Chronic Wasting Disease:
A Review and working hypothesis, the Agent and its Transmission

PART I: A Logical Causative Agent

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Abstract: (Part I) Transmissible Spongiform Encephalopathies (TSE) and in particular, Chronic Wasting Disease are devastating neuropathologic diseases caused by a unique, but unknown infective agent with high degree of refractivity to ordinary disinfectant and sterilization procedures. Three criteria are used to identify TSEs 1) neuronal vacoulation, 2) Scrapie Associated Fibrils, and 3) proteins resistance to proteinase-K digestion as detected by Western Blot and immunostaining or via more abbreviated ELISA tests. All three ID criteria are misleading in their ability to identify TSE disease, and evidence of such fallibility is presented herein together with annotation. Neurotropic, sterol ingesting bacteria of the Class Mollicutes, generally termed Mycoplasma and more specifically various strains of Spiroplasma can, and do, produce the defined TSE identification criteria including proteinase-K resistant proteins of the 25 to 30 kDa range, fibrils of 4 to 6 nm diameter, and will upon intracerebral inoculation of rodents, produce disease symptoms, agent refractivity and the infectivity conditions associated with the putative TSE disease agent. As a working infection hypothesis, Transmissible Spongiform Encephalopathies are amitotic Spiroplasma-induced, intracellular prionic, cytoplasmic, and mitochondrial superoxide dismutase and glutathione red-ox aberrations with resultant neuronal membrane lysis, followed by subsequent cytoapoptosis, or cytonecrosis initiated by concordant, but unrequited reactive oxygen species stress. Conclusively the failure of the scientific community to diligently pursue Mycoplasma and Spiroplasma in their role of disease pathogenesis, particularly in TSEs is a failure in our investigative system of scientific curiosity and prudence.

INTRODUCTION

Chronic Wasting Disease (CWD) is a Transmissible Spongiform Encephalopathy (TSE) affecting both wild and domestic cervidea, including elk, mule deer, black-tailed deer, and white-tailed deer and white-tail hybrids. All TSE diseases are now grouped under the term of "Prion" diseases in recognition of the disease's destructive affect upon a protective protein sheath shielding nervous

tissue, however prion diseases are fundamentally diseases of membranaceous tissue, although not necessarily neuronal tissue. Laboratory-created, transgenic, prion-deficient mice either failed to develop, or developed TSE disease much more slowly when inoculated with scrapie infectivity, while all produced antibodies against the innoculum (Prusiner SB, et al, 1993 and Weissmann C, et al, 1994). Ablation of normal prion protein (PrP^{c}) results in reduced mitochondrial numbers, unusual mitochondrial morphology, and elevated levels of mitochondrial manganese-dependent superoxide dismutase antioxidant enzyme (Miele, 2002). A proteinaceous infective particle (or abnormal "Prion", PrP^{res}) has been widely postulated, even virtually assumed as the suspected disease pathogen, while lesser theories of slow acting virus, filamentous virus, retro virus, viroids, virinos or a bacterial origin have waned. Excessive environmental manganese has been suggested to play a role in TSE pathogenesis (Purdy, 2000), while Kaneko (1997) suggests the necessary presence of a protein X associated with prions in order to initiate TSE infectivity

The unusual characteristics of TSE infections easily distinguish TSEs from more conventional infections. These include: unusually long incubation periods (from months to years); progressive central nervous system degeneration with characteristic histopathological lesions; the lack of an immune or inflammatory response; and unconventional biological and physical properties of the envisaged etiologic agent. Considerable controversy still concerns the nature of the TSE causative agent. A scientific literature review together with accumulated disease observational data herein provides substanciative clues to the nature of the TSE disease agent.

CWD was first recognized in 1967 as a clinical weight loss syndrome (deemed "chronic wasting") in wild mule deer held captive in Colorado Division of Wildlife research facilities and the Wildlife Disease Research Center managed by Colorado State University, both in Ft. Collins, Colorado. In 1981, distinctive brain lesions were found associated with "chronic wasting" and were recognized as the pathological signs of a TSE. The clinical signature of CWD includes several non-specific, non-diagnostic symptoms, such as: weight loss, behavioral changes, excessive water consumption, salivation and urinating, together with erratic teeth grinding (Williams and Young, 1982).

Controversy surrounds the origins of CWD and whether it is a naturally occurring, sporadic disease in the wild, or may have been man-induced during dietary research of co-habited deer and sheep at the government-operated research facilities in northeast Colorado. For the past 3 decades CWD has been erratically found within wild cervidea herds around limited regions of northeast Colorado and southeast Wyoming, and now more recently in northwestern Colorado, southern Wisconsin and southern New Mexico Domestic cervidea have been infected for at least 15 years but have recently sustained an expanded incidence of the disease. Infected domestic cervidea have been found in eight states, two Canadian provinces and in South Korea. Most, but not all disease

occurrences of both wild and domestic cervidae have been traced back to a Colorado or Wyoming derivation, while poor or limited background information prevents the remainder from being specifically tracked.

As a TSE, CWD is grouped via its diagnostic symptoms with several other TSE diseases, some more well known to the public, including Creutzfeldt-Jakob Disease and Kuru of humans, Scrapie in sheep and goats, Bovine Spongiform Encephalopathy ("BSE") of beef or dairy cows (a/k/a Mad Cow Disease), as well as, mink encephalopathy and several other more minor maladies. Some are thought to be spontaneous induced, others under genetic control, and still others readily transmissible. All can be transmitted through direct intracerebral injection of infected tissue.

As a naturally occurring TSE with equally natural transmissibility, CWD presents a unique opportunity to examine a TSE disease in the wild, as well as in captive populations without man's substantial influence or interference in the genetic makeup of the infected species. As such, CWD represents a rare opportunity to achieve a better understanding of a unique form of disease, one potentially devastating to the cervidea industry and natural wildlife populations, in addition to a genre of diseases, capable of affecting domestic ruminants and man, himself.

IDENTIFYING TSE DISEASES

A critical aspect of defining any TSE disease relies on the use of cumulative, non-specific diagnostic procedures. Unfortunately, the majority of symptoms presented by a TSE disease are not individually diagnostic and must be objectively quantified and cumulatively applied to obtain a firm disease diagnosis confirmation.

As a matter of reliability and dependability, one would assume that the testing or analytical procedures, i.e. the assays, used to define and identify selected TSE disease recognition criteria would be beyond reproach so as to facilitate correct disease conclusions. Such is not the case with TSE diseases. Despite decades of dedicated research, Nobel Prize awards and many declarations of proof and success, the scientific community has failed to specifically identify a provable TSE causal agent. Many theories still abound as to the true nature of the TSE pathogen. Competition for funding and the lopsided allocation of funding to those theories regarded as "award winning", "politically" or "popularly correct" have skewed research results toward theory conformity rather than true innovation in determining the authentic TSE causal agent. Complicating the issue, a critical assessment of TSE diagnostic procedures reveals spurious behavior in assay procedures inevitably leading to a myriad of incorrect conclusions. The inability to identify the causative agent has materially hindered development of more specific and refined diagnostic procedures, the creation of symptomolytic or prophylactic medicines and potential vaccines.

A critical review of TSE disease diagnostic procedures is fundamental to the understanding the underlying causal recognition problem. In essence, three major processes are used for disease identification. 1) Observation of brain vacuoles, 2) The presence of scrapie-associated fibrils (SAF), and 3) The presence of proteinase-K resistant (PK^{res}) protein, also know by some as abnormal prion protein (PrP^{res}). Undesirably, each of the three individual identification steps has potential interference, and when taken individually can produce false negative or false positive results. Formerly, all three criteria were generally required for a positive TSE identification, although more recently, shortened steps, focused solely on identification of PK^{res} have come into vogue.

The first identification criterion, that of observing vacuoles in neuronal tissue is fairly distinctive, but is weak in its ability to discern TSE at its early stages. Vacuoles are simply the residual locations of nerve tissue undergoing or having undergone pathogenesis. At the latter stages of disease, vacuoles are easily identified via microscopic thin section observation, yet may not be present or difficult to recognize during earlier disease stages. This gives rise to the potential for early-stage, false negative results. Lamentably, vacuoles can also be found quite totally unrelated to TSEs, hence therefore may not then be independently indicative of a TSE disease. Large numbers of vacuoles are generally not found in normal, healthy brain or nervous tissue.

The second, more evolved, criterion involves the visual identification of specific structures found only in TSE-infected brain and organ tissues. These structures are known as Scrapie-Associated Fibrils or SAF's when found in scrapie, or CJD fibrils (CJDF's) when found in human disease. Fibrils are characteristically present in diseased brain tissue and are found in the spleen as well as other organs. These fibrils commonly possess a physical dimension of approximately four nanometers (nm = one-billionth of a meter) in width and approximately 150 to several hundred nanometers in length. Quite distinctive and excellent micrographic work by P. A. Mertz (1981) initially identified and qualified these unique fibrils, which have now become synonymous with TSE disease. SAF-like fibrils are not found in normal, healthy organ tissue although some other diseases such as Alzheimer's can produce similar looking fibular structures.

The third criterion for the recognition of TSE disease is that of locating proteinase-K resistant proteins (PK^{res}). PK^{res} material is not found in normal, healthy mammalian nervous tissue. The presence of PK^{res} is the cornerstone of the currently popular Prion theory of TSE diseases, promulgated by Dr. Stanley Prusiner of the University of California at San Francisco, for which he has received the Nobel Prize for Medicine. According to Prusiner (1999) prion diseases are aberrations in protein conformation whereby a normal cellular glyoprotein (PrP^{c}), which forms a protective antioxidant sheath over neuronal and other organ cells, is converted into abnormal prion protein (PrP^{res}) without a nucleic code directing the creation of adjacent new, abnormal, but conformable PrP^{res}. PrP^{res} is thought to be the transmissible agent and is regarded as a pathogenic

product common to all TSEs. Interestingly, Naslavsky, (1999) reported that cholesterol and sphingomyelin (phospholipids) support and perhaps regulate the formation of PrP^{res}, and thus presumably the propagation of disease producing prions. As presented herein, however, PrP^{res} is not necessarily synonymous with PK^{res}.

Both PrP^{res} and PK^{res} are identified via digestion of suspect tissue via proteinase-K. Proteinase-K (PK) is a powerful protein-dissolving enzyme, active in strong detergents in a pH range of 2.0 to 12.0 (optimum pH: 7.5 to12.0) and at the proper concentration has the ability to digest most proteins, both native and denatured, in generally less than 2 hours (McKinley, 1983). Only a few unusual proteins can survive PK treatment, with PK itself and PrP^{res} being the two most well known. Under a codified regimen of detergent dissolution and PK digestion, followed by Western Blot gel electrophoresis and immunostaining, a TSE-diseased tissue will reveal the residual presence of suspected abnormal prion protein (PrP^{res} or other PK^{res}) of a molecular weight in the 20 to 30 kiloDalton (kDa) range. PrP^{res} - PK^{res} determination may also use less-involved immunohistostaining or immunochemical techniques.

Initially, under earlier TSE identification protocol, four selection criteria were used to identify TSE diseased tissue. Under the old four criteria system, the immunohistoassays were considered as separate and distinct from the Western Blot. However, given that immunochemistry responds to PK^{res}, such a differentiation is likely unwarranted. While still regarded by some investigators as its own separate criterion, immunohistostaining (IHS) detection for the presence of PrP^{res} is a sub-type of third criterion, that of PK^{res} identification. Effectively, IHS is a micro-procedure whereby a chemical stain hitches a ride on a serum soup composed of poly- and/or monoclonal antibodies specifically created to react to or attach to the desired material, in this case, the PrP^{res} (or PrP^{res}) present in suspected TSE-infected brains. Generally, it can be assumed that the response to IHS in TSE-infected tissue is, in fact, a response to the same PK resistant proteins identified in the Western Blot test for which the staining antibody was intimately created.

Recently, several new, more rapid, Enzyme-Linked ImmunoSorbent Assays (ELISA) have evolved around detecting the presence of PrP^{res} material. ELISA tests rely on the affinity of a contrived antibody or pool of antibodies together with a chemical marker to seek out and attach to the desired disease agent, in this case presumed to be PrP^{res}, making for rapid identification via visual or photometric means without excessive sample preparation. ELISAs have greatly enhanced the productivity of TSE diagnostic workers.

Prior to the year 2000, most TSEs were identified with the cumulative positive results derived from at least three of the four ID criterions. A bit subjective, it was generally accepted that three of the four criteria being positive would result in a positive TSE diagnosis. Two or one positive result would suggest the presence of a TSE, but would be relegated to a "suspect" status, in

need of additional scrutiny. The complete lack of all criteria was indicative of a negative response to TSE, and hence was reported as a negative. However, one must recognize that the lack of all four criteria does not necessarily eliminate a TSE disease, but in actuality represents only the failure to identify any outward TSE disease-related symptoms. In a known disease environment, a serious problem of false negatives at the very early stages of disease development is present. Ultimately, the failure to properly identify the putative TSE causal agent has rendered all TSE diagnostic procedures fallible as they are focused on the symptoms of disease rather than the underlying etiological agent.

If one operates under the premise that a TSE disease is classified as a prion disease, a disease of prions, then, what in fact is the causative agent? Many would suggest that the prions themselves are the causative agent as per Prusiner (1999). However, decades of work have failed to fully or logically explain the nature of prions as it pertains to TSE disease variants and its strains and the occasional presence of nuclear proteins (Sklaviadis, 1989). Abundant evidence exists that abnormal prions are certainly the result of a TSE disease, but proofs are insufficient to assure that prions are the actual cause of TSE disease. Baron, et al (2002) goes so far to suggest that abnormal prions (PrP^{res}) are only present after a complex interaction of normal cellular prion protein (PrP^{c}) by PrP^{res}, hence suggesting a membrane replacement process of unknown evolution.

Plentiful research has demonstrated that the detection of abnormal prions can only be accomplished many weeks after infection. In the case of deer, experimental CWD infections using immunohistochemistry assay procedures enhanced for sensitivity, have shown PrP^{res} development within alimentary-tract-associated lymph nodes as early as 42 days after oral inoculation (Sigurdson, 1999), while more than 12 months may be necessary before outward clinical signs appear. With elk, the time frame can easily exceed sixteen to eighteen months before the presence of any PrP^{res} material can be established. One can rightfully assume that the causative agent is present in the host animal from the time of infection, yet is not readily expressed through PrP^{res} development, or if developed, is of insufficient quantity to be detectable by current methodology.

In Radebold, et al (2001) mice injected with CJD infectivity developed distinct changes in PrP in all abdominal lymphoid tissues 28 to 32 days after inoculation. No changes in PrP in the brain or spinal column appeared until 90 to 120 days post inoculation, and that is despite intracerebral injection. Race and Ernst (1992) found that scrapie infected mice had detectable PrP^{res} in the spleen only one week after inoculation which increases 65-fold 3 weeks post-inoculation, while brain PrP^{res} was not detected until 8 weeks, but later achieved a 200-fold increase in 13 weeks increasing infectivity by 10,000-fold. The agent is evidently lymphotropic or splenotropic initially and only neurotropic with time.

TSE disease variants are characterized by strains, i.e. scrapie 236K, sc237, ME7, 22A, etc. Each strain has distinctive physical parameters, which normally would be regarded as under some

sort of genetic code control: (1) the extent of vacuolation in various regions of the brain; (2) the time period after inoculation for the manifestation of clinical symptoms; (3) the quantity of amyloid plaques in the brain; (4) the occurrence of increased weight in the preclinical phase of disease; (5) the development of aberrant glucose tolerance; (6) the area of the brain which yields the shortest incubation period after injection; and (7) the physical-chemical and immunological characteristics of scrapie associated fibrils. In several well-documented instances differences between scrapie strains were evident after repeated passages of the strains in the same host species (Carp,1989). Yet, no genetic code is present in PrP Prionic causal agent theories. Occasional reports of RNA or DNA being found in TSE preparations, however, are common (Sklaviadis,1989, Narang, 1998).

Interestingly, white blood cells without identifiable prions have been found to contain CJD infectivity shortly after cerebral inoculation and will cause disease in donor recipients (Manuelidis, 1985). Tamai (1992) injected mice intracerabrally with tissue samples collected from a CJD-infected pregnant human mother. The brain, placenta and cord leukocytes were infective, as was the mother's colostrum. Ingrosso (1999) found that gingival tissue was higher in infectivity than dental pulp or ganglia further suggesting that blood more than neurons may be a source of early infectivity.

Hunter and Houston (2002) demonstrated that it is possible to transmit bovine spongiform encephalitis (BSE) to a sheep by transfusion with whole blood taken from another sheep during the pre-clinical phase of an experimental BSE infection when the donor animal appears healthy. But Holada (2002) found that the infectivity of hamster scrapie strain 263K was not in platelets isolated from blood pooled from six hamsters with clinical scrapie. He found a larger proportion (98.4%) of the total infectivity was recovered from the mononuclear leukocyte fraction but little or no PrP^{res} was detected.

Both Pattison & Millson (1962) and more sensationally Bosque (2002) have found TSE infectivity (called by different names and albeit in very small quantities) in muscle tissue of goats and prion-enhanced transgenic mice, respectively.

The gastrointestinal tract appears to be the natural route of infection of TSEs in response to the oral exposure to the infectious agent. The favored interpretation being that the agent spreads by lymphatics from the gut to the spleen and on into the spinal cord via enteric and splenic sympathetic neurons. But the occurrence of abnormal PrP in gut lymphoid tissue suggests that blood transport may be a more potent transportation method than neural routes (Radebold, 2001). The TSE agent is obviously not restricted to neuronal tissue and may, in fact, be found throughout the entire body. Normal prion proteins (PrP^{c}) are found through the body, so is it not to be expected that in infected animals that abnormal PrP^{res} would be found throughout as well?

As such, the three major criteria of TSE identification, that of defining the presence of PrP^{res}

plus the identification of SAF and that of neuronal vacuoles, all have the potential to be flawed. Significantly, all tests currently used are incapable of detecting the presence of the putative pathogenic agent prior to manifestation of the disease symptoms.

THE QUEST FOR A MORE LOGICAL CAUSAL AGENT

PK Resistance

The corner stone of current TSE identification lies with immunohistostaining and, now more recently, ELISA tests for the detection of PrP^{res} proteins confirmed by Western Blot, and followed up with histology to identify SAFs and vacuoles. Does this cornerstone and its subsequent building blocks form a reliable foundation upon which to gauge all TSEs? Unfortunately, like TSEs themselves, the procedures used to evaluate TSE are far from well understood. The cornerstone of TSE detection, the presence of proteinase-K resistant proteins is seemingly a rare phenomenon, but is not exclusive to TSE disease. Other natural processes can fabricate the elusive PK^{res}.

Butler, et al, (1991) found proteinase-K resistant proteins of 40 kiloDaltons (kDa) molecular weight in the outer membrane of a porcine pathogen, *Mycoplasma hyorhinis*. Additional non-immunological, cross reactive PK resistant proteins of 46 kDa were also found in *M. pneumoniae*, *M. orale*, *M. aginini* and *M. salivarium*.

At least one PK resistant protein at the 18 kDa molecular weight range and perhaps more bands of PK^{res} material of prokaryotic origin was found in Leptospria by Nicholson and Prescott (1993). Bastian, et al (1993) found that GT-48 stain of *Spiroplasma mirum* yielded PK^{res} protein bands of 28, 30, 66, and 76 kDa upon Western Blot analysis. The two lower PK^{res} bands mimic those considered diagnostic of PrP^{res} (prions) found in TSE disease. Not surprisingly, the ME-7 antibody used for the detection of SAF proteins in TSE-infected brains (Rubenstein, 1986) reacts positively against all four of Bastian's PK^{res} Spiroplasma proteins at 28, 30, 66 and 76 kDa (Bastian, 1987). Brodeur (1997) found a proteinase-resistant, low molecular weight protein of 22 kDa in the outer membrane of *Neisseria mengingitidis*, the putative agent in some meningitidis diseases.

The seeming abundance of PK^{res} material, particularly in bacteria of the Class Mollicutes-Genera Mycoplasma and Spiroplasma, suggests that PK resistance may be not be an unusual trait of Mollicutes or even other morphologically spiral bacteria. Very few studies have specifically attempted to find natural PK^{res} materials and Mollicutes like TSE disease are very poorly understood. The following is excepted from Bove (1988) and from www.zmbh.(2002)

> Mollicutes are bacteria with the smallest known genomes. This class presently comprises the six eubacterial genera Acholeplasma, Anaeroplasma, Asteroleplasma,

> Mycoplasma, Spiroplasma and Ureaplasma (however, the term mycoplasma has been frequently used to denote any species included in the class Mollicutes). The common characteristics are the complete lack of a bacterial cell wall, osmotic fragility, colony shape and filterability through 450-nm pore diameter membrane filters. The relatively close phylogenetic relationship of these genera was measured by comparative sequence analysis of the 5S and 16S ribosomal RNA (rRNA). The rRNA sequence analyses also revealed that the Mollicutes are not at the root of the bacterial phylogenetic tree, but rather developed by degenerate evolution from gram-positive bacteria with a low mol% G+C (guanine plus cytosine) content of DNA, the Lactobacillus group containing Lactobacillus, Bacillus, Streptococcus and two Chlostridium species. The Mollicutes lost during the process of evolution a substantial part of their genetic information. This is reflected by significantly smaller genome sizes as low as 600 kbp and extending to 2300 kbp as compared with 2500-5700 kbp long genomes of their ancestor bacteria. The loss of coding capacity could probably be tolerated because of the parasitic life style of the Mollicutes. They have never been found as freely living organisms. In nature Mollicutes depend on a host cell, respectively on a host organism. For instance, Mycoplasmas and Ureaplasmas are parasites in different vertebrates, from which they obtain essential compounds such as fatty acids, amino acids, precursors for nucleic acid synthesis and cholesterol, a compound normally not found in bacteria
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> The plant pathogenic mollicutes are transmitted by insect vectors. Many varieties of insects carry Mollicutes, particularly Spiroplasmas, and deposit these organisms on plant surfaces where other insects pick them up. New acholeplasma, mycoplasma and Spiroplasma species have been identified in insect hosts or on plant surfaces. Some Mollicutes are pathogens of animals, some produce diseases in humans. Mollicutes are also frequent contaminants of animal cell cultures.

Spiroplasmas, a division of Mollicutes, are equally not well understood. These unique bacteria were discovered in 1972 when helical microorganisms were envisioned by phase microscopy from the sap of plants infected with corn stunt disease. The taxonomy of the genus *Spiroplasma* actually began in 1973, when Saglio, et al. published a description of the cultivated filamentous mycoplasma-like agent isolated from tissues of citrus plants affected with Stubborn Disease. Other Spiroplasmas, discovered earlier and studied under various aliases, then turned up. One was from *Drosophila* (sex-ratio organism) and two were from ticks (strain 277F and the suckling mouse cataract agent "SMCA"). Of the all unnamed Spiroplasmas, only one (strain 277F) was readily cultivable but that may have been originally derived from plant spiroplasma (First Internet Conference on Phytopathogenic Mollicutes, Invited Lecture, S*piroplasma* Taxonomy).

The proteinaceous makeup of Spiroplasma is critical for understanding of these unique prokaryotes. Wroblewski, (1977) noted that Spiralin protein of a molecular weight of 26 kDa is a major protein of the *Spiroplasma citri* membrane and speculated that spiralin-like proteins are probably present in all Spiroplasma. Le Henaff and Fontenelle (2000) determined that spiralin is a "classical" lipoprotein (i.e. is triacylated) and composed of acylated S-glyceryl cysteine.

Using antiserum, Townsend and Archer (1983) purified Spiroplasma proteins and determined that a single 55 kDa protein was unique to Spiroplasmas together with a lesser abundant protein in the 25 kDa range. Additional protease digestion of this exclusive 55 kDa protein yielded residual peptides of 27 kDa and 28 kDa while in particular the SMCA strain of *Spiroplasma mirum* cleaved to produce an additional 21 to 23 kDa resistant product. Townsend and Plaskitt (1985) found that spiralin protein of a 25 kDa mole weight was localized in the plasma membrane and as extracellular strands.

Clearly, spiralin protein has +/- 25-26 kDa mole weight protein at least a portion of which is PK-resistant protein of its own accord (Brenner, 1997). Since spiralin is a major proteinaceous component of the Spiroplasma wall membrane, selected Spiroplasma spp. can and do respond similarly to: 1) PK-resistant Western blots and 2) associated immunohistochemistry preformed upon TSE tissue. An affirmative ELISA response can be expected from spiralin, but is yet to be tested. Two of the four original TSE diagnostic criteria: PK resistance and positive immunostaining can be met by Spiroplasma.

Scrapie-Associated Fibrils and Other Physical Characteristics Of The TSE Agent.

Scrapie-Associated Fibrils (SAF) are archetypical of TSE disease. Numerous studies have documented SAF presence, which has continually been re-confirmed in all versions of TSE disease. A chronological review of pertinent data is appropriate:

In early research only rudimentary procedures were available. Accordingly, Gibbs (1967) and Kimberlin (1971) virtually no scrapie infectivity passed through 27 to 42 nm filters, while abundant infectivity penetrated 43 to 50 nm filters of the same type.

In Bastian (1979), a biopsy of CJD-infected tissue revealed widespread spongiform degeneration, patchy neuron loss and gliosis without discernable inflammatory response. Electron microscopy revealed rare spiral inclusions primarily within dilated presynaptic terminals and contracted mitochondria. These inclusions were characterized by a coiled membrane configuration with 5 to 8 twists measuring 850 to 1000 nm in length and from 75 to 137 nm in width. .

According to Merz (1981) abnormal fibrillary structures, were observed using negative stain techniques in sub-fractions of brains from scrapie-affected animals. SAF was observed in all combinations or strains of scrapie agent, and strains or species of host examined, regardless of their histopathology, or, in particular, the presence or absence of amyloid plaques. SAF consist either of two or four filaments. They are morphologically dissimilar to the normal brain fibrils--microtubules, neurofilaments, glial filaments, and F actin. However, SAF did bear a resemblance to amyloid.

In 1981, Reyes and Hoenig reported that brain biopsy specimens from two patients with Creutzfeldt-Jakob disease revealed the presence of intracellular membranous spiral inclusions in the processes of cortical cells. These inclusions were 375 nm to 660 nm in length and 50 nm to 88 nm in width. Bolton (1982) observed that abnormal PrP is resistant to proteinase-K digestion and upon detergent lysis seemed to aggregate to form fibrils. In turn, Prusiner and McKinley (1983) similarly found that purified preparations of infected hamster prion proteins of mole weight 27 to 30 kDa consisted exclusively of rod-shaped particles 10 to 20 nm in width and 100 to 200 nm in length, additionally suggesting that they may be amyloid.

Merz (1983) observed that the fibrils isolated from brains with senile dementia of Alzheimer type were 4-8 nm in diameter, narrowing every 30-40 nm and apparently composed of two 2-4 nm filaments. The fibrils from a Gerstmann-Straussler Syndrome brain (a variety of CJD) were 7-9 nm in diameter, narrowing every 70-80 nm and with a suggestion that they are composed of two 3-5 nm filaments. Fibrils isolated from 87V scrapie-affected mouse brains were 4-8 nm in diameter with a twist every 15-25 nm presumably composed of two 2-4 nm filaments. The fibrils from the scrapie brains were usually observed in pairs

Further, abnormal fibrils were observed in synaptosomal preparations of scrapie-infected brain. but never in control animals. SAFs are present in CJD brain fractions in the experimentally transmitted disease, as well as, in a few naturally occurring human cases of CJD. SAF are also present in spleen extracts of animals experimentally infected with scrapie or CJD. This close association of SAF with these two diseases and two different organ tissues (brain and spleen) known to contain titers of infectivity, suggest that the SAF are: (1) a unique pathological response to the disease or (2) the infectious agent of these diseases (Merz, 1983)

Diringer (1983) was able to fractionate fibrils from scrapie-infected golden hamsters visually similar to SAF discovered by Merz. Such fibrils under high magnification were found to be short rods composed mainly of two helically twisted filaments 50 to 300 nm long and 4 to 6 nm in width possessing a molecular weight of 26 kDa. Infectivity measurements were directly proportional to visual fibril density. Additionally, a scrapie agent infectivity size range of about 20 nm to 55 nm was found.

In 1985, Kascsak isolated and purified three different scrapie strains each having different morphology, sedimentation rate and protein composition. SAF from three scrapie agents were distinguishable from each other by their sensitivity to proteinase-K digestion. SAF co-purified with infectivity. SAF appeared to be a unique class of structures, which are related but specific for each individual scrapie strain.

Bode (1985) found, through a modified ELISA technique used on SAF proteins obtained from hamster, mouse, and from patients who died of Creutzfeldt-Jakob disease, that the antisera predominantly detected five bands in a Western blot analysis with apparent molecular weights of 26K, 24K, 20K, 18K and 16K. By gel electrophoresis these antigens seemed to be identical in mouse, hamster and man, but the amount of material in the various bands varied according to host or agent.

This time using 4 scrapie strains, Kascsak (1986) found that SAF proteins were antigenically distinct from those of paired helical filaments or amyloid isolated from patients with Alzheimer disease. Distinct Western blot profiles were demonstrated for SAFs isolated from animals infected with each scrapie strain. Differences seen among SAFs were independent, at least in part, of host species or genotype, implying that certain specific structural and molecular properties of SAFs are mediated by the strain of scrapie agent. Bastian, (1987) found 4 to 5 nm width, fibrillar proteins in the synaptosomal-mitochondrial fraction of scrapie infected sheep brains arranged in twisted, intertwined collections.

Narang, in1988 and again in 1993 describes "tubulofilamentous' particles in CJD brains under electron microscopy. Sodium docecyl sulphate detergent treatment revealed twisted fibril cores, which were identified as scrapie associated fibrils. Treatment of the tubulofilamentous particles with enzymes demonstrated that each larger tubule possessed an outer coat of protease-sensitive material, a middle coat of nuclease-sensitive material and an inner protease-resistant protein identical in morphology to the Merz SAFs.

Liberski, P.P. (1990) found that Swiss white mice inoculated with CJD infectivity produced tubulovesicular structures measuring 20 – 50 nanometers in diameter.

Ruberstein and Merz (1991) found that SAF derived from scrapie-infected spleen tissue was virtually indistinguishable from infected brain tissue. Further, since brain tissue undergoes various gross histopathologic changes while spleens do not, if the SAF was derived from the pathologic changes, then the SAF in spleen should have originated from the brain. However, the earliest detection and maximum yield of infectivity and SAF is found in the spleen although several hundred fold levels of SAF, abnormal PK^{res} and infectivity where later found in the brain. Hence they concluded that SAF and abnormal PK^{res} appear to be a direct result of agent replication rather than pathologic changes.

Nanang (1992) speculated that PrP molecules aggregate to form SAF, which are wrapped around with a single stranded DNA, which then acquires an outer undetermined protein coat. The creation of SAF deprives the cell membrane of needed PrP eventually producing cell disruption.

Guiroy et al, (1993) isolated abnormal fibrils in CWD-infected elk brain tissue. The isolate fibrils resembled those found in scrapie-infected hamster brains, were immuno-reactive to scrapie antibodies and possessed proteinase-K resistant protein bands of 26 to 30 kDa mole weight, confirming that CWD and scrapie were closely related TSEs.

Fundamentally, the presence of SAF is intimately associated with TSE disease. While considered diagnostic, one must reflect on the nature of the fibrils and the procedures through which they are identified. Certainly the microscopic determination of their presence seems to be definitive for two sizes of particles, 1) fibers, tubules or rods from 20 to +100 nm in width, and 2) thin, sometimes helical filaments 3 to 6 nm in width. The SAF were not artifacts of preparation, but are affected by preparation actions. Interestingly, length appears to be a function of physical preparation procedures, the less sample homogenization, the greater the particle length, hence the particles can be broken apart by agitation. Additionally, chemical preparation also seems to affect particle width. The smaller particles generally appear after sonic or detergent treatment, suggesting their release from within the larger width particles.

Knowing the morphology and nature of SAFs, one can then attempt to find comparable natural structures, simply through comparative morphology. Are there any potential causative agents that could produce the presence of SAF within diseased tissue?

In the mid-1970's Spiroplasma, a wall-free prokaryote, were first recognized as a newly discovered variety of Mollicutes, a cell-wall-less (membrane bound) parasitic bacterium containing the smallest of living, self-replicating gnomes. Freeman (1976) found that Spiroplasma requires sterols for growth and incorporates sphingomyelin (phospholipids) when grown in culture.

Both Stalheim (1978) and Williamson (1974) found that osmotic shock and non-ionic detergents can induce a Spiroplasma cell membrane breakdown (normally +/- 100 nm width), which then release long, flexuous fibrils 3-4 nm in width, some helical in form. Williamson suggested the presence of 3 or 4 individual intertwined 4 nm filaments producing a 12-15 nm ribbon used for cell form stability. In 1979, Bastian compared photomicrographic images of spiral CJD inclusions with recently discovered Spiroplasma bacterial images, and discovered that the visual comparison was striking in both size and shape similarities.

Townsend in 1983 observed via thin section, and then proved via detergent cell lysis that 80

to 100 nm wide *Spiroplasma melliferum* (BC-3, honey bee stain), when subjected to detergents and agitation released internal fibrils arranged in helically twisted ribbons 3 to 6 nm in diameter and that the fine fibrils were composed of 55 kDa mole weight protein together with a lesser 26 kDa protein. Bastian (1984) micrographs of *Spiroplasma mirum* (GT-48, tick strain) from pure broth cultures showed abundant bleb forms and occasional distinctive, and sometimes crescent, tubule-like organisms enclosing fine sinuous filaments.

Several researchers, including, Kascsak (1986) have compared SAFs with Alzheimer's amyloid plaque fibers and, while finding some similarity, termed the amyloid plaque fibers antigenically distinct.

Through agent density studies, Sklaviadis (1989) suggested that the CJD agent is likely made up of a protein-nucleic acid complex approximately the size of known animal viruses perhaps ranging from 28 to 75 nm. Further Sklaviadis, indicated that a large portion of putative normal prion protein (Gp34, 34 kDa, PrP^c) can be separated from CJD infectivity suggesting that PrP^c may not needed for infectivity. Preparations of the suspected CJD infectious agent yielded 61% of the infectious material at a density of 1.27 g/ml. This confirmed the Marsh (1984) report that most of the scrapie infectivity from hamster brain and neural retina tissue had a density of 1.280 g/ml. This compares with suspected Spiroplasma purified fibrila (filaments only) at about 1.22 g/ml (Townsend, 1980)

Itot (1989) found that *Spiroplasma mirum* (SMCA tick strain) was present in filtrates above 100 nm. *S. mirum* was observed to undergo several morphological manifestations over its life cycle. Early helical filaments are followed by small spherical or bleb-like bodies attached to filaments in turn followed by larger spheres originating from entangled filaments. Upon large sphere breakdown, granular bodies represented the smallest reproductive units continued the life cycle. No detergent treatment was used.

Clearly, at least one natural agent, Spiroplasma, has been found to create fibrils virtually identical to those found in TSE disease. As such, Spiroplasma has been shown to: 1) contain 26 kDa PK-resistant proteins 2) respond affirmatively to immunohistochemistry and now 3) can form 4-6 nm fibrils. Three of the four TSE diagnostic criteria are met by Spiroplasma.

Neuropathology And TSE Disease

The neuro-degenerative pathology of TSE is undisputed. All TSEs are neuropathologically similar. In particular, Chronic Wasting Disease like other TSEs is characterized by intraneuronal vacuolation, spongiform change of the neuropil and astrocyte embellishment. Neuropathological findings consist of (1) extensive vacuolation in neuronal processes, within myelin sheaths, formed by splitting at the major dense lines or within axons; (2) dystrophic neurites (dendrites, axonal preterminals and myelinated axons containing degenerating mitochondria and pleomorphic, electron-

dense inclusion bodies); (3) prominent astrocytic gliosis; (4) amyloid plaques; and (5) giant neuronal autophagic vacuoles. Other findings include activated macrophages and occasional spheroidal structures containing electron-dense fibrillar material of unknown origin, abundant structures suggestive of degenerating microtubules entrapped in filamentous masses, vacuoles and myelin figures (Guiroy,1993a). Liberski, (1989) noted that pleomorphic, electron-dense inclusion bodies were found as early as 2 weeks post-inoculation of hamsters with 263K scrapie. Inclusion numbers increased with the incubation period, and their highest density was observed at the terminal stage of disease. A distinct feature of TSE disease is that subsequent passages of the disease through an identical host species, generally produces a more virulent, shorter-incubation pathogen (Hadlow, 1987).

While several potential pathogens can create all or a variable portion of the symptoms described by Guiroy(1993a) and others, can these symptoms found in virtually all TSE disease be created specifically by Spiroplasma? The answer is affirmative.

Select versions of Spiroplasma have a distinct affinity for neuronal cells. Elizan, (1972) found that mice inoculated with *Spiroplasma mirum* (SMCA) developed prominent microcystic encephalitis localized in the subpial and subependymal zone and within deep gray matter with locally prominent astrocytes.

Tully (1982) inoculated suckling rats with either *Spiroplasma mirum* GT-48 or SMCA and found that a fatal dose due to septimia approached 10^9 organisms for SMCA, but that lesser dose of 10^7 to 10^8 organisms showed a high incidence of ocular cataracts. The GT-48 strain required only 10^4 to produce pathological septimic results. A third strain TP-2 initially mimicked SMCA but became more pathogenic for rats and showed less propensity for cataracts as it was passaged and purified in artificial media.

Utilizing rabbit-tick-derived strain, GT-48, Tully (1984) intracerebrally inoculated one-day-old neonatal rats. Rats receiving the largest challenge dose (300 organisms) in seven days had larger numbers (10^7 to 10^9) of Spiroplasmas in the brain and smaller numbers in the spleen (10^1 to 10^4). Since only two rats out of ninety receiving the 300-organism dose survived more than 14 days, apparently a lethal septemic dose of GT-48 is easily attained. Those given an order of magnitude less (30 organisms) showed moderate to abundant brain Spiroplasma (10^4 to 10^9) at 21 days but a much lesser spleen presence and only a few infection deaths. Undoubtedly *S. mirum* is neuropathic.

In conjunction with Tully (1984), Bastian (1984) evaluated infected brain material of GT-48 inoculated rats. Histopathology at 14 days post intracranial inoculation revealed microcystic encephalitis with Spiroplasmas recognized as filaments, crescents and membrane blebs. Surprisingly, at and after 25 days post inoculation electron microscopy showed little inflammation,

some neuronal vacuolization, but widespread dilation of neuronal processes and an apparent non-existence of detectable Spiroplasma organisms despite assay titers approaching 10^4 organisms per gram of brain material. Immunostaining using GT-48 antisera showed marked localization to superficial and deep gray matter (cerebral cortex and basal ganglia), yet no visible GT-48 organisms. Cerebellum staining was in close association with Purkinje and granular cells. Despite positive immunostaining Spiroplasma organisms were not detected in any +25 day biopsies. The authors postulated that the membrane bleb form of Spiroplasma was cloaked within the host neuropil.

In 1987 Bastian demonstrated that intraperitoneal or subcutaneous inoculation with the vertebrate virulent GT-48 strain of *Spiroplasma mirum* alleviated short term mortality but produced alopecia (localized hair loss) and a reduction in body weight. Titers were found in both spleen and brain tissue, initially building up in the spleen but then later exceeding in the brain but without vacuolar encephalopathy as found in previous intracranial inoculation. A significant development of cataracts (15 out of 38 rats) both unilateral and bilateral appeared in contrast to prior studies where cataracts were not found with the GT-48 strain. The migration from peripheral tissue into central nervous system indicated at Spiroplasma GT-48 is neurotropic

Humphrey-Smith and Chastel (1988) point out that while most Spiroplasmas are non-pathogenic, antibodies for some strains have been found in humans including at least one with amyotropic lateral sclerosis, a neurologic disorder known as Lou Gehrig's disease and linked with gene mutant copper-zinc dismutase enzyme toxicity.

Tamai (1989) found that the transmissible agent of CJD is closely associated with surface membranes of neuronal and/or glial cells, including their processes; and the CJD agent is diffusely present intracellularly, including in the surface membranes, but for manifestation of infectivity the agent needs membrane components as prerequisite factors.

Select strains of Spiroplasma are able to survive up to nine months in intracerebally-inoculated mice and are associated with significant runting syndrome and an increase incidence of mortality and neurological symptoms generally without the appearance of antibody mimickingand immunological tolerance (Chastel,1991). Humphrery-Smith (1992) found distinct Spiroplasma mitochondrial pathogenicity in both *in vitro* and *in vivo* situations cultivated at 38°C.

In 1998, Kern, documented the only known case of a Spiroplasma infection of a human, describing the etiology of a rapidly progressing unilateral cataract associated with severe anterior uveitis (inflammation of the iris) in a 27th-week Caesarean-delivered, premature baby, then at 4 months of age. While cell cultures were negative, PCR DNA sequencing identified a positive marker for Spiroplasma. Prior to birth, recurrent maternal vaginal infections presumably due to mycoplasmas were treated with erythromycin and the baby sustained a bout of pneumonia of unknown cause at age three months.

Bastian and Foster (2001) found the first direct evidence of an association of TSEs with a bacterium. Initially, three oligonucleotide primers specific to Mollicute 16S rDNA were utilized via polymerase chain reaction ("PCR") and DNA sequence analysis to systematically study diseased CJD brain tissue and scrapie-infected tissue. Preliminary studies of two CJD-brains produced PCR products with 96% to 99% homology to *Spiroplasma mirum*, while control brains had no response. Additionally, and seperately various Mycoplasma and Spiroplasma controls produced recognized PCR products. Continued testing of refined primers toward a Spiroplasma model yielded no PCR products from Mycoplasma and a 276-bp PCR product in 7 out of 8 Spiroplasma species tested. A third CJD-brain tested using the new primers yielded a +99.5% homology to *Spiroplasma mirum*. A GenBank blast search of the product sequences failed to find any sequences over 20-bp in a human gene, hence the defined products were seemingly of non-human origin. Subsequently, sixty-five brains (50 controls and 13 CJD positives) were subjected to the newly refined primers. All 13 positive brains produced the predicted 276-bp PCR products, while none of the 50 controls had a response, strongly suggesting the presence of foreign bacterial rDNA within diseased tissue.

Five of nine scrapie-infected tissues had similar responses with at least two scrapie brains having a +99% homology with *S. mirum*. Normal sheep brains produced no products. The failure to identify 100% of the scrapie-brains was attributed to the diversity of the scrapie agent and the likelihood of over 20 individual scrapie strains. Interestingly, according to USDA personnel one of the detected scrapie positives was very early in its incubation period (Bastian, PC, 2002).

Conclusively, Spiroplasma are neurotropic, have an appetite for sterol, an affinity for gray matter and have been positively identified in diseased brain tissue. Several strains have a documented history of producing neuronal degeneration in vertebrates. The neonatal human symptoms described by Kern (1998) mimic the conditions of SMCA-strain Spiroplasma infections in neo-natal laboratory animals, which show cataracts, posterior uveitis, encephalitis and death. The rat brain degeneration described by Bastian (1984) mimics the symptoms found in all TSE diseases. Vacuolation in neuronal processes, mitochronial degeneration, pleomorphic inclusion bodies are all possible products of Spiroplasma infection. Astrocytes have been documented. Only amyloid plaques have yet to be identified within Spiroplasma infections, however, one must recognize that Spiroplasma vertebrate pathology studies has only scratched the surface of possible disease manifestations.

Spiroplasma have now been shown to: 1) contain 26 kDa PK-resistant proteins 2) respond affirmatively to PrP^{res} immunohistochemistry, 3) form 4-6 nm SAF-like fibrils and; 4) demonstrate vertebrate neurotropic and neuropathologic tendencies virtually identical to TSE diseases. All four of the four TSE diagnostic criteria are met by Spiroplasma.

Agent Destruction Refractivity

While all the TSE diagnostic criteria can be met by Spiroplasma, can some of the other more unusual characteristics of the suspected TSE agents be ascribed to Spiroplasma as well?

Disinfectants:

TSE and in particular scrapie agent has several unique resistive properties. It can survive boiling water for several hours (Stamp,1959, Pattison and Millson 1961) 5 % chloroform and 2% phenol (Stamp, 1959), numerous freeze-thaw cycles (Pattison and Millson, 1961), 20% formalin solutions for 18 hours (Pattison, 1965) and treatment with various protein enzymes (Pattison and Millson, 1960).

Alternatively, cell-wall-less Spiroplasma particularly the insect-hosted varieties, display a considerable resistance to disinfectants compared to wall-bearing organisms (Stanek,G. 1981). Several strains survived 40% ethanol solutions for over 10 minutes. Tick-derived Spiroplasma survived more than 40 minutes at 30% ethanol. Several stains survived 0.5% formalin solution for over 10 minutes and 1.0% glutaraldehyde or 0.5% phenol for over 40 minutes. Most were generally more resistant than the *E. coli* and *Staph. aureus* comparisons. Spiroplasma have been observed to be active in 50% glutaraldehyde, a potent tissue fixative used normally at 3% concentration (Bastian, written press statement, Feb, 2001).

Interestingly, Almagor M. (1983) notes that mycoplasma-produced hydrogen peroxide degrades cell membranes yielding malonyldialdehyde as an indicator of membrane lipid peroxidation. Perhaps a resistance to the aldehyde radical is endemic in Spiroplasma.

Sterilization or Heat Deactivation:

Flechsig (2001) determined that prions (PrP^{res}) (or perhaps infectivity agents) are readily and tightly bound to stainless steel surfaces and even after washing and 10% formaldehyde submersion can transmit scrapie to recipient mice after short exposure times. This substantiates the possibility of contaminated surgical instruments and the potential transmission of CJD to human patients.

Interestingly, early work by Stanek, (1981) found that some Spiroplasma could survive in dried media on smooth surfaces such as plastic, glass and ceramic for over 3 weeks. While Watanabe and Yukitake, (1990) found that some species of mycoplasma could attach to glass surfaces and suggested that electrostatic bonds were involved in the attachment and that bivalent metal ions played a role.

Despite sometimes sensational past claims of heat-treatment resistance, Taylor (1996) found

that while scrapie agent was not completely de-activated by dry heat at up to 180 degrees C for one hour, the titer of surviving infectivity reduced progressively as the temperature was increased with no infectivity after one hour at 200 degrees C.

Work by Somerville, et al, (2002) showed the temperatures at which substantial inactivation of scrapie agent first occurred varied according to TSE strain, ranging from 70 degrees C to 97 degrees C.

Alternatively, viable Spiroplasma organisms have been recovered from boiling water (Bastian, written press statement, Feb, 2001).

Ground Contamination:

Brown and Gajdusek (1991) have demonstrated that scrapie infectivity homogenate can survive for over 3 years in buried soil conditions.

Zhang, et al (2000) discovered that several plant pathogenic Spiroplasma species were capable of surviving up to 9 years in a liquid medium, and up to 17 years in dry soil as well as in farmed soils, generally spherical structures 60 nm in diameter and aggregating into filaments up to 2500 nm long.

Resistance to Antibiotics

Most antibiotics and anti-virals do not modify experimentally-induced Creutzfeldt-Jakob disease in mice, nor have they been effective on naturally occurring cases (Tateishi, 1981). Roikhel (1984) found that multiple exposure of scrapie-infected mice to hyberbaric oxygenation during the incubational period led to a certain aggravation of infection as evidenced by a greater accumulation of the agent in the central nervous system and spleen, as well as by more pronounced ultramicroscopic changes in neuronal tissue.

Tremblay (1998) noted that in transgenic tetracycline PrP regulator mice inoculated with scrapie, the mice developed progressive ataxia at approximately 50 days after inoculation with prions unless they were maintained on doxycycline which delayed ataxia indefinitely.

Josefson D. (2001) noted in a controversial finding that CJD patients have a decrease in PrP^{res} after treatment with Chloropromazine, an anti-psychotic drug, commonly known as Thorazine, and also with Quinacrine an anti-malarial drug with known bactericidal properties. Chloropromazine is virtually unstudied, but those to date support bacteriastat properties. Interestingly, Quinacrine is a coal-tar derived synthetic replacement for natural anti-malarial quinine

of which a derivative Ubiquinol is a strong mitochondrial-oriented antioxidant.

Amphotericin B, a polyene antibiotic, increased the incubation time of scrapie disease in animals infected by either the intraperitoneal or intracerebral route (Pocchiari,1987). This drug, approved as an anti-fungal agent, acts by binding to steroidal alcohols in the cell membrane of susceptible fungi resulting in an increase of membrane permeability that allows leakage of the cellular contents.

Forloni, 2002 found that Syrian hamsters injected intracerebrally with 263K scrapie-infected brain homogenate and a tetracycline-treated inoculum showed a significant delay in the onset of clinical signs of disease and prolonged survival time. These effects were paralleled by a delay in the appearance of magnetic-resonance abnormalities in the thalamus, neuropathological changes, and PrP^{res} accumulation. When tetracycline was pre-incubated with highly diluted scrapie-infected inoculums, one third of hamsters did not develop disease. Tetracycline and its derivatives delay the onset of prion disease and can seemingly assist host immunoreactivity against disease.

Alternatively, Abalain-Colloc (1986) found that six strains of mosquito-derived Spiroplasma were highly susceptible to tetracycline, oxytetracycline, doxycycline, erythromycin, chloramphenicol and pefloxacin. Being a cell-wall-less prokaryote most bactericides are ineffective against Spiroplasma.

Goldstein, (1976) tested the polyene macrolide antibiotic amphotericin B and its chemically modified derivative amphotericin B methyl ester for in vitro activity against Acholeplasma laidlawii, Spiroplasma citri and Mycoplasma gallisepticum. Both polyene macrolide preparations demonstrated anti-mycoplasmal activity. However, the methyl ester derivative was mycoplasmacidal toward all three strains of Mycoplasma. Bastian (1989) demonstrated that hyperbaric oxygen aggravated GT-48 Spiroplasma-induced rat brain encephalopathy. Oxygenation seemingly increased disease virulence under conditions generally detrimental to microbial proliferation.

Manuelidis, (1998) creatively challenged conventional prion theory by intracerebally inoculating mice with low doses of a slow-incubation strain of CJD infectivity followed 80 days later by high dose inoculations of a fast-incubating strain of CJD infectivity. All mice demonstrated the slow infectivity incubation periods yielding disease over 110 days later than predicted for the fast strain. Further, the neurological symptoms were comparable with the slow incubating strain. None of the pathology of the fast strain was detectable. This suggests specific antibody production in response to the TSE agent, much like that experienced with an attenuated agent vaccine.

Nature of "UNKNOWN" TSE Agent	Nature of "KNOWN" Spiroplasmas
1. Unidentified yet reproduces at 30º-38º C.	1. Fastidious, but reproduces at 5º-45º C.
2. Filterable at 40 to 50 nm.	2. Cell widths from 30 to 100 nm, filterable at 40 nm
3. Smaller than most bacteria or virus.	3. Contains the smallest of known living geonomes.
4. Artifacts include fibrils, tubules, electron-dense inclusions or rods.	4. Blebs, electron-dense capsules, spheres or fibril helical morphology.
5. Tubules & rods 50 nm wide, +1000nm long.	5. Helical cells are +/- 50 nm in width, +500 nm length.
6. Tubules have three layers, two broken down by enzymes or detergents. The third as filaments.	6. Cell have outer membrane dissociated by detergents, and inner multiple filaments (SpF).
7. Inner tubule is PK resistant and helical ("SAF").	7. Internal SpF filaments are PK resistant and helical.
8. SAFs 4-6 nm wide, 50-400 nm long.	8. SpFs 3 to 6 nm wide, 50 to over 200 nm long.
9. Upon PK digestion yields resistant, variable 20 to 30 kDa protein bands under Western Blot.	9. Upon PK digestion yields resistant 28 and 30 kDa protein bands under Western Blot. "Spiralin Protein?".
10. Reactivity to PK resistant protein antibodies.	10. Cross reactivity with SAF antiserum.
11. Attacks nervous tissue preferentially, but found in other tissues at lesser titers.	11. With time, neurotropic but found in other tissue at lesser titers.
12. Capable of surviving at least 3 to over 5 years in soil.	12. Capable of surviving up to 17 yrs in soil.
13. Survives autoclaving and heat treatments.	13. Resistant to heat via glyco- or lipo- protein coating.
14. Resistant to most disinfectants.	14. Survives ethanol, formalin, glutaraldehyde, phenol.
15. Untreatable with most antibiotics.	15. Resistant to most bactericides.
16. Hindered by Tetracycline and its derivatives.	16. Susceptible to Tetracycline and other bacteriastats.
17. Produces spongiform brain lesions in vertebrates with an affinity for gray matter.	17. Produces spongiform brain lesions in rodents with an affinity for gray matter.
18. No associated inflammatory response.	18. Initial microcystic inflammation, later no response.
19. Associated with neuron membranes, destroys cytoplasma.	19. Affinity for neuronal membranes, pathogenic for mitochondria.
20. Scrapie transmitted via hay mites & fly extracts.	20. Found in the hemolymph of most insects.
21. Affects ruminants and humans.	21. Strains found in ruminants and humans.

Table 1: A comparison of known characteristics of the putative TSE agent, and those known characteristics of Spiroplasma

DISCUSSION

Two points reveal the epitome of the established scientific community's failure to harness TSE diseases over the last two decades. 1) The fallibility of its TSE diagnostic procedures and; 2) The lack of diverse due-diligence in pursuit of the casual agent. Seemingly, Prion Theory, and the government-provided research money made available for it, have eclipsed unbiased scientific thought and have led a significant majority of the scientific community down a one-way path without momentous reward to date.

Clearly, the current procedures used to identify and define TSE disease are lacking in dependability for properly identifying TSE disease unless used cumulatively without regard to

potential interference or the causal agent. Diagnostic interference can and is expected; hence the results obtained may not be what they are presumed to be by the investigator. By focusing on the disease symptoms rather then a specific, yet putative causal agent, the majority of the scientific community has managed to focus the majority of its efforts on understanding and dissecting a symptom of TSE disease rather than the cloaked agent behind it. While great strides have been made in the understanding of the role of normal prions in mammalian tissue, prions have confused and diverted diligent researchers from the universal insidious causative agent likely to be present in all TSE disease. Of all the potential agents described in the scientific literature, Spiroplasma retains the distinction of harmonizing with the most known TSE agent characteristics, harmonizing to an extent much greater than abnormal prion theory.

Spiroplasmas are extremely unusual, parasitic and fastidious prokaryotes lacking cell walls, having an affinity for mammalian host cell membranes, an appetite for sterol and phospholipids while possessing a pleiomorphic character and the capability of slipping into an intracellular "stealth" mode in their bleb phase of morphogenesis. Spiroplasma appears to have a propensity for the collection of viral infectants, and hence a bacteriaphage, a bacterial-viral symbiotic relationship, may have an underlying role in both host invasion and the pathogenic *modus operandi*.

Under the very limited studies conducted to date, diverse varieties of Spiroplasma have demonstrated plant pathogenicity, while others are capable of producing neuropathic conditions in vertebrates. Many plant pathogenic Spiroplasmas, i.e. citrus blight and corn stunt rely upon insect passage and multiplication within the insect gut epithelium and salivary glands, while still others are pathogenic to the insects, as well as, affecting the sex ratios of the hosts themselves (Tully, 1982). Inoculation of vertebrates and mammals with insect-derived Spiroplasma has revealed a neuropathogenicity, but for only select Spiroplasma species, particularly those derived from ticks. A single known case of a natural Spiroplasma infection in a human had devastating affects upon various autonomic organs.

Upon meticulous investigation, virtually all insects are found to be carriers of one or more Spiroplasma species, in most cases a specific bacteria species or strain within each individual insect species. Even with our limited understanding, the range of arthropods host is astounding. Bees, ticks, leafhopper, flies, wasps, beetles, mosquitoes, all have specific Spiroplasma affiliates. The range of insect hosts is enormous and, hence the suspected pool of insect Spiroplasmas is equally enormous. Compound the potential Spiroplasma reservoir by the possible role of bacteriaphage in disease propagation and the range of pathogens is expanded immensely. To date, the scientific community has barely scratched the surface in the taxonomy, hosts and pathogenicity of Spiroplasma organisms.

Spiroplasmas are but a small part of a much large phylum of bacteria, those of the Mollicutes

and the Mycoplasmas. Recent scientific inquiry has found links, albeit tentative and perhaps proximal ones between Mycoplasmas and a cornucopia of potent aliments, i.e. AIDS, Gulf War Syndrome, Crohn's Disease, chronic fatigue syndrome, rheumatoid arthritis and even some forms of cancer.

While Mycoplasma are a common microbial constituent of healthy mammals, their sinister counterparts complicate and distort diagnostic procedures, instigate acute and chronic diseases at multiple sites with wide ranging complications and have been implicated as effective co-factors in disease. As adroitly described by Baseman & Tully (1997) various innate properties of Mycoplasmas exacerbate disease virulence. These include: 1) the generation of hydrogen peroxide and superoxide radicals increasing oxidation stress particularly on host cell membranes: 2) competition for and depletion of host cell nutrients disputing cell maintenance and function; 3) forming protective, electron-dense structures assisting in immunoregulatory activities; 4) regular and rapid morphological and antigenic variation promoting avoidance of host immune defenses and frustrating etiological identification; 5) creation of endogenous enzymes selectively initiating host cell tissue disruption; and lastly 6) intracellular habitation sequestering Mycoplasmas from host immune response and most drug therapies, while providing for latent or chronic disease manifestation.

Niang (1998) indicated that Mycoplasma are capable of producing electron-dense polysaccharide capsules with variable thickness that are dependent upon culture conditions and strain. Dallo and Baseman (2000) found that pathogenic mycoplasmas reside and replicate intracellularly over extended periods in human cells, consistent with the ability of mycoplasmas to circumvent antibiotic therapy and immune surveillance and establish chronic infections.

The ability of Mycoplasma and their likely surrogates Spiroplasma to generate hydrogen peroxide, organic peroxides (Salman, 1993) and superoxide radicals, as well as, surpressing host cell catalase (Almagor 1984 and Kalane 1984) and corresponding host-cell superoxide dismutase capability is particularly telling. Borovsky (1998) found that under prolonged infection conditions of newly discovered AIDS-associated *Mycoplasma penetrans* resulted in intensive vacuolation of the invaded host cells and a pronounced increase of intracellular organic peroxide levels. Kahane (1984) noted that superoxide anions are generated during the infection and they drastically inhibit the catalase activity of the host cells. With much of the catalase inhibited, oxidation of cell components occurs, among which are membrane lipids, which may lead to membrane leakage and to the ultimate cytopathology of mycoplasma infection.

Both Meyer (1990) and Chen (2000) interestingly document self-protective superoxide dismutase, and copper-zinc superoxide dismutase activity, respectively, in various Mycoplasma species enabling survival under host-induced phagocyte attack. Coincidentally, Sher (1990) found that membranes of *Spiroplasma spp*. strain MQ-1 induce tumor necrosis factor alpha (TNF alpha)

secretion by human bone marrow macrophages. Wong & Goeddel (1988) found that Tumor necrosis factor-alpha (TNF-alpha) a 26 kDa protein was found to induce the messenger RNA for manganous superoxide dismutase which scavenges potentially toxic superoxide radicals produced in the mitochondria.

This fits nicely with trace element studies performed by Wong (2001) on the brains of sporadic CJD patients where he found a 50% decrease in normal brain copper levels and a striking 10-fold increase in the manganese content together with elevated zinc levels. Both copper and manganese changes were pronounced in CJD subjects homozygous for methionine at codon 129 and anti-oxidation activity of PrP^{res} was reduced by up to 85%.

Brown, et al (2000) found that normal prion protein (PrP) binds copper and has anti-oxidant activity, which assists the survival of neurons in vitro. Only manganese could be substituted for copper in PrP^{c} structures and after manganese-loading PrP^{c} exhibited similar structure and approximately 50% of the anti-oxidant activity to copper-loaded PrP^{c}. However, after two weeks aging the manganese-loaded PrP^{c} became proteinase-K resistant (PK^{res}) and had lost almost 100% of its function as an anti-oxidant. Further, he suggests that manganese is apparently used as an electron acceptor in manganese superoxide dismutase, which is found in the mitochondria.

Thackray (2002) noted a major increase in blood manganese in the early stages of TSE disease with a proportional decrease in antioxidant activity between 30 and 60 days post-inoculation. He postulates that alterations in trace-element metabolism as a result of changes in metal binding to PrP are central to the pathological modifications in prion disease. Aschner (1992) discovered that astrocytes play a pivotal role in manganese (Mn) regulation in the central nervous system. Hazell (2002) suggested that astrocytes are the site of early dysfunction and damage in manganese neurotoxicity and that astrocytes accumulate manganese by a high affinity, high capacity, specific transport system. Liao and Chen (2001) determined that neuronal astrocytes differentiated into process-bearing stellate cells following exposure to Mn ++. but was dependant on the presence of extracellular free manganese.

Milhavet (2000) found that PrP^{sc} infected cells presented an increased lipid peroxidation and signs of apoptosis associated with a dramatic reduction in the activities of the glutathione-dependent and superoxide dismutase antioxidant systems. Glutathione is composed of glutamate, cysteine and glycine. Both Kim (2001) and Wong (2001) postulated that the vacoulation of neuronal cells in TSE disease is caused by oxidation stress. Wong further reported dramatic depression of superoxide dismutase-like activity by abnormal prions from scrapie-infected brains, suggesting that brain metal imbalances, especially copper, in scrapie infection is likely to affect the anti-oxidation functions of PrP^{c} and superoxide dismutase, which, together with other cellular dysfunctions, predispose the brains to oxidative impairment and eventual degeneration

A WORKING INFECTION HYPOTHESIS

Transmissible Spongiform Diseases are amitotic Spiroplasma-induced, intracellular prionic, cytoplasmic, and mitochondrial superoxide dismutase, and glutathione redox aberrations with resultant neuro-membrane lysis followed by subsequent cyto-apoptosis, or cytonecrosis initiated by concordant, but unrequited reactive oxygen species stress.

In a generalized scenario, a host-specific species of parasitic Spiroplasma bacteria, most logically supplied by an insect, invades a mammalian host via a variety of potential access routes, but most probably via ingestion to the alimentary system passing through the gut membranes into the lymph and circulatory system. Possessing an affinity for sterols Spiroplasma coats itself in host cholesterol to confound the host's immune system while inducing the production of proinflammatory cytokines, hence stimulating the production of lymphocytes. With innate superoxide dismutase (oxidation-reduction, red-ox) capability Spiroplasma invades leukocytes (B lymphocytes, as per Klein, 1998) without assimilation, allowing it to be transported throughout the host including transgression of the blood-brain barrier. Once ensconced, Spiroplasma induces tumor necrosis factor alpha secretion by bone marrow macrophages and microglia. TNF-alpha in turn induces the mitochondrial messenger RNA for production of manganese superoxide dismutase. TNF-α has been implicated directly in astrogliosis (Liberski, 1994). Being neurotropic Spiroplasma eventually settles into a cloaked bleb stage or as an electron-dense polysaccharide capsule entirely emplaced within host neuronal cells escaping detection and immune response. With time in its reproductive efforts, Spiroplasma assumes a morphological progression culminating in a short-lived, but distinctive helical shape and co- temporally through host-membrane parasitosis acquires the necessary proteins and in particular, the requisite cationic building blocks to convey dismutase ability to its progeny, essentially scavenging the available copper, but lesser the zinc, from the host cell's oxi-protective cellular prion sheath and cytoplasma, while at the same time exacerbating the mitochondrial manganese dismutase production with induced TNF- α. The generation of progenal spiralin membrane demands a cysteine resource potentially robbing intercellular glutathione building blocks further degrading cyto-oxi-protective ability. Newly created Spiroplasma cells (carrying the scavenged copper and cysteine) are available to attack new cells or be exported from the host, again most likely from the alimentary tract to seek new hosts, the virulence or dosage increasing as neuro-degeneration escalates. Shedding of agent effectively diminishes the host-animal's overall copper quotient and hence mimics a resident host clinical copper deficiency. In the subsequent copper-deficient cyto-environment, sporadic, incidental or Spiroplasma-induced manganese replacement of scavenged copper in the residual or newly formed normal cellular prion protein (PrP^{c}) creates the diagnostic Western-Blot and immunostainable abnormally folded prion protein (PrP^{sc}) with attendant proteinase-K resistance at several different mole weights representing both host-derived, variously glycosylated ~20 to ~30 kDa PrP^{sc} and non-host 26 kDa PK-resistant spiralin protein derived from

residual Spiroplasma membranes and from internal 4-6 nm Spiroplasma fibrils visible upon detergent lysis as the diagnostic scrapie-associated-fibrils (SpF = SAF). The host cell's diminished copper-zinc dismutase and catalase capacity, together with exaggerated mitochondrial manganese red-ox capability, disrupts cellular red-ox homeostasis, seriously degrading the host cell's ability to protect itself against continuing oxidation stress while mimicking neuronal manganese necrosis. Naturally occurring oxidation events combined with indigenous Spiroplasma-produced hydrogen peroxidation activity, dissociates the weakened cell membranes, allowing plasma leakage and in turn leading to cyto-apoptosis or cell death, freeing additional dismutase divalent (Mn^{++}) manganese available to displace or replace lost divalent copper-zinc in the remaining prionic sheath and cell plasma, (or if in excess, to enter the blood stream). This leads to the proliferation of manganese-scavaging astrocytes prior to additional significant PrP^{sc} development, further degradation of host glutathione-dependant and superoxide dismutase antioxidant ability, and culminating in more cytonecrosis with the eventual proliferation of the diagnostic sponge-like vacuoles in neural tissue.

The peculiar and unusual nature of Spiroplasma and their infection abilities produce a skewed version of an autoimmune-like disease. Spiroplasma insidiously mobilizes the host's own immune defense mechanisms against itself, and a paradoxical turn of events, Spiroplasma conveniently uses those defense mechanisms to its own reproductive advantage. The stimulation of lymphocytes and then the resultant intracellular invasion of leukocytes allows Spiroplasma convenient transport and dissemination while protecting it from other host cell defense strategies and many intravenous drug therapies, yet eventually allowing it the fulfillment of its own neurotropic tendencies. The early stimulation of pro-inflammation cytokines proliferates host antioxidant activities increasing host superoxide dismutase and glutathione red-ox abilities, effectively creating the building blocks and environment necessary for Spiroplasma's own procreation, when it profitably feeds upon the same building blocks necessary for the host to defend itself. Spiroplasma grows stronger as the host is degraded, and with time, opportunistically uses that host to dilatorily export the pathogen into the host's environment at an appropriate time and in an appropriately encapsulated and refractive form ready to re-infect upon the approach of a suitable primary or perhaps secondary host or vector.

Quite simply, the biomechanics of Mycoplasmas and their surrogate Spiroplasmas are a template for Transmissible Spongiform Encephalopathies. Unfortunately, despite abundant evidence of their pervasive pathogenic potential, Mycoplasmas and Spiroplasma are predominately ignored by the majority of the scientific community. The neglect to pursue Mycoplasma and Spiroplasma in their role of disease pathogenesis, particularly in TSEs is a failure in our investigative system of scientific curiosity and prudence.

A follow-on paper: **Chronic Wasting Disease -** Part II **Observations on TSE Transmissibility – Host Implications** will address the observations of several CWD-infected cervidea herds, possible secondary hosts and their interaction with the now disclosed more logical

causal agent.

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