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A 25 nm Virion Is the Likely Cause of Transmissible Spongiform Encephalopathies

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Abstract The transmissible spongiform encephalopathies (TSEs) such as endemic sheep scrapie, sporadic human Creutzfeldt-Jakob disease (CJD), and epidemic bovine spongiform encephalopathy (BSE) may all be caused by a unique class of ''slow'' viruses. This concept remains the most parsimonious explanation of the evidence to date, and correctly predicted the spread of the BSE agent to vastly divergent species. With the popularization of the prion (infectious protein) hypothesis, substantial data pointing to a TSE virus have been largely ignored. Yet no form of prion protein (PrP) fulfills Koch's postulates for infection. Pathologic PrP is not proportional to, or necessary for infection, and recombinant and ''amplified'' prions have failed to produce significant infectivity. Moreover, the ''wealth of data'' claimed to support the existence of infectious PrP are increasingly contradicted by experimental observations, and cumbersome speculative notions, such as spontaneous PrP mutations and invisible strain-specific forms of ''infectious PrP'' are proposed to explain the incompatible data. The ability of many ''slow'' viruses to survive harsh environmental conditions and enzymatic assaults, their stealth invasion through protective host-immune defenses, and their ability to hide in the host and persist for many years, all fit nicely with the characteristics of TSE agents. Highly infectious preparations with negligible PrP contain nucleic acids of 1–5 kb, even after exhaustive nuclease digestion. Sedimentation as well as electron microscopic data also reveal spherical infectious particles of 25–35 nm in diameter. This particle size can accommodate a viral genome of 1–4 kb, sufficient to encode a protective nucleocapsid and/or an enzyme required for its replication. Host PrP acts as a cellular facilitator for infectious particles, and ultimately accrues pathological amyloid features. A most significant advance has been the development of tissue culture models that support the replication of many different strains of agent and can produce high levels of infectivity. These models provide new ways to rapidly identify intrinsic viral and strain-specific molecules so important for diagnosis, prevention, and fundamental understanding. J. Cell. Biochem. 100: 897–915, 2007.

Key words: Creutzfeldt-Jakob disease; scrapie; BSE; viral particle; latent infection; prion pathology

"*I dislike arguments of any kind. They are always vulgar, and often convincing*."
Oscar Wilde

The epidemic of bovine spongiform encephalopathy (BSE) in the UK, as well as the increasing spread of a comparable infectious encephalopathy among domestic and wild cervids in the USA (chronic wasting disease or CWD), make it important to resolve the nature of the infectious agents that cause these neurodegenerative diseases. Knowledge of intrinsic agent molecules can facilitate rapid and sensitive diagnosis, and ensure adequate preventive measures for both animals and humans. The infectious agents that cause transmissible spongiform encephalopathies (TSEs) typically lead to neurodegeneration only after a long asymptomatic period, with the concomitant risk of transmission from apparently healthy individuals [Manuelidis, 1994b]. This includes inadvertent person-to-person transmissions from tissue transplants, blood [Manuelidis et al., 1978b, 1985; Tateishi, 1985], and possibly even by dental procedures [Manuelidis, 1997]. The first positive transmissions from circulating blood cells from both animals and humans more than 25 years ago already indicated that a larger population than those expressing neurodegenerative disease might

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silently carry these agents. Such iatrogenic transmissions from asymptomatic individuals are finally beginning to be appreciated with cases of the BSE linked vCJD agent acquired by transfusion [Peden et al., 2004]. The observation that infected blood carries the infectious agent to the intestinal tract, a direction opposite to that commonly assumed, also raises the likelihood of shedding these infectious agents in feces, with further environmental contamination [Radebold et al., 2001]. This is a common mode of spread for many viruses such as the enteroviruses and hepatitis B.

Currently the most favored TSE hypothesis is that the infectious agent is composed of a host protein, known as the prion protein (PrP), which becomes infectious by interacting with itself. This presumably infectious protein or "prion" form is defined by its abnormal aggregation and resistance to limited proteolytic digestion in a test tube assay with detergents. The partially resistant form is commonly designated PrP^{Sc}, PrP^{CJD}, PrP^{BSE} and so forth, or PrP-res. The latter designation is more objective because PrP-res patterns are defined predominantly by the host species and cell type rather than by the infectious agent. In any case, these are all equivalent terms used to describe partially digested PrP bands on Western blots stained with PrP antibodies. The corresponding pathologic form of PrP is detected microscopically as amyloid deposits or fibrils in tissues, cells, and subcellular preparations. None of these molecular and microscopic PrPs show significant infectivity. This is a major, but not the sole problem for the prion concept.

There are many distinct strains of TSE agents that can be discriminated by their virulence in different species, by their doubling times, and by the distribution and severity of neuropathological lesions they provoke. In contrast, vastly different PrP-res patterns can be found in different tissues of a single animal yet each tissue yields only a single identical strain on reinoculation. Thus, the agent rather than the PrP-res pattern breeds true. Experimental changes in PrP-res, achieved by infecting different cell types in culture, also fail to alter agent strain characteristics, as detailed below. There are further caveats to the prion hypothesis at the molecular level. For example, abnormal PrP does not behave as a particle of homogeneous size, unlike the infectious agent. Thus the term "prion particle" is misleading. Since PrP is host encoded, it also does not activate innate immune responses whereas the infectious agent does. In TSEs robust host responses, as found in other viral infections, have now been documented, and become apparent a few weeks after infection, well before PrP-res is detectable [Lu et al., 2004]. The term replication, commonly used to describe the rapid conversion of normal PrP to PrP-res in a test tube, also bears little, if any, resemblance to the observed 3- to 8-day doubling time of different TSE agents in the brain [Manuelidis and Fritch, 1996].

These discrepancies bring us to the other compelling counter argument that the causal agent in TSEs is a virus with its own independent genome. The epidemiology of TSE spread indicates these infectious agents exist in the environment and do not originate from some spontaneous, and as yet undetectable change in host-encoded PrP. For example, the UK BSE agent progressively spread to countries where contaminated materials and/or animals were imported, such as the rest of Europe and Japan. Environmental barriers can also prevent spread of these agents. Australia, a country with strict inspection and embargoes on scrapie-infected sheep, has had no cases of scrapie, a common infection of many European flocks of the same genetic stock [Hunter and Cairns, 1998]. The natural route of spread within an animal is also classically viral, with stopping points in the lymphoreticular system [Manuelidis, 2003]. As most other viruses, TSE agents must utilize and depend on host-cell molecules for entry, replication, and maturation. PrP genetic and antibody knockout studies show that host PrP is required for at least one step in this infectious process [Büeler et al., 1993; Mallucci and Collinge, 2004]. Although PrP knockout experiments supposedly "proved" the prion hypothesis, temporal and other studies had instead suggested that PrP acts as a host susceptibility factor or "viral receptor" rather than the infectious agent [Manuelidis et al., 1987, 1988]. This logical and still viable conclusion can also help to explain why PrP pathology is a relatively late, and not always inevitable response to infection.

The purpose of this review is to briefly examine whether the claims made for prions are justified, particularly in the light of recent reports, and to consider whether the central data actually indicate a conventional viral particle that is unlikely to arise from the host

genome. This paper also predict details of the most probable TSE viral structure from the evidence to date, and points out some old tricks these viruses probably perform. These predictions can be evaluated experimentally, and can also lead to the identification of true infectious candidates.

PRIONS REMAIN HYPOTHETICAL

"A wealth of data" presumably supports the notion that a host-protein transforms itself into an infectious agent [Prusiner, 1998]. This hypothesis was acclaimed by a Lasker Award given to Stanley Prusiner "for demonstrating how a genetic mutation can misfold ordinary proteins, turning them into infectious agents that mimic viruses," He was subsequently awarded a Nobel Prize in 1997 for "his discovery of prions—a new biological principle of infection" and, in the words of the committee they gave the following explication: "What is a prion? It is a small infectious protein capable of causing fatal dementia-like diseases in man and animals. The most remarkable feature of prions is that they are able to replicate themselves without possessing a genome.... Until prions were discovered, duplication without a genome was considered impossible.... In 1976, when Gajdusek received his Nobel Prize, the nature of the infectious agent was completely unknown. At this time, these diseases were assumed to he caused by a new unidentified virus, termed a slow or unconventional virus. During the 1970s, no significant advances regarding the nature of the agent were made, that is, not until Stanley Prusiner took on the problem. After 10 years of hard work he obtained a pure preparation.... Strangely enough, he found that the protein was present in equal amounts in the brains of both diseased and healthy individuals" [KarolinskaInstitute, 1997].

Infectious proteins or prions thereby became canonized, although careful review of the data revealed many discrepancies, such as the presence of abundant nucleic acids in "pure" prion preparations, the discovery of many distinct TSE strains during the period of "of no significant advances," and the separation of virus-like infectious particles from abnormal PrP [Manuelidis et al., 1995]. In addition, Koch's well-established principles to identify infectious pathogens (see below) were not fulfilled. The embrace of the prion hypothesis is apparent from the following recent and representative introductory statements to research papers in the TSE field such as

(1) "The pathogenic PrP^{Sc} has the unique property of being a self-replicating and infectious agent that lacks nucleic acid" [Pan et al., 2004].
(2) "There is considerable evidence (not cited) that PrP^{Sc} is an infectious protein and that conversion of PrP^{C} into PrP^{Sc} is the central event in the propagation of prions, the infectious agents in these diseases" [Stewart and Harris, 2005].
(3) "There is little doubt that the main component of the transmissible agent of spongiform encephalopathies—the prion—is a conformational variant of the ubiquitous host protein PrP^{C}" [Weissmann, 2004].
(4) "The causative agent of TSEs such as scrapie is PrP^{Sc}, a misfolded, protease resistant version of the normal PrP^{C} protein" [Aguzzi, 2005].
(5) "Prions are infectious pathogens principally composed of abnormal forms of a protein encoded in the host genome.... Remarkably, distinct strains of prions occur despite absence of an agent-specific genome: misfolded proteins themselves may encode strain diversity" [Collinge, 2005].
(6) "Even now, despite the overwhelming evidence supporting it, some maintain that the infectious agent must be a virus or a virino (agent containing its own nucleic acid enveloped in host-encoded protein) or that PrP^{Sc} must contain a small amount of host-derived nucleic acid (the "co-prion," or molecule that specifies prion infectivity). These alternative theories are maintained even though, as with the miasma, no one has ever demonstrated the presence of these agents. It is demanded that the prion hypothesis satisfy the prion version of the Koch's postulate" [Zou and Gambetti, 2005].

The last comment is interesting not only because it fails to cite any of the primary data that conflicts with the prion hypothesis, but also because it suggests we should abandon proven infectious principles since they do not fit the prion hypothesis. Moreover, the primary assumption that PrP is infectious can be self-fulfilling and circular, especially when it

is continuously reiterated, as for example, with the defective claim that PrP-res is proportional to infectivity. The failure to examine nucleic acids in infectious preparations with the tools of modern molecular biology, such as RT-PCR and specific labeling techniques, also diminishes absolute statements about the lack of an agent genome, especially when coding nucleic acids of >1 kb have been identified in nuclease-treated infectious preparations as detailed below. Moreover, there are few published reports on nucleic acid sequences in infectious TSE preparations during the last 25 years, falsifying the claim that "intense efforts in many laboratories" have been made to identify a TSE-specific (or any other) nucleic acid [Weissmann, 2004]. Through a web of misleading language it has also guided policies and conclusions that have been detrimental for human and animal health [Manuelidis, 2000, 2003].

Before examining the critical data that calls into question the verity of the prion hypothesis, Koch's fundamental postulates, in an up to date form to include current molecular capabilities, are here stated. First, the infectious agent must be invariably present in a characteristic and constant form. Second, the pathogen must be isolated and grown in pure culture, for example, in an unrelated tissue culture, or preferably in a recombinant form. Third, this cultured material must be shown to reproduce the infectious disease experimentally. Fourth, the identical pathogen should be reisolated from the experimentally inoculated subject.

PrP Infectivity Is Questionable, and Probably Non-Existent

Table I lists the key prion claims that are contradicted by data. Most critical are those that fail to fulfill Koch's postulates for an infectious agent, for example, claims 1–4 as well as the many features of prions that are incompatible with those shown by the infectious agent. First, there are numerous independent animal model, subcellular-molecular, and tissue culture studies demonstrating that PrP-res is not required for infection, and it is also not proportional to the infectious titer. Animal transmissions from brains that lack PrP-res, including cross-species transmissions, have been positive [Manuelidis and Manuelidis, 1985; Lasmezas et al., 1997; Manuelidis et al., 1997; Manuelidis and Lu, 2000; Race et al., 2002]. The absence of detectable prions in material that is clearly infectious goes against Koch's first requirement that the agent must always be present. Amphotericin B treatment also highlights discrepancies between PrP-res and infectivity. This drug arrests PrP-res accumulation in scrapie-infected hamsters while the infectious agent continues to replicate exponentially [Xi et al., 1992]. The reverse pattern of dissociation has also been reported. Infectivity declines while PrP-res accumulates in salivary glands [Sakaguchi et al., 1993]. Such data, under normal circumstances would be considered convincing evidence that PrP-res is a secondary pathological response to infection. Infectivity and PrP-res are also not proportional across strains. One Creutzfeldt-Jakob disease (CJD) agent produces 100,000-fold more infectivity than a second CJD strain, yet these brains show only 10-fold difference in PrP-res, a 10,000-fold discrepancy [Manuelidis, 1998]. Thus many different animal models show PrP-res is a poor marker for infectious titers, and a lack of PrP-res does not preclude infection. In terms of public health then, it is important to recognize that asymptomatic but infected individuals without detectable PrP-res may continue to spread infection.

Marked discrepancies between PrP-res and infectivity are also observed during subcellular and chromatographic purifications of the infectious agent as previously reviewed [Manuelidis, 2003]. In such studies, viruslike infectious

TABLE I. Major Claims and Assumptions of the Prion Hypothesis

Claim	Experimentally	Comment
(1) "PrP^{Sc} is proportional to titer," and is infectious	False	In animal, culture, and molecular studies
(2) "Prion diversity is enciphered by PrP^{Sc}"	False	Agent strains breed true, but not PrP^{Sc}
(3) "PrP gene mutations cause spontaneous transmissible disease"	False	Transmissions not reproducible
(4) "Procedures that modify or hydrolyze PrP^{Sc} inactivate prions"	True	They also inactivate viruses
(5) "No evidence exists for a virus-like particle"	False	25–35 nm viruslike particles
(6) "Transmissible particles are devoid of nucleic acid"	False	Infectious particles with >500 nt lengths
(7) "No sign of an immune response to foreign agent"	False	Early innate immune responses
(8) "Accumulation of PrP^{Sc} associated with pathology"	True	PrP^{Sc} is a late host response

For experimental details see text. Quotes are from Prusiner, 1998, 1999.

particles separate from abnormal but non-infectious PrP (vide infra). This again emphasizes the limitations of prion assays for the infectious agent. More recently, infected cell and tissue culture experiments have also shown a poor correlation of PrP-res and infectivity. For example, purified living microglia from CJD-infected brain have no detectable prions, yet these myeloid cells contain maximal levels of infectivity that are equivalent to that of starting brain [Baker et al., 2002]. Additionally, immortalized neural cells infected with a CJD agent in vitro can show a progressive 650-fold increase in infectious titer while their PrP-res levels remain constant [Arjona et al., 2004]. The most parsimonious interpretation would be that the infectious agent is different than abnormal PrP. Indeed, the many PrP-res to infectivity discrepancies, as well as the propagation of distinct strains of TSE agents that breed true, regardless of inconstant PrP conformations, has led to additional speculations to preserve the prion hypothesis. These have included the now apparently abandoned "protein X" co-factor for infection, glycosylation-induced infectious folding patterns of PrP that determine strains, or some yet to be discovered tertiary conformation of PrP amyloid that is infectious [Aguzzi and Weissmann, 1997; Prusiner, 1998; Collinge, 2005]. The apparent lack of infectivity of PrP-res may also underlie more recent descriptions of TSEs as "pseudoinfections," though the epidemic spread of BSE obviously contradicts this terminology.

It is also continues to be evident that no recombinant PrP-res (recPrP) molecules, and no PrP amyloid preparations, are sufficient to transmit infection. The most recent experiments with truncated recPrP [Legname et al., 2004] show similarities to previous (transgenic) Tg PrP mouse transmissions that were compromised by laboratory contamination [Manuelidis et al., 1997]. Rather than producing the predicted novel properties that should be encoded by this recPrP, serial passage of mouse brains showed incubation times and neuropathological characteristics only of the Chandler (RML) scrapie strain used in that laboratory. Even prion believers have realized contamination as the most likely cause of these transmissions [Couzin, 2004; Castilla et al., 2005; Nazor et al., 2005]. Others, however, have been more enthusiastic, with a celebratory call for "the birth of a prion: spontaneous generation revisited" [Weissmann, 2005].

In a newer approach to prove PrP-res is infectious, one serially dilutes scrapie-infected brain homogenates with normal brain, and at each sequential dilution sonicates and incubates these mixtures under conditions that promote PrP to PrP-res conversion in the normal brain sample [Castilla et al., 2005]. A tiny amount of infectivity was ultimately recovered in the final "amplified" PrP-res samples. These minute titers were most consistent with carry over contamination on the sonication probe, as found on other stainless steel probes exposed to infected brain [Zobeley et al., 1999], or with intrinsically imperfect brain homogenate dilutions. Although the amyloid characteristics and abundance of PrP-res were identical in the starting undiluted and the final dilution tubes, the infectivity titer decreased from about 1 million to less than 10. The reported conclusion, that these results prove the infectivity of PrP-res, may instead exemplify Languimer's description of pathological science based on negligible small effects [Langmuir, 1989]. Other investigators have also been unable to find significant replication of infectivity using the same published PrP-res conversion methodology [Bieschke et al., 2004].

Agent Strains do not Display Any Constant Individual PrP Conformation

The second major problem with the prion hypothesis is that it does not account for TSE strain diversity nor for the fidelity of these individual strains in mammals with different PrP sequences. Even as late as 1997, Prusiner minimized the number of TSE strains, and contended "the primary structure of PrP encoding prions during the passage history, rather than the original source of inoculum, determines strain characteristics in any particular host" [Scott et al., 1997]. The epidemic BSE agent surely does not follow this rule. It maintains its singular identity in every species it has infected even though the abnormal PrP in those species is quite variable. Representative BSE-linked isolates from the many infected species including primates, felines, canines, gazella, kudu, caprines, and bovines all have yielded the same BSE strain-specific profile in inbred indicator mice [Bruce, 2003]. Moreover, it has long been known that natural sheep scrapie strains preserve their identity during serial passages in mice, and can reinfect sheep to produce the same original scrapie incubation

and neuropathology characteristics [Zlotnik and Rennie, 1965] despite major PrP differences between sheep and mice. Conversely, most of the different scrapie strains show no PrP-res differences when propagated in inbred mice, and in hamsters there is only a single TSE strain, isolated from a mink, that provokes a different brain PrP-res pattern than the various other scrapie agents in this host [Scott et al., 1997; Bartz et al., 2000]. The slow progressive evolution of a TSE strain by repeated serial propagation in a single species has also been documented, and it is difficult to envision how or why the unchanging PrP of that species would modulate itself to produce these progressive changes. Viruses clearly do this to enhance their survival.

To account for strains, different conformational folding patterns of PrP have been hypothesized to "encode" or "encipher" strain-specific information. Each hypothetical conformation would propagate its own uniquely folded form of PrP-res (Table I). Many experiments show this suggestion to be unlikely because disaggregating or unfolding PrP (so that it loses its proteinase K resistance), does not reduce infectivity and also does not alter strain characteristics [Sklaviadis et al., 1989]. Enzymatic removal of sugars also has no effect on either infectivity or strain characteristics [Manuelidis et al., 1987]. Furthermore, experimentally changing the PrP-res and glycosylation band pattern has no effect on a strain's phenotype. For example, when different TSE agents in brain are propagated for extended passages in monotypic cell cultures with very different PrP and PrP-res patterns than brain, these agents retain and reproduce their original very distinctive strain characteristics, that is, the infectious agent but not the PrP-res conformation breeds true when the infected tissue culture homogenates are reinoculated into animals [Arjona et al., 2004]. In contrast, PrP-res characteristics are largely determined by cell type and species and do not breed true. Thus PrP-res folding differences are not required for either the definition or the maintenance of strain-specific properties. The variability of PrP-res in different cell types bearing a single agent strain also does not fulfill Koch's requirement that the causal agent, that is, PrP-res, must have "constant properties." High-resolution structural analyses of PrP have also failed to resolve any strain-specific features. Nevertheless, hypothetical folding differences that presumably encode strains continue to be illustrated by color-coded cartoons [e.g., Weissmann, 2005].

PrP Mutations can Alter Susceptibility to Agent Strains but do not Reproduce TSEs

A single amino acid mutation in a host protein can change the ability of a virus to infect a cell (reviewed in Manuelidis, 1994b). The classification of rare "familial prion" CJD in people with a 102 L PrP mutation assumes that this gene mutation causes a dominantly inherited (germline) infection rather than an increased susceptibility to an environmental TSE pathogen [Prusiner et al., 1995; Prusiner, 1998]. This now textbook "familial" designation (Table I) rests on data that is irreproducible. The frequent accompanying reasoning, that inherited infections cannot be caused by a conventional viral structure, is also false because retroviruses, for example, can be inherited through the germline. Moreover, the assumption that human TSE agents, unlike scrapie, are vertically transmitted rather than endemic, is also not in accord with studies of several human agents in experimental animals. For example, a sporadic CJD agent produced no maternal transmission during 12 years of observation of guinea pigs born to and housed with CJD-infected parents [Manuelidis and Manuelidis, 1979b]. The disappearance of kuru after the cessation of cannibalism [Gajdusek, 1977] also makes the germline inheritance of this geographically distinct CJD agent unlikely. Moreover, the rare human 102 L mutation in PrP, proposed to cause an "inherited" form of transmissible CJD known as Gerstmann–Straussler–Scheinker disease (GSS), should, according to the prion hypothesis, define a unique agent strain that is geographically independent, and also cause a transmissible disease when inserted transgenically. Instead, geographically distinct CJD agents have been isolated from GSS patients with this 102 Leu PrP mutation [Nishida et al., 2005]. Furthermore, this mutation has been incapable of producing either PrP^{Sc} or transmissible disease in mice with Tg copies of human 102 L PrP. The initial transmissions reported in these Tg mice, as well as the spongiform pathology [Hsiao et al., 1990], were irreproducible when Tg mice with normal rather than freakishly high copies of this 102 L transgene were developed [Barron

and Manson, 2003]. Additionally, neither the high, nor the normal copy mutants produced PrP^{Sc}, and this is an important reminder that spongiform and vacuolar change can be caused by factors other than a transmissible agent. There are also many different non-infectious and infectious causes of amyloid formation, a β-pleated conformation acquired by various types of proteins, not just PrP. None of these other amyloids have shown infectivity.

TSE Agents and Prions Have Different Inactivation Profiles

Inactivation is an indirect and crude way to define an infectious agent because very different structures can display common inactivation features. For example, treatments that destroy TSE infectivity, such as extended proteolysis, also destroy many viruses by digesting the nucleocapsid coats that protects the viral genome (Table I). Digestion of nucleocapsid proteins by broad spectrum proteolytic enzymes such as proteinase K, or disruption of viral protein-nucleic acid cores by harsh chemicals treatments such as $\geq$2 M GdnHCl or boiling in SDS, also markedly reduce viral titers [Manuelidis et al., 1995]. Therefore, one cannot conclude that these disruptive treatments prove the TSE agent is a prion rather than a virus. It is also often stated that "unusual properties" of PrP^{Sc} "mimic" those of prions, that is, infectivity. In fact, however, properties of the infectious agent and PrP-res often diverge significantly. Even Prusiner's group has shown >99% of infectivity can be destroyed by concentrations of selected chemicals that have no effect on PrP-res [Wille et al., 1996]. Additionally, the properties of PrP^{Sc} responsible for proteinase K resistance do not correlate with those conferring thermostability on TSE agents [Somerville et al., 2002]. The heat sensitivities for most TSE strains are, moreover, quite conventional, with substantial inactivation of several strains between 70 and 84°C. Many extraordinary inactivation claims for TSE agents also rest on assays for PrP-res without examination of infectivity. Others emphasize sterilization levels of infectivity (to 0%) that create an unbalanced picture of a fantastical agent resistance, one that does not reflect the more representative 99.9% of the infectious agent population. Indeed, sterilization levels are difficult to achieve with many conventional viruses such as hepatitis B. Other physical differences between the prion and the infectious agent are detailed below.

Prion assumptions based on indirect tests and weak correlative arguments also have ramifications for public health. It is often assumed that PrP-res is so resistant that it will survive the harsh digestive conditions of the gastrointestinal tract to invade the mucosa and infect Peyer's patches, a common entry pattern for a number of sturdy viruses. Obviously, viruses such as enteroviruses are designed to withstand digestive conditions, but until recently no one checked to find if prions could actually infect animals via this likely route for the spread of BSE. A recent study, where huge amounts of infected brain were loaded directly in the gut, showed that PrP-res was rapidly destroyed by alimentary tract fluids. It was also clear that some different infectious structure invaded to provoke de novo accumulation of pathologic PrP-res only after a 30-day hiatus [Jeffrey et al., 2006]. This evidence should also bring to mind the unexamined assumption that PrP-res aggregates can cross the blood-brain barrier in a naked state, or propagate a brain specific PrP-res conformer from infected reticuloendothelial cells that produce a different cell type specific PrP-res conformer.

In summary, although like-to-like host PrP sequences may be needed to convert PrP to PrP-res amyloid, this interaction and its consequences are epiphenomena that are insufficient, and probably not required for the replication of infectious particles. The above data also suggests that mechanisms of amyloid seeding or conversion are relevant for pathogenesis in TSEs and in non-transmissible Alzheimer's disease, but are not essential for encoding strain-specificities. Although pathological PrP has proven to be a reliable surrogate marker for TSE infection, especially during end stage neurodegenerative disease, a lack of PrP-res does not rule out infection. This caveat needs to be recognized in diagnostic PrP-res assays, especially those currently used to assess infection of livestock and human tissues that may be transplanted, or that may inadvertently contaminate surgical instruments [Manuelidis, 1997].

TSE VIRAL PARTICLES

While no form of PrP has proven infectivity, there is substantial direct evidence for infectious

viral particles despite firm statements to the contrary (Table II). To fully appreciate this, one would have to look at data not cited in the prion centric literature, including many scientific publications more than 8 years old with important primary data currently unavailable on the Internet. Particles with the density and size of viruses have been repeatedly found in more purified infectious preparations, and particles of similar diameter have also been identified in many independent ultrastructural studies of infected brain. Subcellular fractionation studies show TSE infectivity concentrates with 25–30 nm particles from which PrP has been largely removed. Moreover, disruption of the protein-nucleic acid components of these particles destroys $\geq$99.5% of the starting infectivity. Ultrastructural studies from the 1960s onwards have also repeatedly shown what are probably corresponding 25–35 nm particles in their natural state in infected, but not in normal brain cells. These viruslike structures continue to be documented in many different TSE models as detailed below.

Viruslike Structures Are Components of the Most Infectious Subcellular Preparations

Between 1989 and 1995, sucrose gradient analyses of CJD-infected hamster brain fractions revealed a narrow, homogeneous 120 S peak of infectivity that separated from non-infectious cellular material at $\leq$10 S. In contrast to this viruslike peak of infectivity, more than 75% of the loaded abnormal PrP was found in the non-infectious cellular protein region [Sklaviadis et al., 1989, 1992]. In other words, non-denatured PrP^{Sc} was separated from infectivity despite the claim that it had not been and could not be separated [Prusiner, 1999]. Subsequent experiments by others with higher titer 263 K (237) scrapie-infected hamster brains reproduced the same sucrose gradient separations of infectivity [Shaked et al., 1999], and Prusiner's team was even more successful in removing PrP-res from infectious particles because no PrP was detectable in their most infectious rapidly sedimenting sucrose fractions [Riesner et al., 1996]. Sucrose equilibrium gradients further demonstrated that the 120 S infectious particles have a density of 1.28 g/cc. This density mirrors conventional viral cores constituted by nucleic acid-protein complexes. Thus it was already likely that the infectious TSE particle was a protein-nucleic acid complex, rather than a protein.

Micrococcal nuclease digestion removed co-purifying nucleic acids, which are easily visualized by silver staining on gels before digestion. However, this treatment did not alter either the viruslike density or size of TSE infectious particles [Sklaviadis et al., 1990, 1992; Manuelidis, 2003]. Thus neither the conventional viral size nor the viruslike density of infectivity could be explained by non-specific binding of extrinsic nucleic acids. Moreover, contrary to the claim that infectious preparations are devoid of nucleic acid (Table I), nucleic acids up to 5,000 nt long were extracted from nuclease-treated particles in the 120 S infectious peak. One of these sequences derived from a co-sedimenting endogenous retrovirus [Akowitz et al., 1994]. Its cognate protective nucleocapsid protein of $\sim$60 kDa was also visualized on 2D gels using nucleocapsid antibodies, further confirming the extracted retroviral nucleic acid sequence was bundled as a true viral particle, albeit not the TSE agent. Thus, as in many viral studies, nuclease resistance fails to exclude a protected TSE viral sequence in particles of the infectious 120 S peak. Nuclease digestion instead can enhance the purification of 25 nm viruslike TSE particles from host nucleic acids.

The TSE infectious agent has not yet been purified to homogeneity, and infectious preparations often contain a large amount of nucleic acids as well as other brain material. Early prion claims of a nucleic acid free agent were put forth despite the fact that the infectious material described had enough DNA to encode for a complete human being [Manuelidis, 2003]. Others, including the Weissmann and Marsh groups also found nucleic acid sequences >300 nt long in Prusiner's later more purified fractions that were claimed to have none (reviewed in Manuelidis [2003]). Additionally, a recent publication from Prusiner's group reconfirms the presence of significant quantities of nucleic acids in their current most "purified prion" preparations. Despite uncontrolled recovery and inadequate detection methods, 10–20 ng of nucleic acid was extracted from their 263 K scrapie brain preparation that contained 10^8–10^9 infectious doses. For a genome $\sim$1,000 nt in length (the length of the hepatitis δ virus), 1 ng is sufficient to code for $\sim 10^9$ infectious particles, and thus 10–20 ng, as recovered from that material was 10- to 200-fold greater than the

measured infectious doses. Hence, tortuous assumptions and calculations were used to exclude a viral genome. No attempts were made to examine the sequences retrieved, or to evaluate retroviral sequences already shown to co-purify with the TSE agent. This required only a simple and rapid RT-PCR test with the primers already described [Akowitz et al., 1994]. The preparative recovery of infectivity in their prion rich fraction was also poor, and yielded only ~0.1% of the starting brain infectivity. In contrast, 15% of the starting brain titer has been reproducibly recovered in the 120 S sucrose peak [Sklaviadis et al., 1992]. Rather than examining the nature of the nucleic acids recovered, indirect and inconclusive radiation effects were cited to exclude a viral genome even though several types of conventional viral particles are highly resistant to radiation (reviewed in Manuelidis, 1994b). Nevertheless, the claim that TSE agents cannot be viral because they have no genetic material has persisted, and has prematurely narrowed the scope of TSE research. Virtually no investigators during the last 15 years have reported on the nucleic acid sequences in their infectious preparations. There is also a paucity of analysis of proteins other than PrP. These include nucleic acid binding and other proteins with no affinity for PrP antibodies [Sklaviadis et al., 1993].

Discrete 25–30 nm Particles in Infectious Subcellular Fractions

There are additional experimental results supporting a viral particle rather than prion as the infectious agent in TSEs. Filtration data claimed to show the infectious agent is a small protein of <100 kDa with a diameter of <15 nm have been irreproducible whereas a number of filtration studies have found a minimal agent size of ~25 nm [Sklaviadis et al., 1992]. By 1992, fast field flow sedimentation and high-pressure liquid chromatography (HPLC), two independent methods, revealed more exact viral dimensions. Field flow sedimentation of the nuclease-treated 120 S infectious peak was compared with marker spheres of different sizes. The infectious peak continued to have a homogeneous and narrow distribution, corresponding to spheres of 25–30 nm in diameter, well within the spectrum of conventional viruses. Particles of this size were also visible ultrastructurally in this fraction, with representative thin sections of the 120 S peak shown in Figure 1. These particles, as shown, did not bind PrP antibodies. Parallel HPLC studies further showed these infectious particles had an M_r of $\sim 10^6$–10^7 Da, an M_r also compatible with viruses of 25–30 nm in diameter. A very recent field flow analysis of a less pure 263 K scrapie preparation has similarly shown that infectivity co-migrates with round particles of 17–27 nm, although these were interpreted as infectious PrP multimers [Silveira et al., 2005]. Since the 25–30 nm particles in our subcellular 120 S fractions do not bind PrP antibodies (Fig. 1) they are probably not prions. Instead, they resemble the viruslike particles observed in intact infected fixed brain. Those intracellular particles, arranged in arrays, also lack PrP (vide infra).

Disruption of Viruslike Particles Destroys Infectivity

A second strong reason to consider these ~25 nm particles are viral rather than prionic, is that disrupting their nucleic acid-protein structure destroys their infectivity [Manuelidis et al., 1995]. Brief exposure to 2 M GdnHCl releases nucleic acids and cognate nucleic acid binding proteins into the supernatant. Infectivity was demonstrably destroyed because it could not be recovered from the solubilized proteins and nucleic acids in the supernatant, nor from the residual particulate material. Yet these GdnHCl-treated fractions continued to show intact abnormal PrP multimers and aggregates, the supposedly infectious prion form. In contrast, low concentrations of SDS stripped off the residual contaminating PrP from the infectious 120 S particles, but did not reduce the infectious titer. These SDS-treated particles continued to sediment at 100,000 g × 1 h, and retained their intrinsic infectivity and intact nucleic acid-protein complexes as assessed by ^{32}P nucleic acid labeling. In sum, subcellular infectious fractions are most consistent with a viral particle of 25–30 nm in diameter that possesses a genome of >1 kb and a protective nucleocapsid-like protein. PrP is not the major intrinsic protective element of these particles because it can be removed without affecting agent properties.

These findings are also not consistent with the "virino" hypothesis. The virino hypothesis assumes a small subviral "informational molecule" [Dickinson and Outram, 1988], presumably

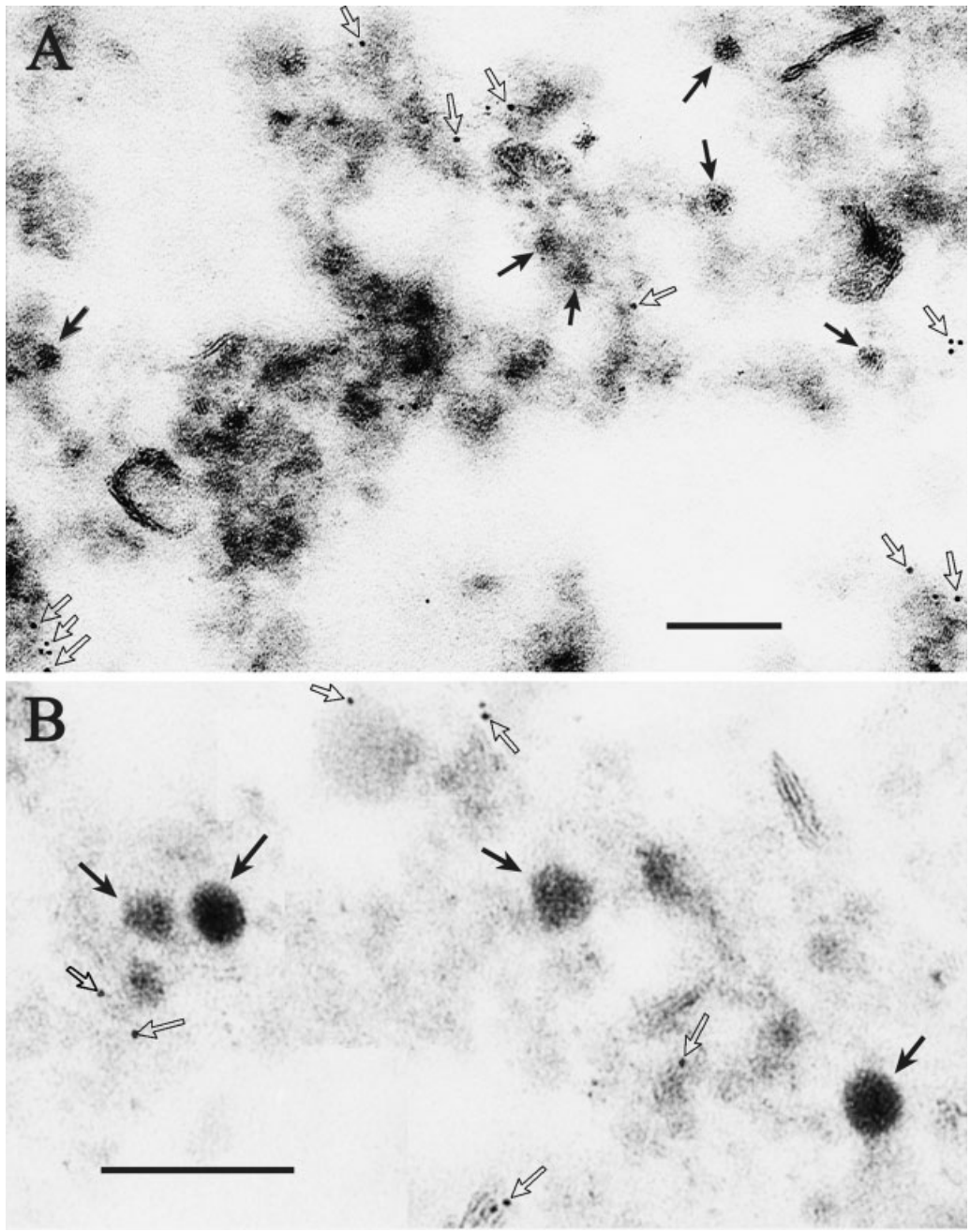

Fig. 1. Electron micrographs of 120 S infectious material fractionated from hamster CJD brain in thin sections. The representative field **A** shows 25 nm diameter particles with dense cores stained by uranyl acetate (arrows) but without PrP associated gold particles (open arrows). PrP antibodies instead bind fluffy proteinaceous material. **B** shows higher magnification, and the dense spherical viruslike particles appear to be attached to membranous (lighter grey) stems and channels. The 120 S fraction was fixed, embedded in LR white, and thin sections were incubated with primary antibodies followed by ~3 nm gold labeled secondary antibodies as described [Manuelidis et al., 1987]. Bars are 100 nm.

smaller than the 1 kb hepatitis δ viral genome. This virino genome is thought to be protected by a host protein, with PrP the main contender. While the virino proposition is an understandable attempt to reconcile PrP and viruslike strain findings, it remains unsupported by any direct experimental data, such as an analysis of specific small nucleic acids in infectious preparations. In addition, structural features of a TSE virus derived from the above subcellular, molecular,

and electron microscopic studies also reasonably match the features of the viruslike particles repeatedly observed in perfusion-fixed brain tissue of many different species infected with a variety of TSE strains.

Viruslike Particles in Infected Brain Tissue

The first electron microscopic description of arrays of viruslike particles of ~35 nm in diameter in infected brains dates back to a beautifully clear and systematic study of experimental scrapie in 1968 [David-Ferreira et al., 1968]. This study excluded papovavirus contaminants. Lampert identified the same "viruslike" particle arrays in synapses in experimental primate CJD, and considered them to be papovaviruses. This interpretation, and his accompanying commentary, indicates a lack of ambiguity about their conventional viral structure [Lampert et al., 1971]. Similar viruslike particles were identified in natural scrapie [Bignami and Parry, 1971], and a number of investigators including Narang [1974] suspected these particles might be the TSE agent. Uninfected controls did not display these viruslike particles. Baringer and Prusiner [1978] also subsequently reported similar crystalline arrays of osmiophilic ~25 nm particles in scrapie-infected mice that "were a size consistent with sedimentation and filtration data for the scrapie agent." These observations contradict later claims of "the lack of evidence for a virus of any shape" [Prusiner, 1999]. With our successful passages of human CJD to guinea pigs and hamsters by the 1970s, I looked for similar particles in perfused and well-fixed CJD-infected brains [Manuelidis et al., 1976, 1978a; Manuelidis and Manuelidis, 1979a], but could not differentiate dense viruslike particles from late neurodegenerative synaptic changes.

Since that time many additional studies carried out by Liberski [2004] have extended and solidified the presence of these viruslike particles in a variety of TSE-infected brain samples, including human and experimental CJD, GSS, BSE linked vCJD, as well as many different sheep derived scrapie strains propagated in various inbred mice [Gibson and Doughty, 1989]. These particles are often called tubovesicular particles or structures because they collect in an array of connected vesicular channels in the synaptic region of neurons. In most animal models they also appear at levels consistent with infectious titers, and are less numerous in lower titer brains [Jeffrey and Fraser, 2000]. In contrast, PrP-res can be a million fold greater than the experimentally determined titers of "purified prion" preparations. The exception to the above generalization is the high titer 263 K scrapie-infected hamster brain. Although 25 nm particle arrays have been identified in this model, they are far less numerous than expected. Whether packaging and/or collection of these particles is different in hamsters is not known. However, conventional viral particles can be very difficult to find in chronic viral brain infections, and may not always correspond to levels of infection. In Rabies, for example, brainstem viral particles (Negri bodies) are obvious, but they may not be apparent in infected cerebrum neurons actively synthesizing abundant viral transcripts [Fu et al., 1993].

Two additional findings make the intracellular 25–35 nm particles reasonable TSE viral candidates, aside from their likeness to the particles observed in high titer subcellular fractions. First, they do not bind PrP antibodies, whereas adjacent cellular PrP amyloid does. This suggests their structure is independent and distinct from PrP. However, given the popularity of the prion hypothesis and the insistence that PrP must be a part of the infectious agent [Prusiner, 1998, 1999], it is not surprising that these particles were considered unlikely TSE viral candidates [Liberski et al., 1997]. Nevertheless, without PrP they appear remarkably similar to the 25 nm particles found within the infectious 120 S peak. These particles also do not bind PrP antibodies (Fig. 1) and can be stripped of residual PrP without loss of infectivity (vide supra). Second, tubulovesicular particles have been identified prior to the onset of neurodegenerative changes, and were less numerous during this preclinical period of lower brain infectivity, as would be expected for a TSE virion [Jeffrey and Fraser, 2000]. Their early appearance further indicates they can be primary causal agents rather than secondary pathological structures generated by neuronal failure. Thus far these particles have only been observed in synaptic regions of neurons. Notably, synaptosomal fractions made without detergents and verified for purity by electron microscopy, have likewise displayed the highest percent of brain infectivity in two different CJD models [Manuelidis and Manuelidis, 1983], further linking the 25–35 nm synaptic particles to infectivity. If they are the

TSE virions, then they should probably be present in high titer infected cell cultures that lack synaptic differentiation. I have recently identified 25–30 nm particles in arrays within TSE-infected cells in culture, but not in mock-infected controls. These show a strong resemblance to those found by others in synaptic processes of infected brains (manuscript in preparation).

TSE Agents Follow the Route of Viruses and Evoke Early Host-Immune Responses

This brings us to the involvement of the reticuloendothelial immune system and its ability to recognize and respond to the TSE infectious agent as a foreign invader. In a classic 1967 paper, Eklund et al. [1967] showed that a scrapie agent inoculated intramuscularly adjacent to the sciatic nerve first replicated in distant lymphoreticular tissues such as spleen before invading neural tissue. The most likely carrier of agent would be white blood cells and this first demonstrated in 1978 in experimental CJD [Manuelidis et al., 1978b]. This is a typical route of dissemination for the vast majority of known human viruses, including those such as poliovirus that eventually spread to the CNS, as well as those that evade immune recognition. The presence of infectious TSE agents in myeloid cells, such as migratory macrophages, microglial cells, and dendritic cells [Manuelidis et al., 2000; Aucouturier et al., 2001; Baker et al., 2002] therefore recapitulates this viral pattern of tissue preference and progressive spread. Moreover, infected lymphoid tissues provide a conduit and source for latent accumulation, as well as for subsequent reactivation and dissemination of agent [Manuelidis, 2003]. In lymphoid tissues, specialized follicular dendritic cells (FDC) of the spleen display pathological PrP [Muramoto et al., 1993] that largely accumulates at the surface of the FDC [Manuelidis et al., 2000]. This is a place known to trap several types of viruses including HIV. This overall pattern of agent spread to lymphoreticular tissues with a lag to produce appreciable titers in specific types of target tissues is difficult to explain for a host-encoded prion that is more widely distributed.

Although the dependence of different TSE strains on FDC can be variable, as determined by infection of Lymphotoxin-β and other knockout mice with compromised genes affecting B cell and FDC development [Klein et al., 1997; Manuelidis et al., 2000; Shlomchik et al., 2001; Aucouturier and Carnaud, 2002], FDC are involved in all experimental TSE models examined thus far, and FDC and antigen presenting myeloid cells are built to facilitate recognition of foreign pathogens. The presence of TSE agents in myeloid cells also raised the issue of an immune system response to TSE agents, despite blanket dismissals of this possibility [Manuelidis et al., 1997]. Therefore, we began to look at inflammatory and innate immune responses in experimental CJD, realizing that a lack of neutralizing antibodies in TSEs represented only a small facet of the complex interplay between the host's immune system and foreign pathogens, particularly for latent and persistent viruses that remain covert.

Early Innate Immune Response of Microglia and Infected Brain

The early microglial recruitment in rat CJD that preceded PrP-res accumulation [Manuelidis et al., 1997] suggested microglia would be a good place to begin analyses of the innate immune system, particularly because these cells with undetectable PrP-res are highly infectious [Baker et al., 2002]. To reduce the enormous cellular heterogeneity of brain tissue it was advantageous to first separate and briefly grow purified microglia. We compared cDNA of CJD-infected versus uninfected microglia using arrays spotted with immune pathway genes, and identified many virus-linked inflammatory changes. Exposing normal microglia to large amounts of partially purified brain PrP-res did not mimic these changes. However, the application of standard inflammatory stimulators such as lipopolysaccharide did activate a select group of immune pathways with a pattern that partially overlapped the infectious TSE pattern [Baker and Manuelidis, 2003]. These innate immune responses to TSE infection were activated at substantial and significant levels (5 to $>40\times$ normal). Additional experiments were designed to look at interferon and interferon pathways that are often recruited in a number of viral infections, especially those that involve ds RNAs. Moreover, interferon is part of a more universal innate host-defense strategy that is not limited to microglia, and is not activated by abnormal PrP. TSE infection activated particular interferon pathways, but did not lead to interferon production. This abortive interferon

pattern has also been observed with other latent and persistent viruses [Baker et al., 2004].

To further test the biological significance of the activated transcripts, these innate immune markers were evaluated on complex brain tissue by RT-PCR. Studies of samples collected at 10-day intervals post-inoculation showed many of the transcripts identified in purified microglia were accurate markers of progressive TSE brain infection. Statistically significant and obvious elevation of 13 transcripts preceded PrP-res accumulation by 60 days, and other transcripts were recruited during ensuing disease progression. Thus different groups of host responses were specific for each particular stage of infection. Additionally, these host responses varied with the agent used for infection, and could distinguish infection by two different CJD agents in whole brain samples [Lu et al., 2004]. In sum, the host can recognize a TSE agent and recruit its innate immune system to respond as early as 20–30 days after inoculation, a time at which the infectious agent begins to replicate. In contrast, PrP-res begins to accumulate only at 90 days post-inoculation, and is incapable of activating the same immune pathways. Aside from the incompatibility of these host responses with the prion hypothesis, immune molecular markers can provide useful diagnostic information and suggest new therapeutic approaches to prevent disease progression. While PrP pathology is also clearly diagnostic at later stages of infection, the involvement of other host molecules will probably provide tests and insights into the early and asymptomatic phases of infection that are medically valuable.

Viruslike Interference and the Rapid Diagnosis of Different TSE Strains by Co-Culture

Viral interference is a well-known phenomenon documented for classes of viruses as elementary as bacterial plasmids and as sophisticated as complex poxvirus infections of mammals. Interference occurs when infection by one viral strain prevents superinfection by a second related challenge agent. Although antibodies provide the major mechanism of interference in acute viral infections of mammals, and are the basis for many effective vaccines, interference is complicated and incompletely understood even for simple bacterial plasmids [Ho et al., 2002]. The mechanisms of interference for various mammalian viruses grown in non-lymphoid cell cultures, where antibody responses are excluded, can also involve several cellular pathways and cell surface molecules [Kristal et al., 1993]. Although understanding TSE interference mechanisms may take years to clarify, the capacity of specific TSE agents to interfere with superinfection by related members of its class strongly implicates a virus rather than a prion. Tests of superinfection in vivo, as well as in newly developed cell culture models, show protection is dependent on the continued presence of specific agent strains, and that PrP-res is irrelevant for effective interference.

A slow and avirulent strain of CJD, typical of sporadic CJD isolates, prevents superinfection by a more virulent Asiatic CJD isolate in vivo even though the slow strain provokes no detectable PrP-res. This interference was so dramatic [Manuelidis, 1998; Manuelidis and Lu, 2003] reviewers had difficulty accepting the results. The presence of the suppressed challenge agent in these brains became evident on further secondary passages [Manuelidis and Lu, 2000]. Interference could also last for the lifetime of the host. With low doses of the protective agent, mice survived free of disease until they began to die of old age at >650 days, even though they were challenged with moderate doses of the more virulent agent. Unprotected control mice inoculated only with the challenge agent all died ~ 350 days earlier than the protected mice. PrP-res was not involved in this protection since PrP-res was undetectable during the prolonged time of challenge. Furthermore, brain factors but not serum factors appeared to be involved in protection, a finding consistent with innate immune responses provoked by the first protective agent. Finally, despite the simultaneous propagation of two distinct CJD strains in each animal for over a year, no "chimeric" or intermediary TSE strains were produced as predicted by the prion hypothesis [Scott et al., 1997]. Doubly infected brains gave rise only to the two original strains, and each agent bred true with no mixed or chimeric agent phenotype by neuropathology and incubation time [Manuelidis and Lu, 2000]. The prion hypothesis has not explained these positive interference results, shown in several experiments, and using different routes of challenge, for example, intracerebral and intravenous.

To find if neural cell cultures that are free of immune system cells could support interference,

we developed a rapid and more accessible co-culture test. This format was used to evaluate a variety of CJD and scrapie agent strains that had similar incubation times and widespread brain lesions, and thus could not be easily discriminated from each other in doubly infected mice [Nishida et al., 2005]. In vitro, neomycin resistant target cells infected with one agent are exposed to cells infected with a second challenge agent, and after a few days of co-cultivation the infected challenge cells are removed by antibiotic selection. The pure target cells can then be assayed for superinfection. These experiments demonstrated that: (1) interference between TSE agents can take place in a simplified cell system without lymphoreticular cells, (2) some sheep derived scrapie strains can interfere with human-derived CJD agents, and visa versa, and (3) interference is dependent on the individual agent strains, but not on the presence or absence of PrP-res. The relationship between different scrapie and CJD agents was surprising because it emphasized interference was unrelated to the natural host species for these agents. For example, two scrapie strains with similar titers showed very different abilities to protect cells from superinfection by a CJD agent isolated in Japan. A low titer CJD agent from the USA also effectively prevented superinfection by scrapie agents. Clearly some scrapie strains have more in common with a human agent than with other sheep agents, at least with respect to their interfering and/or superinfecting capacities. All this leads one to question any rigid distinctions between sheep and human agents, an assumption underlying many public health conclusions about the inability of sheep scrapie to spread to humans. These experiments also underscore the potential and rarely considered possible spread of at least some human TSE agents to other mammals [Manuelidis and Manuelidis, 1993].

The amount and pattern of PrP-res is completely irrelevant to interference in cell culture, as had been shown previously in animals. PrP-res is not necessary to prevent superinfection, since cells infected by an agent that provokes no PrP-res were resistant to challenge by CJD and scrapie agents. Conversely, agents that induced very large amounts of PrP-res failed confer protection as would be expected if different forms of abnormal PrP-res competed for the small amount of remaining normal host PrP. Indeed, interference depended only on the continued presence of the protective infectious agent. This was demonstrated by "curing" target cells of their protective infection by Pentosan polysulfate. They then became susceptible to challenge superinfection [Nishida et al., 2005]. This data shows that the continued presence of the agent itself, rather than any single host response, is the essential determinant underlying interference. These co-culture tests are also remarkable because they can rapidly discriminate among different agent strains. In the past, long and expensive mouse incubations for 120 to more than 350 days, in conjunction with evaluation of subtle neuropathologic differences, were needed to discriminate among strains, especially those without unique PrP-res profiles. In contrast, co-culture interference experiments can resolve strains in as little as 25 days. Additionally, if a strain changes significantly, this may be rapidly detected in these simplified culture interference models. The effect of selected drugs and antisense molecules can also be rapidly evaluated in vitro.

While the exact mechanisms of interference are not known they probably involve cellular pathways used by the agent, as well as agent specified molecules, and/or variant forms of the agent itself such as defective interfering particles. Host molecules may limit agent entry or replication, and/or enhance the clearance of the challenge agent from the cell. In terms of viral-based mechanisms, specific viral molecules such as interfering RNA transcripts may provide interference against invading members with similar genomes. Strain-specific targeting of particular intracellular compartments and organelles may also contribute to interference. Even in protists, intracellular positioning has been considered to be critical in interference or strain incompatibility, with plasmid positions a major determinant for successful cohabitation (reviewed in Ho et al. [2002]). The primacy of strain characteristics in interference further emphasizes the need to look at these agents directly, and to characterize their intrinsic molecules. Host responses, including PrP changes, of course are important for the details of pathogenesis, and when present are very helpful for confirming infection. But without fundamental characterization of the TSE agent itself, the central and probably most critical viral mechanisms will remain obscure.

Predictions for a TSE Virus Based on Biological, Physical, and Molecular Data

Although the molecular nature of the infectious TSE agent remains unknown, there is substantial evidence for predicting that a 25–30 nm round or dodecahedral particle with a protected viral (nucleic acid) genome of 1–4 kb will define specific TSE agent strains. TSE agents depend on normal host PrP in their life cycle, but the cumulative data indicate that PrP is unlikely to be an intrinsic part of the infectious particle. To summarize: (1) infectious particles of 25–30 nm can be substantially separated from all forms of PrP. (2) Disruption and solubilization of the nucleic acid-protein complexes of these particles destroys their infectivity. (3) Viruslike structures of 25–30 nm can be identified in nuclease-treated 120 S infectious fractions. (4) Similar viruslike particles of 25–35 nm are observed in the brains of various species infected with different TSE strains, but not in any uninfected controls. (5) PrP antibodies do not bind to either the subcellular 120 S viruslike particles or to the viruslike structures identified in intact cells. (6) The "virion" arrays in brain increase with the infectious titer. (7) TSE agents spread by the same lymphoreticular pathways as viruses. (8) They also provoke innate immune responses diagnostic of a non-host-encoded environmental pathogen. (9) TSE agents display individual strain characteristics and viruslike interfering capacities that are independent of pathological PrP-res expression.

These key viral features are summarized in Table II. The predicted genome size derives from the physical and molecular data. This genome size has a potential to encode a nucleocapsid protein for protection, and/or an enzyme necessary for replication of the viral genome. Thus one may uncover a viral nucleic acid binding protein, or a sequence motif involved in nucleic acid replication. Predicting the features of a TSE virus can be helpful to future research since it provides specific guidelines for judging if an isolate or structure obtained is a reasonable infectious candidate.

It is also important to recognize some potential pitfalls in looking for a non-host-nucleic acid sequence in TSEs. If these viruses are more common, or commensal, as previously suggested (e.g., Manuelidis and Manuelidis, 1979a; Manuelidis, 1994a; Manuelidis and Lu, 2003, and Table II legend) then they may also be present in "normal" brains at some low level. There are a number of typically non-pathogenic commensal viruses that persist in the host for a lifetime, such as the JC papova virus that has been identified in the brains of over 50% of the asymptomatic human population [White et al., 1992]. This problem, in part, may be overcome by sequencing various agent strains in vivo and in vitro and in different species and tissues. The counter argument can be made, that different agents in various models will not be sufficiently similar to identify a shared or common viral sequence. However, the stability of all these agents and their ability to breed true, indicate a highly conserved and invariant genome. Examples include the unchanging characteristics of the BSE agent after passage through widely divergent species, the maintenance of distinct CJD agent strains despite passage in the same monotypic cell line [Arjona et al., 2004], the stability of sheep scrapie for

TABLE II. Key Biologic, Physical, and Molecular Features of TSE Agents From the Cumulative Experimental Data

Viral properties	Comment
Preference for lymphoid tissues and brain	Typical hematogenous viral spread to specific tissues
Pathogenic and non-pathogenic variant strains	Virulent versus predominantly asymptomatic and species limited
TSE specific members of class	Need host PrP
25–30 nm diameter infectious particle without PrP	From field flow fractionation and morphology
M_r of 10^6–10^7 Da	By HPLC of nuclease-treated 120 S sucrose peak
Can cantain a foreign viral genome of 1–4 kb	May encode replication and/or protective capsid protein

The prediction that the TSE agent will have a viral genome of 1–4 kb is based on the empirical physical properties of infectious particles, the presence of nucleic acids of these lengths that are resistant to nuclease digestion, and the requirement for specifying individual TSE strains. Normal host PrP is a known required susceptibility factor as shown by PrP knockout studies [Büeler et al., 1993], but there are also other tissue and cell type specific factors, unrelated to PrP, that determine susceptibility. The extraordinarily long latency and asymptomatic persistence of most TSE infections in humans, as in kuru [Gajdusek, 1977], the appearance of CJD as long as 30 years after exposure to contaminated growth hormone in only some of the exposed people [Manuelidis, 1994b], and the low incidence of late onset sporadic CJD are also some of the reasons to consider relatively avirulent or non-pathogenic TSE members that may be commensal, or more common than realized. Non-specific stress, as well as other infections and diseases, or even aging itself [Manuelidis, 1994a] may allow non-pathogenic commensal TSE agents to recrudesce from a latent carrier state to one causing disease.

centuries, and the slow evolution of these agents in cross-species infection [Manuelidis et al., 1997]. The overall similarity of different TSE strains with respect to tissue preference, spongiform lesions, and PrP pathology also suggest they will have a common consensus or shared sequence.

New Impartial and Rapid Tests That can Prove if a Virus or a Prion Is the Infectious Agent

Few scientists have attempted to characterize the infectious agent, whereas most have chosen instead to follow all the twists and turns of pathological PrP, and this in part has been due to the length and expense of the TSE infectious assay. With the new infected cell culture models that are capable of supporting high levels of agent replication and a variety of agents strains, as well as the development of rapid tissue culture assays to discriminate among these strains [Arjona et al., 2004; Nishida et al., 2005], the impediments of in vivo infectious assays can be minimized. Cell cultures that have been shown to support levels of infectivity that are comparable to brain provide new opportunities to purify and rapidly evaluate the essential infectious particle from non-degenerating cells. Because cultured monotypic cells do not carry complex brain tissue elements such as myelin, collagen, glial fibers, and vascular material, it is likely that agent purification will be simplified. Molecules in more purified infectious particles from these cells should become obvious above a simplified background of material from one cell type.

Susceptible tissue culture cells can also provide the essential assay system for rapidly screening infectious fractions. Although it remains to be seen if cells in culture are as quantitatively accurate as serial dilution endpoints determined in animals, they can be useful for assessing the relative infectivity of subcellular fractions within 30 days (unpublished observations). Thus one should be able to trace if a newly identified candidate viral molecule corresponds to, and predicts, infectious titers in increasingly purified preparations. Ultimately agent specific molecules should become apparent. If a TSE specific nucleic acid sequence is identified, then the whole TSE field will be opened on a fundamental as well as an exquisitely sensitive diagnostic level, given the power of modern molecular techniques such as PCR. If a nucleocapsid-like protein is found, antibody responses of the host may be enhanced for prevention of infection by vaccination. The failure to find neutralizing antibodies in TSEs does not rule out the elaboration of antibodies against a TSE virion, since neutralization is a relatively insensitive test for covert and evasive viruses.

The development of TSE susceptible cells also trumps all other methods of objectively evaluating whether the infectious agent is a prion or a virus by Koch's criteria. This involves more than just the greater simplicity and rapid readout of cell cultures. Various purified molecules can be delivered much more efficiently to cells in culture than in brain. More than 95% of the inoculum is lost from brain in the first few hours of infection [Manuelidis and Fritch, 1996] whereas cells in culture can be exposed to high concentrations of agent for extended periods. The development of susceptible cells also provides a chance to evaluate small amounts of pure molecules that would be degraded shortly after inoculation in an animal. Viral candidate molecules include naked nucleic acid sequences, as in an engineered plasmid that may encode sufficient information for TSE particle synthesis in the cell. Recombinant PrPs in various states that have not produced clear infection by brain inoculation can also be rapidly tested to see if they can reproduce infectivity in cell cultures. These cell-based infectivity assays are more biologically meaningful than non-physiological test tube manipulations to produce non-infectious PrP-res. Finally, cells that show the hallmarks of infection after delivery of candidate agent molecules can be inoculated as back into animals. This would be the ultimate proof that the molecule(s) identified fulfill Koch's postulates. In animals, these infected cells should produce both transmissible infection and a specific disease phenotype [Arjona et al., 2004]. Modification of these molecules, as by targeted mutagenesis of nucleic acids should also clarify the origin of different strains. This strategy seems the most objective way to systematically clarify intrinsic agent molecules, viral, prion, or otherwise.

CONCLUDING REMARKS

Cell culture models provide an exciting new opportunity to perform critical and fundamental experiments that can elucidate the intrinsic

molecular components of infectious TSE agents. Candidate viral or prion molecules can be evaluated for their infectivity and strain determining properties. The development of in vitro cell assays should please everyone, including those who favor prion, viral, or other hypotheses, because these assays can be used to objectively and efficiently test many different isolates and preparations according to Koch's postulates.

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