Prion diseases, blood and the immune system: concerns and reality

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ABSTRACT

There is a great amount of uncertainty about the nature of the agent which causes spongiform encephalopathies. In recent years the occurrence of bovine spongiform encephalopathy and of new variant-Creutzfeldt-Jakob disease, has raised concerns that prions may, under certain circumstances, contaminate the blood supply. This review article illustrates the problems with which research in this field is fraught, and presents some of the arguments which are controversially discussed in the field.

Key words: prions, Creutzfeldt-Jakob disease

According to all available evidence, the agents causing transmissible spongiform encephalopathies, termed prions, are devoid of informational nucleic acids and consist of an infectious protein (termed PrPSc) capable of converting a normal host protein called PrPc into a likeness of itself (Figure 1). The only organ system in which histopathologic damage and its clinical sequelae can be demonstrated as a consequence of infection with prions is the nervous system.1 This consideration applies to both the human transmissible spongiform encephalopathies, such as Creutzfeldt-Jakob disease, Gerstmann-Sträussler-Scheinker syndrome, Kuru and fatal familial insomnia, and all known prion encephalopathies of animals.2 The latter comprise scrapie in sheep, bovine spongiform encephalopathy, and chronic wasting diseases of mule, deer and exotic ungulates.3

However, there is no doubt that prions, herewith operationally defined as the infectious agents causing transmissible spongiform encephalopathies, can colonize organs other than the central and peripheral nervous systems, and can be demonstrated in extracerebral compartments.4

The problem of which organ systems can harbor infectivity is further complicated by the existence of prion strains. Just like strains of conventional viruses, prions can come in various different flavors, each one of which has its specific preferences with regard to the host range which can be infected and also to the type of cells in which it replicates.5

One paradoxical situation, which is of immediate relevance to the question of blood safety, is exemplified by the radically different organ tropism of the BSE agent in cows and in humans. BSE prions seem to be largely confined to the neural compartment of cows, even after oral exposure.6 A very accurate study of the pathogenesity of experimental BSE in cows upon feeding 100 grams of infected brain has disclosed that there is only a short and transient period during which infectivity can be demonstrated in the terminal ileum.6 At later time points, BSE prions can be shown only in brain, spinal cord, and dorsal root ganglia. The exact localization of BSE in the terminal ileum is not known. It is being discussed whether infectivity resides in Peyer's patches or in the neural compartment which comprises the plexus submucosus M.issner and the plexus myentericus Auerbach.

There is a great body of circumstantial evidence that BSE prions can provoke new variant Creutzfeldt-Jakob disease (nvCJD),7-10 but no absolutely conclusive evidence has been produced. For the purpose of the following discussion we will regard the evidence that BSE and new variant Creutzfeldt-Jakob disease are caused by the same agent as sufficiently verified.11 Upon passage into humans, and consecutive progression to manifest nvCJD, prions experience a dramatic shift in their organotropism. Instead of remaining confined mainly to neural structures, they can be detected in many organs belonging to the immune system including, most notably, tonsils, spleen, and as recently demonstrated, the appendix.12 It is, therefore, unavoidable to conclude that the tropism of the infectious agent for various structures depends both on the strains of prions in question (and therefore it is in part autonomous to its carrier) and on the species in which the prion disease manifests itself.13

These considerations are not only of academic interest. In fact, the transmissibility of the agent by iatrogenic manipulations (i.e. blood transfusions, organ transplants, etc.) is crucially affected by such parameters.

Horizontal transmissibility of human prions

Prion diseases of humans are undoubtedly transmissible. However, transmission is achieved only under particular circumstances. One could say that in this respect prion diseases fulfill the characteristics of transmissibility delineated by Semmelweiss for puerperal fever: these diseases are infectious but not contagious. Direct transmissions of brain-derived material from a patient suffering from Creutzfeldt-Jakob disease to other persons have resulted in documented transmission of disease. A particularly tragic case occurred in the early seventies in Zurich, when electrodes used for cortical recordings from Creutz-
feldt-Jakob patients were sterilized (formaldehyde and alcohol) and then used for other patients. Disease was transmitted to the young recipients.\textsuperscript{14} Transplantation of cornea has most likely also resulted in transmission of disease.\textsuperscript{15}

Despite these tragic dimensions, cases of iatrogenic transmission of CJD via neurosurgical procedures have remained rather rare. This is, in my view, not totally understood, given that the frequency of subclinical CJD must be much higher than that of manifest disease, and that most neurosurgical instruments are not sterilized in a way that would reliably inactivate prions. Therefore, the quite rare occurrence of iatrogenic transmission is likely to indicate that host factors, in addition to the virulence of the prion, may affect the probability of an infection taking place. This notion is strengthened by the epidemiology of iatrogenic CJD (iCJD) upon transmission of contaminated dura mater. It has been estimated that several thousands of patients, predominantly in Japan, may have been exposed to the CJD agent via preparations of cadaveric dura mater which had been contaminated with prions. However, it appears that less than 2\% of those exposed have so far developed disease. While we can rejoice about this low efficiency in the take of infectivity, we do not fully understand the biological basis for the apparent protection enjoyed by most subjects exposed to CJD prions.

The largest problem with iatrogenic transmission has occurred as result of administration of pituitary hormones of cadaveric origin.\textsuperscript{16} Preparations of growth hormone and of gonadotropins contaminated with human prions have caused the death of more than 80 persons, predominantly children. Due to the long latency which can be expected when the agent is introduced into extracerebral sites, such as via intramuscular injection, it must be assumed that further cases from this procedure, which was stopped more than a decade ago, will arise in the future.

Besides its tragic human dimension and the harm that it has done to the patients and to their physicians, the pituitary hormone disaster needs to be

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Figure 1. Current hypotheses about the nature of the prion, the infectious agent causing transmissible spongiform encephalopathies.

A. The refolding model. The conformational change is kinetically controlled: a high activation energy barrier prevents spontaneous conversion at detectable rates. As a result of an interaction with exogenously introduced PrP\textsuperscript{Sc}, PrP\textsuperscript{C} undergoes an induced conformational change to form PrP\textsuperscript{Sc}. This reaction may involve extensive unfolding and refolding of the protein to explain the postulated high energy barrier and could be dependent on an enzyme or chaperone. The process leads to an exponential conversion cascade. Sporadic CJD may come about when an extremely rare event (occurring in one in a million individuals per year) leads to spontaneous conversion of PrP\textsuperscript{C} to PrP\textsuperscript{Sc} and gives rise to a conversion cascade.\textsuperscript{49,50}

B. The seeding model. The conformational change is thermodynamically controlled: the conformational change between PrP\textsuperscript{C} and PrP\textsuperscript{Sc} or a PrP\textsuperscript{Sc}-like molecule is reversible. PrP\textsuperscript{Sc} is only stabilized when it adds onto a crystal-like seed or aggregate of PrP\textsuperscript{Sc}. Seed formation is extremely slow; once a seed is present, monomer addition can ensue at a rapid rate.\textsuperscript{51,52}
understood in detail, because the anterior lobe of the pituitary gland is not a part of the central nervous system. Therefore, these events may serve as a paradigm for transmission of prions via contaminated extracerebral tissue that does not belong to the canonical sites of replication of prions.

The observation that latency after intracerebral contamination is much shorter than latency after peripheral infection is in good agreement with experimental data from various animal models, and suggests that a rather lengthy phase of extracerebral events (which may include replication of the agent, and invasion of specific extraneuronal systems) may be a precondition to prion neuroinvasion.17

Factors influencing the neurotropism of prions

There is good reason to suspect that neuroinvasive processes in the course of prion infections are very tightly controlled. Perhaps the best argument in this respect derives from the observation that the incubation times of disease in experimental animals inoculated intraperitoneally with scrapie prions are extremely reproducible. Upon inoculation with a known amount of standard inoculum, the experience in our laboratory and in many others has been that latencies between inoculation and first clinical symptoms display standard variations in the order of only a few percent points.18 If prion neuroinvasion were a totally random process, one would expect a large variability in the incubation times, which would depend on processes governed by chance. However, if some rate-limiting processes control neuroinvasion, these may be responsible for the remarkable precision of the incubation times. Indeed, we very much hope that this interpretation is correct because if such processes exist they might be amenable to manipulation, which in turn may represent a post-exposure strategy to prevent overt prion disease. Indeed, we and others have explored various mechanisms by which neuroinvasion may be accomplished.

Our current working hypothesis is that neuroinvasion has two phases. In the first phase, widespread colonization of the immune system is achieved. This can be visualized by homogenizing spleen, lymph nodes, tonsils, and also appendix, and injecting the homogenates into suitable experimental animals. The dilution of the homogenates at which 50% of the experimental animals become sick, contains one ID50 of the infectious agent in each inoculum.

The second phase of neuroinvasion seems to be dependent upon a compartment which cannot be replaced by adoptive bone marrow transfer19 and which may be represented by the peripheral nervous system and/or the follicular dendritic cells (FDC) resistant to germinal center of secondary lymphatic organs. It appears that this second compartment necessitates the expression of normal prion protein in order to support neuroinvasion.19

Neuroinvasion is dependent on a functional immune system, and immunodeficient mice do not develop disease after inoculation with a moderate dose of the agent.20-23 One crucial component of the immune system necessary for neuroinvasion has been traced to the physical presence of terminally mature B-lymphocytes. To date, it is not clear whether B-cells are required because they bind physically prions and carry them to sites of neuroinvasion, or whether B-cells produce factors, or induce processes, which are indirectly responsible for facilitating neuroinvasion.18

Given the requirement for B-lymphocytes secreting lymphokinin for the maturation of follicular dendritic cells, and the fact that follicular dendritic cells accumulate large amounts of scrapie prions in experimental situations, it is tempting to speculate that the main function of B-lymphocytes in the aforementioned process consists in allowing FDCs to mature (Figure 2).

How to detect prions: an analytical nightmare

In the age of real-time kinetic polymerase chain reaction (PCR), we have become very spoiled with respect to the detection thresholds which we demand from assays geared at detecting viral contaminants in blood. Consider the case of HIV: here the introduction of quantitative PCR technologies has pushed the limit of detection in blood and blood products down to quasi-perfection. Even when PCR techniques have not proved that useful, or have not yet met with such widespread acceptance, ultrasensitive immunochemical methods, such as time-resolved fluorescent ELISA, have progressed to a degree of sophistication that is highly satisfactory for most screening applications.

So why do we still have a problem with prion detection in blood?

The most formidable problem derives from the unique biology of the prion. According to more-or-less accepted wisdom, prions are likely to consist solely of the PrPSc protein, which has exactly the same amino acid structure as the normal cellular protein PrPC. A more noncommittal way of wording this fact would be to state that PrPSc is the only known surrogate marker for prion infectivity: this latter statement is likely to be acceptable to both the proponents of the protein-only hypothesis and to those who still believe that the infectious agent is a virus.

The consequence of the fact mentioned above for prion detection is obvious: if prion-specific nucleic acids do not exist, any PCR-based screening assay will not be an option. Therefore, we are left with immunochemical assays. Besides being less sensitive than PCR by several orders of magnitude, these are also fraught with a series of prion-specific problems. The biggest trouble, again, derives directly from the peculiar biology of BSE agents. As explained above, PrPSc possesses the same chemical composition as PrPC, and the latter is a membrane-bound protein that is normally found in many cell types of healthy individuals including white blood cells.14 Although PrPSc and PrPC differ in a number of physical properties, it appears to be extremely difficult, if not impossible, to develop immunoologic reagents which reliably differentiate between these two isoforms. Only one monoclonal antibody has been described to react with PrPSc but not with PrPC, and its practical usefulness remains to be demonstrated since fourteen months after its publication no follow-up studies have appeared and even the company which developed this reagent in the first place does not appear to use it in its in-house screening assay for BSE prions.

Prion diseases, blood and the immune system

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The cellular and molecular basis of prion neuroinvasion

Following experimental inoculation of mice with prions at peripheral sites, there is typically a prolonged, clinically silent replication phase of the infectious agent within the lymphoreticular system (LRS). This occurs prior to detectable neuroinvasion by prions and the subsequent occurrence of neurologic symptoms. During this preclinical latency period, prions may replicate to high titers within lymphoreticular tissues. Elucidating the cell types in which prions replicate within the peripheral lymphoid tissue and – crucially – how prions are transported to the central nervous system (CNS) is of great interest and clinical importance.

Despite considerable evidence implicating the role of the immune system in peripheral prion pathogenesis, there have been few studies on the identity of the cells involved in this process. It was shown many years ago that whole-body irradiation of mice with gamma rays fails to influence prion pathogenesis or the incubation time of scrapie. This has been taken as an argument against significant involvement of proliferating cells in the lymphoreticular phase of prion propagation. Instead, follicular dendritic cells (FDC) have been considered as the prime cell type for prion replication within lymphoid tissue since PrP \textsuperscript{Sc} accumulates in the follicular dendritic network of scrapie infected wild-type and nude mice. In addition, severe combined immunodeficient mice (SCID), which lack mature B- and T-cells and which do not appear to have functional FDCs, are highly resistant to scrapie after intraperitoneal inoculation and fail to replicate prions in the spleen. Interestingly, bone-marrow reconstitution of SCID mice with wild-type spleen cells restores full susceptibility to scrapie after peripheral infection. These findings suggest that an intact, or at least partially functional, immune system comprising lymphocytes and FDC is required for efficient transfer of prions from the site of peripheral infection to the CNS.

The time course for the development of scrapie disease following intracerebral or intraperitoneal inoculation is highly reproducible and is primarily dependent on the dose of the inoculum. Therefore, neuroinvasion by prions migrating from peripheral lymphoid tissue may depend on tightly controlled, rate-limiting reactions. In order to identify such rate-limiting steps during prion neuroinvasion, PrP \textsuperscript{Sc} deficient...
mice bearing PrP-overexpressing cerebral neurografts were infected intraperitoneally (i.p.). No disease was observed in the grafts, suggesting that neuroinvasion depends on PrP expression in extracerebral sites. This was further underlined by reconstitution of the lymphoid system with PrP<sup>C</sup> expressing cells, which restores infectivity in the lymphoid tissue, but still fails to transport prions to the nervous system.

To identify the lymphoid cells responsible for accumulation and transport of the infectious agent in secondary lymphoid organs, we investigated prion disease in various sets of immunocompromised mice. We used B- and T-cell deficient mice (RAG-2<sup>–/–</sup>, RAG-1<sup>–/–</sup> and SCID) and AGR<sup>–/–</sup> mice which lack receptors for interferon-αβ and interferon-γ in addition to B- and T-cells. The role of T-cells was investigated by use of mice with a targeted disruption of the gene encoding for either CD4, CD8, β<sub>2</sub>-microglobulin or perforin. Selective ablation of B-lymphocytes was studied in µMT<sup>–/–</sup> mice with a targeted disruption of the transmembrane exon of the immunoglobulin µ-chain gene. These mice do not produce any immunoglobulins and suffer from a B-cell differentiation block at the large-to-small pre-B-cell transition, yet bear complete and functional T-cell subsets.

Intracerebral (i.c.) challenge of each strain of immune deficient mice with scrapie prions resulted in the development of clinical symptoms of disease with a comparable time course to that seen in wild-type mice (Table 1). Disease was confirmed by histopathologic analysis, western blot and by transmission of disease to tg20 mice, which overexpress the normal prion protein (PrP<sup>C</sup>) and are hypersensitive to mouse prions.<sup>26</sup> We concluded from this part of the study that after prions have gained access to the nervous system, scrapie pathogenesis and prion expansion in the brain proceed without the immune status of the host having any detectable influence.

In contrast, after i.p. inoculation of the panel of immunodeficient mice no clinical disease was observed in mice with either a B-cell defect or with a combined B- and T-cell deficiency (Table 1). Importantly, no prion infectivity was detectable in the spleens of disease-free mice. In SCID mice which also lack B- and T-lymphocytes scrapie disease was marginally prolonged after i.p. challenge. This may be due to an incomplete immune deficiency in SCID mice on a C57BL/6 genetic background. Mice with various T-cell defects (including deficiency of perforin, β<sub>2</sub>-microglobulin, CD4, CD8, and of the T-cell receptor α-chain) exposed to prions via the i.p. route developed scrapie disease – however, at the time of writing we are still in the process of investigating the susceptibility of T-cell receptor β<sub>2</sub>δ chain knockout mice which lack all subsets of T cells. These data implicate B-cells as a critical cell type involved in peripheral scrapie pathogenesis.

However, mice devoid of B-cells fail to produce antibodies, and FDCs fail to develop in their lymphoid organs. To distinguish which of these three factors may be responsible for prion pathogenesis, two further mouse strains were investigated. To elucidate the

Table 1. Mice with various immune defects were inoculated with RML scrapie prions intracerebrally or intraperitoneally. While all intracerebrally inoculated mice developed scrapie with a latency similar to that of wild-type mice, intraperitoneal inoculation resulted in scrapie in T-cell deficient mice, but not in mice displaying defects in the maturation of B-lymphocytes. SCID mice bred on the C57Bl/6 genetic background developed scrapie: these mice are however “leaky”<sup>39,40</sup> and tend to contain a residual population of lymphocytes.

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role of immunoglobulins we analyzed mice producing antibody exclusively of the IgM subclass (t11µMT), which had no detectable specificity for PrPC. The role of FDCs was addressed using mice which lacked functional FDCs (TNFR1–/–) but had differentiated B-cells. Both strains developed scrapie after peripheral inoculation demonstrating a crucial role for differentiated B-cells per se in neuroinvasion of scrapie.18

Since the replication of prions27 and their transport from the periphery to the CNS19 is dependent upon expression of PrP C, which is expressed by lymphocytes,28 we further examined whether expression of PrP C in B-cells is necessary to support neuroinvasion. Mice with various immune defects were repopulated by adoptive transfer of hematopoietic stem cells which expressed or lacked expression of PrP C.

Adoptive transfer of Prnp+/+ and of Prnp o/o fetal liver cells (FLCs) induced formation of germinal centers in spleens of all mice, including differentiation of FDCs as visualized by staining with antibody FDC-M1.29 However, no follicular dendritic cells were found in B and T cell deficient mice reconstituted with FLCs from µMT embryos (B-cell deficient), consistent with the notion that B-cells or products thereof are required for FDC maturation.30

Reconstituted mice were challenged i.p. with scrapie prions. Surprisingly, all mice that received FLCs of either genotype, Prnp+/+ or Prnp o/o, from immunocompetent donors succumbed to scrapie after inoculation with a high dose of prions, and most mice after a low dose. Transfer of FLCs from µMT donors, as well as omission of the adoptive transfer procedure, did not restore susceptibility to disease in any of the immune deficient mice challenged with the low dose. We also confirmed that, by using high dose inoculum, susceptibility to scrapie could be restored even in the absence of B-cells and FDCs. However reconstituted mice which received bone marrow from TCR+/+ mice donors (these mice contain B-cells and lack all T-cells but those expressing TCRγδ) regained susceptibility to scrapie, underlining the dependency of infectibility upon the presence of B-cells. By transmitting individual samples of brain and spleen from the scrapie inoculated bone marrow chimeras we observed restoration of infectious titer and PrPSc deposition in spleens and brains of recipient mice carrying either Prnp+/+ or Prnp o/o donor cells.

In a further step we investigated whether spleen PrPSc was associated with FDCs in repopulated mice. Double-color immunofluorescence confocal microscopy revealed deposits of PrP-immunoreactive material in germinal centers which appeared largely co-localized with the follicular dendritic network, in spleens of reconstituted mice (Figure 3).

These results are compatible with the hypothesis that cells whose maturation depends on B-cells are responsible for accumulation of prions in the spleen. FDCs, although their origin remains obscure, are a likely candidate because their maturation correlates with the presence of B-cells and the formation of immune complexes.

However, it is equally possible that the follicular dendritic network serves as a reservoir for the accumulation and multiplication of prions and that other B-cell dependent processes are involved in transferring the agent to autonomic nerve terminals. Prions may be transported on or within B-cells directly within peripheral lymphoid tissue. Alternatively antibodies or other factors may bind prions, especially since PrP can be detected by immunohistochemistry in the germinal center area where immune complexing occurs, and this formation may be of importance for mediating neuroinvasion and facilitating the access to peripheral nervous system terminals.

Figure 3. Confocal double-color immunofluorescence analysis of splenic germinal centers in scrapie-inoculated wild-type mice (top row), non-inoculated Prnp o/o mice (middle row), and RAG-1–/– mice reconstituted with Prnp o/o FLCs and sacrificed 184 days after i.p. inoculation with RML prions (bottom row). Sections were stained with hemalaun (left column), with antibody FDC-M1 to follicular dendritic cells (green, second column from left), and with antiserum R340 to PrP (red, third column). Regions in which both signals are detectable appear yellow in superimposed images (right column). Most of the PrP signal was in germinal centers and appeared to co-localize with FDCs.
An outlook for the near future

As prions can be detected in lymphoreticular tissues, an understanding of the peripheral pathogenesis is of immediate importance in assessing risks of iatrogenic transmission of human BSE via exposure to blood or tissues from preclinical cases, and possibly from contaminated surgical instruments, or even blood and blood products. Additionally, such advances might pave the way for the development of sensitive diagnostic tests and the means to block prion neuroinvasion.

Given the current state of knowledge, one might wonder why contamination of the blood supply with prions should be an issue at all. After all, very thorough epidemiological surveys over two decades have not evidenced that blood transfusions, or administration of blood products, are risk factors for prion diseases.

The problem is, of course, new variant CJD. For one thing, we do not know by far as much about the epidemiology and iatrogenic transmissibility of this new disease as we do about sporadic CJD (sCJD). What is most unsettling, the distribution of preclinical disease in Great Britain and possibly in other countries is totally obscure, and the little knowledge that is being gathered is far from reassuring. Moreover, there is every reason to believe that nvCJD may be much more lymphoinvasive than its sporadic counterpart. In particular, nvCJD prions can be easily detected in lymphatic organs such as tonsils and appendix, a fact that was previously demonstrated to be true for scrapie, but not for sCJD prions. While all available evidence points to follicular dendritic cells as the prion reservoir in lymphatic organs, splenic lymphocytes of experimentally inoculated mice can be infected with prions. Although prion infectivity of circulating lymphocytes appears to be at least two logs lower than that detected in splenic lymphocytes, the possibility that circulating lymphocytes may be in equilibrium with their splenic siblings calls for cautionary measures. The nature of the latter is still matter of controversy and debate: leukodepletion has had some positive effects on prion removal, the latter possibility can be regarded as worst-case scenario.

The second consideration applies to secondary prophylaxis. Given the very large numbers of infectious BSE materials that have entered the human food chain, it is possible that many individuals harbor preclinical nvCJD. It is imperative and urgent to develop strategies that will help control the spread of the agent and that will prevent the clinical outbreak of symptoms in these persons. Possible targets for the interference with neuroinvasion are rate-limiting processes that control prion replication within the infected individual. In light of the knowledge discussed above, treatments that target the neuroimmune interface of prion replication and neuroinvasion seem promising areas for research aimed at post-exposure prophylaxis.

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