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DISSERTATION

CHRONIC WASTING DISEASE OF MULE DEER: TRANSMISSION AND PATHOGENESIS

Submitted by

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In partial fulfillment of the requirements

for the Degree of Doctor of Philosophy

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY CHRISTINA JENNY SIGURDSON ENTITLED CHRONIC WASTING DISEASE OF MULE DEER: TRANSMISSION AND PATHOGENESIS BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

CHRONIC WASTING DISEASE IN MULE DEER: TRANSMISSION AND PATHOGENESIS

Chronic wasting disease (CWD) is a naturally occurring, horizontally transmitted prion disease of deer and elk. Understanding mechanisms and pathways of prion transmucosal entry and subsequent neuroinvasion is critical for development of diagnostic assays and intervention strategies; thus, we have studied CWD of deer as a model for TSE pathogenesis. CWD PrP^{res} (PrP^{CWD}), the abnormal isoform of the prion protein, is abundant in lymphoid tissue of infected deer. Fawns exposed orally to a CWD brain homogenate revealed PrP^{CWD} in regional lymphoid germinal centers (retropharyngeal lymph node, Peyer's patches, and ileocecal lymph node) by 42 days post-exposure—indicating that a substantial lymphoid phase of PrP^{CWD} amplification occurs prior to CNS involvement and clinical disease. This lymphoid tissue phase is similar to vCJD in humans and scrapie in sheep and may reflect the initial pathway of CWD infection in deer. Using tonsil sections dually-labelled for lymphoid cell phenotype markers and PrP^{CWD}, we have identified a population of cells associated with PrP^{CWD} as follicular dendritic cells (FDC), B cells, and tingible body macrophages in the germinal center. PrP^{CWD} appears to co-localize with a cell surface marker indicating that PrP is located on the surface of the FDC,

possibly associated with immune complexes or complement receptors. Relatively little is known of the initial prion trafficking pathways to lymphoid tissue or the CNS. We determined that the peripheral nervous system plays a role in CWD prion spread to the CNS. PrP^{CWD} was demonstrated in the myenteric plexus and peripheral nerves, including the vagus nerve which innervates the alimentary tract, as well as the adrenal medulla, pancreatic islet cells, and in the pituitary using IHC. The results of these studies will contribute to understanding PrP trafficking in the lymphoid system and could provide a basis for development of blood-based diagnostic assays and intervention strategies.

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DEDICATION

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INTRODUCTION

Perspective on TSEs:

The studies described are framed in the context of the most widely accepted paradigm for the etiology of transmissible spongiform encephalopathies (TSEs)--the protein-only hypothesis--which postulates that TSEs result from the post-translational conversion of the normal cellular protein (PrP^{c}) into the abnormal protease-resistant isoform (PrP^{res}) (Prusiner, 1982, Prusiner, 1998). PrP^{res} detection has been shown to strongly correlate with infectivity and disease (Bolton et al., 1991, McKinley et al., 1983, Race et al., 1998), although it is unclear whether PrP^{res} is the transmissible agent itself, a component of the agent, or a by-product of either the infectious process or the disease (Caughey et al., 1990). PrP^{res} accumulates in the brain over a long incubation period, ultimately causing death. Infectious prions appear to be transmitted by ingestion (or iatrogenic exposure), although the exact mechanisms of prion exposure and disease induction remain to be fully elucidated (Cervenakova et al., 1998, Marsh & Bessen, 1993, Wells et al., 1998).

Chronic wasting disease (CWD): natural history:

Chronic wasting disease (CWD) is a TSE that was first recognized in captive mule deer (*Odocoileus hemionus hemionus*) in northcentral Colorado in 1967 (Williams & Young, 1980). Since then, the disease has been described in captive Rocky Mountain

elk (*Cervus elaphus nelsoni*) and in free-ranging cervids within a 100 km radius of Fort Collins (Spraker et al., 1997, Williams & Young, 1982). CWD is transmitted with extreme efficiency in nature—at a rate that appears unparalleled in other TSEs. This remarkable transference of disease is documented at a Colorado Division of Wildlife facility wherein ~90% of deer (n=60 animals) resident for two years or longer developed CWD between 1970 and 1981 (Williams & Young, 1992). Other TSE's, such as BSE, appear to be spread through oral ingestion of the abnormal prion protein. The most likely horizontal route of infection in deer is through ingestion of forage or water contaminated by saliva or feces of infected animals. Experimental transmission of CWD into deer has occurred only by the most efficient route of agent transfer--intracerebral inoculation of infected tissue--with an incubation period of 17-21 months (Williams & Young, 1992). In free-ranging mule deer, the prevalence of CWD averages 5% within the endemic area but ranges up to 15% at some sites, and occurs slightly more in males (Miller et al., 2000). From population modeling studies based on prevalence data and spatial case distribution, it is estimated that CWD originated ~30 years ago (Miller et al., 2000).

Clinical disease

The clinical picture in deer with CWD differs from other animals with TSEs in that signs are nonspecific and do not include obvious neurologic disease. Deer in advanced disease stages may show signs including polyuria/polydipsia, emaciation, drooping ears, excessive salivation, teeth grinding, ruminal regurgitation, and occasionally flaccid hypotonia of facial muscles (Williams & Young, 1980).

Behavior alterations are common and are characterized in free-ranging animals by lack of fear of humans. Captive deer often have a lowered head and a fixed stare (Williams & Young, 1980). Clinical signs persist from several weeks to eight months and ultimately lead to death.

Pathology

Gross pathology of CWD infected deer is unremarkable. Deer are consistently emaciated with serous atrophy of visceral and bone marrow fat and often have watery or foamy rumen contents sometimes containing sand suggestive of a gastrointestinal motility disturbance. Abomasal or omasal ulcers are common and perhaps the cause of the teeth grinding. Esophageal flaccidity and/or bronchopneumonia are occasionally seen (Spraker et al., 1997, Williams & Young, 1980).

The only significant consistent clinical pathologic abnormality is a low urine specific gravity, likely a result of the polyuria and polydipsia. The severe degenerative lesions in the hypothalamus may decrease anti-diuretic hormone produced and result in a diabetes insipidus like syndrome (Williams & Young, 1980).

Histologic brain and spinal cord lesions and PrP^{CWD} deposition (by IHC) are similar in free-ranging and captive deer populations (Spraker et al., 2002a). The spongiform encephalopathy in CWD is characterized by spongiform degeneration of gray matter, intracytoplasmic neuronal vacuolation, neuronal degeneration and loss, astrocytic hypertrophy and hyperplasia, amyloid plaques, and notable lack of inflammation.

Lesion severity and distribution pattern in the central nervous system is most similar to scrapie and BSE; the most severe lesions being evident in the olfactory bulbs and stria, thalamus, supraoptic nucleus and paraventricular nucleus, hypothalamus, tegmental nuclei, parasympathetic vagal nucleus, hypoglossal, medial and lateral cuneatus nuclei, and nucleus of the spinal tract of the trigeminal nerve (Spraker et al., 1997). The severe spongiform change in the olfactory tubercle in CWD is in contrast to other TSEs, including scrapie and BSE.

CWD diagnosis:

CWD diagnosis in symptomatic wild cervids and hunter-killed deer surveys has been made possible by development of immunohistochemistry (IHC) to demonstrate PrP^{CWD} in brain. The IHC methodology was developed through collaboration of several laboratories, including those at Colorado State University, the University of Wyoming, the Wyoming Fish and Game, the Colorado Division of Wildlife, the U.S. Department of Agriculture, and the National Veterinary Services Laboratory using an antibody which recognizes PrP^{CWD} with high specificity. During this time several modifications of the methodology have been made to increase sensitivity. The discovery of PrP^{CWD} in brain and lymph nodes has provided further evidence that CWD is a TSE and has in addition led to recognition of peripheral (lymphoid) PrP^{CWD} accumulation.

Lymphoid tissues of affected mule deer have extensive PrP^{CWD} accumulations similar to scrapie and vCJD (Hill et al., 1999, Sigurdson et al., 1999, Spraker et al., 2002b,

van Keulen et al., 1996). Kimberlin (Kimberlin & Walker, 1989a) and others have associated infection of the lymphoreticular system with the high transmissibility of scrapie among sheep. Perhaps the abundant presence of PrP^{res} in alimentary mucosa-associated lymphoid tissues promotes prion shedding into the environment or direct transfer into fluids such as saliva or feces, thus accounting for efficient transmission of CWD and scrapie.

Experimental transmission of CWD:

The transmissibility of CWD was demonstrated by Williams and colleagues (Williams & Young, 1992) by intracerebral (IC) inoculation of deer. Incubation period to clinical disease was 17-21 months. The CWD agent transmitted poorly if at all to hamsters and mice but did infect ferrets and mink (Marsh, R. unpublished data, M. Bruce, pers. comm.). More recently, the susceptibility of ferrets was demonstrated by IC inoculation, resulting in a 17-21 month incubation period--similar to that in deer (Bartz et al., 1998). In addition, CWD has been transmitted to 3 of 13 cattle by IC inoculation. The incubation period was 24-27 months and PrP^{res} was detected in the brains of all animals by Western blot and immunohistochemistry (Hamir et al., 2001). This study is continuing. Thus, CWD has been transmitted experimentally by IC inoculation to deer, ferrets, goats and mink, but mice and hamsters were relatively resistant to infection (Williams & Young, 1992).

Early events in prion infection: lymphoid PrP^{res} accumulation:

In at least a subset of the TSEs, PrP^{res} accumulates in lymphoid tissues prior to neuroinvasion (Hadlow et al., 1974, Hadlow et al., 1982, Ikegami et al., 1991, O'Rourke et al., 1998, Rubenstein et al., 1991). Although much insight has been gained into the structure of the infectious prion protein, little is known of the cellular targets and the pathways whereby prions invade the nervous system.

The pioneering work of Hadlow and colleagues (Hadlow et al., 1982) studying natural sheep scrapie infections demonstrated that tonsil, lymph nodes, and intestine of sheep contained scrapie infectivity at 10-14 months of age, suggesting a pre-clinical lymphoid phase of prion amplification and an oral route of exposure. Additional experiments in rodent models demonstrated scrapie agent in the spleen and lymph nodes prior to the CNS, supporting the initial findings in sheep (Eklund et al., 1967, Kimberlin & Walker, 1989a). Amazingly, even in IC inoculated mice, infectivity in lymphoid tissue has been shown as early as 7 days post-inoculation (Rubenstein et al., 1991). The significance of the lymphoid replication phase is highlighted by the prolonged incubation period observed in splenectomized (Fraser & Dickinson, 1970, Kimberlin & Walker, 1989b) or genetically asplenic mice (Dickinson & Fraser, 1972). Moreover, studies in SCID mice have shown that after a low dose exposure to scrapie, CNS infection occurred only in mice in which bone marrow had been reconstituted (Fraser et al., 1996), further suggesting that a lymphoid phase is crucial in the pathogenesis of prion disease. Nevertheless, prion transit routes from the lymphoreticular to the central nervous system remain obscure.

Relevance of CWD as a model of prion lymphoid pathogenesis:

The similarities in early lymphoid tissue infection suggests that the pathways of PrP^{res} transport may be the same in CWD, scrapie, and vCJD. Thus, study of lymphotropism in CWD as a model of TSE lymphoid pathogenesis offers advantages, including: (1) infection and cell targets can be studied in the naturally infected species; (2) lymphoid PrP^{res} is abundant and thus is more likely to be detected and tracked; (3) large amounts of blood and bone marrow can be harvested for culture and flow cytometry; and (4) the dynamics, cell targets, dissemination, and potential intervention for oral prion infection can be studied.

Early target cells in prion infection:

The follicular dendritic cells (FDC) were first postulated as candidates for prion accumulation based on IHC studies, thereby serving as an important peripheral reservoir of PrP^{res} in early disease. In one study of CJD-inoculated mice, PrP^{res} immunostaining was detected in cells which co-labelled with FDC markers. However, PrP^{res} staining in other germinal center cells (such as B cells) was not evaluated (Kitamoto et al., 1991). In scrapie-infected mice, PrP^{res}-positive cells were identified as putative FDC by their location, appearance, and immune complex trapping function, and later by dual labeling (McBride et al., 1992, Raeber et al., 1999). Until recently, little was known of PrP^{res}-bearing cells in naturally infected species. This issue takes on greater significance with the recent co-localization of PrP^{res} in tonsillar FDC of vCJD patients (Hill et al., 1999).

DC are potent antigen presenting cells that are essential for inducing primary immune responses (Avigan, 1999). FDC are mature cells which retain immune complexes on their processes and sustain the differentiation of activated B cells (Banchereau & Steinman, 1998). In addition to stationary FDC in the germinal centers, germinal center dendritic cells (GCDC) arrive in lymph nodes via afferent lymphatics carrying antigen from peripheral tissues (Banchereau & Steinman, 1998). These two DC have distinguishable cell marker profiles and likely arise from different precursors (Banchereau & Steinman, 1998). While relatively little is known at present of DC dynamics, these cells could potentially phagocytose and ferry PrP^{res} from mucosal surfaces to draining lymph nodes (Avigan, 1999, Pope, 1999) . It would be important to know whether the PrP^{res} accumulates within or is merely displayed on FDC surfaces, perhaps even in association with antibody or complement.

Some critical questions regarding CWD to which this dissertation research pertains:

Some of the central questions regarding CWD are: (1) how is the disease so readily transmitted among animals, (2) how does the agent enter the body and how does neuroinvasion occur, and (3) what role does the lymphoid system play in pathogenesis? We investigated the routes of infection, tissue tropism, and the earliest stages in the sequence of progression of the CWD agent in the natural host, mule deer. Although previous pathogenesis studies in other prion diseases have revealed clues to the possible means of entry and cells harboring the agent, many questions remain unanswered. In the mouse model of scrapie, PrP^{res} is detected in lymphoid

tissue early in the course of infection, prior to invasion of the nervous system and clinical signs (Kimberlin & Walker, 1989a). The role of the lymphoid cells in the course of infection, however, remains unclear.

Dissertation research:

This backdrop of TSE research provided the basis for the specific aims of this dissertation research. The first objective was to define the early lymphoid events in CWD. We hypothesized that PrP^{CWD} is transported across mucosal lymphoepithelial tissues (tonsils and Peyer's patches) to systemic lymphoid tissues before entry into the peripheral and central nervous systems. We hoped to determine whether mule deer are orally susceptible to CWD and to track PrP^{CWD} in mucosal and systemic lymphoid tissues of deer. This was approached by orally exposing mule deer fawns to CWD infected brain homogenate followed by early serial necropsies in order to assay for PrP^{res} in situ using PrP^{CWD} specific immunohistochemistry.

Little is known regarding the early PrP^{res} target cells in prion diseases, particularly in natural hosts. Knowledge of the initial infected cells is crucial to understanding prion dissemination pathways and to designing intervention strategies. Therefore, the second objective was to characterize PrP^{CWD}-bearing cells in lymphoid tissue of deer with CWD and to localize the PrP^{CWD} to an intracellular or extracellular compartment within the cell using multi-immunofluorescent labelling of PrP^{CWD} and the lymphoid phenotype in tissue sections and confocal microscopy to localize the PrP^{CWD}.

Several potential mechanisms are possible for prion spread from the mucosal entry sites to the CNS and include transit via blood, lymph or nerve. A growing body of evidence comprising both experimental and natural studies implicates PrP^{res} dissemination from the entry site via peripheral nerves. The third objective of this work, therefore, was to determine whether PrP^{CWD} might traffic in the peripheral nervous system as one potential mechanism of spread to the CNS. We searched for PrP^{CWD} in the peripheral nerves and endocrine organs of 6 mule deer with advanced CWD using immunohistochemistry. Our principal goal was to elucidate the stages of TSE infection in the natural host animal with the rationale that this would yield insight into prion transport to the brain and as well as provide a basis for an ante-mortem CWD diagnostic and potential intervention strategies.

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CHAPTER 1

ORAL TRANSMISSION AND EARLY LYMPHOID TROPISM OF CHRONIC WASTING DISEASE PRP^{RES} IN MULE DEER FAWNS

ABSTRACT

Mule deer fawns *(Odocoileus hemionus)* were inoculated orally with a brain homogenate prepared from mule deer with naturally occurring chronic wasting disease (CWD), a prion-induced transmissible spongiform encephalopathy. Fawns were necropsied and examined for PrP^{res}, the abnormal prion protein isoform, at 10, 42, 53, 77, 78, and 80 days post-inoculation (pi) using an immunohistochemistry (IHC) assay modified to enhance sensitivity. PrP^{res} was detected in alimentary-tract-associated lymphoid tissues (one or more of the following: retropharyngeal lymph node, tonsil, Peyer's patch, and ileocecal lymph node) as early as 42 days post inoculation (pi) and in all fawns examined thereafter (53 to 80 days pi). No PrP^{res} staining was detected in lymphoid tissue of three control fawns receiving a control brain inoculum, nor was PrP^{res} detectable in neural tissue of any fawn. PrP^{res}-specific staining was markedly enhanced by sequential tissue treatment with formic acid, proteinase-K, and hydrated autoclaving prior to immunohistochemical staining with monoclonal antibody F89/160.1.5. These results indicate that CWD PrP^{res} can be

detected in lymphoid tissues draining the alimentary tract within a few weeks after oral exposure to infectious prions and may reflect the initial pathway of CWD infection in deer. The rapid infection of deer fawns following exposure by the most plausible natural route is consistent with the efficient horizontal transmission of CWD in nature and enables accelerated studies of transmission and pathogenesis in the native species.

BACKGROUND

Chronic wasting disease (CWD) is a fatal prion disease affecting mule deer *(Odocoileus hemionus)*, white-tailed deer *(Odocoileus virginianus)*, and Rocky Mountain elk *(Cervus elaphus nelsoni)*. This transmissible spongiform encephalopathy (TSE) has been reported in captive and free-ranging deer and elk from northeastern Colorado and southeastern Wyoming (Spraker *et al.*, 1997, Williams & Young, 1980, Williams & Young, 1982, Williams & Young, 1992). Although the pathology of CWD is well-described (Williams & Young, 1993), little is known about CWD transmission. Epidemiologic evidence from captive animals suggests that horizontal transmission may occur at a level apparently unparalleled in other prion diseases (Miller *et al.*, 1998, Williams & Young, 1992). Other non-familial transmissible spongiform encephalopathies (TSE's), such as kuru, transmissible mink encephalopathy (TME), and bovine spongiform encephalopathy (BSE) appear to be transmitted via ingestion of PrP^{res} infected tissue (Cervenakova *et al.*, 1998, Marsh & Bessen, 1993, Wells *et al.*, 1998).

Few studies of early preclinical TSE infections have been performed in natural hosts or using probable natural routes of exposure, however, the results have been intriguing. BSE has been orally transmitted to cattle with infectivity detectable in the ileum of calves at 26 weeks post-inoculation (pi) (by mouse bioassay) (Wells *et al.*, 1994). In another study, scrapie agent infectivity was first detected in the prescapular lymph nodes of goats at 24 weeks post subcutaneous inoculation (Hadlow *et al.*, 1974). However, mice inoculated intragastrically with scrapie had detectable infectivity in Peyer's patches and cervical lymph nodes as early as one week pi (Kimberlin & Walker, 1989). Thus it appears that prions can cross the mucous membranes of the digestive tract to initiate infection in lymphoid tissue prior to invasion of the central nervous system and development of clinical disease.

Oral exposure is the most plausible pathway by which the CWD prion may be introduced to deer in nature. Consequently, we chose this means of inoculation in attempt to demonstrate the feasibility of CWD transmission by this route and to study early lymphoid tissue tropism of the PrP^{res} in deer. Each deer was repeatedly exposed to a known infectious CWD inoculum over a 5 day period because recent results with scrapie in hamsters indicate repeated oral exposure increases the incidence of infection (Diringer *et al.*, 1998). Because mice are relatively resistant to CWD (Moira Bruce, pers comm) precluding bioassay, and because several studies have shown that PrP^{res} strongly correlates with disease (McKinley *et al.*, 1983, Race *et al.*, 1998), we employed an enhanced immunostaining method (formic acid, proteinase-K, and hydrated autoclaving) to detect PrP^{res} in situ. Formic acid and hydrated

autoclaving have been previously described for PrP^{res} epitope exposure prior to IHC (Miller *et al.*, 1994, van Keulen *et al.*, 1995). Using these methods, we demonstrate PrP^{res} in regional lymph nodes as early as 6 weeks after oral exposure of deer fawns to the CWD agent.

METHODS

Animals

Nine free-ranging mule deer fawns (*Odocoileus hemionus*) were acquired from >100 km outside of the CWD endemic area. Control and inoculated animals were housed in separate indoor rooms which had not previously held deer. All fawns were bottle-fed evaporated milk throughout the study and had free access to calf manna, alfalfa hay, mineralized salt, and water per a protocol previously established for raising deer (Wild & Miller, 1991, Wild *et al.*, 1994). Animals received *Clostridium* bacterin-toxoid and iron dextran injections.

Oral inoculation of deer

Six fawns were inoculated orally with 5 ml of a 40% (w/v) brain homogenate (2 g brain) daily for five days using a small syringe inserted into the diastema of the oral cavity. Fawns typically licked and swallowed the material. The homogenate was prepared in normal saline solution from brains of 26 captive mule deer naturally infected with CWD. These deer had characteristic clinical signs and histologic lesions of CWD in the brain. The homogenate had characteristic PrP^{res} bands by

western blot and scrapie associated fibrils by negative stain electron microscopy (Elizabeth Williams, pers comm). Using the same protocol, three control fawns were inoculated in like manner with a 40% brain homogenate from free-ranging mule deer outside the CWD endemic area; these deer were collected from a heavily monitored herd with no immunohistochemical or histologic lesions of CWD (Michael W. Miller, unpublished data).

Necropsy and tissue collection

Infected deer were euthanized with sodium pentobarbital given intravenously and necropsied sequentially at 10, 42, 53, 77, 78, and 80 days pi (n=6). Control deer were necropsied at 27, 70, and 74 days pi (n=3). Pi days were calculated from the last day of exposure. Numerous tissues were collected, including ten lymph nodes (mesenteric, ileocecal, sublumbar, popliteal, prescapular, retropharyngeal, submandibular, parotid, ruminal, and abomasal nodes), spleen, bone marrow, thymus, Peyer's patches, tonsil, conjunctiva, spinal cord, and brain. Tissues were preserved in neutral buffered 10% formalin and then trimmed, processed and embedded in paraffin blocks within 7 days.

Immunohistochemical staining

Prior to staining the fawn tissues, various pre-treatments were tested on tissue sections of obex and tonsil from a positive control CWD mule deer to produce optimal stain enhancement. This was done to maximize staining sensitivity to detect anticipated early accumulation of PrP^{res} in tissues. Sections were treated as follows:

(1) hydrated autoclaving at 121 °C for 20 minutes, (2) immersion of slides in 88% formic acid for 30 minutes followed by hydrated autoclaving for 20 minutes, (3) immersion in 25 μg/ml proteinase-K for 10 minutes at 26 °C followed by hydrated autoclaving, (4) immersion in 12.5 μg/ml proteinase-K for 10 minutes followed by hydrated autoclaving, and (5) immersion in 88% formic acid for 30 minutes, then 25 μg/ml proteinase-K for 10 minutes followed by hydrated autoclaving for 20 minutes. Immunohistochemical staining on the treated sections followed immediately. Staining intensity and specificity was determined by light microscopy. Of these, protocol (5) resulted in the greatest PrP^{res} staining.

Tissue sections were mounted onto positively charged glass slides, deparaffinized, and hydrated in preparation for immunohistochemistry (IHC). Tissue treatment performed prior to IHC consisted of slide immersion in 88% formic acid solution for 30 minutes followed by a rinse in water and immersion in 25 μg/ml proteinase-K solution at 26 °C for 10 minutes. Tissue sections were then autoclaved for 20 minutes at 121 °C in Tris buffer solution and cooled for 30 minutes. The treatments were extensive in order to maximally expose epitopes and enhance staining.

IHC employed an automated immunostainer (Ventana Medical Systems) and PrP^{res} monoclonal antibody (Mab) F89/160.1.5, a biotinylated secondary antibody, an alkaline phosphatase-streptavidin conjugate, a substrate chromagen, and a hematoxylin and bluing counterstain (Ventana Medical Systems). Mab F89/160.1.5 recognizes a conserved epitope on the prion protein of mule deer, elk, sheep, and

cattle (O'Rourke *et al.*, 1998). Positive and negative control tissue sections were included in each run.

Several IHC controls were performed on lymphoid tissues with Mab F89/160.1.5. Lymphoid tissues from 50 deer (collected outside the CWD endemic area) were immunostained using the same methodology as performed on the fawn tissues. IHC on known positive and negative deer tonsil sections was done using Mab F89/160.1.5 substituted by mouse serum or an irrelevant isotype-matched Mab diluted to the same protein concentration as Mab F89/160.1.5. In addition, IHC was performed on a retropharyngeal node section from each fawn with an irrelevant Mab substitution.

RESULTS

1. Enhanced immunostaining.

We assessed five tissue pre-treatment protocols (see Methods) in attempt to maximize immunohistochemical staining sensitivity yet preserve sufficient histologic detail to permit localization of PrP^{res}. Using positive control tissue from deer with naturally occurring CWD, we found that detection of PrP^{res} was markedly enhanced by slide immersion in either formic acid or proteinase-K prior to hydrated autoclaving. Maximal staining was achieved using sequential pre-treatments with formic acid and proteinase-K followed by hydrated autoclaving (Figure 1.1).

Deer tonsil sections from known positive and negative CWD cases immunostained with an irrelevant antibody or with mouse serum substituted for the primary antibody were uniformly negative. No immunostain was detected in lymphoid sections from 48 CWD negative deer originating from non-CWD endemic geographic regions control cases (Mab F89/160.1.5) or in fawn retropharyngeal nodes (irrelevant Mab substitution). In two of the negative deer control cases, a small focus of greyish pink stain was observed in < 5 follicles. The CWD positive control tissue had strong positive staining in the follicular areas when stained with Mab F89/160.1.5.

2. Earliest detection of PrP^{res} in orally exposed deer fawns.

PrP^{res} was not detectable in any tissue of the fawn necropsied at 10 days pi. However, in the fawn necropsied at day 42 pi, PrP^{res} was detected in follicular germinal centers of the retropharyngeal lymph nodes, Peyer's patches, and ileocecal nodes. Of 119 follicles examined in the retropharyngeal nodes, 8 (6.7% of follicles) were PrP^{res} positive. PrP^{res} also was detected in the retropharyngeal node follicles of all infected fawns examined at later time intervals pi (53, 77, 78, and 80 days) (Table 1.1).

3. Tissue distribution of PrP^{res}.

In 6 fawns examined between days 10 and 80 pi, PrP^{res} was detected in the retropharyngeal lymph node follicles of 5, Peyer's patches of 3, tonsil of 2, and ileocecal node of 1 (Table 1.1). PrP^{res}-specific staining consistently appeared as bright granular deposits (red using fast red A substrate) arranged in patterns suggestive of dendritic cells within germinal centers of well-developed secondary

follicles. Staining often occurred in clusters of adjacent follicles (Figure 1.2). In all fawns, the quantity of PrP^{res} estimated by subjective evaluation of stained product was substantially less than that seen in symptomatic cases of CWD, consistent with early foci of formation.

PrP^{res} was detected in 2.7 to 27.3% of the retropharyngeal lymph node follicles in fawns necropsied between days 42 and 80 pi (Table 1.1). At 42 days pi, PrP^{res} was visible in 0.53% of follicles in Peyer's patches. As in lymph nodes, the stain deposits were localized to the germinal centers of the lymphoid aggregates. In tonsil, stain was only seen at the two final timepoints (78 and 80 days pi), in 0.49% and 2.3% of follicles, respectively (Figure 1.3).

PrP^{res} was not detected in brain (obex region), spinal cord, or salivary gland examined from the inoculated animals. No PrP^{res} staining was detected in any tissue of the sham-inoculated control fawns (Figures 1.2 and 1.3).

Clinical signs.

No clinical signs of CWD occurred in any of the inoculated deer throughout the course of the study. One fawn incidentally developed severe laryngeal swelling which resolved completely with antibiotic therapy and two fawns developed mild diarrhea; otherwise fawns remained healthy throughout the study.

DISCUSSION

These results indicate that mule deer fawns develop detectable PrP^{res} after oral exposure to an inoculum containing CWD prions. In the earliest post exposure period, CWD PrP^{res} was traced to the lymphoid tissues draining the oral and intestinal mucosa (i.e. the retropharyngeal lymph nodes, tonsil, ileal Peyer's patches, and ileocecal lymph nodes), which likely received the highest initial exposure to the inoculum. Hadlow (1982) demonstrated scrapie agent in the tonsil, retropharyngeal and mesenteric lymph nodes, ileum, and spleen in a 10 month old-naturally infected lamb by mouse bioassay (Hadlow *et al.*, 1982). 8 of 9 sheep had infectivity in the retropharyngeal lymph node. He concluded that the tissue distribution suggested primary infection via the gastrointestinal tract. The tissue distribution of PrP^{res} in the early stages of infection in the fawns is strikingly similar to that seen in naturally infected sheep with scrapie. These findings support oral exposure as a natural route of CWD infection in deer and support oral inoculation as a reasonable exposure route for experimental studies of CWD.

Cells associated with PrP^{res} were within germinal centers of lymphoid follicles. The staining pattern was morphologically consistent with that of follicular dendritic cells. Experimental inoculation of mice with scrapie or Creutzfeldt-Jakob disease prions resulted in similar localization of PrP^{res} to follicular dendritic cells (Kitamoto *et al.*, 1991, McBride *et al.*, 1992). Peyer's patches have extensive accumulations of lymphoid cells in most species examined (Reynolds & Pabst, 1984). Assuming deer

are similar to sheep, it seems probable that initial uptake and propagation of PrP^{res} could occur in the tonsils and ileal Peyer's patches, and within dendritic cells emigrating via lymphatics to the retropharyngeal and ileocecal lymph nodes.

Studies in mice show rapid accumulation of dendritic cells bearing antigen within regional lymph nodes hours after the skin was painted with contact allergens (Cumberbatch & Kimber, 1990). In that PrP^{res} from inoculum would be expected in draining lymph nodes by 10 days pi and in that the day 10 fawn did not have the immunohistochemically detectable PrP^{res} in any lymphoid tissue, the PrP^{res} staining in fawns examined at later timepoints likely represented accumulating PrP^{res} vs. residual inoculum. Interestingly, and in contrast to the sequence postulated above, PrP^{res} was visible in the tonsil only in the two fawns with the longest pi intervals, 78 and 80 days. This may indicate lower initial quantities of PrP^{res} in tonsil as compared with retropharyngeal node, perhaps due to the migration route of initially infected dendritic cells, resulting in a longer lag before PrP^{res} accumulates in the tonsil to levels detectable with immunohistochemistry.

We detected PrP^{res} by immunohistochemistry as early as 6 weeks pi--an extraordinarily brief period. Detection of PrP^{res} stain in lymphoid tissues by 6 weeks post-inoculation suggests that PrP^{res} accumulates at early disease stages. In goats experimentally infected with scrapie, infectivity was not detected until $\geq$ 3 months pi (Hadlow *et al.*, 1974). Given the repeated exposure to a relatively large amount of inoculum over 5 days, it seems logical to presume that infection in these orally

inoculated fawns may be accelerated, enabling earlier PrP^{res} detection compared to naturally infected deer. Nevertheless, the present study provides proof of principle that CWD PrP^{res} is detectable after oral exposure. Although the present study design precluded the development of clinical disease, the presence of PrP^{res} has been shown to be strongly correlated with infectivity with other TSE's (Race *et al.*, 1998).

Chronic wasting disease in deer is similar to scrapie in that the PrP^{res} is disseminated throughout lymphoid tissues (Terry Spraker, unpublished data). This peripheral reservoir may be responsible for shedding the agent into the environment, or for horizontal transmission among animals, perhaps through saliva or feces. This disseminated lymphoid infection is unlike some other TSE's, such as BSE, in which PrP^{res} is detected only in the ileal Peyer's patches or not at all (Wells *et al.*, 1998). Kimberlin *et al.* (1989) and others have made an association between infection of the lymphoreticular system and the high transmissibility of scrapie among sheep, similar to the findings described in deer and elk (Kimberlin & Walker, 1989, Williams & Young, 1992). It is possible that localization of PrP^{res} to lymphoid tissues adjacent to mucosal surfaces promotes prion shedding into the environment via fluids such as saliva or feces, although the pathway of CWD shedding and potential contagion requires further study.

The exact mode of CWD transmission in nature remains unknown. Scrapie in sheep has been demonstrated in experimental studies to be transmissible via ingestion of fetal membranes from scrapie-positive ewes (Pattison *et al.*, 1972). Nevertheless,

scrapie transmission is nature remains incompletely understood (Detwiler, 1992). Understanding mechanisms of shedding and transmission will be important in management of CWD and in providing insights into the pathogenesis of other TSE's

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Table 1. Immunohistochemical detection of PrP^{res} in fawn lymphoid tissue.

deer no.	days p.i.	retropharyngeal node*	tonsil	Peyer's patches	ileocecal node	mesentaric node	sublumbar node
4	10	0/9	0/14	0/194	0/26	0/37	0/5
5	42	**8/119 (6.7%)++**	0/165	**1/190 (0.53%)**	**1/62 (1.6%)**	0/15	0/3
1	53	**6/104 (5.8%)**	0/128	0/535	0/108	0/81	0/4
7	77	**3/111 (2.7%)**	0/208	**3/549 (0.55%)**	0/72	0/177	0/52
2	78	**49/179 (27.3%)**	**1/205 (0.49%)**	**3/611(0.49%)**	0/57	0/201	0/61
8	80	**5/99 (5.1%)**	**7/300 (2.3%)**	0/542	0/102	0/227	0/214
9	27	0/219	0/72	0/245	0/17	0/19	0/9
3	70	0/135	0/179	0/83	0/40	0/70	0/5
6	74	0/136	0/304	0/385	0/22	0/37	0/11

*** Deer were orally inoculated with CWD positive (deer no.'s 4, 5, 1, 7, 2, 8) or negative control (deer no.'s 3, 6, 9) brain tissue.**
+ Results are expressed as number of positive follicles over total number of follicles counted. Positive IHC staining follicles are bolded.
++ Multiple cross-sections of each lymphoid tissue were examined; low follicle count reflects diffuse lymphocyte distribution and fewer formed follicles.

Table 1. Immunohistochemical detection of PrP^{res} in fawn lymphoid tissue.

deer no.	days p.i.	popliteal node	prescapular node	submandibular node	parotid node	ruminal node	abomasal node	conjunctiva	bone marrow	thymus	spleen
4	10	0/3	0/29	0/6	0/50	0/3	0/4	0/0	0	0/34	0/58
5	42	0/0	0/1	0/45	0/25	0/0	0/30	0/2	0	0/29	0/27
1	53	0/16	0/35	0/46	0/64	0/27	0/50	0/0	0	0/118	0/15
7	77	0/45	0/136	0/112	0/56	0/33	0/34	0/0	0	0/26	0/20
2	78	0/46	0/95	0/49	0/79	0/37	0/39	0/3	0	0/48	0/42
8	80	0/76	0/233	0/126	0/103	0/38	0/97	0/22	0	0/57	0/22
9	27	0/5	0/23	0/81	0/6	0/2	0/27	0/0	0	0/97	0/52
3	70	0/54	0/56	0/96	0/48	0/1	0/35	0/0	0	0/120	0/8
6	74	0/42	0/188	0/118	0/19	0/2	0/4	0/1	0	0/20	0/31

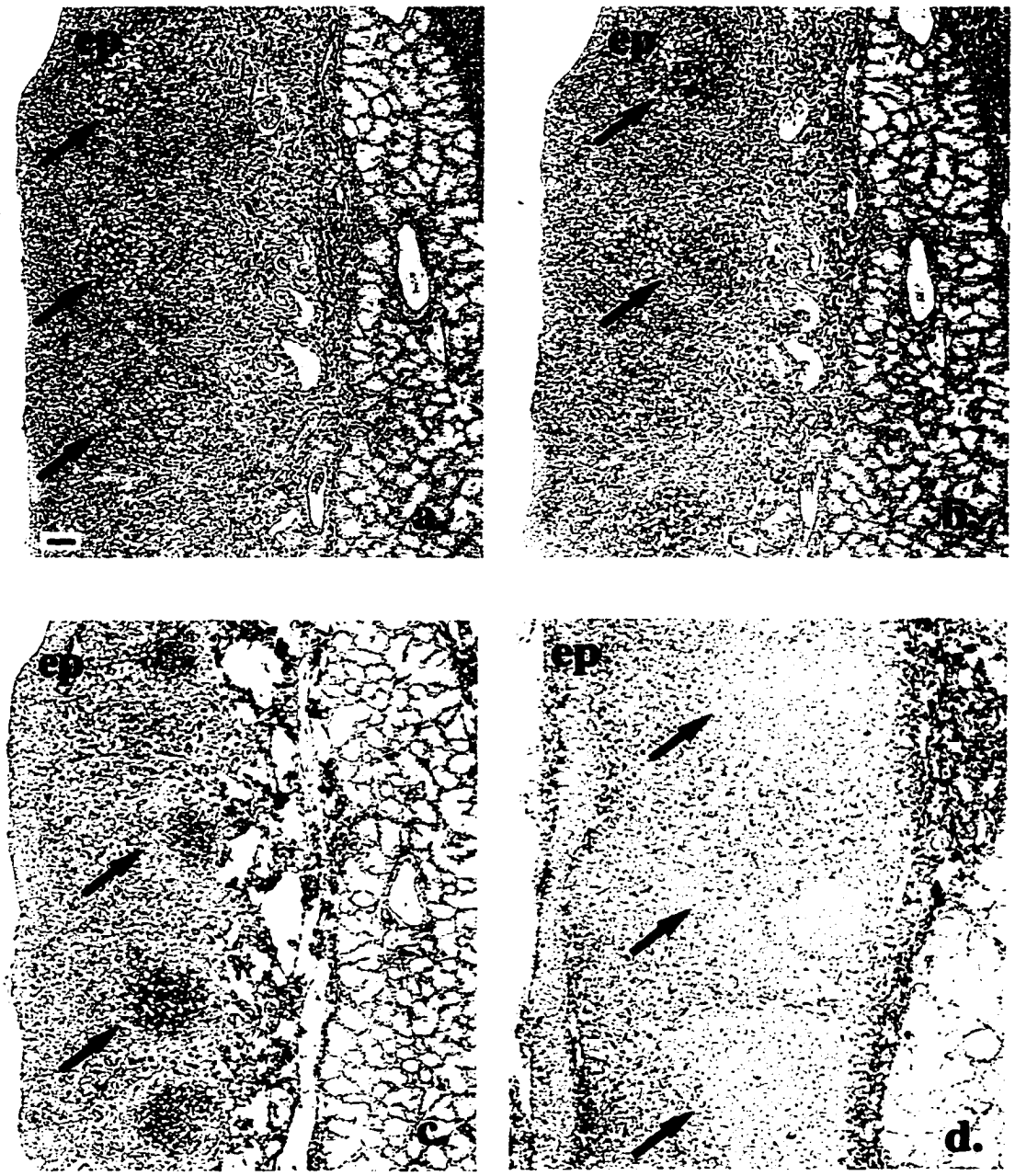

Figure 1.1. Enhanced immunohistochemical detection of PrP^{res} (red, arrows) within tonsillar lymphoid follicles of a known CWD+ deer. Tissue was treated with either: (a) hydrated autoclaving only, (b) formic acid+hydrated autoclaving, or (c) formic acid+proteinase-K+hydrated autoclaving prior to IHC using mAb F89/160.1.5 and an alkaline phosphatase-based system. Best staining was achieved with protocol (c). No PrP^{res} staining was present in CWD-negative control deer tonsil (d). ep=epithelium. bar=100μm.

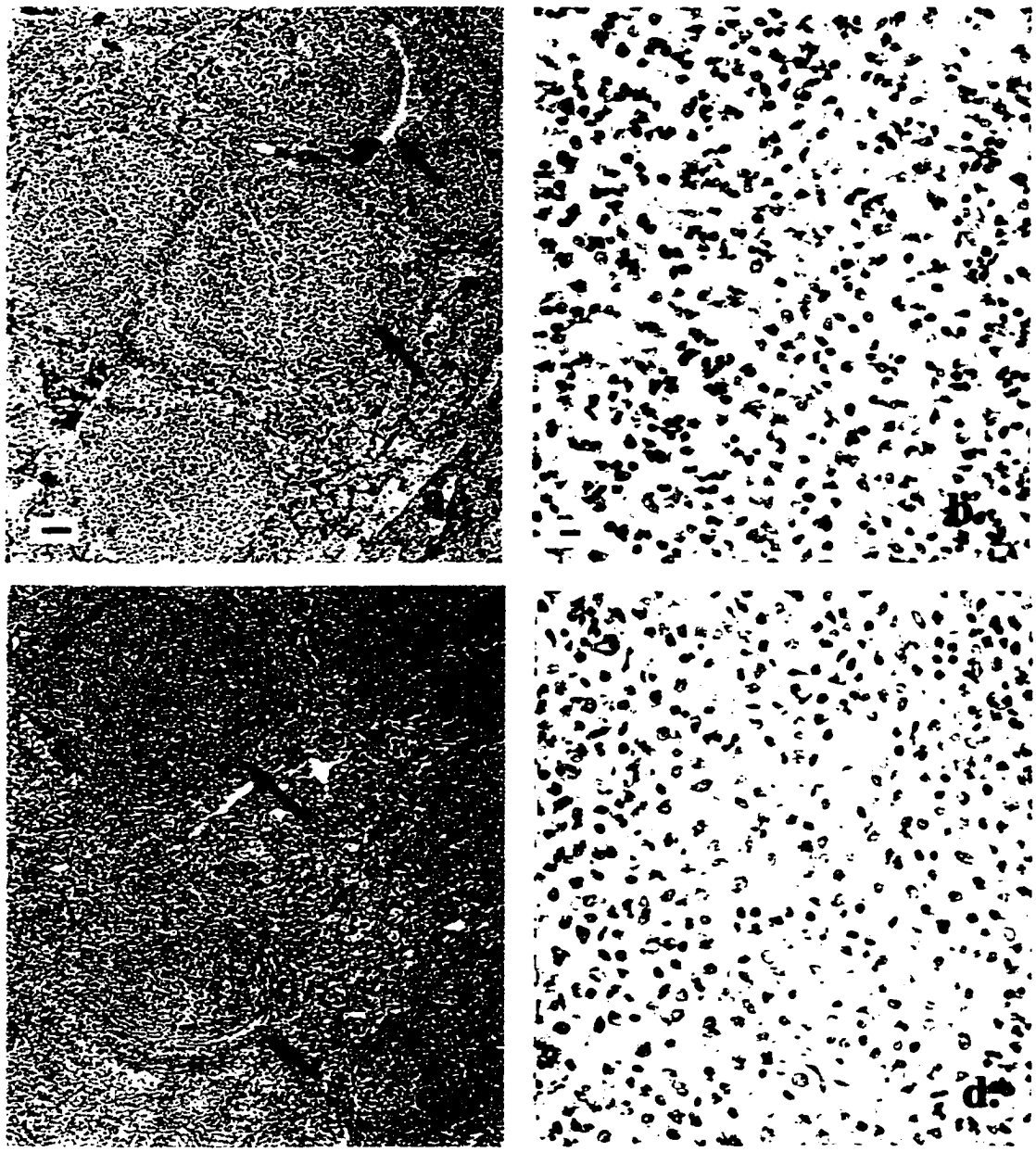

Figure 1.2. Immunohistochemical detection of PrP^{res} in retropharyngeal node lymphoid follicles (red, arrows) of a fawn exposed orally to CWD-positive brain inoculum (panels a & b). No PrP^{res} staining was detected in the retropharyngeal node follicles (arrows) of fawns exposed to CWD-negative brain inocula (panels c & d). bar=100μm (panels a & c) or 10μm (panels b & d).

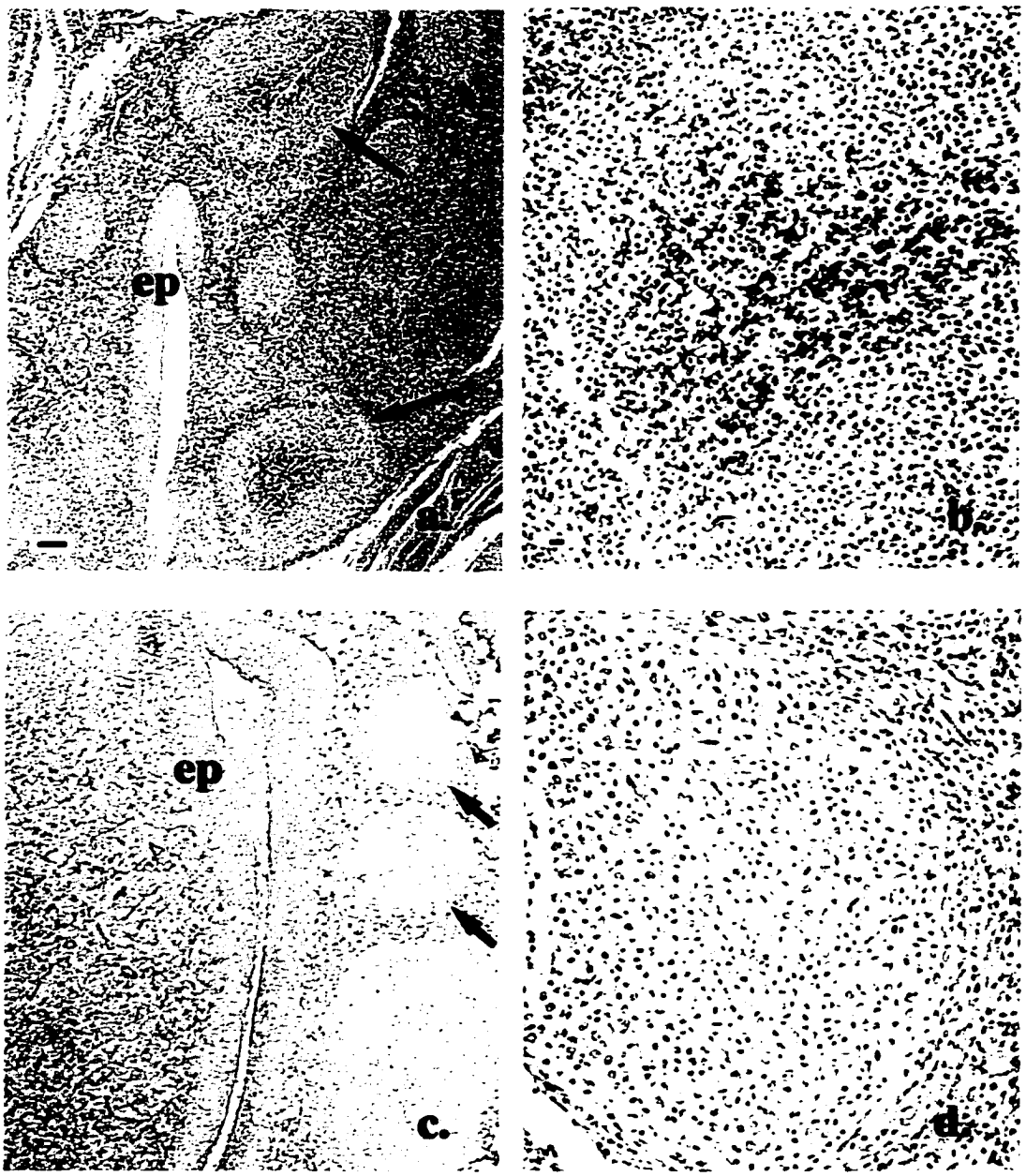

Figure 1.3. Immunohistochemical detection of PrP^{res} in tonsillar lymphoid follicles (red, arrows) of a fawn exposed orally to CWD-positive brain inoculum (panels a & b). No PrP^{res} staining was detected in the tonsillar follicles (arrows) of fawns exposed to CWD-negative brain inocula (panels c & d). bar=100μm (panels a & c) or 10μm (panels b & d).

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CHAPTER 2

PRP^{CWD} LYMPHOID CELL TARGETS IN EARLY AND ADVANCED CHRONIC WASTING DISEASE

ABSTRACT

Up to 15% of free-ranging mule deer in northeastern Colorado and southeastern Wyoming 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 vCJD 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 amplification interposed between mucosal entry and central nervous system accumulation. To identify the lymphoid target cells associated with PrP^{CWD} which may amplify the pathogenic prion agent, we used multi-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, and 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 conversion of PrP^{C} by FDC. In addition, scattered tingible body macrophages (TBM) in the germinal center 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} amplification within TBM. To compare lymphoid cell targets in early and advanced disease, we also examined: (i) PrP^{CWD} lymphoid cell distribution in 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 B cells--a finding which raises questions as to the cells responsible for pathologic prion production.

BACKGROUND

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, Williams & Young, 1993). In endemic areas of Colorado and Wyoming, 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 epidemiologic 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 sheep (SS) (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, SS, and vCJD (Hill et al., 1999, Spraker et al., 2002, van Keulen et al., 1996). However, experimental BSE in 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, e.g. humans with vCJD have lymphoid

CJD 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 (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 centers (GC) 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. 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 and/or tingible body 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 multi-immunofluorescent labelling and laser scanning confocal microscopy. We found--somewhat surprisingly--PrP^{CWD} was almost exclusively in association with cell membrane surfaces. In addition, smaller deposits of PrP^{CWD} were detected intracytoplasmically within CD68-positive macrophages or dendritic cells (DC) within GC and much less commonly within the paracortical zone of lymph nodes. These results suggested to us that either: (a) PrP^{CWD} conversion may occur at the surface rather than within FDCs or (b) that 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 3 groups of captive mule deer (Odocoileus hemionus) in various stages of infection: (1) tonsils from 6 deer with naturally occurring, clinical CWD, (2) retropharyngeal lymph nodes from 2 fawns orally inoculated with a CWD brain homogenate and euthanized at 42 and 78 days post-inoculation, (3) tonsil biopsies from 3 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 for PrP^{CWD}). Tonsils were fixed in 10% neutral

buffered formalin for 1-3 days then immersed in 88% formic acid for 1 hour and embedded in paraffin.

The clinically affected CWD-positive deer were diagnosed by: (1) histologic 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. Deer were confirmed as CWD-negative by the absence of histologic 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 2 sources: (1) adult deer from the CWD non-endemic area (non-endemic area established by methods in (Miller et al., 2000) and (2) 2 mule deer inoculated with CWD-negative brain homogenate from a previous study (Sigurdson et al., 1999). Tissues were similarly fixed and processed.

Phenotype antibodies. Because PrP^{CWD} deposits accumulate within germinal centers of primary and secondary lymphoid follicles, we focused on phenotype marker antibodies which would target follicular dendritic cells, B and T lymphocytes, and tingible body macrophages (Fig. 2.1). To ensure that the 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.

Lymphoid cells in the GC include follicular dendritic cells (FDC), tingible body macrophages (TBM), T and B lymphocytes, and germinal center dendritic cells (GCDC). GCDC, a dendritic cell subset in the tonsil that presents antigen to germinal center B cells, have been described in humans (Grouard et al., 1996, Summers et al., 2001) but not in ruminants.

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 or 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 TBM (Giorno, 1985) and FDC (Tsunoda et al., 1990), (7) anti-FDC (DAKO™), targets an 120kD epitope in FDC of human (Raymond et al., 1997), and (8) S100 (DAKO™), a calcium binding protein present in FDC and/or TBM, depending on the species (Carbone et al., 1988).

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 minutes at 121 °C, and cooled for 5 minutes. Sections were rinsed in phosphate buffered saline (PBS) and immersed in 3% H_2O_2 for 15 minutes to quench endogenous peroxidase. Sections were then briefly rinsed in PBS and incubated in TNB blocking solution (NEN Sciences™) for 30 minutes followed by exposure to 1-2 lymphoid phenotype antibodies and anti-PrP antibody 6H4 (monoclonal, IgG) (kindly donated by Bruno Oesch) or R522 (polyclonal) (kindly donated by Drs. Langeveld and van Keulen) for 30 minutes 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).

Since heat shock protein (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}, dual 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 488nm, HeNe 543nm, and HeNe 622 nm; these emit in the green, red, and far red spectra, respectively. Two secondary follicles were selected from each tonsil section and sequentially scanned at 900X using the three lasers.

Quantitation of co-localization of PrP^{CWD} and phenotype marker. Images from each deer were analyzed using Metamorph™ software (Universal Imaging Corp., West Chester, PA) applying the color 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 (900X magnification) were analyzed for PrP^{CWD} and phenotype marker co-labelling, and the results were averaged. Data were analyzed using the Student's T-test. Significance was defined at $P<0.05$.

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 $2mm^3$ sections in serum-enriched medium containing collagenase, dispase, and DNAase at 37 °C with agitation to digest the stroma and release the cells. The cells were pelleted by centrifugation, washed in phosphate-buffered saline, and then cytocentrifuged onto positively-charged glass slides. Cells were fixed in 10% buffered formalin for 15 minutes 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 2 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 monoclonal antibody (Mab) 99/97.6.1 (Spraker et al., *In Press.* 2002) (generously provided by K. O'Rourke), a biotinylated secondary antibody, an alkaline phosphatase-streptavidin conjugate, a substrate chromagen (fast red A), and a hematoxylin and bluing counterstain (Ventana Medical Systems™). 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.

RESULTS

PrP^{CWD} in lymphoid germinal centers

In tonsils of all CWD infected deer examined by immunohistochemistry (IHC), PrP^{CWD} was concentrated primarily in lymphoid follicle GC. However, PrP^{CWD} was also detected occasionally in cells within perifollicular areas (Fig. 2.2). Tonsils from deer with clinical CWD or tonsil biopsies from preclinical, CWD-infected deer had a high frequency of PrP^{CWD} positive follicles, ~80-100%. By contrast, in fawns examined 7 to 11 weeks after oral CWD exposure, <30% of retropharyngeal lymph node follicles contained detectable PrP^{CWD}.

PrP^{CWD} accumulates on follicular dendritic cell and B cell membranes

To study the association of PrP^{CWD} with GC cells we co-labelled tonsil sections for PrP^{CWD}, FDC, and other lymphoid cell phenotypes. Three phenotype markers were used to identify FDC: S100, vimentin, and anti-FDC. While PrP^{CWD} appeared in close association with FDC (Figs. 2.3, 2.4, 2.5), co-localization of PrP^{CWD} with any of the intracellular markers was rare in dually labelled sections. To investigate whether PrP^{CWD} accumulated extracellularly on FDC membranes, we then dual-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)(Figs. 2.3, 2.4, 2.5). Co-localization with these extracellular labels

was frequent, and documented by quantitative analysis, to be ~4x higher than with intracellular FDC markers ($p<.05$, Fig. 2.6). These observations led us to conclude that PrP^{CWD} accumulated primarily on membranes associated with FDC or B lymphocytes.

PrP^{CWD} in the cytoplasm of tingible body macrophages

We investigated whether tingible body macrophages (TBM) were involved in PrP^{CWD} accumulation based on earlier experiments in which PrP^{CWD} staining was visualized in cells morphologically characteristic of TBM containing cytoplasmic apoptotic bodies (Fig 2.10). To determine whether TBM in germinal centers accumulated PrP^{CWD}, we used three intracellular antibody markers for TBM: CD68, HSP70, and ferritin. We identified 2 populations of macrophages which contained PrP^{CWD}: (1) TBM within GC and (2) isolated macrophages in the perifollicular area (Fig. 2.7). We found PrP^{CWD} was closely associated and occasionally co-localized spatially with macrophage phenotype markers CD68, ferritin, and HSP 70. Serial incremental imaging through the perpendicular axis of the dually labelled sections by high resolution confocal microscopy revealed PrP^{CWD} adjacent to the intracellular TBM phenotype markers--indicating that PrP^{CWD} was intracellular (Figs. 2.8, 2.9).

PrP^{CWD} in separated lymphoid cells

To determine whether membrane associated PrP^{CWD} was affiliated with B cells or 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 2.10). PrP^{CWD} containing cells also stained positively for ferritin, a trait most compatible with macrophages, and occasionally for lambda light chain and vimentin, traits most suggestive of FDC, TBM or B cells.

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

We compared PrP^{CWD} lymphoid target cells from deer in early, asymptomatic stages of infection to deer manifesting 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 (pi), PrP^{CWD} was distributed primarily on FDC and B cell membrane surfaces with less involvement of TBM. One fawn (6 weeks pi) had no apparent PrP^{CWD} in TBM; PrP^{CWD} was primarily associated with cell membranes. In a second fawn (11 weeks pi) PrP^{CWD} was detected in both the cell membrane (FDC/ B cells) and intracellular (TBM) patterns.

DISCUSSION

A prominent feature of CWD in mule deer is the abundant PrP^{CWD} accumulation in lymphoid germinal centers (GC), similar to that in variant CJD in humans (Bruce et al., 2001, Hill et al., 1999, Hilton et al., 1998) and scrapie in sheep (SS) (Andreoletti et al., 2000, Heggebo 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, 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 amplification and trafficking to the central nervous system (CNS) remains unclear.

Our observations indicate that PrP^{CWD} accumulates in close association with FDC. 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 and colleagues (Jeffrey et al., 2000) used immunogold labelling to elegantly demonstrate ME7 PrP^{Sc} on the plasmalemma of splenic FDC. Secondly, Manuelidis et al. (Manuelidis et al., 2000) used confocal microscopy to localize strain FU CJD PrP^{res} on FDC membranes. Interestingly, localization of infectious agent to FDC is not

unique to TSEs. Other infectious agents, especially viruses, have been described on FDC surfaces, including bovine virus diarrhea (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 harbor prions remains controversial. For example, Montrasio et al. (2000) demonstrated that inhibition of FDC development virtually eliminated splenic PrP^{Sc} (Montrasio et al., 2000). While Mabbott et al (2000) found similar results if mice had FDC deleted prior to scrapie challenge, when FDC were deleted after challenge mice developed high levels of splenic infectivity (Mabbott et al., 2000). Moreover, in experiments using chimeric mice in which PrP^{C} expression between FDC and other lymphoid cells was mismatched, Brown et al. (Brown et al., 1999) found that only those mice expressing PrP^{c} in FDC were susceptible to scrapie, strongly suggesting prion propagation in FDC. By contrast, Manuelidis et al. (Manuelidis et al., 2000) concluded that limiting i.p. 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) (Klein et al., 2001, Mabbott et al., 2001).

Unlike the membrane-associated PrP^{CWD} of FDC, intracytoplasmic large, dense aggregates of PrP^{CWD} were detected in tingible body macrophages (TBM). This finding is reminiscent of studies showing PrP^{Sc} deposits associated with CD68 positive cells (Andreoletti et al., 2000) or cells morphologically consistent with TBM in naturally infected scrapie sheep (van Keulen et al., 1996). Moreover, Jeffreys et al. (2000), described PrP^{Sc} in lysosomes of TBM, consistent with immunogold electron microscopy studies localizing PrP^{Sc} in neurons (Laszlo et al., 1992).

There are several potential roles for the TBM in prion pathogenesis. It is possible that CD68-positive DC or macrophages transport PrP^{CWD} into the germinal center and expose the FDC, T and B cells to PrP^{CWD}. CD68-positive cells harboring PrP^{CWD} or PrP^{Sc} (Andreoletti et al., 2000) have been localized adjacent to germinal centers. However, TBM are in close contact with FDC and are known to phagocytose immune complex coated bodies (iccosomes) (Szakal et al., 1988). In addition, TBM 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). Likewise, TBM phagocytose apoptotic B cells, which also could serve as a potential source of PrP^{CWD} exposure.

Although PrP^{CWD} was primary localized to germinal centers, 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. Whether these cells traffic in or out of the lymph node is pertinent to know due to the critical question of whether PrP^{res} circulates in blood. The potential association of T cells with PrP^{CWD} was difficult to assess due to their low numbers within GCs. Scrapie studies in transgenic and immunodeficient mice suggest T cells do not affect disease susceptibility or splenic infectivity (Klein et al., 1997, Klein et al., 1998).

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 pi had PrP^{CWD} extracellularly with no detectable involvement of TBM. We speculate that the TBM may be phagocytosing extracellular PrP^{CWD} iccosomes and that there is a short lag before TBM contain PrP^{CWD}. This scenario could explain why 1 fawn (6 weeks pi) had no apparent PrP^{CWD} in TBM versus a second fawn (11 weeks pi). The immunogold cell labelling of PrP^{Sc} was similar in scrapie inoculated mice at 70 and 170 days post-inoculation (Jeffrey et

al., 2000). In contrast, in sheep naturally infected with scrapie PrP^{Sc} was apparent in CD68 positive cells prior to detection in FDC (Andreoletti et al., 2000).

The close association of PrP^{CWD} with the membrane surfaces of FDC and/or B cells and the presence of intracytoplasmic PrP^{CWD} in TBM 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 pathways emanating from mucosal surfaces and could be critical to diagnostic and intervention measures during the preclinical stages of prion infections.

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Figure 2.1. Lymphoid germinal centers consist of follicular dendritic cells (FDC), B cells in various stages of maturation, T cells, and tingible body macrophages (TBM).

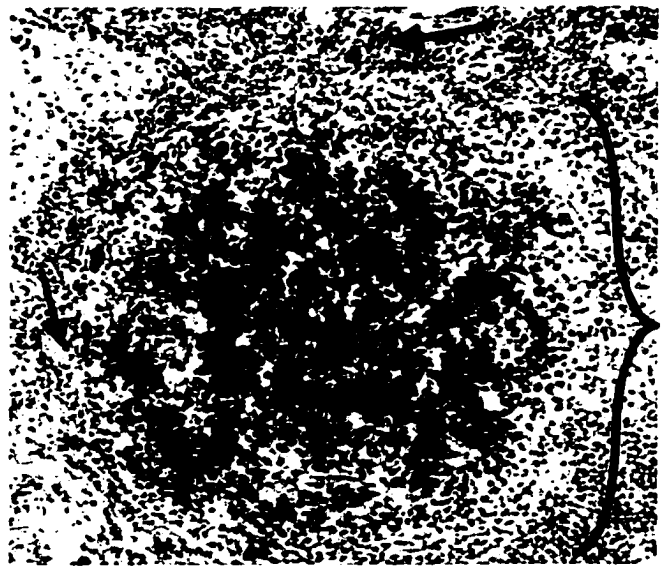

Figure 2.2. Typical abundant follicular PrP^{CWD} deposition in a lymph node follicle from a deer with advanced CWD. Note the network-like lattice of PrP^{CWD} deposition defining the central zone of the follicle (bracket) and isolated PrP^{CWD} positive cells at the periphery of the follicle (arrows).

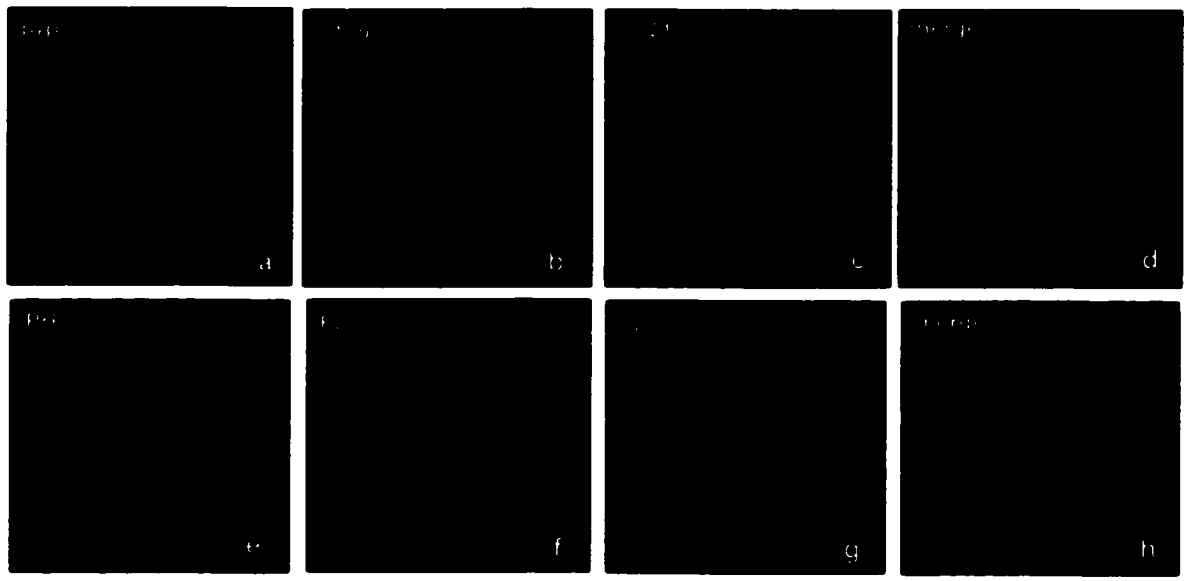

Figure 2.3. PrP^{CWD} is on the cell surface of FDC. Lymphoid follicle within a CWD positive deer tonsil stained using 2 multilabelling protocols. PrP^{CWD} (panels a,e, red) co-localizes with cell membrane markers for cc21 and immunoglobulin (Ig) (panels c,g, blue) visible as pink in the merged image (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).

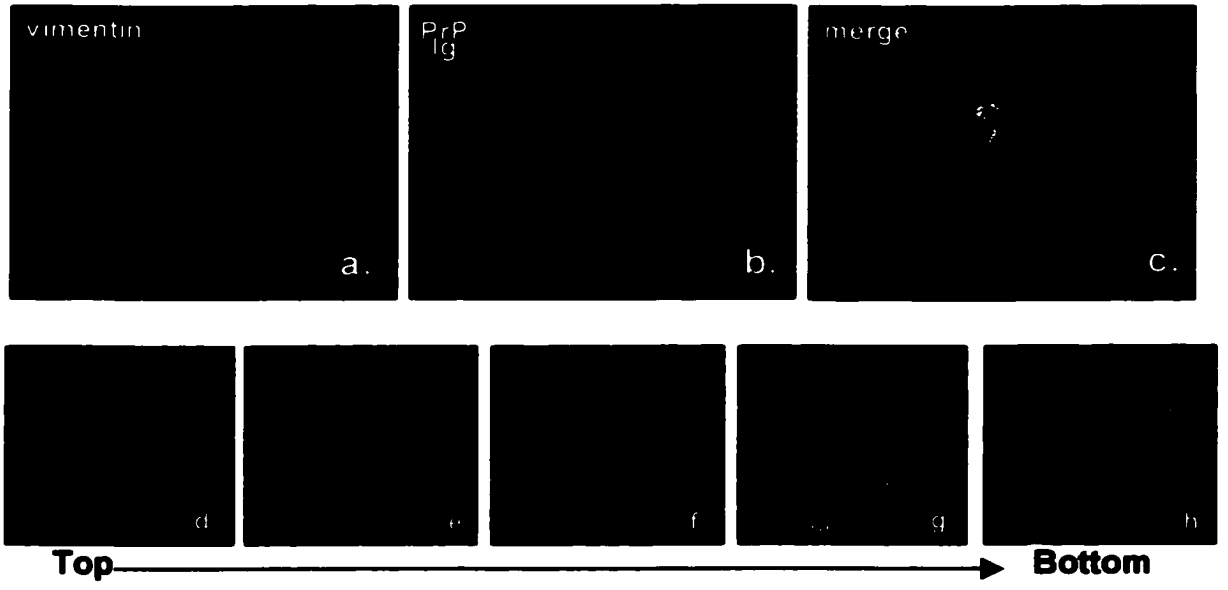

Figure 2.4. PrP^{CWD} (panel b, red) co-localizes strongly with membrane bound lambda light chain of immunoglobulin (Ig, blue) and poorly with the intracellular marker vimentin (panels a,c). Lymphoid follicle, CWD positive deer tonsil, high magnification. Serial sections from the same field show different 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 section.

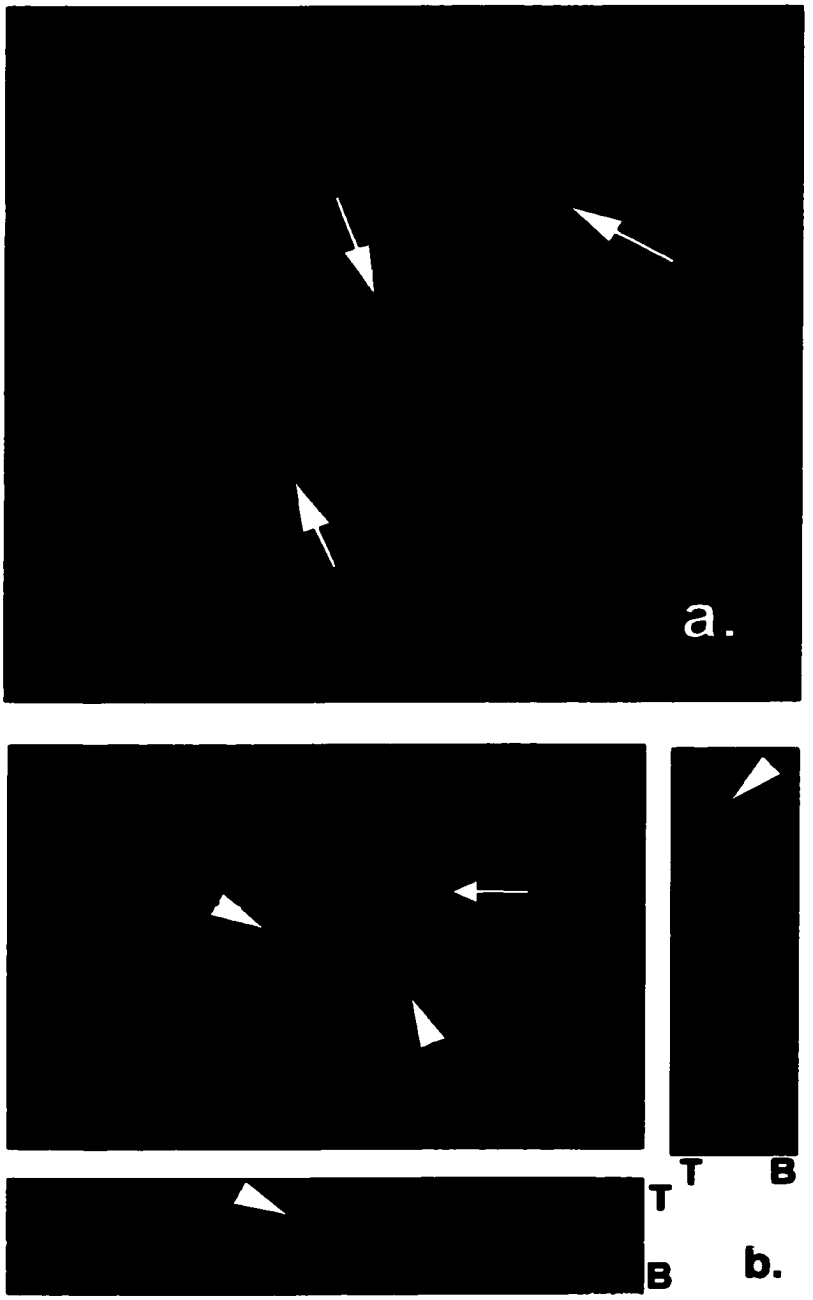

Figure 2.5. PrP^{CWD} associates with cell membranes on follicular dendritic and B cells. Tonsillar germinal center, high magnification. (a) PrP^{CWD} is on membrane surfaces and dendritic processes of FDCs (arrows). (b) PrP^{CWD} co-localizes with cc21 (CD21) on membrane surfaces (arrowheads, pink) 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 perpendicular to the plane of 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 discernable PrP^{CWD} within the cytoplasm of the FDC, PrP^{CWD} is between the cells.

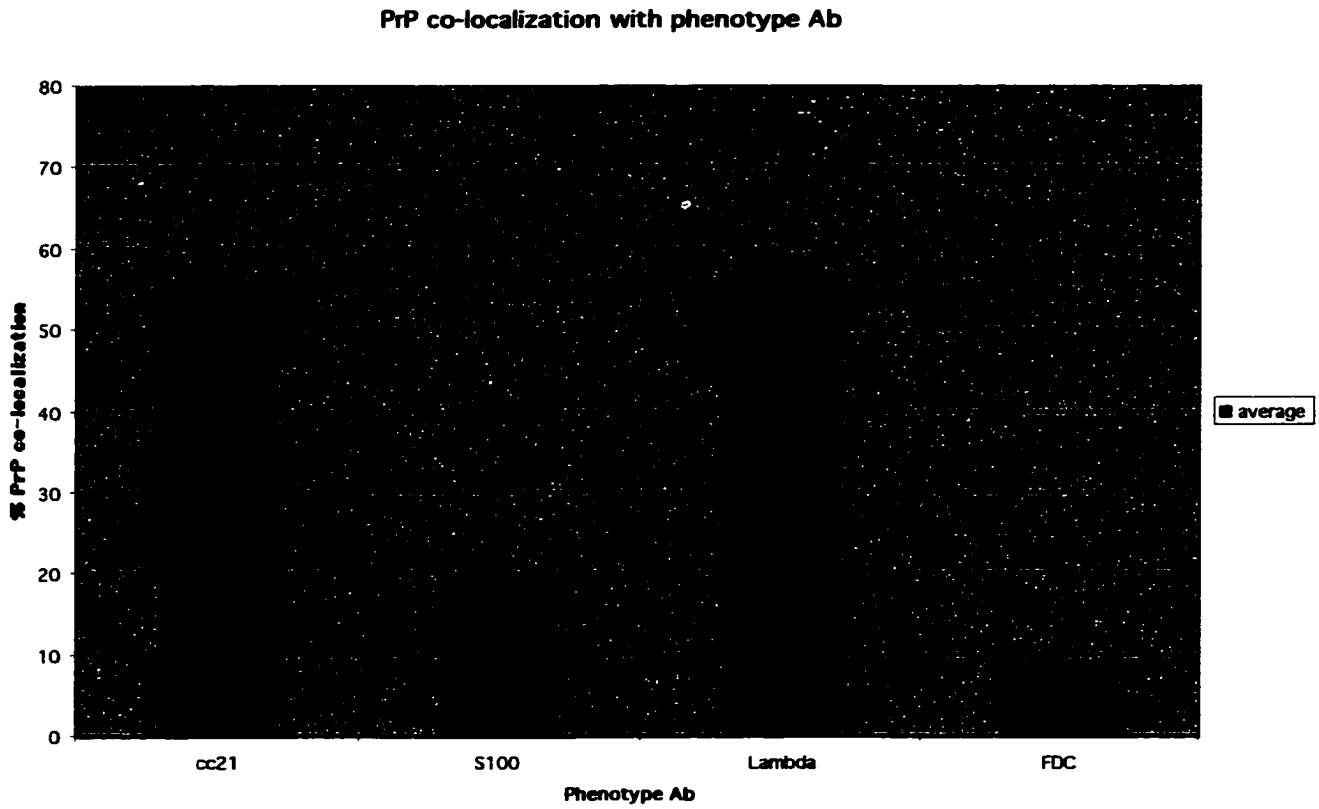

Figure 2.6. Percent PrP^{CWD} co-localization with intracellular markers for FDC (S100, FDC) or cell membrane markers (immunoglobulin, cc21). N=12.

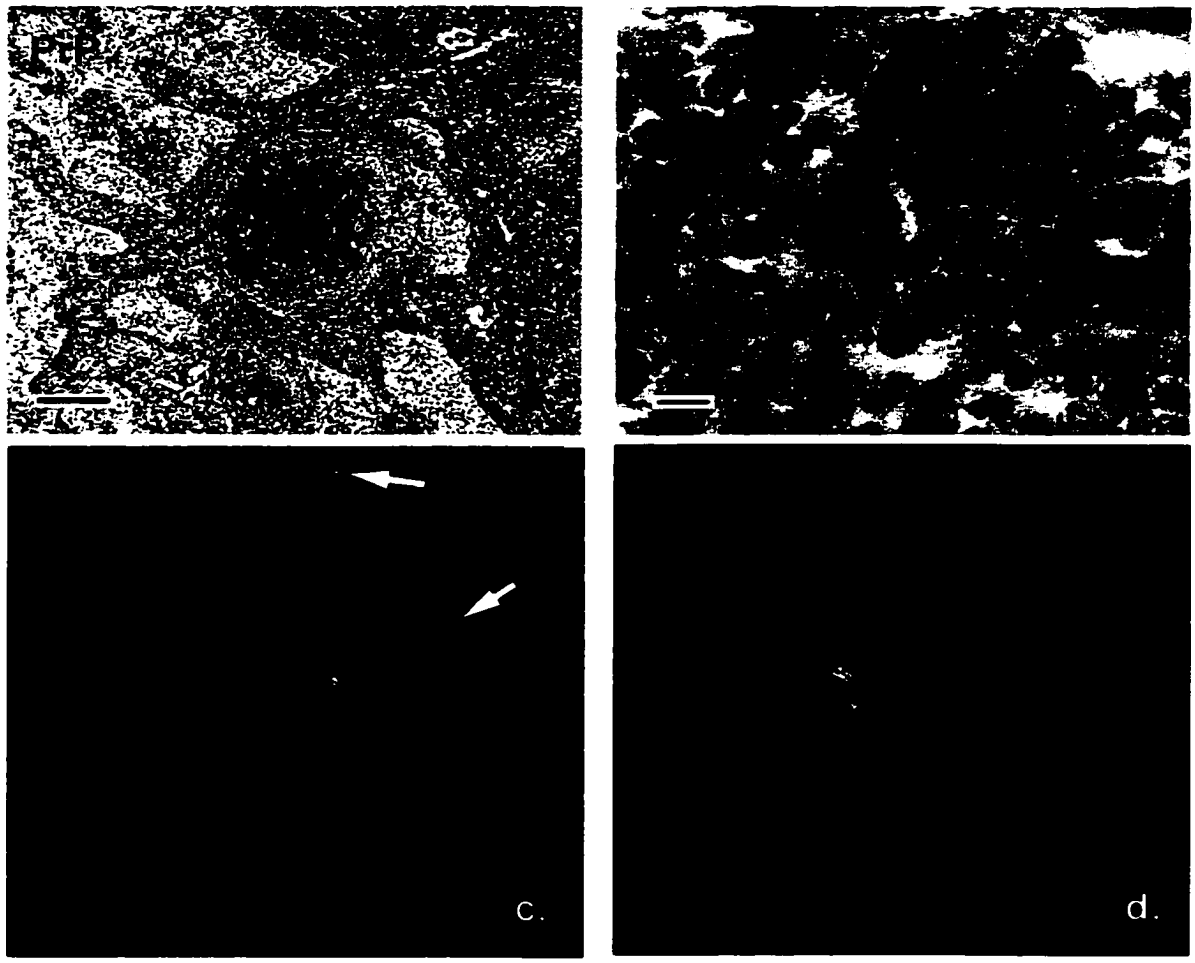

Figure 2.7. PrP^{CWD} containing macrophages (arrows) are peripheral to the lymph node follicule labelled by immunohistochemistry (a, b) or by multi-immunofluorescence demonstrating PrP^{CWD} (red), CD68 (green, macrophages or DC), and nuclei (blue) (c,d), in confocal microscopy. It is likely that the cells in panels a and b are also CD68 positive macrophages. Bar=1mm (a) or 10μm (b).

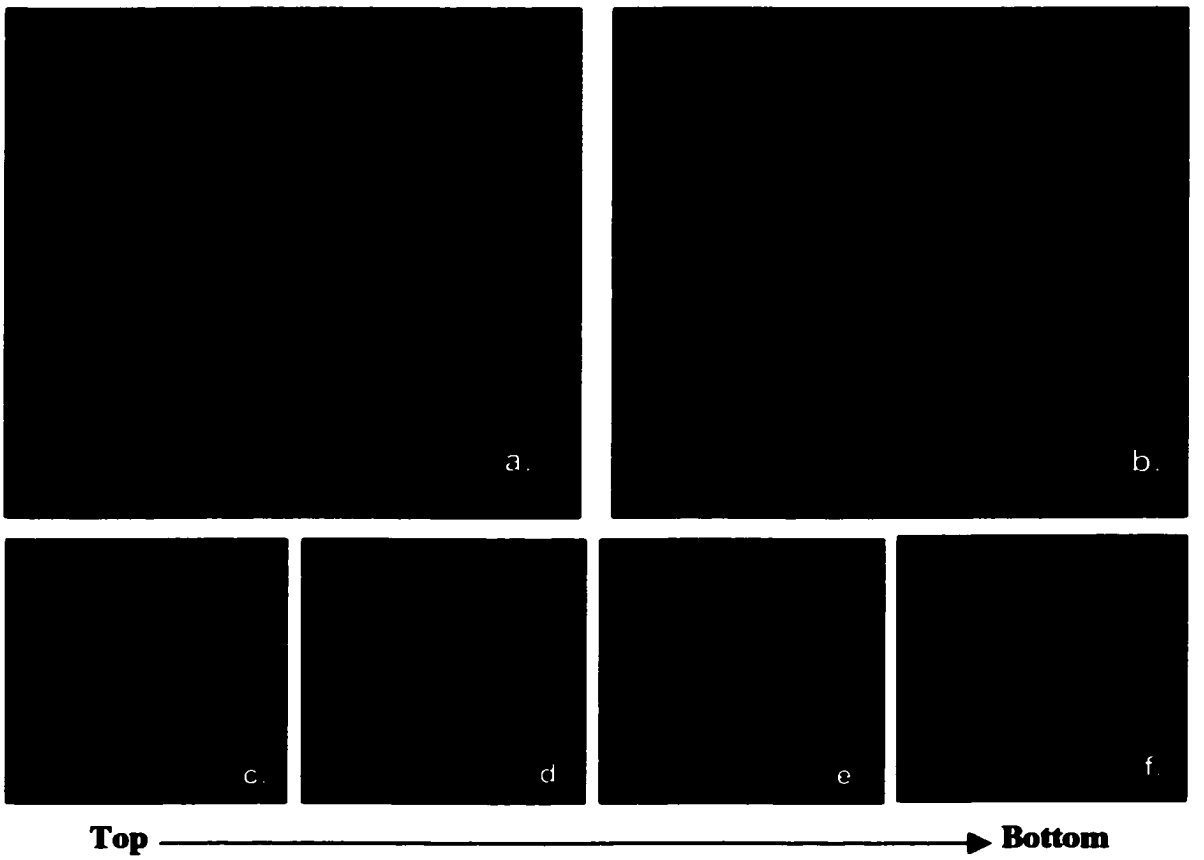

Figure 2.8. Tonsillar follicle from a CWD-positive deer. PrP^{CWD} (red) is detected within cells positive for HSP (green), presumably macrophages (a,b). Serial sections of a single cell from top to bottom were obtained ~1μm apart and demonstrate the presence of PrP^{CWD} and HSP70 within the cytoplasm (c-f). Nuclei are labelled blue.

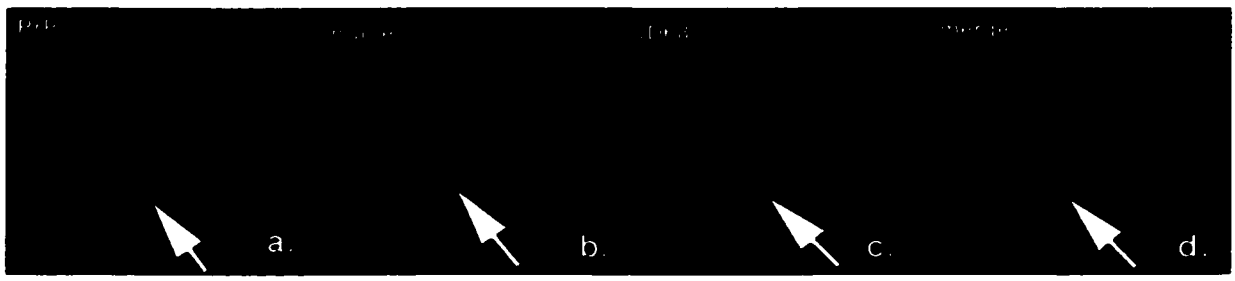

Figure 2.9. CD68 positive cell (green) within the germinal center contains PrP^{CWD} (red, arrow). Nuclei are labelled blue.

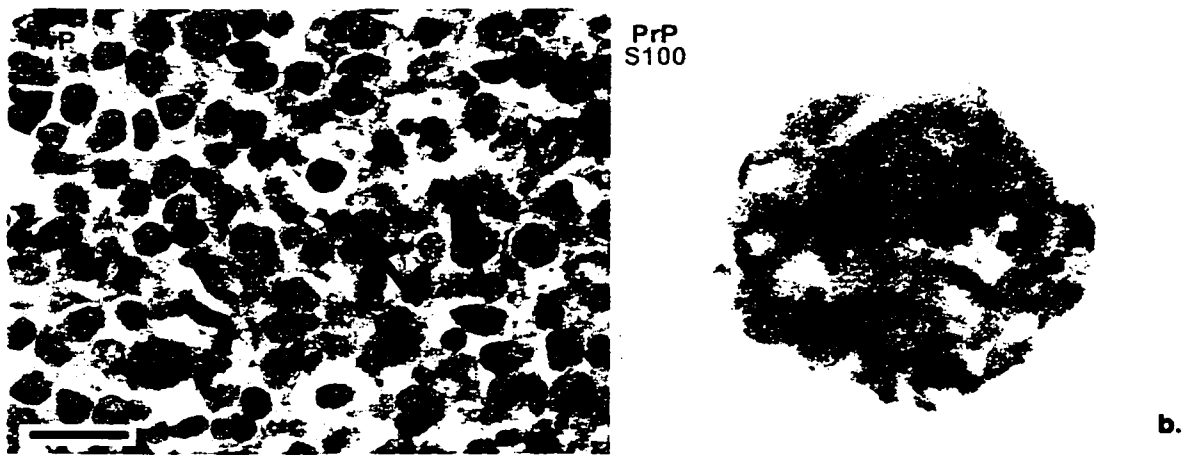

Figure 2.10. Tingible body macrophages (TBM) contain PrP^{CWD}. (a) Tonsil biopsy from a deer with pre-clinical CWD demonstrating PrP^{CWD} in cells morphologically consistent with TBM. Note condensed fragmenting nuclei within the cytoplasm (arrows). (b) S100 positive cell (brown) dually labelled for PrP^{CWD} (red) from a cytospin preparation of an enzymatically dissociated CWD positive deer lymph node.

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CHAPTER 3

PRP^{CWD} IN THE MYENTERIC PLEXUS, VAGOSYMPATHETIC TRUNK, AND ENDOCRINE GLANDS OF DEER WITH CHRONIC WASTING DISEASE

ABSTRACT

Accumulated evidence in experimental and natural prion disease systems supports a neural route of infectious prion spread from peripheral sites of entry to the central nervous system. However, little is known about prion trafficking routes in cervids with a naturally-occurring prion disease known as chronic wasting disease (CWD). In the brain, the pathogenic isoform of the prion protein (PrP^{CWD}) accumulates initially in the dorsal motor nucleus of the vagus nerve (DMNV). To assess whether alimentary-associated neural pathways may play a role in prion trafficking, we examined neural and endocrine tissues from mule deer naturally infected with CWD (n=6) by immunohistochemistry. We detected PrP^{CWD} in the myenteric plexus, vagosympathetic trunk, nodose ganglion, pituitary, adrenal medulla, and pancreatic islets. No to scant PrP^{CWD} staining was detected in other nerves or ganglia (brachial plexus, sciatic nerve, gasserian ganglion, celiac ganglion, cranial cervical ganglion, spinal nerve roots) of CWD-positive deer nor was any PrP^{CWD} detected in nerves or

endocrine tissues from 11 control deer. These findings suggest that: (a) PrP^{CWD} transit in nerves, either centrifugally or centripetally, is one route of prion trafficking and organ invasion and (b) endocrine organs may also be targets for cervid pathogenic prion accumulation.

BACKGROUND

Chronic wasting disease (CWD) is an endemic transmissible spongiform encephalopathy (TSE) of captive and free-ranging mule deer, white-tailed deer, and Rocky Mountain elk in Colorado and Wyoming (Williams & Young, 1980, Williams & Young, 1982, Spraker et al., 1997). CWD is transmitted efficiently in nature, with the prevalence reaching 15% in some subpopulations (Miller et al., 2000). Relatively little is known, however, about the mode of transmission or the pathogenesis of this naturally occurring TSE. TSEs are characterized by the accumulation of a pathogenic, partially protease-resistant isoform (PrP^{res}) of a normal cellular protein (PrP^{c}) (Prusiner, 1982). PrP^{res} deposition occurs principally in the central nervous system (CNS) resulting in a neurodegenerative disease and in some prion infections, e.g. scrapie and variant Creutzfeldt-Jakob disease, PrP^{res} deposition or infectivity also occurs in lymphoid tissues (Hadlow et al., 1982, Hill et al., 1999, Brown et al., 1999). Understanding routes of agent spread from peripheral entry sites to the CNS is fundamental to developing strategies to block neuroinvasion.

A growing body of evidence comprising both experimental and natural studies strongly implicates PrP^{res} dissemination from the entry site via peripheral nerves. Pioneering studies by Kimberlin et al. in intrasciatic or intragastrically inoculated mice demonstrated scrapie transport from the peripheral nervous system (PNS) directly to the brain or from the intestinal tract to the thoracic spinal cord via splanchnic nerves (Kimberlin et al., 1983, Kimberlin & Walker, 1989). Additionally, in hamsters orally challenged with 263K scrapie, PrP^{res} was detected by immunohistochemistry (IHC) in early infection within the myenteric and submucosal plexuses (Beekes & McBride, 2000) as well as the dorsal motor nucleus of the vagus nerve (DMNV) in the brain (Beekes et al., 1998) and terminally in the vagus nerve, the nodose, dorsal root, and celiac ganglia—findings consistent with spread via vagal and splanchnic routes (McBride & Beekes, 1999). van Keulen et al. demonstrated PrP^{res} deposits in the enteric nervous system in natural sheep scrapie which supported the alimentary tract as the site of neural invasion (van Keulen et al., 1999, van Keulen et al., 2000). Moreover, nerve infectivity or PrP^{res} has been detected in natural (Hadlow et al., 1982, Groschup et al., 1996) and experimental scrapie (Groschup et al., 1999), BSE infected lemurs (Bons et al., 1999), and in a natural case of CJD (Hainfellner & Budka, 1999).

Recent findings have highlighted the significance of spread via peripheral nerves. Glatzel and Aguzzi compared PrP^{res} tissue distribution patterns in wild type versus transgenic PrP^{c} overexpressing mice. They demonstrated that elevated PrP^{c} expression in the peripheral nervous system (PNS) biased PrP^{res} transit pathways

toward intranerval spread in transgenic mice versus lymphoreticular spread in wild-type mice (Glatzel & Aguzzi, 2000). Race et al. demonstrated that transgenic mice expressing hamster PrP^{c} in neural but not lymphoid tissues developed brain PrP^{res} infection after oral or intraperitoneal inoculation with hamster scrapie--establishing a vital role for PrP^{c} peripheral nerve expression in neuroinvasion (Race et al., 2000).

As with other prion diseases (scrapie, bovine spongiform encephalopathy, kuru, and variant Creutzfeldt-Jakob disease), CWD infections are suspected to arise from oral exposure to the causative agent. In deer and elk infected orally with brain containing PrP^{res} (PrP^{CWD}), PrP^{CWD} deposition was first detected in alimentary-associated lymphoid

tissues (Sigurdson et al., 1999) and subsequently in the DMNV in the medulla oblongata (Williams & Miller, 2000). In naturally infected deer, histologic lesions of TSE and PrP^{CWD} were present in the hypothalamus, thalamus, brainstem, and spinal cord gray matter with lesser amounts in the cerebral cortex, and cerebellum (Spraker et al., 1997, Spraker et al., 2001). PrP^{res} correlates closely with infectivity and serves as a surrogate marker for prion infection (McKinley et al., 1983, Race et al., 1998). Thus, immunohistochemistry (IHC) can be used to localize PrP^{res} in tissues.

Because the DMNV is the initial target site for PrP^{CWD} in the brain (Williams & Miller, 2000) and the vagosympathetic trunk carries vagal nerve fibers innervating the alimentary tract, we examined alimentary nerves and ganglia from mule deer with naturally occurring CWD using IHC. For comparison to non-alimentary nerves, we

examined brachial plexus and sciatic nerve which innervate the forelimb and hindlimb, respectively. To investigate components of the sympathetic splanchnic circuitry, celiac ganglia and thoracic spinal cord with associated nerve roots were assessed. Gasserian ganglia were included to explore potential PrP transit via the trigeminal nerve. We report that the myenteric plexus, vagosympathetic trunk, and to a lesser degree the other peripheral nerves of deer contain PrP^{CWD}, indicating that nerve transport may be one route of PrP trafficking in CWD. An unexpected finding was the detection of PrP^{CWD} in pancreatic islet cells, adrenal medulla, and pituitary, suggesting nerve-vectored transit may also occur to endocrine organs.

METHODS

CWD infected deer and tissue collection. Six captive mule deer (Odocoileus hemionus) with naturally occurring, clinical CWD were euthanized and the following tissues collected for assessment by immunohistochemistry: brain, ~15 cm of cervical vagosympathetic trunk, 15-20 cm of sympathetic trunk from the thoracic vertebral region, 8-10 cm of sciatic nerve, 8-10 cm of brachial plexus, a 4 cm^2 section of pancreas, pituitary, adrenal gland, small intestine, celiac and gasserian ganglia, and thoracic spinal cord (Fig. 1). Tissues were fixed in 10% neutral buffered formalin for 1-3 days then immersed in 88% formic acid for 1 hour and embedded in paraffin.

Negative control deer and tissues. Brain and vagosympathetic trunk from 8 free-ranging mule deer from a CWD non-endemic area [non-endemic area established by

methods in (Miller et al., 2000)] and brain, sciatic nerve, adrenal gland, pancreas, small intestine, celiac and gasserian ganglia, thoracic spinal cord, and pituitary of 3 mule deer inoculated with CWD-negative brain homogenate from a previous study (Sigurdson et al., 1999) were similarly fixed and processed.

Immunohistochemical staining. Tissue sections were mounted onto positively-charged glass slides, deparaffinized, hydrated, autoclaved in a citrate buffer solution (DAKO™ Target Antigen Retrieval) for 20 minutes at 121 °C, and cooled for 5 minutes.

The IHC protocol employed an automated immunostainer (Ventana Medical Systems™) and PrP monoclonal antibody (Mab) 6H4 (generously provided by B. Oesch) or 99/97.6.1 (generously provided by K. O'Rourke), a biotinylated secondary antibody, an alkaline phosphatase-streptavidin conjugate, a substrate chromagen (fast red A), and a hematoxylin and bluing counterstain (Ventana Medical Systems™). Mab 6H4 recognizes a conserved sequence of the prion protein, corresponding to the human amino acid sequence 144-152, and recognizes PrP epitopes of rabbit, mink, sheep, cattle, and primates (Korth et al., 1997). Mab 99/97.6.1 recognizes residues 220-225 of the ovine prion protein (O'Rourke et al., 2000). An isotype matched, irrelevant antibody was substituted in the IHC protocol as a negative control. Each immunostained nerve section was examined 1-2 times per antibody.

RESULTS

PrP^{CWD} in nerve and ganglia

In that alimentary exposure is likely in CWD, we focused on two major autonomic nerve tracts as potential PrP^{CWD} conduits: (1) the vagosympathetic trunk and (2) the splanchnic neural circuitry. The vagosympathetic trunk includes parasympathetic vagal nerve fibers, which have nerve cell bodies in the DMNV, pass through the nodose ganglion, and synapse with the myenteric plexus of the small intestine. The splanchnic nerves, which have nerve cell bodies in the intermediolateral cell column of the thoracic spinal cord, carry sympathetic fibers which synapse directly in the adrenal medulla, or synapse in the celiac ganglion and innervate the esophagus, stomach, and small intestine (Fig. 3.1).

CWD positive deer were diagnosed by: (1) histologic lesions of CWD in the medulla oblongata including perikaryonic neuronal vacuoles, spongiform degeneration of the neuropil, and astrocytosis, and (2) abundant PrP^{CWD} stain in the DMNV using IHC. Deer were confirmed as CWD negative by the absence of histologic lesions and negative staining for PrP^{CWD} in all tissues. In one deer, abundant PrP^{CWD} stain was present in the DMNV, the radix tract, and the vagus nerve exiting the obex (Fig. 3.2a). PrP^{CWD} was detected in the vagosympathetic trunk (n=6 deer), sciatic nerve (n=1), sympathetic trunk (n=1), and brachial plexus (n=1) (Table 3.1). The stain deposits appeared as scattered coarse granules within the nerve fascicles and

occasionally within axons (Fig. 3.2b). The staining detected in the sciatic nerve and brachial plexus was scant compared with the vagosympathetic trunk. No stain deposits were visible in the nerves from CWD-negative deer from known non-endemic geographic regions using Mab 6H4 (Fig. 3.2c). Three stain granules were detected in one nerve of one negative control deer using Mab 99/97.6.1; therefore only nerves with >3 stain granules were considered positive. PrP^{CWD} was present in the myenteric plexus (n=5, Fig.3.2d), nodose ganglia (n=2) and in the intermediolateral cell column of the thoracic spinal cord (n=4), but not in the cranial cervical, celiac or gasserian ganglia (Table 3.2). Stain deposits in the nodose ganglia appeared primarily along nerve fibers and in satellite cells with scant to no stain within the ganglion cell body. However, coarse stain deposits were present in the myenteric plexus neurons (Fig. 3.2d).

Minor differences in the tissue staining positivity were observed between Mab 6H4 and 99/97.6.1 (Table 3.1, 3.2). The IHC stain deposits in nerve were irregularly distributed and widely spaced along the fascicle. Stain was quantified in the nerve only by the number of red PrP stain granules present in a section and recorded as: + (4-10), ++ (10-20), or +++ (>20). Nerve tissues incubated with an irrelevant Mab were negative.

PrP^{CWD} deposits in pituitary, islets of Langerhans, and adrenal medulla

The pancreases of 5 of 6 CWD-positive deer contained diffuse coarse granular PrP^{CWD} deposits confined to the islets of Langerhans (Fig. 3.3a). Although fewer than one

half of the islets were affected in any pancreas section in 4 of 5 deer, PrP^{CWD} stain was abundant in the affected islets. Such deposits were not detected in pancreases of CWD-negative deer (Fig. 3.3b). In the pituitaries of all CWD-positive deer, PrP^{CWD} deposits were evident primarily in the pars nervosa and intermedia (Fig. 3.3c) and were not seen in the CWD-negative deer (Fig. 3.3d). Likewise, PrP^{CWD} staining was identified in the adrenal medulla in 3 of 5 CWD-positive deer and not the controls.

DISCUSSION

We detected PrP^{CWD} in the myenteric plexus, vagosympathetic trunk, and intermediolateral cell column of the spinal cord of naturally infected CWD deer consistent with previous findings in experimental and natural TSE. Likewise, scrapie PrP^{res} has been demonstrated in submucosal and myenteric plexuses in orally inoculated hamsters (Beekes & McBride, 2000) and in naturally infected sheep (van Keulen et al., 2000). These findings suggest that prion trafficking may occur by centripetal or centrifugal nerve transport. In orally challenged deer euthanized sequentially from 3-28 months post inoculation (n=20), the PrP^{CWD} was initially detected within the DMNV of the brain by immunohistochemistry (Williams & Miller, 2000). The initial appearance of PrP^{CWD} in the DMNV implicates the vagus nerve as a potential route for PrP^{CWD} transit from the presumed site of exposure in the alimentary tract to the CNS.

Abundant PrP^{CWD} was detected in the vagosympathetic trunk and in nerve fibers in the nodose ganglion as compared with scant or no deposition of PrP in the cranial cervical ganglion (sensory), celiac ganglion, sciatic nerve, or brachial plexus which suggests that the vagus nerve could serve as a major transit route of PrP^{CWD}. PrP^{CWD} was detected in myenteric ganglion cell bodies, along nerve fibers and in satellite cells as has been described in other studies (Groschup et al., 1999, McBride & Beekes, 1999). Nevertheless, other routes of PrP^{CWD} transit, such as via blood, sensory or cranial nerves innervating the oral mucosa (IX, X), or sympathetic splanchnic nerves cannot be excluded. In hematogenous dissemination it might be expected that PrP^{CWD} amplification would occur initially in richly vascular neural domains with fenestrated endothelium (e.g., area postrema of the medulla oblongata, hypophysis, pineal body, hypothalamic regions, subfornical organ) as opposed to the DMNV. Dissemination via cranial nerves IX and X might be expected to result in initial PrP^{CWD} amplification in the nucleus solitarius.

The gastrointestinal tract receives parasympathetic vagal nerve fibers from the DMNV and sympathetic nerve fibers from the spinal cord via the celiac ganglion (splanchnic circuitry). In light of PrP^{CWD} detection in the intermediolateral column of the spinal cord and adrenal medulla, which is innervated by splanchnic nerves, it is plausible that PrP^{CWD} may also traffic to the CNS via the splanchnic circuitry. If this is the case, it is surprising that we did not detect PrP^{CWD} in the celiac ganglion, in which preganglionic splanchnic nerve fibers to the intestine synapse. It is possible that PrP^{CWD} in the intermediolateral column resulted from spread within the CNS and

then spread centrifugally to the adrenal medulla, a route which does not involve the celiac ganglion.

Neurotropic viruses which enter the host via the gastrointestinal tract have been investigated to determine route of entry into the CNS. The pathogenesis of prion infections has been compared to pseudorabies virus (Beekes et al., 1998) which spreads retrograde along parasympathetic vagal efferents to the DMNV (Card et al., 1990). Similarly, in mice inoculated perorally with a neurotropic reovirus, the virus spread
to the myenteric plexus and then retrograde along the vagus efferent nerve to the DMNV regardless of the amount of virus in the bloodstream. Moreover, subcutaneous inoculation over the forehead resulted in virus detection in the facial and trigeminal
nuclei of the brain, but not the DMNV, establishing that virus detection in the DMNV depended on an oral inoculation route (Morrison et al., 1991).

Substantial deposits of PrP^{CWD} were detected in the pancreatic islet cells, which are innervated by the vagus nerve (Loewy et al., 1994). PrP-containing islets often were adjacent, which might suggest infection from a common nidus, such as innervation by a common nerve branch. Infectivity in the pancreas has been previously documented in natural and experimental scrapie infections (Pattison & Millson, 1960, Ye et al., 1994b) and PrP has also been demonstrated in islets from uninfected and scrapie infected mice (McBride et al., 1992). Alterations in islet function have not been

examined, although hamsters infected with the 139H scrapie strain develop obesity and hypoglycemia-hyperinsulinemia with extensive pituitary and pancreatic vacuolation (Ye et al., 1994a, Ye & Carp, 1996). Pancreatic lesions were localized to islet beta cells, amyloid deposits were not found, and scrapie infectivity was extremely low (Ye et al., 1997).

The PrP^{CWD} detected in the adrenal medulla could be derived from PrP transport via the splanchnic nerves arising from nerve cell bodies in the intermediolateral column of the spinal cord, which has demonstrable PrP^{CWD}. We also demonstrated PrP^{CWD} in the pars intermedia and nervosa of the pituitary in CWD-infected deer. Potentially, PrP^{CWD} could transit via nerve fibers from the hypothalamus to the pars nervosa, as deer with CWD have abundant PrP^{CWD} deposition in the hypothalamus (Spraker et al., 2001). Histologic lesions were not evident in either the adrenal or pituitary; however, it is not known whether functional disturbances such as altered hormone synthesis are associated with PrP^{CWD} deposition.

In summary, the findings reported here in CWD-infected deer provide circumstantial evidence for: (a) trafficking of PrP^{CWD} in the peripheral nerves and (b) localization of the pathogenic prion protein in the endocrine system of CWD-infected deer. These observations are consistent with assembled findings in other experimental and natural TSEs and may lend insight into the potential pathways of prion trafficking in cervid chronic wasting disease.

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		Deer case number						
		V92	Za93	B93	Mb97	W97	H92	% pos.
Tissue	Mab							
Vagosympathetic trunk	6H4	+	+	+	++	++	++	100
Vagosympathetic trunk	99/97.6.1	+	++	-	++	+++	+	83
Sciatic nerve	6H4	-	nd	-	+	-	-	20
Sciatic nerve	99/97.6.1	-	nd	-	++	-	-	20
Sympathetic trunk	6H4	-	nd	-	-	+	nd	25
Sympathetic trunk	99/97.6.1	-	nd	-	-	++	nd	25
Brachial plexus	6H4	-	-	nd	nd	-	-	0
Brachial plexus	99/97.6.1	-	++	nd	nd	-	-	25

Table 3.1. Immunohistochemical detection of PrP^{CWD} in nerves from CWD-infected mule deer. 6H4 and 99/97.6.1 Mabs were used to detect the prion protein. IHC stain was quantified as number of positive stain granules in a section: += <10, ++= 10-20, +++= >20 positive stain granules. nd=not done.

Tissue	Mab							
Medulla oblongata	6H4	+	+	+	+	+	+	100
Medulla oblongata	99/97.6.1	+	+	+	+	+	+	100
Intermediolateral cell column of spinal cord	99/97.6.1	+	nd	nd	+	+	+	100
Pituitary	6H4	+	+	+	+	+	+	100
Pituitary	99/97.6.1	+	+	+	+	+	+	100
Pancreas	6H4	+	+	-	+	+	-	67
Pancreas	99/97.6.1	+	+	+	+	+	-	83
Adrenal medulla	99/97.6.1	nd	-	+	-	+	+	60
Myenteric plexus	99/97.6.1	+	+	-	+	+	+	83
Celiac ganglion	99/97.6.1	nd	nd*	-	-	-	-	0
Nodose ganglion	99/97.6.1	nd	+	nd	nd	+	nd	100
Cranial cervical ganglion	99/97.6.1	nd	nd	nd	-	nd	nd	0
Gasserian ganglion	99/97.6.1	-	nd*	-	-	-	-	0
Spinal nerve roots, dorsal and ventral	99/97.6.1	-	nd	nd	-	-	-	0
*associated nerve is negative								

Table 3.2. Immunohistochemical detection of PrP^{CWD} in neural and endocrine tissues from CWD-infected mule deer. 6H4 and 99/97.6.1 Mabs were used to detect the prion protein. nd=not done.

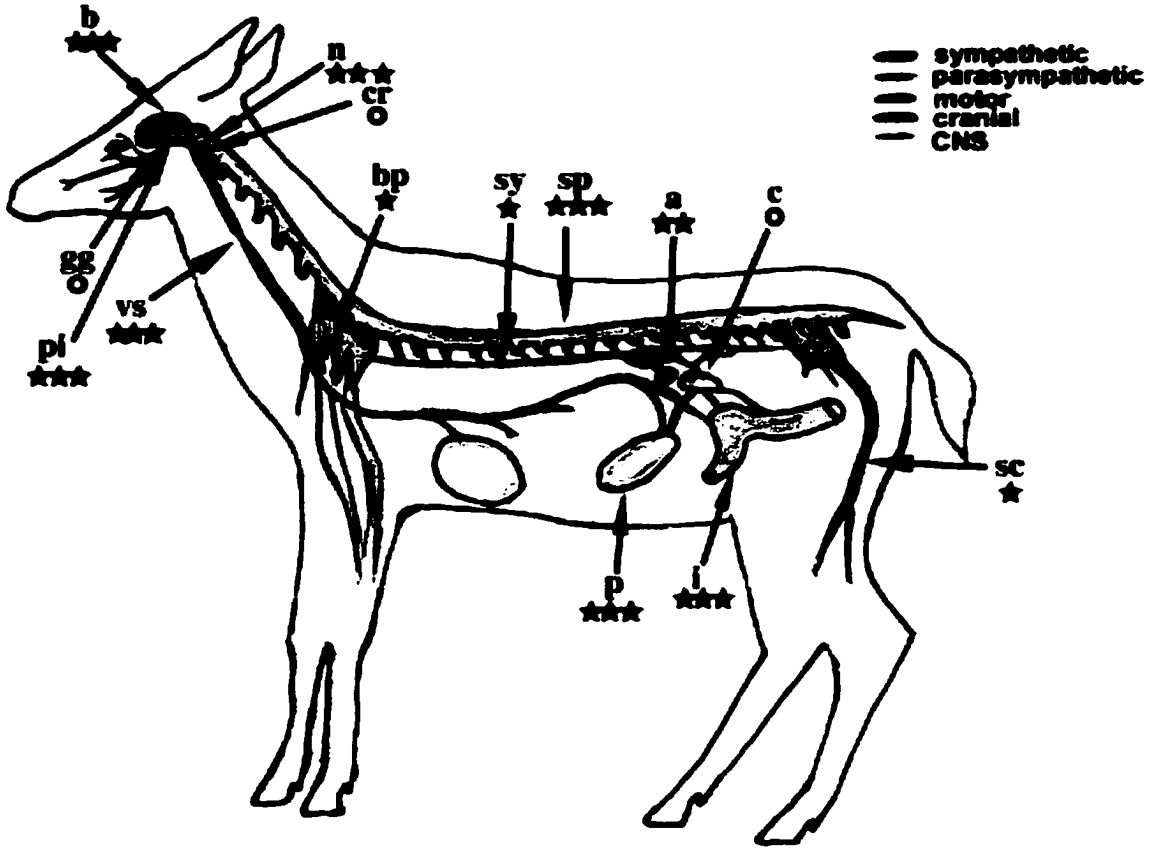

Figure 3.1. Distribution of sympathetic, parasympathetic, cranial, and motor nerves examined for the pathogenic isoform of the prion protein. Arrows indicate sites sampled. Number of stars indicates the incidence of PrP^{CWD} IHC positivity in the 6 deer, defined as number of positive/ total for each tissue sampled X 100 (0=0%, 1 star=1-50%; 2 stars=51-75%; 3 stars=75-100%).
b=brain. gg=gasserian ganglion. n=nodose ganglion. cr=cranial cervical ganglion. pi=pituitary. vs=vagosympathetic trunk. bp=brachial plexus. sy=sympathetic trunk. sp=spinal cord. a=adrenal. c=celiac ganglion. sc=sciatic. p=pancreas. i=intestine/ myenteric plexus.

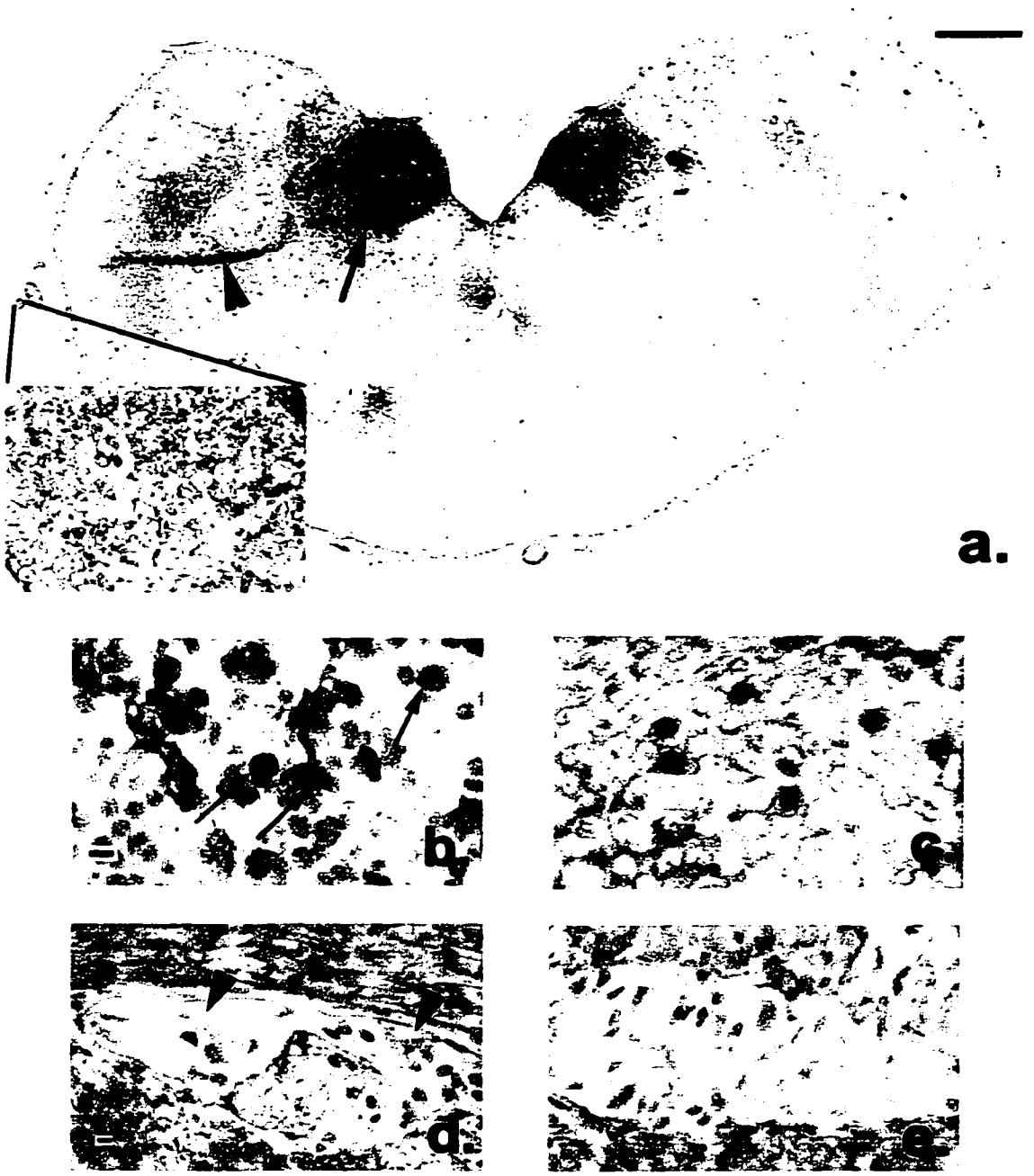

Figure 3.2. Immunohistochemical detection of PrP^{CWD} in the central and peripheral nervous system of naturally infected mule deer using monoclonal antibody 6H4. (a) Brain at medulla oblongata. PrP^{CWD} stain in the dorsal motor nucleus of the vagus (arrow), vagal radix (arrowheads), and presumably the vagus nerve exiting the section (inset). PrP^{CWD} labelling is detected in the vagosympathetic trunk (b, arrows) and in the myenteric plexus of the small intestine (d, arrowheads), but not in the control CWD negative deer (c,e). Bar, 1mm (a), or 10 μ (b,c,d,e).

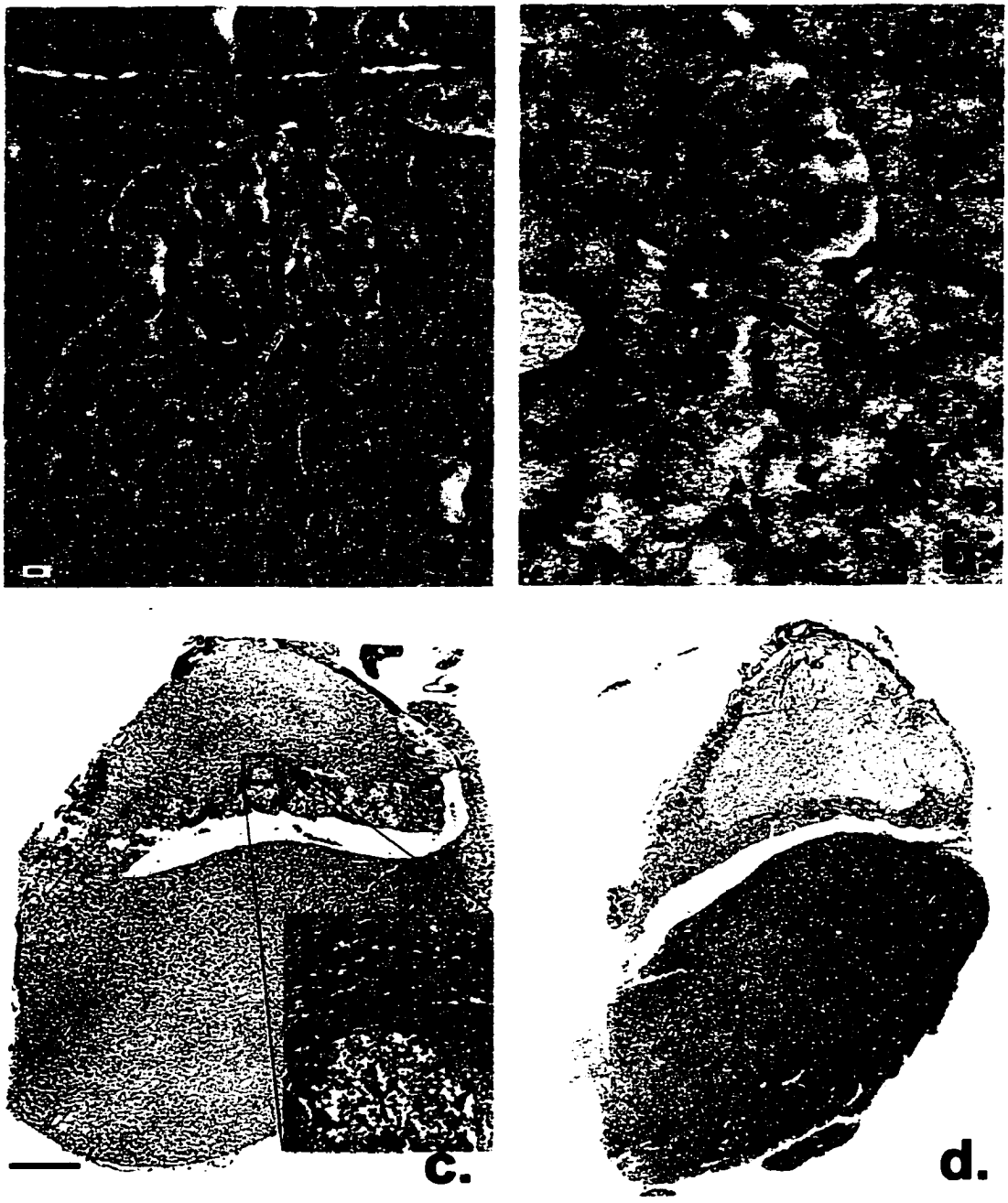

Figure 3.3. Immunohistochemical detection of PrP^{CWD} in endocrine organs of naturally infected mule deer. Pancreatic islets of Langerhans (arrow) accumulate PrP^{CWD} in CWD-infected (a) but not uninfected deer (b). Pars intermedia and nervosa of the pituitary accumulate PrP^{CWD} in CWD-infected (c), but not control deer (d). Bar, 10 μm (a, b), or 1mm (c,d).

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CONCLUSION AND FUTURE DIRECTIONS

We have learned from this work that (1) PrP^{CWD} can cross the mucosa and amplify in lymphoid tissues draining the alimentary tract and (2) PrP^{CWD} can be detected in alimentary associated lymphoid tissues as early as 6 weeks post oral exposure to CWD brain homogenate. These findings indicate that ingestion of the prion agent could represent a natural route of transmission in deer. In addition, PrP^{CWD} amplifies rapidly in mucosa associated lymphoid tissues and thus could serve as a route of prion entry and exit. This would provide a route of agent shedding into the environment via prion contaminated saliva or feces. One might continue this work by searching for PrP^{CWD} at even earlier stages after exposure, such as mucosal entry. Questions to be addressed would include: Do M cells mediate transepithelial transport? Are dendritic cells or macrophages involved in spread of PrP^{CWD} to Peyer's patches or to draining lymph nodes? Are immune factors involved in transport? Does PrP^{CWD} traffic via lymph to alimentary associated nodes or are other routes involved?

The lymphoid cells that are associated with PrP^{CWD} include follicular dendritic cells (FDC), B cells, and tingible body macrophages (TBM) within follicular germinal centers. Our finding of PrP^{CWD} in association with the cell membrane of FDC or B cells implies that: (1) conversion may be occurring on the membrane surface or

intracellularly with rapid exocytosis or (2) immune factors, such as complement or immunoglobulin, may be associated with PrP^{CWD} and result in trapping by FDC Fcγ or complement receptors. This is an important distinction because the latter possibility would indicate that the PrP^{CWD} is replicated and released by a different cell.

The observation that PrP^{CWD} is contained intracellularly in TBM might suggest that these cells are likely exposed to PrP^{CWD} via phagocytosis and may or may not be amplifying PrP^{CWD}. Future directions of this work might entail a closer examination of the roles of TBM and FDC in the pathogenesis. These issues might be investigated: Are FDC or TBM replicating the PrP^{CWD}? If so, where does conversion occur within FDC? Do macrophages contain PrP^{CWD} when they migrate into the germinal center? Does PrP^{CWD} amplification in the lymphoid follicle play a role in neuroinvasion? Experiments that could be performed to address these questions might include transfecting FDC in vitro with a PrP plasmid containing a recognizable epitope tag, or creating a PrP knock-out mice that expresses PrP only in FDC or TBM and neural tissue and determining the susceptibility of those mice to TSE.

Neuroinvasion might occur through several routes, including via blood or nerves innervating the alimentary tract or lymphoid tissue. We found that in deer with advanced CWD, PrP^{CWD} was detectable within the myenteric plexus of the intestine, the vagosympathetic trunk, the nodose ganglia and the parasympathetic nucleus of the brain (dorsal motor nucleus of the vagus-DMNV). This could indicate a direct route of PrP^{CWD} spread to the CNS via retrograde transport through parasympathetic nerve

channels innervating the GI tract, especially in light of the DMNV as the initial site of prion protein amplification in deer. In addition, PrP^{CWD} was detected in the intermediolateral column of the spinal cord which might suggest PrP^{CWD} retrograde spread via splanchnic nerves, sympathetic connections to the spinal cord. These findings are highly suggestive of either centrifugal or centripetal PrP^{CWD} nerve transit and could serve as a mechanism for prion spread to organs such as the pancreas or adrenal medulla, which harbor PrP^{CWD}. Studies that may arise from these findings include a pathogenesis experiment to examine whether PrP^{CWD} is present in the vagus nerve prior to the DMNV as well as studies to determine whether PrP^{CWD} could traffic from lymph node germinal centers via nerves.

The studies comprising this thesis have contributed to understanding the transmission and pathogenesis of CWD. There remain many important questions which remain to be addressed in the CWD research field. Answering these questions will be key to managing the disease in captive and free-ranging cervid populations.