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

A Logical Causative Agent PART Ib: The Quest for an Agent

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Abstract: (Part Ib) Transmissible Spongiform Encephalopathies, 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. Amazingly, a fully described comparison of 28 TSE causal agent characteristics match remarkably well with 28 documented characteristics of Spiroplasma bacteria and/or their lesser cousins, the mycoplasma bacteria. A close resemblance is present for physical structure, such as size and density; agent refractivity to both disinfectants and heat; the unusual characteristics of surface adherence and ground survivability; reaction to hyperbaric oxygen; resistance to bactericidal antibiotics, yet susceptibility to bacteriastatic antibiotics; a comparable response to attenuated agent vaccines characteristics; and lastly comparable infectivity conditions found in blood and lymph. Importantly, B-cells have been implicated in both TSE infectivity and in the mycoplasmal disease process. Blood born bacterial characteristics match extraordinarily well with specifically determined "prion" characteristics. Stunning electron micrographs of helical, CJD-brain inclusions and incontrovertible PCR-DNA results virtually dictate that Spiroplasma and TSE disease are intimately linked. Conclusively, the failure of the scientific community to diligently pursue Mycoplasma and Spiroplasma and their likely role of disease pathogenesis, particularly for 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 cervidae, 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 effect upon protective protein particles shielding nerve cells. Prion diseases are fundamentally diseases of membrane tissue, although not necessarily neuronal tissue. A proteinaceous infective particle (or abnormal "Prion", PrP^{res}) has been widely postulated as the TSE causal agent, yet after two decades of intensive research, definitive proof has yet to be established. Contemporaneously, due to poor results, or more plausibly, a lack of results stemming

from a dearth of funding, lesser theories of slow acting virus, filamentous virus, retrovirus, viroids, virinos or a bacterial origin have waned.

Part 1a "TSE Testing Fallibility" (Forrest 2003e) defined how four selective criteria are used to identify TSE disease: 1) brain tissue vacuolation, 2) Scrapie Associated Fibrils, 3) protein resistance to proteinase-K digestion, as detected by Western Blot and, 4) direct immunostaining of diseased tissue, or identification via abbreviated ELISA tests. Criterion four was relegated to subset of criterion three, as both procedures demonstrate the existence of PK-resistant tissue. Unfortunately, all three of the primary TSE diagnostic criteria are ambiguous in their ability to identify TSE disease. False positive and false negative results are possible and can be occasionally expected. Specifically, numerous and various genera of mammalian-inhabiting bacteria have been demonstrated to contain PK-resistant proteins.

Neurotropic, sterol ingesting bacteria of the Class Mollicutes, generally termed Mycoplasma and more specifically, various strains of Spiroplasma bacteria 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, such as central nervous system neuron vacuolation, agent refractivity, and the infectivity conditions generally associated with TSE disease and its suspected causal agents.

THE AGENT QUEST

Part 1a (Forrest 2003e) defined that potential testing interference is possible with each of the three, well-used TSE testing procedures. Therefore, we know that one or both of: (1) the suspected abnormal prion protein (PrP^{res} or PK^{res}) and/or, (2) the bacteria Spiroplasma could or might be present in any tissue testing positive. While certainly all the necessary 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?

Physical Characteristics of the Agent

Size: According to Gibbs (1967) and Kimberlin (1971) virtually no scrapie infectivity passed through 27 to 42 nanometer (one "nm" = one billionth of a meter) filters, while abundant infectivity penetrated 43 to 50 nm filters (and larger) of the same type. Diringer (1983) found that scrapie agent infectivity size ranged from about 20 nm to 55 nm. In turn, Prusiner and McKinley (1983) similarly found that proteinase digested and 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 suggesting additionally, that they may be amyloid. However, this was partially digested material, the original agent was likely to be larger.

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 nanometers. Liberski, P.P. (1990) found that Swiss white mice inoculated

with CJD infectivity produced tubulo-vesicular structures measuring 20 – 50 nanometers in diameter.

Alternatively, 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.

Bastian (1979) noted that CJD brains contained unusual inclusions which 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. These coiled structures resembled Spiroplasma bacteria. See electron micrographs below:

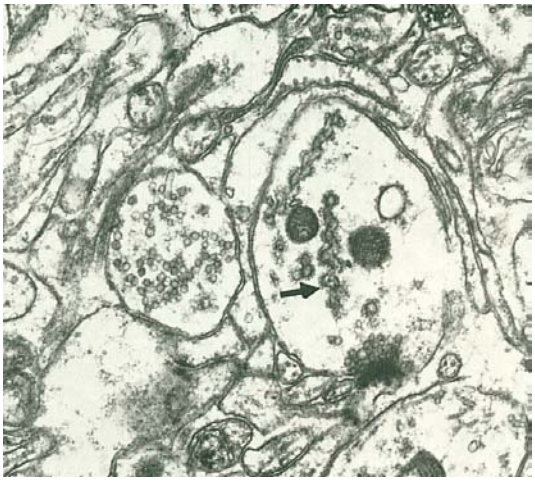

Figure 1: *Electron micrograph of two spiral inclusions present within swollen pre-synaptic terminal of a human CJD brain. (Original magnification X 37,400 (from Bastian, 1979)*

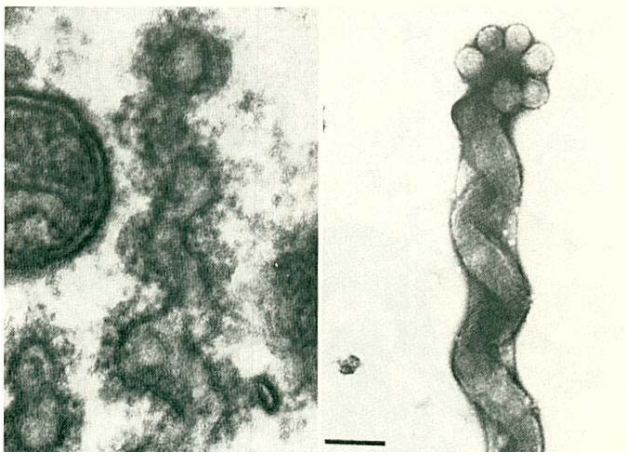

Figure 2:

Left, Electron micrograph of lower spiral inclusion of Figure 1 above (see arrow). (original magnification X 126,000 (from Bastian, 1979)

Right, Cultured Spiroplasma organism at approximately the same magnification (bar = 100 nm) (from Williamson, 1977)

Townsend (1983) observed via thin section, 80 to 100 nm wide *Spiroplasma melliferum* which appeared to be tubular in shape. Zhang (2000) discovered that several Spiroplasma species survived in dry soil, generally as spherical structures 60 nm in diameter and aggregating into filaments up to 2500 nm long. Bastian (PC 4/2002) noted that Spiroplasma can range from 50 nm to over 150 nm in width and will generally pass through the same filter widths as the suspected TSE agents.

Itot (1989) found that *Spiroplasma mirum* (SMCA tick strain) was always 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. See *Figure 3*

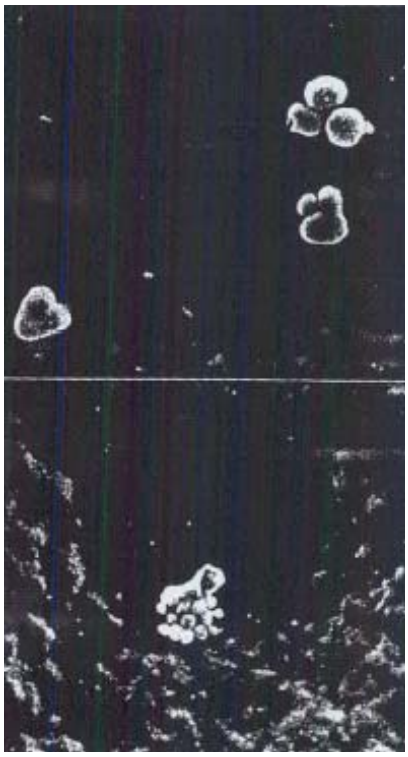

Figure 3
TOP: *Cultured +200 nm spherical Spiroplasma mirum bodies about to burst.*
BOTTOM: *Burst spherical Spiroplasma bodies releasing small granular Spiroplasma bodies*
(Source: Itoh, 1989)

Density: Through agent density studies, Sklaviadis (1989) indicated that a large portion of normal prion protein (34 kDa, PrP^c) can be separated from CJD infectivity suggesting that normal 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.

Alternatively, detergent-dissociated Spiroplasma, as partially purified fibrils (filaments with membrane) weigh in at about 1.22 g/ml, while fully purified fibril protein has a density of 1.27 g/ml, but aggregating only 0.8% to 1.0% of the total whole cell weight (Townsend, 1980). Note that both prepared, protease-digested TSE agent and protease-digested Spiroplasma have identical density measurements.

Agent Destruction Refractivity

Disinfectants: TSE agent and, in particular, scrapie agent has several unique disinfectant resistant properties. The agent can survive; 1) 5 % chloroform and 2 % phenol (Stamp, 1959), 2) 20% formalin solutions for 18 hours (Pattison, 1965), and 3) treatment with various less aggressive protein enzymes (Pattison and Millson, 1960).

Brown, P. (1982) found that sodium hypochlorite reduced scrapie infectivity by 99.99%, and was superior to sodium metaperiodate, chlorine dioxide, Lysol, iodine, potassium permanganate, and hydrogen peroxide. Most inactivation occurred within 15-30 min of exposure to hypochlorite, and little, if any, additional inactivation occurred after one hour. Brains processed for histopathologic examination (formalin fixation followed by

dehydration in methanol, clearing in chloroform, and embedding in paraffin) still retained a portion of the infectivity present in unprocessed control tissues.

BSE-infected bovine brains were exposed for not over 120 minutes to solutions of sodium hypochlorite or sodium dichloroisocyanurate containing up to 16,500 ppm available chlorine. There was no detectable survival of infectivity after the hypochlorite treatments but none of the dichloroisocyanurate solutions produced complete inactivation (Taylor, 1994). Apparently the free chlorine and not the sodium is the effective disinfectant. Next, BSE-infected bovine brain, and rodent brain infected with the 263K and ME7 strains of scrapie agent, were exposed for up 120 minutes to 1M or 2M sodium hydroxide, but hydroxide did not completely inactivate infectivity (Taylor,1994).

Alternatively, cell-wall-less Spiroplasma, particularly the insect-hosted varieties, display a considerable resistance to the normal bactericidal disinfectants, particularly when compared to wall-bearing organisms (Stanek, 1981). Several Spiroplasma 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 Spiroplasma species were more resistant than the *E. coli* and *Staph. aureus* comparisons. Spiroplasma has even been observed to be active in 50% glutaraldehyde, a potent tissue fixative used normally at 3% concentration (Bastian, written press statement, Feb, 2001).

While Spiroplasmas have not been tested, numerous varieties of Mycoplasma bacteria are completely killed by hypochlorite solution containing 25 ppm. available chlorine in just 15 seconds in the absence of other organic material. They are easily killed with 50 ppm available chlorine in five minutes, when in the presence of 1% protein (Lee 1985). Larger quantities of organic material will delay complete disinfection, potentially paralleling the TSE agent characteristics. Interestingly, Almagor (1983) notes that mycoplasma-produced hydrogen peroxide degrades cell membranes yielding malonyldialdehyde as an indicator of membrane lipid peroxidation. As such, the Spiroplasma organism likely possesses an innate tolerance to both the normally disinfective agent hydrogen peroxide and the normally toxic aldehyde radical found in many disinfectants and preservatives.

Sterilization or Heat Deactivation: TSE scrapie agent can survive in boiling water for several hours (Stamp, 1959, Pattison and Millson 1961a), and numerous freeze-thaw cycles (Pattison and Millson, 1961b). Brown, P. (1982) found that one hour in an autoclave at 121 C reduced the titer of scrapie infectivity but did not eliminate minor residual infectivity. He concluded that a combination of exposure to chemicals and autoclaving maybe necessary to sterilize high-titered scrapie-infected tissue. Continuing his work, Brown, P. (1990) found a small amount of infectivity still survived a one-hour exposure to temperatures as high as 360 degrees C.

Taylor (1994), using bovine brain infected with bovine spongiform encephalopathy (BSE) agent, and rodent brain infected with the 263K or ME7 strains of scrapie agent were subjected to porous-load autoclaving at temperatures between 134 and 138 degrees C for up to 60 minutes. Bioassay in rodents showed that none of the cooking procedures

produced complete inactivation. 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. Somerville suggested that the causal agent contains a macromolecular component that is structurally independent of the host, and that the agent varies covalently between TSE strains, and that the agent is further protected by other macromolecular components. The model is in accord with the virino hypothesis, which proposes a host-independent informational molecule protected by the host protein PrP.

The most controversial TSE heat deactivation studies found that a small portion of infectivity was retained despite reported 600 degree C temperatures (Brown, 2000). However, the procedures used for temperature modulation, anomalous conflicting results, and the failure to include negative controls renders such studies suspect.

Alternatively, comparable Spiroplasma wet-heat survivability studies have not been conducted. However, viable Spiroplasma organisms have been recovered from boiling water (100ºC) (Bastian, written press statement, Feb, 2001). Further, no studies have been conducted upon Spiroplasma survivability at high dry-heat temperatures, particularly if Spiroplasma was ensconced within an inert form such as an electron-dense polysaccharide capsule or perhaps as in a separate, but unknown spore phase.

Bacterial heat resistance is not unusual. Bacterial spores are often used to test the effectiveness of equipment and procedures for autoclaves and incinerators. Wet spores of several bacterial species have survived brief exposures between 270 to 340 degrees C, while dry spores are known to persist after exposure to 370 degrees C. (Barbeito, 1968).

Zhang (2000) noted that several plant pathogenic Spiroplasma species were capable of surviving up to 17 years in dry soil as well as in farmed soils even when subjected to harsh weather conditions containing abundant freeze-thaw cycles.

Surface Adherence: Flechsig (2001) determined that prions (PrP^{res}) (or perhaps their underlying infectivity agent) can readily and tightly bind 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 as documented for human surgical 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.

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

Alternatively, 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 as spherical structures 60 nm in diameter and aggregating into filaments up to 2500 nm long.

Hyperbaric Oxygenation: Roikhel (1984) found that multiple exposure of scrapie-infected mice to hyperbaric 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.

Alternately, Bastian (1989) demonstrated that hyperbaric oxygen aggravated GT-48 Spiroplasma-induced rat brain encephalopathy. Oxygenation seemingly increased disease virulence under conditions generally assumed to be detrimental to microbial proliferation.

Resistance to Antibiotics, Tetracycline: 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). 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.

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 immuno-reactivity 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 bacteria most bactericides, those which destroy bacterial cell walls, are ineffective against Spiroplasma. Conversely, bacteriastats, which generally attack microbial reproduction, are shown to be therapeutic.

Resistance to Antibiotics, Amphotericin B: 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.

Alternatively, Goldstein, (1976) tested the 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-bacterial activity. However, the methyl ester derivative was especially effective toward all three strains of the tested bacteria.

Resistance to Other Antibiotics: Josefson (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 studies to date do support bacteriastatic 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.

Alternatively, cell-wall-deficient Spiroplasma has a noted susceptibility to bacteriastat antibiotics, however no studies of Thorazine or quinacrine have been documented.

Attenuated agent vaccine: Manuelidis, (1998) creatively challenged conventional prion theory by intracerebrally 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.

Conversely, Tola (1999) discovered that sheep ewes vaccinated with phenol- and saponin-inactivated *Mycoplasma agalactiae* bacteria resisted experimental disease inoculation challenge. These results suggest that attenuated agent vaccines are effective and can limit or eliminate mycoplasma infections.

A very important and timely study linking scrapie and Mollicutes, conducted by Zanusso (2003), found an accidental intra- and interspecies transmission of scrapie to both sheep and goats following exposure to a vaccine against *Mycoplasma agalactiae*. Apparently the scrapie agent co-purified with the mycoplasmal agent during attenuated vaccine manufacture. Fascinatingly, Zanusso's detailed investigation suggested the co-presence of two prion strains in the mammary glands and in the brain homogenates of the scrapie-infected animals used to derive the vaccines.

Agent Infectivity Characteristics

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 immuno-histochemistry assay procedures enhanced for sensitivity have shown that PrP^{res} development within alimentary-tract-associated lymph nodes can be detected as early as 42 days after oral inoculation (Sigurdson, 1999). 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 (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 was 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 increased 65-fold three weeks post-inoculation. Brain PrP^{res} was not detected until 8 weeks. The agent is evidently lymph- or spleen-seeking initially and only brain- or neuron tissue- oriented 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. Those include: (1) the extent of vacoulation in various regions of the brain; (2) the time period after inoculation for the manifestation of clinical symptoms; (3) the quantity of amyloid-type 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 the 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). However, no genetic code is supposed to be present in classic prion causal agent theories. Interestingly, occasional reports of unknown, non-host-derived RNA or DNA have been found in TSE preparations (Sklaviadis, 1989, Narang, 1998).

Following intracerebral or peripheral inoculation of mice with scrapie prions, infectivity accumulates first in the spleen and only later in the brain. In the spleen of scrapie-infected mice, prions were found in association with T and B lymphocytes and to a somewhat lesser degree with the stroma, which contains the follicular dendritic cells (FDCs) (Weissmann, 2001).

Now, alternatively, Tully (1984) and Bastian (1987) demonstrated that with Spiroplasma intracerebral inoculation of rats, spleen infections appeared first, generally within two days, but central nervous system deterioration did not commence immediately. Fourteen days after inoculation, notable visually detectable Spiroplasma were present in the CNS. However, after 25 day, no visibly detectable Spiroplasma were present, despite significant residual infectivity (Bastian, 1984). Apparently, an intracellular stealth mode had been

achieved.

One must recognize that no rapid, consistent test is available for detection of Spiroplasma, nor for that matter, most mycoplasmal infections. Due to their fastidious nature, little testing has been conducted, let alone comprehensive studies. However, Spiroplasma, being a typical bacterial agent, can and is expected to present itself in the form of varieties or strains, i.e. Spiroplasma mirum, GT-48 and SMCA strains. Each strain would be capable of infecting a species, or group of species with specific characteristics and features to be expected from a more conventional biological agent.

Blood-born Characteristics

Blood-borne TSE characteristics are fundamental in defining a potential agent of TSE transmissibility. White blood cells without identifiable prions have been found to contain CJD infectivity shortly after cerebral inoculation, and are capable of causing disease in donor recipients (Manuelidis, 1985). Tamai (1992) injected mice intracerebrally 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 (dental gum) tissue was higher in infectivity than dental pulp or nerve endings further suggesting that blood more than neurons may be a source of early infectivity. Manuelidis (2000) determined that white cell macrophages can sequester appreciable levels of infectivity and hence act as reservoirs for prolonged latent infection.

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 each with clinical scrapie. He found a larger proportion (98.4%) of the total infectivity was recovered from the mononuclear leukocyte (white blood cell) fraction, yet no PrP^{res} was detected.

Important studies by Klein (1997) using a panel of variously immune-deficient mice inoculated with infective scrapie prions intraperitoneally found that artificially disabled immune system dysfunction affecting only T lymphocytes had no effect on scrapie transmissibility, however, all mutations that disrupted the differentiation and response of B lymphocytes prevented the development of clinical scrapie. As an absence of B cells and of antibodies correlates with severe defects in follicular dendritic cells, a lack of any of these three components hinders scrapie development, hence concluding that differentiated B cells are crucial for neuro-invasion by scrapie. Klein (1998) then demonstrated that prion infectivity accumulates in lymphoid organs, and the absence of mature B lymphocytes prevents peripherally administered prions from inducing central nervous system disease, indicating that cells whose maturation depends on B cells or their products, such as follicular dendritic cells, may enhance neuro-invasion. Alternatively, Klein concluded that B cells might actually transport prions to the nervous system by a PrP-independent mechanism.

Sigurdson (1999) demonstrated that CWD PrP^{res} is intimately associated with follicular dendritic cells where B-cells are stimulated to mature and divide. PrP^{res} can be detected in lymphoid tissues draining the alimentary tract within a few weeks after oral exposure to infectious prions, and such circumstances may reflect that the gut is the initial pathway of CWD infection in deer. Interestingly, prion protein (PrP) apparently co-localized with a minority of macrophages in tumor necrosis factor receptor (TNFR) 1(-/-) lymph nodes. Hence prion pathogenesis can be restricted to portions of the lymph system, but mature follicular dendritic cells are not necessary for this process (Prinz, 2002).

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 lymph system from the gut to the spleen and on into the spinal cord via associated nerve cells. 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). Hence, the TSE agent is not restricted to neuronal tissue and should be found throughout the body.

From a bacteriological perspective, the importance of mycoplasma host invasion methodology is critical to the understanding of a potential Mycoplasma-Spiroplasma role in TSE disease. Spiroplasma and the larger group of mycoplasma are immune cell activators. In vitro studies have demonstrated the ability of mycoplasmas to be mitogenic for lymphocytes, increasing cell numbers substantially. Further, mycoplasma proteins can induce B-cell differentiation and trigger secretion of cytokines including interleukin-1 (Il-1), Il-2, Il-4, Il-6, tumor necrosis factor alpha (TNF-a), interferons and granulocyte macrophage-colony stimulating factor "GMCS" (Brenner, 1996).

Spiralin, an abundant Spiroplasma protein has polyclonally stimulated spleen-derived mouse B-cells, suggesting that a strong and specific humoral (blood-borne) immune response is developed against spiralin in natural infections (Brenner, 1996 unpublished). However many mycoplasma species undergo antigenic variation by which they alter their surface protein structure so as to evade immune system recognition (Wise, 1993). Interestingly, spiralin bearing Mollicutes seemingly possess the ability to both stimulate the host's immune system and then have the capability to hide from the same immune response via surface membrane protein variation.

Abundant evidence notes aggressive cell parasitism by Mycoplasmas and Spiroplasma. In the late 1970's, complete and reproducing Spiroplasma inclusions were noted within CJD patient brain neurons (Bastian, 1979). Lo (1989) had fully documented the ability of *Mycoplasma fermentans incognitus* to intracellularly invade and eventually kill host cells, but was unable to determine if such mycoplasmas were reproductively active. Immunohistological identification was made in thymus, liver, spleen, lymph node, or brain from 22 patients and 2 placentas delivered by patients with AIDS. Cell death was sometimes present without inflammatory response. Taylor-Robinson (1991) confirmed the presence of intracellular *Mycoplasma fermentans* in cervix cancer cells. *M. fermentans* was generally found in the cytoplasma and within membrane-bound vacuoles. Stadtländer (1993) noted pronounced cell invasion characteristics defined by strain differences, with

M. fermentans incognitus being the most aggressive. Co-infection with *Mycoplasma fermentans incognitus* enhances the ability of human immunodeficiency virus type-1 (HIV-1) to induce cytopathic effects on human T lymphocytes in vitro (Lo, 1991b).

In 1993, Lo demonstrated that *M. penetrans* adheres to cell surfaces, deeply penetrates into the cell, strongly hemadsorbs human red blood cells, and cytadsorbs human CD4+ (T) lymphocytes and monocytes *Mycoplasma fermentans* also fuses with peripheral blood lymphocytes. Cheek (1997) confirmed binding of *M. fermentans* to 10%-15% of peripheral blood lymphocytes with minimal granulocyte or monocyte binding detected. Cytometric analysis showed that binding appears predominantly directed towards B-lymphocytes (up to 97%) and this binding process could not be blocked by antibodies directed towards common B lymphocyte cell surface antigens. Conversely, binding to T-lymphocytes was minimal (<5% positive).

While investigating eye disease, Johnson (1993) determined that Mycoplasma-like organisms ("MLO" or Spiroplasma) have been found to cause chronic human eye disease sometimes resulting in cataracts with other autoimmune features. Eye-localized leukocytes displayed pervasive MLO parasitization. MLOs also disseminated to produce randomly distributed lethal systemic disease including chronic hepatitis, Crohn's Disease and other inflammatory bowel diseases suggesting that the gut as the possible source of the MLO infection (Johnson 1989).

In conjunction with Johnson, Wirostko (1990) found that MLO are cytopathogenic, and are detectable in all diseased organs, causing host cell proliferation, destruction, and dysfunction by parasitized leukocytes, monocytes and lymphocytes, altering the host cell nucleus, replacing the cytoplasm, and destroying organelles.

Other Agent Characteristics

Klein (1998) found that the sphingolipids, galactosylceramide and sphingomyelin were consistently observed in chloroform/methanol extracts of PrP^{res} prion rods. Higher infectivity values had lower sphingolipid ratios. Naslavsky, (1999) further reported that cholesterol and sphingomyelin (phospholipids) support and perhaps regulate the formation of PrP^{res}, and thus presumably the propagation of disease producing prions.

Interestingly, Freeman (1976) found that Spiroplasma requires sterols for growth and incorporates sphingomyelin (phospholipids) when grown in culture.

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Tamai (1989) found that the transmissible agent of CJD is closely associated with surface membranes of nervous tissue 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.

Alternatively, in 1996, Bastian noted that Spiroplasmas are similar to their relatively docile cousins, the mycoplasmas, both of which are capable of penetrating host cell, blending with cell membranes. Bastian further suggested that Spiroplasmas can fully fuse with host-cell membranes, thereby entirely blending in with the cell background rendering them

virtually impossible to recognize by microscopic examination.

Direct DNA Evidence

As previous described in Forrest (2003e), Spiroplasma bacteria, as strains of *Spiroplasma mirum* or a closely associated affiliate have been directly identified in both CJD and scrapie brain tissue sourced from divergent regional locations. Utilizing well-defined oligonucleotide primers specific to Mollicutes 16S rDNA, Bastian and Foster (2001) performed polymer chain reaction amplification and DNA sequence analysis of diseased tissue. They obtained definitive PCR products with 96% to 99% homology to *Spiroplasma mirum*, while control brains had no response. All 13 positive CJD-brains produced the predicted 276-bp PCR products, while none of the 50 negative control brains had a response. Such a response is strongly suggestive of the presence of foreign bacterial rDNA within diseased tissue.

Spiroplasma was undoubtedly present in those CJD brains. While some might suggest that the presence of Spiroplasma was solely of an opportunistic or commensal nature, the diversity and preeminence of such a notably and regular occurrence in all 100% of the CJD positive brains makes such an assumption quite arbitrary. Scrapie-brain tissue PCR-DNA analysis was less spectacular, defining only five out of nine positive cases. One must recognize, however, that the samples submitted for analysis were both regionally divergent, and did not attempt to differentiate potential strain differences, of which at least 20 scrapie strains are well recognized in the literature.

Discussion

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 or its strains, or 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 cause of TSE disease.

Upon review the data presented herein, one can conclude that mycoplasmas in general, and Spiroplasma in specific, fully retains the capability and could conceivably possess the ability to infect and create disease characteristics and symptoms virtually identical to the mysterious TSE diseases. Further, Spiroplasmas ability to produce such disease conditions, together with the astonishingly similar attendant agent physical characteristics and comparable agent refractivity demands that greater attention be allocated to Spiroplasma's possible role in TSE disease. While the capacity to produce disease is not direct evidence, certainly one cannot ignore the direct DNA presence of Spiroplasma within diseased tissue (Bastian and Foster, 2001).

Figure 1 stunningly summarized the commonality between the suspected TSE agent and the characteristics of Spiroplasma bacteria.

Nature of "UNKNOWN" TSE Agent	**Nature of "KNOWN" Spiroplasma-Mycoplasma**
1. Unidentified yet reproduces at 30º-38º C.	1. Fastidious, but reproduces at 5º-45º C.
2. Filterable at 40 to 50 nm.	2. Filterable cell widths from 30 to 100 nm,
3. Smaller than most bacteria or virus.	3. Contains the smallest living geonomes.
4. Artifacts include fibrils, tubules, electron-dense inclusions or rods.	4. Blebs, electron-dense capsules, spheres or fibrils with 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 (SAF).	6. Cell can have a outer capsule, a cell membrane dissociated by detergents, and inner filaments ("SpF").
7. Inner tubule is PK resistant and helical (scrapie-associated fibrils, or "SAF").	7. After detergent lysis, 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 +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 antibody or antiserum.
11. Attacks nervous tissue preferentially, but found in other tissues at lesser titers.	11. With time, neuro-invasion occurs, but is found in other tissue at lesser titers.
12. Survives at least 3 to over 5 years in soil.	12. Capable of surviving up to 17 yrs in soil.
13. Survives autoclaving and dry and wet heat treatments.	13. Resistant to heat via glyco- or lipo- protein coating. (sugar coating) or via a "spore" phase.
14. Resistant to most disinfectants including acholol, formalin and phenol	14. Capable of survival in ethanol, formalin, glutaraldehyde, phenol.
15. 99.99% killed by hypochlorite	15. 100% of mycoplasmas killed by hypochlorite.
16. Untreatable with most antibiotics.	16. Resistant to most bactericides.
17. Hindered by Tetracycline and its derivatives.	17. Susceptible to Tetracycline and other bacteriastats.
18. Hyperbaric oxygen increases prion accumulation	18. Hyperbaric oxygen increased Spiroplasma virulence.
19. Seemingly susceptible to attenuated agent vaccines	19. Susceptible to attenuated agent vaccines.
20. Produces spongiform brain lesions in vertebrates with an affinity for gray matter.	20. Produces spongiform brain lesions in rodents with an affinity for gray matter.
21. Generally no associated inflammatory response.	21. Initial microcystic inflammation, later no response.
22. When injected peripherally, infectivity is first found in spleen, then brain.	22. When injected peripherally, Spiro infection is first observed in spleen, then brain
23. Blood is highly infective as are other tissues.	23. Blood is infective as well as other tissues.
24. Blood infectivity associated with B-cells	24. Mycoplasmas fuse with B-cells within the blood.
25 Infectivity concentrates contain sphingomyelin which apparently regulates "prion" formation	25. Spiroplasma incorporates sterols and sphingomyelin, which are necessary for growth.
26. Associated with neuron membranes, destroys cytoplasma.	26. Affinity for neuronal membranes, pathogenic for mitochondria within cytoplasma.
27. Transmitted via hay mites & fly extracts.	27. Found in the hemolymph of most insects.
28. Affects ruminants and humans.	28. Strains found in ruminants and humans.

Table 1: A comparison of 28 characteristics of the suspected but "unknown" TSE agent, and those similar characteristics of "known" Spiroplasma-Mycoplasma bacteria.

Quite simply put, the biomechanics of Mycoplasmas and their surrogate Spiroplasmas are a template for Transmissible Spongiform Encephalopathies. Unfortunately, despite abundant evidence of their pervasive pathogenic potential, such potential literally acknowledged for two decades, Mycoplasmas and Spiroplasma have been predominately ignored and still remain 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 classical failure in our investigative system of scientific curiosity and prudence.

A continuing paper: **Chronic Wasting Disease - A Logical Causative Agent**
Part Ic: "A Working Infection Hypothesis" will examine the possible role of Spiroplasma in the creation of disease, and how its modes operandi can affect a mammalian host and create the various accompanying TSE symptoms and syndromes.

REFERENCES:

Abalain-Colloc ML, et al, 1986, Pathol Biol (Paris) May; 34(5): pp 360-3
Almagor, M., et al, 1983, Infect Immun Jul;41(1): pp 251-256
Barbeito, M.S., et al, 1968, Appl Microbiol 1968 Mar;16(3): pp 490-495
Bastian, F.O., 1979, Arch Pathol Lab Med V103 pp 665-669
Bastian, F.O., et al, 1984, Am J. Patholol V114 pp 496-514
Bastian, F.O., et al, 1987, Ann Inst Pasteur Microbiol;138(6): pp 651-655
Bastian, F.O., et al, 1989, Res Microbiol 1989 Feb;140(2): pp151-158
Bastian, F.O., 1996, Inf Dis News v6
Brenner, C., et al, 1996, Frontiers in Biosci. 1 e42-54
Brown, P., 1982**,** J Infect Dis May;145(5): pp 683-687
Brown, P. et al, 1990, J Infect Dis Mar;161(3): pp467-72
Brown, P., & Gajdusek, D.C., 1991, the Lancet V337 #8636 pp169
Brown, P., et al, 2000, Proc Natl Acad Sci USA. Mar 28, 97 (7): pp 3418-3421
Carp, R.I., et al, 1989, Prog Clin Biol Res 1989; 317: pp 567-82
Cheek, R.F., et al, 1997, Cytometry , May 1;28(1): pp 90-95
Flechsig E, et al, 2001, Mol Med 2001 Oct; 7(10): pp 679-684
Forloni, G. et al, 2002, Proc Natl Acad Sci USA Aug 6; 99(16): pp 10849-54
Forrest, R.A., 2003b, Part Ia, http://www.stopcwd.org/library/cwd_paper1a.cfm
Freeman, B.A., et al, 1976, .J Bacteriol. Mar; 125 (3): pp 946-54.
Gibbs, C.J., 1967, Cur Top Microbiol. V40 pp 44-58
Goldstein, N.I., et al,1976, J Antibiot (Tokyo) Jun;29(6): pp 656-661
Holada K, et al, 2002**,** J Virol May;76(9): pp 4649-50
Hunter N, & Houston F., 2002, Dev Biol (Basel); 108: pp 93-98
Ingrosso, L. etal, 1999, J Gen virology V80 pp 3043-3047
Itoh, K.,et al, 1989, Microbiol Immunol V33 #10: pp 821-832
Johnson LA, et al, 1989, Am J Clin Pathol, Mar;91(3): pp 259-264
Johnson, L.A., et al, 1993, Int J Exp Pathol Aug;74(4): pp 325-331
Josefson D., 2001, BMJ Aug 25; 323(7310): pp 416.
Kimberlin, R.H. et al, 1971, J. Comp. Pathol. V81 pp383-391
Klein M.A., et al, 1997, Nature Dec 18-25;390(6661): pp 687-690
Klein M.A. et al, 1998, Nat Med 1998 Dec; 4(12): pp 1429-1433
Klein, T.R. et al, 1998 **,** Biol Chem, Jun;379(6): pp 655-666
Lee, D.H., et al, 1985, J Hyg (Lond) Oct; 95(2): pp 243-253
Liberski, P.P. et al, 1990, Acta Neuropatholgica V79 #4 pp 349-354
Lo, S. C., et al, 1989, Am J Trop Med Hyg 40, pp 213-226
Lo, S.C. et al, 1991a, *Lancet,* 338, pp 1415-1416

Lo SC, et al, 1991b Science Mar 1;251(4997): pp 1074-1076
Lo, S.C., et al, 1993, Mod Pathol, May;6(3): pp276-280
Manuelidis,E.E., et al, 1985, The Lancet 2 pp 896-897
Manuelidis, L., 1998, PNAS Online V95 #5 pp 2520-2525
Manuelidis L, et al, 2000, J Virol Sep;74(18): pp 8614-8622
Marsh R.F., et al, 1984, J Gen Virol Feb;65 (Pt 2): 415-21
Miele, G., et al, 2002, Biochem Biophys Res Commun 2002 Feb 22;291(2): pp 372- 377
Narang, H.K., 1998, Res Virol. Nov-Dec;149(6):pp 375-82.
Naslavsky N., et al, 1999, J Biol Chem Jul 23; 274(30): pp 20763-71
Pattison, IH, 1965, J. Comp. Path V75 pp 159
Pattison, IH. & G.C. Millson, 1960, J. Comp. Path V70 pp 182
Pattison, IH. & G.C. Millson, 1961a, J. Comp. Path V71 pp 101
Pattison, IH. & G.C. Millson, 1961b, J. Comp. Path V71 pp 350
Pocchiari, M., et al, 1987, J Gen Virol Jan;68 (Pt 1):219-23
Prinz M, et al, 2002**,** Proc Natl Acad Sci U S A Jan 22;99(2): pp 919-924
Prusiner, S.B, M.P. Mckinley, et al,1983 Cell v35 pp349
Prusiner S.B., et al, 1993, Proc Natl Acad Sci U S A Nov 15; 90(22): pp 10608-12
Pruisner, S.B. (Editor) Prion Biology and Disease, Cold Spring Harbor Lab Press, 1999.
Race, R.E. amd Ernst, D., 1992, J Gen virology V73, pp 3319-3323
Radebold, Klaus, et al, 2001 BMC Infect Dis 2001 1:20
Reyes, J.M., & Hoenig, E.M., 1981, J Neuropathol Exp Neurol Jan; V40#1: pp1-8
Roikhel VM, et al, 1984, Acta Virol Jul;28(4): pp 294-299
Sigurdson, C.J., et al, 1999, J Gen Virol V80 :pp2757-2764
Sklaviadis, I.K., et al, 1989, J. Virology, V63 #3, pp 1212-1222
Somerville RA, et al, 2002, J Biol Chem Mar 29;277(13):11084-9
Stadtländer, C.H., et al, 1993, Clin Infect Dis 17, S289-301
Stamp, J.T, et al, 1959, J. Comp. Path V69 pp 268
Stanek, G., et al ,1981, Zbl. Bakt. Hygiene 1 Abt. B174 pp 348-354
Tateishi J., 1981, J Neurol Neurosurg Psychiatry. 1981 Aug; 44(8): pp 723-4
Tamai Y., 1989**,** Microbiol Immunol 1989;33(1):pp35-42
Tamai,Y, 1992, New England J Med, 1992, Aug 27; V327(9): pp 649
Taylor, D.M., et al, Arch Virol 1994; 139(3-4): pp313-26
Taylor, D.M. et al, 1996, J Gen Virology V77 pp3161-3164
Taylor-Robinson D., et al, 1991, Int J Exp Pathol Dec; 72(6): pp 705-714
Tola S., et al, 1999, Vaccine Jul 16;17(22): pp 2764-8
Townsend R. et al, 1980, J Bacteriol. May; 142(2):pp 694-700
Tremblay P, Proc 1998, Natl Acad Sci USA Oct 13;95(21): pp12580-5
Tully, J.G. et al, 1984, Ann. Microbiol V135 pp111-117
Watanabe, T., & Yukitake, H., 1990, Microbiol Immunol;34(10): pp 819-824
Williamson, D.L., 1977, NY Academic Press, pgs 465-472
Weissmann C, et al, 1994, Ann N Y Acad Sci , Jun 6;724: pp235-240
Weissmann C, et al, 2001, Philos Trs R Soc Lon B Bio Sci Feb 28;356(1406):pp 177-184
Wirostko E, et al, 1990, J Submicrosc Cytol Pathol 1990 Apr;22(2): pp 231-239
Wise, K.S., 1993, Trends Microbiol ,V1, pp 59-63
Zanusso, G., et al, 2003 J Gen Virol Apr;84(Pt 4): pp 1047-1052
Zhang Yueji, et al, 2000, Acta Argiculturae Zhejiangensis, V12 #1 pp 11-15