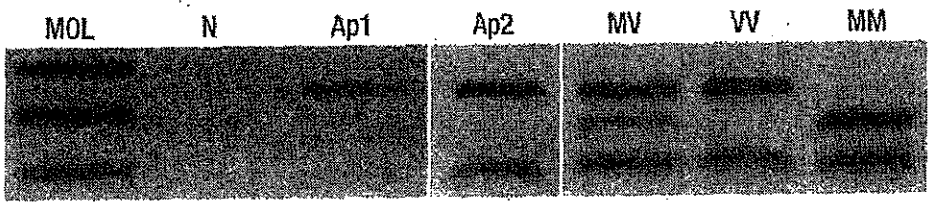

Restriction digest pattern for *PRNP* codon 129 genotype analysis in two paraffin section tissue samples (shown combined). The test sample results clearly show banding patterns equivalent to the VV genotype control (Mol=molecular weight ladder, N=PCR negative control, Ap1=appendix tissue from positive case 2, Ap2=appendix tissue from positive case 3, positive control samples from *PRNP* codon 129 MV, VV, and MM genotypes)

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this genetic locus. People infected with vCJD with a valine homozygous codon 129 *PRNP* genotype may have a prolonged incubation period, during which horizontal spread of the infection could occur either from blood donations or from contaminated surgical instruments used on these individuals during the asymptomatic phase of the illness.

Introduction

In a prevalence study for variant Creutzfeldt-Jakob disease (vCJD), we identified three appendixes that stained positively for disease associated prion protein (PrP). We looked at 12 674 specimens (11 109 appendices, 1565 tonsils) removed from 1995-2000. Most of the patients (83%) were aged 10-30 years at the time of operation.[1 2] This number of positive results is greater than would be predicted from the numbers of patients diagnosed with vCJD in the United Kingdom (161 to date). Furthermore, the annual incidence of new cases of vCJD has declined from a peak in 1999. As all patients with vCJD belong to the methionine homozygous subgroup, determined by the codon 129 polymorphism in the prion protein gene (*PRNP*),[2] one possible explanation for this apparent discrepancy could be a different *PRNP* genotype in the three positive cases (the prevalences of *PRNP* codon 129 genotypes in the general UK population are about 40% methionine homozygous, 10% valine homozygous, and 50% heterozygous). This possibility was supported by a slightly different pattern of immunoreactivity in the second and third positive appendix cases in comparison with clinical cases of vCJD.[2] We recently identified a case of asymptomatic vCJD infection that seemed to have been transmitted by red cell transfusion in a *PRNP* codon 129 heterozygote, demonstrating that the methionine homozygous genotype is not uniquely susceptible to vCJD infection.[3]

Methods

We analysed the *PRNP* codon 129 polymorphism in the three samples of appendix tissue embedded in paraffin that stained positively for disease associated prion protein in the prevalence study. In the first case, a transmission study is currently under way using material from the remaining unstained sections. This meant that only immunostained sections were available for genotype studies and the extracted DNA was not good enough for further analysis. In the two remaining cases, as there was not sufficient material available for both transmission studies and genotype studies, and in view of possible *PRNP* influences on the staining pattern of disease associated prion protein in these cases, we used the remaining material for DNA analysis. A single 6 µm unstained paraffin section was available from each case, and these were de-paraffinised and scraped into individual microcentrifuge tubes for DNA extraction with the Puregene DNA Purification Kit (Gentra Systems, USA). Pelleted DNA was rehydrated for one hour at 65°C and then used as a template for amplification by the polymerase chain reaction (PCR), along with positive and negative control samples. PCR primers used were specific for a 506 bp region of *PRNP* containing the polymorphic sequence for the codon 129 residue. PCR products were digested at 37°C with the restriction enzyme Nsp1 (New England Biolabs, UK), which specifically recognises changes at the *PRNP* codon 129 polymorphic DNA sequence. Digest products were analysed on 1.5% agarose gels with positive controls for the codon 129 variants (MV, VV, and MM).

Results

For both cases the genotype was confirmed as homozygous for the valine allele (VV) (figure). This method has been previously validated[4 5] and was controlled in our laboratory by studying the *PRNP* codon 129 genotype in both paraffin embedded sections and frozen tissues from 25 other cases.

Discussion

These results give the first indication that *PRNP* codon 129 valine homozygotes may be susceptible to vCJD infection. Though the immunohistochemical technique used in our earlier study seems to be specific for disease associated prion protein,[6] it is unlikely to be 100% sensitive, suggesting that the true prevalence of vCJD infection in the UK population may be even higher than earlier estimated (3/12 674).[2] Genetic studies of kuru, another orally transmitted human prion disease, found that *PRNP* codon 129 MV and VV genotypes were associated with longer incubation periods than the MM genotype.[7] As the ethical approval for our study placed restraints on the identification of individual cases, we are not able to state with certainty the age of the patients in the positive cases at the time of surgery. We can, however, state that they were aged 20-29 years at the time of surgery, which took place in 1996-9. No clinical cases of vCJD at any age have yet been identified in *PRNP* codon 129 valine homozygotes, indicating the need for continued surveillance of all cases of vCJD in the UK.

What is already known on this topic

A recent prevalence study of accumulation of prion protein (as a marker for vCJD infection) in appendix and tonsil specimens in the UK found 3/12 674 positive cases, which is more than expected from the current number of clinical cases of vCJD

What this study adds

Analysis of DNA from two of the three positive samples found them to be valine homozygotes at codon 129 in the prion protein gene, indicating that this genetic subgroup (which is a different subgroup from that in which all cases of vCJD have so far occurred) is susceptible to vCJD infection

Individuals with this genotype may have a prolonged incubation period with subclinical infection and could cause secondary spread of vCJD by blood transfusion or surgery

Though it is inadvisable to overinterpret the data from only three positive cases in this study, it is perhaps surprising (given the relative prevalences of *PRNP* codon 129 genotypes in the general population) that both the positive cases analysed here were valine homozygotes. Though this may represent a chance finding, we should consider the possibility of differences in the peripheral pathogenesis of vCJD that depend on the *PRNP* codon 129 genotype. The patient who developed asymptomatic vCJD infection after red blood cell transfusion was a codon 129 heterozygote in whom both tonsil and appendix tissues were negative on staining for disease associated prion protein with identical methods as used in this study, though the spleen and lymph nodes gave positive results.[3] *PRNP* polymorphisms in sheep infected with scrapie also have a major influence on the incubation period and timing and distribution of disease associated prion protein in lymphoid tissues during the incubation period.[8]

A prolonged incubation period after infection with vCJD is likely to result in an asymptomatic carrier state (which cannot yet be identified), which represents a potential risk for horizontal transmission of vCJD infection by blood transfusion, blood products, or contaminated surgical instruments. These uncertainties further underline the need for continued surveillance of vCJD in the UK (including surveillance for subclinical or asymptomatic infection[9]), a requirement to continue to reduce the possibility of secondary iatrogenic transmission, and the inclusion of carrier states and susceptibility to vCJD infection in all *PRNP* codon 129 genotypes in future disease modelling.

Contributors: JWI (guarantor) and DAH were responsible for the prevalence study and the analysis of the results, including the selection of the cases for analysis, and drafted and modified the manuscript. MTB established the methods for DNA extraction and analysis, designed and executed the validation study, and drafted and modified the manuscript. KC and DH performed the DNA extraction on the test materials and in the validation study and modified the manuscript. MLeG, SL, DLR, and LMcC identified cases for the validation study and prepared the paraffin sections for DNA analysis and modified the manuscript.

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Competing interest: None declared.

Ethical approval: The prevalence study received approval from the South and West multi-centre research ethics committee (MREC reference 99/6/32) and for each of the centres included, appropriate local research ethics committee approval.

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Prescribing for RITA

And so it ends—a decade in the training grade. The last rites performed with a final RITA (record of in training assessment): I'm finally grown up, the authorities deem. In fact, my consultant job starts tomorrow.

After 10 years with it, do I have positive suggestions for postgraduate training in the NHS? Of course, dozens, most involving workforce, reorganisation, and resources. But, as in life, the best tonics are free. I vote for a fresh culture that values and grows people. It is remarkable that NHS doctors deliver their high quality service for no immediate tangible gain. More extraordinary is that this work receives not a trace of the positive feedback and moral incentive that would be critical to the health of any comparable organisation.

I worked for some time in a prestigious institution of a more advanced healthcare system. What made their people tick? True, they had impressive buildings, state of the art technology, and good salary prospects; but, really, I think they were primarily driven by an ethos that valued excellence and individuality—initiated, fostered, and rewarded it. Right down to the artwork that lined the corridors—oversized portraits of the previous month's star employees, proud pictures of "graduating" trainees, plaques of senior faculty.

A bit over the top perhaps, but preferable to the anonymous passage of generations of juniors through Britain's many worthy hospitals. In addition, attitudes of derision towards the less skilled and suspicion of those who seem too good or creative are all too common. The end result? Blunted clones coming off an assembly line: competent, yes; extraordinary, no. Tragic for individuals and undesirable for a healthcare system that confronts extraordinary problems.

There, I've had my shout. Tomorrow I step into a new world, recognising that to change it is to change myself. I will not forget my morning dose of free tonic.

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研究報告の概要

1．血液製剤のプリオンに対するリスク（ｖCJDの発生状況）
・BSE の発生は少なくとも英国では減少しつつある。BSE に感染したウシからのvCJD伝播の危険性は低下傾向にある。他方、輸血によるヒトーヒト伝播の危険性は依然として続いている。

2．血漿分画製剤の安全対策
・血液中のプリオン検出のための高感度なスクリーニング方法は現在のところまだない。従って、今のところ、血漿分画製剤のプリオンに対する安全対策は、主として地理的条件によるドナーの排除と製造工程でのプリオンの除去である。

3．プリオン除去試験の留意点と試験結果
・プリオン除去のための個々の製造工程は、実際の製造条件を実験室での条件にスケールダウンさせ、確立されているスクレイピー株をモデル系として用いて通常は評価されている。血液中のプリオン蛋白の存在形態が不明なので、評価実験のためのスパイク材料としてのプリオンの調製方法は注意深く考慮しなければならない。
・プリオンアッセイする方法としては2 つの異なる方法がある。その2 つとはウエスタンブロッティングなどの方法でプリオンを検出するin vitro 試験と、サンプルを動物に接種することによって感染性プリオンを検出するin vivo 試験である。ごく少量のプリオン、例えばウエスタンブロッティング法の限界（例えば、抗体の不適切な使用など）によって陰性の結果を示すような量のプリオンであっても、サンプル中に感染性を検出することができることもある。従って、製造工程評価には、試験方法の間の相異を注意深く考慮しなければならない。
・製造工程条件の如何によって、除去パターンが類似したものであっても、その製造工程のプリオン除去能は変わりうる。従って、評価試験のデザインはきわめて重要である。各製造工程は個別に評価しなければならない。
・現在のところ、血漿分画製剤の製造工程のうち、エタノール分画、PEG 分画、カラムクロマトグラフィー、ウイルス除去膜およびデプスろ過膜でのろ過、その他がプリオン除去に有効であると考えられている。
・製造工程で安定してプリオン除去を行うためには、プリオン除去に寄与する複数の製造工程を組み合わせることが必要である。
・これらのことから、プリオンのスクリーニングと除去のための新しい技法の開発と製造工程評価方法の改良が切望される。

使用上の注意記載状況・その他参考事項等

2．重要な基本的注意
(1)略
1)略
2)現在までに本剤の投与により変異型クロイツフェルト・ヤコブ病（vCJD）等が伝播したとの報告はない。しかしながら、製造工程において異常プリオンを低減し得るとの報告があるものの、理論的な vCJD 等の伝播のリスクを完全には排除できないので、投与の際には患者への説明を十分行い、治療上の必要性を十分検討の上投与すること。

報告企業の意見	今後の対応
血漿分画製剤の製造工程におけるプリオン除去に関する総説論文であり、文献中の表に弊社が行なったプリオン除去試験結果を提示している。 これまで血漿分画製剤によってvCJDを含むプリオン病が伝播したとの報告はない。しかしながら、万一vCJD感染者の血漿が本剤の原料に混入した場合には、製造工程においてプリオンを低減し得るとの報告があるものの、製剤から伝播する可能性を完全には否定し得ない。そのため、弊社の血漿分画製剤の製造工程におけるTSE感染性低減に関する検証実験を加速し、自社データを早期に取得し、工程評価を行い、必要に応じて工程改善を実施する予定である。	vCJD に関連する情報については、今後も注視することとする。

REVIEW

Possible removal of prion agents from blood products during the manufacturing process

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Blood products prepared from human blood theoretically risk contamination with infectious pathogens. Since recent reports now confirm the likely transmission of pathogenic prions through blood transfusion, effective measures to prevent transmission are required globally, although the prevalence of variant Creutzfeldt–Jakob disease outside of the UK is extremely low. Many studies evaluating the manufacturing process have been conducted for the potential removal of the prion protein from plasma derivatives. In this review, we discuss the possibility of removing prions via several processing steps, especially depth and virus-removal filtration. Through a discussion of the limitations and issues associated with such studies, we hope our review will be of help for better study design in the future.

Keywords: blood products, blood transfusion, bovine spongiform encephalopathy, clearance study, depth filter, prion, variant Creutzfeldt–Jakob disease, virus-removal filter

future medicine

The onset of illness in the first case of variant Creutzfeldt–Jakob disease (vCJD), which was published in 1996, occurred in early 1994. vCJD most probably results from the consumption of beef products contaminated by central nervous system tissue derived from bovines infected with bovine spongiform encephalopathy (BSE), which began in the UK sometime prior to 1986 [1]. The worldwide incidence of BSE and vCJD was approximately 190,000 animals and 185 patients (including 159 patients in the UK), respectively, in December 2005. In the UK, where the highest incidence of BSE and vCJD was reported, their peak incidences were observed in 1992 and 2000, respectively. Since these peaks, the incidence in the UK has decreased gradually [101]. There is also the possibility of continuing person-to-person transmission of vCJD through certain forms of healthcare (e.g., through surgery, blood transfusion or treatment with plasma products). Therefore, it is essential to maintain and promote active surveillance of vCJD and CJD (hereafter vCJD/CJD) to evaluate potential transmission by this route [2–4,102,103].

Since blood products are prepared from human blood, they may involve risks of contamination with infectious pathogens including pathogenic prions. Therefore, besides the measures for ordinary pathogens, effective measures implemented globally to prevent transmission of pathogenic prions (especially to prevent vCJD) are also required. Measures to prevent contamination by viruses/prions in plasma derivatives consist of donor plasma sourcing/screening and the elimination of viruses/prions during the manufacturing process. Currently, geographical deferral of blood donors before donation is the only method of identifying donors at higher risk for vCJD, since sensitive and rapid screening methods for prions in blood with the ability to handle many specimens have not currently been developed. The risks to a recipient from fractionated plasma products are probably less than from blood transfusion, not least owing to potential removal during the manufacturing process, as well as the volume of material to which an individual is exposed, which are likely to be important determinants of the level of risk [2]. However, the pooling of plasma donations and the large number of recipients from any given plasma pool complicates any calculations of residual risk.

Little is known regarding the native form of prion protein in blood, especially in plasma, although this information is essential for evaluating the safety of blood products. Under these circumstances, the regulatory agencies of several countries issued guidelines regarding measures to be taken to prevent or reduce the potential for prion transmission through pharmaceutical products. Manufacturers have implemented their measures according to these guidelines [5–7,104–107]. Recently, Brown reviewed prion infectivity in blood, prion removal by the manufacturing process and the current status of the development of prion-screening methods [8]. The removal of prions by partitioning during the manufacturing process is expected to be a practical and effective approach, particularly because effective methods for prion inactivation that are applicable to the manufacture of protein products, have not been developed to date. At present, the removal of prions by physical means is the main measure towards preventing prion contamination. There are numerous reports describing the partitioning and possible removal of prion

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during the manufacturing process. Processes that possibly remove prions include fractionation, using ethanol and/or polyethylene glycol (PEG), and filtration through virus-removal and/or depth filters. Many studies have been performed on the efficacy of ethanol fractionation processes to remove prion and demonstrated similar removal ability, regardless of differences in study conditions and the research institutions. By contrast, studies on depth filtration revealed that the efficacy of depth filters to remove prions is highly dependent on the composition of the solution and/or characteristics of the filters. In this review, we discuss the possibilities and limitations of several manufacturing steps to remove prions using evaluation data from several manufacturers. We hope our discussion will be of help to determine better study design in the future. Cleaning (inactivation) of equipment should also be considered in parallel to the removal of prions during the manufacturing process. However, we will not discuss this area, and the reader is referred to Lee and colleagues who have already discussed this matter in details [9,10,108].

Study design for prion removal ability

Procedures for safety evaluation of plasma derivatives for prions are basically similar to those used for viruses. Regarding the virus clearance study (also termed virus validation study), the first regulatory guidance was issued by the European Community in 1989. Since then, manufacturers have performed virus clearance studies in accordance with this guideline as well as other related guidelines. On the other hand, the European Medical Agency issued guidelines regarding prion clearance in 2004 [104]. Their guideline was largely based on the concept of the virus validation guidelines [109], although care was taken to refer to such studies as investigational, as opposed to validation.

The following should be considered when performing prion-clearance studies.

Model agents

The main purpose of the clearance study is to assess and identify the manufacturing process(es) that can be considered to be effective in eliminating prions using various model agents, such as scrapie. Based on the results with model agents, the partitioning of specific human pathogenic agents, such as vCJD, can be speculated. In this sense, the purpose of the study is to evaluate the risk of the pathogen itself. However, if the pathogen in question is significantly different from the model agent, partitioning of the specific human pathogen may yield incorrect data. Therefore, for clearance studies and related studies, it is indispensable to carefully consider the possible differences between the pathogens in question and model agents.

Assay method

The detection of protease-resistant prion antigen (*in vitro* study) is performed as the first step (e.g., by western blotting [WB], and then, for certain process(es), an infectivity assay using animals (*in vivo* study) is also recommended. The *in vivo* assay remains the only possible option to confirm the quantitative infectivity titration of prions following inoculation of samples into animals. It should also be understood that, in some instances, there could be some discrepancy between *in vitro* and *in vivo* study results.

Simulation of manufacturing process

It may be impossible to exactly simulate all of the manufacturing process parameters on a laboratory scale. For experimental downscaling, it is impossible to use equipment and conditions that are identical to the actual manufacturing process. First, prion proteins are added intentionally, thereby changing the matrix. Second, the processes are downsized to laboratory scale. Therefore, in some instances, the best result that can be achieved is to approximate the behavior of the manufacturing process. Furthermore, it is important not to overestimate the prion removal ability of the manufacturing processes based on the data obtained under scaled-down conditions.

Native form of abnormal prion proteins

The native form of the abnormal prion proteins in blood is still unknown. Abnormal prion proteins in blood remain largely undetermined and may exist as various forms with different particle sizes or aggregation states. Therefore, the preparation method of the prion material used as a spiking agent for process evaluation studies would be an important factor.

Choice of spiking prion agent

Of the various prion diseases, vCJD is the primary concern for manufacturing plasma derivatives. It is difficult to use tissue samples taken from vCJD/CJD patients for the evaluation of manufacturing processes. Therefore, in general, laboratory strains of scrapie (e.g., 263K and ME7) and those of BSE (e.g., 301V) are used in place of vCJD materials [11–15].

Spiking materials are prepared from the brain of infected animals, and there are several methods of preparation. Brain homogenate (BH) has been widely used for a long time, because BH is easy to prepare and contains a high titer of infectivity. However, the uniformity of particle size is not ideal for evaluation purposes. The microsomal fraction (MF) is partially purified from BH, and the titer of MF may be slightly lower than BH. In addition, caveolae-like domains and semipurified scrapie prion protein (purified fibrils) may also be used in spiking studies. The partitioning of abnormal prion protein prepared by different preparations behaves in a similar manner, with the exception of purified fibrils [16–18]. Although the appropriateness of the materials used for spiking experiments was described in reported studies, there has been no discussion of their particle size.

In 2003, Yunoki reported that the particle size of MF used as spiking material was 800 nm on average, and that the particle size of MF fell to less than 220 nm through high-power sonication or detergent treatment [19]. At present, researchers tend to add steps such as sonication, detergent treatment and prefiltration in order to prepare MF or BH for spiking studies. However, discussion regarding the appropriateness of spiking materials is limited because the status of the abnormal prion protein in blood is not currently clear. Various preparation methods have been used in the above reports. Therefore, it is necessary to carefully consider such preparation methods used in individual reports in order to evaluate the removability of prion agents during the process.

As our knowledge regarding the form and characteristics of abnormal prion protein in blood accumulates, these problems are expected to be resolved. A new preparation method utilizing exosomes might be proposed because a recent report described that prions also exist in association with exosomes [20]. In addition, spiking materials derived from cultured cells producing abnormal prion protein may become one possible source for spiking prion material [21]. If strains of vCJD/CJD with a high titer are prepared in cultured cells, it would become possible to use such materials as spiking agents.

Evaluation methods

To estimate the prion levels in samples, two different methods are used: one to detect abnormal prion protein in samples by WB, conformation-dependent immunoassay or enzyme-linked immunosorbent assay (*in vitro* study) [16,22–24]; and the other to detect pathogenic prion protein by inoculating animals with the samples (*in vivo* study). Although WB is widely used, assay conditions are different in every laboratory and there is no standard protocol for the assay. In general, samples taken from manufacturing processes contain plasma proteins at a high level, which sometimes disturb the specific detection of a small amount of prion by WB. To avoid these problems, optimization of assay conditions and/or adjustment of pretreatment conditions for each sample are necessary. Due to these assay variables, the sensitivity of the assay not only differs in every laboratory, but even from sample to sample. When an identical sample is used for comparison, WB generally gives a lower sensitivity than the *in vivo* method. To improve the sensitivity and specificity of WB, several methodologies have been performed; for example, the elimination of plasma proteins that disturb the assay, by heating at 80°C before proteinase K treatment, followed by ultracentrifugation to concentrate prion [25].

For viruses, it is required that clearance studies are performed following the detection of infectivity of process samples as an indicator. However, for prions, according to the guidelines [104,105,109], infectivity experiments *in vivo* are not always required for processes where the relationship between *in vivo* and *in vitro* results has been established. For processes where the relationship is unknown, such as new processes, it may be necessary to check the infectivity of samples *in vivo* following initial testing of samples *in vitro*.

In general, experimental conditions for the detection of infectivity using animals differ at every institution. Even if animals are the same species and age, the amount of inoculums given to the animals and/or incubation period of the animals after inoculation may be different. Symptoms of prion diseases are monitored by clinical signs during the incubation period in animals, although the monitoring procedure may also differ at each institution. Some institutions monitor abnormal behavior only, whereas other institutions use a scoring system for monitoring. However, such observation of clinical signs may not be regarded as a definitive indicator of disease. Classically, histopathology has been used to confirm disease lesions in brain samples taken from infected animals [26]. Similarly, different criteria in pathological examinations are used by institutions to determine prion lesions. Some institutions judge prion diseases by