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Review

Transmissible Encephalopathies: Speculations and Realities

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ABSTRACT

Virtually all transmissible encephalopathies (TSEs), such as scrapie, CJD, and BSE, are caused by a type of infectious particle that remains enigmatic. The language of prion theory supersedes the reality of what is, and what is not known. This review questions the predictive value, consistency and accuracy of this now dominant assumption. Many people believe the normal cellular prion protein (PrP) self-converts into an infectious amyloid protein or prion. Although the amyloidogenic capacity of proteins is well established, the concept of an infectious protein without nucleic acid was "revolutionary." Diverse experiments have repeatedly shown, however, that this protein alone, in any form, is incapable of reproducing transmissible infection. In contrast, the infectious agent copurifies with many other molecules, including nucleic acids, while it separates from the majority of PrP. The infectious particle has a homogeneous viral size of ~25 nm, and infectivity is markedly reduced by conditions that disrupt viral core components but do not disrupt multimers of PrP amyloid. Additionally, the infectious agent replicates to high levels before any PrP abnormalities can be detected. Hence, we initially proposed that PrP changes are part of the host's pathologic response to high levels of infectious agent, but not the agent itself. Newer data clarifying a role for myeloid cells in the spread of infection, the unique character of two different agent strains propagated in a single animal, and the demonstration of long nucleic acids in a variety of simplified high titer preparations continue to raise serious questions for the prion hypothesis. Moreover, the epidemic spread of TSEs, and the activation of host innate immune mechanisms by infection, further indicate these agents are recognizably foreign, and probably viral.

INTRODUCTION

In Vienna, where I was asked to give a lecture on viral perspectives in TSEs, there is a painting by Breugel the younger depicting the unfinished tower of Babel. It suggests a parable for the current state of terminology in TSEs. This tower was supposed to reach God, which both the artist and the scientist might also

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call truth. To realize this the path to this zenith, a common language is central. For the artist, words can be used evocatively to recreate a state of knowledge. In science the aim is similar. However, regardless of whether the language is mathematics, or borrowed from our everyday speech, substances as well as ideas must be defined precisely to test their validity. In our current descriptions of TSEs we are in danger of losing our path to the top, having allowed speculative words to dominate the experimental realities, or be changed at whim. When definitions become progressively corrupted, because the emerging data do not fit, or when they are applied to phenomena and organisms for which they have no biologic meaning, they lose their scientific validity and instead become words of faith. The word prion is representative. Such words slip away if we try to embrace them or challenge their reality by experimental means. The truth must be simpler and more permanent.

The purpose of this review is to further scientific understanding in the often contentious field of TSEs. To do this I use the popular words of our field, as materially defined, to clarify the scientific problem. Ultimately the data, unadorned by preconceptions, should suggest new experimental tests to enhance our understanding of the causal infectious agents. I use the plural here purposely, because there are many individual strains of the scrapie agent that encode information that is independent of the host (9). CJD agents are also demonstrably individual, as is the epidemic BSE agent. I here contrast the viral and prion views, realizing that some completely new agent paradigm may be found.

A virus, by definition, contains a stretch of unique nucleic acid, typically protected by protein, but needs a host to complete its life cycle and replication. While the word virus can also be applied to a computer, a viral infection of a person or horse or caterpillar, as everyone knows, is different. Computer viruses do not make mammals sick (except emotionally). The application of the word prion to phenotypes of maternal inheritance in yeast cells similarly diverts us from the essential realities of mammalian TSE infections. Thus I will restrict the term prion to its original scientific definition where an abnormal form of a 34-kd host-encoded protein (prion protein or PrP) is believed to be the causal infectious agent. The pathologic form of PrP is physically evident as amyloid in some mammals, or can be seen by western blotting after limited digestion with proteinase K (PrP-res) in detergent solutions. This abnormal or β-pleated form of PrP (without nucleic acid) was sanctified as the infectious agent by the Nobel Prize in 1997. Nevertheless, I am not so certain the prion exists. Abnormal PrP has repeatedly failed to fulfill Koch's postulates when tested experimentally. At the very least, it has become increasingly clear that a crucial element for infection is missing. This element, often denoted by an asterisk, or by a hypothetical protein X in the more recent articles of the prion proponents (1,52), stares back from the page reminding us all of what we have not yet grasped. It is a physical infectious entity that ignites the disease process.

While there is convincing evidence that host PrP is important in susceptibility to infection and the progression of disease (7,10), there is little persuasive data that this protein, in any form, is infectious. Indeed, statements that "overwhelming" evidence favors the prion hypothesis have been steadily eroded by observations from the most straightforward of experiments. First, in many animal models, as well as in simplified test tube conditions, abnormal PrP does not correlate with infectivity as initially claimed (36). Second, abnormal PrP made in many different systems, including cell culture and transgenic mice, has failed to transmit significant infection. It is important to emphasize this failure because claims of infectivity in transgenic mice have not been reproducible (7). Indeed, the best kept secret in this field is the repeated inability of abnormal PrP, even amplified $>$100-fold from infected brain (46), to show increased infectivity.

I will refer to a few of the PrP/infectivity discrepancies that early on made me, with the members of our small laboratory, reexamine the infectious agent itself through painstaking infectivity assays of more purified preparations. In these experiments we also evaluated many components present, not just abnormal PrP (23). I would encourage those entering the TSE field to see for themselves that these other molecules exist, despite claims that "no proteins other than PrP have been consistently found in fractions enriched for prion infectivity" (42). Since we have not delineated all such molecules in infectious preparations there is a great deal new to be learned. Third, if you retest some presumably irrefutable observations, particularly with respect to (i) agent resistance, (ii) transmission across species, and (iii) the classification of different forms of TSEs, you will find reproducible data that contradict the most popular claims. Moreover, explanations of data are often singular when alternate explanations seem equally reasonable. In particular, the

word virus has been assiduously avoided, and even dropped ignominiously from our TSE lexicon. Yet a viral hypothesis remains the simplest and most elegant way to explain all the accumulated data. The viral concept also made it possible to predict the evolution and emergence of a more virulent agent strain of BSE, one that could affect many species regardless of their somewhat different PrPs (26,27,30). Currently no reproducible data exclude a viral particle, especially because there are known viruses that can be highly resistant to inactivation, even to radiation (26). Nevertheless, as I have stated, we still do not know the substance of the causal particle with any intimacy.

That verb is missing from our language, the infectious agent that initiates the sentence of disease in TSEs. Without it we can not progress or make correct solutions to limit the spread of infection. I here discuss the evidence and the logical questions or paradoxes raised by the data. Questions fall on the shoulders of both sides of the controversy, and I hope to equitably present some of the obstacles we have met in our own work. In particular, these center on our difficulties in identifying a nucleic acid sequence that is specific for the infectious agent and that has no likeness elsewhere. Because "purified" preparations of the infectious particle have never been made, I use the neutral word agent, allowing it to be whatever it is. This is reasonably fair or objective, but does not alter our view that abnormal PrP probably develops as a pathologic response to infection, perhaps by direct interaction with the infectious particle (23). On the other hand I may be wrong, and ultimately PrP itself may be made infectious. But I would like to see the proof of this transformation of lead into gold before abandoning fundamental principles of genetics, infection and pathology developed over the last hundred years.

BIOLOGIC FEATURES OF INFECTION

Invasion, latency, and natural infection. It is essential to recognize the biologic features of the TSE agents. The most important show a viral character. They include infectivity, exponential replication, and tissue specificity. Tissue specificity does not always correlate with PrP expression levels. Hence, susceptibility must be based on something more than PrP. Some people see the long time required for disease expression, and the lack of immune response to the infectious agent as strong reasons to dismiss a viral particle. However, over the last 20 years much has been learned about how slow viruses escape classical B and T cell dependent surveillance mechanisms. Acute viral infections typically induce rapid immune cell and antibody responses specific for the invading virus. However, these responses are not present in several slow infections. We have also come to appreciate the large number of viruses that live in a latent state, or in persistent co-existence with us, only rarely causing disease. Such a state should not be surprising since we live in symbiosis with many organisms. Innocuous viral infections that recrudesce, such as the commonly carried JC papovavirus, can be relevant for the long inapparent CJD infections of humans (25). This new viral knowledge makes the TSE agents less unusual in the virologic spectrum. It is with some insight that Sigurdsson coined the term slow virus while studying both lentiviral and TSE infections.

In a natural setting, TSE agents can invade via an oral/enteric route. In BSE for example, the agent can be found in the distal ileum, a region rich with submucosal lymphatics. The oral/enteric pathway and lymphatic collection of infectivity is characteristic of many viruses, and from these lymphatic sites in the gut they often seed the bloodstream. Other potential natural routes for TSE agents include direct inoculation via abrasions of the skin or of the mucosal surfaces. In such instances TSE agents can also be widely distributed by blood. Figure 1 shows a diagram presented in several meetings for the last 4 years to highlight how a TSE agent might behave if it were a virus. In this scheme, macrophages and other myeloid cells would be likely reservoirs of agent. We proposed myeloid cells would be the most capable of carrying agent to distant sites (30), even though these cells may contain or produce low intrinsic levels of PrP. These phagocytic cells would also be the ones to clean up extraneous or dead materials, as for example from the experimental inoculum. We therefore tested this idea and isolated myeloid (macrophage and dendritic) cells of spleen at sequential times after intraperitoneal (ip) inoculation. These myeloid cells (distinct from follicular dendritic cells that accumulate surface PrP) harbor reasonably abundant levels of infectivity by 28–34 days after intracerebral (ic) inoculation in experimental CJD (35). Interestingly, abnormal PrP was also

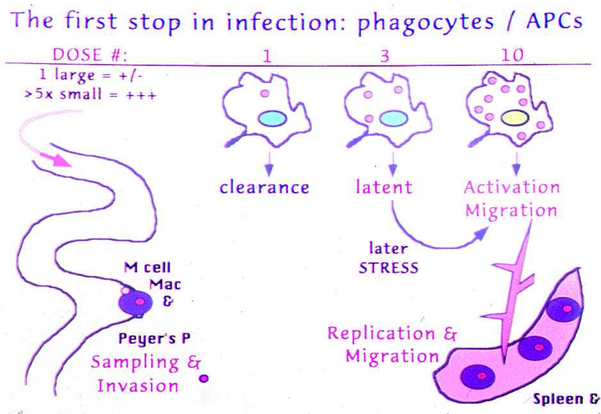

FIG. 1. Schematic showing probable cellular gastrointestinal route for entry of the infectious agent (small pink circles) in naturally occurring disease such as BSE. Agent may be carried across mucosa by M cells to Peyer's patches, but we suspect from other CJD work on M cell–deficient Tg mice that additional cells are involved in local agent transport since dendritic cells also display pathologic PrP (48). Antigen processing cells (APC), such as macrophages, will then sample and clear or else sequester and seed agent to other susceptible tissues. The outcome will be variable depending on the dose of the agent (top row). Low doses can be cleared effectively, moderate doses of agent may be sequestered in a latent state, but high doses will start a more consistent cycle of replication and spread, resulting in a defined incubation time for disease. The bloodstream has been demonstrated as a conduit for infected white blood cells, and latent or sequestered agent may be brought out of hiding by other physiologic stresses. Mucosal abrasions at any point would also allow unobstructed agent penetration with phagocyte dispersion via blood.

found in migratory myeloid cells in the gut soon after ic inoculation (44), and recent experiments have similarly documented infection of dendritic cells in scrapie (3).

These positive findings of myeloid cell infection are in keeping with the first demonstration of "viremia" in 1978 by our laboratory (22), and was later reproduced using scrapie models and human CJD blood (26). These experiments showed infectivity was associated with white blood cells at several periods after ic inoculation, and significant infectivity was recovered during the first few weeks after inoculation. More than 95% of infectivity exits the brain immediately after ic inoculation (29), and since there are no lymphatics in the brain, the trauma would typically allow myeloid and phagocytic cells from blood to distribute infectivity widely to susceptible tissues. Such tissues include spleen, which becomes infectious after ic inoculation. Since macrophages are long lived, agent penetrating the bloodstream by trauma or other means can be sequestered in a latent state if it is not destroyed or cleared. The latent or sequestered state is depicted in Figure 1 by three pink agent particles. It might take years for such low level agent to reactivate or escape.

Because our initial blood experiments were done in guinea pigs, a species much larger than rodents, we could collect and inject many white blood cells, and therefore detect low but reproducible levels of infection in with blood cells. Inoculation of far fewer cells (as in mice) would be a less sensitive test, and one that has led others, such as Weissmann group, to dismiss the problem of blood contamination by many TSE agent strains. However, none have used larger animals such as guinea pigs or sheep to examine standard scrapie transmission via white blood cell concentrates. Direct neural invasion is instead often presented as the only route for agent spread to the brain. Yet classic experiments show the scrapie agent in mice migrates to spleen from a subcutaneous inoculum in the thigh 56 days *before* the spinal cord is infected (15). Neural (sciatic nerve) agent propagation from the nearby inoculation site is not compatible with this progression. The cellular conduits for infection in the bloodstream can be critical for wide epidemic spread. Indeed, our original concerns about the potential spread of TSEs by transfusion has now gained added momentum with the recent demonstration of BSE transmission by transfusion in sheep (19).

The viruslike features of agent clearance, latency and recrudescence (new replication from dormancy) are diagrammed in Figure 1. Understanding the processes involved may further clarify the insidious de-

velopment of these infections and identify new molecular targets for early therapy. We proposed macrophages/myeloid cells (including dendritic-like microglial cells of the brain) could act in several capacities during infection. First, these cells can be central in the process of agent clearance. Notably, microglia can process and accumulate PrP (4,30). Purified microglia also show infectivity levels similar to whole brain although their PrP levels are extremely low (6). Thus microglia probably clear agent, but may also spread agent at early stages when PrP pathology is not apparent. Effective clearance by myeloid or any other cells is dependent on dose, and far more infectious doses (>1,000-fold) are needed to achieve peripheral takes as compared to the ic route. With such overwhelming assault, the agent will be sufficient to resist complete clearance, and thus have the opportunity to reproduce. This is depicted in Figure 1 by myeloid cells with more numerous agent particles. With minimal agent, myeloid cells may clear the agent, or else provide a long-lived reservoir of residual agent, one that can be reactivated years later to spread, for example by unrelated physiological stresses.

These myeloid reservoirs are distinct from the accumulations of abnormal PrP on follicular dendritic cells (FDC) that were claimed to be required for peripheral agent spread (38). However, we showed positive infection and disease progression, rather than resistance, in several transgenic models where FDC and B cell functions are ablated (35,48). Hence, other cells are clearly involved. Further studies showed spleen cells from these mice lacking FDC were clearly infectious, although all recipient mice succumbed later than those inoculated with FDC containing wild-type spleen controls (Manuelidis and Shlomchik, unpublished data). Peripherally, myeloid cells can also accumulate abnormal PrP. Moreover, the myeloid reservoir, since it is scattered in different tissues of the body, would also predict widely variable incubation times due to imperfect and variable agent clearance at different sites. Individual differences in a species, as physiologic health, could further modify agent clearance by myeloid cells. In fact, wide variability in incubation time to disease is what is observed when lower doses of agent are injected peripherally. Similarly, in iatrogenic growth hormone infections of humans, the CJD agent seems to have been effectively cleared by most recipients, while the few affected recipients had incubation times spanning 8–30 years. It is difficult to explain or predict clearance, latency, or the variable temporal pattern of late agent spread with any coherent PrP mechanism.

Infection activates specific pathways of host innate immunity. Since PrP is a host molecule it should not be recognized by the immune system, and infected animals do not develop antibodies to abnormal PrP. Yet, if the agent is not PrP, but a foreign virus, one might expect to see characteristic host responses. Latent viruses often escape immune surveillance and avert specific antibody recognition. Nonetheless, they often activate host innate immune responses. To begin to test for innate immunity in CJD, as well as other cellular reactions to infection, we isolated microglia from infected mouse brain. This simplification was advantageous for tracking these myeloid derived cells, the ones most likely to display molecules related to innate immunity. The close apposition of morphologically activated microglia to blood vessels after ip injection (35) as well as their migration into the brain parenchyma, also suggested some of them might be derived from peripherally infected myeloid cells. Not only were microglia highly infectious despite their low PrP content, but they also displayed activated genes that included those linked to antigen processing and migration such as Cathepsin S and various chemokines (6).

Using arrays, it was further possible to show ~30 transcripts, not previously examined in TSEs, were significantly induced in infected microglia. Moreover, the activated CJD expression profile was unique, and contrasted strongly with that of uninfected microglia exposed to high concentrations of brain PrP-res (5). On the other hand, prototypic inflammatory stimuli such as lipopolysaccharide and IFN-γ could induce a few of the same transcripts identified in CJD-infected microglia. Since the microglial inflammatory profile after infection differs from more simplified and stereotypic secondary responses, it may be useful in early diagnosis, as well as for the design of new therapeutic approaches that enhance these host defense mechanisms. Indeed this may be the basis for interference (see below). We have also found that microglia can show an activated phenotype well before PrP pathology is detectable in brain, and ongoing studies indicate that some of the inflammatory transcripts we have delineated are induced quite early in infected brain (Baker and Manuelidis, in preparation). It should also be emphasized that studies of other cell types may reveal additional early transcripts that are part of the host's defense mechanisms.

To summarize, these agents are cleared, carried and hidden in myeloid, and probably other cell types as well. The entire picture fits within the framework of many latent viruses, and the cellular response to in-

fection indicates a defensive response to an exogenous infectious agent. This response is incompatible with the PrP prion model.

Currently accepted TSE classifications and their validity. Because PrP is encoded by a chromosomal gene, it was advantageous for the prion hypothesis to dismiss arguments, first posed by Dickinson, that host genes modulate susceptibility to an endemic infectious agent (43), and instead to concentrate on so-called "spontaneous" familial, and rare "sporadic" forms of disease. Thus one could "argue that the only difference among the various prions is the sequence of PrP which is dictated by the host and not the prion" (42). All TSEs are infectious by definition and by experimental demonstration. Hence the word transmissible. An infectious etiology is the most important single criterion for grouping these diseases and separating them from other non-transmissible neurodegenerative conditions. The details and minor distinctions of neurodegeneration and host susceptibilities operate within this unifying infectious theme. Surely, the epidemic of BSE and the recent spread of chronic wasting disease (CWD) to wild animals underscores their endemic and apparently changing virulence. Virtually all the major variations in pathology are most simply accounted for by realizing these agents are exogenous, and can have unique characteristics of virulence. Such variant agent strains have designated names and behaviors that are remarkably reproducible in genetically stable populations.

Virulence is most often measured experimentally by incubation time, severity of lesions, and the ability to infect many species. Few animals develop disease if the agent or strain is relatively avirulent, that is to say, largely cleared and/or carried innocuously without causing disease. Our own experimental evidence indicates this is the likely paradigm for the low incidence of typical CJD worldwide. Notably, more than 20 independent agent isolates from these typical "sporadic" CJD patients in Europe and the USA have been uniformly avirulent for mice (32,39). Moreover, all show the same highly circumscribed medial thalamic lesions as the transmissible isolate from "familial" fatal insomnia (31,51). In contrast, the prion hypothesis proposes the infectious agent in these CJD cases arises spontaneously by somatic mutation. However, there is no evidence for spontaneous infection of long-lived mammals, and genetically susceptible (PrP identical) sheep remain free of disease in scrapie free environments (20). Spontaneous infection raises numerous logistical problems for prion propagation models, including its inability to explain spontaneous generation of only one basic agent strain in an inbred species. Other problems, such as the improbable chance of PrP mutation in non-dividing brain cells, and the question of how transmission then propagates to other non-mutated cells have been reviewed (23).

The other classification that sticks out is based on the idea of a genetic form of infection, a diverse group of so called "familial" CJD types associated with unusual inherited PrP mutations. One can not distinguish an exogenous from an endogenous infection in these families. Indeed, we originally suggested TSE agents might have retrovirus-like properties because retroviral elements can integrate into the genome and act as endogenous viruses (32). This could explain the apparent high incidence of Gerstmann-Straussler Scheinker disease (GSS) in families with a rare PrP 102Leu mutation. However, we isolated an agent from a GSS brain that strongly resembled the typical low virulence CJD agent seen in normotypic PrP individuals. In accord with this agent characterization, typical standard CJD agent was isolated from this same brain in England. This finding indicates GSS family members probably have an increased susceptibility to infection by a common, and normally avirulent exogenous agent, rather than an agent inherited through the germline. In contrast, another GSS patient with the identical PrP mutation, who lived in Asia, yielded a markedly different CJD agent. This Japanese agent induced widespread deposits of PrP in the brain, and was very fast or virulent for mice. Thus GSS family members can have enhanced susceptibility to several different exogenous agent strains. Furthermore, if the PrP 102 Leu mutation encoded and specified the agent, in accord with prion theory, one would have expected the same agent strain to be isolated from all GSS patients, but it was not. This undermines the whole concept of an inherited strain of CJD determined by the host's PrP sequence. However, there are diseases with PrP pathology that are not transmissible or infectious, and these rightly belong in a separate category because they are so fundamentally different by cause. In 1994 we suggested such rare genetic diseases that are not transmissible deserve the bone fide name of prion diseases because they seem to be caused only by the mutant protein (26). An example of this is the 178 PrP mutation (17) and this accepted PrP disease underscores the generation of abnormal PrP without infection. This data should also give pause to those who think abnormal PrP is always diagnostic of a TSE infection.

Agent strains with individual identities that are not host-encoded. One of the most important biological properties of TSE agents is that of strains, and strain realities have been "perplexing" for prion proponents. What is an individual agent strain and how is it known? Basically, when agents that evoke unique clinical or neuropathologic sequelae are isolated from a single species or group they can be defined as distinct strains. Each TSE strain elicits its own unique phenotype, even when passed to a very distant species. Thus the BSE agent evokes a characteristic pathologic profile when passed to cats and mice, and this profile is different than that found for a typical scrapie agent (8). These distinct TSE agent properties are similar to those shown by viral variants. As noted above such agents can slowly evolve and change in virulence with passage. In contrast, PrP folding is confined and determined by the species-specific PrP sequence. In prion theory then, how can a single strain propagate its identity among vastly different species, each of which shows a unique and different PrP folding or prion? Again, it seems most likely that each strain contains an exogenous (non-host) component that can encode distinctive traits, that is, a nucleic acid. In their entirety, strain observations present a plethora of additional logistical and experimental problems that are increasingly insurmountable for the prion hypothesis. Therefore, I will bring to your attention a few more necessary details of evidence, and then focus on new initiatives that show the fidelity and power of individual strains.

Pattison first recognized "Hyper" and "Drowsy" strains in scrapie-infected sheep, and the British carried this work further over the next 20 years by showing individual scrapie strains induce different lesion profiles in inbred genetically identical mice (9). With the serial transmission of CJD to mice in our laboratory in the 1970s, it also became obvious that the various CJD strains were different from those of scrapie. Yet Prusiner strongly denied the existence of strains through 1989, and thereby avoided problematic aspects of strains for the prion theory. TSE strains are often poorly appreciated by the academic community, and were also ignored by policy makers during the spread of BSE in cows. Perhaps this is because the word prion obfuscated the diversity of agent strains while it elevated the singular importance of host PrP. Nevertheless, the overwhelming evidence in BSE eventually established the evolution of an agent with enhanced virulence, a characteristic of conventional viruses that change when their nucleic acids mutate under selective pressure.

In addition to species differences in PrP folding noted above, strain data are problematic because *identical* bands of abnormal PrP-res are found after infection with many *different* agent strains, provided the same host is compared. Furthermore, PrP-res patterns depend on the tissue or cell type sampled rather than the agent strain (11,40). PrP glycosylation patterns, sometimes proposed to encode strain specific properties, can also be determined by cell type rather than the agent strain, and deglycosylation of PrP does not affect infectivity levels or agent characterisics (34). Recent attempts to show that PrP-res band patterns are unique and specific for the BSE-linked vCJD agent as compared to the "sporadic" CJD agent are similarly undermined by PrP-res differences among tissues. Tonsils and brain from individuals with BSE linked vCJD show different PrP-res profiles (18), yet there is no evidence that the agent is different in these two tissues. Finally, although some agent strains may induce different folding patterns in PrP, as with the Hyper and Drowsy scrapie strains, it is remarkable that many different strains, and probably most, can not be distinguished on the basis of PrP-res patterns of folding or glycosylation.

It is this point in particular that I would like to illustrate using two very different strains of CJD as a powerful example. The abbreviation SY designates an agent isolated from an American with "sporadic" CJD. It is very avirulent or slow in mice, and takes ~360 days to elicit even minimal lesions after ic inoculation of high agent doses. In contrast, the FU agent, isolated from a GSS patient in Japan, produces widespread disease at a much faster rate (~130 days to terminal signs). Yet SY produces the identical PrP-res band pattern as FU. Furthermore, FU produces ~10,000 more infectious units than SY in the brain, but the amount of PrP-res is only 10-fold different at terminal stages of disease (28). This represents an *in vivo* example of the discrepancy between abnormal PrP and infectious titer, and there are many other examples of this dissociation *in vivo* (16,29,53). The SY and FU CJD strains show even more remarkable interference properties that are reminiscent of some types of conventional viral interference, for example as exhibited by certain retroviruses. Retroviral interference is especially pertinent because it is not antibody dependent in tissue culture studies. In our CJD model, it is also clear that abnormal PrP can not be the basis of interference because PrP changes are undetectable during the period of active interference (28,31a). An

overview of some of these studies, including newer data, is given to illustrate the profound and large effects seen in this situation.

Strain interference without agent "conversion" in the single animal. Figure 2 shows inoculation of the avirulent agent can prevent superinfection by a more virulent agent. Mice were first inoculate ic with an attenuated slow agent (SY), or normal brain as a control (Nl) and then challenged 80 days later with the fast FU agent. FU induced disease at the predicted time when mice were first injected with normal brain, and the same incubation was seen when there was no preinoculation (lines 4, 5, and 6). However, if mice were first injected intracerebrally (ic) with SY, and 80 days later challenged intracerebrally with the same substantial doses of FU (either 10^4 or 10^3 infectious units), they failed to develop FU signs at ~250 days. These protected mice are represented on lines 2 and 3, and lived ~150 days longer than their counterparts not exposed to SY. Remarkably, these protected mice showed symptoms very late, and at the identical time as mice inoculated with only the slow SY strain (line 1). Moreover, the protected mice also developed a scratching syndrome found in SY but not in FU-infected mice, again indicating SY effectively suppressed FU infection.

The pathology of 16 of 18 of the SY mice that were superinfected with FU showed only the restricted thalamic lesions characteristic for SY. Furthermore, abnormal PrP-res was not detectable in these mice until just before clinical disease at ~360 days. Thus a limited availability of normal PrP, either for prion conversion, or for receptor like binding of a virus, can not be the mechanism underlying the suppression observed. The abnormal folding of PrP itself also could not be inhibitory, again because it was not found during the major period of FU suppression. The most obvious mechanism for suppression would center on the infectious agent itself and the host defense mechanisms it elicits early on.

The first question was whether SY suppression was complete in the protected mice, or if some viable FU agent remained. Further, was there any evidence for direct interaction between agent strains when both were replicating vigorously? We were given a unique opportunity to evaluate FU replication and strain interaction because two of 18 challenged mice had somewhat more PrP-res, and slightly more extensive spongiform changes, than seen in typical SY mice. This suggested FU had been suppressed and then reappeared. As calculated from the incubation time, this recrudescence probably occurred during the more prolonged clinical phase of SY disease in these two mice. The neurologic damage during the clinical phase might act as a non-specific stress, or alternatively, might compromise cellular defense mechanisms.

To test for the recrudescence of FU, we passaged these two brains, and compared the isolates retrieved from two random mice that exhibited apparently complete FU suppression. These studies gave unambiguous results. First, they showed that no FU was recovered from the completely suppressed mice, and thus FU could be eliminated by some SY dependent process. Second, either SY or FU, but not an intermediate or "chimeric" strain was present in the two incompletely suppressed mice. Therefore each strain could propagate itself independently, and strain "conversion" or mixing of strains, as postulated by prion theory did not take place. The retrieved FU also appeared to have been kept in a latent state for at least 150 days be-

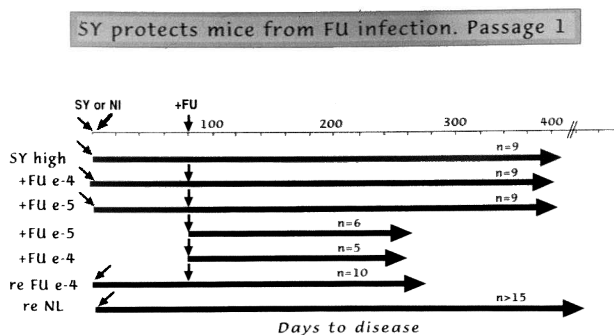

FIG. 2. The ability of a slow agent strain, SY, to prevent superinfection by a more virulent agent, FU. Nl represents inoculation with normal brain as a control and shows this has no effect on FU progression. For second passage data for complete SY suppression of FU in most mice, see text and Manuelidis and Lu (31,31a).

cause the titer of these brains was relatively low. I do not know how any currently hypothesized PrP mechanisms can explain this defined period of long latency and reactivation.

Implications of strain interference for commensal or cleared infection. The SY agent might have a direct suppressive effect, or more likely, induce a cellular response that limited FU replication. We are currently trying to determine the underlying mechanisms that limit the more virulent agent, and experiments to date do not favor antibody mediated inhibition. Instead, they suggest some factors elaborated by brain could be involved. These protective mechanisms apparently can be overcome, especially during clinical illness, when brain functions are probably compromised. This agent-induced defense can also be defeated. Recent experiments in our laboratory show that very high dose FU superinfections can overwhelm the suppressive effects of SY.

Nevertheless, the mechanisms of TSE strain defense may clarify new pathways to control TSEs. Recent unpublished data in our laboratory show that SY suppression can be remarkably extended beyond the normal lifetime. We have found that when the SY protective dose is lowered, not only is FU suppressed, but also mice begin to die of old age before showing signs of SY disease (31a). These protective effects of SY keep mice healthy for as long as 400 days after control unprotected mice have succumbed to FU disease. This is far longer than any drug or PrP-interacting chemical has been shown to exert protective effects, particularly for infection delivered directly into the brain. Given the microglial reactions, as well as other cellular responses to infection early on, we suspect that primed host defense mechanisms of innate immunity can be sufficient to clear low doses of FU. Since we have also found interference after peripheral inoculation, mechanisms of innate immunity might be protective in a natural setting.

Finally, our results and histological findings (31) support our hypothesis that some CJD strains may be widespread, commensal and essentially avirulent in people. The "sporadic" CJD strain, exemplified by SY, may be almost completely lacking in pathogenicity except in a few people, and may in fact protect people from low doses of more virulent strains. Such a widespread infection may be completely cleared in most people yet prime their myeloid cells sufficiently to give protection. However, without a marker that accurately and quantitatively pinpoints the infectious agent, these types of questions cannot be fully addressed. Therefore I would like to turn now to what we have learned about these agents from direct studies on the infectious particles. Their physical and chemical properties are quite different from modeled prion aggregates. On a linguistic note, I would also like to add that the term "proteinaceous particle" is meaningless, since viral particles also contain protein, often as a shell protecting their precious genomes.

Direct studies on the shape and components of the infectious agent

1. What is the shape and dimension of these agents?

Both Heino Diringer and our group recognized early the potential importance of scrapie associated fibrils (SAF) first described by Pat Merz, as well as by the PrP-res first identified by Bolton and Prusiner in hamster scrapie brains. In 1983, Diringer showed PrP was the major component of SAF by developing an amyloid purification procedure in scrapie (13). In the same issue of Nature we showed SAF in membrane rich CJD brain preparations, thus generalizing the finding of these amyloids to a broad spectrum of TSEs. We considered two possibilities at the time. Either PrP amyloid (called SAF or PrP-res) was a pathologic product elicited by the infectious agent, or alternatively, it could be an integral component of the agent (37). To understand more about PrP, we defined its glycosylation patterns in preparations not artificially degraded with proteases. First with a medical student, Sue Valley, and later with a post-doctoral fellow, Theo Sklaviadis, we used sugar binding lectins to characterize and separate PrP-rich fractions in CJD brains. We found it was possible to separate two fractions with very different lectin binding properties, although both fractions contained the same amount of abnormal PrP. We therefore assumed both would be equally infectious. However, they were not. One had >100-fold more infectivity than the other (34).

This data, together with the new knowledge that PrP was coded for by normal cells by Weissmann's group led us to conclude as early as 1986 that PrP itself could not be the infectious agent. I believe this conclusion has not been undermined by any of the numerous more sophisticated molecular experiments of my colleagues during the next 15 years, particularly in view of the failure of PrP-res alone, and of transgenic brains overexpressing constructs of PrP, to transmit infection.

After realizing that abnormal PrP was not a reliable marker for infectivity, our lab became interested in impartially determining the physical characteristics of the infectious agent rather than devising ways to fol-

low or manipulate PrP. Nevertheless, we did assay abnormal PrP in our preparations. This yielded further evidence that abnormal PrP could separate from infectivity under a variety of conditions. These repeated independent approaches showed infectivity levels did not correlate with abnormal PrP. During these studies we further asked to find if the agent exhibited homogeneous viral characteristics.

The most important and central properties of viruses is their defined homogeneous size and viral density. A virus will have a density greater than a pure protein, but less than a nucleic acid, because it contains both components. Because these agents could not be recognized ultrastructurally, the size of the agent had to be determined by biochemical means. The size of the agent was in itself a formidable problem. Prusiner's lab had determined the agent had a heterogeneous size ranging from 4 to 400,000 S. That range includes practically everything short of the kitchen sink. While such an extraordinary range could be consistent with amyloids of variable length, we suspected that (i) the abnormal PrP, (ii) the inherent molecular complexity of brain, and (iii) the pathological changes of vacuolization and cellular response all compromised the accurate determination of particle size. By exploiting the isoelectric point of PrP, and modifying methods to minimize PrP aggregation, we were able to successfully separate a homogeneous peak of infectivity with high recoveries on standard sucrose gradients. Such gradients have been used classically to define and separate many types of viruses.

The infectious agent banded at a density of 1.28 g/cc (49). This density implicates a viral particle or at least a molecular complex composed of both nucleic acid and protein. Notably retroviral cores have the same density. Because we thought that CJD might be an unusual truncated retroviral form, or might associate with a retroviral particle, we set up assays to search for representative retroviral ("strong stop") sequences. Remarkably, some of these retroviral markers purified and fractionated specifically with the infectious agent (39). One of them, from the endogenous retroviral IAP particle, was later followed extensively, and shown to be of substantial length (5,000 nt). Moreover, exhaustive nuclease digestion failed to alter the density of the CJD agent or affect the cosedimenting IAP retrovirus particles and their genomes, although it did reduced extraneous non-viral nucleic acids significantly. These results strongly indicated that the infectious agent, as well as the cosedimenting endogenous retrovirus, consisted of protein-protected nucleic acid. In contrast to the infectious agent, PrP had a heterogeneous density in these equilibrium gradients and about half separated cleanly from the peak of infectivity. Similar discrepancies have been reported in scrapie (50). The finding of long protected nucleic acids also suggested these agents could have viral-size genomes of $>$1,000 nt.

Sedimentation velocity gradients for size further separated the infectious agent from PrP. While most PrP was found at the top of the gradient with other proteins, the infectious agent, as assayed in animals, formed a single peak of ~120 S as shown in Figure 3. Obviously, the agent did not sediment as a heterogeneous smear of non-specific or artifactually induced aggregates. This separation has now been repeated in several laboratories with the same observation of infectious agent separating from most PrP. The latter stays at the top of the gradient (45,47). Further experiments using other methods, including chromatography, showed an agent diameter of ~25 nm, a reasonable dimension for a virus. Finally, when we took the 120 S infectious peak and attempted further purification, we could separate the infectious particle from almost all of the residual contaminating PrP. The details of this have been reported and are accessible on the web (33).

2. Nucleic acids and capsid proteins

The use of the infectious ~120 S peak, or of more purified derivatives thereof, also allowed us to determine if conditions that disrupted intact viral particles destroyed infectivity. In this case, the reproducibly cosedimenting IAP retroviruses provided a useful internal control. When these particles were disassociated into their component nucleic acid and protective capsid proteins, CJD infectivity was reduced by ~1,000-fold. This observation led to the development of more effective ways to chemically decontaminate the agent (24). On the other hand, solubilization and separation of residual PrP (without IAP virus disruption) did not reduce infectivity. Essentially, all the infectivity sedimented as an intact particle in the 100,000$g \times$ 1-h pellet (33). While these results strongly implicate a TSE viral particle, it is not possible to exclude a non-viral complex that is infectious. However, at the very least, the infectious particle must contain components other than abnormal PrP.

There are many molecules other than PrP in the most purified infectious fractions, including unidentified capsid-like binding proteins. If one does not use sensitive methods to detect nucleic acids one can con-

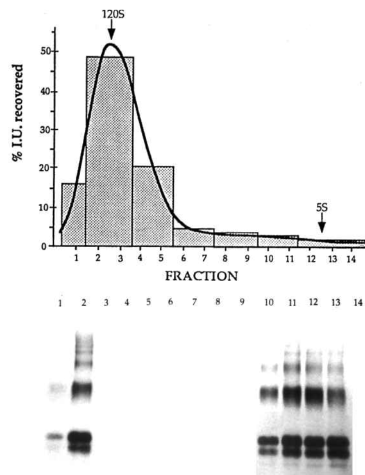

FIG. 3. Homogeneous peak of infectivity (bars) is seen in sucrose gradient fractions for size. Most abnormal PrP separates from this peak and bands with other proteins at ~5 S as shown in the corresponding western blot assay of fractions. Fraction 1 is from the bottom of the gradient. (Data from Akowitz et al., Microb. Pathog. 9:33–45, 1990.)

tinue to claim there are none in their infectious fractions. This is the claim of Prusiner for fractions that have lost >99.9% of their starting infectivity, and where adequate extraction of nucleic acids is disputable. Moreover, simple and standard procedures such as RT-PCR to examine nucleic acid sequences in nuclease treated CJD fractions, such as the long IAP sequences, continue to be unexamined or unreported by this group. Their claims are also suspect because nucleic acid sequences of >300 nt have been cloned from preparations made in Prusiner's lab by both the Weissmann and Marsh groups (2,41), and neither Heino Diringer nor I have been allowed to examine such "pure" fractions independently to evaluate infectious titer or nucleic acid components.

In summary, direct physical and molecular analyses of more purified infected fractions is consistent with data from in vivo studies. Both tell us PrP itself, in any detectable form, cannot be the infectious agent, because PrP (i) does not correlate with infectious titer and (ii) has no demonstrable infectious properties in the absence of other molecules such as nucleic acids. It is surely time to look at other possibilities by examining the components that actually exist in more purified infectious fractions with reasonably high titers. We have looked at nucleic acids to some degree, but no one has done either a systematic or exhaustive search. In this regard, I review the types of approaches we have used thus far to examine nucleic acid. This also underscore several experimental caveats and limitations of such searches.

Nucleic acid: strategies and caveats

Our first approach was to use small amounts of size fractionated CJD hamster brain. Although reasonably purified in terms of nucleic acid and PrP amyloid aggregates, we probably used insufficient amounts to do the robust types of experiments we planned. My first approach was to try looking at RNA using the then new subtractive approach known as representational difference analysis or RDA (21). We decided random primers were likely to give us the most broad based coverage of nucleic acid present. While we pulled out cDNAs that were specific or limited to the fractions from CJD brain, none of these cDNAs had strong homology to any known viral sequence in the database (14). This raised the problem, still unsolved, of how to specifically identify the agent sequence if it lacks homology to any previously recognized virus. Interestingly, I subsequently pulled out additional sequences of 200–500 nt that were intriguing because they had a predicted secondary structure with high stability. This feature could confer special binding and resistance properties on a viral RNA, a relevant property given the relative stability of TSE agents. Nevertheless, random priming did not provide the unambiguous orientation needed to test multiple sequences isolated, and I was concerned that we might have lost less numerous agent-specific sequences with RDA from limited starting material.

Futhermore, in repeating these RDA experiments on new CJD preparations, I found that the unique sequences uncovered were not consistent or the same as in the first experiment, although the common cellular contaminants were. This suggested this subtractive approach might not be very reproducible or representative of the starting material. In retrospect, this is not surprising because PCR amplification can be very variable, especially with low amounts of starting RNA.

To overcome some of these problems as well as to find if preparations in other laboratories contained some of the same sequences, I began to study Heino Diringer's preparations of the 263-K scrapie agent propagated in hamsters. The agent in these preparations was more abundant and more purified than in any other models reported (12), and 263K hamsters produce ~50-fold more agent than CJD-infected hamsters. While it was impossible for me to know the efficiency of nucleic acid extraction from the 263-K scrapie preparations, it seemed reasonable to determine if they contained the endogenous IAP retroviral elements we had found in CJD. Furthermore, it seemed worthwhile to take a more limited and systematic approach to evaluating potential viral sequences. In this context I chose to first examine only single-stranded RNAs that had a poly A tail. Such sequences can sometimes be found together with resistant RNA viruses, such as enteroviruses. Synthesis of cDNA from the defined 3′ end would additionally give a reasonable handle on orientation, making it possible to design simpler RT-PCR experiments for a variety of different brain preparations. I designed a strategy to limit the inherent variability of PCR and used instead the more representative amplification provided by T7 RNA polymerase. This strategy, with non-exhaustive subtraction of normal sequences, is outlined in the flow diagram of Figure 4. Zhi Yun Lu in my laboratory carried out most of the subcloning and screening of the cDNAs I had generated with this approach.

To date, we have sequenced 97 independent cDNA sequences from these screened colonies. They range in size from 200 to 700 nt in length, again confirming that sequences of far greater than 50 nt in length are present in the most purified infectious preparations. All cloned inserts had the expected A+ tails verified by sequence. When homologies to well-known sequences were identified through comparisons with the genetic database, they were strong, typically with a probability of e^{-60} to e^{-120}. This is equivalent to a discrepancy of ≤2–3 bases per 300–600 nt. Thus the strategy introduced few if any T7, PCR or other sequence artifacts. Notably, the endogenous IAP sequence was identified in this independent 263K scrapie preparation, as it had been in all our more purified CJD brain samples, and the homology was e^{-111}.

Other retroviral sequences were also present. This is quite interesting because it corroborates our previous finding that a specific group of retroviral strong stop sequences, in addition to those from IAP, copurify with these agents. We have not yet examined these in any detail. Other clones sequenced had open reading frames. They did not show 5′ MET start sites for synthesis of long proteins, and this is probably due to the artificially short 5′ end retrieved through cDNA synthesis. Nevertheless, ~30% of the clones matched well-known cDNAs. Another ~30% matched new cDNAs in the database. Notably these new entries were mainly from large brain cDNA libraries, and some homologies matched our clones with a probability as high as e^{-108}. Finally, the remaining 30% of independent clones showed no significant sequence matches even though they had well-defined A+ tails as well as internal sequences that looked genuine.

While these experiments further solidify the positive findings of specific nucleic acids, including coding RNAs, in more purified infectious brain fractions, the identification of agent-specific nucleic acids remains daunting for several reasons briefly mentioned. These include the small amounts of starting RNA and the lack of complete purification from brain material. The trapping of nucleic acids in PrP amyloid is an additional caveat that is rarely confronted, and PrP aggregates can severely compromise the retrieval of agent molecules. It is also worth remembering that there are certain types of RNA, as for example double stranded RNAs, that cannot be represented or even detected with current standard cDNA methods. These have some of the most interesting potential as agent molecules, because double stranded RNAs can be unusually resistant to denaturation and inactivation. Similarly, there are other more unusual nucleic acid forms, including those with variant bases, that have not been tracked in any experiments. Thus it would seem wise to include a more systematic evaluation of nucleic acids when attempting to find the missing molecules that are critical for infection. Finally, with recent infections of tissue cultures that provide reasonably high titers without morphologic damage (40), one has the opportunity to evaluate agent sequences without the complexity and problems inherent in pathological brain fractions.

A+ RNA strategy
(extracted nucleic acids from normal and infected brain fractions)

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dt Synthesize cDNA
(unique primers on both strands, 5′end by blunt ligation)

↓

Amplify cDNA with T7 polymerase

↓

Subtract normal cDNA (biotinylated)

↓

Limited PCR of TSE cDNA

↓

Size select TSE cDNAs >300bp

↓

CLONE

Select clones that do not hybridize to normal

↓

PCR check size of inserts

↓

Amplify clones 250-750bp

↓

SEQUENCE
(97 non-redundant clones)

DATABASE HOMOLOGY SEARCH

FIG. 4. Overview of molecular strategy used to amplify and clone poly A+ cDNA sequences specific for 263-K scrapie fractions made and extracted by Heino Diringer (12). T7 polymerase was used to achieve a more faithful representation of all RNA molecules in the preparation.

MAJOR QUESTIONS RAISED BY THESE DATA FOR PRION THEORY

In summary, several questions help focus the data that undermine the prion hypothesis, as listed below. The first set of questions relates to the fact that PrP-res and infectivity do not correlate. (i) If abnormal PrP is infectious, why does most of it separate from infectivity under a variety of experimental conditions? These include methods as different as affinity column chromatography and sucrose gradients. (ii) Why are animal brains with no detectable PrP-res infectious? While one might argue that assays for PrP-res are relatively insensitive, many different animal models show PrP-res does not predict or even reflect titer in any accurate way *in vivo*. Actinomycin D and PrP transgenic models are two examples from other laboratories (16,53) that complement our studies on SY and FU strains of CJD. (iii) Abnormal PrP can be found in animals with genetically modified PrP. Since these animals are not infectious, abnormal PrP would seem to be a pathological phenotype. Thus, is it not likely that abnormal PrP is the result, rather than the primary cause of the infectious disease?

Even more questions arise when one considers the irrefutable evidence for agent properties that transmit through various species with different PrPs. In prion theory, the current explanation is that folding or glycosylation patterns of host PrP "encode" the strain. Then (iv) How can one explain glycosylation and folding patterns that are indistinguishable between two markedly different strains? The slow and fast CJD strains shown here are dramatic examples of this discrepancy. (v) Why are PrP-res patterns believed to be diagnostic for a given strain when such differences are known to be tissue specific, that is, the identical agent in lymphoid and brain tissue gives a different PrP profile? (vi) How can a strain evolve slowly or require many passages to alter its virulence if this property is determined only by the host PrP? Notably, in one study, the PrP-res pattern did not change during sequential passages over 5 years, while the agent became progressively more virulent (30).

These experimental strain problems are also particularly relevant for BSE. (vii) How did the BSE agent evolve into a highly virulent form for many different species? (viii) How does this new BSE strain maintain its identity while being passed or "adapted" to all these different species? (ix) If PrP interactions are the basis for "adaptation," how do two strains as SY and FU in the same animal maintain their individual identities, and show no chimeric forms by either biologic behavior or PrP as predicted by prion theory?

There are further additional problems of a direct physical and molecular nature that must be addressed, or at least acknowledged. (x) Why have all transgenic, recombinant, and *in vitro* forms of PrP failed to reproducibly infect animals? (xi) How does one explain the numerous conformations of abnormal PrP made *in vitro* in 24 h that can be reversible, whereas the infectious agent shows no similar replication time or significant reversibility back and forth between infectious and non-infectious forms? (xii) Why are there always so many non-PrP molecules, such as nucleic acids and viral capsid proteins, in "purified" infectious fractions with high titer, and why is there a loss of infectivity when these non-PrP molecules are disrupted or separated? Experiments showing vast discrepancies between abnormal PrP and agent have been reproduced in many different settings, including Prusiner's laboratory, and cannot be dismissed by citation of only highly selected data (23). And finally, to return to the infected mammal, one sees (xiii) host responses that strongly suggest recognition of an exogenous foreign agent rather than a host encoded entity.

CONCLUSION

In light of the above questions, one may well ask why the scientific community as a whole, in addition to the public at large, believes so totally in the prion model of these infectious agents. I think the casual or expansive use of words, especially those taken out of their properly defined scientific context, has obscured the truth of evidence. It is especially hard then to sort out and clarify for others all the different features ascribed to "prions." Prions have become everything, the agent that causes disease, the pathologic response of disease, the spontaneous self-inflicted pathology of a protein, and the susceptibility to infection, to name a few. Thus, the prion is the only proper thing to evaluate if we are to gain insight into all meaningful mechanisms of infection and disease. But what is a prion? If the prion is confined to its original definition (an abnormal form of PrP), one begins to see difficulties that cannot be overlooked. In contrast, as ever-changing definitions are invented, ad hoc, to fit each experimental context, it yields a muddle that is as hard to escape as quicksand. And this does not help the formulation of good predictions. PrP is, of course, a central player in susceptibility to infection and pathology. But what is the real infectious agent? What is missing is the most intriguing, and when it is found I suspect it will be obvious, and not require a special metaphoric language all of its own. While I may be incorrect, the biologic and physical evidence all points to a virus with a protected nucleic acid core. It is time to look at the actual molecules that could be part of this structure, rather than making believe that nothing else is needed, and nothing else is there.

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