DISSERTATION

# TRACKING INFECTIOUS PRIONS IN THE BODY FLUIDS OF DEER INFECTED WITH CHRONIC WASTING DISEASE

Submitted by

Candace Kay Mathiason

Department of Microbiology, Immunology and Pathology

In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Spring 2010

SF997.5
.C46
.M375
2010

COLORADO STATE UNIVERSITY

February 4, 2010

WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY CANDACE KAY MATHIASON "TRACKING INFECTIOUS PRIONS IN THE BODY FLUIDS OF DEER INFECTED WITH CHRONIC WASTING DISEASE" BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

Committee on Graduate Work

Anne Avery

Anne Avery

James R. Bamburg

James R. Bamburg

Mark Zabel

Mark Zabel

Edward A. Hoover

Advisor: Edward A. Hoover

Edward A. Hoover

Department Head: Edward A. Hoover

COLORADO STATE UNIV. LIBRARIES

ABSTRACT OF DISSERTATION

TRACKING INFECTIOUS PRIONS IN THE BODY FLUIDS OF DEER INFECTED WITH CHRONIC WASTING DISEASE

Chronic wasting disease (CWD) is a prion disease of cervid (elk, moose and deer) with unusually high transmission efficiency. While the nidus of disease was described in a captive herd of cervid in northern Colorado/southeastern Wyoming in the late 60's, it has now been detected in both captive and free-ranging populations in 17 states and 2 Canadian provinces of North America and one Asian country. CWD is unique in being the only transmissible spongiform encephalopathy (TSE) described in a free-ranging population of animals. The etiology of CWD, like all prion diseases, is the conversion of the normal host-encoded cellular prion protein ($PrP^{C}$) to an aberrantly folded protease resistant isoform ($PrP^{RES}$/$PrP^{CWD}$). An intriguing aspect of prion diseases is their ability to be transmitted from one organism to the next. In this dissertation work, we ask—By what means are infectious prions transmitted from one host to the next? In particular to

CWD—What do infected cervids share or leave behind that contain sufficient infectious particles to initiate disease in the next cervid?

We addressed this question by bioassay of body secretions and excretions—'secreta'— (saliva, blood, urine and feces) in the native white-tailed deer host and in transgenic mice expressing the normal cervid prion protein (Tg(CerPrP) mice). Cohorts of deer were exposed by oral (PO) ingestion of 'secreta', or intraperitoneal (IP)/intravenous (IV) transfusion of blood components. To replicate a more natural/realistic exposure to CWD in which a deer might travel into a contaminated area and feed for a short period of time, an additional cohort of deer was exposed to fomites (bedding, feed and water buckets) from the suites of CWD-infected deer—without direct contact with infected deer. These variously exposed deer were monitored for a minimum of 19 months post inoculation (mo pi) for CWD infection and disease by immunohistochemical (IHC) and western blot (WB) detection of $PrP^{CWD}$ in serial tonsil biopsies and in multiple tissues after necropsy. Parallel studies were conducted in Tg(CerPrP) mice with the addition of an intracranial (IC) inoculation group for each body fluid.

We found that sufficient infectious prions were present in the saliva, whole blood, the B cell- and platelet-enriched fractions of blood, and in fomites from infected deer premises to transmit CWD. Conversely, $PrP^{CWD}$ was not detected in the brain or lymphoid tissues of deer or mice inoculated with urine and feces, cell-free plasma or CD14+ monocytes from CWD-infected donor deer.

The results of this work: 1) suggest that the efficient transmission of CWD may be due in part to the sharing of saliva between cervids and its deposition upon surfaces frequented by cervids; 2) establish a hematogenous dissemination of infectious prions in CWD associated with the cellular fraction of blood— in particular B cells and platelets— in CWD-infected deer; 3) extend previous work localizing $PrP^{CWD}$ to the interface of follicular B cells and dendritic cells; 4) provide insights to $PrP^{CWD}$ trafficking and CWD pathogenesis; and 5) establish saliva and blood cells as viable substrates for $PrP^{CWD}$ antemortem detection.

Candace Kay Mathiason
Department of Microbiology,
Immunology, and Pathology
Colorado State University
Fort Collins, CO 80523
Spring 2010

## ACKNOWLEDGEMENTS

The path to completing this endeavor can be compared to the trek down Oz's yellow brick road… …an idea, some heart, some determination… and the gathering of many friends and collaborators along the way. Every member, past and present, of the Prion and Retrovirus Research Laboratory (PRRL) has provided contributions to my growth as a scientist, and perhaps more importantly as a human being. I am grateful for their willingness to share their time, skills, and expertise. Past: I would especially like to thank Andrea Torres, Mary Jo Burkhard, Wendy Sprague, Catherine Hageman, Jen Keane, Nord Zeidner, Christina Sigurdson, Robin Allison, and Sharon Robinson for their scientific curiosity, their 'can do' attitudes, and countless words of encouragement. Present: Kevin O'Halloran and Sherry Colton have made it possible for me to redirect my focus – I thank you both. I gratefully acknowledge Jeanette Hays-Klug for her expertise as CSU's Satellite Colony Manager, her dedication to the PRRL project, and the plethora of "other duties as described" she encountered to keep this project on course. I thank Amy Nalls and Kelly Anderson for their dedication and willingness to take on new projects with gusto. In particular I appreciate the comradery that has developed between the four of us (JHK, AVN, KA, CKM) as a team. I thank Davis Seelig, Nicholas Haley, and Gary Mason for mastery of the blade and sharp-edged wit. Nathaniel Denkers and Timothy Kurt for inquisitive banter and their friendship in the process.

The development of collaborations outside of the PRRL has been one of many rewards associated with this project. In particular, Kathi Delahoy, Hank Gardner, Terry Nett, and Tony Frank for their support in facility development. Sinnett Building Inc. and Brian Hood for perseverance in timely facility construction. Jenny Powers and Margaret Wild for their early consultations regarding white-tailed deer; and Jenny for sharing her cervid veterinary skills. David Osborn for not hanging up on me during our first telephone conversation describing the studies we were interested in, and Sallie Dahmes for her gracious gift of nurturing newborn fawns. Julie Langenberg, Terry Nett, Terry Spraker, and the Colorado Division of Wildlife for providing access to CWD-infected cervid tissues. Katherine O'Rourke for her generosity in genotyping deer. Alan Young for expertise in ruminant immunology. Glenn Telling and Tanya Seward for transgenic mice. I thank each of you for your contribution to these studies.

My committee members have been supportive throughout this project. Anne Avery and Mark Zabel have provided advice and immunological insight. Jim Bamburg has developed a challenging cell biology curriculum and encouraged the ideas I brought to this project.

A special thank you to Sue VandeWoude for her continued support and advice about all things CAPU and Kelly Hall for "pretending" to like science.

My mentor, Ed Hoover. To whom I owe a great deal… for his leadership, his trust, his ability to play the opposite side of any equation. He has provided a space… where asking questions is encouraged …and pondering the 'other' side of the equation is the norm. He inspires one to do their best… with humor. I believe he has found his

niche. I am grateful for his mentorship and more importantly, his friendship. I've certainly enjoyed the tangents along the way.

Financial support for this work was provided by contract number N01-AI25491 from the National Institute of Allergy and Infectious Disease, National Institutes of Health.

## DEDICATION

I dedicate this endeavor to—

My son, Chase Parker DuBard. Thank you… for your high fives when I passed an exam …your patience with my study habits and willingness to share me with this project. I appreciate most your sense of humor, your laughter, and your persistence in sharing your thoughts and feelings with me. Rock On.

feels like
some kind of ride
but it's turning out
just to be life
going absolutely
perfectly
Brian Andreas

Lance Masoner. I thank you for your alacrity—whether it be to listen to me drone on about prions, to inquire about my day, or accept the fact that I was bringing my books and notes on outings.

My parents, Ron and Cherry. Thank you for encouraging me to observe the world around me and to set high goals for myself. Your work ethic and dedication to nature and animals has been an inspiration to me. Thanks Mom… Thanks Dad.

## TABLE OF CONTENTS

# INTRODUCTION

***Perspectives on Transmissible Spongiform Encephalopathies (TSEs):***

Many fatal and irremediable diseases are associated with the fibrillar protein aggregates known as amyloid. These fibrils are formed by the conversion of normal soluble proteins into an alternate conformation that is rich in beta-sheets and promotes well-ordered aggregation of the proteins (15, 20, 86). Amyloid is characterized by a cross-beta sheet quaternary structure, ie. beta-strands of the stacked beta-sheets come from different protein monomers and align perpendicular to the axis of the fibril (93). The core of this amyloid structure is protease resistant, rendering it insoluble, due to limited access within the densely packed structure. Beta-sheet plaque formation can be detected by Thioflavin T or Congo red staining, Transmission Electron Microscopy (TEM), and Fourier transform infrared (FTIR) spectroscopy (68). Sustained plaque generation is thought to interfere with normal cellular function, eventually leading to deterioration of the affected neural cell function, culminating in clinical disease. The basis for the pathology in these diseases may be a cellular inability to degrade misfolded and damaged proteins and formation of cytotoxic intra- or extracellular oligomers and aggregates (12). This aberrant amyloid formation can be generated by spontaneous means (Creutzfeldt-Jakob Disease (CJD); 1 in $10^6$ individuals world wide), genetic inheritance (Huntington's, Fatal Familial Insomnia, Parkinson's), transmitted via iatrogenic means (surgical interventions) or contracted by way of entry through the oral cavity nervous

system or alimentary tract (Bovine Spongiform Encephalopathy (BSE), variant Creutzfeldt-Jakob Disease (vCJD), Scrapie, Chronic Wasting Disease (CWD)).

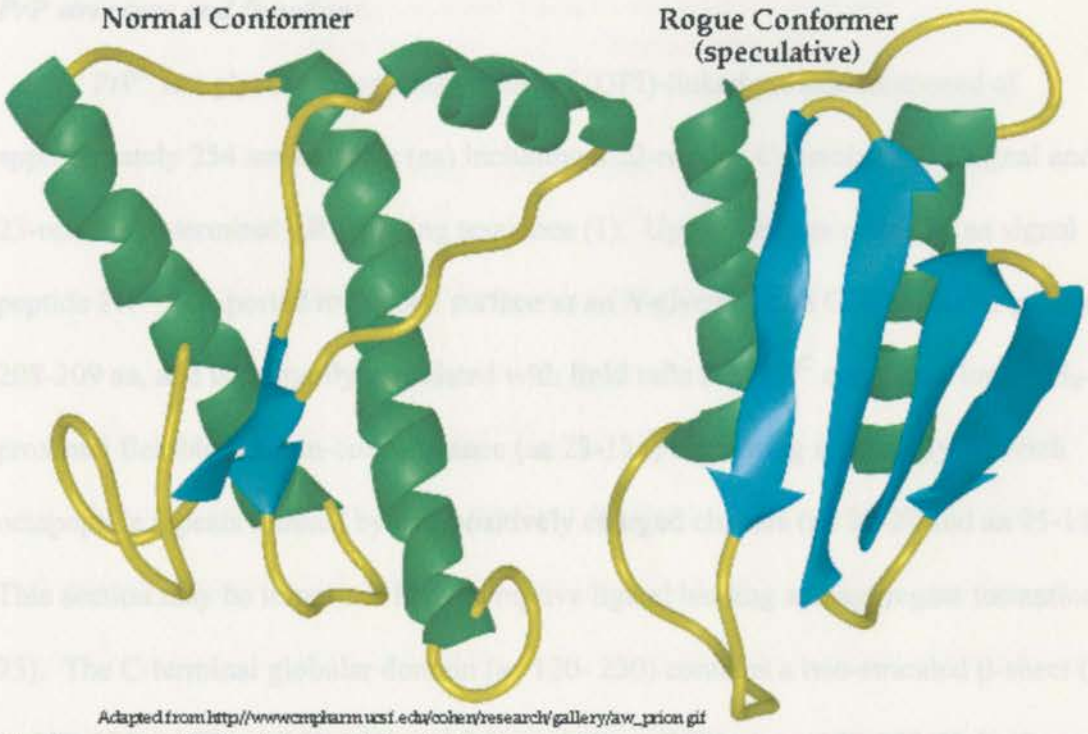
While most amyloid proteins are not infectious, those associated with prion disease are capable of being transmitted from one organism to another (74, 75). Prion diseases, also known as TSE's, are a group of fatal neurodegenerative disorders that affect the brain and nervous system of mammals. The studies posed by this dissertation are held within the framework of the widely accepted paradigm for the etiology of TSEs— the protein-only or 'prion' hypothesis. The premise of this hypothesis revolves around the infectious nature of a cascading post-translational conversion of the normal cellular prion protein ($PrP^{C}$) to abnormal partially protease resistant prion protein ($PrP^{RES}$)— without the aid of nucleic acid (69, 74, 75). Detection of $PrP^{RES}$ is highly correlated with infection which is ultimately fatal(1, 74).

***The 'protein-only' hypothesis:***

Early biochemical studies conducted by T. Alper (3) demonstrated that the infectious agent associated with scrapie was resistant to treatments that inactivated bacteria or viruses. These studies were expanded upon by Prusiner et al. (74) with the purification of diseased brain homogenates such that infectious particles could be separated and fully tested. This work facilitated advanced molecular and genetic understanding of TSEs and led to the term 'prion', short for *pro*tein *only in*fectious particle— denoting an infectious agent devoid of nucleic acids (The 'o' and 'i' were switched to ease pronunciation).

The normal prion protein is a highly conserved host-encoded protein that is protease sensitive. Upon conversion from a mostly α-helical structure (40% α:3% β) to the β-sheet rich conformation (30% α:40% β) associated with TSEs, the protein becomes partially resistant to protease and readily aggregates (69) (Fig. I.1).

Fig. I.1 Prion protein conformations.




http://www.stanford.edu/group/virus/prion/normal_rogue.gif

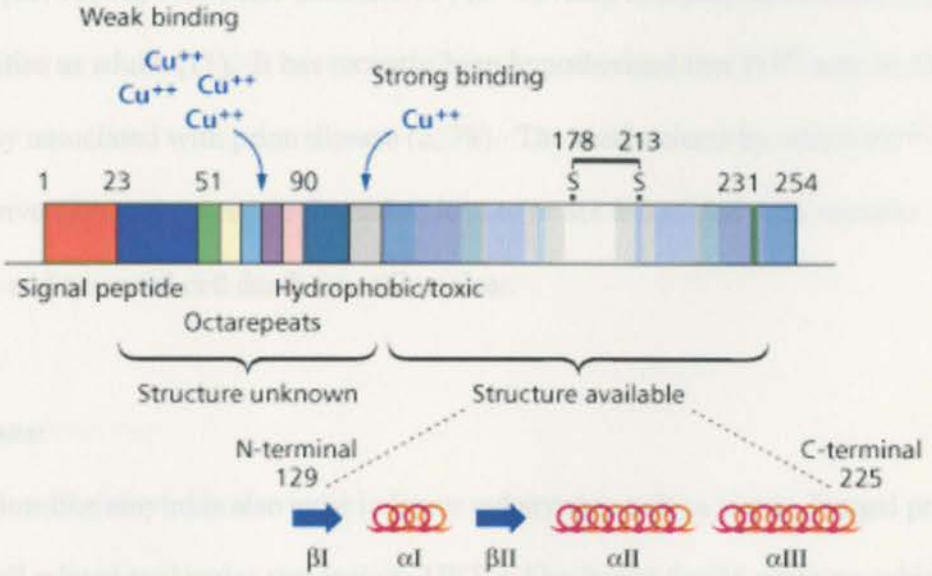
To determine the role of the prion protein in TSE disease, mice devoid of the host encoded protein were inoculated and were found to be resistant to TSE infection (10, 11). Observations made in parallel studies of heritable prion diseases suggested that specific mutations within the *Prnp* gene that encodes $PrP^{C}$ resulted in spontaneous conversion of $PrP^{C}$ to $PrP^{RES}$ (28, 64). This work provided evidence that the host encoded cellular

prion protein was an obligate component in $PrP^{RES}$ conversion and subsequent disease. Crucial support for the 'protein-only' hypothesis was produced by means of *de novo* generation of infectious particles from purified mammalian $PrP^{C}$ that were capable of infecting hamsters (19). Collectively this body of data has led to the acceptance of the 'protein-only' or prion hypothesis.

***PrP structure and function:***

$PrP^{C}$ is a glycosylphospatidyl-inositol (GPI)-linked protein composed of approximately 254 amino acids (aa) including a 22-residue C-terminal GPI signal and a 23-residue N-terminal ER targeting sequence (1). Upon cleavage of the 22 aa signal peptide $PrP^{C}$ is exported to the cell surface as an *N*-glycosylated GPI anchored protein of 208-209 aa, and is primarily associated with lipid rafts (1). $PrP^{C}$ contains a long $NH_2$-proximal flexible random-coil sequence (aa 23-124) containing several glycine-rich octapeptide repeats flanked by two positively charged clusters (aa 23-27 and aa 95-110). This section may be important in presumptive ligand binding and aggregate formation (1, 75). The C-terminal globular domain (aa 120- 230) contains a two-stranded β-sheet (β1: aa 128-131and β2: aa 161-164) and three α-helices (Helix 1: aa 143-153, Helix 2: aa 172-191, Helix 3: aa 199-227). Two of the α-helices are connected by a disulfide bond between Cys179 and Cys214 (75) (Fig. I.2).

Fig. I.2 Prion protein sequence and properties.




http://bioquest.org/bedrock/problem_spaces/prion/background.php

The structural properties of $PrP^{RES}$ are not available due to its insolubility and inclination to aggregate. $PrP^{RES}$ can however be detected by electrophoretic mobility post protease digestion and western blot analysis. Both $PrP^{C}$ and $PrP^{RES}$ can be glycosylated at aa 181 or 197 which has led to the characteristic western blot depictions of di-, mono- and unglycosylated PrP forms of the protein (8). Upon partial protease digestion a loss of 60-70 aa from the N-terminus of $PrP^{RES}$ provides evidence of the protease resistant infectious component associated with prion disease.

Several cellular functions for $PrP^{C}$ have been proposed, including roles in cell signaling and cellular responses to reactive oxygen species (1, 58), but the full picture is not entirely understood. Perhaps this suggests that the physiological function of $PrP^{C}$ is complex, with each established function representing a fraction of the whole. $PrP^{C}$ expression is highest on cells of the central nervous system (neurons and glial cells) (23,

58, 66) with variable expression on many additional cell types (dendritic cells, B cells, platelets) (23, 35, 92, 97). Mice deficient of $PrP^{C}$ develop normally and exhibit few abnormalities as adults (11). It has recently been hypothesized that $PrP^{C}$ may be linked to the toxicity associated with prion disease (2, 78). The mechanisms by which $PrP^{C}$-to-$PrP^{RES}$ conversion and aggregate formation lead to or are associated with vacuole formation and neuronal cell death are still unclear.

***Yeast prions:***

Prion-like amyloids also exist in lower eukaryotes such as yeast. Fungal prions are non-PrP related molecules that include HET-s, Ure2p and Sup35 proteins, which can adopt both nonamyloid and self-perpetuating amyloid structures. In contrast to $PrP^{RES}$, the conversion of these proteins into their prion-like conformations has been shown to have important physiological functions in yeast. The conversion of Ure2p and Sup35 into their amyloid forms (URE3 and PSI+) regulates the transcription and translation of specific yeast genes (47, 95). Aggregated HET-s regulates heterokaryon incompatibility, a fungal self/non-self recognition phenomenon that prevents various forms of parasitism (99).

***CWD:***

Chronic Wasting Disease (CWD) is a TSE of cervids (elk, moose and deer) that was first recognized in the late 1960's in northern Colorado and southeastern Wyoming (103). Since that time CWD has been detected in 16 states and 2 Canadian provinces of North America (98), and Korea (48, 85). The origin of Korea's initial case of CWD was

traced back to a North American sourced cervid that had been exported from Canada to Korea. CWD has remarkably high transmission efficiency. The sharing of body fluids (saliva, blood, urine and feces) by direct contact between cervid and environmental contamination of feeding, watering and bedding areas is presumed to contribute to transmission efficiency. Prevalence of CWD in the wild varies, and has reached as high as 30% in some localized areas (102).

***CWD clinical disease:***

Clinical disease develops in free-ranging and captive cervid over time (2.5-4 years) and is characterized by progressive emaciation, polydipsia/polyuria, excessive salivation, and stereotypic behaviors such as a lowered head and fixed stare, drooping ears, teeth grinding, ruminal regurgitation and flaccid hyptonia of facial muscles (103, 104). Clinical signs are ongoing for 2 weeks to 8 months with all cervid eventually succumbing to death (103).

***CWD pathology:***

Gross pathology, histologic lesions and $PrP^{CWD}$ distributions are similar in free-ranging and captive deer.

Gross pathologic finding are typified by emaciation, a marked absence of visceral adipose tissue and bone marrow fat, and excess fluid mixed with sand or gravel in the rumen (88, 103)

The predominant histologic lesions are found in the brain and spinal cord (88, 90, 103) with widespread microcavitation of neuropil (predominantly of gray matter),

intracytoplasmic vacuoles in neuronal perikaryons and neuronal degeneration (103). Areas with evidence of the most severe lesions include the olfactory bulbs and stria, hypoglossal, supraoptic nucleus and paraventricular nucleus, hypothalamus, thalamus, tegmental nuclei, medial and lateral cuneatus nuclei, nucleus of the spinal tract of the trigemental nerve and parasympathetic vagal nucleus (88, 90). Detection of $PrP^{CWD}$ deposits is found in all areas of spongiform degeneration as well as in some areas where degeneration is absent, especially the cerebrum (90).

***CWD diagnosis:***

CWD is conventionally diagnosed in affected cervid by postmortem detection of $PrP^{CWD}$ via IHC of brain and lymphoid tissue (retropharyngeal lymph node and tonsil) (88). More recently, using similar IHC methodologies and emphasis on the ability to detect $PrP^{CWD}$ during the subclinical phase of disease, a live test has been developed to establish CWD status in rectal/anal mucosal associated lymphoid tissue (RAMALT) tissues of elk (87, 89). Currently there are two United States Department of Agriculture (USDA) approved enzyme-linked immunosorbent assays (ELISAs) available on the market for the detection of $PrP^{CWD}$—one developed by IDEXX (Herdcheck$^{TM}$) and the other by BioRad Inc. (TeSeE$^{TM}$).

***CWD experimental transmission history:***

Williams et al (104) first demonstrated the transmissibility of CWD by intracranial (IC) inoculation of deer with brain from naturally infected deer. Clinical disease was observed in these deer 17-21 months post exposure, similar to that seen in

naturally infected cervid. Per os (oral) experimental transmission of CWD has been shown in mule deer (83), and white-tailed deer (60, 61), as well as transmission via environmental contamination without direct animal-to-animal contact (60, 65) and transmission by blood transfusion (60, 61). Many infectious disease cycles are perpetuated by the presence of secondary hosts that act as reservoirs of infection. To determine if non-cervid reservoirs of CWD infection exist in nature, multiple additional species have been exposed to CWD. Cattle, sheep, goats and ferrets can support CWD infection post IC inoculation. Ferret susceptibility to CWD results in a 15-21 month incubation period—similar to that observed in deer (7, 82) Cattle IC inoculated with CWD became positive (36), while a second passage of brain culminated in both a decrease in time to clinical disease onset (from 5 years to 10-12 mo pi), and an increase in attack rate (37). The first reported transmission of CWD to primates was reported in squirrel monkeys (57, 76). Voles and *Peromyscus spp.* mice are also susceptible to CWD infection via IC inoculation (39). Limited susceptibility to CWD was seen in mink (38, 104), orally inoculated moose (51), Syrian golden, Chinese, Siberian and Armenian hamsters and transgenic mice expressing the Syrian golden hamster prion protein (79). Wild-type mouse strains, Djungarian hamsters and cynomolgus macaques were relatively but not totally resistant to CWD infection (76, 79). While many non-cervid species are susceptible to CWD infection, these studies (with the exception of one orally infected moose) have been limited to the IC route and little is known about susceptibility by *natural* routes of infection.

***Body fluids associated with TSE infection:***

While it has been demonstrated that prion infection can be transmitted from one animal to another by experimental inoculation, natural transmission of the various TSEs is still under investigation. It is postulated that several TSEs, including BSE, scrapie, vCJD, Kuru and CWD, are contracted via ingestion of infectious prions contaminating the environment or food substances (4, 25, 32, 60, 61, 70, 72, 83, 100, 101). It is hypothesized that infectious prions are taken up within the oral cavity or cross the mucosal lining of the gut associated lymphoid tissue (GALT) to establish infection (65, 83). Evidence in BSE and scrapie suggest mucosal uptake/crossing occurs in the lower gastrointestinal tract (LGIT). In CWD there is evidence for oro-pharyngeal crossing in that the peripheral lymph node is the first tissue that tests positive for $PrP^{RES}$. It may be that CWD also crosses from the LGIT but the evidence for this is at present less clear.

Bioassay of the various body fluids (saliva, urine, feces, blood) in the host, rodent and transgenic mouse species has led to an increased awareness of the infectious nature of these fluids. But due to the small volumes that can safely be administered to these species, assay sensitivity and consistency of results are compromised. Nevertheless, body fluids and excretions are proven to contain infectious prions— i.e. saliva (31), urine (18, 30, 33, 34, 84), feces (33, 52, 80, 94) and blood components (9, 17, 45, 96).

***TSE's and peripheral blood:***

The search for blood borne prion transmission has long been sought. Seminal studies were conducted in the 60's and 70's (17, 22, 26, 31) in which bioassays were performed on serial blood collections from mouse-adapted scrapie inoculated mice and

goats. The results of these bioassays were mixed. Gibbs and Clarke (17, 26) reported scrapie infection in serum inoculated mice, although with a delayed onset of disease when compared to brain inoculated mice, while Hadlow and Eklund (31) reported negative findings which they thought were possibly due to assay sensitivity limitations. The authors of all four studies noted scrapie detection in lymphoid tissue and suggested that thus so, blood must be involved in scrapie dissemination. More recent precedent for blood borne prion association can be found in the work of Hunter and colleagues (45, 46) who reported transmission of BSE and scrapie to naïve sheep via transfer of 500ml of blood or buffy coat white blood cells from infected sheep. Studies published as part of this thesis demonstrated that CWD could be transmitted by direct blood transfusion of whole blood and blood cellular fraction from CWD-infected mule and white-tailed deer (60, 61). Moreover, sparse but compelling evidence accrued from people receiving blood transfusions from donors later diagnosed with vCJD, argues for the transmission of vCJD prions in blood of asymptomatic donors (14, 55, 73).

***Blood cells harboring prions:***

Immunohistological detection of $PrP^{RES}$ in lymphoid tissues of scrapie-infected sheep provided some of the first evidence for lymphoid system involvement in TSE diseases (22, 24, 71, 91). While B cells have long been associated with $PrP^{RES}$ transport and/or deposition within the lymphoid system (13, 14, 21, 27, 29, 40, 50, 63, 77, 81, 105), subsequent studies utilizing confocal microscopy confirmed an association between $PrP^{RES}$ and immune cells (follicular dendritic cells (FDCs), tingible body macrophages

(TBMs) and B cells) and extended the repertoire of prion diseases with lymphoid involvement to include CWD and vCJD (49, 62, 67, 81, 83).

$PrP^C$ is produced endogenously by cells of the platelet lineage, which could implicate platelets in $PrP^{RES}$ propagation or trafficking within the body or transmission in contaminated blood products (16, 41-43, 56). 43-53%, 63-95% and 69-93% of bovine (5), ovine (6) and white-tailed deer (59) platelets respectively express $PrP^C$, and given the number of circulating platelets vs. leukocytes, the majority of blood-borne $PrP^C$ expression is platelet associated (16, 56, 92). This blood component could be largely responsible for the transmission of vCJD by transfusion (53, 54). Evidence associating prion infectivity with platelets has been variable— from no detection in sCJD platelets (16), to reported infectivity in hamster scrapie (44), GSS, and vCJD (14).

***Relevance of CWD as a model of prion transmission and pathogenesis:***

Hematogenous infectivity has been demonstrated in CWD, scrapie and vCJD suggesting that this mode of $PrP^{RES}$ peripheral trafficking may be common to TSE diseases. Thus study of blood cell infectivity in CWD as a model of TSE blood-borne pathogenesis offers advantages, including: 1) a unique and informative animal model exhibiting high transmission efficiency; 2) ample amplification of the prion protein within the lymphoreticular system (LRS) enhancing the prospect of peripheral $PrP^{RES}$ detection; 3) availability of large amounts of blood that can be harvested for detection of $PrP^{RES}$ by cell separation, Enzyme Linked Immunosorbent Assay (ELISA), western blot, and/or Protein Misfolding Cyclic Amplification (PMCA) analysis.

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

1) How is CWD transmitted among animals?

2) Do body fluids/excreta contain CWD infectivity?

3) Are CWD prions blood-borne? And if so which blood compartment/fraction harbors this infectivity?

4) If CWD blood transmission is cell-associated, which specific blood-cell subset(s) support or traffic infection?

5) If blood-cell subsets traffic prion infectivity how might they be associated with disease pathogenesis?

***Dissertation research:***

The above-described body of TSE research provided the basis for the specific aims of this dissertation research. Our first objective was to define the body fluid(s) associated with CWD transmission and the relative time to clinical disease onset. We hypothesized that infectious CWD is transmitted among cervid in body secretions/excretions ('secreta') shared between individuals or left as contaminants in the environment. Our hope was to identify 'secreta' containing infectious CWD. This was addressed by orally (saliva, urine, feces) or intravenously (blood) exposing naïve white-tailed deer fawns to body fluids acquired from CWD-infected deer, followed by serial tonsil biopsy collection to assay for $PrP^{CWD}$ by conventional immunohistochemical (IHC) analysis and continuous observation for CWD clinical disease.

While mounting evidence exists for scrapie and vCJD blood transmission, little is known about the infectious nature of blood for CWD. Therefore, upon the detection of

infectious prions in the blood of CWD-infected deer the second objective was to characterize the nature of this infectivity. Our intent was to determine if CWD blood infectivity is associated with the cellular, non-cellular, or both fractions of blood. As a clear distinction between cell vs non-cell association of CWD blood infectivity began to unfold we turned our attention to specific cell subsets known to either co-localize with $PrP^{CWD}$ or express large quantities of the normal cellular prion protein ($PrP^{C}$) as potential targets for prion infectivity/trafficking. The third objective of this work, therefore, was to determine whether these cell subsets (B cells, monocytes or platelets) were traffickers of disease. This was approached by bioassay of these blood components in the native white-tailed deer host and a mouse model expressing the cervid prion protein (Tg(CerPrP)). Our primary goal was to clarify CWD blood infectivity with the rationale that this knowledge would provide insight to prion pathogenesis as well as provide basis for a blood derived antemortem diagnostic.

## REFERENCES


1. **Aguzzi, A., Calella, AM.** 2009. Prions: Protein aggregation and infectious diseases. Physiol Rev **89:**1105-52.
2. **Aguzzi, A., Steele, AD.** 2009. Prion topology and toxicity. Cell **Jun 12;137:**994-996.
3. **Alper, T., Camp, WZ, Haig, DA, Clarke, MC.** 1967. Does the agent of scrapie replicate without nucleic acid? Nature **May 20;214:**764-6.
4. **Alpers, M.** 1979. Slow transmissible diseases of the nervous system, vol. 1. Academic Press, NY.
5. **Barclay, G., Hope, J, Birkett, CR, Turner, ML.** 1999. Distribution of cell-associated prion protein in normal adult blood determined by flow cytometry. Br J Haematol **Dec;107:**804-14.

6. **Barclay, G., Houston, EF, Halliday, SI, Farquhar, CF, Turner, ML** 2002. Comparative analysis of normal prion protein expression on human, rodent, and ruminant blood cells by using a panel of prion antibodies. Transfusion **42:**517-26.
7. **Bartz, J., Marsh, RF, McKenzie, DI, Aiken, JM.** 1998. The host range of chronic wasting disease is altered on passage in ferrets. Virology **Nov 25;251:**297-301.
8. **Bolton, D., McKinley, MP, Prusiner, SB.** 1985. Scrapie PrP 27-30 is a sialoglycoprotein. J Virol **Feb;53:**596-606.
9. **Brown, P., Cervenakova, L, Diringer, H.** 2001. Blood infectivity and the prospects for a diagnostic screening test in Creutzfeldt-Jakob disease. J Lab Clin Med **137:**5-13.
10. **Bueler, H., Aguzzi, A, Sailer, A, Greiner, RA, Auteried, P, Aguet, M, Weissmann, C.** 1993. Mice devoid of PrP are resistant to scrapie. Cell **Jul 2;73:**1339-47.
11. **Bueler, H., Fischer, M, Lang, Y, Bluethmann, H, Lipp, HP, DeArmond, SJ, Prusiner, SB, Aguet, M, Weissmann, C.** 1992. Normal development and behaviour of mice lacking the neuronal cell-surface PrP protein. Nature **Apr 16;356:**577-82.
12. **Carrell, R., Lomas DA.** 1997. Conformational disease. Lancet **Jul 12;350:**134-8.
13. **Casaccia, P., LA, Xi YG, Pocchiari, M.** 1989. Levels of infectivity in the blood throughout the incubation period of hamsters peripherally injected with scrapie. Arch Virol **108:**145-9.
14. **Cervenakova, L., Yakovleva, O, McKenzie, C, Kolchinsky, S, McShane, , Dorohan, WN, Brown, P.** 2003. Similar levels of infectivity in the blood of mice infected with human-derived vCJD and GSS strains of transmissible spongiform encephalopathy. Transfusion **Dec;43:**1687-94.
15. **Chiti, F., Taddei N, Baroni F, Capanni C, Stefani M, Ramponi G, Dobson C.** 2002. Kineic partitioning of protein folding and aggregation. Nat. Struct. Biol. **Feb; 9:**137-43.
16. **Choi, E., Geschwind, MD, Deering, C, Pomeroy, K, Kuo, A, Miller, BL, Safar, JG, Prusiner. SB.** 2009. Prion proteins in subpopulations of white blood cells from patients with sporadic Creutzfeldt-Jakob disease. Lab Invest **Jun;89:**624-35.
17. **Clarke, M., Haig, DA.** 1967. Presence of the transmissible agent of scrapie in the serum of affected mice and rats. Vet Rec **Apr 22;80:**504.
18. **Dabaghian, R., Zerr, I, Heinemann, U, Zanusso, G.** 2008. Detection of proteinase K resisant proteins in the urine of patients with Creutzfeld-Jakob and other neurodegenerative diseases. Prion **Oct;2:**170-8.
19. **Deleault, N., Harris, BT, Rees, JR, Supattapone, S.** 2007. Formation of native prions from minimal components in vitro. Proc Natl Acad Sci **Jun 5;104:**9741-6.
20. **Dobson, C.** 2001. The structural basis of protein folding and its links with human disease. Philos. Trans. R. Soc. Lond. B. Biol. Sci. **Feb 28; 356:**133-45.
21. **Eklund, C., Kennedy, RC.** 1963. Some properties of the scrapie agent and its behavior in mice. Proc Soc Exp Biol Med **112:**974-979.
22. **Eklund, C., Kennedy, RC, Hadlow, WJ.** 1967. Pathogenesis of scrapie virus infection in the mouse. J Infec Dis **117:**15-22.

23. **Ford MJ, B., LJ, Morris, RJ, Hall, SM.** 2002. Selective expression of prion protein in peripheral tissues of the adult mouse. Neuroscience **113:**177-92.
24. **Fraser, H., Dickinson, AG.** 1970. Pathogenesis of scrapie in the mouse: the role of the spleen. Nature **May 2;226:**462-3.
25. **Georgsson, G., Sigurdarson, S, Brown, P.** 2006. Infectious agent of sheep scrapie may persist in the environment for at least 16 years. J Gen Virol **Dec;87:**373-40.
26. **Gibbs, C., Gadjusek, CD, Morris, JA.** 1965. Slow, Latent and Temperate Virus Infections, vol. 2. NINDB Monograph, Washington.
27. **Glatzel, M., Abela, E, Maissen, M, Aguzzi, A.** 2003. Extraneural pathologic prion protein in sporadic Creutzfeldt-Jakob disease. N Eng J Med **Nov 6;349:**1812-20.
28. **Goldfarb, L., Peterse, RB, Tabaton, M, Brown, P, LeBlanc, AC, Montagna, P, Cortelli,P, Julien, J, Vital, C, Pendelbury, WW, et al.** 1992. Fatal familial insomnia and familial Creutzfeldt-Jakob disease: disease phenotype determined by a DNA polymorphism. Science **258:**806-808.
29. **Gordon, W.** 1957. Discussion to Palmer, A.C. studies in scrapie. Vet Rec **69:**1324-1327.
30. **Gregori, L., Kovacs, GG, Alexeeva, I, Budka, H, Rohwer, RG.** 2008. Excretion of transmissible spongiform encephalogpathy infectivity in urine. Emerg. Infect. Dis. **Sept;14:**1406-12.
31. **Hadlow, W., Eklund, CM, Kennedy, RC, Jackson, TA, Whitford, HW, Boyle, CC.** 1974. Course of experimental scrapie virus infection in the goat. J Infec Dis **May;129:**559-67.
32. **Hadlow, W., Kennedy, RC, Race, RE.** 1982. Natural infection of Suffolk sheep with scrapie virus. J Infec Dis **Nov;146:**657-64.
33. **Haley, N., Mathiason, CK, Zabel, MD, Telling, CG, Hoover, EA.** 2009. Detection of subclinical CWD infection in conventional test-negative deer long after oral exposure to urine and feces from CWD+ deer. PLoS One **Nov 24;4:**e7990.
34. **Haley, N., Seelig, DM, Zabel, MD, Telling, GC, Hoover, EA.** 2009. Detection of CWD prions in urine and saliva of deer by transgenic mouse bioassay. PLoS One **4:**e4848.
35. **Halliday, S., Houston, F, Hunter, N.** 2005. Expression of PrPC on cellular components of sheep blood. J Gen Virol **May;86:**1571-9.
36. **Hamir, A., Kunkle, RA, Cutlip, RC, Miller, JM, O'Rourke, KI, Williams, ES, Miller, MW, Stack, MJ, Chaplin, MJ, Richt, JA.** 2005. Experimental transmission of chronic wasting disease agent from mule deer to cattle by the intracerebral route. J Vet Diagn Invest **May;17:**276-81.
37. **Hamir, A., Kunkle, RA, Miller, JM, Greenlee, JJ, Richt, JA.** 2006. Experimental second passage of chronic wasting disease (CWD(mule deer)) agent to cattle. J Comp Pathol **Jan;134:**63-9.
38. **Harrington, R., Baszler, TV, O'Rourke, KI, Schneider, DA, Spraker, TR, Liggitt, HD, Knowles, DP.** 2008. A species barrier limits transmission of chronic wasting disease to mink (Mustela vison). J Gen Virol **Apr;89:**1086-96.

39. **Heisey, D., Mickelsen, NA, Schneider, JR, Johnson, CJ, Johnson, CJ, Langenberg, JA, Bochsler, PN, Keane, DP, Barr, DJ.** 2009. Chronic wasting disease (CWD) susceptibility of several North American rodents that are sympatric with cervid CWD epidemics. J Virol **Oct 14**.
40. **Herzog, C., Riviere, J, Lescoutra-Etchegaray, N, Charbonnier, A, Leblanc, V, Sales, N, Deslys, JP, Lasmezas, CI.** 2005. PrPTSE distribution in a primate model of variant, sporadic, and iatrogenic Creutzfeldt-Jakob disease. J Virol **Nov;79:**14339-45.
41. **Holada, K., Mondora, TH, Muller, J, Vostal, JG.** 1998. Increased expression of phosphatidylinositol-specific phospholipase C resistant prion proteins on the surface of activated platelets. Br J Haematol **Oct;103:**276-82.
42. **Holada, K., Simak, J, Brown, P, Vostal, JG.** 2007. Divergent expression of cellular prion protein on blood cells of human and nonhuman primates. Transfusion **Dec;47:**2223-32.
43. **Holada, K., Vostal, JG.** 2000. Different levels of prion protein (PrPc) expression on hamster, mouse and human blood cells. Br J Haematol **Aug;110:**472-80.
44. **Holada, K., Vostal, JG, Theisen, PW, MacAuley, C, Gregori, L, Rohwer, RG.** 2002. Scrapie infectivity in hamster blood is not associated with platelets. J Virol **2002:**9.
45. **Houston, F., Foster, JD, Chong, A, Hunter, N, Bostock, CJ.** 2000. Transmission of BSE by blood transfusion in sheep. Lancet **Sep 16;356:**999-1000.
46. **Hunter, N., Foster, J, Chong, A, McCutcheon, S, Parnham, D, Eaton, S, MacKenzie, C, Houston, F.** 2002. Transmission of prion diseases by blood transfusion. J Gen Virol **Nov;83:**2897-905.
47. **Immel, F., Jiang, Y, Wang, Y, Marchal, C, Maillet, L, Perrett, S, Cullin, C.** 2007. In virto analysis of SpUre2p, a prion-related protein, exemplifies the relationship between amyloid and prion. J Biol Chem **282:**7912-7920.
48. **Kim, T., Shon, HJ, Joo, YS, Mun, UK. Kand, KS, Lee, YS.** 2005. Additional cases of Chronic Wasting Disease in imported deer in Korea. J Vet Med Sci **Aug;67:**753-9.
49. **Kitamoto, T., Muramoto, T, Mohri, S, Doh-Ura, K, Tateishi, J.** 1991. Abnormal isoform of prion potein accumulates in follicular dendritic cells in mice with Creutzfeldt-Jakob disease. J Virol **Nov;65:**6292-5.
50. **Klein, M., Frigg, R, Flechsig, E, Raeber, AJ, Kalinke, U, Bluethmann, H, Bootz, F, Suter, M, Zinkernagel, RM, Aguzzi, A.** 1997. A crucial role for B cells in neuroinvasive scrapie. Nature **Dec 18-25;390:**687-90.
51. **Kreeger, T., Montgomery, DL, Jewell, JE, Schultz, W, Williams, ES.** 2006. Oral transmission of chronic wasting disease in captive Shira's moose. J Wildl Dis **Jul;42:**640-5.
52. **Kruger, D., Thomzig, A, Lenz, G, Kampf, K, McBride, P, Beekes, M.** 2009. Faecal shedding, alimentary clearance and intestinal spread of prions in hamster fed with scrapie. Vet Rec **Jan-Feb;40:**4 E pub 2008 Oct 3.
53. **Lefrere, J., Danic, B.** 2009. Pictorial representation of transfusion over the years. Transfusion **May;49:**1007-17.

54. **Lefrere, J., Hewitt, P.** 2009. From mad cows to sensible blood transfusion: the risk of prion transmission by labile blood components in the United Kingdom and in France. Transfusion **Apr;49:**797-812.
55. **Llewelyn, C., Hewitt, PE, Knight, RS, Amar, K, Cousens, S, Mackenzie, J, Will, RG.** 2004. Possible transmission of variant Creutzfeldt-Jakob disease by blood transfusion. Lancet **Feb 7;363:**417-21.
56. **MacGregor, I., Hope, J, Barnard, G, Kirby, L, Drum,ond, O, Pepper, D, Hornsey V, Barclay, R, Bessos, H, Turner, M, Prowse, C.** 1999. Application of a time-resolved fluoroimmunoassay for the analysis of ormal prion protein in human blood and its components. Vox Sang **77:**88-96.
57. **Marsh, R., Kincaide, AE, Bessen, RA, Bartz, JC.** 2005. Interspecies transmission of chronic wasting disease prions to squirrel monkeys (Saimiri sciureus). J Virol **Nov;79:**13794-6.
58. **Martins, V., Linden, R, Prado, MA, Walz, R, Sakamoto, AC, Izquierdo, I, Brentani, RR.** 2002. Cellular prion protein: on the road for functions. FEBS Lett **Feb 13;512:**25-8.
59. **Mathiason, C.** 2009. Unpublished findings. Detection of the normal cellular prion protein (PrPc) on blood cells of white-tailed deer. Colorado State University.
60. **Mathiason, C., Hays, SA, Powers, J, Hayes-Klug, J, Langenberg, J, Dahmes, SJ, Osborn, DA, Miller, KV, Warren, RJ, Mason, GL, Hoover, EA.** 2009. Infectious prions in pre-clinical deer and transmission of chronic wasting disease solely by environmental exposure. PLoS One **Jun 16;4:**e5916.
61. **Mathiason, C., Powers, JG, Dahmes, SJ, Osborn, DA, Miller, KV, Warren, RJ, Mason, GL, Hays, SA, Hayes-Klug, Seelig, DM, Wild, MA, Wolfe, LL, Spraker, TR, Miller, MW, Sigurdson, CJ, Telling, GC, Hoover, EA.** 2006. Infectious prions in the saliva and blood of deer with chronic wasting disease. Science **Oct 6;314:**133-6.
62. **McBride, P., Eikelenboom, P, Kraal, G, Fraser, H, Bruce, ME.** 1992. PrP protein is associated with follicular dendritic cells of spleens and lymph nodes in uninfected and scrapie-infected mice. J Pathol **Dec;168:**413-8.
63. **McGovern, G., Jeffrey, M.** 2007. Scrapie-specific pathology of sheep lymphoid tissues. PLoS One **Dec 12;2:**e1304.
64. **Medori, R., Tritschler, HJ, LeBlanc, A, Villare, F, Manetto, V, Chen, HY, Xue, R, Leal, S, Montagna, P, Cortelli, P, et al.** 1992. Fatal familial insomnia a prion disease with a mutation at codon 178 of the prion gene. N Engl J Med **326:**444-449.
65. **Miller, M., Williams, ES, Hobbs, NT, Wolfe, LL.** 2004. Environmental sources of prion transmission in mule deer. Emerg. Infect. Dis. **Jun;10:**1003-6.
66. **Moleres, F., Velayos, JL.** 2005. Expression of PrP(C) in the rat brain and characterization of a subset of cortical neurons. Brain Res **Sep 14;1056:**10-21.
67. **Muramoto, T., Kitamoto, T, Tateishi, J, Goto, I.** 1992. The sequential development of abnormal prion protein accumulation in mice with Creutzfeldt-Jakob disease. Am J Pathol **Jun;140:**1411-20.
68. **Nilsson, M.** 2004. Techniqus to study amyloid fibril formation in vitro. Methods **Sept;34:**151-60.

69. **Pan, K., Baldwin M, Nguyen J, Gasset M, Serban A, Groth D, Mehlhorn I, Huang Z, Fletterick RJ, COhen FE, et al.** 1993. Conversion of alpha-helices into beta-sheets features in the formation of the scrapie prion proteins. Proc Natl Acad Sci **Dec 1;90:**10962-6.
70. **Pattison, I., Hoare, MN, Jebbett, JN, Watson, WA.** 1972. Spread of scrapie to sheep and goats by oral dosing with foetal membranes from scrapie-affected sheep. Vet Rec **Apr 22;90:**465-8.
71. **Pattison, I., Jones, KM.** 1968. Detection of the scrapie agent in tissues of normal mice and in tumours of tumour-bearing but otherwise normal mice. Nature **Apr 6;21:**102-4.
72. **Pattison, I., Millson, GC.** 1961. Scrapie produced experimentally in goats with special reference to the clinical syndrome. J Comp Pathol **Apr:71:**101-9.
73. **Peden, A. H., M. W. Head, D. L. Ritchie, J. E. Bell, and J. W. Ironside.** 2004. Preclinical vCJD after blood transfusion in a PRNP codon 129 heterozygous patient. Lancet **364:**527-9.
74. **Prusiner, S.** 1982. Novel proteinaceous infectious particles cause scrapie. Science **Apr 9;216:**136-44.
75. **Prusiner, S.** 1998. Prions. Proc Natl Acad Sci **Nov 10;95:**13363-83.
76. **Race, B., Meade-White, KD, Miller, MW, Barbian, KD, Rubenstein, R, LaFauci, G, Cervenakova, L, Favara, C, Gardner, D, Long, D, Parnell, M, Striebel, J, Priola, SA, Ward, A, Williams, ES, Race, R, Chesebro, B.** 2009. Susceptibilities of nonhuman primates to chronic wasting disease. Emerg. Infect. Dis. **Sept;15:**1366-76.
77. **Raeber, A., Klein, MA, Frigg, R, Flechsig, E, Aguzzi, A, Weissmann, C.** 1999. PrP-dependent associatio of prions with splenic but not circulating lymphocytes of scrapie-infected mice. EMBO **May 17;18:**2702-6.
78. **Rane, N., Kang, SW, Chakrabarti, O, Feigenbaum, L, Hegde, RS.** 2008. Reduced translocation of nascent prion protein during ER stress contributes to neurodegeneration. Dev Cell **Sep;15:**359-70.
79. **Raymond, G., Raymond, LD, Meade-White, KD, Hughson, AG, Favara, C, Gardner, D, Williams, ES, Miller, MW, Race, RE, Caughey, B.** 2007. Transmission and adaptation of chronic wasting disease to hamsters and transgenic mice: evidence for strains. J Virol **Apr;81:**4305-14.
80. **Safar, M., Lessard, P, Tamguney, G, Greyman, Y, Deering, C, Letessier, F, Dearmond, SJ, Prusiner, SB.** 2008. Transmission and detection of prions in feces. J Infec Dis **July 1;198:**81-9.
81. **Sigurdson, C., Barillas-Mury, C, Miller, MW, Oesch, B, van Keulen, LJ, Langeveld, JP, Hoover, EA.** 2002. PrP(CWD) lymphoid cell targets in early and advanced chronic wasting disease of mule deer. J Gen Virol **Oct;83:**2617-28.
82. **Sigurdson, C., Mathiason, CK, Perrott, MR, Eliason, GA, Spraker, TR, Glatzel, M, Manco, G, Bartz, JC, Miller, MW, Hoover, EA.** 2008. Experimental chronic wasting disease (CWD) in the ferret. J Comp Pathol **May;138:**189-96.
83. **Sigurdson, C., Williams, WS, Miller, MW, Spraker, TR, O'Rourke, KI, Hoover, EA.** 1999. Oral transmission and early lymphoid tropism of chronic

wasting disease PrPres in mule deer fawns (Odocoileus hemionus). J. Gen. Virol. **Oct;80:**2757-64.

84. **Siso', S., Jeffrey, M, Steele, P, McGovern, G, Martin, S, Finlayson, J, Chianini, F, Gonzalez, L.** 2008. Occurrence and cellular localization of PrPd in kidneys of scrapie-affected sheed in the absence of inflammation. J Pathol **Jun;215:**126-34.
85. **Sohn, H., Kim, JH, Choi, KS, Nah, JJ, Joo, YS, Jean, YH, Ahn, SW, Kim, OK, Kim DY, Balachandran, A.** 2002. A case of chronic wasting disease in an elk imported to Korea from Canada. J Vet Med Sci **Sep;64:**855-8.
86. **Soto, C.** 2001. Protein misfolding and disease; protein refolding and therapy. FEBS Lett. **Jun 8;498:**204-7.
87. **Spraker, T., Gidlewski TL, Balachandran A, VerCauteren KC, Creekmore L, Munger RD.** 2006. Detection of PrP(CWD) in postmortem rectal lymphoid tissues in Rocky Mountain elk (Cervus elaphus nelsoni) infected with chronic wasting disease. J Vet Diagn Invest **Nov;18:**553-7.
88. **Spraker, T., Miller MW, Williams ES, Getzy DM, Adrian WJ, Schoonveld GG, Spowart RA, O'Rourke KI, Miller JM, Merz PA.** 1997. Spongiform encephalopathy in free-ranging mule deer (Odocoileus hemionus), white-tailed deer (Odocoileus virginianus) and Rocky Mountain elk (Cervus elaphus nelsoni) in northcentral Colorado. J Wildl Dis **Jan;33**.
89. **Spraker, T., VerCauteren, KC, Gidlewski, TL, Munger, RD, Walter, WD, Balachandran, A.** 2009. Impact of age and sex of Rocky Mountain elk (Cervus elaphus nelsoni) on follicle counts from rectal mucosal biopsies for preclinical detection of chronic wasting disease. J Vet Diagn Invest **Nov;21:**686-70.
90. **Spraker, T., Zink RR, Cummings BA, WIld MA, Miller MW, O'Rourke KI.** 2002. Comparison of histological lesions and immunohistochemical staining of proteinase-resistant prion protein in a naturally occurring spongiform encephalopathy of free-ranging mule deer (Odocoileus hemionus) with those of chronic wasting disease of captive mule deer. Vet Pathol **Jan;39:**110-9.
91. **Stamp, J., Brotherston, JG, Zlotnik, I, Mackay, JM, Smith, W.** 1959. Further studies on scrapie. J Comp Pathol **Jul;69:**268-80.
92. **Starke, R., Harrison, P, Mackie, I, Wang, G, Erusalimsky, JD, Gale, R, Masse, JM, Bramer, E, Pizzy, A, Biggerstaff, J, Machin, S.** 2005. The expression of prion protein (PrP(C)) in megakaryocyte lineage. J Thromb Haemost **Jun;3**.
93. **Sunde, M., Serpell LC, Bartlam M, Fraser PE, Pepys MB. Blake CC.** 1997. Common core structure of amyloid fibrils by synchrotron X-ray diffraction. J Mol. Biol. **Oct 31;273:**729-39.
94. **Tamguney, G., Miller, MW, Wolfe, LL, Sirochman, TM, Glidden, DV, Palmer, C, Lemus, A, DeArmond, SJ, Prusiner, SB.** 2009. Asymptomatic deer excrete infectious prions in feces. Nature **Sep 24;461:**529-32.
95. **Tanaka, M., Collins, SR, Toyama, BH, Weissman, JS.** 2006. The physiological basis of how prion conformations determine strain phenotypes. Nature **442:**585-589.
96. **Taylor, D., Fernie, K, Reichl, HE, Somerville, RA.** 2000. Infectivity in the blood of mice with a BSE-derived agent. J Hosp Infect **Sep;46:**78-9.

97. **Thielen, C., Melot, F, Jolois, O, Leclercq, F, Tsunoda, R, Frobert, Y, Heinen, E, Antoine, N.** 2001. Isolation of bovine follicular dendritic cells allows the demonstration of a particular cellular prion protein. Cell Tissue Res **Oct;306:**49-55.
98. **USGS.** 2009. Map of Chronic Wasting Disease in North American.
99. **Wasmer, C., Lange, A, Van Melckebeke, H, Siemer, A, Riek, R, Meier, B.** 2008. Amyloid fibrils of the HET-s(218-289) prion form a beta solenoid with a triangular hydrphobic core. Science **319:**1523-1526.
100. **Wilesmith, J., Wells, GA, Cranwell, MP, Ryan, JB.** 1988. Bovine spongiform encephalopathy: epidemiological studies. Vet Rec **Dec 17;123:**638-44.
101. **Will, R., Ironside, JW, Zeidler, M, Cousens, SN, Estibeiro, K, Alperovitch, A, Poser, S, Pocchiari, M, Hofman, A, Smith, PG.** 1996. A new variant of Creutzfeldt-Jakob disease in the UK. Lancet **Apr6;347:**921-5.
102. **Williams, E.** 2005. Chronic wasting disease. Vet Pathol **Sept;42:**530-49.
103. **Williams, E., Young S.** 1980. Chronic wasting disease of captive mule deer: a spongiform encephalopathy. J Wildl Dis **Jan;16:**89-98.
104. **Williams, E., Young S.** 1992. Spongiform encephalopathies in Cervidae. Rev Sci Tech **Jun;11:**551-67.
105. **Zabel, M., Heikenwalder, M, Prinz, M, Arrighi, I, Schwarz, P, Kranich, J, von Teichman, A, Haas, KM, Zeller, N, Tedder, TF, Weis, JH, Aguzzi, A.** 2007. Stromal complement receptor CD21/35 facilitates lymphoid prion colonization and pathogenesis. J Immunol **Nov 1;179:**6144-52.

## CHAPTER 1*

### Infectious Prions in the Saliva and Blood of Deer with Chronic Wasting Disease.

***Previously published in part as:**

**Candace Mathiason, Jenny G. Powers, Sallie J. Dahmes, David A. Osborn, Karl V. Miller, Robert J. Warren, Gary L. Mason, Sheila A. Hays, Jeanette Hayes-Klug, Davis M. Seelig, Margaret A. Wild, Lisa L. Wolfe, Terry R. Spraker, Michael W. Miller, Christina J. Sigurdson, Glenn C. Telling and Edward A. Hoover.** 2006. Infectious Prions in the Saliva and Blood of Deer with Chronic Wasting Disease. Science Oct. 6;314(5796):133-6.

## ABSTRACT

A critical concern in the transmission of prion diseases [including chronic wasting disease (CWD) of cervids] is the potential presence of prions in body fluids. To address this issue directly, we exposed cohorts of CWD-naïve deer to saliva, blood, or urine and feces from CWD-positive deer. We report the presence of infectious prions capable of transmitting CWD in saliva (by the oral route) and in blood (by transfusion). The results

help explain the facile transmission of CWD among cervids and prompt caution concerning contact with body fluids in prion infections.

## INTRODUCTION

The prion diseases, or transmissible spongiform encephalopathies (TSEs), are chronic degenerative neurological diseases with uniformly fatal outcomes. TSEs are characterized by the conversion of the normal cellular prion protein ($PrP^{c}$) to an aberrant insoluble partially protease resistant isoform ($PrP^{res}$). Chronic wasting disease (CWD), a transmissible spongiform encephalopathy of cervids (deer, elk, moose), was first observed in the 1960's in captive deer and free ranging deer and elk in northeastern Colorado and southeastern Wyoming (20, 24-26). CWD now has been identified in 14 states in the United States and two Canadian provinces. Despite its facile transmission the exact mode of CWD infection has not been determined. In fact, surprisingly little is known about the transmission of naturally occurring TSEs. For example, scrapie of sheep has been known for centuries yet the precise mode of natural transmission remains unclear (8, 12).

## MATERIALS AND METHODS

To determine whether infectious prions capable of transmitting CWD are present in body fluids and excreta of CWD-infected deer (CWD+), we exposed five cohorts (n=2-4/cohort) of six-month-old CWD-naïve, hand-raised white-tailed deer (*Odocoileus virginianus*) fawns from Georgia, USA (Table 1.1) to blood, saliva, and a combination of

urine and feces from CWD+ mule deer (*Odocoileus hemionus*) from Colorado, USA (Supportive Online Material (SOM) Table SOM 1.1). Deer cohorts #1 to #4 were inoculated with either brain, blood, saliva, or urine and feces from confirmed CWD-infected (CWD+) free-ranging or captive mule deer from Colorado, USA (materials provided from the Colorado Division of Wildlife (CDOW), the Colorado State Veterinary Diagnostic Laboratory (CSUVDL), and the National Park Service (NPS)). Deer cohort #5 received matching control inocula collected from confirmed CWD-negative white-tailed deer from Georgia, USA (provided by the Warnell School of Forestry and Natural Resources, University of Georgia, Athens (UGA)). Each individual deer fawn in cohorts #1-#3 received an inoculum from a different CWD+ source animal (in SOM 1.1).

Table 1.1. CWD prion bioassay inoculation cohorts.

| Animal cohort | n | Inoculum | Route (n) | Amount | No. of inoculations |
|---|---|---|---|---|---|
| 1 | 3 | Blood | IV (1), IP (2) | 250 ml | 1 |
| 2 | 3 | Saliva | PO (3) | 50 ml | 3 |
| 3 | 3 | Urine + Feces | PO (3) | 50 ml + 50 gm | 3-14 |
| 4 | 4 | Brain | IC (2), PO (2) | 1 gm (IC), 10 gm (PO) | 1 (IC), 3 (PO) |
| 5 | 2 | All of the above | PO (2) | All of the above | 1-14 |

Each fawn in cohort #1 received either a single intraperitoneal (IP) inoculation of 250ml of frozen citrated blood (n=2) or an intravenous (IV) transfusion with 250ml fresh citrated whole blood (n=1) each from a single CWD+ donor. Each fawn in cohort #2 received a total of 50ml saliva, each from a different CWD+ donor, orally (PO) in 3 doses over a 3-day period. Each fawn in cohort #3 received a total of 50ml urine + 50 grams feces PO, each from a different CWD+ donor, in divided doses over a 3 to 14 day period. As positive controls, each fawn in cohort #4 was inoculated with a 10% brain homogenate from a CWD+ donor deer via either a single intracranial (IC) injection of 1 gram equivalent of brain (n=2) or PO with a total of 10 gram equivalents of brain (n=2) divided over a 3-day period. Cohort #5 fawns (n=2) were inoculated with equivalent amounts of each of the above materials from a single CWD-negative donor deer to serve as negative controls for the study.

Because polymorphism in the normal prion protein gene (PRNP) may influence CWD susceptibility or incubation time in white-tailed deer, PRNP codon 96 genotype for each deer was determined in the laboratory of Dr. Katherine O'Rourke (16) (SOM Table 1.2).

The deer fawns were housed in separate isolation suites under strict isolation conditions to exclude adventitious sources of prion exposure, thus permitting conclusions based on only the point source exposure. Exacting protocols were enacted to preclude extraneous exposure or cross-contamination among cohorts and to protect personnel (e.g. shower-in, Tyvek™ clothing, face masks, head covers, footwear, traffic flow, sourcing of feeds and bedding, and animal-specific biopsy and sample collection instruments). Sham-inoculated control deer (cohort #5) were housed in the same building as sentinels to assure freedom from cross or adventitious contamination.

After inoculation, the deer were monitored for CWD infection by serial tonsil biopsy performed at 0, 3, 6, and 12 months post inoculation (pi), and at termination (18-22 months pi). Equal portions of tonsil tissue were stored at -70C or fixed in 10% formalin for 24 hours before processing for immunohistochemistry (ihc) or western blotting (wb) for the detection of the protease-resistant abnormal prion protein associated with CWD ($PrP^{CWD}$). At the same sampling intervals, blood, saliva, feces, and urine were collected from each animal and stored at -70C. At necropsy, palatine tonsils, brain, and retropharyngeal lymph nodes, and sections from all major organs were collected as above.

## RESULTS

Serial tonsil biopsy of each recipient deer revealed that infectious CWD prions were present in saliva and blood from CWD+ donor deer (Table 2). As expected $PrP^{CWD}$ was demonstrated between 3-12 months pi in tonsil biopsies of all 4 animals inoculated either orally or IC with CWD+ brain (positive control cohort #4). Of greater interest was the detection of $PrP^{CWD}$ in tonsil biopsies of 2 of 3 deer each in both the saliva and blood cohorts (#'s 1 and 2) at 12 months pi. By contrast, deer in the urine+feces inoculation cohort #3 remained tonsil biopsy negative for $PrP^{CWD}$ throughout the 18 month study. Likewise, animals in the negative control inoculation cohort #5 also remained tonsil biopsy negative throughout the study.

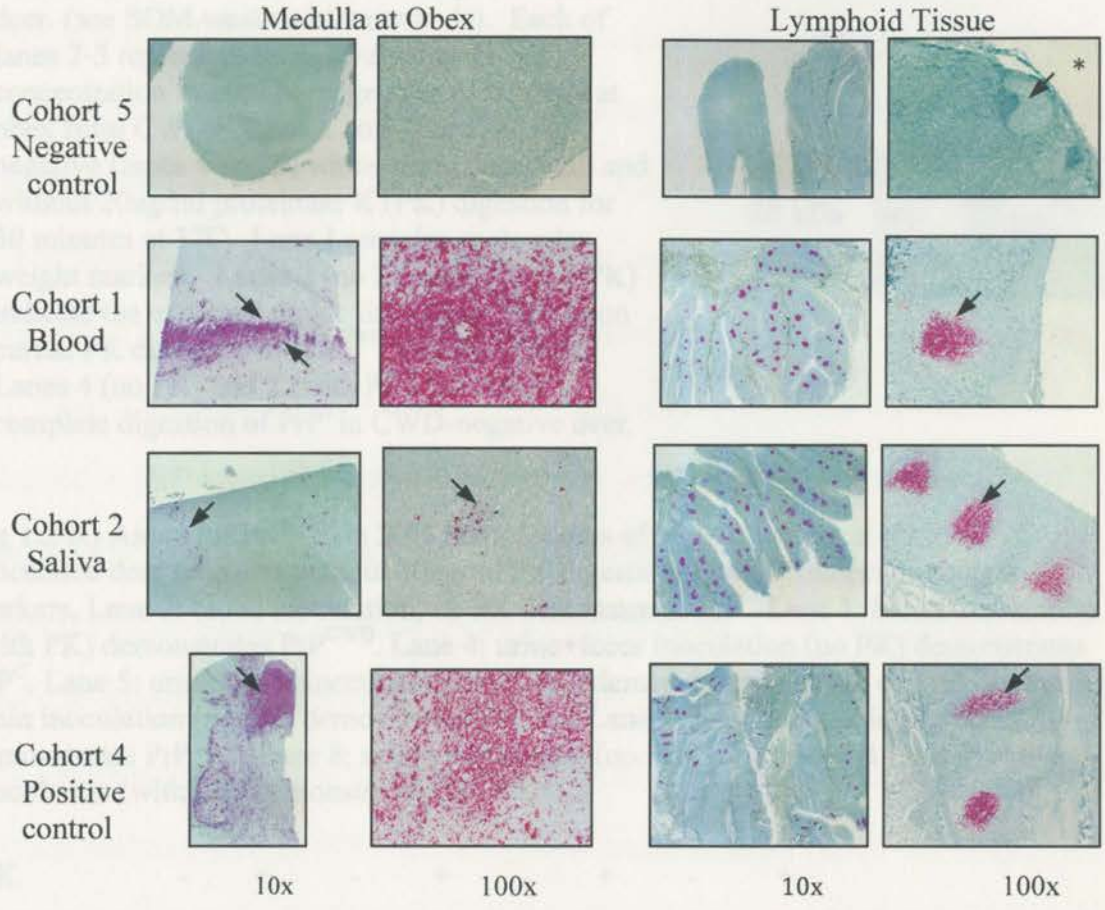
Deer cohorts #1-blood, #2-saliva and #3-urine+feces were electively euthanized at 18 months pi to permit whole body examination for $PrP^{CWD}$. Greatest scrutiny was directed toward those tissues previously established to have highest frequency of $PrP^{CWD}$ deposition in infected deer and generally regarded as the most sensitive indicators of infection, i.e., medulla oblongata and other brainstem regions, tonsil, and retropharyngeal lymph node. We found unequivocal evidence of $PrP^{CWD}$ by both ihc and wb assays in brain and lymphoid tissue of all 6 tonsil biopsy-positive deer in cohorts #1 (blood) and #2 (saliva), whereas all deer in cohorts #3 and #5 were negative for $PrP^{CWD}$ in all tissues (Table 1.2, Figs. 1.1 and 1.2b).

Table 1.2. $PrP^{CWD}$ detection by longitudinal tonsil biopsy and necropsy of deer exposed to body fluids or excreta from CWD+ deer.

| Animal cohort | Inoculum | Biopsy collection 3 mo. | Biopsy collection 6 mo. | Biopsy collection 12 mo. | Termination | | |
|---|---|---|---|---|---|---|---|
| | | T[d] | T[d] | T[d] | T[d] | B[e] | RLN[f] |
| 1 | Blood | 0/3[*] | 0/3 | 2/3[i] | 3/3[a] | 2/3 | 3/3 |
| 2 | Saliva | 0/3 | 0/3 | 2/3[j] | 3/3[a] | 2/3 | 3/3 |
| 3 | Urine + Feces | 0/2[**] | 0/2 | 0/2 | 0/2[a] | 0/2 | 0/2 |
| 4 | Brain | 1/4[g] | 2/4[h] | 4/4 | 2/2[b] | 2/2 | 2/2 |
| 5 | Negative samples | 0/2 | 0/2 | 0/2 | 0/2[c] | 0/2 | 0/2 |

*The number of deer in which $PrP^{CWD}$ was detected (by ihc and wb) over the total number of deer in the cohort. **1 of the 3 original animals inoculated with urine and feces was euthanized prematurely 61 days pi due to a bacterial infection. [a] The deer in cohorts #1, 2 and 3 were terminated at 18 months (mo.) pi. [b] Two of 4 cohort #4 deer were terminated at 20 and 21 mo. pi. [c] Two of 2 cohort 5 deer were terminated at 22 mo. pi. [d]$PrP^{CWD}$ assay results (ihc and wb) for tonsil (T), [e]brain (B)--medulla oblongata at obex, and [f]retropharyngeal lymph node (RLN) are reported. [g]The first of 4 CWD+ brain-inoculated deer was $PrP^{CWD}$ tonsil biopsy positive by 3 months pi (PO—TS989), [h]the second deer in this cohort was $PrP^{CWD}$ positive by 6 months pi (IC—TS989). [i]Two of 3 blood-inoculated deer were $PrP^{CWD}$ tonsil biopsy positive at 12 mo. pi.—one of these had received an IV transfusion of blood from CWD+ donor deer LA01 and the other frozen blood IP from CWD+ donor animal 0428. [j]Two of 3 saliva-inoculated deer were $PrP^{CWD}$tonsil biopsy positive at 12 mo. pi—one was inoculated PO with saliva from donor CWD+ donor JA01, the other from donor 0428.

Fig 1.1. $PrP^{CWD}$ demonstrated by immunohistochemistry in tonsil, brain (medulla oblongata at obex), and retropharyngeal lymph node of deer receiving saliva or blood from CWD-infected donors.




CWD Immunohistochemistry panels at 10x and 100x magnifications of medulla at obex (2 left panels) and lymphoid tissue, either tonsil or retropharyngeal lymph node (2 right panels) (see SOM for immunohistochemistry methods). Arrows indicate $PrP^{CWD}$ staining (red) within brain and lymphoid follicles. Arrow with asterisk indicates lymphoid follicle negative for $PrP^{CWD}$.

Fig 1.2. Western blot demonstration of $PrP^{CWD}$ in brain (medulla) of CWD+ and CWD- negative control white-tailed deer.

Fig 1.2a.) $PrP^{CWD}$ in positive and negative control deer. (see SOM western blot methods). Each of lanes 2-5 represents an equivalent protein concentration in 20% homogenates of medulla at obex from CWD+ (lanes 2 and 3) or CWD-negative (lanes 4 and 5) white-tailed deer, with and without 20ug/ml proteinase K (PK) digestion for 30 minutes at 37C. Lane 1 contains molecular weight markers. Lanes 2 (no PK) and 3 (with PK) indicate the expected molecular weight shift upon partial PK digestion of $PrP^{CWD}$ in CWD+ deer. Lanes 4 (no PK) and 5 (with PK) show the complete digestion of $PrP^{c}$ in CWD-negative deer.

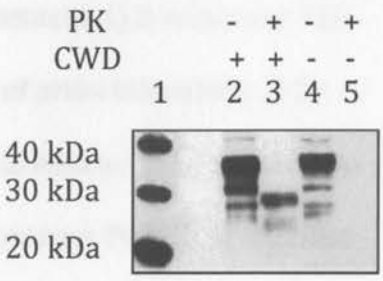
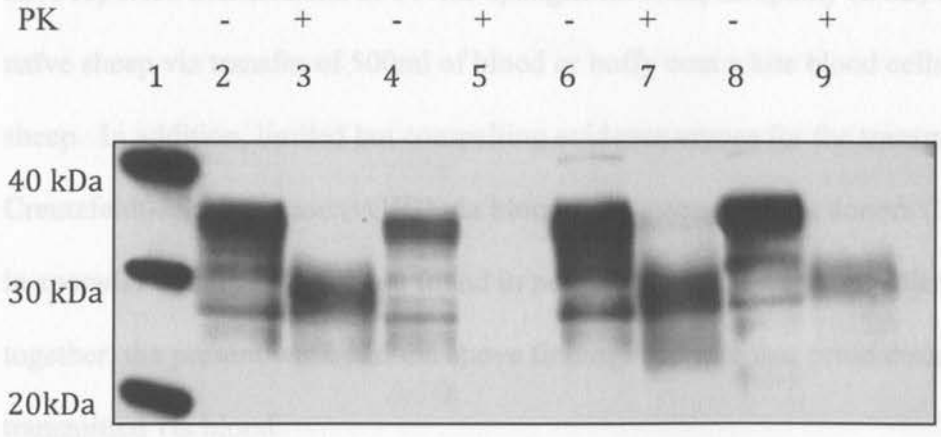



Fig 1.2 b.) Assay for $PrP^{CWD}$ in 20% homogenates of brain (medulla at obex) in inoculated deer with and without 20ug/ml PK digestion. Lane 1: molecular weight markers. Lane 2: blood inoculation, no PK demonstrates PrP. Lane 3: blood inoculation (with PK) demonstrates $PrP^{CWD}$. Lane 4: urine+feces inoculation (no PK) demonstrates $PrP^{C}$. Lane 5: urine+feces inoculation (with PK) demonstrates the lack of $PrP^{CWD}$. Lane 6: brain inoculation (no PK) demonstrates PrP and Lane 7: brain inoculation (with PK) demonstrates $PrP^{CWD}$. Lane 8: saliva inoculation (no PK) with PrP, and Lane 9: saliva inoculation (with PK) demonstrates $PrP^{CWD}$.



## DISCUSSION

The transmission of CWD by a single blood transfusion from 2 symptomatic and 1 asymptomatic CWD+ donor is important in at least three contexts: 1) It reinforces that no tissue from CWD-infected cervids can be considered free of prion infectivity; 2) It poses the possibility of hematogenous spread of CWD, e.g. via insects, and 3) It provides basis for seeking in vitro assays sufficiently sensitive to demonstrate $PrP^{CWD}$ or alternate prion protein conformers in blood—one of the grails of prion biology and epidemiology.

Prior to the present work with CWD, identification of blood borne prion transmission has long been sought. Hadlow et. al. performed bioassays on serial blood collections from goats ic or subcutaneously inoculated with mouse-adapted scrapie. Although Hadlow reported negative findings in these studies, Clarke and Eklund, had previously been able to demonstrate transmission of mouse-adapted scrapie via blood (3, 5, 7). More recent precedent is found in the work of Hunter and colleagues (9, 10) who have reported transmission of bovine spongiform encephalopathy (BSE) and scrapie to naïve sheep via transfer of 500ml of blood or buffy coat white blood cells from infected sheep. In addition, limited but compelling evidence argues for the transmission of variant Creutzfeldt-Jakob disease (vCJD) via blood from asymptomatic donors (2, 13, 17). Even in sporadic CJD $PrP^{res}$ has been found in peripheral organs of some patients (6). Taken together, the present work and the above findings indicate that prion diseases can be transmitted via blood.

The presence of infectious CWD prions in saliva may explain the facile transmission of CWD. Grooming interactions, shared water sources, salt licks, scrape sites, and forage sites, especially those in which cervids are in greater density, e.g. during the breeding season, low predation territories and captivity (e.g. cervid farms), all would be expected to facilitate salivary cross contact. Due to the association of $PrP^{res}$/$PrP^{CWD}$ accumulation with the alimentary tract (e.g. tonsils), saliva has previously been suggested as a possible vehicle for CWD transmission (4, 7, 14). Salivary dissemination of prions may not be limited to CWD, since the protease-resistant prion protein of transmissible mink encephalopathy has been demonstrated in the oral mucosa lamina propria, taste buds, lingual squamous epithelium, vomeronasal organ and olfactory mucosa of infected hamsters (4). We have made similar observations in the olfactory mucosa of ferrets infected with CWD (18). While no instance of CWD transmission to humans has been detected, the present results emphasize the prudence of using impervious gloves during contact with saliva or blood of cervids that may be CWD-infected.

Environmental contamination by excreta from infected cervids has traditionally seemed the most plausible explanation for the dissemination of CWD (15). However, in the current study we could not detect $PrP^{CWD}$ in cohort #3 deer inoculated repeatedly with urine and feces from CWD+ deer and examined up to18 months pi (Table 2). There are several reasons to view this negative finding cautiously, including small sample size, elective preclinical termination, and potential variation in individual susceptibility that may be associated with the 96 G/S polymorphism in the PRNP gene (16, 22). This study was already in progress when the findings of O'Rourke, et. al. were published indicating that although no genotype of white-tailed deer is resistant to CWD infection, PRNP

genotypes S/S or G/S at codon 96 appear to have reduced susceptibility manifest by longer survival (16). Both deer in cohort #3 (urine+feces) were subsequently shown to be of the PRNP 96 G/S genotype. It is therefore possible, although we think unlikely, that these deer had a prolonged incubation period (>18 mo. pi) before the amplification of $PrP^{CWD}$ became detectable in tissues. Interestingly, recent studies have shown that $PrP^{res}$ is poorly preserved after incubation with intestinal or fecal content (11, 19). Further research using cervid and surrogate cervid PrP transgenic mice are indicated to continue to address the presence of infectious CWD prions in excreta of CWD+ deer and provide a more substantial basis for re-consideration of the assumption that excreta are the chief vehicle for CWD dissemination and transmission. In this respect, we have in progress a second series of bioassays employing body fluids and excreta collected from CWD+ deer in the present study using PRNP 96 G/G recipient deer and cervidized transgenic mice (1).

In summary, the results reported here provide a plausible basis for the efficient transmission of CWD in nature. We demonstrate for the first time that blood and saliva in particular are able to transmit CWD to naïve deer and produce incubation periods consistent with those observed in naturally acquired infections (23, 25). The time from exposure to first detection of $PrP^{CWD}$ by tonsil biopsy was variable--as short as 3 months but as long as 18 months (likely these are underestimates due to sampling frequency). The results also reinforce a cautious view of the exposure risk presented by body fluids, excreta, and all tissues from CWD+ cervids. The results also offer hope and direction, however, for eventual clinical diagnosis of CWD and other prion infections based on examination of blood and/or saliva. Our continuing work focuses on these issues.

## SUPPORTIVE MATERIALS

SOM Table 1.1. CWD bioassay inocula sources

| Deer source | Donor animal ID numbers | | | |
|---|---|---|---|---|
| | Brain | Urine + Feces | Saliva | Blood |
| CSUVDL | TS-989 | | | |
| NPS | | 0428 | 0428 | 0428 |
| CDOW | LA01 | LA01/JA01 | LA01/JA01 | LA01 |
| | | JA01/D10 | JA01 | 33a02 |
| UGA | 1/2 | 1/2 | 1/2 | 1/2 |

CWD positive brain, blood, saliva, urine and feces from terminal free ranging mule deer were provided by the Colorado State University Veterinary Diagnostic Laboratory (CSUVDL) and the National Park Service (NPS). The Colorado Division of Wildlife (CDOW) provided similar inocula from terminal captive mule deer. CWD negative control inocula source was provided by the University of Georgia, Athens (UGA).

SOM Table 1.2. Deer cohort #1-#5 PRNP codon 96 genotype

| Animal cohort | Route of inoculation | Donor ID No. | PRNP 96 | Months pi 1st CWD+ status |
|---|---|---|---|---|
| Cohort #1-blood | IP | 33a02 | G/G | 18 |
| blood | IP | 0428 | G/G | 12 |
| blood | IV | LAO1 | G/G | 12 |
| Cohort #2-saliva | PO | JA01 | G/G | 12 |
| saliva | PO | JAO1/LAO1 | G/G | 18 |
| saliva | PO | 0428 | G/G | 12 |
| Cohort #3-urine+feces | PO | JAO1/D10 | G/S | Negative |
| urine+feces | PO | 0428 | G/S | Negative |
| urine+feces | PO | LAO1/JAO1 | nd[a] | nd |
| Cohort #4-brain | IC | LAO1 | G/S | 12 |
| brain | IC | TS-989 | G/S | 6[b] |
| brain | PO | LAO1 | G/S | 12[b] |
| brain | PO | TS-989 | G/G | 3 |
| Cohort #5-negative | All routes | UGA 1/2 | G/G | Negative |
| negative | All routes | UGA 1/2 | G/G | Negative |

All deer in cohorts #1-blood, #2-saliva and #5-negative control were PRNP 96 G/G. Three deer in cohort #4-brain (n=2 IC and n=1 PO) were PRNP 96 G/S while n=1 PO was PRNP 96 G/G. Both cohort #3 urine+feces deer were PRNP 96 G/S. [a]One of 3 deer in cohort #3 was euthanized prematurely 61 days pi due to a bacterial infection; PRNP 96 genotype was not determined. [b] Two of 4 deer in cohort #4 (n=1 IC and n=1 PO) are alive and asymptomatic 24 mo. pi..

***Clinical signs:*** To detect and monitor clinical manifestations of CWD (14, 24, 26) deer were observed daily by project-dedicated caretakers intimately familiar with each animal. Onset of subtle clinical signs consistent with CWD were detected at 15-20 months pi in 2 of 4 positive control animals (cohort # 4). The disease onset was manifest primarily by perceived body muscle mass reduction and was measured by gradual weight loss, which reached ≥20% of maximum body weight over 5 to 8 months (SOM Advanced clinical signs). Animals were euthanized when they displayed advanced clinical signs of CWD. Clinical signs were not observed in the CWD+ deer from cohorts #1-#3, probably because these animals were electively euthanized and necropsied at 18 months pi, although 7 of the 8 had $PrP^{CWD}$ demonstrable in neural and lymphoid tissues at necropsy. Additional clinical signs included a rough-appearing hair coat due to piloerection and a body stance characterized by a lower position of the head and a wider lateral separation of the limbs. Changes in behavior included hyperphagia and polydipsia in the face of weight loss, a head tossing motion, repetitive exaggerated lifting of the legs, diminished alertness, and occasionally aggressive behavior in the advanced stage of disease.

***Western blotting methods:*** Tissue homogenates were prepared from the obex region of the medulla oblongata encompassing the dorsal motor vagal nucleus. 20% w/v homogenates were prepared in NP-40 buffer (10mM Tris-HCl buffer pH 7.5, 0.5% NP-40, 0.5% sodium deoxycholate) by Fastprep ™ disruption at setting 6.5 for 45 seconds. Twenty-five µl of each homogenate was mixed with 5µl proteinase K to a final concentration of 20 µg/ml and incubated for 30 minutes at 37C with shaking. Proteinase

K activity was stopped with 4µl 200mM Pefablock and an equivalent protein concentration of each sample was mixed with 10 µl sample buffer (Invitrogen-20% NP0009, 50% NP0007), 5µl NP-40 buffer, was heated to 95C for 5 minutes and underwent separation by 12% Bis-Tris precast polyacrylamide gel electrophoresis (PAGE) (Invitrogen) at 150 volts for 2.5 hours in 1x MOPS (Invitrogen). PAGE separated proteins were transferred to polyvinylidene fluoride (PVDF) membrane for 1 hour at 100 volts in transfer buffer (0.025M Trizma base, 0.2M glycine, 20% methanol, pH 8.3). After the PVDF membranes blocked overnight at room temperature in Pierce Blocker™ they were probed with the PrP specific antibody BAR224 (kindly supplied by Dr. J. Grassi) followed by hrp-goat anti-mouse IgG diluted in Blocker™. Membranes were washed for 1 hour after blocking and between antibodies with wash buffer (0.1M Tris, 0.15M NaCl pH 8.0). To visualize PrP bands the PVDF membranes were developed with the Amersham™ ECL detection system.

***Immunohistochemistry methods*:** IHC was performed by the CSUVDL employing the Ventana™ Nexus autostainer and Ventana ™ $PrP^{CWD}$ specific antibody as described by Spraker et. al. (21).

**Acknowledgements**

We are grateful to J. Grassi for his gift of PrP monoclonal antibody, M. Perrott for his technical expertise with the NaPTA methodology, K. O'Rourke and L. Hamburg for performing the PRNP genotype analysis, and C. Tedford for his expertise in deer

behavior, facility design, and transport. We thank the office of the CSU Vice President for Research (A. Frank and K. Delehoy) for their support in establishing the CWD deer isolation facility. Animals were housed and cared for in accordance to CSU Animal Care and Use Committee regulations. This work was supported by NIH, NIAID, contract N01-AI-25491.

## REFERENCES

1. **Browning, S. R., G. L. Mason, T. Seward, M. Green, G. A. Eliason, C. Mathiason, M. W. Miller, E. S. Williams, E. Hoover, and G. C. Telling.** 2004. Transmission of prions from mule deer and elk with chronic wasting disease to transgenic mice expressing cervid PrP. J Virol **78:**13345-50.
2. **Cervenakova, L., O. Yakovleva, C. McKenzie, S. Kolchinsky, L. McShane, W. N. Drohan, and P. Brown.** 2003. Similar levels of infectivity in the blood of mice infected with human-derived vCJD and GSS strains of transmissible spongiform encephalopathy. Transfusion **43:**1687-94.
3. **Clarke, M. C., and D. A. Haig.** 1967. Presence of the transmissible agent of scrapie in the serum of affected mice and rats. Vet Rec **80:**504.
4. **DeJoia, C., B. Moreaux, K. O'Connell, and R. A. Bessen.** 2006. Prion Infection of Oral and Nasal Mucosa. Journal of Virology **80:**4546-4556.
5. **Eklund, C. M., R. C. Kennedy, and W. J. Hadlow.** 1967. Pathogenesis of scrapie virus infection in the mouse. J Infect Dis **117:**15-22.
6. **Glatzel, M., E. Abela, M. Maissen, and A. Aguzzi.** 2003. Extraneural pathologic prion protein in sporadic Creutzfeldt-Jakob disease. N Engl J Med **349:**1812-20.
7. **Hadlow, W. J., C. M. Eklund, R. C. Kennedy, T. A. Jackson, H. W. Whitford, and C. C. Boyle.** 1974. Course of experimental scrapie virus infection in the goat. J Infect Dis **129:**559-67.
8. **Hadlow, W. J., R. C. Kennedy, and R. E. Race.** 1982. Natural infection of Suffolk sheep with scrapie virus. J Infect Dis **146:**657-64.
9. **Houston, F., J. D. Foster, A. Chong, N. Hunter, and C. J. Bostock.** 2000. Transmission of BSE by blood transfusion in sheep. Lancet **356:**999-1000.
10. **Hunter, N., J. Foster, A. Chong, S. McCutcheon, D. Parnham, S. Eaton, C. MacKenzie, and F. Houston.** 2002. Transmission of prion diseases by blood transfusion. J Gen Virol **83:**2897-905.
11. **Jeffrey, M., L. Gonzalez, A. Espenes, C. M. Press, S. Martin, M. Chaplin, L. Davis, T. Landsverk, C. MacAldowie, S. Eaton, and G. McGovern.** 2006.

Transportation of prion protein across the intestinal mucosa of scrapie-susceptible and scrapie-resistant sheep. J Pathol **209:**4-14.

12. **Johnson, R. T.** 2005. Prion diseases. Lancet Neurol **4:**635-42.
13. **Llewelyn, C. A., P. E. Hewitt, R. S. Knight, K. Amar, S. Cousens, J. Mackenzie, and R. G. Will.** 2004. Possible transmission of variant Creutzfeldt-Jakob disease by blood transfusion. Lancet **363:**417-21.
14. **Miller, M. W., and E. S. Williams.** 2004. Chronic wasting disease of cervids. Curr Top Microbiol Immunol **284:**193-214.
15. **Miller, M. W., E. S. Williams, N. T. Hobbs, and L. L. Wolfe.** 2004. Environmental sources of prion transmission in mule deer. Emerg Infect Dis **10:**1003-6.
16. **O'Rourke, K. I., T. R. Spraker, L. K. Hamburg, T. E. Besser, K. A. Brayton, and D. P. Knowles.** 2004. Polymorphisms in the prion precursor functional gene but not the pseudogene are associated with susceptibility to chronic wasting disease in white-tailed deer. J Gen Virol **85:**1339-46.
17. **Peden, A. H., M. W. Head, D. L. Ritchie, J. E. Bell, and J. W. Ironside.** 2004. Preclinical vCJD after blood transfusion in a PRNP codon 129 heterozygous patient. Lancet **364:**527-9.
18. **Perrott, M. P.** 2006.
19. **Scherbel, C., R. Pichner, M. H. Groschup, S. Mueller-Hellwig, S. Scherer, R. Dietrich, E. Maertlbauer, and M. Gareis.** 2006. Degradation of scrapie associated prion protein (PrP(Sc)) by the gastrointestinal microbiota of cattle. Vet Res **37:**695-703.
20. **Spraker, T. R., M. W. Miller, E. S. Williams, D. M. Getzy, W. J. Adrian, G. G. Schoonveld, R. A. Spowart, K. I. O'Rourke, J. M. Miller, and P. A. Merz.** 1997. Spongiform encephalopathy in free-ranging mule deer (Odocoileus hemionus), white-tailed deer (Odocoileus virginianus) and Rocky Mountain elk (Cervus elaphus nelsoni) in northcentral Colorado. J Wildl Dis **33:**1-6.
21. **Spraker, T. R., R. R. Zink, B. A. Cummings, C. J. Sigurdson, M. W. Miller, and K. I. O'Rourke.** 2002. Distribution of protease-resistant prion protein and spongiform encephalopathy in free-ranging mule deer (Odocoileus hemionus) with chronic wasting disease. Vet Pathol **39:**546-56.
22. **Supattapone, S., T. Muramoto, G. Legname, I. Mehlhorn, F. E. Cohen, S. J. DeArmond, S. B. Prusiner, and M. R. Scott.** 2001. Identification of two prion protein regions that modify scrapie incubation time. J Virol **75:**1408-13.
23. **Williams, E. S., and M. W. Miller.** 2002. Chronic wasting disease in deer and elk in North America. Rev Sci Tech **21:**305-16.
24. **Williams, E. S., and S. Young.** 1980. Chronic wasting disease of captive mule deer: a spongiform encephalopathy. J Wildl Dis **16:**89-98.
25. **Williams, E. S., and S. Young.** 1992. Spongiform encephalopathies in Cervidae. Rev Sci Tech **11:**551-67.
26. **Williams, E. S., and S. Young.** 1982. Spongiform encephalopathy of Rocky Mountain elk. J Wildl Dis **18:**465-71.

## CHAPTER 2*

# Infectious Prions in Pre-clinical Deer and Transmission of Chronic Wasting Disease Solely by Environmental Exposure.

***Previously published in part as:**

**Candace Mathiason, Sheila A. Hays, Jenny Powers, Jeanette Hayes-Klug, Julia Langenberg, Sallie J. Dahmes, David A. Osborn, Karl V. Miller, Robert J. Warren, Gary L. Mason, and Edward A. Hoover.** Infectious Prions in Pre-clinical Deer and Transmission of Chronic Wasting Disease Solely by Environmental Exposure. 2009. PLoS One. Jun 16;4(6):e5916.

## ABSTRACT

Key to understanding the epidemiology and pathogenesis of prion diseases, including chronic wasting disease (CWD) of cervids, is determining the mode of transmission from one individual to another. We have previously reported that saliva and blood from CWD-infected deer contain sufficient infectious prions to transmit disease upon passage into naïve deer. Here we again use bioassays in deer to show that blood and saliva of pre-symptomatic deer contain infectious prions capable of infecting naïve

deer and that naïve deer exposed only to environmental fomites from the suites of CWD-infected deer acquired CWD infection after a period of 15 months post initial exposure. These results help to further explain the basis for the facile transmission of CWD, highlight the complexities associated with CWD transmission among cervids in their natural environment, emphasize the potential utility of blood-based testing to detect pre-clinical CWD infection, and could augur similar transmission dynamics in other prion infections.

## INTRODUCTION

Chronic wasting disease (CWD) is a fatal transmissible spongiform encephalopathy (TSE), or prion disease, marked by a high transmission efficiency among cervids. Since its identification in cervid populations of northern Colorado and southeastern Wyoming [1-3], CWD has now been identified in 15 states, 2 Canadian provinces, and one Asian country [4-6]. Captive deer studies and epidemiological models of CWD prevalence and risk, suggest that the facile transmission of CWD between cervids occurs primarily by horizontal/lateral means [7-10]. CWD in farmed and free-ranging cervids has caused substantial economic, ecological, trade, and cultural impact and carries with it the potential for human and domestic animal health risk [11-20]. The latter, while at present theoretical, is shaped by the occurrence of human variant Creutzfelt Jacob Disease (vCJD) arising from consumption of bovine spongiform encephalopathy (BSE) [21-23] contaminated beef [24]. As with BSE, humans appear to be separated from CWD by a demonstrable species barrier [25,26]. Nevertheless, the

presence of infectious prions in the saliva and blood of terminal CWD-infected deer [27] provide a possible mechanism for natural transmission among cervids and provide an exposure risk to humans and domestic livestock. Additionally, demonstrated CWD infectivity in saliva and blood raises questions regarding the potential for horizontal transmission of other prion infections, especially during the subclinical or pre-symptomatic phase of disease as has been reported with blood transfusion of human vCJD [28-30], sheep scrapie [31,32], and BSE infection [33-35].

We undertook the present studies to confirm our earlier findings on prion excretion through bioassays in white-tailed deer, but also to determine: (a) whether CWD prions are present in body fluids and excretions during the pre-clinical phase of infection, and (b) whether repeated environmental fomite exposure alone, vs. direct animal-to-animal contact, was sufficient to transmit CWD in deer.

## MATERIALS AND METHODS

***Ethics Statement:*** All animals were handled in strict accordance with good animal practice as defined by relevant national and/or local animal welfare bodies, and all animal work was approved by Colorado State University Animal and Care Use Committee (ACUC approval number 08-175A-01).

***White-tailed deer:*** White-tailed deer fawns were provided by the Warnell School of Forestry and Natural Resources, University of Georgia, Athens (UGA)—a region where

CWD has not been detected. The deer fawns were hand-raised and human and indoor-adapted before overnight transport directly to the Colorado State University (CSU) CWD research indoor isolation facility without contact with the native Colorado environment. The 4-month-old fawns were adapted to the facility housing conditions and diet for 2 months before study start.

***Genotyping:*** All white-tailed deer were genotyped to determine GG/GS (codon 96) status by the laboratory of Dr. Katherine O'Rourke, USDA-ARS, Pullman, WA.

***Biocontainment protocols:*** Protocols to preclude extraneous exposure and cross-contamination between cohorts of animals as previously described [27] incorporated protective shower-in requirements, Tyvek™ clothing, masks, head covers, and footwear, while maintaining stringent husbandry. Tonsil biopsy and terminal sample collections were taken with animal-specific biopsy and sample collection instruments to minimize possibility of cross contamination. Bedding and liquid waste from each suite was either incinerated or collected in a dedicated outdoor underground holding tank and denatured by alkaline digestion.

***Inoculation cohorts*:** Groups of n=2 to 4 six-month-old fawns (Table 2.1) were housed in separate isolation suites throughout the study. Suite-dedicated protective clothing, utensils, and waste disposal were incorporated to exclude cross contamination by fomites, bedding, food, excretions, or contact.

Table 2.1. CWD bioassay inoculation cohorts.

| Animal cohort | *n* | Inoculum | Route of inoculation | Dosage (total volume) | No. of inoculations | *prnp* codon 96 genotype *n* GG/ *n* GS |
|---|---|---|---|---|---|---|
| 1 | 3 | Whole blood | IV | 1 (225ml) | 1 | 2/1 |
| 2 | 3 | Saliva | PO | 10ml/day for 5 days (50 ml) | 5 | 3/0 |
| 3 | 3 | Urine and Feces | PO | 90 daily doses (85ml+112.5 gr) | 90 | 3/0 |
| 4 | 2 | Environmental contact[a] | PO | 19 mos. continuous exposure | Refreshed daily for 570 days (19 mos.) | 1/1 |
| 5 | 4 | Brain | PO | 1gr/day for 5 days (5gr) | 5 | 2/2 |

[a] Water, feed buckets and bedding from CWD+ deer.

***Inocula (route of inoculation; volume of inocula; months pi of donor animals)***: The following inocula were used: Blood (intravenous- IV; 1 transfusion of 225ml; 10-12 mo pi), saliva (oral- PO; 10ml/day for 5 days = 50ml total; 6-13 mo pi) were obtained from serial sample collections of pre-clinical CWD-inoculated, tonsil biopsy positive deer, while urine and feces (PO; 90 daily doses totaling 85ml urine + 112.5gr feces; 3-24 mo pi) were obtained from serial sample collections of pre-clinical and clinical CWD-inoculated, tonsil biopsy positive deer housed in the CSU CWD isolation facility[27]. Positive control brain (PO; 1gr/day for 5 days = 5gr total; terminal field samples) homogenates (medulla oblongata at the level of the obex) that were confirmed as CWD+ by immunohistochemistry (IHC) were from one of two sources-- either free-ranging white-tailed deer collected by the Wisconsin Department of Natural Resources (WDNR) (n=2) or a free-ranging mule deer utilized in a previous bioassay study[27] provided by

the Colorado State Veterinary Diagnostic Laboratory (CSVDL) (n=2). For the environmental exposure study, water, feed buckets, and bedding were collected on a daily basis from CWD+ deer (3 to 24 months pi) that were part of ongoing studies in the CSU CWD research facility. The cohort of environmentally exposed deer was in uninterrupted contact with a continually refreshed supply of CWD+ fomites for 19 months (Table 2.2).

Table 2.2. CWD bioassay inocula sources.

| Animal cohort | Inoculum | Donor animal (n inoculated) | Donor animal clinical status | Donor animal mo. pi. |
|---|---|---|---|---|
| 1 | Whole blood | CSU Yr I 112[b] (2)<br>CSU Yr I 121[c] (1) | Pre-clinical | 10-12 |
| 2 | Saliva | CSU Yr I pool 112+121 (3) | Pre-clinical | 6-13 |
| 3 | Urine and feces | CSU Yr I pool 112+121 (3) | Transitioning[f] | 3-24 |
| 4 | Environmental contact[a] | CSU Yr I 121, 104, 106 (2) | Transitioning[f] | 3-24 |
| 5 | Brain | WDNR[d] (2)<br>CSVDL TS 989[e] (2) | Mixed[g]<br>Pre-clinical | Terminal |

[a] Water, feed buckets and bedding from CWD+ deer suites.
[b] Colorado State University deer number 112 (PO brain inoculated deer from previous study[27]).
[c] Colorado State University deer number 121 (IC brain inoculated deer from previous study [27]).
[d] Wisconsin Department of Natural Resources, Madison, WI, USA.
[e] Colorado State Veterinary Diagnostic Laboratory, Fort Collins, CO, USA.
[f] Transitioning from pre-clinical to clinical.
[g] Contains brain tissue from deer of pre-clinical and clinical status at terminal field collection.

***Monitoring and sample collection:*** All animals were monitored for evidence of CWD infection by serial tonsil biopsies taken at 3, 6, 12 months pi, and at study termination. Tonsil tissue was divided and equal portions either stored at -70C or fixed in 10% formalin for 24 hours before processing for IHC. At the same sampling intervals, blood,

saliva, feces, and urine were collected from each animal and stored at -70C. At necropsy, palatine tonsils, brainstem (medulla at the obex) and retropharyngeal lymph nodes, as well as other tissues, were collected for examination by IHC and western blotting (WB) to identify the presence of the protease-resistant prion protein associated with CWD ($PrP^{CWD}$).

***Western blotting:*** Tissue homogenates were prepared from the obex region of the medulla oblongata encompassing the dorsal motor vagal nucleus (medulla at the obex). Twenty percent (20%) w/v homogenates were prepared in NP-40 buffer (10mM Tris-HCl buffer pH 7.5, 0.5% NP-40, 0.5% sodium deoxycholate) by Fastprep ™ disruption at setting 6.5 for 45 seconds. Twenty-five µl of each homogenate was mixed with 5µl proteinase K (PK) (Invitrogen) to a final concentration of 25 µg/ml and incubated for 30 minutes at 37C with shaking. Proteinase K activity was stopped with 4µl 200mM Pefablock SC and an equivalent volume of each sample was mixed with 10 µl sample buffer (Invitrogen-20% Reducing agent 10x, 50% LDS Sample buffer 4x), 5µl NP-40 buffer (10mM Tris-HCl pH 7.5, 0.5% deoxycholic acid, 0.5% nonylphenoxylpolyethoxylethanol), heated to 95C for 5 minutes and separated by 12% Bis-Tris precast polyacrylamide gel electrophoresis (PAGE) (Invitrogen) at 150 volts for 2.5 hours in 1x MOPS (Invitrogen). PAGE separated proteins were transferred to polyvinylidene fluoride (PVDF) membrane for 1 hour at 100 volts in transfer buffer (0.025M Trizma base, 0.2M glycine, 20% methanol, pH 8.3). After the PVDF membranes were blocked overnight at room temperature in Pierce Blocker™ they were probed with the PrP specific antibody BAR224 (kindly supplied by Dr. J. Grassi)

followed by horse radish peroxidase (HRP)-goat anti-mouse IgG diluted in Pierce Blocker™. Membranes were washed for 1 hour after blocking and between antibodies with wash buffer (0.1M Tris, 0.15M NaCl, 0.2% Tween 20 pH 8.0). To visualize PrP bands the PVDF membranes were developed with the Amersham™ ECL detection system and a digital GelDoc™ (Fuji Intelligent dark box) using LAS-3000 Lite ImageReader software.

***Immunohistochemistry (IHC)*:** was performed by employing protocols as described by Spraker *et al* [3]. Briefly, 3-5 mm sections of formalin fixed formic acid treated tissues were deparafinized at 60-70°C for 1 hour, rehydrated via a series of xylene/ethanol baths, and treated in formic acid a second time (5 minutes) prior to a 20 minute antigen retrieval (Dako Target Retrieval Solution 10x) cycle in a 2100 Retriever™ (PickCell Laboratories). Slides were further processed with the aid of a Ventana Discovery™ autostainer utilizing the Ventana Red Map™ stain kit, the $PrP^{CWD}$ specific primary antibody BAR224 and a biotinylated secondary goat anti mouse antibody (Ventana). After autostaining, the slides were quickly rinsed in a warm water detergent solution, passed through a series of dehydration baths and cover-slipped.

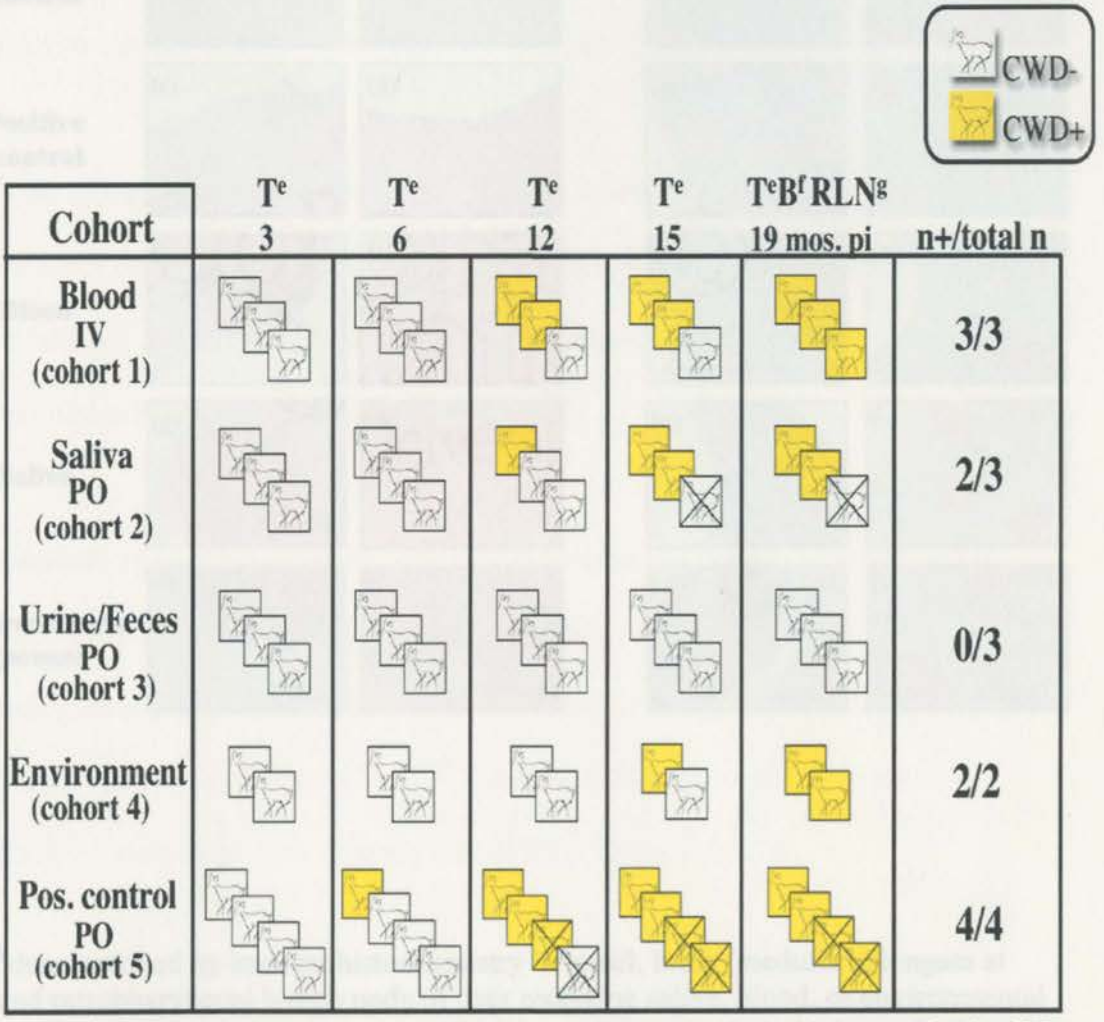
## RESULTS

***Detection of CWD infection in exposed animals:***

***Blood (cohort 1):*** Each naïve deer received blood by the IV route from pre-clinical CWD+ source deer that were 10-12 months post inoculation (pi)(Tables 2.1, 2.2). Two

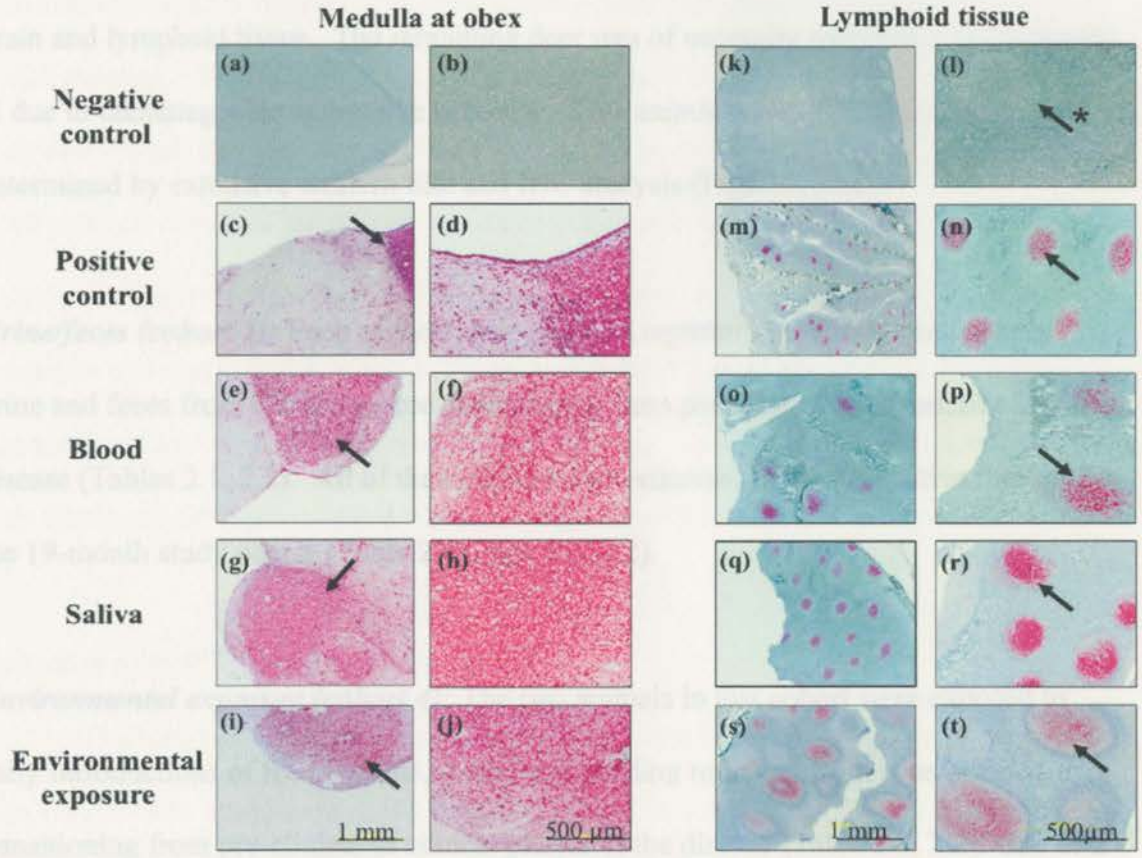
of three recipient deer became $PrP^{CWD}$ tonsil biopsy positive at 12 months pi, but not at earlier sampling intervals (Fig. 2.1). At 19 months pi, when the cohort was necropsied, all three deer were CWD+, as indicated by detection of $PrP^{CWD}$ in the medulla oblongata at the level of the obex (medulla at obex) and in lymphoid tissue (Figs. 2.2).

Figure 2.1. $PrP^{CWD}$ detection by longitudinal tonsil.




Te=tonsil, Bf=Brain at obex, RLNg=Retropharyngeal lymph node, X= animal euthanized for reason other than CWD.

Figure 2.2. Immunohistochemistry results of deer exposed to body secretions and excreta from CWD+ deer.




$PrP^{CWD}$ demonstrated by immunohistochemistry in tonsil, brain (medulla oblongata at obex), and retropharyngeal lymph node of deer receiving saliva, blood, or environmental exposure from CWD-infected donors. CWD immunohistochemistry is shown in the medulla at obex (a-j) and either tonsil or retropharyngeal lymph node (k-t). Arrows indicate $PrP^{CWD}$ staining (red) within brain and lymphoid follicles. Arrow with * indicates lymphoid follicle negative for $PrP^{CWD}$.

***Saliva (cohort 2)***: Each of the 3 deer in this cohort received saliva from pre-clinical CWD+ donors that were 6 to 13 months pi (Tables 2.1, 2.2). $PrP^{CWD}$ was detected in tonsil of 1 of the 3 inoculated deer at 12 months pi, but not at earlier time points. By 19 months pi, study termination, a second animal was CWD+, by detection of $PrP^{CWD}$ in brain and lymphoid tissue. The remaining deer was of necessity terminated at 16 months pi due to unmanageable aggressive behavior. This animal was CWD negative as determined by extensive western blot and IHC analysis (Figs. 2.1, 2.2).

***Urine/feces (cohort 3):*** Each of the 3 deer received repeated (90 daily) oral doses of urine and feces from CWD+ source deer ranging from pre-clinical to advanced clinical disease (Tables 2.1, 2.2). All of the recipient deer remained $PrP^{CWD}$-negative throughout the 19-month study course (Table 2.1, Figs. 2.1, 2.2).

***Environmental exposure (cohort 4)***: The two animals in this cohort were exposed to daily introductions of feed buckets, water, and bedding removed from pens housing deer transitioning from pre-clinical to clinical phases of the disease (Table 2.1, 2.2). One of 2 exposed deer became tonsil biopsy $PrP^{CWD}$-positive at 15 months pi. At study termination, 19 months pi, both animals were CWD+ (Figs. 2.1, 2.2).

***Positive controls (cohort 5)***: After oral inoculation with CWD+ brain homogenate, $PrP^{CWD}$ was detected in the tonsil of 4 of 4 inoculated deer at either 6 (n = 2), or 12 months (n = 2) pi (Figs. 2.1, 2.2 and Tables 2.1, 2.2).

***Clinical signs of CWD:***

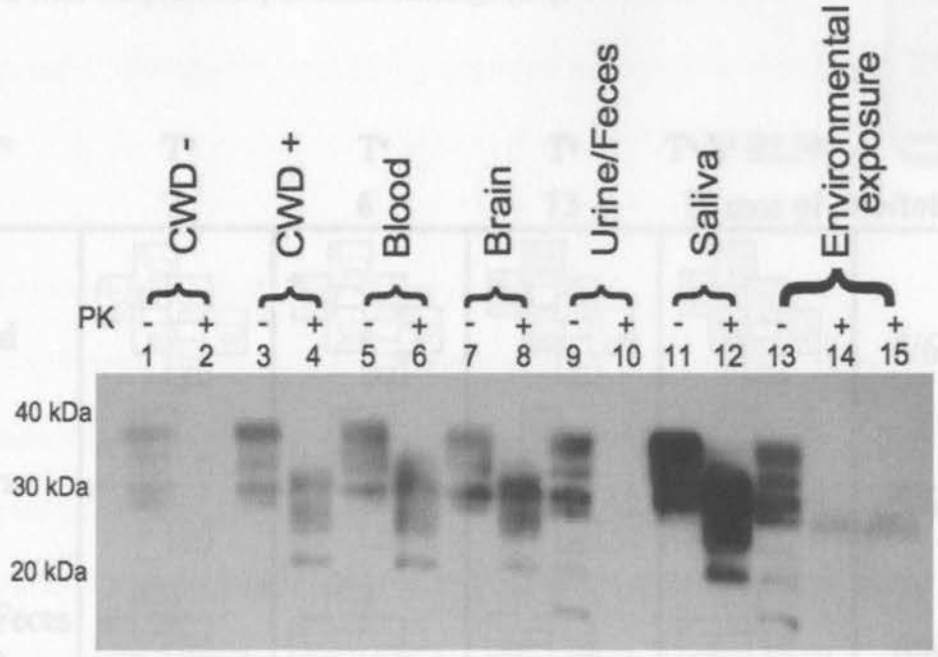
Project-dedicated caretakers intimately familiar with each animal observed the deer daily. Subtle clinical signs consistent with CWD were detected 12-20 months pi in 3 of 4 positive control animals (cohort 5), 2 of 3 animals receiving blood transfusion (cohort 1) and 1 of 3 deer orally inoculated with saliva (cohort 2). Clinical disease onset manifested primarily as perceived body muscle-mass reduction and gradual weight loss, which reached ≥20% of maximum body weight over 2 to 8 months. Other clinical signs included: rough hair coat due to piloerection and a body stance characterized by a low head position and wide leg stance. Changes in behavior included hyperphagia and polydipsia despite weight loss, and stereotypic movements including head tossing, repetitive and exaggerated lifting of the legs, diminished alertness, and occasionally aggressive behavior in the advanced stage of disease. Animals were euthanized when they displayed advanced clinical signs of CWD or at 19 months pi.

***Detection of $PrP^{CWD}$ in tissues at necropsy:***

Nineteen months after inoculation, all animals testing CWD- by tonsil biopsy in cohorts 1-4 were euthanized and necropsied. Two of 4 cohort 5 (positive control) deer and 2 of 3 cohort 1 (blood) tonsil-biopsy-positive deer were euthanized due to advanced clinical signs of CWD at 13, 24, 26 and 29 months pi, respectively. Earlier studies established that medulla at obex, tonsil and retropharyngeal lymph node had highest frequency of $PrP^{CWD}$ deposition in infected deer [36]. Therefore, these tissues were rigorously examined in all animals. The results of immunohistochemistry (IHC) and western blot (WB) assays for $PrP^{CWD}$ correlated with each other and with previous

positive tonsil-biopsy results in 10 of 13 animals (Fig. 2.1). In 3 animals, however, (one each in the blood, saliva and positive control inoculation cohorts) in which previous tonsil biopsies had been negative, $PrP^{CWD}$ was detected in tonsil, retropharyngeal lymphoid tissues and brain collected at necropsy (Fig. 2.1). Western blot analysis confirmed IHC results (Fig. 2.2) in all cases, and demonstrated the characteristic proteinase K-resistant 28-35kd bands typical of $PrP^{CWD}$ (Fig. 2.3).

Figure 2.3. Western blot results of deer exposed to body secretions and excreta from CWD+ deer.



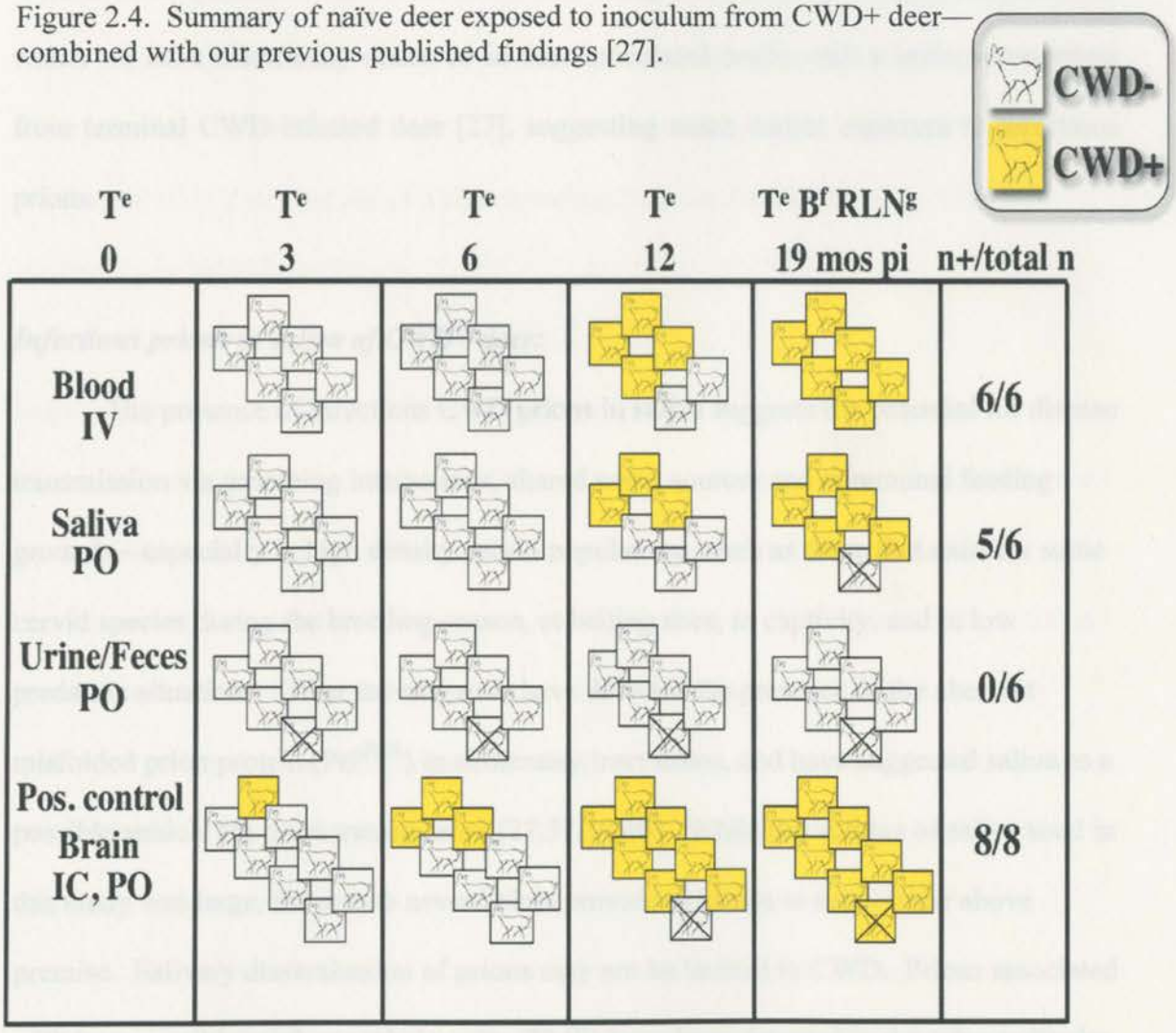

Western blot demonstration of the typical PK digestion band shift (28-35 kD) associated with prion infection in the brain (medulla oblongata at obex) of deer receiving blood, brain, saliva or environmental exposure, but not urine and feces from CWD-infected donors. Lanes 1-4 represent CWD+/- deer controls (10% brain homogenate of medulla at obex) without and with PK digestion at 25µg/ml. Lanes 5-12, 10% brain homogenate of blood, brain, urine/feces or saliva inoculated deer without and with PK digestion at 25µg/ml. Lanes 13-15, brain homogenate from deer environmentally exposed to CWD+ fomites without and with PK digestion at 25 and 50 µg/ml.

## DISCUSSION

***Time interval to detection of CWD infection by tonsil biopsy:***

The high transmission rate of CWD among cervids in their natural environment sets CWD apart from other prion diseases. The results of this study help provide a plausible basis for this facile transmission and extend our earlier findings [27] (Fig. 2.4) in demonstrating infectious prions in blood and saliva of pre-clinical CWD+ donors.

Figure 2.4. Summary of naïve deer exposed to inoculum from CWD+ deer—combined with our previous published findings [27].




[e]T=Tonsil, [f]B=Brain, [g]RLN=Retropharyngeal lymph node, X=animal euthanized for reasons other than CWD

The time from exposure to first detection of $PrP^{CWD}$ by tonsil biopsy was variable—as short as 6 months but as long as 18 months. We assume that the time until appearance of $PrP^{CWD}$ in tonsil is an underestimate due to the inherent variability in prion deposition kinetics [36] and the logistical limitations of tonsil biopsies, which require general anesthesia. The incubation periods prior to clinical CWD in our study were similar to those observed previously in experimental and naturally acquired infections [27,37,38]. While we can not exclude horizontal transmission from the first positive deer in each cohort, the timeframe for detection in the remaining deer (3 months) is less than half that which we have historically observed in deer inoculated orally with a brain homogenate from terminal CWD-infected deer [27], suggesting much earlier exposure to infectious prions.

***Infectious prions in saliva of CWD+ deer:***

The presence of infectious CWD prions in saliva suggests the potential for disease transmission via grooming interactions, shared water sources and communal feeding grounds—especially in high density cervid populations, such as those that exist for some cervid species during the breeding season, at baiting sites, in captivity, and in low predation situations. Other investigators have detected the presence of the aberrant misfolded prion protein ($PrP^{RES}$) in alimentary tract tissue, and have suggested saliva as a possible vehicle for prion transmission [27,37,39,40]. While the volume of saliva used in this study was large, the results nevertheless provide evidence to support the above premise. Salivary dissemination of prions may not be limited to CWD. Prions associated with transmissible mink encephalopathy (TME) have been detected in the submandibular

salivary gland of mink [41] and TME protease-resistant prion protein has been detected in the lamina propria of the oral cavity, taste buds and squamous epithelium of the tongue, and the vomeronasal organ and olfactory mucosa of infected hamsters [40]. Hamster-adapted scrapie agent has been found in the tongue and taste buds of prion-infected hamsters [42]. Vascellari *et al* reported the presence of the pathological prion protein in both major and minor salivary glands of naturally and experimentally infected sheep [43], and we have made similar observations in the olfactory mucosa of ferrets experimentally infected with CWD [44] and in the taste buds of deer (Haley, NJ, personal communication). The exact source of prions shed in saliva remains speculative; possible sources include centrifugal/retrograde passage from nerve fiber terminations in the oral-nasal mucosa, or from lymphoid cells emanating from infected tonsilar or other alimentary lymphoid tissues.

***Infectious prions in blood of CWD+ deer:***

Blood-borne transmission of TSEs has long been feared, and the identification of a prion pathogen associated with blood-borne transmission has been pursued with disparate results [33,45,46]. Here we report the induction of CWD infection by a single blood transfusion from each of two pre-clinical CWD+ blood donors. This result is consistent with previous findings in substantiating the transmission of infectious prions by the blood of asymptomatic animal [27,32] and human [28-30,47,48] donors, thus providing support for a subclinical hematogenous carrier state in TSE infections.

Direct detection of blood-borne $PrP^{RES}$ has been difficult. Saa *et al* were the first to use protein misfolding cyclic amplification assay (PMCA) [49,50] to detect protease-

resistant prion protein in the blood of asymptomatic scrapie-infected hamsters [51]. More recently, Thorne *et al* reported PMCA amplification of $PrP^{SC}$ from the blood of scrapie-infected sheep [52]. Continued efforts toward the development of sensitive, noninvasive, diagnostic tools are paramount. We are presently re-examining by serial PMCA the tissues of exposed but conventional $PrP^{CWD}$ test negative animals that may harbor infectious prions not manifested in the observation periods used in our CWD studies.

Hunter and colleagues [33,34] provided the first evidence for blood-borne TSE transmission for bovine spongiform encephalopathy (BSE) and scrapie by transfusion of whole blood [33,34] and buffy-coat white blood cells [34] from infected donor sheep to naïve sheep. Sparse but compelling evidence has accumulated for blood transmission of variant Creutzfeldt-Jakob Disease (vCJD) [28-30,48] and $PrP^{RES}$ has been found in peripheral organs of some sporadic CJD patients [53], raising the possibility that peripheral distribution of $PrP^{RES}$ is not limited to vCJD. In an ongoing study of sixty-six individuals who received blood products from asymptomatic blood donors who later developed vCJD [54], three of the 66 blood transfusion recipients developed vCJD 6.5 to 8.5 years after receiving blood [28,30,48] and a fourth blood recipient died of causes unrelated to vCJD five years after receiving the blood donation. Upon autopsy of this individual, $PrP^{RES}$ was detected in lymphoid tissue but not brain, thus providing presumptive evidence for a case of subclinical infection [29]. Our findings with CWD further support the tenet that blood products from subclinical prion-infected individuals may transmit disease.

Additional cases of subclinical human prion disease may exist. While *in vitro* conversion studies have indicated an inefficient conversion of human PrP into a protease-resistant form [26,55] and no evidence exists of CWD transmission to non-cervid species cohabitating with or on CWD contaminated environments [24,56-58], it is reasonable to surmise that cross-species transmission of prions may require extenuating circumstances, i.e. origin of specific strains [59,60], prolonged incubation time [61], and permissive genotypes [62]. At least two studies provide information bearing on these concerns. The first study, an ongoing longitudinal study to closely monitor 81 Americans who inadvertently consumed, or were exposed to, CWD+ venison at an upstate New York sportsman's feast, will conduct health evaluations of these individuals over the next six years [63]. The second, a retrospective study using western blot analysis of human tonsil and appendix samples collected in the United Kingdom (UK) to investigate possible exposure to the BSE agent, reported the detection of abnormal prion protein in three of 12,674 samples [64]. Mathematical modeling based on the results of this study predicts a minimum estimate of 3000 BSE infected people in the UK between 10-30 years of age. If this model is accurate, it predicts that 93% of these individuals could develop long-term subclinical infection [65].

***Environmental sources of CWD infection:***

Previous studies have confirmed direct animal-to-animal contact—horizontal transmission—as an efficient mode for prion disease transmission [9,66]. Moreover, Miller and colleagues [9,67,68] have provided substantial evidence for environmental contamination as a source of CWD infection. Our bioassay study inocula doses (50 ml

saliva/deer), while efficient in establishing the infectious nature of saliva, are likely unrealistic doses to be acquired in a natural setting. To emulate a more feasible natural environment-associated dose, while negating direct animal-to-animal contact, we exposed naïve deer to repeated exposures to fomites from the suites of CWD-infected deer. The study design was meant to mirror the daily habits and movements of a deer in its natural setting in which it may return to an area contaminated with small amounts of infectious prions over time. Here we provide the first report that under controlled indoor conditions CWD-naïve deer can acquire infection by exposure to fomites from the environment of CWD-infected deer, supporting the findings of Miller *et al* in the natural environment [9,67,68], in demonstrating that there are sufficient infectious prions in bedding and water to transmit CWD. Efficient transmission, as evidenced by tonsillar lymphoid $PrP^{CWD}$ detection, was seen in as little as 15 months post initial exposure. These results are also consistent with the findings of Georgsson [69] and Miller [67] as part of their attempts to decontaminate areas heavily contaminated with scrapie and CWD. Animals reintroduced to these areas after decontamination developed clinical signs of prion disease within two years. The presence of infectious CWD prions in the environment therefore strongly suggests that natural prion infection occurs by routes additional to direct animal-to-animal contact. Based on the present and our previous findings [27], we speculate that saliva may harbor the greatest concentration of CWD prions available for horizontal transmission and environmental contamination, but recognize that other routes of excretion at lower concentration and greater volume still remain plausible.

***Lack of detectable infectious prions in the urine and feces of CWD+ deer:***

Previous studies have postulated that environmental contamination by excreta from infected cervids seems the most plausible explanation for the dissemination of CWD [70], yet at 19 months pi we were not able to detect $PrP^{CWD}$ in the three deer inoculated with urine and feces. Our earlier report [27] indicated that 2 of 2 deer expressing the *prnp* gene G/S polymorphism at codon 96 remained negative 19 mo. pi. In the present study all three deer inoculated with urine and feces expressed the G/G polymorphism at *prnp* codon 96, which is associated with susceptibility to CWD infection [71]. We report no detection of $PrP^{CWD}$ in the obex or lymphoid tissues of deer with either G/G or G/S polymorphisms at 19 mo pi. Although both of our bioassay studies in deer have failed to transmit CWD infection by oral exposure to urine and feces from CWD-infected deer, these results must still be interpreted with caution in light of ongoing PMCA and cervid transgenic mouse intracerebral bioassay studies which suggest that very low concentrations of prions may be present in urine and feces of CWD+ cervids [72-74]. Perhaps an incubation time longer than 19 months is necessary for a detectable accumulation of lymphoid $PrP^{CWD}$, or a larger dose of inoculum by the oral route is necessary for efficient passage of prions across the alimentary mucosa.

In summary, the results reported here reconfirm that blood and saliva are sources of infectious CWD prions, consistent with previous findings [27], and further support a mechanism for efficient CWD transmission in nature. We also show that infectious prions shed into the environment by CWD+ deer are sufficient to transmit the disease to naïve deer in the absence of direct animal-to-animal contact. These observations

reinforce the exposure risk associated with body fluids, excreta, and all tissues from CWD+ cervids and suggest that similar dynamics may exist in other prion infections.

**Acknowledgements**

We thank Katrina Albarado, Danyahl Robison, Jennifer Finn and Heidi Kinsell for their dedication to the PRRL project, Nathaniel Denkers, Davis Seelig and Nicholas Haley for their necropsy expertise, and Katherine O'Rourke and Linda Hamburg for generously genotyping the deer.

## REFERENCES

1. **Williams ES, Young S.** 1980. Chronic wasting disease of captive mule deer: a spongiform encephalopathy. J Wildl Dis **16**:89-98.
2. **Williams ES, Young S.** 1992. Spongiform encephalopathies in Cervidae. Rev Sci Tech **11**:551-567.
3. **Spraker TR, Miller MW, Williams ES, Getzy DM, Adrian WJ, et al.** 1997. Spongiform encephalopathy in free-ranging mule deer (Odocoileus hemionus), white-tailed deer (Odocoileus virginianus) and Rocky Mountain elk (Cervus elaphus nelsoni) in northcentral Colorado. J Wildl Dis **33**:1-6.
4. **United States Geographical Survey NWHC.** 2009. Map of Chronic Wasting Disease in North America. <http://www.nwhc.usgs.gov/disease_information/chronic_wasting_disease/index.jsp>. Accessed
5. **Sohn HJ, Kim JH, Choi KS, Nah JJ, Joo YS, et al.** 2002. A case of chronic wasting disease in an elk imported to Korea from Canada. J Vet Med Sci **64**:855-858.
6. **Kim TY, Shon HJ, Joo YS, Mun UK, Kang KS, et al.** 2005. Additional cases of Chronic Wasting Disease in imported deer in Korea. J Vet Med Sci **67**:753-759.
7. **Miller MW, Wild MA, Williams ES.** 1998. Epidemiology of chronic wasting disease in captive Rocky Mountain elk. J Wildl Dis **34**:532-538.
8. **Miller MW, Williams ES, McCarty CW, Spraker TR, Kreeger TJ, et al.** 2000. Epizootiology of chronic wasting disease in free-ranging cervids in Colorado and Wyoming. J Wildl Dis **36**:676-690.

9. **Miller MW, Williams ES.** 2003. Prion disease: horizontal prion transmission in mule deer. Nature **425**:35-36.
10. **Williams ES, Miller MW.** 2002. Chronic wasting disease in deer and elk in North America. Rev Sci Tech **21**:305-316.
11. **Belay ED, Maddox RA, Williams ES, Miller MW, Gambetti P, et al.** 2004. Chronic wasting disease and potential transmission to humans. Emerg Infect Dis **10**:977-984.
12. **Joly DO, Samuel MD, Langenberg JA, Blanchong JA, Batha CA, et al.** 2006. Spatial epidemiology of chronic wasting disease in Wisconsin white-tailed deer. J Wildl Dis **42**:578-588.
13. **Wisconsin Department of Natural Resources.** 2008. A Plan for Managing Chronic Wasting Disease in Wisconsin: The Next 10 Years.
14. **Arkansas Game and Fish Commission A.** 2007. Chronic Wasting Disease Response Plan. In: Commission GaF, editor.
15. **New York State Department of Environmental Conservation NY**. 2009. Chronic Wasting Disease. In: Conservation E, editor.
16. **The Texas Animal Health Commission and The Texas Parks and Wildlife Department T.** 2003. Texas Chronic Wasting Disease Management Plan. In: Department TAHCaTPaW, editor.
17. **Pennsylvania Inter-agency CWD task force; Governor's Office PGC, the state Department of Agriculture, the US Department of Agriculture, the state Department of Health, the state Department of Environmental Protection and the Pennsylvania Emergency Management Agency.** 2007. Pennsylvania's Chronic Wasting Disease Response Plan.
18. **Michigan Department of Natural Resources- Wildlife Division MDoA-AID.** 2002. Michigan Surveillance and Response Plan for Chronic Wasting Disease of Free-Ranging and Privately-Owned/Captive Cervids. In: Michigan Department of Natural Resources MDoA, editor.
19. **Michigan Department of Natural Resources MDoA.** 2008. Chronic Wasting Disease in Michigan. In: Michigan Department of Natural Resources MDoA, editor.
20. **Ontario Ministry of Natural Resources O.** 2008. Ontario's CWD Surveillance and Response Plan. In: Resources N, editor.
21. **Will RG, Ironside JW, Zeidler M, Cousens SN, Estibeiro K, et al.** 1996. A new variant of Creutzfeldt-Jakob disease in the UK. Lancet **347**:921-925.
22. **Bruce ME, Will RG, Ironside JW, McConnell I, Drummond D, et al.** 1997. Transmissions to mice indicate that 'new variant' CJD is caused by the BSE agent. Nature **389**:498-501.
23. **Hill AF, Desbruslais M, Joiner S, Sidle KC, Gowland I, et al.** 1997. The same prion strain causes vCJD and BSE. Nature **389**:448-450, 526.
24. **Bosque PJ.** 2002. Bovine spongiform encephalopathy, chronic wasting disease, scrapie, and the threat to humans from prion disease epizootics. Curr Neurol Neurosci Rep **2**:488-495.
25. **Kong Q, Huang S, Zou W, Vanegas D, Wang M, et al.** 2005. Chronic wasting disease of elk: transmissibility to humans examined by transgenic mouse models. J Neurosci **25**:7944-7949.

26. **Raymond GJ, Bossers A, Raymond LD, O'Rourke KI, McHolland LE, et al.** 2000. Evidence of a molecular barrier limiting susceptibility of humans, cattle and sheep to chronic wasting disease. Embo J **19**:4425-4430.
27. **Mathiason CK, Powers JG, Dahmes SJ, Osborn DA, Miller KV, et al.** 2006. Infectious prions in the saliva and blood of deer with chronic wasting disease. Science **314**:133-136.
28. **Llewelyn CA, Hewitt PE, Knight RS, Amar K, Cousens S, et al.** 2004. Possible transmission of variant Creutzfeldt-Jakob disease by blood transfusion. Lancet **363**:417-421.
29. **Peden AH, Head MW, Ritchie DL, Bell JE, Ironside JW.** 2004. Preclinical vCJD after blood transfusion in a PRNP codon 129 heterozygous patient. Lancet **364**:527-529.
30. **Wroe SJ, Pal S, Siddique D, Hyare H, Macfarlane R, et al.** 2006. Clinical presentation and pre-mortem diagnosis of variant Creutzfeldt-Jakob disease associated with blood transfusion: a case report. Lancet **368**:2061-2067.
31. **Hunter N.** 2003. Scrapie and experimental BSE in sheep. Br Med Bull **66**:171-183.
32. **Houston F, McCutcheon S, Goldmann W, Chong A, Foster J, et al.** 2008. Prion diseases are efficiently transmitted by blood transfusion in sheep. Blood **112**:4739-4745.
33. **Houston F, Foster JD, Chong A, Hunter N, Bostock CJ.** 2000. Transmission of BSE by blood transfusion in sheep. Lancet **356**:999-1000.
34. **Hunter N, Foster J, Chong A, McCutcheon S, Parnham D, et al.** 2002. Transmission of prion diseases by blood transfusion. J Gen Virol **83**:2897-2905.
35. **Peden AH, Ritchie D, Ironside JW.** 2005. Risks of transmission of variant Creutzfeld-Jakob disease by blood transfusion. Folia Neuropathol **43**:271-278.
36. **Sigurdson CJ, Barillas-Mury C, Miller MW, Oesch B, van Keulen LJ, et al.** 2002. PrP(CWD) lymphoid cell targets in early and advanced chronic wasting disease of mule deer. J Gen Virol **83**:2617-2628.
37. **Miller MW, Williams ES.** 2004. Chronic wasting disease of cervids. Curr Top Microbiol Immunol **284**:193-214.
38. **Williams ES.** 2005. Chronic wasting disease. Vet Pathol 42:530-549.
39. **Hadlow WJ, Eklund CM, Kennedy RC, Jackson TA, Whitford HW, et al.** 1974. Course of experimental scrapie virus infection in the goat. J Infect Dis **129**:559-567.
40. **DeJoia C, Moreaux B, O'Connell K, Bessen RA.** 2006. Prion infection of oral and nasal mucosa. J Virol **80**:4546-4556.
41. **Hadlow WJ, Race RE, Kennedy RC.** 1987. Temporal distribution of transmissible mink encephalopathy virus in mink inoculated subcutaneously. J Virol **61**:3235-3240.
42. **Bartz JC, Dejoia C, Tucker T, Kincaid AE, Bessen RA.** 2005. Extraneural prion neuroinvasion without lymphoreticular system infection. J Virol **79**:11858-11863.
43. **Vascellari M, Nonno R, Mutinelli F, Bigolaro M, Di Bari MA, et al.** 2007. PrPSc in salivary glands of scrapie-affected sheep. J Virol **81**:4872-4876.

44. **Sigurdson CJ, Mathiason CK, Perrott MR, Eliason GA, Spraker TR, et al.** 2008. Experimental chronic wasting disease (CWD) in the ferret. J Comp Pathol **138**:189-196.
45. **Eklund CM, Kennedy RC, Hadlow WJ.** 1967. Pathogenesis of scrapie virus infection in the mouse. J Infect Dis **117**:15-22.
46. **Clarke MC, Haig DA.** 1976. The effect of homogenization on the level of scrapie activity detected in cells from spleens of scrapie affected mice. Res Vet Sci **20**:106-107.
47. **Cervenakova L, Yakovleva O, McKenzie C, Kolchinsky S, McShane L, et al.** 2003. Similar levels of infectivity in the blood of mice infected with human-derived vCJD and GSS strains of transmissible spongiform encephalopathy. Transfusion **43**:1687-1694.
48. **EuroSurveill.** 2007. Fourth case of transfusion-associated vCJD infection in the United Kingdom. Euro Surveill **12**:E070118 070114.
49. **Soto C, Saborio GP, Anderes L.** 2002. Cyclic amplification of protein misfolding: application to prion-related disorders and beyond. Trends Neurosci **25**:390-394.
50. **Saborio GP, Permanne B, Soto C.** 2001. Sensitive detection of pathological prion protein by cyclic amplification of protein misfolding. Nature **411**:810-813.
51. **Saa P, Castilla J, Soto C.** 2006. Ultra-efficient replication of infectious prions by automated protein misfolding cyclic amplification. J Biol Chem **281**:35245-35252.
52. **Thorne L, Terry LA.** 2008. In vitro amplification of PrPSc derived from the brain and blood of sheep infected with scrapie. J Gen Virol **89**:3177-3184.
53. **Glatzel M, Abela E, Maissen M, Aguzzi A.** 2003. Extraneural pathologic prion protein in sporadic Creutzfeldt-Jakob disease. N Engl J Med **349**:1812-1820.
54. **Hewitt P.** 2006. vCJD and blood transfusion in the United Kingdom. Transfus Clin Biol Nov;**13**:312-316.
55. **Raymond GJ, Hope J, Kocisko DA, Priola SA, Raymond LD, et al.** 1997. Molecular assessment of the potential transmissibilities of BSE and scrapie to humans. Nature **388**:285-288.
56. **Gould DH, Voss JL, Miller MW, Bachand AM, Cummings BA, et al.** 2003. Survey of cattle in northeast Colorado for evidence of chronic wasting disease: geographical and high-risk targeted sample. J Vet Diagn Invest **15**:274-277.
57. **Anderson CA, Bosque P, Filley CM, Arciniegas DB, Kleinschmidt-Demasters BK, et al.** 2007. Colorado surveillance program for chronic wasting disease transmission to humans: lessons from 2 highly suspicious but negative cases. Arch Neurol **64**:439-441.
58. **Mawhinney S, Pape WJ, Forster JE, Anderson CA, Bosque P, et al.** 2006). Human prion disease and relative risk associated with chronic wasting disease. Emerg Infect Dis **12**:1527-1535.
59. **Benestad SL, Sarradin P, Thu B, Schonheit J, Tranulis MA, et al.** 2003. Cases of scrapie with unusual features in Norway and designation of a new type, Nor98. Vet Rec **153**:202-208.
60. **Manuelidis L, Fritch W, Xi YG.** 1997. Evolution of a strain of CJD that induces BSE-like plaques. Science **277**:94-98.

61. **Manson JC, Cancellotti E, Hart P, Bishop MT, Barron RM.** 2006. The transmissible spongiform encephalopathies: emerging and declining epidemics. Biochem Soc Trans **34**:1155-1158.
62. **Mead S, Poulter M, Uphill J, Beck J, Whitfield J, et al.** 2009. Genetic risk factors for variant Creutzfeldt-Jakob disease: a genome-wide association study. Lancet Neurol **8**:57-66.
63. **Garruto RM, Reiber C, Alfonso MP, Gastrich H, Needham K, et al.** 2008. Risk behaviors in a rural community with a known point-source exposure to chronic wasting disease. Environ Health **7**:31.
64. **Hilton DA, Ghani AC, Conyers L, Edwards P, McCardle L, et al.** 2004. Prevalence of lymphoreticular prion protein accumulation in UK tissue samples. J Pathol **203**:733-739.
65. **Clarke P, Ghani AC.** 2005. Projections of the future course of the primary vCJD epidemic in the UK: inclusion of subclinical infection and the possibility of wider genetic susceptibility. J R Soc Interface **2**:19-31.
66. **Ryder S, Dexter G, Bellworthy S, Tongue S. 2004.** Demonstration of lateral transmission of scrapie between sheep kept under natural conditions using lymphoid tissue biopsy. Res Vet Sci **76**:211-217.
67. **Miller MW, Williams, E.S., Hobbs, NT, Wolfe, LL.** 2004. Environmental sources of prion transmission in mule deer. Emerg Infect Dis June;**10**:1003-1006.
68. **Dickinson AG, Young GB, Stamp JT, Renwick CC.** 1965. An analysis of natural scrapie in Suffolk sheep. Heredity **20**:485-503.
69. **Georgsson G, Sigurdarson S, Brown P.** 2006. Infectious agent of sheep scrapie may persist in the environment for at least 16 years. J Gen Virol **87**:3737-3740.
70. **Miller MW, Wild MA.** 2004. Epidemiology of chronic wasting disease in captive white-tailed and mule deer. J Wildl Dis **40**:320-327.
71. **O'Rourke K, Spraker, R., Hamburg, K., Besser, T., Brayton, K., Knowles, D.** 2004. Polymorphisms in the prion precursor functional gene but not the psuedogene are associated with susceptibility of chronic wasting disease in white-tailed deer. J Gen Virol May;**85**:1339-1346.
72. **Gonzalez-Romero D, Barria MA, Leon P, Morales R, Soto C.** 2008. Detection of infectious prions in urine. FEBS Lett **582**:3161-3166.
73. **Murayama Y, Yoshioka M, Okada H, Takata M, Yokoyama T, et al.** 2007. Urinary excretion and blood level of prions in scrapie-infected hamsters. J Gen Virol **88**:2890-2898.
74. **Safar JG, Lessard P, Tamguney G, Freyman Y, Deering C, et al.** 2008. Transmission and detection of prions in feces. J Infect Dis **198**:81-89.

## CHAPTER 3*

**B cells and platelets harbor prion infectivity in the blood of CWD–infected deer.**

***Previously published in part as:**

**Candace Mathiason, Jeanette Hayes-Klug, Jenny Powers, David A. Osborn, Sallie J. Dahmes, Karl V. Miller, Robert J. Warren, Gary L. Mason, Glenn C. Telling, Alan J. Young, and Edward A. Hoover.** B cells and platelets harbor prion infectivity in the blood of CWD–infected deer. 2010. Journal of Virology. March 10;ePub ahead of print.

## ABSTRACT

Substantial evidence for prion transmission via blood transfusion exists for many TSE diseases. Determining which cell phenotype(s) are responsible for trafficking infectivity has important implications in understanding dissemination of prions as well as their detection and elimination from blood products. We used bioassay studies in the native white-tailed deer and transgenic cervidized mice to determine: (a) if chronic wasting disease (CWD) blood infectivity is associated with the cellular vs. the cell-free/plasma fraction of blood, and (b) in particular if B cell (MAb2-104+), platelet (CD41/61+) or CD14+ monocyte blood cell phenotypes harbor infectious prions. All four

deer transfused with the blood mononuclear cell fraction from CWD+ donor deer became $PrP^{CWD}$- positive by 19 months post inoculation, whereas none of the deer (0/4) inoculated with the same source cell-free plasma developed prion infection. All deer (4/4) injected with B cells, and 3/4 deer receiving platelets from CWD+ donor deer became $PrP^{CWD}$-positive in as little as 6 months post inoculation, whereas none (0/4) of the deer receiving blood CD14+ monocytes developed evidence of CWD infection (immunohistochemistry and western blot analysis) after 19 months of observation. Results of the Tg(CerPrP) mouse bioassays mirrored those in the native cervid host. These results indicate that CWD blood infectivity is cell-associated and suggest a significant role for B cells and platelets in trafficking CWD infectivity *in vivo* and support earlier tissue-based studies associating putative follicular B cells with $PrP^{CWD}$. Localization of CWD infectivity with leukocyte subpopulations may aid in enhancing sensitivity of blood-based diagnostic assays for CWD and other TSEs.

## INTRODUCTION

Chronic wasting disease (CWD) is an infectious protein-misfolding disease, or transmissible spongiform encephalopathy (TSE), affecting cervids in North America (59, 76-79) and one Asian country (41, 68). CWD is unique among prion diseases in affecting free-ranging wildlife populations (deer, elk and moose). Early and subsequent observations made by Williams and Miller (58, 79) related CWD transmission to direct contact with clinically affected deer, as well as indirect contact with environments

previously populated by infected deer (57). Bioassay studies in white-tailed deer have demonstrated that body fluids and excreta (saliva, urine, feces and blood) contain infectious prions (53, 54). Both clinical and preclinical CWD-infected deer harbored sufficient infectious prions to produce CWD in naïve white-tailed deer following ingestion of saliva or transfusion of whole blood (53, 54).

The detection of blood-borne infectious prions has important implications in understanding the spread of prions among and within individuals as well as to eliminating prions from blood products (13, 15, 31, 45), given the evidence for CJD transmission via blood transfusion (16, 37, 47, 50, 62, 72, 73). Identifying the cell phenotype or cell-free protein fractions that harbor prion infectivity would contribute importantly to this understanding and to the development of blood-based assays to detect prion infection. We undertook the present studies to address these issues.

## MATERIALS AND METHODS

### *Bioassay studies in deer*

***White-tailed deer:*** White-tailed deer fawns were provided by the Warnell School of Forestry and Natural Resources, University of Georgia, Athens (UGA)—a region in which CWD has not been detected. The deer fawns were hand-raised and human- and indoor-adapted before overnight transport directly to the Colorado State University (CSU) CWD research indoor isolation facility without contact with the native Colorado

environment. The 4-month-old fawns were adapted to the facility housing conditions and diet for 2 months before study start.

***Genotyping:*** All white-tailed deer were genotyped to determine GG/GS (codon 96) status by the laboratory of Dr. Katherine O'Rourke, USDA-ARS, Pullman, WA. Deer were allocated into inoculation cohorts of n=4 without knowledge of G96 genotype.

***Biocontainment protocols:*** Protocols to preclude extraneous exposure and cross-contamination between cohorts of animals as previously described (53, 54) incorporated protective shower-in requirements, Tyvek™ clothing, masks, head covers, and footwear, while maintaining stringent husbandry. Tonsil biopsy and terminal sample collections were taken with animal-specific biopsy and sample collection instruments to minimize possibility of cross contamination. Bedding and liquid waste from each suite was either incinerated or collected in a dedicated outdoor underground holding tank and denatured by alkaline digestion.

***Deer inoculation cohorts*:** Groups of six-month-old fawns (usually a n=4/group)(Table 3.1) were housed in separate isolation suites throughout the study. Suite-dedicated protective clothing, utensils, and waste disposal were incorporated to exclude cross contamination by fomites, bedding, food, excretions, or contact. *Deer Cohorts 1-6* were inoculated by intravenous (IV) route with blood components from CWD-infected donor deer housed in the CSU CWD isolation facility as follow:

Table 3.1. White-tailed deer cohorts IV inoculated with blood components.

| Cohort # | No./cohort | Donor status | Inoculum source | Inoculum |
|---|---|---|---|---|
| 1 | 8 | CWD+ | Whole blood | 250 ml CWD+ blood |
| 2 | 4 | " | Blood mononuclear cells | $1x10^7$-$1.24x10^8$ WBC[a] plus platelets |
| 3 | 4 | " | Cell-free plasma | 140-150 ml recovered from 250ml blood |
| 4 | 4 | " | B cells | $1\text{-}5x10^6$ 2-104+ cells (98% purity) |
| 5 | 4 | " | Platelets | $6\text{-}35x10^9$ CD41/61+ cells (99% purity) |
| 6 | 4 | " | Monocytes | $4x10^5$ CD14+ cells (98% purity) |
| 7 | 2 | CWD- | Negative control | 250 ml CWD- blood |

[a] white blood cells.

*Cohort 1*: Whole blood (250ml); *Cohort 2*: blood mononuclear cell fraction (1 x $10^7$-1.24 x $10^8$ white blood cells plus platelets); *Cohort 3*: cell-free plasma fraction (140-150 ml) recovered from 250 ml citrated whole blood; *Cohort 4*: B cells (1-5 x $10^6$) separated by Dynatec™ magnetic bead separation (98% purity) using anti-sheep B cell monoclonal antibody (Mab) 2-104, which identifies peripheral blood B cells and may identify FDCs in lymphoid germinal centers (80); *Cohort 5*: CD41/61+ platelets (6-35 x $10^9$) magnetically separated to 99% purity using Mab CAPPA 2A (VMRD, Pullman, WA USA); *Cohort 6*: CD14+ cells (4 x $10^5$) magnetically separated to 98% purity using anti-sheep CD14 Mab clone VPM65 (Fitzgerald Industries Inc., Concord, MA USA); and *Cohort 7:* naïve white-tailed deer inoculated with blood from CWD-negative deer served as negative controls for the study and were housed in a separate suite at the same facility.

***Blood donor deer:*** Six experimentally inoculated CWD+ and 2 CWD negative (CWD-) deer housed at the CSU indoor research facility were recruited from previously described studies (54) for use as blood donors for these studies (Table 3.2).

Table 3.2. CWD+/CWD- blood cell component donor history.

| Recipient Cohort # | Donor CWD status | Number of donors | Donor source history | Donor Incubation time/Clinical status | Donor Codon 96 G/S |
|---|---|---|---|---|---|
| 1, 2, 3 | CWD + | n=2 | IC inoculated; brain homogenate from naturally infected CWD+ deer TS-989-09147 | 14 mo pi/ terminal clinical disease | G/S |
| 1, 4, 5, 6 | CWD + | n=2 'Blood pool' | IV blood transfusion; 250ml whole blood from inoculated CWD+ deer TS989-CSU112 | 24 mo pi/ late stage clinical disease | G/G |
| | | n=2 'Brain pool' | PO inoculated; (2 gr/day for 5 days) CWD+ brain from naturally infected CWD+ deer TS-989-09147 or WDNR (n=1/ea) | 23 mo pi/ late stage clinical disease | n=1 G/G n=1 G/S |
| 7 | CWD- | n=2 | IV infused 250ml CWD- whole blood from deer residing at the Warnell School of Forestry and Natural Resources, Athens, GA USA. | CWD- | G/G |

***Blood donors for Cohorts 1, 2 and 3:*** Two CWD-infected deer previously inoculated IC with 1gram (gr) whole brain homogenate collected from a naturally infected CWD+ deer (TS-989-09147) were the source animals.

***Blood donors for Cohorts 1, 4, 5 and 6:*** Four CWD-infected deer. Two donors (designated 'Brain pool') had been orally inoculated with 10gr (2gr/day for 5 days) brain from naturally infected field isolates (TS-989-09147 or WDNR). The remaining two

***Cohorts 1, 4, 5, and 6*:** Similarly, a portion (250ml) of the whole blood collected from the 'Blood pool' and 'Brain pool' donors (Table 3.2) was administered IV to n=2 recipient Cohort 1 deer/donor. (Table 3.1) (n=4 recipients). The remaining blood collected from the 'Blood pool' was pooled as was the remaining blood from the 'Brain pool' and each pool was further processed to harvest specific cell phenotypes by magnetic separation (as described below) that were then inoculated by IV infusion to Cohorts 4, 5 or 6 (Table 3.1).

***Deer monitoring and sample collection:*** All animals were monitored for evidence of CWD infection by serial tonsil biopsies taken at 3, 6, 12, 15 months post inoculation (pi), and at study termination (19 months pi). Tonsil tissue was divided and equal portions either stored at -70C or fixed in 10% formalin for 24 hours before processing for immunohistochemistry (IHC). At the same sampling intervals, blood, saliva, feces, and urine were collected from each animal and stored at -70C. At necropsy, palatine tonsils, brainstem (medulla at the obex) and retropharyngeal lymph nodes, as well as other tissues, were collected for examination by IHC and western blotting (WB) to identify the presence of the protease-resistant prion protein associated with CWD ($PrP^{CWD}$).

***Cervid PrP transgenic mouse bioassay studies***

***Cervid PrP transgenic mice:*** Tg(CerPrP-E226)5037$^{+/-}$ mice (2), which express the elk PrP coding sequence, were generated in the Telling laboratory at the University of Kentucky. Mice were inoculated and maintained in accord with Colorado State University IACUC guidelines.

***Genotyping:*** All mice were screened at weaning for the presence of the cervid/elk *Prnp* transgene by both conventional and real-time PCR. All inoculated mice that tested negative for cervid $PrP^{RES}$ at the completion of bioassay studies were rescreened to confirm the presence of cervid *Prnp* transgene.

***Biocontainment protocols:*** The protocols for white-tailed deer above also applied to cohorts of mice housed in filter-top isolation cages.

***Mouse inoculation cohorts*:** Groups of n= 5 to 9 weanling mice (Table 3.3) were housed in separate cages throughout the study. Suite-dedicated protective clothing, utensils, and waste disposal were incorporated to exclude cross contamination by fomites, bedding, food, excretions, or contact. *Cohorts 8-9*: Naïve Tg(CerPrP-E226)$5037^{+/-}$ mice served as IC brain inoculate positive or negative controls each receiving 30µl of a 1% brain homogenate prepared in PBS of either CWD+ deer D10 or CWD negative deer UGA. *Cohorts 10-25*: Naïve Tg(CerPrP-E226)$5037^{+/-}$ mice were inoculated by intracranial (IC), intravenous (IV), intraperitoneal (IP) or per os (PO) route using the same CWD+ blood components described for white-tailed deer inoculations above. *Cohorts 10-13*: Whole blood, *Cohorts 14-17*: blood mononuclear cell fraction ($10^6$ white blood cells plus platelets), *Cohorts 18-21*: cell-free plasma fraction, *Cohort 22-23:* B cells ($10^6$) harvested from retropharyngeal lymph node or spleen, *Cohort 24*: CD41/61+ cells ($10^9$) plus platelets, *Cohort 25*: CD14+ cells ($10^5$), *Cohorts 26-41*: Cohorts of n=5-9 naïve Tg(CerPrP-E226)$5037^{+/-}$ mice served as the negative controls for this study and were IC,

donors (designated 'Blood pool') had received 250 ml of blood via IV infusion from an experimentally inoculated CWD+ deer (TS989-CSU112). Two deer from each Cohort (1, 4, 5 and 6) were inoculated with whole blood or specific cell phenotypes from the 'Brain pool' donors while the other two deer from each cohort were inoculated with similar components from the 'Blood pool' donors.

***Blood donors for Cohort 7:*** One CWD-negative deer housed at the Warnell School of Forestry and Natural Resources, University of Georgia, Athens (UGA)—a region where CWD has not been detected served as donor for n=2 negative control donors that each received 250 ml whole blood via IV infusion.

Thus the inocula used reflected both a conscious attempt to assess the universality of the results obtained given the constraints of a limited number of recipient animals and limited amounts of inoculum materials (cell fractions etc.) available.

***Blood collections, harvests, and inoculations:***

***Cohorts 1-3*:** One liter sodium citrate treated whole blood was collected from each of n=2 CWD+ donor deer (Table 3.2) for Cohort 1-3 inoculations (Table 3.1). The blood was not pooled. Half (500ml) of each whole blood collection was immediately administered IV to n=2 recipient Cohort 1 deer (250 ml/deer)/donor deer (n=4 recipients). Plasma and blood mononuclear cells harvested from 250ml non-pooled aliquots of whole blood were administered IV to Cohorts 2 and 3 (Table 3.1).

IV, IP or PO inoculated with blood components from the same negative control white tailed deer donors (Table 3.2) as per the negative control white-tailed deer inoculations (Table 3.1).

Table 3.3. Tg(CerPrP) mouse cohorts inoculated with blood components from CWD+ donor deer.

| Cohort # | No./cohort | Donor status | Inoculum | Volume/concentration/ # of cells | Route of Inoculation |
|---|---|---|---|---|---|
| 8 | 10 | CWD+[a] | Brain control | 30µl 1% | IC |
| 9 | 10 | CWD-[b] | " | " | IC |
| 10 | 9 | CWD+ | Whole blood | 30µl | IC |
| 11 | 9 | " | " | 100µl | IV |
| 12 | 9 | " | " | 150µl | IP |
| 13 | 9 | " | " | 50µl/day x 3 days | PO |
| 14 | 9 | " | BMC[c] | $10^6$ + platelets[d] | IC |
| 15 | 9 | " | " | $10^6$ + platelets | IV |
| 16 | 9 | " | " | $10^6$ + platelets | IP |
| 17 | 9 | " | " | $10^6$ + platelets | PO |
| 18 | 9 | " | Cell-free plasma | 30µl | IC |
| 19 | 9 | " | " | 100µl | IV |
| 20 | 9 | " | " | 150µl | IP |
| 21 | 9 | " | " | 50µl x 3 days | PO |
| 22 | 9 | " | B cells (RLN) | $10^6$ | IC |
| 23 | 5 | " | B cells (Splenic) | $10^6$ | IC |
| 24 | 5 | " | Platelets | $10^9$ | IC |
| 25 | 5 | " | Monocytes | $2x10^5$ | IC |
| 26 | 5 or 9 | CWD- | Neg. controls | One group each as per animal cohorts 10-25 using CWD- inoculum | IC or IV or IP or PO |

[a] CWD positive. [b] CWD negative. [c] Blood Mononuclear Cells. [d] $10^6$ BMC plus platelets.

***Mouse monitoring and sample collection:*** All mice were monitored daily for evidence of CWD clinical disease. Upon detection of clinical disease mice were euthanized and necropsied. Brain tissue was collected, divided in equal portions and either stored at -70C for western blotting (WB) or fixed in 10% formalin for 24 hours before processing

for IHC to identify the presence of the protease-resistant prion protein associated with CWD ($PrP^{CWD}$).

***Blood cell and plasma harvests (white-tailed deer and Tg(CerPrP-E226)5037$^{+/-}$ mouse inocula):*** Total blood cell populations were collected from 250 ml sodium citrate treated whole blood by centrifugation at 1200rpm for 15 minutes at 4C. The plasma fraction was collected and set aside on ice after an additional centrifugation at 3000rpm for 15 minutes at 4C to remove any residual cellular material. The cell fraction from this initial centrifugation was diluted 1:1 in 1x phosphate buffered saline (PBS) (Gibco, Inc) and layered over Histopaque 1088 (Sigma) at a 1:1 ratio. These Histopaque™ gradients were centrifuged without brake at 2500rpm for 30 minutes at room temperature. The discrete bands of white blood cells (wbc) were collected, diluted in equal volume 1x PBS, and further centrifuged for 10 minutes at 2500 rpm at 4C (washed). The cell pellets were washed in wash buffer (1x PBS/0.2% fetal bovine serum (FBS)/2mM EDTA) three times. Platelets were collected from the plasma fraction by centrifugation at 3000 rpm for 15 minutes. Cells and plasma recovered from Histopaque-1088 gradient separations and plasma centrifugations were either directly inoculated into deer and mouse bioassay studies, or were further processed to separate cell phenotypes.

***Retropharyngeal lymph node and spleen cell harvests (Tg(CerPrP-E226)5037$^{+/-}$* mouse inocula):** Retropharyngeal and spleen tissue was pressed through a fine wire mesh (0.45μm) and wbcs were collected as per above histopaque gradient protocol.

*Cell phenotype labeling and flow cytometry:*

Cell phenotype monoclonal antibodies were used to recover and determine the purity of 2-104+B cells, CD14+ monocytes and CD41/61+ platelets from the blood donor sources described above. Leukocyte and platelet blood cells were collected by centrifugation and Histopaque-1088 separation as described above and were then labeled with one of three (3) antibodies (Table 3.4); either anti-sheep pan B cell Mab 2-104 (equivalent to Mab 2-8 as described by Young et al) (80) (cell supernatant used neat), anti-sheep CD14 Mab clone VPM65 (cell supernatant used neat) (Fitzgerald Industries Inc., Concord, MA USA) or anti-sheep CD41/61 Mab CAPPA 2A (1:100 dilution of a 1mg/ml stock) (VMRD, Pullman, WA USA).

Table 3.4. Monoclonal antibodies used for cell specific phenotype harvests

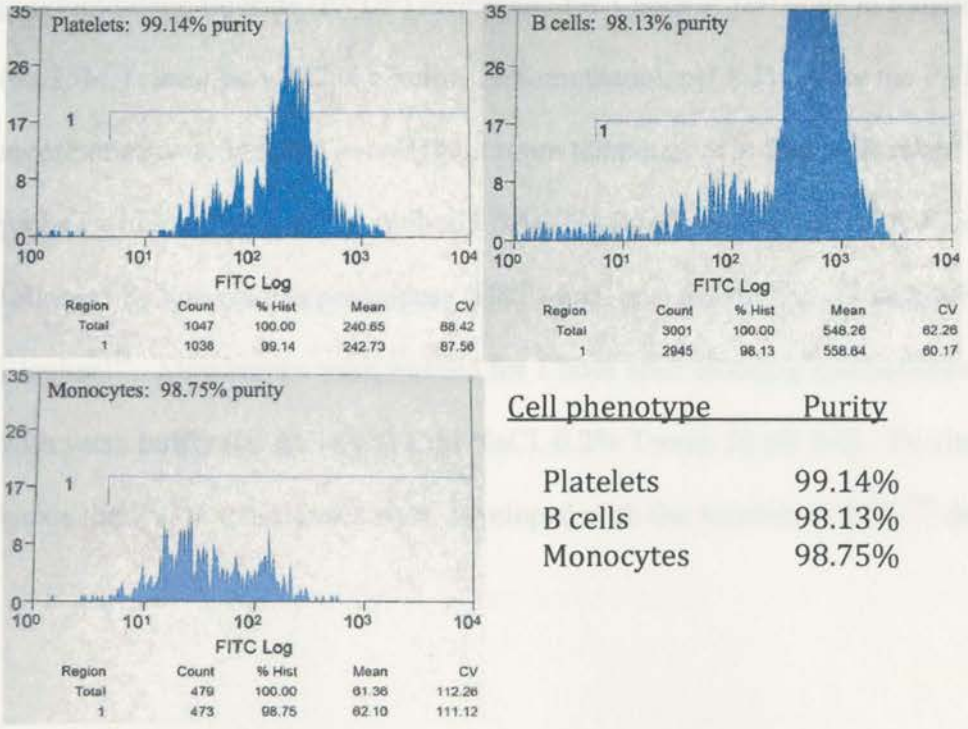
| Name | Target | Source |
|---|---|---|
| 2-104[a] | CD72 B cells, lymphoid germinal center FDCs | Alan Young, SDSU[b], Brookings, SD USA |
| VPM65 | CD14 monocytes/macrophages | Fitzgerald Ind., Concord, MA USA |
| CAPPA 2A | CD41/61 platelets | VMRD[c], Pullman, WA USA |

[a] MAb 2-104 is equivalent to MAb 2-8[81].
[b] South Dakota State University
[c] Veterinary Diagnostics, Monoclonal Antibodies

Cell aliquots were incubated with primary antibody for 20 minutes on ice followed by 3 washes in wash buffer. Secondary antibody, goat anti-mouse IgG or IgM FITC, was diluted 1:100 in 1x PBS/0.2% FBS and placed on the cells for 20 minutes on ice. Cells were again washed 3 times in wash buffer. The cells were then labeled with anti-FITC beads at 10µl beads/$10^7$ cells, again incubated on ice for 20 minutes, and passed over LS or LD Dynatec™ magnetic bead separation columns (as per manufacturer instructions). Cell populations of interest were eluted in 1x PBS containing 0.1% FBS.

Eluted (FITC-labeled) cells were analyzed by flow cytometry (Dako:Becton Dickinson). To determine purity (Fig. 3.1), the cells were gated by forward and side scatter (FSC/SSC) to include primarily lymphocytes, which were counted and volumes adjusted to equal to or greater than the total number of each cell specific phenotype populating 1 x $10^7$ peripheral blood mononuclear cells (PBMC) (as determined by prior flow cytometric analysis of specific cell phenotype populations in white-tailed deer, i.e. 2-104+B cell populations = ~10% and CD14+ cells = ~ 2% of the total leukocyte population). This was done to equate the total number of phenotype specific cells (2-104+, CD14+) to that found in the total blood mononuclear cell fraction inoculum (Cohort 2) that established infection ($10^7$ blood mononuclear cells) (see Tables 3.1, 3.5). The cells were either directly inoculated by IV inoculation into deer bioassay or frozen for future IC mouse bioassay.

Fig. 3.1. FACS analysis to verify purity of B cell, platelet and monocyte cell phenotype populations post magnetic bead positive selection fractionation.




| Cell phenotype | Purity |
|---|---|
| Platelets | 99.14% |
| B cells | 98.13% |
| Monocytes | 98.75% |

***Western blotting:*** Tissue homogenates were prepared from the obex region of the medulla oblongata encompassing the dorsal motor vagal nucleus (medulla at the obex). Ten percent (10%) w/v homogenates were prepared in NP-40 buffer (10mM Tris-HCl buffer pH 7.5, 0.5% NP-40, 0.5% sodium deoxycholate) by Fastprep™ disruption at setting 6.5 for 45 seconds. Twenty-five µl of each homogenate was mixed with 5µl proteinase K (PK) (Invitrogen) to a final concentration of 20 µg/ml and incubated for 30 minutes at 37C with shaking. Proteinase K activity was stopped with 4µl 200mM Pefablock SC™ and an equivalent volume of each sample was mixed with 10 µl sample buffer (Invitrogen-20% Reducing agent 10x, 50% LDS Sample buffer 4x), 5µl NP-40 buffer (10mM Tris-HCl pH 7.5, 0.5% deoxycholic acid, 0.5% nonylphenoxylpolyethoxylethanol), heated to 95C for 5 minutes and separated by 12% Bis-Tris precast polyacrylamide gel electrophoresis (PAGE) (Invitrogen) at 150 volts for 2.5 hours in 1x MOPS™ (Invitrogen). PAGE separated proteins were transferred to polyvinylidene fluoride (PVDF) membrane for 1 hour at 100 volts in transfer buffer (0.025M Trizma base, 0.2M glycine, 20% methanol, pH 8.3). After the PVDF membranes were blocked overnight at room temperature in Pierce Blocker™ they were probed with the PrP specific antibody BAR224 (kindly supplied by Dr. J. Grassi) followed by horseradish peroxidase (HRP)-goat anti-mouse IgG diluted in Pierce Blocker™. Membranes were washed for 1 hour after blocking and between antibodies with wash buffer (0.1M Tris, 0.15M NaCl, 0.2% Tween 20 pH 8.0). To visualize PrP bands the PVDF membranes were developed with the Amersham ECL™ detection

system and a digital GelDoc™ (Fuji Intelligent dark box) using LAS-3000 Lite ImageReader software.

***Immunohistochemistry (IHC)*:** Immunohistochemistry was performed by employing protocols as described by Spraker *et al (69)*. Briefly, 3-5 mm sections of formalin fixed formic acid treated tissues were deparafinized at 60-70°C for 1 hour, rehydrated via a series of xylene/ethanol baths, and treated in formic acid a second time (5 minutes) prior to a 20 minute antigen retrieval (Dako Target Retrieval Solution 10x) cycle in a 2100 Retriever™ (PickCell Laboratories). Slides were further processed with the aid of a Ventana Discovery™ autostainer utilizing the Ventana Red Map™ stain kit, the $PrP^{CWD}$ specific primary antibody BAR224 and a biotinylated secondary goat anti mouse antibody (Ventana). After autostaining, the slides were quickly rinsed in a warm water detergent solution, passed through a series of dehydration baths and cover-slipped.

## RESULTS

***White-tailed deer bioassays (Tables 3.5, 3.7, Figs. 3.2, 3.3):***

***Cell vs non-cell associated inoculates; Cohorts 2-3:*** Four of 4 deer (Cohort 2) inoculated with blood mononuclear cells (leukocytes plus platelets) from 250ml CWD+ deer blood ($1 \times 10^7$-$1.24 \times 10^8$), became tonsil biopsy $PrP^{CWD}$ positive [by immunohistochemistry (IHC) and western blot (WB)] between 6 and 19 months pi (mo pi) (Table 3.5). By contrast, $PrP^{CWD}$ was not detected in any tissue (tonsil, retropharyngeal lymph node or

medulla at obex) of any of n=4 deer (Cohort 3) that received cell-free plasma portion (140-150 ml plasma) from this same 250ml of CWD+ blood.

Table 3.5. Bioassay results from naïve deer cohorts inoculated with CWD+ blood components.

| Deer Cohort # | Inoculum | Tonsil $PrP^{CWD}$ result at the listed month post inoculation (n+/total n) | | | Terminal necropsy $PrP^{CWD}$ result[a] (n+/total n) 19 mo pi | % CWD+ |
|---|---|---|---|---|---|---|
| | | 3 | 6 | 12 | | |
| 1 | Whole blood | 0/8 | 4/8 | 8/8 | 8/8 | 100 |
| 2 | Blood mononuclear cells | 0/4 | 1/4 | 2/4 | 4/4 | 100 |
| 3 | Cell-free plasma | 0/4 | 0/4 | 0/4 | 0/4 | 0 |
| 4 | B cells 2-104+ | 0/4 | 1/4 | 2/4 | 4/4 | 100 |
| 5 | Platelets CD41/61+ | 0/4 | 0/4 | 1/4 | 3/4 | 75 |
| 6 | Monocytes CD14+ | 0/4 | 0/4 | 0/4 | 0/4 | 0 |
| 7 | CWD- whole blood | 0/2 | 0/2 | 0/2 | 0/2 | 0 |

[a]Retropharyngeal lymph node, tonsil and medulla oblongata at obex.

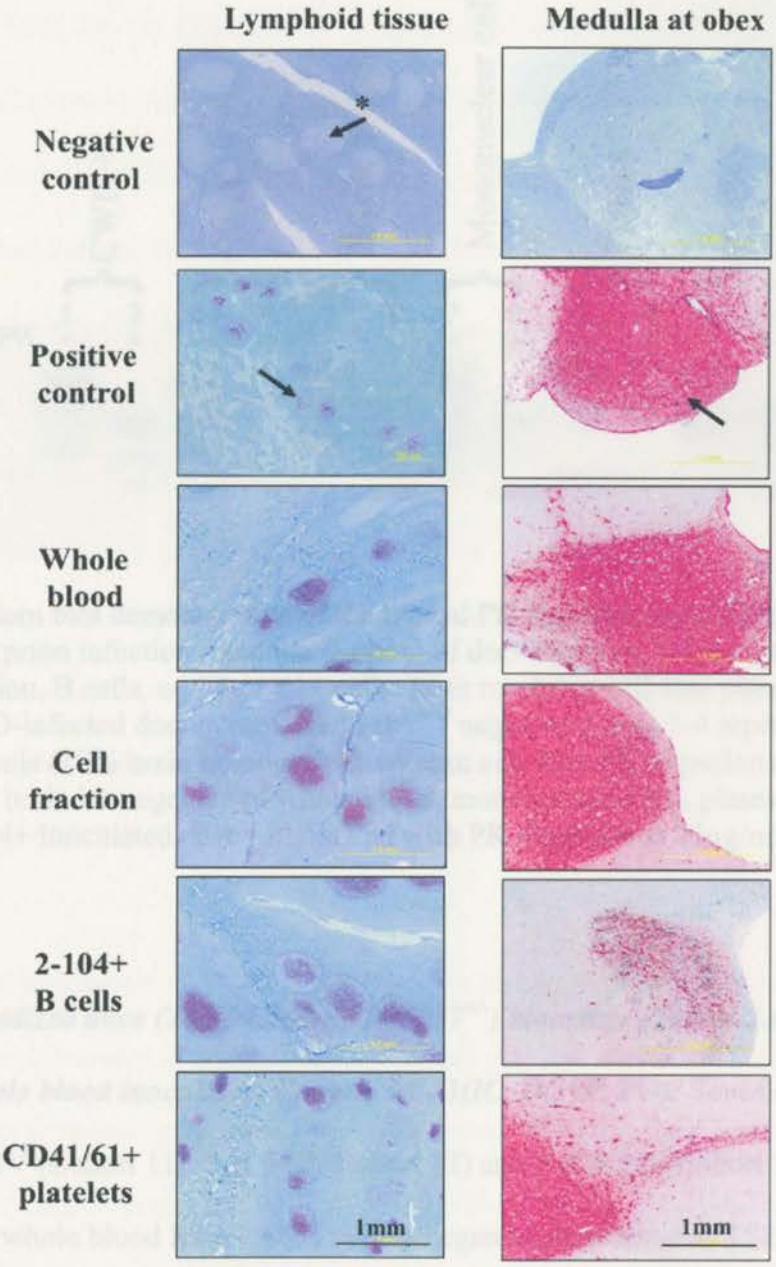
***Specific blood cell phenotype (2-104+B, CD14+, CD41/61+) inoculates; Cohorts 4-6:*** Four of 4 deer (Cohort 4) inoculated with 2-104+B cells (1-5 x $10^6$) and 3 of 4 deer (Cohort 5) receiving CD41/61+ platelets (6-35 x $10^9$) became tonsil biopsy $PrP^{CWD}$ + between 12 and 19 mo pi. All four deer (Cohort 6) inoculated with CD14+ monocytes (4 x $10^5$) remained CWD negative through the observation period of 19 months.

***Whole blood control deer inoculates; Cohorts 1 and 7:*** $PrP^{CWD}$ was detected in tonsil biopsies of all 8 deer (8/8) inoculated with 250ml whole blood from CWD+ deer (Cohort 1) between 6 and 12 mo p.i.. All deer began to show signs of TSE disease between 15-26

mo pi including wasting, hyperphagia, polydipsia, lowered head with wide leg stance and lethargy. Both negative control deer (Cohort 7) remained CWD negative as determined by IHC and WB analysis.

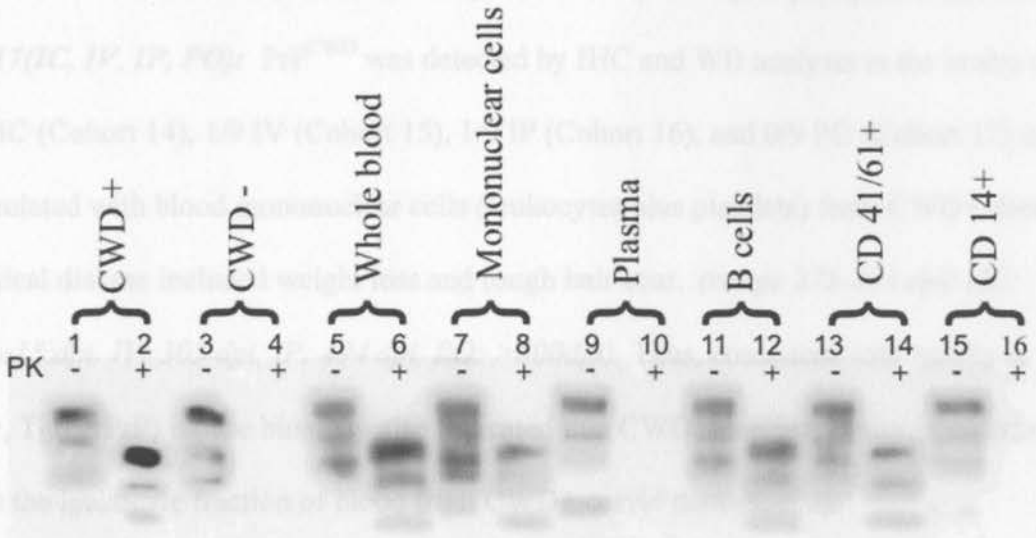
***White-tailed deer immunohistochemistry and western blot:*** Immunohistochemical analysis (Fig. 3.2) of terminal lymphoid tissue and medulla at obex from deer inoculated with either whole blood, blood mononuclear cells (leukocytes plus platelets), 2-104+B cells or CD41/61+ platelets demonstrated punctate $PrP^{CWD}$ staining within lymphoid follicles and brain tissue typical of that found associated with CWD-infected cervid controls. The Cohort 4 (2-104+B cell) deer brain IHC shown in Fig. 3.2 had overall less demonstrable $PrP^{CWD}$ in the medulla than the other CWD+ deer cohorts. Confirmatory western blot analysis (Fig. 3.3) of brain tissue (medulla at obex) showing the presence of PK resistant bands in lanes 6, 8, 12 and 14, corroborates CWD infection. Similar tissues from deer inoculated with cell-free plasma and CD14+ monocytes did not reveal $PrP^{CWD}$ staining by either conventional test (IHC/WB) used to verify CWD infection.

Fig. 3.2 Terminal lymphoid and brain (medulla at obex) immunohistochemistry results of naïve deer cohorts inoculated with CWD+ blood components.




$PrP^{CWD}$ demonstrated by ihc in tonsil, brain and, retropharyngeal lymph node of deer receiving whole blood, cell fraction, B cells or CD41/61+ cells from CWD-infected donors. Arrows indicate $PrP^{CWD}$ staining (red) within brain and lymphoid follicles. Arrow with * indicates lymphoid follicle negative for $PrP^{CWD}$

Fig. 3.3 Western blot analysis of naïve deer cohorts inoculated with CWD+ blood components.




Western blot demonstration of the typical PK digestion band shift (28-35 kD) associated with prion infection (medulla at obex) of deer receiving whole blood, mononuclear cell fraction, B cells, or CD41/61+ cells Deer receiving cell-free plasma or CD14+ cells from CWD-infected donors remained $PrP^{CWD}$ negative. Lanes 1-4 represent CWD+/- deer controls (10% brain homogenate) without and with PK digestion at 25µg/ml. Lanes 5-16, 10% brain homogenate of whole blood, mononuclear cells, plasma, B cell, CD41/61+, CD14+ inoculated deer without and with PK digestion at 25µg/ml.

***Cervidized mice (Tg(CerPrP-E226)5037$^{+/-}$) bioassays (Tables 3.6, 3.7, Figs. 3.4, 3.5):***

***Whole blood inoculates; Cohorts 10-13(IC, IV, IP, PO):*** Seven of 9 IC (Cohort 10), 1 of 9 IV (Cohort 11), 5 of 9 IP (Cohort 12) and 2 of 9 PO (Cohort 13) mice inoculated with whole blood from a CWD+ deer began to show signs of TSE clinical disease including weight loss, circling, rigid tail, hyperactivity or inactivity 270-490 days post infection (dpi). Upon termination (2-4 wks post initial clinical signs), $PrP^{CWD}$ was detected in the brain of all mice exhibiting clinical disease by IHC and WB analysis.

*(range 270-490 dpi) (IC: 275±5 dpi, IV: 312 dpi, IP: 340±10 dpi, PO: 482±8 dpi).*

***Blood mononuclear cell (cell-associated) inoculates (leukocytes plus platelets); Cohorts 14-17(IC, IV, IP, PO):*** $PrP^{CWD}$ was detected by IHC and WB analysis in the brains of 6/9 IC (Cohort 14), 1/9 IV (Cohort 15), 1/9 IP (Cohort 16), and 0/9 PO (Cohort 17) mice inoculated with blood mononuclear cells (leukocytes plus platelets) from CWD+ deer. Clinical disease included weight loss and rough hair coat. *(range 275-494 dpi) (IC: 290±15 dpi, IV: 303 dpi, IP: 494 dpi, PO: >600dpi).* Thus, consistent with results in deer, Tg(cerPrP) mouse bioassay also indicated that CWD prion infectivity is associated with the leukocyte fraction of blood from CWD+ cervid donors.

***Non-cell associated (cell-free) plasma inoculates; Cohorts 18-21 (IC, IV, IP, PO):*** As with bioassay results in deer, $PrP^{CWD}$ was not detected in the brains of mice inoculated with cell-free plasma from CWD+ deer, which were monitored for their natural life span. *(range 623-862 dpi).*

***B cell inoculates; Cohorts 22-23 (IC):*** In that harvest of sufficient B cells from blood to permit bioassays in deer and mice was not possible, 2-104+B cells harvested from retropharyngeal lymph node and spleen were analyzed. Five of 5 mice IC inoculated with retropharyngeal lymph node 2-104+B cells from a terminal CWD+ deer (Cohort 22) began to show clinical TSE disease including hyperactivity and circling 282±7 dpi. One of 5 mice IC inoculated with 2-104+B cells from the spleen of this same CWD+ deer (Cohort 23) developed with signs of TSE disease at 180 dpi (weight loss, rough hair coat,

rigid tail). All 6 mice demonstrating TSE clinical disease were $PrP^{CWD}$ positive by IHC and WB analysis of brain tissue. *(range 275-289 dpi).*

***Platelet inoculates; Cohort 24 (IC):*** Three of 5 mice IC inoculated with CD41/61+ platelets were $PrP^{CWD}$ positive by IHC and WB analysis of brain tissue at 305±10 dpi. All three of these mice exhibited TSE signs of wasting and rough hair coats. *(range 295-315 dpi).*

***Monocyte blood cell inoculates; Cohort 25 (IC):*** Neither TSE clinical disease nor $PrP^{CWD}$ could be detected in the 5 mice (0/5) IC inoculated with CD14+ blood cells upon termination at 600-862 dpi. *(range 600-862 dpi),* thus paralleling results in deer bioassay.

***Blood component negative control inoculates; Cohorts 26 -41:*** All mice inoculated with cell components or cell-free plasma from CWD- white-tailed deer donors remained free of TSE clinical disease for up to 862 dpi and were $PrP^{CWD}$ negative upon analysis of brain tissue by IHC and WB.

***Positive and negative control brain inoculates; Cohorts 8 and 9:*** Clinical disease progression (wasting, circling, inability to right self, hyperactivity or inactivity) and $PrP^{CWD}$ was detected by IHC and WB in the brain of 10/10 IC CWD+ brain homogenate inoculated mice at 168 dpi ± 4 days, while 0/10 negative control mice were $PrP^{CWD}$+ (>600 dpi).

Table 3.6. Bioassay of blood components from CWD+ deer in Tg(CerPrP) mice.

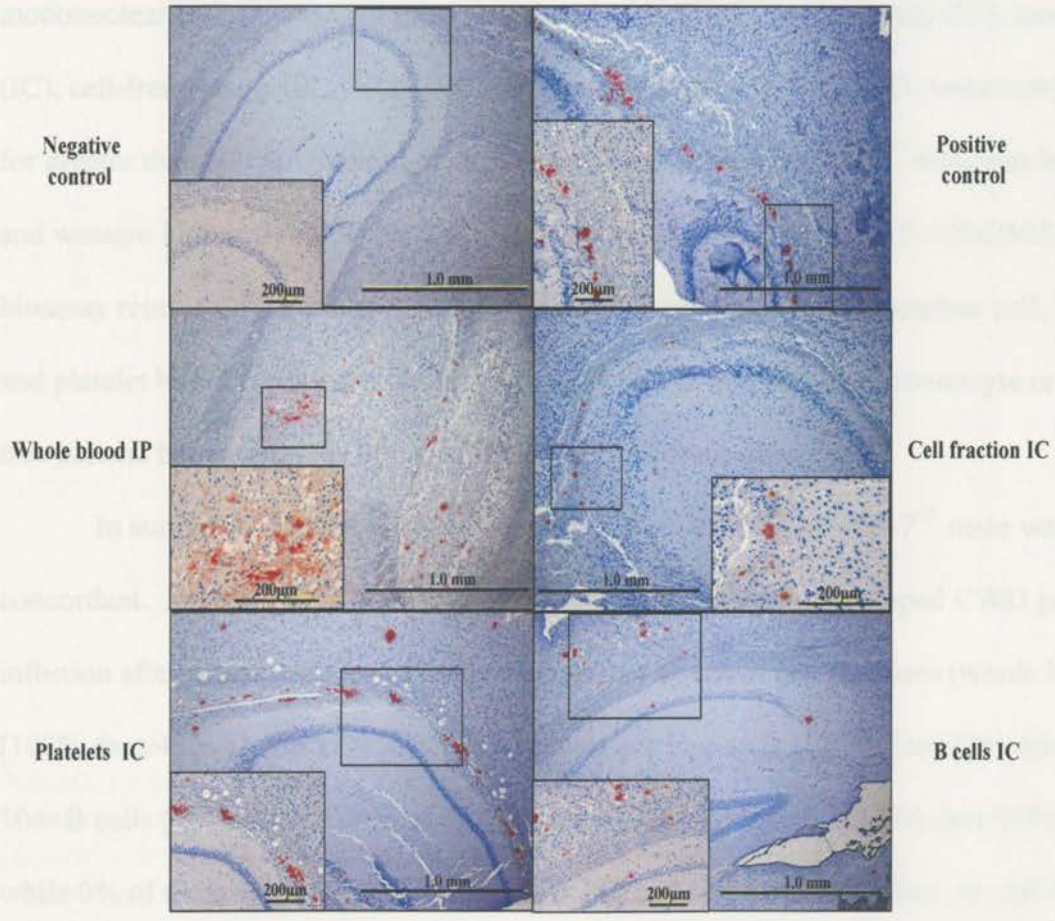
| Cohort # | Donor status | Inoculum | Route of inoculation | Terminal $PrP^{CWD}$ result[a] (n+/total n) | DPI observed for clinical disease[b] | % CWD+ |
|---|---|---|---|---|---|---|
| 8 | CWD+ | Brain | IC[c] | 10/10 | 168±4[i] | 100 |
| 9 | CWD- | " | IC | 0/10 | >600 | 0 |
| 10 | CWD+ | WB[j] | IC | 7/9 | 270±5 | 78 |
| 11 | " | " | IV[d] | 1/9 | 312 | 11 |
| 12 | " | " | IP[e] | 5/9 | 340±10 | 56 |
| 13 | " | " | PO[f] | 2/9 | 482±8 | 22 |
| 14 | " | BMC[g] | IC | 6/9 | 290±15 | 67 |
| 15 | " | " | IV | 1/9 | 303 | 11 |
| 16 | " | " | IP | 1/9 | 494 | 11 |
| 17 | " | " | PO | 0/9 | >600 | 0 |
| 18 | " | CF plasma[k] | IC | 0/9 | >600 | 0 |
| 19 | " | " | IV | 0/9 | >600 | 0 |
| 20 | " | " | IP | 0/9 | >600 | 0 |
| 21 | " | " | PO | 0/9 | >600 | 0 |
| 22 | " | RLN[h] B cells | IC | 5/5 | 282±7 | 100 |
| 23 | " | Splenic B cells | IC | 1/5 | 180 | 20 |
| 24 | " | Platelets | IC | 3/5 | 305±10 | 60 |
| 25 | " | Monocytes | IC | 0/5 | >600 | 0 |
| 26-29 | CWD- | Whole blood | IC,IV,IP,PO | 0/9 | >600 | 0 |
| 30-33 | " | BMC | IC,IV,IP,PO | 0/9 | >600 | 0 |
| 34-37 | " | CF plasma | IC,IV,IP,PO | 0/9 | >600 | 0 |
| 38-41 | " | RLN B cells, Splenic B cells, Platelets, or Monocytes | IC | 0/5 | >600 | 0 |

[a]Immunohistochemistry/western blot.
[b]The natural lifespan of Tg(CerPrP-E226)5037$^{+/-}$ mice ranged from 601-862.
[c]intracranial.
[d]intravenous.
[e]intraperitoneal.
[f]per os (oral).
[g]Blood Mononuclear Cells.
[h]Retropharyngeal lymph node.
[i]days post inoculation standard deviation (sd).
[j] whole blood.
[k]cell-free plasma.

***Cervidized mice (Tg(CerPrP-E226)5037$^{+/-}$) immunohistochemistry:***

Immunohistochemical analysis (Fig. 3.4) of sagittal brain tissue sections from terminal mice inoculated with either whole blood, blood mononuclear cells (leukocytes plus platelets), 2-104+B cells or CD41/61+ platelets demonstrate punctate $PrP^{CWD}$ staining typically found associated with CWD infection (28). Similar $PrP^{CWD}$ detection was not seen in mice inoculated with cell-free plasma or CD14+ monocytes.

Fig. 3.4. Brain immunohistochemistry results in Tg(CerPrP-E226)5037$^{+/-}$ mice inoculated with CWD+ blood components.



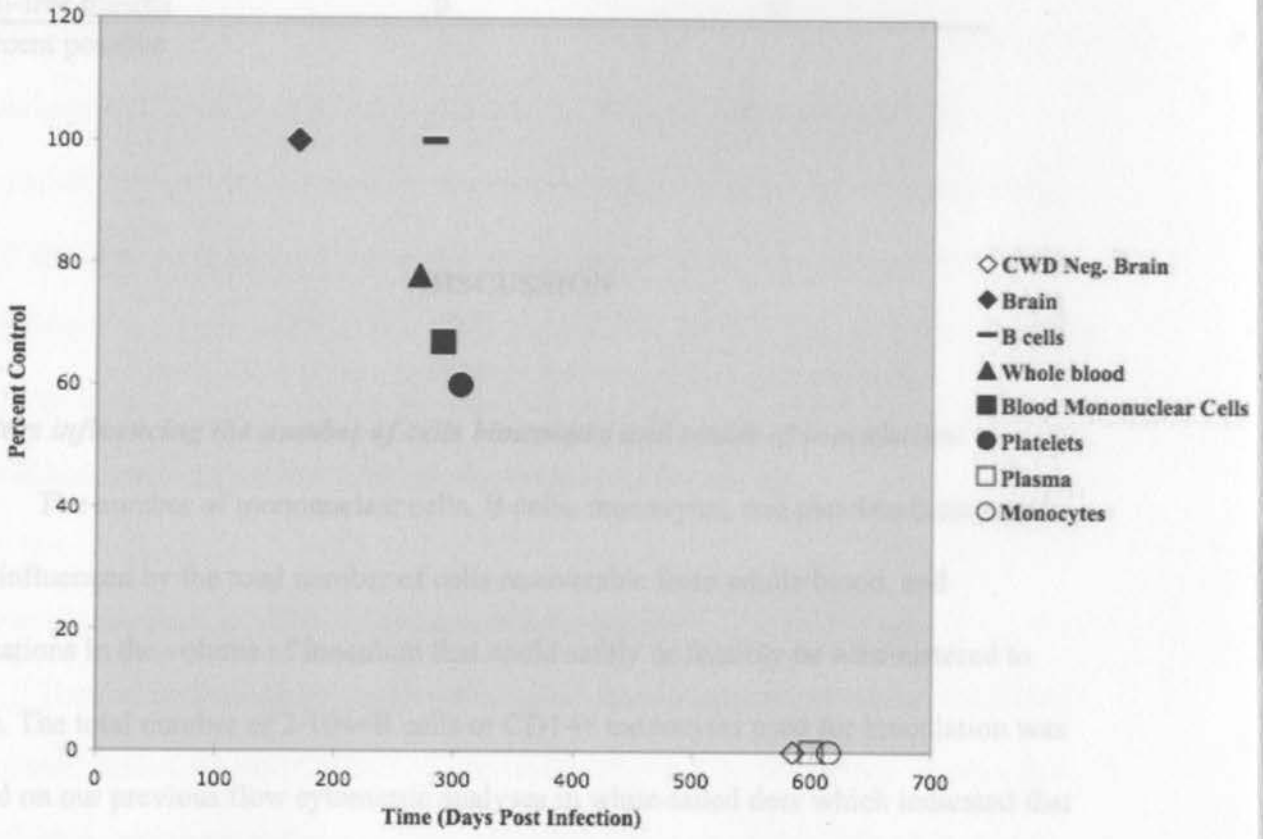

$PrP^{CWD}$ demonstrated by immunohistochemistry in sagittal brain sections of Tg(CerPrP-E226)5037$^{+/-}$ mice receiving whole blood, cell fraction, CD41/61+ or B cells from CWD-infected donors. $PrP^{CWD}$ plaque deposits are typical of those previously described in CWD infection in white-tailed deer (69) and Tg(CerPrP-E226)5037$^{+/-}$ mice (28).

***Cervidized mice (Tg(CerPrP-E226)5037$^{+/-}$) as a bioassay tool:*** Multiple routes of inoculation (IC, IV, IP, PO) were utilized to assess the infectivity of CWD blood fractions in cervid PrP transgenic (TgCerPrP) mice. Clinical disease and $PrP^{CWD}$ were detected in TgCerPrP mice inoculated IC with: (a) CWD+ brain (100% attack rate), (b) whole blood (78%), (c) blood mononuclear cells (67%), (d) platelets (60%), (e) retropharyngeal lymph node 2-104+ B cells (100%), and (f) splenic 2-104+ B cells (20%) (Fig. 3.5). CWD prion infection was also detected in mice inoculated with whole blood by the IP (56%), or oral (PO) (22%) routes and mice inoculated IV or IP with blood mononuclear cells (11%). All mice inoculated with CWD negative brain (IC), monocytes (IC), cell-free plasma (IC, IV, IP, PO), or blood mononuclear cells (PO) were maintained for greater than 600 dpi without evidence of clinical disease or $PrP^{CWD}$ detection by ihc and western blot analysis (Table 3.6). Thus the results from Tg(CerPrP-E226)5037$^{+/-}$ bioassay reinforced the infectious nature of whole blood, blood mononuclear cell, B cell and platelet blood fractions, and did not detect prion infectivity in the monocyte or cell-free plasma blood fractions of blood from CWD-infected deer.

In summary, bioassay results in deer and Tg(CerPrP-E266)5037$^{+/-}$ mice were concordant. Namely: 79% of deer and 35% of cervidized mice developed CWD prion infection after inoculation with blood or blood mononuclear cell fractions (whole blood [100% deer/42% Tg(CerPrP) mice], blood mononuclear cells [100% deer/22% mice], 2-104+B cells [100% deer /60% Tg(CerPrP) mice], CD 41/61+ cells [75% deer/60% mice], while 0% of those inoculated with either CD 14+ cells [0% deer/0% mice] or cell-free plasma became $PrP^{CWD}$ positive (Table 3.7). Thus, CWD infectivity segregated with

mononuclear cell, platelet, 2-104+B cell-enriched fractions, and not with either the CD14+ monocyte or plasma compartments of blood from CWD+ deer.

Fig. 3.5. Attack rate in Tg(CerPrP-E226)5037$^{+/-}$ mice intracranially inoculated with CWD+ blood components.




Percentage of Tg(CerPrP-E226)5037$^{+/-}$ mice affected with prion disease post IC inoculation with brain ◆, B cells -, whole blood ▲, cell fraction ■, platelets ●, cell-free plasma □ or monocytes ○ from CWD-infected donor deer or brain from a CWD negative donor deer ◇.

Table 3.7. Summary of white-tailed deer and Tg(CerPrP) mouse blood component bioassay studies.

| Inoculum | White-tailed deer (% +)[a] | Tg(CerPrP-E226)5037$^{+/-}$ mice (% +) |
|---|---|---|
| Whole blood | 100 | 42 |
| Blood mononuclear cells | 100 | 22 |
| B cells (2-104+) | 100 | 60 |
| Platelets (CD41/61+) | 75 | 60 |
| Monocytes (CD14+) | 0 | 0 |
| Cell-free plasma | 0 | 0 |

[a] percent positive

## DISCUSSION

***Factors influencing the number of cells bioassayed and routes of inoculation:***

The number of mononuclear cells, B cells, monocytes, and platelets bioassayed was influenced by the total number of cells recoverable from whole blood, and limitations in the volume of inoculum that could safely or feasibly be administered to mice. The total number of 2-104+B cells or CD14+ monocytes used for inoculation was based on our previous flow cytometric analyses in white-tailed deer which indicated that ~10% of mononuclear leukocytes were 2-104+B cells and CD14+ cells accounted for ~2% of the total mononuclear leukocyte population. Because CWD-infection was generated by $10^7$ total blood mononuclear cells (Cohort 2), we surmised that a minimum of $10^6$ 2-104+B cells (10%) or $2x10^4$ CD14+ cells (2%) would be sufficient to determine whether this cell phenotype may carry blood-borne prion infectivity. Thus these cell numbers were used for both deer and mouse cohorts. To mimic blood transfusion

dynamics all deer were inoculated by IV infusion. At the time this study was initiated very little was known about peripheral trafficking of CWD in Tg(CerPrP-E226)5037$^{+/-}$ mice. We therefore used multiple routes of inoculation (IC, IV, IP, PO) to explore the ability of these mice to support CWD infection. While attack rates were incomplete, likely due to the limited volume of blood or blood cells we could administer or innate differences in sensitivity of deer vs. Tg(cerPrP) mice, we did see a similar pattern of CWD infectivity associated with cell vs cell-free blood components (Fig. 3.5). These results suggest that while infection may not be as robust as that incurred post IC inoculation, Tg(CerPrP-E226)5037$^{+/-}$ mice are capable of establishing and maintaining CWD infection via peripheral routes of inoculation. Due to logistical reasons and animal availability it was not possible in this study to determine the minimum infectious dose for any of the inocula, although this information would surely be of interest and could be approached in subsequent more specific-inoculum-focused studies.

***Interval to detection of CWD infection by tonsil biopsy:***

Transmission of infectious prions by blood transfusion has now been established for scrapie, BSE, CJD, and CWD (3, 6, 36, 38, 39, 47, 54, 62). Identifying whether this infectivity is associated with the cellular, cell-free, or both fractions of blood has been a bit more challenging. Inherent limitations associated with the volume of inoculum that can be introduced by IC inoculation in rodent bioassay models impose constraints on assay sensitivity.

We were able to intravenously inoculate large volumes of CWD-infected whole blood and equivalent concentrations/volumes of blood constituents [leukocytes plus

platelets, cell-free plasma, or cell phenotype fractions (2-104+B cell, CD14+ monocytes, CD41/61+ platelets)] harvested from the same donor pool into cohorts of naïve white-tailed deer. In recipient deer, the time to tonsil biopsy positivity after inoculation of blood, blood mononuclear cells plus platelets, 2-104+B cells, or CD41/61+ platelets was variable—as early as 6 months to as late as 19 months p.i.. We have observed similar $PrP^{CWD}$ detection kinetics in previous cervid bioassay studies employing several routes of inoculation and inocula from CWD+ deer (53, 54). While we can not rule out horizontal transmission from the first positive animal in each cohort, the time frame for detection in the remaining deer (6 months) is half that which we have historically observed in deer inoculated with large quantities of saliva from CWD-infected deer (53, 54), suggesting much earlier exposure to infectious prions, i.e. more likely to relate to the experimental inoculum rather than to animal to animal transfer.

***Infectious prions in cell-associated (leukocyte plus platelet) blood fraction:***

Although identification of blood cell-associated TSE infectivity has been sought with disparate results, in CWD we detected infectivity in the cellular but not the cell-free plasma fraction of blood. While some bioassay studies have yielded negative findings (7, 72), likely due to the restricted sample volume assayable in rodent models, the transmission of infectious prions associated with buffy-coat white blood cells of TSE infected-donors has been well documented. Kuroda (44), Manulidis (50), and Brown (4-6) were the first to demonstrate this association in rodent models for Creutzfeldt-Jakob disease (CJD) and Gertsmann-Straussler-Scheinker disease (GSS). Subsequent to these

studies, scrapie prion infectivity in leukocyte populations has been detected by several researchers utilizing bioassay in rodent models (3, 10, 34, 50) and in sheep (39).

The replication of prions in the lymphoid tissues precedes CNS infection in several TSE's (CWD, scrapie, vCJD) (19, 27, 66, 67), raising the potential for haematogenous spread via recirculting lymphocytes. Consistent with this is the fact that leukodepletion reduces blood-borne prion transmission (24, 63).

***Infectious prions in Mab2-104+B cells:***

Immunohistological detection of $PrP^{RES/Sc}$ in lymphoid tissues of scrapie-infected sheep provided some of the first evidence for lymphoid system involvement in TSE diseases (19, 20, 61, 70). Subsequent studies utilizing confocal microscopy confirmed an association between $PrP^{RES}$ and immune cells (follicular dendritic cells (FDCs), tingible body macrophages (TBMs) and B cells) and extended the repertoire of prion diseases with lymphoid involvement to include CWD and vCJD (42, 55, 60, 66, 67). B cells have been associated with $PrP^{RES}$ transport and/or deposition within the lymphoid system (9, 10, 18, 22, 23, 30, 43, 56, 64, 66, 81). The present study supports this contention in demonstrating that Mab2-104+ primarily B cells harvested from peripheral blood or retropharyngeal lymph nodes contain sufficient infectious prions to transmit CWD to native or transgenic hosts.

B cells harvested for the deer bioassay studies were collected from peripheral whole blood. However, as noted earlier, due to cell loss associated with ficol and magnetic bead separation, we were not able to harvest sufficient 2-104+B cells from peripheral blood to adequately analyze the infectivity of these cells in both deer and

*Tg(CerPrP-E226)5037*$^{+/-}$ mice. We therefore harvested B cells from spleen and the retropharyngeal lymph nodes from the terminal harvest of one of the donor deer for bioassays in mice. The B cells harvested from these tissues (by mechanical disruption/filtration) (for mouse bioassay) and those collected from whole blood ficol separations (for deer bioassay) were sorted using Mab2-104 known to be specific for all peripheral blood B cells in sheep and has been found to cross react in a similar fashion in cervid species (Fig. 3.S1). Molecular studies suggest that the target antigen is the sheep homologue of CD72 (80) with more recent findings verifying that Mab 2-104 may identify FDCs, but does not recognize CD21, T cells, monocytes, or granulocytes (Fig. 3.S1). It is therefore feasible that Mab 2-104 could label germinal center FDCs in addition to B cells. It would be expected that most FDCs would either remain within the connective tissue stroma, be rendered non-viable during mechanical disruption, or be removed by the gradient separation (17). The morphology of the cells harvested for these studies by these methods supported their identity as B-lymphocytes. We cannot, however, exclude the possibility that CWD infectivity may have been sheared from dendritic processes of FDCs by mechanical disruption and therefore be a constituent within the B cell harvests from retropharyngeal lymph node for the mouse bioassay study. As FDCs are not found in the peripheral blood this is not a concern for the B cell harvests from whole blood for deer bioassay.

Differences exist between lymphatic recirculating and non-recirculating lymphocyte populations found in peripheral blood and lymphoid tissues. The reassortment of lymphocytes into these two subsets correlates with lymphocyte lineage and the expression of maturation and/or adhesion markers. Not all cells in the peripheral

blood have equal access to the lymphatic recirculation pathway (80). Recirculating lymphocytes constitute 60% of peripheral blood lymphocytes (PBLs) in the blood while non-recirculating PBLs (40%) are, by definition, excluded from lymph and confined to blood and the spleen. CD21 expression correlates with the recirculation competency of these subsets. B cells expressing the CD21 molecule ($CD21^+$) preferentially migrate across the high endothelial venules (HEV) and are able to recirculate between the peripheral blood and lymphatics, while those not expressing CD21 ($CD21^-$) do not cross the HEV and therefore remain in peripheral blood (non-recirculating). Based on previous data, the spleen would therefore contain representative populations of both recirculating and non-recirculating B cells, whereas the lymph node would only be expected to contain recirculating B cells and therefore represent 60% of total peripheral blood B cells (25, 80). Given that blood, lymph node and splenic B cells were competent to induce infection in recipient Tg(cerPrP) mice or deer, it is likely that the recirculating B cell population (i.e. CD21+) was responsible. However, it cannot be excluded that a unique population of germinal center resident B cells was present in samples harvested from spleen and lymph node, which would not be present in the peripheral blood. As well, although platelet contamination was not observed in this cell fraction, it can not be completely ruled out as a possible contributor to infectivity.

***Infectious prions in CD41/61+ platelets:***

Evidence associating prion infectivity with platelets has been variable— from no detection in sCJD platelets (11), to reported infectivity in hamster scrapie (34), GSS, and

vCJD (10). Here we report transmission of infectious CWD via CD41/61+ platelets in both naïve white-tailed deer and cervid transgenic mouse bioassays.

$PrP^C$ is produced endogenously by cells of the platelet lineage, which could implicate platelets in $PrP^{RES}$ propagation or trafficking within the body or transmission in contaminated blood products (11, 32, 33, 35, 48). 43-53%, 63-95% and 69-93% of bovine (3), ovine (4) and white-tailed deer (unpublished findings) platelets respectively express $PrP^C$, and given the number of circulating platelets vs. leukocytes, the majority of blood-borne $PrP^C$ expression is platelet associated (11, 48, 71). This blood component could be largely responsible for the transmission of vCJD by transfusion (45, 46).

***Absence of infectious prions in the plasma from CWD+ deer:***

The documentation of TSE infectivity (5, 6, 12, 21, 24, 74), or lack thereof (7, 19, 26, 51, 52), associated with the cell-free serum or plasma is proportionate historically. These discrepancies may be explained by the inherent limits on the volume of fluid that can be inoculated in rodent bioassays or the presence/absence of contaminating cells, or variation in the biology of prion diseases. Here we report absence of infectious CWD prions in cell/platelet-free plasma collected from an equivalent volume of whole blood (250ml) shown capable of infecting naïve white-tailed deer (Cohort 1). The virtual elimination of vCJD transmission by leuko-reduction argues strongly that as for CWD, prion infectivity is strongly leukocyte- and/or platelet-associated in vCJD (14, 15, 45, 46, 75).

***Absence of infectious prions in CD14+ cells from CWD+ deer:***

The presence of $PrP^{RES}$ in lymph node tingible body macrophages of scrapie-infected sheep (1, 29, 40, 56) and CWD-infected deer (66) led us to investigate the possibility that circulating CD14+ monocytes may contain infectious prions capable of transmitting disease. Somewhat to our surprise, the results indicated that in chronic wasting disease this is not the case. We were not able to detect $PrP^{CWD}$ in brain or lymphoid tissues of white-tailed deer or cervidized mice inoculated with up to $4 \times 10^5$ CD14+ macrophages (twice the number of cells present in the blood mononuclear cell inoculum that produced CWD).

*In vitro* experiments have determined that bone marrow derived macrophages can acquire and degrade $PrP^{BSE}$ (65), which in an *in vivo* setting could lead to decreased rates of infection (8). Maignien et al (49) found that depletion of macrophage numbers at the gut/follicle interface prior to TSE infection leads to an increase in infection rate. While macrophages are capable of receptor-mediated uptake of infectious particles, in particular infectious prions, it appears that their role may be associated with lysosomal degradation vs. sites of prion amplification or trafficking.

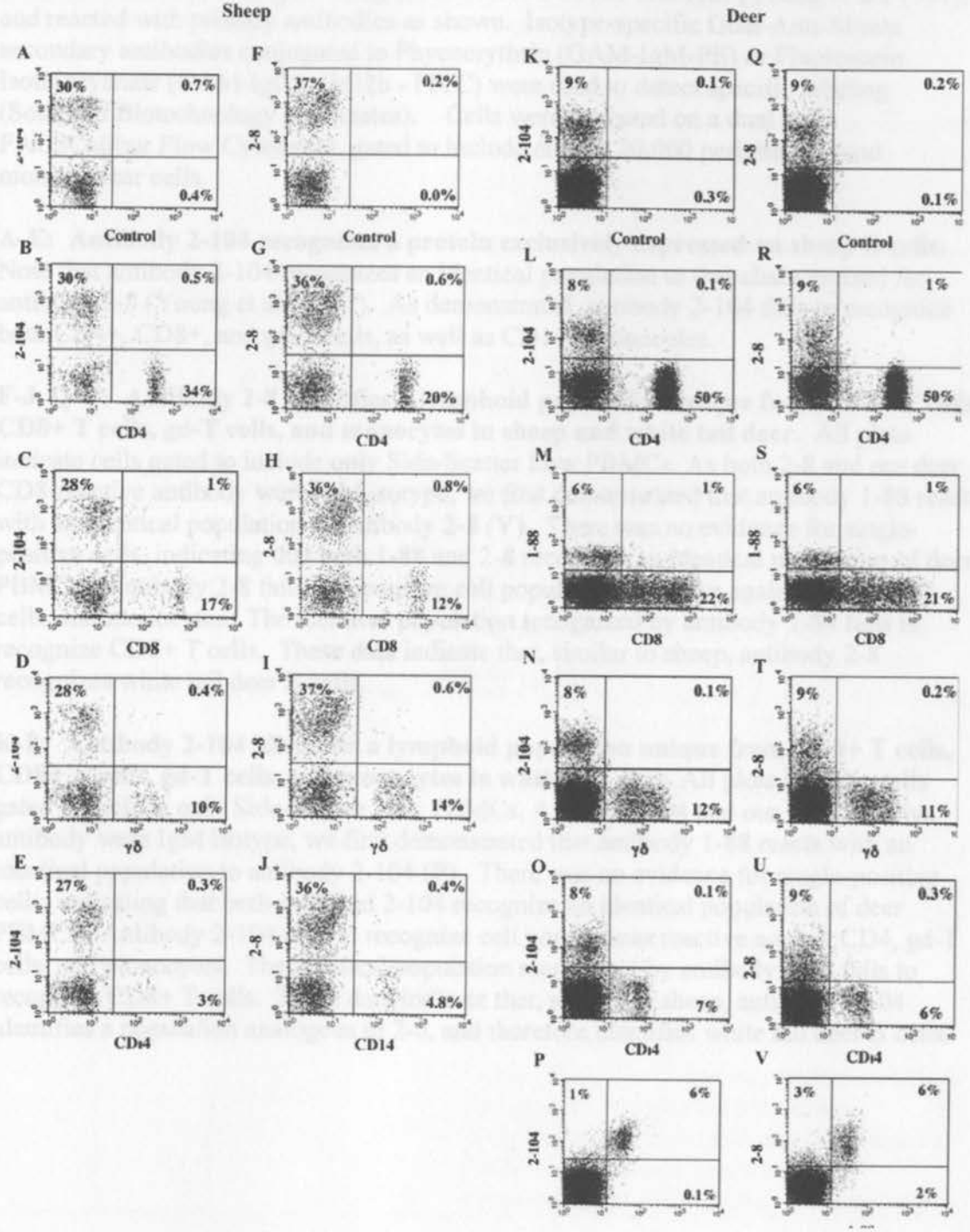
## SUMMARY

We have detected infectious prions in the cellular fraction (mononuclear leukocytes plus platelets) and not the cell-free plasma fraction of blood from CWD+ deer. B cells from blood or retropharyngeal lymph node and platelets, but not CD14+ monocytes or plasma, contained infectious prions capable of transmitting CWD. These

results: 1) support the identity of a hematogenous route of CWD infection and reinforce that all tissues are exposed to infection, 2) help in understanding the pathogenesis and trafficking of CWD prions, and 3) highlight the utility of CWD as a model in the development of antemortem assays to detect prion infections.

## SUPPORTIVE MATERIALS

Fig. 3.S1. Comparative FACS analysis of monoclonal antibodies 2-104 and 2-8 in sheep and white-tailed deer blood.



FACS analysis to verify specificity and equivalence of MAbs 2-104 and 2-8 in white-tailed deer were performed by Dr. Alan Young, South Dakota State University. All antibodies used are cited in Table S1. Peripheral blood leukocytes were harvested from peripheral blood by cell lysis using 0.16MNH4Cl/0.17M Tris-HCl (Young et al., 1997), and reacted with primary antibodies as shown. Isotype-specific Goat-Anti-Mouse secondary antibodies conjugated to Phycoerythrin (GAM-IgM-PE) or Fluorescein Isothiocyanate (GAM-IgG1, IgG2b - FITC) were used to detect specific labeling (Southern Biotechnology Associates). Cells were analyzed on a dual laser FACSCalibur Flow Cytometer, gated to include at least 20,000 peripheral blood mononuclear cells.

**A-E: Antibody 2-104 recognizes a protein exclusively expressed on sheep B cells.** Note that antibody 2-104 recognizes an identical population to that characterized for antibody 2-8 (Young et al., 1997). As demonstrated, antibody 2-104 fails to recognize both CD4+, CD8+, and gd-T cells, as well as CD14+ monocytes.

**F-J, Q-V: Antibody 2-8 identifies a lymphoid population unique from CD4+ T cells, CD8+ T cells, gd-T cells, and monocytes in sheep and white tail deer.** All plots indicate cells gated to include only Side-Scatter Low PBMCs. As both 2-8 and our deer CD8-reactive antibody were IgM isotype, we first demonstrated that antibody 1-88 reacts with an identical population to antibody 2-8 (**V**). There was no evidence for single-positive cells, indicating that both 1-88 and 2-8 recognize an identical population of deer PBMCs. Antibody 2-8 fails to recognize cell populations reactive against CD4, gd-T cells, and monocytes. The identical population recognized by antibody 1-88 fails to recognize CD8+ T cells. These data indicate that, similar to sheep, antibody 2-8 recognizes white tail deer B cells.

**K-P: Antibody 2-104 identifies a lymphoid population unique from CD4+ T cells, CD8+ T cells, gd-T cells, and monocytes in white tail deer.** All plots indicate cells gated to include only Side-Scatter Low PBMCs. As both 2-104 and our CD8-reactive antibody were IgM isotype, we first demonstrated that antibody 1-88 reacts with an identical population to antibody 2-104 (**P**). There was no evidence for single-positive cells, indicating that both 1-88 and 2-104 recognize an identical population of deer PBMCs. Antibody 2-104 fails to recognize cell populations reactive against CD4, gd-T cells, and monocytes. The identical population recognized by antibody 1-88 fails to recognize CD8+ T cells. These data indicate that, similar to sheep, antibody 2-104 identifies a population analogous to 2-8, and therefore identifies white tail deer B cells.

Table 3.S1. Antibodies used to detect Sheep and White Tail Deer PBMC subsets.

| Clone | Target | Isotype | Reactivity | Reference |
|---|---|---|---|---|
| 2-8 | B cells | IgM | Sheep, Deer | Young, A.J., Marston, W.L., Dessing, M., Dudler, L., Hein, W.R. 1997. Distinct recirculating and non-recirculating B-lymphocyte pools in the peripheral blood are defined by coordinated expression of CD21 and L-selectin. *Blood* 90(12):4865-4875. |
| 2-104 | B cells | IgM | Sheep, Deer | Halliday, S., Houston, F., Hunter, N. 2005. Expression of $PrP^c$ on cellular components of sheep blood. *J. Gen. Virol.* 86:1571-1579. |
| 1-88 | B cells | IgG1 | Sheep, Deer | N/A. Manuscript in Preparation. |
| 17D | CD4 | IgG1 | Sheep, Deer | Mackay, C.R., Hein, W.R., Brown, M.H., Matzinger, P. 1988. Unusual cexpression of CD2 in sheep: Implications for T cell interactions. *Eur. J. Immunol.* 18:1681-1688. |
| 7C2 | CD8 | IgG1 | Sheep | Young, A.J., Marston, W.L., Dessing, M., Dudler, L., Hein, W.R. 1997. Distinct recirculating and non-recirculating B-lymphocyte pools in the peripheral blood are defined by coordinated expression of CD21 and L-selectin. *Blood* 90(12):4865-4875. |
| ST8 | CD8 | IgM | Sheep, Deer | McClure, S.J., Hein, W.R. 1989. Functional Characteristics of 197+ CD4- CD8- sheep T lymphocytes: Expansion and differentiation of peripheral T cells. *Immunol. Cell Biol.* 67:223-231. |
| 197 | T19 (gd-T cells) | IgG2b | Sheep, Deer | McClure, S.J., Hein, W.R. 1989. Functional Characteristics of 197+ CD4- CD8- sheep T lymphocytes: Expansion and differentiation of peripheral T cells. *Immunol. Cell Biol.* 67:223-231. |
| 3C10 | CD14 | IgG2b | Sheep, Deer | Van Voorhis, WC. Steinman, R.M., Hair, L.S., Luban, J., Witmer, M.D., Koide, S., Cohn, Z.A. 1983. Specific antimononuclear phagocyte monoclonal antibodies. Application to the purification of dendritic cells and the tissue localization of macrophages. *J. Exp. Med.* 158(1):126-145. |

**Acknowledgements**

We wish to thank A. Avery and M. Zabel for technological conversations and input in this work; K. O'Rourke and L. Hamburg for performing the *Prnp* genotype analysis; J. Grassi and J. Langeveld for their generous gifts of antibodies; N. Albarado and E. McNulty for sample collection and tireless dedication to animal welfare; A. Nalls for insightful editing; and fellow graduate students D. Seelig, N. Denkers and N. Haley for necropsy banter, support and study critique.

This work was supported by the NIH, National Institute of Allergy and Infectious Diseases, contract N01-AI-25491.

## REFERENCES

1. **Andreoletti, O., P. Berthon, E. Levavasseur, D. Marc, F. Lantier, E. Monks, J. M. Elsen, and F. Schelcher.** 2002. Phenotyping of protein-prion (PrPsc)-accumulating cells in lymphoid and neural tissues of naturally scrapie-affected sheep by double-labeling immunohistochemistry. J Histochem Cytochem **50:**1357-70.
2. **Angers, R., T. Seward, D. Napier, M. Green, E. Hoover, T. Spraker, K. O'Rourke, A. Balachandran, and G. Telling.** 2009. Chronic wasting disease prions in elk antler velvet. Emerg Infect Dis **15:**696-703.
3. **Bons N, L. S., Mestre-Frances N, Dormant D, Brown P.** 2002. Brain and buffy coat transmission of bovine spongiform encephalopathy to the primate Microcebus murinus. Transfusion **42:**513-516.
4. **Brown, P.** 1995. Can Creutzfeldt-Jakob disease be transmitted by transfusion? Curr Opin Hematol **2:**472-7.
5. **Brown P, C. L., McShane P, Rubenstein BR, Drohan WN.** 1999. Further studies of blood infectivity in an experimental model of transmissible spongiform

encephalopathy, with an explanation of why blood components do not transmit Creutzfeldt-Jakob disease in humans. Transfusion **39:**1169-1178.

6. **Brown P, R. R., Dunstan BC, MacAuley C, Gajdusek DC, Drohan WN.** 1998. The distribution of infectivity in blood components and plasma derivatives in experimental models of transmissible spongiform encephalopathy. Transfusion **38:**810-816.
7. **Bruce ME, M. I., Will RG, Ironside JW.** 2001. Detection of variant Creutzfeldt-Jakob disease infectivity in extraneural tissues. Lancet **358:**208-209.
8. **Carp RI, C. S.** 1981. In vitro interaction of scrapie agent and mouse peritoneal macrophages. Intervirology **16:**8-13.
9. **Casaccia P, L. A., Xi YG, Pocchiari M.** 1989. Levels of infectivity in the blood throughout the incubation period of hamsters peripherally injected with scrapie. Arch Virol **108:**145-149.
10. **Cervenakova, L., O. Yakovleva, C. McKenzie, S. Kolchinsky, L. McShane, W. N. Drohan, and P. Brown.** 2003. Similar levels of infectivity in the blood of mice infected with human-derived vCJD and GSS strains of transmissible spongiform encephalopathy. Transfusion **43:**1687-94.
11. **Choi EM, G. M., Deering C, Pomeroy K, Kuo A, Miller BL, Safar JG, Prusiner SB.** 2009. Prion proteins in subpopulations of white blood cells from patients with sporadic Creutzfeldt-Jakob disease. Lab Invest **89:**624-635.
12. **Clarke MC, H. D.** 1967. Presence of the transmissible agent of scrapie in the serum of affected mice and rats. Vet Rec **80:**504.
13. **Clarke P, G. A.** 2005. Projections of the future course of the primary vCJD epidemic in the UK: inclusion of subclinical infection and the possibility of wider genetic susceptibility. Journal of the Royal Society Interface **2:**19-31.
14. **Cleemput, I., Leys M, Ramaekers D, Bonneux L.** 2006. Balancing evidence and public opinion in health technology assessments: the case of leukoreduction. Int J Techol Assess Health Care **22:**403-7.
15. **Coste, J., C. Prowse, R. Eglin, and C. Fang.** 2009. A report on transmissible spongiform encephalopathies and transfusion safety. Vox Sang **96:**284-91.
16. **Deslys, J., C. Lasmezas, and D. D.** 1994. Selection of specific strains in iatrogenic Creutzfeldt-Jakob disease. Lancet **2:**848-9.
17. **Eaton S, A. M., Hamilton S, Gonzalez L, Sales J, Feffrey M, Reid HW, Rocchi MS, Chianini F.** 2009. CD21 B cell populations are altered following subcutaneous scrapie inoculation in sheep. Veterinary Immunology and Immunopathology:1-5.
18. **Eklund CM, H. W., Kennedy RC.** 1963. Some properties of the scrapie agent and its behavior in mice. Proc Soc Exp Biol Med **112:**974-979.
19. **Eklund CM, K. R., Hadlow WJ.** 1967. Pathogenesis of scrapie virus infection in the mouse. J Infectious Disease **117:**15-22.
20. **Fraser, H., and A. G. Dickinson.** 1970. Pathogenesis of scrapie in the mouse: the role of the spleen. Nature **226:**462-3.
21. **Gibbs, C. Gajdusek, and Morris.** 1965. Slow, latent, and temperate virus infections. NINDB Monograph No.2., Washington D.C.

22. **Glatzel, M., E. Abela, M. Maissen, and A. Aguzzi.** 2003. Extraneural pathologic prion protein in sporadic Creutzfeldt-Jakob disease. N Engl J Med **349:**1812-20.
23. **Gordon, W.** 1957. Discussion to Palmer, A.C. Studies in scrapie. Vet Record **69:**1324-1327.
24. **Gregori, L., N. McCombie, D. Palmer, P. Birch, S. O. Sowemimo-Coker, A. Giulivi, and R. G. Rohwer.** 2004. Effectiveness of leucoreduction for removal of infectivity of transmissible spongiform encephalopathies from blood. Lancet **364:**529-31.
25. **Gupta, V., McConnell, I, Dalziel, RG, Hopkins, J.** 1998. Two B cell subpopulations have distinct recirculation characteristics. Eur J Immunol **May;28:**1597-603.
26. **Hadlow WJ, E. C., Kennedy RC.** 1974. Course of experimental scrapie virus infection in the goat. The Journal of Infectious Diseases **129:**559-567.
27. **Hadlow WJ, K. R., Race RE.** 1982. Natural infection of Suffolk sheep with scrapie virus. Journal of Infectious Disease **146:**657-64.
28. **Haley, N., D. Seelig, M. Zabel, G. Telling, and E. Hoover.** 2009. Detection of CWD prions in urine and saliva of deer by transgenic mouse bioassay. PLoS One **4:**e4848.
29. **Herrmann LM, C. W., Davis WC, Knowles DP, O'Rourke KI.** 2003. CD21-positive follicular dendritic cells: A possible source of PrPsc in lymph node macrophages in scrapie infected sheep. Am J Pathol **162:**1075-1081.
30. **Herzog, C., J. Riviere, N. Lescoutra-Etchegaray, A. Charbonnier, V. Leblanc, N. Sales, J. P. Deslys, and C. I. Lasmezas.** 2005. PrPTSE distribution in a primate model of variant, sporadic, and iatrogenic Creutzfeldt-Jakob disease. J Virol **79:**14339-45.
31. **Hilton DA, G. A., Conyers L, Edwards P, McCardle L, Ritchie D, Penney M, Hegazy D, Ironside JW.** 2004. Prevalence of lymphoreticular prion protein accumulation in UK tissue samples. Journal of Pathology **203:**733-739.
32. **Holada K, M. T., Juller J, Vostal JG.** 1998. Increased expression of phosphatidylinositol-specific phospholipase C resistant prion proteins on the surface of activated platelets. Br J Haematol **103:**276-282.
33. **Holada, K., J. Simak, P. Brown, and J. G. Vostal.** 2007. Divergent expression of cellular prion protein on blood cells of human and nonhuman primates. Transfusion **47:**2223-32.
34. **Holada K, V. J., Theisen PW, MacAuley C, Gregori L, Rohwer RG.** 2002. Scrapie infectivity in hamster blood is not associated with platelets. J Virology **76:**4649-4950.
35. **Holada, K., and J. G. Vostal.** 2000. Different levels of prion protein (PrPc) expression on hamster, mouse and human blood cells. Br J Haematol **110:**472-80.
36. **Houston F, F. J., Chong A, Hunter N, Bostock CJ** 2000. Transmission of BSE by blood transfusion in sheep. Lancet **356:**999-1000.
37. **HPA, E.** 2007. Fourth case of transfusion-associated vCJD infection in the United Kingdom.
38. **HPA, E.** 2009, posting date. vCJD abnormal prion protein found in a patient with haemophilia at post mortem. [Online.]

39. **Hunter, N., J. Foster, A. Chong, S. McCutcheon, D. Parnham, S. Eaton, C. MacKenzie, and F. Houston.** 2002. Transmission of prion diseases by blood transfusion. J Gen Virol **83:**2897-905.
40. **Jeffrey, M., G. McGovern, S. Martin, C. M. Goodsir, and K. L. Brown.** 2000. Cellular and sub-cellular localisation of PrP in the lymphoreticular system of mice and sheep. Arch Virol Suppl:23-38.
41. **Kim TY, S. H., Joo YS, Mun UK, Kang KS, Lee YS** 2005. Additional cases of Chronic Wasting Disease in imported deer in Korea. J Vet Med Sci **67:**753-759.
42. **Kitamoto T, M. T., Mohri S, Doh-Ura K, Tateishi J.** 1991. Abnormal isoform of prion protein accumulates in follicular dendritic cells in mice with Creutzfeldt-Jakob disease. J Virology **65:**6292-6295.
43. **Klein, M. A., R. Frigg, E. Flechsig, A. J. Raeber, U. Kalinke, H. Bluethmann, F. Bootz, M. Suter, R. M. Zinkernagel, and A. Aguzzi.** 1997. A crucial role for B cells in neuroinvasive scrapie. Nature **390:**687-90.
44. **Kuroda Y, G. C., Herbert LA, Gajdusek DC.** 1983. Creutzfeldt-Jakob disease in mice: Persistent viremia and preferential replication of virus in low-density lymphocytes. Infection and Immunity **41:**154-161.
45. **Lefrere, J., Hewit P.** 2009. From mad cows to sensible blood transfusion: the risk of prion transmission by labile blood components in the United Kingdom and in France. Transfusion **49:**797-812.
46. **Lefrere, J. J., and B. Danic.** 2009. Pictorial representation of transfusion over the years. Transfusion **49:**1007-17.
47. **Llewelyn CA, H. P., Knight RS, Amar K, Cousens S, Mackenzie J, Will RG.** 2004. Possible transmission of variant Creutzfeldt-Jakob disease by blood transfusion. Lancet **363:**417-421.
48. **MacGregor, I., J. Hope, G. Barnard, L. Kirby, O. Drummond, D. Pepper, V. Hornsey, R. Barclay, H. Bessos, M. Turner, and C. Prowse.** 1999. Application of a time-resolved fluoroimmunoassay for the analysis of normal prion protein in human blood and its components. Vox Sang **77:**88-96.
49. **Maignien T, S. M., Calvo P, Marce D, Sales N, Fattal E, Deslys JP, Couvreur P, Lasmezas CI.** 2005. Role of gut macrophages in mice orally contaminated with scrapie or BSE. Int J Pharm **298:**293-304.
50. **Manuelidis EE, K. J., Mericangas JR, Manuelidis L.** 1985. Transmission to animals of Creutzfeldt-Jakob disease from human blood. Lancet **2:**896-897.
51. **Marsh, R. F., and R. P. Hanson.** 1969. Transmissible mink encephalopathy: neuroglial response. Am J Vet Res **30:**1637-42.
52. **Marsh, R. F., J. M. Miller, and R. P. Hanson.** 1973. Transmissible mink encephalopathy: studies on the peripheral lymphocyte. Infect Immun **7:**352-5.
53. **Mathiason, C. K., S. A. Hays, J. Powers, J. Hayes-Klug, J. Langenberg, S. J. Dahmes, D. A. Osborn, K. V. Miller, R. J. Warren, G. L. Mason, and E. A. Hoover.** 2009. Infectious prions in pre-clinical deer and transmission of chronic wasting disease solely by environmental exposure. PLoS One **4:**e5916.
54. **Mathiason, C. K., J. G. Powers, S. J. Dahmes, D. A. Osborn, K. V. Miller, R. J. Warren, G. L. Mason, S. A. Hays, J. Hayes-Klug, D. M. Seelig, M. A. Wild, L. L. Wolfe, T. R. Spraker, M. W. Miller, C. J. Sigurdson, G. C. Telling, and**

**E. A. Hoover.** 2006. Infectious prions in the saliva and blood of deer with chronic wasting disease. Science **314:**133-6.

55. **McBride, P. A., P. Eikelenboom, G. Kraal, H. Fraser, and M. E. Bruce.** 1992. PrP protein is associated with follicular dendritic cells of spleens and lymph nodes in uninfected and scrapie-infected mice. J Pathol **168:**413-8.
56. **McGovern G, J. M.** 2007. Scrapie-specific pathology of sheep lymphoid tissues. PLoS One **2:**e1304.
57. **Miller, M., E. Williams, N. Hobbs, and W. LL.** 2004. Environmental sources of prion transmission in mule deer. Emerg Infect Dis **10:**1003-6.
58. **Miller, M. W., and E. S. Williams.** 2003. Prion disease: horizontal prion transmission in mule deer. Nature **425:**35-6.
59. **Miller MW, W. M., Williams ES.** 1998. Epidemiology of chronic wasting disease in captive Rocky Mountain elk. J Wildl Dis **34:**532-8.
60. **Muramoto, T., T. Kitamoto, J. Tateishi, and I. Goto.** 1992. The sequential development of abnormal prion protein accumulation in mice with Creutzfeldt-Jakob disease. Am J Pathol **140:**1411-20.
61. **Pattison, I. H., and K. M. Jones.** 1968. Detection of the scrapie agent in tissues of normal mice and in tumours of tumour-bearing but otherwise normal mice. Nature **218:**102-4.
62. **Peden AH, H. M., Ritchie DL, Bell JE, Ironside JW** 2004. Preclinical vCJD after blood transfusion in a PRNP codon 129 heterozygous patient. Lancet **364:**527-529.
63. **Prowse, C. V., and A. Bailey.** 2000. Validation of prion removal by leukocyte-depleting filters: a cautionary tale. Vox Sang **79:**248.
64. **Raeber, A. J., M. A. Klein, R. Frigg, E. Flechsig, A. Aguzzi, and C. Weissmann.** 1999. PrP-dependent association of prions with splenic but not circulating lymphocytes of scrapie-infected mice. Embo J **18:**2702-6.
65. **Rybner-Barnier C, J. C., Cuche C, Dorre G, Majlessi L, Gabellec MM, Moris A, Schwartz O, Di Santo J, Cumano A, Leclerc C, Lazarini F.** 2006. Processing of the bovine spongiform encephalopathy-specific prion protein by dendritic cells. J Virol **80:**4656-4663.
66. **Sigurdson, C. J., C. Barillas-Mury, M. W. Miller, B. Oesch, L. J. van Keulen, J. P. Langeveld, and E. A. Hoover.** 2002. PrP(CWD) lymphoid cell targets in early and advanced chronic wasting disease of mule deer. J Gen Virol **83:**2617-28.
67. **Sigurdson, C. J., E. S. Williams, M. W. Miller, T. R. Spraker, K. I. O'Rourke, and E. A. Hoover.** 1999. Oral transmission and early lymphoid tropism of chronic wasting disease PrPres in mule deer fawns (Odocoileus hemionus). J Gen Virol **80 ( Pt 10):**2757-64.
68. **Sohn HJ, K. J., Choi KS, Nah JJ, Joo YS, Jean YH, Ahn SW, Kim OK, Balachandran A** 2002. A case of chronic wasting disease in an elk imported to Korea from Canada. .J Vet Med Sci **64:**855-858.
69. **Spraker TR, Z. R., Cummings BA, Wild MA, Miller MW, O'Rourke KI.** 2002. Comparison of histological lesions and immunohistochemical staining of proteinase-resistant prion protein in a naturally occurring spongiform

encephalopathy of free-ranging mule deer (Odocoileus hemionus) with those of chronic wasting disease of captive mule deer. Vet Pathol **39:**110-119.

70. **Stamp, J. T., J. G. Brotherston, I. Zlotnik, J. M. Mackay, and W. Smith.** 1959. Further studies on scrapie. J Comp Pathol **69:**268-80.
71. **Starke, R., P. Harrison, I. Mackie, G. Wang, J. D. Erusalimsky, R. Gale, J. M. Masse, E. Cramer, A. Pizzey, J. Biggerstaff, and S. Machin.** 2005. The expression of prion protein (PrP(C)) in the megakaryocyte lineage. J Thromb Haemost **3:**1266-73.
72. **Tamai Y, K. H., Kitajima R, Taguchi F, Ohtani Y, Kawaguchi T, Miura S, Sato M, Ishihara Y.** 1992. Demonstration of the transmissible agent in tissue from a pregnant woman with Creutzfeldt-Jakob disease. New England Journal of Medicine **327:**649.
73. **Tateishi, J., T. Kitamato, and H. Hiratani.** 1985. Creutzfeldt-Jakob disease pathogen in growth hormone preparations is eliminatable. Lancet **2:**1299-300.
74. **Taylor, D. M., I. McConnell, and C. E. Ferguson.** 2000. Closely similar values obtained when the ME7 strain of scrapie agent was titrated in parallel by two individuals in separate laboratories using two sublines of C57BL mice. J Virol Methods **86:**35-40.
75. **Turner, M. L., and C. A. Ludlam.** 2009. An update on the assessment and management of the risk of transmission of variant Creutzfeldt-Jakob disease by blood and plasma products. Br J Haematol **144:**14-23.
76. **USGS** January 2009, posting date. Map of Chronic Wasting Disease in North America. [Online.]
77. **Williams, E.** 2005. Chronic Wasting Disease. Vet Pathol **42:**530-49.
78. **Williams, E. S., and M. W. Miller.** 2002. Chronic wasting disease in deer and elk in North America. Rev Sci Tech **21:**305-16.
79. **Williams ES, Y. S.** 1992 Spongiform encephalopathies in Cervidae. Rev Sci Tech **11:**551-567.
80. **Young AJ, M. W., Dessing M, Dudler L, Hein WR.** 1997. Distinct recirculating and non-recirculating B-lymphocyte pools in the peripheral blood are defined by coordinated expression of CD21 and L-selectin. Blood **90:**4865-4875.
81. **Zabel MD, H. M. P. M., Arrighi I, Schwarz P, Kranich J, von Teichman A, Haas KM, Zeller N, Tedder TF, Weis JH, Aguzzi A.** 2007. Stromal complement receptor CD21/35 facilitates lymphoid prion colonization and pathogenesis. Journal of Immunology **179:**6144-6152.

## CONCLUSION

We have learned from this work that— 1) infectious prions are found in the saliva and blood of CWD-infected symptomatic and asymptomatic deer; 2) CWD can be transmitted via multiple routes of infection (PO, IV, IP and IC); 3) sufficient infectious prions are present in the environment (bedding, water and feed containers) of CWD-infected deer to transmit the disease by indirect exposure, negating the need for direct animal-to-animal contact; 4) CWD blood infectivity is cell associated; and 5) B cells and platelets appear to be most responsible for trafficking blood-borne CWD infectivity.

It is reasonable to speculate that the high efficiency of CWD transmission among cervid in free-ranging and captive environments is associated with the sharing and/or contamination of infected saliva. To our surprise this work did not support the dogma that excreta (feces/urine) contamination is a primary contributor in transmission. It is possible we were unable to detect CWD infectivity in excreta because either— 1) insufficient concentrations of infectivity were present in the amounts of feces and urine used as inoculum; 2) study termination was prior to the development of sufficient amounts of $PrP^{RES}$ for conventional detection (IHC, western blot) in brain or lymphoid tissues; 3) the inoculum used for these studies was collected prior to or after optimal prion shedding in excreta; or 4) perhaps the conformation of $PrP^{CWD}$ is sufficiently different in urine/feces to require a longer latent period prior to amplification and detection within the host. Additional questions that could be asked in regard to transmission include: What is the minimum amount of saliva/blood needed to transmit CWD from one animal to the next? Can $PrP^{RES}$ be directly detected in the saliva/blood of

prion-infected animals leading to an antemortem diagnostic? Is assay sensitivity an issue in the lack of detection of $PrP^{RES}$ in asymptomatic animals, and if so, what assays would be better suited for detection (PMCA, NaPTA, non PK requiring assays)?

For those charged with the task of managing free-ranging and captive cervid populations, the role environmental contamination plays in the spread of CWD has consumed great attention. The results of this work expand on the efforts of Miller (CWD) (4) and Georgsson (scrapie) (1), and highlight the fact that sufficient infectious prions can be acquired from the environment without the aid of direct contact with animals infected with CWD. Based on the outcome of our bioassays we suggest that saliva is the main contributor in this route of transmission.

While transmission studies have previously been undertaken to investigate CWD pathogenesis using IC and PO routes of inoculation, these are the first studies in which blood transfusion via IV and IP inoculation generate clinical disease in recipient deer. Demonstration of hematogenous trafficking of prion infection— 1) contributes to our knowledge of CWD pathogenesis; 2) suggests that all tissues are at risk of being contaminated; and 3) establishes similarities in lymphoreticular involvement in CWD, scrapie, and vCJD infection. One might ask: Are disease induction kinetics similar for these routes of inoculation? What cell subsets are responsible for trafficking blood CWD infectivity? Do additional similarities exist between CWD, scarpie, and vCJD blood infectivity (i.e. the role of complement, subsets of infected cells, etc.).

With the demonstration of vCJD transmissibility via blood transfusion came a concerted effort to define blood infectivity in TSE diseases. We have demonstrated that the cellular fraction, but not the cell-free plasma fraction of blood from CWD-infected

donors contains sufficient infectious prions to establish infection and clinical disease progression. We were further able to define at least two cell subsets responsible for ferrying this infection—B cells and platelets.

The detection of B cell CWD infectivity expands on the work of Sigurdson, et al (6) who established follicular dendritic cell (FDC), B cell and tingible body macrophage (TBM) association with $PrP^{RES}$ via confocal microscopy. CD14+ monocytes did not establish infection in bioassay suggesting that a key role for these cells may be in the up-take of $PrP^{RES}$ for lysosomal degradation vs trafficking or replication. One line of continued investigation derived from this work centers on B cell infectivity. The B cell marker used to harvest cells for these studies was $CD72^{+}$ $CD21^{-}$. Based on the work of Young, et al (7) cells lacking CD21 expression are excluded from lymphoid circulation and remain in the periphery… it is therefore reasonable to hypothesize that there are additional subsets of B cells harboring CWD infection. Can these cells be differentiated, harvested, and assayed for their infectivity?

Holding with the observation that $PrP^{C}$ is an obligate component to $PrP^{RES}$ conversion, another cell type with high $PrP^{C}$ expression in the blood of humans, sheep, and deer was found to harbor sufficient infectious prions to transmit CWD—platelets. The majority of blood-borne $PrP^{C}$ expression is platelet associated. This blood component could be largely responsible for the transmission of CWD, scrapie and vCJD infections by transfusion. The results of this thesis work does not distinguish whether the infectious prions associated with platelets are amplified on or within these cells, or whether platelets merely act as transporters of infection to cells proficient in $PrP^{RES}$ conversion. Due to their high $PrP^{C}$ expression and the relative ease of collection,

platelets could be exploited for many purposes. In particular, platelets could be used as a substrate for a blood antemortem diagnostic, or could be used for the study of a prion receptor.

The findings of the work presented in this dissertation provide several advances in our knowledge of CWD infection and portend similar dynamics in all prion diseases. To highlight, the information gained— 1) establishes saliva as an efficient transmitter of CWD infection; 2) will contribute to management of CWD in free-ranging and captive cervid populations; 3) substantiates prior work establishing a hematogenous phase in TSE disease; 4) establishes at least 2 subsets of blood cells as traffickers of CWD infection; and 5) provides a basis for antemortem diagnostics within saliva and blood of infected individuals.

## FUTURE DIRECTIONS

Future studies born of this work include— 1) Antemortem diagnostic testing utilizing saliva or blood cells from CWD-infected deer as substrate. This work is underway with the use of PMCA, western blot analysis, an ELISA based kit (IDEXX) or a combination of 2 or more of these assays to increase sensitivity for the detection of $PrP^{CWD}$; 2) Determining the minimum dose necessary to generate infection from saliva and blood cell components. Titration of saliva and blood cell component infectivity would be most efficiently done by bioassay in transgenic mice expressing the cervid prion protein. Alternatively, the use of a smaller deer species being developed as a model

for CWD (muntjac) would allow the studies to continue in a cervid species. In vitro analysis of these body fluids by ELISA based kit, NaPTA and western blot analysis or PMCA would provide insight to correlation between *in vivo* and *in vitro* assays; 3) Comparing blood transfusion and orally acquired CWD infection kinetics to determine pathogenesis similarities/differences. A current bioassay study in white-tailed deer is underway to assess this kinetic; 4) Detection of additional subsets of B cells capable of transmitting CWD infection. In the work presented here, we have demonstrated the infectivity of a $CD72^{+}CD21^{-}$ B cell population. Efforts directed toward harvesting and inoculating a $CD21^{+}$ B cell population for bioassay would enhance understanding of lymphoid recirculating B cell involvement in CWD infectivity; 5) Because $PrP^{CWD}$ has been detected in the feces (by PMCA) (5) and urine (by bioassay in transgenic mice) (3) of CWD-infected deer, and has been detected in brain homogenates from the urine/feces inoculated deer described in this thesis (by bioassay in transgenic mice) (2) we continue to seek infectious prions in the excreta of CWD-infected cervid by bioassay in deer. Two cohorts of deer have been inoculated for this ongoing study—serial lymphoid biopsies will be collected from these deer and tested for the detection of $PrP^{CWD}$ for a timeframe greater than 19 months pi.

As is the seeking of answers to all questions… one answer provides fodder for a plethora of new questions. While this work provides insight, it as well provides fodder.

## REFERENCES


1. **Georgsson, G., Sigurdarson, S, Brown, P.** 2006. Infectious agent of sheep scrapie may persist in the environment for at least 16 years. J Gen Virol **87:**3737-3740.
2. **Haley, N., Mathiason, CK, Zabel, MD, Telling, GC, Hoover, EA.** 2009. Detection of sub-clinical CWD infection in conventional test-negative deer long after oral exposure. PLoS One **4:**e7990.
3. **Haley, N., Seelig, DM, Zabel, MD, Telling, CG, Hoover, EA.** 2009. Detection of CWD prions in urine and saliva of deer by transgenic mouse bioassay. PLoS One **4:**e4848.
4. **Miller, M., Williams, ES, Hobbs, NT, Wolfe, LL.** 2004. Environmental sources of prion transmission in mule deer. Emert Infect Dis **10:**1003-1006.
5. **Safar, J., Lessard, P, Tamguney, G, Freyman, Y, Deering, C, Letessier, F, Dearmond, SJ, Prusiner, SB.** 2008. Transmission and detection of prions in feces. J Infect Dis **198:**81-89.
6. **Sigurdson, C., Barillas-Mury, C, Miller, MW, Oesch, B, van Keulen, LJ, Langeveld, JP, Hoover, EA.** 2002. PrP(CWD) lyphoid cell targets in early and advanced chronic wasting disease of mule deer. J Gen Virol **83:**2617-28.
7. **Young, A., Marston, WL, Dessing, M, Dudler, L, Hein, WR.** 1997. Distinct recirculating and non-recirculating B-lymphocyte pools in the peripheral blood are defined by coordinated expression of CD21 and L-selectin. Blood **90:**4865-75.