Unexpected prion phenotypes in experimentally transfused animals: predictive models for humans?

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ABSTRACT
The recently reevaluated high prevalence of healthy carriers (1/2,000 in UK) of variant Creutzfeldt-Jakob Disease (v-CJD), whose blood might be infectious, suggests that the evolution of this prion disease might not be under full control as expected. After experimental transfusion of macaques and conventional mice with blood derived from v-CJD exposed (human and animal) individuals, we confirmed in these both models the transmissibility of v-CJD, but we also observed unexpected neurological syndromes transmissible by transfusion: despite their prion etiology confirmed through transmission experiments, these original cases would escape classical prion diagnosis, notably in the absence of detectable abnormal PrP with current techniques. It is noteworthy that macaques developed an original, yet undescribed myelopathic syndrome associating demyelination and pseudo-necrotic lesions of spinal cord, brainstem and optical tract without affecting encephalon, which is rather evocative of spinal cord disease than prion disease in human medicine. These observations strongly suggest that the spectrum of human prion diseases may extend the current field restricted to the phenotypes associated to protease-resistant PrP, and may notably include spinal cord diseases.

The current apparent situation towards BSE and v-CJD is reassuring

European population was widely exposed to Bovine Spongiform Encephalopathy (BSE), or mad cow disease, through the consumption of contaminated alimentary products during a period presumably extending from 1986 to 2001. Indeed, it is now admitted that at least two million of contaminated undiagnosed cattle (10-fold more animals than the reported clinical cases) would have entered the human food chain in United Kingdom, the most affected country, whereas exposure would have been ten-fold lower in France, the main worldwide country that imported British cattle during BSE epizooty.

In 1996 were described the first cases of variant of Creutzfeldt-Jakob disease (v-CJD), linked to the transmission of this bovine prion strain to humans [1], leading to an unprecedented sanitary crisis known as ‘the mad cow crisis’. Several initial mathematic projections had a huge media impact as their worst scenario [2] predicted up to several hundred thousands of victims after primary exposure. Notwithstanding, twenty years after the onset of the first cases of v-CJD, a total of less than 250 clinical v-CJD cases has been worldwide reported. This apparent discrepancy between the prevalence in animal and human population is commonly considered as due to a highly protective cattle-to-human species barrier [3].

According to this limited impact on human health on one hand, and the quasi-disappearance of BSE cases in Europe on the other hand, it has been decided to relax the main measures set up to protect animal and human health from this peculiar prion strain. In a similar way, safety of blood products is ensured through indirect precautionary measures only (specific deferral of blood donors and leukodepletion of all blood-derived products initially set up against blood-borne viruses) [4], with the justification that the number of secondary cases is very limited (between 2002 and 2006 were reported 4 cases of contamination supposed to be transfusion-transmitted, and none since this period) [5].

Prevalence studies apparently support a consequent contingent of healthy carriers

In 2013 was published a large retrospective study in UK based on samples derived from patients born before
Ten million people were exposed to BSE during the period at risk. Sixteen samples were found positive (presence of PK-resistant, abnormal PrP deposition) among more than 30,000 appendix samples issued from asymptomatic people [6]. All the genotypes were concerned, whereas all the reported v-CJD clinical cases are up to now Met/Met homozygous (129th amino-acid of PrP), except one recently described Met/Val case [7]. This study demonstrated that healthy carriers of v-CJD infection occur with a prevalence of 493 per million (i.e. 1/2,000) in the British population, i.e. around two-fold more than the presumed prevalence initially estimated in a first study published in 2004 and based on 12,000 appendix samples [8]. It is interesting to note that in a third study, no appendix was found positive among samples removed before 1977 (0/8,827) or issued from patients born after 2001 (0/4,750); conversely, similar prevalence of positivity was observed in appendix samples removed between 1977 and 1979 (2/5,865) or derived from patients born between 1996 and 2000 (5/10,074) [9], extending thus the presumed period during which consumers were exposed to a significant risk of BSE.

Theoretically the situation towards BSE/vCJD should be worse than apparently

Taken together with a recent publication that estimates that up to ten million people would have been exposed to BSE [10], those observations suggest thus that we are facing a Matryoshka situation towards v-CJD in UK:

- Ten million people were exposed to the BSE risk;
- Up to 40,000 (i.e. less than 1%) of those exposed people are infected according to the current criteria of v-CJD diagnosis (accumulation of PK-resistant abnormal PrP). Those people, as carriers of peripheral infectivity, may be the source of a risk of secondary transmission notably through blood transfusion during their silent incubation phase (as illustrated by the 4 aforementioned transfusion-transmitted cases). Would their blood be infectious, up to 1,000 blood donations might be supposed at risk every year in UK.
- Less than 250 (i.e. less than 1%) of those infected people were recorded as developing a clinical v-CJD. A doubling of clinical cases with heterozygous patients should not significantly modify the concept.

In other countries, the corresponding prevalence rates have not been subjected to such extensive analyses, but according to the respective initial BSE exposures, 10- to 100-fold lower prevalence has been estimated for all Western Europe countries, prevalence in Canada has been expected to be 4 to 5 logs lower, and prevalence in USA to be 5 to 6 logs lower (SEAC January 2009). However, one may presume that if the aforementioned Matryoshka situation with proportions between exposure, infection and clinical disease are maintained outside UK, up to 20,000 supplementary healthy carriers might be expected in Western Europe (without UK), and some healthy carriers may live in Canada and USA.

In summary, we are facing a paradox between an apparent reduced prevalence of clinical cases of v-CJD, which incites to limit the expensive measures of protection towards BSE/v-CJD, and a potential Damocles' sword constituted by a wide exposure of consumers, a supposed high prevalence of healthy carriers and consecutive levels of blood donations at risk which encourages to pursue the risk assessment and the development of protective strategies.

An alternative hypothesis based on macaque experiments: a heterogeneous exposure of consumers

Experimental infection of cynomolgus macaque currently constitutes the closest model of human situation towards BSE, notably since macaques share with humans similar specific lesions and efficient oral [11] and intravenous [12-14] transmissions contrarily to other primate models [15,16] that are moreover phylogenetically more distant from humans. In studies of our group [11] and others [17], the oral LD50 of BSE in macaques was achieved with around 5 grams of infected brain, that is only around 30-fold higher than in cattle (estimated cattle oral ID50 = 150 mg) [18]: such results indicate a relatively low cattle-to-macaca species barrier.

Do macaques and humans present similar susceptibility to BSE/vCJD agent? Taking together those experimental observations and the epidemiological situation of BSE and v-CJD, one might assume at first sight that macaques would be highly susceptible to BSE contrary to humans, and therefore those non-human primates would be a very worst-case model of the human situation.

An alternative hypothesis might explain the discrepancy between the high global exposure of the human population and its relative low level of infection: humans might be considered as (highly) susceptible to BSE as macaques, but v-CJD prevalence would remain limited because individual exposure of consumers would have been highly heterogeneous and mainly composed of low doses. The Scientific Steering Committee of the European Commission supports this hypothesis in its ‘opinion on
the human exposure risk via food with respect to BSE published in December 1999 (Figure 1): this report indicates that the exposure of consumers to BSE depends on the size of batches in industrial food process, leading to a difference of exposures with 5 logs of magnitude between both extremes. Thus, a low number of people would have been exposed to a high amount of infectivity, whereas a high number of people would have been exposed to a very low amount of infectivity. One could then hypothesize that the reported v-CJD clinical cases might correspond to highly-exposed and/or highly susceptible consumers. However, the evolution towards infection of the majority of (low-exposed) consumers, probably including the healthy carriers identified by the prevalence studies [6,9], remain to be determined. It is to note that a similar configuration has already been observed in cattle: despite an absence of species barrier, the incidence of BSE within affected herds was low (often only one case) because ‘the variation in feed and offal processing methods and... per-animal feed uptake’ [19].

**Experimental evaluation of the transfusional transmission of classical vCJD: a risk under control**

In this context, we evaluated the transfusional risk of v-CJD in two susceptible experimental models [14]: conventional Swiss mice and cynomolgus macaques were either transfused with different blood products derived from v-CJD exposed donors (mice and macaque donors for mice transfusion, macaque and human donors for macaque transfusion), or intravenously exposed to soluble extracts of v-CJD-contaminated brains that are classically used to model blood infectivity [20]. The classical v-CJD picture was observed in a faint proportion of mice and macaque recipients, confirming that, at least in these models, v-CJD is transmissible through transfusion but requires optimal conditions including the absence of species barrier, the transfusion of non deleukocyted samples, donors with high levels of peripheral replication and donations sampled during the clinical phase. The extrapolation of these results to the human situation suggests that the protective measures that have been set-up to secure blood transfusion (specific deferral of blood donors and systematic leukodepletion) should be of nature to prevent such situations.

**Occurrence of alternative disease phenotypes in non-optimal conditions of transmission**

Alongside, we observed a higher proportion of animals (2- to 5-fold more depending on the couple animal-inoculum) which developed fatal neurological conditions.
syndromes distinct from v-CJD. In mice, these atypical diseases corresponded to incomplete v-CJD phenotypes (incomplete topographical distribution of spongiform changes, inconstant presence of abnormal PrP with variable resistance to proteolysis) focused either on brain or spinal cord, whereas the classical v-CJD profiles affects the whole CNS. In macaques, recipients with atypical diseases developed sensitive, sensorial and locomotor deficiencies that were in coherence with the demyelinating and pseudonecrotic lesions that we observed in their spinal cord (mainly lower cervical level), whereas their medulla and their optical tracts whereas brain was poorly involved. We defined this syndrome as a myelopathy. In comparison to the transfusional-transmitted v-CJD cases that we observed in these experiments, the occurrence of this myelopathic syndrome or incomplete phenotypes was linked to less optimal conditions for prion transmission, which included deleukocytes samples, donors with low levels of peripheral replication and inter-specific (human to macaque or macaque to mice) transfusion. Moreover, the occurrence of these incomplete phenotypes was rather related to soluble supports of infectivity (supernatant of clarified brain, plasma). It is also noteworthy that myelopathy occurred in two macaque recipients transfused with blood derived from healthy carriers (i.e. orally BSE-exposed macaques sampled during their asymptomatic incubation phase): such non-optimal conditions correspond to the situations more plausible to arise in human transfusion.

A peripheral selection of different prion substrains

The vCJD prion strain is reputed to have a stable unique signature [23,24]. Our results rather support the idea that vCJD may express under different phenotypes as previously suggested in humanized transgenic mice [25]. A major common feature of the atypical phenotypes that we observed in both mice and macaques is the apparent absence of abnormal PrP within the lymphoid follicles of the spleen of any affected animal: we hypothesized that these atypical prion diseases would be sustained by soluble prions with a tropism towards spinal cord (‘myelotropic’), that would be preferentially promoted by certain mechanisms taking place in peripheral organs which remain to be identified. This theory of differential peripheral selection of prion substrains depending on the physical support of the initial infectivity finds an echo among the recent publication of Aguilar-Calvo et al [26], which demonstrated a dichotomy of transmission efficiency between fibrillar and sub-fibrillar prion strains after peripheral exposure, and notably they evidenced the generation of novel prion strains after intravenous challenge.

Confirmation of the prion nature of these unexpected phenotypes

Such syndromes were never previously described in conventional mice and macaques, but it should also be noted that reports on experimental transmission of prions through the intravenous route within these models are very limited [21,22]. Even if none of our control animals exhibited any of these unexpected pictures, our first reaction was to seek an alternative, non-prion etiology to these atypical profiles. Several hypotheses of metabolic, environmental, dietary, auto-immune or (non-prion) infectious origins were long-lastingly tested, but no obvious element was found to sustain any of them. Besides, several elements support a prion etiology to these atypical diseases. Firstly, these atypical diseases are transmissible: mice exposed to blood or brains derived from myelopathic primates developed incomplete vCJD profiles, mainly focused on spinal cord. These incomplete vCJD profiles are transmissible from mice to mice after secondary transmission, and can lead to the emergence of vCJD in some recipients with the presence of PrPres. Secondly, accumulation of abnormal PrP was evidenced in the CNS of certain rodents and macaques affected with these atypical profiles, by using either IHC or amplification techniques (QuIC, PMCA). Thirdly, the depletion of PrP in the initial blood inoculum prevented the onset of myelopathy: after transfusion with leukodepleted red blood cell concentrates (LR-RBCC) derived from vCJD donors, two macaques developed myelopathy after 30 months of incubation, whereas the three macaques exposed to the same LR-RBCC after passage through an anti-prion filter, are still asymptomatic 118 months post exposure [13]. At the opposite, vCJD was observed in the macaque transfused with the corresponding whole blood.

Is the emergence of atypical phenotypes restricted to transfusion?

We described these syndromes in the frame of transfusion with labile blood-derived components, but their potential occurrence within other contexts needs to be evaluated: firstly at the level of extractive plasmatic derivatives, since the nanofiltration step implemented in the corresponding processes of production has been described of poor efficiency against soluble prions [27–30]. Secondly, Holznagel et al. also described in macaques orally challenged with BSE the occurrence of atypical prion diseases with specific involvement of spinal
cord in the absence of cerebral involvement [17]: these observations suggest that the BSE-related syndromes that we described may also be selected within the frame of primary BSE contaminations through the oral route. By extension, the absence of abnormal PrP detectable by current classical techniques in these myelopathic animals questioned the criteria of inclusion used to identify healthy carriers, i.e. the presence of abnormal PrP in lymphoid organs. Accordingly, the proportion of healthy carriers may currently be underestimated in a proportion that remains to be determined. Thirdly, in accordance with the theory that the variety of prion strains is supported by the variety of aggregation/quaternary structures profiles [31], such atypical phenotypes linked to soluble forms of prions may emerge from other prion strains than v-CJD.

**Human health consequences of atypical phenotypes**

The observations of such diseases raise several questions with important public health and fundamental consequences. Under the consideration that those experimental models are predictive of the human situation, the following questions rely on the temporality of the occurrence of such diseases in humans – will they occur in the future, or are they already present? – and their phenotypical manifestations. As vCJD macaques exhibit similar clinical signs and lesions as vCJD patients, one may assume at first that it will be also the case for atypical phenotypes. It is to note that the clinical signs exhibited by the macaques developing this myelopathy are evocative of certain human spinal cord diseases already described, including the rare flail arm syndrome of ALS [32,33], neuromyelitis optica (NMO) spectrum disorders [34], the recently described FOSMN [35] or necrotizing myelopathies [36]. However, the pathognomonic lesions of these aforementioned human spinal cord diseases under their classic forms are clearly distinct from the lesions that we observed in our myelopathic macaques, *a priori* infirming this comparison; nevertheless, the proportion of patients suffering from spinal cord diseases that are nowadays autopsied is very faint, and the diagnosis is thus mainly based on clinical and complementary examinations. In the field of human prion diseases, the clinical suspicions are 4- to 10-fold more numerous than the definite cases, and post mortem confirmation through neuropathological examination or biochemical analyzes of brain samples are compulsory for a definite classification as a prion disease. This reasoning can be extended to spinal cord diseases: since the accuracy of all the ante mortem suspicions cannot be confirmed through an autopsy, it cannot be ruled out that certain of those cases would be of prion origin.

Indeed, the definition of certain spinal cord diseases is broad and their criteria of inclusion tend to be modified with time. Notably, Flail Arm Syndrome has initially been described as a form of Amyotrophic Lateral Sclerosis, but it is now considered as a peculiar, separate pathological entity according to its neuropathological specificities [37]. It is to note that the MRI images we observed in the brainstem of our myelopathic primates are reminiscent of ‘owl eyes’ images observed in certain cases of Flail Arm Syndromes [38] or ‘snake eyes’ images associated to certain lower motor neuron diseases [39]. Concerning NMO spectrum disorders, the discovery in 2004 of aquaporin 4 autoantibodies classified this syndrome as an autoimmune disease [40], but the occurrence of seronegative cases [41] questioned the unicity of this pathological entity [42].

It could also be objected to this hypothesis that there is no obvious epidemiological evidence of increasing prevalence of spinal cord diseases due to a novel emergent phenotype during the last years. It should be nevertheless remembered that the prevalence of those diseases (around 1/100.000 for ALS or NMOSD) are exceeding by several magnitudes the prevalence of human prion diseases (1 per million for sporadic CJD every year, while a total of 221 cases of vCJD in 20 years): even if myelopathy would affect ten-fold more human patients than vCJD, the epidemiological analyzes and surveillances would be unable to detect them.

**Fundamental consequences of atypical phenotypes**

Independently of the relevance of these observations for the human situation and their consequences for public health, the existence of these atypical prion phenotypes has fundamental consequences in our conception of prion diseases. We described here prion diseases devoid of obvious accumulation of proteinase K-resistant abnormal PrP, whereas this peculiar property of resistance to proteolysis is often considered as an ultimate diagnostic criteria of prion diseases. In a similar way, the techniques of PrP amplification developed by several teams for proposing new generations of diagnostic tests were inefficient for identifying these original prion strains. We may hypothesize that within the wide field of prion diseases, that correspond to diseases with abnormal PrP, the currently available techniques have allowed us to identify the diseases that were the easiest to detect (Figure 2), i.e. those which modified the normal PrP towards forms with ‘higher’ properties (half-life, hydrophobicity, aggregation, resistance to proteolysis…), but other diseases, including diseases focused on other organs than brains [43,44], may
have a dysfunction of PrP that have to be identified now with new tools based on different properties and that have to be built up. Other groups have already, ‘recently’ according to the history of prion diseases, described natural prion diseases that also exhibit abnormal PrP accumulation with limited resistance to proteolysis, as variably protease-sensitivity proteinopathy in humans [45,46] or Nor-98 [47] in small ruminants.

At the experimental level, we described 20 years ago the development of clinical prion disease in the absence of detectable PrPres in conventional mice exposed to BSE [48], whereas PrPres accumulation was acquired with secondary passage with adaptation to the host, acting as a factor of virulence: more and more evidences support a quantitative dissociation between infectivity and PrPres [20,31,49], but also a dissociation between prion seeding activity and regions of neurodegeneration [50]. This dissociation between pathological proteins and neurodegeneration is also evidenced in other prion-like neurodegenerative diseases [51,52].

In conclusion, our observations of these atypical phenotypes related to vCJD open new fields to the current global revision of the classification and physiopathology of the neurological disorders, initiated several years ago with the extension of the ‘prion-like’ concept to almost all neurodegenerative diseases.

**Notes**

1. [http://webarchive.nationalarchives.gov.uk/20110316162913/http://www.seac.gov.uk/papers/102-3annex.pdf](http://webarchive.nationalarchives.gov.uk/20110316162913/http://www.seac.gov.uk/papers/102-3annex.pdf).
2. [https://ec.europa.eu/food/sites/food/files/safety/docs/sci-com_ssc_out67_en.pdf](https://ec.europa.eu/food/sites/food/files/safety/docs/sci-com_ssc_out67_en.pdf).

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