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Review

The pathogenesis of transmissible spongiform encephalopathy: routes to the brain and the erection of therapeutic barricades

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Abstract. Classical and modern studies of the pathogenesis of transmissible spongiform encephalopathy are reviewed, with particular attention paid to recent investigations of the routes of neuroinvasion. In various experimental models, a heirarchy of paths to the brain includes direct neural transit from the site of infection, replication in the spleen and neural entry through the spinal cord, and hematogenous spread. Possible modes and sites of therapeutic intervention are suggested.

Key words: Transmissible spongiform encephalopathy; Creutzfeldt-Jakob disease; scrapie; variant Creutzfeldt-Jakob disease; prion disease; follicular dendritic cells; B lymphocyte.

Introduction

The transmissible spongiform encephalopathies (TSEs) constitute a small group of fatal neurodegenerative disorders affecting humans and animals. The most important human disorder is Creutzfeldt-Jakob disease (CJD), which is characterized by progressive dementia, disturbances of gait and coordination, visual deterioration, and involuntary movements, terminating after several months in a helpless bedridden state of mutism and death. Typically affecting older adults, with an annual incidence of 1 per million population, the sporadic form of CJD accounts for approximately 90% of all cases, and has no identifiable cause. The remaining 10% is subdivided into familial disease (due to any one of multiple mutations in the PRNP gene on chromosome 20), and environmentally acquired disease that until recently was entirely the fault of iatrogenic events. Within the past few years, however, environmental causes have been extended to include a zoonotic variant of CJD (vCJD) resulting from the consumption of tissue from cattle afflicted with bovine spongiform encephalopathy (BSE).

First steps

Although the brain is the target organ for the pathogenic agents causing TSE, and is the only organ to show microscopic signs of disease, lower levels of infectivity can be detected in many other organs and tissues of the body, and it may properly be said that the recognition in the late 1950s of the presence of infectivity in several non-neural tissues of sheep and goats that were naturally infected with scrapie (analogous to CJD in humans) inaugurated the pathogenetic study of TSE [1–3]. In retrospect, it is interesting that the first such peripheral tissue shown to contain infectivity was the spleen [1].

The successful adaptation of scrapie to laboratory rodents opened the way to much further investigation, culminating in the 1960s and 1970s in an extraordinary series of reports by Hadlow and colleagues on the bodily distribution and levels of infectivity in naturally and/or experimentally infected sheep, goats, and mice [4–7]. These observations provided the scientific foundation for the chronology of oral TSE infections in which infectivity is detectable in the gastrointestinal tract and its associated lymphatic tissue (including the tonsil and Peyer's patches), before finding its way to the central nervous system (CNS). This sequence was later verified in other experimental models with different pathogen strains and host species, using bioassays to measure infectivity [8, 9] or immunochemistry to measure the 'prion protein' (PrP), a pathognomonic marker of the infectious agent[1] [10]. After other types of peripheral route infections (e.g., after intraperitoneal inoculation), the spleen has been found to be an important site of agent replication, and Kimberlin and Walker [8, 9] showed that infectivity from the spleen traverses the splanchic nerve plexus to attain the spinal cord and ascend to the brain. The importance of this early peripheral phase of infection can hardly be over-emphasized in view of current concern about orally acquired vCJD from BSE-infected cattle.

The straight and narrow path

The role of the spleen in neuroinvasion was further highlighted by the observation that splenectomy prolongs the incubation period following peripheral infection of wild-type mice [8, 11–13], and by the discovery in the early 1980s that several chemical compounds (e.g., dextran sulfate) inhibit scrapie agent replication in the spleen and other reticuloendothelial tissues, and can sometimes prolong the incubation period beyond the natural lifespan of the rodent host [14–18].

In these studies, we also find the first attempts to determine whether any particular cell types within the spleen were responsible for harboring, replicating, or transferring the infectious agent to the nervous system. Evidence was presented that 'splenic macrophages' contained more infectivity than lymphocytes [14], and that dextran sulfate persisted for many months in 'mononuclear phagocytes' [15]. Other studies in mice infected with either scrapie or human TSE reported highest levels of infectivity in gradient fractions containing 'low-density, non-adherent lymphocytes' [19, 20], or in physically separated fractions containing 'stromal cells' [21]. Immunohistological studies of spleens and lymph nodes from these same mouse models showed that almost all visible PrP was confined to 'large cells that interdigitated with lymphocytes' [22, 23]. As these studies were concluding, it became apparent that different names were being used to describe what in reality was a single cell type – the follicular dendritic cell (FDC).

Within the past few years, the subject of spleen cell interaction with the infectious agent has been further elucidated through the use of genetic strategies involving inbred mice with a variety of immunologic defects [especially severe combined immunodeficiency (SCID) mice], and genetically manipulated 'knock-out' or null mice that do not express the precursor of PrP. The first use of immunodeficient mice was reported by Japanese workers in 1991, who found that in contrast to wild-type mice, SCID mice inoculated peripherally with a human strain of TSI had no immunostainable PrP in splenic FDCs and did not develop disease [22]. Intracerebrally inoculated SCID mice behaved like wild-type mice. This finding was soon confirmed in experimental scrapie and BSE infections in mice by workers in the United States [24], France [25, 26], and Scotland [27–29]. In addition, these studies also showed that spleens from peripherally inoculated SCID mice had no detectable infectivity [24], and that partial reconstitution of immunocompetence by the injection of normal spleen cells [25] or bone marrow cells [28] partially restored both splenic PrP positivity and infectivity.

Alternative paths

By the mid-1990s, a fairly consistent picture had emerged about the pathogenetic fundamentals of oral and other peripheral route infections, about the primary involvement of the spleen, and more particularly, the crucial participation of FDCs in the transfer of infectivity from the periphery to the CNS. Partially blocking the view, however, were signs that suggested the existence of additional (or alternative) pathways. For example, although splenectomized scrapie-infected wild-type mice showed prolonged incubation periods, neuroinvasion and death did eventually occur, and in both a scrapie-infected splenectomized transgenic mouse model, and in congenitally asplenic mice infected with a human TSE, incubation periods were identical to those of normal mice [30, 31].

Furthermore, direct axonal neuroinvasion had been shown to follow experimental infection of either the optic or sciatic nerves [32–34], and time studies of the ap-

[1] Over the years, PrP terminology has become unneccessarily burdened with superscripts. Different authors refer to the normal full-length cellular precursor as PrP^{c} (for cellular), or PrP^{sen} (because it is sensitive to proteinase K). The corresponding terms for the abnormal isoform (which is insoluble and proteinase-K resistant) are PrP^{sc} (for scrapie), PrP^{CJD} (for CJD), or PrP^{res} (for proteinase resistance). This article takes a simpler approach. The abbreviation PrP will *always* be used in its original sense of proteinase-resistant-protein, i.e., the pathological amyloid isoform, and the precursor will always be spelled out as such.

pearance of PrP in the CNS of hamsters that had been infected orally or intraperitoneally with scrapie strongly suggested neuroinvasion via the vagus nerve, bypassing the spleen and splanchnic plexus altogether [35, 36].

Further evidence for direct neuroinvasion comes from recent experiments in sheep with naturally acquired scrapie, and in experimentally infected transgenic mice. In sheep sacrificed at various ages, PrP was first detected in the lymphoid and enteric nervous systems beginning at 5 months, and by 10 months was also found in the thoracic spinal cord and, separately, in the dorsal motor nucleus of the vagus nerve (personal communication, B. Schreuder, Institute for Animal Science and Health, Lelystad, The Netherlands). Thus, neuroinvasion was progressing by both the splenic-spinal cord and direct vagal routes.

The mouse experiment was more complicated: chimeras were created that either expressed hamster PrP precursor protein in all tissues, or in which a neuron-specific enolase promotor limited precursor expression to nervous system tissues (including the peripheral nerves). Infection with the hamster scrapie agent produced similar mortality rates in both chimeric groups, although the incubation period was much longer after intraperitoneal infection in mice with expression limited to the nervous system. Thus, replication in the spleen was a facilitative but not obligatory step in neuroinvasion [31].

These many experimental studies would suggest that neuroinvasion can be unpredictably influenced by the choice of pathogen strain, host species, route of infection, and infecting dose, and, if so, may help to explain the controversy that has recently arisen over the importance of FDCs and B lymphocytes in the neuroinvasive process.

Detour

In 1997, researchers in Zurich refined the study of splenic cell types involved in scrapie neuroinvasion by using inbred mice with a variety of specific immunological defects, rather than just the globally deficient SCID mice. These included defects limited to B lymphocytes, T lymphocytes, B and T lymphocytes, and FDCs. Neuroinvasion was unaffected in mice with T lymphocyte or FDC deficiency, but was prevented or severely inhibited in mice with a deficiency of mature B lymphocytes. These results were interpreted as suggesting that B lymphocytes may transport prions from lymphoid organs to nervous tissue, 'disproving a prime role for FDCs in peripheral pathogenesis' [37]. The gauntlet was thus dramatically thrown down to all previous work indicating that FDCs were the critical cellular element.

Surprisingly, a follow-up study published in 1998 showed that neuroinvasiveness was restored equally well by reconstitution with fetal liver cells that either expressed or did not express the PrP precursor [38]. The implications were clear: if PrP is inseparable from the infectious agent (or is the infectious agent), and non-precursor-producing B lymphocytes were necessary for neuroinvasion, they must act either as carriers of PrP originating in another cell type, or they must in some way mediate the neural transfer of infectivity from a precursor-expressing cell. (A third possibility is that PrP is separable from the infectious agent). The authors acknowledged the possibility that this cell could be the FDC, or some other cell population not identified by FDC-M1 and M2 markers.

A further publication in the following year reported that irradiation of normal mice, followed by reconstitution with precursor-negative fetal liver cells and intraperitoneal inoculation of the scrapie agent, produced infectivity in the splenic stroma but not splenic lymphocytes, reinforcing the previous suggestion that FDCs (known to be radiation resistent) would be a prime candidate for agent replication [39]. Circulating lymphocytes were also reported to contain no detectable infectivity, but the small number of inoculated assay animals would have been insufficient to detect the very low levels shown to be present in the buffy coat of other rodent models [40, 41].

The next question to be addressed was whether or not the subclinical infection produced in the B lymphocyte-deficient mice might be due to some neurotoxic factor in the scrapie brain inoculum prepared from wild-type mice [42]. However, these mice, when inoculated with homogenate prepared from B lymphocyte-deficient mice, not only had similar amounts of PrP and infectivity in their brains, but also developed symptomatic disease. A neurotoxic or other factor in some way related to B lymphocytes was thus definitively excluded, and suggested that 'secondary events, such as maturation of cells induced by B lymphocytes, e.g., FDCs, in lymphoid organs or immune cells resident in the CNS may contribute to scrapie neurotoxicity'.

Taken as a whole, this series of artfully planned and executed experiments was looking more and more like a confirmation, rather than a discrediting, of the essential role of FDCs in neuroinvasion.

Back on track

Interspersed with this quartet of papers from the Zurich group was a trio of papers from workers in Edinburgh, who had taken up the gauntlet thrown down by Zurich. In the first of these studies [43], chimeric mouse lines were created by crossing SCID mice with wild-type or knockout mice to produce SCID mice with positive and negative PrP precursor genotypes. Maturation of the host FDCs was accomplished in a checkerboard fashion by grafting negative SCID mice with bone marrow from positive wild-type mice, and positive SCID mice with

marrow from negative knock-out mice. This experimental design permitted a clear distinction between the origins of FDCs (from the host) and lymphocytes (from the graft), and showed that the precursor developed only in FDCs of the genotypically positive chimera, irrespective of the genotype of the lymphocyte-containing graft. Thus, the normal precursor was produced exclusively by FDCs. The chimeric lines were also inoculated intraperitoneally with the scrapie agent, and PrP, splenic infectivity, and neuroinvasion occurred only in the genotypically positive SCID mice, irrespective of the graft cell genotype.

A second study examined the same inbred line of tumor necrosis factor alpha-deficient (TNF-$\alpha^{-/-}$) that had earlier been used by the Zurich group [44]. This deficiency results in an absence of FDCs and germinal centers, but B lymphocytes remain functional. Intraperitoneal inoculation of the scrapie agent failed to transmit disease to most of the mice, and the few mice that became infected had prolonged incubation periods. None of the mice, symptomatic or not, had any PrP or infectivity detectable in the spleen. Parallel experiments using mice deficient in interleukin-6 (IL-$6^{-/-}$), in which FDCs are intact but germinal center development is diminished, showed splenic PrP accumulation, infectivity titers, and disease transmission with an efficiency equal to that in wild-type mice. The results further implicated FDCs as the critical cellular element in neuroinvasion from the spleen, and suggested that, occasionally, the spleen might be bypassed en route to the brain.

The third study approached the problem via immunoelectronmicroscopy [45]. Spleens from wild-type mice were examined during the incubation (70 days) and symptomatic (170 days) stages of disease after intraperitoneal inoculation of the scrapie agent. Anti-PrP immunogold staining was seen in the lysosomes of germinal center cells morphologically identified as tingible body macrophages, and at the surface of the labyrinthine dendritic processes of activated FDCs, where they were often associated with amorphous electron-dense deposits resembling immune complexes. Staining was more intense and widespread in the spleens of symptomatic than incubating mice, and was absent in uninfected controls. No staining was associated with splenic lymphocytes in any mouse. Overall, the findings suggested that 'most of the PrP detected at the cell surface of FDCs and within electron-dense material at the cell surface was initially expressed at the FDC surface and then released into the extracellular space,' where some PrP was phagocytosed by the tingible body macrophages.

Both the Zurich and Edinburgh groups have used ingenious and at times overlapping methods in their attempts to define the cell (or cells) involved in neuroinvasion, and in the evolution of their studies appear to have come to nearly the same conclusions, even though each group has worked with a different strain of the scrapie agent and, in some cases, different mouse strains. There is ample precedent for such differences to influence many aspects of TSE, including pathogenesis, and they may be responsible for the comparatively minor points about which these mostly complementary studies still disagree.

Therapeutic barricades

Considered in its entirety, the literature of pathogenesis indicates that after oral infection, infectivity first appears in the gastrointestinal tract and its associated lymphatic tissue. From these tissues, infectivity may proceed directly through the vagus nerve to the brain, or through the spleen, splanchnic nerve plexus, and spinal cord en route to the brain. Other peripheral route infections usually, but not always, reach the brain via the splenic route (fig. 1).

Current studies of splenic cell types involved in neuroinvasion confirm the less sophisticated earlier studies of wild-type and SCID mice in which the FDC was identified as the principle and perhaps only splenic cell type directly involved in the replication and neural transfer of PrP and infectivity. The role of the B lymphocyte is limited to its requirement by FDCs for functional maturation, and as such could be considered equally important to the overall schema of neuroinvasion. In consequence, the

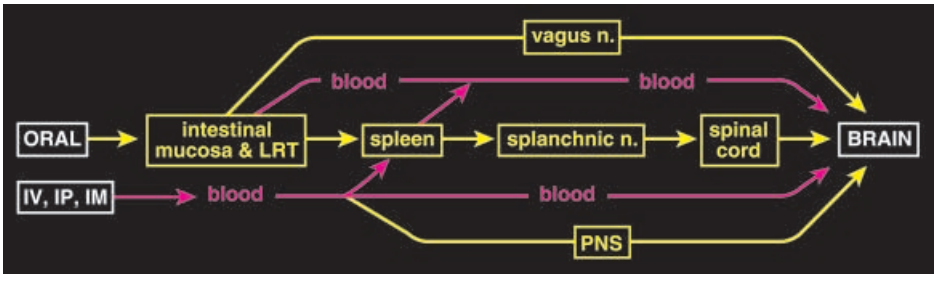

Figure 1. Neuroinvasive pathways from peripheral sites of TSE infection. The peripheral nervous system (with and without splenic involvement) has been well documented in experimental models. Hematogenous routes have been inferred to occur under some circumstances. IV, intravenous; IP, intraperitoneal; IM, intramuscular; LRT, lymphoreticular tissue; n, nerve; PNS, peripheral nervous system.

argument about spleen cell primacy is reduced to a question of semantics.

At what possible points might therapeutic intervention be possible, and what kinds of compounds might be effective? From all we know about the relationship between PrP and infectivity, it is reasonable to assume that any cell involved in the pathogenesis of TSE must be capable of expressing the normal precursor molecule. At the tissue level, the precursor has been found to have a nearly ubiquitous distribution in the bodies of normal hamsters, including brain (where it occurs in highest concentration), stomach, intestine, spleen, thymus, and buffy coat [46]. At the cellular level, a recent study has documented precursor expression in a number of potentially important locations in uninfected 'bovinized' transgenic mice: endothelial cells within mucosal capillaries and venules of the lamina propria mucosa of the intestinal villae; neurons within peripheral nerves, including Auerbach's and Meissner's plexus of the gut; medullary epithelial cells in the thymus; isolated dendritic cells in the spleen; mesenteric lymph nodes; Peyer's patches; and circulating T and B lymphoctes [47].

Extending these observations to infected animals, an immunohistochemical study of lemurs that had been orally infected with BSE detected PrP in tonsillar epithelial cells and lymphoid nodules; epithelial cells and connective tissue lymphocytes of the esophogus, small intestine, and colon; cells in the periphery of Peyer's patches and spherical lymph nodes; splenic red pulp cells, and neurons in the dorsal and ventral roots of the spinal cord [10].

Cells that process, replicate, or shuttle PrP (and infectivity) between the initial site of infection and the brain can all in principle be considered as possible therapeutic targets. Considering the extensive list of organs and cells that could be involved in the pathogenesis of neuroinvasion following peripheral route infections, any effective therapeutic approach will need to block the production and/or transfer of PrP and infectivity in multiple sites, and probably in more than one cell type.

Amphotericin B is one of a handful of chemical compounds that can prolong the incubation period following experimental peripheral route scrapie infections, and in a recent study, SCID mice were used to deduce the site of action of an amphotericin B congener called MS-8209 [26]. The proportion of untreated SCID mice that became infected after intraperitoneal inoculation of the scrapie agent was dramatically reduced by a 2-week drug treatment regimen, but mice that did become infected had an incubation period similar to untreated mice. In contrast, treatment of reconstituted SCID mice did not reduce the rate of infection, but significantly prolonged the incubation period. These results were interpreted to mean that the drug was at least partially effective in blocking infection at the first step of the neuroinvasive process (rather than at a subsequent stage of replication), e.g., at the point of infectious agent uptake by peripheral nerve endings. A treatment combining this type of drug with one that interfered with replication in the lymphoreticular system was suggested to offer the best chance of success.

Very recent work by the research groups in Zurich and Edinburgh has, in almost coincidental reports, identified a drug that might fit the description. Because splenic FDCs are the cells directly implicated in the PrP/infectivity neuroinvasive process, because they require lymphotoxin-α/β (LT-α/β) signals from B lymphocytes to maintain their differentiated (functional) state, and because the receptor for these signals can be blocked by an immunoglobulin fusion protein (LTβR-Ig), a signal-blocking therapeutic strategy appeared both logical and practical.

In the Zurich experiment, a single 300-µg dose of LTβR-Ig dose given 1 week before or after intraperitoneal scrapie infection abolished PrP accumulation and infectivity in the spleens of most mice during an 8-week observation period [48]. Weekly injections of 100 µg of LTβR-Ig beginning 1 week before or after inoculation and then repeated at weekly intervals for up to 8 weeks significantly prolonged the incubation periods. In the Edinburgh study, a single 100 µg dose of LTβR-Ig was administered 3 days before, 14 days after, or 42 days after intraperitoneal infection. Incubation periods of the treated mice were significantly prolonged by 29%, 23%, and 12%, respectively, compared to untreated mice [49].

Combination drug therapy has never been used to treat experimental TSE infections, and it would now seem worthwhile to design a study of different treatment regimens employing amphotericin B (or one of its congeners) and LTβR-Ig (with or without another lymphoreticular blocking agent, such as dextran sulfate) in an effort not merely to prolong the onset of disease, but to prevent it altogether. However, prevention will in all likelihood require not only the successful interruption of the entire peripheral nervous transport system, but also of hematogenous neuroinvasion.

Two different experimental rodent models have repeatedly documented low levels of buffy coat infectivity [40, 41], and a third rodent model comparing scrapie inoculation of intact and transected sciatic nerve suggested hematogenous rather than neural invasion of the brain [50]. A human growth hormone patient who died of pneumonia without any neurological symptoms was found to have neuropathological changes of CJD limited to the hypothalamus, which is not the area that would be predicted if neuroinvasion had proceeded along nervous pathways originating at or around the usual hormone inoculation site of the lateral thigh [51]. Furthermore, the only plausible explanation for the presence of infectivity in many different bodily tissues of humans and animals with TSE is that there is, at some point during its evolu-

tion, a 'viremic' (or 'prionemic') phase of disease. This could as easily occur during the incubation as during the symptomatic stage, and even if less efficient than a neural pathway to the brain, would ultimately lead to disease and death.

If a drug (or drug combination) existed today that would in a single dose permanently block – by whatever route – the entry of infectivity into the brain, it would be given to all individuals considered at risk for environmentally acquired disease, including recipients of corneal and dural grafts, native pituitary hormones, and blood transfusions, individuals who had undergone procedures with instruments previously used on (unsuspected) CJD patients, and the entire population of Great Britain. If the drug had to be given on a continuing regimen for the lifetime of the individual, or if it had serious side effects, it would probably still be considered on a case-to-case basis.

However, at the first sign of illness (and for an indeterminate period in advance of illness), the utility of such a drug would be nil, because the infectious agent would already be present and active in the brain. None of the drugs under study have been shown to have any effect when given at or near the onset of symptomatic disease. And if, as seems increasing likely, the sporadic and familial forms of TSE, which account for over 95% of all cases, arise de novo within the brain itself, rather than from without, drugs that block neuroinvasion would be purposeless. In this situation, the only effective therapeutic modality would be drugs that either inhibit or reverse the process of amyloid formation in the target cells themselves – the neurons. A few examples of this type of drug are currently showing signs of promise in experimental models of disease, but they are still a long way from testing in humans. Valhalla may be around the bend, but we are not there yet.

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