar to that described in natural scrapie of

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sheep (van Keulen *et al.*, 1996) and variant Creutzfeldt–Jakob disease (vCJD) of humans (Hill *et al.*, 1999).

Lymphoid tropism differs among the TSEs – these differences possibly reflect variants of prion disease pathogenesis. For example, in bovine spongiform encephalopathy (BSE) no detectable PrP^{res} or infectivity is detectable in spleen (Somerville *et al.*, 1997) or lymph nodes (Wells *et al.*, 1998), unlike CWD, sheep scrapie and vCJD (Hill *et al.*, 1999; Spraker *et al.*, 2002; van Keulen *et al.*, 1996). However, experimental inoculation of BSE into sheep does result in detectable lymphoid PrP^{res} (Foster *et al.*, 2001; Jeffrey *et al.*, 2001). Moreover, lymphotropism appears to be determined not only by host species, but also by prion strain: for example, humans with vCJD have lymphoid PrP^{res} accumulation or infectivity (Bruce *et al.*, 2001; Hill *et al.*, 1999; Hilton *et al.*, 1998) whereas humans with sporadic or iatrogenic CJD do not have lymphoid PrP^{res} accumulation (Hill *et al.*, 1999).

Naturally infected deer with advanced CWD have CWD PrP^{res} (PrP^{CWD}) disseminated throughout lymph nodes, spleen, tonsils and Peyer's patches. In tonsils, PrP^{CWD} accumulation is restricted primarily to germinal centres and is present in $> 50\%$ of secondary follicles (Spraker *et al.*, 2002). In fawns orally inoculated with CWD brain homogenate, PrP^{CWD} was detected in alimentary-associated lymphoid tissues as early as 6 weeks post-inoculation (p.i.). In these early stages of infection, PrP^{CWD} was limited to $< 30\%$ of secondary follicles, which were typically clustered, suggesting a common conduit or seeding site into the draining lymph node (Sigurdson *et al.*, 1999).

The mechanisms of lymphoid tissue PrP^{CWD} accumulation remain uncertain, although studies in natural and experimental scrapie (Andreoletti *et al.*, 2000; Brown *et al.*, 2000; Jeffrey *et al.*, 2000; Kitamoto *et al.*, 1991; McBride *et al.*, 1992; Montrasio *et al.*, 2000), CJD in mice (Manuelidis *et al.*, 2000) and vCJD in humans (Hill *et al.*, 1999) provide evidence for PrP^{res} association with follicular dendritic cells (FDC) and/or tingible body (TB) macrophages. With the abundant PrP^{CWD} in lymphoid tissues of deer, it seems possible that PrP^{CWD}-containing lymphoid cells could traffic into the blood. Several studies have established that PrP^{res} strongly correlates with infectivity (Bolton *et al.*, 1991; McKinley *et al.*, 1983; Race *et al.*, 1998). Therefore, with the hope of gaining insight into potential trafficking, conversion or capture sites of PrP^{CWD}, we studied the spatial relationship of the protease-resistant prion protein to lymphoid cell phenotypes in the tonsils and lymph nodes of mule deer naturally or experimentally infected with CWD by triple-immunofluorescent labelling and laser scanning confocal microscopy. We found PrP^{CWD} almost exclusively in association with cell membrane surfaces. In addition, smaller deposits of PrP^{CWD} were detected intracytoplasmically in $CD68^+$ macrophages or dendritic cells within germinal centres and much less commonly within the paracortical zone of lymph nodes. These results are reminiscent of those of Jeffrey *et al.* (2000) regarding PrP^{Sc} and suggest to us that either: (a) PrP^{CWD} conversion occurs at the surface rather than within FDCs or (b) PrP^{CWD} formation occurs at distant sites and is concentrated at FDC surfaces.

Methods

■ **CWD-infected deer and tissue collection.** Tonsils or retropharyngeal lymph nodes from CWD-positive deer were acquired from three groups of captive mule deer (*Odocoileus hemionus*) in various stages of infection: (1) tonsils from six deer with naturally occurring, clinical CWD, (2) retropharyngeal lymph nodes from two fawns orally inoculated with a CWD brain homogenate and euthanized at 42 and 78 days p.i., and (3) tonsil biopsies from three naturally infected, asymptomatic deer from a captive herd with endemic CWD. The asymptomatic deer eventually developed clinical signs of CWD and were euthanized (CWD confirmed with brain immunohistochemical staining (IHC) for PrP^{CWD}). Tonsils were fixed in 10% neutral buffered formalin for 1–3 days then immersed in 88% formic acid for 1 h and embedded in paraffin.

The clinically affected CWD-positive deer were diagnosed by: (1) histological lesions of CWD in the medulla oblongata including perikaryonic neuronal vacuoles, spongiform degeneration of the neuropil and astrocytosis, and (2) abundant PrP^{CWD} staining in the medulla oblongata by IHC (methods described in Sigurdson *et al.*, 2001). Deer were confirmed as CWD-negative by the absence of histological brain lesions and negative staining for PrP^{CWD} in brain and tonsil.

■ **Negative control deer and tissues.** Tonsils from CWD-negative mule deer were acquired from two sources: (1) adult deer from the CWD non-endemic area (non-endemic area established by methods in Miller *et al.*, 2000) and (2) two mule deer fawns inoculated with CWD-negative brain homogenate from a previous study (Sigurdson *et al.*, 1999). Tissues were similarly fixed and processed.

■ **Phenotype antibodies.** Several antibodies which recognize lymphoid epitopes on deer lymphoid cells were used. These included antibodies which recognize: (1) lambda light chain (DAKO), present in antigen–antibody complexes on FDC membrane surfaces and on B cells, (2) cc21 (CD21 or complement receptor type 2) (antibody generously donated by Dr Chris Howard), a receptor that traps immune complexes on FDC surfaces also expressed by B cells (Zabel & Weis, 2001), (3) CD68 (Serotec), an intracytoplasmic, lysosome-associated epitope within macrophages and human DC (Betjes *et al.*, 1991), (4) ferritin (DAKO), a large protein surrounding a core of ferric oxide which functions to store and detoxify iron (Morikawa *et al.*, 1995) in macrophages (Kindblom *et al.*, 1982), (5) heat shock protein 70 (HSP70) (DAKO) in macrophages (Bachelet *et al.*, 1998), (6) vimentin (DAKO), an intermediate filament in TB macrophages (Giorno, 1985) and FDC (Tsunoda *et al.*, 1990), (7) anti-FDC (DAKO), which targets a 120 kDa epitope in FDC of humans (Raymond *et al.*, 1997) and has been shown to cross-react with sheep FDC (Lezmi *et al.*, 2001), (8) S100 (DAKO), a calcium-binding protein present in FDC and/or TB macrophages, depending on the species (Carbone *et al.*, 1988), and (9) CD3 (DAKO), an intracytoplasmic domain of the CD3 epsilon chain of T cells.

■ **Immunofluorescent staining.** Tissue sections (6 μm) were mounted onto positively charged glass slides, deparaffinized, hydrated, autoclaved in a buffer solution (DAKO Target Antigen Retrieval) for 12 min at 121 °C, and cooled for 5 min. Sections were rinsed in PBS and immersed in 3% H_2O_2 for 15 min to quench endogenous peroxidase. Sections were then briefly rinsed in PBS and incubated in TNB blocking solution (NEN Sciences) for 30 min followed by exposure to 1–2 lymphoid phenotype antibodies and anti-PrP antibody 6H4 (monoclonal, IgG, 1:200 dilution) or R522 (polyclonal, 1:1500 dilution) for 30 min at

room temperature. mAb 6H4 recognizes a conserved sequence of the prion protein, corresponding to the human amino acid sequence 144–152 (Korth *et al.*, 1997). R522 recognizes ovine PrP 94–105 (Garssen *et al.*, 2000; van Keulen *et al.*, 1995). Antibodies were diluted in a protein block containing goat serum (Biogenex).

Since HSP epitopes appear to be destroyed by autoclaving, slides stained for HSP and PrP were initially labelled for HSP, followed by autoclaving and labelling for PrP^{CWD}. In general, phenotype antibodies were labelled with FITC or Alexa 488 (Molecular Probes) and PrP labelled with CY3. In sections labelled for HSP or CD68, PrP^{CWD} was labelled with FITC. Tyramide amplification (NEN Sciences) was used to enhance stain signal on R522, ferritin and HSP labels. Slides were coverslipped using anti-fade mounting media (Molecular Probes). CWD-negative deer tissues were incubated with an anti-PrP antibody and an isotype- and concentration-matched rabbit or mouse antibody to control for the phenotype antibody.

Confocal microscopy. To co-localize the cell phenotype marker and PrP^{CWD}, triple immunofluorescently labelled sections were examined using an Olympus FLUOVIEW laser scanning confocal microscope equipped with 12-bit resolution which allows for data acquisition from three fluorescent channels using three lasers, Argon 488 nm, HeNe 543 nm and HeNe 622 nm; these emit in the green, red and far-red spectra, respectively. Secondary follicles were selected from each tonsil section and sequentially scanned using the three lasers.

Quantification of co-localization of PrP^{CWD} and phenotype marker. Images from each deer were analysed using Metamorph software (Universal Imaging Corp., West Chester, PA) applying the colour thresholding tool to differentiate the positively stained cells from the unstained cells. Percent co-localization of PrP^{CWD} with the phenotype marker stain was measured using the co-localization tool and recorded on a Microsoft Excel spreadsheet. For each tissue section, two follicles (900 × magnification) were analysed for PrP^{CWD} and phenotype marker co-labelling, and the results were averaged. Data were analysed using Student's *t*-test. Significance was defined at $P < 0{\cdot}05$.

Dual immunocytochemical (ICC) staining. To determine whether PrP^{CWD} could be associated with individual cells from a CWD-infected lymph node, we collected the retropharyngeal lymph node into cold cell culture medium immediately after euthanasia. Single cell suspensions were prepared by mincing and incubating 2 mm^3 sections in serum-enriched medium containing collagenase, dispase and DNase at 37 °C with agitation to digest the stroma and release the cells. The cells were pelleted by centrifugation, washed in PBS, and then cytocentrifuged onto positively charged glass slides. Cells were fixed in 10% buffered formalin for 15 min and pretreated by hydrated autoclaving if necessary immediately prior to immunostaining.

The ICC protocol employed an automated immunostainer (Ventana Medical Systems) and was separated into two stages. First, the cells were labelled with a phenotype marker using the appropriate phenotype antibody, a biotinylated secondary antibody, a horseradish peroxidase–streptavidin conjugate and a diaminobenzadine chromagen. Second, hydrated autoclaving was performed on cell preparations not previously autoclaved and the cells were labelled for PrP^{CWD} using PrP mAb F99/97.6.1 (generously provided by Dr Katherine O'Rourke) (Spraker *et al.*, 2002), a biotinylated secondary antibody, an alkaline phosphatase–streptavidin conjugate, a substrate chromagen (fast red A), and a haematoxylin and bluing counterstain (Ventana Medical Systems). mAb F99/97.6.1 reacts with a conserved epitope (residues QYQRES) on the prion protein of mule deer, Rocky Mountain elk, domestic sheep and cattle (Spraker *et al.*, 2002). An isotype-matched, irrelevant antibody was substituted in the ICC protocol as a negative control for the phenotype marker. The anti-PrP antibody was applied to both CWD-negative and -positive deer cell preparations.

IHC was performed on lymphoid tissue as described for the ICC utilizing anti-PrP mAbs F89/160.1.5 and F99/97.6.1. mAb F89/160.1.5 recognizes a conserved epitope of the prion protein of mule deer, elk, sheep and cattle (residues IHFG) (O'Rourke *et al.*, 1998).

Results

Lymphoid cells in the germinal centres include FDC, TB macrophages, T and B lymphocytes, and germinal centre dendritic cells. Germinal centre dendritic cells, a dendritic cell subset in the tonsil that presents antigen to germinal centre B cells, have been described in humans (Grouard *et al.*, 1996; Summers *et al.*, 2001) but not in ruminants.

Because PrP^{CWD} deposits accumulate within germinal centres of primary and secondary lymphoid follicles, we focused on phenotype marker antibodies which would target FDC, B and T lymphocytes, and TB macrophages. To ensure that the human antigen-derived phenotype antibodies recognized the appropriate target epitope, we compared the cell staining patterns of our phenotype antibodies in human and deer tonsil sections and determined that the antibodies identified lymphoid cells with similar morphology and anatomical distribution.

PrP^{CWD} in lymphoid germinal centres

In tonsils of all CWD-infected deer examined by IHC, PrP^{CWD} was concentrated primarily in lymphoid follicle germinal centres (Fig. 1). Tonsils from deer with clinical CWD or tonsil biopsies from preclinical, CWD-infected deer had a

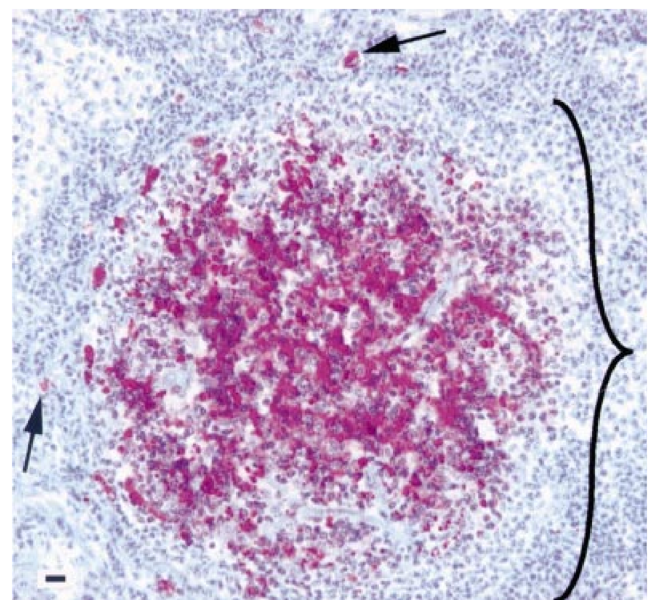

Fig. 1. Typical abundant follicular PrP^{CWD} deposition in a lymph node follicle from a deer with advanced CWD. Note the network-like lattice of PrP^{CWD} deposition defining the central zone of the follicle (bracket) and isolated PrP^{CWD}-positive cells at the periphery of the follicle (arrows). Anti-PrP antibody used: F99/97.6.1. Bar, 10 µm.

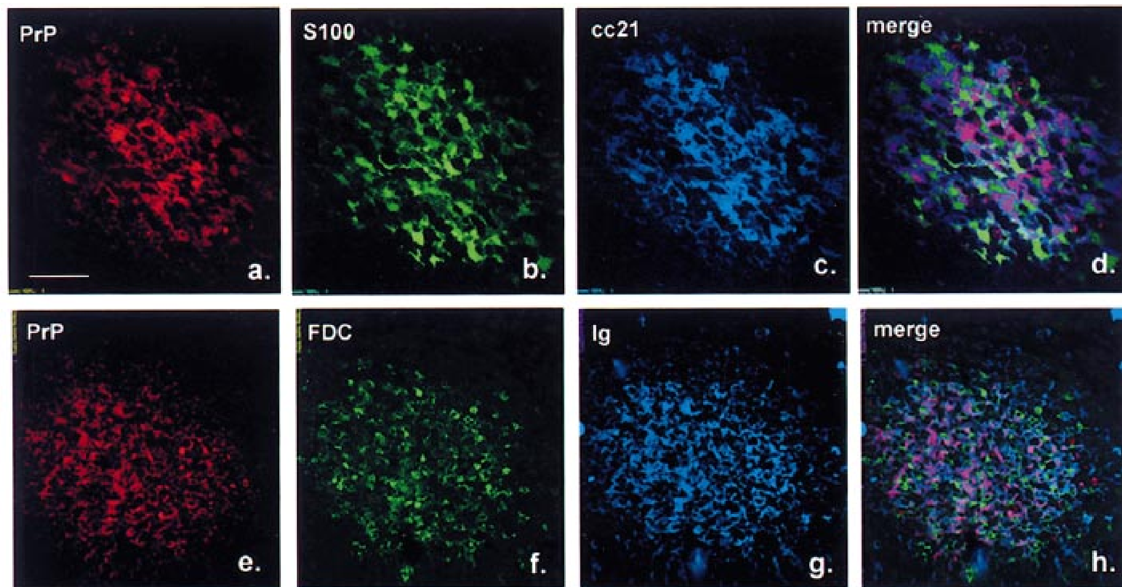

Fig. 2. PrP^{CWD} is on the cell surface of FDC. Lymphoid follicle within a CWD-positive deer tonsil stained using two triple-labelling protocols. PrP^{CWD} (panels a, e, red, antibody 6H4) co-localizes with cell membrane markers for cc21 and immunoglobulin (Ig) (panels c, g, blue) visible as pink in the merged image (panels d, h). PrP^{CWD} (red) does not co-localize with intracellular FDC labels, S100 and FDC (green), as apparent by the lack of yellow in merged images (panels d, h). Bar, 50 μm.

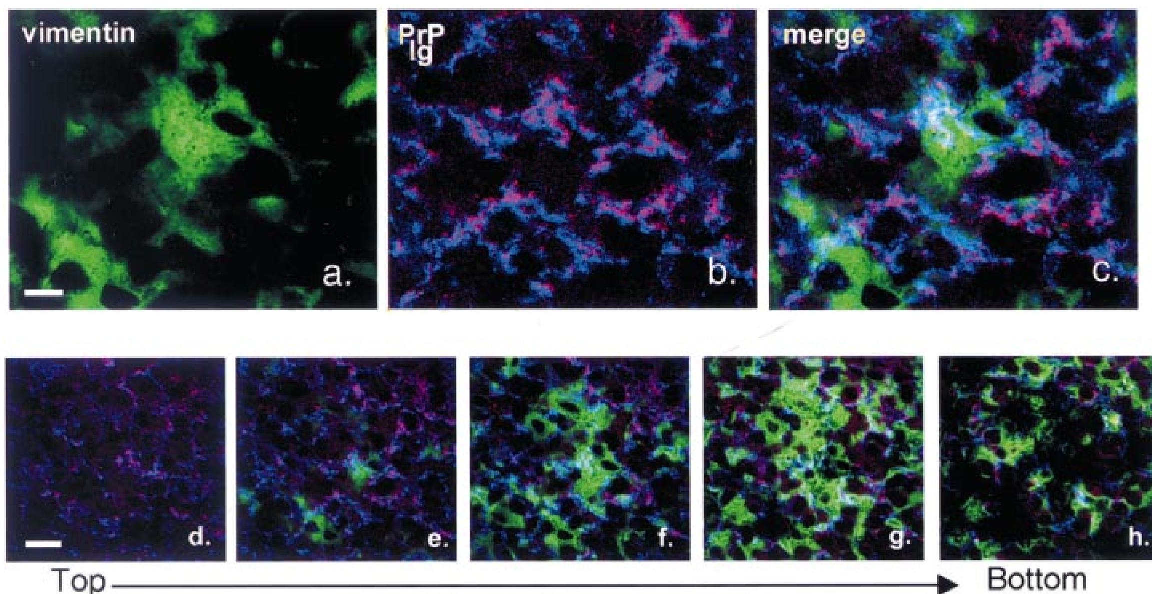

Fig. 3. Lymphoid follicle, CWD-positive deer tonsil. PrP^{CWD} (panel b, red, antibody 6H4) co-localizes strongly with membrane-bound lambda light chain of immunoglobulin (Ig, blue) and poorly with the intracellular marker vimentin (panels a, c). Lower magnification, serial sections from the same field show different planes ~ 1 μm apart from top to bottom and demonstrate the strong co-localization of PrP^{CWD} and Ig and the poor co-localization of PrP^{CWD} and vimentin through the specimen. Bars, 10 μm (a), 20 μm (b).

high frequency (~ 80–100%) of PrP^{CWD}-positive follicles. By contrast, in fawns examined 7 to 11 weeks after oral CWD exposure, < 30% of retropharyngeal lymph node follicles contained detectable PrP^{CWD}. Although PrP^{CWD} was found primarily within the germinal centres, it was also detected occasionally in cells within perifollicular areas (Fig. 1).

PrP^{CWD} accumulates on FDC membranes

To study the association of PrP^{CWD} with germinal centre cells, we co-labelled tonsil sections for PrP^{CWD}, FDC and other lymphoid cell phenotypes. Three cytoplasmic phenotype markers were used to identify FDC: S100, vimentin and anti-

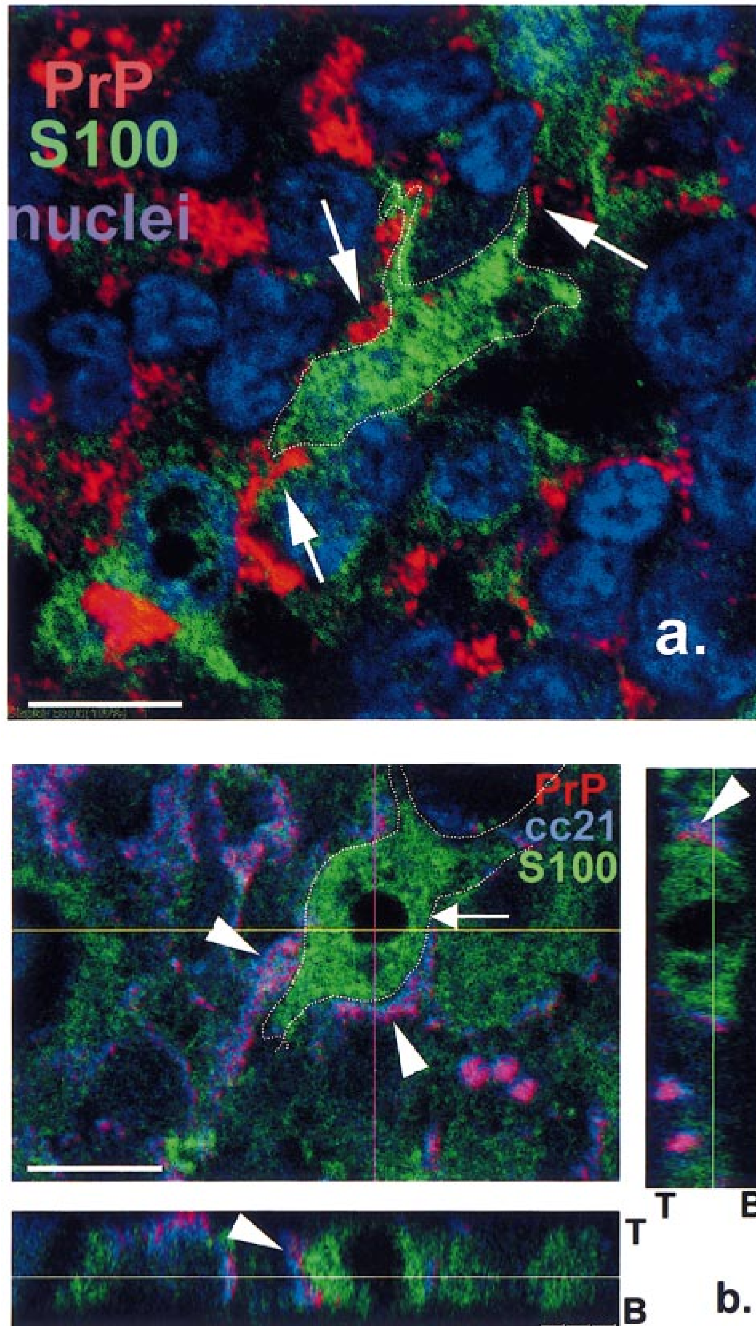

Fig. 4. PrP^{CWD} associates with cell membranes on follicular dendritic and B cells. Tonsillar germinal centre, high magnification. (a) PrP^{CWD} is on membrane surfaces and dendritic processes of FDCs (arrows). (b) PrP^{CWD} co-localizes with cc21 (CD21) on membrane surfaces (arrowheads, pink) and poorly with intracellular S100 (note no yellow) indicating that PrP^{CWD} accumulates on cell surfaces. Nucleus is likely the central round black structure (arrow). FDC cell membrane is designated by the white dotted line. Side panel to the right of main panel is the same cell viewed on a perpendicular plane across the pink line and the lower panel shows a section perpendicular to the yellow line in the main panel (T = top, B = bottom). These views demonstrate that there is no discernible PrP^{CWD} within the cytoplasm of the FDC; PrP^{CWD} is between the cells. Anti-PrP antibody used: 6H4. Bar, 10 µm.

FDC (see Methods for details on antibodies). To investigate whether PrP^{CWD} accumulated on the cell membrane or cytoplasmically with respect to FDC, we also triple-labelled tonsil sections with antibodies targeting two membrane-bound epitopes associated with FDC and B cell membranes: lambda light chain and cc21 (CD21 or complement receptor type 2). Using confocal microscopy, we found that co-localization of PrP^{CWD} with the FDC intracellular phenotype markers was rare, though PrP^{CWD} appeared in close association with FDC (Fig. 2).

Although the lack of co-localization of PrP^{CWD} with the FDC cytoplasmic markers (S100 and anti-FDC) was visually apparent as assessed by the lack of yellow stain, we quantified and compared the co-localization of PrP^{CWD} with intracytoplasmic and membrane markers using Metamorph software. PrP^{CWD} co-localization with the extracellular markers was approximately four times higher than with intracellular FDC markers ($P < 0{\cdot}05$ by Student's t-test; Fig. 5). We concluded that PrP^{CWD} accumulated primarily on membranes associated with FDC and B lymphocytes.

To verify our results, we analysed tonsil sections labelled with the intracytoplasmic marker vimentin at a higher

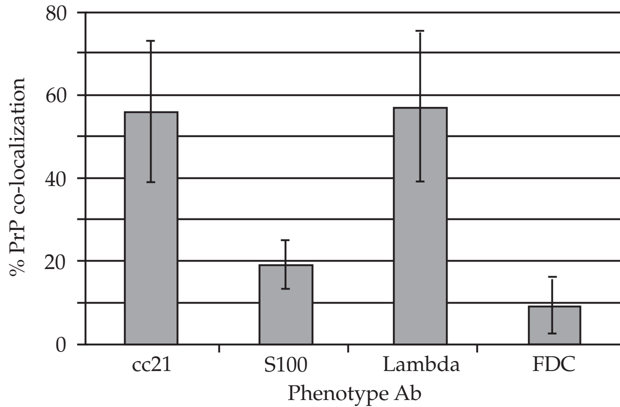

Fig. 5. Percentage PrP^{CWD} co-localization with intracellular markers for FDC (S100, FDC) or cell membrane markers (immunoglobulin, cc21). $n = 12$.

magnification (900 × with zoom) and created a stack of individual optical sections ~ 0·3 microns apart through the tissue. At high magnification, PrP^{CWD} co-localized strongly with lambda light chain and poorly with vimentin (Fig. 3a–c); this labelling pattern was consistent throughout the thickness of the tissue section (Fig. 3d–h).

We then characterized PrP^{CWD} in relation to individual cells at high magnification. Using the FDC intracytoplasmic label (S100) and a nuclear stain, we found that PrP^{CWD} was present on the plasma membrane surface and on the fine processes of the FDC (Fig. 4a). Perpendicular sections through a cell labelled with S100 and cc21 antibodies indicated that even in three-dimensional views PrP^{CWD} co-localized only with the plasma membrane marker and did not appear to be intracytoplasmic (Fig. 4b).

To determine whether T cells may harbour PrP^{CWD}, we co-labelled tonsil sections for PrP^{CWD} and T cell receptor CD3. We found that relatively low numbers of T cells were present and no consistent intimate association was evident between these CD3$^+$ cells and PrP^{CWD} (data not shown).

PrP^{CWD} in the cytoplasm of TB macrophages

Perifollicular cells containing PrP^{CWD} were seen in chromagen-based IHC staining of lymph nodes (Fig. 6a, b). To

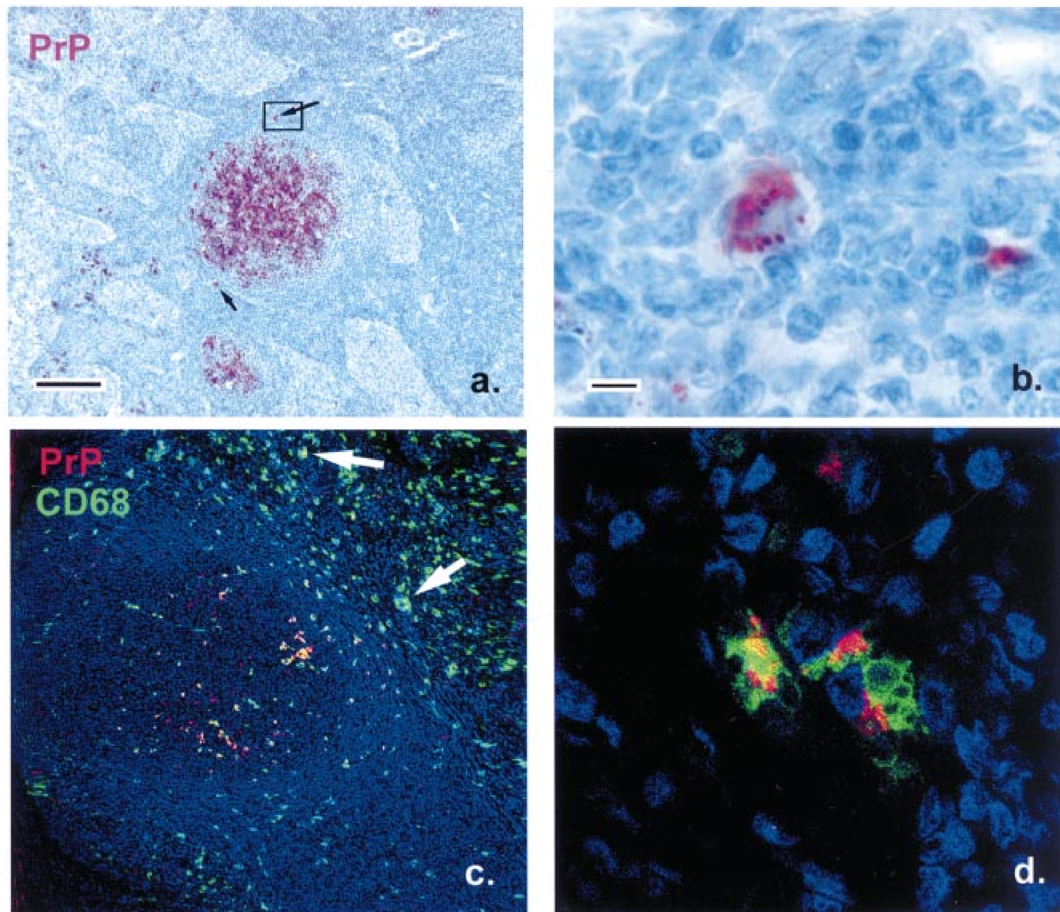

Fig. 6. PrP^{CWD}-containing cells (arrows) are peripheral to the lymph node follicle. Cells are labelled by immunohistochemistry (a, b) or by triple-immunofluorescence (c, d). Panels (c) and (d) demonstrate PrP^{CWD} labelling (red, antibody R522) in CD68$^+$ cells (green, macrophages or dendritic cells) using confocal microscopy. Nuclei are labelled blue. Bars, 1 mm (a) or 10 µm (b).

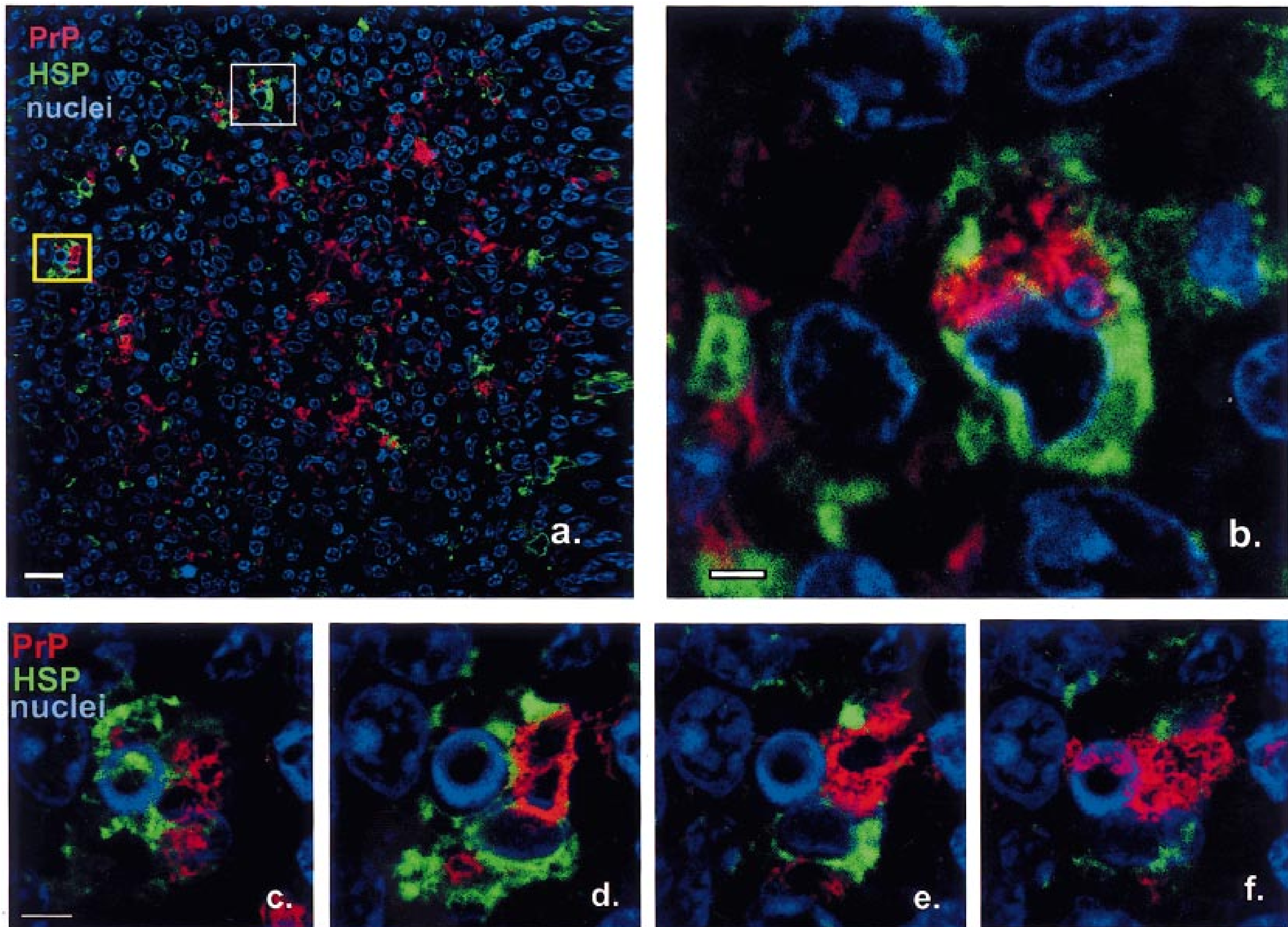

Fig. 7. Tonsillar follicle from a CWD-positive deer. PrP^{CWD} (red, antibody 6H4) is detected within cells positive for HSP (green), presumably macrophages (a, b). (b) Higher magnification of cell from panel (a) (white box) shows close approximation of PrP^{CWD} and HSP likely within a single cell. Serial sections of a single cell (yellow box, panel 1) from top to bottom were obtained ~ 1 µm apart and demonstrate the presence of PrP^{CWD} and HSP70 within the cytoplasm (c–f). Nuclei are labelled blue. Bars, 15 µm (a), 2·5 µm (b), 5 µm (c–f).

phenotype these cells, we triple-labelled a tonsil section using antibodies against PrP^{CWD}, nuclei and CD68, which labels a lysosomal epitope of macrophages and human dendritic cells, and found that PrP^{CWD} was associated with CD68^{+} macrophages or dendritic cells (Fig. 6c, d).

We investigated whether TB macrophages were involved in PrP^{CWD} accumulation based on earlier experiments in which PrP^{CWD} staining was visualized in cells morphologically characteristic of TB macrophages (see Fig. 9). To determine whether TB macrophages in germinal centres accumulated PrP^{CWD}, we used three intracellular antibody markers for TB macrophages: CD68, HSP70 and ferritin.

In HSP70-labelled sections examined at high magnification, we found that PrP^{CWD} was closely associated with HSP70 (Fig. 7a, b). Serial optical sections through a single cell consistently demonstrated PrP^{CWD} adjacent to the intracellular TB macrophage marker (HSP70), indicating that PrP^{CWD} was intracellular (Fig. 7c–f). Similar results were seen with the ferritin label (data not shown). At high magnification, PrP^{CWD} occasionally co-localized with the macrophage phenotype marker CD68 (Fig. 8). To conclude, two populations of macrophages contained PrP^{CWD}: (1) TB macrophages within germinal centres and (2) isolated macrophages or possibly dendritic cells in the perifollicular area.

PrP^{CWD} in separated lymphoid cells

In a further attempt to determine whether PrP^{CWD} was membrane associated and affiliated with FDC, we examined lymphoid cells enzymatically digested from a CWD-infected retropharyngeal lymph node. Using cytospin preparations of lymphoid cells stained for S100 and PrP^{CWD}, we found that many PrP^{CWD}-bearing cells labelled for S100, identifying them as FDC (Fig. 9). In addition, however, some PrP^{CWD}-containing

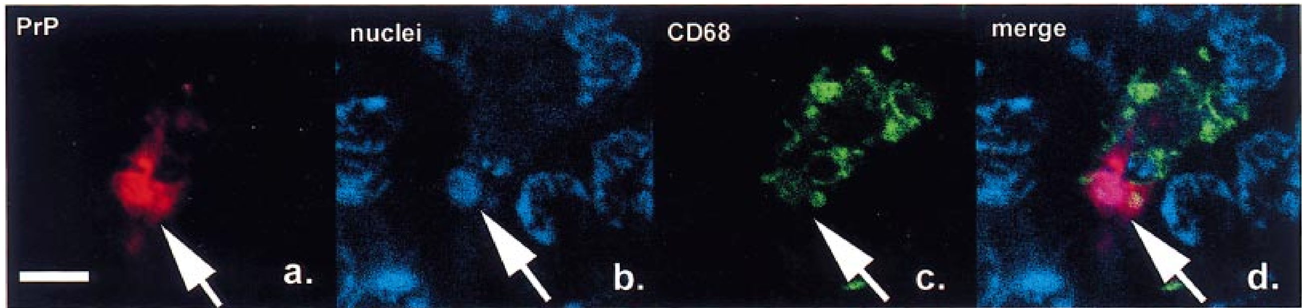

Fig. 8. CD68^{+} cell (green) within the germinal centre contains PrP^{CWD} (red, arrow, antibody R522). Nuclei are labelled blue. Bar, 5 µm.

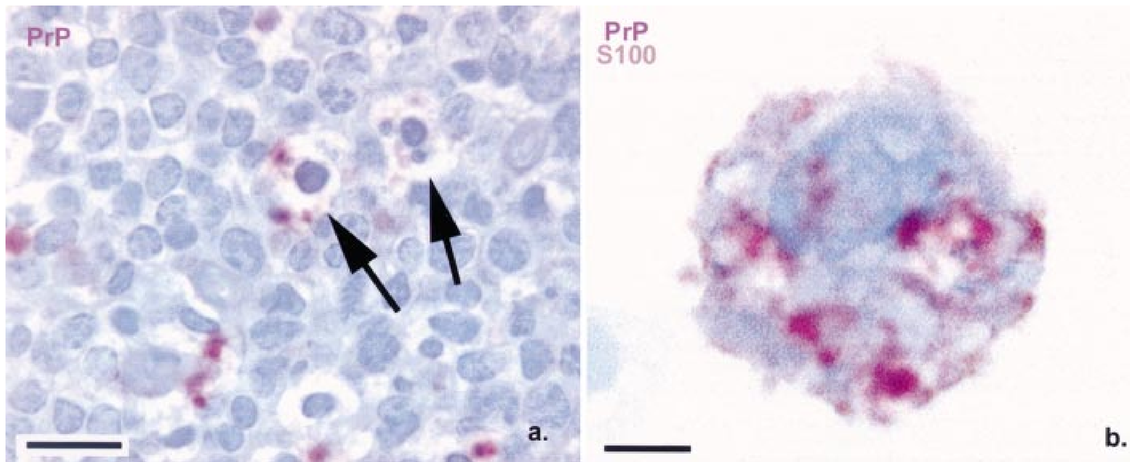

Fig. 9. TB macrophages contain PrP^{CWD}. (a) Tonsil biopsy from a deer with pre-clinical CWD demonstrating PrP^{CWD} in cells morphologically consistent with TB macrophages. Note condensed fragmenting nuclei within the cytoplasm (arrows). (b) S100-positive cell (brown) dually labelled for PrP^{CWD} (red) from a cytospin preparation of an enzymatically dissociated CWD-positive deer lymph node. Anti-PrP antibodies used: F89/160.1.5 (a); F99/97.6.1 (b). Bar, 10 µm.

cells also stained positively for ferritin, a trait most compatible with macrophages. Occasionally PrP^{CWD}-positive cells also co-labelled for lambda light chain or vimentin, traits compatible with FDC, TB macrophages or B cells. These experiments demonstrated that: (1) PrP^{CWD} was cell associated, and (2) PrP^{CWD}-harbouring cells were positive for either S100, ferritin, vimentin or lambda light chain, confirming that at least FDC and macrophages were accumulating PrP^{CWD}.

PrP^{CWD} lymphoid cell association in preclinical CWD-infected deer

To determine whether the lymphoid cell association of PrP^{CWD} changed through the course of infection, we compared PrP^{CWD} lymphoid target cells from deer in early, asymptomatic stages of infection to deer with clinical signs of advanced CWD. The PrP^{CWD} distribution in tonsil biopsies from asymptomatic, naturally exposed deer was similar to that in the tonsils from clinically affected deer. In contrast, in fawns sacrificed 6–11 weeks post-oral inoculation, PrP^{CWD} was distributed primarily on FDC and B cell membrane surfaces with less involvement of TB macrophages. One fawn (6 weeks p.i.) had no apparent PrP^{CWD} in TB macrophages; PrP^{CWD} was primarily associated with cell membranes. In a second fawn (11 weeks p.i.) PrP^{CWD} was detected in both the cell membrane (FDC/B cells) and intracellular (TB macrophages) patterns. These studies suggested that PrP^{CWD} accumulated first in association with FDC vs macrophages and that no additional cell associations were apparent in early pre-clinical stages of infection.

Discussion

A prominent feature of CWD in mule deer is the abundant PrP^{CWD} accumulation in lymphoid germinal centres, similar to that in variant CJD in humans (Hill *et al.*, 1999; Hilton *et al.*, 1998) and scrapie in sheep (Andreoletti *et al.*, 2000; Heggebø *et al.*, 2000; van Keulen *et al.*, 1996). PrP^{Sc}/PrP^{CWD} or infectivity is initially detectable in alimentary-associated lymphoid tissue within weeks following oral exposure and months before detection in the brain (Andreoletti *et al.*, 2000; Hadlow *et al.*, 1982; Kimberlin & Walker, 1989;

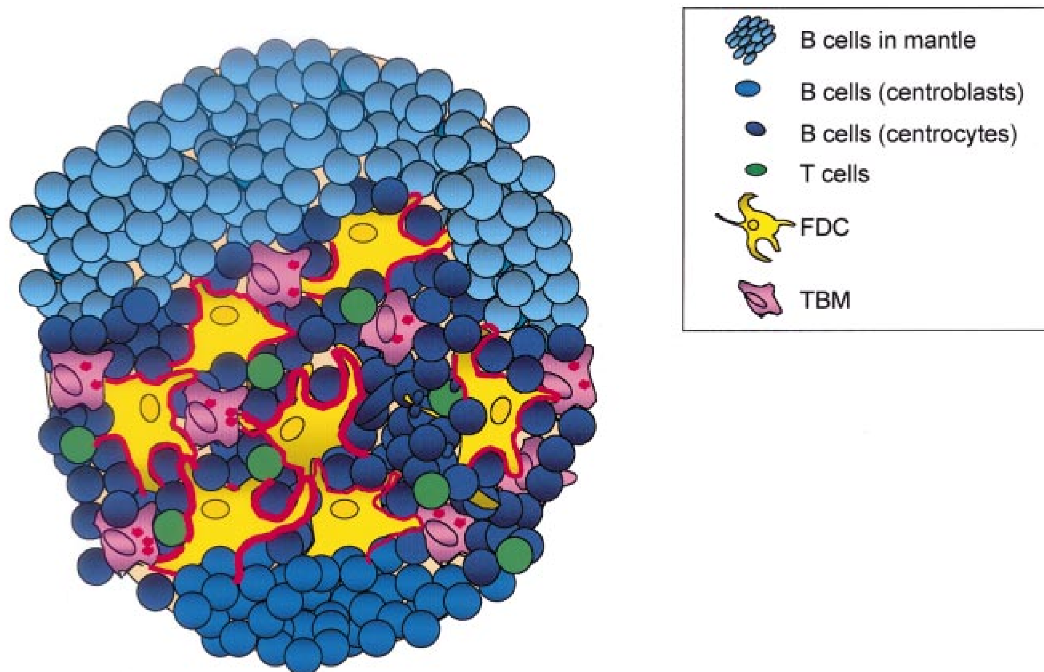

Fig. 10. Working model for lymphoid cells associated with PrP^{CWD}. PrP^{CWD} (red) accumulates on the cell membrane or extracellularly in association with the FDC and/or B cells and accumulates within the cytoplasm of TB macrophages.

Sigurdson *et al.*, 1999; van Keulen *et al.*, 2000; Williams & Miller, 2000). While PrP^{CWD} accumulates in lymph nodes in these early stages of infection, the role of specific immune system cells in prion replication and trafficking to the central nervous system (CNS) remains unclear.

Our observations indicate that PrP^{CWD} accumulates in close association with FDC (Fig. 10). Due to the close contact of FDC processes with numerous B cells (emperiopolesis), it is possible that PrP^{CWD} is also on B cell membranes, or is in the extracellular space between FDC and B cells. This finding is consistent with two recent studies in the mouse TSE models demonstrating FDC membrane-associated PrP^{Sc}: Jeffrey *et al.* (2000) used immunogold labelling to elegantly demonstrate ME7 PrP^{Sc} on the plasmalemma of splenic FDC. Secondly, Manuelidis *et al.* (2000) used confocal microscopy to localize strain FU CJD PrP^{res} on FDC membranes. Interestingly, the localization of infectious agent to FDC is not unique to TSEs. Other infectious agents, especially viruses, have been described on FDC surfaces, including bovine viral diarrhoea (Fray *et al.*, 2000) and human immunodeficiency viruses (Fujiwara *et al.*, 1999; Joling *et al.*, 1993; Schmitz *et al.*, 1994).

Although FDC have been associated with PrP^{Sc} (Brown *et al.*, 1999; Hill *et al.*, 1999; Kitamoto *et al.*, 1991; McBride *et al.*, 1992; Ritchie *et al.*, 1999), whether FDC replicate or merely harbour prions remains controversial. For example, Montrasio *et al.* (2000) demonstrated that inhibition of FDC development virtually eliminated splenic PrP^{Sc}. Mabbott *et al.* (2000) found similar results if mice had FDC deleted prior to scrapie challenge; however, when FDC were deleted after challenge, mice developed high levels of splenic infectivity. Moreover, in experiments using chimeric mice in which PrP^{C} expression between FDC and other lymphoid cells was mismatched, Brown *et al.* (1999) found that only those mice expressing PrP^{C} in FDC were susceptible to scrapie, strongly suggesting prion propagation by FDC. In contrast, Manuelidis *et al.* (2000) concluded that limiting intraperitoneal doses of CJD into FDC-deficient mice resulted in only a slightly prolonged incubation period over wild-type controls, suggesting FDC do not play a key role in this model. In our confocal microscopy study of PrP^{CWD} in deer tonsils, serial images through FDC failed to reveal intracytoplasmic PrP^{CWD}, which might indicate that FDC do not uptake or convert appreciable PrP^{CWD} in the cytoplasmic compartment. This finding suggests that FDC may convert PrP^{C} at the cell membrane or that intracellular conversion may be followed by rapid PrP^{CWD} exocytosis. Another possibility would be that the FDC could act as scaffold for passive capture of PrP^{CWD} on the cell membrane, potentially in association with complement or Fc- γ receptors. The association of PrP^{CWD} on cell membranes is consistent with recent evidence for complement involvement in prion pathogenesis, shown by Klein *et al.* (2001) and Mabbott *et al.* (2001).

Unlike the membrane-associated PrP^{CWD} of FDC, intracytoplasmic large, dense aggregates of PrP^{CWD} were detected in TB macrophages. This finding is reminiscent of studies showing PrP^{Sc} deposits associated with CD68^{+} cells (Andreoletti *et al.*, 2000) or cells morphologically consistent with TB macrophages in naturally infected scrapie sheep (van

Keulen *et al*., 1996). Moreover, Jeffrey *et al*. (2000) described PrP^{Sc} in lysosomes of TB macrophages, consistent with immunogold electron microscopy studies localizing PrP^{Sc} in lysosomes of neurons (Laszlo *et al*., 1992).

There are several potential roles for the TB macrophages in prion pathogenesis. It is possible that $CD68^+$ dendritic cells or macrophages transport PrP^{CWD} into the germinal centre and expose the FDC, T and B cells to PrP^{CWD}. $CD68^+$ cells harbouring PrP^{CWD} or PrP^{Sc} (Andreoletti *et al*., 2000) have been localized adjacent to germinal centres. However, TB macrophages are in close contact with FDC and are known to phagocytose immune complex-coated bodies (iccosomes) on FDC membranes (Szakal *et al*., 1988). TB macrophages may phagocytose PrP^{CWD}-retaining FDC cell fragments (Heinen *et al*., 1993) and extracellular PrP^{CWD} amyloid, and may or may not replicate PrP^{CWD}, as suggested by Jeffrey *et al*. (2000). In addition, TB macrophages phagocytose apoptotic B cells, which also could serve as a potential source of PrP^{CWD} exposure. Therefore, PrP^{CWD} accumulation in TB macrophages may be a secondary event which follows FDC PrP^{CWD} accumulation.

While $CD3^+$ T cells were present in germinal centres, a consistent association between these cells and PrP^{CWD} deposits was not detected, although this association was difficult to assess due to the low number of T cells. Studies with scrapie in transgenic and immunodeficient mice suggest that T cells do not affect disease susceptibility or splenic infectivity (Klein *et al*., 1997, 1998). Nevertheless, the involvement of T cells in CWD pathogenesis remains an open question.

Although PrP^{CWD} was primarily localized to germinal centres, PrP^{CWD} was not restricted to follicles in all lymphoid tissue studied. Scattered cells in the paracortical zone and medullary cords of lymph nodes occasionally contained PrP^{CWD}. These cells invariably labelled for CD68, indicating that they were either macrophages or dendritic cells.

Surprisingly few differences in the lymphoid cells associated with PrP^{CWD} were seen in fawns weeks after oral exposure to CWD when compared to naturally infected deer with advanced CWD. One fawn at 6 weeks p.i. had PrP^{CWD} extracellularly with no detectable involvement of TB macrophages. We speculate that the TB macrophages may be phagocytosing extracellular PrP^{CWD} iccosomes and that there is a short lag before TB macrophages contain PrP^{CWD}. This scenario could explain why 1 fawn (6 weeks p.i.) had no apparent PrP^{CWD} in TB macrophages versus a second fawn (11 weeks p.i.). In scrapie-inoculated mice at 70 and 170 days p.i., the cell labelling of PrP^{Sc} was similar at both time-points (Jeffrey *et al*., 2000). In contrast, in sheep naturally infected with scrapie, PrP^{Sc} was apparent in $CD68^+$ cells prior to detection in FDC (Andreoletti *et al*., 2000).

The close association of PrP^{CWD} with the membrane surfaces of FDC and B cells and the presence of intracytoplasmic PrP^{CWD} in TB macrophages raises questions as to the contribution of each of these cell types to PrP^{CWD} replication and trafficking. Our findings in naturally infected deer add to those in CJD- and scrapie-infected mice, and may lend insight into the lymphoid cell targets in vCJD. Understanding peripheral lymphoid reservoirs may be central to deciphering prion trafficking routes from mucosal surfaces and could be critical to diagnostic and intervention measures during the preclinical stages of prion infections.

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