DISSERTATION

ORAL AND NASAL MUCOSAL PATHWAYS OF PRION INFECTION IN CHRONIC WASTING DISEASE

Submitted by

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ABSTRACT

ORAL AND NASAL MUCOSAL PATHWAYS OF PRION INFECTION IN CHRONIC WASTING DISEASE

Chronic wasting disease (CWD) is a fatal neurodegenerative prion disease of deer, elk, and moose. The unique feature of CWD as a prion disease is its efficient transmission among cervids in nature. As with other prion infections, CWD disease inception relies on the conversion of the normal host cellular prion protein (PrP^{C}) to the abnormal, protease-resistant isoform (PrP^{CWD})--the diagnostic hallmark of prion diseases. Since its detection in Colorado in 1967, CWD has spread to captive and free-ranging cervid species in 16 additional states, 3 Canadian provinces, and one Asian country. CWD is also exceptional as the only prion disease to afflict a free-ranging, wildlife population. Understanding the facile means by which CWD is transmitted from animal to animal is important not only in understanding prion transmission overall but also in elucidating the potential public health consequences of cross species prion transmission. This dissertation work asks whether and how CWD prions are able to cross the oral and nasal mucosa to induce infection and disease.

The above questions were addressed through the use of two strains of transgenic mice that express the normal cervid prion protein [Tg(CerPrP)1536, Tg(CerPrP-

E226)5037+/-] and prion protein knockout mice [FVB $PrP^{0/0}$] exposed by either aerosol, nasal, or oral route to CWD prions. In the first series of studies, cohorts of Tg(CerPrP)1536 mice were exposed to brain homogenates from either CWD-infected or CWD-naïve deer via either aerosolization or direct nasal installation. In the second series of studies, cohorts of Tg(CerPrP-E226)5037+/- mice were exposed to the same inocula via installation onto the lingual mucosal surface which had or had not been previously subjected to superficial abrasions. Mice were then observed and tissues from each cohort, at time points ranging from weeks to as long as 2 years post inoculation, were examined for the presence of the abnormal prion protein of CWD (PrP^{CWD}) using western blotting and immunohistochemistry assays. The final studies employed the same inoculation techniques used in the first two studies to seek to identify early (less than 4 hours) sites of prion entry via the mucous membranes.

The results of these dissertation studies demonstrated; 1) that CWD could be transmitted by aerosol exposure with high efficiency compared with direct inoculation onto the nasal mucosa; and 2) that micro-abrasions to the lingual surface greatly facilitated CWD prion transmission. Finally and perhaps surprisingly, we were unable to detect PrP^{CWD} in either the nasal or oral mucosa shortly after inoculation or at any time, even after the onset of clinical symptoms of CWD. The results from these studies suggest that; 1) CWD prions can be transmitted by aerosol exposure; thus exposure to the respiratory system merits increased consideration in prion transmission and biosafety; 2) minor oral mucosal injury does greatly facilitate prion infection—a potentially significant co-factor in CWD transmission of foraging cervids; and 3) these mucosal pathways may

explain how and why CWD is transmitted with high efficiency in animals exposed to low concentrations of prions in nature.

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DEDICATION

I dedicate this work to:

My wife Natalie and my three children – Devin, Allie and Ryan.

Thank you for your flexibility and willingness to share me over the last 6 years. I could not have done this without your support, patience, love, and understanding.

I Love You All!

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INTRODUCTION

Prions and the 'Protein-Only' Hypothesis:

The term 'prion' was coined by Dr. Stanley Prusiner in 1982 to describe the "proteinaceous infectious particles" thought to be the cause of transmissible spongiform encephalopathies (TSE's) (97). At the time, this statement was highly controversial as prion diseases had been considered slow-virus diseases (114) for nearly 40 years. Yet the inception of the term was warranted given the following observations; 1) formalin and heat did not inactivate the scrapie agent (49); 2) high doses of ionizing and UV radiation also did little to inactivate the scrapie agent (3, 4); and 3) purified scrapie agent subjected to protease and electrophoretic studies indicated the infectious components were proteins that lacked nucleic acids (100, 101). The culmination of the previous studies led Prusiner to postulate the 'protein-only' hypothesis (97) as an alternative to the virus theory.

Prion Protein Structure and Function:

The normal host cellular prion protein (PrP^c) is endogenous, expressed in all tissues with the highest concentrations within the central nervous system (CNS) (11, 17, 37, 90). PrP^c is approximately 250 amino acids in length, contains key motifs including a series of five peptide repeats and a central hydrophobic core, flanked by a carboxy-terminal glycosylphosphatidylinositol (GPI) anchor and an amino-terminal signaling sequence (121, 127), and is susceptible to protease digestion (Fig. 1). Primarily localized

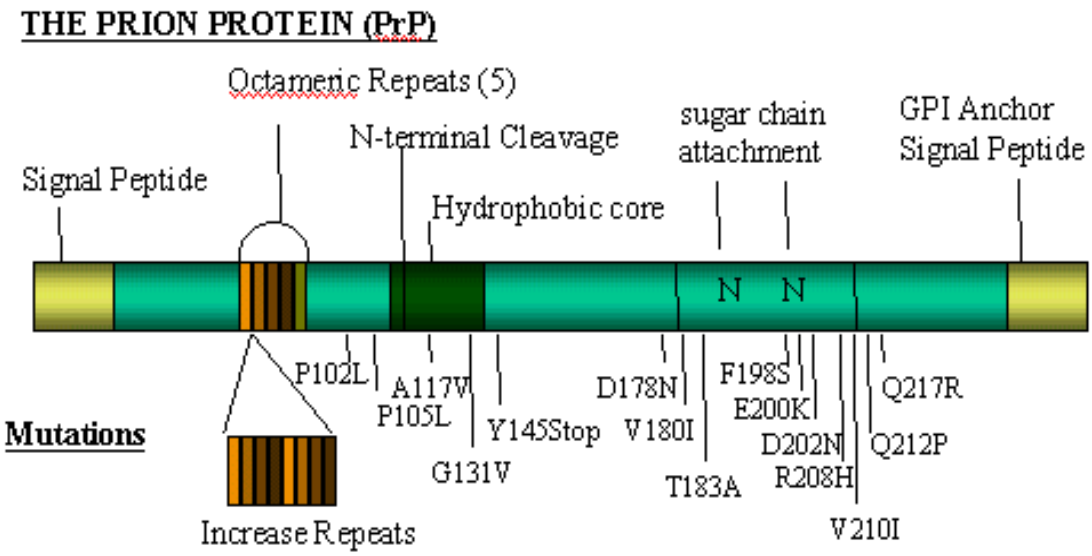

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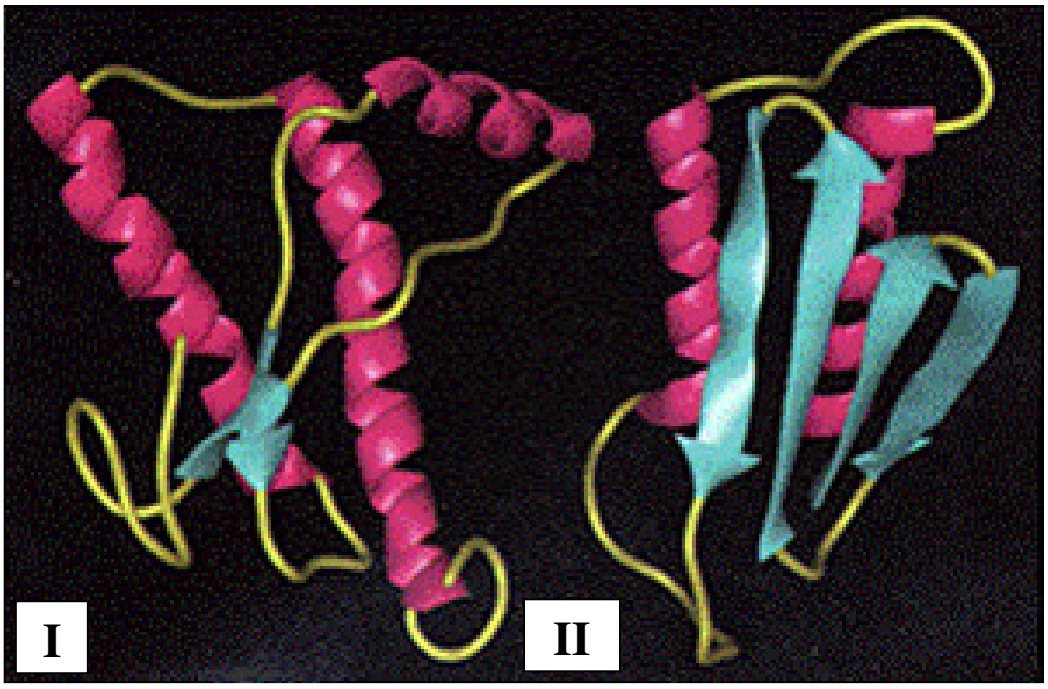
Fig. 1 Illustration representing the structural motifs of the prion protein

within lipid rafts of cell membranes, the function of PrP^{c} is thought to be related to copper (Cu^{2+}) binding, cell signaling, or adhesion (36).

The entire principle of prion diseases resides on the recruitment and conversion of PrP^{c} to the disease or protease resistant protein form – PrP^{res} (103). The mechanism behind the conversion process is still not fully understood. Initially, structural modification experiments revealed the scrapie agent to be a protein (84, 100, 101). Upon gene identification and amino acid sequencing, PrP^{c} and PrP^{res} were shown to be identical (9, 92) indicating post-translational modifications accounted for the structural differences. While implications that cellular factors such as nucleic acids, lipids, proteoglycans, and proteins have been considered in assisting the conversion process, no chemically modifying features have been identified (1, 16, 25, 120). Conversion was therefore believed to come about strictly through conformational change.

Evidence supporting this theory was revealed by the partially determined secondary structures of PrP^{c} and PrP^{res}. Results on both protein isoforms from Fourier-transform infrared (FTIR) spectroscopy, circular dichroism (CD) spectroscopy, and electron microscopy (EM) analysis demonstrated PrP^{c} consists predominantly of α-helical (~ 43%) content with a small β-sheet (~ 5%) [Fig. 2.I] while PrP^{res} is an enriched β-sheet (~ 40%) isoform with reduced α-helical (~ 30%) content [Fig. 2.II] (94, 105). This β-helix conformational shift renders the protein resistant to protease digestion (84) and allows for polymerization and aggregation (108).



www.udel.edu/chem/bahnson/chem645/websites/Janas/

Fig. 2 Proposed partial structural representations of the cellular [PrP^{c}] (I) and resistant [PrP^{res}] (II) prion protein.

Two possible scenarios for conversion have emerged; 1) a template-assistance (refolding) model and; 2) a nucleation-polymerization (seeding) reaction. The template-

assistance model states spontaneous conversion of PrP^{c} to PrP^{res} cannot occur due to high-activation energy barriers and that introduction and binding of exogenous PrP^{res} to PrP^{c} induces the conformational shift (102, 103). Host cellular factor(s)/chaperone(s) are required for conversion (24, 26, 104). Proposed structural intermediates (denoted PrP*) (28) formed by the interaction of PrP^{c} and unknown 'protein X' (123) are thought to lower the activation energy and facilitate conversion when bound to PrP^{res}, yet no compound has been found (1). Nucleation-polymerization assumes PrP^{c} and PrP^{res} are in thermodynamic equilibrium and when multiple PrP^{res} monomers assemble, a stable aggregate is formed (20). Initial aggregate formation is slow, yet once established, allows for faster recruitment and conversion of additional monomers (20). Whether aggregate formation within the central nervous system (CNS) is the root cause of the neuropathies associated with prion diseases remains unclear.

Transmissible Spongiform Encephalopathies (TSE):

While 'priontology' is a relatively new field in science, descriptive accounts of TSE's (scrapie) trace back over 200 years. In the late 50's, Klatzo et. al. (72) and Hadlow (51) recognized the similarities in histopathology between scrapie, Kuru, and Creutzfeldt-Jakob disease. The responsible agent was unaffected by filters designed to eliminate bacteria, therefore deemed a virus, and due to the long latency periods involved, these conditions were classified as 'slow virus' diseases (114).

Prion diseases are neurodegenerative disorders that are invariably fatal in both humans and animals, with no evidence of a febrile or humoral immune response (45, 51, 80). Clinical disease in humans typically manifest as progressive dementia with

involuntary tremors whereas animals usually present with noticeable ataxia (129). Histopathologic characteristics for both species include spongiform changes and astrocytic gliosis (98), with the true hallmark of prion disease being the accumulation of PrP^{RES} in the brain (84). Variations in clinical presentation and histological features exist in most prion diseases.

Mammalian prion proteins are highly conserved within species; differences in amino acid sequences and host cellular factors appear to mediate the species barrier or susceptibility of disease transmission between species (22, 78, 102). The existence of prion strains provides an additional explanation for disease variations seen within a species. Prion strains have distinct biological characteristics which can be classified by incubation period (30), pattern of PrP^{RES} deposition (57), histopathology (39), and differences in behavior (96). Biochemical characteristics, such as protease sensitivity and molecular weight differences after protease digestion can also be used in classifying prion strains (12).

Creutzfeldt-Jakob Disease (CJD):

Creutzfeldt-Jakob disease was first described in the early 1920's by Creutzfeldt and Jakob (76). CJD can arise spontaneously (133), affecting ~1 in 1 million individuals worldwide (81). Approximately 5 – 15% of diagnosed CJD cases are familial (18); i.e. associated with known mutations in the PRNP gene (60). Familial Fatal Insomnia (FFI) (48) and Gerstmann-Straussler-Scheinker disease (GSS) (60) are two examples of inherited prion diseases, but as many as 16 different polymorphisms with upwards of 55 pathogenic mutations have been identified (74). In 1968, Gibbs et. al. (47) demonstrated

that CJD was transmissible. Subsequent investigations revealed iatrogenic transmission of CJD occurred through corneal transplants (34), dura mater grafts (124), and hormone replacement therapy with human pituitary tissue (73). While the infectious component and routes are known in these cases, the transmission of other prion diseases remains highly variable.

Kuru:

Kuru is a prion disease of the Fore people of Papua New Guinea that primarily affected adult females and children of all ages, yet rarely adult males (44). Experiments in chimpanzees demonstrated that Kuru was transmissible (43). Natural disease transmission and familial patterns were explained by the practice of ritualistic cannibalism, where a mourning family, consisting only of women and children consumed the entire relative as a sign of respect (40). With the cessation of cannibalism, the incidence rate has decreased to almost zero. The origin of Kuru is not known yet one theory speculates that a sporadic case of CJD was responsible for the epidemic (42).

Transmissible mink encephalopathy (TME):

Outbreaks of transmissible mink encephalopathy in the United States and Europe are associated with the consumption of contaminated ovine or bovine feed (56, 79), although sporadic cases, albeit rare, do occur. Transmission studies into several species using TME material from a single outbreak demonstrated the existence of multiple strains (80). Expansive work from the previous study demonstrated the structure of PrP^{RES} is largely responsible for determining prion strains (12, 13).

Bovine Spongiform Encephalopathy (BSE):

Bovine spongiform encephalopathy, or 'mad cow' disease, was first recognized in the United Kingdom in 1986 (129). Evidence based on simultaneous countrywide outbreaks, the young age of affected animals, and method of calf rearing led investigators to the dietary supplement, meat and bone meal (MBM) as the potential source (131). Omission of organic solvents in the rendering process of MBM was thought to allow prion survival (131). Initiation of a ruminant feed ban effectively decreased the incidence rate, supporting the idea that MBM contained the infectious agent. During the epidemic, BSE transmission by contaminated MBM was confirmed in 43 exotic species (71) and the direct consumption of infected cattle caused variant CJD in humans (132).

Variant Creutzfeldt-Jakob Disease (v-CJD):

Variant CJD emerged in the mid 1990's exhibiting marked differences compared to sporadic CJD, such as age of onset (29 vs. 66 years), duration of illness (14 vs. 4.5 months), and behavior changes (132). Comparative analysis of v-CJD and BSE deposition of PrP^{RES} were similar compared to other CJD forms (29). Transmission of BSE to mice demonstrated neuropathology, deposition patterns of PrP^{RES}, and incubation times were identical to v-CJD (21). The delayed onset of v-CJD compared to the BSE epidemic saw the same initial increase in cases and has declined in accordance with the ruminant feed ban and decreased BSE cases. Kuru, TME, BSE, and v-CJD are similar in that they were all transmitted by the oral route.

Scrapie:

Index case reports of scrapie date back to the early 1700's. Unlike the previous TSE's that are artificially transmitted, scrapie is contagious (114), perpetuated by either vertical and/or horizontal transmission (31, 95). Scrapie pathogenesis involves a lymphatic phase prior to CNS involvement (128). Scrapie was never a public health concern until BSE emerged as an epizootic disease in Great Britain (129). Even then, no causative links between scrapie outbreaks and CJD cases were found (18). A major resurgence in the interest of prion diseases and the concern over the safety of human food came with the diagnosis of v-CJD (132). Consequently, chronic wasting disease (CWD) has fallen under intense scrutiny by the demonstration of BSE transmission to humans.

Chronic Wasting Disease:

Chronic wasting disease (CWD) is a TSE that affects mule and white-tailed deer (*Odocoileus hemionus and virginianus*), Rocky Mountain elk (*Cervus elaphus nelsoni*), and most recently moose (*Alces alces shirasi*) (6, 75, 106, 117, 137). CWD was first described as a spongiform encephalopathy of captive Colorado mule deer in 1967 (134). Within a decade, sporadic cases of CWD were reported in free-ranging deer and captive elk populations in Northern Colorado and Southeastern Wyoming. Shortly thereafter, Williams et. al. (136) intracranially (IC) inoculated infectious brain material into deer to establish that CWD was a TSE. At present, CWD has been identified in 17 states, 3 Canadian provinces, and the country of South Korea (33, 61, 68). Prevalence rates in wild herds can vary from < 1% to over 30%, but can exceed 90% in captive herds (85,

86, 89, 130, 134, 137). Interestingly, the cervid family is the only known free-ranging wildlife species affected by a TSE.

CWD Clinical Presentation:

Clinical presentation of CWD includes behavior changes (nervousness or hyper-excitability), excessive salivation, polyuria/polydypsia, emaciation, poor coat quality, drooping head and ears, repetitive patterning, and ataxia (117, 135). Gross pathology is noted as excessive fluid mixed with sand and gravel within the rumen along with a total loss of subcutaneous and abdominal adipose tissue (86, 117, 134). Clinical symptoms can vary greatly among animals. Manifestation of disease occurs in ages ranging from 18 months to 10+ years (average between 3-5 years) with the duration of clinical disease lasting from 2 weeks up to almost 1 year (6, 75, 86, 106, 117, 135, 136).

CWD Histological Features:

Histological evidence of disease was initially characterized solely by gross examination of central nervous system (CNS) tissue, as non-neural tissues exhibited no discernible lesion profile (135, 136). Gross lesions within the CNS include intraneuronal vacuolization, neuronal degeneration, and spongiosis of the grey matter, accompanied by a reactive gliosis, astrocytic hypertrophy and hyperplasia, and the presence of eosinophilic amyloid plagues (134-137). The extent of disease dissemination was greatly expanded upon by the discovery that the scrapie fibril antibody used in immunohisto-chemistry (IHC) cross-reacted against the CWD prion (50).

CWD Diagnosis:

An essential technique in diagnosing CWD by immunochemical means requires pre-treatment of test samples with proteinase K (PK) to eliminate cellular prion proteins (PrP^{C}), leaving the protease resistant core (PrP^{CWD}) which is detected with prion antibodies. Current diagnostic methods include conventional immunohistochemistry (IHC) for post-mortem analysis of obex and retropharyngeal lymph nodes and/or ante-mortem analysis of tonsil and rectal lymphoid tissue biopsies (116, 117, 130) and a United States Department of Agriculture (USDA) approved enzyme-linked immunosorbent assay (ELISA) for tissue analysis (59). An additional laboratory method used to visually delineate PrP^{C} from PrP^{CWD} is the standard western blot (WB). This assay demonstrates in uninfected material the absence or presence of PrP^{C} in PK vs. non-PK treated samples (respectively), whereas the molecular weight difference of the truncated PrP^{CWD} protein of infected material is shown in PK vs. non-PK treated samples, respectively.

CWD Pathogenesis:

With the advent of an immunological marker to detect PrP^{CWD} aggregates, studies were undertaken to investigate the pathogenesis of CWD. Initial experiments confirmed the presence of PrP^{CWD} aggregates within the retropharyngeal lymph node (RLN) , tonsils, and Peyer's patch of orally inoculated deer (113) and in the CNS of terminal, naturally exposed deer (117). These findings concurred with scrapie studies which had shown equivalent early accumulation and terminal patterns of PrP^{Sc} after oral inoculations and naturally occurring disease, respectively (5, 52, 58). Complete tissue

analysis of free-ranging and captive, naturally infected animals confirmed that the CNS and lymphatic tissues contained the highest concentration of CWD infectivity (77, 118, 119), with the observation that oropharyngeal and gut associated lymphoid tissue (GALT) infections appeared] to precede CNS involvement. Subsequent study of naturally occurring and oral inoculation cases reaffirmed that lymphoid tissues were the primary sites of PrP^{CWD} accumulation (38, 75, 115, 130). Involvement of the myenteric plexus, vasosympathetic trunk, and nodose ganglion (10, 112) provided evidence for theoretical, internal trafficking routes from the enteric nervous system to the CNS, yet no definitive mechanism for initial uptake has been elucidated.

CWD Transmission:

While the trafficking of prions from lymphatic sites to the CNS is becoming well defined, the question of *how* prions are naturally acquired remains unknown. CWD is thought to be transmitted horizontally, as current evidence does not support vertical transmission (87-89). Bodily fluids and excreta, including urine, feces, saliva, and blood have all been shown to contain infectious CWD prions, albeit small quantities (53, 67, 83, 122). Assumptions that CWD can be transmitted through direct contact during social interactions have been made; however studies demonstrated that indirect mechanisms (animals housed on previously contaminated pastures, fomite transmission) proved highly effective (82, 88). While prion amounts excreted into the environment are minimal, confounding evidence related to prion – soil interactions, persistence, and infectivity suggests soil as a significant reservoir for scrapie and CWD infectivity.

Scrapie prions bind to soil particles with high affinity (66), possibly enhancing initial infectivity (65), and remain infectious for 3 years under experimental conditions (19, 110) with suggested environmental persistence of at least 16 years (46). Environmental contamination could therefore play a large role in disease transmission as soil consumption is part of natural cervid behavior (15, 63). In any case, exposure and entry of CWD prions through the oral and/or nasal mucosa seems assured.

Cervids have a highly-developed olfactory epithelium which is used to monitor their environment for food, predators, prey, and pheromones (reproductive cues) (35, 125). The Flehmen response is used in particular to draw in pheromones and other aerosolized and solid compounds into the vomeronasal organ (VNO), which is a specialized neurosensory organ in the anterior nasal mucosa (32). Aside from detecting environmental odors, respiratory epithelium can be exposed to disease causing agents through normal respiration.

Studies have demonstrated that cervids are highly susceptible to *Mycobacterium bovis* and foot-and-mouth disease via aerosol transmission (2, 27, 93). In 1995, Shaw (111) suggested the occurrence of shortened incubation periods for CJD in farmers could have come from "breathing the dust from feed containing prions." While this theory was never investigated, subsequent studies employing scrapie and TME prions demonstrated intranasal inoculations were effective, and surprisingly more efficient than *Per os* (oral) inoculations (14, 55, 70, 107). It is therefore reasonable that the nasal passages of cervids are likely exposed to CWD prions when performing the Flehmen response and social nuzzling.

Prior to the studies contained in this dissertation, no information pertaining to the potential risk posed by aerosolized prions or the exposure of the nasal mucosa to CWD prions existed. Demonstrating aerosol transmission of CWD prions could warrant the re-evaluation of respiratory system exposure in prion disease transmission. Additionally, re-evaluation of biosafety protocols might be required depending on the potential risk. Current CDC recommendations are that human and animal prions should be manipulated at biosafety level (BSL)-2, with certain BSE-human circumstances in which splashes and/or aerosolized infectious material could be created requiring additional physical containment or BSL-3 (62).

Oral prion inoculation studies have been conducted in attempt to mimic a more natural route of infection. Consistent outcomes in these experiments, when compared to the conventional intracranial inoculations, were that a substantially larger inoculum dose was required, incubation times were longer, and disease transmission was less efficient (54, 69, 99, 109, 113, 126). These studies were also problematic in that brain tissue, most likely an atypical source of shed prions in nature, was used as the inoculum. When infectious excreta were administered orally, saliva was sufficient to initiate clinical disease whereas urine and feces produce an asymptomatic disease that was not detectable by conventional means (53, 83). These findings suggest that the amount of infectious prions were substantially less in urine and feces and that longer incubation times are likely required to establish an infection.

CWD is most assuredly transmitted through the oral route, yet the amount of infectious material in excreta indicates that animals would either have to ingest a single, massive quantity, be exposed for an extended amount of time, or perhaps be exposed at

some permissive mucosal portal or site. The latter situation implies that prions require assisted entry. A possible co-factor, such as an abraded mucosal surface might play a role in facilitating transmission. In 1985, Gajdusek (41) speculated that Kuru could be passed to conjunctiva by eye rubbing, nose picking, or through mucosal injury. A study in mice (23) and hamsters (64) demonstrated that infection by the scrapie agent was enhanced if the inoculum was applied to scarified gingival tissue. Subsequent studies demonstrated that inoculation of TME or scrapie agent onto abrasions on the tongue or by intra-lingual injection produced up to a 100,000-fold more efficient transmission rate than standard oral inoculation (7, 8, 14, 54, 91). The above studies were seminal for this dissertation research on the transmission of CWD.

Questions addressed in this dissertation:

1.) Can CWD prions be transmitted in an aerosolized form?

2.) Are the nasal passages a viable route for initiating CWD infection?

3.) Do lingual abrasions facilitate the transmission of CWD?

4.) Can the site(s) of CWD prion entry in the oral and/or nasal mucosa be discerned shortly after inoculation?

Dissertation Research:

The background of TSE research provided the basis for the specific aims of this dissertation research. The first objective was to determine whether CWD prions could be transmitted via exposure of the nasal mucosa and/or VNO. Our goal was to investigate pathways whereby CWD prions are passed efficiently from one animal to the next. This

objective was addressed by using transgenic mice that express the normal cervid prion protein [Tg(CerPrP)1536] exposed by either aerosolized particles or direct nasal instillation and assessed for disease by clinical presentation and detection of PrP^{CWD} by western blotting and immunohistochemistry assays.

The second objective was to determine whether CWD prions could be transmitted via exposure of the intact or disturbed oral mucosa. Our hypothesis was that the transit of CWD prions across the alimentary tract mucosa would be substantially enhanced by minor abrasions to the oral mucosa—a phenomenon especially pertinent to cervids that commonly subsist on abrasive forage. Our rationale in the studies conducted was to determine whether lingual abrasions lead to shorter incubation periods, thereby providing insight into one mechanism for the facile transmission of CWD under natural conditions. We addressed this objective by again using cervidized transgenic mice [Tg(CerPrP-E226)5037+/-] exposed to CWD by a single inoculation to the surface of the tongue in mice that had or had not received superficial mucosal abrasions. We then monitored and assessed these animal cohorts for CWD infection by clinical criteria and examination for PrP^{CWD} in tissues by western blotting and immunohistochemistry assays.

The third objective was to determine the early tissue site(s) of CWD prion transit across the mucosal surface after inoculation. We hypothesized that; 1) PrP^{CWD} could be detected in the olfactory epithelium, stimulated VNO, or NALT after respiratory exposure; and 2) PrP^{CWD} could be detected at the abrasion site of the lingual mucosa after oral exposure. Our goal was to determine the cellular association of PrP^{CWD} in the respective mucosa immediately or within hours after inoculation in order to elucidate the mechanism(s) behind early pathogenesis. We addressed this objective through the use of

cervid, prion protein expressing transgenic mice [Tg(CerPrP)1536, Tg(CerPrP-E226)5037+/-] and a prion protein knockout mouse [FVBPrP$^{0/0}$] exposed by aerosol, nasal, or oral routes. Serial sacrifices were performed within hours and tissues were assessed by the tyramide signal amplification immunohistochemistry assay in attempt to reveal sites of early prion entry.

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CHAPTER 1*

Aerosol and Nasal Transmission of Chronic Wasting Disease in Cervidized Mice

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ABSTRACT

Little is known regarding the potential risk posed by aerosolized prions. Chronic wasting disease (CWD) is transmitted horizontally, almost surely by mucosal exposure, and CWD prions are present in saliva and urine of infected animals. However, whether CWD may be transmissible by the aerosol or nasal route is not known. To address this question, FVB mice transgenetically expressing the normal cervid PrP^{C} protein [Tg(cerPrP) mice] were exposed to CWD prions by either nose-only aerosol exposure or by drop-wise instillation into the nostrils. Mice were monitored for signs of disease for up to 755 days post inoculation (dpi) and by examination of tissues for lesions and PrP^{CWD} after necropsy. In particular, nasal mucosa, vomeronasal organ, lungs, lymphoid

tissue, and the brain were assessed for PrP^{CWD} by western blotting and immunohistochemistry. Six of 7 aerosol-exposed Tg(cerPrP) mice developed clinical signs of neurologic dysfunction mandating euthanasia between 411 and 749 dpi. In all these mice, CWD infection was confirmed by detection of spongiform lesions and PrP^{CWD} in the brain. Two of 9 intra-nasally inoculated Tg(cerPrP) mice also developed TSE associated with PrP^{CWD} between 417 and 755 dpi. No evidence of PrP^{CWD} was detected in CWD-inoculated Tg(cerPrP) mice examined at pre-terminal time points. These results demonstrate that CWD can be transmitted by aerosol (as well as nasal) exposure and suggest that exposure via the respiratory system merits consideration for prion disease transmission and biosafety.

INTRODUCTION

Chronic wasting disease (CWD) is an efficiently transmitted prion disease [transmissible spongiform encephalopathy (TSE)] affecting deer, elk, and moose. Although discovered in northern Colorado and southern Wyoming, CWD has since been identified in 13 additional states, 3 Canadian providences and Korea (7, 15, 20). CWD is unique as the only TSE that occurs in wild animal populations.

Available information indicates that CWD is transmitted by some horizontal means, most likely involving trans-mucosal entry (26, 30, 38). CWD prions are known to be present in saliva and urine of infected cervids (10, 24) and excreted prions can contaminate the environment and persist for years, given that clay components of soil bind prion proteins with high affinity (16). Exposure of the nasal or other respiratory

mucosa via aerosolization or direct contact to CWD prions is sufficient to initiate infection and disease remains unproven, yet plausible. Precedent for nasal transmission of prion disease can be found in studies of experimental scrapie in sheep and mice and transmissible mink encephalopathy (TME) in hamsters (11, 21, 28).

Olfactory sensory nerve endings in the olfactory epithelium (4) of animals such as cervids are highly-developed to monitor the environment, e.g. to locate food, predators and prey, and detect the pheromones initiating reproductive behaviors (8, 36). Reproductive cues in particular are detected by the Flehmen response which draws the trapped particulates into the vomeronasal organ (VNO), a specialized neurosensory region in the anterior nasal mucosa (6). The Flehmen response and social nuzzling both provide opportunities for CWD prions to enter the nasal passages.

To determine directly whether CWD is transmissible by the respiratory route, cervid PrP-expressing transgenic mice (Tg(CerPrP) mice) were exposed to CWD prions by aerosolization or intranasal instillation. We present evidence that exposure of the respiratory mucosa to CWD prions is sufficient to transmit the disease after long incubation periods.

MATERIAL AND METHODS

Tg(CerPrP) Mice:

The cervid PrP-expressing transgenic mice and their susceptibility to CWD infection after intracerebral inoculation have been described previously (3). All mice were cared for in accordance with Colorado State University ACUC guidelines.

Confirmation of the cervid PrP^C gene insert was performed by western blot and PCR. The Tg(cerPrP) mice were 5 to 12 weeks of age at inoculation. After inoculation, mice were examined for evidence of neurologic abnormality every 2 days and weighed weekly (starting at 3 months post inoculation). Criteria for assessing CWD symptoms included ataxia, lethargy, tail rigidity, poor coat quality, and weight loss. Once the onset of clinical signs was observed, mice were isolated into individual cages to prevent cannibalism from cage mates. Mice were euthanized when distinct signs of neurologic disease were evident, accompanied by an age-matched sham control. CWD and sham-inoculated mice were housed in separate rooms to minimize potential for cross contamination.

Inocula and Inoculation Routes:

The CWD inoculum consisted of brain homogenate from CWD-infected mule deer (D10), obtained through the courtesy of Dr. Michael Miller, Colorado Division of Wildlife. CWD-negative (sham) control brain homogenate was from a CWD-naive white-tailed deer brain (UGA) obtained through the courtesy of Drs. David Osborn, Carl Miller, and Robert Warren at the University of Georgia Warnell School of Forestry. Brain homogenates were prepared in 1X phosphate-buffered saline (PBS) to a final concentration of 10% (w/v) for intranasal, 5% (w/v) for aerosol and 1% for intracerebral (IC) and per os (PO) inoculations. All mice (except IC and PO) were administered a 0.01 mL of epinephrine intraperitoneally, immediately post inoculation to stimulate the Flehmen response.

In two separate experiments, two cohorts of Tg(CerPrP) mice (n=6 CWD and n=6 sham) were inoculated by exposure to an aerosol of 5% (w/v) CWD brain homogenate for 4-minutes in a custom designed chamber providing nose-only exposure. The mice were monitored until clinical symptoms were detected or until study termination at 749 days. Two additional cohorts of Tg(CerPrP) mice (n=24 CWD and n=24 sham) were inoculated with 10 µL (5 µL per nostril) of the 10% weight to volume (w/v) extracts by direct pipet instillation into the nasal passages. A total of 12 mice in each cohort (n=4/time point) were sacrificed at 7, 14 and 28 days post inoculation (dpi) and analyzed for early PrP^{cwd} detection. The remaining mice (12/cohort) were monitored either until clinical symptoms became apparent or to study termination at 755 days. Per os inoculations into Tg(CerPrP) mice were administered in 2-50µl doses of a 1% extract given on 2 consecutive days. Intracerebral inoculations into Tg(CerPrP) mice of 30µl of a 1% extract from the same positive mule deer (D10) or naïve white tailed deer (UGA) served as positive and negative controls.

Aerosolizing Chamber:

Aerosolizing chambers commonly expose the entire animal to the agent being investigated. Our goal to expose only the nose/nasal passages to CWD prions vs. the entire animal. Thus we (NDD/JHK) fashioned an exposure chamber using a 473 ml, rubber-sealed lid container (Rubbermaid) into which 4 – 1" diameter holes were drilled into the side walls. Four, 50 ml conical tubes (BD) with the tips removed were inserted and sealed into the holes. Each tube was supported by an additional tube that collectively functioned as legs. A 3/4" x 1/2" hole was also cut into the container to accommodate

the mouth of the Omron Nebulizer (NE-C21, J. H. Inc.). The conical tubes and the nebulizer were sealed in place with silicone adhesive (GOOP). Finally, a 0.22 µm filter unit (Sterivex™, Millipore) was attached and sealed to the lid of the container to serve as an air vent and trap aerosolized particles (Fig. 4a). Once the mice and the inocula were loaded into the apparatus (Fig. 4b), the entire chamber was placed in a secondary enclosure and exposure occurred in a separately vented room.

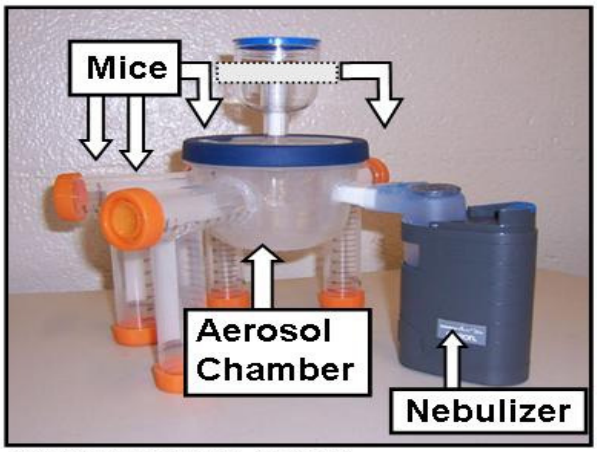

Fig. 4a Aerosolizing chamber

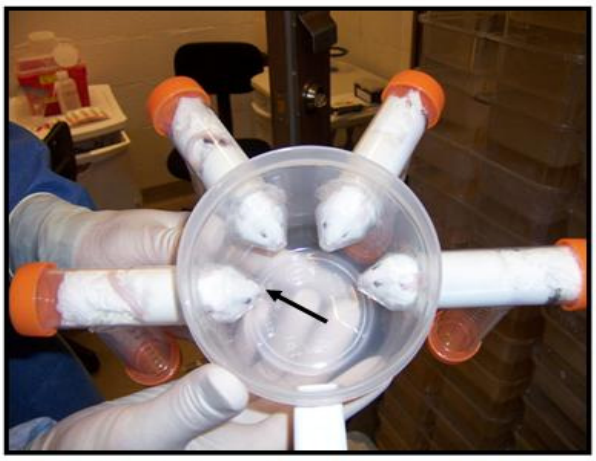

Fig. 4b Mice in Aerosolizing chamber

Fig. 4a. Aerosolizing chamber with nebulizer chamber and four plastic enclosures to accommodate anesthetized Tg(CerPrP) mice.

Fig. 4b. Top view of aerosol chamber with lid removed showing Tg(CerPrP) mice inserted in place to provide nose-only exposure to the chamber (arrow).

Statistical Analysis:

Statistics (Fisher Exact test) were performed using the software package Graphpad Prism™ 4.

Delivered Dose Estimations:

To approximate the total solid mass of brain inoculum deposited onto the mucous membranes of each aerosol-exposed mouse, we used a lognormal distribution generator program created by Dr. J. Volckens (Department of Environmental and Radiological Health Sciences, Colorado State University), using modified equations (14) for aerosolized particles. Using particle sizes generated by the nebulizer of 0.1 to 10 μm (log distribution), the estimated number of particles per each size generated and a designated mass for each particle size, the total mass for each particle size was calculated. The total mass was then multiplied by a deposition fraction (percentage of particles deposited in the respiratory tract based on size) and the fraction of total air inhaled by each mouse (1.2%; [(mouse respiration rate (163) multiplied by tidal volume (.00015 L)) / air flow through the system (2 lpm)]) to give the total deposited particle mass. Using a 5% brain homogenate, with brain material containing approximately 10% solid material, we calculated the mass fraction of solids per particle to be 0.005g (0.05 x 0.10). This fraction multiplied by the total deposited particle mass for each particle size produced the total mass of solid material deposited. The sums from each particle size deposited mass where then added to generate the total amount of solid inocula deposited into each mouse.

Similar algorithms were used to estimate the total amount of solid inoculum deposited into each intranasally inoculated mouse based on 10 µL of a 10% brain homogenate.

Western Blotting:

Harvested tissues were prepared at 10% (w/v) in a 1X PBS/1% Triton-X 100 mixture. Glass beads (2.5mm, Biospec) were added to each tube and samples subjected to one Fastprep™ (Biosalvant) cycle for 45 seconds at a speed setting of 5.5, followed by a 2 minute cool down at -20º. Samples were then centrifuged for 5 minutes at 2000 rpm to remove tissue debris and supernatants stored at -20º until further use.

Samples were incubated with 50 mg/µL of Proteinase K (Invitrogen) for 30 minutes at 37º, shaking at 1000 rpm. Samples were then combined with a reducing agent/sample buffer (Invitrogen) to a final concentration of 1X and run through NuPage 10% Bis-Tris gels (Invitrogen) for 2.5 hours at 100 volts. Proteins were then transferred to 0.22µm PVDF membranes (Millipore) over 1.5 hours at a setting of 110 volts. Membranes were blocked in a casein/TBS (Thermo Scientific) + 0.2% Tween-20 mixture for 45 minutes, shaking at room temperature. Monoclonal antibody BAR-224 (Spi-Bio) conjugated with HRP was used to detect the PrP^{CWD} (1:20,000 dilution in casein/TBS +0.2% Tween- 20). Membranes were washed 3 times and developed with ECL Plus Western Blotting Detection kit (GE). Blots were viewed and photographed with a Gel Doc system (LAS-3000, Fujifilms).

Immunohistochemistry:

Tissues were fixed in 10% formalin for 5 days, transferred to 60% ethanol (with the exception of the nasal passages), embedded in paraffin, and sectioned at 5μm for staining. Nasal tissues were placed in a 10% tetra-sodium EDTA (Sigma) solution for 10 days, with a fresh solution change occurring on the fifth day. Slides were de-paraffined through a series of xylene/ethanol baths, treated in 89% formic acid for 30 minutes, rinsed in running water for 5 minutes, and then subjected to a 15 minute, antigen-retrieval process (Pickcell Laboratories, Netherlands). The Dako Autostainer was used for conventional immunohistochemistry. Briefly, slides were blocked with 3% H_2O_2 in methanol (30 minutes), blocked with TNB buffer (30 minutes), incubated with a 1:250 dilution of HRP-conjugated BAR-224 in TNB (45 minutes), developed with chromagen AEC [3-Amino-9-Ethylcarbazole, Dako] (10 minutes), and counterstained with hematoxylin (5 minutes) and bluing reagent (1 minute). Both H&E and IHC sections were evaluated for the presence of TSE lesions which were characterized by neuronal loss, spongiform change, and gliosis.

RESULTS

Intracerebral (IC) exposure:

All IC inoculated mice exhibited clinical symptoms of CWD at 168 +/- 4 days post inoculation (dpi) and were euthanized. PrP^{CWD} was detected in all positive controls by western blot (WB) and immunohistochemistry (IHC), thus confirming the infectivity of the inoculum (data not shown).

Aerosol exposure:

In our first aerosol exposure of Tg(cerPrP) mice, 4 of 6 CWD and 3 of 6 sham-exposed mice died within 0 to 6 days post inoculation. The cause of death was not determined with certainty. After modification to the exposure protocol no similar fatalities occurred. Of a total of the 7 mice exposed to CWD by aerosol, 6 developed TSE between 411 and 749 dpi. None of the sham-inoculated mice developed evidence of prion infection through 749 dpi of observation. Neurological signs, survival times, weight loss, and development of TSE lesions (primarily neuronal loss/vacuolation and gliosis) within the brains of aerosol-exposed mice are summarized in Table 1 and Fig. 1.

Table 1 Aerosol Transmission of CWD in Tg(cerPrP) mice

Animal*	Neurologic Signs†	Survival (DPI)	Weight loss (% max body wt)	PrP^{CWD} WB	PrP^{CWD} IHC	TSE Lesions
Sham n=5	-	411-749	2 (average)	-	-	-
384	+	411	31	+	+	+
746	+	417	32	+	+	+
739	+	457	35	-	-	-
732	+	524	27	+	+	+
734	+	563	31	+	+	+
738	-	708	11	-	+	-
380	+	749	12	+	+	+

* One sham control was sacrificed when a positive mouse was euthanized (if possible)
† Animals were considered positive if they exhibited at least 3 of the following symptoms: Ataxia, lethargy, tremors, weight loss, poor coat quality, rigid tail

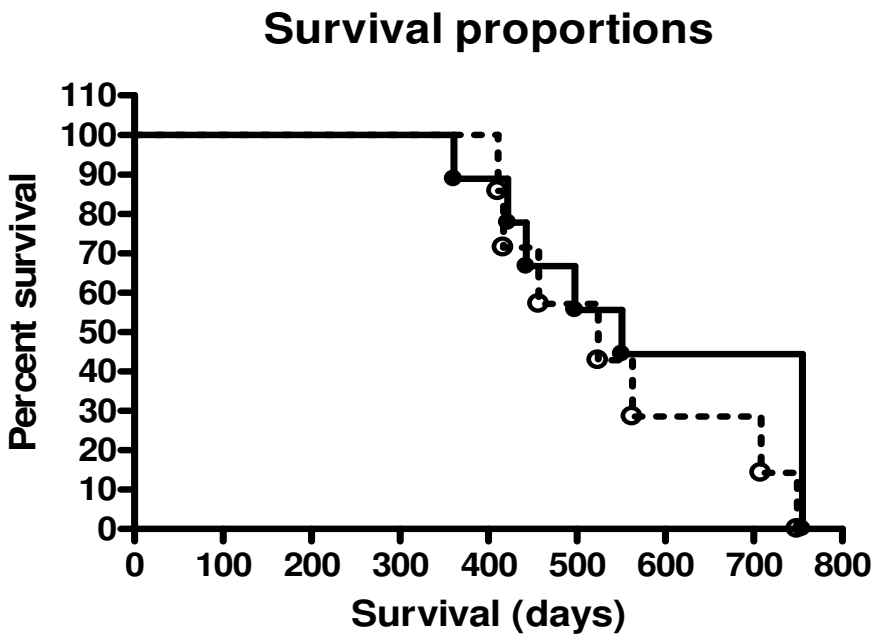

Fig. 1 Kaplan-Meier survival plot of Tg(CerPrP) mice exposed to CWD by aerosol (○, black dotted line) vs. intranasal instillation (●, black solid line).

Western blot and immunohistochemistry analysis of brains from the 7 CWD-aerosol-exposed mice demonstrated PrP^{CWD} in 6 (Fig. 2a & 3a). All of the mice exposed by aerosol to CWD-negative control deer brain inoculum were negative for PrP^{CWD} by WB (Fig. 2a; lane 1 & 2) and IHC (Fig. 3b). Immunohistochemistry of the nasal passages, including the VNO, olfactory and respiratory epithelium, and nasal-associated lymphoid tissue (NALT), as well as the olfactory bulb of the brain and spleen, did not reveal PrP^{CWD} (data not shown).

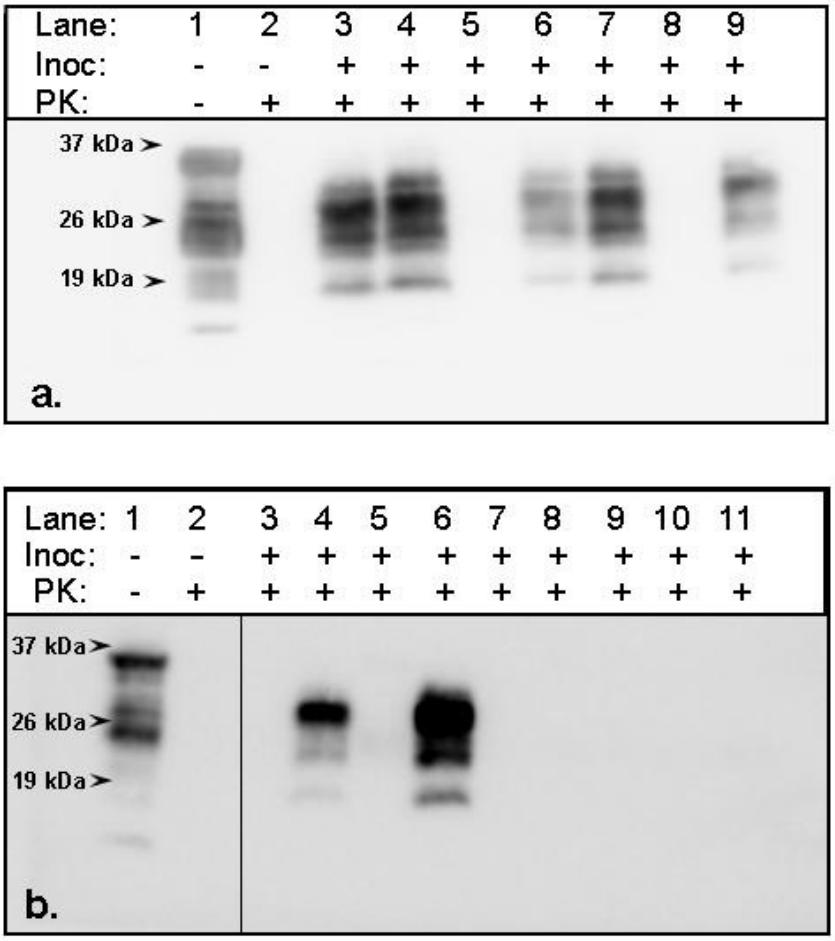

Fig. 2

Fig. 2a. PrP^{CWD} (lanes 3, 4, 6, 7, & 9) in brains of Tg(CerPrP) mice exposed to aerosolized CWD prions demonstrated by western blot. Aerosolized sham control (lane 1 & 2) showing no PrP^{CWD}.

Fig. 2b. PrP^{CWD} (lanes 4 & 6) in brains of Tg(CerPrP) mice exposed to CWD prions intra-nasally demonstrated by western blot. Intra-nasal sham control (lane 1 & 2) showing no PrP^{CWD}.

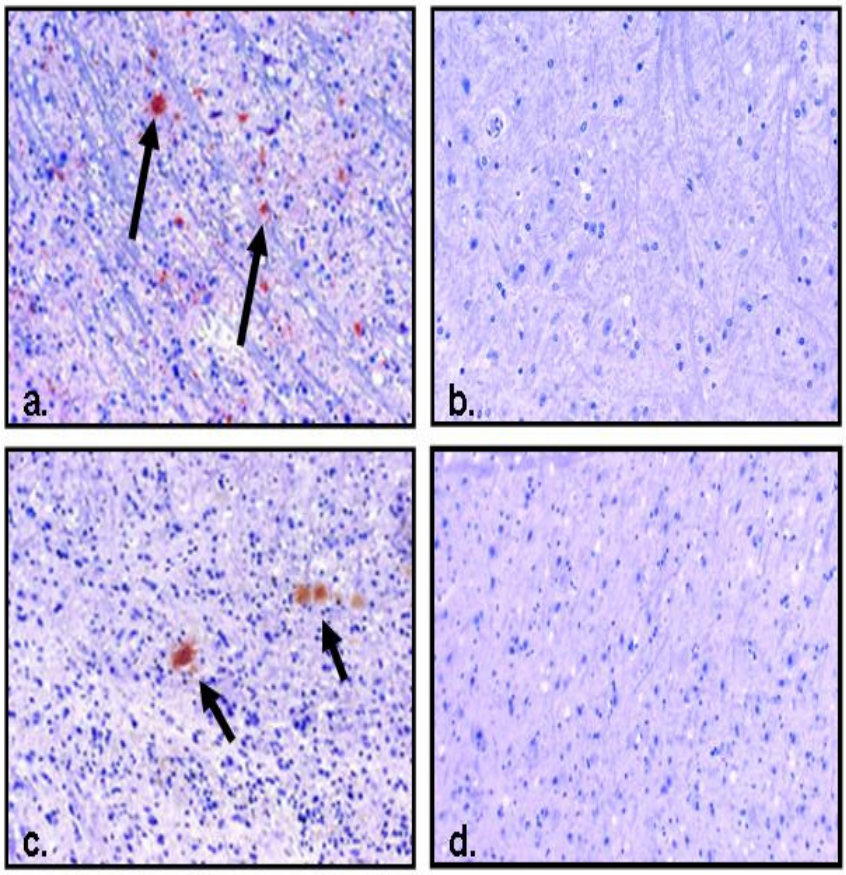

Fig. 3

Fig. 3. Immunohistochemistry (IHC) from the obex region of the medulla from Tg(CerPrP) mice (20X). (a) PrP^{CWD} (arrows) in a mouse exposed to CWD by aerosol vs. (b) mouse exposed to sham inoculum. (c) Mouse exposed to CWD by intranasal route demonstrating PrP^{CWD} aggregates (arrows) vs. (d) mouse exposed intra-nasally to sham inoculum.

Intranasal (IN) exposure:

Nine of 12 Tg(cerPrP) mice exposed to CWD by intra-nasal (IN) inoculation survived past 250 dpi. Two of these 9 mice developed TSE at 422 and 498 dpi, respectively. A summary of neurological signs, survival times, weight loss, and development of TSE lesions (primarily neuronal loss/vacuolation and gliosis) is in Table 2 and Fig. 1.

Table 2 Intranasal Transmission of CWD in Tg(cerPrP) mice

Animal*	Neurologic Signs†	Survival (DPI)	Weight loss (% max body wt)	PrP^{CWD} WB	PrP^{CWD} IHC	TSE Lesions
Sham n=9	-	361-755	5 (average)	-	-	-
635	+	361	39	-	-	-
641	+	422	43	+	+	+
646	+	443	50	-	-	-
631	+	498	17	+	+	+
633	-	551	+14	-	-	-
630	-	755	23	-	-	-
632	-	755	15	-	-	-
645	-	755	19	-	-	-
648	-	755	35	-	-	-

* One sham control was sacrificed when a positive mouse was euthanized
† Animals were considered positive if they exhibited at least 3 of the following symptoms: Ataxia, lethargy, tremors, weight loss, poor coat quality, rigid tail

Both IN-inoculated mice that developed TSE had PrP^{CWD} accumulation in the brain, as detected by WB (Fig. 2b; lane 4 & 6) and IHC (Fig. 3c). None of the mice exposed IN to CWD-negative control deer brain inoculum displayed clinical signs of CWD. All were negative for PrP^{CWD} by WB (Fig. 2b; lane 1 & 2) and IHC (Fig. 3d). In none of the negative control or CWD-inoculated mice sacrificed at planned time points or

dying spontaneously was evidence of PrP^{CWD} or histopathologic lesions of TSE detected (data not shown). PrP^{CWD} was also not detected in the nasal passages, olfactory bulb, or spleen of any mice (data not shown).

Statistical Analysis:

Using Fisher's Exact test demonstrated the attack rates in Tg(CerPrP) mice exposed to CWD by aerosol vs. nasal inoculation were significantly different (Fisher's Exact test, p value = 0.0406).

Dose Estimations:

To estimate the quantity of brain material delivered per mouse by aerosol exposure, we used a lognormal distribution generator assumptions and calculations as described in Methods. Based on particle sizes ranging from 0.1 to 10μm (log distribution) generated by the nebulizer we estimated that 24 μg of solid brain material was deposited on the mucous membranes of each mouse. Applying the same algorithm to intranasal exposure produces an estimated 100 μg of brain material delivered per mouse.

DISCUSSION

In 1995, Shaw (29) suggested that the occurrence of a shorter incubation period for Creutzfeldt-Jacob disease in farmers could have come from "breathing the dust from feed containing prion". The present studies were done to simulate the inhalation of

airborne particles and the direct contamination of the nasal passages with CWD prions. The finding that CWD prions can be transmitted via inhalation (perhaps even more effectively than by nostril contact only) while unique for CWD infection, extends precedent for transmission of prions via the respiratory system. Sheep and hamsters inoculated with scrapie intra-nasally (11, 28) and hamsters inoculated with TME extra-nasally (21) have been shown to develop TSE. Results in the latter study suggested that the nasal passages may even be more effective than the oral route in transmitting prion disease (21).

In the present study, 86% (6/7) of Tg(cerPrP) mice exposed to CWD via aerosol developed CWD vs. 22% (2/9) of IN-exposed mice (Fisher's Exact test, p value = 0.0406). It may be argued that the early deaths of 4 mice in the initial CWD-aerosol exposure group could bias the statistical significance of the results. After modifications to the exposure procedure, early mortalities were eliminated. Moreover, 2 of 2 surviving mice in the initial exposure and 4 of 5 mice in the second exposure study developed TSE.

One possible explanation for the enhanced infectivity after aerosol exposure might be the disruption and dispersion of infectious PrP^{CWD} aggregates during aerosolization to yield more small infectious particles or seeds (31). These smaller aggregates might be more readily taken up by lymphoid or distal airway epithelial cells not typically accessible by either nasal contact or drop-wise instillation of prions.

Another potential explanation for enhanced infection after aerosolization could be that a larger prion dosage was delivered by aerosol vs. nasal exposure. Based on a random distribution of infectivity in the aerosol material, the respiratory tidal volume of mice and the average anesthetized respiratory rate for a 4 min period, we calculated that

aerosol-exposed mice would receive approximately 1.2% of the available inoculum, or a maximum of 24µg of particulate inoculum deposited in the respiratory system. Mice inoculated intra-nasally received 10µl of a 10% brain homogenate, or 100µg of particulate inoculum--4 times that delivered by aerosol. Given that only the nostril region (< 5mm) of each mouse was exposed during aerosolization, we estimate that even if up to an additional 15µl of inoculum were to be ingested due to nasal region grooming, the total dosage would not surpass (and more likely would never attain) that delivered intra-nasally.

Clearly inoculation by either the aerosol or nasal route would result in inoculum entering the alimentary tract via the nasopharynx. Thus oral exposure and potential uptake cannot be avoided or excluded. However, oral inoculation of Tg(cerPrP) mice with 100µg CWD particulate brain homogenate failed to transmit CWD infection or disease after > 700 days of observation (Seelig, DM, Mason, GL, Telling, GC, Hoover, EA, unpublished results)(Table 3). Thus it is unlikely that CWD transmission by the aerosol or nasal routes reflects infection via the alimentary tract.

Table 3 Summary of CWD Inoculation Results in Tg(cerPrP) mice

Route	Exposure Dose	Attack Rate*
Aerosol	24µg †	6/7 (86%)
Intranasal	100µg ‡	2/9 (22%)
Oral	100µg §	0/10 (0%)

*No. affected/total (% positive)
† 4 min aerosol of 5% w/v homogenate
‡ 10µl of 10% w/v homogenate
§ 100µl of 1% w/v homogenate

Mice are an excellent model for studying airborne and direct nasal contact transmissions because they are obligate nasal breathers (22). Odorants and particles inhaled into the nasal passage are subject to a number of cell surfaces. Thus multiple sites of CWD prion entry are plausible, including the nasal-associated lymphoid tissue (NALT), the mucosal associated macrophages and/or dendritic cells, respiratory epithelium, olfactory epithelium, and vomeronasal organ (VNO). The NALT typically incorporates the retropharyngeal lymph nodes, palatine, and lingual tonsil (23) and is similar in structure and function to the gut-associated lymphoid tissue (GALT) in that it is responsible for antigen uptake and presentation by M cells, B cells, and follicular dentritic cells (13, 23). The GALT, especially the Peyers patches are considered to be the primary site of PrP^{Sc} uptake for BSE, vCJD, and scrapie (1, 9, 12, 27, 35, 37).

Mice do not have tonsils or retropharyngeal lymph nodes per se, but rather a bi-symmetrical NALT structure that lines the floor of the nasal cavity (13). Kinkaid and Bartz (21) found that hamsters inoculated via uptake of droplets or TME inoculum via the external nares had shorter incubation periods using lower dose of infectious prions than those inoculated orally. Studies in deer inoculated orally or naturally exposed to CWD indicate that the primary structures that accumulate PrP^{CWD} early in infection are the retropharyngeal lymph nodes and tonsils (18, 30, 32). Nevertheless, in the present study we were unable to demonstrate PrP^{CWD} in the NALT of early, pre-terminal, or terminal Tg(cerPrP) mice after aerosol or nasal exposure to CWD.

Another seemingly likely site for prion entry and infection is the olfactory mucosal epithelium, which contains odor receptors that provide a direct neural connection from the nasal cavity to the olfactory bulbs of the brain. Previous

immunohistochemistry studies of deer terminally infected with CWD have demonstrated PrP^{CWD} depositions in the olfactory bulbs, which also show marked spongiform degenerative changes (33, 35, 39, 40). However in deer, sequential PrP^{CWD} accumulation in the brain appears to occur in a caudal (brainstem) to rostral (frontal cortex) fashion as the disease progresses (34). In the present study, PrP^{CWD} was not detected in the olfactory bulbs of terminal CWD-infected Tg(cerPrP) mice, although aggregates were identified in the frontal cortex immediately dorsal to the olfactory bulbs in some mice. This was especially surprising since the olfactory bulb glomeruli are sites of substantial cervid PrP^{C} expression in naïve Tg(cerPrP) mice.

Given that CWD prions have been demonstrated in saliva (24), urine (10, 17) and soil (16), it is possible that prion entry could involve the vomeronasal organ (VNO)--a region of the anterior ventral nasal passages specialized to detect non-volatile molecules such as pheromones by a process known as the Flehmen response (36);(19); (25). Nevertheless, neither we nor DeJoia (5) were able to detect PrP^{RES} in the VNO of terminal CWD-inoculated Tg(cerPrP) mice or TME-inoculated hamsters, respectively. Thus while all of the above exposure studies failed to identify $PrP^{CWD/RES}$ in mucosal sites, it remains likely that early prion trafficking involves relatively few potentially protease-sensitive oligomeric molecules which may not be identifiable with the detection methods used.

Our inability to detect PrP^{CWD} outside the central nervous system (CNS) in the present studies was somewhat perplexing. This finding could reflect a more limited peripheral expression of PrP^{C} expression in the Tg(cerPrP) mice vs. deer, although other studies in our laboratory have demonstrated PrP^{C} in many peripheral tissues of

Tg(cerPrP) mice (Seelig, DM, Mason, GL, Telling, GC, Hoover, EA, unpublished results). Additionally, over-fixation of our tissues could eliminate possible PrP^{CWD} aggregates. An alternative explanation would be that TSE induced by nasal exposure to CWD prions in Tg(cerPrP) mice in mediated largely by non-lymphoreticular system pathways. Such pathway is supported by the recent work of Bessen and colleagues (2) demonstrating that intra-nasal inoculation of RML scrapie into immunodeficient transgenic mice transmits TSE without lymphoreticular system involvement.

In summary, the present study demonstrates the transmissibility of prions via aerosolization. Several aspects of respiratory transmission of CWD prions remain to be refined and no evidence of a peripheral or lymphoid phase of the infection was detected. The results suggest that prion exposure via the respiratory system merits consideration in prion transmission and biosafety.

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CHAPTER 2*

Minor Oral Lesions Facilitate Transmission of Chronic Wasting Disease

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ABSTRACT

While the exact mechanisms of chronic wasting disease (CWD) prion transmission, entry, and trafficking remain incompletely elucidated, transmission by exposure of the oral and/or nasal mucous membranes seems certain. As part of foraging, cervids commonly sustain minor lesions in the oral mucous membranes which could have an impact on susceptibility to prion infection. To explore this potential co-factor, we studied cohorts cervid PrP transgenic mice with or without superficial abrasions to the lingual mucosa, to determine whether minor oral mucosa lesions may enhance susceptibility to CWD infection. Accordingly, cohorts of orally inoculated mice were observed for signs of neurologic disease or for a maximum of 2 years. A subset of mice

from each group were sacrificed at 90 days post inoculation (dpi) to assess early evidence of CWD transmission. Tissues were assessed for spongiform lesions and for the CWD abnormal prion protein (PrP^{CWD}) by western blotting and immunohistochemistry. Between 296 and 515 dpi, all CWD-inoculated mice with lingual lesions developed clinical signs of progressive neurologic dysfunction, mandating euthanasia. Spongiform lesions and PrP^{CWD} were detected in the brains of all these mice. Conversely, mice without oral lesions did not develop neurologic disease after 738 dpi and all were negative for PrP^{CWD}. No evidence of PrP^{CWD} was detected in mice of either group examined at 90 dpi. The results demonstrate that minor abrasions to the lingual mucosal surface substantially facilitate CWD transmission, a co-factor that may be significant in cervids and perhaps other species.

INTRODUCTION

Chronic wasting disease (CWD) is a fatal neurodegenerative prion disease affecting cervid species (white-tailed deer, mule deer, elk and moose). The efficiency by which CWD spreads suggests transmission occurs primarily by horizontal means (21-24). Body fluids and excreta, including blood, saliva, urine, and feces from infected cervids, have been shown to contain infectious CWD prions (11, 19, 33). While the exact mechanisms of CWD prion transmission, entry, and trafficking remain incompletely elucidated, transmission by exposure through direct or indirect contact of the oral and/or nasal mucous membranes seems certain. Whether prion infection occurs after mucous

membrane exposure may be influenced by co-factors beyond dose, such as particle association with soil and status of the mucous membrane barrier (10, 15, 16, 29).

Oral inoculation studies with sheep Scrapie and CWD have indicated that early amplification of protease resistant protein ($Pr^{PRES/Sc}$) occurs within the tonsils, retropharyngeal lymph nodes, and/or peyers patches (5, 8, 30). Prion dissemination can also occur through lymphoreticular system (LRS)-independent pathways (3, 6, 27). The oral cavity is highly innervated and contact between prions and free nerve endings of the cranial-facial nerves might provide a direct site for prion uptake. Foraging cervids experience lesions in the oral mucous membranes that could have impact on susceptibility to prion entry by facilitating direct contact with exposed nerves or blood vessels. The present work was prompted by that of Bessen and Bartz (4, 6) who demonstrated in the Transmissible Mink Encephalopathy (TME) and scrapie systems that lesions on or injection into the tongue enhanced susceptibility to these prion infections. We therefore explored the hypothesis that CWD transmission may be facilitated by small lesions/micro-abrasions in the oral epithelial surface.

MATERIAL AND METHODS

Transgenic Mice and Ethics Statement:

Transgenic mice expressing elk prion protein (Tg(CerPrP-E226)5037+/-) were created in the Telling laboratory (University of Kentucky). The susceptibility of this line of mice to CWD infection has been described previously (1). 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).

Monitoring and Clinical Symptoms:

Mice were examined for evidence of neurologic abnormality every 2 days and weighed weekly (starting at 3 months post inoculation). Clinical criteria for assessing CWD symptoms included ataxia, lethargy, tail rigidity, poor coat quality, and weight loss. Upon onset of clinical symptoms, mice were isolated into individual cages to prevent cannibalism from cage mates. Mice were euthanized when distinct signs of neurologic disease were evident. CWD and sham-inoculated mice were housed in separate rooms to minimize potential for cross contamination.

Inoculum and Inoculation Routes:

The CWD inoculum consisted of brain homogenate from a CWD-infected white-tailed deer (#104) and negative control brain homogenate from a CWD-naive white-tailed deer brain (#123). Brain homogenates were prepared in 1X phosphate-buffered saline (PBS) to a final concentration of 10% weight to volume (w/v).

Two cohorts of (Tg(CerPrP-E226)5037+/-) mice (n=12 CWD and n=12 control) were inoculated per os (PO) by direct pipet instillation of 10 µL of the 10% (w/v) brain homogenate onto the lingual surface of each mouse. Two additional cohorts of (Tg(CerPrP-E226)5037+/-) mice (n=12 CWD and n=12 sham) were anesthetized using a ketamine/xylazine mix and 3 minor abrasions were created by lightly scratching the lingual surface using a 27-gauge needle. Mice were then inoculated PO by pipet

instillation of 10 µL of inoculum onto the surface of the tongue including those regions containing abrasions. Three mice from each cohort were sacrificed at 90 days post inoculation and analyzed for early PrP^{cwd} detection. The remaining mice (9/cohort) were monitored either until clinical symptoms became apparent or to study termination at 738 days.

Western Blotting:

Harvested tissues were prepared at 10% (w/v) in a 1X PBS/1% Triton-X 100 mixture. Glass beads (2.5mm, Biospec) were added to each tube and samples subjected to one Fastprep™ (Biosavant) cycle for 45 seconds at a speed setting of 5.5, followed by a 2 minute cool down at -20º. Samples were then centrifuged at room temperature for 5 minutes in a Microfuge® 18 (Beckman Coulter - F241.5P Microfuge Fixed-Angle Polypropylene Rotor) at 2000 rpm to remove tissue debris and supernatants stored at -20º until further use.

Samples were incubated with 50 mg/µL of Proteinase K (Invitrogen) for 30 minutes at 37º, shaking at 1000 rpm (Eppendorf Thermomixer R). Samples were then combined with a reducing agent/sample buffer (Invitrogen) to a final concentration of 1X and electrophoresced through NuPage 12% Bis-Tris gels (Invitrogen) for 1.5 hours at 125 volts. Proteins were then transferred to 0.22µm PVDF membranes (Millipore) over 1.0 hour at a setting of 125 volts. Membranes were processed through the actively driven SNAP i.d. system (Millipore). Briefly, membranes were blocked in a 0.5% casein/TBS (Thermo Scientific) + 0.1% tween-20 (Sigma) mixture for 3 minutes, incubated with monoclonal antibody BAR-224 (Spi-Bio) conjugated with HRP at a 1:20,000 dilution in

0.5% casein/TBS + 0.1% tween-20 for 7 minutes, and finally washed 3 times with 1X tris-buffered saline (TBS) + 0.02% tween-20. Membranes were developed with ECL Plus Western Blotting Detection kit (GE). Blots were viewed and photographed with a Gel Doc system (Fujipics).

Immunohistochemistry:

Tissues were fixed in 10% formalin for 2-to-5 days, transferred to 60% ethanol, embedded in paraffin, and sectioned at 5μm for staining. Slides were de-paraffinized through a series of xylene/ethanol baths, treated in 89% formic acid for 15 minutes, rinsed in running water for 5 minutes, and then subjected to a 15 minute, antigen-retrieval process (Pickcell laboratories, Netherlands). All tissues were stained by hand using a Tyramide Signal Amplification (TSA) Plus DNP-HRP kit (Perkin Elmer). Briefly, slides were blocked with 3% H_2O_2 in methanol (45 minutes), blocked with either TNB buffer or a mouse-on-mouse (M.O.M™) IgG blocking reagent (Vector Laboratories) (45 minutes) with a secondary M.O.M™ protein concentrate block (5 minutes), incubated with a 1:500 dilution of HRP-conjugated BAR-224 in TNB or the M.O.M™ protein concentrate (90 minutes), amplified with a 1:50 dilution of 1X DNP amplification reagent in amplification diluent (15 minutes), enhanced with a 1:100 dilution of α-DNP-HRP in TNB (30 minutes), developed with chromagen AEC (30 minutes), and counterstained with hematoxylin (5 minutes) and bluing reagent (1 minute). Brain sections stained by H&E and IHC were assessed for TSE lesions which included primary neuronal loss, vacuolation, and gliosis. All images processed using Adobe Photoshop™ 7.0.1.

RESULTS

Oral exposure of Tg(CerPrP-E226)5037+/- mice without lingual mucosal abrasions:

Of the cohort (n=9) of CWD-inoculated (Tg(CerPrP-E226)5037+/-) mice that did not receive lingual lesions, 2 were euthanized at 568 and 673 dpi due to neoplasias unrelated to CWD, as both were negative for PrP^{CWD} by western blot and immunohisto-chemistry; the remaining 7 mice survived until study termination at >700 dpi (Fig. 1).

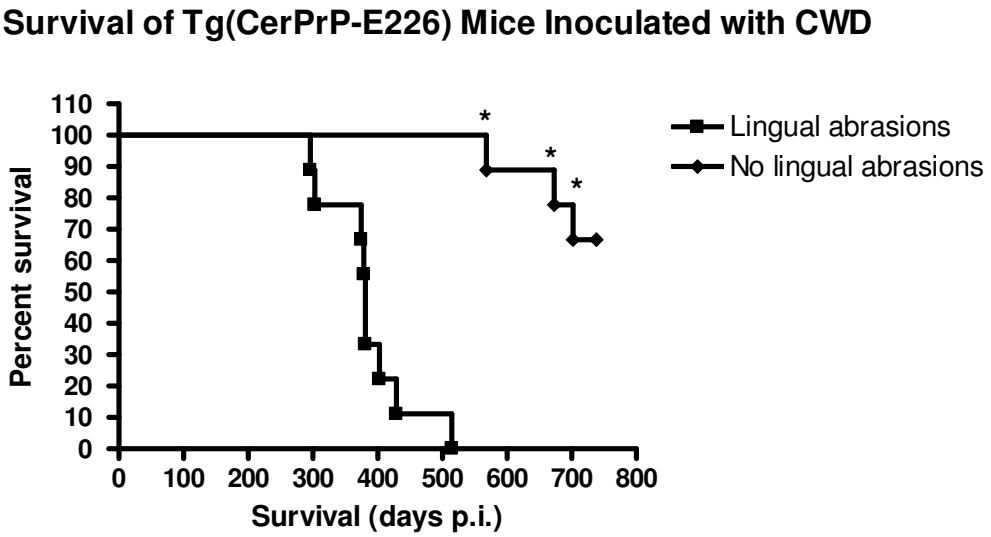

Fig. 1. Kaplan-Meier survival plot of Tg(CerPrP-E226) mice with (solid square) vs. without (solid diamond) lingual abrasions inoculated orally with CWD. (*) denotes mice that died of causes unrelated to CWD.

None of the inoculated mice exhibited clinical signs of CWD infection. Likewise, none of the mice without lingual lesions orally administered negative control-inoculum developed signs of TSE, although 3 were euthanized between 507 and 598 dpi due to

neoplasias unrelated to CWD. Western blot (WB) analysis on brains from both early and terminal CWD and sham-inoculated mice were negative for PrP^{CWD} (data not shown). Despite application of the sensitive Tryamide Signal Amplification (TSA) immunohistochemistry (IHC) methodology, no PrP^{CWD} could be demonstrated in the brain, tongue, salivary glands, trachea, esophagus, spleen, stomach or gastrointestinal tract in either the early sacrificed or terminal mice (data not shown). A summary of these results can be found in Table 1.

TABLE 1. Summary of CWD inoculation results in Tg(CerPrP-E226)5037+/- mice

	Inoculum	Attack rate[a]	Survival (days p.i.)	PrP^{CWD} (+)[b]
Lingual abrasions	**CWD + brain**	**9/9 (100%)**	**385 ± 62**	**100%**
	CWD - brain	**0/9 (0%)**	**388 ± 61[c]**	**0%**
No Lingual abrasions	**CWD + brain**	**0/9 (0%)**	**708 ± 57**	**0%**
	CWD - brain	**0/9 (0%)**	**607 ± 212[c]**	**0%**

a = No. affected/total (%)
b = Positive by western blot and immunohistochemistry
c = One control mouse was sacrificed when each symptomatic mouse was euthanized

Oral exposure of Tg(CerPrP-E226)5037+/- mice with lingual abrasions:

Nine of 9 CWD-inoculated mice with lingual abrasions developed clinical symptoms consistent with TSE between 296 and 515 dpi and were euthanized (Fig. 1). None of the negative control-inoculated mice (n=9) displayed evidence of TSE throughout the observation period of 515 days.

PrP^{CWD} was demonstrated in the brain of all 9 CWD-inoculated mice by WB (Fig. 2; lanes 3-11). All negative control-inoculated mice were negative for PrP^{CWD} (Fig. 2; lane 2). IHC confirmed the presence of PrP^{CWD} aggregates in the brain tissue of all 9 CWD-inoculated mice, with predominant staining occurring within the obex and cerebellum (Fig. 3A). Both H&E and stained IHC sections contained evidence of neuronal loss and gliosis consistent with CWD infection (data not shown). All negative control-inoculated mice were negative for PrP^{CWD} (Fig. 3B) and TSE lesions. TSA IHC analysis on peripheral tissues was again unable to detect PrP^{CWD}. A summary of these results can be found in Table 1.

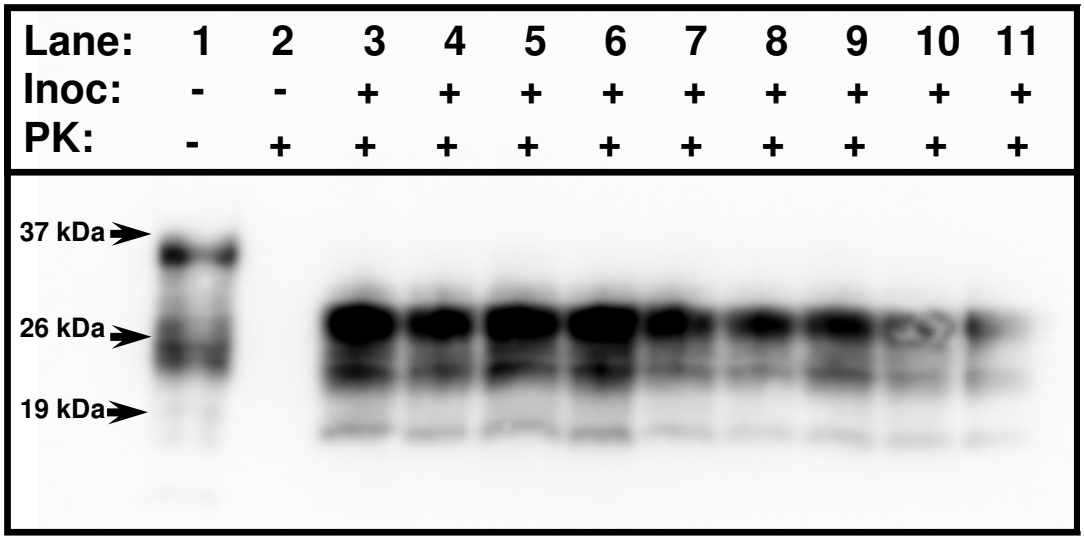

Fig. 2. Western blot detection of PrP^{CWD} (lanes 3-11) in brains of Tg(CerPrP-E226)5037+/- mice with lingual lesions exposed to CWD prions. Lesioned mice exposed to negative control inoculum (lane 1 & 2) show no evidence of PrP^{CWD}.

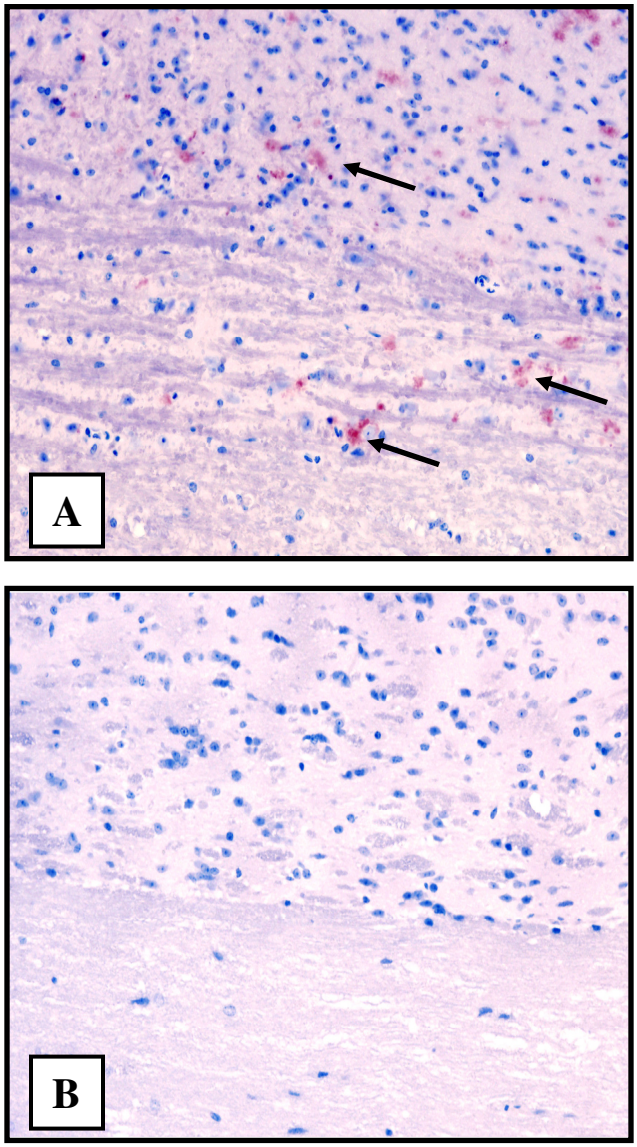

Fig. 3. Immunohistochemistry (IHC) from the obex region of the medulla of: (A) a Tg(CerPrP-E226)5037+/- mouse with lingual lesions (20X) demonstrating aggregates of PrP^{CWD} (arrows) vs. (B) a lesioned mouse exposed to negative control inoculum.

DISCUSSION

Transmission of CWD prions to deer by oral inoculation has been well demonstrated and the oral route is considered the most plausible site of access in nature (8, 12, 18, 19, 23, 30). In addition, cervids naturally acquire minor oral lesions as part of foraging, providing a potential cofactor for prion entry that might help explain the facile transmission of CWD (3, 31). The present study models this natural event in cervidized transgenic mice [Tg(CerPrP-E226)5037+/- mice]. Available evidence indicates that cervidized transgenic mice are much less susceptible to oral CWD infection than are cervids, and thereby require a potent inoculum to induce infection with lower attack rates (28), (35). Here we show that this resistance is negated by the presence of minor breeches in the lingual epithelium--enhancing CWD infection from 0 to 100%. In addition, the lack of demonstrable PrP^{CWD} in non-neural tissues of the affected mice suggests that transit to the brain occurred by a lymphoreticular system (LRS)-independent pathway.

Twenty five years ago Gajdusek (9) suggested that lesions in the conjunctiva, skin, or mucosal injury might facilitate the passage of Kuru. Support of this hypothesis has accrued. Scarification of either skin or gingival tissues has been shown to enhance the transmission of scrapie (7, 10, 14, 34). Likewise, scarification of the lingual surface in hamsters exposed to scrapie or TME produced a significantly increased attack rates, shortened survival periods, and facilitated infection with substantially smaller prion doses compared with conventional per os inoculation (4, 6, 13). The present study suggests that enhancement of CWD infection is even greater.

We surmise that mucosal lesions may have made possible direct neural contact as the tongue is heavily innervated (4, 36). However, we were not able to detect PrP^{CWD} immunostaining in any histologic structure in the tongue or lymphoid tissues at 90 days pi or in terminally affected mice. In that CWD is a lymphotropic TSE (8, 30, 32), the lack of discernible PrP^{CWD} in LRS tissues suggests that prion ascension to the brain occurred independent of the LRS. Of course caveats to this conclusion include that small PrP^{CWD} aggregates in peripheral tissues might have been below the sensitivity limits of TSA IHC protocols or that such aggregates exist in a formalin- or protease- sensitive form. Nevertheless, while entry via a lymphatic or hematogenous route cannot be excluded, the most plausible pathway would *seem* to be trafficking along lingual and facial nerves—a prion phenomenon well demonstrated by the work of several investigators (4, 14, 25).

Dose dependency in prion infections has been extensively demonstrated (2, 17, 20, 26, 28). While environmental contamination almost surely plays a role in CWD transmission, available information indicates that prion concentrations in excretions, soil, and the environment are below the detection limits of conventional assays (11). Results of the present study may help explain how exposure to low concentrations of prions in urine, feces, and soil (11, 19, 33) may be able to transit the mucosal barrier to initiate infection in foraging cervids, other ruminants, and perhaps in other species.

In summary, superficial lesions to the lingual surface of cervid transgenic mice significantly enhanced susceptibility to oral CWD infection. The absence of PrP^{CWD} detection in the tongue, lymphoid tissues, or any peripheral tissue site suggested a direct

neural route of invasion. These results implicate one co-factor that could facilitate CWD infection in cervids after oral exposure to very low concentrations of prions in nature.

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CHAPTER 3

A Search for Early Mucosal Sites of Chronic Wasting Disease Prion Entry

ABSTRACT

Chronic wasting disease is horizontally transmitted, most assuredly with involvement of oral and nasal mucosa as the obvious first contact site for infectious prions. Cervids continually monitor their environment by exposing highly-sensitive olfactory epithelium and/or the vomeronasal organ to particulates drawn in via inhalation and the Flehmen response. Moreover, cervids commonly acquire minor lesions on oral mucous membranes when foraging. Exposing prions to nasal passages or compromised oral mucosa has proven exceedingly effective at transmitting disease; however *where* the initial site of prion uptake within mucous membranes occurs remains unknown. To address this question, two lines of FVB mice trangenetically expressing the cervid prion protein [Tg(CerPrP)1536 and Tg(CerPrP-E226)5037 +/-] and a prion protein knockout mouse [FVB $PrP^{0/0}$] were exposed via aerosol, nasal and oral routes to CWD prions. Cohorts of mice were serially sacrificed up to 4 hours post inoculation. Respective tissues based on route of inoculation were assessed for PrP^{CWD} by tryamide signal

amplification immuno-histochemistry. Detection of PrP^{CWD} was largely inconclusive as immunoreactive staining appeared in both CWD and sham-inoculated cohorts and Tg(CerPrP) and $PrP^{0/0}$ mice, all irrespective of sacrifice times. These results demonstrate further optimization for both fixation and detection techniques are required.

INTRODUCTION

Transmissible Spongiform Encephalopathies (TSE's), or prion diseases, are neurodegenerative disorders that affect humans and a variety of animal species with an inevitably fatal outcome. TSE's are believed to be transmitted via a horizontal route (24, 25), due to the lack of evidence supporting sustainable maternal transmission. The oral and nasal mucosas have been implicated as the primary site of contact for infectious prions. While the transmission of Kuru (15), transmissible mink encephalopathy (TME) (17), bovine spongiform encephalopathy (BSE) (32), and new-variant Creutzfeldt-Jakob disease (nvCJD) (8, 33) have been shown to occur primarily through oral ingestion, the exact mechanism for scrapie and chronic wasting disease (CWD) transmission remains unknown.

CWD, as well as other TSE's are lymphotropic. Studies tracking infectious prions after oral and nasal inoculations confirmed accumulation first within the tonsil, retropharyngeal lymph node, and gut associated lymphoid tissue (GALT) (3, 5-7, 14, 20, 21, 29, 30), therby indicating that lymphatic amplification preceded neural invasion and disease onset. Proposed mechanisms involving platelets, B cells, dendritic cells, and macrophages that might mediate the trafficking of prions to lymphatic structures have

been explored, yet the exact mechanism has not been elucidated (1, 22, 28). Interestingly, while these studies provided valuable information about trafficking and early lymphoid pathogenesis, events earlier than one week post inoculation in oral or nasal prion infections have not been examined.

How prions transit the mucosal barrier is of considerable importance in understanding prion pathogenesis. Previous studies have demonstrated that exposing prions to nasal passages or compromised oral mucosa enhanced prion infection and required a smaller dose to induce TSE diseases compared to conventional routes (5, 13, 19) (Denkers ND, Telling, GC, Hoover EA, unpublished results). Here we extend those studies by focusing on the first hours after prion exposure to the mucous membranes in of cervidized transgenic [Tg(CerPrP)] and prion protein knockout [FVB $PrP^{0/0}$] mice with the goal of identifying sites of CWD prion uptake.

MATERIAL AND METHODS

Transgenic Mice and Ethics Statement:

Two lines of transgenic mice created in the laboratory of Dr. Glenn Telling (University of Kentucky) were used for these studies: Both lines express the cervid PrP gene [Tg(CerPrP)1536+/-] and [Tg(CerPrP-E226)5037+/-] on a FVB mouse background. The PrP knockout mouse, in which the expression of mouse prion gene had been deleted [FVB $PrP^{0/0}$] were generated from heterozygous 5037 mice in the Zabel laboratory (Dr. Mark Zabel, Colorado State University). The susceptibility of these mice to CWD infection or lack thereof after intracerebral inoculation has been previously described (2, 10), (Zabel, personnel communication). Confirmation of the presence or lack of the

cervid PrP^C gene insert was performed by PCR. All mice were 5 to 12 weeks of age at time of inoculation. 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).

Inoculum:

The CWD inoculum consisted of brain homogenate from a CWD-infected white-tailed deer (#104) and sham-control brain homogenate from a CWD-naive white-tailed deer brain (#123), both of which were infected at Colorado State University. Brain homogenates were prepared in 1X phosphate-buffered saline (PBS) to a final concentration of 10% weight to volume (w/v) for PO and IN inoculations and a 5% (w/v) for aerosol exposure.

Oral (PO) Inoculation:

Two cohorts of Tg(CerPrP-E226)5037+/- and FVB $PrP^{0/0}$ mice (n=9 CWD and n=9 sham for each line) were used in these experiments. Mice were anesthetized using a ketamine/xylazine mix and 3 minor abrasions were created by lightly scratching the lingual surface using a 27-gauge needle. Mice were then inoculated by direct pipet instillation of 10 µL of the 10% (w/v) homogenate onto the surface of the tongue including the regions containing abrasions. Mice (n=3/cohort) were sacrificed immediately (T=0), 1 hour post inoculation (T=1), and 4 hours post inoculation (T=4)

and all tissues were immersion fixed in Paraformaldehyde/ Lysine/Periodate (PLP) for 24 hours.

Intranasal (IN) Inoculation:

Two cohorts of (n=9 CWD and n=9 sham for each line) were used in these experiments. Mice were inoculated with 10 µL (5 µL per nostril) of the 10% (w/v) extracts by direct pipet instillation into the nasal passages. Mice (n=3/cohort) were sacrificed immediately (T=0), 1 hour post inoculation (T=1), and 4 hours post inoculation (T=4). Tg(CerPrP-E226)5037+/- mouse tissues were immersion fixed in 10% formalin for 4 days, Tg(CerPrP)1536+/- mouse tissues were immersion fixed in PLP for 24 hours, and two sets of FVB $PrP^{0/0}$ mouse tissues were fixed in both 10% formalin for 4 days and PLP for 24 hours.

Aerosol Inoculation:

Two cohorts of Tg(CerPrP-E226)5037+/- and FVB $PrP^{0/0}$ mice (n=9 CWD and n=9 sham for each line) were used in these experiments. Mice were anesthetized using a ketamine/xylazine mixture, placed in a custom designed chamber as previously described (13), providing nose-only exposure then subjected to aerosolized 5% (w/v) brain homogenate for 4-minutes. All aerosol exposed mice were administered 0.01 mL of epinephrine intraperitoneally immediately post inoculation to stimulate the Flehmen response. Mice (n=3/cohort) were sacrificed immediately (T=0), 1 hour post inoculation (T=1), and 4 hours post inoculation (T=4). Tg(CerPrP-E226)5037+/- and FVB $PrP^{0/0}$ mouse tissues were immersion fixed in 10% formalin for 4 days.

Immunohistochemistry:

Post fixation, tissues were transferred to 60% ethanol. Trimmed in tissue was subjected to a 5 minute formic acid (89%) treatment, thoroughly washed in water, embedded in paraffin, and sectioned at 5μm for staining. Slides were de-paraffinized through a series of xylene/ethanol baths, treated in 89% formic acid for 5-45 minutes, rinsed in running water for 5 minutes, and then subjected to a 15 minute, antigen-retrieval process (Pickcell laboratories, Netherlands). All tissues were stained by hand using a Tyramide Signal Amplification (TSA) Plus DNP-HRP kit (Perkin Elmer). Briefly, slides were blocked with 3% H_2O_2 in methanol (60 min), blocked with either TNB buffer or a mouse-on-mouse (M.O.M's™) IgG blocking reagent (Vector Laboratories) (60 min) with a secondary M.O.M.™ protein concentrate block (5 minutes), incubated with a 1:500 dilution of HRP-conjugated BAR-224 in TNB or the M.O.M.™ protein concentrate (90 minutes), amplified with a 1:50 dilution of 1X DNP amplification reagent in amplification dilulent (15 minutes), enhanced with a 1:100 dilution of α-DNP-HRP in TNB (30 minutes), developed with chromagen AEC (30 minutes), and counterstained with hematoxylin (5 minutes) and bluing reagent (1 minute). A minimum of 5 sections per tissue were examined for PrP^{CWD}.

RESULTS

Orally inoculated Tg(CerPrP-E226)5037+/- mice:

Tissues analyzed included the tongue, salivary gland, submandibular lymph node (if present), esophagus, trachea, stomach, small intestine, and spleen. No PrP^{CWD}-specific staining was detected in any tissue at any of the time points examined. Immunoreactivity was observed in both sham and prion-inoculated Tg(cerPrP) mice within the circumvallate papillae of the tongue and in the myenteric plexuses of the stomach and small intestine. This staining was attributed to incomplete removal of PrP^{C}. Staining was also present in mast cells of the tongue in both cohorts, irrespective of formic acid treatment times.

Orally inoculated FVB $PrP^{0/0}$ mice:

Tissues analyzed included the tongue, salivary gland, submandibular lymph node (if present), esophagus, trachea, stomach, small intestine, and spleen. No PrP^{CWD}-specific staining was detected in any tissue at any of the time points examined. In these mice, as might be expected, no immunoreactivity was observed in the circum vallate papillae or myenteric plexus but was present in mast cells (as seen previously) in both cohorts, irrespective of formic acid treatment times.

Intranasally inoculated Tg(CerPrP-E226)5037+/- and Tg(CerPrP)1536+/- mice:

Tissues analyzed included the nasal cavity containing the vomeronasal organ (VNO), nasal associated lymphoid tissue (NALT), respiratory and olfactory epithelium,

the olfactory bulb and the accessory olfactory bulb (AOB) when present. Additionally, the trachea, esophagus, lung, and spleen were assessed. No PrP^{RES}-specific staining was detected in any tissue at any of the time points examined. In both sham and prion-inoculated Tg(cerPrP) mice, immunoreactivity was present in the glomeruli of the olfactory bulb, the olfactory nerves below the olfactory epithelium, and the ventral-medial aspect of the nasal septum just below the respiratory epithelium. This staining was therefore considered to represent incompletely quenched PrP^{C} immunoreactivity in these high PrP^{C}-containing tissue sites. In neither the NALT nor the VNO was immunoreactivity detected.

Intranasally inoculated FVB $PrP^{0/0}$ mice:

Tissues analyzed included the nasal cavity containing the vomeronasal organ (VNO), nasal associated lymphoid tissue (NALT), respiratory and olfactory epithelium, the olfactory bulb and the accessory olfactory bulb when present. As expected, no PrP^{RES}-specific staining was detected in any tissue at any of the time points examined. Immunoreactive staining was detected in ventral-medial aspect of the nasal septum just below the respiratory epithelium, similar to observations in the Tg(CerPrP-E226)5037+/- and Tg(CerPrP)1536+/- mice.

Aerosol inoculated Tg(CerPrP-E226)5037+/- mice:

Awaiting results: Oct./Nov. 2010

Aerosol inoculated FVB $PrP^{0/0}$ mice:

Awaiting results: Oct./Nov. 2010

DISCUSSION

The transmission of CWD to cervids and transgenic mice expressing the cervid prion protein through oral and nasal inoculations has been demonstrated (13, 14, 20, 23, 27, 29, 31) and likely represents the most natural routes for prion acquisition. In addition to these studies, work involving scrapie, TME, and CWD have provided substantial evidence that the oral and nasal route are much more efficient (require a considerably lower infectious prion dose) at transmitting disease, especially if the mucosal surface is compromised (4, 5, 7, 12, 18, 19) (Denkers ND, Telling GC, Hoover, EA unpublished results). The present studies were undertaken in an attempt to localize CWD prions at sites within the oral and nasal mucosa at the earliest possible times (T=0, 1, 4 hours pi) using [Tg(CerPrP)1536+/-], [Tg(CerPrP-E226)5037+/-] and PrP knockout [FVB $PrP^{0/0}$] mice. The results of these experiments were inconclusive in that any immunoreactivity detected was present in both CWD and sham-inoculated mice for all cohorts, at all time points, and attributable to incomplete removal of PrP^{C} in high expression sites such as taste and olfactory structures.

Both lines of transgenic mice used in this study have been shown to over-express the cervid prion protein (~ 5x more) in the CNS, with the Tg(CerPrP-E226)5037+/- line providing greater peripheral expression. Due to the overabundance of prion protein, abrogating PrP^{C} might require more stringent applications of formic acid. We addressed this issue by varying the exposure times the slides were in formic acid from 5 to 45 minutes. This was problematic, as PrP^{CWD} is degraded by formic acid. With such small

inoculation doses, we ran the risk of destroying our initial inoculum. Inoculum amount proved inconsequential, as increased formic acid times were insufficient at removing the immunoreactivity seen in the glomeruli of the olfactory bulb, the circum vallate papillae, and the myenteric plexi of the small intestine. Supporting evidence that the immunoreactivity seen in the above tissues was PrP^{c} came from the FVB $PrP^{0/0}$ mice that were negative for staining in all these tissues. An ongoing experimental approach to try and correct this problem includes a formic acid treatment after the antigen retrieval process.

Limited studies have previously attempted to detect PrP^{RES} immediately after inoculation. These experiments exposed sheep to scrapie(16) and mice to Creutzfeldt-Jakob disease (26) via the intracranial route, then analyzed brain sections for PrP^{RES} one and five hours post-inoculation, respectively. Surprisingly, dissemination of the original inoculum was rapid, leading to limited or no detection of PrP^{RES} in both studies. These findings in part explain our inability to detect PrP^{CWD} as detecting inoculum distributed over a mucosal surface is more challenging.

Another potential cause of the non-specific immunoreactivity could have come from the fixative we used. Cohorts of mice were originally fixed in 10% formalin buffer for 4 days. Following experimental initiation, information was presented that over-fixation with formalin could lead to decreased sensitivity for PrP^{res} and that PLP was potentially better (9, 11), although no direct comparative study was done. None the less, a second cohort of mice were inoculated, sacrificed, and fixed using PLP (immersed for 24 hour). Comparative immunohistochemistry's (IHC) were performed between formalin and PLP fixed tissues, to which no discernable difference could be made due to

the inability to eliminate PrP^{C} staining from either transgenic mouse line (irrespective of time spent in formic acid). There was no change in ability to detect PrP^{CWD} in the FVB $PrP^{0/0}$ mouse line based on fixation method. Further evaluation is required to optimize the fixation of tissue in order to detect prions in early inoculation experiments.

The most perplexing staining of these experiments was located in the mast-like cells of the tongue and the ventral-medial aspect of the nasal septum. Immunoreactivity in these two areas was almost uniform in both CWD and sham-inoculated cohorts of all three transgenic mouse lines, regardless of fixative used. One explanation for these results was that staining was caused by an immunological response to inoculum placed in nasal passages and the trauma of lingual abrasions. Steps taken to eliminate this staining included varying treatment times of the hydrogen peroxide and/or blocking reagents to quench any endogenous peroxidases. These treatments were ineffective. We are currently investigating the use of R505 and unconjugated-BAR to alleviate this possible cross-reactive staining.

In summary, we were not able to locate early entry sites for prion invasion in the oral and nasal mucosa. This result is unfortunately consistent with the limited, available published references indicating that very early, post infection in situ demonstration of protease-resistant prion protein is problematic, a circumstance compounded when inoculum is distributed over a mucosal surface.

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CONCLUSIONS AND FUTURE DIRECTIONS

This work has demonstrated that; 1) CWD can be transmitted via the respiratory route through direct nasal contact; 2) aerosolized prions appear to be more efficacious when inhaled vs. direct contact to the nasal mucosa passages; 3) lesions to the oral mucosa greatly facilitate the transmission of CWD; 4) detecting prions in mucosal surfaces immediately after exposure requires more sensitive methodology than that employed here.

These findings in Tg(CerPrP) mice suggest that the respiratory system could be a natural transmission route of CWD in cervids. Exposure to inhaled prions could provide immediate access to free-nerve endings in the olfactory or VNO epithelium, or to lymphoid structures such as the retropharyngeal lymph node or nasal associated lymphoid tissue. Moreover, the finding that CWD can be transmitted by aerosolization is novel and implies that direct contact between animals is not necessary to transmit disease, albeit prions could still be inhaled from contaminated environment sources or from nasal contact with infected animals.

The efficiency by which aerosolized CWD was transmitted suggests that smaller, more infectious particles were created during the process and that the host could process these smaller particles easier through mucosal or lymphoid surfaces. This finding has implication for the transmission of other prion diseases as well as the need to reevaluate

prion biosafety protocols. Protocol changes would primarily be applicable to prion material known to infect humans and might include; 1) that all manipulations are done in biosafety cabinets or physical containment devices; and 2) the use of full face respirators instead of masks, goggles, and face shields.

This work is highly relevant to the probability of nasal and/or aerosol transmission of CWD in nature. Throughout fall, deer, elk, and moose participate in breeding rituals known as the rut. During this time, males and females frequently urinate to mark territory or assert reproductive status (5, 9, 12). The Flehmen response is performed by both genders to decipher these chemosensory cues. By nuzzling, sniffing, and licking urine as it is voided and/or sampling voided urine on the ground (3), the respiratory mucosa are being exposed to urine and soil particulates by direct nasal contact as well as by aerosolization. Considering CWD prions remain infectious in urine and soil (6, 8), it is possible that rutting behaviors provide a plausible mechanism for natural CWD transmission and should be investigated further.

Future directions of this work might involve brain homogenate titration experiments to determine a minimum infectious dose required for respiratory route exposure. Determining the efficacy of nasal mucosal exposure could better explain the relationship between prion dose in the environment and the ease in which CWD is transmitted. To mimic possible natural infections, directly inoculating or aerosolizing urine and saliva might demonstrate the capability of excreta to transmit disease. Additionally, if prions remain infectious in the soil for years, aerosolizing lyophilized urine and saliva or urine and saliva dried on soil, might help determine if environmental factors facilitate transmission.

We found that small abrasions to the lingual surface of Tg(CerPrP) mice significantly enhanced the transmission rate of CWD. This work expands upon the precedent set by Bartz, Bessen, and others (1, 7) showing lingual inoculations transmitted disease with greater efficiency than standard oral inoculations. Oral lesions could allow ingested prions to enter directly into the circulatory, lymphatic, or nervous system. Lack of involvement of the lymphoreticular system in our model implies entry most likely occurred through exposed free nerve endings.

Our results may directly contribute to the understanding of natural oral transmission of CWD, in that cervids that experience mucosal abrasions, possibly due to tooth eruption, harsh forage, or disease, may be more susceptible to CWD infections. Gingival tissues in deer are constantly disrupted in the first 24 months of life by tooth eruptions (10). Gingival inoculations studies with scrapie demonstrated higher attack rates than the conventional oral route (2); therefore it is plausible that CWD might be transmitted via the dental route in nature.

Oral cavity foreign bodies (presumed to be lodged forage) have been documented in cervids at time of necropsy (11). Penetration of contaminated feed or open wounds caused from foraging could allow trans-mucosal entry and CWD transmission. Moreover, assumptions that primary infections from an ancillary disease, such as epizootic hemorrhagic disease (EHD), could facilitate CWD transmission are reasonable. Acute and chronic clinical presentation of EHD includes lesions/ulcers to the tongue and dental pad (4). The previous observations that oral lesions occur naturally validates our claim that CWD could be transmitted through abraded mucosal surfaces.

Future directions of this work might again involve titration experiments to determine a minimum infectious dose. Once more, this might help clarify the relationship between environmental prion dose and CWD transmission. The use of urine, saliva, soil, and feces to mimic a natural exposure might expand our knowledge on the ability of excreta to transmit disease. Investigating the length of time between the initial lesion and prion exposure required to establish sufficient disease transmission could provide a time frame of susceptibility.

We found our capacity to detect PrP^{CWD} in Tg(CerPrP) mice immediately after inoculation suboptimal. The quick dissemination of infectious prions immediately after exposure combined with our inability to eliminate PrP^{C} indicates that further adjustments in tissue and slide processing are required. The benefit of this work, if successfully optimized, would greatly enhance our understanding of initial prion pathogenesis. Co-localizing the infectious prions to specific cell types could elucidate how trans-mucosal entry occurs, whether immune cells are involved initially or are secondary carriers, and in a broader spectrum, which systems (circulation, lymphatic, nervous) are involved in initial uptake.

Future directions of this work might or currently include; 1) altering fixation time or method of fixation (perfusion vs. immersion); 2) varying hydrated-autoclaving and formic acid treatment procedures; 3) using different prion PrP antibodies to eliminate cross-reactivity; 4) employing alternate detection methods (immunofluorescence); and 5) utilizing purified, pre-labeled prions rods for tracking studies.

A limitation of these studies involves the use of cervidized-transgenic mice instead of a cervid species. While transgenic mice allow for easy experimental

manipulations, anatomical and physiological differences between mice and cervids make correlations for disease transmission and pathogenesis difficult to assess. An additional limitation of these studies includes the use of brain material as inoculum instead of naturally excreted material. While the largest concentration of infectious prions resides within the CNS and lymphoid structures, accessibility to this material in nature is limited compared to the constant secretion of prions in 'secreta'.

In summary, the results presented in this dissertation suggest new paradigms for how CWD is naturally acquired. We demonstrated that the respiratory route (through direct nasal contact) is a viable site for CWD transmission, with a significant increase in efficacy if exposed via aerosolization. In addition, we demonstrated that lingual lesions greatly facilitated the transmission of CWD. Finally, we established that more sensitive methods might be required to detect prions immediately after inoculation. Continuing to build on this foundation should further elucidate transmission and early pathogenesis mechanisms for CWD.

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