numerous European countries (Austria, Czech Republic, Finland, Germany, Greece, Holland, Italy, Luxembourg, Poland, Slovakia, Slovenia, Spain), as well as in Japan, Canada, and the USA. They have also shown that many countries with no recorded case up to 2000 (e.g. Germany, Italy, and Spain), and which denied the presence of BSE, had an incidence equivalent to or higher than that of France and Switzerland, which have been recording cases since the early 1990s. Lastly, rapid tests allow much more precise epidemiological follow-up, allowing the measurement of trends at low prevalence, and which has more clearly shown a spectacular decline in the BSE epizootic in Europe.

Active monitoring of TSE in small ruminants (sheep and goats) was set up in Europe in March 2002, essentially to gather epidemiological data, and obliges member states of the European Community to test a quota of animals slaughtered normally or from at-risk populations. Between 2002 and 2006, nearly three million tests were performed, which led to the detection of over 13 000 cases of scrapie[4]. Note that this active surveillance resulted in the detection of a great many cases of so-called atypical scrapie among European livestock. This form of scrapie, which very likely corresponds to strain Nor98 [7] identified in 1998 in Norway, now accounts for over 50% of TSE cases in small ruminants in many countries (France, Germany, Portugal, UK, etc.). The PrP^{Sc} associated with this strain is characterised by increased sensitivity to proteinase K, which makes its detection more difficult and explains why numerous rapid tests perform poorly in diagnosis. In practice, the vast majority of cases of atypical scrapie were identified using the tests from Bio-Rad (TeSeE since 2002) and IDEXX (post-mortem test, since 2005).

In view of the diversity of TSE strains present in small ruminants, the European Commission set up biochemical typing in 2005, mainly designed to identify the BSE strain in small ruminant populations[5]. Testing, performed exclusively in national reference laboratories, is based on Western blotting techniques [74, 76]. It is worth noting that, to date, only a single case of BSE, in a goat, was observed in France [21] before the implementation of this new regulation.

3. NEW APPROACHES TO ANTE-MORTEM TESTS

As we have seen, diagnosis of prion diseases depends principally on the detection of the abnormal form of PrP (PrP^{Sc} or PrP^{res}). This approach has been very useful in reacting to the BSE epizootic and in setting up active surveillance for TSE in ruminants, but to date has not met all the requirements of the diagnosis of prion diseases. These tests are only applicable to tissues collected after the death of the animal and so cannot be used for early preclinical diagnosis. So far, no test can give a reliable diagnosis using a readily available sample from a living animal or person, such as blood or urine. The problem is particularly acute for blood transfusion, insofar as it is now well established that vCJD can be transmitted by blood. Considerable effort has been devoted to the search for alternative markers enabling earlier diagnosis of TSE (for a review see Parveen et al. [55]).

3.1. The search for new markers

The search for alternative markers has grown greatly in recent years, boosted by the development of postgenomic approaches, which can be used for large-scale parallel analysis of the transcriptome, proteome, and metabolome of tissues. Attention naturally first turned to neuronal markers, which include protein 14-3-3 [28, 78], neurone-specific enolase [1], the protein S100B [5, 29], glial acidic fibrillar protein [44, 50], Tau protein [51], and prionins [59]. However, none of these markers has proved usable as a basis for a sufficiently sensitive and specific test allowing early preclinical diagnosis.

[5] Commission Regulation (EC) No 36/2005 of 12 January 2005 amending Annexes III and X to Regulation (EC) No 999/2001 of the European Parliament and of the Council as regards epidemio-surveillance for transmissible spongiform encephalopathies in bovine, ovine and caprine animals: http://ec.europa.eu/food/food/biosafety/bse/legisl_en.htm [consulted 11 January 2008].

Metabolic markers, such as fatty acid-binding proteins, interferon γ, prostaglandin E2, C-reactive protein, interleukin 6, cystatin C, and corticosteroids, have also been studied, but with no more success (for a review see Parveen et al. [55]).

Transcriptomic studies have revealed potential markers [70, 85], but to date none has proved of practical use in the diagnosis of prion diseases. Erythroid differentiation-related factor, for example, initially seemed highly promising (downregulation [43]), but its value was not confirmed in subsequent work [25].

Finally, some research groups have developed an approach based on serum analysis by Fourier transform infrared spectroscopy combined with data processing by the neural network method [13, 39, 40, 69, 77]. This approach has shown high (> 90%) sensitivity and specificity in cattle populations, but it remains to be seen whether it is usable under routine conditions, and can be used to make an early diagnosis of TSE.

3.2. Protein misfolding cyclic amplification

To facilitate preclinical detection of prions in peripheral tissues, notably blood, Claudio Soto's group developed an original approach in which the PrP^{Sc} in a sample is amplified by means of protein misfolding cyclic amplification (PMCA) [62]. In this approach, which seeks to mimic pathological processes and is akin to the polymerase chain reaction used to amplify DNA (but without addition of exogenous polymerase enzyme), PrP^{Sc} is incubated in the presence of excess PrP^{C} to allow expansion of aggregates of PrP^{Sc} which are then dispersed by sonication to generate smaller units and to encourage the formation of new aggregates. The quantity of PrP^{Sc} formed depends on the number of expansion/sonication cycles performed. In early articles [62, 72], amplification was modest (10- to 50-fold), but optimisation and automation subsequently enabled amplifications of several million fold [61]. In most studies, amplification is achieved by using as a source of PrP^{C}, a brain extract from the same species as that which produced the PrP^{Sc} to be amplified. Recent works [18, 19] have shown that PrP^{Sc} can be replicated in a more controlled "minimal" system in the presence of highly purified PrP^{C} (the only identified contaminant being lipids) and polyanions (polyA RNA in these studies).

Although most of the work by Soto's group concerns a hamster model infected by strain 263K, significant amplification has been achieved with the PrP^{Sc} produced by various mammalian species, including mice [47, 72], sheep, goats and cattle [72], cervids [38] and humans [36]. The PrP^{Sc} newly formed by PMCA has all the properties of the original PrP^{Sc}, notably its infectious character [14, 82]. Lastly, early detection of PrP^{Sc} in hamster blood fractions (buffy coats) was achieved at a sensitivity ranging between 0 and 89% and a specificity of 100% [15, 60].

PMCA has great potential and is certainly the most promising approach from the viewpoint of developing a blood test. It is, though, hampered by various fundamental and technical difficulties. Given the requirements imposed by a blood test (see paragraph below), notably in terms of practicability, sensitivity, and specificity, several technical improvements are needed. For adaptation to routine analysis, there is a need for simplification, reduction of the duration, and better control. Moreover, the obligatory requirement for a concentrated source of PrP^{C} (brain extract or purified PrP^{C}) of the same species as the target to be amplified constitutes an important practical handicap. This specific problem could be resolved by the use of recombinant PrP and accelerated procedures as recently shown [4], assuming the results obtained with the hamster model can be extended to other mammalian species. However, PMCA must also prove effective in terms of diagnosis (sensitivity and specificity close to 100%) using blood sample series more representative than those obtained with the hamster model. Finally, recent results from Supattapone's group show that infectious PrP^{Sc} can be generated de novo and stochastically by PMCA [18] in the absence of pre-existing prions, and this raises concerns about the specificity of this approach when used in routine conditions.

3.3. Blood tests: state of the art

As we have emphasised several times in this review, the development of a blood test is the top priority in prion disease diagnosis, notably to ensure the safety of blood transfusion in humans. Numerous difficulties, however, have to be overcome, which explains why no test is yet operational. Whereas blood from vCJD infected patients is clearly infectious [41, 56, 84], its concentration of infectious material is very likely much lower than that in the central nervous system, and its concentrations of PrP^{Sc} are estimated to be in the range of pg/mL [8, 9]. Given the efficacy of disease transmission by the intravenous route, and the large volume (commonly > 400 mL) of packed red blood cells transfused in humans, transmission can occur with very low levels of infectious material, and, as a consequence, candidate tests must have excellent analytical sensitivity. Also, blood is a complex tissue rich in cells and proteins, and little is known of the distribution of prions (and of PrP^{Sc}). Several studies indicate that the bulk of the infectious material is in the white blood cells, but the plasma is also clearly infectious [8, 9]. In a healthy individual, significant levels of PrP^{C} are present in white blood cells, red blood cells, platelets, and plasma, probably at much higher concentrations than PrP^{Sc}. A candidate test must therefore also be very selective. Also, we know very little about the biochemical properties of the PrP^{Sc} in the different blood fractions. Given its low concentration and its environment, it is not certain, for instance, that it can form aggregates resistant to proteinase K, the treatment on which most current rapid tests are based.

In terms of the risk of vCJD infection by blood transfusion, because the incidence of the disease is assumed to be very low, a highly specific test is needed, or it could lead to more false-positive results than detection of real cases. Such a situation would be very difficult to manage ethically, given that vCJD is a fatal disease for which at present there is no treatment. There is clearly a great need for at least one very specific confirmation test, which does not exist today.

Due to the above mentioned difficulties it is not surprising that very few publications report on blood tests for TSE.

The first promising results were obtained, as early as 1996, by the group of Mary-Jo Schmerr, which combined capillary electrophoresis with a competitive immunoassay to detect a PK resistant C-terminal sequence of PrP in the blood of sheep infected with scrapie [66–68]. The technique was subsequently improved and applied to more relevant series of scrapie infected sheep [34, 35, 42, 86], but despite achieved improvement, the method appeared insufficiently robust for routine use [22].

In recent years, many research groups or companies have developed original strategies to try to overcome the intrinsic difficulties associated with the blood test. These include:

- The use of ligands for a specific capture of PrP^{Sc} possibly present in blood fractions, which include the 15B3 antibody produced by Prionics [37, 48] and the Seprion resin of the Microsense company (already used in the IDEXX test for post-mortem diagnosis). In both cases, the idea is to concentrate abnormal PrP by immunoprecipitation, taking advantage of its polymerisation state (aggregate? polymers? oligomers?) to allow a more sensitive and more specific detection by ELISA or flow cytometry. Another approach developed by the bioMérieux company (Marcy l'Étoile, France) involves binding and aggregation of abnormal PrP in plasma by streptomycin [45], followed by a specific capture on calyx-Arenes "molecular basket" immobilised onto a solid phase, and final detection with an appropriate anti-PrP antibody.
- The development of immunoassays designed to detect polymerised PrP (AS-ELISA, for aggregate specific ELISA) and based on the use of the same monoclonal antibody for capture and detection [52]. The sensitivity of AS-ELISA was increased by combining signal amplification (fluorescence) and target amplification (prion amplification using a simplified PMCA like procedure). Using

this approach (named Am-A-FACTT) the group of Man-Sun Sy succeeded in detecting prion aggregates in plasma from mice or deer infected with scrapie or CWD respectively [16]. A similar approach has been developed by the Korean company PeopleBio (Seoul, Korea, Multimer Detection System (MDS)) without amplification of signal and target but details remain unpublished.

- The use of fluorescence labelled palindromic PrP peptides to detect misfolded PrP (MPD for misfolded protein diagnostic). In this approach, when the labelled peptide is in contact with PrP^{Sc}, it undergoes a large coil to a β-sheet conformational change which largely modifies the fluorescence properties of the pyrene label [30]. This method allowed discrimination between TSE infected and uninfected animals, albeit on a rather small series of blood samples [53].

However, even if some of these approaches seem promising, for the moment none of these tests has fulfilled the very strict analytical and diagnostic requirements described above. With the passage of time (some of these approaches were initially described a few years ago) it becomes apparent that they are facing real difficulties in establishing routine and robust assays, and that much more time and development is needed to achieve the goal of an operational blood test for TSE.

4. CONCLUSIONS

The successive "mad cow" crises of 1996 and 2000 have clearly boosted very significantly research in the field of prion diseases, and more data have been accumulated during the last ten years than during the previous century. This has considerably improved our knowledge on prion biology, but also provided much more relevant tools including: transgenic mice ($PrP^{0/0}$ or over-expressing various forms of wild-type or mutated PrP), cellular models of TSE infection, a large series of well characterised monoclonal antibodies and, of course, much more relevant analytical methods and diagnostic tests. As far as diagnosis is concerned, very significant progress has been made in the post-mortem detection of PrP^{Sc}, with the development of reliable and very sensitive methods suitable for routine analysis (results available within less than three hours, more than 20 000 tests performed every day throughout the world), having the capacity of diagnosing TSE before the onset of clinical signs. These tests have been used efficiently for managing the mad cow crisis, and are still very useful for monitoring the BSE epizootic as well as the various forms of TSE in small ruminants and cervids. The analytical sensitivity of these tests can now be considerably improved by coupling PMCA amplification with the appropriate detection techniques (ELISA, CDI, Western-blot), and this allows detection of minute amounts of PrP^{Sc} in the brain or in peripheral lymphoid tissues. However, so far, there is no test that delivers an early and specific diagnosis of TSE in live animals or patients, i.e. a test which can be easily applied to a body fluid like blood or urine. This is particularly critical for ensuring the safety of blood transfusion in countries that have experienced a large BSE epizootic (UK and Western Europe). We have seen that PMCA has shown a good potential, in terms of sensitivity, for achieving such an aim but its use in routine conditions and its actual specificity are questionable. There is thus a place for another approach, and the development of a blood test for TSE diagnosis remains the most important challenge for the years coming in this field of prion research.

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別紙 3-3

識別番号・報告回数		回	報告日 年　月　日	第一報入手日 2008 年 2 月 28 日	新医薬品等の区分 該当なし	総合機構処理欄
一般的名称			研究報告の公表状況	Approaches to investigating transmission of spongiform encephalopathies in domestic animals using BSE as an example. Simmons, M. M. et al, Vet. Res., 39, 34 (2008).	公表国 英国	
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研究報告の概要

本稿では，伝染性海綿状脳症（TSE）の伝播性を調べるための実験的アプローチ法を要約し，実験における所見と自然発生する TSE〔主にウシ海綿状脳症（BSE）及びスクレイピー〕との関連性を考察している。BSE はこれまで影響を受けなかった動物種における新規の海綿状脳症であり，点感染源の特徴を有する。本実験では，人工的感染経路（脳内接種）及び自然感染経路（経口）を用いて伝播の効率ならびに宿主の感受性を，特に食用動物種に焦点を当てて特定した。実験的伝播が認められても，曝露時の動物の年齢等，種々のパラメータの影響を受けることから結果の解釈は常に困難であった。しかしながら，ヒツジでは，BSE 陽性ウシの脳を経口投与した雌ヒツジから，その仔ヒツジへの BSE 伝播が示された。

これとは対照的に，スクレイピーは英国で数世紀にわたりヒツジ個体群において地域固有のものであった。それにもかかわらず，スクレイピーの真の垂直（子宮内）伝播は確認されておらず，一方で水平伝播が確認されている。すなわち，疾患を引き起こすには汚染された環境に曝露するだけで十分であると考えられる。特に胎盤はスクレイピーの自然伝播の原因とされており，感染性の PrP^{Sc} プリオンを含むことが立証されている。現時点では多くの疑問が依然として解明されておらず，結論として著者らは，様々な分野の研究者らに対して TSE の特性をより理解するため協力及び支援を強く呼び掛けている。

使用上の注意記載状況・その他参考事項等

BYL-2008-0312

報告企業の意見	今後の対応
本概論は，TSE 研究の複雑さを明らかにしており，反芻動物でない，生物学上遠隔種のトランスジェニックマウスを用いた研究であっても，全ての研究結果は有益であり，疾患管理の向上及び公衆衛生を守る上で役立つであろう。	ヒトに影響するプリオン関連疾患伝播のメカニズムの更なる理解に関連した調査の情報を収集する以外，現時点で新たな安全対策上の措置を講じる必要はないと考える。

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